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Influence of Angiotensin II on α_1-Adrenergic Receptors Function in Rat Aorta and Expression in Vascular Smooth Muscle Cells

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Abstract

Angiotensin II (Ang II) is the main mediator of the Renin-Angiotensin-System acting on AT_1 and other AT receptors. It is regarded as a pleiotropic agent that induces many actions, including functioning as a growth factor, and as a contractile hormone, among others. The aim of this work was to examine the impact of Ang II on the expression and function of α_1-adrenergic receptors (α_1-ARs) in cultured rat aorta, and aorta-derived smooth muscle cells. Isolated Wistar rat aorta was incubated for 24 h in DMEM at 37˚C, then subjected to isometric tension and to the action of added norepinephrine, in concentration-response curves. Ang II was added (1×10^{-5} M), and in some experiments, 5-Methylurapidil (α_{1A}-AR antagonist), AH11110A (α_{1B}-AR antagonist), or BMY-7378 (α_{1D}-AR antagonist), were used to identify the α_1-AR involved in the response. Desensitization of the contractile response to norepinephrine was observed due to incubation time, and by the Ang II action. α_{1D}-AR was protected from desensitization by BMY-7378; while RS-100329 and prazosin partially mitigated desensitization. In another set of experiments, isolated aorta-derived smooth muscle cells were exposed to Ang II and α_1-ARs proteins were evaluated. α_{1D}-AR increased at 30 and 60 min post Ang II exposure, the α_{1A}-AR diminished from 1 to 4 h, while α_{1B}-AR remained unchanged over 24 h of Ang II exposure. Ang II induced an increase of α_{1D}-AR at short times, and BMY-7378 protected α_{1D}-AR from desensitization.

Keywords

Angiotensin II, α_{1D}-AR, α_1-AR Expression, Rat aorta, Smooth Muscle Cells

1. Introduction

Angiotensin II (Ang II), the main product and mediator of the Renin-Angiotensin System (RAS), is recognized as a pleiotropic agent involved in numerous physiological actions, including its significant role in elevating blood pressure (hypertension), by acting on the AT_1 receptor (AT_1R) [1] [2] [3] [4]. The AT_1R responds to Ang II stimulation provoking pressor effects and growth of cardiac myocytes, and vascular smooth muscle cells, as well as aldosterone secretion, renal tubular Na^+ reabsorption, thirst, activation of sympathetic nervous system, cardiac ionotropic and chronotropic actions and cardiovascular inflammation, hypertrophy and fibrosis [4]. Thus, diminution of Ang II synthesis by inhibitors of the angiotensin-converting enzyme, or AT_1R antagonism leads to the decrease of blood pressure and reversion of cardiac hypertrophy [4].

Previous studies have demonstrated that Ang II upregulates the expression of α_1-adrenergic receptors (α_1-ARs), particularly α_{1D}-AR, promoting growth in rat vascular smooth muscle cells [5], and contributing to cardiac hypertrophy and increased aorta contraction in the $AHR^{-/-}$ null mouse [2] [6]. Continuous Ang II exposure has been reported to induce aortic vascular hypertrophy in the rats, which could be prevented and reverted by the α_{1D}-AR antagonist BMY-7378 [3]. This phenomenon was associated with an enhanced contractile response to the α_1-AR agonist, phenylephrine, and correlated with aorta hypertrophy, and a reduction in both mRNA and protein of the α_{1D}-AR [3], suggesting that Ang II desensitized the α_{1D}-AR *in vivo*, following the hypertrophic process, without significantly affecting α_{1A}- or α_{1B}-ARs [3]. Furthermore, Godínez *et al.* reported that captopril diminished the expression and function of the α_{1D}-AR in young, pre-hypertensive SHR [7]; whereas Rodríguez *et al.* showed that cardiac hypertrophy observed in the aged SHR was reverted by captopril and by BMY-7378, suggesting the interplay between ACE/AT_1R and α_{1D}-AR during heart hypertrophy [4] [8]. It is not clear if the increase in blood pressure and cardiovascular hypertrophy are due solely to Ang II acting on AT_1R, or if it is added to noradrenergic action on α_{1D}-AR [3] [8]. Our recent findings indicate that endogenous norepinephrine (NE) desensitizes α_{1D}-AR when the aorta is cultured 24 h in DMEM, whereas the α_{1D}-AR antagonist, BMY-7378 protects the α_{1D}-AR from desensitization [9]. Consequently, this study aims to elucidate the influence of Ang II on the expression and function of α_1-adrenergic receptors in rat aorta and vascular smooth muscle cells.

2. Materials and Methods

2.1. Animals and Ethical Statement

Male Wistar rats, aged 3 months and weighing 250 - 300 g, were housed under

pathogen-free conditions with controlled parameters (40% - 60% humidity, 22˚C ± 2˚C, and a 12 h light/dark cycle), in our vivarium. They had *ad libitum* access to food and water. All animal care and experimental procedures were conducted in accordance with the Mexican Regulations of Animal Care and Use (NOM-062-ZOO-1999, SAGARPA, Mexico), and were consistent with the Guide for the Care and Use of Laboratory Animals, as promulgated by the U.S. National Institutes of Health [10]. The Institutional Ethics Committee of FES Iztacala, UNAM, approved all procedures (Protocol 1497).

2.2. Procedures

2.2.1. Incubation Conditions

Rats were euthanized, and the thoracic aortas were carefully dissected and cleaned of surrounding adipose tissue. In a laminar flow hood, the isolated aortas were sectioned into rings measuring 4 - 5 mm in length. To exclude the influence of endothelium-derived factors on the contractile response, the endothelium was gently removed with a rugged metal. The effectiveness of the endothelium removal was verified by the absence of relaxation to carbachol (1×10^{-6} M) [11]. Subsequently, the arterial rings were immersed in 3 ml of Dulbecco's Modified Eagle Medium (DMEM), within a 6-well culture plate. These plates were incubated in a CO_2 incubator at 37˚C (model BB 150, Thermo Scientific, Waltham, MA, USA), maintaining an atmosphere of 95% air and 5% CO_2, for 24 h [9].

2.2.2. Concentration-Response Curves (CRC)

The arterial rings were placed in 10 ml organ chambers filled with Krebs-Henseleit solution, maintained at 37˚C and pH 7.4. The solution had the following composition (in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 11.1 [3]. It was continuous bubbling with a gas mixture of 95% O_2 and 5% CO_2. Each arterial ring was connected to an isometric FT03E Grass force displacement transducer (Astro-Med, Inc., West Warwick, RI, USA). This transducer, in turn, was connected to a MP100A data acquisition system (Biopac Systems Inc., Santa Barbara, CA, USA), which recorded the isometric tension response. The aortic rings were adjusted to an optimal tension of 3 g [3] [12].

2.2.3. α_1-Adrenergic Receptor Stimulation in Aorta Exposed to Angiotensin II

Upon completion 24 h of incubation in DMEM, the aortic rings were transferred to the recording chamber. They were then exposed to norepinephrine (1×10^{-7} M) in the presence of rauwolscine (1×10^{-7} M) and propranolol (1×10^{-7} M), to antagonize α_2- and β-adrenergic receptors, respectively. This solution was changed every 30 min over a 2 h period to allow for stabilization. Subsequently, a reproducible cumulative concentration-response curve (CRC) to norepinephrine was established, with concentrations ranging from 1×10^{-10} M to 1×10^{-4} M, increasing in half logarithm increments to establish a control curve.

In a parallel set of experiments, aortic rings were incubated in DMEM, supplemented with a constant concentration of Ang II (1×10^{-5} M) for 24 h. After this period, the aortic rings were transferred to the recording chamber and subjected to incremental half-logarithm concentrations of norepinephrine from 1×10^{-10} M to 1×10^{-4} M [3] [7].

2.2.4. α_1-Adrenergic Receptor Antagonism

To evaluate the effect of Ang II on α_1-AR-mediated response, aortic rings were first incubated in DMEM for 24 h. Subsequent to this incubation, the rings were exposed to selective α_1-ARs antagonists prior to being challenged with escalating concentrations of norepinephrine. The antagonists employed were 5-Methylurapidil for α_{1A}-AR, AH11110A for α_{1B}-AR, and BMY-7378 for α_{1D}-AR [13] [14] [15]. The purpose of this protocol was to identify the specific α_1-AR contributing to the contractile response to norepinephrine following 24 h incubation with Ang II (1×10^{-5} M).

2.2.5. Isolation and Culture of Aorta Smooth Muscle Cells

The aorta was obtained as described in section 2.2.1, followed by the removal of the endothelium via gently rubbing. The arterial segments were treated with collagenase II (2 mg/ml) during 15 min at 37˚C to facilitate the mechanical removal the adventitia layer, under a stereoscope (Zeiss Stemi 2000-C; Carl Zeiss, Oberkochen, Baden-Württemberg, Germany). Subsequently, smooth muscle cells were disaggregated using a combination of collagenase II and elastase (5 mg/ml and 0.1 mg/ml, respectively). Afterwards, the cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 U/ml) (Gibco), at maintained at 37˚C in a humidified 5% CO_2 atmosphere. The medium was replenished every two days until the cells attained 90% - 95% confluency. The cells were kept quiescent in DMEM without FBS, after which they were exposed to Ang II (1×10^{-7} M) for various durations: 0.5, 1, 2, 4, 8, 12, and 24 h [4]. Smooth muscle cell morphology was verified through immunofluorescence using α-actin as a marker [16].

2.2.6. Angiotensin II Influence on α_1-ARs Protein Expression in Smooth Muscle Cells

The expression of α_1-ARs proteins was detected by Western Blot analysis following the exposure of smooth muscle cells to Ang II (1×10^{-7} M), using specific antibodies (kindly provided by Dr. JA García-Sáinz) (10 μg per sample) were resolved on 10% SDS-PAGE under denaturing conditions, and subsequently transferred to a PDVF membrane using a Semi-Dry Transfer Blot system (Bio-Rad Labs., Hercules, CA, USA).

Blocking of non-specific binding was achieved with 5% non-fat milk dissolved in TBST. The membranes were incubated overnight at 4˚C with rabbit polyclonal antibodies to each α_1-AR or to β-actin (Santa Cruz Biotechnology), at dilutions of 1:3000 and 1:1000, respectively, in non-fat milk. After thorough washing, membranes were exposed to goat anti-rabbit secondary antibodies

conjugated to horseradish peroxidase (Zymed Laboratories Inc., San Francisco, CA, USA) at a dilution of 1:1000 for 1 h at room temperature, followed by extensive washing. Detection was conducted using chemiluminescence with Luminol and captured on Hyperfilm (Amersham Biosciences, GE Healthcare, Buckinghamshire, UK). Densitometry was performed on bands corresponding to α_{1A}-AR and α_{1D}-AR (~72 kDa) and α_{1B}-AR (~60 kDa) using a FLA-5000 scanner (Fujifilm).

2.2.7. Materials

All reagents were prepared either in Krebs-Henseleit solution or distilled water. Solutions were freshly prepared for every experiment. The compounds used, including Angiotensin II, (±)-Norepinephrine-HCl, (±) Propranolol-HCl, Rauwolscine-HCl, Carbachol-HCl, 5-Methylurapidil (5-MU, 5-Methyl-6[[3- [4-(2-methoxyphenyl)-1-piperazinyl]propyl]amino]-1,3-dimethyluracil), AH11110A (AH, 1-[Biphenyl-2-yloxy]-4-imino-4-piperidin-1-yl-butan-2-ol hydrochloride), BMY-7378 (BMY, 8-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro [4.5]decane-7,9-dione dihydrochloride), collagenase II, elastase, dithiotreitol, were obtained from Sigma-Aldrich (St. Louis, MO, USA). DMEM, fetal bovine serum, penicillin and streptomycin were purchased from Gibco (Thermo Fisher Scientific). All other reagents were of analytical grade and were obtained from local sources.

2.2.8. Statistical Analysis

Values for pD_2 (-log EC_{50}) were derived using nonlinear regression, while pA_2 values were determined through Schild analysis, or pK_B [17] [18]. Data are expressed as means ± standard error of the mean (SEM), based on observation from 8 rats per experimental group. Statistical evaluations were conducted by analysis of variance (ANOVA) followed by Bonferroni's or Dunnett's *post hoc* test, with differences statistically significant set at $p < 0.05$.

3. Results

To assess the viability of aortic rings after incubation of 24 h at 37˚C in DMEM, contractions were induced using high KCl (80 mM), which depolarizes the membrane, promoting Ca^{2+} entry into muscle cells and thus inducing contraction independent of receptor activation [19]. **Figure 1** demonstrates that high KCl induced contraction in aortic rings following a 24 incubation at both 37˚C and 4˚C in DMEM, indicating that the incubation conditions did not modify tissue responsiveness.

The concentration-response curve (CRC) for norepinephrine and the α_1-ARs antagonism was explored; **Figure 2** displays control curve response of aortic rings incubated with different α_1-ARs antagonists. **Figure 2A** shows the norepinephrine CRC and the rightward shift by the α_{1A}-AR antagonist 5-Methylurapidil (5-MU), suggesting the presence of multiple receptor populations as inferred from a non-unitary slope in Schild analysis; the pK_B was subsequently calculated to be 8.2. **Figure 2B** shows a rightward CRC shift in response to the α_{1B}-AR antagonist AH11110A, with a pK_B of 6; while **Figure 2C** demonstrates that BMY-7378

(α_{1D}-AR antagonist) caused a rightward CRC shift with a pA_2 of 8.9, whereas the average pD_2 for norepinephrine was 8.3 ± 0.1.

In an attempt to identify which α_1-AR was involved in the desensitization, aortic tissue was incubated for 24 h in separate assays, with RS-100329 ($1 \times 10^{-8.5}$ M, a highly selective α_{1A}-AR antagonist, $pA_2 = 9.2/pK_i = 9.6$, [20]), prazosin (1×10^{-9} M, nonselective α_1-ARs antagonist, $pA_2 = 9.2$, [21]), or BMY-7378 (1×10^{-7} M, a highly selective α_{1D}-AR antagonist, $pA_2 = 8.9/pK_i = 9.4$, [14]). All three α_1-ARs antagonists protected, in a different pattern the α_1-ARs from desensitization; where BMY-7378 avoided desensitization, followed by partial protection by RS-100329, and by prazosin (Figure 3).

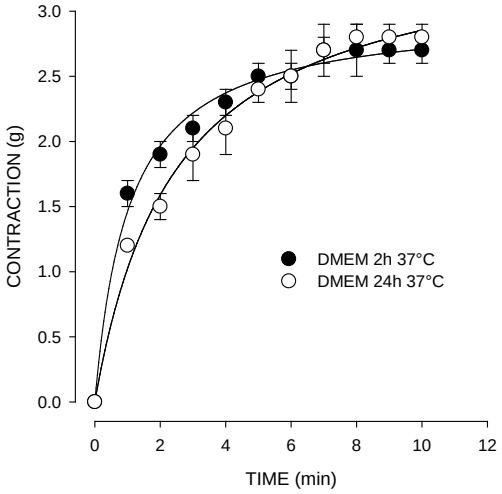

Figure 1. Time-course of aortic contraction induced by high KCl (80 mM) following incubation for 24 h at 4˚C (○) and 24 h at 37˚C (●), both in DMEM. n = 8 rats.

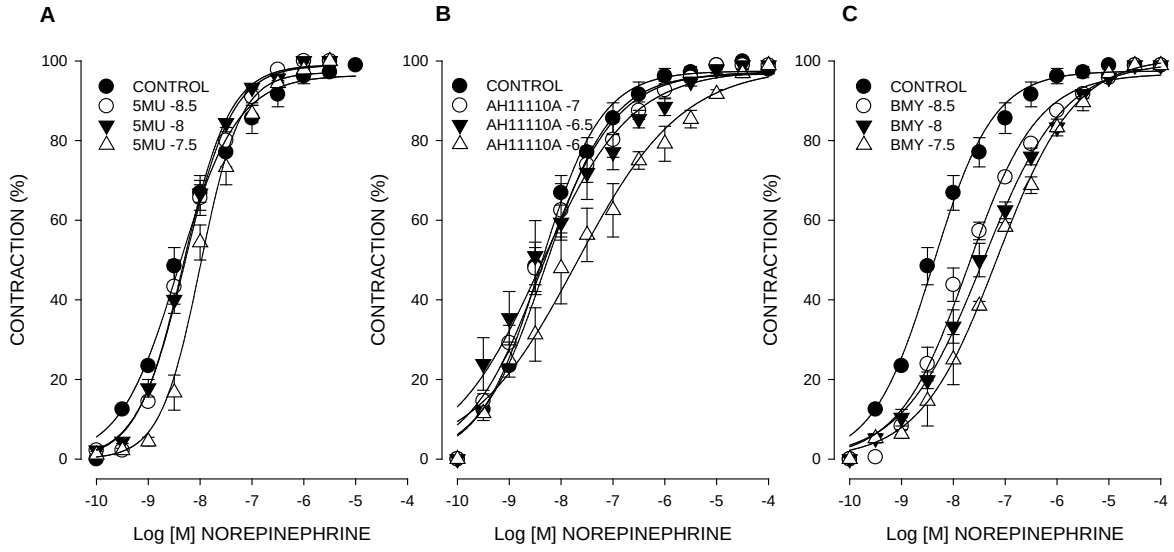

Figure 2. Concentration-Response Curves to norepinephrine (NE) in the aorta, and the displacement due to α_1-ARs antagonists. The control curve represents NE-induced contraction (●), while the curves with (○, △, ▼) depict NE-induced contraction in the presence of (A) 5-Methylurapidil, (B) AH11110A, and (C) BMY-7378. n = 8 rats.

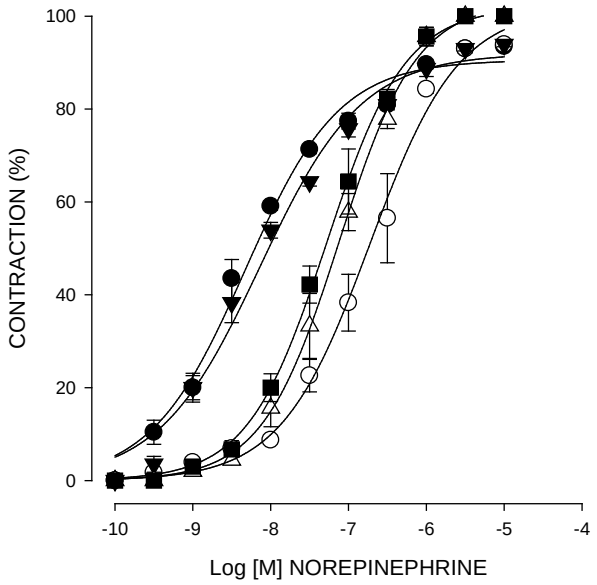

Figure 3. Desensitization of α_1-ARs and protection by antagonists: Concentration-Response Curves to norepinephrine in the aorta incubated at 37˚C in DMEM at zero time (Control, ●), or for 24 h alone (○), or with added RS-100329 ($1 \times 10^{-8.5}$ M, ■), prazosin (1×10^{-9} M, △), or BMY-7378 (1×10^{-7} M, ▼). n = 8 rats.

After a 24 h incubation at 37˚C in DMEM, a rightward shift of the norepinephrine CRC and reduction in maximal contraction were observed, indicative of α_1-ARs desensitization. The calculated pD_2 for noradrenaline under these conditions was 6.6 ± 0.1 *vs.* 8.3 ± 0.1, while the maximal effect was reduced to 2.5 ± 0.2 *vs.* 3.5 ± 0.1 in the non-incubated arteries.

Aortic arteries incubated for 24 h at 37˚C in DMEM with Ang II (1×10^{-5} M), exhibited a rightward CRC shift to norepinephrine and a reduced maximal contraction (pD_2 = 6.6 ± 0.1), demonstrating desensitization to the catecholamine. The presence of Ang II further decreased the maximal norepinephrine response (E_{max}, 3.0 ± 0.3 g *vs.* 2.2 ± 0.2 g, **Figure 4**), without changing the pD_2 for norepinephrine (6.7 ± 0.1). The α_1-ARs antagonism did not produce further CRC shifts to norepinephrine, with pK_B values of 6.5 for 5-MU and 7.4 for AH11110A, which unexpectedly caused a leftward shift; while BMY-74378 showed a pK_B of 7.3 (**Figures 5A-C**), confirming the modulatory effect of Ang II on α_1-ARs function.

This result prompted us to evaluate the action of Ang II on smooth muscle cells derived from rat aorta. As observed in **Figure 5**, Ang II (1×10^{-7} M) diminished the protein expression of α_{1A}-AR between 1 and 4 h, restoring the expression at the basal value from 8 to 24 h (**Figure 6A**). α1B-AR expression remained unchanged over a 0.5 to 24-hour incubation period (**Figure 6B**), whereas α_{1D}-AR was upregulated from 0.5 to 2 h reaching basal values afterwards (**Figure 6C**). Additional experimentation revealed that inhibiting protein synthesis with cycloheximide (CHX 10 μg/ml), as well as antagonizing the AT_1 receptor with losartan (1×10^{-5} M), diminished Ang II-induced α_{1D}-AR expression below basal value, with a more pronounced effect observed with CHX (**Figure 6D**).

4. Discussion

Angiotensin II, a pleiotropic agent, is implicated in various pathologies, including cardiovascular hypertrophy, hypertension, renal damage, among other pathologies [1] [2] [3] [4] [6] [7] [8], and has been reported to upregulate α_1-ARs in vascular smooth muscle cells and tissue, and in aryl hydrocarbon receptor ($AHR^{-/-}$) null mouse aorta [5] [6] [7]. Contrary to our expectations that Ang II would enhance α_1-ARs function in aorta after 24 h incubation in DMEM, however,

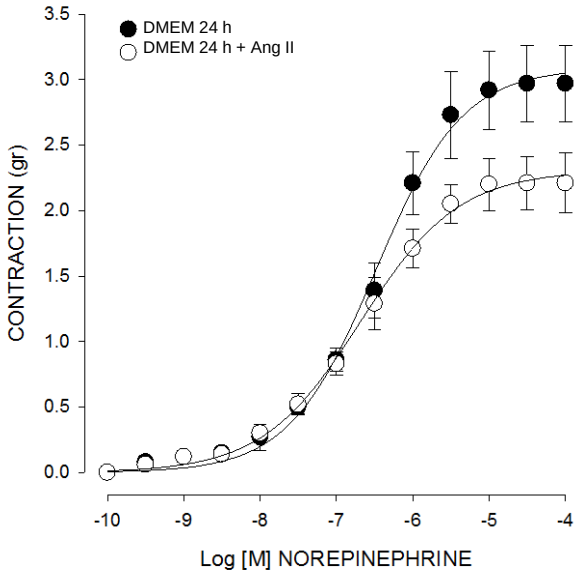

Figure 4. Desensitization of α_1-ARs due to incubation at 37˚C in DMEM with Ang II (○) or without (Control, ●). n = 8 rats.

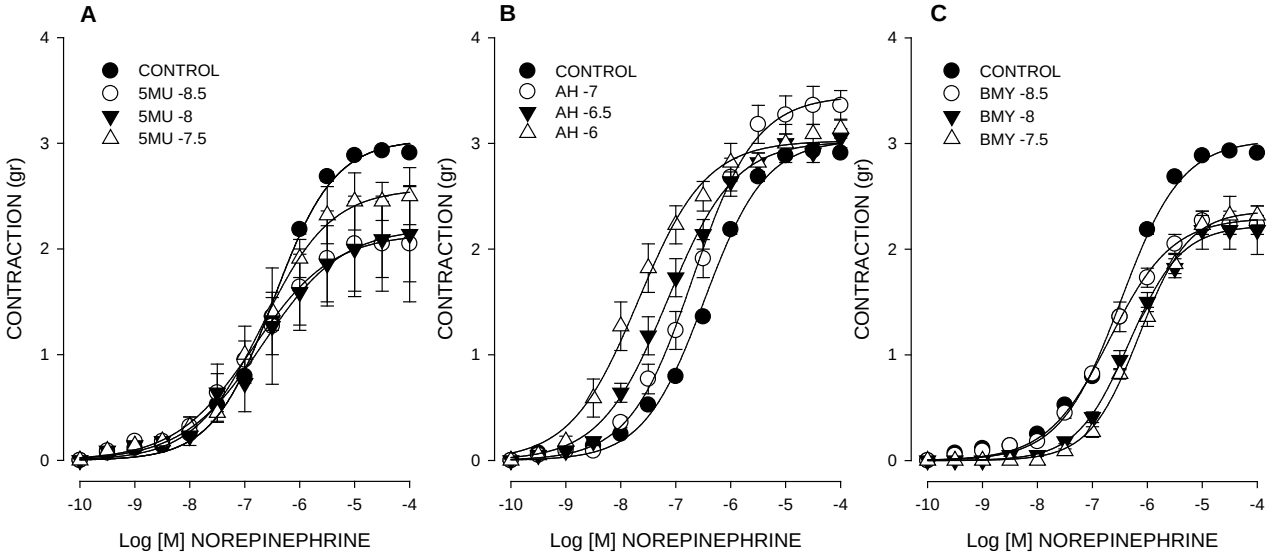

Figure 5. Concentration-Response Curves to norepinephrine (NE) in aorta incubated at 37˚C in DMEM 24 h with Ang II (1×10^{-5} M), and the displacement due to α_1-ARs antagonists. The control curve represents NE-induced contraction (●), while the curves with (○, Δ, ▼) depict NE-induced contraction in the presence of (A) 5-Methylurapidil (5 MU), (B) AH11110 (AH), and (C) BMY-7378 (BMY). n = 8 rats.

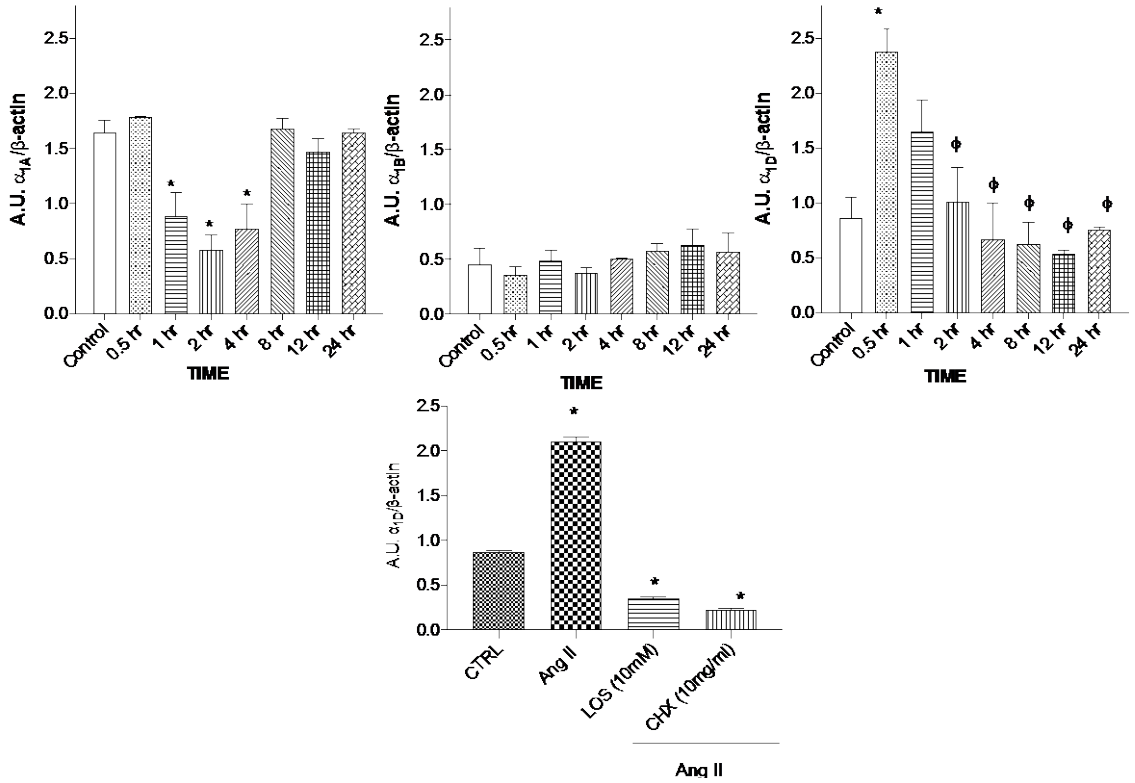

Figure 6. Effect of Ang II on α1-ARs protein expression in aorta smooth muscle cells culture. Upper panels represent the action of Ang II on α1A-, α1B-, and α1D-ARs protein expression along 24 h. ANOVA followed by Bonferroni's multiple comparison test: ϕ $p < 0.05$ compared to 0.5 hr and *$p < 0.05$ compared to control. The lower panel represents the effect of losartan (LOS, AT1R antagonist) or Cycloheximide (CHX, protein translation inhibitor), on the Ang II-induced increase of α1D-AR protein expression. ANOVA followed by Dunnett's multiple comparison post hoc test: * $p < 0.05$ compared to control. The sample size was n = 8 rats.

we observed two phenomena: incubation *per se* decreased both maximal effect and affinity of α_1-ARs in vascular tissue [9], and Ang II addition decreased further the contractile maximal response to norepinephrine without affecting affinity.

Previous reported contrasting results showed that an increase in circulating Ang II, either through $AHR^{-/-}$ knockout, or continuous infusion, provoked augmented maximal contractions to phenylephrine or noradrenaline in isolated aorta, suggesting that *in vivo*, constant Ang II exposure integer a whole animal's response *versus* what is observed in isolated aorta [3] [6].

The absence of a significant shift with α_{1A}- and α_{1D}-ARs antagonists in Ang II-treated tissue suggests that norepinephrine-induced contraction might be mediated by α_{1B}-AR activation. However, competitive antagonism of α_{1B}-AR with AH11110A resulted in a leftward CRC shift, indicating that α_{1B}-AR might be modulating the action of norepinephrine on the other α_1-ARs. This hypothesis could be supported with previous findings of no response to norepinephrine with the α_{1B}-AR alkylating antagonist, chloroethyl clonidine (CEC), described previously [9] and confirmed in this study (not shown).

These discrepancies prompted us to evaluate Ang II action on the α_1-ARs ex-

pression in isolated smooth muscle cells. Hu *et al.* reported that Ang II (1×10^{-7} M) increased α_1-ARs RNA up to 70% above basal, in a time-dependent manner with a maximal effect at 8 h in vascular smooth muscle cells [5]. Similarly, they observed a significant transient increase in $\alpha_{1A/D}$-AR (currently identified as α_{1D}-AR) expression after Ang II exposure (~2.5 fold above basal at 2 h after treatment), which returned to baseline by 24 h [5]. Our results show a similar pattern at the earlier times, the α_{1D}-AR was overexpressed 30 min after Ang II treatment followed by a time-dependent decrease until basal values, suggesting that the peptide effects on the α_{1D}-AR occur soon after its interaction with AT_1R.

Furthermore, Hu *et al.* showed that blocking α_1-ARs with the irreversible antagonist phenoxybenzamine (PBZ), significantly reduced α_1-ARs, (~6 times; from 70 to 12 fmol/mg protein, control *vs.* PBZ), yet Ang II was able to increase eight times α_1-ARs after PBZ treatment (from 12 to 96 fmol/mg protein). In line with this, our study reveals that both losartan, an AT_1R antagonist, and cycloheximide (CHX), a protein synthesis inhibitor, acting on different targets diminished the action of Ang II on the α_{1D}-AR expression below basal value [5]. This suggests that AT_1R blockade leads to downregulation of α_{1D}-AR expression and that prevention of protein translation inhibits the expression of α_{1D}-AR. It is not clear at what step of signal amplification these two pathways interact, but it is known that receptor heterodimerization occurs between AT_1R and α_{1D}-AR [22]; then it would be interesting to define if these receptors' interaction promotes α_{1D}-AR activation in the absence of catecholamines, that leads to muscle growth. It is important to mention that integration of hormone signaling between two pathways, *i.e.*, RAS and *a*1-adrenergic, the so-called cross-talk, with physiology or pathophysiology leads to a better understanding of how the neural and cardiovascular systems work to keep body homeostasis.

5. Conclusion

Angiotensin II exerts a biphasic action on α_1-ARs, at early times it increases α_{1D}-AR, diminishes α_{1A}-AR, and has no effect on α_{1B}-AR; while at longer times it adds to incubation-induced desensitization on maximal aorta contraction. This initial increase of α_{1D}-AR may trigger later effects on cellular machinery that promotes growth; so, it is interesting to block enzymatic steps downstream of signal amplification, in order to identify those steps involved in the gene expression, both of receptors and of proteins related to muscle growth.

These observations highlight the necessity for further studies to elucidate the apparently different actions of Ang II on cells *vs.* aortic tissue, specifically in terms of α_1-ARs expression and functionality.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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