shown). Further studies are required for elucidating the mechanism of this effect.

The conformations of APN were not compared in our study. It has been reported that the proteolytically cleaved product of APN, its globular form, is present in circulation at an extremely low concentration and exhibits much more extensive biological activity than the full-length form of APN.[26] Furthermore, AdipoR1 and AdipoR2 resulted in different expressions in our study. AdipoR1 exhibits a strong affinity for globular APN and a weak affinity for full-length APN, whereas AdipoR2 exhibits an intermediate affinity for both.[3] AdipoR1 is ubiquitously expressed in mice, whereas AdipoR2 is abundantly expressed only in the liver of mice.[33] In our study, these differences could be associated with their phenotype. It has been shown that their ratio changes in the situation of MI, but it is still controversial whether AdipoR1 or AdipoR2 is more protective against HF.[34] Further studies are necessary to resolve these issues by assessing AdipoR1-KO and AdipoR2-KO mice.

In conclusion, APN induces AMPK expression, activates its phosphorylation, and partially inhibits DOX-induced apoptosis in cardiomyocytes. In addition, this study demonstrates that the inhibition of AMPK phosphorylation completely reduces the cardioprotective effects of APN. Taken together, we suggest that APN is cardioprotective in DOX-induced chronic cardiomyopathy via its anti-apoptotic effects through AMPK signalling. APN could be a therapeutic target of DOX-induced cardiomyopathy and HF in mice; however, further studies must be conducted in other animals to confirm how to utilize APN in clinical HF.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Conflict of interest: none declared.

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Mitochondrial Lon protease regulates mitochondrial DNA copy number and transcription by selective degradation of mitochondrial transcription factor A (TFAM)

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Lon is the major protease in the mitochondrial matrix in eukaryotes, and is well conserved among species. Although a role for Lon in mitochondrial biogenesis has been proposed, the mechanistic basis is unclear. Here, we demonstrate a role for Lon in mtDNA metabolism. An RNA interference (RNAi) construct was designed that reduces Lon to less than 10% of its normal level in Drosophila Schneider cells. RNAi knockdown of Lon results in increased abundance of mitochondrial transcription factor A (TFAM) and mtDNA copy number. In a corollary manner, overexpression of Lon reduces TFAM levels and mtDNA copy number. Notably, induction of mtDNA depletion in Lon knockdown cells does not result in degradation of TFAM, thereby causing a dramatic increase in the TFAM• mtDNA ratio. The increased TFAM• mtDNA ratio in turn causes inhibition of mitochondrial transcription. We conclude that Lon regulates mitochondrial transcription by stabilizing the mitochondrial TFAM• mtDNA ratio via selective degradation of TFAM.

AAA+ protease • mtDNA maintenance • quality control

Lon is the major protease in the mitochondrial matrix and is well conserved among species (1–4). Lon is a member of the super family of ATPases associated with diverse cellular activities •AAA• ATPases•[1], and forms a homooligomeric, ring-shaped structure (5, 6). Lon contributes to protein quality control surveillance in mitochondria by degrading preferentially oxidatively-modified or misfolded proteins before they aggregate (7–9). In bacteria, in addition to proteolysis of damaged proteins, Lon also plays a key role in turnover of specific unstable proteins involved in a variety of biological processes (3, 4). Similarly, the steroidogenic acute regulatory protein StAR, several subunits of cytochrome c oxidase, and oxidized mitochondrial aconitase are known to be Lon substrates in animal mitochondria (10–14).

In addition to its proteolytic function, mitochondrial Lon has the ability to bind DNA in vitro (15–17), and has been shown to interact with mtDNA in human cultured cells (18). However, the physiological role of DNA binding by Lon is not clear. In yeast, loss of PIM1, which is the ortholog of animal Lon protease, causes mtDNA deletion, impairs mitochondrial gene expression and results in respiratory deficiency (19, 20). A role for Lon has been postulated in mtDNA replication, transcription, and/or maintenance, but this remains to be validated. Lon was demonstrated to be a component of mitochondrial nucleoids, which are protein: DNA complexes formed to package mtDNA (21, 22). The major protein component of mtDNA nucleoids is mitochondrial transcription factor A (TFAM or mtTFA) (23, 24). TFAM contains two high mobility group (HMG) amino acid sequence boxes; it binds to mtDNA both specifically and nonspecifically (25). TFAM is essential for mtDNA transcription and for mtDNA packaging in mtDNA maintenance (26–30). Interestingly, mtDNA and TFAM levels are interdependent, such that knockdown of TFAM results in mtDNA depletion, and reduction of mtDNA copy number causes reduction of TFAM levels (26, 29, 31).

In this study, we investigated the role of Lon protease in regulating mtDNA maintenance and transcription, and the protein components of mitochondrial nucleoids in cultured cells. Our results argue strongly that Lon modulates mtDNA biogenesis by the selective degradation of TFAM.

Results

Overexpression of Lon Reduces TFAM Levels and mtDNA Copy Number. Mitochondrial localization of the Drosophila Lon gene product (CG8798) was confirmed in Schneider cells by fluorescence microscopy (Fig. S1). Next, Drosophila Lon was subcloned into the inducible expression vector pMt/Hy under the control of the metallothionein promoter. The resulting expression vector, pMt/Lon/Hy, was introduced into Schneider cells, and stable cell lines harboring this plasmid were cultured in media with or without 0.2 mM $CuSO_4$. After 10 d of incubation in the presence of copper, immunoblot analysis indicated a fivefold increase in Lon relative to that in the uninduced control cells (Fig. 1A). In contrast, expression of •-tubulin, used as a control protein, was unchanged. Levels of protein components of the mitochondrial nucleoid were measured by immunoblotting of cells carrying no plasmid, pMt/Hy, or pMt/Lon/Hy.

Overexpression of Lon reduced the level of TFAM to 75% of that in the control cells (Fig. 1 A, B). Levels of other proteins localized in mitochondrial nucleoids, including mtTFB2, mtDNA helicase, pol •-•, and mtSSB, were not changed. We used Southern blots to quantify relative mtDNA copy number in the Lon overexpression cells. We found that the relative mtDNA copy number in the overexpression cells was • 0.7-fold of that in the control cells (Fig. 1C). Northern blots were used to quantify the relative expression of the Cytb, ND4, and 12S rRNA genes in cells grown for 10 d in the presence or absence of copper. Overexpression of Lon did not show any significant changes on these mitochondrial transcript levels as compared to the control cells (Fig. 1D). Furthermore, TFAM mRNA levels were unchanged by Lon overexpression, indicating that the reduction in TFAM protein levels in the knockdown cells does not result from the reduction of TFAM mRNA.

RNAi-Dependent Knockdown of Lon Increases TFAM, Mitochondrial DNA Copy Number, and Mitochondrial Transcription. We reduced the abundance of Lon by expressing a metallothionein-inducible Lon-targeted RNAi species from the plasmid pMt/invLon/Hy. The RNA species produced a form of dsRNA hairpin homolo-

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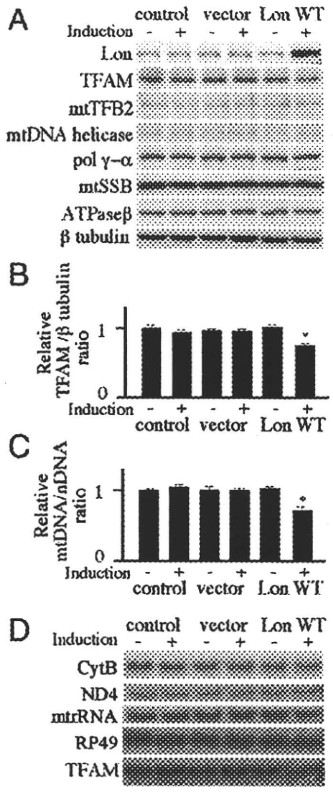

Fig. 1. Expression of Drosophila Lon protease in Schneider cells. Schneider cells with no plasmid (control) or carrying pMt/Hy (vector) or pMt/Lon/Hy (Lon WT) were cultured for 10 d in the presence or absence of 0.2 mM CuSO4. (A) Protein extracts (20 • g) were fractionated by 7.5%, 10.5%, or 13.5% SDS-PAGE, transferred to nitrocellulose filters and probed with antibodies against Lon protease, TFAM, mtTFB2, mtDNA helicase, pol •-•, mtSSB, ATPase •, or • tubulin as indicated. (B) The TFAM/ • tubulin ratio was quantitated by normalizing TFAM protein levels to • tubulin protein levels as described under Materials and Methods. Error bars indicate means • standard error of three independent experiments. The asterisk indicates $P < 0.05$ in comparison to control. (C) Total DNA (10 • g) was extracted from Schneider cells or Schneider cells carrying pMt/Hy or pMt/Lon/Hy that were cultured for 10 d in the presence of 0.2 mM CuSO4. DNA was digested with XhoI, fractionated in a 0.7% agarose/ TBE gel, and then blotted to a nylon membrane. The membrane was hybridized with a radiolabeled probe for CytB, and then stripped and rehybridized with radiolabeled probe for the histone gene cluster as a control. The relative mtDNA copy number was quantitated as described under Materials and Methods. Error bars indicate means • standard error of three independent experiments. The asterisk indicates $P < 0.05$ in comparison to control. (D) Total RNA (10 • g) was extracted from Schneider cells or Schneider cells carrying pMt/Hy or pMt/Lon/Hy after 10 d of culture in the presence or absence of 0.2 mM CuSO4. RNA was fractionated in a 1.2% agarose/formaldehyde gel, blotted to nylon membrane, and hybridized with radiolabeled probes for the mitochondrial transcripts 12S rRNA, ND4, and Cytb, the nuclear transcript RP49, and TFAM.

gous to Lon. Schneider cells stably expressing pMt/invLon/Hy showed the accumulation of oxidized proteins in mitochondria (Fig. S2), but the knockdown cells could be maintained for at least 6 mo under normal culture conditions. Schneider cells stably expressing pMt/invLon/Hy were cultured for 10 d in the absence or presence of 0.2 mM $CuSO_4$. Immunoblot analysis of copper-treated cells showed that cells carrying pMt/invLon/Hy expressed >10-fold less Lon than cells carrying the control vector (Fig. 2A). Even in the uninduced condition, the cells carrying pMt/invLon/Hy suppressed expression of Lon by >10-fold, most likely due to leaky expression (32–35). In contrast, expression of •-tubulin was unchanged. Again levels of mitochondrial nucleoid proteins were measured by immunoblotting of cells carrying no plasmid, pMt/Hy, or pMt/invLon/Hy. Depletion of Lon increased the protein levels of TFAM and mtTFB2 • 1.4-fold relative to their levels in the control cells (Fig. 2 A, B). At the same time, the levels of other mitochondrial nucleoid proteins were not changed significantly. Next, relative mtDNA copy number was measured in the knockdown cells in the presence or absence of copper and found to be • 1.3-fold higher than in the control cells (Fig. 2C). These results suggest that the increase in mtDNA copy number results from the increased TFAM levels.

Northern blots were used to quantitate relative expression of the Cytb, ND4, and 12S rRNA genes in cells grown for 10 d in the presence or absence of copper. Basal expression of Lon-targeted RNAi increased the transcript levels of Cytb, ND4, and 12S rRNA to • 1.4-fold that of the control cells (Fig. 2D). This increase in the mitochondrial transcripts may result from either the increase in mtTFB2 or mtDNA copy number, or both. In contrast, the level of transcripts from the nuclear gene RP49 was unchanged by Lon knockdown. Similar to that observed upon the overexpression of Lon, TFAM mRNA levels were unchanged in the Lon knockdown cells (Fig. 2D), indicating that the increase in TFAM pro-

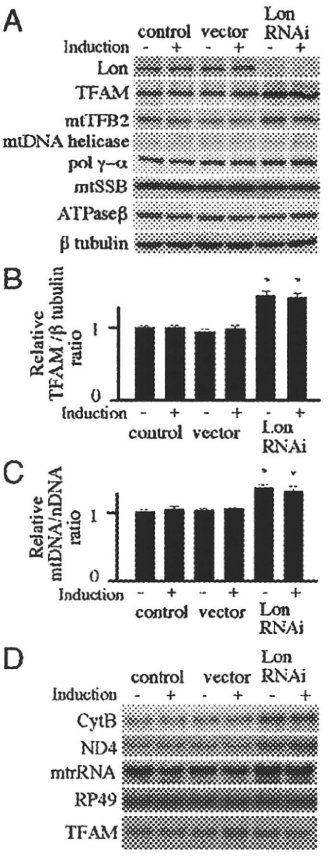

Fig. 2. Expression of Drosophila Lon-targeted RNAi in Schneider cells. Schneider cells with no plasmid (control) or carrying pMt/Hy (vector) or pMt/invLon/Hy (Lon RNAi) were cultured for 10 d in the presence or absence of 0.2 mM CuSO4. (A) Immunoblot analysis was carried out as described in the legend to Fig. 1A. (B) The TFAM/• tubulin ratio was quantitated by normalizing TFAM protein levels to • tubulin protein levels as described under Materials and Methods. Error bars indicate means • standard error of three independent experiments. The asterisk indicates $P < 0.05$ in comparison to control. (C) Relative mtDNA copy number was determined as described in the figure legend to Fig. 1C. Error bars indicate means • standard error of three independent experiments. The asterisk indicates $P < 0.05$ in comparison to control. (D) Northern blot analysis using 12S rRNA, ND4, Cytb, RP49, and TFAM was carried out as described in the legend to Fig. 1D.

tein in the knockdown cells does not result from an increase in the steady-state level of TFAM transcripts.

Lon Regulates mtDNA-Dependent TFAM Degradation in Schneider Cells. Our data show that Lon regulates TFAM levels and mtDNA copy number. Because of the interdependent relationship of TFAM and mtDNA, we asked whether or not Lon degrades TFAM directly. To do so, we cultured the cell lines in the presence of ethidium bromide (EtBr), an inhibitor of mtDNA replication. In the control cells, EtBr treatment results in a rapid reduction of mtDNA copy number, and TFAM protein levels were also reduced, albeit more slowly than that of mtDNA (Fig. 3 A, B). At the same time, TFAM mRNA levels were unchanged (Fig. S3). Because the levels of other mitochondrial nucleoid proteins were not affected by mtDNA depletion with EtBr, we conclude that TFAM is depleted selectively following mtDNA reduction. In the Lon overexpression cells, EtBr treatment also resulted in the reduction of mtDNA (Fig. 3B). Interestingly, here the reduction of TFAM was faster than in the control cells (Fig. 3A). EtBr treatment also caused mtDNA depletion in the Lon knockdown cells. However in this case, the relative level of TFAM protein was increased 1.2-fold (Fig. 3A), while again TFAM mRNA levels were unchanged (Fig. S3). Similar to the control cells, the levels of other mitochondrial nucleoid proteins were unchanged in the EtBr treated Lon knockdown cells.

To demonstrate conclusively the proteolytic role of Lon in mtDNA-dependent TFAM degradation, we established a cell line expressing a Lon mutant carrying a S880A amino acid substitution, in which the conserved serine in the proteolytic active site

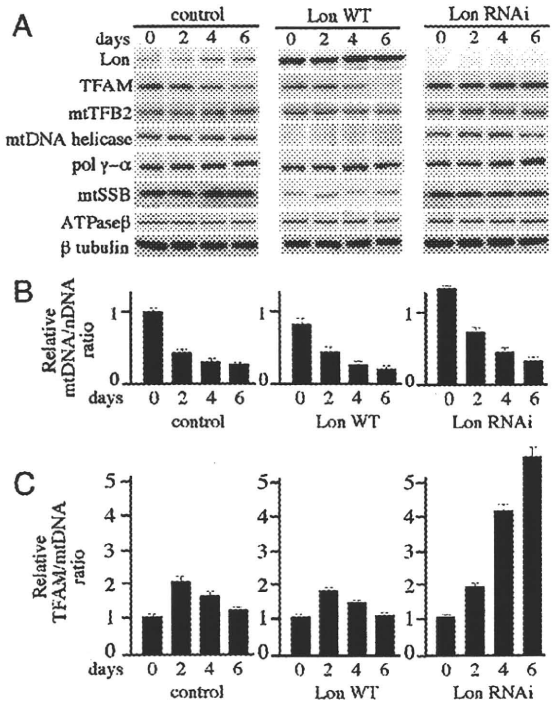

Fig. 3. Dynamics of mitochondrial nucleoid proteins during mtDNA depletion in Lon overexpressing or knockdown Schneider cells. Schneider cells with no plasmid (control) or carrying pMt/Lon/Hy (Lon WT) or pMt/invLon/Hy (Lon RNAi) were cultured for 6 d in the presence of 200 ng• mL EtBr. The cells were harvested prior to EtBr treatment (0 d) and after 2, 4, and 6 days of EtBr treatment. (A) Immunoblot analysis was carried out as described in the legend to Fig. 1A. (B) Relative mtDNA copy number was determined as described in the legend to Fig. 1C. Error bars indicate means • standard error of two independent experiments. (C) The TFAM/ mtDNA ratio was quantitated by normalizing TFAM levels to relative mtDNA copy number. Error bars indicate means • standard error of two independent experiments.

was replaced by alanine. The cell line expressing Lon S880A showed severe retardation of TFAM degradation following mtDNA depletion, likely because overexpression of Lon S880A results in a dominant negative phenotype that is caused by the formation of mixed oligomeric forms (Fig. S4). Taken together, our data show clearly that Lon is responsible for specific degradation of TFAM.

What Is the Physiological Role of Lon Degradation of TFAM? We sought to investigate the functional significance of the specific degradation of TFAM in mtDNA-depleted cells. In control cells treated with EtBr, the TFAM• mtDNA ratio was raised transiently, and then reverted to normal levels within 6 d (Fig. 3C). However, Lon knockdown cells did not recover a normal TFAM• mtDNA ratio. In mammalian cells, overexpression of TFAM causes suppression of mitochondrial transcription (36). We confirmed this phenomenon in Schneider cells overexpressing TFAM under the control of the metallothionein promoter (Fig. S5), and find that suppression occurs in cells containing a TFAM• mtDNA ratio >2 (Fig. 4). Interestingly, the highest overexpression level showed depletion of mtDNA copy number in addition to transcriptional suppression. We thus hypothesized that Lon regulates mtDNA transcription by stabilizing the cellular TFAM• mtDNA ratio. To document this hypothesis, we measured mitochondrial transcript levels in mtDNA-depleted cells. Because EtBr also inhibits mtDNA transcription, we instead induced mtDNA depletion by knockdown of mtDNA replication factors and in particular, the catalytic subunit of mitochondrial DNA polymerase and the mtDNA helicase. Control and Lon knockdown cells were cultured for 10 d with dsRNA targeted against these proteins or GFP as a control, and the relevant protein levels were then evaluated by immunoblotting (Fig. 5A). The cells cultured with GFP dsRNA showed no change in these protein levels, whereas in the cell lines cultured with the mtDNA helicase or mtDNA polymerase dsRNAs, mtDNA copy number was reduced to • 60% of that in the control cells (Fig. 5B). After the dsRNA treatments, the TFAM levels decreased to 75% in control cells, but increased 1.2-fold in the Lon knockdown cells (Fig. 5A). After the dsRNA treatments, the TFAM• mtDNA ratio in the control cells was increased • 1.3-fold relative to the control cells alone, and the ratio in the Lon RNAi cells was increased • 2.5-fold (Fig. 5C). Mitochondrial transcripts in the control cells were unchanged with or without dsRNA treatment (Fig. 5D). However, the transcript levels in mtDNA-depleted Lon RNAi cells were reduced to 47%–60% of those in the mtDNA-depleted cells that showed transcript levels equivalent to 66%–84% of the

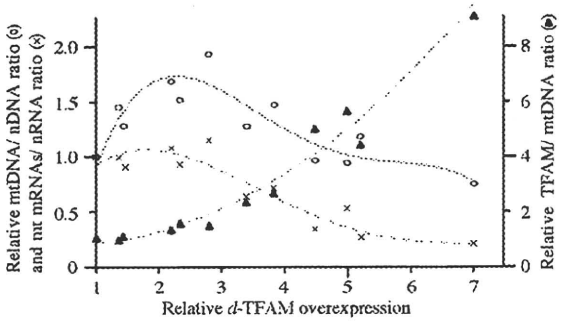

Fig. 4. Expression of TFAM in Schneider cells. Relative ratio of mtDNA copy number (open circles, solid line), mt mRNAs/nRNA (crosses, dotted line), and TFAM/mtDNA (filled triangles, dashed line) were measured at different overexpression levels of TFAM in Schneider cells as indicated. Relative mtDNA copy number was determined as described in the legend to Fig. 1C. The mt mRNAs/nRNA ratio was quantitated by normalizing mitochondrial transcript abundance (ND4 and Cyt b) to that of nuclear Rp49. TFAM/mtDNA ratio was quantitated by normalizing TFAM protein levels to relative mtDNA copy number.

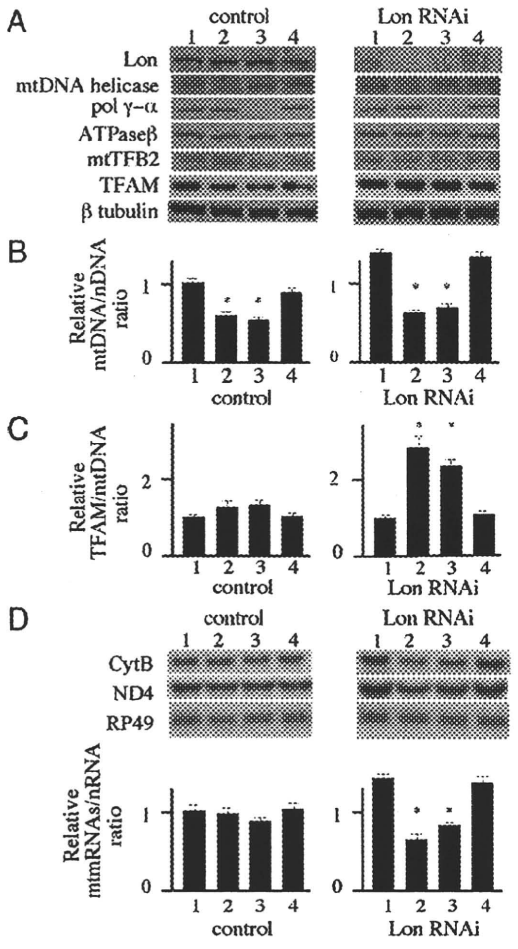

Fig. 5. Effects of Lon knockdown on mitochondrial transcript levels after mtDNA depletion in Schneider cells. Schneider cells carrying no plasmid (control) and or pMt/invLon/Hy (Lon RNAi) were cultured for 10 d in the presence or absence of dsRNA of mtDNA helicase, pol •-•, or GFP as control. (A) Immunoblot analysis was carried out as described in the legend to Fig. 1A. (B) Relative mtDNA copy number was determined as described in the legend to Fig. 1C. Error bars indicate means • standard error of two independent experiments. The asterisk indicates $P < 0.05$ in comparison to the control. (C) The ratio of TFAM/mtDNA was determined as described in the legend to Fig. 3C. Error bars indicate means • standard error of two independent experiments. The asterisk indicates $P < 0.05$ in comparison to each cell that was cultured in the absence of dsRNA. (D) Northern blot analysis using ND4, Cytb, and RP49 was carried out as described in the legend to Fig. 1D. Relative mitochondrial mRNA levels were quantitated by normalizing ND4 and Cytb abundance to that of RP49. Error bars indicate means • SE of two in dependent experiments. The asterisk indicates $P < 0.05$ in comparison to the control.

control cells. Interestingly, the reduced mitochondrial transcript levels were lower than that of control cells. Together, these results indicate that Lon regulates mitochondrial transcription by controlling the TFAM•mtDNA ratio.

Discussion

We have established that Lon knockdown cell lines express <10% of endogenous Lon protein levels, yet these cells grow for at least 6 mo. In human fibroblast cells, depletion of Lon over 4 d resulted in apoptotic cell death (37, 38), whereas Lon knockdown in human colon carcinoma cells allows survival for at least 15 d (18). Thus, the effects of Lon depletion may be species- or cell type- specific. As in a recent report with human rhabdomyosarcoma cells (39), depletion of Lon protease in Drosophila Schneider cells results in an increase in the levels of oxidized proteins in mitochondria, indicating that Lon is responsible for degradation of oxidized mitochondrial proteins, and suggesting that variations in cell viability as a result of Lon depletion may reflect varying cellular tolerances for oxidative damage to mitochondrial proteins.

It is known that TFAM protein levels are reduced coincident with mtDNA depletion in animal cells, but the mechanism for this phenomenon is unclear (31). Here, we show that Lon is responsible for degradation of TFAM upon mtDNA depletion. Moreover, Lon may be also responsible for TFAM degradation under normal conditions, because the cellular level of TFAM varies in concert with Lon levels. These findings imply that TFAM turnover is strongly dependent on Lon protease function. In addition to TFAM, we found that the mtTFB2 level is increased in Lon knockdown cells, so it may also be a specific substrate for the Lon protease. Other mitochondrial biogenesis proteins tested were unchanged appreciably in either Lon knockdown or overexpression cells. In Escherichia coli, some substrates for Lon overlap those of other AAA$^+$ proteases such as the ClpP protease; notably, the ClpP ortholog in animal cells comprises another major protease in the mitochondrial matrix space (2, 3, 40). Therefore, the depletion of mitochondrial Lon may be compensated partially by mitochondrial ClpP protease. Alternatively, the residual Lon in knockdown cells may be sufficient to degrade its protein targets.

We found that mitochondrial transcripts in Lon knockdown cells are increased moderately in association with an increase of TFAM and mtTFB2 levels. Previous studies showed that the relative levels of mtDNA and TFAM are not critical to observe stimulation of mitochondrial transcription in Drosophila Kc167, and Schneider cells (29, 35). We suggest that in contrast, increase of mtTFB2, which is essential for mitochondrial transcription, may be responsible for up-regulation of mitochondrial transcription.

We show that degradation of TFAM by Lon protease is facilitated by mtDNA depletion. Interestingly, a similar phenomenon was reported in Bacillus subtilis. B. subtilis LonA, which is the ortholog of Lon, is involved in degradation of the structural maintenance of chromosomes protein (SMC), and the degradation of SMC is facilitated by DNAase treatment (41). Although the mechanisms by which Lon recognizes its target proteins are not well understood, a recent report showed that E. coli Lon recognizes specific aromatic residue-rich sequences that are hidden in the hydrophobic cores of native structures, but are accessible in unfolded structures (42). Interestingly, the HMG boxes in TFAM contain four conserved aromatic residues within a hydrophobic core, and these residues may be masked when TFAM binds DNA (25, 27, 29). Another possible explanation is that TFAM not bound to mtDNA becomes exposed to oxidative stress, whereas TFAM bound to mtDNA comprises part of the core of the mitochondrial nucleoids, and is thus surrounded by other proteins (43). Mitochondrial Lon has the ability to bind DNA and localizes in mitochondrial nucleoids (15–18). Our current hypothesis is that excess, free TFAM is degraded by Lon before the TFAM binds DNA. Alternatively, it seems possible, albeit more complicated, that excessive DNA compaction resulting from binding of high TFAM levels signals the degradation of TFAM by DNA-bound Lon. Further experiments are warranted to address these and other possibilities, and to clarify the link between the DNA-binding activity of Lon and TFAM turnover.

Suzuki and colleagues showed that mtDNA copy number was unchanged after 15 d of Lon knockdown in human colon carcinoma cells (18). This model differs from ours, in which we observe an increase in mtDNA copy number. We established our Lon knockdown cells over a period of 8 wks and because of leaky expression from the inducible promoter in the RNAi vector, Lon depletion was already ongoing prior to the 10 d induction

period. Thus, one possible explanation for the difference we observe is that TFAM accumulation may be slower in their model and consequently, increased mtDNA copy number might not have been apparent. Another possible explanation is that compensation by other proteases such as ClpP (2, 40) might effect TFAM degradation in the Lon-depleted human colon carcinoma cells.

We found that upon EtBr treatment, the TFAM• mtDNA ratio is nearly restored within 6 d in both control and Lon overexpressing cells, whereas restoration did not occur in Lon knockdown cells. Moreover, TFAM turnover resulting from mtDNA depletion by EtBr treatment is strongly reduced in cells overexpressing a protease-deficient Lon variant, which shows a dominant negative effect. Similar results involving restoration of normal TFAM/ mtDNA ratios were produced in the case of knockdowns of mtDNA helicase and mtDNA polymerase, though there the reduction of mtDNA is lower as compared to that upon EtBr treatment. Until stabilization occurs, excess overexpression of TFAM results in reduction of mitochondrial transcript levels; notably, in TFAM overexpressing cells, ratios of TFAM• mtDNA >2 show inhibitory effects on mitochondrial transcription. In the case of knockdown of mtDNA helicase or mtDNA polymerase, mitochondrial transcription levels are unchanged in control cells upon mtDNA depletion, whereas Lon knockdown cells showed a similar reduction in mitochondrial transcripts. Upon knockdown of mtDNA helicase or mtDNA polymerase, the TFAM• mtDNA is • 1.3 in control cells and >2 in Lon knockdown cells, consistent with the observations in TFAM overexpression cells. Because there are environmental conditions under which mtDNA copy number is known to vary, such as during development and upon drug treatment (44, 45), transient Lon degradation of excess TFAM would be important to maintain normal mitochondrial transcription levels. We conclude that the TFAM• mtDNA ratio is crucial for both mtDNA biogenesis and homeostasis, and that Lon stabilizes the TFAM• mtDNA ratio by degradation of excess TFAM. With the example of TFAM, we provide direct evidence of the physiological role of Lon protease activity in mtDNA maintenance in animal cells, warranting future study of its potential involvement in the surveillance and turnover of other proteins involved in mtDNA replication, transcription, and translation.

Materials and Methods

Preparation of Lon Antibody. A recombinant protein corresponding to amino acids Asp613 to Ser838 of Drosophila Lon (CG8798) was used to immunize rabbits to obtain polyclonal antibody.

Preparation of Inducible Plasmids Expressing Lon, TFAM, and Lon-Targeted RNAi. The plasmid pMt/Lon/Hy, in which Lon is regulated by the metallothionein promoter and the plasmid pMt/invLon/Hy, which carries an inverted repeat of a nucleotide sequence from Lon cDNA that is transcribed from the metallothionein promoter were constructed as described in the SI Text. Plasmid pMt/TFAM/Hy is as described previously (35).

Generation and Induction of Stable Cell Lines. Drosophila Schneider S2 cells were cultured at 25 °C in Drosophila Schneider Medium (Invitrogen) supplemented with 10% FBS. Cells were subcultured to 3 • 10^6 cells• mL every fifth day. Cells were transfected using Effecten (QIAGEN). Hygromycin-resistant cells were selected with 200 • g• mL hygromycin. Cells were passaged for 8 wks in hygromycin-containing medium and then cultured in standard medium. The cell lines were grown to a density of 3 • 10^6• mL and then treated with 0.2 mM $CuSO_4$ to induce expression from the metallothionein promoter.

Immunoblotting. Total cellular protein (20 •g per lane) was fractionated by 13.5%, 10.5%, or 7.5% SDS-PAGE and transferred to nitrocellulose filters. Immunoblotting was performed as described previously (46). Protein bands were visualized using ECL Western blotting reagents (Amersham). ECL luminescence was quantified on a Kodak Image Station 4000R. Antibodies against Drosophila mtSSB (47), TFAM (29), Pol •-• (48), mtDNA helicase (32), ATPase • (32), mtTFB2 (35), and • tubulin (E7) (Developmental Studies Hybridoma Bank) were prepared and used as described. The ratio of the signals for TFAM and • tubulin was used to estimate the relative protein levels of TFAM. The TFAM immunoblotting experiments shown in Figs. 1, 2, 3, and 5 were performed two or thee times with each of the two or three independent cell lines carrying each plasmid construct, including control (no plasmid). The data presented represent one such experiment, and the quantitation is provided for the duplicate or triplicate experiments from one of two or thee cell lines.

Northern and Southern Blotting. Northern blotting was performed as described previously (46), and the data were analyzed using a PhosphoImager (Molecular Dynamics). The signal for RP49 was used to normalize mitochondrial transcripts.

Southern blotting was performed as described previously (32), and the data were analyzed using a PhosphoImager (Molecular Dynamics). Blots were probed with radiolabeled DNAs for the mitochondrial gene Cytb and the nuclear histone gene cluster. The ratio of the signals for these two genes was used to estimate the relative copy number of mtDNA. The Northern and Southern blot experiments shown in Figs. 1, 2, 3, 4, and 5 were performed two or thee times with each of the two or three independent cell lines carrying each plasmid construct, including control (no plasmid). The data presented represent one such experiment, and the quantitation is provided for the duplicate or triplicate experiments from one of two or thee cell lines.

RNA Interference. To generate double-stranded RNA (dsRNA) for RNAi, sequences directed against the protein to be silenced were amplified by PCR from each cDNA. Each primer used in the PCR contained a T7 RNA promoter followed by sequences specific for the targeted genes. The following primer sets were used for each protein: mtDNA helicase for GTAATACGACTCACTATAGGGCATGGAAAATGAGACGCGC and GTAATACGACTCACTATAGGGGATTGCTGACTAGAACCGC; DNA polymerase •-• for TAATACGACTCACTATAGGGTGCCTACGCCTGCGGTGAGC and TAATACGACTCACTATAGGGCTCCAATGCTCGACTAAGAC; GFP for GTAATACGACTCACTATAGGGGGAGAAGAACTTTTCACTGG and GTAATACGACTCACTATAGGGTCTGCTAGTTGAACGCTTC. PCR products were used as templates for in vitro transcription using the T7 Megascript RNAi kit (Ambion). 3 • 10^7 S2 Drosophila tissue culture cells were plated into a T25 flask in 5 mL of medium without FBS. 100 • g of dsRNA was added and mixed by swirling. After 30 min, 5 mL of media containing 20% FBS was added. The cells were collected after 5 d culture and repeat dsRNA treatment. After 5 d after second dsRNA treatment, the cells were harvested for the analysis.

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Supporting Information

Matsushima et al. 10.1073/pnas.1008924107

SI Text

SI Materials and Methods. *Preparation of inducible plasmids expressing cMyc-Lon and the Lon S880A mutant.* The plasmid pMt/Lon/Hy, in which Lon is regulated by the metallothionein promoter, was constructed as follows: a fragment of Drosophila Lon cDNA was amplified by PCR using as 5•-primer 5•- GGGCTCGAGTGCGAGTGGATATTGCTTTC -3• and as 3•-primer 5•- GCGCACTAGTATTACAAGTCTTCTTCAGAAATAAGCTTTTGAGAATAAGGCCACGTCTC -3•. The PCR fragment was cleaved by XhoI and SpeI and subcloned.

pMt/S880A/Hy was constructed from pMt/Lon/Hy by site directed mutagenesis using the following pair of primers: 5•-AGATGGCCCCGCTGCGGGCATC -3• and 5•- GATGCCCGCAGCGGGGCCATCT -3•.

Detection of carbonylated proteins. The OxyBlot procedure (Millipore) was used to perform immunoblot detection of oxidatively modified proteins by the generation of carbonyl groups. Ten micrograms of protein were used for each reaction. Carbonyl groups in mitochondrial protein samples (10 • g) were derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine. Carbonylated proteins were detected by immunoblot analysis using an anti-DNP antibody.

Indirect immunofluorescence. Indirect immunofluorescence was performed as described (1) with anti-c-Myc monoclonal antibody (Sigma) and Alexa Fluor 488 anti-mouse IgG (Molecular Probes).

Preparation of inducible plasmids expressing Lon, TFAM, and Lon-Targeted RNAi. The plasmid pMt/Lon/Hy, in which Lon is regulated by the metallothionein promoter, was constructed as follows: a fragment of Drosophila Lon cDNA was amplified by PCR using as 5•-primer 5•- GGGCTCGAGTGCGAGTGGATATTGCTTTC -3• and as 3•-primer 5•- GCGCACTAGTCTAAGAATAAGGCCACGTCTC -3•. The PCR fragment was cleaved by XhoI and SpeI and subcloned. The plasmid pMt/TFAM/Hy was as described previously (2). The plasmid pMt/invLon/Hy carries an inverted repeat of a nucleotide sequence from Lon cDNA that is transcribed from the metallothionein promoter. The insert in pMt/invLon/Hy was generated from two PCR-amplified fragments of Lon cDNA. One fragment has terminal XhoI and EcoRI sites and was prepared using the following pair of primers: 5•- GCGCCTCGAGACTAGTGGGATGATTCCAACGGGGAT -3• (forward) and 5•- GCGCGAATTCGGGATCGATTCCGCTTGATCAGTGCTTTG -3•(reverse). A second fragment has terminal SpeI and EcoRI sites and was prepared using the primers 5•- GCGCCTCGAGACTAGTGGGATGATTCCAACGGGGAT -3• (forward) and 5•- GCGCGAATTCAAAAAGCTTTCCGCTTGATCAGTGCTTTG -3• (reverse). The two PCR products were ligated and cloned into the pMt/Hy vector cleaved with XhoI and SpeI.

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2. Matsushima Y, Garesse R, Kaguni LS (2004) Drosophila mitochondrial transcription factor B2 regulates mitochondrial DNA copy number and transcription in schneider cells. J Biol Chem 279:26900–26905

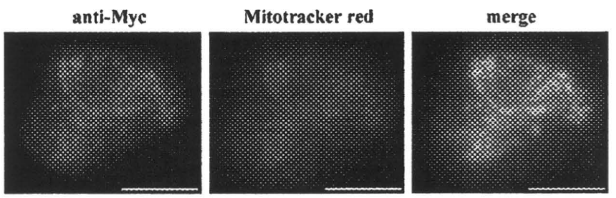

Fig. S1. Overexpression of Drosophila Lon in Schneider cells. Immunocytochemistry was performed on Schneider cells that were transiently transfected with pMK/Lon-Myc/Hy using anti-c-Myc monoclonal antibody (Sigma), and counterstained with Mitotracker Red.

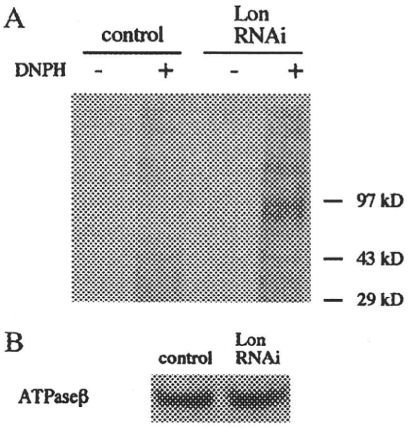

Fig. S2. Protein oxidation status in mitochondria from Lon knockdown cells. Mitochondrial fractions were prepared from Schneider cells carrying no vector (control) or pMt/invLon/Hy (Lon RNAi). (A) Detection of oxidized protein was performed using the Oxyblot protocol (Millipore). Mitochondrial protein extracts (10 • g) were incubated in the presence or absence of 2,4-dinitrophenylhydrazine (DNPH) to derivatize protein carbonyl groups. After fractionation by 4%–10% gradient SDS-PAGE, the proteins were transferred to a nitrocellulose filter, and oxidized proteins were detected with antibody against DNP. (B) Mitochondrial protein extracts (10 • g) were fractionated by 12% SDS-PAGE, transferred a nitrocellulose filter and probed with affinity-purified rabbit antiserum against ATPase • as a control.

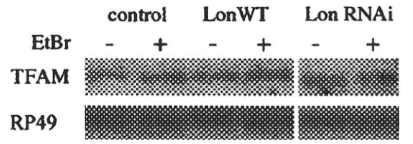

Fig. S3. Unchanged TFAM mRNA levels in Schneider cells upon EtBr treatment. After 6 d culture, Total RNA was isolated from Schneider cells with no plasmid (control) or carrying pMt/Lon/Hy (Lon) or pMt/invLon/Hy (Lon RNAi) that were cultured for 6 d in the presence or absence of 200 ng• mL EtBr. Northern blot analysis using TFAM and RP49 probes was carried out as described in the legend to Fig. 1D.

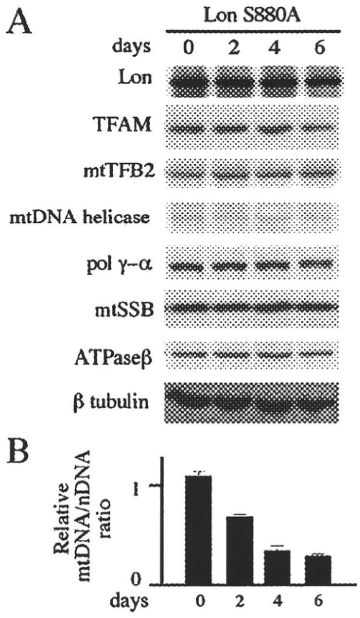

Fig. S4. Steady-state levels of mitochondrial nucleoid proteins during mtDNA depletion in Lon S880A- overexpressing Schneider cells. Schneider cells carrying pMt/S880A/Hy (S880A) were cultured for 6 d in the presence of 200 ng• mL EtBr. The cells were harvested prior to and after EtBr treatment at 0, 2, 4, and 6 days. (A) Immunoblot analysis was carried out as described in the legend to Fig. 1A. (B) Relative mtDNA copy number was determined as described in the legend to Fig. 1C.

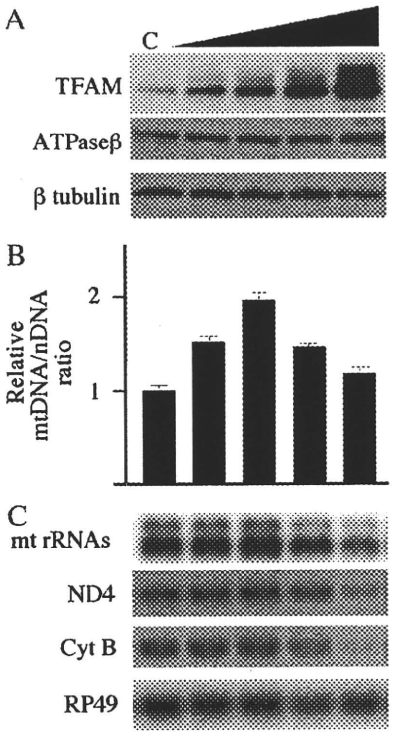

Fig. S5. Expression of Drosophila TFAM in Schneider cells. Schneider cells (C) or cells carring pMt/TFAM/Hy were grown for 10 d in the presence of 0, 0.05, 0.1, or 0.4 mM CuSO4. (A) Immunoblot analysis was carried out as described in the legend to Fig. 1A. (B) Relative mtDNA copy number was determined as described in the legend to Fig. 1C. Error bars indicate means • standard error of two independent experiments. (C) Northern blot analysis using 12S rRNA, ND4, Cytb, and RP49 was carried out as described in the legend to Fig. 1D.

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ORIGINAL ARTICLE
Molecular Cardiology

Impact of Renin-Angiotensin System Polymorphisms on Development of Systolic Dysfunction in Hypertrophic Cardiomyopathy

– Evidence From a Study of Genotyped Patients –

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Background: Although the renin–angiotensin system (RAS) can affect the development of left ventricular (LV) hypertrophy, few data exist regarding the relationships between RAS polymorphisms and alteration of LV function. The effect of RAS polymorphisms on LV function in genotyped hypertrophic cardiomyopathy (HCM) was examined in the present study.

Methods and Results: The study group comprised 126 carriers with sarcomere gene mutations from 49 HCM families (64 males, mean age 51±21 years). LV morphology and function were evaluated by echocardiography. In angiotensin-converting enzyme (ACE) insertion/deletion (I/D), the D allele (n=81) exhibited significantly larger LV end-systolic dimension (LVDs) (32±11 mm) and lower ejection fraction (56±15%) than those with the II genotype (28±7 mm and 62±12%, respectively, P<0.05; n=45). Although angiotensin II type 1 receptor (AT_1-R) A/C^{1166} polymorphism did not affect echocardiographic parameters, the presence of the ACE D allele with the AT_1-R C^{1166} allele (n=9) was associated with larger LVDs (37±17 mm) and lower ejection fraction (48±20%) compared with other genotypes (30±9 mm and 58±14%, respectively, P<0.05; n=117). Under these conditions, severe LV hypertrophy was frequently associated with LV wall thinning.

Conclusions: The presence of both the ACE D and AT_1-R C^{1166} allele is associated with LV dilation with systolic dysfunction in genotyped HCM. In addition to the severity of LV hypertrophy, screening for these RAS polymorphisms could contribute to further risk stratification of patients with HCM, although other genetic polymorphisms should be further examined. (*Circ J* 2010; **74:** 2674–2680)

Key Words: Angiotensin-converting enzyme; Hypertrophic cardiomyopathy; Left ventricular remodeling; Renin–angiotensin system; Systolic dysfunction

Hypertrophic cardiomyopathy (HCM) is a primary cardiac disorder that has been defined as a left ventricular (LV) hypertrophy without other cardiovascular disease, and often transmits with heterogeneous clinical and morphological expression. Although many HCM patients experience a relatively benign course, there still remains a high risk of adverse cardiac events, such as sudden death, heart failure and embolism because of atrial fibrillation. Therefore, a major challenge in the management of the broad spectrum of HCM disease has been identifying the subsets of patients predisposed to sudden death and unexpected or heart failure-related death.[1–3] In fact, some patients show progression of systolic dysfunction with and without the LV dilatation that is characteristic of dilated cardiomyopathy,[4–6] although heart failure in HCM is largely the consequence of diastolic dysfunction.[7] However, the predisposing or precipitating causes of developing systolic dysfunction are not fully determined.

Differences in the clinical manifestations of HCM can be related to the presence of different disease-causing genes that encode sarcomeric proteins or different mutations within a given gene. However, the phenotypic expression and clinical course of HCM are also heterogeneous, even within families with an identical etiological sarcomere gene mutation.[8–10] This may be influenced by additional genetic factors, such as renin–angiotensin system (RAS) polymorphisms,

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which can affect both LV hypertrophy and remodeling.[11] It is also intriguing to speculate whether the severity of LV hypertrophy (LVH) itself can be associated with development of LV dysfunction.[4-6] Therefore, the aim of this study was to examine the potential relationship between RAS polymorphisms and development of systolic dysfunction in HCM patients based on a molecular genetic diagnosis.

Methods

Study Population

This study included 49 unrelated patients with HCM exhibiting disease-causing mutations in genes such the beta-myosin heavy chain (*MYH7*), cardiac myosin binding protein-C (*MYBPC3*), cardiac troponin T (*TNNT2*), and cardiac troponin I (*TNNI3*). In addition, their family members were clinically and genetically examined and 77 carriers with the same etiological sarcomere gene mutation as each patient were identified. Thus, total 126 genetically-affected patients (64 males, mean age 51±21 years) comprised the study population. All patients were identified at the Kanazawa University Hospital or its affiliated hospitals between 1998 and 2006.

The diagnosis of HCM was based on echocardiographic findings, such as maximal LV wall thickness ≥13 mm and the absence of any other cause of LVH as specified in the criteria of Maron et al.[12] Patients with systolic dysfunction were also included in this study: patients with (1) progression from typical HCM to systolic dysfunction during follow-up, and (2) typical HCM with an identical sarcomere gene mutation identified in the same family without any other cause of systolic dysfunction. To further examine whether hypertrophy itself can be a risk factor for developing LV dysfunction, the changes in the echocardiographic parameters from initial to last evaluation divided by follow-up period were additionally adopted as a sub-analysis. Written informed consent was given by all patients in accordance with the guidelines of the Bioethical Committee on Medical Research, School of Medicine, Kanazawa University, Kanazawa, Japan.

Genetic Studies

Genomic DNA was purified from patients' white blood cells, after which in vitro gene amplification was performed by polymerase chain reaction (PCR). Oligonucleotide primers were used to amplify all exons and exon/intron boundaries of 4 sarcomere genes, namely, *MYH7*, *MYBPC3*, *TNNT2*, and *TNNI3*, using standard protocols, as described previously.[13-16] Single-strand conformational polymorphism analysis of amplified DNA was then performed. For abnormal single-strand conformational polymorphism patterns, the nucleotide sequences of the cloned PCR products were determined on both strands (bidirectional sequencing) by the dye terminator cycle sequencing method using an automated fluorescent sequencer (ABI PRISM™ 310 Genetic Analyzer, PE Biosystems, Foster City, CA, USA). The sequence variation was confirmed by restriction enzyme digestion.

Determination of RAS-Related Gene Polymorphisms

In this study, we chose the angiotensin-converting enzyme (ACE) insertion/deletion (I/D) and the angiotensin II type 1 receptor (AT_1-R) A/C^{1166} from among the many RAS polymorphisms because these 2 polymorphisms (ie, the ACE D allele (DD and ID genotypes) and the AT_1-R C^{1166} allele (CC and AC genotypes)) are reported to be associated with increased LVH in HCM.[17-19] Gene polymorphisms of ACE I/D were identified by PCR performed with a set of oligonucleotide primers flanking the polymorphic site in intron 16. To avoid mistyping, each sample found to have the DD genotype was subjected to a second round of independent PCR amplification with a primer pair that recognized an insertion-specific sequence, as described previously.[20] The AT_1-R A/C^{1166} polymorphism was determined by PCR and restriction fragment length polymorphisms, as previously reported.[21] PCR was performed to amplify a fragment encompassing the polymorphic site at nucleotide position 1166 in the 3' untranslated region of the human *AT_1-R* gene. PCR products were then digested with restriction enzyme DdeI (Toyobo, Osaka, Japan) and the cleaved products were separated by electrophoresis.

Table 1. Demographic and Clinical Characteristics of the Study Population

n	126
Age (years)	51±21
Male (%)	64 (51)
Echocardiography	
LAd (mm)	40±9
IVST (mm)	15±6
PWT (mm)	10±2
MWT (mm)	16±6
LVDd (mm)	46±8
LVDs (mm)	30±10
EF (%)	58±14
Disease-causing gene (%)	
MYH7	17 (14)
MYBPC3	35 (28)
TNNT2	23 (18)
TNNI3	51 (40)
Renin-angiotensin system polymorphisms (%)	
ACE I/D	
DD	25 (20)
ID	56 (44)
II	45 (36)
AT_1-R A/C^{1166}	
AA	112 (89)
AC	14 (11)
CC	0 (0)
Combination of ACE I/D and AT_1-R A/C^{1166}	
ACE D allele with AT_1-R C allele	9 (7)
Others*	117 (93)

Values are mean±SD unless otherwise shown.
LAd, left atrial dimension; IVST, interventricular septal thickness; PWT, posterior wall thickness; MWT, maximal wall thickness; LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; EF, ejection fraction; *MYH7*, β-myosin heavy chain; *MYBPC3*, cardiac myosin binding protein-C; *TNNT2*, cardiac troponin T; *TNNI3*, cardiac troponin I; ACE, angiotensin-converting enzyme; I, insertion; D, deletion; AT_1-R, angiotensin II type 1 receptor; D allele, DD and ID genotype; C allele, CC and AC genotype.
*Others comprises ACE D allele with AT_1-R AA^{1166} genotype, ACE II genotype with AT_1-R C^{1166} allele and ACE II genotype with AT_1-R AA^{1166} genotype.

Echocardiographic Examinations

Standard transthoracic M-mode and 2-dimensional echocardiographic studies were performed to identify and quantify

Table 2. Differences in Patients' Characteristics Among 4 Sarcomere Gene Mutations

	MYH7 (n=17)	*MYBPC3* (n=35)	*TNNT2* (n=23)	*TNNI3* (n=51)	P value
Age (years)	57±18	52±21	53±19	46±22	0.272
Echocardiography					
LAd (mm)	38±9	38±8	41±8	42±9	0.145
IVST (mm)	16±6	15±3	16±7	13±5	0.128
PWT (mm)	11±3	11±2	10±2	10±2	0.513
MWT (mm)	16±6	16±6	18±7	14±5	0.160
LVDd (mm)	46±9	44±6	48±12	45±7	0.380
LVDs (mm)	30±12	28±7	34±15	30±8	0.184
EF (%)	59±4	61±12	54±4	57±13	0.313
Renin-angiotensin system polymorphisms					
Frequency of ACE I/D polymorphisms					
D allele (%)	12 (86)	16 (46)	15 (65)	38 (75)	0.048
II (%)	5 (14)	19 (54)	8 (35)	13 (25)	
Frequency of AT_1-R A/C^{1166} polymorphisms					
C allele (%)	1 (6)	2 (6)	6 (3)	5 (10)	0.078
AA (%)	16 (94)	33 (94)	17 (97)	46 (90)	
Frequency of combination of ACE I/D and AT_1-R A/C^{1166} polymorphisms					
ACE D allele with AT_1-R C allele (%)	1 (6)	1 (3)	3 (13)	4 (8)	0.521
Others (%)	16 (94)	34 (97)	20 (87)	47 (92)	

Values are mean±SD unless otherwise shown.
Abbreviations as in Table 1.

the morphologic features of the LV. LV end-diastolic dimension (LVDd) and end-systolic dimension (LVDs), and the thicknesses of the interventricular septum (IVST) and LV posterior wall (PWT) were measured at the level of the tips of the mitral valve leaflets. Additionally, we measured the anterior and lateral walls of the LV at the same levels used for measuring the septum and posterior walls. From these measurements, we defined the maximal LV wall thickness (MWT). Ejection fraction (EF) was calculated by Teichholz's method and by modified Simpson's method when LV dilatation or regional decrease of LV wall motion occurred. The left atrial dimension was measured at end-systole.

Statistical Analysis

Values are expressed as mean±SD. Measured values were compared between 2 groups with an unpaired Student's t-test. Comparison between groups was performed using a one-way analysis of variance followed by Scheffé's method. Categorical variables were compared by the chi-square test for independence. A P-value<0.05 was considered statistically significant. Statistical analyses were carried out with the computer software StatView for Windows version 5.0 (Abacus Concepts, Inc, Berkeley, CA, USA).

Results

Genetic Results and Clinical Characteristics of the Study Population

The demographic and clinical characteristics of the study population are presented in Table 1; 15 different mutations were identified in 126 patients and among them, 17 patients had mutations in *MYH7* (Ala26Val, n=4; Arg204His, n=1; Arg858Cys, n=4; Arg870Cys, n=1; Gly733Glu, n=2; Met822Leu, n=2; and Glu935Lys, n=3), 35 patients had mutations in *MYBPC3* (Arg820Gln, n=15; c.2067+1G>A, n=15; and Del593C, n=5), 23 patients had mutations in *TNNT2* (Arg92Trp, n=10; Lys273Glu, n=10; Val85Leu, n=1; and Phe110Ile, n=2), and 51 patients had a mutation in *TNNI3* (Lys183Del; n=51). All of these mutations have been identified and described elsewhere.[22,23]

As for the frequencies of ACE I/D, 36% were II, 44% were ID and 20% were DD. For the AT_1-R A/C^{1166}, 89% were AA and 11% were AC (Table 1); 9 of the 126 patients had both the ACE D and AT_1-R C^{1166} alleles and of these 9 patients 7 were from 7 unrelated kindred with genetic mutations (*MYH7*=1, *MYBPC3*=1, *TNNT2*=1 and *TNNI3*=4), and the other 2 were from a kindred with a *TNNT2* mutation in which 1 was typical HCM and the other had systolic dysfunction.

The differences in the underlying sarcomere gene mutations are shown in Table 2. There were no differences between the 4 genes with respect to echocardiographic parameters. Regarding the relationship between sarcomere gene mutations and RAS polymorphisms, the proportion of the II genotype of ACE I/D polymorphisms was higher than that of the D allele only in association with *MYBPC3* mutation (Table 2).

Echocardiographic Parameters and RAS Polymorphisms

Among the ACE I/D polymorphisms, the presence of the dominant D allele (DD and ID genotypes) was associated with significantly decreased PWT (10±2mm vs 11±3mm, P<0.05), larger LVDs (32±11mm vs 28±7mm, P<0.05) and lower EF (56±15% vs 62±12%, P<0.05) than in those with the II genotype. We demonstrate 2 representative patients who showed entirely different clinical manifestations, despite having an identical mutation of Arg92Trp in *TNNT2*.[24] One had dilated cardiomyopathy-like features with the ACE D

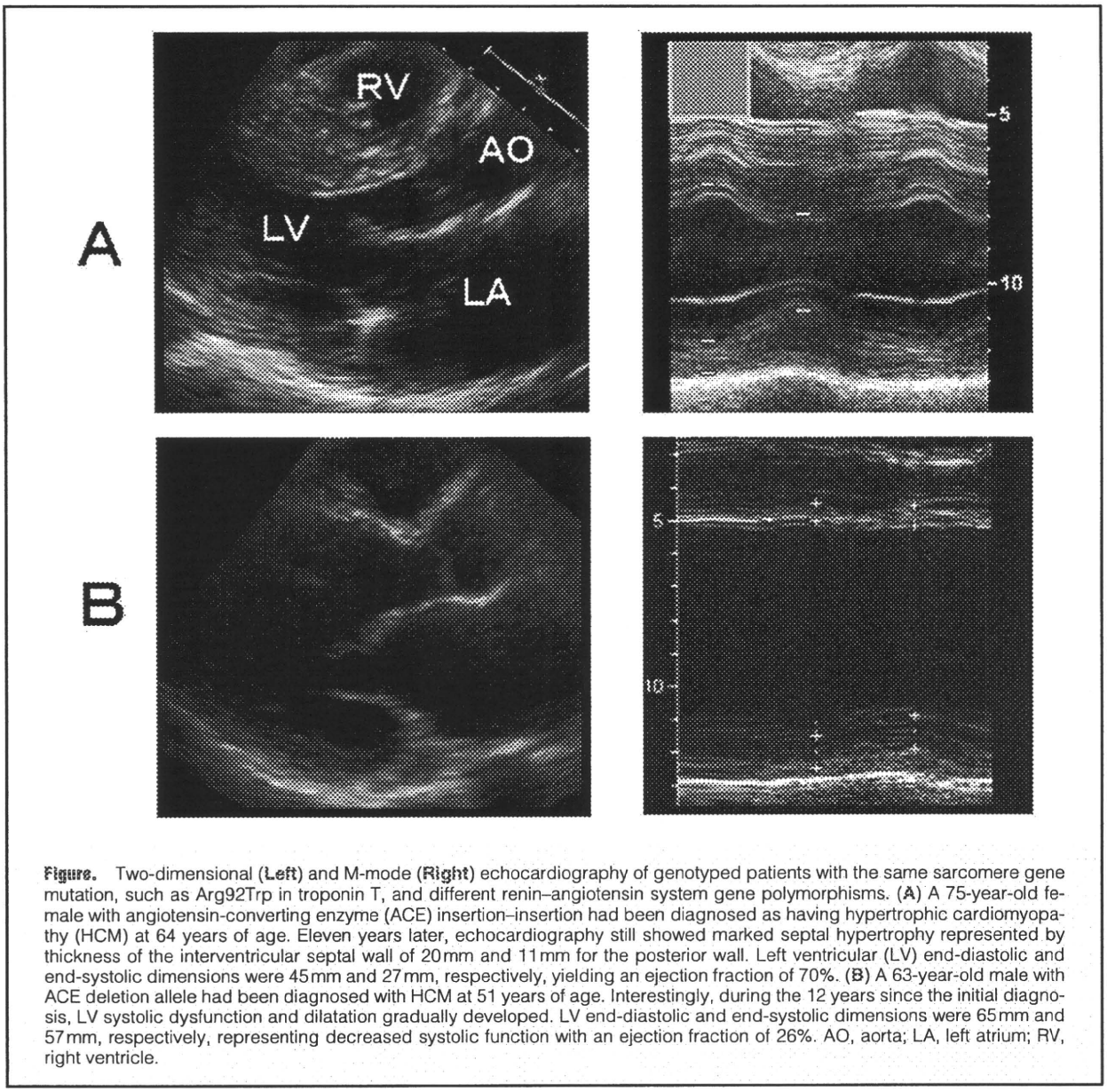

Figure. Two-dimensional (**Left**) and M-mode (**Right**) echocardiography of genotyped patients with the same sarcomere gene mutation, such as Arg92Trp in troponin T, and different renin–angiotensin system gene polymorphisms. (**A**) A 75-year-old female with angiotensin-converting enzyme (ACE) insertion–insertion had been diagnosed as having hypertrophic cardiomyopathy (HCM) at 64 years of age. Eleven years later, echocardiography still showed marked septal hypertrophy represented by thickness of the interventricular septal wall of 20 mm and 11 mm for the posterior wall. Left ventricular (LV) end-diastolic and end-systolic dimensions were 45 mm and 27 mm, respectively, yielding an ejection fraction of 70%. (**B**) A 63-year-old male with ACE deletion allele had been diagnosed with HCM at 51 years of age. Interestingly, during the 12 years since the initial diagnosis, LV systolic dysfunction and dilatation gradually developed. LV end-diastolic and end-systolic dimensions were 65 mm and 57 mm, respectively, representing decreased systolic function with an ejection fraction of 26%. AO, aorta; LA, left atrium; RV, right ventricle.

allele and the other with an ACE II genotype had typical HCM (**Figure**). Interestingly, the presence of the ACE D allele with the AT1-R C^{1166} allele was associated with significantly larger LVDs (37±17 mm vs 30±9 mm, P<0.05) and lower EF (48±20% vs 58±14%, P<0.05) compared with others (**Table 3**), although AT1-R A/C^{1166} polymorphism itself did not influence the echocardiographic parameters.

When patients were divided into 3 groups, such as ACE D allele with AT1-R C^{1166} allele (n=9), ACE D allele with AT1-R AA^{1166} genotype (n=72) and ACE II genotype with AT1-R C^{1166} allele or AT1-R AA^{1166} genotype (n=45), those with the ACE D and AT1-R C^{1166} alleles showed the largest LVDs dimension (37±17 mm vs 31±10 mm vs 28±7 mm, P<0.05) and the lowest EF (48±20% vs 56±14% vs 62±12%, P<0.05) among the 3 groups.

Effect of LVH on the Relationship Between LV Dysfunction and RAS Polymorphisms

To study the development of LV dysfunction, 59 of the 126 patients underwent serial echocardiography at least 2 years apart. After excluding 15 patients with LV dysfunction such as EF<50% and/or LVDd>55 mm at initial evaluation, the data from the remaining 44 patients (25 males, mean age 48±16 years and mean follow-up 6.7±3.5 years) were analyzed. When patients were divided into 2 groups by median MWT of 19 mm, there were 23 patients with MWT≥19 mm and 21 patients with MWT<19 mm. There was no significant difference between the 2 groups in the frequencies of disease-causing genes and RAS polymorphisms. Interestingly, MWT and IVST decreased by 0.46±1.01 and 0.35±1.14 mm/year, respectively, in patients with MWT≥19 mm, although MWT and IVST increased by 0.43±0.78 and 0.43±0.75 mm/year, respectively, in those with MWT <19 mm (P<0.05, respec-

Table 3. Differences Among Genotypes of RAS Gene Polymorphisms

	ACE I/D			AT_1-R A/C^{1166}			Combination of ACE I/D and AT_1-R A/C^{1166}		
	D allele (n=81)	II (n=45)	P value	C allele (n=14)	AA (n=112)	P value	ACE D with AT_1-R C (n=9)	Others (n=117)	P value
Age (years)	53±20	47±23	0.146	53±18	50±21	0.634	58±18	50±21	0.308
Echocardiography									
LAd (mm)	41±8	39±10	0.412	42±10	40±8	0.579	45±10	40±8	0.113
IVST (mm)	14±6	16±5	0.108	15±7	15±6	0.740	12±6	15±6	0.209
PWT (mm)	10±2	11±3	0.048	10±2	10±2	0.942	11±3	10±2	0.835
MWT (mm)	15±6	16±5	0.223	17±6	15±6	0.471	15±5	16±6	0.606
LVDd (mm)	47±9	44±7	0.057	47±13	46±8	0.677	49±15	45±7	0.157
LVDs (mm)	32±11	28±7	0.025	32±15	30±9	0.489	37±17	30±9	0.043
EF (%)	56±15	62±12	0.021	56±18	58±14	0.612	48±20	58±14	0.042

Values are mean±SD.
ACE D with AT_1-R C=ACE D allele with AT_1-R C allele; other abbreviations as in Table 1.

Table 4. Effect of Degree of LV Hypertrophy on LV Dysfunction

	MWT≥19 (n=23)	MWT<19 (n=21)	P value
Age (years)	50±12	46±20	0.49
Male (%)	13 (57)	12 (57)	0.97
Follow-up (years)	6.9±3.8	6.5±3.3	0.72
Echocardiography at initial evaluation			
IVST (mm)	20±3.7	14±2.6	<0.0001
PWT (mm)	11±2.8	11±2.2	0.32
MWT (mm)	22±3.0	14±2.6	<0.0001
LVDd (mm)	43±5.7	44±3.3	0.36
LVDs (mm)	26±4.5	26±3.6	0.77
EF (%)	65±7.0	66±6.7	0.57
Echocardiography at last evaluation			
IVST (mm)	18±6.5	16±4.3	0.16
PWT (mm)	11±2.7	11±2.4	0.75
MWT (mm)	20±6.2	16±4.5	0.03
LVDd (mm)	46±6.0	45±6.4	0.73
LVDs (mm)	29±6.9	29±7.3	0.86
EF (%)	61±11	61±12	0.93
Annual change in echocardiographic parameters			
ΔIVST/year	−0.35±1.14	0.43±0.75	0.011
ΔPWT/year	−0.06±0.65	−0.05±0.56	0.96
ΔMWT/year	−0.46±1.01	0.43±0.78	0.002
ΔLVDd/year	0.41±1.33	0.09±1.05	0.38
ΔLVDs/year	0.48±1.19	0.28±1.24	0.59
ΔEF/year	−0.67±2.19	−0.57±2.08	0.87

Values are mean±SD unless otherwise shown.
Δ, change from initial evaluation to last evaluation. All abbreviations as in Table 1.

tively) (Table 4). Thus, patients with MWT≥19mm showed higher annual EF decrease and larger annual LV dimension increase than patients with MWT<19mm, although results did not reach statistical significance.

Discussion

The major findings of this study are that, in addition to severity of LVH, the ACE D allele is associated with development of systolic dysfunction and LV dilatation in genotyped HCM. Furthermore, the presence of the ACE D allele with the AT_1-R C^{1166} allele appears to be a risk factor for further systolic dysfunction and LV dilatation.

The ACE D allele has been reported to be associated with higher plasma ACE levels than the II genotype,[25] and subjects with the AT_1-R C^{1166} allele exhibit higher angiotensin II responses than those with the AA genotype.[26] Therefore, the ACE D and AT_1-R C^{1166} alleles cause an increase in RAS

activity, which is closely related to myocardial hypertrophy and subsequent LV remodeling.[11] Indeed, the ACE D and the AT1-R C[1166] alleles have been reported as associated with increased LVH in HCM.[17–19] In previous follow-up studies of the predictors of LV systolic dysfunction in HCM, patients who developed systolic dysfunction had a greater LV wall thickness than patients who maintained normal systolic function at initial evaluation.[4–6] Thus, it is possible that these 2 RAS polymorphisms are associated not only with LVH but also with subsequent LV remodeling that can lead to development of systolic dysfunction and LV dilatation. However, few data exist regarding the relationship between these polymorphisms and the progression of systolic dysfunction in HCM patients because of methodological difficulties in collecting both phenotypic and genetic data from such cases.

In the present study, the ACE D allele was associated with decreased PWT, decreased EF and increased LVDs. In addition, the presence of the ACE D allele with the AT1-R C[1166] allele was associated with further decrease in EF and increase in LVDs, both of which reflect LV remodeling. Therefore, one might speculate that the ACE D and AT1-R C[1166] alleles are responsible for LVH and subsequent LV remodeling. The present study is the first to demonstrate the novel modifying effects of these RAS polymorphisms as a risk factor for progression of systolic dysfunction and LV dilatation in the clinical setting. RAS polymorphisms partially account for the inter- and intrafamilial variability in the phenotypic expression of HCM.

It was quite interesting that the combination of the ACE D and AT1-R C[1166] alleles contributed to the development of systolic dysfunction and LV dilatation. The ACE I/D and AT1-R A/C[1166] polymorphisms are known as those that cause LVH. Indeed, studies have revealed a direct relationship between the burden of polymorphisms and the degree of LVH in HCM.[8,9] In a histologic study, the burden of gene polymorphisms related to LVH was associated with the extent of cardiomyocyte hypertrophy in HCM.[27] Those results support the hypothesis of different polymorphic genotypes forming a compound unit, the components of which act in a related manner.[8] In the present study, the presence of the ACE D and AT1-R C[1166] alleles was associated with further decrease in EF and increase in LVDs compared with the ACE D allele alone. It is possible that the combined effects of polymorphisms related to LVH are associated with not only LVH but also the resultant further LV remodeling that can lead to greater systolic dysfunction and LV dilatation.

In contrast to the results of previous studies,[17–19] neither the ACE D nor the AT1-R C[1166] allele itself was related to the presence of LVH in the present study. This may be explained by the fact that almost all of the previous studies had limited numbers of patients and the inclusion criteria differed from ours. In particular, the patients enrolled in previous studies were diagnosed as HCM only by echocardiography without the genetic analysis that was an important part of the inclusion criteria for the present study. Inclusion of HCM with systolic dysfunction in the present study might also have contributed to the different results. Indeed, 25 of the 126 (20%) patients has decreased EF (<50%). The AT1-R CC[1166] genotype was not found in the present study, probably because of the small number of patients with the AT1-R CC[1166] genotype in the general Japanese population.[21]

During the follow-up, MWT and IVST in patients with MWT≥19 mm decreased, although those in patients with MWT<19 mm increased, which suggests that LV remodeling is prominent in accordance with LVH. Indeed, patients with MWT≥19 mm showed higher annual EF decreases and larger annual LV dimension increases than patients with MWT<19 mm. Under these conditions, RAS polymorphisms were not associated with LVH and development of LV dysfunction, probably because we excluded patients with LV dysfunction at initial evaluation, as they could be highly affected by RAS. It is also possible that LV dysfunction because of hypertrophy reflects remodeling caused by ischemia, myocardial fibrosis, small vessel disease and myocardial disarray, suggesting that other genetic or environmental factors could be involved.[5,6]

Clinical Implications and Study Limitations

It has been recently demonstrated that angiotensin II type 1 receptor blockers decreased myocardial fibrosis in a transgenic mouse model of human HCM,[28] and this class of drug has improved LV diastolic function and prevented progression of LVH in clinical HCM patients.[29,30] In the present study, the presence of the ACE D and AT1-R C[1166] alleles was shown to be a possible maker for effective prediction of systolic dysfunction and LV dilatation in HCM. For pharmacological intervention, we suggest that RAS blockade could be effective in patients with these RAS polymorphisms, although the rationale and hypothesis need further investigation.

There remain several limitations to the present study. First, we did not include all of the disease-causing genes, only screening for the 4 major disease-causing genes because their frequencies are higher than the others and comprise most of the genotyped HCM cases, even in the Japanese HCM population.[22,31] Second, 51 of the 126 (40%) genetically-affected patients had a mutation in *TNNI3*, which has caused less than 6% of HCM in most large series studied to date. Therefore, the distribution of mutations in our population was somewhat different from those previously reported.[22,31]

Third, we studied only 2 gene polymorphisms related to LVH: ACE I/D and AT1-R A/C[1166]. Therefore, further investigation of the other polymorphisms related to LVH, such as angiotensinogen M235T, an A/G exchange at position-1903 of the cardiac chymase A gene, and a C/T exchange at position-344 in the aldosterone synthase gene, is necessary in future to confirm the association between RAS polymorphisms and the development of systolic dysfunction.[8,9,27] Fourth, there may be familial bias, because gene-specific genotype–phenotype correlations have been reported.[24,32,33] However phenotypic variability of HCM is not completely explained by disease-causing gene mutation alone.[8–10] From that we infer that the final phenotype is likely the consequence of interaction between the disease-causing gene mutation and other gene variants in the genetic background, such as RAS polymorphisms and environmental factors.

Finally, a proportion of the original study population did not undergo serial evaluation. Therefore, we could not precisely demonstrate a relationship between RAS polymorphisms and life-long LV remodeling from hypertrophy to dysfunction. However, it can be speculated that LV dysfunction would be more prominent in patients with previously greater LV wall thickness and this process would be modified by RAS polymorphisms, as shown in the present study. Long-term follow-up of a large number of patients may confirm this hypothesis.

Conclusions

The ACE D allele is associated with systolic dysfunction and LV dilatation in genotyped HCM. Furthermore, the pres-

ence of the ACE D and AT_1-R C^{1166} alleles is closely related to further systolic dysfunction and LV dilatation, although LVH itself may contribute to the development of LV dysfunction. We suggest that, in addition to screening for disease-causing gene mutations, detection of these RAS polymorphisms may assist further risk stratification and better pharmacological therapy in genotyped HCM patients.

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Analysis of Metabolic Remodeling in Compensated Left Ventricular Hypertrophy and Heart Failure

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Background—Congestive heart failure (CHF) is associated with a change in cardiac energy metabolism. However, the mechanism by which this change is induced and causes the progression of CHF is unclear.

Methods and Results—We analyzed the cardiac energy metabolism of Dahl salt-sensitive rats fed a high-salt diet, which showed a distinct transition from compensated left ventricular hypertrophy to CHF. Glucose uptake increased at the left ventricular hypertrophy stage, and glucose uptake further increased and fatty acid uptake decreased at the CHF stage. The gene expression related to glycolysis, fatty acid oxidation, and mitochondrial function was preserved at the left ventricular hypertrophy stage but decreased at the CHF stage and was associated with decreases in levels of transcriptional regulators. In a comprehensive metabolome analysis, the pentose phosphate pathway that regulates the cellular redox state was found to be activated at the CHF stage. Dichloroacetate (DCA), a compound known to enhance glucose oxidation, increased energy reserves and glucose uptake. DCA improved cardiac function and the survival of the animals. DCA activated the pentose phosphate pathway in the rat heart. DCA activated the pentose phosphate pathway, decreased oxidative stress, and prevented cell death of cultured cardiomyocytes.

Conclusions—Left ventricular hypertrophy or CHF is associated with a distinct change in the metabolic profile of the heart. DCA attenuated the transition associated with increased energy reserves, activation of the pentose phosphate pathway, and reduced oxidative stress. **(*Circ Heart Fail.* 2010;3:420-430.)**

Key Words: heart failure ■ hypertrophy ■ glycolysis ■ dichloroacetate ■ pentose phosphate pathway

Congestive heart failure (CHF) is associated with a significant change in energy metabolism of the heart, and the altered energetics is hypothesized to play an important role in the progression of CHF.[1] Cardiac energy metabolism consists of 3 main components.[2] The first is substrate utilization that includes the uptake and metabolism of fatty acids and carbohydrate (glucose and lactate) and the entry of the resulting intermediates into the tricarboxylic acid (TCA) cycle. Several studies have analyzed substrate utilization in failing hearts, with conflicting results.[3] The second component is oxidative phosphorylation in the mitochondria to produce ATP with reduced nicotinamide adenine dinucleotide produced by the TCA cycle. Energy production and oxygen consumption decrease when associated with a decrease in activities of the respiratory chain and ATP synthase.[4] The third component is the transfer of ATP from the mitochondria to the myofibrils by the creatine kinase system. Creatine kinase activity and phosphocreatine (PCr) decrease in patients with CHF.[5] The ratio of PCr to ATP, a marker of the energetic state of the heart, is correlated with the symptoms or prognosis of patients with CHF.[2]

Clinical Perspective on p 430

Among the changes in energy metabolism associated with CHF, modulation of substrate utilization seems to be a promising target of intervention.[3] Dichloroacetate (DCA) is a chemical that inhibits pyruvate dehydrogenase (PDH) kinase, thereby stimulating PDH and carbohydrate oxidation. Since its discovery in 1969, DCA has been used to treat lactic acidosis complications of congenital mitochondrial diseases and diabetes.[6,7] Recently, it has been suggested that DCA may be useful for treating pulmonary hypertension or cancer.[8,9] DCA improves cardiac function after ischemia in ex vivo perfused hearts.[10] The effects of short-term administration of DCA on left ventricular performance have been studied in patients with CHF, with conflicting results.[11,12]

Despite all research efforts, the mechanism by which the alteration of cardiac energy metabolism is induced and causes

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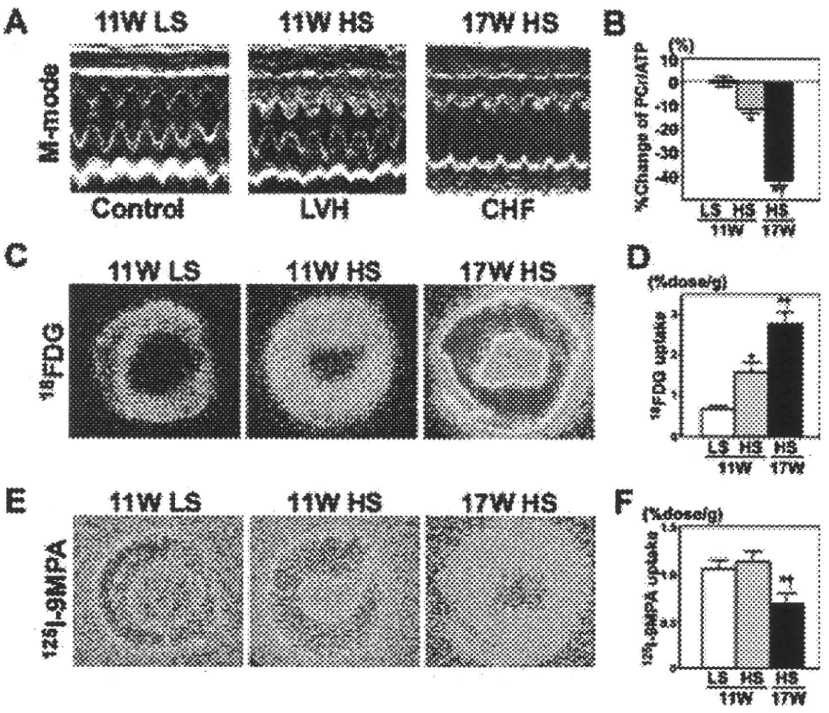

Figure 1. Analysis of cardiac energy metabolism in DS with LVH or CHF. A, Representative images of echocardiography. DS rats that were fed an HS diet developed hypertension, showing concentric LVH at 11 weeks (W) of age. The rats eventually developed CHF with systolic dysfunction at ≈17 weeks of age. DS rats that were fed an LS diet were used as controls. B, PCr/ATP decreased by 12% in LVH rats and 42% in CHF rats compared with control rats; n=8–16 for each group. C, Representative images of myocardial uptake of ^{18}FDG. D, Uptake of ^{18}FDG increased by 1.4-fold in LVH rats and 2.4 fold in CHF rats compared with control rats. E, Representative images of myocardial uptake of ^{125}I-9MPA. F, Uptake of ^{125}I-9MPA did not change in LVH rats but decreased by 36% in CHF rats; n=6–12. *P<0.05 vs control rats. †P<0.05 vs CHF.

progression of CHF is unclear. One reason for this is that current knowledge is based on studies analyzing different parameters of metabolism in different animal models or in patients with different clinical backgrounds. Thus, we simultaneously analyzed cardiac function, substrate uptake, cardiac energy reserve, gene and protein expression, and amounts of metabolites by metabolome analysis in a rat model of CHF that shows a distinct transition from compensated left ventricular hypertrophy (LVH) to CHF. The effect of long-term treatment with DCA on this transition was also analyzed to examine the role of glucose metabolism in the progression of CHF.

Methods

See the online-only Data Supplement for additional details.

Animals

Inbred male Dahl salt-sensitive (DS) rats were fed a 0.3% NaCl (low salt; LS) diet until the age of 6 weeks, after which they were fed an 8% NaCl (high salt; HS) diet.[13] DS rats that were fed an LS diet were used as controls. At 11 weeks of age, the rats fed an HS or LS diet were randomly assigned to groups to receive either vehicle or DCA (Sigma-Aldrich Co, St. Louis, Mo) at a dose of 80 mg/(kg · d) in drinking water.[8] Animal care and the experiments were approved by the institutional animal care and use committee of Kyoto University.

Statistical Analysis

Values are expressed as means±SEM. The survival of the animals was analyzed by the Kaplan-Meier method with a log-rank test. Nonparametric analysis with Dunn's procedure was used to analyze metabolome data. Other data were analyzed by ANOVA followed by post hoc comparisons with the Bonferroni test. When there were 2 crossed factors, differences between groups were analyzed by 2-way factorial ANOVA followed by post hoc comparisons with the Bonferroni test. In all tests, a value of P<0.05 was considered significant.

Results

Transition From LVH to CHF in Dahl Rats

As we reported previously, DS rats that were fed an HS diet developed hypertension at 11 weeks of age (supplemental Table I).[13] On echocardiographic examination, left ventricular posterior wall thickness was significantly increased and fractional shortening was preserved in DS rats at 11 weeks of age, indicating the establishment of concentric LVH (Figure 1A and supplemental Table II). At ≈17 weeks of age, the DS rats that were fed an HS diet showed signs of CHF and showed decreased fractional shortening.

Myocardial Energy Reserve Measured by In Situ ^{31}P Magnetic Resonance Spectroscopy

We analyzed the change in cardiac energy reserves during the transition from cardiac hypertrophy to heart failure by measuring cardiac high-energy phosphates with in situ ^{31}P magnetic resonance spectroscopy. The cardiac PCr/ATP decreased by 12% at the LVH stage and by 42% at the CHF stage, compared with that for control rats (Figure 1B and Online Supplemental Figure I).

Myocardial Uptake of Glucose and Fatty Acids

To estimate the change in substrate utilization during the transition from LVH to CHF, we examined the myocardial uptake of glucose and fatty acids by using ^{18}F-deoxyglucose (FDG) and ^{125}I-15-(*p*-iodophenyl)-9-*R,S*-methylpentadecanoic acid (9MPA), respectively (Figure 1C and 1E and supplemental Table III).[14] In DS rats that were fed an HS diet, the uptake of ^{18}FDG increased by 1.4-fold at the LVH stage and by 2.4-fold at the CHF stage compared with the levels in control rats fed an LS diet (Figure 1D). The uptake of ^{125}I-15-(*p*-iodophenyl)-9MPA did not change at the LVH stage and decreased by 36% at the CHF stage compared with the levels in control rats (Figure 1F).

Expression of Molecules Related to Energy Metabolism

To examine the mechanisms underlying the change in cardiac energy reserves and substrate uptake, we profiled the expres-

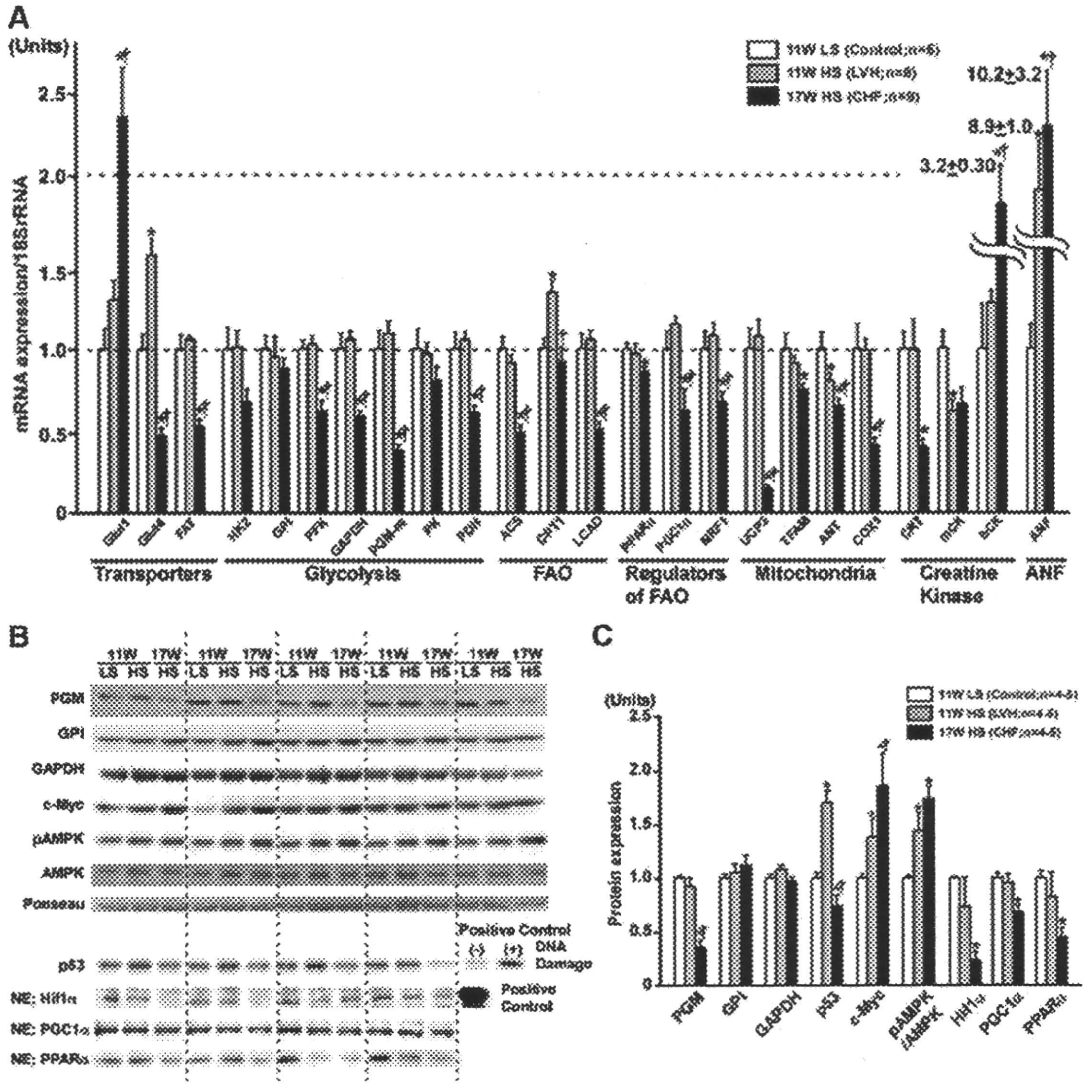

Figure 2. Expression of molecules related to energy metabolism during the transition from LVH to CHF. A, Gene expression related to cardiac energy metabolism was measured by quantitative reverse transcription–polymerase chain reaction. 18S rRNA was used as an internal control. B and C, The levels of proteins related to energy metabolism were examined by Western blotting and quantified by densitometry. Hypoxia-inducible factor-1α, peroxisome proliferator–activated receptor-γ coactivator-1-α, and peroxisome proliferator–activated receptor-α were examined in nuclear extracts. Total cell lysate was used for other proteins. The mean value for control rats was expressed as 1 unit. *$P<0.05$ vs control rats. †$P<0.05$ vs LVH rats.

sion of molecules related to energy metabolism. The gene expression of glucose transporter 4 was increased at the LVH stage, whereas the expression of glucose transporter 4 was decreased and that of glucose transporter 1 was increased at the CHF stage (Figure 2A and supplemental Table IV). The expression of fatty acid transporter was preserved at the LVH stage but decreased at the CHF stage.

The gene expression of glycolytic enzymes was preserved at the LVH stage, and levels of some glycolytic enzyme mRNAs were decreased at the CHF stage (Figure 2A). We examined the expression of transcription factors that may regulate the expression of glycolytic enzymes.[15,16] The decrease in glycolytic enzyme mRNAs at the CHF stage seemed to be correlated with the decrease in hypoxia-inducible factor-1α protein (Figure 2B and 2C).

The gene expression of several enzymes related to fatty acid oxidation (FAO) and mitochondrial function was preserved at the LVH stage but decreased at the CHF stage.[17] The reduction in expression of FAO-related genes was associated with decreased expression of transcriptional regulators of FAO and mitochondrial function (Figure 2A through 2C), such as peroxisome proliferator-activated receptor-α, peroxisome proliferator-activated receptor-γ coactivator-1α, and nuclear respiratory factor 1.[18,19] Creatine transporter mRNA was decreased at the CHF stage. Shift of the creatine kinase isoform from muscle type to brain type was observed at the CHF stage (Figure 2A), which is consistent with a previous report.[20] Another key regulator of energy metabolism in the heart is AMP-activated protein kinase.[21] AMP-activated protein kinase is activated by an increase in the AMP-ATP ratio and is thought to be a marker of cellular energy status. Phosphorylated AMP–activated protein kinase, an activated form, increased at the LVH and CHF stages (Figure 2B and 2C).[22]