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ROLE OF ENDOTHELIN-1 AND ITS RECEPTORS ET_A AND ET_B IN THE SURVIVAL OF HUMAN VASCULAR ENDOTHELIAL CELLS.

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Short title: ET-1 receptors in vascular endothelial cell survival

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Abstract

Our previous work showed the presence of endothelin-1 (ET-1) receptors, ET_A and ET_B, in human vascular endothelial cells (hVECs). In this study, we wanted to verify whether ET-1 via the activation of its receptors ET_A and/or ET_B (ET_AR and ET_BR respectively) play a role in the survival of hVECs. Our results showed that treatment of hVECs with ET-1 prevented apoptosis induced by genistein, an effect that was mimicked by treatment with ET_BR specific agonist IRL1620. Furthermore, blockade of ET_BR with the selective ET_BR antagonist A192621 prevented the anti-apoptotic effect of ET-1 in hVECs. However, activation of ET_A receptor alone did not seem to contribute to the anti-apoptotic effect of ET-1. In addition, the anti-apoptotic effect of ET_BR was found to be associated with caspase 3 inhibition and does not depend on the density of this type of receptor.

In conclusion, our results showed that ET-1 possesses an anti-apoptotic effect in hVECs and that this effect is mediated, to a great extent, via the activation of ET_BR. This study revealed a new role of ET_BR in the survival of hVECs.

Keywords: Endothelin-1, human vascular endothelial cells, ET_A receptor, ET_B receptor, apoptosis

Introduction

The vascular endothelium forms a continuous monolayer of cells lining the inner vascular wall (Mudrovic et al. 2017). This type of cell presents a large surface area for molecule exchange between the blood and tissues (Baldwin and Thurston 2001). The endothelial cells respond to chemical and physical stimuli by synthesizing and releasing a variety of vasoactive substances (Cines et al. 1998; Triggle et al. 2012; Mudrovic et al. 2017). The equilibrium between these substances is necessary for the regulation of vascular tone (Shepro and D'amore 1984; Triggle et al. 2012; Radenkovic et al. 2016). Among the most important vasoconstrictors secreted by the endothelium is endothelin-1 (ET-1) (Yanagisawa et al. 1988). ET-1 mediates its physiological actions via two types of G-protein coupled receptors namely ET_A receptors (ET_ARs) and ET_B receptors (ET_BRs) (Arai et al. 1990; Sakurai et al. 1992; Avedanian et al. 2010; Houde et al. 2016). Both ET_A and ET_B receptors are reported to be present in vascular smooth muscle cells (VSMCs) and are mainly responsible for the vasoconstrictor effects of endothelin-1 (Davenport and Maguire 1994; Sudjarwo et al. 1993; Bigaud and Pelton 1992; Cristol et al. 1993; Bkaily et al. 2011, 2015). Both types of ET-1 receptors were reported to be present in human vascular (Avedanian et al. 2010) and endocardial endothelial (Abdel-Samad et al. 2016; Jules et al. 2015) cells. Furthermore, these two receptors contribute to ET-1 induced increase of intracellular calcium in human endocardial endothelial cells (EECs) (Abdel-Samad et al. 2016; Jules et al. 2015) whereas this effect is mediated by only ET_AR in human vascular endothelial cells (hVECs) (Avedanian et al. 2010). Although the role of ET_BR in hVECs function is still to be determined, literature in the field reports a controversial role of ET_BR in survival of different cell types. For example, ET_BR has no effect on survival of rat cardiomyocytes (Ren et al. 2008) whereas in other

cell types including human and pulmonary arterial smooth muscle cells, glioma cell lines and rat aortic endothelial cells, ET_BR (not ET_AR) is anti-apoptotic (Shichiri et al. 1997; De Miguel et al. 2017; Sakai et al. 2016; Paolillo et al. 2010). As for ET_BRs, the role of ET_ARs in cell survival is also controversial (Shichiri et al. 1997; Ren et al. 2008; De Miguel et al. 2017, Maffei et al. 2014; Azova et al. 2012; Paolillo et al. 2010). As for its receptors, ET-1 was found to be pro-apoptotic or anti-apoptotic depending on cell type (Ren et al. 2008).

Taking into account all the above, in the present study, the specific ET_AR antagonist (ABT-627) and ET_BR antagonist (A-192621) were used to investigate the effect of ET-1 on survival of hVECs.

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Materials and Methods

All methods used for cell isolation and culture, indirect immunofluorescence, confocal microscopy setting, Fura-2 loading, nuclear SYTO-11 staining, solutions, and sources of all compounds, as well as statistical analysis, were the same as those reported previously (Bkaily et al. 1997, 2008; Avedanian 2010).

Isolation and culture of vascular endothelial cells

All work was done in accordance with the requirements of the institutional review committee for the use of human material. Human VECs were isolated from non-diseased aortas of healthy donors (16-45 years old) supplied by Quebec Transplant. The freshly isolated and cultured aortic endothelial cells used in this study originated from at least 3 different donors and at low passages (2-4). Briefly and as previously described (Bkaily et al. 1997, 2008; Avedanian et al. 2010), the aortas were placed for 15 min in M199 medium (Gibco-BRL, Burlington, Ont.) containing antibiotics and 0.1% (50 U/mL) collagenase. Next, the medium was replaced by a fresh, collagenase-free M199 solution containing antibiotics. The VECs were then gently scraped with a sterile scalpel blade and the cell-containing medium was collected and centrifuged. Cells were resuspended and cultured in a medium composed of M199 medium containing 10% fetal bovine serum and 50 IU/mL penicillin-G-potassium (Ayrest, Toronto, Ont.). A routine check of quality and purity of the hVECs was done using specific probes as indicated elsewhere (Bkaily et al. 1997; Avedanian et al. 2010). The aortic hVECs derivation was also confirmed by assessing the presence of the Von Willebrand factor (Bkaily et al. 1997; Avedanian et al. 2010). Furthermore, functional quality control of freshly cultured (used

within 12 h of culture) and long-term cultured (passage 4 to 6) cells was routinely done by ensuring that both had similar densities and distribution of ET-1 and Ang II receptors and responded similarly to ET-1, Ang II, and high extracellular K^+ (30 mmol/L) by increasing $[Ca^{2+}]_i$ (Bkaily et al. 1997; Avedanian et al. 2010).

Annexin V labeling

The Annexin V labeling was performed according to the protocol provided with “Annexin-V-fluos Staining Kit” (Roche Applied Science). Briefly, hVECs were cultured on 25 mm round cover slips until they reach the desired confluence (~ 70 %) and then the cells were treated according to each protocol. The extracellular medium in each Petri dish was then injected with 75 μ l of the Annexin-V-Fluos working solution prepared by mixing 20 μ l of stock probe in 1 ml of incubation buffer. The cells were incubated in the presence of the probe for 30 min at room temperature. The coverslips were then observed by a confocal microscope using the 488-nm wavelength line.

Terminal deoxynucleotidyl transferase dUTP mediated nick end labeling (TUNEL)

The *in situ* TUNEL technique was performed according to the protocol provided with the “ApopTag fluorescein *in situ* detection kit” (Cedarlane Laboratories limited, Ontario, CA) with some modifications (Beauséjour et al. 2013). Briefly, cultured hVECs (on 12-well plates) were fixed with a 2% formaldehyde/phosphate buffer (0.2 M Na_2HPO_4 pH 7.4) for 45 min at 4°C. The fixation reaction was stopped by quenching with PBS-glycine (150 mM pH 7.2) for 15 min at RT. The cells were then permeabilized with a fresh 0.1% PBS-Triton-X-100 solution for 3 min at RT. The non-specific sites were blocked with a non-fat dried milk (NFDM) (10%) solution containing Na^+ -azide followed by incubation with an equilibrium buffer for 5 min at RT. The reaction was then started by adding the

enzyme solution (TdT diluted 2.4 times in the reaction buffer) and incubating at 37 °C for 1 hour. After that, the reaction was stopped by incubating the cells in the stop buffer (diluted 1/34 in water) during 10 min at RT followed by blocking with an NFDM solution (10%) containing Na⁺-azide (0.05%) for 15 min at RT. The cells were then incubated with the anti-dioxigenin antibody coupled to fluorescein (diluted 2.13 times in the blocking solution) for 45 min at RT in a humid chamber. After that, the cells were colored with an Evans blue solution (0.01%) (Sigma-Aldrich, St-Louis, MO) for 30 sec and in a DAPI solution (dilution 1/50 000 from a 10 mg/ml solution) for 5 min and washed twice with PBS 1X for 10 min. Finally, the coverslips with cells were mounted into a solution of glycerol-PBS (9:1) containing 0.1 % of paraphenylenediamine (Sigma-Aldrich, St-Louis, MO) and observed with a Leica DM-RxA (Leica, St-Laurent, Qc) fluorescence microscope.

Assessment of Apoptosis

After obtaining the images from confocal microscopy (for Annexin V) and fluorescence microscopy (for TUNEL), the number of total and apoptotic cells was counted, in five different fields of vision, in order to obtain a minimum of 500 cells per treatment. After that, the percentage of apoptotic cells to total number of cells was calculated for control and treated conditions.

Indirect immunofluorescence for ET_BR

The protocol for indirect immunofluorescence was previously described (Avedanian et al. 2010). In brief, aortic hVECs were fixed for 10 min in ice cold 4% paraformaldehyde followed by washing with PBS 1X. The cells were then incubated for 10 min with PBS

containing sodium borohydride (2 mg/mL), permeabilized and blocked with 0.1% Triton X-100, 7% normal serum (NS), and 5% non-fat dry milk (NFDM) for 30 min. Finally, the cells were washed twice in PBS and incubated overnight at 4 °C with PBS containing 1.4% NS, 1% NFDM, and 0.1% Triton X-100 in the presence of a rabbit polyclonal anti-ET_B antibody (1/200) (Alamone labs, Jerusalem, Israel). After two PBS washes, the cells were incubated for 1 h at room temperature with the same buffer as the primary antibody in addition to an anti-rabbit secondary antibody coupled to Alexa Fluor 488 (1 µg/mL) (Molecular Probes, Eugene, Ore.). The cells were examined using 3D imaging confocal microscopy.

Indirect immunofluorescence for caspase 3

Briefly, cultured hVECs were fixed with paraformaldehyde (2% in PBS 1X) for 45 min at 4°C, washed three times (5 min each) with PBS 1X and then incubated with PBS 1X/Glycine (150 mM pH 7.2) for 45 min at 4 °C. The cells were then permeabilized with fresh 0.2% PBS-Triton-X-100 solution for 5 min at RT and then washed twice with PBS 1X (5 min each). After that, blocking was performed with an NFDM (10%) solution containing Na⁺-azide (0.05%) for 30 min at RT followed by two washes. The cells were then incubated with the cleaved caspase-3 (Asp175) antibody (Cell Signaling, Boston, MA) at a dilution of 1:100 in an NFDM 5% solution for 30 min at RT in a humid chamber. After that the cells were washed for three times and incubated with the anti-mouse secondary antibody conjugated to AlexaFluor 488 (Molecular Probes, Eugen, OR) diluted at 1:400 in the same solution as the primary antibody. After two washes with PBS 1X, the cells were colored with an Evans blue solution (0.01%) (Sigma-Aldirch, St-Louis, MO) for 30 sec and with a DAPI solution (dilution 1/50 000 from a 10 mg/ml

solution) for 5 min and washed twice with PBS 1X for 10 min. Finally, the coverslips with cells were mounted into a solution of glycerol-PBS (9:1) containing 0.1 % of paraphenylenediamine (Sigma-Aldrich, St-Louis, MO) and observed with a Leica DM-RxA (Leica, St-Laurent, Qc) and/or a Nikon eclipse E 1000 (Nikon, Mississauga, ON) fluorescence microscope.

Confocal microscopy

Cells were examined with a Molecular Dynamics (Sunnyvale, Calif.) Multi Probe 2001 confocal argon laser scanning (CSLM) system equipped with a Nikon Diaphot epifluorescence inverted microscope and a 60× (1.4 NA) Nikon Oil Plan achromat objective as described previously (Bkaily et al. 1997, 2017). In brief, 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% to 3% neutral density filter to reduce photobleaching. Pinhole size was set at 100 μm . The image size was set at 512 pixels $\times$ 512 pixels with a pixel size of 0.34 μm . Laser line intensity, photometric gain, PMT settings, and filter attenuation were kept rigorously constant throughout the experimental procedures. For each sample, 16 to 20 sections were recorded covering the entire volume of the cell and constituting a 3D 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, Eugene, Ore.) 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. Reconstruction of 3D images was performed on unfiltered serial sections and images were represented as top-view maximum intensity 3D projections. $\text{ET}_\text{A}\text{R}$ and $\text{ET}_\text{B}\text{R}$ distribution images were represented as pseudo-colored

representations according to an intensity scale of 0 to 255 nm with zero intensity in black and maximum intensity in white.

Volume rendering and fluorescence intensity measurement

Measurement of receptor or protein densities within the cytosol and nucleus was performed on quantitative real 3D images as described previously (Bkaily et al. 1997, 2017). In brief, the nuclear area following SYTO-11 staining was isolated from the rest of the cell by setting a lower intensity threshold filter to confine relevant pixels. A 3D binary image series of the nuclear volume was then generated for each cell using the exact same x , y , and z set planes. By applying these binary image patterns of the nucleus to the same cell but labeled for the peptide or the receptors (the binary image serves a cookie cutter), a new 3D projection was created depicting fluorescence intensity levels exclusively within the nucleus. Hence, by removing the nucleus from the surrounding cytoplasm, we were then able to measure mean fluorescence intensity values in the entire nuclear or cytosolic volume.

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. Following the apoptosis-inducing protocols, the extracellular medium was collected and centrifuged at in order to conserve the suspended cells. The cells were then resuspended in 1 ml of PBS 1X followed by centrifugation. Then 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). The laemmli solution containing the cells was

then transferred to the microtube containing the pellet of resuspended cells. Proteins (70 µg) were then separated by SDS-PAGE gel electrophoresis (10% gels) and transferred on nitrocellulose membranes (Amersham-Pharmacia Biotech, Baie d'Urfé, Que.) Membranes were blocked for 2 h at room temperature in PBS 1X containing 6% NFDM and 0.05% Tween and then incubated overnight at 4 °C with an anti-ET_B antibody (10 µg/ml) (Alomone Labs, Jerusalem, Israel). After washing in PBS containing 0.05% Tween, PBS with 5% NFDM buffer was used for incubation with anti-rabbit IgG for ET_B (1/5000) (GE Healthcare Bio-Sciences Inc., Baie d'Urfé, Que.) for 1 h at RT. The immune complexes were then detected by chemiluminescence (ECL, Roche Diagnostics Co, Indianapolis, Ind.) and visualized by autoradiography (BIOMAX MR Eastman Kodak Company, Rochester, N.Y.). A positive control was performed using A549 cell extracts which strongly express ET_BR. In addition, a negative control using the peptide antigen control of the anti-ET_BR antibodies was done which showed the specificity of the antibody used.

For each western blot, in order to insure that the quantities of proteins loaded in the wells are equivalent, a control was obtained where the membrane was incubated for 30 min at RT with a mouse monoclonal anti-β-actin antibody (dilution 1/3000).

Densitometry

In order to quantify the levels of protein expression, densitometric analyses were performed using the MCID Basic-M5 program (Imaging Research, St. Catherines, Ontario, Canada). The Western blot films were digitalized using a camera (MTI CCD72; Imaging Research, St. Catherines, Ontario, Canada) and the density of each band was

measured over the band's area. The background value was subtracted from the value obtained. Hence, the final result was $[(\text{Density} \times \text{Area of band}) - \text{Background}]$.

Statistics

Measurements of fluorescence intensities were presented as mean intracellular fluorescence intensity values. All values were expressed as means $\pm$ S.E.M (standard error of the mean), where 'n' represents the number of cells in at least 3 different experiments. Statistical significance was determined using the Student's *t* test and/or one-way ANOVA followed by posthoc Bonferroni analysis where applicable (GraphPad Prism). A *p* value < 0.05 was considered as significant.

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Results

Effect of ET-1 and its receptors on apoptosis induced by genistein

In this series of experiments, we wanted to study the effect of ET-1 and its receptors on genistein-induced apoptosis in hVECs using two complementary methods namely Annexin V labeling and *in situ* TUNEL assays.

In the first series of experiments, cultured hVECs were treated with genistein (300 μ M) for 24 hours in the presence and absence of ET-1 (10^{-7} M) or the specific ET_B receptor agonist IRL1620 (10^{-7} M). Figure 1A-D shows examples and figure 1E summarizes the results. As can be seen in this figure, a 24-hr treatment with genistein induced an apparent increase in the number of cells labeled with annexin V (Fig. 1B) compared to control (Fig. 1A). However, in the presence of ET-1 (10^{-7} M), treatment with genistein did not cause an apparent change in the number of annexin V-labeled cells (Fig. 1C) compared to control (Fig. 1A). Similarly, a 24-hr treatment with genistein in the presence of the specific ET_B receptor agonist IRL1620 (10^{-7} M) had no effect on the number of annexin V-labeled cells (Fig. 1D) compared to control. Figure 1E shows that there was a significant increase ($p < 0.001$) in the apoptotic index of hVECs (the percentage of annexin V-labeled cells with respect to total number of cells) following genistein treatment for 24 hours compared to control. However, the presence of ET-1 attenuated significantly ($p < 0.01$) the apoptotic index of hVECs. Similarly, in the presence of the ET_BR specific agonist IRL1620 the apoptotic index of hVECs was significantly decreased ($p < 0.01$) back to control level.

In order to identify the ET-1 receptor subtype mediating the anti-apoptotic effect of ET-1, hVECs were treated for 24 hours with genistein in the presence of ET-1 (10^{-7} M) and either the ET_AR antagonist, ABT-627 (10^{-7} M) or the ET_BR antagonist, A-192621 (10^{-7} M). As figure 1E shows, pretreatment with ET_AR antagonist, ABT-627, did not affect the anti-apoptotic effect of ET-1 on genistein-induced apoptosis. In a last series of experiments, the cells were treated with the specific ET_BR antagonist A-192621 followed by addition of Et-1 and genistein. As can be seen in figure 1E, blockade of ET_BR completely prevented ET-1 from preventing apoptosis induced by genistein.

In the next series of experiments, the same protocols described above were repeated but apoptosis was assessed using *in situ* TUNEL assays. Figure 2A-L shows examples and figure 2M summarizes the results. As can be seen in this figure, treatment for 24 hours with genistein (300 μ M) caused an apparent increase in the number of TUNEL positive (TUNEL+) cells compared to control (Fig. 2M). Moreover, in the presence of ET-1 there was a significant ($p < 0.05$) decrease of the apoptotic index compared to genistein alone. Similar to ET-1, pre-treatment with the ET_BR agonist IRL1620, also significantly ($p < 0.05$) prevented genistein from increasing the apoptotic index. In another series of experiments, the cells were first treated with the ET_AR antagonist, ABT627, and then ET-1 and genistein were added for 24 hours. As can be seen, blockade of ET_AR did not prevent ET-1 from decreasing the apoptotic index induced by gensitein ($p < 0.05$). In the last series of experiments, pretreatment with the ET_BR antagonist, A192621, followed by exposure of the cells to ET-1 and genistein for 24 hours completely prevented ET-1 from preventing genistein from inducing apoptosis of hVECs (Fig. 2M).

Effect of genistein on ET_BR relative density

In the first series of experiments, we used Western blot in order to determine whether genistein-induced apoptosis was accompanied by changes in total ET_BR relative density. In addition, we verified whether ET-1 or the ET_BR agonist IRL1620 (10⁻⁷M) prevented these changes. Figure 3A shows an example and figure 3B summarizes the results. As seen in this figure, a 24-hr treatment with genistein induced a significant decrease ($p<0.01$) in ET_BR relative density. Neither ET-1 nor the ET_BR agonist prevented genistein from significantly decreasing the total ET_BR relative density.

In another series of experiments, we used the quantitative 3D imaging technique in order to verify whether the observed decrease of relative ET_BR density was at the cytosolic (including plasma membrane, PM) and/or nuclear (including nuclear envelope membranes, NEM) levels. Figure 4A-F shows examples of the effects of genistein and ET-1 and figure 4G summarizes the results. As can be seen in figure 4A, and as expected, the relative density of ET_BR in hVECs was very high at the nuclear level and treatment for 24 hours with genistein apparently decreased the relative density of ET_BR (Fig. 4C) which is significant only at the nuclear level (Fig. 4G). Treatment with ET-1 alone induced an increase in ET_BR relative density only at the cytosolic (including PM) level ($p<0.001$) (Fig. 4G). As can be seen in figure 4E and G, ET-1 prevented the decrease in nuclear (including NEM) ET_BR relative density along with increasing the relative density of this type of receptor at the cytosolic level ($p<0.01$, genistein vs. ET-1+genistein). However, treatment with the ET_BR agonist did not prevent genistein from inducing a significant decrease in nuclear (including NEM) relative density of ET_BR (genistein vs. IRL1620+genistein). In another series of experiments, blockade of ET_AR with ABT627 significantly ($p<0.05$) reduced the ET-1 prevention of genistein induced decrease in

ET_BR relative density. In another series of experiments, blockade of ET_BR with A-192321 did not affect ET-1 from preventing the genistein induced decrease in ET_BR relative density at both the cytosolic (including PM) and nuclear (including NEM) levels.

Effect of genistein in the absence and presence of ET-1 and IRL1620 on caspase-3.

In this series of experiments, using indirect immunofluorescence technique, our results showed that a 24-hr treatment with genistein significantly ($p < 0.001$) increased the percentage of cells with active caspase-3 (Fig. 5A-D and I). Pre-treatment with either ET-1 or the ET_BR agonist IRL1620 significantly decreased ($p < 0.001$) the genistein-induced increase of the percentage of cells with active caspase-3 (Fig. 5E-H and I).

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Discussion and conclusion

Survival of vascular endothelial cells in response to mechanical and chemical damage is crucial for maintaining normal functioning of the cardiovascular system. Several circulating cardiovascular active ligands may directly or indirectly affect life and death of VECs. These cells secrete several factors including ET-1 in different cardiovascular pathologies such as hypertension (Taddei et al. 1996), heart failure (Monnink et al. 2002) and atherosclerosis (Turk et al. 2005). This endothelial factor is reported to be either pro-apoptotic or anti-apoptotic. In this study, we used genistein, a well-known pan-inhibitor of protein tyrosine kinase activity to induce apoptosis (Anderson 1997) of VECs. It is often used to induce caspase-dependent apoptosis. In addition, it is used as an experimental "positive control" of induced caspase-dependent apoptosis (Gauthier et al. 2001a,b; Vachon et al. 2002; Dufour et al. 2004; Harnois et al. 2004).

Our results show that in human VECs, ET-1 promotes survival by preventing apoptosis of this cell type. It is difficult to make a generalization concerning the role of ET-1 in cell survival since it is reported to be pro-apoptotic or anti-apoptotic depending on cell type as well as on the ratio of ET_A/ET_B receptor subtypes (Ren et al. 2008). It is worth mentioning that the finding that ET-1 as anti-apoptotic mediator in hVECs is similar to that reported in rat VECs (Shichiri et al. 1997; Jankov et al. 2006). However, our results show that the anti-apoptotic effect of ET-1 in hVECs is mediated via activation of ET_{BR} and not ET_{AR} which is different from what is reported in rat VECs (Ren et al. 2008; Jankov et al. 2006). In addition, the anti-apoptotic effect of ET_{BR} activation was found to be associated with caspase 3 inhibition. This study looked at caspase-dependent apoptosis (also known as "intrinsic" or "common" or "apoptosome-driven" pathway of apoptosis

(Kroemer et al. 2009; Vachon, 2011). Hence, there are no other mechanisms to be taken into account experimentally in this study since caspase 3 is a predominant executioner caspase in caspase-dependent apoptosis (Julien and Wells 2017; Galluzzi et al. 2015; Vachon, 2011).

Thus, it is possible to postulate that the role of ET-1 in survival and the type of receptor implicated may also vary depending on the species studied. Our results also suggest that, although an increase of ET-1 can contribute to hypertension and VSM proliferation, this increase can also protect VECs from undergoing apoptosis. Our results also suggest that ET_BRs, in addition to their role in releasing NO from VECs, can also promote survival and remodeling by preventing caspase-3 activation.

Our results also show that apoptosis in hVECs induced by genistein is accompanied by a decrease in the total relative density of ET_BRs. Using the very powerful technique of quantitative 3D imaging for measuring the relative density of receptors demonstrated that this decrease of ET_BR relative density by genistein takes place at the nuclear level (including NEM). This technique also revealed that ET-1 does prevent the decrease of nuclear ET_BRs induced by genistein. These results suggest that nuclear membranes ET_BRs may play an important role in life and death of hVECs. Our results also show that the prevention of genistein-induced decrease in nuclear ET_BRs by ET-1 is mediated via the activation of ET_ARs. These results suggest that the anti-apoptotic effect of ET-1 is due to both activation of ET_BRs as well as to maintaining the density of the nuclear ET_BRs via activation of ET_ARs which is reported to increase cytosolic and nuclear calcium in hVECs (Avedanian et al. 2010). These results are in accordance with the reported concept that ET-1 does modulate the synthesis of its own receptors.

Finally, our results also suggest that a crosstalk between ET_ARs and ET_BRs does exist and this can take place as a receptor monomer or as a heterodimer. In addition, ET-1 may also play a role in the suppression of caspase-independent apoptosis, autophagy and/or necroptosis. These latter concepts need to be explored.

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Figure 1. Effect of ET-1 and its receptors ET_A and ET_B on apoptosis induced by genistein assessed by annexin V staining.

Real 3D confocal microscopy images of annexin V staining performed in hVECs treated for 24 hours with a control medium (A), or with genistein (300 μ M) alone (B) or together with ET-1 10^{-7} M (C) or with the ET_BR specific agonist IRL-1620 (10^{-7} M) (D). The white scale bar is in μ m. (E) The histogram shows the effect of different treatments on apoptosis induced by genistein (300 μ M). Annexin V staining was done in order to establish the apoptosis index which is expressed as the percentage of apoptotic cells with respect to the number of total counted cells for a given treatment. A minimum of 500 cells per experiment were counted for at least 3 independent experiments. The values are expressed as mean $\pm$ S.E.M. * $p < 0.05$ and *** $p < 0.001$ vs. Ctrl. ♦ $p < 0.05$, ♦♦ $p < 0.01$ vs. genistein treatment.

Figure 2. Role of ET-1 receptors in genistein-induced apoptosis of hVECs assessed by *in situ* TUNEL assays.

Representative micrographs of *in situ* TUNEL performed in hVECs treated for 24 hours with a control medium (A), or with genistein (300 μ M) (C), ET-1 (10^{-7} M) + genistein (300 μ M) (E), IRL-1620 (10^{-7} M) + genistein (300 μ M) (G), ET-1 (10^{-7} M) + ABT-627 (10^{-7} M) + genistein (300 μ M) (I), or ET-1 (10^{-7} M) + A-192621 (10^{-7} M) + genistein (300 μ M) (K). The positive cells in the panels A, C, E, G, I and K are stained with anti-dioxigenin coupled with FITC. The panels B, D, F, H, J and L show the nuclear staining with DAPI of the cells in A, C, E, G, I and K respectively. Magnification = 10X. The white scale bar is in μ m. (M) Histogram showing the effect of the different treatments on

apoptosis induced by genistein. The apoptosis index is expressed as the percentage of apoptotic cells with respect to the total number of counted cells for a given treatment. A minimum of 500 cells per experiment were counted for at least 3 independent experiments. The values are expressed as mean $\pm$ S.E.M. * $p < 0.05$ vs. Ctrl. ♦ $p < 0.05$ vs. genistein treatment.

Figure 3. Western blot showing the effect of ET-1 and IRL1620 on the genistein-induced decrease of ET_B receptor density in hVECs.

(A) Autoradiography showing the relative density of ET_B receptors in hVECs in the absence or presence of genistein alone or together with ET-1 or IRL-1620. The band for the ET_B receptor is situated around 50 kDa. Total lysates of A549 cells were used as positive control. The negative control (or control peptide, CP) was performed by incubating the membrane in the presence of the antibody directed against the ET_B receptor blocked with the peptide (1/20) from the supplier. The β -actin band indicates that equal quantities of proteins were loaded in all wells. (B) Histogram summarizing the densitometric measurements of the bands obtained by Western blot. The ET_B/ β -actin ratio was calculated using the densitometric measurements of the corresponding bands. The values are expressed as mean $\pm$ SEM from at least 3 different experiments. * $p < 0.05$, ** $p < 0.01$ vs. control.

Figure 4. Effect of genistein, ET-1 and IRL1620 on the relative density of the ET_B receptor in hVECs.

Real 3D confocal microscopy images (top view) of hVECs showing the immunoreactivity of the ET_B receptor in control condition (A) and following a 24-hr

treatment with genistein (300 μ M) alone (C) or in the presence of ET-1 (10^{-7} M) (E). Panels B, D and F show the nuclear staining using Syto-11 of the cells in A, C and E respectively. The pseudocolor scale represents the level of fluorescence intensity from 0 (Black, absence of fluorescence) to 255 (White, max fluorescence) in panels A, C and E. The white scale bar is in μ m. (G) Histogram showing the effect of different treatments on the decrease of ET_B receptor relative density induced by genistein treatment. The hVECs were treated for 24 hours with a control medium, or with ET-1 (10^{-7} M), or with genistein, G, (300 μ M) alone, or in the presence of ET-1 (10^{-7} M), IRL-1620 (10^{-7} M), ET-1 (10^{-7} M) + ABT-627 (10^{-7} M), or ET-1 (10^{-7} M) + A-192621 (10^{-7} M). The values are expressed as mean $\pm$ S.E.M. * p < 0.05 and *** p < 0.001 vs. Ctrl. ♦♦ p < 0.01 and ♦♦♦ p < 0.001 vs. genistein treatment. ⁺ p < 0.05 vs. ET-1+G. n is the number of cells in N=8 different experiments.

Figure 5. Effects of ET-1 and ET_B receptors on the increase of the relative density of active caspase-3 induced by genistein treatment in hVECs.

Images obtained by fluorescence microscopy showing, in hVECs, the immunoreactivity of the active form of caspase-3 in control condition (A) and following 24-hr treatments with genistein (300 μ M) alone (C) or in the presence of ET-1 (10^{-7} M) (E) or IRL-1620 (10^{-7} M) (G). Panels B, D, F and H show the nuclear staining using DAPI of the cells in A, C, E and G respectively. The white scale bar is in μ m. (I) Histogram summarizing the changes in the relative density of active caspase-3 induced by genistein alone or in the presence of ET-1 or IRL-1620. The values represent the percentage of apoptotic cells with respect to total counted cells for a given treatment. A minimum of 500 cells per experiment was counted for at least 3 different experiments. The values are expressed as

mean + S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Control and ♦♦♦ $p < 0.001$ vs. genistein treatment.

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Figure 1. Effect of ET-1 and its receptors ETA and ETB on apoptosis induced by genistein assessed by annexin V staining.

Real 3D confocal microscopy images of annexin V staining performed in hVECs treated for 24 hours with a control medium (A), or with genistein (300 μM) alone (B) or together with ET-1 10⁻⁷ M (C) or with the ETBR specific agonist IRL-1620 (10⁻⁷M) (D). The white scale bar is in μm. (E) The histogram shows the effect of different treatments on apoptosis induced by genistein (300 μM). Annexin V staining was done in order to establish the apoptosis index which is expressed as the percentage of apoptotic cells with respect to the number of total counted cells for a given treatment. A minimum of 500 cells per experiment were counted for at least 3 independent experiments. The values are expressed as mean ± S.E.M. ↔ p < 0.05 and ↔↔↔ p < 0.001 vs. Ctrl. ∪ p < 0.05, ∪∪ p < 0.01 vs. genistein treatment.

239x135mm (300 x 300 DPI)

Figure 2. Role of ET-1 receptors in genistein-induced apoptosis of hVECs assessed by in situ TUNEL assays. Representative micrographs of in situ TUNEL performed in hVECs treated for 24 hours with a control medium (A), or with genistein (300 μ M) (C), ET-1 (10⁻⁷ M) + genistein (300 μ M) (E), IRL-1620 (10⁻⁷ M) + genistein (300 μ M) (G), ET-1 (10⁻⁷ M) + ABT-627 (10⁻⁷ M) + genistein (300 μ M) (I), or ET-1 (10⁻⁷ M) + A-192621 (10⁻⁷ M) + genistein (300 μ M) (K). The positive cells in the panels A, C, E, G, I and K are stained with anti-dioxigenin coupled with FITC. The panels B, D, F, H, J and L show the nuclear staining with DAPI of the cells in A, C, E, G, I and K respectively. Magnification = 10X. The white scale bar is in μ m. (M) Histogram showing the effect of the different treatments on apoptosis induced by genistein. The apoptosis index is expressed as the percentage of apoptotic cells with respect to the total number of counted cells for a given treatment. A minimum of 500 cells per experiment were counted for at least 3 independent experiments. The values are expressed as mean $\pm$ S.E.M. $\leftrightarrow$ p < 0.05 vs. Ctrl. $\uparrow$ p < 0.05 vs. genistein treatment.

187x190mm (300 x 300 DPI)

Figure 3. Western blot showing the effect of ET-1 and IRL1620 on the genistein-induced decrease of ET_B receptor density in hVECs.

(A) Autoradiography showing the relative density of ET_B receptors in hVECs in the absence or presence of genistein alone or together with ET-1 or IRL-1620. The band for the ET_B receptor is situated around 50 kDa. Total lysates of A549 cells were used as positive control. The negative control (or control peptide, CP) was performed by incubating the membrane in the presence of the antibody directed against the ET_B receptor blocked with the peptide (1/20) from the supplier. The β-actin band indicates that equal quantities of proteins were loaded in all wells. (B) Histogram summarizing the densitometric measurements of the bands obtained by Western blot. The ET_B/β-actin ratio was calculated using the densitometric measurements of the corresponding bands. The values are expressed as mean ± SEM from at least 3 different experiments. ↔p < 0.05, ↔↔p < 0.01 vs. control.

146x141mm (300 x 300 DPI)

Figure 4. Effect of genistein, ET-1 and IRL1620 on the relative density of the ETB receptor in hVECs. Real 3D confocal microscopy images (top view) of hVECs showing the immunoreactivity of the ETB receptor in control condition (A) and following a 24-hr treatment with genistein (300 μM) alone (C) or in the presence of ET-1 (10-7M) (E). Panels B, D and F show the nuclear staining using Syto-11 of the cells in A, C and E respectively. The pseudocolor scale represents the level of fluorescence intensity from 0 (Black, absence of fluorescence) to 255 (White, max fluorescence) in panels A, C and E. The white scale bar is in μm . (G) Histogram showing the effect of different treatments on the decrease of ETB receptor relative density induced by genistein treatment. The hVECs were treated for 24 hours with a control medium, or with ET-1 (10-7M), or with genistein, G, (300 μM) alone, or in the presence of ET-1 (10-7 M), IRL-1620 (10-7 M), ET-1 (10-7 M) + ABT-627 (10-7 M), or ET-1 (10-7 M) + A-192621 (10-7 M). The values are expressed as mean $\pm$ S.E.M. $\leftrightarrow$ $p < 0.05$ and $\leftrightarrow\leftrightarrow$ $p < 0.001$ vs. Ctrl. $\leftrightarrow\leftrightarrow\leftrightarrow$ $p < 0.01$ and $\leftrightarrow\leftrightarrow\leftrightarrow\leftrightarrow$ $p < 0.001$ vs. genistein treatment. $\leftrightarrow$ $p < 0.05$ vs. ET-1+G. n is the number of cells in N=8 different experiments.

242x164mm (300 x 300 DPI)

Figure 5. Effects of ET-1 and ETB receptors on the increase of the relative density of active caspase-3 induced by genistein treatment in hVECs.

Images obtained by fluorescence microscopy showing, in hVECs, the immunoreactivity of the active form of caspase-3 in control condition (A) and following 24-hr treatments with genistein (300 µM) alone (C) or in the presence of ET-1 (10⁻⁷M) (E) or IRL-1620 (10⁻⁷M) (G). Panels B, D, F and H show the nuclear staining using DAPI of the cells in A, C, E and G respectively. The white scale bar is in µm. (I) Histogram summarizing the changes in the relative density of active caspase-3 induced by genistein alone or in the presence of ET-1 or IRL-1620. The values represent the percentage of apoptotic cells with respect to total counted cells for a given treatment. A minimum of 500 cells per experiment was counted for at least 3 different experiments. The values are expressed as mean + S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001 vs. Control and ****p < 0.001 vs. genistein treatment.

231x165mm (300 x 300 DPI)