Clinical research

N-Methylethanolamine attenuates cardiac fibrosis and improves diastolic function: inhibition of phospholipase D as a possible mechanism

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Aim Ventricular fibrosis is promoted by many effectors that chronically activate phospholipase D (PLD), and induces cardiac dysfunction and heart failure in cardiovascular diseases. Since ethanolamine is a product of PLD, we hypothesised that an administration of an analogue of ethanolamine, N-methylethanolamine (MEA), decreases PLD activity through a negative feedback mechanism, suppresses collagen accumulation, and thus prevents organ dysfunction.

Methods and results In human fibroblasts 1-butanol inhibited collagen synthesis and enhanced collagenase production, but iso-butanol did not. These indicate crucial roles of PLD in collagen synthesis and degradation. In fibroblasts, MEA dose-dependently decreased PLD activity, inhibited collagen synthesis and enhanced collagenase production. In a hypertensive heart failure model using Dahl–Iwai salt-sensitive rats, PLD activity increased with progressive ventricular fibrosis, leading to myocardial stiffening and overt heart failure. Long-term administration of MEA did not significantly decrease blood pressure, however, but decreased PLD activity and collagen content with inhibited gene expression of collagens, leading to the prevention of myocardial stiffening and haemodynamic deterioration. MEA also attenuated ventricular hypertrophy, another detrimental structural alteration.

Conclusion MEA may exert therapeutic effects on cardiac disorders due to ventricular fibrosis through suppression of PLD activity and modulation of the fibrosis pathway even without relief from mechanical stress.

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KEYWORDS
Collagen;
Diastole;
Heart failure

Introduction

Progression of left ventricular (LV) fibrosis induces cardiac dysfunction such as myocardial stiffening, and results in heart failure.1–4 Although evidence has been...
accumulated about the treatment of heart failure, prevalence of hospitalisation due to heart failure is still increasing and heart failure is a major public health problem in the developed countries. One of the targeted goals for the treatment of heart failure in order to achieve a better prognosis is the attenuation and/or prevention of LV fibrosis; however, there is currently no established therapeutic regimen to attenuate organ fibrosis.

Collagen synthesis is stimulated in fibroblasts by growth factors and agonists binding to G-protein-coupled receptors, whose important downstream signaling component is phospholipase D (PLD). PLD is also activated by other effectors that are involved in the pathogenesis of heart failure such as platelet-derived growth factor, epidermal growth factor and oxidant stress. Specifically, PLD hydrolyses phosphatidylcholine and phosphatidylethanolamine into phosphatic acid. Phosphatic acid induces the Akt-dependent activation of the mammalian target of rapamycin-p70 S6 kinase 1 and the activation of Ras/Raf/MEK/Erk signalling cascade and is involved in inflammatory responses. Through these or other signalling pathways, PLD is involved in cell proliferation, protein production and exocytosis. Therefore, PLD is likely to contribute to the pathogenesis of heart failure including progressive ventricular fibrosis.

A concomitant product in the hydrolysis of phosphatidylethanolamine by PLD is ethanolamine. We hypothesised that an analogue of ethanolamine decreases PLD activity through a negative feedback mechanism and suppresses collagen production. Our preliminary in vitro study demonstrated that N-methyl ethanolamine (MEA), an analogue of ethanolamine, enhanced pro-collagenase production in human fibroblasts (unpublished data). Thus, MEA may be expected to suppress collagen production and to enhance collagen degradation, leading to the prevention of LV dysfunction due to collagen accumulation. The current study aimed to explore the effects of MEA in fibroblasts and rats with hypertensive heart failure (HHF) as well as to examine whether PLD plays a crucial role in collagen synthesis and degradation in fibroblasts.

**Methods**

**In vitro study**

**Effect of MEA on PLD activity in fibroblasts**

PLD activity was assessed by formation of choline from pre-labelled phospholipids. Human skin fibroblasts (Detroit-551 (ATCC CCL 110)) in the Eagle’s MEM, comprising 10% (v/v) FBS, 2 mM glutamine, 1% (v/v) non-essential amino acids solution and 1 mM sodium pyruvate were placed at a density of 8 x 10⁴ cells/well (12-well plate) and incubated under 5% CO₂, 95% air, at 37 °C. After 48 h, 0.1 µCi/ml of [³²P]choline chloride was added to each well and incubated for an additional 48 h. Cells were washed and incubated for 3 h in fresh medium without FBS. Then, cells were treated with or without MEA (n = 4 for each concentration) for 18 h in the medium without FBS.

Incubations were stopped by addition of ice-cold methanol. Cells were scraped off the plates, and choline was extracted and separated from the cells with methanol/chloroform/water (1:1:0.9). The aqueous phase was dried under vacuum, resuspended in water, and separated by TLC (Whatman LK60F) using the solvent system methanol/0.5% NaCl/13 N ammonia (50/50/1 v/v/v). The band containing choline was visualised by autoradiography of the TLC plate and quantified by densitometry with a BAS1500 system (Fuji film).

**Effects of PLD inhibition and MEA on collagen and collagenase production in fibroblasts**

Detroit-551 cells were seeded at a density of 2 x 10⁵ cells/well (24-well plate) and cultured as described above. After 72 h, the cells were washed with PBS (pH 7.4), and treated with butanol or MEA in the medium supplemented with 0.6% (v/v) FBS with ascorbic acid at 50 µg/ml for 48 h. Ascorbic acid concentration in cell culture medium decreases rapidly and affects collagen synthesis and secretion. Through these experiments, the experiments were conducted with the addition of ascorbic acid. For the estimation of collagen production, cells were labeled for 8 h (for butanol) or 24 h (for MEA) of treatment (n = 4 for each concentration) with [³¹P]proline per well in the presence of β-aminopropionitrile at 50 µg/ml. After labelling, collagen was extracted from each well by addition of acetic acid and pepsin at final concentrations of 0.5 M and 0.5 mg/ml, respectively, followed by standing at 4 °C overnight. Collagen was purified, and total radioactive proline was calculated to assess pro-collagen and collagen.

For the assessment of total protein synthesis, cells were labelled for 8 h with 0.2 µCi of [³¹P]proline per well, and total radioactive cell-associated proteins were quantified.

For the estimation of collagenase production, conditioned medium was collected at 48 h after treatment with butanol or MEA (n = 3 for each concentration), and then analysed by ELISA kit (Daiichi Fine Chemical, Takaoka, Japan) according to the manufacturer’s instructions. This kit detects both pro-matrix metalloproteinase (MMP)-1 and activated MMP-1 (a principal collagenase in human).

**In vivo study**

**Effects of long-term administration of MEA in HHF model**

Male Dahl salt-sensitive rats fed 8% NaCl from 7 weeks of age were used as a HHF model. They were randomly divided at 8 weeks of age into two groups: rats with or without oral administration of MEA at 200 mg/kg/day (n = 7, respectively). Rats fed 0.3% NaCl served as the control (n = 5). Systolic blood pressure was measured with a tail cuff system. We determined the dose of MEA following our previous result that its long-term administration at 200 mg/kg/day was effective against chronic liver injury induced by CCL₄ (unpublished data), and a lack of toxicity was preliminarily confirmed by its 2-week administration at 400 mg/kg/day in male Wister rats (Table 1). This study conforms to the guiding principles of Osaka University Graduate School of Medicine with regard to animal care.

**Haemodynamic study**

Echocardiographic studies were performed at 19 weeks of age under anaesthesia with intra-peritoneal administration of ketamine HCl (50 mg/kg) and xylazine HCl (10 mg/kg). Then, a 1.5 F high-fidelity manometer-tipped catheter was introduced...
through the right carotid artery into the left ventricle to calculate peak positive and negative values of the first derivative of LV pressure (peak $+\frac{dP}{dt}$ and peak $-\frac{dP}{dt}$) and the time constant of isovolumetric LV pressure fall (Tau) determined with a non-zero asymptote method. Simultaneous recordings of LV pressure and LV M-mode echogram were obtained to calculate the myocardial stiffness constant as previously described. The mean value of the myocardial stiffness constant of the septum and the posterior wall was used for statistical analysis. After the haemodynamic studies, the heart was harvested. The LV and lung weights were corrected for tibial length. LV specimens used for measurement of hydroxyproline content and mRNA levels, evaluation of PLD activity and zymography were immediately placed in liquid nitrogen and stored at $-80^\circ C$. Samples for immunohistochemistry were immersed in OCT compound and frozen at $-80^\circ C$.

**Histological study**
Following fixation, LV specimen was embedded in paraffin and slices were stained with hematoxylin and eosin for measurement of myocyte diameter. Sections stained with Azan Mallory staining were used to evaluate the percent area of fibrosis using NIH image programs. Sections were also stained with picrosirius red and were assessed with a standard light microscopy and polarization microscopy. LV hydroxyproline content was measured and corrected for tissue weight. Immuno-histo-chemical staining for collagens was conducted as previously described.

**Quantification of mRNA**
Expression of mRNAs of GAPDH, collagen type I and III, pre-pro-endothelin-1 (ppET-1), ET converting enzyme-1 (ECE-1) and angiotensin converting enzyme (ACE) was quantified with real-time quantitative PCR. Each mRNA level was normalised for GAPDH mRNA level.

**PLD assay**
PLD activity in microsomal protein from the LV specimen was assessed by quantification of PLD-catalysed $[^{14}C]$palmitoyl-phosphatidyethanol using 1-palmitoyl-2-$[^{14}C]$palmitoyl-sn-glycero-3-phosphocholine as the substrate. The amount of microsomal protein used for the reaction was normalised for total protein. $[^{14}C]$palmitoyl-phosphatidyethanol was separated by TLC (Whatman LK6DF) using the solvent system chloroform/methanol/acetic acid (13/3/1 v/v/v) and visualised by autoradiography of the TLC plate. The band intensities were quantified by densitometry with a BAS1500 system.

**Table 1**
| & MEA(−) & MEA(+) |
|---|---|---|
| $n$ & 6 & 6 |
| Body weight (g) & $366 \pm 8$ & $339 \pm 7$ |
| Aspartate aminotransferase (mU/mL) & $124 \pm 11$ & $117 \pm 6$ |
| Alanine aminotransferase (mU/mL) & $53 \pm 7$ & $27 \pm 3$ |
| Alkaline phosphatase (mU/mL) & $533 \pm 49$ & $380 \pm 12$ |
| $\gamma$-glutamyl-transpeptidase (mU/mL) & $1.3 \pm 0.3$ & $1.5 \pm 0.3$ |
| Total bilirubin (mg/dL) & $0.10 \pm 0.00$ & $0.10 \pm 0.00$ |
| Total protein (g/dL) & $6.67 \pm 0.09$ & $7.38 \pm 0.05$ |
| Albumin (g/dL) & $2.98 \pm 0.03$ & $3.47 \pm 0.04$ |
| Creatinine (mg/dL) & $0.5 \pm 0.04$ & $0.5 \pm 0.04$ |
| Urea nitrogen (mg/dL) & $18.7 \pm 0.6$ & $21.0 \pm 0.6$ |

Values are expressed as means ± SEM.

**Zymography**
In vitro gelatin zymography was performed as previously described.

**MEA concentration**
Serum concentrations of MEA were measured by capillary electrophoresis with a Hewlett Packard G1600A with cation solution kit. Conditions were as follows: indirect UV-detection was employed at 310 nm, using a 50 μm internal diameter non-coated 50 cm fused silica capillary. The capillary was thermostatted at 25 °C. The samples diluted with water were pressure-injected into the capillary at a pressure of 50 mbar for 2 s. The separation

![Fig. 1](image_url) Roles of PLD in collagen synthesis (a) and human collagenase (MMP-1) production (b) in fibroblasts. The presence of 1-butanol, not iso-butanol, suppressed collagen synthesis and enhanced MMP-1 production. *$p < 0.05$ vs. 0%, #$p < 0.05$ vs. 0.3%, †$p < 0.05$ vs. 0.6%.
Voltage was $\pm$ 30 kV. Prior to injection, all samples were filtered through a 0.45 μm PVDF filter.

Statistics
Results are expressed as means ± SEM. All statistical analyses were performed using commercially available statistical software (STATVIEW version 5.0, SAS Institute Inc.). All possible pairwise comparisons were assessed using one-factor ANOVA and Fisher’s protected least significant difference test when the comparisons were conducted among three groups. When the comparisons were conducted among four different doses of 1-butanol or iso-butanol, all possible pairwise comparisons were assessed using ANOVA with Tukey analysis to avoid type I error. $P < 0.05$ for two-sided comparisons was considered statistically significant.

Results
Role of PLD in collagen and collagenase synthesis in fibroblasts
A possible role of PLD in the synthesis of collagen and collagenase was tested in cultured fibroblasts by use of primary and secondary alcohols. Previous work demonstrated that primary alcohols selectively inhibit PLD activity.26,27 The presence of a primary, not secondary, alcohol inhibits production of phosphatidic acid by PLD, and primary alcohols have been used to implicate PLD activity in a variety of cellular functions. We used 1-butanol as a primary alcohol and iso-butanol as a secondary alcohol. Collagen synthesis in fibroblasts was inhibited by 1-butanol (Fig. 1(a)), and a ratio of collagen synthesis to total protein synthesis dose-dependently decreased in the presence of 1-butanol (by 21% at 0.3% 1-butanol, by 39% at 0.6% 1-butanol, by 51% at 1.0% 1-butanol, $p < 0.05$ vs 0% 1-butanol, respectively). Production of human collagenase (MMP-1) was enhanced by 1-butanol (Fig. 1(b)). In contrast, iso-butanol did not significantly affect collagen or collagenase synthesis in fibroblasts (Fig. 1). Although there was a tendency that iso-butanol at high concentrations decreased collagen synthesis, a ratio of collagen synthesis to total protein synthesis was not affected by iso-butanol. This indicates that the reduction of collagen synthesis and the enhancement of collagenase production by 1-butanol were attributed to inhibition of PLD-dependent synthesis of phosphatidic acid but not to non-specific effects of alcohols, and that the inhibition of collagen synthesis was not provided as a part of inhibition of total protein production.

Effects of MEA on PLD activity, collagen synthesis and collagenase production in fibroblasts
The effects of MEA on PLD activity, collagen synthesis and collagenase production in fibroblasts were tested next. Human fibroblasts were incubated with $[^{14}C]$choline chloride to label cellular phosphatidylcholine, and the release of $[^{14}C]$choline was used as an index of PLD activity. As compared with the control, MEA reduced the release of $[^{14}C]$choline (Fig. 2(a)), indicating that MEA decreased PLD activity in fibroblasts. MEA inhibited the collagen synthesis (Fig. 2(b)) and enhanced the collagenase production (Fig. 2(c)).

Effects of MEA in the HHF model
In vivo effects of long-term administration of MEA were examined in the HHF rat model. An untreated rat died at 17 weeks of age due to heart failure, and the data of the other six untreated rats were analysed. None of the age-matched control rats or the rats treated with MEA died during the study.

Characteristics of the HHF model (Table 2)
At 19 weeks of age the HHF rats showed signs of overt heart failure such as tachypnoea, laboured respiration and loss of activity, and increases in lung weight/tibial...
length and LV end-diastolic pressure indicated the presence of pulmonary congestion due to congestive heart failure. As the phenotype of heart failure of this model is diastolic heart failure, abnormalities in Tau, peak $-\frac{dP}{dt}$, and myocardial stiffness constant were not associated with changes in indices of systolic function.

In the HHF rats PLD activity increased to 150% of the control (Fig. 3). LV hydroxyproline content, area of fibrosis, LV mass/tibial length and myocyte diameter were greater in the HHF rats than in the control rats (Table 2, Fig. 4). The progressive LV fibrosis was associated with enhanced gene expression and accumulation of collagens (Fig. 5). The 72 kDa gelatinase (MMP-2) activity increased

![Phosphatidylethanol](image)

**Fig. 3** PLD activity determined by the productive activity of phosphatidylethanol in the control (Control), the HHF rats (HHF), and the HHF rats treated with MEA (HHF + MEA) at 19 weeks.

![Control HHF HHF+MEA](image)

**Fig. 4** Ventricular tissue sections at 19 weeks stained with (a) Azan Mallory staining, (b) picrosirius red viewed using standard light microscopy, and (c) picrosirius red viewed using polarisation microscopy.

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Values are expressed as means ± SEM.

Abbreviations: Tau = the time constant of isovolumetric LV pressure fall.

* $p < 0.05$ versus HHF.

$^*$ $p < 0.05$ versus Control.
by 18% (Fig. 6). Gene expression of ACE, ppET-1 and ECE-1 also increased (Fig. 7).

**Effects of MEA**

Serum MEA was detectable only in the rats treated with MEA (Table 2), and long-term administration of MEA did not affect blood pressure. No rats treated with MEA showed signs of overt heart failure at 19 weeks of age, and LV end-diastolic pressure and lung weight/tibial length were normal. MEA administration decreased PLD activity to 46% of the control (Fig. 3), normalised hydroxyproline concentration and area of fibrosis in the left ventricle, and decreased LV mass/tibial length and myocyte diameter (Table 2, Fig. 4). The MEA-induced prevention of LV fibrosis was accompanied with decreases in mRNA and protein levels of collagens (Fig. 5).

MEA administration decreased the 72 kDa gelatinase activity (Fig. 6) and mRNA levels of ACE, ppET-1 and ECE-1 (Fig. 7).

The administration of MEA did not affect indices of LV relaxation such as Tau and peak \(-\text{dP}/\text{dt}\), and the prevention of haemodynamic deterioration was accompanied with a decrease in myocardial stiffness constant (Table 2). MEA administration slightly, although significantly, increased LV end-diastolic dimension; however, LV end-systolic stress was still within the normal range.

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**Fig. 5** LV mRNA level and immuno-histo-chemical staining of collagens type I (a, c) and type III (b, d) at 19 weeks. *p < 0.05 vs. Control, #p < 0.05 vs. HHF.
Discussion

The current study indicated that PLD is a critical component of the fibrosis pathway. MEA suppressed PLD activity and collagen synthesis in vitro and in vivo. However, effects of MEA on collagen degradation were not consistent. MEA enhanced human collagenase (MMP-1) production in human fibroblasts. Rats lack MMP-1, and MMP-13 is the principal collagenase in rats. MEA decreased the MMP-13 mRNA level in the HHF model rats (data not shown). The gelatin zymography represented a MEA-induced decrease in the 72 kDa gelatinase (MMP-2) activity, but did not present a band for MMP-13 (55–57 kDa) in any groups (Fig. 6). The discrepancy may be explained by several possibilities. First, collagen degradation by MEA might be temporarily enhanced at an early stage but overlooked by the assessment at 19 weeks of age. The degradation of extracellular matrix by MMPs is considered to lead to not only to a decrease in the amount of extracellular matrix but also a breakdown of the architecture of the myocardial collagen matrix. The collagen network is necessary to keep ventricular structure, and its breakdown induces ventricular dilatation. MMP expression was found to be enhanced in the dilated ventricle,28,29 and the MMP inhibition attenuated LV dilatation.30 Thus, a slight increase in LV end-diastolic dimension in the rats treated with MEA might partly support the possibility of temporary enhancement of collagen degradation. Activity of MMPs is regulated by their intrinsic inhibitor, tissue inhibitors of matrix metalloproteinase (TIMPs), and a genetic deletion of TIMPs led to ventricular dilatation.31 Although we assessed mRNA levels of MMP-2, MMP-9, MMP-13, TIMP-1 and TIMP-2 at 19 weeks of age, they changed in almost the same way. They increased in the HHF rats and decreased with the MEA administration (data not shown). Besides the inhibitory effects of TIMPs on MMPs, TIMPs may actually stabilise, localise and even activate MMPs under certain conditions,32 and it is currently difficult to explain the discrepancy between the in vitro and in vivo results from the viewpoint of the interaction between MMPs and TIMPs. Second, the gelatin zymography is widely used to assess collagen degradation in vivo, but the gelatinase...
activity might not be equal to the collagenase activity. The present in vitro study assessed the production of MMP-1 and may not correlate to the enzyme activity. However, assessment of collagenase activity has not been established.

The HHF model is a model of diastolic dysfunction. LV relaxation abnormality occurs at an early stage and does not progress after a compensatory hypertrophic stage (13 week of age) in this model. In contrast, myocardial stiffening occurs at an advanced stage and is closely related to haemodynamic deterioration. The administration of MEA did not improve peak –dP/dt or Tau, which are indices for LV relaxation, but significantly decreased the myocardial stiffness constant (Table 2). Thus, the decrease in LV end-diastolic pressure and the prevention of pulmonary congestion in the MEA-treated rats are well explained by the MEA-induced decrease in myocardial stiffness that is principally attributed to the prevention of ventricular fibrosis.3

The MEA administration attenuated LV hypertrophy. This may also be attributed to suppression of PLD, because PLD is involved in cell growth as well as protein production.14

One may argue that the MEA-induced decrease in PLD activity was secondary to the prevention of haemodynamic deterioration rather than its primary effect. However, MEA decreased PLD activity below the control level, at least suggesting that the MEA-induced decrease in PLD activity cannot be explained solely by the secondary effect. The mRNA levels of ppET-1, ECE-1 and ACE increased in the HHF rats and MEA prevented the increases. Our additional experiment showed that MEA decreased mRNA levels of ppET-1, ECE-1 and ACE in neonate rat ventricular myocytes by 45%, 36% and 57%, respectively. However, it remains unclear whether the inhibition of their gene expression was provided by the MEA-induced inhibition of PLD or not. Choline kinase activity and ethanalamine kinase activity reside on the same protein, and ethanalamine inhibits this enzyme.33 Choline kinase catalyses phosphorylation of choline to form choline phosphate, which activates p70 S6 kinase and phosphatidylinositol 3-kinase and may contribute to fibrosis and myocyte hypertrophy. MEA inhibited the production of choline phosphate as well as choline in fibroblasts (data not shown); however, it is unclear whether the decrease in choline phosphate is secondary to decreased PLD-dependent production of choline or due to inhibition of choline kinase. Further studies are necessary to clarify actions of MEA.

MEA slightly, although not significantly, reduced blood pressure in the HHF rats (Table 2). Our previous study showed that a much larger decrease in blood pressure provided by a calcium antagonist administration did not attenuate LV fibrosis in this model. Thus, such slight reduction in blood pressure cannot explain the effects of MEA.

Conclusions

PLD plays a crucial role in the fibrosis pathway. MEA suppressed PLD activity, LV fibrosis and LV hypertrophy in the HHF model rats without anti-hypertensive effects and prevented myocardial stiffening and pulmonary congestion. These findings will permit further studies to elucidate the therapeutic efficacy of MEA in heart failure.

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References


