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**Angiotensin II induces apoptosis of human right and left ventricular endocardial
endothelial cells by activating the AT₂ receptor**

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Abstract

Endocardial endothelial cells (EECs) form a monolayer lining the ventricular cavities. Studies from our laboratory and the literature have shown differences between EECs isolated from the right and left ventricles (EECRs and EECLs respectively). Angiotensin II (Ang II) was shown to induce apoptosis of different cell types mainly via AT₁ receptor activation. In this study, we verified whether Ang II induces apoptosis of human EECRs and EECLs and via which type of receptor. Using the Annexin V labeling and *in situ* TUNEL assays, our results showed that Ang II-induced apoptosis of both hEECRs and hEECLs in a concentration-dependent manner. Our results using specific AT₁ and AT₂ receptor antagonists showed that the Ang II-induced apoptosis in both hEECRs and hEECLs is mediated mainly via the AT₂ receptor. However, AT₁ receptor blockade partially prevented Ang II-induced apoptosis, particularly in hEECRs. Hence, our results suggest that mainly AT₂ receptors mediate Ang II-induced apoptosis of hEECRs and hEECLs. The damage of EECs would affect their function as a physical barrier between the blood and cardiomyocytes thus affecting cardiomyocyte functions.

Keywords: human endocardial endothelial cells, apoptosis, angiotensin II, AT₁ receptor, AT₂ receptor, angiotensin II receptor antagonists.

Introduction

The heart cavities, including the trabeculae and papillary muscles, are lined with a thin monolayer of endothelial cells about 0.5 μm thick, called the endocardial endothelium (EE) (Brutsaert et al. 1996; Jacques and Bkaily, 2018; Jacques et al. 2017). The extracellular matrix between the EE and cardiomyocytes consists of basal lamina, a reticular lamina, and a fibroblastic layer. The latter contains blood vessels and a neuronal plexus (Marron et al. 1995) composed of nerves and sympathetic fibers capable of releasing several peptides including angiotensin II (Ang II), endothelin-1 (ET-1) and neuropeptide Y (NPY) (Jacques et al. 2003a, 2003b, 2017a; Jacques and Abdel-Samad, 2007; Abdel-Samad et al. 2016; Marron et al. 1994). Furthermore, endocardial endothelial cells themselves are capable of secreting cardioactive factors such as NO (Andries et al. 1998; SHAH, 1996), Ang II (Paul and Ganten, 1992), ET-1 (Mebazaa et al. 1993; Abdel-Samad et al. 2012, 2016) and NPY (Jacques et al. 2003b). Studies from our laboratory (Jacques et al. 2000a, 2003b, 2005, 2006a, 2006b, 2017a, 2017b; Jacques and Abdel-Samad, 2007; Abdel-Samad et al. 2012, 2016; Jules et al. 2015), as well as from the literature (Brutsaert, 2003; Nosaka et al. 1997; Ekman et al. 1997; Kim et al. 1999; Discigil et al. 2009) showed differences between endocardial endothelial cells (EECs) isolated from the right and left ventricles (EECRs and EECLs respectively). Among these differences, our group has shown that the distribution of Ang II and its receptors, AT_1 and AT_2 , is different between EECRs and EECLs (Jacques et al. 2003a, 2017b). Also, EECLs are more sensitive to Ang II stimulation than EECRs (Jacques et al. 2017b).

Evidence for the involvement of Ang II in apoptosis is abundant. In fact, the octapeptide has been shown to induce apoptosis of several cell types including cardiomyocytes (Cigola et al. 1997, Goldenberg et al. 2001, Kajstura et al. 1997), human umbilical vein endothelial cells (HUVECs) (Kou et al. 2007, Shan et al. 2008), bovine pulmonary artery endothelial cells (PAECs) (Lee et al. 2010), and human vascular smooth muscle cells (hVSMCs) in culture (Li et al. 2006). However, there is no consensus concerning the Ang II receptor subtype mediating the Ang II-induced apoptosis (Stoll and Unger 2001, Yamada et al. 1996; Dimmeler et al. 1997; Yamada et al. 1998; Kajstura et al. 1997; Li et al. 2002; Bascands et al. 2001; Li et al. 1999; Zhai et al. 2005; Kaschina and Unger, 2003; Wassmann and Nickenig, 2006; Pei et al. 2017; Liu et al. 2018). This controversy could be species and/or cell type-dependent (Namasolleck et al. 2014). In addition, the apoptotic effect of the AT₂ receptor was observed in heart failure, particularly when the ratio of AT₁/AT₂ receptor density was reduced (Nio et al. 1995, Tsutsumi et al. 1998) and when the AT₁ receptor was internalized and desensitized by high levels of circulating Ang II (Griendling et al. 1987; Lassegue et al. 1995). Hence, both Ang II receptors can be involved in the Ang II-induced apoptosis. However, nothing is known about the effect of Ang II on the apoptosis of EECs and more particularly those of human origin. Thus, in the present study, we wanted to verify whether Ang II induces apoptosis of human EECRs and EECLs and via which type of receptor.

Materials and methods

Isolation and culture of human endocardial endothelial cells

The method for the isolation and culturing of human EECs was previously described (Jacques et al. 2003b, 2006a, 2017, 2018; Abdel-Samad et al. 2012, 2016). The work was done in compliance with the requirements of the institutional review committee for the use of human material. Briefly, EECs were isolated from the right and left ventricles of fetal human donor hearts within 30 min of the extraction of the human tissue. After carefully opening the ventricles, the latter were cleaned from blood with sterile M199 medium containing penicillin and streptomycin. For isolating EECRs and EECLs, the heart ventricle lumens were subjected to trypsin (0.05%) for 5 min and then washed with M199 medium supplemented with 5% FBS (v/v). The endocardial endothelial layer was gently scraped with the blade of a scalpel, centrifuged for 10 min at 200g and then resuspended in M199 medium supplemented with 5% FBS. The isolated EECRs and EECLs were cultured as monolayers in culture flasks under the conditions previously reported (Abdel-Samad et al. 2012). Upon confluence, cells were trypsinized and reseeded in medium M199 supplemented with 10% FBS (Bkaily et al. 1997, Abdel-Samad et al. 2012). Cells were conserved frozen at -80°C or in liquid nitrogen, then thawed and plated when needed. The cells were routinely checked for quality and purity using specific fluorescent probes such as FITC-conjugated Lectin Ulex Europaeus Agglutinin-1, which binds to alpha-linked fucose residues on the surface of endothelial cells.

Induction of apoptosis by Ang II

EECRs and EECLs were cultured on 25 mm coverslips and exposed to 10^{-16} , 10^{-13} and 10^{-8} M Ang II for 72 hours at 37 ° C. These experiments were carried out in the absence or in the presence of specific AT₁R and AT₂R antagonists, losartan (10^{-7} M) or PD123319 (10^{-7} M), respectively. The cells were then subjected to annexin V or *in situ* TUNEL labeling.

Annexin V labeling

Annexin V labeling was performed using the "Annexin-V-fluos Staining kit" according to the protocol provided by the manufacturer (Roche Applied Science, Indianapolis, IN, USA) as previously described (Mikhail et al. 2017). Briefly, the cells were first cultured at approximately 70% confluence on 25 mm diameter glass coverslips (Thermo Fisher Scientific, Ottawa, ON, Canada). Following the different treatments, 20 µl of "Annexin-V-Fluos" was added to 1 ml of the incubation buffer, and 100 µl of the mixture was then added directly into 1 ml of culture medium. The cells were incubated for 30 min at room temperature in the dark. The coverslips were then observed by confocal microscopy, keeping the cell culture medium in order to observe the cells in suspension.

Confocal microscopy

Cells were examined with a Bio-Rad confocal krypton–argon and ultraviolet laser system, as previously described (Bkaily et al. 1997, 2004, 2017; Ahmarani et al. 2013). Briefly, the 488 nm argon laser line (9.0 mV) was directed to the sample via a 510 nm primary dichroic filter and attenuated with a 1%–3% neutral density filter to reduce photobleaching. All the confocal microscope settings (pinhole size, image size, pixel size, laser line intensity, photometric gain, photomultiplier tube settings, and filter attenuation)

were kept constant throughout the experimental procedures. For each sample, 16–20 sections were recorded covering the entire volume of the cell, constituting a 3-dimensional serial section. At the end of each experiment, the nucleus was stained with 100 nmol/L of live cell nucleic acid stain SYTO 11 (Molecular Probes) 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 μm^3 . Images were represented as top-view maximum intensity real 3D projections (not deconvolution) (Bkaily et al. 2017).

***In situ* Terminal deoxynucleotidyl transferase dUTP mediated nick end labeling (*in situ* TUNEL)**

In situ TUNEL was performed according to the protocol provided by the "ApopTag fluorescein in situ detection kit" (Cedarlane Laboratories Limited, ON, Canada) with several modifications (Mikhail et al. 2017). In brief, cells were grown at about 70-80% confluence on 12 mm glass coverslips (Thermo Fisher Scientific, Ottawa, ON, Canada). The cells were fixed with 4% paraformaldehyde (10 min at RT), washed twice (5 min) with PBS (1X: 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4 , 1.8mM KH_2PO_4 , pH 7.4) and incubated with NaBH_4 (2 mg/ml) for 10 min at RT. After two washes with PBS 1X for 5 min, the cells were permeabilized with a solution of PBS 1X containing 0.1% Triton, 0.5% sodium azide and 10% NFDM for 30 min at RT then washed twice with PBS 1X for 5 min. After that, the coverslips were incubated in the equilibration buffer (kit) for 5 min at RT. The TdT enzyme solution, which was diluted 2.4 fold in the

reaction buffer, was added for one hour at 37 °C. The reaction was terminated by incubation for 10 min at RT in the stop buffer (kit) diluted to 1:34 in water. After washing for 5 min with 1X PBS, the coverslips were incubated with the fluorescein-coupled anti-digoxigenin antibody, which was diluted 2.13 times in the blocking solution, for 45 min at RT in a humid chamber. The coverslips were then washed twice for 5 min with 1 × PBS and then stained with a DAPI solution (1: 50,000 dilution of a 10 mg/ml solution) for 5 min, then with a solution. 0.1% Evans blue (Sigma-Aldrich, St. Louis, MO, USA) for 30 sec. Two washes with PBS 1X (10 min each) were carried out. Finally, the coverslips were mounted on microscope slides in 9:1 glycerol-PBS containing 0.1% paraphenylenediamine (Sigma-Aldrich, St. Louis, MO, USA) (Mikhail et al. 2017). The coverslips were then observed using a Nikon Eclipse 2000 fluorescence microscope.

Statistics

All values were expressed as mean $\pm$ SEM. Statistical significance was determined using the ANOVA repeated measures test for partial values followed by the Student-Newman-Keuls test with the multiple comparisons. Probability values (P) less than 0.05 (95% confidence interval) were considered significant.

Results

Effect of Ang II on apoptosis of right and left human ventricular endocardial endothelial cells.

In order to test the effect of Ang II on apoptosis of hEECRs, the cells were treated with different concentrations of Ang II (known to induce increases in intracellular calcium, Jacques et al. 2017b) (10^{-16} , 10^{-13} or 10^{-8} M) for 72 hours and then apoptosis was assessed using the annexin V labeling technique coupled with quantitative 3D confocal microscopy. Figure 1a shows examples of annexin V labeling in hEECRs and figure 3A summarizes the results. As seen in figure 1a, following chronic treatment with a very low concentration of Ang II (10^{-16} M, for 72 hours), there was no apparent change in the number of annexin V-labeled hEECRs (Fig. 1 aB) compared to control (Fig. 1 aA). On the other hand, 72-hour treatments with concentrations of 10^{-13} M (Fig. 1 aC) and 10^{-8} M (Fig. 1 aD) of Ang II induced an apparent concentration-dependent increase in the number of annexin V-labeled hEECRs compared to controls (Fig. 1 aA). As seen in figure 3A, the Ang II-induced apoptosis reached a significant effect at 10^{-13} M ($p < 0.01$) and further significant increase took place at 10^{-8} M Ang II.

Using the same protocol described previously, human left ventricular EECs were used and figure 1b shows examples whereas figure 3B summarizes the results. As seen in figure 1b A-D, as was shown for EECRs, a very low concentration of Ang II had no apparent effect on annexin V labeling of hEECLs. However, there was an increase in the number of annexin V-labeled hEECLs in the presence of 10^{-13} and 10^{-8} M Ang II. Figure 3B shows that 10^{-13} M Ang II induced a significant increase ($p < 0.05$) in the number of

annexin V-labeled cells which further significantly increased following an increase in the concentration of Ang II to 10^{-8} M ($p < 0.001$).

In order to verify the type of Ang II receptor is implicated, we used specific AT_1 R and AT_2 R antagonists, losartan and PD123319 respectively. Figure 2 shows examples, and figure 3 summarizes the results. As can be seen in figure 2a, using a concentration of Ang II that induces the highest significant increase in apoptosis (10^{-8} M), we first treated the cells for 72 hours with either the AT_1 receptor antagonist losartan (10^{-7} M) or with the AT_2 receptor antagonist PD123319 (10^{-7} M) in order to verify if blockade of one receptor type induces apoptosis.

Chronic treatment with losartan did not affect the apoptosis of hEECRs, however, losartan significantly ($p < 0.05$) induced apoptosis in hEECLs (Fig. 3A and B).

We treated hEECRs and hEECLs with different concentrations of Ang II (10^{-16} , 10^{-13} and 10^{-8} M) in the presence of losartan and Figure 3 summarizes the results whereas figure 2 shows examples of the effect of 10^{-8} M of Ang II. As seen in figure 3, losartan partially but significantly prevented Ang II-induced apoptosis in EECRs. However, losartan significantly potentiated the Ang II-induced apoptosis in hEECLs.

Replacing losartan with the AT_2 receptor antagonist PD123319 showed that blockade of the AT_2 receptor did not affect apoptosis of both hEECRs and hEECLs. Also, pretreatment with the AT_2 receptor antagonist completely prevented the Ang II-induced apoptosis in both hEECRs and hEECLs.

The same protocols described previously were used in order to confirm the results obtained using annexin V labeling but using the *in situ* TUNEL technique. Figure 5

summarizes the results and figure 4 shows examples. As seen in figure 5, a 72-hour treatment with losartan (10^{-7} M) alone did not affect apoptosis. Ang II alone had no effect at 10^{-16} M on the apoptosis of hEECRs and hEECLs. However, treatment with 10^{-13} M of Ang II significantly induced apoptosis in both right and left ventricular hEECs. Increasing the Ang II concentration to 10^{-8} M further induced apoptosis in both cell types. Pretreating the cells with either the AT_1 receptor antagonist, losartan, or the AT_2 receptor antagonist, PD123319, in the presence of different concentrations of Ang II partially but significantly prevented the Ang II-induced apoptosis in hEECRs (Fig. 5). However, the AT_1 receptor antagonist did not affect Ang II-induced apoptosis of hEECLs. Pretreatment with the AT_2 receptor antagonist, PD123319 (10^{-7} M) completely prevented the Ang II-induced apoptosis at all the concentrations used (10^{-13} and 10^{-8} M).

Discussion

Using annexin V and *in situ* TUNEL techniques, our results showed that chronic treatment with Ang II (72 hours) induced apoptosis in both hEECRs and hEECLs. Our results using annexin V and *in situ* TUNEL showed that AT_2 receptor blockade with PD123319 prevented Ang II-induced apoptosis in both right and left hEECs. These results clearly demonstrate the implication of the AT_2 receptor in life and death decisions in response to Ang II. Although the contribution of the AT_2 receptor to Ang II-induced apoptosis is similar in both types of EECs, the contribution of the AT_1 receptor seems to be cell type-dependent. The latter Ang II receptor type seems to partially prevent the Ang II-induced apoptosis in hEECRs. However, it seems to potentiate the apoptotic effect of Ang II in hEECLs. This suggests that, in EECs, the AT_1 receptor participates in apoptosis since annexin V is a more sensitive technique than *in situ* TUNEL and detects

earlier stages of apoptosis than the *in situ* TUNEL technique (Vermes et al. 1995). Similar results have been reported showing AT₁ receptor involvement in the apoptosis of rat cardiomyocytes (Kajstura et al. 1997, LI et al. 2002) and VSMCs (Bascands et al. 2001) in culture. Also, our results show that PD123319 completely prevents Ang II-induced apoptosis in both EECRs and EECLs. This suggests that the AT₂ receptor is the primary mediator of apoptosis in EECs in general. These results are similar to those reported in PC12W cells (rat pheochromocytoma cell line) (Yamada et al. 1996), rat granular follicular cells (Tanaka et al. 1995), neonatal rat neurons in culture (Shenoy et al. 1999) and R3T3 cells (mouse fibroblast cell line) (YAMADA et al. 1996). It is also shown in the literature that both receptors participate in the apoptosis of HUVECs (Dimmeler et al. 1997) and endothelial cells of the human coronary artery (Li et al. 1999). These observations are in agreement with the results that we obtained in EECRs.

Interestingly, our results suggest that in EECLs, AT₁ receptor inhibition and AT₂ receptor activation alone induce apoptosis and that Ang II amplifies this effect. These results suggest that the AT₁ receptor may act as a physiological antagonist of the AT₂ receptor. This interaction between the two receptors was also reported in the literature, where it has been proposed that the AT₂ receptor may act as an AT₁ receptor antagonist in PC12 cells, in rat fetal fibroblasts (Abdalla et al. 2001) and rat cardiomyocytes (Masaki et al. 1998). Moreover, it was reported that the ratio of AT₁/AT₂ receptors is a determining factor in the effect of Ang II in endothelial cells and that disrupting this ratio can induce apoptosis of these cells (Watanabe et al. 2005). Also, Miura et al. proposed that Ang II-induced AT₁ receptor signaling may be blocked by AT₂ receptor signaling via their negative cross-signaling rather than by their membrane surface heterodimerization. (Miura et al.

2010). The same group proposed that the balance of AT₁/AT₂ receptor ratio is critical in the antagonistic actions between the two receptors (Miura et al. 2010). Hence, the difference in the receptor type involved in Ang II-induced apoptosis between EECRs and EECLs in our study could be due to the difference in the ratio of AT₁/AT₂ receptors at the plasma membrane level in these two cell types (Jacques et al. 2017b).

Our results also show that, as in EECRs, at the level of EECLs, PD123319 prevents apoptosis induced by Ang II. Thus, the AT₂ receptor mediates apoptosis induced by Ang II at the level of EECRs. The *in situ* TUNEL technique labels the 3'-OH residues, which are found at the ends of broken DNA fragments, indicating that the cells have already entered apoptosis (Gavrieli et al. 1992). It is, therefore, possible to postulate that chronic activation of the AT₂ receptor is responsible for entry into apoptosis as well as the final phase of apoptosis. On the other hand, the chronic activation of the AT₁ receptor is involved in the entry of EECRs into apoptosis and the prevention of EECLs from entering apoptosis. The results from this study are additional proof to the presence of differences in properties between EECRs and EECLs as previously demonstrated by our laboratory (Jacques et al. 2000a, 2003b, 2005, 2006, 2007, 2017; Jacques and Abdel-Samad, 2007; Abdel-Samad et al. 2012, 2016; Jules et al. 2015).

In conclusion, our results show that cardiac hypertrophic factors, such as Ang II, induce apoptosis of EECs and thus exposing cardiomyocytes to circulating factors which may result in hypertrophy and severe arrhythmia. Our study also supports the concept of using AT₂ receptor antagonists to protect EECs physical and secretory properties which may prevent Ang II-induced cardiac hypertrophy and arrhythmia. However, more studies are

needed in order to elucidate the downstream signaling events induced by Ang II leading to the apoptosis of EECRs and EECLs.

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Figure legends

Figure 1: Effect of treatment with different concentrations of Ang II on Annexin V labeling in hEECRs and hEECLs. Quantitative 3D confocal microscopy images showing Annexin V labeling in EECRs (a) and EECLs (b) before (control) (A) and after 72 hours of treatment with increasing concentrations of Ang II: 10^{-16} (B), 10^{-13} (C) and 10^{-8} M (D),

Figure 2: Effect of AT₁ and AT₂ receptor antagonists on Ang II-induced apoptosis of hEECRs and hEECLs using annexin V labeling. Quantitative 3D confocal microscopy images showing Annexin V labeling in hEECRs (a) and hEECLs (b) pretreated with 10^{-7} M of losartan (A) or PD123319 (C) followed by treatment with Ang II 10^{-8} M (B and D, respectively).

Figure 3: Effect of treatment with Ang II and its receptor antagonists on the Annexin V labeling of REECs and LEECs. Graphs showing the ratio of the number of REECs (A) or LEECs (B) labeled with Annexin V to the total number of REECs or LEECs respectively. The results were obtained in REECs and LEECs in the absence of Ang II (control) and after treatment for 72 hours with increasing concentrations of Ang II (10^{-16} , 10^{-13} and 10^{-8} M, orange curve), or in cells pre-treated with losartan (10^{-7} M, green curve), or pretreated with PD123319 (10^{-7} M, blue curve), followed by treatment with Ang II at the three concentrations used. The values are expressed as mean $\pm$ S.E.M. * $p < 0.05$; *** $p < 0.001$ vs. control without Ang II. \$ $p < 0.05$; \$\$\$ $p < 0.001$ losartan + Ang II vs. losartan without Ang II. + $p < 0.05$; ++ $p < 0.01$; +++ $p < 0.001$ losartan + Ang II 10^{-8} M vs. Ang II 10^{-8} M. ### $p < 0.001$ PD123319 + Ang II vs. Ang II. N is the number of different experiments.

Figure 4: Effect of treatment with Ang II and its receptor antagonists on EECR and EECL apoptosis assessed by *in situ* TUNEL assay. Fluorescence microscopy images showing TUNEL-positive EECRs (a) and EECLs (b) before (control, A) and after 72-hour treatment with Ang II (10^{-8} M) alone (C), or in the presence of losartan (10^{-7} M, E) or PD123319 (10^{-7} M, G). The panels B, D, F and H show the labeling of the nuclei of the cells in panels A, C, E and G respectively with dapi. Each image is a representative of four different experiments.

Figure 5: Effect of treatment with Ang II and its receptor antagonists on the ratio of TUNEL positive REECs and LEECs to the total number of cells. The graph presents the ratio of the number of TUNEL-positive REECs (A) or LEECs (B) to the total number of REECs or LEECs respectively. The results were obtained in REECs and LEECs in the absence of Ang II (control) and after the treatment for 72 hours with increasing concentrations of Ang II (10^{-16} , 10^{-13} and 10^{-8} M, orange curve), or cells pretreated with losartan (10^{-7} M, green curve), or pretreated with PD123319 (10^{-7} M, blue curve), followed by treatment with Ang II at the three concentrations used. The values are expressed as mean $\pm$ S.E.M. ** $p < 0,01$; *** $p < 0,001$ vs. control without Ang II. \$\$ $p < 0,01$; \$\$\$ $p < 0,001$ losartan + Ang II vs. losartan without Ang II. +++ $p < 0,001$ losartan + Ang II vs. Ang II. ## $p < 0,01$; ### $p < 0,001$ PD123319 + Ang II vs. Ang II. N represents the number of different experiments.

Figure 1: Effect of treatment with different concentrations of Ang II on Annexin V labeling in hEECRs and hEECLs. Quantitative 3D confocal microscopy images showing Annexin V labeling in EECRs (a) and EECLs (b) before (control) (A) and after 72 hours of treatment with increasing concentrations of Ang II: 10⁻¹⁶ (B), 10⁻¹³ (C) and 10⁻⁸ M (D),

186x102mm (300 x 300 DPI)

Figure 2: Effect of AT1 and AT2 receptor antagonists on Ang II-induced apoptosis of hEECRs and hEECLs using annexin V labeling. Quantitative 3D confocal microscopy images showing Annexin V labeling in hEECRs (a) and hEECLs (b) pretreated with 10⁻⁷ M of losartan (A) or PD123319 (C) followed by treatment with Ang II 10⁻⁸ M (B and D, respectively).

185x108mm (300 x 300 DPI)

Figure 3: Effect of treatment with Ang II and its receptor antagonists on the Annexin V labeling of REECs and LEECs. Graphs showing the ratio of the number of REECs (A) or LEECs (B) labeled with Annexin V to the total number of REECs or LEECs respectively. The results were obtained in REECs and LEECs in the absence of Ang II (control) and after treatment for 72 hours with increasing concentrations of Ang II (10⁻¹⁶, 10⁻¹³ and 10⁻⁸ M, orange curve), or in cells pre-treated with losartan (10⁻⁷ M, green curve), or pretreated with PD123319 (10⁻⁷ M, blue curve), followed by treatment with Ang II at the three concentrations used. The values are expressed as mean ± S.E.M. * p < 0.05; *** p < 0.001 vs. control without Ang II. \$ p < 0.05; \$\$\$ p < 0.001 losartan + Ang II vs. losartan without Ang II. + p < 0.05; ++ p < 0.01; +++ p < 0.001 losartan + Ang II 10⁻⁸ M vs. Ang II 10⁻⁸ M. ### p < 0.001 PD123319 + Ang II vs. Ang II. N is the number of different experiments.

166x230mm (300 x 300 DPI)

Figure 4: Effect of treatment with Ang II and its receptor antagonists on EECR and EECL apoptosis assessed by in situ TUNEL assay. Fluorescence microscopy images showing TUNEL-positive EECRs (a) and EECLs (b) before (control, A) and after a 72-hour treatment with Ang II (10^{-8} M) alone (C), or in the presence of losartan (10^{-7} M, E) or PD123319 (10^{-7} M, G). The panels B, D, F and H show the labeling of the nuclei of the cells in panels A, C, E and G respectively with dapi. Each image is a representative of four different experiments.

179x143mm (300 x 300 DPI)

Figure 5: Effect of treatment with Ang II and its receptor antagonists on the ratio of TUNEL positive REECs and LEECs to the total number of cells. The graph presents the ratio of the number of TUNEL-positive REECs (A) or LEECs (B) to the total number of REECs or LEECs respectively. The results were obtained in REECs and LEECs in the absence of Ang II (control) and after the treatment for 72 hours with increasing concentrations of Ang II (10-16, 10-13 and 10-8 M, orange curve), or cells pretreated with losartan (10-7 M, green curve), or pretreated with PD123319 (10-7 M, blue curve), followed by treatment with Ang II at the three concentrations used. The values are expressed as mean ± S.E.M. ** p < 0,01; *** p < 0,001 vs. control without Ang II. \$\$ p < 0,01; \$\$\$ p < 0,001 losartan + Ang II vs. losartan without Ang II. +++ p < 0,001 losartan + Ang II vs. Ang II. ## p < 0,01; ### p < 0,001 PD123319 + Ang II vs. Ang II. N represents the number of different experiments.

169x216mm (300 x 300 DPI)