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# Mas receptor is involved in the estrogen-receptor induced nitric oxide-dependent vasorelaxation

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ABSTRACT

The Mas receptor is involved in the angiotensin (Ang)-(1–7) vasodilatory actions by increasing nitric oxide production (NO). We have previously demonstrated an increased production of Ang-(1–7) in human umbilical vein endothelial cells (HUVEC) exposed to estradiol (E2), suggesting a potential cross-talk between E2 and the Ang-(1–7)/Mas receptor axis. Here, we explored whether the vasoactive response and NO-related signalling exerted by E2 are influenced by Mas. HUVEC were exposed to 10 nM E2 for 24 h in the presence or absence of the selective Mas receptor antagonist A779, and the estrogen receptor (ER) antagonist ICI182780 (ICI). E2 increased Akt and endothelial nitric oxide synthase (eNOS) mRNA and protein expression, measured by RT-PCR and Western blot, respectively. Furthermore, E2 increased Akt activity (determined by the levels of phospho-$Ser^{473}$) and eNOS activity (by the enhanced phosphorylation of $Ser^{1177}$, the activated form), resulting in increased NO production, which was measured by the fluorescence probe DAF-2-FM. These signalling events were dependent on ER and Mas receptor activation, since they were abolished in the presence of ICI or A779. In *ex-vivo* functional experiments performed with a small-vessel myograph in isolated mesenteric vessels from wild-type mice pre-contracted with noradrenaline, the relaxant response to physiological concentrations of E2 was blocked by ICI and A779, to the same extent to that obtained in the vessels isolated from Mas-deficient. In conclusion, E2 induces NO production and vasodilation through mechanisms that require Mas receptor activation.

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## 1. Introduction

Estradiol (E2) exerts a wide range of vascular actions by interacting with estrogen receptors (ER), which are expressed by both endothelial and smooth muscle cells [1]. Microarray analysis performed by our group in human umbilical vein endothelial cells (HUVEC) revealed that E2 regulates relevant vasoactive pathways, including the nitric oxide (NO) or the renin–angiotensin system (RAS) by interacting with ER [2,3].

NO is a major regulator of the vascular tone generated by endothelial NO synthase (eNOS) whose activity is tightly regulated through different intracellular events, including protein phosphorylation. Two main regulatory sites of eNOS phosphorylation are $Ser^{1177}$ and $Thr^{495}$, which result in increased or diminished enzymatic activity, respectively [4]. In endothelial cells, the PI3K/Akt pathway is involved in prototypical endothelial functions such as the regulation of vascular tone, angiogenesis, control of adhesion, and recruitment of leucocytes to the vessel wall [5]. Indeed, eNOS phosphorylation by PI3K/Akt is associated with enhanced NO production and release [5].

The RAS is a key systemic regulator of blood pressure and electrolytic homeostasis, but it also exerts paracrine inflammatory, trophic and vasoactive effects within the vasculature. While angiotensin (Ang) II is the main biologically active compound of the RAS, a number of other active Ang peptides have been identified during the last years. Among them, Ang-(1–7) is a heptapeptide generated from Ang I and Ang II by different enzymatic pathways involving neprilysin (NEP; neutral endopeptidase) and angiotensin-converting enzyme (ACE) 2, among others [6]. Ang-(1–7) binds to

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the G protein-coupled receptor Mas and is considered nowadays a physiological antagonist of Ang II [7].

In previous studies, we have described that, even in the absence of Ang-(1–7), the inactivation of Mas receptors by genetic deletion or pharmacological blockade deregulates the vasoactive responses elicited by several endothelium-dependent vasodilators [8]. Hence, Mas blockade blunts the NO-dependent vasorelaxation elicited by acetylcholine or bradykinin in isolated microvessels [9].

Recently, we have demonstrated that E2 stimulates production of Ang-(1–7) in HUVEC by increasing the expression and activity of angiotensin converting enzymes via ER [3]. This suggests the existence of a cross-talk between E2 and the Ang-(1–7)/Mas receptor arm of the RAS.

Since E2 shares common vasodilatory mechanisms with bradykinin and acetylcholine based on endothelial NO production and release, we aimed to investigate whether Mas receptors were also required for E2 to properly exert its vasorelaxant actions. For this purpose, we have studied the influence of Mas blockade on Akt levels, eNOS expression, and NO production, as well as on microvascular reactivity using both human endothelial cell cultures and isolated murine mesenteric arteries stimulated with E2.

## 2. Material and methods

### 2.1. Cell culture and experimental design

Primary HUVEC were isolated by collagenase (GIBCO, Invitrogen, Paisley, UK) treatment of human umbilical veins as described earlier [2]. Briefly, HUVEC were grown in 75-cm$^2$ flasks (Orange Scientific, Waterloo, Belgium) in human endothelial cell specific Medium EBM-2, (Lonza, Verviers, Belgium) supplemented with EGM-2 (Lonza), in an incubator at 37 °C with 5% $CO_2$.

Cells were identified as endothelial by their characteristic cobblestone morphology and the presence of von Willebrand factor by immunocytochemistry using a specific antibody (F-3520; Sigma, Madrid, Spain) as previously described [10]. Cells from passages 4–6 were seeded onto 6-well plates with fibronectin-treated coverslips for immunocytochemistry, onto 6-well plates for NO measurement and onto 25 cm$^2$ flasks for Western blot and mRNA isolation. When cells reached 75% of confluence, culture medium was exchanged for a phenol red–free Medium 199 (GIBCO) supplemented with steroid-deprived (by charcoal/dextran treatment) 20% fetal bovine serum (GIBCO) and maintained for 24 h. Then, culture medium was eliminated and immediately replaced with phenol red–free Medium 199. The desired concentrations of E2 (Sigma) were obtained by successive dilutions of a stock solution with ethanol. The desired concentrations of the ER antagonist ICI182780 (ICI; Tocris Bioscience, Ellisville, MI, USA), and A779 (Bachem, Bubendorf, Switzerland), were obtained by successive dilutions of a stock solution with DMSO and phosphate buffered saline (PBS), respectively. Control cells were exposed to the same vehicles (less than 0.1% ethanol, 0.1% DMSO or 0.1% PBS, respectively).

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

HUVEC were treated in 25-cm$^2$ flasks with the desired products. After 24 hours, total cellular RNA was extracted by using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription (RT) was carried out using SuperScriptTM First-Strand Synthesis System for RT-PCR (Invitrogen). One microgram of total RNA was reverse transcribed to cDNA following the manufacturer's instructions. Taqman probes for eNOS, Akt and GAPDH (endogenous control) were Hs00167166_m1, Hs00178289_m1 and 4326317E, respectively and were from Applied Biosystems (Fosters City, CA, USA). PCR was performed with TaqMan Universal Mastermix (Applied Biosystems) in a 7900HT Fast Real-Time PCR System (Applied Biosystems). Each sample was amplified in triplicate for each gene. Data were analyzed with the ABI PRISM 1.7 analysis software (Applied Biosystems).

### 2.3. Immunoblotting

Treated HUVEC were collected in RIPA buffer (Sigma) containing protease inhibitors (complete ULTRA Tablets, Roche Diagnostics, Madrid, Spain) and phosphatase inhibitors (PhosStop Tablets, Roche Diagnostics). Protein content was measured by the BCA method (Thermo Scientific Inc., Rockford, IL, USA) and samples were frozen at −20 °C until assayed.

Equal amounts of protein (ranging 15–50 μg) were then separated by NuPAGE®Noves 4–12% Bis Tris midi gel, 20-well (Invitrogen), and protein was then transferred to PVDF sheets (PVDF Transfer Membrane, Bio-Rad, Madrid, Spain). Immunostaining was achieved using the following specific antibodies: anti-eNOS (#9570), anti-eNOS phosphorylated at Ser$^{1177}$ (#9571), anti-eNOS phosphorylated at Thr$^{495}$ (#9574), anti-Akt (#9272) and anti-Akt phosphorylated at Ser$^{473}$ (#4058) (all from Cell Signaling Technology, Danvers, MA, USA). Development was performed with horse rabbit peroxidase-linked antibodies (Sigma), followed with Supersignal Chemiluminescent Substrate (Thermo Scientific Inc.). Signal density was analyzed with ImageGauge 4.0 software. Equivalent protein loading and transfer efficiency were verified by staining for β-actin (Sigma).

### 2.4. Nitric oxide production

Intracellular NO was monitored with the fluorescence probe DAF-2, as described earlier [9]. Briefly, HUVEC were seeded on 24-well plates, and at 80% confluence, cells were exposed to the desired treatments. Then, cells were loaded with 2.5 μmol/l 4-amino-5-methylamino-2,7-difluorofluorescein diacetate (DAF-2-FM diacetate; Molecular Probes, Invitrogen) for 30 min. After loading, cells were rinsed three times with culture medium. To quantitate the DAF-related fluorescence, the cells were observed under an inverted fluorescence Nikon Eclipse Ti-S microscope. Fluorescence from five different fields per well was measured (excitation wavelength: 488 nm; emission wavelength: 515 nm). Fluorescence signals were quantified using NIS-Elements 3.0 software (Nikon Izasa S.A, Barcelona, Spain). NO synthase inhibitor Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME, 100 μM; Sigma) was used to confirm and validate that the fluorescent signal to DAF was originated by NO.

### 2.5. Cell viability measurement

The possible toxic effect of the used compounds on HUVEC was discarded by the measurement of cell respiration, an indicator of cell viability, that was assessed by mitochondrial dependent reduction of 3–(4.5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan [11]. Cell viability after all treatments was the same as control cells maintained without treatments (data not shown).

### 2.6. Microvascular reactivity

Six-month-old male C57Bl/6 mice or Mas-deficient mice on a C57Bl/6 background [12] were used in the experiments. Animals were maintained under standardized conditions with an artificial 12-h dark–light cycle, with free access to food and water. All animal studies were performed according to national guidelines and approved by the institutional animal care committees.

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