Antisense to Epidermal Growth Factor Receptor Prevents the Development of Left Ventricular Hypertrophy

Shuntaro Kagiyama, Keping Qian, Tomoko Kagiyama, M. Ian Phillips

Abstract—We previously demonstrated that left ventricular hypertrophy (LVH) induced by angiotensin II infusion requires epidermal growth factor receptor (EGFR) activation to mediate the mitogen-activated protein kinase/extracellular signal–regulated kinase (MAPK/ERK) pathway. To test whether the EGFR-mediated MAPK/ERK activation plays an important role in development and maintenance of LVH in spontaneously hypertensive rats (SHR), we investigated the effects of antisense oligodeoxynucleotide to EGFR (EGFR-AS) on LVH and blood pressure in young and adult SHR. EGFR-AS, sense oligonucleotide to EGFR (EGFR-S; 1.5 mg/kg), or vehicle control (5% dextrose) with liposome was injected once a week for 2 months in 5- or 13-week-old SHR. The effect of EGFR-AS on the expression of EGFR and phosphorylated ERK in the heart were examined by Western blots. After treatment, EGFR-AS significantly (P<0.05) decreased left ventricular weight/body weight and blood pressure in young SHR compared with EGFR-S or control-treated rats. In adult SHR, EGFR-AS did not affect left ventricular weight/body weight and blood pressure. EGFR and phosphorylated ERK significantly declined from 5 to 20 weeks (P<0.05). EGFR-AS, but not EGFR-S, significantly (P<0.05) decreased the expression of EGFR and phosphorylated ERK in young SHR, but had no significant effect in adult SHR. These results suggest that EGFR-mediated ERK activation is critically important for LVH in young SHR. This may be related to the high levels of EGFR and phosphorylated ERK in young SHR, suggesting a critical role of the EGFR-activated ERK pathway in cardiovascular development but not in the maintenance of established LVH in adult SHR. (Hypertension. 2003;41[part 2]:824-829.)

Key Words: antisense ■ gene therapy ■ receptors, epidermal ■ protein kinases ■ blood pressure ■ hypertrophy, left ventricular

In patients with essential hypertension in which plasma renin activity is normal, ACE inhibitors or angiotensin (Ang) II type 1 receptor (AT1R) antagonists lower blood pressure (BP) and regress left ventricular hypertrophy (LVH).1,2 This indicates the importance of the local renin angiotensin system in cardiovascular regulation and remodeling. Ang II promotes cell growth in vitro and induces LVH in rats without the elevation of BP.3 The induction of cell growth by Ang II requires the activation of mitogen-activated protein kinase (MAPK) pathway through tyrosine kinase activation.4 However, AT1R itself does not have a tyrosine kinase domain. We previously reported that the epidermal growth factor receptor (EGFR), which is a receptor-type tyrosine kinase, plays an important role in LVH in Ang II–infused hypertensive rats through the activation of extracellular signal–regulated kinase (ERK), one of the MAPK family members.5

A greater reactivity of blood vessels6 and enhanced mitogenic responses of aortic myocytes7 to Ang II have been reported in spontaneously hypertensive rats (SHR). Ang II infusion increases EGFR mRNA8 and protein in the aorta and heart.5 EGFR protein levels are elevated in the kidney and aorta from genetically hypertensive rats.9 The activities of ERKs in the heart were elevated in young SHR and decreased with age.10,11 However, there is no direct evidence regarding the contribution of EGFR to ERK activation in the development and maintenance phase of hypertension and LVH in SHR. To address this question, we developed an effective antisense oligonucleotide (AS-ODN) to inhibit EGFR expression. We have shown that the EGFR AS-ODN effectively inhibited the protein synthesis of EGFR both in vitro and in vivo.3 Thus, the purpose of the present study is to investigate the role of EGFR and ERK activation in LVH of young and adult SHR by using the EGFR AS-ODN, and to determine the contribution of this pathway on the development and maintenance of hypertension and LVH.

Methods

All experimental procedures on animals were approved by the Institutional Animal Care and Uses Committee at the University of Florida.

Oligodeoxynucleotides

AS-ODNs (targeted to bases −9 to +7 of EGFR mRNA, GeneBank AB 025197) and its sense oligodeoxynucleotides (S-ODNs) were
Effects of EGFR-AS on EGFR Synthesis

To confirm the inhibitory effects of the single injection of the AS-ODN on EGFR mRNA and protein in vivo, 8 week-old male Sprague-Dawley rats (Harlan, Indianapolis, Ind; n=12) were anesthetized by 5% isoflurane, and EGFR-AS (1.5 mg/kg) was injected in the tongue vein with liposome. The rats were euthanized on either day 0 (no injection) or 1, 3, or 7 days after injection. At each time point, the left ventricles were dissected out to measure EGFR mRNA and protein.

Effects of EGFR-AS on LVH and BP in SHR

Two different ages of SHR (Charles River, Wilmington, Mass) were used. To examine the effect of EGFR-AS on development and maintenance phase of hypertension, we used 5- and 13 week-old rats as young (n=15) and adult (n=14) SHR, respectively. The rats were treated with EGFR-AS or EGFR-S for 8 weeks. Oligodeoxynucleotides (1.5 mg/kg) were injected via the vein once a week with lipid (1,2-bis[oleoyloxy]-3-[trimethylammonio] pro-pane [DOTAP, Avanti Polar Lipids] combined with L-α-dioleoyl phosphatidylethanolamine [DOPE, Avanti Polar Lipids]) under anesthesia by 5% isoflurane. The DNA/lipid ratio was 1:2.5, based on results in our previous study.\(^1\) For controls, 5% dextrose with lipid was injected. Systolic BP was measured by the tail-cuff method. Eight weeks after treatment, the rats (13 and 20 weeks) were deeply anesthetized, and the left ventricles were dissected on ice and homogenized for Western blot analysis and quantitative real-time polymerase chain reaction (PCR). To test the age-dependency of the EGFR and ERK activity, the left ventricles were also taken from 5-week-old SHR (n=4).

Western Blot Analysis

The left ventricle was homogenized in ice-cold lysis buffer (in mmol/L) Tris-HCl 25 at pH 7.4, NaCl 25, NaF 10, sodium pyrophosphate 10, EGTA 0.5, sodium orthovanadate 1, okadaic acid 10, and phenylmethylsulfonyl fluoride 1, with 0.8\(\mu\)g/mL leupeptin, 10\(\mu\)g/mL aprotinin, and 10\(\mu\)g/mL \(\gamma\)-nitosopropyl phosphate) and centrifuged for 10 minutes at 13 000 rpm at 4°C. Aliquots of proteins (150\(\mu\)g) were run in sodium dodecyl sulfate-polyacrylamide gel (10%), and transferred onto nitrocellulose membranes (ECL nitrocellulose, Amersham). Immunoblotting was performed with enhanced chemiluminescence (ECL, Amersham) using anti-EGFR antibody, anti-ErbB2 antibody, anti-phosphor-specific ERK2 (p-ERK2) antibody, anti-ERK2 antibody, and anti-AT1R antibody (Santa Cruz Biotechnology). Quantification of the blots was determined by Quantity One Ver:4.0.1 (Bio Rad).

Quantitative Real-Time PCR

Total RNA was isolated with Trizol (Life Technologies) followed by DNase I treatment. One\(\mu\)g of total RNA was reverse transcribed by using a Taqman reverse transcriptase kit (Applied Biosystems). EGFR mRNA expression was analyzed by real-time quantitative PCR performed with the Taqman system based on real-time detection of accumulated fluorescence (ABI PRISM 7700 Sequence Detector System, Perkin-Elmer Inc) by using a SYBR Green Master Mix (Applied Biosystems). EGFR gene expression was evaluated with the endogenous control, 18S ribosomal RNA (rRNA) gene. Primers for EGFR and 18S rRNA were constructed with the help of Primer Express (ABI Prism 7700, Perkin-Elmer Inc). For EGFR, the forward primer was 5’-AGATGGCAAGGCGATGAACTAC-3’ and the reverse primer was 5’-ACATTTCCCTGGCTGGCATAAGTC-3’; for 18S rRNA, the forward primer was 5’-AGTCCCT- GCCCTTTTGACACA-3’ and the reverse primer was 5’-CAGGGGCCCTGCAAAACC-3’. The amplification was performed with the following time course: 2 minutes at 50°C, 10 minutes at 95°C; and 40 cycles of 94°C for 20 seconds and 60°C for 1 minute. Each sample was tested in triplicate. Results were expressed as relative to EGFR-S-treated rats, which were arbitrarily assigned a value of 1.0.

Statistics

Data are expressed as mean\(\pm\)SEM. Differences in BP between the treatments were analyzed by 2-way ANOVA, and other statistical analyses were performed by Student \(t\) test or 1-way ANOVA followed by the Fisher least significant difference method. \(P<0.05\) was viewed as statistically significant.

Results

Effects of EGFR-AS on EGFR Synthesis

Figure 1 shows the effects of a single injection of EGFR-AS to Sprague-Dawley rats (n=12) on EGFR protein and mRNA in the heart. At 3 and 7 days after injection, EGFR-AS significantly \((P<0.05)\) decreased EGFR protein (Figure 1A). EGFR mRNA was significantly \((P<0.05)\) decreased at 3 and 7 days after EGFR-AS injection (Figure 1B).

Effects of EGFR-AS on LVH and BP in SHR

Young SHR showed the expected increase in BP from 5 weeks, reaching a stable level at 12 weeks (Figure 2A).\(^1\)
EGFR-AS treatment from 5 weeks to 12 weeks, significantly ($P<0.05$) attenuated an increase in BP of young SHR compared with either EGFR-S or control treatment. There was no effect of the EGFR-AS treatment (13 to 20 weeks) on adult SHR (Figure 2A). Left ventricle weight/body weight was decreased in the EGFR-AS–treated young SHR compared with the control and EGFR-S–treated SHR ($P<0.05$). In adult SHR, there is no difference in left ventricle weight/body weight between EGFR-AS and EGFR-S treatments (Figure 2B).

Western blot analysis of the heart showed EGFR protein was age-dependently decreased (Figure 3A and 3B). In young SHR treated from 5 to 12 weeks, EGFR-AS significantly decreased EGFR expression, whereas in adult SHR (treated from 13 to 20 weeks), EGFR-AS did not alter EGFR expression. Also, in adult SHR, the EGFR protein level was significantly lower than in young SHR ($P<0.01$). ErbB2, which is a subtype of the EGFR family, was the same at 5 and 8 weeks of age, whereas it was significantly decreased in adult SHR ($P<0.05$) (Figure 3A). EGFR-AS did not affect the ErbB2 protein level. AT1R did not show any changes in 3 different groups of age and was not affected by antisense. EGFR mRNA was decreased in the EGFR-AS–treated young SHR compared with the control or EGFR-S–treated young SHR (Figure 3C). In adults, there was no significant difference between treatments. Figure 4 shows the relative density of p-ERK2 at 5, 12, and 20 weeks. The p-ERK2 antibody is cross-reactive with p-ERK1 to a lesser extent. The p-ERK signals were also age-dependently decreased, and EGFR-AS significantly decreased the p-ERK signal in young SHR ($P<0.05$) but not in adult SHR.

**Discussion**

The present study demonstrated that LVH could be attenuated by EGFR-AS with subsequent reduction of phosphorylation of ERK in young SHR. However, in the adult SHR, EGFR antisense had no effect of either EGFR expression or LVH. These results indicate that EGFR-mediated phosphorylation of ERK plays an important role in the development of LVH in SHR.

Enhanced ERKs phosphorylation by Ang II has been reported in vascular smooth muscle cells (VSMCs) from SHR.
compared with Wistar Kyoto rats, and these responses were inhibited by AT1R antagonists. Furthermore, Touyz et al reported that these augmented responses of ERKs to Ang II in VSMCs from SHR were attenuated by a selective inhibitor of EGFR kinase. Taken together, ERK phosphorylation by Ang II through EGFR transactivation is enhanced in VSMCs from SHR. In cultured cardiac myocytes, activation of ERKs is indispensable for protein synthesis induced by Ang II.21 Depletion of ERKs by AS-ODN to ERK1 and ERK2 decreased the phenylephrine-induced hypertrophic response in cardiac myocytes.22 Aoyagi et al reported that pressure overload to the isolated heart increased the p-ERK in young SHR; however, the increase in ERKs activity by pressure overload was diminished in aged rats. The question of how AT1R stimulation activates EGFR transactivation appears to be owing to heparin-binding epidermal growth factor (EGF)–like growth factor (HB-EGF) has been reported in VSMCs and in the heart.23 HB-EGF is cleaved from cell membranes by metalloproteinase. Antagonism of HB-EGF action inhibits EGFR transactivation by Ang II in VSMCs.23 LVH is inhibited by antagonism of metalloproteinase processing of HB-EGF, when LVH is induced by aortic banding and Ang II infusion.24 We demonstrate here that the inhibition of EGFR by AS-ODN decreased LVH and p-ERK in the SHR heart. The specificity of the antisense was confirmed because neither AT1R nor ErbB2 was affected by the EGFR-AS treatments. Although we did not test the effect of EGFR-AS in Wistar Kyoto rats, our report is the first to show that the EGFR transactivation of ERKs is a requirement in the development of LVH in SHR.

The present study also demonstrates an age-dependent decrease in EGFR levels in the heart of SHR. Fujino et al
reported that EGFR mRNA levels were elevated in SHR but did not report a change with age. However, we demonstrated the decrease in protein level of EGFR, whereas their study examined only the mRNA. In addition to a decrease in EGFR with age, we also demonstrated that p-ERKs were decreased with age. An age-dependent decrease in phosphorylation of ERK in the heart has been observed in stroke-prone SHR. We did not examine the decrease in protein level of EGFR, whereas their study reported that EGFR mRNA levels were elevated in SHR but did not report a change with age. However, we demonstrated the decrease in protein level of EGFR, whereas their study examined only the mRNA. In addition to a decrease in EGFR with age, we also demonstrated that p-ERKs were decreased with age. An age-dependent decrease in phosphorylation of ERK in the heart has been observed in stroke-prone SHR and aged normotensive rats.

Antisense inhibition although highly specific, however, does not result in a total knockout of the gene expression, but rather in a partial decrease. The fact we could not show any reduction of EGFR in adult SHR with EGFR-AS was because EGFR in the adult SHR is so low that it cannot be effectively decreased by EGFR-AS. Although increasing the doses of EGFR-AS might reduce EGFR protein further in the adult SHR, higher concentrations of EGFR-AS were not used because AS-ODN may be precipitated with lipid and have a non-specific hypotensive effect.

The primary ligand of the EGFR, EGF is a vasoconstrictor and can induce a mitogenic response in VSMCs. This vasoconstrictor response is enhanced in experimental hypertension and LVH.

**Perspectives**

The data from the present study indicate that the EGFR-activated ERK pathway has an important role in the development of LVH and hypertension. Inhibition of MAPK/ERKs pathway by EGFR-AS effectively decreased LVH and hypertension in young SHR but did not affect the maintenance of LVH. Our data suggest that EGFR is an appropriate target for the prevention of hypertension and LVH. The use of antisense to specifically inhibit EGFR and reduce its effects might offer a possible gene therapy approach for genetically prone LVH.

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**References**


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