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AT1 receptor blocker potentiates shear-stress induced nitric oxide production via modulation of eNOS phosphorylation of residues Thr^{495} and Ser^{1177}

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ABSTRACT

We tested the hypothesis that AT1R blockade modulates the shear stress-induced (SS) synthesis of nitric oxide (NO) in endothelial cells (EC). The AT1R blocker Candesartan in the absence of the ligand angiotensin II (ang II) potentiated SS-induced NO synthesis accompanied by increased p-eNOS^{Ser1177} and decreased p-eNOS^{Thr495}. Candesartan also inhibited SS-induced ERK activation and increased intracellular calcium transient in a time-dependent manner. To confirm the role of ERK to modulate p-eNOS^{Thr495} and calcium to modulate p-eNOS^{Ser1177}, the MEK inhibitor U0126 and the calcium chelator BAPTA-AM were used, respectively. Pre-treatment of EC with U0126 completed abrogated basal and SS-induced ERK activation, inhibited p-eNOS^{Thr495} and increased NO production by SS. On the other hand, pre-treatment of EC with BAPTA-AM decreased the effects of SS alone or in combination with Candesartan to induce p-eNOS^{Ser1177} and partially inhibited the effects of Candesartan to potentiate NO release by SS. The AT1R blockers Losartan and Telmisartan were also tested but only Telmisartan potentiated NO synthesis and blocked SS-induced AT1R activation. Altogether, we provide evidence that Candesartan and Telmisartan potentiate SS-induced NO production even in the absence of the ligand ang II. This response requires both the inhibition of eNOS phosphorylation at its inhibitory residue Thr^{495} as well as the increase of eNOS phosphorylation at its excitatory residue Ser^{1177}. In addition, the response is associated with inhibition of SS-induced ERK activation as well as increasing intracellular calcium transient. One may speculate that these yet undescribed events may contribute to the benefits of ARBs in cardiovascular diseases.

1. Introduction

Endothelial cells (EC) are constantly exposed to and interact with mechanical forces, such as shear stress (SS) and stretch, which play a role on the maintenance of vascular homeostasis [1]. The mechanotransduction and endothelial cell coupling involves a variety of candidates including ion channels [2], integrins [3], G-protein coupled receptors [4] and enzymes [5]. We and others have recently described that AT1 receptor (AT1R), a member of the Renin angiotensin system (RAS), is activated by SS independent of its ligand ang II [6,7].

Endothelial dysfunction [8] characterized by impaired endothelium-dependent vasodilatation and anti-proliferative, anti-thrombotic and anti-inflammatory functions [9] may be a central step underlying cardiovascular diseases. Endothelial isoform of nitric oxide synthase (eNOS) regulates nitric oxide (NO) production in EC in response to SS and to a variety of other stimuli, such as vascular endothelial growth factor (VEGF), estrogen, insulin, ascorbate and bradykinin [10–14]. Phosphorylation status of eNOS at different sites allows rapid changes in eNOS activity, and several signaling cascades and kinases have been implicated in eNOS phosphorylation [9,15,16].

SS-induced cellular responses are mediated by diverse signaling pathways, including stimulation of the MAP kinase cascade. In EC it appears that ERK1/2 induced phosphorylation of Thr^{495} decreases eNOS activity [14]. The proximity of eNOS and MAP kinases within the caveolae may facilitate their interactions and coordinate the regulation of these complex signaling pathways, both of which are known to be activated in response to SS [17]. Also, we have previously demonstrated that AT1R activation by SS leads to ERK phosphorylation by G-protein dependent and independent pathways [6].

We now tested the hypothesis that ang II receptor blockers (ARB) modulate the SS-induced activation of eNOS and NO

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production in EC. Our data suggest that ARBs, in the absence of ang II, can potentiate SS-induced eNOS activation while also inhibiting ERK phosphorylation.

2. Methods

2.1. Cell culture

Primary EC culture was obtained as previously described (saphenous vein endothelial cells, SVEC) [18]. Human saphenous veins were obtained from patients undergoing aortocoronary bypass surgery at the Heart Institute (InCor/USP). All individuals gave written informed consent to participate in the study, which was reviewed and approved by the local Ethics Committee (SDC 2454/04/074–CAPPesq 638/04). Cells were cultured in Human Endothelial-SFM (Invitrogen) supplemented with 20% newborn calf serum, 20 ng/mL FGF, 10 ng/mL EGF, 10U/mL penicillin, 10 mg/mL streptomycin, and 10U/mL heparin. All the experiments were performed with cells up to the 8th passage.

2.2. Shear stress protocol

SVECs underwent controlled SS as previously described [5,6,18,19]. Cells were plated in 100-mm dishes pre-coated with gelatin 1% and serum starved for 24-hours before SS using a cone plate viscometer (15 dyne/cm^2). Cells were washed with cold phosphate buffered solution and lysed to further analysis and culture medium was collected before and after the stimuli. Candesartan was a kind gift from AstraZenica (São Paulo, SP, Brazil). The selective inhibitor of MEK1 and MEK2, U0126 was from Sigma–Aldrich. BAPTA-AM was used as an intracellular calcium chelator and was from Life Technologies. Candesartan, U0126, and BAPTA-AM were added to the cells 30 min before SS stimuli.

2.3. Nitric oxide production

These analyses were performed in a Sievers NOA TM-280 nitric oxide analyzer. Briefly, the analysis solution responsible for reducing nitrite (NO_2^-) present in the samples to nitric oxide contained potassium iodide (45 mM), iodine (10 mM), decanol (ca. 5%) in glacial acetic acid and was maintained at 60 °C. The analyzer was calibrated daily with a freshly prepared nitrite solution (0.01–5 μM).

2.4. Western blot analysis

Cells were lysed in RIPA buffer with protease and phosphatase inhibitor cocktails (Sigma–Aldrich). Samples were centrifuged at 10,000*g* for 10 min to remove cellular debris. Cell lysates (5–40 μg) were heated in SDS–PAGE sample buffer, fractioned by SDS–PAGE, and transferred to Hybond membranes (GE-Healthcare). Blotted membranes were blocked with 5% non-fat milk for 2 h at room temperature, and then proteins were detected using their respective antibodies. The p-ERK and ERK antibodies were from CellSignaling (1:1000), the p-eNOS^{Ser1177}. p-eNOS^{Thr495} and eNOS were from BD Bioscience (1:1000). Horseradish peroxidase-conjugated antibody was used as a secondary antibody, and signals were detected using the ECL detection kit (GE-Healthcare).

2.5. Intracellular calcium determination

SVEC were labeled for 20 min at 37 °C by incubating 4×10^5 cells suspended in 0.5 mL HEPES buffer (NaCl 135 mM, KCl 5 mM, HEPES 10 mM, $MgCl_2$ 1 mM, glucose 2 mM, $CaCl_2$ 2 mM, pH 7.2) with Fluo-3/AM at a final concentration of 1 μM (Life Technologies). Cells were washed with HEPES before experiments. Candesartan was added immediately before processing via flow cytometry. For calcium-free experiments, cells were suspended in HEPES buffer containing BAPTA-AM (10 mM). Measurements of calcium transients were performed using FACS Calibur flow cytometer (BD) excitation at 488 nm and emission at 526 nm. After starting cell stimulation with Candesartan, the fluorescence intensity was measured in at least a 5×10^4 cell at each time point (0, 10, 20, 30, 45, and 60 min). The data are presented as the percentage of cells with fluorescence intensity above DMSO cells at time zero.

2.6. Angiotensin II measurement

Ang II levels were determined by ELISA, according to the manufacturer's instructions using a commercially available kit (SPI-BIO, Montigny-le-Bretonneux, France). The protein content was determined by the Bradford methods by using bovine serum albumin as the standard (Bio-Rad Protein Assay).

2.7. Statistical analysis

All data represent at least four independent experiments. Numerical data are expressed as mean ± SEM. Comparisons among

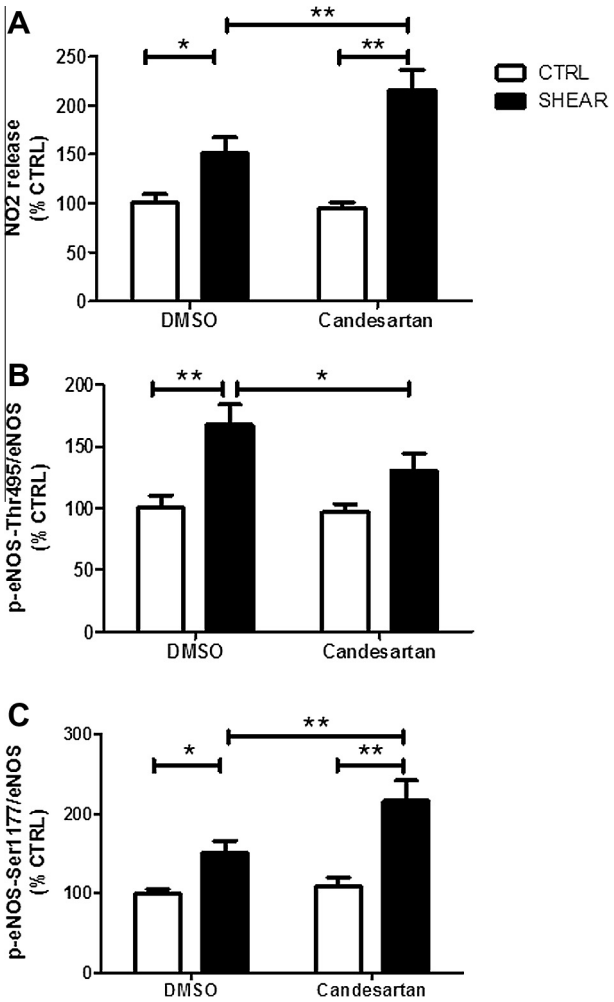

Fig. 1. AT1R blocker Candesartan potentiates NO production by Shear Stress through eNOS post-translational modification. SS-induced (A) NO_2^- production; (B) p-eNOS^{Ser1177} and (C) p-eNOS^{Thr495} in the presence or absence of Candesartan; SVEC were exposed to laminar shear stress (10 min, 15 dyne/cm^2); Candesartan 1 μM was administered 30 min before the onset of SS stimulus. Each bar represents mean ± SEM of 4–6 separate experiments. $^*p < 0.05$ and $^{**}p < 0.01$.

groups were performed using two-way (treatment versus drug) analysis of variance (ANOVA) followed by the Fisher LSD post-hoc test when appropriate. Values of $p < 0.05$ were considered significant. The Statistica 8.0 package for Windows (StatSoft Inc., Tulsa, OK, USA) was used for analysis.

3. Results

3.1. Candesartan potentiates SS-induced NO synthesis by post-translational modifications on eNOS

SS (10 min, 15 dyn/cm^2) in human SVEC is associated with an increased synthesis of NO (Ctrl, 100 ± 12% vs. SS, 151 ± 24%, $p < 0.05$; Fig. 1A) that was potentiated in the presence of the ARB Candesartan (SS, 151 ± 24% vs. SS + Cand, 216 ± 28%, $p < 0.01$; Fig. 1A). eNOS is the main enzyme responsible in EC to convert L-arginine and molecular oxygen to NO and L-citruline; it has many phosphorylation target sites that rapidly modulate the activity of the enzyme. SS-increases p-eNOS^{Thr495} (Ctrl, 100 ± 9% vs. SS, 168 ± 19%, $p < 0.01$; Fig. 1B) and p-eNOS^{Ser1177} (Ctrl, 100 ± 14% vs. SS, 151 ± 18%, $p < 0.01$; Fig. 1C), respectively (Fig. 2A). In the presence of Candesartan, SS-induced both a higher p-eNOS^{Ser1177} (SS, 151 ± 18% vs. SS + Cand, 227 ± 32%, $p < 0,01$; Fig. 1C) and also blocked the p-eNOS^{Thr495} (SS, 168 ± 19% vs. SS + Cand, 121 ± 22%, $p < 0.05$; Fig. 1B). Candesartan alone did not modulate the NO synthesis or the basal levels of p-eNOS (Ser^{1177}: Ctrl, 100 ± 14% vs. Cand, 111 ± 18%; Fig. 1C. Thr^{495}: Ctrl, 100 ± 9% vs. Cand, 97 ± 16%; Fig. 1B).

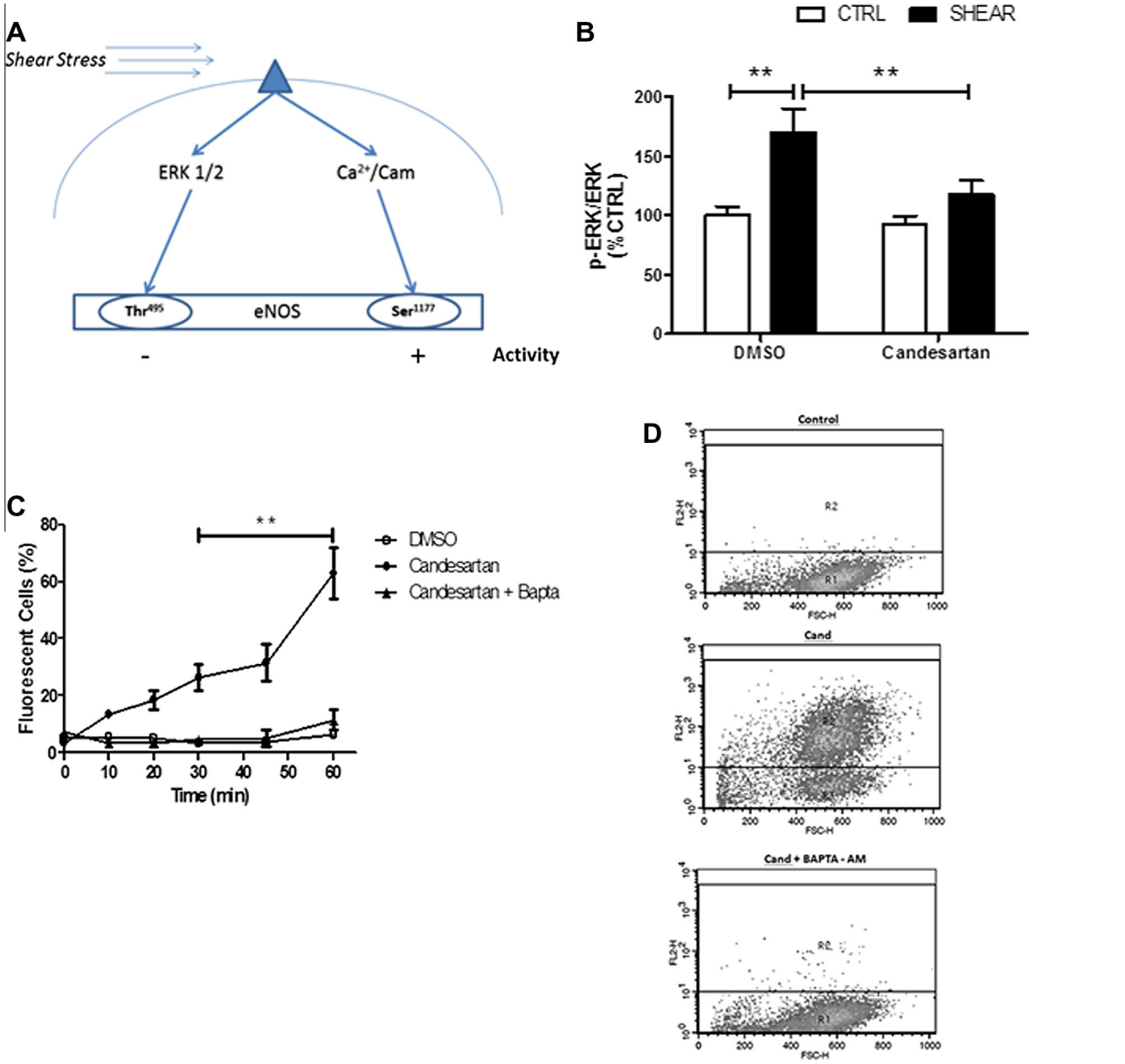

Fig. 2. AT1R blocker Candesartan modulates 2 upstream pathways of eNOS phosphorylation. (A) Representative figure showing the 2 main functionally established phosphorylation sites of eNOS modulated by ERK and calcium investigated in our model. Threonine^{495} (Thr^{495}) is a negative regulatory site and its phosphorylation is associated with decreased enzyme activity, while Serine^{1177} (Ser^{1177}) is a positive regulatory site and its phosphorylation increases enzyme activity; (B) Candesartan blockade of ERK activation by SS; (C) time-dependent intracellular calcium transient by Candesartan. SVEC were exposed to laminar SS (10 min, 15 dyne/cm^2); Candesartan 1 μM was administered 30 min before the onset of SS. BAPTA 10 μM was administered 30 min before Candesartan for the calcium transient assay. Each bar represents mean ± SEM of 4–6 separate experiments. $^*p < 0.05$ and $^{**}p < 0.01$.

3.2. Candesartan modifies eNOS upstream molecules that affect its phosphorylation status

The two main established phosphorylation sites of eNOS are schematically represented in Fig. 2A. eNOS phosphorylation at Thr^{495} is associated with lower activity of the enzyme, and ERK1/2 is considered the main upstream activation kinase, whereas increased cytoplasmic calcium levels activate calmodulin (CaM)/CaM-Kinase II to phosphorylate the stimulatory site of $eNOS^{Ser1177}$ [20].

We have previously demonstrated that Candesartan blocks SS-induced ERK activation in the absence of ang II [6]. Data in Fig. 2B confirm this effect in a human EC culture model (Ctrl, 100 ± 17%; SS, 170 ± 15%, $p < 0.01$; SS + Cand, 118 ± 20%; Fig. 2B). In addition, data on Fig. S1 show that no SS-induced release of ang II to the cell culture media occurs, which confirms our previous data showing that SS directly activates AT1R independently of its ligand, and the effect of Candesartan to block this mechanical activation of AT1R. These data suggest a possible mechanism by which Candesartan potentiates the SS-induced NO synthesis: By blocking the SS-induced ERK activation (Fig. 2B), Candesartan blocks p-eNOS at its inhibitory residue Thr^{495}(Fig. 1B) and increases NO synthesis (Fig. 1A) as previously suggested by agonist-stimulated EC [21].

In addition, we observed by flow cytometry that Candesartan itself could increase cytoplasmic calcium influx in EC (Fig. 2C), which may explain the increased eNOS phosphorylation observed at Ser^{1177} (Fig. 1C). Next, we tested the role of Candesartan on these two upstream molecules to explain the changes in eNOS phosphorylation status associated with shear stress.

3.3. Role of SS-induced ERK activation on NO synthesis and eNOS phosphorylation status

Candesartan blocked SS-induced ERK activation (Fig. 2B) and $p\text{-}eNOS^{Thr495}$ (Fig. 1B), while potentiating NO production (Fig. 1A). To further understand the role of ERK1/2 activation by SS and its effect on eNOS phosphorylation status, the MEK 1 and MEK 2 inhibitor, U0126, was used. Data in Fig. 3A show that U0126 blocked p-ERK1/2 at basal condition and under SS (Ctrl, 100 ± 10%; SS, 159 ± 14%; U0126 4 ± 1%; SS + U0126, 5 ± 2%). NO synthesis was further increased by SS in the presence of U0126 (SS, 152 ± 15%; SS + U0126, 216 ± 18%; Fig. 3B), and no significant eNOS phosphorylation at Thr^{495} was observed (Ctrl, 100 ± 12% vs. SS + U0126, 125 ± 14%; Fig. 3C). There were no significant changes at $p\text{-}eNOS^{Ser1177}$ in the presence of U0126 by SS (Ctrl, 100 ± 12%; SS, 151 ± 23%; SS + U0126, 135 ± 17%; Fig. 3D). These data show that SS-induced ERK1/2 phosphorylation of eNOS at its inhibitory residue Thr^{495} may partly contribute to the decrease of SS-induced NO synthesis in EC. Therefore, Candesartan blockade of SS-induced p-ERK and $p\text{-}eNOS^{Thr495}$ is consistent with the potentiation of NO synthesis (Fig. 2A).

3.4. Role of Candesartan-induced calcium transient on NO synthesis and eNOS phosphorylation status by shear stress

As depicted in Fig. 2A, another important eNOS phosphorylation residue is Ser^{1177}. Elevated intracellular calcium transient is a short stimulus to induce the phosphorylation of this site by CaM-Kinase II [22]. Since Candesartan increased calcium transient (Fig. 2C), we tested whether calcium transient blockade with BAPTA-AM inhibited Candesartan-induced NO synthesis by SS. Data in Fig. 4A show

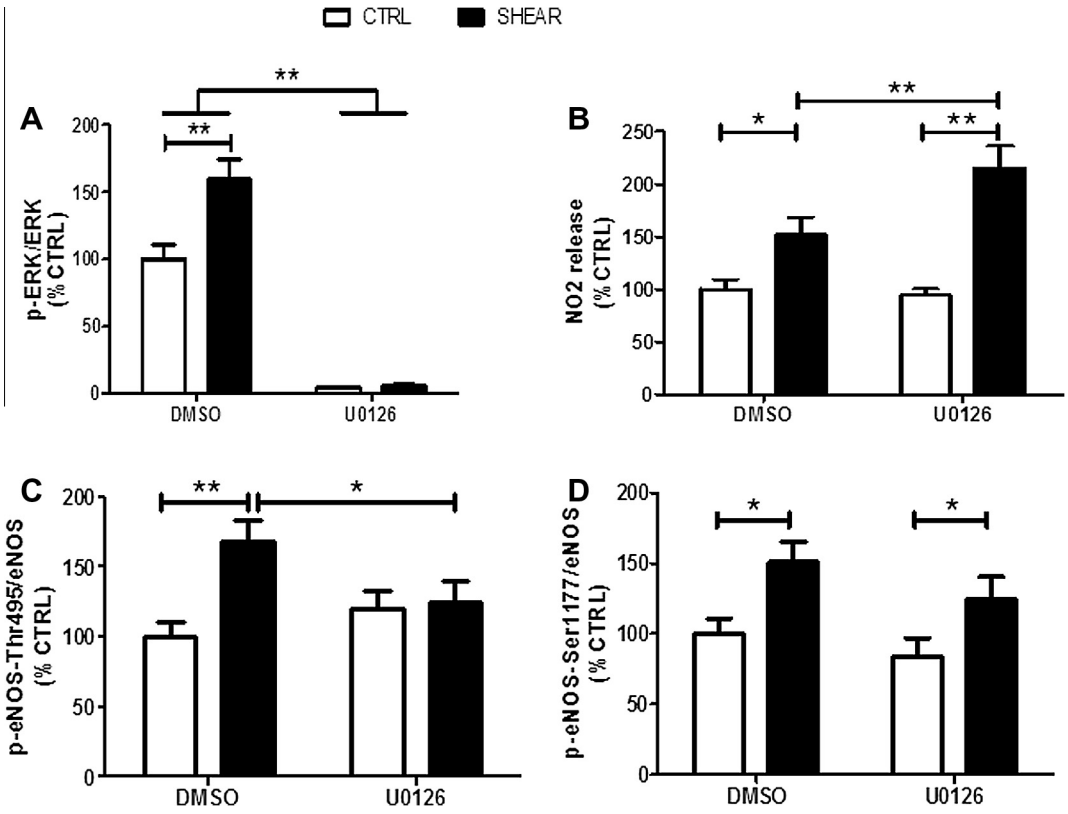

Fig. 3. Inhibition of shear stress-induced ERK activation potentiates NO synthesis by blocking $p\text{-}eNOS^{Thr495}$. Effects of the MEK1 and MEK2 inhibitor, U0126, on SS-induced (A) ERK activation, (B) NO_2^- production and phosphorylation status of (C) $eNOS^{Thr495}$ and (D) $eNOS^{Ser1177}$. SVEC were exposed to laminar shear stress (10 min, 15 dyne/cm^2); U0126 10 μM was administered 30 min before the onset of SS. Each bar represents mean ± SEM of 4–6 separate experiments. $^*p < 0.05$ and $^{**}p < 0.01$.

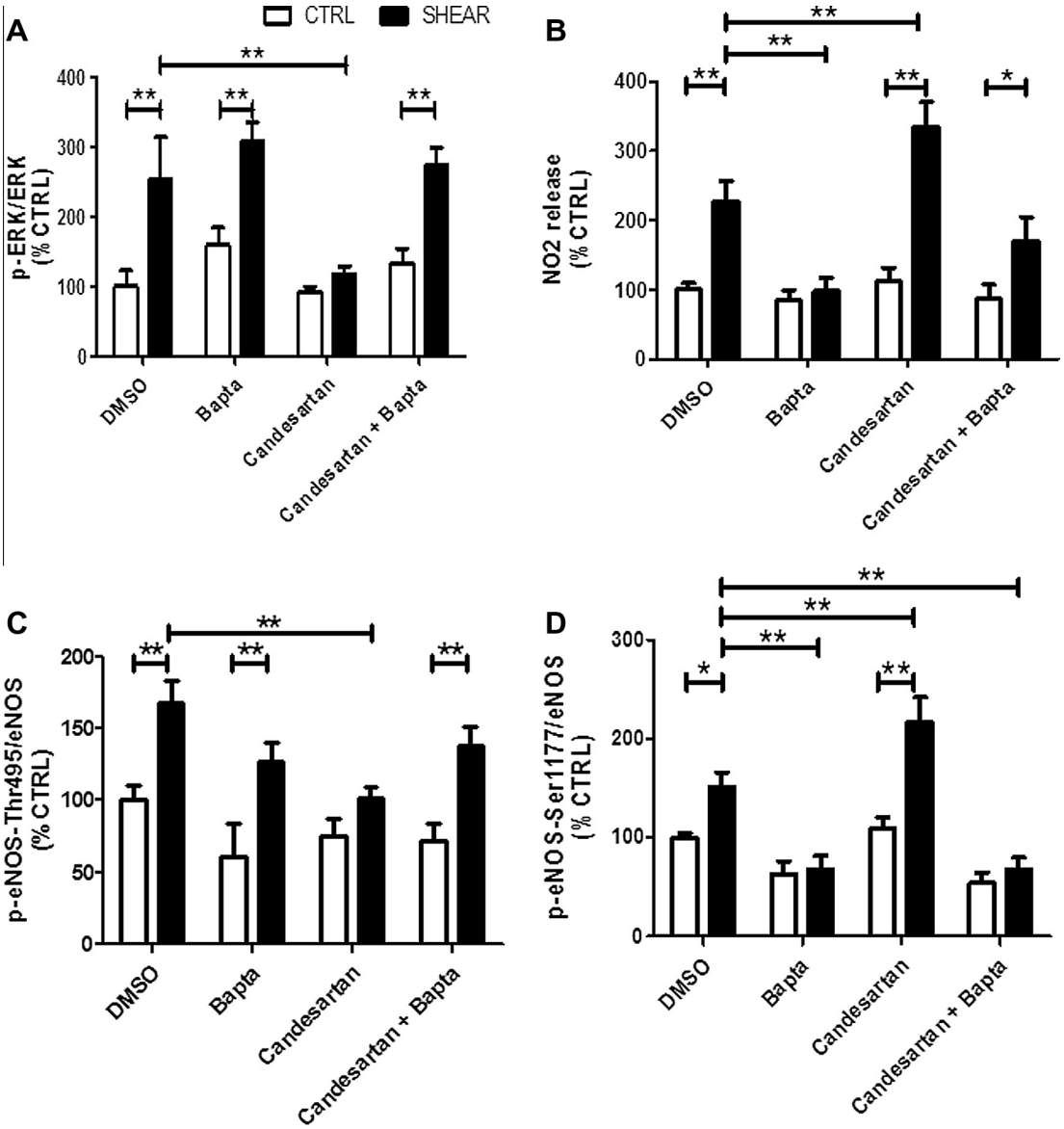

Fig. 4. Inhibition of Candesartan-induced calcium transient blocks NO synthesis potentiation effect by decreased p-eNOS^{Ser1177}. Effects of BAPTA-AM on SS-induced (A) p-ERK1/2 (B) NO_2^- production (C) phosphorylation status of (C) eNOS^{Thr495} and (D) eNOS^{Ser1177} and its combinatory effect with Candesartan. SVEC were exposed to laminar SS (10 min, 15 dyne/cm^2); Candesartan 1 μM was administered 30 min before the onset of SS. BAPTA-AM 10 μM was administered 30 min before candesartan. Each bar represents mean ± SEM of 4–6 separated experiments. $^*p < 0.05$ and $^{**}p < 0.01$.

that BAPTA-AM did not affect SS-induced ERK activation (Ctrl, 100 ± 15%; SS, 253 ± 39%; SS + BAPTA-AM 308 ± 22%). However, as seen in Fig. 4B, BAPTA-AM alone blocked SS-induced NO production (Ctrl, 100 ± 16%; SS, 227 ± 27%; SS + BAPTA-AM 99 ± 12%; Fig. 4B) while significantly reducing the stimulatory effect of Candesartan (Ctrl, 100 ± 16%; SS, 227 ± 27%; SS + Cand 334 ± 32%; SS + Cand + BAPTA-AM 170 ± 27% Fig. 4B). When eNOS phosphorylation status was analyzed, it was observed that BAPTA-AM prevented p-eNOS^{Ser1177} by SS both in the absence or presence of Candesartan (Ctrl, 100 ± 14%; SS, 152 ± 13%; SS + BAPTA-AM 68 ± 19%; SS + Cand + BAPTA-AM, 67 ± 18%; Fig. 4D). It is also remarkable that BAPTA-AM had no effect on SS-induced p-ERK activation (Fig. 4A), but increased p-eNOS^{Thr495} could still be observed (Ctrl, 100 ± 14%; SS, 168 ± 24%; SS + BAPTA-AM 128 ± 8%; Fig. 4C), consistent with the suggested crosstalk depicted in Fig. 3. Moreover, it is also interesting that BAPTA-AM inhibited the Candesartan effect to block SS-induced p-ERK activation (SS + Cand, 118 ± 10%; SS + Cand + BAPTA-AM, 274 ± 26%; Fig. 4A) and the phosphorylation of eNOS^{Thr495} (Fig. 4C), which suggests possible crosstalk between Candesartan-induced increase on calcium transient and ERK inhibition.

Data in Supplementary Fig. S2 show that the SS-induced NO potentiation response is also seen with Telmisartan (Fig. S2B). Thus, Telmisartan, similarly to Candesartan but unlike Losartan in vitro inhibited SS-induced ERK activation (Fig. S2A).

4. Discussion

The results of the present study reveal three main findings: first, that blockade of the AT1R, independently of the ang II ligand, potentiates the SS-induced NO synthesis in EC; second, that SS-induced ERK activation in EC negatively regulates eNOS activity by phosphorylation of its residue Thr^{495}; and third, that Candesartan potentiates the SS-induced NO synthesis via modulation of eNOS phosphorylation at the residues Thr^{495} and Ser^{1177}.

The role of ERK1/2 in eNOS regulation is complex and it seems to be cell- and stimulus-specific. In pulmonary artery EC and in uterine artery EC, inhibition of MEK1/2 attenuated estrogen-stimulated eNOS activity [11]. Also using uterine artery EC, Cale and Bird [23] showed that ATP-stimulated eNOS activity was potentiated by treatment with the MEK1/2 inhibitor U0126. Other studies in bovine aortic EC have shown that inhibition of MEK1/2 results in no change or enhancement in stimulated eNOS activity with bradykinin, green tea or sphingosine 1-phosphate [14,24,25]. We have shown here for the first time that inhibition of SS-induced ERK activation with U0126 leads to increased eNOS activity by $eNOS^{Thr495}$ dephosphorylation. This is in contrast with Boo et al findings that showed no changes on p-$eNOS^{Thr495}$ by SS in bovine aortic endothelial cells [26]. However, in bovine aortic EC, VEGF also did not stimulate dephosphorylation of $eNOS^{Thr495}$, which is in contrast with other studies using human umbilical vein EC where VEGF stimulate dephosphorylation of $eNOS^{Thr495}$ [27].

Post-translation modifications on eNOS, together with the presence of cofactors, are the most important modulators of eNOS enzymatic activity. Although there are many potential phosphorylation sites on eNOS, functionally most is known about the phosphorylation of the Ser^{1177} residue in its reductase domain and the Thr^{495} within the CaM-binding domain [9,15,16]. Our data show that Candesartan potentiates SS-induced NO production by affecting the dual phosphorylation status of both residues in human EC.

In unstimulated cultured EC, $eNOS^{Ser1177}$ is not phosphorylated, but many factors such as VEGF, estrogen, insulin, and bradykinin or mechanical forces either by SS or stretch, leads to increased eNOS activity and NO production. The SS-induced phosphorylation of $eNOS^{Ser1177}$ in EC is classically attributed to upstream activation of phosphatidylinositol 3-kinase and then serine kinases AKT and protein kinase A [28–30]. This is referred to as calcium-independent mechanism of eNOS activation, but chelation of intracellular calcium abolishes the SS-induced increase in eNOS activity, which suggests a calcium dependent component exists [29]. Our observations support the role of basal calcium in the increase of $eNOS^{Ser1177}$ phosphorylation and NO production induced by SS since BAPTA-AM pre-treatment of EC abolished these responses. In addition, we have shown that Candesartan has pleiotropic effects on EC that result in increased calcium transient. This increased basal calcium concentration may explain the higher phosphorylation of $eNOS^{Ser1177}$ residue in response to SS. Although we have not assessed whether Candesartan also potentiated the SS-induced calcium transient, BAPTA-AM administration completely abrogated the effects of candesartan on p-$eNOS^{Ser1177}$.

Moreover, eNOS at the residue Thr^{495}, which plays a negative regulatory influence, is constitutively phosphorylated in EC. ERK1/2 has been attributed as the main kinase that phosphorylates this residue [14]. The close proximity of this residue and the CaM-binding domain on eNOS gives further support to the link of its phosphorylation and NO production. It has already been described that decreases in Thr^{495} phosphorylation facilitates the accessibility of the CaM-binding domain to CaM, which appears to increase phosphorylation of $eNOS^{Ser1177}$ and NO production [21]. We have provided evidence that this mechanism may also work in EC exposed to SS. SS induces p-ERK activation, p-$eNOS^{Thr495}$ and p-$eNOS^{Ser1177}$. However, when ERK activation was blocked either by U0126 or Candesartan, SS-induced p-$eNOS^{Thr495}$ was inhibited (Figs. 3C and 1B) and p-$eNOS^{Ser1177}$ was potentiated (Figs. 3D and 1C) resulting in increased NO synthesis by EC (Figs. 3B and 1A).

Therefore, it is tempting to suggest that the maximal activation of eNOS at a physiological concentration of calcium and CaM requires the simultaneous phosphorylation of $eNOS^{Ser1177}$ and dephosphorylation of $eNOS^{Thr495}$ [21]. The $eNOS^{Thr495}$ site is often considered intrinsically linked to the Ser^{1177} site, regulating its phosphorylation status reciprocally though mechanisms that are incompletely understood [31].

We also showed that there are differences in the ability of ARBs to influence the SS-induced response. Although it is tempting to suggest that the results may potentially explain therapeutic differences among members of the ARBs families, it is important to emphasize that, *in vivo*, Losartan undergoes significant first-pass metabolism to produce 5-carboxylic acid metabolite (EXP3174) [32]. EXP3174 is 10–40 times more potent in blocking AT1R than Losartan [33,34].

Taken together, we provide evidence that mechanical SS stimulates the synthesis of NO by modulation of eNOS phosphorylation status at residues Ser^{1177} and Thr^{495} in endothelial cells, consistent with our previous observation using CHO-AT1 cells [6]. We have also shown that ARBs potentiate SS-induced NO production by blocking ERK activation, increasing p-$eNOS^{Ser1177}$ and decreasing p-$eNOS^{Thr495}$. Increased calcium transient and decreased SS-induced ERK activation seems to play a role in this dual eNOS phosphorylation status change. Finally, one may speculate that these yet undescribed events may play a role on the beneficial effects associated with ARBs in cardiovascular diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.108.

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