Angiotensin II–Induced Cardiac Hypertrophy and Hypertension Are Attenuated by Epidermal Growth Factor Receptor Antisense

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Background—Angiotensin II (Ang II) is a vasoconstrictor but also a growth factor. However, the Ang II type 1 receptor does not have a tyrosine kinase domain that mediates the cellular signals for mitosis. We have shown that Ang II acts via “trans”-activation of the epidermal growth factor receptor (EGFR) to induce activation of tyrosine kinase and mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) in vascular smooth muscle cells (VSMCs).

To examine whether EGFR is involved in the development of left ventricular hypertrophy (LVH), we inhibited EGFR with a specific antisense oligodeoxynucleotide to attenuate the Ang II–induced cardiovascular hypertrophic effects.

Methods and Results—The antisense oligodeoxynucleotide to EGFR (EGFR-AS) was designed and tested on Ang II–induced ERK activation in cultured VSMCs. We also investigated the effects of EGFR-AS on LVH and blood pressure (BP) in Ang II–infused hypertensive rats. In VSMCs, EGFR-AS (2.5 μmol/L) reduced EGFR expression and inhibited the Ang II–induced phosphorylation of ERK. In rats, Ang II (150 ng/h for 14 days) increased BP compared with controls (184 ± 6 mm Hg versus 122 ± 3 mm Hg; n = 7; P < 0.01). Continuous intravenous infusion of EGFR-AS (2 mg/kg) decreased BP (169 ± 8 mm Hg; n = 8; P < 0.05). Ang II infusion increased the left ventricular/body weight (LV/BW) ratio compared with control rats (2.75 ± 0.08 versus 2.33 ± 0.07; P < 0.01). EGFR-AS, but not EGFR-sense, normalized the LV/BW in Ang II–infused rats (2.32 ± 0.06; P < 0.01) and attenuated Ang II–enhanced EGFR expression and ERK phosphorylation.

Conclusion—Ang II requires EGFR to mediate ERK activation in VSMCs and the heart. EGFR plays a critical role in the LVH induced by Ang II. (Circulation. 2002;106:909-912.)

Key Words: oligodeoxyribonucleotides, antisense ■ receptor, epidermal growth factor ■ hypertrophy ■ angiotensin ■ mitogen-activated protein kinases

Left ventricular hypertrophy (LVH) constitutes an independent cardiovascular risk factor, which is related to a morphological and functional maladaptation. The pathological changes associated with LVH, which are commonly referred to as myocardial remodeling, are characterized by myocyte growth, fibrosis, and cell death. These pathological alterations have been linked, in part, to activation of the renin-angiotensin system. Angiotensin II (Ang II) can induce LVH in rodent models; this effect is independent of the pressor action of Ang II. Angiotensin-converting enzyme (ACE) inhibitors and Ang II type 1 receptor (AT1R) antagonists prevent or reverse LVH after pressure overload. Therefore, it has been proposed that the hypertrophic effects of Ang II in the heart are mainly mediated through AT1R.

For growth factors, tyrosine kinase activation is an essential step in the signaling pathway for mitogenesis, such as in mitogen-activated protein (MAP) kinase. However, the AT1R is not directly linked to tyrosine kinase, and yet it seems to induce tyrosine phosphorylation of multiple signaling proteins, suggesting a cross-talk between AT1R and a tyrosine kinase. Recently, we showed that “trans”-activation of the epidermal growth factor receptor (EGFR) through AT1R plays a key role in Ang II–induced MAP kinase/extracellular signal-regulated kinase (ERK) activation and subsequent protein synthesis in cultured vascular smooth muscle cells (VSMCs). Hence, we hypothesized that the EGFR is indispensable for Ang II–induced hypertrophic effect in vivo. An application of antisense oligodeoxynucleotide (AS-ODN) in vivo has great potential as a new tool to study the function of genes by inhibiting specific protein synthesis. The present study was designed to examine the effects of an AS-ODN to EGFR (EGFR-AS) on the LVH induced by Ang II infusion and on MAP kinase/ERK activation in the heart and VSMCs.
Methods

The Institutional Animal Care and Uses Committee at the University of Florida approved all experimental procedures.

Oligodeoxynucleotides

AS-ODNs and sense oligodeoxynucleotides were designed according to principles previously described as 16-mers targeted to bases −9 to +7 of EGFR mRNA (GeneBank AB 025197), which were confirmed in the GeneBank database for uniqueness. The oligodeoxynucleotides were modified by phosphorothioation and synthesized by GenoMechanix.

In Vitro Experiment in VSMCs

VSMCs were prepared from the thoracic aorta of Sprague-Dawley rats (Harlan, Indianapolis, Ind) by the explant method, as previously described. Cells at ≥80% confluence were made quiescent by incubation in serum-free medium for 2 days, then treated with oligodeoxynucleotides for additional 2 days. In some experiments, VSMCs grown on a 6-well plate were stimulated with Ang II at 37°C for 10 minutes. After the treatment, the medium was replaced with sodium dodecyl sulfate (SDS) sample buffer (120 µL). Then, ~30 µL of the samples that contained same amount of proteins were loaded to SDS-PAGE. Immunoblotting was performed with enhanced chemiluminescence (Amersham) using anti-EGFR antibody, anti-Erb2 antibody, phosphospecific ERK2 antibody and ERK2 antibody (Santa Cruz Biotechnology). The ERK2 antibody is cross-reactive with ERK1 to a lesser extent. All antibodies were used under the skin. Another osmotic minipump for the intravenous treatment of oligodeoxynucleotides. 11,12 DOTAP/protamine sulfate may not be solely due to the inhibition of BP elevation but explained by an inhibition of EGFR/MAP kinase (ERK) activation. This was shown using a pharmacological agent, AG1478, as a specific EGFR tyrosine kinase inhibitor. 17 Although EGFR–AS treatment did not normalize BP, a reduction of BP by EGFR-AS treatment significantly decreased systolic BP compared with Ang II infusion (P<0.05), although systolic BP in EGFR–AS–treated rats was still higher than in sham-operated rats (Figure 2A).

To assess LVH, the left ventricular weight to body weight ratio (LVW/BW) was determined. LVW/BW in Ang II–treated rats was larger than in sham-operated rats (Figure 2B). EGFR–AS treatment significantly (P<0.05) reduced LVW/BW compared with Ang II–infused rats, whereas EGFR-sense treatment did not alter the elevated LVW/BW (Figure 2B). EGFR expression and phosphorylation of ERK in the heart was increased in Ang II–infused rats compared with sham-operated rats, and EGFR–AS decreased EGFR expression and phosphorylation of ERK (Figure 2C). EGFR-sense did not alter the elevated expression of EGFR and phosphorylated ERK.

Discussion

By using EGFR–AS as a selective inhibitor, we demonstrated for the first time that EGFR plays an essential role in Ang II–induced cardiac hypertrophy in vivo. In a previous report, EGFR seemed to be required for Ang II–mediated MAP kinase activation in vitro. 7 This was shown using a pharmacological agent, AG1478, as a specific EGFR tyrosine kinase inhibitor. 7,10 AS-ODN is more specific than pharmacological agents because AS-ODN is a synthetic DNA with a gene-specific sequence that hybridizes to the mRNA of the target gene. 8 Because cationic liposomes and cationic polymers enhance the uptake of AS-ODNs by tissue, 11,12 DOTAP/DOPC liposome and protamine sulfate were used in the present study. We demonstrated that these cationic polymers and lipids are also useful in increasing the antisense action either in vitro or in vivo. Ang II stimulation of the Gq-coupled AT1R rapidly induces intracellular calcium mobilization and protein kinase C activation. 5 This mechanism was the main signaling pathway of the AT1R that elevates BP. However, Carmines et al 13 reported that AG1478 could suppress the renal arteriolar contraction responses to Ang II. To our knowledge, there has been no direct evidence that EGFR inhibition decreases BP in vivo, but a tyrosine kinase inhibitor 14 and MAP kinase kinase inhibitor 15 also attenuated the elevation of BP in Ang II–induced hypertension. The primary ligand of EGFR, epidermal growth factor (EGF), causes the contraction of rat aorta. 16 The contractile response to EGF is augmented in hypertensive rats and is inhibited by AG1478 or MAP kinase inhibitor. 17 Although EGFR–AS treatment did not normalize BP, a reduction of BP by EGFR–AS can be partially explained by an inhibition of EGFR/MAP kinase (ERK)–mediated vasoconstriction and/or a disappearance of the receptor for EGF-mediated contraction.

As we demonstrated, the inhibition of LVH by EGFR–AS may not be solely due to the inhibition of BP elevation but also due to the inhibitory effect of EGFR–AS on EGFR expression and ERK activation in the heart. Ang II infusion...
Figure 1. Western blot analysis of EGFR expression and ERK phosphorylation. A, VSMCs were treated with varying concentrations of EGFR-AS for 48 hours. B, VSMCs were preincubated with EGFR-AS (5 μmol/L) or EGFR-sense (5 μmol/L), or without oligodeoxynucleotides. Then, VSMCs were stimulated with Ang II (100 nmol/L) for 10 minutes and treated with (+) or without (−) oligodeoxynucleotides. IB indicates immunoblots. Data shown are representative of 3 separate experiments.

Figure 2. A, Time course of changes in systolic BP in untreated control rats (open circle) and rats treated with an Ang II infusion (closed triangle), Ang II infusion with EGFR-AS (closed circle), and Ang II infusion with EGFR-sense (closed square). B, LVW/BW in untreated control rats and rats treated with an Ang II infusion, Ang II infusion with EGFR-AS, and Ang II infusion with EGFR-sense. EGFR-AS–treated rats had significantly lower LVW/BW than rats treated with an Ang II infusion alone and with EGFR-sense (t-test, \( P < 0.05 \)). C, Representative Western blots analysis of EGFR expression and ERK phosphorylation in the hearts and their quantitative evaluations expressed as a percentage of sham-operated rats. Data shown are representative of 3 separate blots. IB indicates immunoblot. \( \ast P < 0.05 \), \( ** P < 0.01 \) vs control; \( \dagger P < 0.05 \) vs Ang II infusion with EGFR-AS–treated rats.
increases the expression of several growth factors such as heparin-binding EGF and epiregulin, which promote the growth signal by EGFR. In cultured cardiac myocytes, activation of ERK is indispensable for the protein synthesis induced by Ang II. In VSMCs, heparin-binding EGF generation is involved in Ang II–induced EGFR transactivation and subsequent ERK activation. During the revision of this article, Asakura et al showed that a metalloproteinase inhibitor attenuated the LVH caused by Ang II infusion by blocking heparin-binding EGF production. Thomas et al further reported that AT1R overexpression promoted hypertrophy of cultured cardiac myocytes through an EGFR pathway. Although these publications strongly suggest the importance of EGFR in the development of LVH, our report is the first to show that Ang II activation of the EGFR-ERK pathway may be a requirement in LVH in vivo.

In summary, our results demonstrate that Ang II stimulates ERK/MAP kinase through the EGFR in vivo and in vitro. EGFR-AS inhibits this pathway and reduces both the BP and LVH induced by Ang II. EGFR-AS may therefore offer a new therapeutic intervention for the treatment of LVH and hypertension.

Acknowledgments
This work was supported by NIH MERIT award HL 27334 (Dr Phillips), HL58205 (Dr Inagami) and DK20593 (Dr Inagami), a UNC/merck Postdoctoral Science Research Fellowship (Dr Frank), an AHA Scientist Development Grant (Dr Eguchi), a Vanderbilt University Diabetes Center Pilot and Feasibility Proposal (Dr Eguchi), an AHA Postdoctoral Fellowship Grant (0120346B; Dr Kagiyama), and a Japan Heart Foundation and Bayer Yakuhin Research Grant Abroad (Dr Kagiyama).

References
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*Circulation*. 2002;106:909-912; originally published online August 5, 2002; doi: 10.1161/01.CIR.0000030181.63741.56

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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http://circ.ahajournals.org/content/106/8/909

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