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# Endothelin-converting enzyme-1 gene ablation attenuates pulmonary fibrosis via CGRP-cAMP/EPAC1 pathway

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（課程博士関係）

学 位 論 文 の 内 容 要 旨

# Endothelin-converting enzyme-1 gene ablation attenuates pulmonary fibrosis via CGRP–cAMP/EPAC1 pathway

エンドセリン変換酵素-1 ノックアウトマウスにおけるカルシトニン遺伝子関連ペプチド-cAMP/EPAC1 経路を介した 肺線維症抑制効果の検討

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## INTRODUCTION

Endothelin-1 (ET-1) and endothelin-converting enzyme-1 (ECE-1) were involved in pulmonary fibrosis. Clinical trials with endothelin receptor antagonists had unmet results which opened avenues to other strategies opposing ET-1 pathway, such as ECE-1 suppression. ET-1 is a 21 amino-acid peptide and produced by endothelia, smooth muscle cells, airway epithelia, and alveolar macrophages of lung. ET-1 is generated from big ET-1 after cleavage by ECE-1. Previous studies showed that ECE-1 hydrolizes and biologically inactivates bradykinin, atrial natriuretic peptides (ANP), beta amyloid, and calcitonin gene-related peptides (CGRP) *in vitro*. This *in vitro* studies were confirmed by *in vivo* on ECE-1 knock-out mice, which had higher beta-amyloid and ANP levels physiologically. However, the pathological relevance of ECE-1 activity needs further elucidation.

Complete suppression of ECE-1 in ECE-1 homozygous knock-out mice (ECE-$1^{-/-}$) results in embryonic lethality or premature death because of craniofacial and cardiovascular defects. In contrast, ECE-1 heterozygous knock-out (ECE-$1^{+/-}$) mice having half ECE-1 activity compared to that of wild type littermates (ECE-$1^{+/+}$), survive and develop normally. In view of the above, we studied whether ECE-1 genetic suppression would protect against pulmonary fibrosis induced by bleomycin in ECE-1 heterozygous knock-out mice (ECE-$1^{+/-}$) and further investigated the impact of reduced ECE-1 activity on its related peptides.

## MATERIALS AND METHODS

ECE-$1^{+/-}$ and ECE-$1^{+/+}$ mice under 129SvEv background were used in this experiments (male, 8- to 10-week-old). Pulmonary fibrosis was induced by bleomycin exposure. Mice were anesthetized with 50 mg/kg ip pentobarbital, trachea was exposed by 26G needle insertion. Bleomycin (10 μg/g body weight) or 0.9% saline vehicle in 60 μl volume were instilled into the lung. Mice were allowed to recover from surgery, and then sacrificed at indicated times (7, 14, 21 and 28 days).

Histologic spesimens were used to evaluate pulmonary inflammation and fibrosis. Sections were stained with HE and inflammation was scored using a semi-quantitative scale of 0-3. Fibrosis was determined by Masson trichrome staining and analyzed using Image J program. Macrophages and fibroblasts were detected using immunohistochemistry and immunofluorescence. Whole fields from eight sections per lung in each mice were assessed. Lung collagen content was measured with Sircol assay. Bronchoalveolar lavage fluid was performed for protein quantification using Bradford methods and TNF-α immunoassay.

Lung ECE-1 protein level was measured with western blot. Lung ECE-1 activity was determined from lung membrane fraction. Lung ET-1, CGRP, bradykinin, ANP and cyclic AMP level was measured with enzyme immunoassay. Gene expression was quantified with

real-time qPCR from cDNA-derived reverse transciptase of total RNA. Specific primers for ECE-1, CGRP, TGF-β, CTGF, Col1A1, Col3A1, CD68, Ym1, Arg1, MCP1, RANTES, GAPDH and HPRT-1 were applied. Protein level for S100A4, TGF-β, CRLR, RAMP1, EPAC1, PKAα, GAPDH and β-actin was quantified with western blot. Immunofluorescence were performed for CRLR/RAMP1 and macrophages colocalisation.

For statistical analysis, multiple comparisons were analyzed with ANOVA and Fisher's LSD tests. *P* value of <0.05 was considered statistically significant.

## RESULTS

Lung inflammation with minimal fibrosis occurred on days 7 and 14. Lung fibrosis appeared on days 21 and 28. ECE-$1^{+/-}$ mice had less fibrosis area, lower collagen, fewer fibroblasts, and α-SMA (+) cells. Reduced expressions of TGF-β, CTGF and collagen were also found in ECE-$1^{+/-}$ mice. Fibrosis in the bleomycin animal model is preceded by inflammation. ECE-$1^{+/-}$ mice had lower inflammation score, BALF protein and TNF-α on day 14 following bleomycin instillation.

Given the attenuated inflammation and fibrosis in ECE-$1^{+/-}$ mice, we assessed ECE-1 system in both phases. Lung ECE-1 mRNA, protein and enzyme activity were significantly lower in ECE-$1^{+/-}$ mice, in both phases. We further studied the impact of ECE-1 activity on its related peptides in the lung. During inflammation, ET-1 expression was significantly increased in ECE-$1^{+/-}$ mice, in contrast to a constant expression in ECE-$1^{+/+}$ mice, but the differences was not significant. In fibrosis, ET-1 expression was significantly higher in ECE-$1^{+/+}$ mice. ECE-$1^{+/-}$ mice had significantly higher CGRP levels during inflammation and fibrosis. Bradykinin and ANP levels were not significantly different in both phases.

Because of increased CGRP by reduced ECE-1 activity, we investigated CGRP pathway. CGRP mRNA expression was increased during inflammation phase and no significant differences between both genotypes. CRLR co-expressed with RAMP1 forms active CGRP receptor. Western blot analysis showed more CRLR glycosylation and less RAMP1 dimerisation irrespective of genotypes in inflammation phase, indicating CRLR glycosylation due to RAMP1 binding which confirms that CRLR/RAMP1 is functional. CGRP signaling via CRLR/RAMP1 activates a cAMP pathway. Intracellular cAMP action is mediated through PKA and EPAC. Intracellular cAMP, EPAC1 and PKA was reduced in ECE-$1^{+/+}$ mice in inflammation phase. The cAMP pathway remained unaltered in ECE-$1^{+/-}$ mice.

Recent studies proposed the role of M2 macrophages in regulating pulmonary fibrosis. M2 macrophages was less accumulated in ECE-$1^{+/-}$ mice, suggesting restricted M2-facilitated transition from lung inflammation to fibrosis in ECE-$1^{+/-}$ mice. M2 macrophages in control



mice did not express CGRP receptors. In contrast, M2 macrophages expressed both CRLR and RAMP1 in inflammation phase, confirming that CGRP acted through M2 macrophages and may modulate its function during lung inflammation to fibrosis transition.

## DISCUSSION

Our study showed that ECE-1 genetic suppression prevented bleomycin-induced pulmonary fibrosis. A previous *in vitro* study showed that, in the presence of ECE-1, CGRP formed a complex with CRLR/RAMP1 in the cell membrane, was internalized, and degraded by ECE-1. Strengthening it, our study exposed that ECE-1 genetic suppression may have diminished this process, thereby promoting CGRP signaling and subsequently activating cAMP/EPAC1 and cAMP/PKA pathways. It indicates that CGRP-induced cAMP pathway activation reduces inflammation and fibrosis.

We identified that attenuated lung inflammation by ECE-1 suppression was not attributed to reduced ET-1. Our study confirms that ET-1 does not play a role during transition from inflammation to fibrosis; rather it was upregulated due to fibrosis thereafter. Although lung CGRP levels were significantly increased during lung inflammation, due to peptide degradation by ECE-1, their levels were lower in ECE-$1^{+/+}$ mice. It shows the rate-limiting effect of half-reduced ECE-1 in degrading CGRP in pathological conditions, and hence higher CGRP levels were observed. Further supporting these results, CGRP mRNA expression were similar in both genotypes.

Lung cAMP protects against bleomycin-induced inflammation and fibrosis. Higher levels of intracellular cAMP in ECE-$1^{+/-}$ mice was attributed to CGRP induction. We suggested that more CGRP in ECE-$1^{+/-}$ mice activated the cAMP/EPAC1 and cAMP/PKA pathways. Transition from lung inflammation to fibrosis after bleomycin instillation is ascribed to M2 macrophages. Our result shows less M2 macrophage in ECE-$1^{+/-}$ mice. M2 macrophages expressed CRLR and RAMP1 in inflammation phase. We confirm that in ECE-$1^{+/-}$ mice, excess CGRP acted on M2 macrophages and modulated their function through cAMP/EPAC1 and cAMP/PKA pathways limiting the inflammation and fibrosis.

In conclusion, our results provide novel evidence that ECE-1-mediated degradation of CGRP is necessary for transition from lung inflammation to fibrosis, *in vivo*. Our study identified M2 macrophages to be the target cells for CGRP action during this transition.

神戸大学大学院医学系研究科（博士課程）

# 論文審査の結果の要旨

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| 論文題目<br>Title of Dissertation | Endothelin-converting enzyme-1 gene ablation attenuates pulmonary fibrosis via CGRP–cAMP/EPAC1 pathway<br><br>エンドセリン変換酵素-1ノックアウトマウスにおける<br>カルシトニン遺伝子関連ペプチド-cAMP/EPAC1経路を介した<br>肺線維症抑制効果の検討 | | |
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（要旨は１，０００字～２，０００字程度）

**【研究の背景と目的】**

肺線維症に対する新規治療戦略としてエンドセリン変換酵素-1（ECE-1）阻害という治療アプローチの検証に期待がかかっている。エンドセリン-1(ET-1)は血管内皮細胞、血管平滑筋、気道上皮細胞、肺胞マクロファージより分泌される21アミノ酸残基よりなるペプチドで、前駆体のビッグエンドセリン-1がECE-1のペプチド分解酵素の働きにより代謝され活性型ET-1に変換される。さらにin vitro実験でECE-1のエンドセリン系以外への作用としてブラジキニン(BK)、心房ナトリウムペプチド(ANP)、βアミロイド、カルシトニン遺伝子関連ペプチド(CGRP)への分解作用が報告されているが、病態学的状態における作用に関してはさらなる検証が必要である。ECE-1$^{-/-}$マウスは顔面や心血管系の発生奇形により胎生致死である一方、ECE-1$^{+/-}$マウスは正常に出生発育する。よって本研究ではECE-1$^{+/-}$マウスを用いブレオマイシン誘発肺線維症モデルでECE-1抑制による肺線維症発症抑制効果を検証し、同時に作用機序の解析として関連ペプチド群への代謝効果が検討された。

**【研究方法および結果、考察】**

129SvEv系マウスのECE-1$^{+/-}$マウスとECE-1$^{+/+}$マウス(雄 8～10週齢)を用い、ブレオマイシン(10μg/g体重)の経気管内投与で肺線維症モデルを作成し、薬剤投与後7日、14日、21日、28日の経時的な観察を行った。ブレオマイシン投与後21日目より肺の線維化は顕著となるが、21日目、28日目の両方でECE-1$^{+/-}$マウスはECE-1$^{+/+}$マウスに比して著明な線維化抑制効果をマッソントリクローム染色、Sircolアッセイ、コラーゲン1A1・3A1 mRNAレベルで示した。線維芽細胞数(S100A1による免疫染色)もECE-1$^{+/-}$マウスで明らかに抑制され、線維化促進因子であるTGF-βとCTGFも明らかに抑制されていた。またさらに線維化前段階である7日目、14日目ではECE-1$^{+/+}$マウスで炎症細胞浸潤が著明に認められたのに対し、ECE-1$^{+/-}$マウスではHE染色による組織学的評価でも、マクロファージマーカーであるCD68 mRNAレベルでも炎症が抑制されていた。

ECE-1$^{+/-}$マウスにおいて炎症誘発性の肺線維化が抑制されることが明らかとなったが、メカニズム解析としてペプチドの定量が行われた。肺組織中ET-1ペプチドは、14日目の炎症細胞浸潤期にECE-1$^{+/+}$とECE-1$^{+/-}$マウス間ではほぼ同程度の発現量であったが、CGRPではECE-1$^{+/-}$マウスで顕著に増加しており、BK, ANPも有意差は認めないものの増加傾向にあった。以上の結果よりECE-1$^{+/-}$マウスの炎症抑制・線維化抑制の表現型はエンドセリン系が重要な働きをしているのでなく、CGRP経路が最も重要な経路である事が推察された。肺組織中CGRP mRNAレベルが両群間に差が無かった事よりECE-1$^{+/-}$マウスにおけるCGRPペプチドの増加はECE-1によるCGRP分解抑制が機序である事が推察される。

下流シグナルである肺組織中cAMPレベルもブレオマイシン投与により著明に抑制されるが、ECE-1$^{+/-}$マウスのcAMPレベルはCGRP上昇によりコントロール群と同じレベルを保っていた。さらにその下流シグナルであるPKAとEpacの発現をウェスタンブロッティングで定量したが、いずれもcAMPと同様のパターンを示し特にEpacの経路が著明にECE-1$^{+/-}$マウスで賦活されていた。

炎症細胞を構成するマクロファージの中には2種類のサブクラスが存在し、早期に炎症部位に浸潤し炎症を増悪させるM1マクロファージとそれとは逆に抗炎症作用を持ち組織の線維化により組織修復を担うM2マクロファージがある。特にM2マクロファージは肺線維症発症に重要な働きをしている事が最近の研究で明らかとなってきたが、ECE-1$^{+/-}$マウスではECE-1$^{+/+}$マウスと比べて、M1マクロファージに関しては明らかな差が無かったものの、M2マクロファージの細胞数が著明に抑制されていた。またこのM2マクロファージはその細胞マーカーであるCD206とCGRP受容体であるCRLRとRAMP1の2重免疫染色によりその局在が一致し、M2マクロファージがCGRP経路の標的細胞である事が確認された。よってECE-1抑制により賦活されたCGRP経路は主にM2マクロファージに働き、その細胞浸潤を抑制し結果的に肺の線維化を抑制しているという病態学的機序が考察される。

【結論】

ECE-1 の遺伝学的抑制によるブレオマイシン誘発性肺線維症の発症抑制効果が確認され、機序として CGRP 経路が賦活され下流シグナルの cAMP/Epac 経路、cAMP/PKA 経路を介して線維化促進因子である M2 マクロファージの浸潤を抑制する事が明らかとなった。

本研究は、肺線維症に対する新規治療戦略として ECE-1 阻害という治療アプローチの有効性とその作用機序の解析として関連ペプチド群への代謝効果について重要な知見を得たものとして価値ある業績であると認める。よって本研究者は、博士（医学）の学位を得る資格があるものと認める。