

Original Paper

Mesangial Cells Cultured from Pregnant Rats Display Reduced Reactivity to Angiotensin II: the Role of Relaxin, Nitric Oxide and AT2 Receptor

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Key Words

Pregnancy • Mesangial cells • Intracellular calcium • Relaxin • AT2 receptor • Nitric oxide

Abstract

Background/Aims: Pregnancy is characterized by vasodilatation and increased glomerular filtration rate (GFR), despite overstimulation of the renin angiotensin system (RAS). The mesangial cells (MCs) influences GFR and when cultured from pregnant rats displays refractoriness to Ang II. We evaluated the role of relaxin (RLX) and its receptor (RXFP1), nitric oxide (NO) and the AT2 receptor in this response. **Methods:** MCs cultured from kidneys of virgin (V) and pregnant (P) Wistar rats were treated with RLX or AT2 receptor blocker PD123319 or NO synthase inhibitor L-NAME. After 24 hr, intracellular calcium concentration ($[Ca]_i$) was recorded before and after the addition of Ang II. **Results:** MCs from V group expressed AT2, RLX and RXFP1, whose levels were increased in P cells. Ang II induced a 150% increase in $[Ca]_i$ in the V cells and 85% ($p < 0.05$) in the P cells. V cells treated with RLX displayed a similar response to that observed in P cells, suggesting that RLX can modulate the reactivity of the MCs to Ang II. L-NAME and PD123319 did not interfere in this response. **Conclusion:** Results suggest that RLX is a mediator of the refractoriness of the MCs to Ang II during pregnancy.

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Introduction

Normal pregnancy is characterized by intrarenal vasodilatation, increased renal plasma flow (RPF) and increased glomerular filtration rate (GFR) [1, 2]. Pregnancy-associated vasodilatation has been, in part, attributed to increased circulating vasodilators, such as prostaglandins (PGs) [3], nitric oxide (NO) [3-5], and relaxin, a hormone that has been implicated as an important regulator of renal function during pregnancy [6, 7]. Relaxin is

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primarily synthesized by the corpus luteum, and serum levels increase soon after conception in humans and near day 8 of gestation in rats [8]. It has been suggested that the vasodilator actions of relaxin involve activation of inducible nitric-oxide synthase, (iNOS) [9].

The mesangial cell tone influences the GFR through its contraction and relaxation properties [10]. In a previous study, it was demonstrated that mesangial cells (MCs) cultured from 14-day pregnant rats were less responsive to Ang II when compared with the MCs from virgin rats [11]. This observation is relevant because, plasma Ang II levels are increased during pregnancy [12], in spite of systemic and intrarenal vasodilatation; thus, the MCs, similar to smooth muscle cells, can adapt to pregnancy by maintaining a relatively relaxed tone even in the presence of high levels of Ang II.

In the present study, we evaluated the possible mediators of the attenuated response of mesangial cells derived from pregnant rats to Ang II. Specifically we examined relaxin and nitric oxide as well as the AT₂ receptor because binding of Ang II to this receptor triggers antagonistic effects of the AT₁ receptor, including vasodilation [13, 14].

Materials and Methods

Animals

The experimental protocol was approved by the Ethical Committee of the Federal University of São Paulo (no. 01008/08). Adult female Wistar rats (200-250 g) were obtained from the animal care facility of the Federal University of São Paulo. Animals had free access to standard rat chow and tap water and were maintained in a temperature-controlled room (23°C) in a 12 h light/dark cycle. Female rats were pair-housed with an adult male Wistar rat for 2-3 days, and the presence of sperm in the vaginal smears (verified daily) was considered day 1 of pregnancy. Age-matched virgin and 12-14 days pregnant rats, were anesthetized, and the kidneys were removed for primary mesangial cell culture. This gestation period was chosen because it is characterized by a maximum increase in renal plasma flow. The groups of cells were denoted as V (from virgin rats) and P (from pregnant rats).

Primary culture of mesangial cells (MCs)

The primary MCs were cultured using standard techniques, following glomerular isolation by differential sieving [15]. Glomeruli were isolated from freshly removed kidneys from virgin and pregnant rats and then plated at a density of approximately 300 glomeruli/cm in DMEM supplemented with 20% fetal bovine serum, 50 U/ml penicillin, 2.6 g of HEPES acid, and 2 mM glutamine (Sigma-Aldrich Co., St. Louis, MO, USA). Cells were used between the third and fifth subcultures. The cultures were periodically checked for purity by testing for the presence of α -actin and for the absence of factor VIII and cytokeratin.

Experimental Groups

After the 3rd subculture, mesangial cells isolated from Virgin (n=5) and Pregnant rats (n=5) were maintained in DMEM culture medium containing 1% of FBS for 24 hr and under the following treatments: V and P Control cells with no treatment; V and P cells treated with the AT₂ receptor antagonist PD123319 (10⁻⁶M) for 24 hr; V and P treated with a NOS inhibitor L-NAME (10⁻³M) for 24 hr; V and P treated with relaxin (100ng/ml) for 24 hr. After the respective 24 hr treatment, cells were prepared for determination of [Ca]_i as described below. Additional V and P groups with no treatment were acutely stimulated with relaxin (100ng/ml). Acute experiments were performed as follows: after baseline [Ca]_i determinations relaxin was added to the bath and then [Ca]_i was measured. It was allowed [Ca]_i to return to basal levels when Ang II was added and [Ca]_i estimated again. Human relaxin (RLX) was purchased from Phoenix Pharmaceuticals, Inc. (CA, USA) and the dose of 100ng/ml corresponds to maximum concentration in the blood of pregnant rats [16-18]. PD123319, L-NAME and Angiotensin II were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and the doses used as previously determined [19-22].

Intracellular calcium ([Ca]_i) measurements

The reactivity of the MCs to Ang II was estimated by the increase in [Ca]_i levels induced by addition of 10⁻⁷ M Ang II. Measurement of [Ca]_i was performed by the Fura 2-AM incorporation method as previously

described [11]. After confluence, the MCs (10^6 cells/ml) were resuspended in 2.5 ml of Tyrode buffer (137 mM NaCl, 2.68 mM KCl, 1.36 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.49 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 12 mM NaHCO_3 , 0.36 mM NaH_2PO_4 , and 5.5 mM D-glucose) containing 0.2% bovine serum albumin and left to stand in a CO_2 incubator at 37°C for 30 minutes. The cell suspension was then centrifuged, and the pellet was resuspended in 2.5 ml of albumin-free Tyrode and transferred to a quartz cuvette for autofluorescence determination in a spectrofluorimeter (Photon Technology International, Ontario, Canada). Measurements were made at excitation wavelengths of 340 and 380 nm with emission at 505 nm. The autofluorescence ratio was less than 10%. The MCs were then incubated with 0.01% Pluronic-127 detergent and $2 \mu\text{M}$ of Fura 2-AM. Following this, the samples were read in the excitation range of 300 to 400 nm, with emission at 520 nm. In the esterified form, the maximum fluorescence of Fura 2-AM was observed at 390 nm. Within three hours, the indicator was transformed to its acidic form, Fura-2, and the fluorescence peak shifted to 350 nm, indicating that the maximum amount of indicator was incorporated into the cell suspension. The cells were washed with 15 mL of Tyrode and centrifuged at 1500 rpm for four minutes. The supernatant was discarded, and the pellet was resuspended in 2.5 mL of Tyrode and transferred to a spectrofluorimeter programmed for excitation at two wavelengths (340 and 380 nm) with emission at 505 nm with constant stirring at 37°C . The first reading of this phase corresponded to the basal $[\text{Ca}]_i$ levels. The cells were then stimulated with Ang II (10^{-7} M). At the end of each experiment, $50 \mu\text{M}$ digitonin, 1 mM MnCl_2 manganese chloride and 2 mmol/L ethylene glycol tetraacetic acid were added to the cells. The results are reported as the ratio of the 340 and 380 nm wavelengths, relative to digitonin, which was set at 100%. The $[\text{Ca}]_i$ was estimated by the formula of Grynkiewicz et al. [23].

mRNA expression levels

The mRNA expression levels of relaxin (RLX), its receptor (RXFP1) and the AT2 receptor were measured by quantitative real-time PCR. Total RNA was purified using the phenol and guanidine isothiocyanate-cesium chloride method (TRIzol kit; Life Technologies, Carlsbad, CA, USA). The RNA pellets were resuspended in RNase-free water. Two micrograms of total RNA were pretreated with DNase and then reverse transcribed into cDNA by the addition of a reaction mix, containing 0.5 mg/mL oligo-dT, 10 mM dithiothreitol, 0.5 mM dNTPs (Pharmacia Biotech, Pittsburgh, PA, USA), and 200 U of reverse transcriptase (Superscript RT; Life Technologies, Carlsbad, CA, USA). The mixture was incubated at 37°C for 1 hr and then at 95°C for 5 min. Primer sequences for the amplification of relaxin, RXFP1 and AT2 receptors were based on cDNA sequences obtained from GenBank and were designed using the Designer PCR software (Research Genetics, Huntsville, AL, USA). The following primer sequences were used (forward and reverse, respectively): RLX (ttcgtatgacgttgaaatgcc and aatcctccaagctaacaacgga), RXFP1 (tgggctcattggcgttctg and actccattcgtgacctagtag), AT2 (cagtggtctgctggattgc and ccattcaggtcagagcatcc) and β -actin (cctctatgccaacacagtgc and acatctgctggaaggtggac), which was used as an internal control. For each PCR set, negative controls (for contamination from exogenous sources) were included by replacing cDNA with water. Real-time RT-PCR was performed using the GeneAmp 5700 and ABI Prism 7700 Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA). Real-time PCR product accumulation was monitored using the intercalating dye SYBR Green I (Molecular Probes, Carlsbad, CA, USA). Fluorescence for each cycle was quantitatively analyzed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). At the end of PCR amplification, the temperature was increased from 60°C to 95°C at a rate of $2^\circ\text{C}/\text{min}$. Fluorescence was measured every 15 sec to construct the melting curve, which was used to verify the absence of nonspecific amplification products. The results were normalized to β -actin amplification, and the relative gene expression of each molecule, expressed in arbitrary units, was calculated by using the control group (V cells) as the standard.

Western Blot Analysis

The MCs were lysed in ice-cold purification buffer (50 mM Tris, pH 8.0; 150 mM NaCl; 1% NP-40; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulfate (SDS); 2.5 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; and 44 mM o-phenanthroline). The samples were centrifuged, and the protein concentration was determined by the Folin method using reagents from Bio-Rad (Bio-Rad DC Protein Assay; Hercules, CA, USA). Fifty micrograms of protein were resolved on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA) at 4°C using transfer buffer containing 25 mM Tris-HCl, 192 mM glycine, and 20% methanol. Nonspecific binding was blocked with 5% nonfat dry milk in TBS buffer containing 10 mM Tris-HCl (pH 7.5) and 200 mM NaCl. The immunoblots

Table 1. [Ca]_i values in MCs from Virgin and Pregnant groups. p<0.05: * vs Basal; # vs Virgin. [Ca]_i was measured under basal (unstimulated) conditions and after addition of 10⁻⁷M AngII.

[Ca] _i (nM)	Virgin (n=5)	Pregnant (n=5)
Basal	124±16	159±13
After Ang II	313±19*	297±36*
% of increase	161±12	87±15#

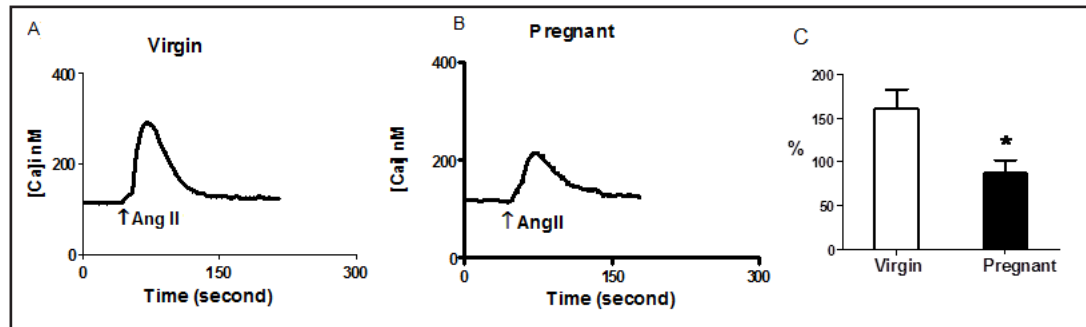


Fig. 1. Effect of Ang II on [Ca]_i. Representative traces of [Ca]_i in response to Ang II (10⁻⁷ M): (A) Cells of the V group (n=5); (B) Cells of the P group (n=5). (C) The peak [Ca]_i, after stimulation with Ang II, was expressed as percentage of increase in [Ca]_i, *p<0.05: vs. control.

were then incubated overnight at 4°C with the primary polyclonal antibodies against the following: relaxin (Abcam Inc., Cambridge, UK), RXFP1 (Abcam Inc., Cambridge, UK), AT2 receptor (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and β-actin (Sigma-Aldrich Co., St. Louis, MO, USA). Subsequently, the membranes were washed 3 times with TBS buffer and then incubated for 1 hr at 4°C with an anti-rabbit peroxidase-conjugated secondary antibody (Sigma-Aldrich Co., St. Louis, MO, USA). Detection of specific protein bands was performed with a Luminol detection system (Immobilon Western, Chemiluminescence HRP substrate; Millipore Corporation, Billerica, MA, USA). Then, each membrane was transferred to a photodocumentation system, the Luminescent Image Analyzer (LAS 4000 - Fuji, Tokyo, Japan). The Image Gauge software, version 3.1 (Fuji, Tokyo, Japan) was used for densitometric analysis of the bands. The results were normalized to β-actin expression, which was used as a positive endogenous control and expressed as a percentage of the control group (V cells).

Statistical Analysis

The results are presented as the means ± standard error. Differences in the basal [Ca]_i concentrations between virgin and pregnant rats were evaluated using unpaired Student's t-test, whereas the [Ca]_i variations in response to Ang II in the same group was analyzed by paired Student's t-test. One-way ANOVA analysis was used to determine the differences among groups. The level of statistical significance was defined as p≤0.05.

Results

Under unstimulated (basal) conditions, the mean [Ca]_i did not significantly differ between virgin and pregnant groups (Table 1), however, the increase in [Ca]_i induced by Ang II was significantly lower in MCs in the pregnant group compared to those from the virgin group as it can be observed in the representative traces shown in Fig. 1. As a result the calculated percentage of increase in [Ca]_i after Ang II stimulus, was significantly lower in P group compared to V cells (control), as depicted in panel C. The mRNA expression levels of relaxin, RXFP1 and AT2 receptors were significantly increased in the MCs from the pregnant group, compared with virgin group (Fig. 2). The protein expression levels, estimated by

Fig. 2. mRNA expression levels. The results were obtained from RT-real time PCR reactions in triplicate. The values for the pregnant (P) group were calculated with the virgin (V) group as the standard for each molecule. Bars represent the mean values for the V (n = 5) and P (n = 5) groups. *p<0.05: vs. virgin.

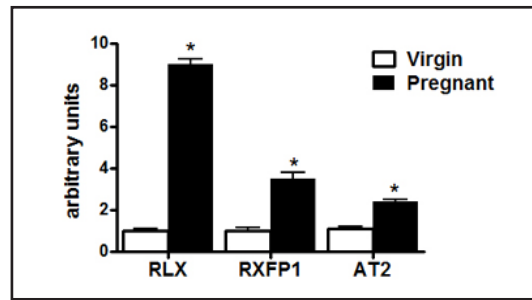


Fig. 3. Protein expression. A: Representative gel electrophoresis for relaxin (RLX), relaxin receptor (RXFP1) and the AT2 receptor (n=4 for each group). B: Graphical representation of the bands quantified by densitometry and normalized for β -actin. The values were expressed as percentage changes compared to the virgin group, set at 100%. *p<0.05: vs. virgin.

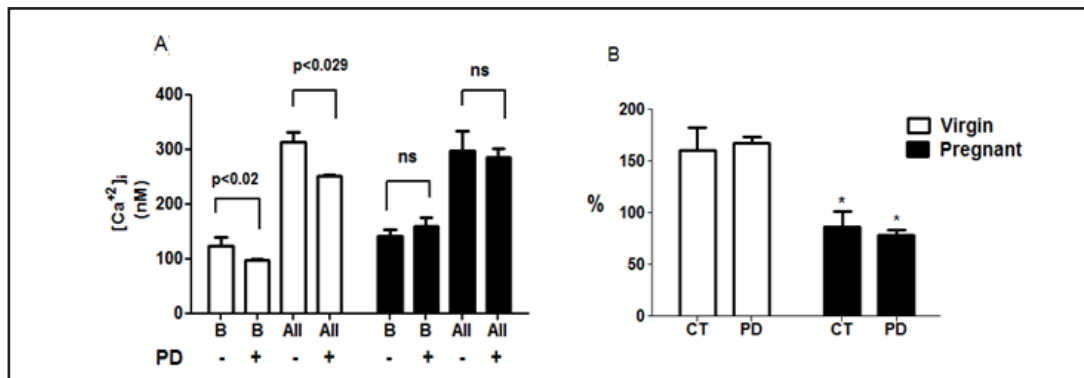
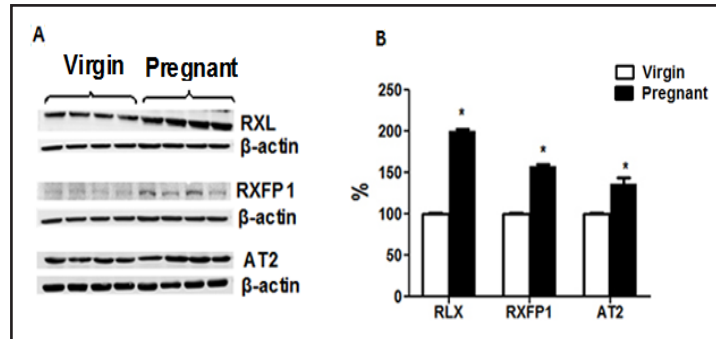


Fig. 4. Effect of PD123319 on [Ca]_i. A: Absolute values of [Ca]_i obtained before (B) and after Ang II (AII) addition to the MCs of the V (n = 5) and P (n = 5) groups, with and without PD123319 (PD) treatment. B: Percentage change in [Ca]_i, after Ang II addition in V and P groups in the absence (CT) and presence of PD. *p<0.05 vs. control.

western blot, coincided with the mRNA profile as shown in Figure 3. Representative blots probed with specific antibody for each molecule are shown in Fig. 3A and the quantification of bands is shown in Fig. 3B. The MCs, under control conditions (V), express both relaxin and its receptor, whereas the MCs cultured from pregnant rats showed significant increases in both. There was also a slightly but significant rise in the AT2 protein in P cells.

As shown in Fig. 4A, treatment of cells with the AT2 antagonist, PD123319 (PD), over 24 hr induced a slight but significant decrease in baseline [Ca]_i in the virgin group. Additionally, the Ang II-induced increase in [Ca]_i was lower in PD-treated V cells compared with untreated V cells; however, the percentage change between the treated and untreated groups was not significant (Fig. 4B). In contrast, PD123319 did not affect either the baseline [Ca]_i or the attenuated response of the P cells to Ang II.

Figure 5 shows the average values of [Ca]_i in groups of cells treated with L-NAME for 24 hr; a significant decrease in the baseline [Ca]_i in V cells was observed. The increase in

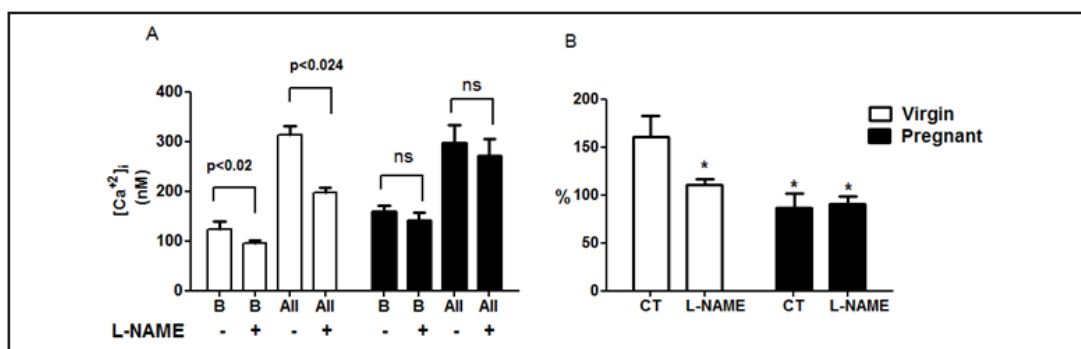


Fig. 5. Effect of L-NAME on [Ca]_i. A: Absolute values of [Ca]_i obtained before and after Ang II (All) addition in MCs of the V (n = 5) and P (n = 5) groups with and without treatment with L-NAME. B: Percentage changes in [Ca]_i after Ang II addition to the V and P groups in the absence (CT) and presence of L-NAME. *p<0.05 vs. control (V).

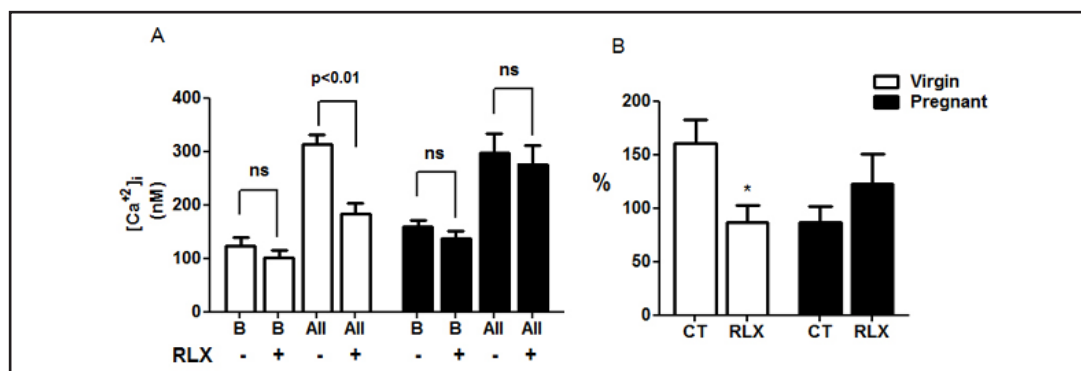


Fig. 6. Effect of relaxin on [Ca]_i. A: Absolute values of [Ca]_i obtained before (B) and after Ang II (All) addition in MCs of the V (n = 5) and P (n = 5) groups with and without treatment with relaxin (RLX). B: Percentage changes in [Ca]_i after Ang II addition in the V and P groups in the absence (CT) and presence of relaxin. *p<0.05 vs. control.

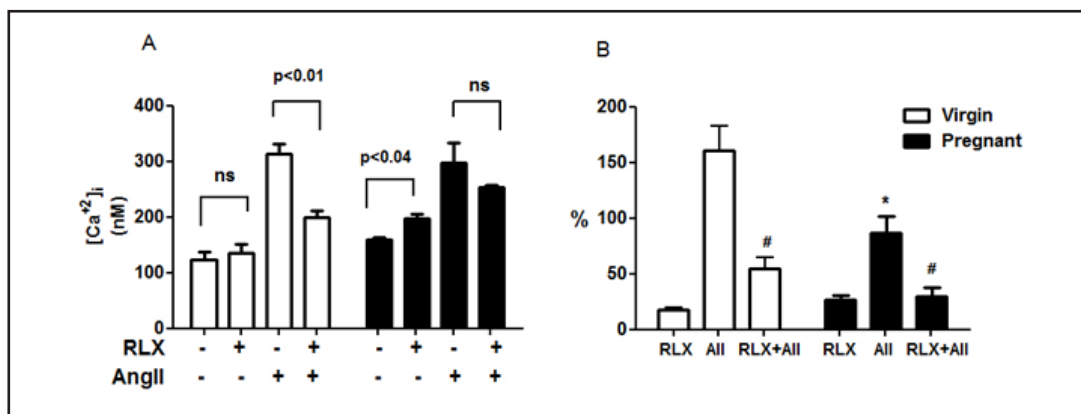


Fig. 7. Acute effects of relaxin. After baseline [Ca]_i measurements, relaxin (100 ng/ml) was added to the cell suspension, followed by the addition of Ang II. A: absolute values; B: Percentage changes (n = 5 for each group), p < 0.05: *vs. V+Ang II; # vs. Ang II.

[Ca]_i was also diminished by L-NAME in V cells (Fig. 5A). This difference still persisted when [Ca]_i was expressed as percentage of increase (Fig. 5B). Similar to the results observed with PD123319, L-NAME did not affect either the baseline [Ca]_i or the response of the P cells to Ang II.

Figure 6 shows the response of the MCs in both groups after stimulation for 24 hr with relaxin. There was no change in the baseline $[Ca]_i$ in either the V or P groups (Fig. 6A). Relaxin caused a lower response to Ang II in V cells but had no effect on the response of P cells to Ang II. The same profile was observed when $[Ca]_i$ was expressed as a percentage change (Fig. 6B)

Acute addition of relaxin to the MCs produced a slight but significant increase in $[Ca]_i$ in the P group (Fig. 7A). However, the increase in $[Ca]_i$ produced by Ang II was significantly attenuated in the presence of relaxin in both V and P cells. This effect can be clearly seen in Fig. 7B, where the values are represented as the percentage changes.

Discussion

The present study aimed to evaluate some potential mediators of the hyporeactivity to Ang II of MCs cultured from pregnant rats [11]. In the present study, we confirmed that the MCs from pregnant rats are under responsive to Ang II treatment, and displayed a slight increase in $[Ca]_i$ when compared with the response of cells from virgin animals. This behavior was similar to that observed in vascular smooth muscle cells from pregnant rats [24], suggesting the presence of a contra-regulatory mechanism, which could maintain the cellular tone even in the presence of high levels of Ang II, typical of pregnancy. The results found here point to relaxin as a potential mediator of the refractoriness observed in the MCs of the P group, as also described in other cell types, including vascular smooth muscle cells and endothelial cells [21].

Interestingly, relaxin was constitutively expressed in the MCs cultured from V rats. It has been demonstrated that relaxin is present outside of the reproductive organs, such as in the kidney, where the function is mainly related to antifibrotic property [25, 26]. This function could explain the presence of relaxin in the MCs cultured from non-pregnant rats and suggests that these cells may constitute a site of intrarenal production of relaxin. During pregnancy, the circulating levels of relaxin are increased, and the relaxin receptor RXFP1 is upregulated in many tissues including the kidney [11], but there is no available data on intrarenal production of relaxin during pregnancy. We found that the levels of relaxin were significantly upregulated in cells of the pregnant group, suggesting a potential role for relaxin in the MCs function during pregnancy, including the reduced reactivity of these cells to vasoconstrictor agents, such as Ang II. In addition, we observed that both acute and 24 hr administration of relaxin induced hyporeactive response to Ang II in the control V cells similar to that displayed by the P cells. This result supports our hypothesis that relaxin is a mediator of the attenuated response of the MCs cultured from pregnant rats to Ang II *in vitro*.

In contrast to relaxin, both the AT2 receptor antagonist (PD123319) and NOS inhibitor (L-NAME) did not significantly change the reduced response of the P cells to Ang II. The AT2 receptor was upregulated in P cells, and since it has been implicated in the gestational decline of systolic blood pressure (SBP), at least in mice [27], we expected to find that the AT2 receptor would have a role in the reduced reactivity of MCs to Ang II. The results, while unexpected, suggest that AT2 activation does not appear to be a relevant pathway in the refractoriness of the pregnant MCs to Ang II. Similarly, NOS inhibition with L-NAME did not modify the low reactivity of the P cells to Ang II. Interestingly, apart from the relaxation property of NO, it has been demonstrated that relaxin can act through activation of NO signaling [20, 28]. Moreover, we have previously observed that MCs cultured from pregnant rats showed a significant increase in the mRNA expression of inducible NOS [11]. While our results are inconsistent with this previous study, there are at least 2 possible reasons for our observations: 1) NO is not involved in the hyporeactivity of P cells; and 2) L-NAME was not effective in inhibiting iNOS in P cells, which is intriguing since L-NAME was effective to reduce the response to Ang II in MCs from V rats. Thus, further studies are needed to clarify this issue.

In summary, the results of this study support a different paradigm regarding the MCs from virgin and pregnant rats, and this *in vitro* model may be useful in evaluating the effects

of certain stimuli induced *in vivo*. Our results suggest that relaxin is a potential mediator of the refractoriness of the MCs to Ang II and may modulate the mesangial cell tone during Ang II stimulation, thereby contributing to keep a higher GFR even under the high activity of RAS typical of pregnancy.

Acknowledgements

This work was supported by grants from Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação Oswaldo Ramos (FOR).

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