Valsartan regulates the interaction of angiotensin II type 1 receptor and endothelial nitric oxide synthase via Src/PI3K/Akt signalling

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Aims Valsartan, a selective angiotensin II type 1 receptor (AT1R) blocker, has beneficial effects in the cardiovascular system in part by its increase of nitric oxide (NO) bioavailability, yet the mechanisms are unclear. We investigated the molecular mechanisms underlying this effect in endothelial cells (ECs).

Methods and results NO production was examined by Griess reagent assay, DAF-2 DA fluorescence staining and cGMP ELISA kits. Protein interaction was determined by western blotting and immunoprecipitation. Treating bovine or human aortic ECs with valsartan increased NO production, as evidenced by the level of stable NO metabolites and intracellular cGMP. Valsartan increased the phosphorylation but not the protein level of endothelial NO synthase (eNOS). Inhibition of phosphoinositide-3 kinase (PI3K)/Akt and Src pathways by specific inhibitors suppressed valsartan-induced NO release. In addition, valsartan increased the tyrosine residue phosphorylation of AT1R, which was attenuated by inhibition of Src but not PI3K activities. Valsartan also suppressed the interaction of eNOS and AT1R, which was blocked by Src or PI3K inhibition.

Conclusion Valsartan-induced NO production in ECs is mediated through Src/PI3K/Akt-dependent phosphorylation of eNOS. Valsartan-induced AT1R phosphorylation depends on Src but not PI3K activities. Valsartan-induced suppression of AT1R–eNOS interaction depends on Src/PI3K/Akt signalling. These results indicate a novel vasoprotective mechanism of valsartan in upregulating NO production in ECs.

KEYWORDS Valsartan; PI3K; Src family kinase; AT1R; eNOS

1. Introduction

Hypertension is a major risk factor of morbidity and mortality in cardiovascular diseases.1,2 Over the past decade, clinical trials have demonstrated that valsartan is an antagonist of the selective angiotensin II (Ang II) type 1 receptor (AT1R) and widely used in therapy for hypertension.3–5 Through its primary pharmacological function, valsartan abolishes the action of Ang II on AT1R, which leads to various anti-hypertensive effects, including relaxation of vascular smooth muscle cells (VSMCs) and inhibition of VSMC proliferation.6,7 In addition, accumulating evidence suggests that valsartan possesses beneficial effects other than AT1R antagonism. These pleiotropic benefits include anti-atherogenic, anti-inflammatory, anti-platelet aggregating, and anti-thrombotic actions,8–10 all of which are linked to the bioavailability of nitric oxide (NO), a crucial molecule for maintaining cardiovascular function under physiological and pathological conditions.11,12

The production of NO in endothelial cells (ECs) is mainly mediated through activation of endothelial NO synthase (eNOS).12 The EC-derived NO subsequently activates the soluble guanylyl cyclase to elevate the production of cGMP, which results in VSMC relaxation.11–13 eNOS activation is mediated through kinase-dependent signalling pathways such as Src kinase, PI3K/Akt, protein kinase A (PKA), PKC, or AMP-activated protein kinase (AMPK).11,13,14 eNOS activation can also be mediated through its physiological interaction with a variety of intracellular proteins.15,16 Valsartan has been shown to increase NO production in vessels.9,17 Additionally, eNOS can interact with AT1R in vitro.18 Whether the valsartan-mediated increase in NO production in ECs is linked to kinase-dependent regulation and the interaction of AT1R and eNOS is unknown.

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In the present study, we elucidated the underlying molecular mechanisms of valsartan in eNOS activation and the AT₁R-eNOS interaction. We examined whether valsartan treatment causes the induction of NO and eNOS phosphorylation in ECs, the role of kinase-dependent regulation in valsartan-mediated eNOS phosphorylation, and the molecular mechanisms involved in valsartan-induced alteration of a possible AT₁R-eNOS interaction. Our data demonstrate that valsartan causes rapid phosphorylation of eNOS accompanied by dissociation of eNOS and AT₁R, which consequently increases NO production via an Src/PI3K/Akt-dependent mechanism. Collectively, our findings suggest that increased eNOS activation may contribute to the beneficial effects of valsartan in ECs.

2. Methods

2.1 Reagents

Valsartan was obtained from Novartis Co., Ltd (Taipei, Taiwan). Rabbit antibodies for phosphorylated eNOS at Thr-495, Ser-617, Ser-635, Ser-1179, eNOS, phoso-Akt at Thr473, Akt, phoso-Src at Tyr416, Src, and phospho-tyrosine were purchased from Cell Signaling Technology (Beverly, MA). The antibody for AT₁R was purchased from Santa Cruz (Santa Cruz, CA). Mouse antibody for α-tubulin, SU6656, NG-Nitro-Larginine methyl ester (L-NAME), and Griess reagent were purchased from Sigma-Aldrich (St Louis, MO). LY294002, wortmannin, diaminofluorescein-2-diacetate (DAF-2 DA), compound C, calphostin, and H89 were obtained from Calbiochem (San Diego, CA).

2.2 Cell culture

Bovine aortic endothelial cells (BAECs) were obtained from Cell Applications (San Diego, CA) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin (HyClone, Logan, UT). Human aortic endothelial cells (HAECs) were purchased from Cascade Biologics (Portland, OR) and cultured in Medium 200 supplemented with 10% FBS and 1% PEN-STREP-AMPHO SOL (penicillin, 10 000 units/mL; streptomycin, 10 mg/mL; amphotericin B, 0.025 mg/mL) and 2% low serum growth supplement (Cascade Biologics, Portland, OR). Cells at passages 3–8 were grown to about 0.025 mg/mL and 2% low serum growth supplement (Cascade Biologics, 10 000 units/mL; streptomycin, 10 mg/mL; amphotericin B, from Cascade Biologics (Portland, OR) and cultured in Medium 200 Logan, UT). Human aortic endothelial cells (HAECs) were purchased from Cell Applications (Beverly, MA).

2.3 Detection of NO and cGMP assay

Accumulated nitrite (NO₂⁻), the stable breakdown product of NO, in culture media, was measured by mixing an equal volume of Griess reagent and then incubating at room temperature for 15 min. The azo dye production was then analysed by using an SP-8001 UV/VIS spectrophotometer (Metertech, Taipei, Taiwan) with absorbance set at 540 nm. Sodium nitrite was used as a standard. NO production was also measured by comparing DAF-2 DA (an NO sensitive dye) fluorescence staining before and after valsartan treatment with use of a Nikon TE2000-U fluorescence microscope. Intracellular levels of cGMP in ECs were assessed by enzyme immunoassay kit (R&D systems, Minneapolis, MN) according to the manufacturer’s instructions and normalized to protein content as determined by the Bradford assay.

2.4 Protein extraction and immunoblot analysis

ECs were washed in phosphate-buffered saline and then lysed by use of SDS-lysis buffer (1% Triton, 0.1% SDS, 0.2% sodium azide, 0.5% sodium deoxycholate) and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 1 μg/mL leupeptin). Western blot analysis was performed as described. Protein bands in the blots were quantified by use of Scion image software (Healthcare Bio-Sciences, Philadelphia, PA).

2.5 Immunoprecipitation

To identify the protein–protein interactions between AT₁R and eNOS, cells were lysed with immunoprecipitation buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 0.1% NP-40) supplemented with 1% Triton X-100, 0.1 mM Na₃VO₄, and protease inhibitors. Cells were sheared by brief sonication on ice, and cellular debris was removed by centrifugation at 10 000g for 10 min. Aliquots (1000 μg) of lysates were incubated with protein A/G-Sepharose for 1 h at 4°C and then incubated with anti-AT₁R or anti-eNOS polyclonal antibodies, or a pre-immune rabbit IgG at a final concentration of 1 μg/mL overnight at 4°C. Protein A/G-Sepharose (Santa Cruz Biotechnology, Santa Cruz, CA) was then added for 2 h at 4°C. Immune complexes were collected by centrifugation, washed three times with cold PBS, disrupted by boiling in 5× SDS loading dye and then subjected to western blot analysis.

2.6 Statistical analysis

Results are presented as mean ± SEM from three independent experiments. Statistical evaluation involved one-way ANOVA followed by the Fisher least significant difference multiple-comparison test as appropriate. A value of *P < 0.05* was considered statistically significant.

3. Results

3.1 Valsartan increases NO production in bovine aortic endothelial cells via increased endothelial NO synthase phosphorylation

To test whether valsartan is capable of inducing NO production in ECs, BAECs were treated with various doses of valsartan or at indicated times, and NO production and intracellular cGMP level were assessed. Compared with control treatment, with 24 h 10 μM valsartan treatment, levels of nitrite and cGMP in cultured medium were significantly increased (Figure 1A and B). However, eNOS protein level was not affected by the valsartan doses tested (Figure 1C). Time course experiments demonstrated a significant increase in nitrite level with 10 μM valsartan as early as 6 h, with peak level at 24 h (Figure 1D). Moreover, we applied DAF-2 DA fluorescence staining to confirm results obtained from the measurement of NO byproducts, and exposure of BAECs to 10 μM valsartan for 12 h results in an increase in DAF-2 DA signals (Figure 1E). Additionally, L-NAME, an eNOS inhibitor, totally abrogated the valsartan-induced increase in NO production (Figure 1F). The increase in NO production might be through the induction of eNOS expression or phosphorylation. Since valsartan did not alter the protein expression of eNOS, increased activity of eNOS could be involved in the valsartan-induced elevation of NO production. It has been reported that phosphorylation of Thr-495, Ser-615/617, Ser-635/635, or Ser-1179/1179 in eNOS plays an important role in the regulation of its enzymatic activity in ECs. We next examined the effect of valsartan on phosphorylations of eNOS at these sites in BAECs. Cells were treated with 10 μM valsartan for up to 240 min. We found that valsartan induced phosphorylation of eNOS only at Ser-1179 and the response occurred as
early as 10 min after treatment, peaked at 30 min and then gradually decreased to the basal level (Figure 2A).

3.2 Valsartan induces endothelial NO synthase phosphorylation through Src/PI3K/Akt signalling

To elucidate the mechanisms underlying the increase in eNOS phosphorylation in response to valsartan, specific inhibitors for PKA, AMPK, PI3K/Akt, or PKC signalling were utilized to clarify their involvement in this response. Valsartan-induced eNOS phosphorylation was significantly attenuated by PI3K inhibitors LY294002 or wortmannin but was unaffected by DMSO (vehicle), PKA inhibitor H89, AMPK inhibitor compound C, or PKC inhibitor calphostin (Figure 2B). Treatment with each of these inhibitors alone did not alter both the expression of eNOS protein and the production of NO (data not shown). Moreover, valsartan significantly increased Akt phosphorylation in a time-dependent manner as early as 5 min after treatment (Figure 2C). These results imply an essential role of PI3K/Akt signalling in valsartan-mediated eNOS activation.

We next examined whether c-Src kinase was involved in valsartan-induced Akt and eNOS phosphorylation. Phosphorylation of Src was increased by valsartan within 5–10 min after treatment and returned to the basal level at 15 min (Figure 3A). Pre-treatment with the c-Src kinase inhibitor SU6656 prevented the valsartan-induced increase in NO production (Figure 3B). SU6656 also hindered valsartan-induced Akt and eNOS phosphorylation (Figure 3C). These data imply that Src family kinases play a critical role in valsartan-induced activation of PI3K/Akt/eNOS signalling.

3.3 Role of c-Src and PI3K/Akt signalling in valsartan-induced dissociation of the AT1R–eNOS complex

The physiological interaction of eNOS with intracellular proteins may play an important role in the regulation of eNOS activity. However, whether AT1R interacts with eNOS and participates in valsartan-mediated eNOS activation remains unclear. Here, we used immunoprecipitation assay to study the interplay between AT1R and eNOS. AT1R was associated with eNOS under normal conditions, and valsartan suppressed the interaction of AT1R and eNOS in a time-dependent manner, with maximal effect at 30 min after treatment (Figure 4).

Because valsartan activated both c-Src and PI3K/Akt signalling in eNOS phosphorylation, we then examined whether valsartan also induces AT1R phosphorylation. Valsartan induced AT1R tyrosine phosphorylation in ECs, which was abrogated by treatment with SU6656 but not
LY294002 (Figure 5A). However, treatment with both SU6656 and LY294002 reversed the valsartan-mediated dissociation of the AT1R-eNOS complex (Figure 5B). These results suggest that c-Src kinase activation is important in the valsartan-induced increase of AT1R phosphorylation and that both c-Src and PI3K/Akt pathways are required for the valsartan-induced dissociation of the AT1R-eNOS complex.

3.4 Involvement of Src/PI3K/Akt signalling in valsartan-induced activation of endothelial NO synthase and dissociation of AT1R-eNOS complex in human aortic endothelial cells

Because valsartan is a clinically used angiotensin receptor blocker (ARB), we thus studied the effect of valsartan in human ECs. Levels of phosphorylated eNOS and Akt were elevated in valsartan-treated HAECs (Figure 6A). In parallel, valsartan treatment significantly enhanced accumulations of nitrite and cGMP, which were attenuated on pre-treatment with LY294002 or SU6656 (Figure 6B). Moreover, valsartan-induced AT1R-eNOS dissociation was blocked by SU6656 or LY294002 administration (Figure 6C). Based upon our observations, a molecular mechanism is proposed (Figure 6D). As shown, in addition to its AT1R antagonist function, valsartan may activate Src/PI3K/Akt signalling leading to a decrease in the interaction of AT1R and eNOS. These two events may work in concert to promote eNOS activation and NO production.

4. Discussion

Clinical trials have demonstrated that application of ARBs such as valsartan and losartan provide plausible anti-hypertensive effects for managing hypertension and related cardiovascular complications.3,20 In the current study, we demonstrated that the AT1R antagonist valsartan increases eNOS phosphorylation and induces NO production in bovine or human aortic ECs through the c-Src/PI3K/Akt pathway (Figures 1 and 6). Valsartan promoted eNOS phosphorylation in a rapid and transient manner. This time course of response is comparable to that promoted by vascular endothelial growth factor (VEGF), shear stress, statins, and estradiol.21,22 Although PI3K/Akt, PKA, AMPK, and PKC are key kinases for eNOS phosphorylation,13,21,23 our results suggest that inhibition of only PI3K/Akt signalling prevented valsartan-induced increase in NO production, whereas AMPK, PKA, and PKC did not appear to mediate
this effect (Figure 2B). We also showed that valsartan up-regulated Akt phosphorylation in a rapid and transient manner (Figure 2C). These data suggest that the PI3K/Akt-dependent pathway is involved in valsartan-mediated eNOS activation and NO production.

Several groups have demonstrated that inhibition of Src family kinases abrogates VEGF-mediated eNOS activation and subsequent NO production.\textsuperscript{14-20} We showed a rapid and transient phosphorylation of Src kinase 5–10 min after valsartan treatment (Figure 3A), which occurred much earlier than valsartan-induced phosphorylation of Akt (5–30 min) or eNOS (10–120 min) (Figure 2A and C). Additionally, pharmacological inhibition of Src kinase activity completely abolished phosphorylation of Akt and eNOS and formation of NO (Figure 3), which suggests that Src kinase is the signalling molecule upstream of the PI3K/Akt pathway required for eNOS activation. Taken together, our observations indicate that Src/PI3K/Akt signalling is essential for valsartan-induced eNOS phosphorylation, NO production, and cGMP accumulation.

Figure 3  Involvement of Src kinase in valsartan-induced NO production and phosphorylation of Akt and eNOS in bovine aortic endothelial cells. (A) Bovine aortic endothelial cells were treated with 10 μM valsartan for the indicated times. Cells were then lysed and subjected to western blotting with antibodies for phosphorylated c-Src (p-Src) at Tyr416 or Src protein. (B) Bovine aortic endothelial cells were pre-treated with SU6656 (20 μM) for 2 h, followed by valsartan (10 μM) in the presence of SU6656 for 24 h. NO production was determined by Griess assay. (C) Bovine aortic endothelial cells were pre-treated with DMSO, LY294002 (10 μM), or SU6656 (20 μM) for 2 h, followed by treatment with valsartan (10 μM) in the presence of LY294002 or SU6656 for 15 min. Cellular lysates were subjected to western blot analysis with antibodies for phosphorylated Akt (p-Akt) at Thr473, phosphorylated endothelial NO synthase (p-eNOS) at Ser-1179, and total Akt and endothelial NO synthase proteins. Data are mean ± SEM from three independent experiments. *P < 0.05 vs. control group, #P < 0.05 vs. valsartan-treated group.

In addition to the AT\textsubscript{1}R antagonizing functions, ARBs may have other biological actions. For example, irbesartan and losartan inhibit the thromboxane A\textsubscript{2}/prostaglandin endoperoxide H\textsubscript{2} receptor and platelet aggregation in BAECs.\textsuperscript{27,28} Telmisartan has anti-inflammatory, anti-oxidant, and neural protective effects through activation of peroxisome proliferator-activated receptor γ and eNOS and suppression of cyclooxygenase-2.\textsuperscript{29-31} Valsartan administration protects rodents against injury of heart failure and improves cardiac function in an eNOS-dependent mechanism.\textsuperscript{32-34} Losartan induces activation of Akt and eNOS in BAECs via its AT\textsubscript{1}R-blocking metabolite EXP3174 and non-AT\textsubscript{1}R-blocking metabolite EXP3179, and this activation is VEGFR2-dependent but AT\textsubscript{1}R-independent manner.\textsuperscript{35} Besides, Kalinowski et al.\textsuperscript{9} reported that the effective concentration of losartan is much lower (1 μM) than valsartan (10 μM) for NO release in platelets.\textsuperscript{3} However, the efficacies of losartan (1 and 10 μM) and valsartan (1 and 10 μM) in NO production are similar in human umbilical vein ECs. Similar to the effects of losartan, we here demonstrated that valsartan also induces eNOS activation and NO production (Figures 1
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Figure 4 Valsartan suppresses the association of AT$_1$R and endothelial NO synthase. Bovine aortic endothelial cells were treated with valsartan (10 $\mu$M) for the indicated times. Cell lysates were immunoprecipitated (IP) with anti-endothelial NO synthase antibody and immuno-probed with anti-AT$_1$R or anti-endothelial NO synthase antibody (IB). The input was 50 $\mu$g non-immunoprecipitated whole cellular extract. Data are mean ± SEM from three independent experiments. *P < 0.05 vs. control group.

Figure 5 Src family kinase and PI3K/Akt signalling is involved in valsartan-induced dissociation of endothelial NO synthase and AT$_1$R. Bovine aortic endothelial cells were pre-treated with or without SU6656 (20 $\mu$M) or LY294002 (10 $\mu$M) for 2 h and then incubated with valsartan (10 $\mu$M) in the presence of antagonist for 30 min. Cell lysates were immunoprecipitated (IP) with phosphorylated tyrosine antibody and then immuno-probed with (A) anti-AT$_1$R antibody or (B) anti-endothelial NO synthase antibody. Data are mean ± SEM from three independent experiments. *P < 0.05 vs. control group, #P < 0.05 vs. valsartan-treated group.

In conclusion, this study demonstrates a unique non-ABR function of valsartan, which involves activation of kinase and PI3K/Akt pathways completely preventing valsartan-induced suppression of the AT$_1$R–eNOS complex (Figures 5B and 6C). That Src kinase rather than PI3K is essential for mediating valsartan-induced phosphorylation of AT$_1$R (Figure 5A) implies that tyrosine phosphorylation of AT$_1$R induced by Src family kinase activation is required for valsartan-induced dissociation with eNOS. Although the exact mechanism remains to be uncovered, our results are the first to indicate that both kinase-dependent regulation and protein–protein interaction are significantly involved in the valsartan-mediated eNOS activation and NO biosynthesis.

Valsartan has been shown to increase NO production in ECs, the underlying mechanism is completely unknown. Our findings indicate a unique and detailed mechanism underlying this non-ARB action of valsartan. Additionally, despite the fact that losartan is known to induce activation of Akt and eNOS in BAECs, this function is mediated through via its metabolites and VEGFR2, a mechanism that is totally different from that underlying the function of valsartan as reported in this study. Furthermore, our finding regarding the interaction of AT$_1$R and eNOS has never been demonstrated previously. Thus, our findings of Src/Pi3K/Akt signaling and the kinase-dependant regulation of interaction of AT$_1$R and eNOS may provide novel mechanisms underlying the valsartan-induced increase in NO production. Our findings certainly are relevant to the scenario that different clinically used ARBs may provide similar therapeutic benefits, but may be mediated via dissimilar mechanisms. Although the effects of valsartan on eNOS activation in ECs appear to be transient (within 2 h), the increase in NO production may locally diffuse to the vascular smooth muscles in the vicinity to promote vasorelaxation, a consequence presumably offering therapeutic benefit in addition to the ARB function of valsartan. The multiple biological functions of valsartan should provide valuable information for therapy and prevention of hypertension and cardiovascular diseases.

In conclusion, this study demonstrates a unique non-ABR function of valsartan, which involves activation of...
Src/PI3K/Akt signalling leading to a decrease in the interaction of AT₁R and eNOS as well as the subsequent eNOS activation and increased NO production in ECs.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.

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References

Figure 6  Valsartan induces endothelial NO synthase and Akt phosphorylation and decreases the interaction of AT₁R and endothelial NO synthase in human aortic endothelial cells. (A) Human aortic endothelial cells were treated with 10 μM valsartan for 15 min, then lysates were subjected to western blot analysis with antibodies for phosphorylated endothelial NO synthase (p-eNOS) at ser-1179 and endothelial NO synthase, and phosphorylated Akt (p-Akt) at Thr-473 and Akt. Human aortic endothelial cells were treated with or without LY294002 (10 μM) or SU6656 (20 μM) for 2 h, followed by treatment with valsartan (10 μM) in the presence of LY294002 or SU6656 for 4 h (B) or 20 min (C). NO production was examined by Griess assay (D). Cell lysates were incubated with anti-endothelial NO synthase antibody for IP assay and then immunoblotted with anti-AT₁R or anti-endothelial NO synthase antibody (C). Data are mean ± SEM from three independent experiments. *P < 0.05 vs. control group, **P < 0.05 vs. valsartan-treated group. (D) Schematic illustration of proposed mechanism underlying valsartan-induced endothelial NO synthase activation and increased NO production in ECs. As shown, in addition to its AT₁R antagonist function, valsartan may activate Src/PI3K/Akt signalling leading to a decrease in the interaction of AT₁R and endothelial NO synthase. These two events may work in concert to promote endothelial NO synthase activation and NO production.
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