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<th>Canadian Journal of Physiology and Pharmacology</th>
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<td>Manuscript ID</td>
<td>cjpp-2017-0416.R1</td>
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<td>Manuscript Type:</td>
<td>Article</td>
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<td>Date Submitted by the Author:</td>
<td>12-Jul-2017</td>
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<td>Complete List of Authors:</td>
<td>Kamal, Maud; Institut Curie Jacques, Danielle; Anatomy and Cell Biology, Bkaily, Ghassan; Département d’anatomie et biologie cellulaire,</td>
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<td>Is the invited manuscript for consideration in a Special Issue?:</td>
<td>IACS Sherbrooke 2016 special issue Part 1</td>
</tr>
<tr>
<td>Keyword:</td>
<td>Angiotensin II, human vascular endothelial cells, AT1 receptor, AT2 receptor, calcium</td>
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ANGIOTENSIN II RECEPTORS’ MODULATION OF CALCIUM HOMEOSTASIS IN HUMAN VASCULAR ENDOTHELIAL CELLS

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Short title: Angiotensin II receptors in human vascular endothelial cells

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Abstract

Angiotensin II (AngII) plays an important role in the regulation of vascular smooth muscle function. However, little is known about AngII and its receptors AT₁ (AT₁R) and AT₂ (AT₂R) and their modulation of intracellular calcium in vascular endothelial cells (VECs) in general and more particularly of human origin. Using western blots, our results showed that AT₁Rs and AT₂Rs are present in human VECs (hVECs). Using quantitative 3D confocal imaging, our results showed that AngII is present at the cytoplasmic and nucleoplasmic levels and its relative density is lower in the nucleoplasm. However, both AngII receptors AT₁ and AT₂ are present at both the plasma and the nuclear envelope membranes (NEMs). AngII (10⁻¹⁰ M) induces a transient decrease of the relative density of cytosolic and nuclear AT₁Rs. Blockade of AT₁Rs with losartan or blocking protein synthesis with cycloheximide does not prevent internalization and nuclear translocation of AT₁Rs but prevents de novo AT₁R synthesis. In addition, AngII induces cytosolic and nuclear increases (EC₅₀ near 5x10⁻¹⁴ M) of calcium via the activation of AT₁Rs. These results demonstrate that both AT₁ and AT₂ receptors are present in hVECs and that only AT₁Rs seem to undergo transcellular trafficking and modulate cytosolic and nuclear calcium homeostasis.

Keywords: Angiotensin II, human vascular endothelial cells, AT₁ receptor, AT₂ receptor, calcium.
Introduction

Angiotensin II is an octapeptide known to affect the cardiovascular system by increasing blood pressure. Although its effect was largely studied at the vascular smooth muscle and renal levels, fewer studies were done in vascular endothelial cells. This octapeptide is known to regulate the function of VECs (Stoll et al. 1995a). It is also known to be implicated in proliferation (Stoll et al. 1995) and survival (Dimmeler et al. 1997; Rossig et al. 2002) of VECs. Studies showed that AngII modulates intracellular calcium of human umbilical vein endothelial cells (Ko et al. 1997). However, no studies were done in normal adult VECs and in particular of human origin. Several studies in vascular smooth muscle cells including those of human origin (hVSMCs) showed the presence of AT$_1$R at the plasma membrane (PM), however, that of AT$_2$R was limited to the nuclear membranes level (Bkaily et al. 2003). Furthermore, in hVSMCs, plasma membrane as well nuclear membranes AT$_1$Rs were shown to undergo internalization and nuclear translocation (Bkaily at al., 2003). However, it is not yet known whether such a phenomenon takes place in hVECs. Internalization of AT$_1$R is highly important since it is implicated in the sustained activation of signaling induced by this type of receptor (Schelling and Linas 1994). In addition, internalization of this receptor contributes to the regulation of the density of the receptor (Hein et al. 1997) as well as the accumulation of AngII inside the cell (Booz et al. 1992; Bkaily et al. 2009, 2012) which contributes to nuclear membrane AngII receptors activation (Booz et al. 1992; Bkaily et al. 2009, 2012). In this study, we report that both AT$_1$ and AT$_2$ receptors are present in hVECs and that only AT$_1$R undergoes internalization and nuclear translocation and modulates cytosolic and nuclear calcium.
Materials and methods

Isolation and culture of hVECs.

hVECs were isolated from aortas of healthy donors (16–45 years old) supplied by Quebec Transplant. All work was done in accordance with the requirements of the institutional review committee for the use of human material. The freshly isolated and cultured hVECs used in this study originate from at least 5 different donors as previously described (Bkaily et al. 1997). In brief, intact aortas, were placed for 15 min in M199 medium (Gibco-BRL, Burlington, ON) containing antibiotics and 0.1% (50 U/mL) collagenase. After that, the medium was replaced by a fresh, collagenase-free M199 solution containing antibiotics and hVECs were gently scraped with a sterile scalpel blade. The cell-containing medium was collected and centrifuged using the same conditions as those described previously (Bkaily et al. 1997; Avedanian et al. 2010). Cells were then cultured in M199 medium containing 10% fetal bovine serum and 50 IU/mL penicillin-G-potassium (Ayrest, Toronto, ON). A routine check of quality and purity of hVECs was done using specific probes (Bkaily et al. 1997; Avedanian et al. 2010). Furthermore, functional quality control of cultured (used within 12 h of culture) and long-term cultured (passages 4–6) cells was routinely done (Bkaily et al. 1997; Avedanian et al. 2010).

Indirect immunofluorescence

The protocol for indirect immunofluorescence was previously described (Bkaily et al. 2003). Briefly, aortic hVECs were fixed with 4% paraformaldehyde followed by washing with PBS 1X and incubation for 10 min with PBS containing sodium borohydride (2 mg/mL). The cells were then permeabilized and blocked with 0.1% Triton X-100, 7%
normal serum (NS), and 5% non fat dry milk (NFDM) for 30 min. Following two washes with PBS, cells were incubated overnight at 4 °C with PBS containing 1.4% NS, 1% NFDM, and 0.1% Triton X-100 in the presence of either a goat polyclonal anti-human AngII antibody (10 µg/ml) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), or rabbit polyclonal anti-AT\(_1\) receptor antibody (1/250) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or rabbit polyclonal anti-AT\(_2\) receptor antibody (10 µg/mL) (Research Diagnostics Inc., Flanders, NJ). After washing twice with PBS, cells were incubated for 1 h at room temperature with the same solution as the primary antibody containing an anti-goat (for AngII) or anti-rabbit (for AT\(_1\) and AT\(_2\) receptors) secondary antibody coupled to AlexaFluor 488 (1 µg/mL) (Molecular Probes, Eugene, OR). The cells were then examined using 3D imaging confocal microscopy. All antibodies were checked for specificity using a control peptide and no primary antibody and figure 1 shows examples.

**Loading with the calcium fluorescent dye Fluo-3/AM.**

The Ca\(^{2+}\) fluorescence dye Fluo-3/AM (Molecular Probes, Eugene, OR) at a final concentration of 13 µM in Tyrode-BSA was used to load the cells as described previously (Bkaily et al. 1997, 1999, 2003, 2004, 2017; Ahmarani et al. 2013). In brief, hVECs were incubated with Fluo-3/AM for 1 hour at room temperature, washed and then left for 15 min to ensure complete hydrolysis of the acetoxymethyl ester groups before starting the experiments. Fluo-3 was found to be homogeneously distributed all through the cells (Bkaily et al. 2001, 2003) and was calibrated to be expressed in absolute concentration of free Ca\(^{2+}\) (Bkaily et al. 2001).
Confocal microscopy, volume rendering and quantitative fluorescence intensity measurement.

Cells were examined with a Multiprobe 2001 confocal krypton–argon laser scanning system (Molecular Dynamics, Sunnyvale, CA) or a Bio-Rad confocal krypton–argon and ultraviolet laser system as previously described (Bkaily et al. 1997, 2004, 2017; Ahmarani et al. 2013). In brief, laser line intensity, photometric gain, PMT settings, and filter attenuation were kept rigorously constant throughout the experimental procedures (Bkaily et al. 1997, 1999, 2017). At the end of each experiment, the nucleus was stained with 100 nmol/L of live cell nucleic acid stain SYTO-11 (Molecular Probes, Eugene, OR) as described previously (Bkaily et al. 1997, 2017). Scanned images were transferred onto a Silicon Graphics workstation equipped with Molecular Dynamics’ ImageSpace analysis and Volume Workbench software modules. The ImageSpace program permits the generation of quantitative 3D images which permits the expression of the measurement per $\mu m^3$. Images were represented as top-view maximum intensity real 3D projections (not deconvolution) (Bkaily et al. 2017).

Western blot

Extraction of proteins to be used for Western blot was performed using the same protocol described elsewhere (Bkaily et al. 2003) with some modifications. Cultured hVECs were scraped and lysed in a Laemmli buffer (62.5 mmol/L Tris–HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.005% bromophenol blue). Following dosage using the Bicinchoninic acid protein assay (Pierce Chemical Co., Dallas, TX), proteins (70 µg) were separated by SDS-PAGE gel electrophoresis (10% gels), transferred on
nitrocellulose membranes (Amersham-Pharmacia Biotech, Baie d'Urfé, Qc.) and stained with ponceau red and then washed. Membranes were blocked for 2 h at room temperature in PBS 1X (for AT_2 receptor) or TBS (10 mM Tris-HCl, 150 mM NaCl, pH 8.0) (for AT_1 receptor) containing 5% NFDM and 0.05% Tween and then incubated overnight at 4 °C with an anti-AT_1 (1/200) or anti-AT_2 antibody (10 µg/ml). After washing in PBS containing 0.05% Tween, PBS with 5% NFDM buffer was used for incubation with anti-rabbit IgG conjugated to peroxidase (1/5000) (Amersham Pharmacia-Biotech, Baie d'Urfé, Qc.) for 18 h at room temperature (RT). The membranes were then washed and the immune complexes were detected by chemiluminescence (ECL, Roche Diagnostics Co, Indianapolis, IND) and visualized by autoradiography (BIOMAX MR Eastman Kodak Company, Rochester, NY). A positive control was performed using PC-12 WCL cell lysates. In addition, a negative control using the peptide antigen control of the anti-AT_1 and anti-AT_2 receptor antibodies was done where only the band of the receptors was blocked by the control peptide antigen. This showed the specificity of the antibody used.

For each western blot, in order to insure equal loading of proteins in all wells, a control was obtained with β-actin. This was done by stripping the membrane used for AT_1 and AT_2 receptors detection with glycine for 30 min at RT. The membrane was then washed 3 times with PBS and blocked with PBS containing NFDM (5%) and Tween-20 (0.05%) for 3 hrs at RT. After that, the membrane was incubated for 30 min at RT with a mouse monoclonal anti-β-actin antibody (dilution 1/20000) (Abcam, Toronto, ON). The membrane was then incubated for 1 hr at RT with an anti-mouse secondary antibody anti-IgG conjugated to peroxidase (Sigma-Aldrich, Saint-Louis, MO), diluted in the blocking
solution at a concentration of 1/1000. The β-actin band was then detected by chemiluminiscence.

Statistics

Values are expressed as means ± SEM, and \( n \) is the number of cells from at least three different experiments of at least three different donors. Results are compared to control values unless indicated differently. Statistical significance was determined using the Student’s t-test or one way repeated measure ANOVA with post-hoc t test (\( P < 0.05 \)) using the Tukey-Kramer or the Newman-Keuls method where applicable for data comparison and analysis by the program Graph Pad Prism.
Results

Presence and distribution of AngII, AT$_1$R and AT$_2$R in hVECs.

In the first series of experiments we used specific antibodies directed against AngII. Figure 1A-B shows an example and figure 2A summarizes the results. As seen in these figures, AngII was present all through the cell including the nucleus. It was distributed in a heterogeneous manner and its relative density was significantly ($p<0.001$) lower in the nucleus (including the perinucleoplasmic envelope) compared to the cytosol.

In another series of experiments, we determined the presence, distribution and relative density of AT$_1$R in hVECs. Figure 1G shows an example and figure 2B summarizes the results. These figures show the presence of AT$_1$R at the PM, cytosol, NEMs as well as nucleoplasm. It was heterogeneously distributed throughout the cell including the nucleus (Fig. 1G) and its relative density is higher ($p<0.001$) in the nucleus (including NEMs) than in the cytosol (Fig. 2B). Its relative density was near six-fold higher in the nucleus compared to the cytosol.

In the last series of experiments, we determined the presence, distribution and relative density of AT$_2$R in hVECs. Figure 1M shows an example and figure 2C summarizes the results. As seen in these figures, AT$_2$R was mainly present at the PM with a lower level in the cytosol. Its distribution in the nucleus seemed to be relatively homogeneous including the NEMs. Its relative density at the nuclear level (including NEMs) was nearly double that in the cytosol (including PM) ($p<0.001$) (Fig. 2C).
Role of AT<sub>1</sub>R and AT<sub>2</sub>R in AngII-induced increase of cytosolic and nuclear free calcium.

In this series of experiments, we tested the effect of different concentrations of AngII (10<sup>-15</sup>-10<sup>-5</sup> M) on cytosolic and nuclear calcium in hVECs. Figure 3 A-K show examples and figure 3 M summarizes the results. As expected, the nuclear level of calcium was higher than that in the cytosol in control condition (Fig. 3A) and this difference was maintained at all concentrations of AngII used. As seen in figure 3A-K, increasing the concentration of AngII induced an increase of cytosolic and nuclear calcium. Figure 3M shows the concentration-dependent effect of AngII on cytosolic and nuclear calcium. As can be seen, the maximum effect of AngII took place at a concentration of 10<sup>-10</sup>M (cytosolic calcium) to 10<sup>-9</sup>M (nuclear calcium) and then a plateau was reached. The concentration of 10<sup>-9</sup>M was used in all the subsequent protocols in order to produce a maximal effect on the increase of cytosolic and nuclear calcium induced by AngII. The calculated EC<sub>50</sub> was similar for cytosolic and nuclear calcium (4.3x10<sup>-14</sup>M and 6.3x10<sup>-14</sup>M respectively).

In the next series of experiments, we tested the effect of AT<sub>1</sub>R specific antagonist, losartan (10<sup>-6</sup>M) on AngII (10<sup>-9</sup>M) induced increase of cytosolic and nuclear calcium. The concentration of losartan used was reported to completely block AT<sub>1</sub>R (Bkaily et al. 2003; Jacques et al. 2003). Figure 4 A shows examples and figure 4 B summarizes the results. Superfusion for 5 min with losartan had no effect on cytosolic and nuclear calcium. In the presence of the AT<sub>1</sub>R antagonist, superfusion with a concentration of AngII (10<sup>-9</sup>M) that induces a maximum effect on intracellular calcium had no effect on cytosolic and nuclear calcium (Figure 4 A and B). However, washout with a solution free
of losartan but containing 10⁻⁹ M of AngII induced, within a minute, a significant increase of cytosolic and nuclear calcium (p<0.001).

In the last series of experiments, we verified whether the effect of AngII on intracellular calcium is also mediated via activation of AT₂R. Figure 5 A shows examples and figure 5 B summarizes the results. As seen in this figure, the presence of the AT₂R specific receptor antagonist, PD123319 (10⁻⁶ M), did not prevent AngII (10⁻⁹ M) from inducing a significant (p<0.001) increase of cytosolic and nuclear calcium.

**Effect of AngII on AT₁R and AT₂R trafficking and levels in hVECs.**

In this series of experiments, we tested the effect of a high concentration of AngII (10⁻¹⁰ M) on AT₂R trafficking and its relative cytosolic (including PM) and nuclear (including NEM) levels. As seen in figure 6 A and B, AngII had no effect on cytosolic and nuclear levels of AT₂R. Pretreatment with PD123319 and stimulation with AngII (10⁻¹⁰ M) did not affect the distribution and the relative level of AT₂R all through the cells (Fig. 6).

In the next series of experiments, we used the same protocol as for AT₂R in order to verify whether AT₁R undergoes internalization upon activation by AngII. As seen in figure 7 A and B, AngII did not affect the distribution and level of AT₁R during the first 15 min of exposure to the octapeptide. However, at 30 min, there was a decrease of both cytosolic (p<0.01) and nuclear (p<0.001) AT₁R levels. At 60 min, the distribution and cytosolic and nuclear levels of AT₁R return back to the normal level. Blockade of AT₁R prevented AngII from inducing a transient decrease of cytosolic and nuclear levels of AT₁R (Fig. 7 A and B).
In the following series of experiments, we verified whether blockade of protein synthesis with cycloheximide (10 µg/mL) would prevent the re-increase of AT\(_1\)R level induced by AngII at 60 min. As seen in figure 8, treatment for 30 min with cycloheximide decreased (p<0.01) the level of cytosolic and nuclear AT\(_1\)R levels. In the presence of cycloheximide, treatment with AngII (10\(^{-10}\)M) had no effect on the distribution and relative levels of AT\(_1\)R during the first 15 min. However, further decrease took place at 30 min at both the cytosolic and nuclear levels. This decrease persisted as long as cycloheximide is present (Fig. 8).

In the last series of experiments, using Western blot and the protocol described above, we wanted to confirm the effect of 60-min exposure to AngII (10\(^{-10}\)M) on the relative density AT\(_1\)R observed using quantitative 3D confocal microscopy. Figure 9 shows examples. As can be seen in figure 9 A, at 30 min, AngII induced a decrease in the relative total AT\(_1\)R protein followed by \textit{de novo} synthesis. This \textit{de novo} synthesis was prevented by the protein synthesis inhibitor cycloheximide (Fig. 9 A and B). In addition, the AT\(_1\)R inhibitor losartan but not the AT\(_2\)R inhibitor PD123319 prevented the decrease of the protein level of AT\(_1\)R at 30 min (Figure 9 A).
Discussion

In this study, contrary to what was reported previously in human aortic smooth muscle cells (Bkaily et al. 2003, 2009, 2012), our results show that, in human aortic endothelial cells (hVECs), both AT\textsubscript{1}R and AT\textsubscript{2}R are present at the cytosolic (including PM) and nuclear (including NEM) levels. However, our results are similar to those reported in human endocardial endothelial cells (hEECs) (Jacques et al. 2003). In addition, AngII is found to be present at both the cytosolic and nuclear levels. The octapeptide and both its receptors are distributed heterogeneously all through hVECs. Contrary to hEECs (Jacques et al. 2003), the level of AngII is significantly higher in the cytosol compared to the nucleus. However, similar to hEECs (Jacques et al. 2003), the relative density of AT\textsubscript{1}R and AT\textsubscript{2}R in hVECs is higher in the nucleus (including NEM) compared to the cytosol (including PM). The presence of AngII at the cytosolic and nucleoplasmic levels will promote activation of its receptors at the nuclear level thus modulating nucleoplasmic calcium homeostasis and gene expression (Bkaily et al. 2003, 2009, 2014, 2017). Our results also show that, as in hEECs (Jacques et al. 2003) and hVSMCs (Bkaily et al. 2003, 2009, 2014), AngII induces a dose-dependent sustained increase of cytosolic and nuclear calcium. However, its EC\textsubscript{50} value is lower in hVECs (near 10\textsuperscript{-14}M) when compared to hEECs (near 10\textsuperscript{-12}M) (Jacques et al. 2003) and hVSMCs (near 10\textsuperscript{-11}M) (Bkaily et al. 2003). This difference in EC\textsubscript{50} between hVECs, hEECs (Jacques et al. 2003) and hVSMCs (Bkaily et al. 2003) could be due to the higher density of AT\textsubscript{1}R in hVECs at both the cytosolic (including PM) and nuclear (including NEM) levels. This is supported by the fact that the absence of AT\textsubscript{1}R at the nuclear membranes level of hVSMCs is associated with a high EC\textsubscript{50} value (Bkaily et al. 2003) when compared to
hVECs and that a low expression of human AT$_1$R increases the EC$_{50}$ value of AngII in hVSMCs (Bkaily et al. 2003). These results suggest that hVECs are highly sensitive to AngII and that hVECs respond to very little changes in circulating AngII when compared to hEECs and hVSMCs. Such a high sensitivity of AngII makes this type of cells the first target for increasing AngII level in cardiovascular pathologies such as hypertension (Regoli and Gobeil 2017). Furthermore, a high sensitivity to AngII will modulate hVECs excitation-secretion coupling and promote the secretion of various calcium-dependent factors such as endothelin-1 (ET-1). This latter, along with the high AngII level will contribute further to the development and maintaining of hypertension and to the development of cardiac hypertrophy.

Although our results show that AT$_2$R is present at both the plasma and nuclear envelope membranes, this type of receptor does not seem to regulate calcium homeostasis in hVECs and also does not undergo internalization and nuclear translocation. The absence of internalization of AT$_2$R upon activation by AngII in hVECs is unexpected and internalization seems to be a property of AT$_1$R. The presence and role of AT$_2$R in regulation of cell function and more particularly in endothelial cells are controversial. In addition, our results confirm that the presence of AT$_2$R is not limited to fetal tissue (Grady et al. 1991). As in many cell types, AT$_1$R in hVECs seems to mediate most of the AngII effects. AT$_2$R often opposes the effects of AT$_1$R such as in survival (Namsolleck et al. 2014). It is possible that AT$_2$R may play a specific role in the modulation of hVEC functions and this needs to be elucidated. Since AT$_2$R did not undergo internalization upon AngII activation, it is possible to postulate that, at least in hVECs, heterodimeric AT$_1$R/AT$_2$R do not undergo internalization and nuclear translocation. Finally, our results
show that $10^{-10}$M of AngII induces, within 15 minutes, internalization of AT$_1$R but not AT$_2$R and this is followed, at 30 minutes, by degradation of the receptor and then by de novo synthesis taking place at 60 minutes. This de novo synthesis is supported by the fact that it is sensitive to the protein synthesis inhibitor cycloheximide. Interestingly, the decrease in the relative density of cytosolic and nuclear AT$_1$R (not AT$_2$R) is prevented by the AT$_1$R antagonist losartan. These results suggest that the AT$_1$R antagonist does not by itself promote internalization of its receptor but prevents it from being internalized by inhibiting AngII binding. It is possible that internalization of a receptor upon its binding to its ligand will contribute to its biological effects. This action of the internalized receptor/ligand complex can, in part, make available the ligand in the cytosol in order to activate its receptors at the NEMs (Bkaily et al. 2003, 2012, 2014). In addition, it is also possible that the internalized phosphorylated receptor/ligand complex will continue its signaling and effects at both the cytosolic and nucleoplasmic levels. This needs to be explored.
Acknowledgements

This work is supported by an NSERC grant.
References


Figure legends

**Figure 1. Distribution of Ang II and its receptors AT\textsubscript{1} and AT\textsubscript{2} in hVECs.**

A) Real 3D confocal microscopic images (top view) showing the immunoreactivity of AngII (A), AT\textsubscript{1} receptor (G) and AT\textsubscript{2} receptor (M) in hVECs. For each antibody, two negative controls were done: one in the presence of the primary antibody preadsorbed with the provided control peptide (C, I and O), and another in the absence of the primary antibody (E, K and Q). Panels B, D, F, H, J, L, N, P, and R show the syto.11 staining of the nuclei of the cells from the panels A, C, E, G, I, K, M, O and Q respectively. The pseudo-color bar represents the fluorescence intensity from 0 (black, no fluorescence) to 255 (white, maximum fluorescence). The white scale bar is in µm.

**Figure 2. Relative density of Ang II and its receptors AT\textsubscript{1} and AT\textsubscript{2} in hVECs.**

Histograms showing the relative density of AngII (A), AT\textsubscript{1}R (B) and AT\textsubscript{2}R (C) in the cytosol (including PM) and the nucleus (including N EM) of adult human VECs. The values are expressed as mean ± SEM. n is the number of cells from at least three different experiments. ***p < 0.001.

**Figure 3. Effect of different concentrations of extracellular Ang II on the sustained levels of cytosolic and nuclear calcium in adult human VECs.**

(A-K) Real 3D confocal microscopic images (top view) showing the effect of different concentrations of extracellular AngII on the sustained levels of cytosolic ([Ca\textsubscript{c}]) and nuclear ([Ca\textsubscript{n}]) calcium in adult human VECs. Panel A shows the basal sustained [Ca\textsubscript{c}] and [Ca\textsubscript{n}] in the absence of Ang II. Panels B to K show the sustained cytosolic and nuclear Ca\textsuperscript{2+} levels in the presence of 10\textsuperscript{-15} M, 10\textsuperscript{-14} M, 10\textsuperscript{-13} M, 10\textsuperscript{-12} M, 10\textsuperscript{-11} M, 10\textsuperscript{-10} M, 10\textsuperscript{-9} M, 10\textsuperscript{-8} M, 10\textsuperscript{-7} M, and 10\textsuperscript{-6} M of Ang II, respectively. As can be seen in panels A to K, Ang II, at concentrations between 10\textsuperscript{-15} and 10\textsuperscript{-6} M, induces a dose-dependent increase of free [Ca\textsubscript{c}] and [Ca\textsubscript{n}] in adult human VECs. Panel L shows the syto.11 staining of the nucleus of the cell from panels A to K. The pseudocolor bar represents the fluorescence intensity from 0 (black, no fluorescence) to 255 (white, maximum fluorescence). The white scale bar is in µm. (M) Dose response curves showing the effect of different concentrations of AngII on [Ca\textsubscript{c}] and [Ca\textsubscript{n}] in adult human VECs. The sustained increase of the free [Ca\textsubscript{c}] and [Ca\textsubscript{n}] levels reaches a maximum at a concentration of 10\textsuperscript{-10} M of Ang II. The EC\textsubscript{50} values are 4.32 x 10\textsuperscript{-14} M for the cytosol and 6.28 x 10\textsuperscript{-14} M for the nucleus. The values are presented as the Mean ± SEM. n is the number of cells observed in 5 different experiments.

**Figure 4. Effect of the AT\textsubscript{1}R antagonist, losartan, on the sustained increase of [Ca\textsubscript{c}] and [Ca\textsubscript{n}] induced by Ang II in adult human VECs.**
(A) Real 3D confocal microscopic images (top view) showing the effect of the AT$_1$R antagonist, losartan, on the sustained increase of [Ca]$\text{c}$ and [Ca]$\text{n}$ induced by AngII in adult human VECs. Panel a shows the basal sustained [Ca]$\text{c}$ and [Ca]$\text{n}$ in an adult human VEC. Losartan (10$^{-6}$ M) does not seem to have an effect on the basal sustained [Ca]$\text{c}$ and [Ca]$\text{n}$ (b) but prevents the sustained increase of [Ca]$\text{c}$ and [Ca]$\text{n}$ induced by AngII (10$^{-9}$ M) (c). The addition of AngII (10$^{-9}$ M) after the washout of the cell with a medium devoid of AngII and its antagonist causes an sustained increase of [Ca]$\text{c}$ and [Ca]$\text{n}$ (d). Panel e shows the syto-11 staining of the nucleus of the cell from panels a to d. (e). The pseudocolor bar represents the fluorescence intensity from 0 (black, no fluorescence) to 255 (white, maximum fluorescence). The white scale bar is in µm. (B) Histogram showing that losartan (10$^{-6}$ M) prevents the sustained increase of [Ca]$\text{c}$ and [Ca]$\text{n}$ induced by 10$^{-9}$ M of Ang II. However, following washout of the losartan containing medium, AngII (10$^{-9}$ M) induced a significant increase of [Ca]$\text{c}$ and [Ca]$\text{n}$. The values are expressed as Mean ± SEM. n is the number of cells from 5 different experiments. *** p < 0.001.

Figure 5. Effect of the AT$_2$R antagonist, PD123319, on the sustained increase of [Ca]$\text{c}$ and [Ca]$\text{n}$ induced by Ang II in adult human VECs

A) Real 3D confocal microscopic images (top view) showing the effect of the AT$_2$R antagonist, PD123319, on the sustained increase of [Ca]$\text{c}$ and [Ca]$\text{n}$ induced by AngII in adult human VECs. Panel a shows the basal sustained [Ca]$\text{c}$ and [Ca]$\text{n}$ in an adult human VEC. PD123319 (10$^{-6}$ M) does not seem to have an effect on the basal sustained [Ca]$\text{c}$ and [Ca]$\text{n}$ (b). In the presence of PD123319, AngII (10$^{-9}$ M) induced an sustained increase of [Ca]$\text{c}$ and [Ca]$\text{n}$ (c). Panel d shows the syto-11 staining of the nucleus of the cell from panels a to c. (e). The pseudocolor bar represents the fluorescence intensity from 0 (black, no fluorescence) to 255 (white, maximum fluorescence). The white scale bar is in µm. B) Histogram showing that in the presence of PD123319 (10$^{-6}$ M), AngII (10$^{-9}$ M) induced a significant increase of [Ca]$\text{c}$ and [Ca]$\text{n}$ in adult human VECs. The values are presented as the Mean ± SEM. n is the number of cells from 7 different experiments. *** p < 0.001.

Figure 6. Effect of extracellular Ang II on the trafficking of AT$_2$R in adult human VECs.

(A) Real 3D confocal microscopic images (top view) showing the distribution of the AT$_2$R in adult human VECs in control condition (a) and at 5 min (b), 15 min (c), 30 min (d) and 60 min (e) following AngII (10$^{-10}$ M) treatment. Panel f shows the distribution of AT$_2$R in VECs treated with PD123319 (10$^{-6}$ M) before treatment with AngII for 15 min. AngII does not seem to induce any change of the cytosolic (including PM) and nuclear (including NEM) distribution of AT$_2$R in the absence or presence of PD123319. The pseudocolor bar represents the fluorescence intensity from 0 (black, no fluorescence) to
255 (white, maximum fluorescence). The white scale bar is in µm. (B) Histogram showing the relative density of AT$_2$R in the cytosol (including PM) and the nucleus (including NEM) of VECs before and at different time intervals following treatment with AngII (10$^{-10}$ M). The values are expressed as the mean ± SEM. n is the number of cells from 4 different experiments.

**Figure 7. Effect of extracellular AngII on the trafficking of AT$_1$R in adult human VECs.**

(A) Real 3D confocal microscopic images (top view) showing the distribution of AT$_1$R in adult human VECs in control condition (a) and at 5 min (b), 15 min (c), 30 min (d) and 60 min (e) following AngII (10$^{-10}$ M) treatment. Panel f shows the distribution of AT$_1$R in VECs treated with losartan (10$^{-6}$ M) before treatment with AngII for 15 min. Following treatment with AngII for 30 min, there is an decrease of both cytosolic (including PM) and nuclear (including NEM) AT$_1$R fluorescence intensity (d) followed by a return to control level at 60 min (e). The effect of Ang II on AT$_1$R level is prevented by losartan (f). The pseudocolor bar represents the fluorescence intensity from 0 (black, no fluorescence) to 255 (white, maximum fluorescence). The white scale bar is in µm. (B) Histogram showing the relative density of AT$_1$R in the cytosol (including PM) and the nucleus (including NEM) of VECs before and at different time intervals following treatment with AngII (10$^{-10}$ M). The values are expressed as the mean ± SEM. n is the number of cells from 4 different experiments. ** p < 0.01, *** p < 0.001 vs. control and +++ p < 0.001 vs. Ang II 30 min.

**Figure 8. Effect of cycloheximide on the trafficking of AT$_1$ receptors induced by Ang II in adult human VECs.**

A) Real 3D confocal microscopic images (top view) showing the distribution of AT$_1$R in adult human VECs in control condition (a), at 30 min following cycloheximide (10 µg/mL) treatment (b), and at 5 min (c), 15 min (d), 30 min (e) and 60 min (f) following AngII 10$^{-10}$ M treatment in the presence of cycloheximide (30 min). Following treatment with cycloheximide for 30 min, there is an decrease of both cytosolic (including PM) and nuclear (including NEM) AT$_1$R fluorescence intensity (b) compared to control (a). There is a further decrease of AT$_1$R fluorescence intensity at 30 min following AngII treatment (e) and this decrease persists after 60 min (f). The pseudocolor bar represents the fluorescence intensity from 0 (black, no fluorescence) to 255 (white, maximum fluorescence). The white scale bar is in µm. (B) Histogram showing the relative density of AT$_1$R in the cytosol (including PM) and the nucleus (including NEM) of VECs before and at different time intervals following treatment with AngII (10$^{-10}$ M) in presence of cycloheximide (10 µg/mL). The values are expressed as the mean ± SEM. n is the number of cells from 4 different experiments. ** p < 0.01; *** p < 0.001 vs. control and + p < 0.05; +++ p < 0.001 vs. cycloheximide 30 min.
Figure 9. Western blots showing the effect of extracellular Ang II on the relative density of AT$_1$R in adult human VECs.

Representative Western blots of AT$_1$R in hVECs (out of 3 different experiments) treated with Ang II ($10^{-10}$ M) in the absence or presence of losartan or PD123319 (A) or cycloheximide (B). The band at ~ 50 kDa corresponds to the AT$_1$R. The band corresponding to actin indicates that equal quantities of proteins were loaded in all wells.
Figure 1. Distribution of Ang II and its receptors AT1 and AT2 in hVECs. A) Real 3D confocal microscopic images (top view) showing the immunoreactivity of AngII (A), AT1 receptor (G) and AT2 receptor (M) in hVECs. For each antibody, two negative controls were done: one in the presence of the primary antibody preadsorbed with the provided control peptide (C, I and O), and another in the absence of the primary antibody (E, K and Q). Panels B, D, F, H, J, L, N, P, and R show the syto-11 staining of the nuclei of the cells from the panels A, C, E, G, I, K, M, O and Q respectively. The pseudo-color bar represents the fluorescence intensity from 0 (black, no fluorescence) to 255 (white, maximum fluorescence). The white scale bar is in μm.
Figure 2. Relative density of Ang II and its receptors AT1 and AT2 in hVECs. Histograms showing the relative density of AngII (A), AT1R (B) and AT2R (C) in the cytosol (including PM) and the nucleus (including NEM) of adult human VECs. The values are expressed as mean ± SEM. n is the number of cells from at least three different experiments. **p < 0.001.
Figure 3. Effect of different concentrations of extracellular Ang II on the sustained levels of cytosolic and nuclear calcium in adult human VECs.

(A-K) Real 3D confocal microscopic images (top view) showing the effect of different concentrations of extracellular AngII on the sustained levels of cytosolic ([Ca]c) and nuclear ([Ca]n) calcium in adult human VECs. Panel A shows the basal sustained [Ca]c and [Ca]n in the absence of Ang II. Panels B to K show the sustained cytosolic and nuclear Ca2+ levels in the presence of 10^{-15} M, 10^{-14} M, 10^{-13} M, 10^{-12} M, 10^{-11} M, 10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M of Ang II, respectively. As can be seen in panels A to K, Ang II, at concentrations between 10^{-15} and 10^{-6} M, induces a dose-dependent increase of free [Ca]c and [Ca]n in adult human VECs. Panel L shows the syto-11 staining of the nucleus of the cell from panels A to K. The pseudocolor bar represents the fluorescence intensity from 0 (black, no fluorescence) to 255 (white, maximum fluorescence). The white scale bar is in µm. (M) Dose response curves showing the effect of different concentrations of AngII on [Ca]c and [Ca]n in adult human VECs. The sustained increase of the free [Ca]c and [Ca]n levels reaches a maximum at a concentration of 10^{-10} M of Ang II. The EC50 values
are $4.32 \times 10^{-14}$ M for the cytosol and $6.28 \times 10^{-14}$ M for the nucleus. The values are presented as the Mean ± SEM. $n$ is the number of cells observed in 5 different experiments.
Figure 4. Effect of the AT1R antagonist, losartan, on the sustained increase of \([\text{Ca}]_c\) and \([\text{Ca}]_n\) induced by Ang II in adult human VECs.

(A) Real 3D confocal microscopic images (top view) showing the effect of the AT1R antagonist, losartan, on the sustained increase of \([\text{Ca}]_c\) and \([\text{Ca}]_n\) induced by AngII in adult human VECs. Panel a shows the basal sustained \([\text{Ca}]_c\) and \([\text{Ca}]_n\) in an adult human VEC. Losartan (10^-6 M) does not seem to have an effect on the basal sustained \([\text{Ca}]_c\) and \([\text{Ca}]_n\) (b) but prevents the sustained increase of \([\text{Ca}]_c\) and \([\text{Ca}]_n\) induced by AngII (10^-9 M) (c). The addition of AngII (10^-9 M) after the washout of the cell with a medium devoid of AngII and its antagonist causes an sustained increase of \([\text{Ca}]_c\) and \([\text{Ca}]_n\) (d). Panel e shows the syto-11 staining of the nucleus of the cell from panels a to d. (e). The pseudocolor bar represents the fluorescence intensity from 0 (black, no fluorescence) to 255 (white, maximum fluorescence). The white scale bar is in µm. (B) Histogram showing that losartan (10^-6 M) prevents the sustained increase of \([\text{Ca}]_c\) and \([\text{Ca}]_n\) induced by 10^-9 M of Ang II. However, following washout of the losartan containing medium, AngII (10^-9 M) induced a significant increase of \([\text{Ca}]_c\) and \([\text{Ca}]_n\). The values are expressed as Mean ± SEM. n is the number of cells from 5 different experiments. *** p < 0.001.
Figure 5. Effect of the AT2R antagonist, PD123319, on the sustained increase of \([\text{Ca}]_{\text{c}}\) and \([\text{Ca}]_{\text{n}}\) induced by Ang II in adult human VECs

A) Real 3D confocal microscopic images (top view) showing the effect of the AT2R antagonist, PD123319, on the sustained increase of \([\text{Ca}]_{\text{c}}\) and \([\text{Ca}]_{\text{n}}\) induced by AngII in adult human VECs. Panel a shows the basal sustained \([\text{Ca}]_{\text{c}}\) and \([\text{Ca}]_{\text{n}}\) in an adult human VEC. PD123319 (10^-6 M) does not seem to have an effect on the basal sustained \([\text{Ca}]_{\text{c}}\) and \([\text{Ca}]_{\text{n}}\) (b). In the presence of PD123319, AngII (10^-9 M) induced an sustained increase of \([\text{Ca}]_{\text{c}}\) and \([\text{Ca}]_{\text{n}}\) (c). Panel d shows the syto-11 staining of the nucleus of the cell from panels a to c. (e). The pseudocolor bar represents the fluorescence intensity from 0 (black, no fluorescence) to 255 (white, maximum fluorescence). The white scale bar is in µm. B) Histogram showing that in the presence of PD123319 (10^-6 M), AngII (10^-9 M) induced a significant increase of \([\text{Ca}]_{\text{c}}\) and \([\text{Ca}]_{\text{n}}\) in adult human VECs. The values are presented as the Mean ± SEM. n is the number of cells from 7 different experiments. *** p < 0.001.
Figure 6. Effect of extracellular Ang II on the trafficking of AT2R in adult human VECs.
(A) Real 3D confocal microscopic images (top view) showing the distribution of the AT2R in adult human VECs in control condition (a) and at 5 min (b), 15 min (c), 30 min (d) and 60 min (e) following AngII (10-10 M) treatment. Panel f shows the distribution of AT2R in VECs treated with PD123319 (10-6 M) before treatment with AngII for 15 min. AngII does not seem to induce any change of the cytosolic (including PM) and nuclear (including NEM) distribution of AT2R in the absence or presence of PD123319. The pseudocolor bar represents the fluorescence intensity from 0 (black, no fluorescence) to 255 (white, maximum fluorescence). The white scale bar is in µm. (B) Histogram showing the relative density of AT2R in the cytosol (including PM) and the nucleus (including NEM) of VECs before and at different time intervals following treatment with AngII (10-10 M). The values are expressed as the mean ± SEM. n is the number of cells from 4 different experiments.
Figure 7. Effect of extracellular AngII on the trafficking of AT1R in adult human VECs. 
(A) Real 3D confocal microscopic images (top view) showing the distribution of AT1R in adult human VECs in control condition (a) and at 5 min (b), 15 min (c), 30 min (d) and 60 min (e) following AngII (10-10 M) treatment. Panel f shows the distribution of AT1R in VECs treated with losartan (10-6 M) before treatment with AngII for 15 min. Following treatment with AngII for 30 min, there is a decrease of both cytosolic (including PM) and nuclear (including NEM) AT1R fluorescence intensity (d) followed by a return to control level at 60 min (e). The effect of Ang II on AT1R level is prevented by losartan (f). The pseudocolor bar represents the fluorescence intensity from 0 (black, no fluorescence) to 255 (white, maximum fluorescence). The white scale bar is in µm. (B) Histogram showing the relative density of AT1R in the cytosol (including PM) and the nucleus (including NEM) of VECs before and at different time intervals following treatment with AngII (10-10 M). The values are expressed as the mean ± SEM. n is the number of cells from 4 different experiments. ** p < 0.01, *** p < 0.001 vs. control and +++ p < 0.001 vs. Ang II 30 min.
Figure 8. Effect of cycloheximide on the trafficking of AT1 receptors induced by Ang II in adult human VECs. A) Real 3D confocal microscopic images (top view) showing the distribution of AT1R in adult human VECs in control condition (a), at 30 min following cycloheximide (10 µg/mL) treatment (b), and at 5 min (c), 15 min (d), 30 min (e) and 60 min (f) following AngII 10-10 M treatment in the presence of cycloheximide (30 min). Following treatment with cycloheximide for 30 min, there is a decrease of both cytosolic (including PM) and nuclear (including NEM) AT1R fluorescence intensity (b) compared to control (a). There is a further decrease of AT1R fluorescence intensity at 30 min following AngII treatment (e) and this decrease persists after 60 min (f). The pseudocolor bar represents the fluorescence intensity from 0 (black, no fluorescence) to 255 (white, maximum fluorescence). The white scale bar is in µm. (B) Histogram showing the relative density of AT1R in the cytosol (including PM) and the nucleus (including NEM) of VECs before and at different time intervals following treatment with AngII (10-10 M) in presence of cycloheximide (10 µg/mL). The values are expressed as the mean ± SEM. n is the number of cells from 4 different experiments. ** p < 0.01; *** p < 0.001 vs. control and + p < 0.05; +++ p < 0.001 vs. cycloheximide 30 min.
Figure 9. Western blots showing the effect of extracellular Ang II on the relative density of AT1R in adult human VECs. Representative Western blots of AT1R in hVECs (out of 3 different experiments) treated with Ang II (10−10 M) in the absence or presence of losartan or PD123319 (A) or cycloheximide (B). The band at ∼50 kDa corresponds to the AT1R. The band corresponding to actin indicates that equal quantities of proteins were loaded in all wells.