Oxidative Posttranslational Modifications Develop LONP1 Dysfunction in Pressure Overload Heart Failure

Hoshino et al: Mitochondrial Protease Compromise in Heart Failure

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Abstract

Background—Mitochondrial compromise is a fundamental contributor to heart failure. Recent studies have revealed that several surveillance systems maintain mitochondrial integrity. The present study evaluated the role of mitochondrial AAA+ protease in a mouse model of pressure overload heart failure.

Methods and Results—The FITC-casein assay and immunoblotting for endogenous mitochondrial proteins revealed a marked reduction in ATP-dependent proteolytic activity in failing heart mitochondria. The level of reduced cysteine was decreased and tyrosine nitration and protein carbonylation were promoted in Lon protease homolog (LONP1), the most abundant mitochondrial AAA+ protease, in heart failure. Comprehensive analysis revealed that electron transport chain (ETC) protein levels were increased even with a reduction in the expression of their corresponding mRNAs in heart failure, which indicated decreased protein turnover and resulted in the accumulation of oxidative damage in the ETC. The induction of catalase targeted to mitochondria (mCAT) ameliorated proteolytic activity and protein homeostasis in the ETC, leading to improvements in mitochondrial energetics and cardiac contractility even during the late-stage of pressure overload. Moreover, the infusion of mitoTEMPO, a mitochondria-targeted superoxide dismutase mimetic, recovered oxidative modifications of LONP1 and improved mitochondrial respiration capacity and cardiac function. The in vivo siRNA repression of LONP1 partially canceled the protective effects of mCAT induction and mitoTEMPO infusion.

Conclusions—Oxidative posttranslational modifications attenuate mitochondrial AAA+ protease activity, which is involved in impaired ETC protein homeostasis, mitochondrial respiration deficiency, and left ventricular contractile dysfunction. Oxidatively inactivated proteases may be an endogenous target for mitoTEMPO treatment in pressure overload heart failure.

Key Words: oxidative posttranslational modifications, LONP1, AAA+ protease, mitochondria, heart failure
Marked improvements have been achieved in survival rates following adverse cardiovascular events over the past several decades. However, subsequent heart failure still remains a public health problem. Although significant advances have been made in the management of heart failure mainly through neurohumoral blockade, which prevents cardiac remodeling with drugs that act on the renin-angiotensin-aldosterone system or adrenergic system, mortality and morbidity in patients with heart failure still remain high. Therefore, a novel therapeutic approach is needed. Mitochondrial dysfunction is involved in the pathogenesis of numerous diseases including heart failure. Impaired ATP production and subsequent cardiac energy compromise are fundamental contributors to the development of heart failure. Moreover, mitochondria play a central role in metabolism, oxidative stress, calcium homeostasis, and cell death, all of which are pathogenic contributors to heart failure.

Mitochondrial quality control systems have been identified at the protein and organelle levels\(^1,2\). We previously reported that the autophagic degradation of mitochondria was attenuated by an inhibitory protein-protein interaction between Parkin and cytosolic p53, which contributed to declines in mitochondrial integrity and cardiac contractility in a mouse model of heart failure induced by doxorubicin treatment or cardiac aging\(^3\). At a basal level, proteolytic degradation is important to maintain the dynamic equilibrium of mitochondrial proteins and destroy damaged proteins. As the main pathway for intramitochondrial protein degradation, the mitochondrial protease is required to sustain biological processing in
mitochondria. Mitochondrial proteases are energy-dependent proteases containing conserved ATPase modules which are characteristic of the AAA+ family (ATPases Associated with a variety of cellular Activities)\textsuperscript{4}. Two soluble proteases, the Lon protease and ClpXP protease, are present in the mitochondrial matrix, and two proteolytic complexes, \textit{m}- and \textit{i}-AAA metalloproteases, are anchored within the inner mitochondrial membrane, which exposes their catalytic domains to the matrix and intermembrane space, respectively\textsuperscript{5}. Lon protease homolog (LONP1), the most abundant AAA+ protease in cardiac mitochondria\textsuperscript{6,7}, forms soluble heptameric ring-shaped assemblies with serine protease activity within the mitochondrial matrix\textsuperscript{8}. LONP1 was previously shown to degrade various misfolded and oxidatively damaged matrix and inner-membrane proteins, thereby preventing their deleterious accumulation\textsuperscript{7,9}. Despite the recognition of proteomic alterations in mitochondrial in heart failure, factors that regulate the rate and mechanisms of mitochondrial protein degradation under physiological and pathological conditions remain obscure.

The present study examined the function of AAA+ mitochondrial protease in a mouse model of pressure overload heart failure following transverse aortic constriction (TAC). The measurement of ATP-dependent proteolytic activity in isolated mitochondria revealed marked reductions in mitochondrial protease activity. The protein carbonylation and tyrosine nitration of LONP1 were promoted in failing heart mitochondria, and the induction of mitochondria-targeted human catalase (mCAT) in failing hearts recovered protease activity.
These results suggested that oxidative posttranslational modifications (OPTM) contributed to the proteolytic deficit of AAA+ proteases. OPTM-mediated protease dysfunction was closely associated with declines in electron transport chain (ETC) protein homeostasis, mitochondrial energetics, and left ventricular (LV) contractility. Moreover, the infusion of mitoTEMPO, a mitochondria-targeted superoxide dismutase mimetic, improved mitochondrial respiration capacity and cardiac function at least partially, through the improvement of mitochondrial protease activity in heart failure.

Methods

Please see the Data Supplement for additional details. The floxed stop sequence preceding mitochondria-targeted human catalase (mCAT) in the inducible BAC model (C57BL/6J background) was kindly gifted from Dr. Rabinovitch (University of Washington) and excised by tamoxifen-induced αMHC promoted Cre. MitoTEMPO (700 μg kg⁻¹ day⁻¹), MG132 (720 μg kg⁻¹ day⁻¹), and epoxomicin (500 μg kg⁻¹ day⁻¹) were delivered for 2 weeks with Alzet 1002 pumps. All animal studies were approved by the Animal Care and Use Committee of Kyoto Prefectural University of Medicine. Data are expressed as mean ± SD. All statistical analyses were conducted using StatMate III (ATMS, Tokyo, Japan). An unpaired t test was used for comparison between 2 groups, and comparisons among multiple groups were performed with 1-way ANOVA and the Tukey post hoc test. Pearson correlation
coefficients were used to assess the relationship between groups in iTRAQ proteomics. 

\( P<0.05 \) was considered to be significant.

Results

Mitochondrial AAA+ protease activity is impaired in pressure overload heart failure.

We examined the intramitochondrial AAA+ protease activity in TAC-operated heart failure. Isolated mitochondria were incubated with a quenched-FITC substrate in ATP-containing buffer to assess their ability to degrade exogenous casein. AAA+ protease activity was attenuated 2 weeks after the TAC operation and further deteriorated in a manner that was dependent on the progression of heart failure (Figure 1A). Endogenous protein degradation was also assessed by immunoblotting for COX5a that was previously shown to be a substrate of LONP1\(^8\). Endogenous protein degradation, which was effectively attenuated by a protease inhibitor, was similarly reduced in heart failure (Figure 1B). Although the ubiquitin-proteasome system (UPS) was also reported to be involved in mitochondrial protein homeostasis through a poorly understood mechanism\(^6\), no significant changes were observed in proteasome activity 2 weeks and 8 weeks after the TAC operation (Supplemental Figure 1).

Enhanced oxidative stress impairs proteolytic activity.

The expression of AAA+ proteases was not decreased in heart failure (Figure 2A and B). Therefore, we hypothesized that oxidative stress may have contributed to AAA+ protease
dysfunction in pressure overload heart failure. Reactive oxygen species (ROS) production in isolated mitochondria was assessed by detecting hydrogen peroxide using the Amplex Red-horseradish peroxidase system. The rate of hydrogen peroxide release measured in the presence of 0.5 mM NADH or 5 mM succinate was promoted in heart failure (Figure 2C). The enhanced production of ROS was associated with increased protein carbonylation in mitochondria (Figure 2D). The ATP-stimulated proteolysis of casein was mostly caused by LONP16,7; therefore, we evaluated the extent of oxidative modifications in LONP1. The level of reduced cysteine in LONP1 was decreased in heart failure (Figure 2E). We also evaluated tyrosine nitration and protein carbonylation in the proteins immunoprecipitated from mitochondrial extracts using an anti-LONP1 antibody, because these oxidative modifications are known to be deleterious to the structures of proteins and lead to the decline of protein function15,16. Nitrotyrosine and carbonylation levels in LONP1 were significantly increased in heart failure (Figure 2F and G). These results indicated that excessive oxidative stress oxidized AAA+ proteases and induced a reduction in proteolytic activity in heart failure. Consistent with this theory, the exposure of isolated mitochondria to exogenous hydrogen peroxide decreased proteolytic activity to degrade FITC-labeled casein (Figure 3A). Endogenous protein degradation was also impaired by excessive oxidative stress (Figure 3B).
The levels of electron transport chain proteins increase, while their corresponding mRNA levels decrease in heart failure

To examine the effects of AAA+ protease dysfunction on mitochondrial remodeling, we analyzed changes in the mitochondrial proteome in pressure overload heart failure. We purified heart mitochondria and performed iTRAQ that enabled the simultaneous identification and relative quantification of mitochondrial proteins through isobaric peptide tagging (Supplemental Figure 2). Three groups of animals were examined: sham-operated mice (Sham), TAC-operated mice at 2 weeks (TAC 2W), and TAC-operated mice at 8 weeks (TAC 8W), and TAC 2W and TAC 8W samples were compared with Sham samples (Supplemental Table 1). A total of 7925 iTRAQ-labeled peptides that mapped to a total of 464 proteins were identified and quantified from purified heart mitochondria. Differences between two two-group comparisons were displayed as a scatter image, revealing a roughly similar pattern of TAC-induced changes (Figure 4A). In contrast, the mRNA and protein levels of individual genes rarely correlated in both TAC 2W and TAC 8W. ETC protein levels were higher in spite of rare alterations or reductions in mRNA levels (Figure 4B and Supplemental Table 2). Blue Native Gel analysis showed that protein carbonylation was elevated in individual ETC components (Supplemental Figure 3). These results indicated that the functional decline in AAA+ protease might be predominantly associated with reduced protein turnover and subsequent accumulation of oxidatively damaged proteins in the ETC in
heart failure.

The induction of mitochondrial catalase restores AAA+ protease activity and cardiac contractility

To demonstrate the contribution of oxidative stress to AAA+ protease dysfunction and subsequent abnormal ETC protein homeostasis, we utilized a system that induced the overexpression of mitochondria-targeted human catalase (i-mCAT) in mouse hearts (Supplemental Figure 4A). An approximately 20-fold increase in catalase activity was observed in the isolated mitochondria of i-mCAT Tg mouse hearts (Supplemental Figure 4B). We injected tamoxifen 6 weeks after the TAC operation to induce mCAT (Figure 5A) and determined whether abolishing oxidative stress recovered proteolytic activity and abnormal protein homeostasis 2 weeks later. The overexpression of mCAT partially restored proteolytic activity to degrade FITC-labeled casein in heart failure (Figure 5B). Similar results were observed in the evaluation of endogenous protein degradation including the other ETC proteins, Ndufa9 and Cyc1 as well as COX5a (Figure 5C).

We also determined whether the enhancement in protein homeostasis contributed to histological and functional improvements. The late-stage and short-term induction of mCAT induced no alteration in interstitial fibrosis, and cardiac hypertrophy at both the whole-heart and cardiomyocyte levels (Supplemental Figure 5A-D). Although ROS signals have been shown to modulate angiogenesis under various conditions, no significant differences were
observed in capillary density in the myocardium (Supplemental Figure 5E). We then examined the functional integrity of the ETC and found that the mitochondrial oxygen consumption rates of complexes II and IV were improved (Figure 6A). We measured hydrogen peroxide release from heart mitochondria isolated from TAC-operated failing hearts using 3-Amino-1,2,4-triazole (3-AT) to eliminate the direct effect of induced catalase. The rate of hydrogen peroxide release measured in the presence of NADH or succinate was attenuated by mCAT induction, whereas succinate-induced hydrogen peroxide generation maximally stimulated by the addition of antimycin A, a complex III inhibitor, was prominent (Figure 6B), which reflected the improved enzymatic activity of the respiratory complex. HPLC studies were then conducted on cardiac tissues to determine changes in the energy state by mCAT induction. Compared with WT mice, i-mCAT Tg mice exhibited a marked improvement of PCr concentrations in TAC-operated heart failure. ATP contents were similar between these mice, although PCr/ATP ratios were significantly recovered by mCAT induction (Figure 6C). Increased ETC protein levels did not contribute to energy production, but promoted the ROS production in heart failure. The recovery of proteolysis in dysfunctional ETC proteins led to a reduction in the production of hydrogen peroxide and improved myocardial high-energy phosphate metabolism. Despite the absence of alterations in the histological examination, the antioxidant intervention led to mitochondrial bioenergetic improvements in heart failure. Consistent with these results, echocardiography revealed that
cardiac contractility was improved even with slight changes in LV size (Figure 6D).

**The inhibition of LONP1 negates the protective effects of mCAT expression**

We determined whether protective effects of mitochondrial catalase induction were mediated by proteolytic activity with the inhibition of LONP1. We used a siRNA-mediated *in vivo* knockdown strategy to specifically inhibit the expression of LONP1. The protein levels of LONP1 remarkably decreased in a time-dependent manner in hearts of mice transfected with siRNA targeting LONP1. The down-regulation of LONP1 protein continued for 4 days, and started to recover at 6 days (Figure 7A). Based on these results, siRNA injections were administrated once every 5 days starting on the first day of tamoxifen injections. The *in vivo* knockdown of LONP1 induced an approximately 40 % decrease in ATP-dependent proteolytic activity in heart mitochondria (Figure 7B), which partially canceled the protective effects induced by mCAT on the mitochondrial oxygen consumption rate (Figure 7C) and cardiac contractility (Figure 7D).

To further strengthen our interpretation, we utilized a well-known cell-permeable protease and proteasome inhibitor, MG132 which has an inhibitory effect on the activity of LONP1 with the selective proteasome inhibitor, epoxomicin as an additional control. TAC-operated mice were infused with MG132, epoxomicin, or the carrier DMSO for 2 weeks starting on the first day of tamoxifen injections. The infusion of MG132 induced a more than 30 % reduction in ATP-dependent proteolytic activity in isolated heart mitochondria.
(Supplemental Figure 6A and B). The preservation of mitochondrial oxygen consumption rate and cardiac function was diminished in MG132 treatment, but not in epoxomicin treatment in i-mCAT Tg mice (Supplemental Figure 6C and D). These results indicated that ameliorating oxidative stress recovered mitochondrial energy production and cardiac contractility, at least partially by restoring AAA+ protease activity.

**Antioxidant therapy recovers protease activity and mitochondrial respiration**

We determined whether a mitochondria-targeted antioxidant molecule had a therapeutic effect on a mouse model of pressure overload heart failure. TAC-operated mice were infused with mitoTEMPO\(^{11}\), a mitochondria-targeted superoxide dismutase mimetic for 2 weeks 6 weeks after surgery (Figure 8A). The infusion of mitoTEMPO restored the level of reduced cysteine in LONP1 and proteolytic activity to degrade FITC-labeled casein (Figure 8B and C). The ATP-dependent endogenous protein degradation of isolated mitochondria also showed improved proteolysis (Figure 8D). This restored proteolytic activity ameliorated mitochondrial oxygen consumption by complexes II and IV (Figure 8E), which resulted in an increase in LV contractile function (Figure 8F). These protective effects were partially eliminated by *in vivo* knockdown of LONP1.
Discussion

Functional integrity and appropriate responses to pathophysiological stimuli are required for mitochondria to meet cellular energy demands. Mitochondrial ETC is a main source of ROS; therefore, mitochondrial proteins are susceptible to oxidative modifications that impair protein function. The enzymatic repair and degradation of oxidized proteins are crucial to maintain mitochondrial integrity and cellular homeostasis. In the present study, we demonstrated for the first time that mitochondrial AAA+ protease activity was impaired in pressure overload heart failure in mice. The expression of AAA+ proteases was not decreased and genetic antioxidant intervention in mitochondria recovered proteolytic activity, which suggested that the OPTM of AAA+ proteases may have been responsible for decreasing proteolytic activity. This hypothesis was supported by the decreased level of reduced cysteine and increased level of carbonylation and nitrotyrosine in LONP1. The decline in AAA+ protease activity was closely associated with decreased protein turnover and increased oxidative damage in the ETC, leading to mitochondrial respiration deficiency and LV contractile dysfunction. This is also the first study showing that mitoTEMPO treatment improved cardiac contractility in heart failure. The infusion of mitoTEMPO recovered the level of reduced LONP1 and ATP-dependent proteolytic activity. The cardioprotective effects of mitoTEMPO were at least partially eliminated by the loss-of-function of LONP1. These results indicate that the OPTM of AAA+ protease is the endogenous target for this small
molecule used as a drug.

The decline in LONP1 activity has been well documented in aged mice. Although LONP1 expression levels increased in aged mouse hearts, AAA+ protease activity remained constant in heart mitochondria during aging\textsuperscript{19}. Another study reported that AAA+ proteolytic activity decreased by 2.5-fold in the livers of 27 month-old rats with no concomitant changes in LONP1 protein expression levels\textsuperscript{20}. These findings suggest that the efficiency of LONP1 may decrease with aging. Because oxidative stress is closely related with aging, especially in post-mitotic tissues, persistent oxidative stress may be associated with a decrease in LONP1 activity. Evidently, both the ATPase and proteolytic activities of LONP1 were highly susceptible to oxidative inactivation when isolated rat brain mitochondria were treated with the reactive nitrogen species peroxynitrite. This inactivation preceded ETC dysfunction and was recovered with reduced glutathione addition\textsuperscript{21}. These evidences indicate that LONP1 undergoes an age-related functional decline that may be mediated by oxidative modifications, and that is likely to contribute to the accumulation of oxidatively inactivated proteins, similar to our findings in heart failure.

Not only AAA+ proteases but also proteasomes contribute to mitochondrial protein degradation. The autophagy machinery is involved in the elimination of whole mitochondria\textsuperscript{6,22}. In most mammalian cells, the UPS conducts approximately 90\% of intracellular proteolysis, ensuring that misfolded, oxidized, or damaged proteins are degraded\textsuperscript{23}. There is
compelling evidence for UPS activity in heart failure. Some studies on human biopsies from failing hearts reported the UPS dysfunction in heart failure\(^{23,24}\), whereas an analysis in mice revealed the bidirectional alteration of UPS function in pressure overload heart failure\(^{25,26}\). The proteasome substrate in mitochondria and its exporting mechanism are also poorly understood. The UPS was previously shown to be involved in the degradation of UCP2 in inner-membrane or intermembrane space proteins\(^{22,27}\). A recent study reported that some ETC proteins and TCA cycle enzymes were susceptible to proteolysis by the 20S proteasome \textit{in vitro}\(^6\). Although we observed no alterations in UPS function in TAC-induced heart failure, the UPS may be involved in ETC protein homeostasis.

We performed a comprehensive analysis to evaluate mitochondrial alterations in heart failure using a microarray and iTRAQ proteomics of purified mitochondria, and showed that mitochondrial ETC protein levels were up-regulated even with a reduction in the expression of their corresponding mRNAs. Proteomic alterations in mitochondria in heart failure have been investigated in different etiologies (myocardial infarction, pressure overload, and pacing-induced) and various severities (hypertrophied and decompensated) using different proteomic strategies (2-DE, DIGE, and iTRAQ)\(^{28}\). These investigations consistently revealed that FAO enzymes were attenuated in a manner that was dependent on the progression of heart failure\(^{29-32}\). In our study, FAO enzymes were downregulated at both the mRNA and protein level (data not shown). In contrast, alterations in ETC proteins have been
inconsistently reported even among studies performed in pressure overload heart failure due to hypertension or aortic constriction. Some studies identified bidirectional changes in the ETC subunit composition\textsuperscript{29-33}. However, a study using the same mouse model as our experiments reported that ETC protein levels were mostly increased in heart failure\textsuperscript{32}, which is consistent with our results.

In the present study, carbonylation, tyrosine nitration, and cysteine oxidation were enhanced in LONP1 in failing heart mitochondria. Under physiological conditions, ROS are constantly generated within cells and controlled at low concentrations; fluctuations in which can affect many cellular functions through redox-signaling. Cellular oxidative damage develops when ROS-generating systems overwhelm ROS-scavenging ones. OPTM can induce alterations in protein structures, leading to bidirectional effects on protein function. Some reversible modifications, such as S-glutathionylation and S-nitrosation, might play a dual role in protection against irreversible oxidation and the modulation of protein function\textsuperscript{15}. Tyrosine nitration is an established marker for oxidative stress and cytotoxicity. Nitrotyrosine is relatively stable and nitration often results in structural and functional abnormalities of proteins\textsuperscript{16}. Protein carbonylation is an irreversible, non-enzymatic modification of proteins, and is formed directly via the oxidation of side chains or indirectly via reactive carbonyl derivatives and aldehydes, leading to the loss of protein function\textsuperscript{15}. Previous studies reported the detrimental effects of OPTM on the progression of heart failure. Cysteine sulfonylation
and tyrosine nitration have been implicated in the pathophysiology of cardiac dysfunction in
Gq-induced cardiomyopathy. A study using human biopsy samples demonstrated that
carbonylation of proteasomes might contribute to defective protein degradation in human
cardiomyopathies. Our results provide additional evidence to support proteolytic systems
being the target of OPTM in heart failure.

Oxidative stress has been implicated in the pathogenesis of many human diseases
including cardiovascular diseases, whereas common antioxidants have proven ineffective in
clinical trials. One of the reasons is that these agents are not adequately delivered to the
subcellular sites of ROS production. Recent experimental studies showed that treatments with
the mitochondria-targeted antioxidant mitoTEMPO prevented hypertension and arrhythmic
events in the setting of renin-angiotensin system activation and reduced
diabetes-attributable mortality after myocardial infarction. This study provides evidence
that this agent improves cardiac function even during the late-stage of pressure overload,
albeit to a modest degree. Oxidatively inactivated mitochondrial AAA+ proteases might be a
target of mitoTEMPO treatment. A greater protective effect might be observed by a more
long term treatment from the early-stage. A recent study reported that other
mitochondria-targeted antioxidant peptides attenuated TAC-induced heart failure and
proteomic alterations of mitochondria, demonstrating the effectiveness of selective delivery
to mitochondria.
Acknowledgments

We are grateful to Dr. Rabinovitch (University of Washington) for providing the mitochondria-targeted human catalase overexpression mice.

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Disclosures

None.

References


Figure Legends

Figure 1. ATP-dependent protease activity was impaired in TAC-operated failing hearts.

A, The FITC-casein assay was performed to assess ATP-dependent proteolytic activity of isolated heart mitochondria 2 and 8 weeks after the TAC operation (n = 6-8 per group, duplicate experiments). B, Isolated mitochondria from sham or TAC-operated hearts were incubated in ATP-containing buffer for 12 hr, followed by immunoblotting to assess the ATP-dependent degradation of endogenous COX5a protein. PI indicates protease inhibitors; ns, not significant. Data are shown as the means ± SD. *P < 0.05; **P < 0.01.

Figure 2. Oxidative stress promoted oxidative modifications of mitochondrial AAA+ protease LONP1 in heart failure. A, Real-time PCR assessing the expression of AAA+ proteases in Sham and TAC 8W (n = 6 per group). B, LONP1 protein expression was assessed by immunoblotting (n = 6 per group). C, Hydrogen peroxide production in isolated mitochondria was assessed using the Amplex Red-HRP system. NADH or succinate-induced hydrogen peroxide production was promoted in TAC-operated failing hearts (n = 4 per group, duplicate experiments). D, Mitochondrial protein carbonylation was measured by immunoblotting with an anti-DNPH antibody (n = 4 per group). E, The extent of cysteine reduction in LONP1 was evaluated with iodoacetamide. Reduced LONP1 protein levels were normalized by the total LONP1 protein level (n = 4 per group). F, Protein carbonylation on
LONP1 was evaluated by immunoblotting with an anti-DNPH antibody in immunoprecipitated samples with an anti-LONP1 antibody (n = 4 per group). G, Tyrosine nitration on LONP1 was evaluated by immunoblotting with an anti-nitrotyrosine antibody in immunoprecipitated samples with an anti-LONP1 antibody (n = 4 per group). Rot indicates rotenone; Suc, succinate; AA, antimycin A. Data are shown as the means ± SD. *P < 0.05; ** P < 0.01; *** P < 0.001.

Figure 3. Exogenous hydrogen peroxide impaired ATP-dependent proteolytic activity. A, The FITC-casein assay with the indicated levels of exogenous hydrogen peroxide. Results from four independent experiments performed in four replicate are shown. B, Immunoblots to assess the ATP-dependent endogenous protein degradation of isolated mitochondria with the indicated levels of exogenous hydrogen peroxide. Representative immunoblots are shown from three independent experiments. Data are shown as the means ± SD. *P < 0.05; *** P < 0.001 versus untreated control.

Figure 4. ETC protein levels were increased in cardiac mitochondria of TAC-operated mice. Comprehensive analysis of mitochondrial remodeling in pressure overload heart failure was performed using a microarray and iTRAQ proteomics of purified mitochondria. A, Correlation of the protein expression ratio between TAC 2W versus Sham and TAC 8W
versus Sham. B, Correlation between protein expression and gene expression changes in TAC-operated hearts relative to sham-operated hearts. ETC indicates electron transport chain.

Figure 5. The induction of mCAT restored ATP-dependent proteolytic activity in failing heart mitochondria. A, Protocol of mCAT induction. We administered an intraperitoneal injection of 4-hydroxytamoxifen (20 mg kg⁻¹ day⁻¹) once daily for 5 days 6 weeks after the TAC operation. B, The FITC-casein assay was performed to assess the effect of mCAT induction on ATP-dependent proteolytic activity in isolated heart mitochondria (n = 6-8 per group, duplicate experiments). C, Immunoblots to assess the ATP-dependent endogenous protein degradation of isolated mitochondria after the induction of mCAT. Data are shown as the means ± SD. *P < 0.05; **P < 0.001.

Figure 6. The induction of mCAT ameliorated the mitochondrial respiration capacity and cardiac contractility in heart failure. A, The sequential state III oxygen consumption rate of isolated heart mitochondria was assessed using the Seahorse XF24 Extracellular Flux Analyzer. With the initial presence of 5 μg mitochondria per well, 0.5 mM NADH and 4 μM FCCP, injections were made as follows: port A, 50 μl of 20 μM rotenone (2 μM final); port B, 55 μl of 100 mM succinate (10 mM final); port C, 60 μl of 40 μM antimycin A (4 μM final); port D, 65 μl of 10 mM ascorbate plus 4 mM TMPD (1 mM and 400 μM final, respectively).
Representative results are shown at the left panel (n = 5 per group, five replicate experiments).

B, Hydrogen peroxide production in isolated mitochondria was assessed by the Amplex Red/HRP system in the presence of the substrate alone or together with respiratory chain inhibitors. To eliminate the effect of catalase, 3-Amino-1,2,4-triazole was used (n = 4 per group, duplicate experiments). C, Myocardial high-energy phosphate metabolism was biochemically measured by high-performance liquid chromatography (n = 5 per group, duplicate experiments). D, Representative images of trans-thoracic M-mode echocardiographic tracings and echocardiographic parameters (n = 12-13 per group). OCR indicates oxygen consumption rate; Rot, rotenone; Suc, succinate; AA, antimycin A; TMPD, N,N,N’,N’-tetramethyl-p-phenylenediamine dihydrochloride; Asc, ascorbate; 3-AT, 3-Amino-1,2,4-triazole; PCr, phosphocreatine. Data are shown as the means ± SD. *P < 0.05; **P < 0.01; *** P < 0.001.

Figure 7. The in vivo knockdown of LONP1 attenuated the protective effects induced by mCAT expression. A, Immunoblots to assess the extent of in vivo knockdown in heart tissues at indicated time points after siRNA injection. B, The FITC-casein assay was performed to assess ATP-dependent proteolytic activity of isolated heart mitochondria from mice transfected with siRNA targeting LONP1 in vivo (n = 4 per group, duplicate experiments). C, The sequential state III oxygen consumption rate of isolated heart mitochondria was assessed
using the Seahorse XF24 Extracellular Flux Analyzer. Representative results are shown at the left panel (n = 5 per group, five replicate experiments). D, Representative images of trans-thoracic M-mode echocardiographic tracings and echocardiographic parameters (n = 7-8 per group). Data are shown as the means ± SD. *P < 0.05; **P < 0.01.

Figure 8. mitoTEMPO improved proteolytic activity and ETC function in heart failure.

A, Protocol of mitoTEMPO infusion. We delivered mitoTEMPO (700 µg kg⁻¹ day⁻¹) for 2 weeks 6 weeks after the TAC operation. B, The extent of cysteine reduction in LONP1 was evaluated with iodoacetamide. C, The FITC-casein assay was performed to assess the effect of mitoTEMPO on ATP-dependent proteolytic activity of isolated heart mitochondria (n = 6-8 per group, duplicate experiments). D, Immunoblots to assess the ATP-dependent endogenous protein degradation of isolated mitochondria after mitoTEMPO infusion. E, The sequential state III oxygen consumption rate of isolated heart mitochondria (300 µg) was monitored with a fiber-optic system. Substrates and inhibitors were added sequentially at the final concentrations indicated as follows. FCCP, 4 µM Carbonylcyanide-p-trifluoromethoxyphenylhydrazone; M/P, 5 mM malate + 5 mM pyruvate; Rot, 2 µM rotenone; Suc, 5 mM succinate; AA, 1 µM antimycin A; TMPD/Asc, 0.4 mM N,N,N’,N’-tetramethyl-p-phenylenediamine + 1 mM ascorbate; KCN, 5 mM potassium cyanide. Representative results are shown at the left panel and the oxygen consumption rate
in each complex was calculated (n = 8 per group). 

F, Representative images of trans-thoracic M-mode echocardiographic tracings and echocardiographic parameters (n = 8-14 per group).

Data are shown as the means ± SD. *P < 0.05; ***P < 0.001.
Figure 1

A

ATP-stimulated proteolytic activity (%)

Sham  TAC 2W  TAC 8W

**

*

B

<table>
<thead>
<tr>
<th>ATP (−)</th>
<th>10 mM ATP 12 hr</th>
<th>ATP + PI</th>
</tr>
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<tbody>
<tr>
<td>Sham</td>
<td>TAC 8W</td>
<td>Sham</td>
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COX5a (complex IV)

Degraded

TOM20
Figure 2

A

B

C

D

E

F

G

Relative expression

ns

ns

H₂O₂ production (pmol per min per mg protein)

***

TAC

Sham

LONP1 fold change

0

0.5

1

1.5

2

2.5

3

3.5

4

Agr211, Agr212, Yma111, LONP1, Clpp, Clpx, BNP

LONP1 fold change

0

0.5

1

1.5

2

2.5

3

3.5

4

Sham

TAC

GAPDH

LONP1

H₂O₂ production (pmol per min per mg protein)

NADH

NADH + Rot

Suc

Suc + AA

Sham

TAC

reduced LONP1

total LONP1

DNPH

LONP1

IP-LONP1

Nitrotyrosine

IP-LONP1

Sham

TAC

Carbonyls, fold change

0

1

2

3

4

0

1

2

3

4

Sham

TAC

Carbonyls on LONP1, fold change

0

0.5

1

1.5

2

2.5

3

4

Sham

TAC

Carbonyls on LONP1, fold change

0

0.5

1

1.5

2

2.5

3

4

Sham

TAC

Nitrotyrosine on LONP1, fold change

0

0.5

1

1.5

2

2.5

3

4

Sham

TAC

Nitrotyrosine on LONP1, fold change

0

0.5

1

1.5

2

2.5

3

4

Sham

TAC

(mitochondrial fraction)
Figure 4

A

B

Circulation

ETC subunits
Others
Figure 5

A

B

C

NDUFA9 (complex I)
Degraded

COX5a (complex IV)
Degraded

Cyc1 (complex III)
Degraded

TOM20

ATP (−) 10 mM ATP 12 hr

Sham

TAC

WT

i-mCAT

WT

i-mCAT

WT

i-mCAT

WT

i-mCAT
Oxidative Posttranslational Modifications Develop LONP1 Dysfunction in Pressure Overload Heart Failure

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Mice experiences

The floxed stop sequence preceding mitochondria-targeted human catalase (mCAT) in the inducible BAC model (C57BL/6J background) was kindly gifted from Dr. Rabinovitch (University of Washington), and excised by tamoxifen-induced αMHC promoted Cre. Overexpression of human catalase targeted to mitochondria was shown to be specific to mitochondria (Online Figure 3). TAC was induced in 10–12-week-old mice. The animals were intubated and ventilated with room air using a small animal respirator (SN-480-7, Shinano Seisakusyo, Tokyo, Japan), and 1.0% isoflurane inhalation was added to maintain anesthesia. The left side of the chest was opened at the second intercostal space. Aortic constriction was performed by ligation of the transverse thoracic aorta between the innominate artery and left common carotid artery with a 28-gauge needle using a 7-0 silk string. Sham operation was performed without constricting the aorta. To measure arterial pressure gradients, a 1.2 French Millar type catheter-tip micromanometer catheter (Scisense) was used. MitoTEMPO (700 μg kg⁻¹ day⁻¹)²⁻³, MG132 (720 μg kg⁻¹ day⁻¹)⁴, and epoxocimim (500 μg kg⁻¹ day⁻¹)⁵ was delivered for 2 weeks with Alzet 1002 pumps.

Study approval

All animal studies were approved by the Animal Care and Use Committee of Kyoto Prefectural University of Medicine.

FITC-casein assay

Proteolytic activity was assayed using Fluoro Protease Assay kit (G-Biosciences). The crude mitochondria (5 μg) was incubated in assay buffer containing 0.05% Triton X-100 and 10 mM
ATP for 1 hr. Fluorescence was red at 485/527 nm.

**Endogenous proteolytic activity measurement**

The crude heart mitochondria (150 μg) were incubated in 100 μM sucrose buffer and 10 mM ATP at 37°C for 12 hr. Protein degradation was assessed by immunoblotting.

**Mitochondrial purification**

Three animals from each experimental group were processed for proteomics. Heart tissues were homogenized with a Dounce Tissue Grinder in cold HE buffer (0.25 M sucrose, 10 mM HEPES, 1 mM EGTA, pH 7.5), and then the homogenate was centrifuged at 800 g twice for 5 min. The supernatant were further centrifuged at 4000 g twice for 5 min at 4°C. The crude pellets were resuspended and underwent three-step Percoll gradients fractionation. The density of gradient centrifugation contained 18%, 29%, and 52% Percoll in 0.3 M sucrose, 10 mM MOPS/KOH pH7.2. After 45 min at 70000 g centrifugation (Beckman SW 40 rotor, Beckman Coulter), intact mitochondria were isolated from the 29%/52% interphase. For iTRAQ proteomics, purified mitochondria were solubilized with the Tissue Protein Extraction Reagent (PIERCE). In this study, 60 μg of protein from each sample in each group was pooled before proteomic analysis because the extensive analysis of well-characterized pooled samples is more productive than analyzing individual samples.

**iTRAQ proteomics**

The commercial iTRAQ analysis serves (Filgen, Nagoya, Japan) was utilized for the mass spectrometric analysis. Briefly, 100 μg of proteins were reduced and alkylated prior to trypsin digestion; the resulting peptides were then lyophilized and reconstituted before labeling, according to the manufacturer's instructions (AB SCIEX). The iTRAQ tags were as follows; sham control-iTRAQ 114; TAC 2W-iTRAQ 115; TAC 8W-iTRAQ 116. The labeled digests were combined into one sample mixture. Protein identification and relative iTRAQ quantitation
were performed using an AB SCIEX TripleTOF 5600 mass spectrometer, with ProteinPilot™ software version 4.5 using Paragon™ Algorithm 4.5.0.0. HUGO Gene Nomenclature Committee (HGN) database was applied to identify ETC proteins (http://www.genenames.org/genefamilies/mitocomplex).

**Microarray**

Three groups of animals were examined: sham-operated mice (Sham), TAC-operated mice at 2 weeks (TAC 2W), TAC-operated mice at 8 weeks (TAC 8W). Total RNA was extracted from hearts using TRIzol reagent (Invitrogen) followed by RNeasy clean-up (Qiagen), according to the manufacturer's instructions. The total RNA sample of each group was combined into 1 sample for analysis. The expression of 36,142 transcripts was profiled by an analysis service (Filgen). Briefly, RNA samples were checked for RNA integrity on a Bioanalyzer2100 (Agilent Technologies). The target RNA for hybridization was prepared using MessageAmp™II-Biotin Enhanced Kit (ambion). Fragmented target aRNA (10 μg) was used for hybridization to CodeLink™ Mouse Whole Genome Bioarray slides (Applied Microarrays). The microarrays were then washed and processed using a direct detection method of the biotin-containing transcripts by a streptavidin-Cy5 conjugate. The slides were scanned using a GenePix 4000B laser scanner (Molecular Devices) and the images were digitized with CodeLink™ Expression Analysis v5.0 (Applied Microarrays). Data were normalized and are expressed as fold increase relative to data from the sham-operated heart using the MicroArray Data Analysis Tool Ver. 3.2 (Filgen).

**Transthoracic echocardiography**

One percent isoflurane inhalation was administered for sedation. Echocardiographic analysis was performed with a VisualSonics Vevo 2100 equipped with an 18- to 38-MHz probe (VisualSonics, , Toronto, ON, Canada) The LV was assessed in the parasternal short-axis view.
End-systole or end-diastole was defined as the phase in which the smallest or largest area of LV, respectively. Diastolic left ventricular internal diameters (LVIDd), systolic left ventricular internal diameters (LVIDs), and diastolic left ventricular posterior wall (LVPWd) were measured from the LV M-mode tracing at the papillary muscle level.

**Myocardial high energy phosphate measurement**

Heart tissues were freeze-clamped with Wollenberger tongs, prechilled in liquid nitrogen. Frozen samples were kept in liquid nitrogen and then lyophilized overnight. Ten milligrams of lyophilized tissue was homogenized in 0.6 N ice-cold perchloric acid and centrifuged at 500 g for 10 min at 4°C. The supernatants were neutralized with KOH to pH 5.0 to 7.0. After 10 min, the extracts were centrifuged to remove the KClO₄, and the supernatants were used for the assays. PCr, Cr, ATP, ADP, and AMP were measured by high-performance liquid chromatography (LC-20AD liquid chromatograph, Shimadzu) with a column of STR ODS-M (Shimadzu)⁷.

**Mitochondrial isolation**

Mouse heart was rapidly minced in ice cold MSE buffer (220 mM mannitol, 70 mM sucrose, 2 mM EGTA, 5 mM MOPS [pH 7.4], 2 mM taurine, and 0.2% BSA). Heart tissue was homogenized in MSE buffer with a polytron type tissue grinder at 11,000 rpm for 2.5 sec followed by 2 quick strokes at 500 rpm with a loose fit Potter-Elvenhjem tissue grinder. The homogenate was centrifuged at 800 g twice for 5 min saving the supernatant. Pellet mitochondria from the supernatant at 4000 g twice, rinsing the pellet with MSE buffer. The final pellet was rinsed and resuspended in 50 μl incubation medium (220 mM mannitol, 70 mM sucrose, 1 mM EGTA, 5 mM MOPS [pH 7.4], 2 mM taurine, 10 mM MgCl₂, 5 mM KH₂PO₄, and 0.2% BSA). Mitochondria were incubated for 15 min on wet ice and protein concentration was determined with BSA as a standard by a Bradford assay. All work is performed on wet ice.
Mitochondrial oxygen consumption measurements using the fiber-optic system

Mitochondrial oxygen consumption measurements were performed in a water-jacketed chamber maintained at 37°C. Oxygen consumption was monitored with a fiber-optic system (Instech model 210 fiber-optic oxygen monitor, Plymouth Meeting, PA) and a digital data-acquisition system (Ocean Optics, Dunedin) as previously described. Isolated mitochondria (300 μg) were resuspended in a sealed, stirred chamber. Sequential electron flow through different complexes of the electron transport chain was examined with sequential addition of substrates and inhibitors in the following order and concentrations: 4 μM Carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP); 0.5 mM NADH; 2 μM rotenone; 5 mM succinate; 1 μM antimycin A; 1 mM ascorbate and 0.4 mM N,N,N’,N’-tetramethyl-p-phenylenediamine dihydrochloride (TMPD) and 5 mM KCN. Oxygen consumption rates were expressed as nmol of O₂/min/mg protein.

Mitochondrial oxygen consumption measurements using XF24

Detailed protocol was previously reported. Briefly, isolated mitochondria 5 μg was suspended in 50 μl of Mitochondrial Assay Solution (MAS: Sucrose 70 mM, Mannitol 220 mM, KH₂PO₄ 5 mM, MgCl₂ 5 mM, HEPES 2 mM, EGTA 1 mM, BSA fatty acid-free 0.2 %, pH 7.4 adjusted with KOH 1 M) and loaded in the V7 plate. After loading, centrifugation of the V7 plate during 20 minutes at 2000 g (4°C) is performed to attach the mitochondria at the bottom of the plate. To analyze the sequential electron flow through different complexes of the electron transport chain, 400 μl of MAS + 0.5 mM NADH and 4 μM FCCP is added on top. The loaded V7 plate was incubated for 5 minutes at 37°C (no CO₂ incubator) before loading it into the XF24. Inhibitors and substrates were injected as follows: port A, 50 μl of 20 μM rotenone (2 μM final); port B, 55 μl of 100 mM succinate (10 mM final); port C, 60 μl of 40 μM antimycin A (4 μM final); port D, 65 μl of 10 mM ascorbate plus 4 mM TMPD (1 mM and 400 μM final, respectively).
Detection of H$_2$O$_2$ Production and catalase activity

The rate of H$_2$O$_2$ production in mitochondria and submitochondrial particles was determined using the oxidation of the fluorogenic indicator Amplex Red in the presence of horseradish peroxidase. The concentrations of horseradish peroxidase, Amplex Red, and CuZn-SOD in the incubation were 0.2 unit/ml, 50 μM, and 50 unit/ml, respectively. Fluorescence was recorded in a microplate reader (Infinite F200, Tecan) with 530 nm excitation and 590 nm emission wavelengths. Standard curves obtained by adding known amounts of H$_2$O$_2$ to assay medium in the presence of the reactants (Amplex Red and horseradish peroxidase) were linear up to 2 μM. Background fluorescence was measured in the absence of mitochondria and presented as fluorescence minus background (pmol per min per mg of protein). In a typical experiment, mitochondria were incubated at 0.1 mg of protein/ml for 30 min at 37 °C. H$_2$O$_2$ production in mitochondria was measured with substrate alone and the addition of respiratory chain inhibitors as follows; 0.5 mM NADH with or without 2 μM rotenone, 5 mM succinate with or without 1 μM antimycin A. To eliminate the effect of catalase, 10 mM 3-Amino-1,2,4-triazole (3-AT) was used. The rate of H$_2$O$_2$ production was linear with respect to mg of mitochondrial protein. Catalase activity was also examined using this Amplex Red/HRP system.

Proteasome activity assay

Fluorogenic substrates Suc-LLVY-AMC and Bz-VGR-AMC (BioMol/Enzo Life Sciences) were used to analyze the chymotrypsin-like activity and the trypsin-activity, respectively. Cytosolic protein (100 μg) was incubated with fluorogenic substrates for 45 min in a 96 well plate with a final volume of 200 μL per well. The plate was scanned at an excitation wavelength of 365 nm and emission wavelength of 460 nm in a microplate reader (Infinite F200, Tecan). All measurements were performed in duplicate and were further replicated in independent experiments.
**Blue native PAGE**

Crude heart mitochondria were resolved on Native PAGE Novex Bis-Tris gels according to the manufacturer’s instructions (Invitrogen). Briefly, a mitochondria pellet was lysed with NativePAGE buffer (Invitrogen) containing 10% n-dodecyl β-D-maltoside (Sigma). The sample was incubated on ice for 30 min with frequent vortexing before pelleting by centrifugation at 15000 rpm for 10 min at 4°C. After centrifugation, 100 μl of lysates adjusted to 1.0 mg/ml was transferred to a new tube containing 5 μl of 5% Coomassie Brilliant Blue G-250 (Tokyo Chemical Industry Co., Ltd.) and then 15 μl of samples underwent electrophoresis. Resolved proteins were also transferred to PVDF membrane (Millipore) to evaluate protein carbonylation.

**Immunoblot and immunoprecipitation**

Antibodies used for immunoblot included mouse anti-NDUFA9 (Invitrogen), mouse anti-COX5a (Invitrogen), rabbit anti-Cyc1 (Abcam), mouse anti-ATP synthase α (Invitrogen), rabbit anti-TOM20 (Santa Cruz), rabbit anti-LONP1 (Sigma), goat anti-GRP75 (Santa Cruz), mouse anti-GAPDH (Millipore), mouse anti-β-actin (Sigma), rabbit anti-human erythrocyte catalase (Athens Research & Technology), and rabbit anti-nitrotyrosine (Molecular Probes). HRP-conjugated secondary antibodies were obtained from Amersham Pharmacia and Millipore. PVDF membrane (Biolad) was used for blotting and signals were revealed by ECL prime (GE healthcare). For immunoprecipitation, the crude mitochondria were treated in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1 mM DTT, 1 mM EGTA, 1 mM Na$_3$VO$_4$, 50 mM NaF, supplemented with mammalian protease inhibitor cocktail: Sigma). Lysates (200 μg protein) were subjected to immunoprecipitation with anti-LONP1 antibody coupled to magnetic beads (Magnosphere MS300/Carboxyl, JSR). After the beads were washed, samples were boiled with sample buffer and subjected to immunoblot or protein carbonylation assay.

**Measurement of protein carbonylation**
Protein carbonylation was detected with commercially available OxyBlot protein oxidation detection kit (Chemicon, Millipore, Temecula, CA). An aliquot of mitochondrial protein or immunoprecipitated protein with anti-LONP1 antibody were derivatized with 2,4-dinitrophenylhydrazine (DNPH) under acid denaturing conditions. Denatured proteins were separated on a SDS-PAGE gel and transferred to PVDF membrane. Membranes were probed with a primary antibody for DNPH supplied in the kit, and a secondary horseradish-peroxidase conjugated antibody, and then developed by ECL prime. In the case of blue native PAGE, DNPH derivatization was performed after protein transfer to PVDF membrane. The membrane was incubated in 2% SDS in 50 mM Tris (pH 6.8) for 15 min before the incubation in 5 mM DNPH in 2 M HCl for 2 min, and then was probed with a primary antibody for DNPH.

**Detection of thiolate cysteines of LONP1**

The crude heart mitochondria from mice treated with or without TAC were lysed with lysis buffer containing 200 μmol/L biotinylated iodoacetamide (Sigma). Biotinylated proteins were pulled down with streptavidin beads (PIERCE). Biotinylated LONP1, representing the reduced form, were detected by immunoblot.

**RNA analysis**

Total RNA was extracted from hearts using TRIzol reagent (Invitrogen) followed by RNeasy clean-up (Qiagen), and then reverse-transcribed using a PrimeScript RT reagent kit with gDNA Eraser (Takara). Quantitative real-time reverse transcription-PCR was performed using SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara) with the Thermal Cycler Dice Real Time System (Takara) according to the manufacturer's suggestions. The expression levels of target genes were normalized by the expression levels of TIF. Sequences of primer are provided in Supplemental Table 3.

**siRNA delivery in vivo**
LONP1 siRNA and negative control siRNA were dissolved in Max Suppressor In Vivo RNA-LANCEr II (Bioo Scientific, Austin, TX) following manufacturer’s instructions; mice were injected three times at days 1, 6, and 11 after tamoxifen injections.

**Histological analysis**

The LV accompanied by the septum was cut into base, middle portion, and apex, fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned at 6 µm thickness. The sections were incubated in 3% H₂O₂ in PBS to prevent endogenous peroxidation and blocked with 5% BSA in PBS. Myocyte cross-sectional area was measured from images captured from Hematoxylin-Eosin staining sections. The outlines of 200 myocytes were traced in each section. Interstitial fibrosis was evaluated by Masson Trichrome staining. Specimens were also stained with CD31 antibody using the DAKO EnVision™ + System (DAKO Co, CA). All images were captured on a computer assisted microscope (Biorevo; Keyence, Japan).
Supplemental Tables

**Supplemental Table 1**  The weights of hearts and lungs in mice after transverse aortic constriction or sham procedure.

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<th>Sham (n=3)</th>
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<th>TAC 8W (n=3)</th>
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<td>Tibia length (mm)</td>
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<td>HW (mg)</td>
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<td>LW (mg)</td>
<td>136±13</td>
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<td>HW/BW (mg g(^{-1}))</td>
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BW indicates body weight; HW, heart weight; LW, lung weight. Data are shown as the means ± SD.
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Supplemental Table 2  Detailed data of iTRAQ and Microarray
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|------|-------------|-----------|------------|-----------|------------|-----------|------------|-----------|------------|-----------|------------|------------|
| Iars2 | Mitochondrial import inner membrane translocase subunit TIM44 | NM_007757 | -0.329588 | NM_053071 | -0.05679 | NM_026322 | -0.663156 | NM_023646 | 0.06350 | NM_023480 | 0.02645 |
| Mitochondrial import inner membrane translocase subunit TIM44 | IPI:IPI00119094.1 | 0.24318 | 0.62368 | -0.18131 | -0.098719 | IPI:IPI00132623.3 | -0.15827 | -0.09511 | Sdr39u1 | 0.02645 |
| ATP synthase, H+ transporting, mitochondrial F1 complex, subunit 9 | IPI:IPI00127598.1 | 0.45754 | -0.270887 | 0.051717 | -0.444355 | IPI:IPI00283203.3 | -0.81087 | IPI:IPI00222526.4 | -0.339338 | -0.21759 | 0.36244 |
| ATP synthase, H+ transporting, mitochondrial F1 complex, subunit 8 | IPI:IPI00133092.3 | -0.1870 | -0.3646 | 0.01722 | 0.346895 | IPI:IPI00274656.6 | 0.346895 | Peptidyl-prolyl cis-trans isomerase F, mitochondrial | Ndufc2 | -0.13163 | phenylalanine-tRNA synthetase 2 (mitochondrial) | Sirt5 | 0.17581 |
| enoyl-CoA hydratase domain containing 2 | IPI:IPI00315302.5 | 0.62368 | -0.18131 | -0.098719 | IPI:IPI00127598.1 | 0.45754 | -0.270887 | 0.051717 | -0.444355 | IPI:IPI00114342.1 | -0.10656 | Ccbl2 | 0.230441 | 0.117917 |
| Peptidase (mitochondrial processing) beta | IPI:IPI00124900.1 | -0.492069 | 0.205415 | NM_178848 | Msra | 0.88386 | IPI:IPI00124900.1 | -0.492069 | 0.205415 | NM_172398 | IPI:IPI00133562.1 | 0.024689 |
| glyoxalase domain containing 4 | IPI:IPI00114342.1 | -0.10656 | Ccbl2 | 0.230441 | 0.117917 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4, beta subunit | IPI:IPI00131584.1 | 0.024689 |
| Phenylalanine-tRNA synthetase 2 (mitochondrial) | Sirt5 | 0.17581 | Peptidase (mitochondrial processing) beta | Msra | 0.88386 | IPI:IPI00124900.1 | -0.492069 | 0.205415 | NM_178848 | Msra | 0.88386 | IPI:IPI00131584.1 | 0.024689 |
| Phenylalanine-tRNA synthetase 2 (mitochondrial) | Sirt5 | 0.17581 | Peptidase (mitochondrial processing) beta | Msra | 0.88386 | IPI:IPI00124900.1 | -0.492069 | 0.205415 | NM_178848 | Msra | 0.88386 | IPI:IPI00131584.1 | 0.024689 |</p>
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**Note:** The table above contains gene symbols, gene names, and expression values. The expression values are given in a way that indicates the fold change or log2 ratio for each gene compared to a control or reference sample. The table is structured to allow for easy reading and comparison of gene expression across different conditions or samples.
### Supplemental Table 3  
**Primer sequences used for real-time PCR**

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Supplemental Figures and Figure Legends

Supplemental Figure 1  Cardiac proteasome activity in TAC-operated hearts. Heart tissue homogenates (100 μg total cytosolic protein) were assayed for proteasome peptidase activity using fluorogenic substrates, Suc-LLVY-AMC and Bz-VGR-AMC for chymotrypsin-like activity and trypsin-activity (n = 6 per group, duplicate experiments). Data are shown as the means ± SD.

Supplemental Figure 2  The experimental work flow of iTRAQ proteomics. A, Three experimental groups (Sham, TAC 2W, and TAC 8W) were processed for proteomics. The crude
mitochondria were purified by three-step Percoll gradients fractionation. Three samples in each group were pooled before iTRAQ proteomic analysis. Protein identification and relative iTRAQ quantitation were performed using an AB SCIEX TripleTOF 5600 mass spectrometer. B, The extent of purification was assessed by immunoblotting for the cytosolic marker β-actin and the mitochondrial marker TOM20. Intact mitochondria were isolated from the 29%/52% interphase. Separated content in 18%/29% interphase contained residual cytosolic protein and broken mitochondria. Mt indicates mitochondria or mitochondrial.

**Supplemental Figure 3** Protein carbonylation in individual ETC components.

Blue Native gelelectrophoresis using crude heart mitochondria isolated from Sham and TAC 8W mice was followed by the evaluation of protein carbonylation in ETC components. DNPH adducts were observed in complexes I, III, and IV and their signals were prominent in TAC 8W mice.
Supplemental Figure 4  αMHC-inducible mitochondria-targeted human catalase (mCAT). The flox-stopped mCAT BAC mouse model was used. Floxed STOP is excised by Tamoxifen induced αMHC-Cre. A, Representative immunoblots of cardiac mitochondrial and cytosolic fractions showing that human catalase was mainly found in mitochondrial fraction. GRP75 and NDUFA9 were used as a mitochondrial marker and GAPDH was as a cytosolic marker. Some contamination of the cytosolic fractions with mitochondrial proteins is due to inadvertent disruption of mitochondria during tissue homogenization. B, The catalase activity examined using the Amplex Red/HRP system showed that αMHC-inducible mCAT provided an approximately 20-fold increase in total catalase activity in heart mitochondria (n = 3 per group, duplicate experiments). WHL indicates whole heart lysate. Data are shown as the means ± SD.
Supplemental Figure 5  The histological examination of TAC-operated hearts in WT and αMHC-i-mCAT mice. A, Representative images of Masson trichrome staining. Scale bars, 1 mm and 500 μm, respectively. B, The area of fibrosis relative to the total area of ventricle in
short axis section was calculated with BZ-Analyzer software (n = 3 per group). C, Representative images of hematoxylin-eosin staining. Scale bars, 1 mm. Heart weight per body weight (n = 3 per group). D, Magnified images of hematoxylin-eosin staining and left ventricular myocyte cross-sectional area. Scale bars, 50 µm (n = 3 per group). E, Representative images of immunostaining for CD31 and left ventricular capillary density. Scale bars, 50 µm (n = 3 per group). Data are shown as the means ± SD.

Supplemental Figure 6 A LONP1 inhibitor attenuated the protective effects of mCAT induction. TAC-operated mice were infused with MG132 (720 µg kg⁻¹ day⁻¹), epoxomicin (500 µg kg⁻¹ day⁻¹), or the carrier DMSO for 2 weeks starting on the first day of tamoxifen injections.
A. The FITC-casein assay was performed to assess the ATP-dependent proteolytic activity of isolated heart mitochondria (n = 4 per group, duplicate experiments). B. Heart tissue homogenates (100 μg total cytosolic protein) were assayed for proteasome peptidase activity using fluorogenic substrates, Suc-LLVY-AMC for chymotrypsin-like activity (n = 4 per group, duplicate experiments). C. The sequential state III oxygen consumption rate of isolated heart mitochondria was assessed using the Seahorse XF24 Extracellular Flux Analyzer (n = 5 per group, five replicate experiments). D. Echocardiographic parameters of trans-thoracic M-mode tracings (n = 8-12 per group). Data are shown as the means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001.

Supplemental References


