Kobe J. Med. Sci., Vol. 58, No. 1, pp. E12-E18, 2012

Physiological Relevance of Hydrolysis of Atrial Natriuretic Peptide by Endothelin-Converting Enzyme-1

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Received 17 January 2012 / Accepted 18 January 2012

Key words: Endothelin-converting enzyme-1, Endothelin-1, Atrial natriuretic peptide, Neutral endopeptidase 24.11

Endothelin-converting enzyme-1 (ECE-1) is a membrane-bound metalloprotease that cleaves biologically inactive big endothelin-1 (ET-1) into active ET-1. ET-1 is involved in the cardiovascular homeostasis and the development of cardiovascular diseases including pulmonary arterial hypertension and heart failure. Atrial natriuretic peptide (ANP) is an endogenous hormone that is released from the heart in response to myocardial stretch and overload. ANP was shown to be hydrolyzed by neutral endopeptidase 24.11 (NEP) which shares important structural features with ECE-1. Previous *in vitro* studies using recombinant soluble ECE-1 suggested that ECE-1 cleaved several biologically active peptides including ANP in addition to big ET-1. However, physiological relevance of ANP-degrading activity by ECE-1 has stayed unclear. Here, we aimed to investigate whether endogenous ECE-1 is able to hydrolyze ANP using live-cell based assay and *ECE-1*–deficient mice. Chinese hamster ovary (CHO) cells, which lack detectable levels of ECE activity, degraded ANP in the medium efficiently when transfected with *ECE-1* cDNA. ANP peptide contents in the E14-15 embryos were significantly higher in *ECE-1*$^{+/-}$ mice compared with *ECE-1*$^{+/+}$ mice. These observations strongly suggest that ECE-1 is involved in the physiological degradation of ANP *in vivo*. Thus, pharmacological inhibition of ECE-1 may provide a novel strategy to treat various cardiovascular diseases by suppressing and potentiating the ET and ANP pathway, respectively.

Endothelin-1 (ET-1) is a multifunctional peptide that is produced from a biologically inactive precursor, big endothelin-1, via a proteolytic processing (8). This activation of ET-1 is catalyzed by a type II membrane-bound metalloprotease termed endothelin-converting enzyme-1 (ECE-1) (5, 18). A series of gene targeting studies revealed that ECE-1 is a bona fide activating protease for big ET-1 and big ET-3 *in vivo* in the developmental stage (2, 3, 6, 11, 19, 20).

Atrial natriuretic peptide (ANP) is a cardiac hormone which is upregulated in heart failure and counteracts the deleterious effects by promoting vasodilatation, natriuresis, and diuresis (12). Exogenous ANP is widely used for the management of acutely decompensated heart failure. ANP has been shown to be metabolized by endopeptidase 24.11 (NEP), which shares structural domains with ECE-1 (15).

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In vitro studies using recombinant soluble ECE-1 suggested that ECE-1 possesses a broad substrate specificity and is potentially involved in the metabolism of many biologically active peptides (7). Recombinant ECE-1 cleaves ANP at multiple sites. However, physiological relevance of this ANP-metabolizing activity by ECE-1 remains elusive. In view of the potential therapeutic intervention, it is important to determine whether endogenous ECE-1 can cleave biologically active peptides other than big endothelins (4).

Here, we aimed to investigate whether ECE-1 presents physiological ability in hydrolyzing ANP *in vivo*. The results obtained from the live cell-based assay and *ECE-1*–gene deficient mice indicated that ECE-1 may degrade ANP in a physiological setting.

MATERIALS AND METHODS

Animals

Mice deficient for the ECE-1 gene were generated by homologous recombination as described previously (19, 20). We backcrossed the founder mice against 129/SvEv mice more than eight times. All of the procedures were conducted according to the Kobe University Graduate School of Medicine Guidelines for Animal Experiments.

Cell culture and Transfection

CHO-K1 cells were cultured as previously described (5, 18). The coding region from human ECE-1 was subcloned into pME18Sf– vector (5, 18). This construct was transiently transfected into CHO-K1 cells by Lipofectamine (Invitrogen). Twelve hours after transfection, cells were refed with CHO-SFM medium (Gibco) containing 1 μM of ANP with or without 100 μM phosphoramidon. The medium was conditioned for the designated time and applied on Fluorescent ANP immunoassay kits (Phoenix Pharmaceuticals).

Gene Expression Analysis

Real-time RT-PCR was performed on an ABI PRISM 7500 system (Applied Biosystems) with a One-Step SYBR PrimeScript RT-PCR kit (Takara) and Superscript III Platinum One-Step qRT-PCR System (Invitrogen). Hypoxanthine-guanine phosphoribosyltransferase-1 (HPRT-1) expression was used as an internal control.

Tissue and Serum Levels of ANP

Tissue and whole embryos were homogenized and centrifuged at 15,000*g* for 30 minutes at 4 degree. The supernatants were applied through Sep-Pak C-18 cartridges (Waters). These samples were dried, lyophilized, and resuspended in assay buffer. The samples and serum were applied on Fluorescent ANP immunoassay kits (Phoenix Pharmaceuticals), and analyzed.

Statistical Analysis

Data are expressed as the mean ± s.e.m. Statistical significance was tested with an unpaired two-tailed Student's t-test and analysis of variance (ANOVA). The difference was considered to be significant if $p<0.05$.

RESULTS

In order to examine whether ECE-1 can hydrolyze ANP in a physiological context, we firstly used transfected CHO cells. Previously, we have shown that CHO cells lack detectable levels of endogenous ECE activity (5, 18). We transiently transfected CHO cells with an ECE-1 expression construct. Synthetic ANP was added in the medium and the concentration of ANP in the medium was determined by enzyme immunoassay. As shown in Fig. 1, concentration of ANP in the medium of CHO/ECE-1 cells was significantly lower than those

of parental CHO cells. Moreover, this effect was inhibited by phosphoramidon, an ECE/NEP inhibitor. Taken together, these observations indicated that transfection of *ECE-1* cDNA conferred these cells the ability to hydrolyze exogenously added ANP.

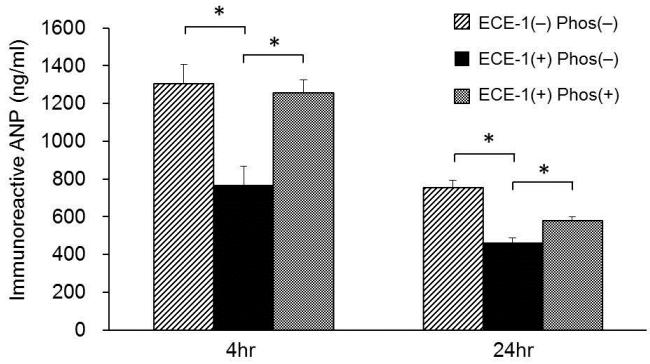

Figure 1. Hydrolysis of ANP by CHO cells transfected with human ECE-1 cDNA. CHO cells were transfected transiently with human ECE-1 cDNA and ANP was added in the medium in the absence of presence of 100 μM phosphoramidon. n=3 each time point; *p<0.05

Since all of *ECE-1*$^{-/-}$ embryos die *in utero* before E13.5, we compared the phenotype between *ECE-1*$^{+/-}$ mice and *ECE-1*$^{+/+}$ mice. In *ECE-1*$^{+/-}$ mice, the expression of *ECE-1* mRNA was approximately half of *ECE-1*$^{+/+}$ mice (Fig. 2). Western blot analysis was performed using membrane preparations from the heart and E14-15 whole embryos. In *ECE-1*$^{+/-}$ mice, the intensity of the 126 kDa band corresponding to ECE-1 protein was approximately half compared with those of *ECE-1*$^{+/+}$ mice (data not shown). The mRNA expression of NEP in the heart, lung, kidney, and whole embryos was not different between *ECE-1*$^{+/-}$ mice and *ECE-1*$^{+/+}$ mice (Fig. 3).

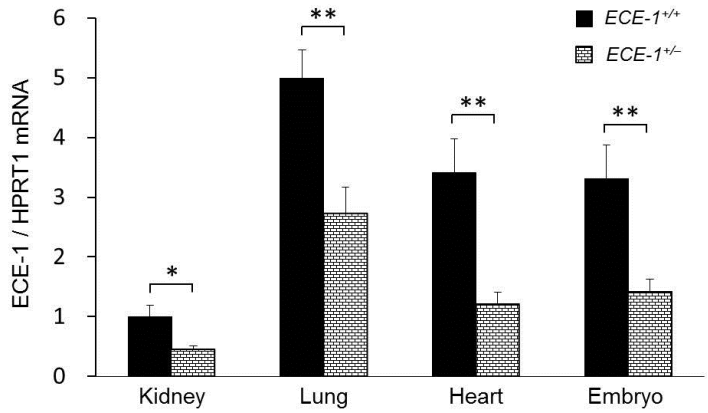

Figure 2. Expression of ECE-1 mRNA in *ECE-1*$^{+/-}$ mice. Transcriptional levels of ECE-1 were assessed by real-time PCR. The expression of ECE-1 mRNA was approximately half of *ECE-1*$^{+/+}$ mice. n=6 each group; *p<0.05, **p<0.01

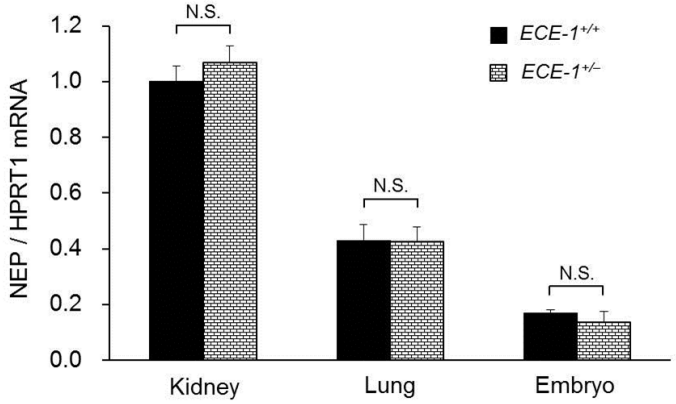

Figure 3. Expression of NEP mRNA in *ECE-1*$^{+/-}$ mice. Transcriptional levels of NEP were assessed by real-time PCR. The mRNA expression of NEP in the lung, kidney, and whole embryos was not different between *ECE-1*$^{+/-}$ mice and *ECE-1*$^{+/+}$ mice. n=6 each group; N.S.: not significant.

The expression of ANP mRNA in the heart and whole embryos were not different between *ECE-1*$^{+/-}$ and *ECE-1*$^{+/+}$ mice (Fig. 4). On the other hand, ANP peptide contents in the embryos were significantly higher in *ECE-1*$^{+/-}$ mice (Fig. 5). Serum ANP levels were increased in *ECE-1*$^{+/-}$ mice compared with *ECE-1*$^{+/+}$ mice as well, although the difference was not statistically significant (Fig. 5). These findings suggest that ECE-1 hydrolyzed ANP *in vivo*.

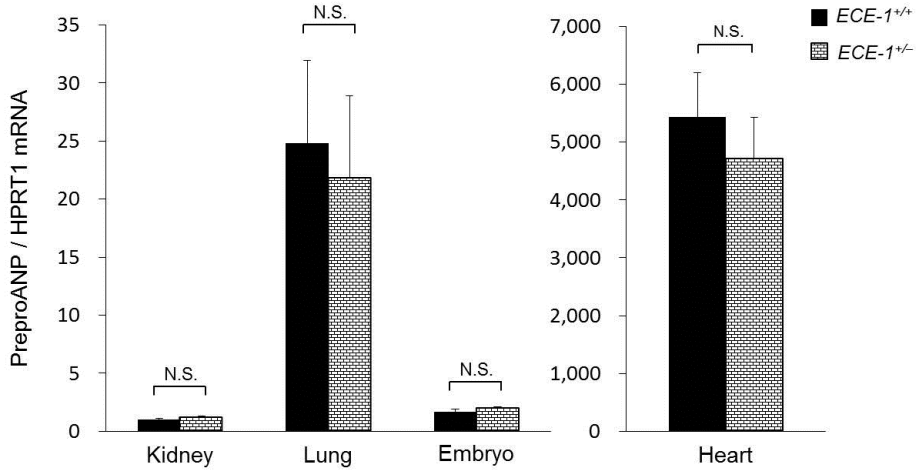

Figure 4. Expression of preproANP mRNA in *ECE-1*$^{+/-}$ mice. Transcriptional levels of preproANP were assessed by real-time PCR. The expression of ANP mRNA in the heart and whole embryos were not different between *ECE-1*$^{+/-}$ and *ECE-1*$^{+/+}$ mice. n=6 each group; N.S.: not significant.

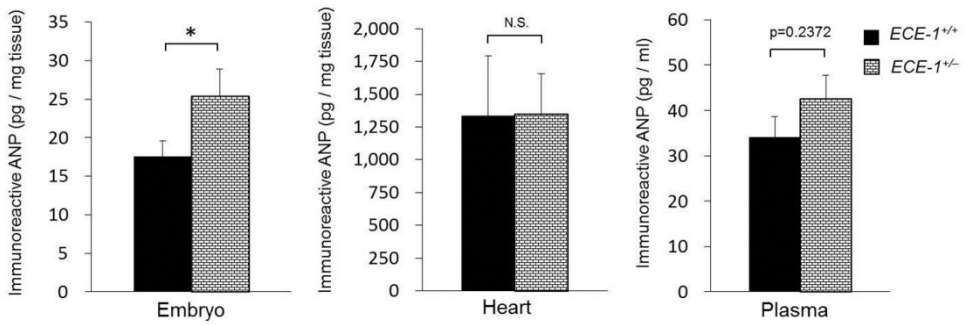

Figure 5. ANP peptide levels in *ECE-1*$^{+/-}$ mice. ANP peptide contents in the embryos were significantly higher in *ECE-1*$^{+/-}$ mice. Serum ANP levels were increased in *ECE-1*$^{+/-}$ mice compared with *ECE-1*$^{+/+}$ mice. n=6 each group; *p<0.05, N.S.: not significant.

DISCUSSION

Studies of ECE-1 peptidase activity have been hampered by the limited availability of the pure enzyme. Therefore, an extensive examination of ECE-1 activity and substrate specificity was performed by using purified recombinant soluble ECE-1 which consists of the extracellular domain of human ECE-1 and signal sequence of human alkaline phosphatase (1, 7). Although recombinant modified ECE-1 was shown to hydrolyze ANP at multiple sites, physiological relevance of this activity was unclear. In the present study, our live cell-based assay showed that full length ECE-1 expressed in CHO cells hydrolyzed ANP. We also demonstrated that genetic reduction of ECE-1 expression in mice contributed to the increase in ANP levels without affecting its transcriptional levels. These observations support that ECE-1 cleaves ANP in a physiological context.

All of *ECE-1*$^{-/-}$ embryos die *in utero* before E13.5 on a 129SvEv background (20). Premorbid *ECE-1*$^{-/-}$ embryos exhibited peripheral vascular dilatation and pericardial effusion which is consistent with heart failure. Since the expression of ANP is increased in heart failure, it is highly likely that *ECE-1*$^{-/-}$ embryos show increased ANP expression. Nevertheless, the early embryonic lethality and the phenotype of heart failure have prevented us from analyzing *ECE-1*$^{-/-}$ mice. Therefore, in the present study, we compared *ECE*$^{+/-}$ and *ECE-1*$^{+/+}$ mice.

Previously, we have reported that heterozygous deletion of the ECE-1 gene did not affect ET-1 levels in serum and the heart of *ECE-1*$^{+/-}$ mice (13). In contrast, we report here that ANP levels in serum and embryos of *ECE-1*$^{+/-}$ mice were increased. These data suggest that half expression of ECE-1 gene has a rate-limiting effect for ANP hydrolysis, but not for big ET-1 processing *in vivo*. This is in agreement with the *in vitro* observations showing that ANP is not hydrolyzed efficiently by ECE-1 compared with big ET-1, as k_{cat}/K_m of ANP was estimated to be 250-fold lower than that of big ET-1 (7). Taken together, these observations strongly suggest that ECE-1, in addition to NEP, is a potential site for clearing circulating ANP *in vivo*.

ECE-1 is a zinc metalloprotease and possesses structural similarity with NEP. ECE-1 and NEP have the greater sequence identity in their COOH-terminal regions, especially in residues involved in zinc binding and catalysis, indicating a similar catalytic mechanism (5, 18). NEP has been extensively characterized and shown to have a broad substrate specificity

(15). In particular, ANP is an excellent substrate for NEP. In the present study, we did not detect the expression of NEP in CHO-K1 cells. We also confirmed that the expression of NEP in heart, lung and kidney of *ECE-1*$^{+/-}$ mice did not differ from those of *ECE-1*$^{+/+}$ mice. Therefore, we concluded that NEP is not involved in the increase of ANP levels in our current setting.

Circulating ET-1 and big ET-1 levels are increased in heart failure and are prognostic indicators of survival in patients with heart failure (14, 16, 17). Therefore, strategies to antagonize the effects of ET-1 were expected to be a rational approach for treating heart failure. In fact, several endothelin receptor antagonists (ERAs) underwent evaluation in the treatment of heart failure (9, 10). Despite favorable hemodynamic and neurohumoral effects obtained with ERAs in animal studies, clinical trials testing ERAs for heart failure failed to demonstrate improvement in patient outcomes. In the present study, we demonstrated the physiological relevance of the degradation of ANP by ECE-1. ECE-1 inhibition could thus potentiate the ANP system by preventing the proteolytic inactivation of ANP. ANP has direct vasodilator effects, antagonizes the renin-angiotensin system, inhibits endothelin secretion, and reduces sympathetic activity (12). Given the favorable cardioprotective actions of ANP, ECE-1 inhibition may be a potential strategy for the treatment of heart failure by potentiating the ANP system in addition to the suppression of the ET system. Considering this, it would be important to determine whether ECE-1 inhibition can improve pathological condition in disease models.

ACKNOWLEDGEMENTS

This study was supported in part by a Grant-in-Aid for Scientific Research (C) to N.E. from the Japan Society for the Promotion of Science.

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