MITOPHAGY IN HEART FAILURE

A SELECTIVE AUTOPHAGIC DEGRADATION OF MITOCHONDRIA

Approved by Supervisory Committee

For the Degree of

DOCTOR OF MEDICINE WITH DISTINCTION IN RESEARCH

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

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MITOPHAGY IN HEART FAILURE

A SELECTIVE AUTOPHAGIC DEGRADATION OF MITOCHONDRIA

by

KAI-CHUN (DANIEL) YANG

DISSERTATION

Presented to the Faculty of the Medical School

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DOCTOR OF MEDICINE WITH DISTINCTION IN RESEARCH

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ABSTRACT

INTRODUCTION: Cardiovascular disease is associated with declines in mitochondrial function. Autophagy is a lysosomal-dependent process through which cytoplasmic proteins and organelles can be degraded and has recently been shown to participate in remodeling of the myocardium in a variety of cardiac pathologies. Autophagy can be either non-selective or selective for damaged protein aggregates or organelles. Reactive oxygen species (ROS) generated in mitochondria causes mitochondrial permeability transition (MPT) and induces selective degradation of mitochondria (mitophagy). We hypothesized that mitophagy contributes to remodeling of the heart under severe oxidative stress.

METHODS: Mice were subjected to hemodynamic overload by severe thoracic aortic constriction (sTAC). qPCR was used to measure the abundance of mtDNA relative to nuclear DNA. Changes in proteins and cardiac function were also assessed.

RESULTS: Decreases in mtDNA abundance were time dependent after sTAC (-47%±17 at day 2, $p<0.1$; -73%±10 at day 4, $p<0.05$) (n=2 each) and correlated with increased mortality (37% at day 2; 75% at day 4). The decline in mtDNA was greater in the basal septum (-88%±2, $p<0.01$) than in the left ventricular free wall (- 42%±15, $p<0.15$) (n=4) day 8 post-sTAC. The basal septum is where we have observed the largest increases in autophagic activity and protein carbonylation, a ROS-mediated protein modification. Daily injections with cyclosporine (CsA), an inhibitor of both MPT and calcineurin, blunted load-induced mtDNA loss (-28%±2 with CsA vs -83%±10 with vehicle treatment, $p<0.01$) (n=3) at 4 days post-sTAC. Furthermore, CsA improved survival at 4 days-post sTAC (40% mortality with CsA vs 75% with vehicle) (n=5-8). Mice with increased ROS

generation due to a disruption of the cardiac isoform of the cytochrome-c oxidase subunit *COXVIaH* were more sensitive to pressure overload-induced loss of mtDNA and mitochondrial proteins.

CONCLUSION: MtDNA abundance declines in this model of load-induced heart failure and is associated with increased autophagic activity and ROS generation. Short-term application of CsA can blunt mtDNA loss and improve survival.

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TABLE OF CONTENTS

PRIOR PRESENTATION AND GRANT

Yang K**,** Tannous P, Hill JA. "Autophagic Elimination of Mitochondria in the Stressed Cardiomyocyte." **Alpha Omega Alpha Carolyn L. Kuckein Student Research Fellowship Grant**, Summer 2006.

Yang K, Tannous P, Hongxin Z, Rothermel B, Hill J. "Autophagic Elimination of Mitochondria in Pressure-Stressed Myocardium." Poster, 1st Place Basic Science Category, **44th Annual UT Southwestern Medical Student Research Poster Competition**, Jan. 17, 2006.

LIST OF FIGURES

INTRODUCTION

Heart failure is the most significant disorder in industrialized countries from the standpoint of both mortality and public health resource utilization [2]. It is estimated that the total cost of heart failure in the United States for 2009 will be around $37 billion [3]. Despite tremendous advances in clinical care and in our basic science understanding, heart failure remains a leading cause of morbidity and mortality, a problem that will only grow as the population ages. In 2006, 73 million Americans were diagnosed with hypertension, a major risk factor for heart failure, and 5.7 million progressed to chronic heart failure [3]. Once diagnosed with heart failure, the one year mortality is around 20% [3]. Metabolic and structural remodeling of the heart occurs during failure, including both reversible and irreversible mitochondrial damage [4, 5]. The ability to more effectively treat heart failure in the future will be dependent upon our continued elucidation of the molecular events that are involved in the pathogenesis of this disease.

The Hill lab has developed pressure-overload surgical models in mice to study the molecular events that govern cardiac hypertrophy and/or failure. They have found that 3 weeks of Thoracic Aortic Constriction (TAC) will result in significant cardiac hypertrophy without a decline in cardiac function, while 1 week of severe Thoracic Aortic Constriction (sTAC) will induce heart failure [6]. Using this model, the Hill lab has shown that when mice are subjected to severe hemodynamic stress, autophagy is upregulated in the heart [7]. Autophagy is a highly conserved lysosomal-mediated mechanism for degrading organelles and cytoplasmic proteins. Increased oxidative stress and the generation of reactive oxygen species (ROS) is a prominent feature of heart failure [4]. In cardiomyocytes, ROS is primarily generated in mitochondria, therefore

mitochondria are a major site of oxidative damage. ROS damage to mitochondria mediates a specific form of autophagy that selectively degrades mitochondria called mitophagy [1, 8-12]. This study tests the hypothesis that increased oxidative stress in response to severe cardiac pressure-overload results in oxidative damage to mitochondria and the subsequent upregulation of autophagy to remove the damaged mitochondria.

RESULTS AND DISCUSSION

Pressure-overload induces mitochondrial DNA loss

To determine whether hemodynamic stress induces the degradation of cardiac mitochondria, mice were subjected to severe Thoracic Aortic Constriction (sTAC), a surgical model for severe hypertension, for five days. Mitochondrial DNA (mtDNA) copy number and mitochondrial protein abundance were used as surrogates for mitochondrial mass [13]. The ventricles were isolated and processed separately for DNA and protein. Real-time PCR analysis of the DNA (Figure 1A) was performed with primers for the mitochondrial gene encoding NADH dehydrogenase subunit 2 (mtND2) and the nuclear gene encoding myxovirus resistance 1 (Mx1). Quantification of the amplicons revealed a statistically significant 44% decrease in mtDNA copy number when normalized to nuclear DNA in the sTAC group compared to the sham group ($P<0.01$). Western blot analysis (Figure 1B) was performed with antibodies to the following three mitochondrial proteins: mitochondrial encoded cytochrome c oxidase subunit 1 (mtCOX1), nuclear encoded cytochrome c oxidase 4 (COX4), and nuclear encoded voltage-dependent anion-selective channel protein 1 (VDAC). mtCOX1 and COX4 were mildly decreased in the sTAC group; however, VDAC was not decreased with banding. Processing of LC3-I (microtubule-associated protein 1 light chain 3) protein to the lipid conjugated form (LC3-II) is an early step in autophagic activation. Increased LC3-II levels are used as an indication of increased autophagic activity [14]. LC3-II was increased in the ventricles of sTAC animals, indicative of increased autophagic activity and consistent with a previous report from our group [7]. We postulate that the decrease in mtDNA copy number in response to sTAC occurs through an autophagic mechanism.

Interestingly, in all experiments, the decrease observed in mtDNA was less than the decrease observed in mitochondrial proteins. This could be due to differential degradation of mtDNA compared to mitochondrial proteins or a differential rate of synthesis of new mtDNA and proteins. The only nuclease in mitochondria is endonuclease G, located in the inner mitochondrial space and in the mitochondrial matrix complexed with mtDNA [15]. It is released to cleave nuclear DNA during apoptosis and has not been shown to act on mtDNA [15]. Autophagic degradation of whole mitochondria is currently thought to be the major route for degradation of mtDNA. Thus it is likely that mitochondrial proteins are being degraded along with mtDNA.

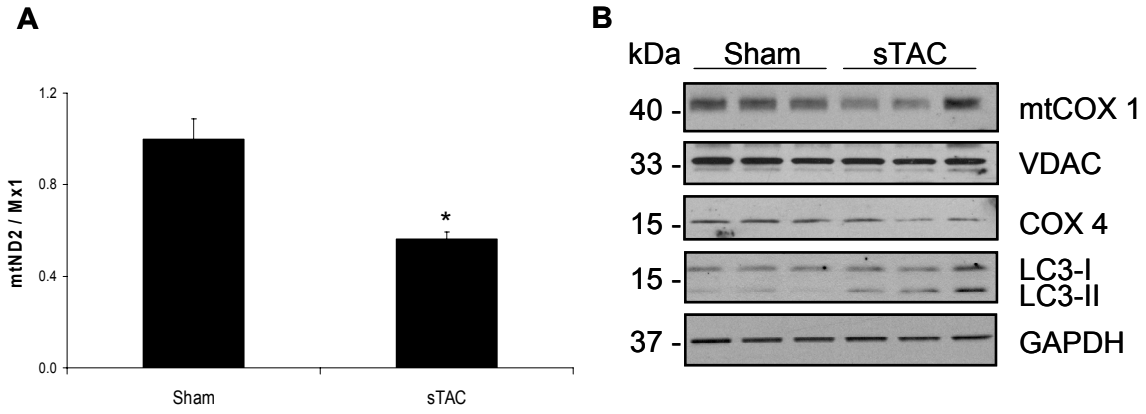

Figure 1. Ventricular Changes in Mitochondrial DNA and Protein with sTAC. Male mice subjected to 5 days of sTAC. **(A)** Real-time PCR analysis of mitochondrial DNA to nuclear DNA ratio demonstrates a significant decrease in mtDNA copy number in the sTAC group. n = 6 sham; n = 3 sTAC. * $P < 0.01$ versus sham. **(B)** Representative immunoblot probed for mitochondrial proteins and LC3 in ventricular lysates as indicated. Lanes were cropped from the same gel.

Mitochondria are continually turned over with a half-life of roughly 10-25 days [9]. Nuclear genes control new mitochondrial synthesis, which adjusts to meet demand.

Increased energy demand in hearts subjected to pressure-overload would activate mitochondrial biogenesis. Loss through mitophagy would further increase this demand. VDAC and COX4 are nuclear encoded and can be synthesized even in the absence of mtDNA in an attempt to compensate for the energy demand. Replacement of mitochondrial encoded proteins such as COX1 may be slower because of reduced template. A recent report has shown that mRNAs of mitochondrial encoded proteins are increased 2-3 fold when mtDNA and mitochondrial mass are decreased [16]. Each of these mechanisms could contribute to the dichotomy observed between real-time PCR measurements of mtDNA copy number and western blot analysis of mitochondrial protein abundance.

Pressure-overload induced mtDNA loss is most robust in the basal septum

To determine if the loss of mitochondria is homogenous throughout the heart or is specific to particular locations in the heart, female mice were subjected to sTAC for 8 days. Tissue from the basal septum and left ventricle were separately isolated and processed for both total DNA and protein from sham and sTAC operated mice. Real-time PCR was performed with mtND2 and Mx1 primers (Figure 2A). With total DNA harvested from the basal septum, mtDNA copy number were found to be decreased by 88% in the sTAC group compared to the sham group ($P<0.01$). Real-time PCR analysis with total DNA harvested from the left ventricle of the same hearts identified a 42% decrease in mtDNA copy number in the sTAC group compared to the sham group ($P<0.15$). Protein isolated from the basal septum and left ventricle was analyzed with western blot techniques and probed with mitochondrial encoded NADH dehydrogenase

subunit 1 (mtND1), mtCOX1, VDAC, COX4, BNIP3, and GAPDH (Figure 2B). Although mtCOX1 was decreased in the sTAC group from tissue harvested from the basal septum and left ventricle, the other mitochondrial proteins, mtND1, VDAC, and COX4, were not decreased in the samples. This result is puzzling, but it may suggest a unique regulation of the mtCOX1 protein and is not necessarily indicative for loss of the entire mitochondria or due to differences in regeneration as discussed earlier. BNIP3 (Bcl-2/adenovirus E1B 19 kDa interacting protein), a protein that has been shown to be capable of promoting mitochondrial autophagy [11], was increased with sTAC (Figure 2B). An increased level of BNIP3 is consistent with the hypothesis that mitochondrial autophagy occurs in the pressure-overload model.

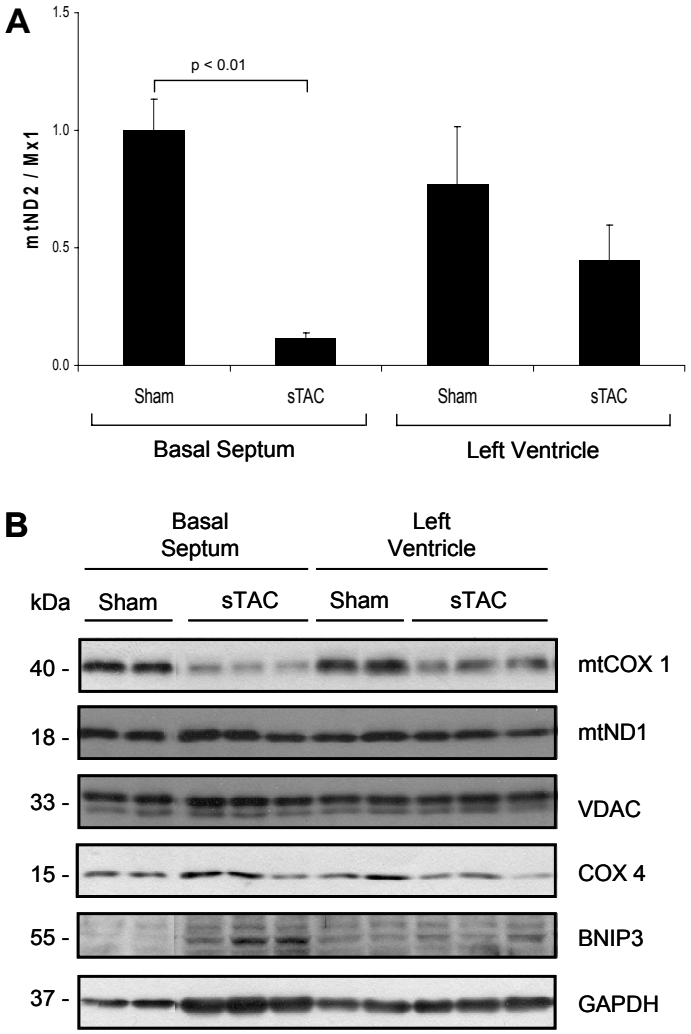

Figure 2. Basal Septal and Left Ventriclar Changes in Mitochondrial DNA and Protein with sTAC. Female mice were subjected to 8 days of sTAC. Tissue was isolated from the basal septum and left ventricle and processed for total DNA and protein. **(A)** Real-time PCR analysis of mitochondrial DNA to nuclear DNA ratio demonstrates a significant decrease in mtDNA copy number in the basal septum of the sTAC group compared to sham. n = 2 sham; n = 4 sTAC. The decrease in mtDNA copy number in the left ventricle of the sTAC group was not statistically significant compared to the sham group. n = 2 sham; n = 4 sTAC. **(B)** Representative immunoblot probed for mitochondrial proteins, BNIP3, and GAPDH as indicated. Lanes were cropped from the same gel.

It is clear from Figure 2A that mtDNA loss is most robust in the basal septum of the pressure-overloaded heart. Using mtDNA is a well established surrogate for mitochondrial number and mass, these results suggest that there is more mitochondrial degradation specifically in the basal septum than in the left ventricle during pressure-overload stress. This is consistent with the finding that there is more autophagy in the basal septum than in the left ventricle in the pressure-overload model [7]. Although mitochondrial proteins are not decreased to the same extent as mtDNA with sTAC,

numerous mechanisms, as outlined above, may account for this. Interestingly, BNIP3 is increased primarily in the basal septum of the pressure-overloaded heart, which is inversely proportional to the decrease in mtDNA copy number that is seen in this particular area of the heart. A similar inverse correlation between BNIP3 levels and mtDNA copy number has been reported in hypoxia treated rat hearts [17] and hypoxia treated mouse embryonic cells [11]. It is tempting to speculate that BNIP3 is mediating an autophagic dependent loss of mitochondria in the basal septum of the pressure-overloaded heart. A future experiment that would address whether the mtDNA loss seen in sTAC animals is BNIP3 mediated is to subject BNIP3 knockout animals to sTAC. The Hill lab is currently collaborating with Dr. Jeff Robbins to obtain these animals.

Mitophagy in BNIP3 overexpressing cells and pressure-overloaded hearts

BNIP3 has also been shown to be important for mitochondrial autophagy in HL-1 myocytes [1]. Hamacher-Brady et al. cotransfected mito-DsRed2 (a red fluorescent protein that is targeted to the mitochondria), BNIP3, and GFP-LC3 plasmids in HL-1 myocytes. Images were captured with a 3D confocal microscope 48 hours after transfection. Figure 3A, adapted from Hamacher-Brady et al. Cell Death and Differentiation 2006 [1], clearly depicts a green GFP-LC3 studded autophagosome engulfing a red mitochondria. From their work, it is reasonable to conclude that physiologically elevated BNIP3 levels can also induce the autophagic degradation of mitochondria in myocytes. When extrapolated to the pressure-overload model, it suggests that BNIP3 upregulation in the basal septum could be mediating autophagic degradation of mitochondria.

To directly demonstrate that mitochondrial autophagy occurs in the pressure-overload model, male mice were subjected to sTAC for 7 days and the basal septum was processed for electron microscopy (EM) (Figure 3B). EM images clearly depict autophagosome engulfment of mitochondria, suggesting that this process occurs in the basal septum of the pressure-overloaded heart, although this is difficult to quantify by EM.

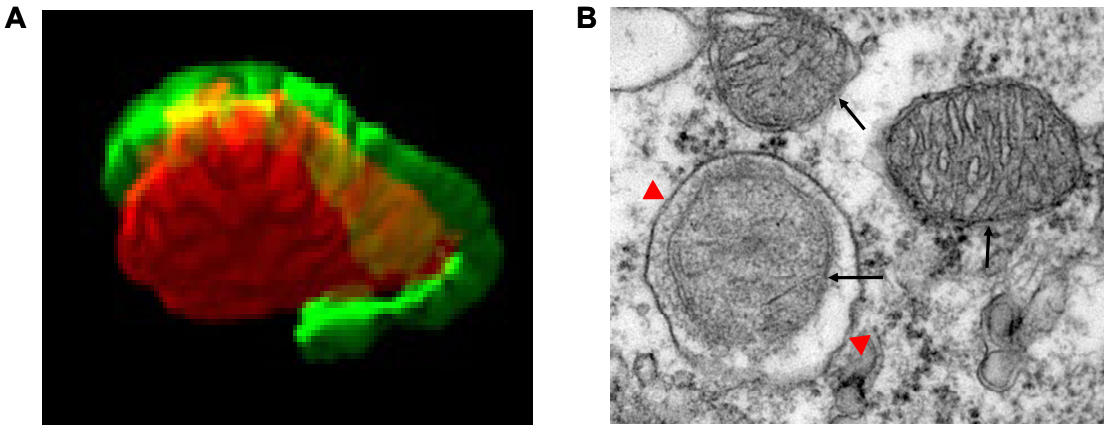

Figure 3. Selective Autophagic Degradation of Mitochondria. (A) BNIP3 overexpression in HL-1 myocytes induces mitochondrial autophagy. HL-1 cells were cotransfected with mito-DsRed2, GFP-LC3 and BNIP3. 48 hours post-transfection, GFP-LC3 and mito-DsRed2 colocalization was captured with 3D confocal microscopy. (Adapted from Hamacher-Brady et al. Cell Death and Differentiation 2006 [1].) **(B)** Autophagosome engulfs mitochondria in the basal septum of mice subjected to sTAC. Male mice were subjected to sTAC for 7 days and the basal septum was processed for electron microscopy. Black arrows delineate mitochondria. Red triangles point out the double membrane autophagosome. (This image was obtained by Janet Johnstone from the Hill lab.)

Quantification of mtDNA copy numbers with different primers

In order to corroborate the real-time PCR data obtained with the mtND2 and Mx1 primers, mtND1 and nuclear-encoded 18S (H19) primers were used as described previously [18]. The same DNA from Figure 2A (total DNA from female 8 day sTAC

animals) was used for real-time PCR with mtND2 and Mx1 primers (Figure 4A) and also with mtND1 and H19 primers (Figure 4B). The mtND2 and Mx1 primers identified a 90% decrease in mtDNA copy number in the sTAC group compared to the sham group (*P<0.01*). Similarly, the mtND1 and H19 primers identified a 78% decrease in mtDNA copy number in the sTAC group compared to the sham group (*P<0.05*). By obtaining the same trend while using two different sets of primers, the data strongly suggests that mtDNA copy number are decreased in the basal septum of the pressure-overloaded heart.

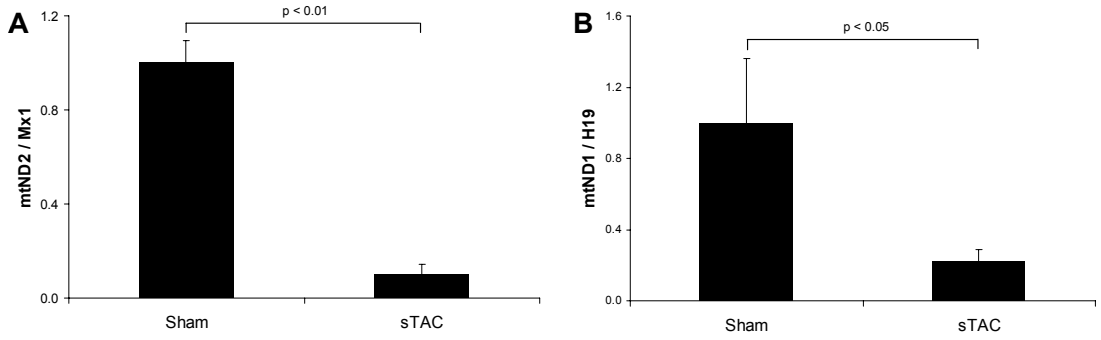

Figure 4. Decrease of Mitochondrial DNA Copy Number in the Basal Septum with sTAC. Female mice were subjected to 8 days of sTAC. Tissue was isolated from the basal septum and left ventricle and processed for total DNA. **(A)** Real-time PCR analysis with mtND2 and Mx1 primers demonstrated a significant decrease in mtDNA copy number in the basal septum of the sTAC group compared to sham. n = 2 sham; n = 4 sTAC. **(B)** Real-time PCR analysis with mtND1 and H19 primers also demonstrated a significant decrease in mtDNA copy number in the basal septum of the sTAC group compared to sham. n = 2 sham; n = 4 sTAC.

Cyclosporine blunts pressure-overload induced mtDNA loss

Lemasters' group has shown that opening of the mitochondrial permeability transition pore (MPTP) promotes mitophagy and that the inhibition of the MPTP prevents the depolarization of the mitochondria and the subsequent selective autophagic degradation of the mitochondria in hepatocytes [9]. Cyclosporine (CsA) is a common

immunosuppressive drug that is known to inhibit calcineurin phosphatase [19]. This autoimmune property has been exploited clinically for the prevention of solid organ transplant rejection and the treatment of various autoimmune diseases [20]. CsA also inhibits the MPTP by binding to cyclophilin D and thereby confers protective properties under the setting of ischemia-reperfusion injury [21-23].

To determine if the decrease in mtDNA copy number in the pressure-overloaded heart is mediated through the MPTP-dependent mitochondrial autophagy, male mice were subjected to sTAC and injected post-surgery subcutaneously with either CsA (10mg/kg) or equal volumes of vehicle twice a day for 4 days. Tissue from the basal septum was isolated at the end of 4 days. One group of vehicle treated sTAC operated animals was sacrificed 1.5 days post-banding to demonstrate the temporal response of mtDNA copy number to banding. Real-time PCR was performed with mtND2 and Mx1 primers (Figure 5A). mtDNA copy number was found to be decreased by 73% in the vehicle group with 4 days of sTAC ($P<0.05$). In the CsA injected group, mtDNA copy number were decreased by only 36% with sTAC, which was a significant blunting of mtDNA copy number loss compared to the vehicle treated sTAC group ($P<0.01$). Protein isolated from the basal septum was analyzed with western blot techniques and probed with mtCOX1, VDAC, COX4, BNIP3, and GAPDH (Figure 5B). No consistent change in mitochondrial proteins was observed with banding and/or CsA injections. However, BNIP3 levels increased in the sTAC group that was treated with vehicle only. Mice subjected to sTAC and injected with CsA did not show an elevation in BNIP3 levels.

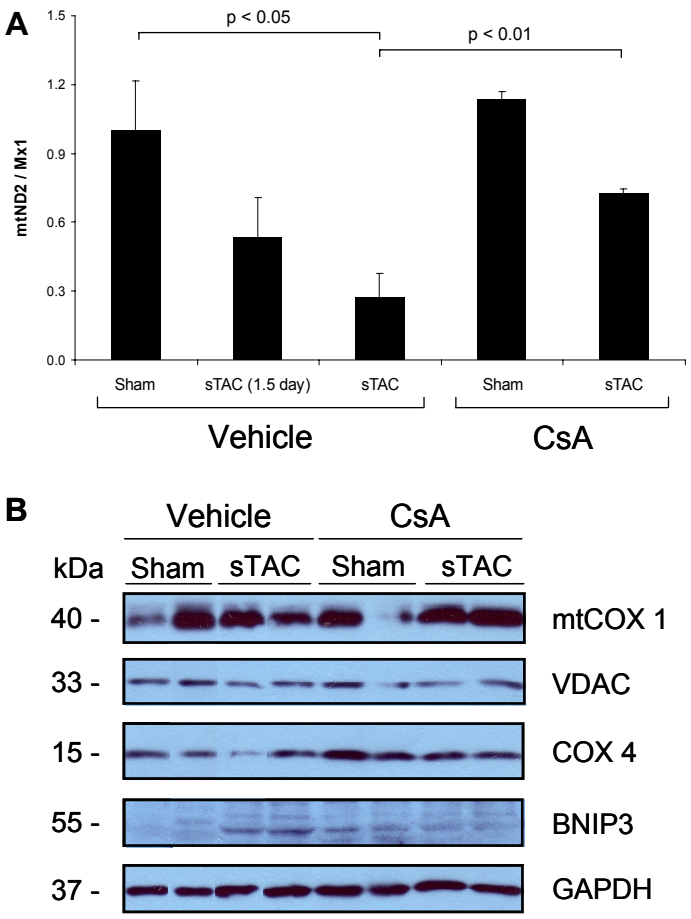

Figure 5. Basal Septal Changes in Mitochondrial DNA and Protein with sTAC and CsA Injections. Male mice were subjected to 4 days of sTAC and injected subcutaneously twice daily immediately after banding with either CsA (10mg/kg) or vehicle. All basal septal samples were collected at 4 days post-banding unless otherwise noted. One group was harvested 1.5 days post-banding. **(A)** Real-time PCR analysis of mitochondrial DNA to nuclear DNA ratio demonstrates a time-dependent decrease in mtDNA to genomic DNA ratio. CsA injections were able to partially inhibit this loss of mtDNA copy number. n=4 sham; n=2 sTAC 1.5 day; n=2 sTAC; n=2 sham CsA; n=3 sTAC CsA **(B)** Representative immunoblot probed for mitochondrial proteins, BNIP3, and GAPDH. Lanes were cropped from the same gel.
CsA, cyclosporine.

The results in Figure 5A suggest a time-dependent decrease in mtDNA copy number occurs in the basal septum secondary to banding. This decrease can be inhibited by the MPTP inhibitor, CsA, suggesting that the depolarization of the mitochondria is important for mtDNA loss. CsA, however, is also known to inhibit calcineurin, an important mediator of cardiac remodeling. Previous reports have used CsA injections of 25mg/kg to fully inhibit calcineurin [24]. CsA 10mg/kg injection was chosen for this experiment because this dose was sufficient to inhibit cyclophilin D, one of the proteins that form the MPTP, while minimizing the inhibition of calcineurin [25]. To distinguish between CsA effects on MPTP from calcineurin, NIM811, a specific inhibitor of the MPTP that does not inhibit calcineurin, will be tested [26].

The results in Figure 5B suggest that BNIP3 levels are increased in the basal septum of pressure-overloaded hearts and that this accumulation of BNIP3 can be inhibited by CsA. It is possible that the depolarization of the mitochondria contributes to increased BNIP3 levels. It is tempting to speculate that the increased BNIP3 level in the basal septum of the pressure-overloaded heart induces mitochondrial autophagy and thereby explains for the decrease in mtDNA copy number observed in this model.

sTAC does not induce mtDNA loss in the right ventricle

Although the greatest decrease in mtDNA copy number occurs in the basal septum of the pressure-overloaded hearts, mtDNA also decreases in the left ventricle. If this is due to differences in hemodynamic stress, then mtDNA copy numbers should not decrease in the right ventricle because it does not experience significant increases in afterload. To test this, tissue from the right ventricle was also isolated from the 4 day sTAC animals that were injected with either CsA or vehicle. Total DNA was obtained and real-time PCR was performed with mtND2 and Mx1 primers (Figure 6). There was no significant decrease in mtDNA copy numbers with sTAC; however, there was a general downward trend in the right ventricle that was blunted by CsA (Figure 5B). Significantly increased lung weights were observed in the sTAC hearts (data not shown). As the most common cause of right heart failure is left heart failure, it is possible that the right ventricle was experiencing failure secondary to pulmonary edema, and the same phenomenon, mtDNA loss, was beginning to manifest even in the right ventricle. If true, this would suggest that cardiac failure is associated with mtDNA loss, and furthermore,

the extent of failure may be associated with the magnitude of which the mtDNA copy numbers are decreased.

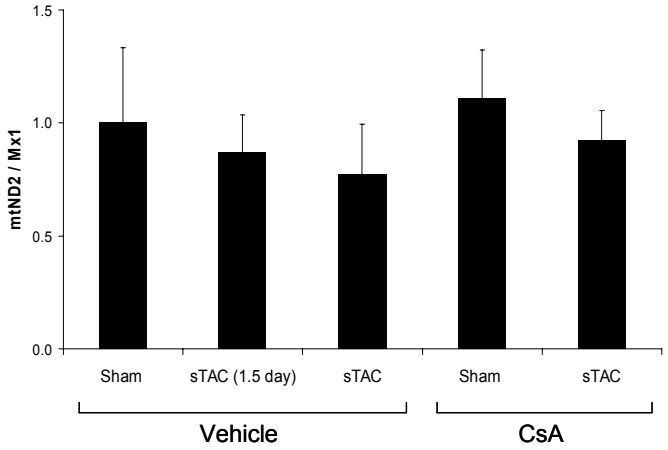

Figure 6. No Changes in Right Ventricle Mitochondrial DNA with sTAC or CsA Injections.
Male mice were subjected to 4 days of sTAC and injected subcutaneously twice daily after banding with either CsA (10mg/kg) or vehicle. All right ventricular samples were collected at 4 days post-banding unless otherwise noted. One group was harvested 1.5 days post-banding. Real-time PCR analysis of mitochondrial DNA to nuclear DNA ratio demonstrated no significant change in mtDNA to nuclear DNA ratio. n=4 sham, n=2 sTAC 1.5 day, n=2 sTAC, n=2 sham CsA, n=3 sTAC CsA.

Short-term inhibition of mitophagy with cyclosporine is beneficial

In order to gauge whether CsA treatments were beneficial post-sTAC, the survival of the 4 day male sTAC mice were followed daily post-surgery for a total of 4 days. All mice were injected with either vehicle or CsA as described previously (twice a day for four days), starting with the first injection immediately after surgery. Four sham mice were injected with vehicle, and all four were alive at post-operation day (POD) 4 (100% survival). Two sham mice were injected with CsA, and both were alive at POD 4 (100% survival). Eight sTAC mice were injected with vehicle, and two were alive at POD 4 (25% survival). Five sTAC mice were injected with CsA, and three were alive at POD 4 (60% survival). These results are depicted in Figure 7. Based strictly on survival, short-

term cyclosporine appears to be beneficial. Furthermore, a recent clinical trial found that immediately after an ST elevated myocardial infarction (STEMI), giving just one dose of CsA prior to a percutaneous coronary intervention was protective [23]. It is important to point out, however, that long-term (3 week) injections of cyclosporine in the pressure-overload model appears to be maladaptive [6]. Taken together, these results suggest that subsequent to a sudden cardiac stressor (sTAC or STEMI), short-term CsA-mediated inhibition of the MPTP is beneficial, but in the long-term, continued CsA-mediated inhibition of the MPTP is maladaptive. A possible explanation for this is that after a sudden cardiac stressor, mitochondrial autophagy occurs too rapidly and cardiomyocytes become relatively depleted of mitochondria. Inhibiting this mitophagy in the short-term and thereby maintaining mitochondrial homeostasis is beneficial. However, long-term suppression of mitophagy after a cardiac stressor may be maladaptive. It has been speculated by various investigators that mitophagy targets mitochondria that are damaged by reactive oxygen species (ROS) [10, 27]. When mitophagy is inhibited for an extended period of time, the long-term accumulation of damaged mitochondria may be maladaptive.

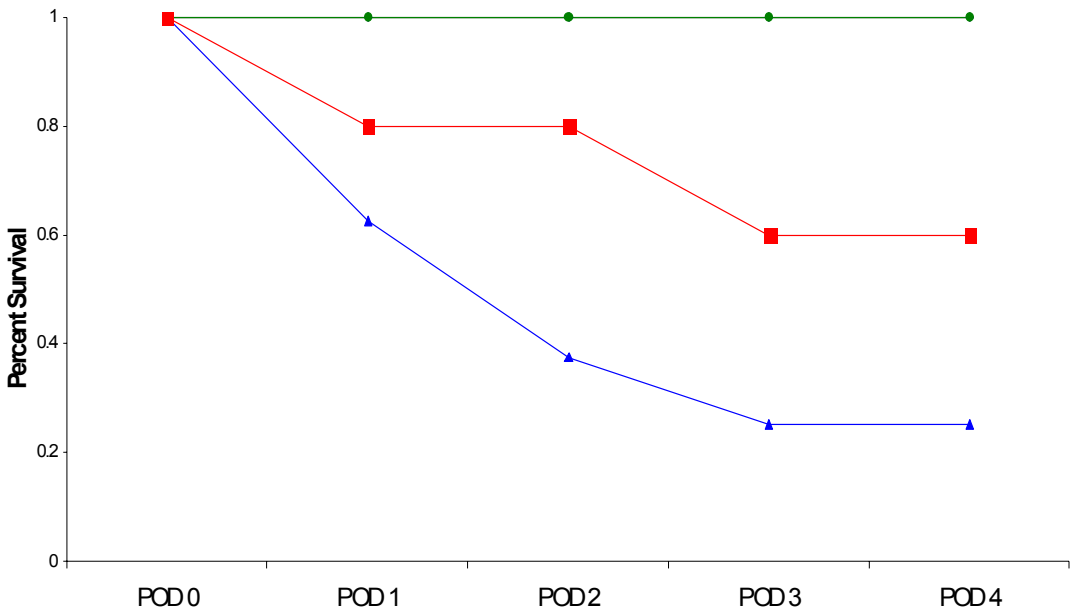

Figure 7. Survival After sTAC is Increased with CsA. Mice were subjected to sham operation versus sTAC. The survival of four groups were followed for a total of four days post-surgery, sham vehicle (green diamond, n=4), sham CsA (green diamond, n=2), sTAC vehicle (blue triangle, n=8), and sTAC CsA (red square, n=5). POD, post-operation day.

sTAC induces ROS-mediated protein damage

Mitochondria are thought to be a major source for ROS, especially in the setting of pressure-overload [10, 27, 28]. Because of proximity, it is hypothesized that mitochondrial proteins are preferentially damaged by ROS. Carbonylation of cellular proteins is a form of post-translational nonenzymatic modification that is induced by ROS [29]. In fact, protein carbonylation has been used as a marker for oxidative damage [30]. Because it is thought that ROS-mediated damage is an inducer of mitophagy, it is hypothesized that sTAC hearts would have increased levels of protein carbonylation. To test this, male mice were randomized to receive either 3 days of MntBAP

[Mn(III)tetrakis(4-benzoic acid)porphyrin Chloride, a superoxide dismutase (SOD) mimetic] or equal volumes of vehicle, phosphate buffered saline (PBS). The animals were then subjected to either sham or sTAC operations. The same injections were then continued for 3 days post-surgery. Basal septal protein was isolated on POD 3 and probed for carbonyl modifications. It is evident from figure 8 that ROS-mediated protein carbonylation is markedly increased in the basal septum of the sTAC operated animal treated with vehicle. MntBAP, a SOD2 mimetic, was successful in reducing the amount of protein carbonylation in the animals that were subjected to sTAC.

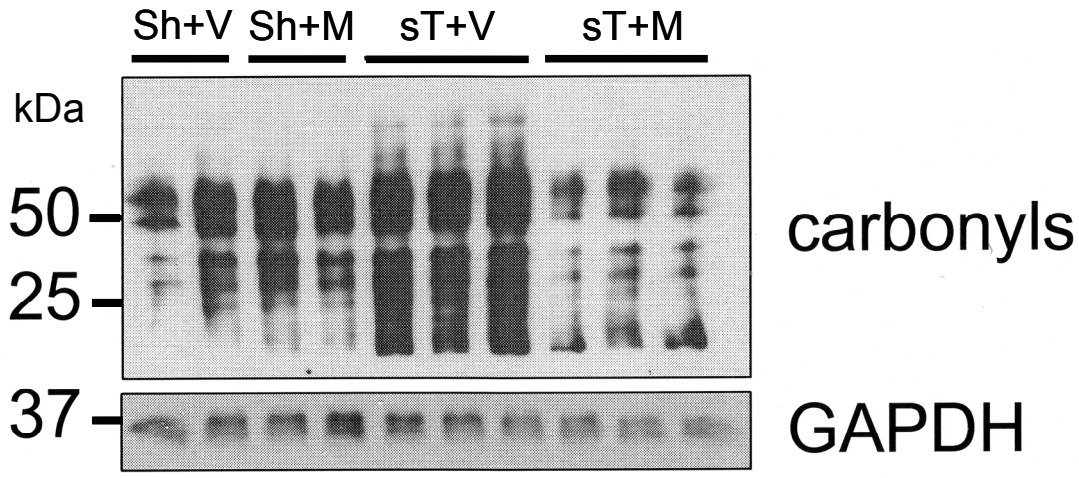

Figure 8. sTAC Induced Carbonylation of Basal Septal Proteins is Inhibited by MntBAP.
Male mice were pre-conditioned daily for 3 days prior to surgery with either 100 ug/kg of MntBAP or vehicle (PBS) and then subjected to either sham or sTAC surgery. The injections were then continued daily for 3 days post-surgery. Basal septal protein was isolated 3 days post-surgery and probed for ROS-mediated protein carbonylation.
Sh, sham. sT, sTAC. V, vehicle. M, MnTBAP. PBS, phosphate buffered saline. (This immunoblot was generated by Andriy Nemchenko from the Hill lab.)

Figure 8 indicates that pressure-overload induces oxidative damage in the basal septum and that this damage can be mitigated by an SOD2 mimetic. Although this result does not implicate ROS-mediated damage as an inducer of autophagy, it does suggest

that ROS-mediated protein damage is present in the basal septum of the pressure-overloaded heart. This correlates with the loss of mtDNA copy number seen in the pressure-overloaded heart and raises the possibility that ROS-mediated damage is the trigger for mitochondrial autophagy. Future experiments will address whether ROS-mediated damage is necessary for mtDNA loss in the pressure-overload model. Because MntBAP is capable of reducing the amount of ROS-mediated protein carbonylation, it is hypothesized that MntBAP will also blunt the loss of mtDNA copy number seen in the basal septum of the pressure-overloaded heart.

COX 6 KO mice are more susceptible to pressure-overload

To determine if increased levels of ROS can increase mtDNA loss and further decrease cardiac function, mice lacking the skeletal muscle and cardiac-specific cytochrome-c oxidase subunit VIaH (COX 6) from the electron transport chain were obtained from Dr. Pradeep Mammen. Phenotypically, these mice have diastolic dysfunction when measured on a Langedorff preparation at high retrograde perfusion pressures [31]. These mice have evidence of increased ROS-mediated damage in the skeletal muscles at baseline (Dr. Mammen's unpublished observations). It was hypothesized that their hearts would also have increased levels of ROS production at baseline and that subjecting them to pressure-overload would further increase the levels of ROS. Therefore, these animals were subjected to the milder procedure of thoracic aortic constriction (TAC) rather than sTAC, and their heart function was assessed with transthoracic echocardiogram. Figure 9 indicates that after 3 days of TAC, cardiac

function is normal in wildtype but is depressed in the COX 6 KO animals from 58% (sham-operated) to 35% (TAC-operated).

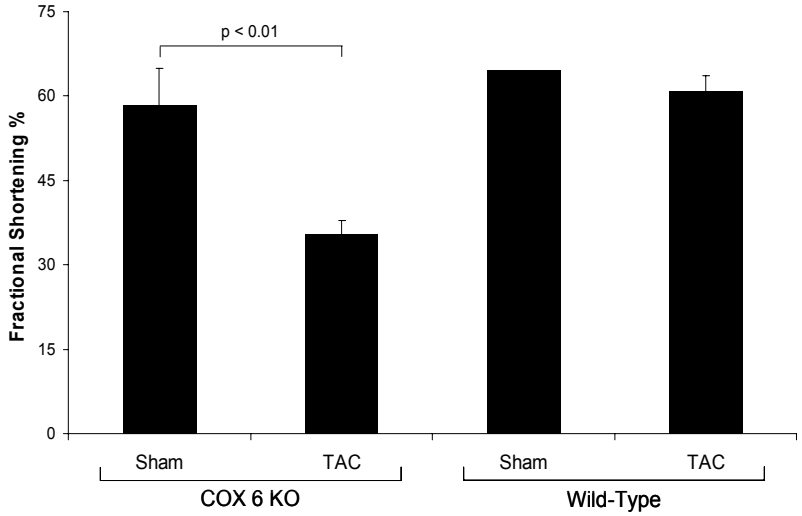

Figure 9. Left Ventricular Fractional Shortening in COX 6 KO and Wild-Type Mice with 3 Days of TAC.
Male COX 6 KO and WT mice were subjected to TAC for 3 days. Echocardiogram of the LV indicates that the LV function in the COX 6 KO mice were depressed. n=2 COX 6 KO sham, n=3 COX 6 KO TAC, n=2 WT sham, n=3 WT TAC. LV, left ventricle. (Echocardiogram data was obtained by Pavan Battiprolu from the Hill lab.)

Pressure-overload induces mtDNA and protein loss in COX 6 KO mice

Next, the COX 6 KO and WT mice were examined for mitochondrial DNA and protein content to determine if increased levels of ROS are associated with decreased levels of mitochondria. Total DNA and protein were isolated from the basal septum of COX 6 KO and WT animals 4 days post-surgery. Real-time PCR analysis of total DNA from COX 6 KO mice subjected to TAC identified a 54% decrease in mtDNA copy number compared to COX 6 KO sham-operated mice (Figure 10A). There was no change in mtDNA copy number in WT TAC-operated mice. Protein isolated from the basal septum was analyzed with western blot techniques and probed with mitochondrial

proteins, superoxide dismutase 2 (SOD2), BNIP3, and GAPDH (Figure 10B). All mitochondrial proteins (mtCOX1, mtND1, VDAC, and COX4) were decreased in the COX 6 KO TAC group compared to the sham-operated group. There were no differences in the WT group. SOD2, a mitochondrially located superoxide dismutase [32], was increased in both COX 6 KO TAC-operated and WT TAC-operated mice, suggesting a potential adaptive response to pressure-overload induced ROS release. Consistent with the previous experiments, BNIP3 was again upregulated. GAPDH was probed for a loading control; however, it is evident that it is upregulated uniformly in the COX 6 KO mice. A ponceau stain is included to demonstrate equal protein loading.

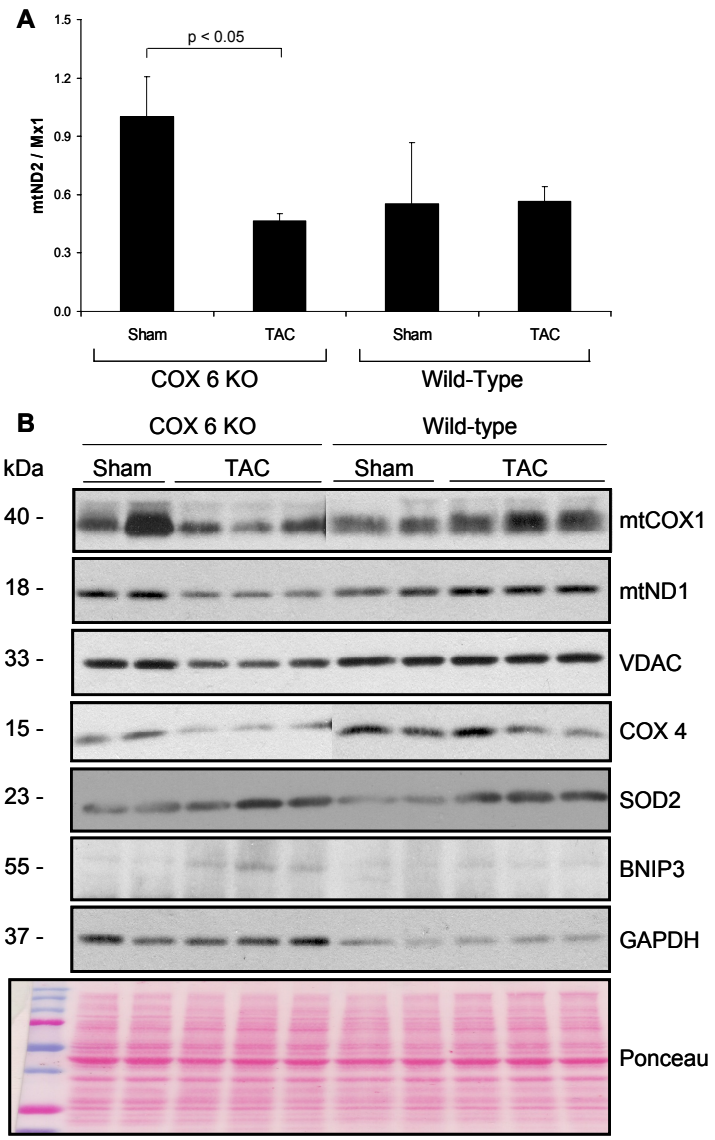

Figure 10. Mitochondrial DNA and Protein are Decreased in the Basal Septum of COX 6 KO Mice Subjected to TAC.
Male COX 6 KO and wild-type mice were subjected to 4 days of TAC. Tissue was isolated from the basal septum and processed for total DNA and protein. **(A)** Real-time PCR analysis of mtDNA to nuclear DNA ratio demonstrates a decrease in mtDNA copy number in the COX 6 KO TAC mice that was not present in the WT TAC mice. n=2 COX 6 KO sham; n=3 COX 6 KO TAC; n=2 WT sham; n=3 WT TAC. **(B)** Representative immunoblot probed for mitochondrial proteins, BNIP3, and GAPDH. A ponceau stain is included to demonstrate equal loading of all lanes. Lanes were cropped from the same gel.
TAC, thoracic aortic constriction. WT, wild-type.

The decrease in mtDNA copy number in the COX 6 KO TAC group compared to the WT TAC group suggests that mtDNA loss is augmented by ROS production. Experiments to test whether MntBAP is able to blunt the loss of mtDNA would further strengthen the role of ROS in mediating the loss of mtDNA. Interestingly, all mitochondrial proteins were decreased in the COX 6 KO TAC group. It is possible that the COX 6 KO mice at baseline were already undergoing a maximal rate of mitochondrial biogenesis because of respiratory insufficiency and increased ROS induced

mitochondrial autophagy. Therefore, when these mice were subjected to further oxidative stress, although mitochondrial autophagy can be further upregulated, mitochondrial biogenesis had reached a maximal rate and could not match the increased mitochondrial degradation rate. Therefore, both mtDNA and mitochondrial proteins were decreased with pressure-overload in these animals. BNIP3 was upregulated only in the COX 6 KO TAC group, the group that had the largest decrease in mtDNA copy number. The increased BNIP3 level correlates inversely with the decrease in LV function identified on echocardiogram in Figure 9 and the decrease in mtDNA copy number in Figure 10A. This suggests that ROS induced BNIP3-mediated mitochondrial autophagy may ultimately be maladaptive. GAPDH was uniformly increased in the COX 6 KO mice. This was not unexpected as these mice have a deficient electron transport chain and, therefore, will upregulate glycolysis in order to maintain ATP homeostasis. In summary, Figures 9 and 10 suggest that genetically engineered mice with increased ROS levels at baseline, when subjected to pressure-overload, developed decreased cardiac function and decreased levels of cardiac mitochondria when compared to wild-type mice subjected to the same operation.

Is autophagy the mechanism for pressure-overload induced mtDNA loss?

The current work suggests that ROS generation secondary to pressure-overload can lead to loss of cardiac mitochondria as quantified by mtDNA copy number; however, the mechanism that mediates the removal of mitochondria is still not fully elucidated. In the pressure-overload model, the number of GFP-LC3 punctate aggregates are increased in the basal septum [7], total LC3-II levels are increased in the ventricles (Figure 1B),

and CsA can inhibit the loss of mtDNA (Figure 5A). This all suggests that autophagy may be the primary mechanism for eliminating mitochondria. To more directly address this, mice deficient for autophagy will be used. Mice lacking the autophagy-related 5 (Atg5) gene specifically in cardiomyocytes where the alpha-myosin heavy chain (alpha-MHC) promoter is expressed were bred by Oktay Rifki from the Hill lab. These mice will be subjected to sTAC and mitochondrial protein and DNA will be assessed. It is hypothesized that these mice, which are deficient in autophagy, will not have decreased levels of mtDNA or mitochondrial proteins after sTAC. This finding would suggest that autophagy is the primary mechanism for mediating the loss of cardiac mitochondria in the setting of pressure-overload. A separate group has independently generated these animals and has reported that when these animals are subjected to 1 week of TAC, cardiac function is severely depressed compared to WT TAC operated animals. They conclude that autophagy is adaptive in the setting of pressure-overload; however, they do not address the effects of autophagy on mitochondrial mass [33]. It is possible that in the setting of pressure-overload, inhibition of all autophagy, as they report, is maladaptive, while the blunting of mitochondrial autophagy with CsA is beneficial (Figure 7). In either case, demonstrating that mtDNA loss, as a surrogate for mitochondrial mass, is blunted in autophagy deficient mice will be instrumental in elucidating the mechanisms that contribute to cardiac hypertrophy and, ultimately, failure. If this process is determined to occur in the pressure-overloaded heart, future studies can then be undertaken to specifically address whether augmenting or inhibiting this process is beneficial.

CONCLUSION

The current "Working Model" for this project is depicted in figure 11. Wild-type mice subjected to sTAC have been shown to have increased levels of ROS in the basal septum as quantified by protein carbonylation (Figure 8). It is hypothesized that COX 6 KO mice subjected to TAC have increased ROS, although this has not been demonstrated definitively. These mice, however, do have a decrease in mtDNA and protein in the setting of pressure-overload (Figure 10). Because sTAC has been shown to increase ROS levels, it may be beneficial to subject COX 6 KO mice to sTAC in order to discern whether COX 6 KO mice do in fact generate more ROS than wild-type. MntBAP, a SOD2 mimetic, inhibits ROS-mediated protein carbonylation (Figure 8). The next step will be to determine if MntBAP can inhibit the loss of mtDNA that is associated with the sTAC operation. If true, this would help strengthen the role of ROS in inducing mitochondrial autophagy.

The increased levels of ROS-mediated protein carbonylation in the basal septum have been associated with increased levels of BNIP3 (Figures 2B, 5B, 10B). BNIP3 has been shown to be important for mitochondrial autophagy [1, 11]. This can help explain the decrease in mtDNA copy number that is observed with pressure-overload. It has been hypothesized that ROS increases the opening of the MPTP [10, 27]. CsA, a known inhibitor of the MPTP, is able to blunt further increases in BNIP3 levels with pressure-overload and is able to blunt mtDNA copy number loss (Figure 5). Furthermore, it is hypothesized that when the MPTP opens, cytochrome c leaves the mitochondria, resulting in further ROS production. This has been termed ROS-induced ROS release (RIRR) [34]. CsA is able to blunt this second wave of ROS release [35] and thereby

mitigate ROS-mediated protein damage. The final, and most important, question to address is whether the loss of mtDNA is Atg5 (autophagy) dependent. If mitochondrial autophagy does occur in the pressure-overload setting, this would reveal a novel pathway that is involved in the pathogenesis of heart failure and, in the future, could provide potentially new targets for therapeutic intervention.

For now, the current work suggests that pressure-overload induces ROS-mediated protein damage in the basal septum of the heart. This damage is associated with an upregulation of BNIP3. mtDNA, a surrogate for mitochondrial mass, is temporally decreased with pressure-overload, and this process can be inhibited by the MPTP inhibitor, CsA. In conclusion, the body of this work is consistent with the hypothesis that mitophagy occurs in the pressure-overloaded heart and that inhibition of mitophagy is adaptive in the short-term.

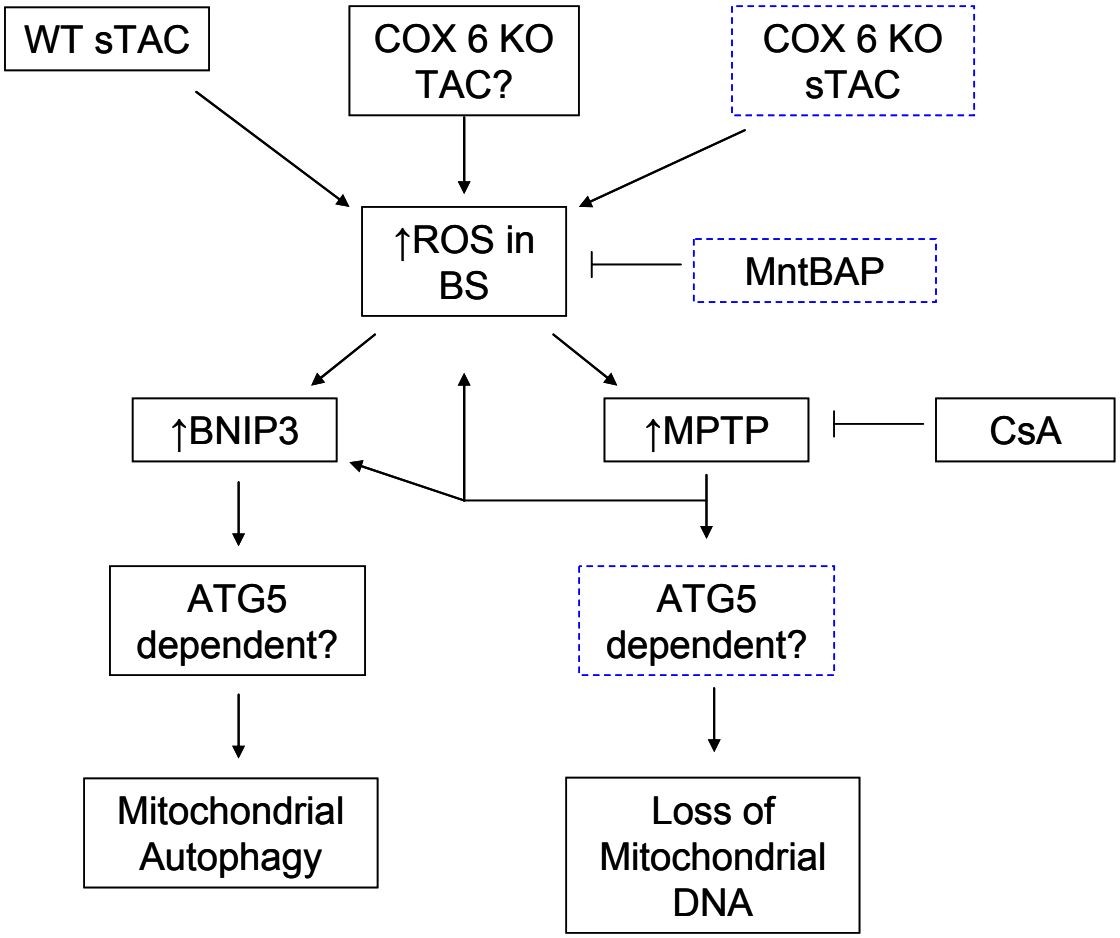

Figure 11. Working Model for Pressure-Overload Induced Mitochondrial Autophagy.
Blue dotted boxes indicate experiments planned for the near future.
BS, basal septum. CsA, cyclosporine. MntBAP, superoxide dismutase mimetic.
MPTP, mitochondrial permeability transition pore.

MATERIALS AND METHODS

Pressure-Overload Hypertrophy Models

Male and female C57BL6 mice (10-14 weeks old) were subjected to pressure-overload by thoracic aortic constriction as previously described [6]. At the designated time point, the animals were anesthetized and the basal septum of the heart was isolated. The basal septum was then cut longitudinally. One half was used for protein isolation; the other half was used for total DNA isolation.

Western Blot Analysis

Tissue was dounce homogenized to obtain total protein lysates. Protein lysates were then denatured with boiling, and 20ug/lane was subjected to polyacrylamide gel electrophoresis. The protein was then transferred overnight onto a Hybond ECL Nitrocellulose membrane before being blocked in 5% milk for 2 hours. Primary antibodies were probed in 5% milk overnight as follows: mtCOX1 (Abcam ab14705, 1:1000), mtND1 (Santa Cruz sc-65237, 1:1000), VDAC (Abcam ab14734, 1:2000), COX4 (Molecular Probes a21347, 1:1000), BNIP3 (Abcam ab28506, 1:500), Tubulin (1:3000), GAPDH (1:2000), SOD2 (1:15,000).

Real-Time PCR Analysis

Total DNA was extracted from isolated basal septal, right ventricular, or left ventricular whole tissue lysates and quantified with real-time PCR with mtND1, mtND2, Mx1, and H19 primers using SYBR green PCR Master Mix and a 7000 Sequence Detection System (Applied Biosystems). The ratio of mtND2 DNA copies to Mx1 DNA copies or mtND1 DNA copies to H19 DNA copies represented the relative mtDNA copy number. The relative fold change was calculated based on the threshold cycle (Ct) as previously described [11].

mtND1 forward = 5’ -CTCTTATCCACGC TTCCGTTACG- 3’
mtND1 reverse = 5’ -GATGGTGGTACTCCCGCTGTA- 3’
mtND2 forward = 5’ -CCCATTCCACTTCTGATTACC- 3’
mtND2 reverse = 5’ -ATGATAGTAGAGTTGAGTAGCG- 3’
H19 forward = 5’ -GTACCCACCTGTCGTCC- 3’
H19 reverse = 5’ -GTCCACGAGACCAATGACTG- 3’
Mx1 forward = 5’ -GACATAAGGTTAGCAGCTAAAGGATCA- 3’
Mx1 reverse = 3’ –TCTCCGATTAACCAGGCTAGCTAT- 3’

Cyclosporine Injections

CsA (Sigma) was diluted in 60% propylene glycol and 40% PBS. Vehicle was 60% propylene glycol in 40% PBS. Mice were injected with 10mg/kg CsA subcutaneously twice a day. Equal volumes of vehicle was injected in the non-CsA treated groups.

MntBAP Injections

MntBAP (Calbiochem) was diluted in 100% PBS. 100% PBS was used as vehicle. Mice were injected with 100ug/kg MntBAP daily 3 days before surgery and 3

days post-surgery. Equal volumes of vehicle was injected in the non-MntBAP treated groups.

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