

## Losartan inhibits EGFR transactivation in vascular smooth muscle cells

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**Background/aim:** Angiotensin II (Ang II)-induced molecular signaling pathways play a significant role in the progression of cardiovascular diseases, including hypertension and atherosclerosis. In addition to the well-known effects of Ang II, it may activate epidermal growth factor receptor (EGFR) in a process known as transactivation, which contributes to vascular pathologies. The aim of this study was to determine whether losartan could reduce EGFR transactivation induced by Ang II. Additionally, we evaluated the roles of heparin-binding epidermal-like growth factor (HB-EGF) and matrix metalloproteinases (MMPs) in Ang II-induced EGFR transactivation.

**Materials and methods:** Vascular smooth muscle cells were isolated from a rat aorta and grown in primary culture. Ang II-induced EGFR phosphorylation (tyrosine 1068) and ERK1/2 MAPK phosphorylation (threonine 202 and tyrosine 204) were evaluated by western blotting.

**Results:** Ang II induced EGFR phosphorylation through the Ang II type I receptor ( $P < 0.05$ ). The transactivation process was inhibited by losartan and mediated by HB-EGF and MMPs. Ang II transactivates EGFR in an AT1R-dependent manner.

**Conclusion:** The results of this study show that losartan, a widely used antihypertensive agent, can suppress EGFR phosphorylation (Y1068) upon Ang II stimulation in vascular smooth muscle cells. EGFR inhibition is a candidate therapy for combating cardiovascular diseases such as hypertension and atherosclerosis.

**Key words:** Angiotensin II, EGF receptor, losartan, transactivation, extracellular regulated kinase, vascular smooth muscle, cardiovascular disease

### 1. Introduction

Cardiovascular diseases (CVDs) are the most common cause of death in developing and developed countries ([www.who.int](http://www.who.int)). CVDs include atherosclerosis, which is becoming more prevalent in the world (1). The balance between vascular smooth muscle cell (VSMC) proliferation and apoptosis is crucial in the progression of atherosclerosis, because excess proliferation of VSMCs contributes to the thickening of the arterial wall at the initial phase of atherosclerotic plaque formation (2). Angiotensin II (Ang II) can act as a growth factor to stimulate the migration and proliferation of VSMCs (3). Ang II mainly shows its effects on VSMCs in the vascular wall (4). Moreover, the discovery of an intracellular renin-angiotensin system (RAS) (5) has made understanding the cellular effects and related signaling pathways of Ang II more important.

Ang II exerts its pathophysiological effects by binding to the Ang II type 1 receptor (AT1R) and activating

downstream signaling pathways in VSMCs. One of these pathways is the mitogenic ERK1/2 (or p44/42) MAPK pathway, which triggers VSMC proliferation upon activation. Surprisingly, studies have demonstrated that Ang II may induce receptor tyrosine kinase (RTK) activity without binding to the receptor directly, a process known as transactivation (6). Membrane-anchored heparin-binding EGF-like growth factor (HB-EGF), A disintegrin and metalloproteinase (ADAM17), and matrix metalloproteinases (MMPs) appear to be responsible for mediating the transactivation process (7–10). Although Ang II-triggered EGFR transactivation could arise in different ways, inhibition of the receptor activation would be the ultimate goal in terms of its crucial role in CVD.

The role of EGFR activation in cancer development is clearly defined. However, the role of EGFR in vascular biology, or more generally in CVD, is less studied despite similarities between cancer and VSMC activation after vascular injury. EGFR activation is associated with

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atherosclerosis, blood pressure regulation, and restenosis in the cardiovascular system (11). The importance of EGFR signaling in atherogenesis has been shown by many studies. It was demonstrated that EGFR and its ligands are expressed in VSMC, with elevated expressions of these molecules in human atherosclerotic plaques (12,13). The role of EGFR in VSMC proliferation and migration (14,15) and alleviation of atherosclerosis progression following deletion of EGFR in myeloid cells were shown (16). Hence, a number of publications highlighted the importance of EGFR inhibition as a potential therapeutic target to treat CVD (17,18). Moreover, the usage of RTK inhibitors to inhibit EGFR activation could be risky in noncancerous people with CVD as a consequence of RTK inhibitors' cardiotoxicity (19).

In this study, we aimed to determine the effect of the AT1R antagonist losartan on Ang II-induced EGFR transactivation, and the roles of HB-EGF and MMPs in this process. Additionally, we assessed the functions of HB-EGF and MMPs in Ang II-induced ERK1/2 MAPK phosphorylation and evaluated the dependence of EGFR transactivation on the ERK1/2 MAPK pathway. Finally, we investigated the roles of HB-EGF and MMPs on EGF-induced ERK1/2 MAPK activation.

## 2. Materials and methods

Dulbecco's modified Eagle's medium (DMEM) and Hank's balanced salt solution (HBSS) were purchased from Biochrom AG (Berlin, Germany). Fetal calf serum, HEPES, elastase, collagenase, AG1478, CRM197, L-glutamine, and penicillin-streptomycin were obtained from Sigma (St. Louis, MO, USA). Ang II and GM6001 were purchased from Calbiochem (Darmstadt, Germany). EGF was purchased from Upstate, Millipore (Burlington, MA, USA). Primary antibodies for anti-phospho ERK1/2 (Thr 202 and Tyr 204) and anti-phospho EGFR (Y1068) and the corresponding secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The AT1 receptor (AT1R) antagonist losartan was a kind gift from Merck (Darmstadt, Germany).

### 2.1. Isolation and primary culture of rat VSMCs

This study was approved by the Akdeniz University Local Committee on Animal Research Ethics. VSMCs were isolated from the thoracic aorta of male Wistar rats (250–350 g) as described previously (20). VSMCs were grown in DMEM supplemented with 10% (v/v) fetal calf serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin. Three to seven passages of VSMCs were used in the experiments and showed 99% positive immunostaining against smooth muscle  $\alpha$ -actin antibody (Sigma). The 80% confluence cultures were incubated with serum-free DMEM for 24 h before each experiment.

### 2.2. Immunoblotting for ERK1/2 MAPK and EGFR phosphorylation

VSMCs at 80% confluence were left in serum-free DMEM for 24 h after washing once with serum-free medium. VSMCs were then incubated with agonist (Ang II or EGF), antagonist/inhibitor (AT1R antagonist losartan, EGFR antagonist AG1478, HB-EGF inhibitor CRM197, or MMP inhibitor GM6001), or both (losartan + Ang II, etc.) at 37 °C in serum-free DMEM for specified durations. Incubation was terminated by replacing the medium with lysis buffer (pH 7.4, 4 °C). After sonication (10 s, Bendelin UW2070), the cell lysate was collected and centrifuged at 10,000  $\times$  g (4 °C, 30 min). The supernatant was mixed in 5X Laemmli sample buffer and 40 µg of protein sample loaded into 10% and 7.5% SDS-polyacrylamide gel for p-ERK1/2 MAPK and p-EGFR, respectively. The gel was transferred to a 0.45-µm nitrocellulose membrane (Whatman Protran, Dassel, Germany). The membrane was blocked with 5% BSA solution, followed by incubation with primary antibody at 4 °C overnight. Blots were probed with a horseradish peroxidase-conjugated secondary antibody. Immunoreactive proteins were detected by enhanced chemiluminescence reagent (Thermo Scientific, Rockford, IL, USA).

### 2.3. Data analysis

Western blots were analyzed using ImageJ software (Version 1.48), and each blot was representative of at least three different experiments. Data are expressed as mean  $\pm$  SD and  $P < 0.05$  was considered significant. One-way ANOVA testing was applied to evaluate the significance of various groups (SPSS 18.0, SPSS Inc., Chicago, IL, USA).

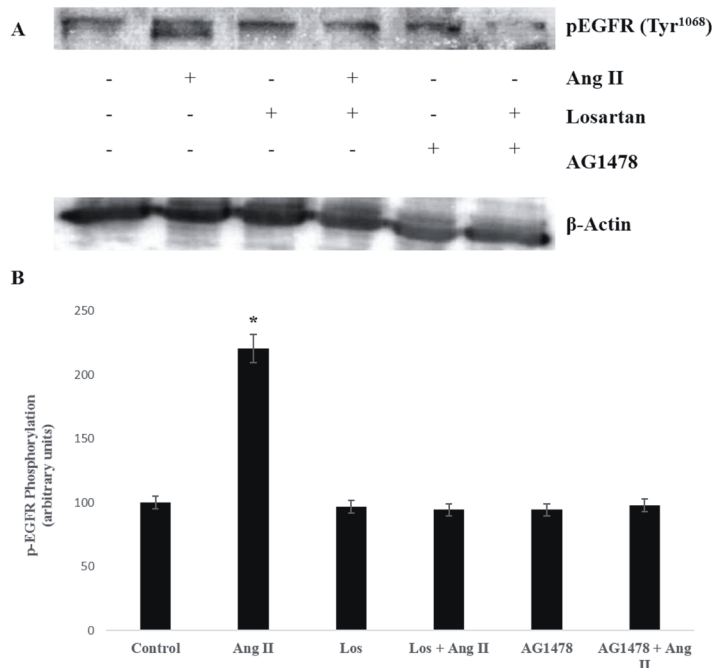
## 3. Results

### 3.1. Losartan inhibits Ang II-induced EGFR transactivation

To determine Ang II-induced EGFR transactivation by phosphorylation and the role of AT1R, VSMCs were treated with either losartan (10 µM) or EGFR kinase inhibitor AG1478 (1 µM) for 30 min. The cells were then induced by 100 nM Ang II for 5 min. Ang II enhanced EGFR phosphorylation (Y1068) approximately twofold compared to the control group. Losartan suppressed EGFR phosphorylation to control levels. The cells treated with AG1478 did not display EGFR phosphorylation (Figure 1,  $P < 0.05$ ).

### 3.2. Ang II-induced EGFR transactivation is mediated by HB-EGF and MMPs

After determining that Ang II-induced EGFR phosphorylation is AT1R-mediated, we searched for possible pathways. VSMCs were treated with either the HB-EGF inhibitor CRM197 (10 µg/mL, 30 min) or the broad-spectrum MMP inhibitor GM6001 (20 µM, 30



**Figure 1.** The effect of losartan on Ang II-induced EGFR phosphorylation (Y1068) in VSMCs. VSMCs were incubated with either 10  $\mu$ M AT1R antagonist losartan or 1  $\mu$ M EGFR kinase inhibitor AG1478 for 30 min before stimulation. Cells were then induced by 100 nM Ang II for 5 min. Losartan and AG1478 decreased EGFR phosphorylation to control levels. A) A representative example of the immunoblotting. B) EGFR phosphorylation as arbitrary units. Bars represent the mean  $\pm$  SD from three independent replicates. The control group was set as 100 units. \* $P < 0.05$ , significant compared to the control group. Ang II: Angiotensin II, Los: losartan.

min) before Ang II stimulation (100 nM, 5 min). Ang II increased EGFR phosphorylation nearly twofold compared to the control group ( $P < 0.05$ ), whereas inhibitor-treated cells did not show a significant increase (Figure 2).

### 3.3. Ang II-induced ERK1/2 MAPK phosphorylation is not EGFR transactivation-dependent

We examined proliferative ERK1/2 MAPK phosphorylation (Thr<sup>202</sup>/Tyr<sup>204</sup>), which is an intracellular target of Ang II. Losartan (10  $\mu$ M, 30 min) and AG1478 (1  $\mu$ M, 30 min) were used as inhibitors before Ang II stimulation (100 nM, 5 min). Ang II raised ERK1/2 MAPK phosphorylation nearly fivefold compared to the control group. Losartan-treated cells did not exhibit ERK1/2 MAPK phosphorylation, indicating that the event is AT1R-mediated. When EGFR intrinsic kinase activity was blocked by AG1478, ERK1/2 MAPK phosphorylation was not terminated (Figure 3,  $P < 0.05$ ).

Pretreatment of VSMCs with HB-EGF inhibitor CRM197 (10  $\mu$ g/mL, 30 min) before Ang II stimulation (100 nM, 5 min) attenuated ERK1/2 MAPK phosphorylation compared to Ang II-induced cells, but not to control levels. MMP inhibitor GM6001-treated cells (20  $\mu$ M,

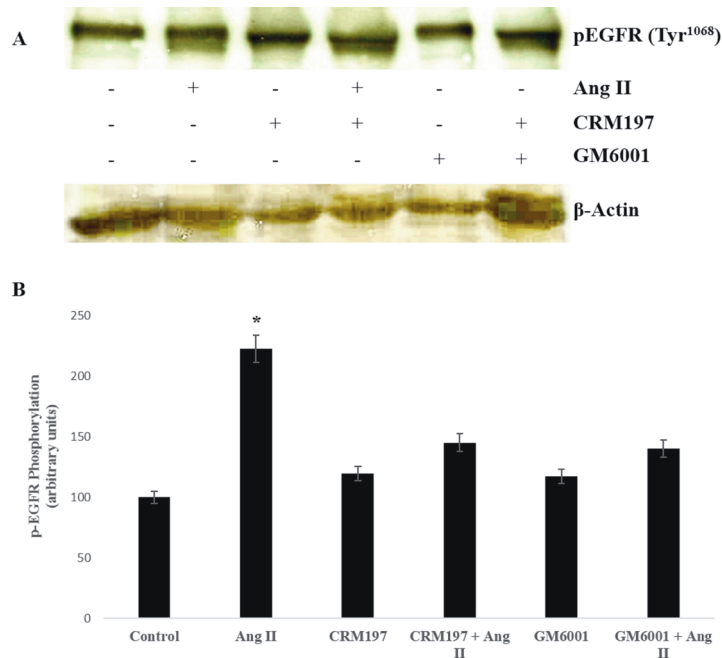
30 min) displayed similarly decreased ERK1/2 MAPK phosphorylation levels upon Ang II induction (Figure 4,  $P < 0.05$ ).

### 3.4. EGF-induced ERK1/2 MAPK activation is independent of HB-EGF release and MMP action in VSMCs

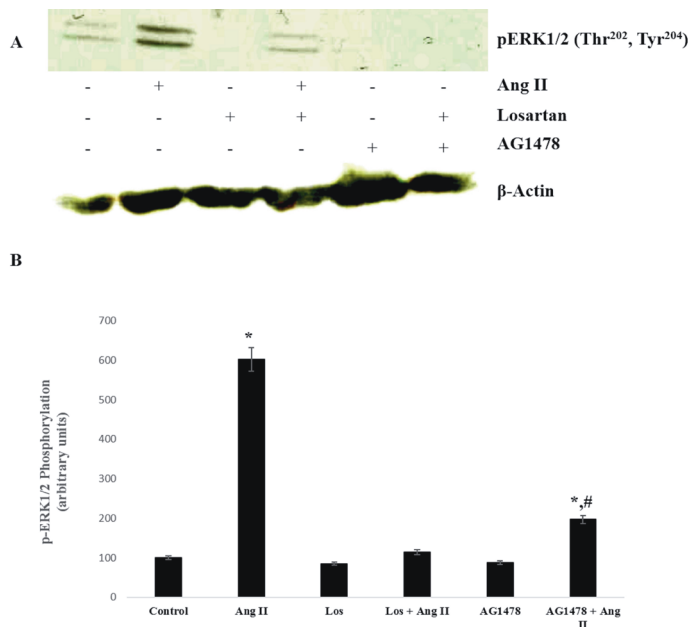
The cells were initially incubated with inhibitors CRM197 (10  $\mu$ g/mL, 30 min) or GM6001 (20  $\mu$ M, 30 min) to assess the effect of HB-EGF release and MMP activation, respectively, on EGF-induced ERK1/2 MAPK phosphorylation. Phosphorylation was then induced by 20 ng/mL EGF ligand for 5 min. EGF enhanced ERK1/2 MAPK phosphorylation approximately eightfold compared to the control group (Figure 5,  $P < 0.05$ ). The cells treated with either inhibitor did not exhibit significant ERK1/2 MAPK phosphorylation upon EGF stimulation, suggesting that neither HB-EGF nor MMP influences EGF-induced ERK1/2 MAPK phosphorylation (Figure 6).

## 4. Discussion

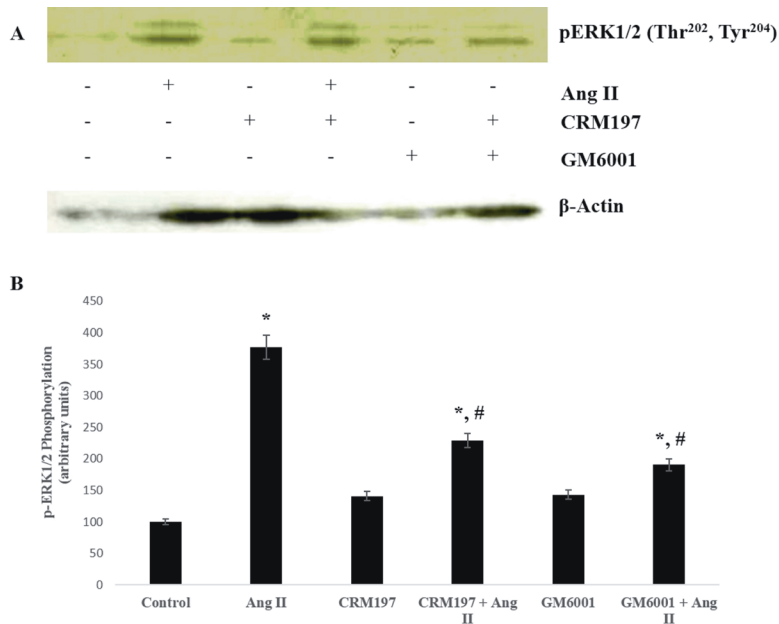
CVDs are serious health problems associated with high mortality rates, particularly in developed countries. Ang



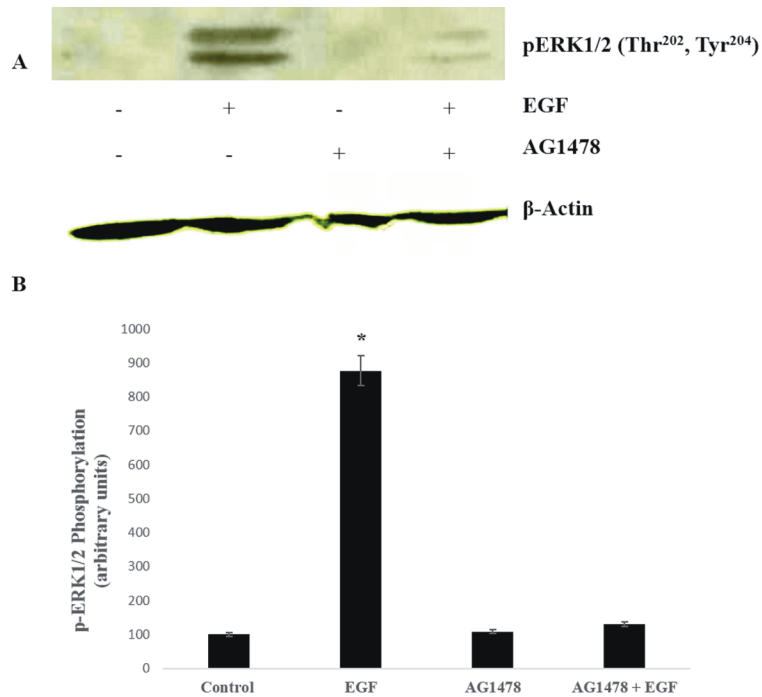
**Figure 2.** The effects of the HB-EGF release inhibitor CRM197 and the MMP inhibitor GM6001 on EGFR phosphorylation after Ang II induction. VSMCs were incubated with either 10  $\mu$ M CRM197 or 20  $\mu$ M GM6001 for 30 min before stimulation. Cells were then induced by 100 nM Ang II for 5 min. CRM197 and GM6001 decreased EGFR phosphorylation to control levels. A) A representative example of the immunoblotting. B) EGFR phosphorylation as arbitrary units. Each well was loaded with 50 mg of protein. Bars represent the mean  $\pm$  SD from three independent replicates. The control group was set as 100 units. \* $P < 0.05$ , significant compared to the control group.



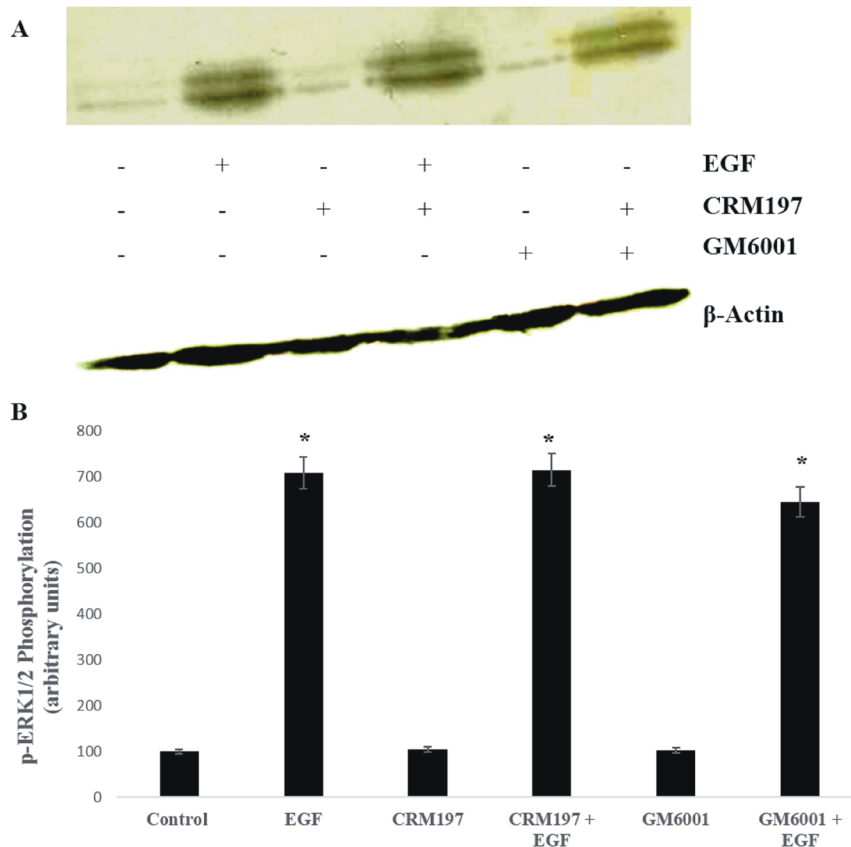
**Figure 3.** The effect of EGFR transactivation on Ang II-induced ERK1/2 MAPK phosphorylation (Thr<sup>202</sup>/Tyr<sup>204</sup>) in VSMCs. Ang II induction at 100 nM for 5 min increased ERK1/2 MAPK phosphorylation nearly fivefold. Whereas losartan (20  $\mu$ M) decreased phosphorylation to control levels, AG1478 (1  $\mu$ M) significantly decreased phosphorylation compared to Ang II-treated cells. A) A representative example of the immunoblotting. B) ERK1/2 MAPK phosphorylation as arbitrary units. Each well was loaded with 50 mg of protein. Bars represent the mean  $\pm$  SD from three independent replicates. The control group was set as 100 units. \* $P < 0.05$ , significant compared to the control group. # $P < 0.05$ , significant compared to Ang II.



**Figure 4.** The effects of the HB-EGF release inhibitor CRM197 and the MMP inhibitor GM6001 on ERK1/2 MAPK phosphorylation after Ang II induction. VSMCs were incubated with either 10 µg/mL CRM197 or 20 µM GM6001 for 30 min before stimulation. Cells were then induced by 100 nM Ang II for 5 min. CRM197 and GM6001 attenuated ERK1/2 MAPK phosphorylation, but not to the control level. A) A representative example of the immunoblotting. B) ERK1/2 MAPK phosphorylation as arbitrary units. Each well was loaded with 50 mg of protein. Bars represent the mean ± SD from three independent determinations. The control group was set as 100 units. \*P < 0.05, significant compared to the control group. #P < 0.05, significant compared to Ang II.



**Figure 5.** The effects of EGF ligand stimulation on ERK1/2 MAPK activation in VSMCs. Cells were induced by 20 ng/mL EGF ligand for 5 min. EGF enhanced ERK1/2 MAPK phosphorylation significantly. AG1478 inhibitor-treated cells (1 µM) did not respond to EGF induction. A) A representative example of the immunoblotting. B) ERK1/2 MAPK phosphorylation as arbitrary units. Each well was loaded with 50 mg of protein. Bars represent the mean ± SD from three independent determinations. The control group was set as 100 units. \*P < 0.05, significant compared to the control group.



**Figure 6.** Determination of the roles of HB-EGF and MMP on EGF-induced ERK1/2 MAPK activation in VSMCs. VSMCs were incubated with either 10  $\mu$ g/mL HB-EGF inhibitor CRM197 or 20  $\mu$ M MMP inhibitor GM6001 for 30 min before stimulation. Cells were then induced by 20 ng/mL EGF for 5 min. Neither CRM197 nor GM6001 treatment affected phosphorylation levels of ERK1/2 MAPK upon EGF induction. A) A representative example of the immunoblotting. B) ERK1/2 MAPK phosphorylation as arbitrary units. Each well was loaded with 50 mg of protein. Bars represent the mean  $\pm$  SD from three independent determinations. The control group was set as 100 units. \* $P < 0.05$ , significant compared to the control group.

II is a vasoactive chemical agent that can exhibit hormone-like effects. Many new functions of Ang II related to CVDs, including hypertension and atherosclerosis, are currently being discovered. In addition to canonical vasoconstriction and the active role of Ang II in RAS, noncanonical growth hormone-like effects have also been defined in recent years (21). Although EGFR activation has been studied extensively in cancer biology, research focusing on its role in CVD pathogenesis and atherosclerotic plaque formation is comparatively new (19). G-protein coupled receptor (GPCR)-mediated EGFR transactivation occurs after binding of the GPCR ligand to the receptor; this results in activation of RTKs including EGFR (22). GPCR-mediated EGFR activation is thought to be to utilize a triple-membrane-passing signal (TMPS) mechanism (23). The proposed TMPS mechanism for Ang

II-induced EGFR transactivation is that Ang II binds to its receptor AT1R, activating intracellular signaling pathways leading to HB-EGF release and MMP activation, which transactivates EGFR.

Ang II-induced EGFR transactivation has long been known, but the effect of losartan on EGFR phosphorylation is less studied. Sandoval et al. reported diminished EGFR Tyr<sup>1173</sup> phosphorylation following losartan incubation in spontaneously hypertensive rats (SHRs) (24). They incubated losartan for a prolonged time period and at a higher concentration. Our study differs from their study in the origin of cultured VSMCs (SHR vs. Wistar), the studied EGFR phosphorylation (Y1173 vs. Y1068), and the duration and concentration of losartan (100  $\mu$ M, 16 h vs. 10  $\mu$ M, 30 min). Our study showed that Ang II cannot transactivate EGFR in the presence of the AT1R

antagonist losartan in parallel with the findings of Chen et al. in renal epithelial cells (7). The experiments carried out in the presence of either HB-EGF or MMP inhibitors demonstrated that Ang II did not transactivate EGFR. This observation indicates that Ang II sends a signal to the extracellular matrix to release HB-EGF and to activate MMPs to trigger EGFR transactivation. These results are similar to those found by Eguchi et al. (8,25), who showed that HB-EGF and MMP may have active roles in the transactivation process. Beyond these, the ability of losartan to inhibit Ang II-induced EGFR transactivation could not be desired at any time since EGFR signaling is a noteworthy cardiac survival pathway, and EGFR inhibition is detrimental to the diabetic or ischemic heart (26).

Intracellular MAPK pathways play a significant role in CVD pathogenesis (27). The results of our study highlight that Ang II-induced ERK1/2 MAPK activation occurs owing to AT1R, and that EGFR transactivation contributes to the activation. These findings contradict those of Eguchi et al. (25), which suggest that Ang II-induced ERK1/2 MAPK activation is dependent on EGFR transactivation. This finding may be based solely upon the use of chemical inhibitor that may have actions other than inhibition of the targeted enzymes. EGFR transactivation plays a role in Ang II-induced ERK1/2 MAPK activation, as ERK1/2 MAPK phosphorylation partially proceeds even when EGFR, MMP, or HB-EGF release is inhibited. However, it is clear that Ang II-induced ERK1/2 MAPK is not activated merely by the transactivation of EGFR. Some other factors,

such as PDGFR activation, intracellular Ca<sup>2+</sup>, and ROS concentrations, are thought to affect the phosphorylation levels of ERK1/2 MAPK (28).

Induction of mitogenesis by EGF ligands was shown by Dreux et al. (12), and EGF-induced ERK1/2 MAPK phosphorylation elevation was demonstrated by other research groups (29). Our results are supported by the findings of Mugabe et al. (30). We determined that EGF-induced ERK1/2 MAPK phosphorylation does not utilize an HB-EGF release- or MMP activation-dependent pathway. However, these findings are not surprising. Although the EGF ligand stimulates ERK1/2 MAPK as a growth factor, its signaling pathway differs from Ang II. Whereas Ang II binds to its receptor and then causes the release of HB-EGF, the EGF ligand binds directly to EGFR and may not need intermediary signaling molecules.

In conclusion, Ang II receptor blockers such as losartan are widely prescribed to treat hypertension and to reduce stroke risk worldwide. Our study shows that losartan can inhibit EGFR transactivation induced by Ang II. The results of this study highlight that losartan is a potential novel candidate to inhibit EGFR phosphorylation. Further studies are needed to fully understand the therapeutic potential of losartan to inhibit EGFR activation in CVD.

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