Ca\(^{2+}\) entry mode of Na\(^{+}\)/Ca\(^{2+}\) exchanger as a new therapeutic target for heart failure with preserved ejection fraction

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Aims
Left ventricular (LV) fibrosis and stiffening play crucial roles in the development of heart failure with preserved ejection fraction (HFPEF). Plasma level of digitalis-like factors (DLFs) is increased in patients with hypertension, a principal underlying cardiovascular disease of HFPEF. Digitalis-like factors inhibit ion-pumping function of Na\(^{+}\)/K\(^{+}\)-ATPase and activate the Ca\(^{2+}\) entry mode of Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCX). Digitalis-like factors are known to promote collagen production in fibroblasts. The aim of this study was to explore whether the pharmacological inhibition of the NCX entry mode is effective in the prevention of LV fibrosis and in the development of HFPEF.

Methods and results
(i) Dahl salt-sensitive rats fed 8% NaCl diet from age 6 weeks served as hypertensive HFPEF model. In this model, 24 h urine excretion of DLFs was greater than that in the age-matched control at compensatory hypertrophic and heart failure stages. (ii) Continuous administration of ouabain for 14 weeks developed LV fibrosis without affecting blood pressure in Sprague–Dawley rats. (iii) Ouabain elevated intracellular Ca\(^{2+}\) concentration through the entry of extracellular Ca\(^{2+}\), increased the phosphorylation level of p42/44 mitogen-activated protein kinases, and enhanced 3H-proline incorporation in cardiac fibroblasts; and SEA0400, the inhibitor of the NCX entry mode, suppressed these effects. (iv) In the HFPEF model, administration of SEA0400 at subdepressor dose improved the survival rate in association with the attenuation of LV fibrosis and stiffening.

Conclusion
Digitalis-like factors and the subsequently activated NCX entry mode may play an important role in the development of hypertensive HFPEF, and the blockade of the NCX entry mode may be a new therapeutic strategy for this phenotype of heart failure.

Keywords
Heart failure  •  Diastole  •  Fibrosis  •  Na\(^{+}\)/Ca\(^{2+}\) exchanger  •  Digitalis-like factors

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Introduction

Heart failure with preserved ejection fraction (HFPEF) has poor prognosis and its prevalence has increased over the past two decades. In spite of its socioeconomic burden, therapeutic strategy has not been established. The progression of left ventricular (LV) stiffening plays a crucial role in the transition from asymptomatic LV hypertrophy to HFPEF and is attributed to myocardial fibrosis. Thus, LV fibrosis is likely a therapeutic target for HFPEF.

Digitalis-like factors (DLFs), such as ouabain and marinobufagenin, exist in humans. The elevation of plasma level of ouabain was observed in patients with hypertension, a principal underlying cardiovascular disease of HFPEF. Digitalis-like factors promote collagen production in fibroblasts, and the activation of p42/44 mitogen-activated protein kinases (MAPKs) plays an important role in collagen synthesis. Previous in vitro studies have reported DLFs-induced the activation of Src-p42/44 MAPKs pathway through non-pumping function of Na+/K+-ATPase signalosome. However, the binding of DLFs to Na+/K+-ATPase also leads to the inactivation of its ion-pumping function, and the Na+/K+-ATPase signalosome.5,7

We hypothesized that DLFs are one of risk factors for the development of HFPEF, and this study aimed at exploring whether the pharmacological inhibition of the Ca2+ entry mode of NCX is effective in preventing the development of LV fibrosis and the transition to hypertensive HFPEF.

Methods

This study consists of following in vivo and in vitro studies, and details are provided in the supplementary data.

Study 1: Urinary excretion of DLFs in the HFPEF model.
Study 2: Effects of ouabain on cardiac structure.
Study 3: Effects of ouabain and the NCX inhibitor on cardiac fibroblast.
Study 4: Effects of NCX inhibitor on the HFPEF model.

Results

Study 1: Urinary excretion of digitalis-like factors in the HFPEF model rats

At the age of 19 weeks of the HFPEF model, the ratio of LV mass to tibial length and the ratio of lung weight to tibial length were increased without the decrease in LV endocardial or mid-wall fractional shortening or the changes in LV end-diastolic dimension (Table 1). Thus, the HFPEF model of this study protocol represents the characteristics of the hypertensive HFPEF model as published in the previous studies.1,11 Urinary excretion of ouabain-like immunoreactivity was significantly higher in the HFPEF model than in the age-matched control at the age of 13 and 19 weeks (Figure 1A). Urinary excretion of marinobufagenin-like immunoreactivity also increased at the age of 13 weeks and tended to be high at the age of 19 weeks in the HFPEF model rats when compared with the age-matched control (Figure 1B).

Study 2: Effects of ouabain on cardiac structure in Sprague–Dawley rats

There was no significant difference in systolic blood pressure, echocardiographic parameters, or a ratio of LV mass to tibial length between the ouabain and the control groups; however, LV area of fibrosis was significantly greater in the ouabain group than in the control group irrespective of NaCl concentration of chow (Table 2, Figure 2).

Study 3: Effects of ouabain and SEA0400 on cardiac fibroblast

Intracellular Ca2+ dynamics

In normal PSS (control), the fibroblasts responded to bath-applied ouabain with robust Ca2+ transient (Figure 3A and D). This Ca2+ signal was completely diminished by removal of Ca2+ from extracellular PSS (Figure 3B and D). Furthermore, SEA0400, an NCX inhibitor that preferentially blocks the Ca2+ entry mode,12 substantially suppressed the ouabain-induced elevation of [Ca2+]i (Figure 3C and D). In quite contrast, Ca2+ release stimulated by angiotensin II normally occurred in the absence of extracellular Ca2+, or in the presence of SEA0400 (Figure 3A–G). These results suggest that the ouabain-induced elevation of [Ca2+]i is largely attributed to the Ca2+ entry mode operation of the NCX.
$^3$H-proline incorporation and p42/44 mitogen-activated protein kinase activity

$^3$H-proline incorporation (Figure 4A) and the phosphorylation of p42/44 MAPK (Figure 4B) were promoted by ouabain administration in cardiac fibroblasts. SEA0400 suppressed these effects of ouabain, but did not affect the baseline levels.

Study 4: Effects of Na$^+$/Ca$^{2+}$ exchanger inhibitor on HFPEF model rats

Survival study

The administration of SEA0400 improved the survival rate of the HFPEF model (Figure 5A). All of the rats of the untreated group which died during the protocol were associated with pulmonary congestion. There were no differences in systolic blood pressure ($P = 0.056–0.801$) or heart rate ($P = 0.091–0.689$) between the two groups at any age (Figure 5B and C).

Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Low-salt diet</th>
<th>High-salt diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control ($n = 4$)</td>
<td>Ouabain ($n = 6$)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>128 ± 7</td>
<td>130 ± 1</td>
</tr>
<tr>
<td>LV end-diastolic dimension (mm)</td>
<td>8.5 ± 0.3</td>
<td>9.0 ± 0.3</td>
</tr>
<tr>
<td>LV posterior wall thickness at end-diastole (mm)</td>
<td>1.4 ± 0.2</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>LV endocardial fractional shortening (%)</td>
<td>38 ± 6</td>
<td>35 ± 2</td>
</tr>
<tr>
<td>LV mid-wall fractional shortening (%)</td>
<td>21 ± 4</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>E/A ratio</td>
<td>1.9 ± 0.4</td>
<td>1.9 ± 0.8</td>
</tr>
<tr>
<td>Deceleration time of mitral E-wave (ms)</td>
<td>46 ± 6</td>
<td>45 ± 4</td>
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</table>

Values are expressed as mean ± SD.

E/A ratio, a ratio of peak early diastolic filling velocity to peak filling velocity at atrial contraction.
was increased by 5.7-fold ($P < 0.05$), and the hydroxyproline content of LV myocardium was higher in the untreated group than in the control group ($4.6 \pm 0.7$ vs. $3.3 \pm 0.5$ μmol/g, $P < 0.05$). The administration of SEA0400 reduced the area of LV fibrosis (Figure 6) in association with the significant decrease in type I collagen mRNA level by 21% ($P < 0.05$). Left ventricular hydroxyproline content in the SEA group ($4.2 \pm 0.4$ μmol/g) tended to be decreased when compared with the untreated group, although the difference was not statistically significant. The activity of 72 kDa gelatinase (matrix metalloproteinase-2) was significantly enhanced in the untreated group compared with the control group and was attenuated in the SEA group (Figure 7).

**Discussion**

The principal findings of the present study are (i) daily urinary excretion of DLFs was increased at compensated hypertrophic and heart failure stages in the HFPEF model, (ii) chronic administration of ouabain promoted cardiac fibrosis without the elevation of blood pressure irrespective of NaCl loading, (iii) ouabain elevated [Ca$^{2+}$], through the entry of extracellular Ca$^{2+}$, and enhanced collagen production with the activation of p42/44 MAPK in cardiac fibroblast, (iv) SEA0400 suppressed these in vitro effects of ouabain, (v) SEA0400 attenuated LV fibrosis and stiffening without the anti-hypertensive effects and prevented pulmonary congestion and the increase in LV filling pressure in hypertensive HFPEF model rats, leading to the improvement of survival rate.

The prevalence of HFPEF has increased, and its prognosis has not improved during the past two decades. Experimental and clinical studies have shown that LV fibrosis plays a crucial role in the development of HFPEF, indicating that LV fibrosis is a therapeutic target for this phenotype of heart failure. Although experimental and small-sized clinical studies have suggested the efficiency of angiotensin receptor blocker or angiotensin-converting enzyme-inhibitor in the prevention of LV fibrosis and/or HFPEF, clinical trials have failed to show the reduction of the primary outcome by these drugs. Thus, another target to prevent fibrosis is awaited to improve the prognosis of HFPEF.

Digitalis-like factors bind to Na$^+$/K$^+$-ATPase and subsequently result in the change of the mode of NCX and the increase in [Ca$^{2+}$]. Previous experimental studies have shown that DLFs activate MAPKs and promote collagen production in fibroblasts. Plasma level of DLFs is elevated in a half of patients with hypertension and also increases in association with the reduction of ejection fraction or physical stress situations. This study showed that the urinary excretion of DLFs was augmented in the hypertensive HFPEF model at compensatory hypertrophic and heart failure stages, which suggests the enhanced secretion of DLFs and the elevation of their plasma level in HFPEF. A close relation between DLFs and the
Figure 3 Representative intracellular Ca\(^{2+}\) transients in cardiac fibroblasts at control (A), Ca\(^{2+}\) free condition (B), and SEA0400 administration (C). The values are expressed as R/R\(_0\) (R: 340/380 nm emission ratio; R\(_0\): R at time 0). Peak R/R\(_0\) was evaluated from 15 cells (3 cells from 5 independent experiments) in each protocol (D). Data are presented as mean ± SD. *P < 0.05 vs. control. †P < 0.05 vs. Ca\(^{2+}\) (−).

Figure 4 (A) Summary data for \(^3\)H-Proline incorporation in fibroblasts. (B) Representative western blot analysis of phosphorylated p42/44 mitogen-activated protein kinase and total p42/44 mitogen-activated protein kinase, and summary data for p42 phosphorylation. Phosphorylated p42 values were normalized against the total p42 signal and expressed as mean ± SD. *P < 0.05 vs. control; †P < 0.05 vs. ouabain.
Previous in vitro studies have shown that DLFs promote collagen production in fibroblasts through the non-pumping function of Na\(^+/\)K\(^+-\)ATPase signalosome,\(^5,7\) and this signalling cascade has been considered to lead to the activation of Src and p42/44 MAPK.\(^5\) Liu et al.\(^6\) reported that the activation of p42/44 MAPK is mandatory in transforming growth factor-\(\beta\)-stimulated collagen synthesis in cardiac fibroblast. These studies suggest that DLFs-induced stimulation of non-pumping function of Na\(^+/\)K\(^+-\)ATPase is responsible for the enhancement of collagen production. Digitalis-like factors also inhibit pumping function of Na\(^+/\)K\(^+-\)ATPase and increase intracellular Na\(^+\) concentration, which in turn raises [Ca\(^{2+}\)]\(_i\), through the entry mode of NCX; however, a previous study showed that the increase in [Ca\(^{2+}\)]\(_i\) was secondary to the activation of p42/44 MAPK in myocytes.\(^10\) If so in fibroblast, the blockade of the entry mode of NCX may not lead to the inhibition of p42/44 MAPK and collagen production. This study showed that the ouabain-induced increase in [Ca\(^{2+}\)]\(_i\) was due to the influx of extracellular Ca\(^{2+}\) through the entry mode of NCX, and that the blockade of the entry mode with SEA0400 attenuated the phosphorylation level of p42/44 MAPK and the collagen synthesis as well as the increase in [Ca\(^{2+}\)]\(_i\). These results suggest that DLFs activate p42/44 MAPK and promote collagen synthesis in fibroblasts at least partly through the inhibition of pumping function of Na\(^+/\)K\(^+-\)ATPase and the secondary promotion of the entry mode of NCX. The entry mode of NCX also enhances migration, contraction, and proliferation of fibroblasts.\(^25\) Thus, the blockade of the entry mode of NCX can be expected to inhibit tissue fibrosis in the condition with enhanced secretion of DLFs.

This study demonstrated that the secretion of DLFs was enhanced in an animal model of hypertensive HFPEF, and that the chronic administration of SEA0400, an inhibitor of the NCX entry mode,\(^12\) attenuated LV fibrosis and myocardial stiffening, inhibited the elevation of LV filling pressure and pulmonary congestion, and improved the survival rate of this model. Iwamoto et al.\(^8\) reported the depressor effects of SEA0400 in Dahl salt-sensitive rats fed on high-salt diet. We used a low dose of SEA0400 to avoid its anti-hypertensive effects and demonstrated that SEA0400 provided the beneficial effects even without the changes in systolic blood pressure. Thus, the blockade of the entry mode of NCX is likely a new therapeutic strategy for the prevention of LV fibrosis, LV stiffening, and HFPEF, and our results suggest that the attenuation of LV fibrosis is provided through the inhibition of collagen synthesis rather than through the enhancement of collagen degradation. Previous studies have suggested that the entry mode of NCX has protective effects on LV systolic function in patients and animals with reduced ejection fraction.\(^26\) However, the administration of SEA0400 did not alter LV endocardial and mid-wall fractional shortenings in HFPEF model rats (Table 3), suggesting that the chronic blockade of the entry mode of NCX does not provide adverse effects on systolic function in HFPEF.

Current guidelines for the treatment of heart failure recommend the use of digitalis in patients with reduced ejection fraction to decrease hospitalization for worsening heart failure. Digitalis Investigation Group (DIG) trial showed that digitalis did not reduce mortality of heart failure patients irrespective of ejection fraction but was not inferior to placebo,\(^27\) indicating that digitalis does...
not provide adverse effects. The current results suggest the adverse effects of digitalis and are likely discrepant from the clinical studies. However, a post hoc analysis of DIG trial demonstrated that high serum digoxin concentration was associated with an unadjusted increase in all-cause mortality, whereas low serum digoxin concentration was associated with its decrease.28 Thus, the adverse effects of DLFs may be exerted in association with the increase in their tissue/serum concentration, which is partly compatible with the current results. Another explanation is that the effects of all of DLFs are not the same.29 Some of DLFs provide counteractive effects against other DLFs. Thus, the effects of exogenous digitalis may not mimic those of endogenous DLFs.

Study limitation
First, the chronic administration of ouabain promoted myocardial fibrosis in Study 2, but we did not directly assess the effects on LV diastolic function. Our previous study showed that myocardial fibrosis proportionally promotes myocardial stiffening,3 suggesting that ouabain exacerbated diastolic function in Study 2. Elkareh et al.7 demonstrated that marinobufagenin, one of DLFs, induced

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**Table 3** Haemodynamic and echocardiographic parameters at the age of 19 weeks in Study 4 (Dahl salt-sensitive rats)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group (n = 8)</th>
<th>Untreated group (n = 8)</th>
<th>SEA group (n = 8)</th>
<th>P-value for NO difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>128 ± 7</td>
<td>217 ± 12*</td>
<td>211 ± 10*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LV end-diastolic dimension (mm)</td>
<td>9.7 ± 0.7</td>
<td>10.6 ± 0.8</td>
<td>10.3 ± 0.7</td>
<td>0.055</td>
</tr>
<tr>
<td>LV posterior wall thickness at end-diastole (mm)</td>
<td>1.1 ± 0.1</td>
<td>1.7 ± 0.1*</td>
<td>1.5 ± 0.1*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LV endocardial fractional shortening (%)</td>
<td>27 ± 3</td>
<td>28 ± 7</td>
<td>30 ± 6</td>
<td>0.690</td>
</tr>
<tr>
<td>LV mid-wall fractional shortening (%)</td>
<td>16 ± 2</td>
<td>14 ± 3</td>
<td>16 ± 3</td>
<td>0.312</td>
</tr>
<tr>
<td>LV end-diastolic pressure (mmHg)</td>
<td>5 ± 5</td>
<td>17 ± 7*</td>
<td>9 ± 5**</td>
<td>0.023</td>
</tr>
<tr>
<td>Time constant of LV relaxation (ms)</td>
<td>18 ± 2</td>
<td>34 ± 12*</td>
<td>28 ± 3*</td>
<td>0.004</td>
</tr>
<tr>
<td>Myocardial stiffness constant</td>
<td>2.9 ± 0.6</td>
<td>7.0 ± 1.4*</td>
<td>5.5 ± 1.4***</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>A ratio of LV mass to tibial length (mg/mm)</td>
<td>21 ± 3</td>
<td>32 ± 2*</td>
<td>31 ± 3*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>A ratio of lung weight to tibial length (mg/mm)</td>
<td>36 ± 2</td>
<td>84 ± 22*</td>
<td>52 ± 16**</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD. *P < 0.05 vs. control group. **P < 0.05 vs. untreated group.

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**Figure 6** Representative Azan Mallory staining of left ventricle (A) and summary data for area of fibrosis (B). Data are expressed as mean ± SD. *P < 0.05 vs. control; †P < 0.05 vs. untreated.
myocardial fibrosis and diastolic dysfunction. The administration of ouabain did not change indices of transmural flow velocity curves (Table 2); however, they are insensitive to changes in diastolic function in subjects with preserved ejection fraction. Therefore, the results of Study 2 may not contradict our conclusion. Second, the increase in LV stiffness of HFPEF is provided by myocyte stiffening as well as interstitial fibrosis. SEA0400 modulates the amplitude of the intracellular Ca\(^{2+}\) transient and myocyte contractility. However, we did not assess the effects of DLFs and SEA0400 on myocyte stiffness, and thus, the beneficial effects of SEA0400 may not be attributed solely to the attenuation of LV fibrosis. Third, we used Dahl salt-sensitive rats fed on high-salt diet as the hypertensive HFPEF model. Exogenous salt loading and an increase in extracellular Na\(^{+}\) concentration may directly increase intracellular Na\(^{+}\) concentration and induce the NCX entry mode independent of DLFs. However, the decrease, not the increase, in the extracellular Na\(^{+}\) concentration elevated [Ca\(^{2+}\)]\(_{i}\) through the NCX entry mode. The profibrotic effects of ouabain were not masked by high-salt intake (Figure 2). Exogenous salt loading may not directly cause the NCX entry mode. Fourth, SEA0400 has been reported as the most potent and selective inhibitor of NCX. However, SEA0400 depressed Ca\(^{2+}\) transients even in the absence of NCX. Therefore, we cannot completely exclude the possibility that in vivo efficiency of SEA0400 was provided through mechanisms other than the blockade of DLFs-induced NCX entry mode. Fifth, the administration of SEA0400 was initiated at the compensatory hypertrophic stage without LV fibrosis, and this study showed the preventive, not therapeutic, effects against LV fibrosis. Left ventricular fibrosis is usually highly prevalent in human HFPEF by the time of diagnosis. To assess the therapeutic effects of the blockade of the entry mode of NCX, future studies are required.

**Conclusions**

The secretion of DLFs was enhanced in the hypertensive HFPEF model. Ouabain activated p42/44 MAPKs and enhanced collagen production with the elevation of [Ca\(^{2+}\)]. via the NCX entry mode. SEA0400, the potent inhibitor of the NCX entry mode, attenuated the development of LV fibrosis and stiffening, and prevented the transition to HFPEF with the improvement of survival rate in the animal models. Thus, DLFs and the subsequently activated the Ca\(^{2+}\) entry mode of NCX may play important roles in the development of LV fibrosis and hypertensive HFPEF, and the blockade of the entry mode of NCX may be a new therapeutic strategy for this phenotype of heart failure. This study was conducted in a rat model for hypertensive HFPEF, and future studies in large animal models or humans are awaited to confirm the clinical relevance of the current findings.

**Supplementary material**

Supplementary material is available at *European Heart Journal* online.

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**Conflict of interest:** none declared.

**References**

6. Liu X, Sun SQ, Hassid A, Ostrom RS. cAMP inhibits transforming growth factor-beta-stimulated collagen synthesis via inhibition of extracellular signal-

**Figure 7** Representative gelatinolytic bands from in vitro gelatin zymography of left ventricle (A) and summary data for 72 kDa gelatinase activity (B). The left line of (A) was a positive control for matrix metalloproteinase-2 (72 kDa) and matrix metalloproteinase-9 (92 kDa). Data are expressed as mean ± SD. *P < 0.05 vs. control; †P < 0.05 vs. untreated.