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Angiotensin II Type 1a Receptor Signals are Involved in the Progression of Heart Failure in *MLP*-Deficient Mice

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Background Angiotensin II (AT) is implicated in the development of cardiac remodeling, which leads to heart failure, and pharmacological inhibition of the AT type 1 (AT_1) receptor has improved mortality and morbidity in patients of heart failure. The aim of this study was to elucidate the role of the AT_1 receptor in disease progression in muscle LIM protein (*MLP*)-deficient mice, which are susceptible to heart failure because of defective function of mechanosensors in cardiomyocytes.

Method and Results Hearts from *MLP* knockout (MLPKO) mice and *MLP-AT1a* receptor double knockout (DKO) mice were analyzed. MLPKO hearts showed marked chamber dilatation with cardiac fibrosis and reactivation of the fetal gene program. All of these changes were significantly milder in the DKO hearts. Impaired left ventricular (LV) contractility and filling were alleviated in DKO hearts. However, the impaired relaxation and downregulated expression of sarcoplasmic reticulum calcium-ATPase 2 were unchanged in DKO hearts.

Conclusions The AT_{1a} receptor is involved in progression of LV remodeling and deterioration of cardiac function in the hearts of MLPKO mice. These results suggest that blockade of the receptor is effective in preventing progression of heart failure in dilated cardiomyopathy. (*Circ J* 2007; **71:** 1958 – 1964)

Key Words: Angiotensin II; Cardiomyopathy; Heart failure; Remodeling; Sarcoplasmic reticulum

A growing body of evidence suggests that blockade of the renin–angiotensin system (RAS) leads to a decrease in the morbidity and mortality of patients with congestive heart failure[1] In addition to the systemic effects, including elevation of blood pressure (BP), sodium and water retention, and activation of the sympathetic nervous system, the activated RAS has unfavorable direct effects on the heart[2] Most of the known functions of angiotensin II (Ang II) in the cardiovascular system are mainly mediated through the Ang II type 1 (AT_1) receptor[3] In mice or rats, the AT_1 receptor has 2 subtypes (AT_{1a} and AT_{1b}), but the AT_{1a} receptor is predominantly expressed and functionally important in cardiomyocytes[4,5] According to the results of in vitro experiments, activation of the AT_1 receptor stimulates diverse intracellular signaling cascades and produces reactive oxygen species, which evoke hypertrophic responses in cardiomyocytes and enhance cellular proliferation and production of extracellular matrix proteins, such as collagen, in cardiac fibroblasts[2,6] These cellular alterations that occur within the heart would promote left ventricular (LV) remodeling and contribute to the progression of heart failure. However, the effects of the AT_1 receptor have been experimentally verified only in animal models of heart failure, such as a myocardial infarction model produced by coronary artery ligation[7–9] a pacing-induced heart failure model[10] Dahl salt-sensitive rats[11] a pressure-overloaded model[12,13] a shunt-induced volume-overloaded model[14] experimental myocarditis[15] and doxorubicin-induced cardiomyopathy[16] A number of reports have suggested that inhibition of the AT_1 receptor prevents LV remodeling after myocardial infarction[7–9] but it remains unknown whether it is also beneficial for dilated cardiomyopathy (DCM).

Mice deficient for the gene for muscle LIM protein (*MLP*) have been characterized as a good model of human DCM[17] MLP, a member of the LIM-only proteins[18] is involved in organization of cytoarchitecture[17] and is proposed to function as a mechanosensor[19] Approximately 35% of *MLP*-deficient mice (offspring of homozygous breeders) exhibit an early phenotype with marked hypertrophy and death within 10–11 days, and remainder survive into adulthood and exhibit a number of phenotypic features of human DCM (adult phenotype)[17] In the present study, we used *MLP*-deficient mice as a model of DCM and examined the effects of AT_1 receptor blockade on disease progression using *AT1a*-deficient mice.

Methods

Animals

G1 pups generated from an $MLP^{+/-}$ heterozygote × $AT_{1a}^{-/-}$ homozygote cross were mated to created the $MLP^{+/-}$/$AT_{1a}^{+/-}$ double heterozygotes (G2). G4 offspring were gen-

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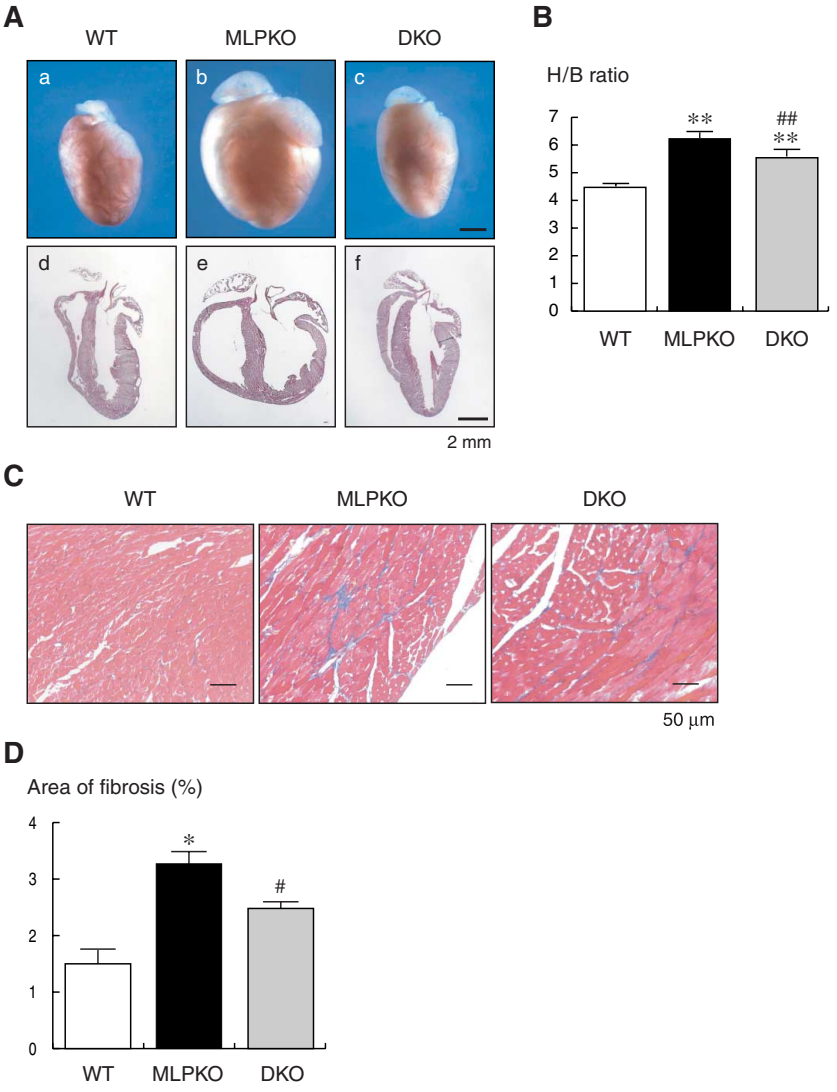


Fig 1. Rescue of the morphological and histological changes in double knockout (DKO) hearts. (A) (a–c) Excised hearts from 24-week-old wild-type (WT), muscle LIM protein-knockout (MLPKO) and DKO mice. (d–f) Histological analysis of 4-chamber sections (hematoxylin-eosin). Marked chamber dilatation in MLPKO hearts (e) was rescued in the DKO hearts (f). (B) Heart-to-body weight ratio (H/B ratio (mg/g)). n=20. (C) Histological analysis of light micrographs of cardiac fibrosis (stained blue) in left ventricle wall sections from WT, MLPKO and DKO mice at 24 weeks of age (Masson's trichrome; ×100). (D) Quantification of fibrotic area in the hearts. Data are mean ± SEM of 6 independent experiments. **p<0.01, *p<0.05: WT vs MLPKO or DKO; ##p<0.01, #p<0.05: MLPKO vs DKO.

erated from $MLP^{+/-}/AT_{1a}^{+/-}$ matings (G3). The genotypes of the gene-targeted crosses were determined by PCR on genomic DNA isolated from tail biopsies as described previously[17,20] We analyzed 24-week-old G4 offspring of the same crosses according to 3 groups: wild-type (WT) mice ($MLP^{+/+}/AT_{1a}^{+/+}$), MLP-knockout (MLPKO) mice ($MLP^{-/-}/AT_{1a}^{+/+}$) and double knockout (DKO) mice ($MLP^{-/-}/AT_{1a}^{-/-}$). All protocols were approved by the Institutional Animal Care and Use Commmittee of Chiba University.

Histological Analysis

Hearts were fixed in 10% neutralized formalin and embedded in paraffin. Serial sections at 4 μm were routinely stained with hematoxylin-eosin for morphological analysis, and with Masson's trichrome for detection of fibrosis[21] The images were acquired by stereomicroscope (MZ12, Leica, Tokyo, Japan) and captured by DC100 program (Leica), or by light microscope (Axioskop 2 plus, Carl Zeiss, Oberkochen, Germany) and captured by Axio Cam CCD camera and Axio Vision 3.0 imaging system (Carl Zeiss). The fibrotic areas by Masson's trichrome staining were calculated with image analysis software Adobe Photoshop (Adobe Systems, CA, USA).

Physiological Studies

BPs and pulse rates were measured in conscious mice noninvasively by a standard tail-cuff method (Softron). After anesthetizing the mice by intraperitoneal injection of a mixture of ketamin (50 mg/kg) and xylazine (2.5 mg/kg), transthoracic echocardiograms were recorded with an echocardiographic system (SONOS 4500, Philips Medical Systems, Andover, MA, USA) using a 12-MHz imaging transducer as described previously[16,21] For hemodynamic measurements, a pressure transducer (Samba Sensors, Göteborg, Sweden) was inserted into the LV via the right carotid artery. Heart rate, LV systolic pressure, LV end-diastolic pressure (LVEDP), positive and negative first derivatives for maximal rates of LV pressure development (max dP/dt and min dP/dt), were acquired digitally by a data acquisition system (SAMBA3000, Samba Sensors). The time constant of LV isovolumetric pressure decay (tau) was calculated according to a variable asymptote method[22]

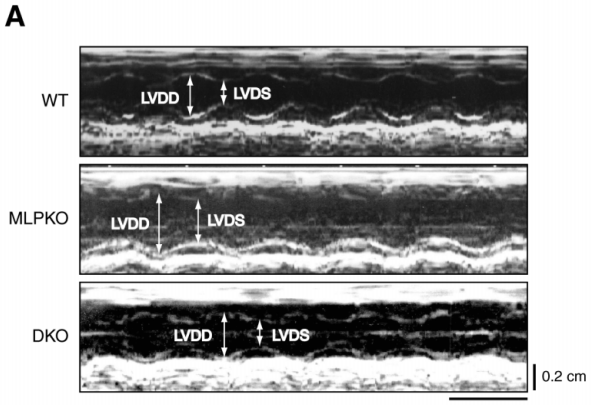
Northern Blot Analysis

Total RNA (10 μg) was extracted from the LV with TRIzol (Invitrogen) and was hybridized with the [α-^{32}P]-dCTP-labeled cDNA probes for brain natriuretic peptide (*BNP*), skeletal α-actin, and sarcoplasmic reticulum cal-

Table 1 BPs and Pulse Rates

	WT	*MLPKO*	*DKO*
N	*5*	*5*	*3*
Pulse rate (beats/min)	*643.9±15.5*	*640.2±7.1*	*636.5±20.9*
Systolic BP (mmHg)	*96.3±3.3*	*80.4±1.6***	*71.9±0.9**,#*
Diastolic BP (mmHg)	*60.3±3.9*	*55.5±1.4*	*39.1±4.6*,##*
Mean BP (mmHg)	*72.3±3.3*	*63.8±0.7**	*50.1±3.4**,##*

BP, blood pressure; WT, wild type; MLPKO, muscle LIM protein knockout; DKO, double knockout.
Data are mean±SEM.
***p<0.01, *p<0.05: WT vs MLPKO or DKO; ##p<0.01, #p<0.05: MLPKO vs DKO.*



B

	WT	MLPKO	DKO
Number	16	16	16
Body weight (g)	29.0±0.8	30.1±1.2	27.2±0.9
Heart rate (beats/min)	302.6±4.6	294.2±6.8	296.9±6.4
LVDD (mm)	3.66±0.08	4.54±0.10 **	3.96±0.09 *, ##
LVSD (mm)	2.13±0.07	3.56±0.11 **	2.77±0.10 **, ##
FS (%)	41.7±1.4	21.8±1.3 **	30.2±1.1 **, ##
LVPWT (mm)	0.52±0.03	0.52±0.04	0.54±0.03
LVDD (mm) / LVPWT (mm)	7.51±0.49	9.48±0.67 *	7.63±0.39 #

Fig 2. Rescue of cardiac chamber dilatation and dysfunction in double knockout (DKO) mice. Representative transthoracic M-mode echocardiograms obtained at the level of papillary muscles. Echocardiographic parameters. Data are mean±SEM. LVDD, left ventricular end-diastolic dimension; LVSD, left ventricular end-systolic dimension; FS, fractional shortening; LVPWT, left ventricular posterior wall thickness; WT, wild-type; MLPKO, muscle LIM protein-knockout; DKO, double knockout. **p<0.01, *p<0.05: WT vs MLPKO or DKO; ##p<0.01, #p<0.05: MLPKO vs DKO.

Table 2 Hemodynamic Characteristics

	WT	*MLPKO*	*DKO*
Number	*5*	*5*	*5*
Heart rate (beats/min)	*368.8±25.9*	*358.1±32.2*	*349.6±20.3*
Mean AoP (mmHg)	*91.4±3.4*	*79.9±6.0*	*59.6±4.35*,#*
LVEDP (mmHg)	*5.4±1.5*	*32.9±7.3**	*7.1±2.2#*
Max dP/dt	*4,669±258*	*2,070±295***	*3,415±307*,#*
–min dP/dt	*4,304±233*	*2,306±208***	*2,561±111***
Tau (ms)	*15.56±2.76*	*44.76±6.12***	*26.78±5.33*

AoP; aortic pressure; LVEDP, left ventricular end-diastolic pressure; max dP/dt, maximal rate of left ventricular pressure rise; min dP/dt, minimal rate of left ventricular pressure fall; tau, time constant of left ventricular isovolumetric pressure decay. Other abbreviations see in Table 1.
Data are mean±SEM.
***p<0.01, *p<0.05: WT vs MLPKO or DKO; ##p<0.01, #p<0.05: MLPKO vs DKO.*

cium (Ca^{2+})-ATPase (*SERCA*) 2 as previously described[21] Hybridized bands were quantified with NIH IMAGE software (NIH, Research Service Branch).

Western Blot Analysis

Total proteins were fractionated by SDS PAGE and transferred to Hybond membranes (GE Healthcare Life Sciences, Buckinghamshire, UK). The blotted membranes were incubated with the following antibodies, as described previously:[21] polyclonal antibody against ryanodine receptor (RyR2) (Oncogene Research Products, rabbit polyclonal antibody against phosphorylated phospholamban (Ser-16) (Upstate Biotech, Charlottesville, VA, USA), mouse monoclonal antibody against phospholamban (PLN) (Oncogene Research Products, San Diego, CA, USA), mouse monoclonal antibody against SERCA2 (Oncogene Research Products), and rabbit polyclonal antibody against Actin (Sigma-Aldrich).

Statistical Analysis

All data are expressed as mean±SEM. Differences in measured values were evaluated with an analysis of variance using Fisher's t-test and unpaired Student's t-test. Values of $p<0.05$ were considered to be statistically significant.

Results

Cardiac Morphological Changes and Fibrosis

Macroscopic inspection and histological sections revealed global chamber dilatation in MLPKO hearts at 24 weeks of age (Fig 1A), as previously described![17] In addition, MLPKO mice displayed a marked increase in the heart-to-body weight ratio (H/B ratio, 6.23±0.21 mg/g), compared with gender-matched WT mice (4.47±0.14 mg/g) (Fig 1B). These apparent cardiomyopathic features were alleviated in the DKO mice. Chamber dilatation was not prominent in DKO mice (Fig 1A), and the H/B ratios were significantly smaller (5.54±0.21 mg/g) than those of the MLPKO mice (Fig 1B).

Masson's trichrome staining revealed more marked cardiac fibrosis in MLPKO hearts (3.27±0.19% of total myocardial area) than in WT hearts (1.5±0.23% of total myocardial area) (Fig 1C). Cardiac fibrosis was also significantly attenuated in the DKO hearts (2.48±0.12% of total myocardial area) (Figs 1C,D). These results suggest that genetic ablation of the AT_{1a} receptor prevents the morphological and histological changes observed in the hearts of MLPKO mice.

Cardiac Function

The BP and pulse rates are shown in Table 1. There were no differences in pulse rate in any of the groups, but the BP was significantly lower in MLPKO mice than in WT mice (mean BP, $p<0.05$), presumably because of the low cardiac output in MLPKO mice. Genetic disruption of the AT_{1a} receptor further lowered BP in MLPKO mice (mean BP, $p<0.01$, MLPKO vs DKO)![20]

To examine whether the deterioration in cardiac performance in MLPKO mice was rescued in DKO mice, we first performed echocardiography with 24-week-old mice (Fig 2). MLPKO mice showed a 1.2-fold increase in LV end-diastolic dimensions (LVDD) and a 1.9-fold decrease in the percent of fractional shortening (%FS) of the LV, when compared with WT mice (LVDD, $p<0.01$; %FS, $p<0.01$) (Fig 2B). However, these parameters showed significant improvement in DKO mice (LVDD, $p<0.01$; %FS, $p<0.01$,

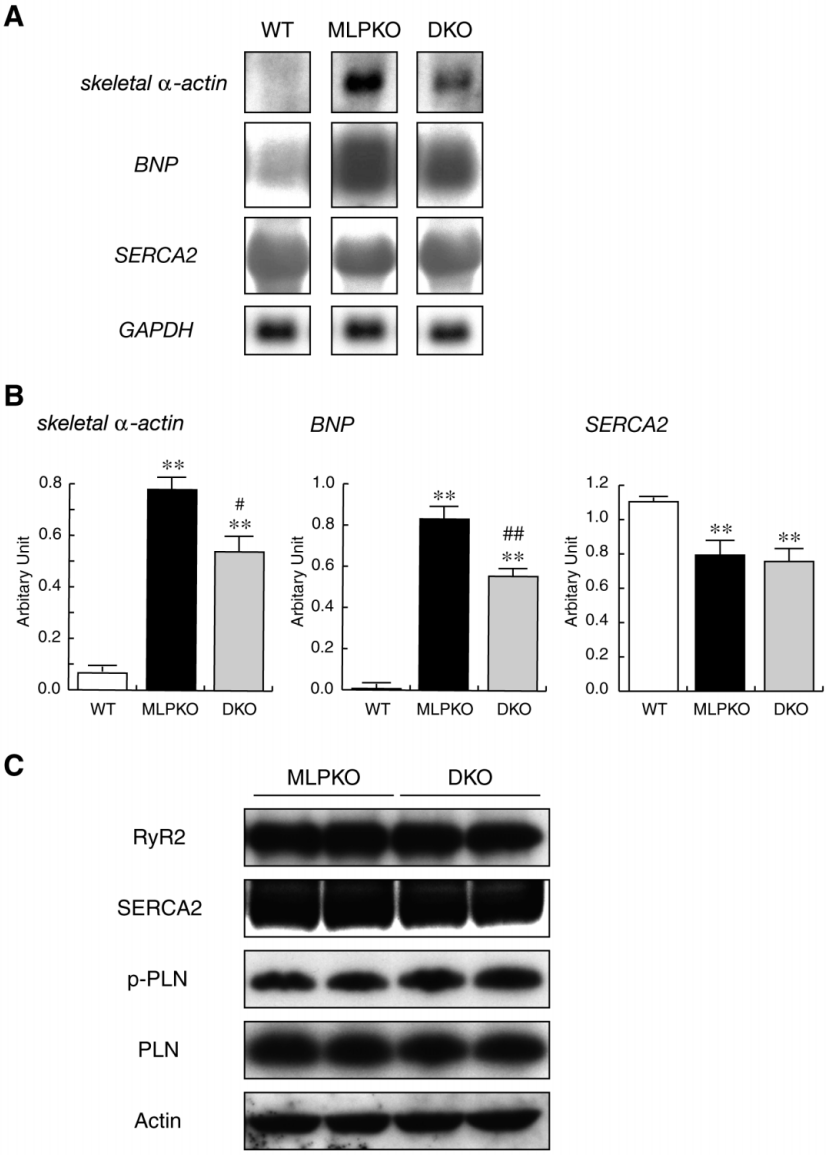


Fig 3. Cardiac gene expression in the left ventricle. (A) Representative autoradiograms of northern blot analysis for expressions of skeletal α-actin, brain natriuretic peptide (*BNP*) and sarcoplasmic reticulum Ca^{2+}-ATPase 2 (*SERCA2*). (B) The intensity of each band is quantified and corrected for the amount of glyceraldehyde phosphate dehydrogenase (*GAPDH*) mRNA. Data are mean ± SEM of 6 independent experiments. WT, wild-type; MLPKO, muscle LIM protein-knockout; DKO, double knockout; RyR2, ryanodine receptor; p-PLN, phosphorylated phospholamban; PLN, phospholamban. **p<0.01: WT vs MLPKO or DKO; ##p<0.01, #p<0.05: MLPKO vs DKO. (C) Immunoblot analysis showing unchanged expression levels of the Ca^{2+} handling proteins in the SR.

MLPKO vs DKO) (Fig 2B), indicating that genetic ablation of the AT1a receptor prevented progression of ventricular dilatation and systolic dysfunction. Although the LV posterior wall thickness (LVPWT) showed no significant difference, the ratio of LVDD to LVPWT was normalized in DKO (Fig 2B), which suggested that the wall stress in DKO hearts was relieved[23] by the absence of the AT1a receptor.

To further evaluate the cardiac performance of the WT, MLPKO and DKO mice, several independent hemodynamic parameters were measured by cardiac catheterization in anesthetized mice (Table 2). Max dP/dt was significantly decreased in MLPKO mice (p<0.01, vs WT mice), indicating depression of LV contractility. Genetic ablation of the AT1 receptor led to a significant increase in max dP/dt in the MLPKO mice (p<0.05, MLPKO vs DKO), confirming improved LV contractility in the DKO mice. LVEDP was significantly elevated in MLPKO mice (32.9±7.3 mmHg, vs 5.4±1.5 mmHg in WT mice, p<0.01), indicating increased cardiac stiffness. Notably, the LVEDP in DKO mice was almost normal (7.1±2.2 mmHg). Likewise, min dP/dt was markedly reduced and tau was significantly prolonged in the MLPKO mice (min dP/dt: p<0.01, tau: p<0.01, vs WT). However, DKO mice showed only a slight improvement in these parameters for LV relaxation (min dP/dt: p=0.31, tau: p=0.06, vs MLPKO).

Gene Expressions in MLPKO and DKO Hearts

To characterize the molecular basis of the effects of AT1a receptor ablation, we examined expression levels of molecular markers for cardiac hypertrophy and failure. Reactivation of the fetal cardiac gene program is a typical cellular response observed during cardiac hypertrophy.[24] The MLPKO hearts showed a remarkable increase in expressions of skeletal α-actin and *BNP*, when compared with WT hearts (Fig 3). In DKO hearts, the upregulation of skeletal α-actin and *BNP* expressions was milder than that in MLPKO hearts (skeletal α-actin, p<0.05; *BNP*, p<0.05, MLPKO vs DKO) (Figs 3A,B). These results suggest that genetic ablation of the AT1a receptor also prevented alterations in gene expression.

Downregulation of *SERCA2* is considered to be a feature characteristic of human patients and animal models of heart failure.[25] In the present study, the MLPKO hearts consistently showed a 1.4-fold reduction in *SERCA2* expression,

when compared with WT hearts (Figs 3A,B). In contrast to the blunted upregulation of skeletal α-actin and *BNP*, the expression level of *SERCA2* in the DKO hearts was comparable to that in MLPKO hearts (Figs 3A,B). We further confirmed that the levels of SERCA2 in MLPKO and DKO hearts were comparable (Fig 3C). These results suggest that blockade of the AT_{1a} receptor had a marginal effect on the expression of *SERCA2* in MLPKO hearts. In addition, MLPKO and DKO hearts showed no significant differences in the levels of Ca^{2+} regulatory proteins in the SR, such as RyR2 and PLN (both phosphorylated and total PLN) (Fig 3C).

Discussion

Many clinical studies have indicated that pharmacological inhibition of the RAS can reduce overall the mortality and morbidity in patients with heart failure.[2] However, the clinical syndrome of heart failure is the final state of a wide spectrum of diseases affecting the heart[1,26] and the effects of RAS inhibition may differ according to the causation, genetic and environmental backgrounds, and disease stage. To gain insights into the in vivo effects of RAS inhibition on the development and progression of heart failure, several animal models have been utilized;[8–16,27] however, most do not perfectly represent human heart failure;[28] and the effects of RAS inhibition are unknown in a mouse model of DCM caused by mutations affecting the cytoskeleton and sarcomere. In this regard, we are the first to analyze the preventive effects of AT_{1a} receptor blockade on progression of heart failure in *MLP*-deficient cardiomyopathic mice.

Genetic explorations have revealed that aberrant force generation or transmission caused by cytoskeletal abnormalities is an important pathogenic factor in DCM.[29] MLP was originally identified as an essential regulator of cardiac muscle development, serving as a scaffold protein promoting the assembly of the actin-based cytoskeleton.[18] A recent report demonstrated that MLP stabilizes a protein complex at the Z disc, which anchors the actin filaments and functions as a key component of the intrinsic mechanosensor in cardiomyocytes.[19] Accordingly, *MLP*-deficient mice exhibit chamber dilatation and cardiac dysfunction,[17] possibly because stretch-induced survival responses are not stimulated in response to increased wall stress.[19] Defects in stabilization of the Z disc complex involving the MLP protein might be important in the pathogenesis of human DCM as well, because a human MLP mutation is associated with DCM[19] and MLP protein levels are decreased in the hearts of patients with idiopathic and ischemic cardiomyopathies.[30] Therefore, *MLP*-deficient mice are considered to be a good model for human DCM.

The present study demonstrated that genetic ablation of the AT_{1a} receptor could rescue the cardiomyopathic phenotype of MLPKO. AT_{1a} receptor blockade suppressed morphological and histological changes characterized by chamber dilatation and cardiac fibrosis (Fig 1). Similarly, hypertrophic gene reprogramming was attenuated in the absence of the AT_{1a} receptor, because reactivation of *BNP* and skeletal α-actin expressions was less prominent (Figs 3A,B). These findings suggest that AT_{1a} receptor blockade prevents the progression of maladaptive structural LV remodeling in MLPKO hearts as in other heart failure models.[2] In addition, precise evaluation of hemodynamic parameters by echocardiography and cardiac catheterization revealed that AT_{1a} receptor blockade led to an improvement of cardiac performance in MLPKO mice (Fig 2, Table 2). Mechanistically, an improvement of LV contractility, as evidenced by increased %FS and max dP/dt, might result from restoration of the geometric changes in the DKO hearts. Alternatively, the negative inotropic effects of Ang II might be alleviated, because it has been reported that Ang II reduces LV contractility in failing hearts through the AT_1 receptor.[31–33] In addition, a recent study reported that pharmacological inhibition of the AT_1 receptor restores cardiac RyR2 function in the isoproterenol-induced failing heart.[34] However, a reduction in BP in the absence of the AT_{1a} receptor may have beneficial effects in DKO hearts. In particular, an improvement of LV contractility may be caused in part by diminished afterload. Further experiments are required to dissect the effects of AT_{1a} receptor blockade on cardiac contractility in vivo, at least by using a control group, to treat MLPKO mice with a BP-lowering agent or by confining gene deletion to the myocardium.

As reported previously,[23,35–37] MLPKO hearts show an increase in passive stiffness. Normalization of the LVEDP in the DKO hearts indicated that the impaired filling in the MLPKO hearts was rectified in the absence of the AT_{1a} receptor (Table 2). It is well established that accumulation of interstitial collagen results in increased wall stiffness, which in turn impairs LV filling and increases the LVEDP. Suppression of cardiac fibrosis might contribute to decreased stiffness of the DKO hearts. In comparison with the LVEDP, min dP/dt and tau showed subtle improvement in the DKO hearts (Table 2). Although it is difficult to assign specific biochemical mechanisms to the impaired lusitropy, these results suggest that AT_{1a} receptor blockade has a marginal effect on relaxation during isovolumic early diastole.[38,39] Slowed relaxation may be caused by declined pumping activities of the SR, which are often coupled to reduced expression of *SERCA2* (reviewed by Kass et al[39]). Consistently, the DKO hearts did not show a significant difference with MLPKO hearts in *SERCA2* expression (Fig 3). In addition, no significant differences were observed in the phosphorylation levels of PLN, an inhibitory regulator for *SERCA2,* in either MLPKO or DKO hearts (Fig 3C). In line with this speculation, it has been reported that genetic ablation of *PLN* normalized min dP/dt, as well as LVEDP, in MLPKO hearts.[23] Insomuch as AT_1 receptor blockade has been reported to restore slowed relaxation during isovolumic diastole in other models of heart failure,[8,11,12,34] insufficient restoration of SR function by AT_{1a} receptor blockade may be a phenomenon occurring specifically in failing hearts caused by abnormalities of the cytoskeleton or sarcomere. On the basis of the functional role of MLP as a key component of the mechanosensor,[19] restoration of *SERCA2* expression might be dependent on the stretch sensor machinery at the Z disc that perceives and mediates the alterations of wall stress. In addition, intracellular Ca^{2+} handling is intricately influenced by multiple signaling pathways in cardiomyocytes,[40] and further experiments are required to elucidate the precise role of AT_{1a} signaling in the regulation of SR function.

Although mechanical stress is the primary trigger that stimulates structural and functional alterations in cardiomyocytes (reviewed by Komuro and Yazaki;[41] Sadoshima and Izumo[42]), it remains unclear how mechanical stress is perceived and converted into active intracellular signaling. Besides the Z disc complex involving MLP, integrins and their associated signaling machinery and stretch-activated ion channels have been reported to be sensors for mechani-

cal stress.[43–46] In addition, we recently demonstrated that the AT1 receptor itself may function as a receptor for mechanical stress.[47] However, it is unknown how the mechanosensors are activated by mechanical stress and how they regulate the wide variety of stretch-induced responses, especially in failing hearts. Although the expressions and activities of RAS components were not examined in MLPKO hearts, our present study results suggest that the AT1a receptor also plays a critical role in the progression of heart failure caused by a defect of the Z disc mechanosensor machinery.

We conclude that genetic ablation of the AT1a receptor prevents progression of LV remodeling and deterioration of cardiac contractility and filling in the hearts of MLPKO mice, which are a good model of DCM caused by defective function of a mechanosensor in cardiomyocytes. In addition, our present study has highlighted distinctive effects of AT1a receptor blockade on impaired lusitropy according to the pathophysiology of the underlying disease.

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