Cardiac hypertrophy is inhibited by antagonism of ADAM12 processing of HB-EGF: Metalloproteinase inhibitors as a new therapy

MASANORI ASAKURA[1], MASAFUMI KITAKAZE[1], SEIJI TAKASHIMA[1], YULIN LIAO[1], FUMINOBU ISHIKURA[5], TSUYOSHI YOSHINAKA[7], HIROSHI OHMOTO[7], KOICHI NODE[1], KOHICHIRO YOSHINO[7], HIROSHI ISHIGURO[6], HIROSHI ASANUMA[1], SHOJI SANADA[1], YASUSHI MATSUMURA[3], HIROSHI TAKEDA[3], SHINTARO BEPPU[5], MICHIHIKO TADA[2], MASATSUGU HORI[1] & SHIGEKI HIGASHIYAMA[4]

Departments of [1]Internal Medicine and Therapeutics, [2]Pathology and Pathophysiology, and [3]Medical Information Science, Osaka University Graduate School of Medicine, Osaka, Japan
Departments of [4]Biochemistry and [5]Echocardiography, School of Allied Health Science, Osaka University Faculty of Medicine, Osaka, Japan
[6]Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Japan
[7]Nippon Organon K.K., Osaka, Japan
Correspondence should be addressed to S.H.; e-mail: shigeki@sahs.med.osaka-u.ac.jp, or S.T.; email: takasima@medone.med.osaka-u.ac.jp

G-protein–coupled receptor (GPCR) agonists are well-known inducers of cardiac hypertrophy. We found that the shedding of heparin-binding epidermal growth factor (HB-EGF) resulting from metalloproteinase activation and subsequent transactivation of the epidermal growth factor receptor occurred when cardiomyocytes were stimulated by GPCR agonists, leading to cardiac hypertrophy. A new inhibitor of HB-EGF shedding, KB-R7785, blocked this signaling. We cloned a disintegrin and metalloprotease 12 (ADAM12) as a specific enzyme to shed HB-EGF in the heart and found that dominant-negative expression of ADAM12 abrogated this signaling. KB-R7785 bound directly to ADAM12, suggesting that inhibition of ADAM12 blocked the shedding of HB-EGF. In mice with cardiac hypertrophy, KB-R7785 inhibited the shedding of HB-EGF and attenuated hypertrophic changes. These data suggest that shedding of HB-EGF by ADAM12 plays an important role in cardiac hypertrophy, and that inhibition of HB-EGF shedding could be a potent therapeutic strategy for cardiac hypertrophy.

Cardiac hypertrophy is an adaptive response of the heart that occurs in various cardiovascular diseases[1], but prolonged hypertrophy typically culminates in chronic heart failure or sudden cardiac death[2]. Elucidation of the mechanisms underlying cardiac hypertrophy is thus important to the field of cardiovascular biology, and may lead to new strategies for the prevention or treatment of cardiovascular diseases.

Vasoactive molecules such as phenylephrine (PE), angiotensin II (Ang II) and endothelin-1 (ET-1) are well-known inducers of cardiomyocyte hypertrophy[3–5], and inhibition of their actions can be beneficial for the treatment of chronic heart failure following cardiac hypertrophy[6,7]. All of these molecules bind to G-protein–coupled receptors (GPCRs), and there is a common cellular response to the activation of such receptors that includes an increase in protein synthesis concomitant with an increase in cell size, induction of immediate early genes and reactivation of fetal genes[8, 9]. The similarity of the response to different agents suggests that the initial signaling pathways triggered by different GPCR agonists converge on a common downstream pathway that leads to cardiac hypertrophy.

Transactivation of the epidermal growth factor receptor (EGFR) has a role in GPCR-mediated signal transduction in various cells[10–13]. Transactivation of EGFR is mediated, at least sometimes, by the EGFR ligand heparin-binding EGF (HB-EGF), which is cleaved from its membrane-anchored form (proHB-EGF) by a specific metalloproteinase[14]. If ectodomain shedding of proHB-EGF is required for GPCR signaling in various cell types, a neutralizing antibody for HB-EGF or specific inhibitors of proHB-EGF shedding might be used therapeutically to block pathological signaling via GPCRs. Here we show that transactivation of EGFR by truncated HB-EGF occurs in cardiomyocytes, and that a disintegrin and metalloprotease 12 (ADAM12) is a key metalloproteinase in this pathway. Recently, there have been several reports indicating beneficial effects of matrix metalloproteinase (MMP) inhibitors on heart failure[15]. We hypothesized that such beneficial effects of metalloproteinase inhibitors are at least partially mediated by antagonism of GPCR signaling via the ADAM12-mediated shedding of HB-EGF in cardiac cells, particularly cardiomyocytes.

EGFR transactivation by hypertrophic stimuli

To assess the ability of HB-EGF to stimulate tyrosine phosphorylation of EGFR, we treated cultured rat neonatal cardiomyocytes with recombinant HB-EGF (1×10^{-8} M). Cells were lysed at specified times, after which the lysates were immunoprecipitated with antibody against EGFR and probed with an anti-phosphotyrosine antibody (4G10). Phosphorylated EGFR (170 kD) was clearly seen

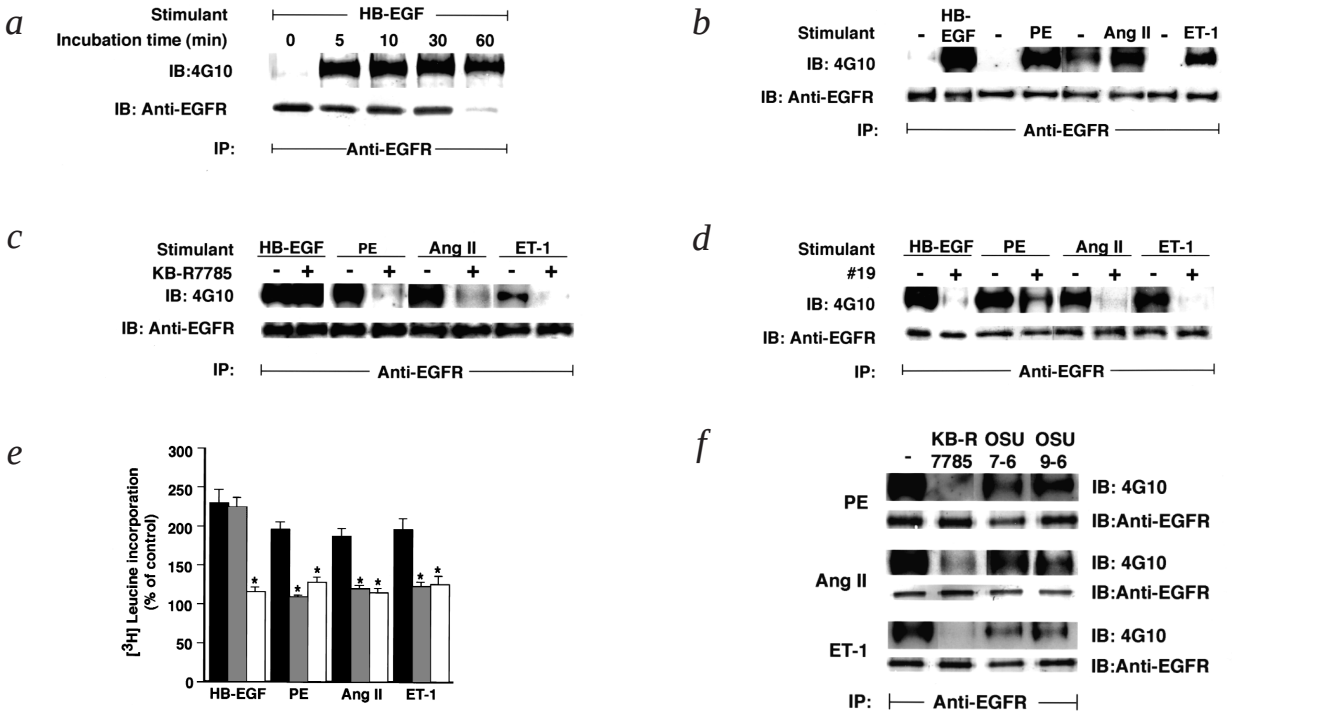


Fig. 1 Effects of KB-R7785 and HB-EGF neutralizing antibody on EGFR transactivation and protein synthesis induced by GPCR agonists in cardiomyocytes. EGFR tyrosine phosphorylation in cultured cardiomyocytes was detected by blotting with a monoclonal antibody against phosphotyrosine, 4G10 (upper panels), or with antibody against EGFR (lower panels). ***a***, Tyrosine phosphorylation of EGFR activated by HB-EGF for the indicated times. ***b***, Tyrosine phosphorylation of EGFR after treatment with or without indicated agents. ***c***, ***d*** and ***f***, Tyrosine phosphorylation of EGFR activated by HB-EGF or GPCR agonists after preincubation with or without KB-R7785 (*c*), the neutralizing antibody #19 against HB-EGF (*d*), or OSU7-6 or OSU9-6 (*f*). ***e***, In protein synthesis experiments, cardiomyocytes were exposed to the indicated agents for 18 h after preincubation for 30 min with KB-R7785 or antibody #19, or after no treatment. Protein synthesis was determined by the incorporation of [^{3}H]leucine after ■, no treatment; ■, KB-R7785; □, #19. Results (mean ± s.e.m., n = 3) are expressed relative to control values for cells not exposed to any agents and not preincubated with any inhibitor or antibody. *, $P < 0.05$ versus control cells.

from five minutes after HB-EGF treatment (Fig. 1*a*). Next we examined whether GPCR agonists transactivated EGFR in cardiomyocytes. When cardiomyocytes were treated with physiological concentrations of PE (1×10^{-5} M), Ang II (1×10^{-8} M) or ET-1 (1×10^{-7} M) for five minutes, significant enhancement of EGFR phosphorylation was induced by all three agonists (Fig. 1*b*).

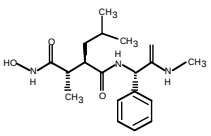
EGFR transactivation by the released ectodomain of HB-EGF

To investigate whether metalloproteinases were involved in EGFR transactivation in cardiomyocytes by GPCR agonists, we examined the effect of a new metalloproteinase inhibitor, KB-R7785. From over 2,000 metalloproteinase inhibitors, we selected KB-R7785 as one of the most potent inhibitors of HB-EGF shedding in a growth factor-alkaline phosphatase (AP) tag assay[16]. Table 1 shows the specificity of this compound: KB-R7785 inhibited HB-EGF shedding at a lower IC_{50} than that for other growth factors of the EGF family or tumor necrosis factor-α (TNF-α). In this study, the KB-R7785 concentration (1×10^{-6} M) was selected to obtain relatively specific inhibition of HB-EGF shedding. Preincubation with KB-R7785 (1×10^{-6} M) for 30 min abrogated the tyrosine phosphorylation of EGFR by PE, Ang II and ET-1, whereas EGFR activation by HB-EGF was unaffected (Fig. 1*c*). The HB-EGF neutralizing antibody (#19) also inhibited the tyrosine phosphorylation of EGFR by PE, Ang II and ET-1 (Fig. 1*d*), indicating that release of soluble HB-EGF by metalloproteinases after GPCR stimulation was responsible for EGFR transactivation.

Effect of HB-EGF on protein synthesis in cardiomyocytes

The exposure of cultured cardiomyocytes to PE, Ang II or ET-1 increased total protein synthesis, a typical hypertrophic response. HB-EGF also stimulated [^{3}H]leucine uptake in cardiomyocytes to

Table 1 Inhibitory activity of KB-R7785

KB-R7785	Cell assay [a] $I C_{50}$ (μM)				Peptide assay [b] $I C_{50}$ (nM)			
	HB-EGF	AR	TGF-α	TNF-α [c]	MMP-1	MMP-3	MMP-9	TACE [c]
	0.23	0.76	2.01	4.50	0.30	3.00	0.30	9.60

[a], Inhibitory activities of the compounds were measured by using HT1080 transfectants. Each value was the mean of 5 time measurements. [b], Inhibitory activities of the compounds for MMP-1 and MMP-9 were measured by using FITC-collagen and FITC-gelatin as the substrate, respectively[16]. IC_{50} values were calculated by the probit method from concentration-% inhibition curves. [c],Since TNF-α is processed by TACE, the difference of IC_{50} values estimated by cell assay and peptide assay indicates ~470-fold difference of their sensitivities.

Table 2 Morphometric and hemodynamic analyses

	TAC (C57BL/6J, 4 weeks)			PE (C57BL/6J, 7 days)			Ang II (FVB, 14 days)		
	Sham	TAC	TAC + KB	Vehicle	PE	PE + KB	Vehicle	Ang II	Ang II + KB
	(*n* = 10)	(*n* = 9)	(*n* = 9)	(*n* = 9)	(*n* = 9)	(*n* = 9)	(*n* = 7)	(*n* = 7)	(*n* = 6)
BW (g)	25.2 ± 1.0	24.4 ± 0.9	26.4 ± 0.4	21.7 ± 0.5	20.7 ± 0.3	21.1 ± 0.6	26.9 ± 0.5	26.4 ± 0.6	25.9 ± 0.4
HW (mg)	128.9 ± 12.3	208.3 ± 11.2*	177.7 ± 10.6**	89.9 ± 2.7	100.9 ± 2.1*	91.6 ± 4.0	109.2 ± 3.3	123.0 ± 2.9*	108.8 ± 5.2**
HW/BW (g/mg)	5.09 ± 0.27	8.62 ± 0.56*	6.69 ± 0.35**	4.19 ± 0.07	4.87 ± 0.07*	4.33 ± 0.10**	4.05 ± 0.09	4.67 ± 0.14*	4.20 ± 0.18**
HR (mg^{-1})	644.1 ± 13.7	667.7 ± 13.9	656.1 ± 14.1	502 ± 35	604 ± 31*	578 ± 33	684 ± 36	686 ± 17	648 ± 89
BP (mmHg)	99.4 ± 2.1	101.8 ± 1.7	101.3 ± 4.0	91.2 ± 3.9	115.9 ± 4.5*	118.0 ± 4.8	102.3 ± 2.8	127.7 ± 4.0*	120.0 ± 5.6

KB, KB-R7785; BW, body weight; HW, heart weight; HR, heart rate; BP, blood pressure. Values are means ± s.e.m. *, $P < 0.05$ versus sham or vehicle; **, $P < 0.05$ versus TAC, PE or Ang II.

twice the control level (Fig. 1*e*). KB-R7785 attenuated the increase of [^{3}H]leucine uptake induced by PE, Ang II- and ET-1, whereas stimulation of [^{3}H]leucine uptake by soluble HB-EGF was unaffected. HB-EGF neutralizing antibody also inhibited [^{3}H]leucine uptake. These results are consistent with the hypothesis that EGFR transactivation resulting from the shedding of HB-EGF is a common event in the pathways promoting cardiac hypertrophy.

ADAM12 is involved in GPCR-induced HB-EGF shedding

We used two additional metalloproteinase inhibitors (OSU7-6 and OSU9-6), which showed similar inhibition of HB-EGF shedding but had different inhibitory effects on MMP-1, MMP-3 and MMP-9 (ref. 16). Both OSU7-6 and OSU9-6 caused a similar attenuation of EGFR transactivation by GPCR agonists (Fig. 1*f*), although OSU7-6 was a more potent inhibitor of MMP-1, -3 and -9 than OSU9-6. These results indicated the involvement of other metalloproteinases besides MMP-1, -3 or -9. Next, we identified the specific protease causing HB-EGF shedding in cardiomyocytes. HB-EGF shedding is regulated by protein kinase C (PKC), especially PKC-δ isoform activation[17,18]. We used the yeast two-hybrid method to screen for proteases binding to the PKC-δ regulatory region in a cDNA library constructed from human heart tissue mRNA; this resulted in the identification of ADAM12. Then a plasmid carrying the cDNA of flag-tagged wild-type ADAM12 (WT-ADAM12) or its metalloprotease-domain deleted mutant (ΔMP-ADAM12) was stably transfected into HT1080/HB-AP cells, and the transfectants of WT-ADAM12 (W46 and W48) and ΔMP-ADAM12 (Δ53 and Δ57) were cloned. After the exposure to PE, the secretion of HB-EGF-AP into the culture medium was measured by detecting AP activity. Although the level of HB-EGF-AP expression was similar (Fig. 2*a*), W46 and W48 showed a 1.3–1.5-fold increase of AP activity in the culture medium after

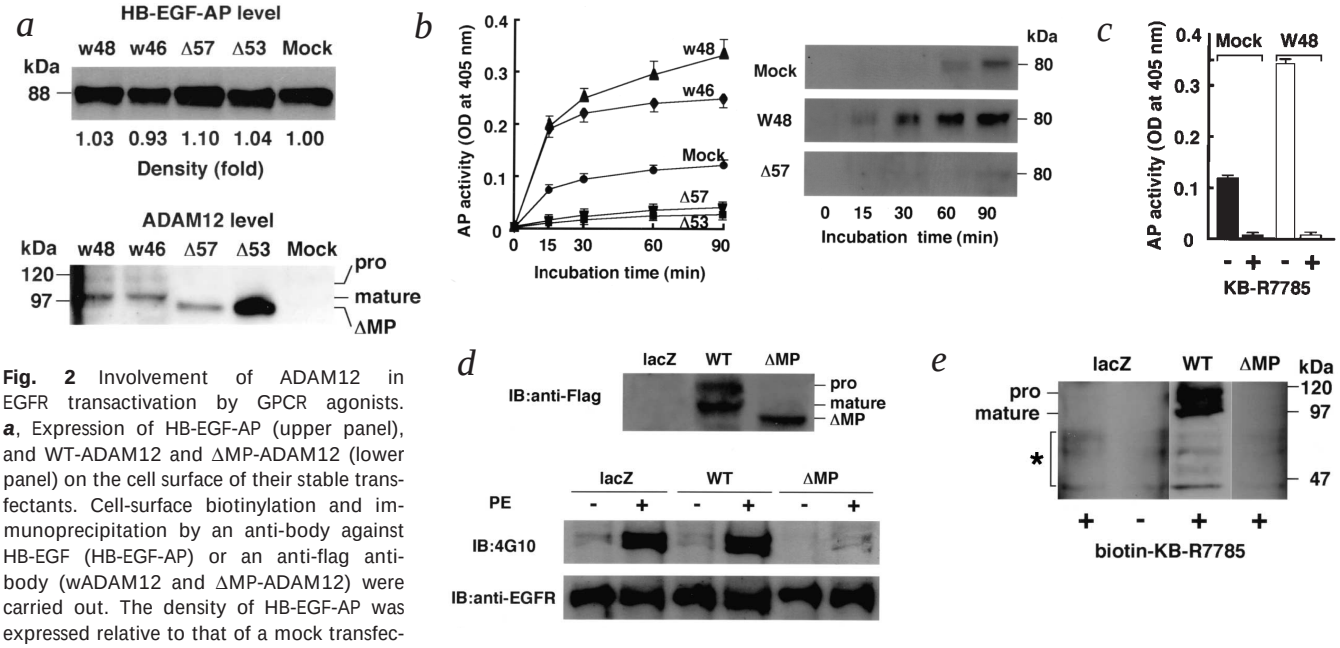


Fig. 2 Involvement of ADAM12 in EGFR transactivation by GPCR agonists. ***a***, Expression of HB-EGF-AP (upper panel), and WT-ADAM12 and ΔMP-ADAM12 (lower panel) on the cell surface of their stable transfectants. Cell-surface biotinylation and immunoprecipitation by an anti-body against HB-EGF (HB-EGF-AP) or an anti-flag antibody (wADAM12 and ΔMP-ADAM12) were carried out. The density of HB-EGF-AP was expressed relative to that of a mock transfectant. ***b***, PE-induced production of soluble HB-EGF-AP activities (left panel) and proteins (right panel) in conditioned media. Each point is the mean of quadruplicate measurements ± s.d. ▲, W48; ◆, W46; ●, mock; ▼, Δ57; ■, Δ53 cells. ***c***, KB-R7785 equally inhibited soluble HB-EGF-AP production induced by phenylephrine in a mock transfectant and W48 cells. ■, mock; □, W48. ***d***, ΔMP-ADAM12 expression blocked EGFR transactivation by phenylephrine in cultured cardiomyocytes. Expression of WT-ADAM12 and ΔMP-ADAM12 in adenovirus-infected cultured cardiomyocytes was shown by western blotting with an anti-flag antibody (upper panel). Immunoprecipitates with the polyclonal antibody against EGFR were immunoblotted with the monoclonal anti-phosphotyrosine antibody 4G10 (middle panel) or with anti-EGFR (lower panel). ***e***, Biotinylated KB-R7785 (biotin-KB-R7785) bound to WT-ADAM12, but not to ΔMP-ADAM12 in cardiomyocytes. *, nonspecific bands of avidin-HRP.

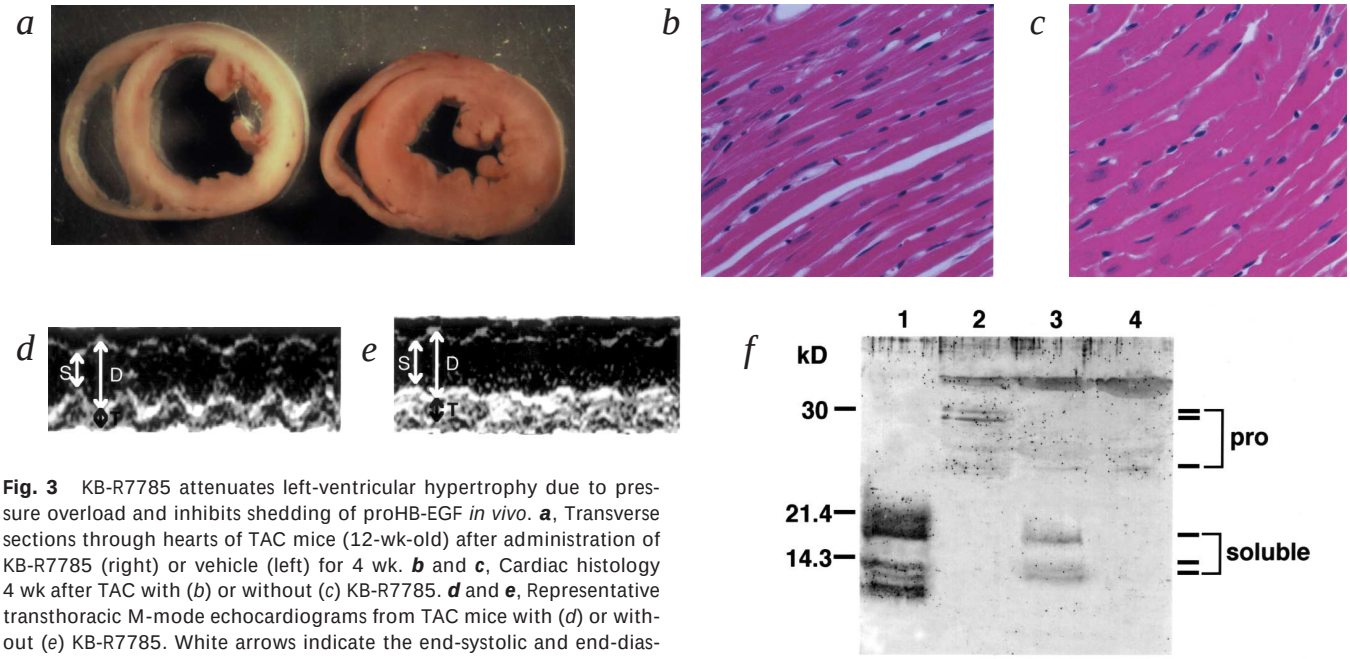


Fig. 3 KB-R7785 attenuates left-ventricular hypertrophy due to pressure overload and inhibits shedding of proHB-EGF *in vivo*. ***a***, Transverse sections through hearts of TAC mice (12-wk-old) after administration of KB-R7785 (right) or vehicle (left) for 4 wk. ***b*** and ***c***, Cardiac histology 4 wk after TAC with (*b*) or without (*c*) KB-R7785. ***d*** and ***e***, Representative transthoracic M-mode echocardiograms from TAC mice with (*d*) or without (*e*) KB-R7785. White arrows indicate the end-systolic and end-diastolic left-ventricular diameters (LVDd (D) and LVDs (S)). The black arrow shows posterior-wall thickness (PWT). ***f***, Detection of HB-EGF protein in mouse hearts by immunoblotting with anti-HB-EGF. Lanes: 1, recombinant soluble HB-EGF; 2, TAC mouse treated with KB-R7785; 3, TAC mouse without KB-R7785 treatment; 4, BL-6 mouse. The heterogeneous bands of soluble HB-EGF and proHB-EGF are derived from multiple N-terminal truncations and heterogeneous sugar chains[22].

90 minutes of PE treatment when compared with mock-transfected cells. In contrast, Δ53 and Δ57 showed 60–70% suppression of AP activity in the medium after PE treatment. AP activity was shown to correspond with the HB-EGF-AP level in the culture medium. A gradual increase of soluble HB-EGF-AP (80 kD) was seen with W48 cells, but was less apparent with Δ57 cells as compared with that of mock cells (Fig. 2*b*). KB-R7785 completely blocked the release of HB-EGF-AP from W48 cells (Fig. 2*c*).

To confirm that endogenous ADAM12 was responsible for the shedding of HB-EGF in cultured cardiomyocytes, we used an adenovirus vector bearing the cDNA of ΔMP-ADAM12 for the analysis of EGFR transactivation by PE. Expression of ΔMP-ADAM12 suppressed EGFR transactivation by PE, whereas overexpression of WT-ADAM12 slightly enhanced EGFR transactivation when compared with LacZ-overexpressing cardiomyocytes (Fig. 2*d*). Direct binding of KB-R7785 to ADAM12 was also confirmed. We transfected adenovirus WT-ADAM12 and ΔMP-ADAM12 to cardiomyocytes and the cells were incubated with biotinylated KB-R7785. Subsequent electrophoresis revealed that biotinylated KB-R7785 showed direct binding to WT-ADAM12, but not to ΔMP-ADAM12 (Fig. 2*e*). These data indicated that, at least in cardiomyocytes, ADAM12 is the specific enzyme of HB-EGF shedding and is a potential target of KB-R7785.

KB-R7785 attenuates pressure overload hypertrophy in mice

Next, we examined the effect of KB-R7785 on cardiac hypertrophy in response to pressure overload on the left ventricle. We subjected mice to thoracic aortic constriction (TAC), which produced a systolic pressure gradient of nearly 45 mmHg between the left and right carotid arteries (right, 90.3 ± 6.5 mmHg; left, 45.8 ± 2.6 mmHg). Pressure overload caused marked thickening of the left-ventricular posterior wall on echocardiography two and four weeks after TAC compared with sham-operated mice (Fig. 3*a*, *c* and *e*). The ratio of heart-weight to body-weight also increased by 69% over that in sham-operated mice at four weeks after TAC. Daily administration of KB-R7785 (100 mg/kg) significantly lessened the increase in left-ventricular wall thickness (2 and 4 wk after TAC) and heart:body-weight ratio (4 wk after TAC) (Tables 2 and 3). KB-R7785 significantly improved fractional shortening four weeks after TAC (Table 3). Both before and after TAC, blood pressure was similar in the mice with and without KB-R7785, suggesting that hemodynamic changes could not explain the beneficial effect of KB-R7785. Histological examination revealed that the reduction of wall thickness by KB-R7785 was due to a decrease of cardiomyocyte diameter, and neither fibrosis nor myofibril degradation was detected in both sham-operated and KB-R7785-treated mice (Fig. 3*a*, *b* and *d*). KB-R7785 also blocked the shedding of HB-EGF *in vivo* since proHB-EGF was detected by western-blot analysis, whereas soluble HB-EGF was predominant in the hearts of untreated mice (Fig. 3*f*).

KB-R7785 attenuates hypertrophy induced by PE or Ang II

We also examined the effect of KB-R7785 on cardiac hypertrophy induced by GPCR agonists (PE and Ang II) in mice with two different genetic backgrounds (C57BL/6J and FVB), since it was unclear whether cardiac hypertrophy was due to the pressure overload model directly mediated through GPCRs. Both PE and Ang II treatment increased heart:body-weight ratio more than 10%. Daily administration of KB-R7785 (100 mg/kg) significantly attenuated the increase of heart:body-weight ratio for both groups (Table 2). The administration of KB-R7785 did not affect blood pressure in either PE- or Ang II-treated mice, suggesting that hemodynamic changes could not explain the effect of KB-R7785. These results confirm our hypothesis that both GPCR-mediated HB-EGF processing and cardiac hypertrophy are blocked by KB-R7785 *in vivo* as well as *in vitro*.

Table 3 Echocardiographic measurements in mice with aortic banding

	0 week			2 weeks			4 weeks		
	Sham (n = 10)	TAC (n = 13)	TAC + KB (n = 10)	Sham (n = 10)	TAC (n = 9)	TAC + KB (n = 9)	Sham (n = 10)	TAC (n = 9)	TAC + KB (n = 9)
PWT (mm)	0.55 ± 0.27	0.54 ± 0.31	0.57 ± 0.29	0.66 ± 0.20	0.87 ± 0.30*	0.69 ± 0.30**	0.66 ± 0.20	1.02 ± 0.33*	0.74 ± 0.12**
EDD (mm)	3.05 ± 0.10	3.06 ± 0.10	3.06 ± 0.09	3.44 ± 0.08	3.05 ± 0.07	3.07 ± 0.15	3.27 ± 0.17	3.24 ± 0.08	3.20 ± 0.15
ESD (mm)	1.98 ± 0.10	1.96 ± 0.08	2.00 ± 0.14	2.23 ± 0.08	1.89 ± 0.08	2.04 ± 1.34	1.94 ± 0.14	2.38 ± 0.10*	2.00 ± 0.15**
FS (%)	35.3 ± 2.5	35.9 ± 2.1	35.4 ± 2.4	35.1 ± 1.4	38.2 ± 1.8	34.3 ± 2.5	40.6 ± 2.5	31.1 ± 1.8*	38.1 ± 2.2**

Echocardiographic measurements obtained from transthoracic M^mode tracings with and without KB-R7785 at 0, 2 and 4 weeks after TAC or sham opereations. PWT, posterior-wall thickness; EDD, end-diastolic diameter; ESD, end-systolic diameter; FS, fractional shortening. Values are means ± s.e.m. *, $P < 0.05$ versus sham; **, $P < 0.05$ versus TAC.

Discussion

We showed that stimulation of cultured rat neonatal cardiomyocytes with PE, Ang II or ET-1 induced the transactivation of EGFR and subsequent increases in protein synthesis after shedding of HB-EGF from the cell surface, based on the complete abrogation of these changes by a neutralizing antibody specific for HB-EGF or a metalloproteinase inhibitor KB-R7785. ADAM12 was identified as the protease causing the shedding of HB-EGF. ADAM12 was also shown to be the target protease for KB-R7785 because of direct binding to this compound, although KB-R7785 may have other effects. The fact that the dominant-negative form of ADAM12 completely abolished the EGFR transactivation induced by a GPCR agonist in cardiomyocytes indicates that, in the heart, ADAM12 is solely involved in this pathway and is also the potential target of KB-R7785. However, because we did not test the dominant-negative forms of other ADAMs or MMPs, we could not completely exclude the possibility of their involvement in the EGFR transactivation induced by a GPCR agonist. Based on the finding that Moss *et al.* successfully cloned a TNF-α shedding enzyme (TACE) from the spleen by specific binding to a metalloproteinase inhibitor (GW9277)[19], and despite the broad spectrum of GW9277 *in vitro*, we suggest that KB-R7785 specifically interacted mainly with ADAM12 in heart and blocked HB-EGF shedding. The main signal transduction pathway leading to cardiac hypertrophy seems likely to be mediated by ADAM12 shedding of HB-EGF in cardiomyocytes.

In our aortic-banding model, we showed that KB-R7785 not only attenuated cardiac hypertrophy resulting from pressure overload, but also improved cardiac function. These results confirm recent reports suggesting the effectiveness of MMP inhibitors for heart failure[15]. Because it is unclear that the cardiac hypertrophy in the aortic-banding model is directly mediated through GPCRs, we further tested whether KB-R7785 attenuates cardiac hypertrophy induced by either PE or Ang II infusion. The results indicate that the metalloproteinase inhibitor blocks cardiac hypertrophy directly mediated through GPCRs. Together, our observations indicate that certain metalloproteinase inhibitors may be clinically effective for delaying the progression of heart failure following cardiac hypertrophy, or even for treating cardiac impairment that develops before the uncompensated phase of cardiac hypertrophy.

Thus, we conclude that cellular signaling in cardiomyocytes following treatment with GPCR agonists is dependent upon EGFR transactivation triggered by ADAM12-mediated cleavage of HB-EGF. This is the first demonstration that inhibition of HB-EGF shedding can lessen cardiac hypertrophy both *in vivo* and *in vitro*, and suggests that both HB-EGF and ADAM12 are potential targets for the treatment of cardiac hypertrophy.

Methods

Materials. PE, Ang II and ET-1 were purchased from Sigma. A mouse monoclonal anti-phosphotyrosine antibody 4G10, and a sheep polyclonal antibody to EGFR were from Upstate Biotechnology (Lake Placid, New York). A goat polyclonal antibody against EGFR was from Santa Cruz Biotechnology (Santa Cruz, California). The HB-EGF neutralizing polyclonal antibody #19 was from J.A. Abraham. KB-R7785 ([4-(*N*-hydroxyamino)-2R-isobutyl-3S-methylsuccinyl]-L-phenylglycine-*N*-methylamide), OSU7-6 and OSU9-6 were synthesized by Organon Japan (Osaka, Japan). Adenovirus carrying genes encoding LacZ, WT-ADAM12 and ΔMP-ADAM12 were prepared using adenovirus expression vector kit (Takara Biomedicals, Tokyo, Japan).

Cell culture. Primary cultures of neonatal rat cardiomyocytes were prepared as described[3]. Cells from hearts of 1–2-day-old Wistar rats were seeded onto 60-mm collagen-coated dishes (2×10^6 cells per dish) or 96-well plates in MEM medium supplemented with 10% FCS. After incubation for 18 h, the medium was replaced with MEM plus insulin, transferin and sodium selenite 24 h before experiments.

EGFR immunoprecipitation in cardiomyocytes. Cultured cardiomyocytes were exposed to the agents (1×10^{-8} M HB-EGF, 1×10^{-5} M PE, 10^{-8} M Ang II, and 1×10^{-7}M ET-1) for 5 min except in Fig. 1 after pretreatment for 30 min with or without reagents (1 μM of KB-R7785 and 10 μg/ml of HB-EGF neutralizing antibody). Cells were lysed by incubation for 20 min at 4 °C in a buffer (50 mM Tris-HCl, pH 7.3; 150 mM NaCl; 2 mM EDTA; 0.5% sodium fluoride; 10 mM sodium pyrophosphate; 0.5 mM Na_3VO_4, 100 μg/ml phenylmethylsulfonyl fluoride; 2 μg/ml aprotinin; protease inhibitor cocktail; and 1 % Nonidet P-40). Immunoblotting analyses using 4G10 or antibody against EGFR were as described[16]. Cultured cardiomyocytes infected with adenovirus vectors (at multiplicity of infection 50) for 24 h were also subjected to EGFR immunoprecipitation analysis.

Incorporation of [^{3}H]leucine. Protein synthesis in cardiomyocytes was evaluated by incorporation of [^{3}H]leucine into cells. Following serum withdrawal, myocytes were exposed to compounds in MEM medium for 18 h, incubated with 1 μCi/ml [^{3}H]leucine for 12 h and washed once with PBS. The cells were attached to glass filter mats by a microharvester. Radioactivity was measured by a liquid scintillation counter (Wallac β-plate, Finland).

Yeast two-hybrid screening and assay. A human heart cDNA library (Clontech Japan, Tokyo, Japan) were used for large-scaled transformation of yeast cells (CG1945) carrying the pPKC-δ RD bait plasmid (gift from Y. Ono). The interacting cDNA clones were selected by growth on plates lacking histidine. Histidine-positive colonies were screened for LacZ expression by a colony lift method according to the manufacturer's instructions.

HB-EGF-AP shedding assay. Plasmids, pcDNA3.1 (Invitrogen, Carlsbad, California), human WT-ADAM12-flag/pcDNA3.1 and human ΔMP-ADAM12-flag/pcDNA3.1 were introduced into HT1080/HB-AP cells using lipofectamine (Gibco BRL, Rockville, Maryland). Stable transfectants were seeded in 24-well plates (1×10^5 cells per well) and then incubated for 24 h. The cells were incubated for an indicated time at 37 °C with 1×10^{-5} M PE. 50-μl aliquots of the conditioned media were transferred to 96-well plates, and AP activity was measured as described[16].

Transverse aortic banding. Pressure-overload cardiac hypertrophy was induced by transverse aortic banding in 8-wk-old male C57BL/6J mice (20–25 g). Suture was tied twice around a 27-gauge needle, which was positioned adjacent to the aorta between the right and left carotid arteries, and was removed after placement of the ligature. This yielded an outer diameter of approximately 0.3 mm (60–80% constriction). Mice were treated i.p. with or without KB-R7785 (100 mg/kg/d) for 4 wk.

Pharmacological induction of hypertrophy. 8-wk-old C57BL/6J male mice or 7-wk-old FVB male mice were treated with PE (30 mg/kg/day), Ang II (200 ng/kg/min) or vehicle by osmotic minipump (Alzet, California) to induce cardiac hypertrophy as reported[20,21]. In the KB-R7785 treatment group, KB-R7785 was administered daily (i.p.) during PE or Ang II treatment. After systemic blood pressure and heart rate were measured (BP-98A, Softron, Tokyo), mice with PE treatment were killed at 7 d after implantation of pumps and mice with Ang II treatment were killed at 14 d.

Echocardiography. Mice were anesthetized by pentobarbital (50 mg/kg) and the extent of cardiac hypertrophy was assessed by 15-MHz pulsed-wave Doppler echocardiography (Philips, SONOS5500, the Netherlands). We measured posterior-wall thickness (PWT), systolic left-ventricular diameter (LVDs), diastolic left-ventricular diameter (LVDd) and fractional shortening (FS).

HB-EGF immunoblotting. Mouse hearts were homogenized in 20 mM Tris-HCl (pH 7.2), 1.5 M NaCl, 1% Triton X-100, 1 mM EDTA (β-amidinophenyl) methanesulfonyl fluoride and 20 μg/ml aprotinin. After centrifugation at 15,000*g* for 10 min at 4 °C, supernatant aliquots (10 μg protein/aliquot) were fractionated by SDS–PAGE and immunoblotted using the polyclonal HB-EGF antibody #2998. HB-EGF was detected by alkaline phosphatase-conjugated secondary antibody.

Data analysis. Numerical data are reported as mean ± s.e.m. Data were analyzed statistically by Student's *t*-test.

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1. Katz, A.M. Cardiomyopathy of overload. A major determinant of prognosis in congestive heart failure. *N. Engl. J. Med.* **322**, 100–110 (1990).
2. Levy, D., Garrison, R.J., Savage, D.D., Kannel, W.B. & Castelli, W.P. Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. *N. Engl. J. Med* **322**, 1561–1566 (1990).
3. Simpson, P., McGrath, A. & Savion, S. Myocyte hypertrophy in neonatal rat heart cultures and its regulation by serum and by catecholamines. *Circ. Res.* **51**, 787–801 (1982).
4. Ito, H. *et al.* Endothelin-1 induces hypertrophy with enhanced expression of muscle-specific genes in cultured neonatal rat cardiomyocytes. *Circ. Res.* **69**, 209–215 (1991).
5. Sadoshima, J., Xu, Y., Slayter, H.S. & Izumo, S. Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. *Cell* **75**, 977–984 (1993).
6. Schmieder, R.E., Martus, P. & Klingbeil, A. Reversal of left ventricular hypertrophy in essential hypertension. A meta-analysis of randomized double-blind studies. *JAMA* **275**, 1507–1513 (1996).
7. Effects of enalapril on mortality in severe congestive heart failure. Results of the Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS). The CONSENSUS Trial Study Group. *N. Engl. J. Med*. **316**, 1429–1435 (1987).
8. Chien, K.R. *et al.* Transcriptional regulation during cardiac growth and development. *Annu. Rev. Physiol* . **55**, 77–95 (1993).
9. Sadoshima, J. & Izumo, S. The cellular and molecular response of cardiac myocytes to mechanical stress. *Annu. Rev. Physiol*. **59**, 551–571 (1997).
10. Daub, H., Weiss, F.U., Wallasch, C. & Ullrich, A. Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature* **379**, 557–560 (1996).
11. Tsai, W., Morielli, A.D. & Peralta, E.G. The m1 muscarinic acetylcholine receptor transactivates the EGF receptor to modulate ion channel activity. *EMBO J.* **16**, 4597–4605 (1997).
12. Zwick, E. *et al.* Critical role of calcium-dependent epidermal growth factor receptor transactivation in PC12 cell membrane depolarization and bradykinin signaling. *J. Biol. Chem.* **272**, 24767–24770 (1997).
13. Eguchi, S. *et al.* Calcium-dependent epidermal growth factor receptor transactivation mediates the angiotensin II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. *J. Biol. Chem.* **273**, 8890–8896 (1998).
14. Prenzel, N. *et al.* EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* **402**, 884–888 (1999).
15. Spinale, F.G. *et al.* Matrix metalloproteinase inhibition during the development of congestive heart failure: effects on left ventricular dimensions and function. *Circ. Res.* **85**, 364–376 (1999).
16. Tokumaru, S. *et al.* Ectodomain shedding of epidermal growth factor receptor ligands is required for keratinocyte migration in cutaneous wound healing. *J. Cell Biol.* **151**, 209–220 (2000)
17. Goishi, K.*et al.* Phorbol ester induces the rapid processing of cell surface heparin-binding EGF-like growth factor: Conversion from juxtacrine to paracrine growth factor activity. *Mol. Biol. Cell* **6**, 967–980 (1995).
18. Izumi, Y. *et al.* A metalloprotease-disintegrin, MDC9/meltrin-γ/ADAM9 and PKCδ are involved in TPA-induced ectodomain shedding of membrane-anchored heparin-binding EGF-like growth factor. *EMBO J.* **17**, 7260–7272 (1998).
19. Moss, M.L. *et al.* Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-α. Nature **385**, 733–736 (1997).
20. Friddle, C.J., Koga, T., Rubin, E.M. & Bristow, J. Expression profiling reveals distinct sets of genes altered during induction and regression of cardiac hypertrophy. *Proc. Natl. Acad. Sci. USA* **97**, 6745–6750 (2000).
21. Saadane, N., Alpert, L. & Chalifour, L.E. Expression of immediate early genes, GATA-4, and Nkx-2.5 in adrenergic-induced cardiac hypertrophy and during regression in adult mice. *Br. J. Pharmacol*. **127**, 1165–1176 (1999).
22. Higashiyama, S., Lau, K., Besner, G., Abraham, J. A. & Klagsbrun, M. Structure of heparin-binding EGF-like growth factor: Multiple forms, primary structure, and glycosylation of the mature protein. *J. Biol. Chem.* **267**, 6205–6212 (1992).