Angiotensin (1–7) counteracts the negative effect of angiotensin II on insulin signalling in HUVECs

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Aims
Angiotensin II participates to the regulation of cardiovascular physiology and it is involved in molecular mechanisms of insulin resistance. Angiotensin (1–7), derived from angiotensin II metabolism, is able to counteract many of the haemodynamic and non-haemodynamic actions of angiotensin II. In this study, we investigated in human umbilical vein endothelial cells (HUVECs) the possible action of angiotensin (1–7) on the insulin signalling pathway.

Methods and results
We stimulated HUVECs with insulin, angiotensin II and angiotensin (1–7), testing the effects on endothelial nitric oxide synthase (eNOS) enzyme activation and on insulin receptor substrate-1 (IRS 1) phosphorylation. Moreover, we analysed the involvement of angiotensin type1, type2, and Mas receptors in these actions. Finally, we measured the nitric oxide (NO) production, the intracellular cGMP and the PKG-related activity in HUVECs, and the subsequent functional vasoactive effect of angiotensin (1–7) in mesenteric arteries of mice. Angiotensin II inhibits the insulin-induced Akt and eNOS phosphorylation, reducing the NO production. On the other hand, angiotensin (1–7) counteracts the inhibitory effect of angiotensin II, being able to restore the insulin-induced Akt/eNOS activation and the NO production. This effect is mediated by the Mas receptor. The inhibitory effects of angiotensin II on insulin signalling are, at least in part, mediated by an increased serine phosphorylation of IRS1. Angiotensin (1–7) inhibits the serine phosphorylation of IRS1 induced by angiotensin II.

Conclusion
In endothelial cells angiotensin (1–7) counteracts the negative effects of angiotensin II on insulin signalling and NO production. The balance between angiotensin II and angiotensin (1–7) could represent a key mechanism in the pathophysiological processes leading to endothelial dysfunction and insulin-resistance.

Keywords
Angiotensin (1–7) • Insulin • Endothelium • Nitric oxide

1. Introduction
Angiotensin (Ang) II is a member of the renin–angiotensin system (RAS), which is involved in the regulation of blood pressure, cardiovascular physiology, and electrolytes homoeostasis. The binding of Ang II to its type-1 receptor (AT1R) is able to activate several signalling pathways into the vasculature leading to vascular inflammation, endothelial dysfunction, contraction, growth and migration of vascular smooth muscle cells, all factors that promote the development and progression of vascular damage.

In addition, we previously demonstrated that the exposure of human umbilical vein endothelial cells (HUVECs) to Ang II, inhibiting the production of nitric oxide (NO) induced by insulin, was associated with impaired tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and its corresponding association with the p85 subunit of phosphatidylinositol 3-kinase (PI3K), leading to a defective activation of Akt and endothelial-NO synthase (eNOS). This occurred via the AT1R, as demonstrated by the capacity of AT1R antagonist losartan to inhibit the actions of Ang II. Moreover, we recently reported that the effects of Ang II on the insulin signalling are...
exclusively mediated by AT,R, supporting the hypothesis that other molecular pathways operate in the transduction of this signal.5

Ang (1–7) is a heptapeptide hormone generated by the cleavage of Ang I by prolylendopeptidase and neutral endopeptidase and also from Ang II by numerous enzymes, in particular by the angiotensin-converting enzyme homologue (ACE2).6–8 Ang 1–7, acting through the G protein-coupled Mas receptor (MasR), counteracts many of the actions of Ang II inducing vasodilatation, diuresis and natriuresis, inhibiting cell growth and norepinephrine release, and enhancing the actions of bradykinin.9

Recently, several studies have revealed a clear association between insulin and Ang II signalling pathways.10–13 In vascular tissues, one of the more interesting molecular mechanisms is the interaction between Ang II signalling and the insulin-IRS/PI3K/Akt/eNOS axis that leads to NO production in endothelial cells. Ang II negatively modulates insulin signalling at multiple levels, such as the insulin receptor (IR), IRS1 and IRS2, PI3K and Akt through an AT,R-mediated mechanism.14 Recently, it has been shown a direct action of Ang II on eNOS activation.15 This results in an inhibition of the vasodilator properties of insulin.

Taken together, we investigated in HUVECs the possible action of Ang (1–7) on the insulin signalling pathway and its ability to restore the NO production. Using selective receptor antagonists, we analysed the role of the AT,R, Ang II type-2 receptor (AT,2R), and MasR in the Ang (1–7) actions. Finally, we explored the effects of Ang (1–7) on Ang II-induced serine phosphorylation of IRS.

2. Methods

2.1 Materials

Culture media and sera for endothelial cells (ECM) were from ScienCell (San Diego, CA, USA). Ang II, insulin, and wortmannin, a selective inhibitor of PI3K, were purchased from Sigma (St Louis, MO, USA); Ang (1–7) was from Calbiochem (San Diego, CA, USA). The specific MasR antagonist (D-Ala7) Angiotensin III (1–7) was purchased from Bachem AG (Bubendorf, CH), whereas PD123319, a selective blocker of AT,2R, was from Sigma (St Louis, MO, USA). Losartan was generously provided by Merck Sharp & Dohme (Whitehouse Station, NJ, USA).

Rabbit polyclonal antibodies against eNOS, phospho-eNOS (Ser1177), Akt, and phospho-Akt (Ser473) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-IRS1 and anti-phospho IRS1 (Ser312 and Ser616) antibodies were obtained from Upstate Biotechnology (Lake Placid, NY, USA).

2.2 Cell culture

HUVECs were seeded in 100 mm tissue culture dishes and cultured until 80% confluent. HUVECs were grown in ECM medium supplemented with 5% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, at 37°C in an atmosphere with 5% CO2 in air in a humidified incubator. The medium was replaced every 48 h.

2.3 Experiments and protein extraction

HUVECs were starved in the ECM medium without FBS and supplements for 18 h before stimulation. In the ‘time course-experiment’, HUVECs were incubated for 1, 2, 5, or 10 min in the presence of Ang II or Ang (1–7) (at the same concentration of 10−7 M). In the ‘combined-experiment’, HUVECs were stimulated both with Ang II (10−7 M) and Ang (1–7) (10−7 M), contemporary or at different times (in this case, the Ang II stimulation preceded the Ang (1–7) one). In the experiments with insulin and wortmannin, these were added to cells, respectively, 10 and 30 min before the angiotensins, at concentrations of (10−7 M) and (50 nM). Finally, the selective receptor antagonism was obtained with the utilization of losartan (200 nM), PD123319 (10−6 M), or D-Ala (10−5 M). The receptor inhibition preceded 20 min the following stimulation with Ang II or Ang (1–7).

After stimulation, cells were washed twice with ice-cold PBS and then lysed in ice-cold lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 10 mM Na3PO4, 100 mM NaF, and 2 mM sodium orthovanadate supplemented with protease inhibitor cocktail). The lysates were put on ice and vortexed every 5 min for 30 min. Lysates were then centrifuged at 14000 g for 30 min at 4°C, and the supernatants were aliquoted and stored at −80°C. The protein content of cell lysates was determined by a BioPhotometer (Eppendorf AG, Hamburg, Germany).

2.4 Immunoprecipitation and immunoblotting

Equal amounts of proteins (300 µg) were incubated at 4°C overnight with anti-IRS1 antibodies. Immunocomplexes were collected by incubation with protein A-Sepharose for 3 h at 4°C and resuspended in Laemmli buffer.

Cell lysates (30 µg of protein) or immunoprecipitated proteins were separated on 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. These were blocked by incubation for 1 h with a blocking buffer composed of TBST buffer (20 mM Tris–HCl, pH 7.4, and 150 mM NaCl with 0.05% Tween 20) containing 5% non-fat dry milk and incubated overnight at 4°C with each primary phosphospecific antibody (1:500 dilution). The membranes were washed three times in TBST buffer, incubated with secondary antibody (HRP-conjugated donkey anti-rabbit IgG at 1:1500) for 1 h at room temperature; the reaction products were then detected by ECL, and their intensities were quantified by digital densitometry.

2.5 eNOS activity assay

The effect of Ang II and Ang (1–7) treatment, on the insulin-stimulated endothelial eNOS activity, was evaluated in HUVECs. The enzymatic activity of eNOS was assayed by using the NOS detect Assay kit (Stratagene Italy) based on the ability of NOSs enzymes to convert [14C]-L-arginine into [14C]-L-citrulline. Following the kit manufacturer protocol, after hormones exposure, cells were homogenized and 100 ng of whole cell lysate was used for the assay. Briefly, triplicate tubes of cell lysates were incubated in reaction buffer (25 mM Tris–HCl, pH 7.4, 3 mM BSA, 1 mM FAD, 1 mM FMN) containing 1 mM NADPH freshly prepared, 0.6 mM CaCl2, 0.1 µM calmodulin, and 1 µCi/mL [14C]-L-arginine in a final volume of 50 mL. The mixture was incubated for 30 min at room temperature with gentle agitation. The amount of [14C]-citrulline produced was purified by column chromatography and measured by liquid scintillation in a beta counter for 1 min each sample. Positive controls are included in the assay, respectively, represented by rat cerebellum extract and adding to the reaction mixture 1 mM of Nω-nitro-L-arginine methyl ester HCl.

2.6 Determination of intracellular cGMP

The effective NO production was assessed by the quantification of intracellular cGMP levels (index of NO production). Briefly, after Ang II and Ang (1–7) treatment, the HUVECs were lysed in 0.1 mol/L of hydrochloric acid. Aliquots (100 µL) of the cell lysates were transferred into well plates and cGMP was quantified by using a colorimetric cGMP enzyme immunoassay kit (ENZKO Lifesciences, Farmingdale, NY, USA), according to the manufacturer’s instructions.

2.7 PKG-1 Activity Assay

cGMP-induced PKG-1 activity was assayed by a colorimetric analysis with a CyclicLex cGMP-dependent protein kinase assay kit (MBL International, Woburn, MA, USA) in HUVECs lysates according to the manufacturer’s
protocol. cGMP and KT5823 were utilized as positive and negative quality controls, respectively.

2.8 Vascular function evaluation
To evaluate the functional effect of Ang (1–7)-related NO production, we performed a myographic assay in vessels. Second-order mesenteric arteries were dissected free of connective tissue from anaesthetized mice and set up on wire myograph (DMT Instruments), in gassed (95% O2–5% CO2) KREBS (NaCl 114, 13 mM, KCl 4.69 mM, MgSO4·7H2O 51 mM, KH2PO4 1.18 mM, NaHCO3 25 mM, C6H12O6 5.99 mM, CaCl2 2.5 mM) pH 7.4, at 37°C, under a normalized tension corresponding to an effective pressure of 75 mmHg, for 1 h to equilibrate. All substances used were from Sigma.

The presence of a functional endothelium was assessed by the ability of acetylcholine (ACh) (1 μM) to induce 70% relaxation of vessels pre-contracted with phenylephrine at 80% of KPSS contraction. Concentration–response curves were performed for Ang II (from 10−9 to 10−5 mol/L) and for Ang (1–7) (from 10−10 to 10−6 mol/L) and ACh (from 10−9 mol/L to 10−5 mol/L) in vessels pre-contracted with phenylephrine at 80% of KPSS contraction, to achieve the same tension level.

To assess the endothelium-dependent vasorelaxation with ACh and Ang (1–7), vessels were pre-incubated in the presence of NG-nitro-L-arginine methyl ester (L-NAME; 100 μM; Sigma).

2.9 Statistical analysis
Differences between groups were compared by the unpaired Student's t test. Results are expressed as means ± SE of at least three experiments. Significant differences were assumed to be at *P < 0.05. All comparisons were performed using the statistical package SPSS 12.0 for Windows.

3. Results

3.1 Time-course effects of Ang II and Ang (1–7) on Akt/eNOS activation
For better understanding the characteristics of Ang II and Ang (1–7) interaction on Akt and eNOS activation, at first we carried out a time-course stimulation to determine the timing of signalling effects for both peptides in HUVECs. These experiments have shown an inhibiting peak for Ang II after 10 min and a stimulating peak after 5 min for Ang (1–7) (Supplementary material online, Figure S1).

3.2 Interactions between Ang II and Ang (1–7) on Akt/eNOS axis
Ang II reduces the level of phosphorylated Akt and eNOS; the addition of Ang (1–7) increases the portion of active Akt and eNOS by 40 and 58%, respectively (Figure 1). Thus Ang (1–7), in addition to stimulate directly the serine phosphorylation of both enzymes, is able to effectively counteract the inhibition of Ang II on the same.

3.3 Receptors involved in Ang II and Ang (1–7) effects
The selective antagonists losartan, PD123319, and D-Ala were, respectively, used to study the involvement of AT1R, AT2R, and MasR in the effects of Ang II and Ang (1–7). As shown in Figure 2, losartan entirely restores the levels of phosphorylated Akt/eNOS after the Ang II inhibition; no effect was observed on Ang (1–7) action. The use of PD123319 produces no change on Ang II effects, while it partially inhibits the actions of Ang (1–7) on Akt/eNOS activation, even if not significantly. In contrast, the blockage of MasR, obtained with the use of D-Ala, significantly attenuates the Ang (1–7) effects, confirming the importance of this receptor in mediating the actions of the peptide. Finally, the use of inhibitors alone does not alter the levels of phosphorylated enzymes in comparison with the basal control.

3.4 PI3K involvement in the Ang (1–7) signalling
To determine whether the Ang (1–7)-induced Akt activation is mediated by the PI3K, the cells were pre-treated with wortmannin.
Figure 2 Effects of losartan (A), PD-123319 (B), and D-ALA (C) on Akt and eNOS serine phosphorylation in HUVECs. To normalize the blots for protein levels, after being immunoblotted with anti-phosphospecific antibodies, the blots were stripped and reprobed with anti-eNOS and anti-Akt antibodies. Data are means ± SE, expressed as relative change in comparison with the basal value (n = 3 for every experiment). *P < 0.05 vs. basal; †P < 0.05 vs. Ang II; ‡P < 0.05 vs. Ang (1–7).
(50 nm, 30 min). As shown in Figure 3, wortmannin inhibits the Ang (1–7)-induced phosphorylation of Akt on Ser473 confirming that the Ang (1–7) effect is mediated by PI3K.

### 3.5 Interactions between Ang II, Ang (1–7), and insulin on Akt/eNOS axis

To study the influence of Ang II and Ang (1–7) on insulin signalling, HUVECs were stimulated with insulin, Ang II, and Ang (1–7) alone, or with a combination of all three hormones. The combined stimulation then was tested in the presence of AT1R, AT2R, and MasR blockade. Insulin stimulates the serine phosphorylation of Akt/eNOS, increasing their active portion by 2.5 times (Figure 4). The addition of Ang II completely inhibits the insulin effects on two enzymes. This inhibitory effect on insulin signalling is almost completely countered by the addition of Ang (1–7). Losartan in combination with Ang (1–7) completely restores the effects of insulin, eliminating the deleterious effects of Ang II. The use of PD123319 does not significantly modify the Ang (1–7) effect, whereas the addition of D-Ala, by inhibiting the actions of Ang (1–7), restores the negative effects of Ang II on insulin signalling (Supplementary material online, Figure S2).

### 3.6 Effects of Ang II and Ang (1–7) on IRS1 serine phosphorylation

It has been shown that serine phosphorylation of IRS1 compromises the ability of this substrate to be phosphorylated in tyrosine by the IR, thereby impairing the ability to activate PI-3K. In consideration of the opposite effects of Ang II and Ang (1–7) on insulin signalling, we tested the effects of both hormones on serine phosphorylation of IRS1. As shown in Figure 5, the exposure of HUVECs to Ang II results in a Ser616 phosphorylation of IRS1. This effect is completely inhibited by the addition of Ang (1–7). Pre-incubation of HUVECs with losartan restores the baseline phospho-serine IRS1 levels, eliminating the effect of Ang II. Inhibition of AT2R partially reduces the effects of Ang (1–7), although not significantly ($P < 0.08$), whereas the use of D-Ala almost completely blocks the inhibitory effects of Ang (1–7) on insulin signalling (Supplementary material online, Figure S2).
3.7 Effects of Ang II and Ang (1–7) on insulin-stimulated NO production

We also determined the effects of Ang II and Ang (1–7) on eNOS activation induced by insulin. eNOS activity was increased by insulin, whereas Ang II treatment resulted in a 85% decrease in insulin-stimulated eNOS activity. The addition of Ang (1–7) to HUVECs reversed the inhibitory effects of Ang II on insulin-stimulated eNOS activity (Figure 6).

3.8 Effects of Ang (1–7)-induced NO production

The downstream NO signalling transduction leads to an increase in intracellular cGMP that in turn is able to activate the PKG. This event is associated with the vasorelaxation in arteries. We tested the effectiveness of the Ang (1–7) eNOS activation and NO production by measuring the intracellular cGMP and the subsequent PKG-1 activity. As observed in Supplementary material online, Figure S3, Ang II has no effect on cGMP production and PKG-1 activity with respect to the control. In contrast, Ang (1–7) is able to increase the intracellular cGMP in HUVECs (A), by inducing a significant increase in PKG-1 activation (B).

3.9 Endothelium-mediated vasodilatory effect of Ang (1–7)

To test the functional effect of Ang (1–7)-mediated NO production, we performed a myograph assay in mesenteric arteries of mice. We utilized the L-NAME as a specific inhibitor of endothelial NO production.

Figure 5 Effects of Ang II and Ang (1–7) on the insulin pathway: induction of Ser616 phosphorylation of IRS1 by Ang II and reversibility of the effect by Ang (1–7). Data are means ± SE, expressed as relative change in comparison with the basal value (n = 3 for every experiment). *P < 0.05 vs. insulin; †P < 0.05 vs. insulin + Ang II; ‡P < 0.05 vs. insulin + Ang II + Ang (1–7).

Figure 6 The eNOS enzymatic activity assay upon insulin stimulation and Ang II and Ang (1–7) addition on HUVECs. Bars represent the average radioactivity associated to the 14C-citrulline amount (means ± SE of three independent experiments) generated with NO production. *P < 0.05 vs. basal value; †P < 0.05 vs. insulin; ‡P < 0.05 vs. insulin + Ang II.
synthesis. As shown in Supplementary material online, Figure S4, ACh was able to induce an endothelium-dependent vasodilation in a dose-dependent manner; this effect was inhibited in the presence of L-NAME (A). Similar effects were obtained with Ang (1–7) (C), demonstrating thus the key role of endothelial NO in the Ang (1–7) functional actions. In contrast, Ang II caused a vasoconstriction at a maximal efficacy dose of 10^-7 mol/L (B).

4. Discussion

It is well established that Ang II is implicated in the pathophysiological mechanisms of both insulin resistance and endothelial dysfunction16–19 that participate to the appearance and progression of atherosclerotic process. Interestingly, the cross-talk between Ang II and insulin signalling transduction may explain, at least in part, the impaired metabolic effects observed in the presence of the insulin resistance status.20–22 At the vascular level, the Ang II-induced inhibition on the IRS3/Pi-3 kinase/Akt/eNOS pathway reduces the endothelial NO production, pre-disposing to the development of vascular damage.23 In recent years, increasing interest has exerted the Ang (1–7), a peptide capable to counteract many actions of Ang II.23–28

In this study, we evaluated the possible mechanisms by which Ang (1–7) may positively interfere in the cross-talk between RAS and the signalling of insulin in HUVECs. Our data demonstrate that the exposure of endothelial cells to Ang (1–7) is directly able to activate the signalling pathway producing NO, a molecule that possesses some vasoprotective effects. In particular, NO production is associated with an increase in intracellular cGMP and PKG-1 activation; this event leads to an effective functional vasodilatory role in the vasculature, as observed in the myographic assay. Akt and eNOS are two crucial enzymes in this signalling pathway. Their activation is related to a serine phosphorylation, which is significantly inhibited by Ang II through the AT1R stimulation. On the contrary, Ang (1–7) counteracts the inhibitory effects of Ang II on them.

The use of receptor antagonists indicates that the effects of Ang II are only attributable to AT1R recruitment, whereas those of Ang (1–7) are largely mediated by MasR and, minimally, by AT2R. These evidences, as also previously demonstrated by us,23,27 demonstrate that the AT1R stimulation does not contribute to the biological effects of the AT2R block in the insulin signalling transduction.

Moreover, the PI3K activation is an important step for the Akt recruitment that it is necessary to activate the metabolic actions of insulin, such as the glycogen synthesis and the membrane glucose transport. Our study confirms that the PI3K/Akt axis is also crucial in the effects mediated by Ang (1–7). In fact, Ang (1–7) is able to promote the Akt and eNOS serine phosphorylation that is inhibited by the wortmannin, a selective PI3K inhibitor. Our data suggest that the exposure of endothelial cells to Ang (1–7) is directly able to activate the signalling pathway producing NO, a key biological molecule involved in the preservation of endothelial function and vascular integrity. The biological relevance of this finding is supported by the demonstration that Ang (1–7) is able to induce an endothelium-dependent vasodilation in mice arteries, similarly to that exerted by muscarinic receptor stimulation. Moreover, Ang (1–7) counterbalances the negative effect of Ang II, by improving the haemodynamic and metabolic actions of insulin. Thus, we can presume that in clinical conditions of insulin resistance, in which Ang II is up-regulated, such as hypertension, diabetes, obesity, and hypercholesterolaemia, Ang (1–7) could have a positive impact on insulin sensitivity and haemodynamic properties of vasculature.

Another biologically relevant finding, obtained in this study, is the demonstration that Ang (1–7) counteracts the inhibitory effects of Ang II on insulin-induced activation of Akt, with a concomitant increase in eNOS activity. MasR mediates this opposite effect of Ang (1–7), as confirmed by the effects observed using its selective inhibitor D-Ala. The importance of this finding consists in the fact that neither AT1R nor AT2R participate in this specific effect of Ang (1–7) that is instead activated by MasR recruitment. All these evidences confirm the hypothesis that RAS is a complex system in which several signalling pathways mediated by AT receptors are involved.

Serine phosphorylation of IRS1, is the most known molecular mechanism responsible for insulin resistance.29,30 Many molecules, including interferons and interleukins, are able to induce serine phosphorylation of IRS1, compromising the ability of the substrate to be tyrosine phosphorylated by IR and to activate PI3K.31–34 We demonstrated that Ang II induces a Ser616 IRS-1 phosphorylation, impairs the downstreaming insulin signalling pathway that is restored by the Ang (1–7) through the MasR activation. These data confirm the crucial role of MasR in the modulation of both metabolic and vascular actions of Ang II. All these evidences have clinical relevance because some of non-haemodynamic effects of sartans are mediated, at least in part, by an increased bioavailability of Ang (1–7).

On the basis of these results, we hypothesized that Ang (1–7) positively interferes with insulin action in the endothelium. Thus, a greater bioavailability of Ang (1–7), opposing the negative effects of Ang II, could have a positive impact on insulin sensitivity and haemodynamic properties of vasculature in course of many cardiovascular diseases. On the other hand, Ang (1–7) has been shown to be also effectiveness-enhancing in cellular systems beyond the endothelium, such as vascular smooth muscle cells and macrophages, all involved in the pathogenetic mechanisms of plaque formation. Obviously, further studies would be necessary to better understand the Ang (1–7) signalling in the anti-atherosclerotic properties of the vessels and its possible role in the future therapeutic targets.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.

References
