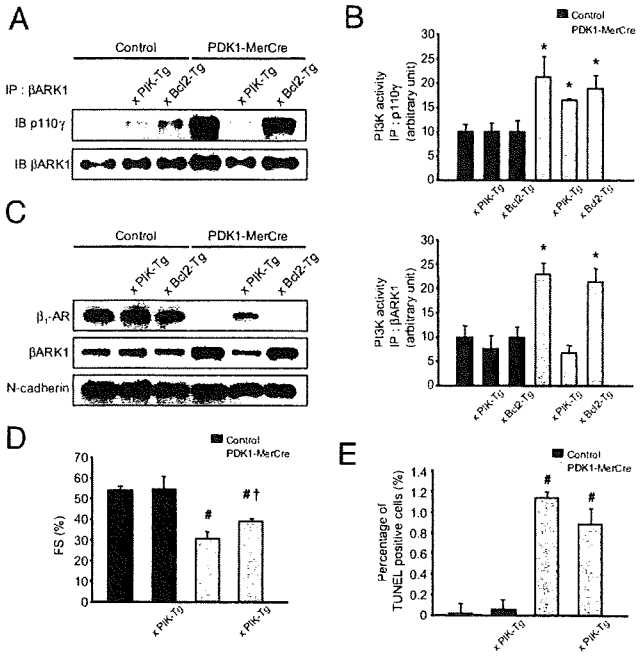


**Fig. 4.** Alleviated cardiac dysfunction in PDK1-MerCre mice by overexpression of PIK domain or Bcl-2 protein. (*A*) Immunoblot analysis of βARK1-associated p110γ protein in the hearts. (*B*) Kinase assays for PI3-K activity in the hearts. The hearts were subjected to immunoprecipitaion with antibody to p110γ (*Upper*) or βARK1 (*Lower*), and the resulting precipitates were assayed for the kinase assay. PI3-K activity of control mice was adjusted to 10 arbitrary units. (*C*) Immunoblot analysis of $\beta_1$-AR and βARK1 in membrane fraction in the hearts. N-cadherin was used as an internal control for the amount of membrane protein. (*D*) Fractional shortening measured by echocardiography. Values represent the mean ± SEM of data from control mice (n = 10), control × PIK-Tg mice (n = 6), PDK1-MerCre mice (n = 10), and PDK1-MerCre × PIK-Tg mice (n = 6). #, $P < 0.01$ versus control mice. †, $P < 0.01$ versus PDK1-MerCre mice. FS, % of fractional shortening. (*E*) Percentage of TUNEL-positive caridomyocytes. Values represent the mean ± SEM (3,000 cardiomyocytes in each group). #, $P < 0.01$ versus control group. †, $P < 0.01$ versus PDK1-MerCre group.

## Discussion

Our present study revealed that PDK1 plays an integrative role in normal cardiac function by coordinating survival signals and β-adrenergic response (Fig. S6). Besides the fundamental role in promoting cell growth and survival observed in many tissues in common (18–21), PDK1 uniquely accommodates β-adrenergic response to prevent cardiac decompensation. In addition, decreased expression of PDK1 protein in experimental models of heart failure raises a possibility that functional alterations of PDK1 may be implicated in the pathogenesis of heart failure, although it remains unclear how PDK1 expression is regulated in stressed hearts.

β-AR signaling plays a pivotal role in the chronotropic and inotropic functions in the hearts (22). In PDK1-MerCre hearts, the activity of βARK1-associated PI3-Kγ was enhanced, which enforced robust $\beta_1$-AR down-regulation. PDK1 is a direct downstream effector of PI3-K and may participate in the negative feedback regulation of PI3-K signaling pathway (20). Importantly, overexpression of PIK-domain prevented $\beta_1$-AR down-regulation by interfering βARK1/PI3-Kγ complex formation, and alleviated cardiac dysfunction in PDK1-MerCre mice. A recent report demonstrated that PI3-Kγ negatively modulates cardiac contractility by promoting phosphodiesterase 3B-mediated destruction of cAMP in a kinase-independent manner (23), but we did not observe significant change in the activity of phosphodiesterase 3B in PDK1-MerCre hearts despite enhanced PI3-Kγ activity (Fig. S7). Therefore, we suppose that impairment of β-adrenergic responsiveness results from intense β-AR down-regulation in PDK1-MerCre hearts.

It remains controversial whether down-regulation and desensitization of β-AR function is beneficial or detrimental in failing hearts. Indeed, clinical trials have indicated that the use of β-AR antagonists improves morbidity and mortality in patients of heart failure (1). Sustained β-AR overstimulation promotes energy consumption and apoptosis in cardiomyocytes (1, 24). But, accumulating evidence has suggested that normalization of β-adrenergic signaling by interfering βARK1 function rescued numerous genetic and experimental models of heart failure in mice (16, 25–28). A possible explanation for this discrepancy is that the therapeutic window for optimal level of β-AR signaling may be narrow in failing hearts (22, 28). It has been reported that the proapoptotic effect of $\beta_1$-AR stimulation is dependent on $Ca^{2+}$/calmodulin-dependent kinase II (CaMKII) (24). The phosphorylation level of CaMKII was decreased in PDK1-MerCre hearts, and restored to a subnormal level by overexpression of PIK domain (Fig. S8). Importantly, normalization of $\beta_1$-AR did not induce excessive activation of CaMKII and cardiomyocyte apoptosis (Fig. 4*E* and Fig. S4). Thus, the $\beta_1$-AR normalization may improve contractile function without evoking a 'fight or flight' reaction, unlike the simple $\beta_1$-AR activation. Alternatively, robust β-AR internalization may activate adverse intracellular signaling pathways through β-arrestins (29) and abrogate the cardioprotective effects mediated by transactivation of epidermal growth factor receptor (30). Further investigations will be required to clarify the entire mechanisms of how normalization of β-AR signaling confers therapeutic benefits on failing hearts.

A growing body of evidence has suggested that cardiomyocyte apoptosis plays an important role in the pathogenesis of heart failure (31). In PDK1-MerCre hearts, the phosphorylation levels of Akt, SGK1 and FOXO3a were reduced, which may give rise to marked increase in cardiomyocyte apoptosis. In addition, PDK1-MerCre hearts showed an increase in expression level of Bax protein, a key molecule that translocates to the mitochondrial membrane and triggers the release of cytochrome *c* into the cytoplasm (31). Overexpression of Bcl-2 attenuated apoptotic loss of cardiomyocytes and alleviated cardiac dysfunction in PDK1-MerCre mice, suggesting that cardiomyocyte apoptosis contributes to the development of heart failure.

The previous paper demonstrated that PDK1-MCKCre mice showed growth retardation and contractile dysfunction of cardimyocytes (5). In our study, PDK1-MerCre mice showed severe heart failure without alterations in cardiomyocyte size. Besides regulation of cell growth, PDK1 controls cardiac homeostasis by promoting cell survival and preserving β-AR response. The phenotypic difference between PDK1-MerCre mice and PDK1-MCKCre mice resulted from the timing of gene disruption. The *Pdk1* gene was deleted within a week in tamoxifen-treated PDK1-MerCre hearts of adult mice, but in contrast, *Pdk1* disruption commenced before birth in PDK1-MCKCre mice. The number of apoptotic cardiomyocytes was pronouncedly increased in PDK1-MerCre hearts, but was unchanged in PDK1-MCKCre hearts (5). Some compensation mechanisms may prevent proapoptotic effects of *Pdk1* disruption in PDK1-MCKCre mice.

In conclusion, PDK1 is a pivotal effector with dual functions to promote survival of cardiomyocytes and to preserve β-AR response in vivo (Fig. S6). In this regard, up-regulation of PDK1 in the hearts may emerge as a potential therapeutic strategy for heart failure.

## Methods

**Generation of PDK1-MerCre Mice.** Mice harboring a *Pdk1*$^{flox}$ allele were previously described (7, 8). Mice expressing MerCreMer under the control of *α-myosin heavy chain* promoter were previously described (9). Details are in *SI Methods*. Bcl2-Tg mice and PIK-Tg mice were kindly gifted by Dr. Michael D. Schneider (Imperial

College, London, U.K.) (15) and Dr. Howard A. Rockman (Duke University Medical Center, Durham, NC) (16). All of the experimental protocols were approved by the Institutional Animal Care and Use Committee of Chiba University.

**Echocardiography and Isolated Heart Preparation.** Transthoracic echocardiography was performed on conscious mice with Vevo 660 Imaging System using a 25-MHz linear probe (Visual Sonics Inc.). For analyses of hemodynamic parameters, hearts were excised rapidly and mounted on a Langendorff perfusion system, and a balloon was inserted into the cavity of the left ventricle (32). Isolated hearts were stabilized for 30 min by perfusion of Krebs-Henseleit buffer followed by perfusion of isoproterenol (NIKKEN Chemical Laboratory) or forskolin (Sigma). For measurement of surface areas of cardiomyocytes, hearts were enzymatically dissociated as described previously (33).

**Histological Analysis and Immunohistochemistry.** Hearts were excised and immediately fixed in 10% neutralized formalin, embedded in paraffin. Serial sections at 5 $\mu$m were stained with hematoxylin and eosin for morphological analysis, and with Masson's trichrome for detection of fibrosis. For immunohistochemistry, Vectastain ABC kit (Vector Laboratories) was used to detect the primary antibodies. TUNEL assay was performed on paraffin sections, using an in situ apoptosis detection kit (Takara Bio Inc.).

**Western Blot Analysis and Subcellular Fractionation.** Protein samples were fractionated by SDS/PAGE, and immunoblot analysis was performed as described previously (34). The membrane and cytosol fractions were isolated from lysate of the hearts as previously described (35).

**Assay for PI3-K Activities.** PI3-K acitivity was measured as previously described (36). We determined Akt activity using a Akt Kinase Assay Kit according to the manufacturer's protocol (Cell Signaling Technology).

**Antibodies.** The following antibodies were used: p110$\gamma$, phosphorylated-SGK, and cleaved caspase-3 (Cell Signaling Technology), $\beta$ARK1, Bax, Bcl-xL, Bcl-2 (Santa Cruz Biotechnology), $\beta_1$-AR (Affinity BioReagents), N-cadherin (Zymed Laboratories Inc.), SGK1, FOXO3a, phosphorylated-FOXO3a (Thr-32), phosphorylated-FOXO3a (Ser-253) (Upstate) and actin (Sigma).

**Statistical Analysis.** All data are presented as means ± SEM. All data were analyzed by one-way ANOVA followed by the Fisher procedure for comparison of means. A probability value of $P < 0.05$ was considered to be statistically significant.

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Review

# Mitochondrial oxidative stress and dysfunction in myocardial remodelling

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Recent experimental and clinical studies have suggested that oxidative stress is enhanced in myocardial remodelling and failure. The production of oxygen radicals is increased in the failing heart, whereas normal antioxidant enzyme activities are preserved. Mitochondrial electron transport is an enzymatic source of oxygen radical generation and can be a therapeutic target against oxidant-induced damage in the failing myocardium. Chronic increases in oxygen radical production in the mitochondria can lead to a catastrophic cycle of mitochondrial DNA (mtDNA) damage as well as functional decline, further oxygen radical generation, and cellular injury. Reactive oxygen species induce myocyte hypertrophy, apoptosis, and interstitial fibrosis by activating matrix metalloproteinases. These cellular events play an important role in the development and progression of maladaptive myocardial remodelling and failure. Therefore, oxidative stress and mtDNA damage are good therapeutic targets. Overexpression of the genes for peroxiredoxin-3 (Prx-3), a mitochondrial antioxidant, or mitochondrial transcription factor A (TFAM), could ameliorate the decline in mtDNA copy number in failing hearts. Consistent with alterations in mtDNA, the decrease in mitochondrial function was also prevented. Therefore, the activation of *Prx-3* or *TFAM* gene expression could ameliorate the pathophysiological processes seen in mitochondrial dysfunction and myocardial remodelling. Inhibition of oxidative stress and mtDNA damage could be novel and effective treatment strategies for heart failure.

## 1. Introduction

Heart failure is a leading cause of morbidity and mortality in industrialized countries.[1] It is also a growing public health problem, mainly because of aging of the population and the increase in the prevalence of heart failure in the elderly. Previous basic, clinical, and population sciences have advanced the modern treatment of heart failure. Despite extensive studies, the fundamental mechanisms responsible for the development and progression of left ventricular (LV) failure have not yet been fully elucidated.

Reactive oxygen species (ROS) such as superoxide anions ($\cdot O_2^-$) and hydroxy radicals ($\cdot OH$) cause the oxidation of membrane phospholipids, proteins, and DNA[2] and have been implicated in a wide range of pathological conditions including ischaemia-reperfusion injury,[3] neurodegenerative diseases,[4] and aging.[5] Under physiological conditions, their toxic effects can be prevented by scavenging enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSHPx), and catalase as well as by other non-enzymatic antioxidants. However, when the production of ROS exceeds the capacity of antioxidant defenses, oxidative stress might have a harmful effect on the functional and structural integrity of biological tissue. ROS cause contractile failure and structural damage in the myocardium. The importance of oxidative stress is increasingly emerging with respect to a pathophysiological mechanism of LV remodelling responsible for heart failure progression.

Recent evidence indicates a prominent role of ROS as signalling molecules in the response to hormones, growth, and coagulation factors, cytokines, and other factors, as well as to changes in oxygen tension.[6] Many previous studies support the notion that an elevation of levels of mitochondrial ROS, during hypoxia, can control the activation of hypoxia-inducible factor (HIF)-1α. HIFs are key players in the cellular response to changes in oxygen tension. Mitochondrial ROS are intrinsically linked to HIF-1α expression during hypoxia and multiple protein kinases have the potential to be involved in the mechanism(s) by which the ROS signal is transduced to HIF-1α.[7] Recently, HIFs have also been shown to respond to non-hypoxic stimuli. However, the specific mechanisms whereby ROS accomplish this remain to be established. In addition, there were several reports that antioxidants worsened heart failure in some cases.[8]

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## 2. Mitochondrial oxidative stress in cardiac failure

Recent experimental and clinical studies have suggested the generation of ROS increases in heart failure.[9-12] Levels of lipid peroxides and 8-iso-prostaglandin F2α, the major biochemical markers of ROS generation, have been shown to be elevated in the plasma and pericardial fluid of patients with heart failure and also are positively correlated to its severity.[9,12] Belch *et al.*[9] reported that there was a significant negative correlation between malondialdehyde and LV ejection fraction ($r = -0.35$). Mallat *et al.*[12] assessed the functional severity of heart failure by use of the NYHA classification and the echocardiographic indices (LV diameters and the derived LV fractional shortening) and correlated these with pericardial fluid levels of 8-iso-$PGF_{2\alpha}$. Pericardial levels of 8-iso-$PGF_{2\alpha}$ were significantly increased in patients with symptomatic heart failure (NYHA II and III) compared with findings in asymptomatic patients (NYHA I) and gradually increased with the functional severity of heart failure ($P = 0.0003$). In addition, pericardial levels of 8-iso-$PGF_{2\alpha}$ were significantly correlated with LV end-diastolic and end-systolic diameters ($P = 0.008$ and 0.026, respectively).[12]

By using electron spin resonance (ESR) spectroscopy combined with the nitroxide radical, 4-hydroxy-2,2,6,6-tetramethyl-piperidine-*N*-oxyl (hydroxy-TEMPO), we definitively and directly demonstrated enhanced generation of ROS in the failing myocardium.[13] $\cdot O_2^-$ is a primary radical that could lead to the formation of other ROS, such as $H_2O_2$ and ·OH, in the failing myocardium. ·OH could arise from electron exchange between $\cdot O_2^-$ and $H_2O_2$ via the Harber-Weiss reaction. In addition, ·OH is also generated by the reduction of $H_2O_2$ in the presence of endogenous iron by means of the Fenton reaction. The generation of ·OH implies a pathophysiological significance of ROS in heart failure because ·OH radicals are the predominant oxidant species causing cellular injury.[14] A decreased antioxidant capacity could further aggravate the ROS accumulation in heart failure. However, the activities of SOD, catalase, and GSHPx were not decreased in the failing hearts,[15] indicating that oxidative stress in heart failure is primarily due to the enhancement of pro-oxidant generation rather than to the decline in antioxidant defenses.

The cellular sources of ROS generation within the heart include cardiac myocytes, endothelial cells, and neutrophils. Within cardiac myocytes, ROS can be produced by several mechanisms including mitochondrial electron transport, NAD(P)H oxidase, and xanthine dehydrogenase/xanthine oxidase. The heart has the highest oxygen uptake rate within the human body, consuming about 0.1 mL $O_2$/g per minute at basal rates.[16] To meet the demand for synthesis of ATP by oxidative metabolism, cardiac myocytes have the highest volume density of mitochondria in the entire body. Mitochondria produce ROS through a single electron transport to molecular oxygen in the respiratory chain. Under physiological conditions, small quantities of ROS are formed during mitochondrial respiration, which, however, can be detoxified by the endogenous scavenging mechanisms of myocytes.

By using ESR spectroscopy with 5,5'-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a spin trap, the inhibition of electron transport at the sites of complex I and complex III in the normal submitochondrial particles resulted in a significant production of $\cdot O_2^-$.[17] Mitochondria from heart failure produced more $\cdot O_2^-$ than normal mitochondria in the presence of NADH, indicating that mitochondrial electron transport could be the predominant source of such $\cdot O_2^-$ production. Furthermore, the failing mitochondria were associated with a decrease in complex enzyme activity. Therefore, mitochondria are an important source of ROS in failing hearts, indicating a pathophysiological link between mitochondrial dysfunction and oxidative stress[18] as has been reported in other disease conditions including aging and neurodegenerative diseases.

Even though mitochondrial electron transport is considered to play an important role in ROS production in heart failure, other enzymatic sources of ROS generation such as vascular endothelial cells (via xanthine oxidase and/or NAD(P)H oxidase) and activated leukocytes (via NAD(P)H oxidase) could also contribute to oxidative stress.[19] In fact, Bauersachs *et al.*[20] have demonstrated that vascular NAD(P)H oxidase is activated in heart failure. NAD(P)H oxidase is the major source of ROS in both the endothelium and vascular smooth muscle. An increase in myocardial activity of NAD(P)H oxidase has been also observed in human heart failure.[21] Recently, by using mice lacking the cytosolic NAD(P)H oxidase component $p47^{phox}$ ($p47^{phox-/-}$ mice), Doerries *et al.*[22] demonstrated that a deficiency of the NAD(P)H oxidase and its subunit $p47^{phox}$ protected the heart from remodelling and dysfunction after myocardial infarction (MI). $p47^{phox}$ deficiency reduced LV cavity dilatation and dysfunction as well as cardiac myocyte hypertrophy, apoptosis, and interstitial fibrosis after MI, all of which contributed to improved survival. Therefore, NAD(P)H oxidase activation might also be involved in increased myocardial oxidative stress in patients with heart failure. They are able to generate ROS in response to angiotensin II, which stimulates the expression of NAD(P)H oxidase.[23,24] Plasma renin activity as well as tissue ACE activity is activated in patients with heart failure.[25,26] Therefore, an enhanced formation of angiotensin II may lead to oxidative stress via this enzyme system. Recently, Doughan *et al.*[27] provided the first evidence that angiotensin II mediate mitochondrial dysfunction in vascular endothelial cells. Angiotensin II increased mitochondrial ROS production, which was associated with decreased endothelial NO bioavailability. Furthermore, angiotensin II-mediated mitochondrial dysfunction was dependent on activation of vascular NAD(P)H oxidases and opening of the $mitoK_{ATP}$ channels.[27]

## 3. Mitochondrial DNA damage, dysfunction, and oxidative stress

Mitochondria have their own genomic system, mitochondrial DNA (mtDNA), a closed-circular double-stranded DNA molecule of ~16.5 kb. MtDNA contains two promoters, the light-strand and heavy-strand promoters (LSP and HSP, respectively), from which transcripts are produced and then processed to yield the individual mRNAs encoding 13 subunits of the oxidative phosphorylation, including seven subunits (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6) of rotenone-sensitive NADH-ubiquinone oxidoreductase (complex I), one subunit (cytochrome b) of ubiquinol-cytochrome c oxidoreductase (complex III), three subunits

(COI, COII, and COIII) of cytochrome c oxidase (complex IV), and two subunits (ATPases 6 and 8) of complex V along with 22 tRNAs and two rRNA (12S and 16S) subunits.[28,29] Transcription from the LSP also produces RNA primer, which is necessary for initiating mtDNA replication. Mitochondrial function is controlled by the mtDNA as well as by factors that regulate mtDNA transcription and/or replication.[30] This raises the possibility that mtDNA damage and the impairment of mitochondrial gene transcription and/or replication are involved in heart failure. Indeed, heart failure is frequently associated with qualitative and quantitative defects in mtDNA.[31–34] Our previous studies have shown that the decline in mitochondrial function and mtDNA copy number plays a major role in the development of heart failure that occurs after MI.[17,35]

ROS can damage mitochondrial macromolecules either at or near the site of their formation. Therefore, in addition to the role of mitochondria as a source of ROS, the mitochondria themselves can be damaged by ROS. The mtDNA could be a major target for ROS-mediated damage for several reasons. First, mitochondria do not have a complex chromatin organization consisting of histone proteins, which may serve as a protective barrier against ROS. Second, mtDNA has a limited repair activity against DNA damage. Third, a large part of $\cdot O_2^-$, which is formed inside the mitochondria, cannot pass through the membranes and, hence, ROS damage may be contained largely within the mitochondria. In fact, mtDNA accumulates significantly higher levels of the DNA oxidation product, 8-hydroxydeoxyguanosine, than nuclear DNA.[36] As opposed to nuclear-encoded genes, mitochondrial-encoded gene expression is largely regulated by the copy number of mtDNA.[37] Therefore, mitochondrial injury is reflected by mtDNA damage as well as by a decline in the mitochondrial RNA (mtRNA) transcripts, protein synthesis, and mitochondrial function.[38,39] We demonstrated that the increased generation of ROS was associated with mitochondrial damage and a dysfunction in the failing hearts, which were characterized by an increased lipid peroxidation in the mitochondria, a decreased mtDNA copy number, a decrease in the number of mtRNA transcripts, and a reduced oxidative capacity due to low complex enzyme activities.[35] Importantly, the enzymatic activities of complexes I, III, and IV all decreased in the failing hearts, whereas there was no decrease in the enzymatic activity of complex II and citrate synthase, both of which were exclusively encoded by nuclear DNA. Chronic increases in ROS production are associated with mitochondrial damage and dysfunction, which thus can lead to a catastrophic cycle of mitochondrial functional decline, further ROS generation, and cellular injury (*Figure 1*). MtDNA defects may thus play an important role in the development and progression of myocardial remodelling and failure.

A number of pathogenic mtDNA base substitution mutations, such as misssense mutations and mtDNA rearrangement mutations (deletions and insertions), have been identified in patients with mitochondrial diseases.[34] An accumulation of the deleted forms of mtDNA in the myocardium frequently results in cardiac hypertrophy, conduction block, or heart failure.[40] Furthermore, there is now a consensus view that mutations in mtDNA and abnormalities in mitochondrial function are associated with common forms of cardiac diseases such as ischaemic heart disease[41] and dilated cardiomyopathy.[42] In these conditions, however, the strict causal relationships between abnormalities in mtDNA and cardiac dysfunction have yet to be fully elucidated.[43] Even though the mechanisms by which mtDNA damage arises in these conditions have not been clarified, ROS have been proposed to be the primary contributing factor. We have provided direct evidence that the abnormalities in mtDNA replication/transcription as well as repair occur not only in a limited small subset of mitochondrial diseases but also in a more common form of heart failure phenotype such as that seen occurring after MI, in cases of cardiomyopathy, or in the diabetic heart.[44–46]

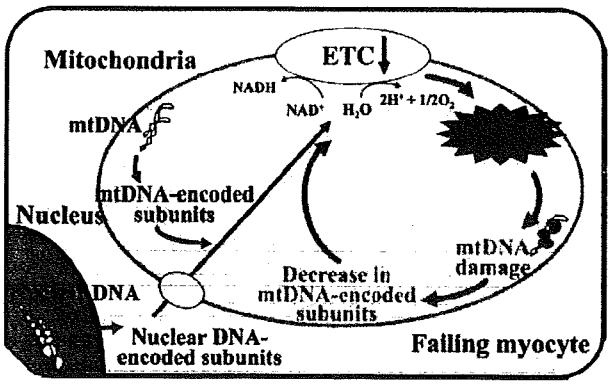


Figure 1 Schematic representation of an intimate link between reactive oxygen species (ROS), mitochondrial DNA (mtDNA) damage, and respiratory chain dysfunction in the mitochondria. Mitochondrial ROS generation may lead to a catastrophic cycle of mitochondrial functional decline, further ROS generation, and cellular injury. ATP, adenosine triphosphate; ETC, electron transport chain.

## 4. Role of mitochondrial oxidative stress in myocardial remodelling

Oxidative stress has direct effects on cellular structure and function and may activate integral signalling molecules in myocardial remodelling and failure (*Figure 2*). ROS result in a phenotype characterized by hypertrophy and apoptosis in isolated cardiac myocytes.[47]

Another potential target of ROS is matrix metalloproteinases (MMPs), a family of proteolytic enzymes. MMPs play a pivotal role in normal tissue remodelling processes, such as cell migration, invasion, proliferation, and apoptosis. They regulate many developmental processes, including branching morphogenesis, angiogenesis, wound healing, and extracellular matrix degradation. This proteolytic system is constitutively expressed in a large number of cell and tissue types and degrades a wide spectrum of extracellular matrix proteins.[48] ROS have also been shown to activate MMP in cardiac fibroblasts.[49] Myocardial MMP activity is increased in the failing hearts.[47,50] Further, an MMP inhibitor has been shown to limit early LV dilatation in a murine model of MI.[51] We have shown significant improvement in survival after MI in MMP-2 knockout mice, which was mainly attributable to the inhibition of early cardiac rupture and the development of subsequent LV dysfunction.[52] Because MMP can be activated by ROS,[53] one proposed mechanism of LV remodelling is the activation of MMPs secondary to increased ROS production. Sustained MMP activation might influence the structural properties of the myocardium by providing an abnormal extracellular environment with which the

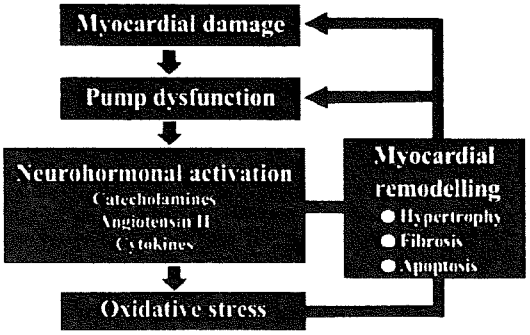


Figure 2 Role of neurohormonal activation and oxidative stress in the onset and progression of myocardial remodelling and failure.

myocytes interact. We have demonstrated that an ·OH scavenger, dimethylthiourea, inhibits the activation of MMP-2 in association with the development of LV remodelling and failure.[54] These data raise the interesting possibility that enhanced oxidative stress after MI can be a stimulus for myocardial MMP activation, which might play an important role in the development of heart failure.

## 5. Role of oxidative stress and skeletal muscle dysfunction in heart failure

The limitation of exercise capacity is a major symptom in patients with heart failure[55] and is independent of the degree of their cardiac dysfunction.[56] Increased oxidative stress has been shown to be related to the limitation of exercise capacity in patients with heart failure.[57] We have demonstrated that ROS are increased in the skeletal muscle of mice with heart failure after MI and that they originate from $\cdot O_2^-$ produced by mitochondria.[58] Recently, Kinugawa *et al.*[59] clarified the relationship between $\cdot O_2^-$ and the limitation of exercise capacity by using heterozygous manganese superoxide anion dismutase (SOD2) gene-knockout mice, in which SOD2, a family of enzymes that catalyse the dismutation of $\cdot O_2^-$, is reduced by 30–80%, increasing $\cdot O_2^-$ production in the mitochondria, associated with altered mitochondrial function. The whole-body oxygen consumption ($VO_2$) and carbon dioxide production ($VCO_2$) at rest were increased in $SOD2^{+/-}$. The work (vertical distance run × body weight) to exhaustion was decreased in $SOD2^{-/-}$. When the maximum $VO_2$ and $VCO_2$ were corrected to per work unit, they were increased in $SOD2^{-/-}$. Tempol, a SOD mimetic, normalized basal $VO_2$ and $VCO_2$ and improved the work to exhaustion and corrected $VO_2$ and $VCO_2$ in $SOD2^{+/-}$. There was a decrease in SOD2 protein levels and a concomitant increase in lucigenin-detectable $\cdot O_2^-$ production in skeletal muscle from $SOD2^{-/-}$. Therefore, exercise capacity was reduced in conditions in which $\cdot O_2^-$ was increased, and this was associated with a greater increase in whole-body oxygen consumption.

Uncoupling proteins (UCPs) are inner mitochondrial membrane proton transporters and decrease the proton electrochemical gradient across the inner mitochondrial membrane. Decreases in the electrochemical gradient reduce the energy force for adenosine triphosphate (ATP) biosynthesis during respiration requiring oxygen and stimulate heat production. Myocardial expression of UCP-2 was significantly increased and levels of the high-energy phosphate creatine phosphate (CrP) were decreased in heart failure.[60,61] In the failing heart, oxygen consumption is disproportionately high despite a decrease in CrP or the ratio of CrP to ATP, indicating a reduction in myocardial energy efficiency.[62] In addition, Echtay *et al.*[63] have shown that ROS inside mitochondria activate the expression of UCPs. These results suggested that ROS could cause the alteration of energy efficiency through the expression of UCP-2, which might have an important role in regulating cardiac as well as skeletal muscle function during the process of heart failure.

## 6. Amelioration of mitochondrial dysfunction and myocardial remodelling

### 6.1 GSHPx

The first line of defense mechanism against ROS-mediated cardiac injury comprises several antioxidant enzymes including SOD, catalase, and GSHPx. Among these antioxidants, GSHPx is an important enzyme that performs several vital functions. GSHPx is a key antioxidant that catalyses the reduction of $H_2O_2$ and hydroperoxides. ·OH arises from electron exchange between $\cdot O_2^-$ and $H_2O_2$ via the Harber–Weiss reaction. In addition, ·OH is also generated by the reduction of $H_2O_2$ in the presence of endogenous iron by means of the Fenton reaction. GSHPx scavenges $H_2O_2$, which results in the prevention of the formation of other more toxic radicals such as ·OH.[14] GSHPx possesses a higher affinity for $H_2O_2$ than catalase. Further, it is present in relatively high amounts within the heart especially in the cytosolic and mitochondrial compartments.[64] These lines of evidence imply the primary importance of GSHPx as a defense mechanism within the heart compared with catalase. Moreover, GSHPx is expected to exert greater protective effects against oxidative damage than SOD because greater dismutation of $\cdot O_2^-$ by SOD may result in an increase of $H_2O_2$. Therefore, compared with SOD or catalase, GSHPx is thought to be more effective in protecting cells, tissues, and organs against oxidative damage.[65]

GSHPx gene overexpression inhibited the development of LV remodelling and failure after MI, which might contribute to the improved survival.[66] These findings not only extended the previous observation that employed antioxidants, but also revealed the major role of ROS in the pathophysiology of myocardial remodelling. These effects were associated with the attenuation of myocyte hypertrophy, apoptosis, and interstitial fibrosis.[66] Similarly, overexpression of the GSHPx gene attenuated myocardial remodelling and preserved diastolic function in diabetic heart.[67] Therefore, therapies designed to interfere with oxidative stress by using GSHPx could be beneficial to prevent myocardial remodelling and failure.

### 6.2 Manganese superoxide dismutase

Manganese superoxide dismutase (MnSOD) is the primary mitochondrial antioxidant enzyme and is essential for maintaining normal cell development and function. Overexpression of the MnSOD gene has been shown to be beneficial in various animal models of cardiac diseases.[68,69] Recently, Shen *et al.*[70] demonstrated that protection of cardiac mitochondria by overexpression of the MnSOD gene reduced the severity of diabetic cardiomyopathy. Crossing *MnSOD*

transgenic mice with a type 1 diabetic mouse model improved respiration and normalized mass in diabetic mitochondria. MnSOD also protected the morphology of diabetic hearts and completely normalized contractility in diabetic myocytes. These results showed that elevating levels of MnSOD provided extensive protection to diabetic mitochondria and provided overall protection to the diabetic heart. Interestingly, MnSOD gene overexpression also elevated levels of myocyte catalase and mitochondrial GSH, which might also act together with MnSOD against oxidative stress.

On the contrary, Nojiri *et al.*[71] reported that heart/muscle-specific *MnSOD*-deficient mice developed progressive heart failure with specific molecular defects in mitochondrial respiration in association with excess formation of superoxide and transcriptional alterations of genes associated with heart failure. Importantly, administration of an SOD mimetic significantly ameliorated these abnormalities.

### 6.3 Peroxiredoxin-3

Peroxiredoxin-3 (Prx-3) is a mitochondrial antioxidant protein and member of the Prx family that can scavenge $H_2O_2$ in cooperation with thiol and peroxynitrite.[72] Among six known mammalian Prxs, Prx-1 to -4 require the small redox protein thioredoxin (Trx) as an electron donor to remove $H_2O_2$, whereas Prx-5 and -6 can use other cellular reductants, such as GSH, as their electron donor.[73] Prx-1, -2, and -6 are found in the cytoplasm and nucleus, whereas Prx-3 contains a mitochondrial localization sequence, is found exclusively in the mitochondria,[74] and uses mitochondrial Trx-2 as the electron donor for its peroxidase activity.[75] Prx-3 functions not only by removing $H_2O_2$ formed after the SOD-catalysed dismutation reaction but also by detoxifying peroxynitrite. Moreover, *in vivo* transfer of *Prx-3* protected neurons against cell death induced by oxidative stress.[76]

We have recently demonstrated that the overexpression of *Prx-3* protects the heart against post-MI remodelling and failure in mice. It reduces LV cavity dilatation and dysfunction as well as myocyte hypertrophy, interstitial fibrosis, and apoptosis of the non-infarcted myocardium. These beneficial effects of the Prx-3 gene overexpression were associated with the attenuation in oxidative stress, mtDNA decline, and dysfunction.[44] The specific localization of Prx-3 in the mitochondria suggests that mitochondrial oxidative stress plays an important role in the development and progression of heart failure and the antioxidant localized specifically within the mitochondria provides a primary line of defense against this disease process.

### 6.4 Mitochondrial transcription factor A

Mitochondrial transcription factor A (TFAM) is a nucleus-encoded protein that binds upstream of the LSP and HSP of mtDNA and promotes transcription of mtDNA. TFAM not only regulates mtDNA transcription and replication,[77] but also maintains mtDNA copy number. In fact, *Tfam* knockout mice, which had a 50% reduction in their transcript and protein levels, exerted a 34% reduction in the mtDNA copy number, 22% reduction in the mitochondrial transcript levels, and partial reduction in the cytochrome *c* oxidase levels in the heart.[78] Moreover, cardiac-specific disruption in Tfam in mice exhibited dilated cardiomyopathy in association with a reduced amount of mtDNA and mitochondrial transcripts.[79] The transfection of antisense plasmids in culture, designed to reduce the expression of *TFAM*, effectively decreased the levels of mtDNA encoded transcripts.[31] On the contrary, the forced overexpression of *TFAM* could produce the opposite effect.[80] These lines of evidence have established a critical role for TFAM in regulation of mtDNA copy number and mitochondrial function as well as maintenance of the physiological function of the heart. In addition, a reduction in *TFAM* expression has been demonstrated in several forms of cardiac failure.[32,35,46,81]

By using transgenic mice that overexpress human TFAM, we examined whether TFAM could protect the heart from mtDNA deficiencies and attenuate LV remodelling and failure after MI.[82] *TFAM* overexpression could ameliorate the decline in mtDNA copy number and preserve it at a normal level in post-MI hearts. *TFAM* overexpression might increase the steady-state levels of mtDNA by directly stabilizing mtDNA. Consistent with alterations in mtDNA, the decrease in oxidative capacities seen in MI was also prevented. Moreover, *TFAM* significantly attenuated cardiac chamber dilatation and dysfunction as well as histopathological changes such as myocyte hypertrophy, interstitial fibrosis, and apoptosis seen in heart failure. Therefore, TFAM was considered to play an important role in myocardial protection against remodelling and failure (*Figure 3*).

Several factors may be attributable to the protective effects conferred by *TFAM* overexpression against myocardial remodelling and failure. First, *TFAM* overexpression prevented the decrease in mtDNA copy number and normalized mitochondrial electron transport function, which may contribute to the decrease in oxidative stress. The decreased oxidative stress could contribute to the amelioration of cardiac hypertrophy, apoptosis, and interstitial fibrosis.[82] A recent study by Ekstrand *et al.*[83] demonstrated that the overexpression of human *TFAM* in the mouse increased mtDNA copy number. These lines of evidence imply the primary importance of TFAM as a regulatory mechanism of

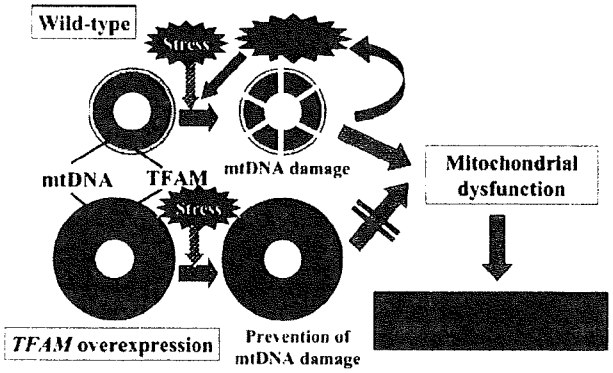


**Figure 3** Proposed mechanisms through which overexpression of the mitochondrial transcription factor A (TFAM) gene prevents mitochondrial DNA (mtDNA) damage, oxidative stress, and myocardial remodelling and failure. In wild-type mice, mitochondrial transcription factor A directly interacts with mitochondrial DNA to form nucleoids. Stress such as ischaemia causes mitochondrial DNA damage, which increases the production of reactive oxygen species (ROS) and thus leads to a catastrophic cycle of mitochondrial electron transport impairment, further reactive oxygen species generation, and mitochondrial dysfunction. *TFAM* overexpression may protect mitochondrial DNA from damage by directly binding and stabilizing mitochondrial DNA and increasing the steady-state levels of mitochondrial DNA, which ameliorates mitochondrial dysfunction and thus the development and progression of heart failure.

mtDNA copy number. TFAM has been shown to directly interact with mtDNA to form nucleoids.[84,85] Therefore, increased levels of TFAM may increase the steady-state levels of mtDNA by directly binding and stabilizing mtDNA in transgenic mice. Secondly, *TFAM* overexpression may induce mitochondrial biogenesis, which, however, is thought to be unlikely because the number and size of the mitochondria assessed by electron microscopy were not altered. Nevertheless, in addition to antioxidant mechanisms, *TFAM* overexpression might have metabolic effects through increased respiratory chain activity and ATP synthesis, which is considered to exert bioenergetic improvements and be cardioprotective against heart failure.

The results obtained from human *TFAM* transgenic mice differ from those from the inducible, cardiac-specific overexpression of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α)) transgene in adult mice, which leads to a modest increase in mitochondrial number and development of reversible cardiomyopathy.[86] PGC-1α is the transcriptional coactivator and acts upstream of TFAM and also has the capacity to increase mtDNA levels as well as mitochondrial mass in cultured cells and in transgenic mice.[87,88] The reason for the discrepant results between *PGC-1α* and *TFAM* transgene overexpression remains unsolved, which, however, may be related to the complex regulatory mechanisms of mitochondrial biogenesis and function by PGC-1α and its downstream factors, including nuclear respiratory factors 1 and 2 and TFAM.[89,90]

## 7. Future perspectives

mtDNA decline, mitochondrial defects, and mitochondrial oxidative stress are now well recognized in a variety of diseases such as neurodegenerative diseases, diabetes mellitus, cancer, and even aging. Therefore, with further knowledge on the mechanisms of TFAM for maintenance of mtDNA copy number and mitochondrial function, it may eventually be possible to develop novel strategies based on the manipulation of TFAM for the treatment of diseases such as heart failure.

The sirtuin family has emerged as therapeutic targets to treat age-related diseases including cardiovascular diseases, type 2 diabetes, and neurodegenerative diseases.[91-93] There are seven human sirtuins (SIRT1-7) that display diversity in cellular localization and function. SIRT1 has been implicated as a key mediator of the pathways downstream of resveratrol and calorie restriction, a dietary regimen that is known to delay the onset and reduce the incidence of age-related diseases. Calorie restriction increases muscle mitochondrial biogenesis through SIRT1 and activation of PGC-1.[94] Recently, small-molecule activators of SIRT1 have been shown to improve glucose homeostasis, increase insulin sensitivity in liver, skeletal muscle, and fat, and increase mitochondrial function in type 2 diabetic mice.[95] The interesting possibility that these interventions can prevent or ameliorate the onset and progression of various age-related diseases including heart failure awaits further experimentation. However, Alcendor *et al.*[92] demonstrated that a high level (12.5-fold) of the Sirt1 gene increased apoptosis and hypertrophy and decreased cardiac function, thereby stimulating the development of cardiomyopathy, whereas low (2.5-fold) to moderate (7.5-fold) overexpression of the Sirt1 gene in transgenic mouse hearts attenuated age-dependent cardiac hypertrophy, apoptosis/fibrosis, cardiac dysfunction, and expression of senescence markers. Moderate overexpression of the Sirt1 gene protected the heart from oxidative stress induced by paraquat, with increased expression of antioxidants, such as catalase, through forkhead box O (Fox O)-dependent mechanisms.[92]

## 8. Summary

To improve the prognosis of patients with heart failure, we need to develop therapeutic strategies based on a novel insight into the pathophysiology of myocardial remodelling and failure. The approach of regulating mitochondrial oxidative stress and mtDNA damage may contribute to the establishment of effective treatment strategies for patients with heart failure. Oxidative stress is involved not only in heart failure, but also in various cardiovascular diseases including atherosclerosis, hypertension, and in the aging process. Therefore, therapeutic strategies to modulate this maladaptive response should become a target for future extensive investigation and could have a broad application.

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