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Estradiol, acting through ERα, induces endothelial non-classic renin-angiotensin system increasing angiotensin 1–7 production

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ABSTRACT

Intracellular renin-angiotensin system (RAS) can operate independently of the circulating RAS. Estrogens provide protective effects by modulating the RAS. Our aim was to investigate the effect of estradiol (E2) on angiotensin converting enzymes (ACE) 1 and ACE2 expression and activities in human endothelial cells (HUVEC), and the role of estrogen receptors (ER). The results confirmed the presence of active intracellular RAS in HUVEC. Physiological concentrations of E2 induced a concentration-dependent increase of ACE1 and ACE2 mRNA expression and ACE1, but not ACE2, protein levels. ACE1 and ACE2 enzymatic activities were also induced with E2. These effects were mediated through ERα activation, since ER antagonists ICI 182780 and MPP completely abolished the effect of E2. Moreover, the ERα agonist PPT mirrored the E2 effects on ACE1 and ACE2 protein expression and activity. Exposure of endothelial cells to E2 significantly increased Ang-(1–7) production. In conclusion, E2 increases Ang-(1–7) production, through ERα, involving increased ACE1 and ACE2 mRNA expression and activity and ACE1 protein levels.

1. Introduction

Renin-angiotensin system (RAS) is a signaling pathway responsible for regulation of extracellular fluid homeostasis and therefore, for the maintaining of blood pressure. The substrate of the circulating system, angiotensinogen, is secreted by the liver and is enzymatically cleaved to angiotensin I (Ang I) by kidney-derived renin. Ang I is then cleaved by angiotensin converting enzyme 1 (ACE1) to the main effector peptide Angiotensin II (Ang II) (Griendling et al., 1993). Ang II effects are mediated through receptor type 1 (AT1R), which is involved in the classical vasoconstrictor and remodeling effects, and Ang II receptor type 2 (AT2R) with opposing effects (Dinh et al., 2001; Montezano et al., 2014). In addition to these classical RAS components, Ang II can also be degraded by angiotensin converting enzyme 2 (ACE2) to the biologically active vasodilator peptide Angiotensin 1–7 (Ang-(1–7)) (Chappel and Ferrario, 2006). Thus, ACE2 counteracts the classical RAS activity towards vasodilator pathway (Crackower et al., 2002; Lovren et al., 2008).

In addition to this circulating RAS, their components are also synthesized and act locally in multiple organs and tissues, some of which are directly involved in cardiovascular regulation, like heart and vessels (Nguyen Dinh Cat and Touyz, 2011). All the RAS components are expressed in the vascular wall, except renin which is taken up from the circulation (te Riet et al., 2015). Therefore tissue RAS can operate independently of the circulating RAS, contributing to a fine-tuning of vascular tone and arterial structure. Also, tissue RAS may amplify the vascular effects of the circulating RAS in disease states such as hypertension (Nguyen Dinh Cat and Touyz, 2011).

Several studies have demonstrated an interaction between the RAS and sex hormones in regulating cardiovascular function and blood pressure (Maric-Bilkan and Manigrasso, 2012). This interaction has been described mainly at the endothelial level, where the classical types of estrogen receptors (ER), ERα, ERβ are expressed (Kim et al., 2014; Mendelsohn, 2002). Estrogens provide vascular protective effects as promotes vasodilation and decreases

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inflammation and atherosclerosis. The estrogenic effect in the vasculature is so far postulated as one of the main drivers behind the beneficial cardiovascular profile of pre-menopausal women (Prabhushankar et al., 2014).

Wealth of evidence shows that estrogens provide protective effects by modulating the RAS. In addition, sex differences in the expression of RAS components have been identified (Sullivan, 2008; Xue et al., 2014).

Our group previously reported estradiol (E2) effects on the endothelial gene expression profile (Sobrino et al., 2009), demonstrating a significant increase in ACE1 mRNA expression in E2 treated human umbilical vein endothelial cells (HUVEC). We hypothesized that estradiol may modify local RAS through modification of the endothelial expression and activity of ACE1 and/or ACE2. Therefore, the aims of the present work were to determine the effect of physiological E2 concentrations on ACE1 and ACE2 expression and activities, and on Ang II and Ang-(1–7) production in HUVEC, delving also in the role of classical ER on the observed effects.

2. Materials and methods

2.1. Cell culture and experimental design

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cord veins by means of collagenase digestion procedure as previously described (Sobrino et al., 2010). HUVEC were grown in Medium 199 (Sigma–Aldrich, Tres Cantos, Madrid, Spain) supplemented with 20% fetal bovine serum (GIBCO, Invitrogen, Barcelona, Spain), endothelial cell growth supplement from bovine neural tissue (ECGS, Sigma–Aldrich) and heparin sodium salt from porcine intestinal mucosa (Sigma–Aldrich). Cells were routinely grown in an incubator at 37 °C with 5% CO_2.

When cells from passages 3 to 5 were at 90% confluence, culture medium was exchanged for a phenol red-free Medium 199 (GIBCO) supplemented with 2% charcoal/dextran-treated fetal bovine serum (GIBCO), ECGS, pyruvic acid and antibiotics ("hormone free medium") to avoid any estrogenic activity and maintained for 24 h.

Cells were exposed for 24 h to 1–10 nmol/l of 17β-estradiol (Sigma–Aldrich), the selective ERα agonist 4,4′,4″-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT, 10 nmol/l; Tocris Bioscience, Ellisville, MI, USA), or the selective ERβ agonist 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN, 10 nmol/l; Tocris Bioscience). In another set of experiments, the ER unspecific antagonist ICI 182780 (1 μmol/l; Biogen, Madrid, Spain) or the specific ERα antagonist methyl-piperidino-pyrazole (MPP) (1 μmol/l; Tocris Bioscience), were added 30 min before E2 to evaluate whether the observed effects elicited by E2 were mediated by ER.

This investigation conforms to the principles outlined in the Declaration of Helsinki, was approved by the Ethical Committee of Clinical Research of the INCLIVA, Hospital Clínico of Valencia, Spain, and written informed consent was obtained from all donors.

2.2. RNA isolation and quantitative real-time PCR (qRT-PCR) assay

Total RNA was extracted by using TRIzol reagent (Invitrogen) following the manufacturer's instructions. Reverse transcription was carried out using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) by using a personal Mastercycler Eppendorf Thermocycler (Eppendorf, Hamburg, Germany). One microgram of total RNA was reverse transcribed to cDNA following the manufacturer's instructions. The qRT-PCR data for ACE1, ACE2 and GAPDH (endogenous control) were obtained with TaqMan assays (Hs00174179_m1, Hs01085333_m1 and Hs99999905_m1 respectively) performed with TaqMan Universal Mastermix (Applied Biosystems). Data were analyzed with the SDS 2.2.2 software (Applied Biosystems).

2.3. Immunoblotting

Treated HUVEC were collected in RIPA buffer (Sigma–Aldrich) containing protease inhibitors (complete ULTRA Tablets, Roche Diagnostics, Madrid, Spain). Protein content was measured and samples were frozen at −20 °C until assay. Equal amounts of protein were then separated by 7.5% of SDS-Polyacrylamide gel electrophoresis. Protein was then transferred to nitrocellulose membranes (Whatman, GE Healthcare Life Sciences). Immunostaining was achieved using specific antibodies anti-ACE1 (ab11734; Abcam, Cambridge, United Kingdom), and anti-ACE2 (sc-20998; Santa Cruz Biotechnology Inc, Heidelberg, Germany). Development was performed with anti-mouse and anti-rabbit peroxidase-linked antibodies (Santa Cruz Biotechnology), followed with Supersignal Chemiluminescent Substrate (Thermo Scientific Inc.). Signal density was analyzed with ImageJ software (NIH Image, NIH, Bethesda, USA). Beta-actin (Sigma–Aldrich) was used as internal control of the amount of protein.

2.4. ACE1 activity assay

Treated HUVEC were suspended in ACE1 reaction buffer (K_2PO_4 100 mmol/l, NaCl 300 mmol/l) plus 0.5% Triton X-100. Suspensions were sonicated on ice, maintained for 15 min in ice and then centrifuged at 16000× *g* for 5 min at 4 °C, using the cell lysate supernatant. ACE1 activity was measured by a modification of the method described earlier (Schwager et al., 2006). Briefly, ACE1 activity was assayed by measuring the hydrolysis of ACE1 substrate N-Hippuryl-His-Leu hydrate (Sigma–Aldrich) after 2 h of incubation at 37 °C. The reaction was stopped with 0.28 mol/l NaOH. The absorbance readings of different His-Leu (Sigma–Aldrich) concentrations, used as standard, were plotted as linear regression graph. ACE1 activity of the samples was obtained by interpolation the absorbance readings of samples with the standards. Lisinopril (100 μmol/l, Sigma–Aldrich) was used as a negative control for ACE1 activity. The substrate hydrolysis was measured (excitation wavelength 360 nm and emission 485 nm) in a spectrofluorimeter SpectraMax Gemini XPS with Softmax Pro 6.2.2 software (Molecular Devices, Wokingham, UK). Results were expressed as nmol per minute and per mg of sample.

2.5. ACE2 activity assay

Treated HUVEC were collected in ACE2 reaction buffer (NaCl 1 mol/l, $ZnCl_2$ 0.5 mmol/l, TrisHCl 75 mmol/l) plus 0.5% Triton X-100. Suspensions were sonicated on ice, maintained for 15 min in ice and then centrifuged at 16000× *g* for 5 min at 4 °C. ACE2 activity was measured in the supernatant by a modification of the method described earlier (Guy et al., 2003). Briefly, the assay is based on the use of the fluorogenic peptide substrate Mca-APK(Dnp) (Enzo Life Sciences, New York, USA). ACE2 removes the Dnp group that quenches the Mca fluorescence resulting in an increase in fluorescence in the presence of ACE2 activity at excitation and emission wavelengths of 328 and 393 nm, respectively. The change in fluorescence was monitored continuously for 4 h using a spectrofluorimeter SpectraMax Gemini XPS with Softmax Pro 6.2.2 software (Molecular Devices). Specific ACE2 activity was calculated by subtracting the activity in the presence of 1 μmol/l DX600 (Phoenix Pharmaceuticals, Karlsruhe, Germany), a specific ACE2 inhibitor (Huang et al., 2003), from the total activity. Specific ACE2 activity was expressed as nmol of substrate converted to product per minute and per mg of sample. Standard curves are generated using

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0–50 μmol/l of the fluorogenic substrate OmniMMP (Enzo Life Sciences).

2.6. Ang II and Ang-(1–7) detection by immunofluorescence

Treated HUVEC were fixed by incubating in 4% paraformaldehyde in PBS for 15 min. To block nonspecific reactions, 1% BSA in phosphate-buffered saline containing 0.2% Triton was added for 30 min. Cells were incubated with anti-Ang-(1–7) polyclonal antibody developed as previously described (Lopez Verrilli et al., 2009), and anti-Ang II (Bachem, Saint Helens, UK) overnight at 4 °C. Cells were then incubated with IgG FITC conjugate (Sigma–Aldrich) and IgG Texas Red conjugate (Abcam) for 1 h at room temperature. In both cases, negative controls were obtained after exposure to secondary antibodies, without anti-Ang-(1–7) or anti-Ang II antibodies. DAPI (BioLegend, San Diego, CA, USA) was used for DNA specific nuclear staining. Microscopic observation was performed with spectral confocal microscope Leica TCS SP2. Pictures were taken with a 40× objective and shown at ×400 magnification. Confocal images were quantified with ImageJ software.

2.7. Statistical analysis

Values shown in the text and figures are mean ± Standard Error of Mean (SEM) from data obtained in 6–9 independent experiments. ANOVA test was applied for comparisons of mean, and then Bonferroni's test was performed. P values < 0.05 were considered significant. The statistical analysis was carried out using the Prism 6 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. E2 increased ACE1 and ACE2 mRNA expression through ERα

To study the role of E2 on endothelial intracellular RAS, we first checked the effect of E2 on ACE1 and ACE2 mRNA expression in HUVEC. Exposure of endothelial cells to two different, physiological and near-physiological concentrations of E2 (1 and 10 nmol/l respectively) for 24 h, resulted in a concentration-dependent increase of both ACE1 (Fig. 1A) and ACE2 (Fig. 1B) mRNA expression, with a maximum of expression for 10 nmol/l of E2 in both cases.

To test whether the increased ACE1 and ACE2 mRNA expression induced by E2 was mediated through ER, HUVEC were exposed to the unspecific ER antagonist ICI 182780 (1 μmol/l) and specific ERα antagonist MPP (1 μmol/l). Blockade of ER completely abolished the increment in ACE1 and ACE2 mRNA induced by estradiol (Fig. 1A and B), suggesting a role of ERα in mediating responses of estradiol in endothelial RAS. Neither ICI 182780 nor MPP had effects on gene expression of ACE1 and ACE2.

3.2. Estradiol increased ACE1 but not ACE2 protein expression

HUVEC expressed both ACE1 and ACE2 proteins, as demonstrated by western blot analysis (Fig. 2). However, only the increase in mRNA ACE1 was accompanied by an increment in the amount of ACE1 protein. Exposure to 10 nmol/l E2 increased ACE1 protein expression by 25% above control values ($^{*}p < 0.05$ vs. control, Fig. 2A). These effects were mediated through ER activation, specifically through ERα, since the treatment of cells with ICI 182780 and MPP completely reverted the effect of E2 (Fig. 2A). On the contrary, ACE2 protein expression remained unaltered after E2 exposure and/or treatment with ER antagonists (Fig. 2B).

3.3. E2 increased ACE1 and ACE2 enzymatic activity through selective ERα activation

Since E2-treated HUVEC presented an increased ACE1 and ACE2 expression, we further investigated whether ACEs activity was also modified by E2 treatment. In control conditions, HUVEC enzymatic activities for ACE1 and ACE2 were 0.66 ± 0.15 and 0.014 ± 0.002 nmol/min × mg protein, respectively. Treatment with E2 (1 and 10 nmol/l) for 24 h, resulted in a concentration-dependent increase of ACE1 and ACE2 enzymatic activities up to 203 ± 26 and 180 ± 19% of control values respectively ($^{**}p < 0.01$ vs. control) (Fig. 3). The increase in ACE1 and ACE2 activities induced by E2 was completely prevented by ICI 182780 and MPP, suggesting the involvement of ERα in the effect elicited by E2.

3.4. The ERα selective agonist mimics the E2 effects on ACE1 and ACE2 protein expression and enzymatic activity

To reinforce the role of ERα on the E2-induced effects, a set of experiments were performed with the selective ERα agonist, PPT,

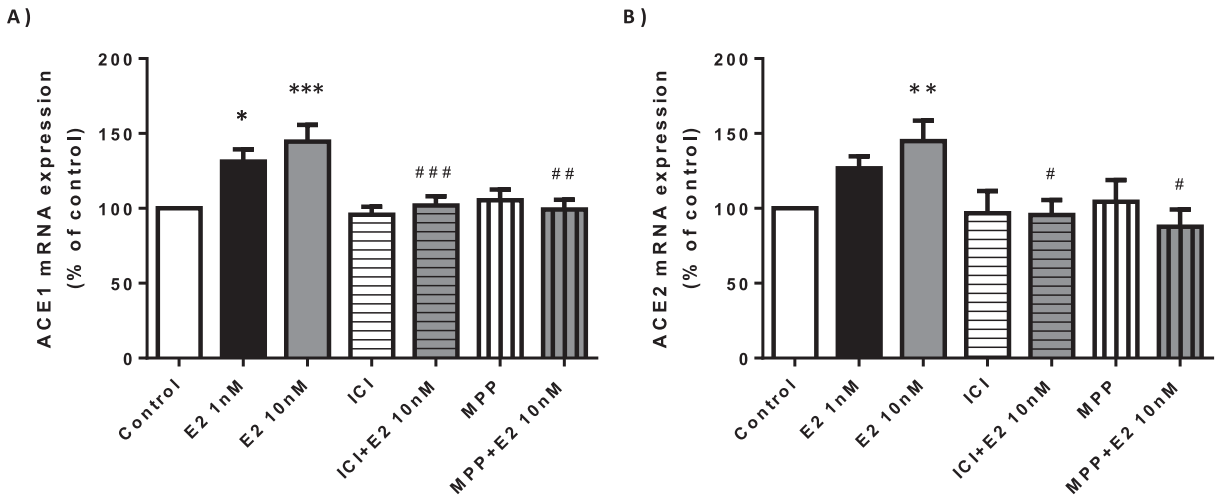

Fig. 1. ACE1 and ACE2 mRNA expression. HUVEC were exposed to 1–10 nmol/l E2, ER antagonist ICI (1 μmol/l) and/or ERα antagonist MPP (1 μmol/l) for 24 h. ACE1 (A) and ACE2 (B) mRNA levels were assessed by qRT-PCR. Data are expressed as the percentage of the control values and presented as mean ± SEM of n = 6–8 from 3 to 4 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control; #p < 0.05, ##p < 0.01 and ###p < 0.001 vs. E2 10 nmol/l alone.

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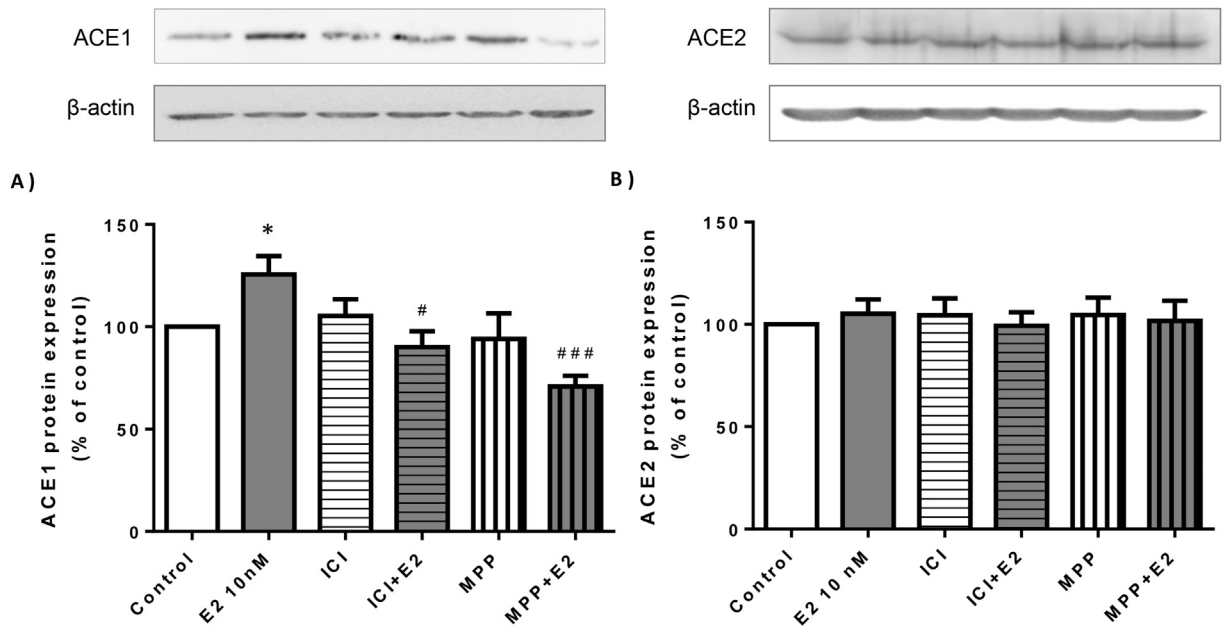

Fig. 2. ACE1 and ACE2 protein expression in HUVEC. Cells were exposed to 10 nmol/l E2, ER antagonist ICI (1 μmol/l) and ERα antagonist MPP (1 μmol/l) for 24 h, and protein expression of ACE1 (A) and ACE2 (B) was measured by immunoblotting. A typical immunoblotting image and relative levels assessed by densitometry of bands of 160 kDa (ACE1) or 90 kDa (ACE2) are presented. Data are expressed as the percentage of the control values and presented as mean ± SEM of n = 6–8. *p < 0.05 vs. control; #p < 0.05 and ###p < 0.001 vs. E2 10 nmol/l alone.

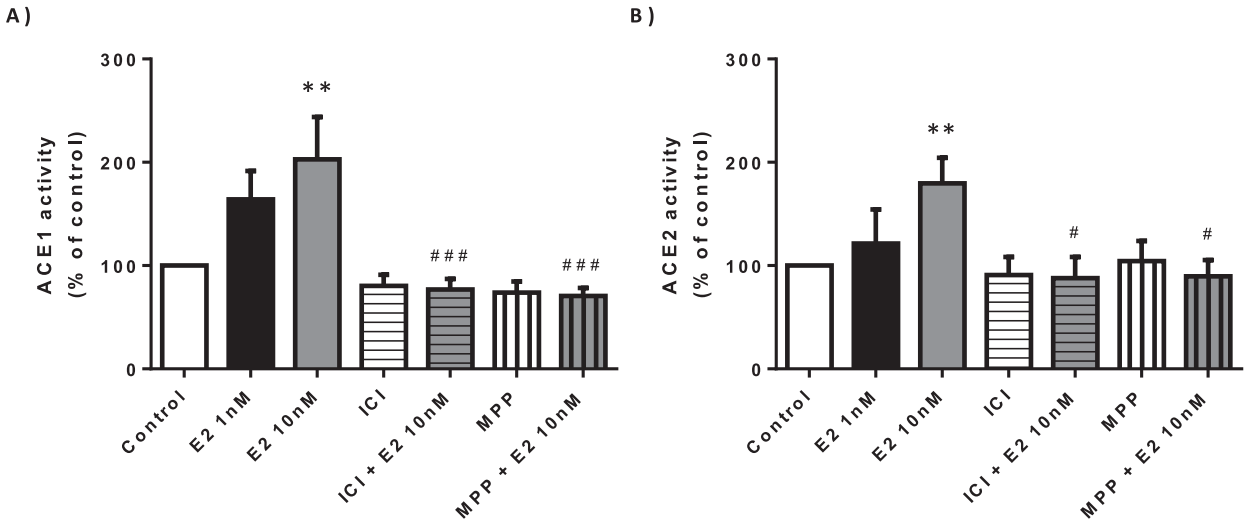

Fig. 3. ACE1 and ACE2 enzymatic activity in HUVEC. Cells were exposed to 1–10 nmol/l E2, ER antagonist ICI (1 μmol/l) and/or ERα antagonist MPP (1 μmol/l) for 24 h. ACE1 (A) and ACE2 (B) specific activity was measured by spectrofluorimetry as described in Materials and methods. Data are expressed as the percentage of the control values and presented as mean ± SEM of n = 6–9 from 3 to 4 independent experiments. **p < 0.01 vs. control; #p < 0.05 and ###p < 0.001 vs. E2 10 nmol/l alone.

and the selective ERβ agonist, DPN. Exposure to 10 nM PPT, but not 10 nM DPN, stimulate ACE1 protein expression to the same extent than E2, without changes on ACE2 protein expression (Fig. 4A and B). PPT also reproduce the effect of E2 on ACE1 and ACE2 activities, whereas DPN did not change these enzymatic activities (Fig. 4C and D). These results strengthen the role of ERα on the increased ACE1 protein expression and the enhanced ACE1 and ACE2 enzymatic activities induced by E2.

3.5. E2 increased Ang-(1–7) production in HUVEC

We next evaluated the E2 treatment on Ang II and Ang-(1–7) levels in HUVEC. Although Ang II levels were detected in HUVEC (Fig. 5A), and were above the negative control, quantification demonstrated there were no differences on Ang II production between E2-treated and control cells (Fig. 5B). The exposure to ICI or MPP, alone or in the presence of E2, did not modify the Ang II levels.

Exposure of endothelial cells to E2 10 nmol/l significantly increased Ang-(1–7) production (Fig. 6A and B). Since the treatment of endothelial cells with ICI 182780 and MPP completely reverted the effect of E2, the E2 increased Ang-(1–7) production was mediated through ERα.

The presence of an ACE1 inhibitor (Lisinopril) prevented the increase in Ang-(1–7) levels induced by E2 treatment. These results

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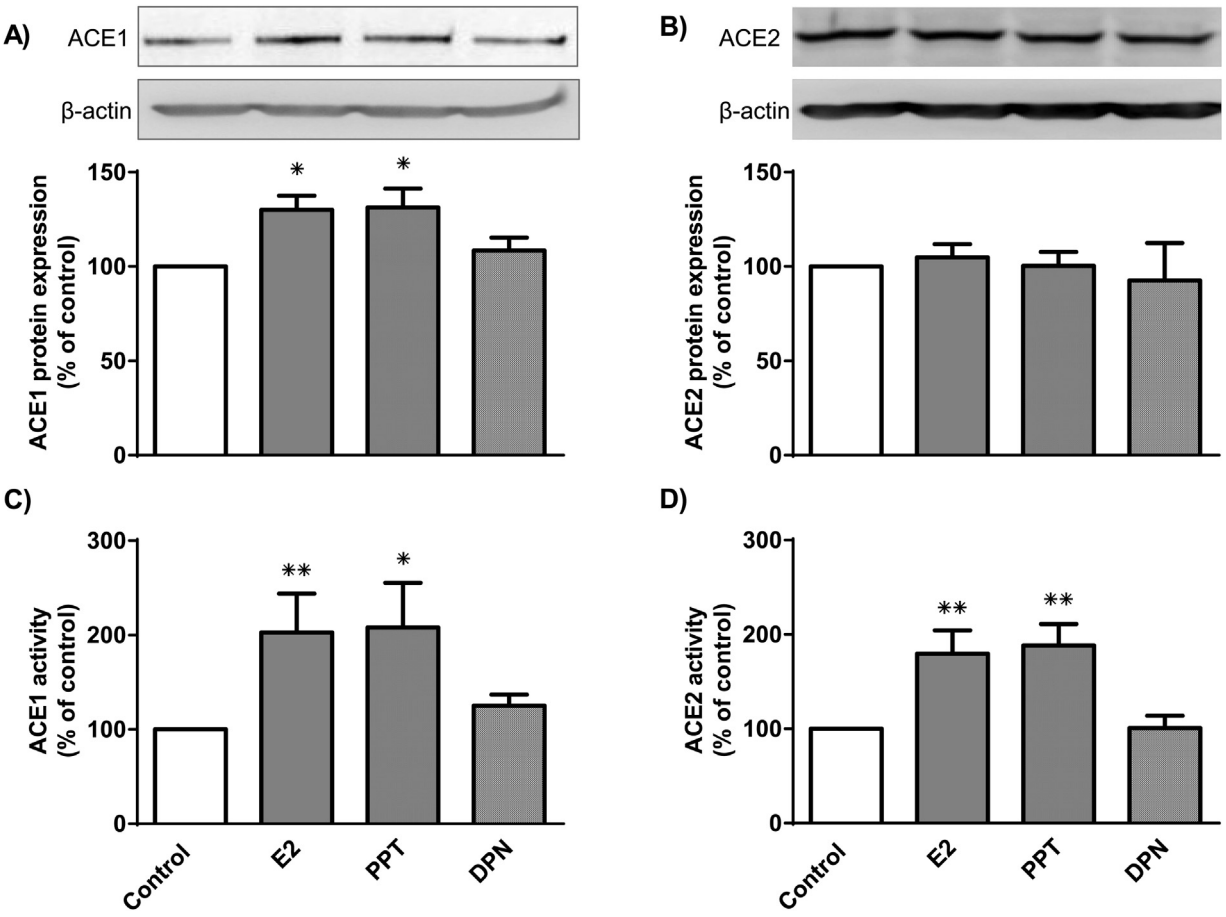

Fig. 4. ERα and ERβ agonists effects on ACE1 and ACE2 protein expression and enzymatic activity in HUVEC. Cells were exposed to 10 nmol/l E2, ERα agonist PPT (10 nmol/l) or the ERβ agonist DPN (10 nmol/l) for 24 h. The protein expression of ACE1 (A) and ACE2 (B) was measured by immunoblotting. A typical immunoblotting image and relative levels assessed by densitometry of bands of 160 kDa (ACE1) or 90 kDa (ACE2) are presented. The ACE1 (C) and ACE2 (D) specific activity was measured by spectrofluorimetry as described in Materials and methods. Data are expressed as the percentage of the control values and presented as mean ± SEM of n = 3–6. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control.

suggest that in response to E2 in endothelial cells, RAS is shifted towards an Ang-(1–7) production, and depends on both ACE1 and ACE2 activities.

4. Discussion

In the present study, we demonstrated that physiological concentrations of E2 increase Ang-(1–7) levels through ERα stimulation in HUVEC. Furthermore, E2 treatment rises ACE1 and ACE2 mRNA levels and activities, increases ACE1 but not ACE2 protein expression, and these facts may explain the increased Ang-(1–7) levels in these cells. The main results obtained in the present study are schematized in Fig. 7.

The results confirm the active presence of intracellular RAS in endothelial cells, confirming the vascular wall not only as an effector organ for the circulating RAS, but as a local RAS operating independently (Paul et al., 2006).

E2 increased ACE1 mRNA expression and activity in a concentration-dependent manner. In addition, E2 augmented ACE1 protein expression. These results confirm our previous studies showing gene expression analysis of E2-estimulated human endothelial cells (Sobrino et al., 2009). In that study, ACE1 mRNA expression was significantly increased by E2, without changes in other RAS proteins, such as angiotensinogen, cathepsin, or chymase 1. Moreover, angiotensin receptor 1 and 2 or Mas receptor expressions remained also unaltered (Sobrino et al., 2009). In spite of the lack of studies in endothelial cells, several studies on vascular and systemic RAS suggest that estrogens decrease ACE1 activity. Plasma ACE1 activity is higher in males, both in humans (Zapater et al., 2004) and mice (Lim et al., 2002). Moreover, estrogen replacement therapy decrease circulating ACE1 activity in healthy postmenopausal women (Proudler et al., 2003) and in ovariectomized rats (Gallagher et al., 1999). In the vascular wall, experimental studies demonstrated an increased ACE1 expression in ovariectomized rats, which was prevented after estrogen administration (Gallagher et al., 1999; Yung et al., 2011). In other tissues, however, ACE1 expression activity was higher in females, as in rat renal cortex (Bhatia et al., 2013). Differences between male and female could be attributed to a higher stimulation of ACE1 activity due to androgens, as it has been demonstrated in men, in which testosterone increases ACE1 activity (Pingili et al., 2015) or even in women suffering polycystic ovary syndrome, in which high androgens are related to high ACE1 activity (Arefi et al., 2013). Discrepancies between our data on E2-exposed HUVEC and data obtained from estrogen replacement therapy experiments could be explained by a counterbalance between circulating and local RAS, or even between a different regulation of smooth muscle cells and endothelium physiology by E2.

In our study, E2 also increased ACE2 mRNA expression and stimulated its activity in a concentration-dependent manner. Surprisingly, the ACE2 protein expression did not appeared increased in immunoblotting analysis. 24 h is enough time to translation, but

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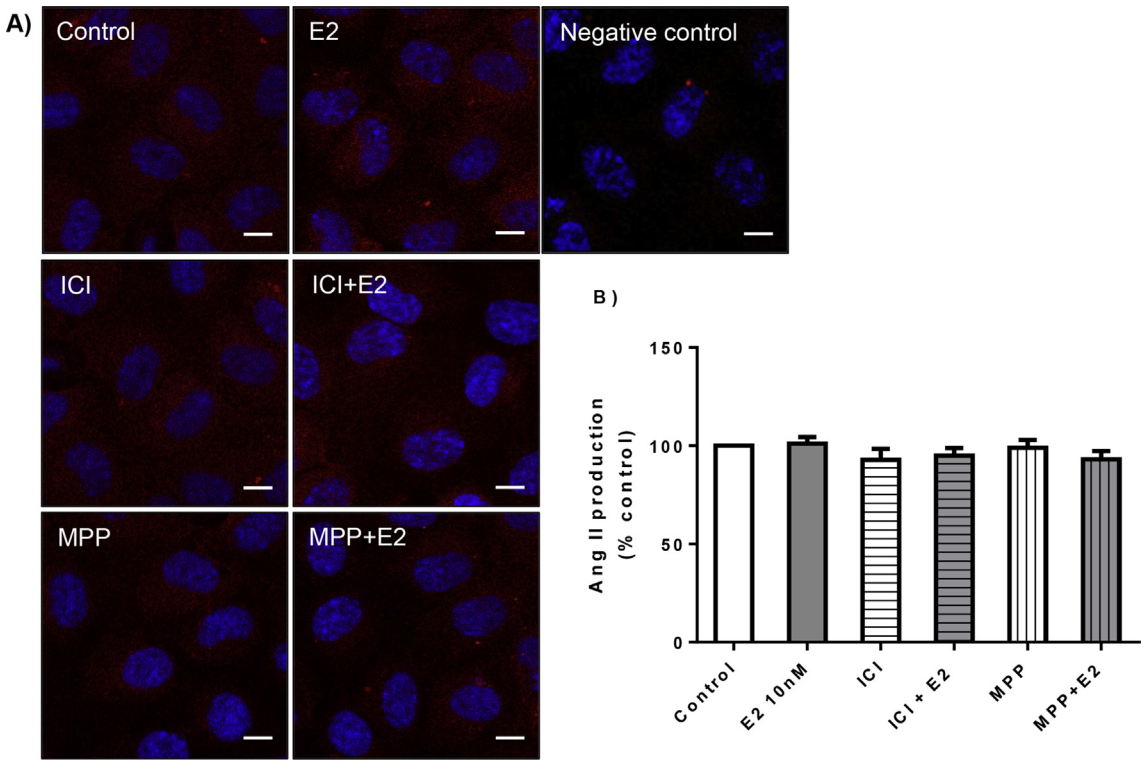

Fig. 5. Ang II levels in HUVEC. (A) Representative confocal images of Ang II production in HUVEC. Cells were exposed to E2 (10 nmol/l) alone or in combination with ER antagonist ICI (1 μmol/l), ERα antagonist MPP (1 μmol/l) for 24 h. DNA specific nuclear staining (DAPI) is shown in blue. Red color (Texas Red) represents Ang II production. Negative control represents the background of secondary antibody. (B) Ang II expression quantification of confocal images in HUVEC. Data are expressed as the percentage of the control values and presented as mean ± SEM. The experiment was performed in 4–6 duplicate experiments. Scale bar represents 10 μm (original magnification ×400). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

a longer time of analysis could be resulted in increased ACE2 protein expression. Alternatively, the translation of ACE2 mRNA on protein could be down-regulated by E2, directly due to increased ACE2 activity of increased Ang-(1–7) production. The increased activity of ACE2 without increased protein expression could be due to a direct E2-induced ACE2 activity, but an indirect activation could not be discarded.

It has been hypothesized that ACE2 might protect against increases in blood pressure and that ACE2 deficiency leads to hypertension (Crackower et al., 2002). There are not studies on the effect of E2 on ACE2 activity in endothelial cells, but experimental data from rats suggest an up-regulation of ACE2 by estrogens. For instance, female rats have significantly greater ACE2 kidney expression compared with male, demonstrating an enhanced vasodilator arm of the RAS in female kidneys (Sampson et al., 2012). Moreover, ACE2 activity increased in the renal cortex and the medulla in pregnant rats, which is correlated with increased E2 in serum (Joyner et al., 2007). In ovariectomized rats, ACE2 activity and protein expression were decreased in the renal cortex and normalized after estrogen replacement (Ji et al., 2008). Furthermore, rat cardiac ACE2 protein was increased after exposure to E2 therapy in rats (Shenoy et al., 2009). Nevertheless, data from mice partially disagree, since male mice have greater renal ACE2 activity, whereas no sex differences were detected in the heart and lung (Liu et al., 2010).

As stated, the majority of reports on E2-induced effects on circulating and even in local RAS are dual, by reducing ACE1 and increasing ACE2. In HUVEC, the activity of both enzymes was increased by E2, and therefore ACE1 provides Ang II to ACE2 to produce Ang-(1–7), as demonstrated by the abolishment of E2-increased Ang-(1–7) production in the presence of lisinopril, a selective inhibitor of ACE1 not acting on ACE2 activity (Donoghue et al., 2000). Therefore, the E2-induced RAS activity resulted in increased Ang-(1–7) concentration, and may prevent the accumulation of Ang II.

Our results are in agreement with several experimental studies describing a relationship between estrogens and elevated local Ang-(1–7). For instance, female rats exhibit higher renal Ang-(1–7) levels than do males, whereas plasma Ang II levels were greater in male than in female WKY rats (Bhatia et al., 2013). Sex differences on plasma and renal Ang II or Ang-(1–7) were not demonstrated in normotensive Lewis rat strain, but in hypertensive mRen2.Lewis rats, male plasma Ang II were higher and Ang-(1–7) lower than in females (Pendergrass et al., 2008; Yamaleyeva et al., 2012).

Ang II/Ang-(1–7) balance is regulated differently in male and female mice with diet induced obesity (Gupte et al., 2012). In this model, low-fat fed females exhibited lower plasma Ang-(1–7) levels whereas high-fat fed decreased plasma Ang II and increased Ang-(1–7) levels in females. Moreover, ovariectomy of high-fat female mice reduced plasma Ang-(1–7) levels, thus supporting a role for E2 on maintenance of Ang-(1–7) levels.

Estrogen modulates cardiovascular function and physiology acting through its classic receptors, ERα and ERβ, both expressed in HUVEC (Mendelsohn, 2002; Sobrino et al., 2009; Wagner et al., 2001) and also in endothelial cells from arterial origin (Novella et al., 2013). Molecular, cellular and animal studies have demonstrated beneficial estrogen effects on vascular cells, most of them mediated by ERα (Arnal et al., 2010; Novella et al., 2012). ERα and ERβ protein expression remained unaltered when HUVEC were exposed to E2 and ER antagonists in the same conditions than in the present work (Sobrino et al., 2009, 2010).

The effects induced by E2 on ACE1 and ACE2 mRNA and protein

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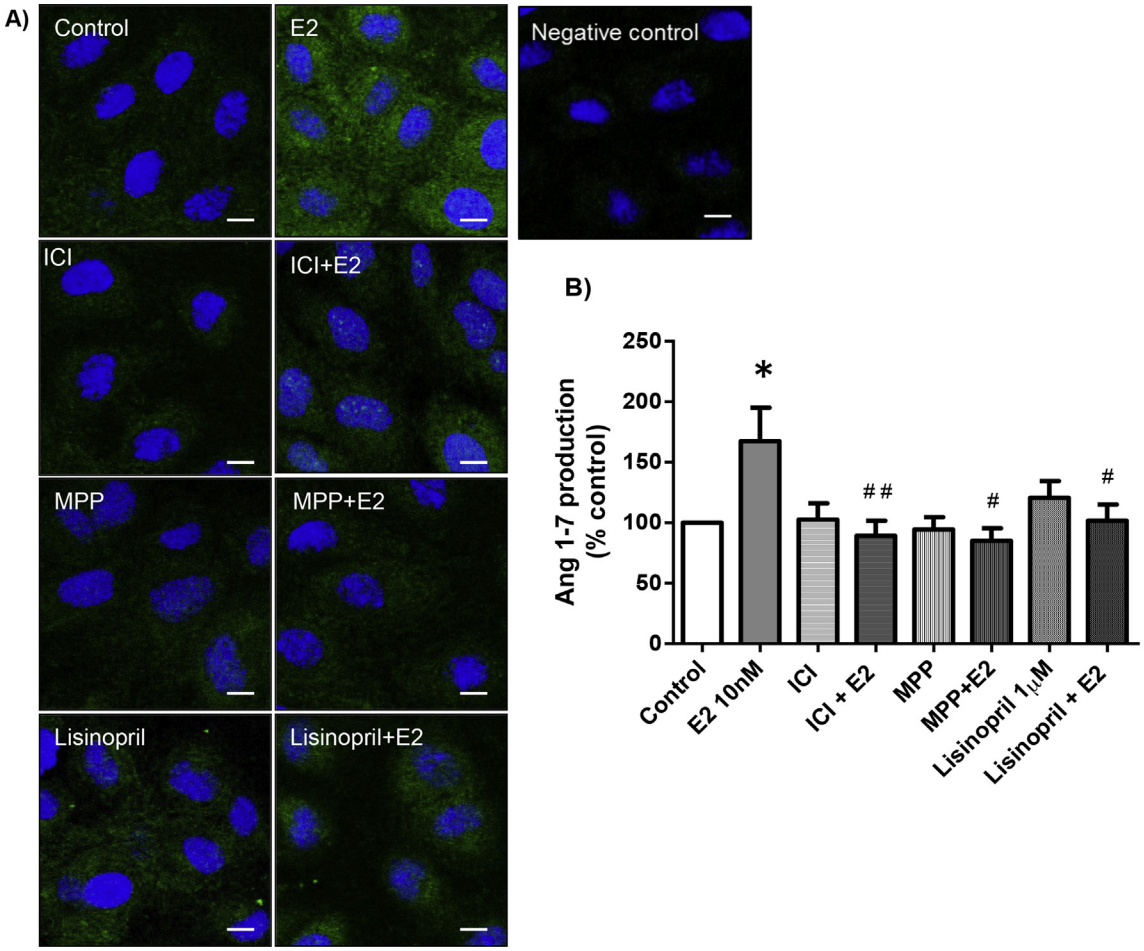

Fig. 6. Ang-(1–7) levels in HUVEC. (A) Representative confocal images of Ang-(1–7) in HUVEC. Cells were exposed to E2 (10 nmol/l) alone or in combination with ER antagonist ICI (1 μmol/l), ERα antagonist MPP (1 μmol/l) or ACE1 inhibitor (Lisinopril; 1 μmol/l) for 24 h. Green color (FITC) represents Ang-(1–7) production. Negative control represents the background of secondary antibody. (B) Ang-(1–7) expression quantification of confocal images in HUVEC. Data are expressed as the percentage of the control values and presented as mean ± SEM. The experiment was performed in 6 duplicate experiments. *$p < 0.05$ vs. control; #$p < 0.05$ and ##$p < 0.01$ vs. E2 10 nmol/l alone. Scale bar represents 10 μm (original magnification ×400). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

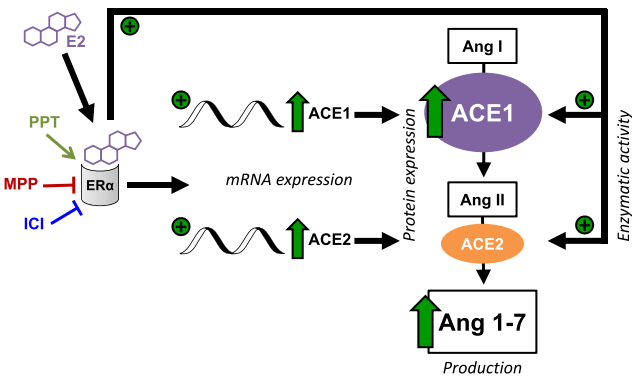

Fig. 7. Schema illustrating the E2-induced effects on HUVEC. E2, acting through ERα, increases ACE1 and ACE2 mRNA expression and activities and ACE1 protein expression, resulting in increased Ang-(1–7) production. PPT is a specific ERα agonist, MPP is a specific ERα antagonist and ICI is an ER unspecific antagonist.

expressions and activities, and on Ang-(1–7) production were abolished by the competitive antagonist of ERα and ERβ, ICI 182780, and by the specific ERα antagonist MPP. Moreover, the ERα agonist PPT, but not the ERβ agonist DPN, mirrored the E2 effects. Taken together, these results demonstrate a selective involvement of ERα in the effect of E2 modulating RAS components in HUVEC.

Considering the cardiovascular beneficial effects of Ang-(1–7), the ACE2-mediated degradation of Ang II and the resulting formation of Ang-(1–7), might represent a protective mechanism. Thus, our results add new information that supports a role for ERα in E2-mediated vasodilatory effects and reinforce the central role of ERα on the estrogen beneficial effects on endothelial physiology (Arnal et al., 2010; Novella et al., 2012, 2013).

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