Divergent Mitochondrial Biogenesis Responses in Human Cardiomyopathy

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Background—Mitochondria are key players in the development and progression of heart failure (HF). Mitochondrial (mt) dysfunction leads to diminished energy production and increased cell death contributing to the progression of left ventricular failure. The fundamental mechanisms that underlie mt dysfunction in HF have not been fully elucidated.

Methods and Results—To characterize mt morphology, biogenesis, and genomic integrity in human HF, we investigated left ventricular tissue from nonfailing hearts and end-stage ischemic (ICM) or dilated (DCM) cardiomyopathic hearts. Although mt dysfunction was present in both types of cardiomyopathy, mt were smaller and increased in number in DCM compared with ICM or nonfailing hearts. mt volume density and mtDNA copy number was increased by ≈2-fold (P<0.001) in DCM hearts in comparison with ICM hearts. These changes were accompanied by an increase in the expression of mtDNA-encoded genes in DCM versus no change in ICM. mtDNA repair and antioxidant genes were reduced in failing hearts, suggestive of a defective repair and protection system, which may account for the 4.1-fold increase in mtDNA deletion mutations in DCM (P<0.05 versus nonfailing hearts, P<0.05 versus ICM).

Conclusions—In DCM, mt dysfunction is associated with mtDNA damage and deletions, which could be a consequence of mutating stress coupled with a peroxisome proliferator-activated receptor γ coactivator 1α–dependent stimulus for mt biogenesis. However, this maladaptive compensatory response contributes to additional oxidative damage. Thus, our findings support further investigations into novel mechanisms and therapeutic strategies for mt dysfunction in DCM. (***Circulation***. **2013;127:1957–1967.**)

Key Words: cardiomyopathy, dilated ■ DNA, mitochondrial ■ heart failure ■ mitochondrial turnover

Mitochondria are the major site of energy production in the cell, thus it is not surprising that energy-dependent tissues such as the heart are particularly sensitive to mitochondrial (mt) dysfunction. Accumulating evidence suggests that mt dysfunction, reflected in the structure, function, and number of mitochondria within cardiac myocytes, leads to diminished energy production, loss of myocyte contractility, and increased cell death during the development of heart failure.[1,2] However, despite extensive animal studies, the fundamental mechanisms behind mt dysfunction contributing to the development and progression of left ventricular (LV) failure in humans have not been fully elucidated.

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Each cardiac myocyte contains numerous mitochondria (50–100), and each mitochondria contains multiple copies of mtDNA (1–10 molecules/mitochondria).[3] The abundance of mitochondria per cell is determined by the rate of mt biogenesis and cell division.[4] It has been reported that limited myocardial mt biogenesis occurs early in response to pathological stimuli, but ultimately proliferation of mitochondria does not match the increased metabolic energy demand of the hypertrophied myocytes, which may contribute to the eventual decompensation of the heart.[5] The abundance of mitochondria per cell is tightly controlled by the activation of specific transcription factors and signaling pathways.[4,6] Recent studies have revealed a central role of peroxisome proliferator-activated receptor γ coactivator (PGC)-1 alpha[7] and c-Myc[8] in mt biogenesis in the heart. Myc activation was shown to trigger mt biogenesis in adult heart by directly activating nuclear genes involved in mt replication and biogenesis, which was protective in response to ischemic stress.[8] Downregulation of PGC-1α and its target genes has been observed in a number of rodent models of heart failure, raising the intriguing possibility that impaired

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mt biogenesis can be a causal mechanism for mt dysfunction in HF.[9,10] However, these models may not recapitulate the pathophysiology of human dilated cardiomyopathic (DCM) hearts. PGC-1α expression levels in human failing hearts has been more variable. Two studies examining the PGC-1α levels in human heart failure (HF) found a decrease in PGC-1α mRNA or protein levels.[11,12] However, more recently it was reported that PGC-1α protein levels are increased in failing human hearts. The difference in findings was attributed to the severity of HF as well as lack of age-matched control group used in previous studies.[13] All of these studies were done in cohorts of failing human hearts from mixed causes, highlighting the need to further characterize mt biogenesis and related signaling pathways with respect to HF cause.

mt injury in failing heart can also be reflected by mtDNA damage, which is tightly associated with the expression of mt transcripts, proteins, and mt function. Human mtDNA is a closed-circular, double-strand DNA molecule encompassing 16 569 bp and encoding 13 proteins that are essential for mt biogenesis and respiratory function.[14] Human mtDNA is more susceptible to oxidative damage and consequently acquires mutations at a higher rate than nuclear DNA[15,16] as a result of exposure to high levels of free radicals and reactive oxygen species generated during respiration, lack of protective histones, and limited capacity for repair of mtDNA damage (for review, see[17,18]). Thus, increased oxidative stress may contribute to alterations in the abundance of mitochondria as well as the copy number and integrity of mtDNA under pathological conditions. Indeed, increased oxidative stress may play a critical role in regulating the decline in mt abundance and mtDNA copy number in stressed cells.[16] When the capacity of the antioxidant system is compromised, higher oxidative stress results in an increase in defective mitochondria and mutated mtDNA, leading to a cyclic increase in reactive oxygen species production and further oxidative damage.[19] Despite strong evidence suggestive of abnormal mt biogenesis and increased mutated mtDNA copy number in aging tissues,[19] the relative importance of this process in HF, particularly with respect to different HF causes, is not known.

We have characterized the changes in mt morphological dynamics, biogenesis, mtDNA content, and damage in subjects with HF of differing causes. We examined LV tissue from nonfailing (NF) and from end-stage ischemic cardiomyopathic (ICM) or DCM human hearts. Mitochondria were fragmented and increased in number in DCM failing hearts compared with ICM and NF hearts. There was increased mtDNA copy number in DCM accompanied by an increase in the expression of mtDNA-encoded genes. In contrast, these parameters were reduced in failing ICM hearts consistent with published report. The stimulus for abnormal mt biogenesis in end-stage DCM hearts appeared to be PGC-1α–dependent. Importantly, mt abnormalities in DCM were associated with a defective mtDNA repair system and increase in mtDNA deletion mutations. Taken together, these observations suggest that mt dysfunction in failing human hearts may be cause-specific. The basis for these differences is uncertain but raises hope that a better understanding of the pathophysiologic mechanisms could lead to novel therapies to prevent HF progression.

Methods

Myocardial Samples From NF and Failing Hearts

The failing heart samples (n=16) were obtained from the LV anterior wall during heart transplantation or implantation of an LV assist device. The NF heart samples (n=8) were obtained from the LV free wall and procured from National Disease Research Interchange (NDRI) and University of Pennsylvania. NF heart donors had no history of macroscopic or laboratory signs of cardiac diseases. The tissue collection was approved by the UCLA Institutional Review Board #11-001053 and #12-000207.

Histochemistry

Hematoxylin and eosin (H&E) staining was performed according to the rapid H&E technique for cryostat sections.[20] Cytochrome C oxidase (COX) and succinate dehydrogenase (SDH) activity staining were performed as previously described.[21]

Mitochondrial Electron Transport Chain Activity Assays

Mitochondrial Complex II activity was determined by standard spectrophotometric enzyme assay as described.[22] Activity of Complex II was measured by changes in absorbance at 600 nm after reduction of artificial electron acceptor, 2,6-dichlorophenolindophenol (DCPIP). The absorbance at 600 nm was recorded continuously for 5 minutes at every 30-second intervals. The relative Complex II activity was defined as the rate of change of absorbance per unit time of incubation.

Complex IV activity was measured using a microplate assay kit from Abcam according to the manufacturer's procedure. Briefly, Complex IV was immunocaptured within the wells and activity was determined calorimetrically after the oxidation of reduced cytochrome C at 550 nm.

Transmission Electron Microscopy

Small pieces from the left myocardium were fixed in 2% glutaraldehyde, 2% paraformaldehyde at room temperature for 2 hours, followed by 24h at 4°C. Specimens were rinsed in 0.1 mol/L phosphate buffer (PB), pH 7.4, fixed in 1% osmium tetroxide in PB (pH 7.4) at 4°C for 1h, dehydrated in graded ethanol followed by propylene oxide and embedded in Eponate 812 (TedPella Redding, CA). Ultrathin sections (around 60–70 nm) from longitudinal parts were cut and contrasted with uranyl acetate followed by lead citrate and examined in a JEOL 10 000× transmission electron microscope (Tokyo, Japan) at 80 kV.

Morphometric Analysis

Cardiac mt volume densities were determined from electron micrographs as described previously.[8] Data were expressed as volume density (volume of mitochondria [μm^3] per cytoplasmic volume [μm^3]).

Protein and RNA Analysis

Western blots were performed on protein extracts from LV anterior wall samples, according to established protocols.[23] Total RNA was extracted from frozen tissue samples using Tri Reagent (Sigma), and first strand cDNA were generated using Omniscript Reverse Transcriptase Kit (Qiagen) as per the manufacturer's instructions. Primer sequences were designed with the OligoPerfect Designer software. Real-time quantitative PCR was conducted using ABI PRISM 7700 Sequence Detection System; (ABI, CA).

Oxyblot Procedures

The Oxyblot Oxidized Protein Detection kit was purchased from Chemicon. Protein homogenates from LV anterior wall samples were derivatized with 1,3-dinitrophenylhydrazine for 15 minutes according to the manufacturer's instructions and followed by electrophoresis SDS-polyacrylamide gel.

Mitochondrial DNA Copy Measurement

DNA was collected from LV anterior wall from NF, DCM, and ICM hearts. Real-time PCR was performed for cytochrome C oxidase subunit 1 (COX1) and peroxisome proliferative activated receptor gamma coactivator-related (PPRC). Absolute COX1 DNA copies were normalized to a nuclear gene, PPRC1.

mtDNA Mutation Assays

The random mutation capture assays were performed as previously described.[21] Briefly, total DNA was digested with TaqI for 5 hours, with the addition of 100 U of TaqI every hour. DNA was then probed with primers flanking the TaqI restriction site to detect mtDNA genomes that contain a mutation in the TaqI restriction site. A second pair of primers was used to determine the amount of mtDNA genomes that was interrogated. The following primers were used for DNA amplification: mtDNA control primer forward, ACAGTTTATGTAGCTTACCTCC; mtDNA control primer reverse, TTGCTGCGTGCTTGATGCTTGT; mtDNA deletion primer forward, GAACCAACACCTCTTTACAG; mtDNA deletion primer reverse, CCTGCTAATGCTAGGCTGCC.

Next generation sequencing was performed on Illumina GAIIx sequencer. Libraries of mtDNA were constructed according to the manufacturer's instructions, and paired-end sequence reads of 75 nucleotides were obtained. To identify candidate deletions, each sequence read was trimmed to 70 nucleotides, and the 15 nucleotides at each end were mapped using Bowtie to the human mtDNA genome. Sequence reads with ends mapping to separate regions of the mtDNA genome were retrieved as candidate deletions. With this algorithm, deletions occurring within the central 40-nucleotide region of a sequence read could be identified. The candidate deletions were then manually verified. To minimize the false-positive rate, only deletions identified by ≥4 independent reads were included in the analysis.

Superoxide Dismutase Activity Assay

To measure the manganese superoxide dismutase (MnSOD) activity, mitochondria were isolated from frozen heart tissue using standard differential centrifugation in 10.0 mmol/L Tris-HCl (pH 7.4), 10.0 mmol/L Hepes, 250 mmol/L sucrose, 0.5 mmol/L EGTA, and 0.02% protease inhibitor cocktail.[24] MnSOD activity was measured using an assay kit from Abcam according to the manufacturer's instruction. The data were presented as the percentage inhibition of the rate of superoxide anion generation from xanthine oxidase.

Statistical Analysis

All data are presented as mean+SEM. Results were compared by using 1-way ANOVA using Fisher protected least significant difference tests as post hoc corrections for multiple testing. Significance was declared at a $P<0.05$. After statistical analysis, mRNA expression data of genes were grouped together based on functional similarity for presentation purposes.

Results

Mitochondrial Functional Integrity and Respiratory Dysfunction in Failing Hearts

The characteristics of the subjects included in the ICM and DCM subgroups recorded at the time of sample collection are shown in Table 1. As a control, mRNA levels of brain natriuretic peptide and α-myosin heavy chain were determined by real-time PCR in all samples (Figure I in the online-only Data Supplement). As expected, upregulation of brain natriuretic peptide and downregulation of α-myosin heavy chain were observed in failing hearts confirming integrity of samples (Figure IA and IB in the online-only Data Supplement).

To determine the functional integrity of the mitochondria, end-stage DCM and ICM hearts were examined for activity of enzymes of the electron transport chain, SDH and COX (Figure 1A). Histological analysis revealed cardiac myocyte degeneration and cytoplasmic vacuolation in both ischemic and dilated cardiomyopathic hearts (Figure 1A-b and 1A-c), which was associated with enlarged cross-sectional area of individual myocytes, particularly in DCM hearts (Figure 1A-c). Histochemical staining for COX and SDH activities was performed to examine qualitative differences in these electron transport activities in ICM and DCM. This demonstrated decreased staining of COX (Figure 1A-e and 1A-f) and SDH in both ICM and DCM hearts (Figure 1A-h and 1A-i) in comparison with NF hearts (Figure 1A-d and 1A-g). To quantitate the observed decrease in electron transport chain activities, spectrophotometric enzyme assays were performed for complex IV and II on mt rich fractions isolated from NF, end-stage DCM, and ICM hearts. As shown in Figure 1B and 1C, both ICM and DCM failing hearts demonstrated decrease in complex IV (Figure 1B; $P<0.05$ versus NF hearts) and complex II (Figure 1C; $P<0.05$ versus NF hearts) activities by almost 50%, consistent with severe dysfunction of mt respiratory chain during HF in humans. The mt respiratory dysfunction in failing hearts prompted us to investigate whether increased levels of oxidation products could be detected in these failing human hearts. Analysis of protein oxidative stress was done by derivatizing carbonyl groups on oxidized proteins from NF and failing hearts with 1,3-dinitrophenylhydrazine on oxyblot using immunoblotting. Representative example of oxyblot shown in Figure 1D revealed increase in oxidized proteins in failing hearts.

Table 1. Preimplantation Clinical Characteristics of the Patients

		Heart Failure	
	NF	ICM	DCM
No.	8	8	8
Age in years, median (range)	55 (50–80)	53(40–60)	57(40–75)
Sex, male/female	4/4	6/2	6/2
Ejection fraction, (%)	60±5*	20±5	20±5
Cardiac index (L/min/m^2)	No data	2.5±0.4	2.2±0.2
PWP, mm Hg	No data	20±5	27±5
Inotropic support, n	–	5/8	7/8
Diabetic, n	0/4*	5/8	1/8
Renal disease, n	0/4*	1/8	3/8
Hypertension, n	0/4*	5/8	1/8
Smoking history, n	5/8*	1/8	0/8

DCM indicates dilated cardiomyopathy; ICM, ischemic cardiomyopathy; NF, nonfailing; and PWP, pulmonary wedge pressure.

*Clinical data for 4 of 8 NF hearts.

Mitochondrial Morphology and Biogenesis in Failing Hearts

To determine whether mt morphology was also altered in end-stage human HF, electron microscopy was performed on myocardial sections from ICM (Figure 2A-a) and DCM (Figure 2A-b) hearts. Mitochondria were more numerous but with abnormal morphology in DCM. The marked mt proliferation was accompanied by myofibrillar displacement and loss in

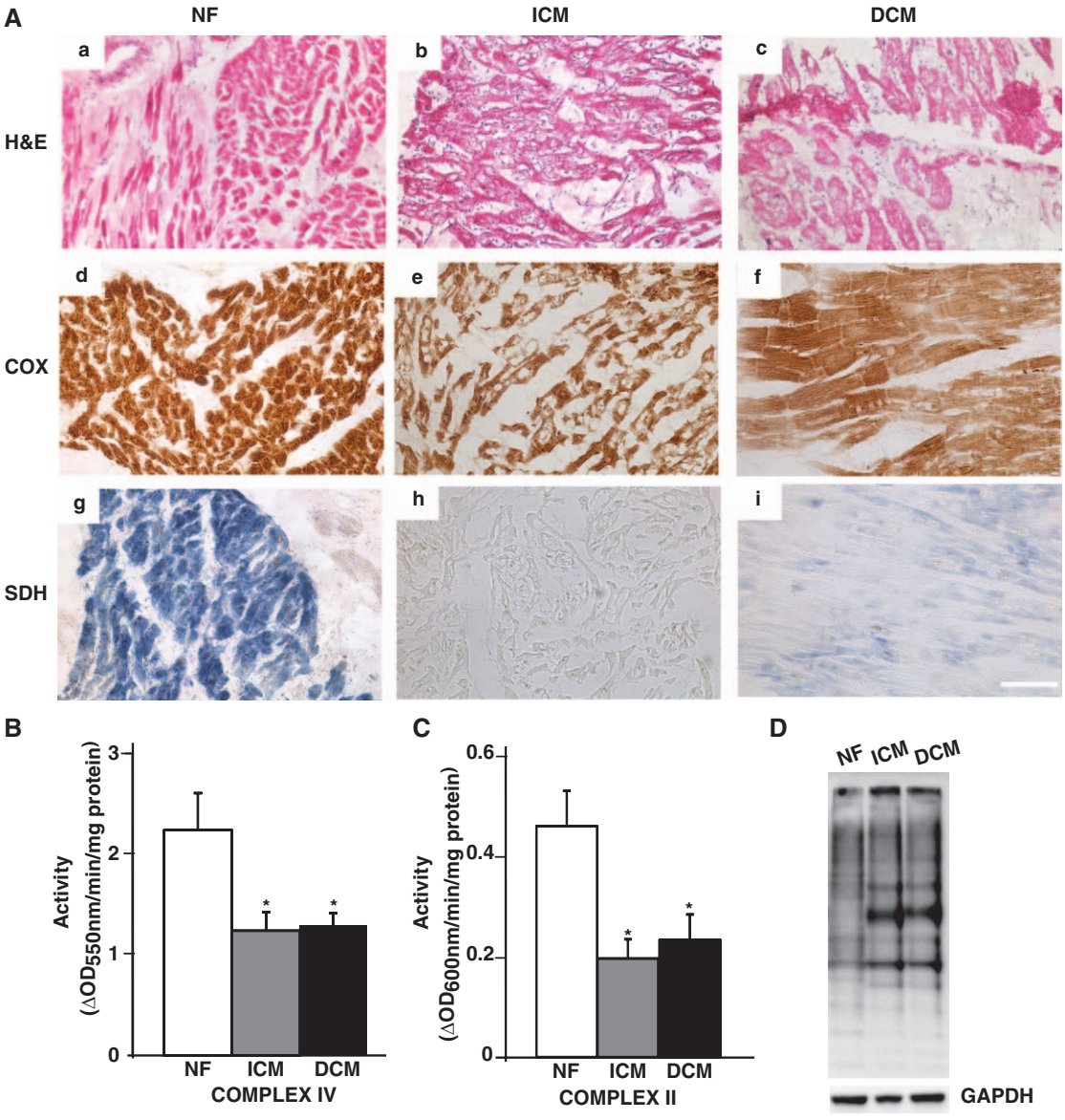

Figure 1. Mitochondrial functional integrity and respiratory dysfunction in failing hearts. Hematoxylin and eosin (H&E; **Aa–Ac**), Cytochrome C oxidase (COX; **Ad–Af**), and succinate dehydrogenase (SDH; **Ag–Ai**) stained frozen sections from nonfailing (NF), ischemic (ICM), or dilated (DCM) cardiomyopathic left ventricular myocardium (n=3/group) showing cardiac myocyte degeneration and reduced functional state of electron transport chain complexes IV and II. Scale bar, 50 μm. Enzymatic activity of electron transport chain complexes (**B**) II and (**C**) IV on mt rich fractions obtained from NF, ICM, and DCM hearts (*P<0.05 vs NF; n=8/group). **D**, Representative micrograph of measurement of oxidized proteins in the failing hearts using oxyblot.

DCM sections. Quantitative morphometric analysis confirmed a 2.2-fold increase in mt volume density in DCM hearts (Figure 2B; P<0.001 versus ICM hearts). In contrast, electron micrographs of ICM myocardium depicted fewer mitochondria with fragmented morphology (Figure 2A-a). To determine whether the observed morphological changes in failing hearts were caused by an imbalance in mt dynamics, we analyzed total ventricular lysates from NF, ICM, and DCM failing hearts for mt fusion and fission proteins. As shown in Figure 2C, mt fusion proteins, such as MFN2 and OPA1, were increased in DCM hearts but decreased in ICM hearts. These observations suggest an imbalance in mt fusion and fission in end-stage HF.

Because mt morphological and functional alterations are often associated with changes in mtDNA,[18,25] we examined total mtDNA content. Total DNA (genomic and mtDNA) isolated from NF, ICM, and DCM ventricles was assayed for levels of the mtDNA-encoded gene, COX1, and compared with the nuclear-encoded gene PPRC (Figure 2D). The observed increase in mt number in DCM failing hearts was associated with a 2.41-fold increase in mtDNA (P<0.001 versus NF hearts). In contrast, ICM hearts displayed a 32% reduction in mtDNA content when compared with NF hearts (P<0.05). To rule out the possibility that the observed increased mtDNA was from a relative increase in the noncardiac myocyte population in the failing hearts, expression levels of fibroblast marker DDR2 (Discoidin domain receptor 2) was determined from total ventricular RNA from NF, ICM, and DCM hearts. There was no significant difference in expression of DDR2 in failing hearts when compared with NF hearts (Figure II in the online-only Data Supplement).

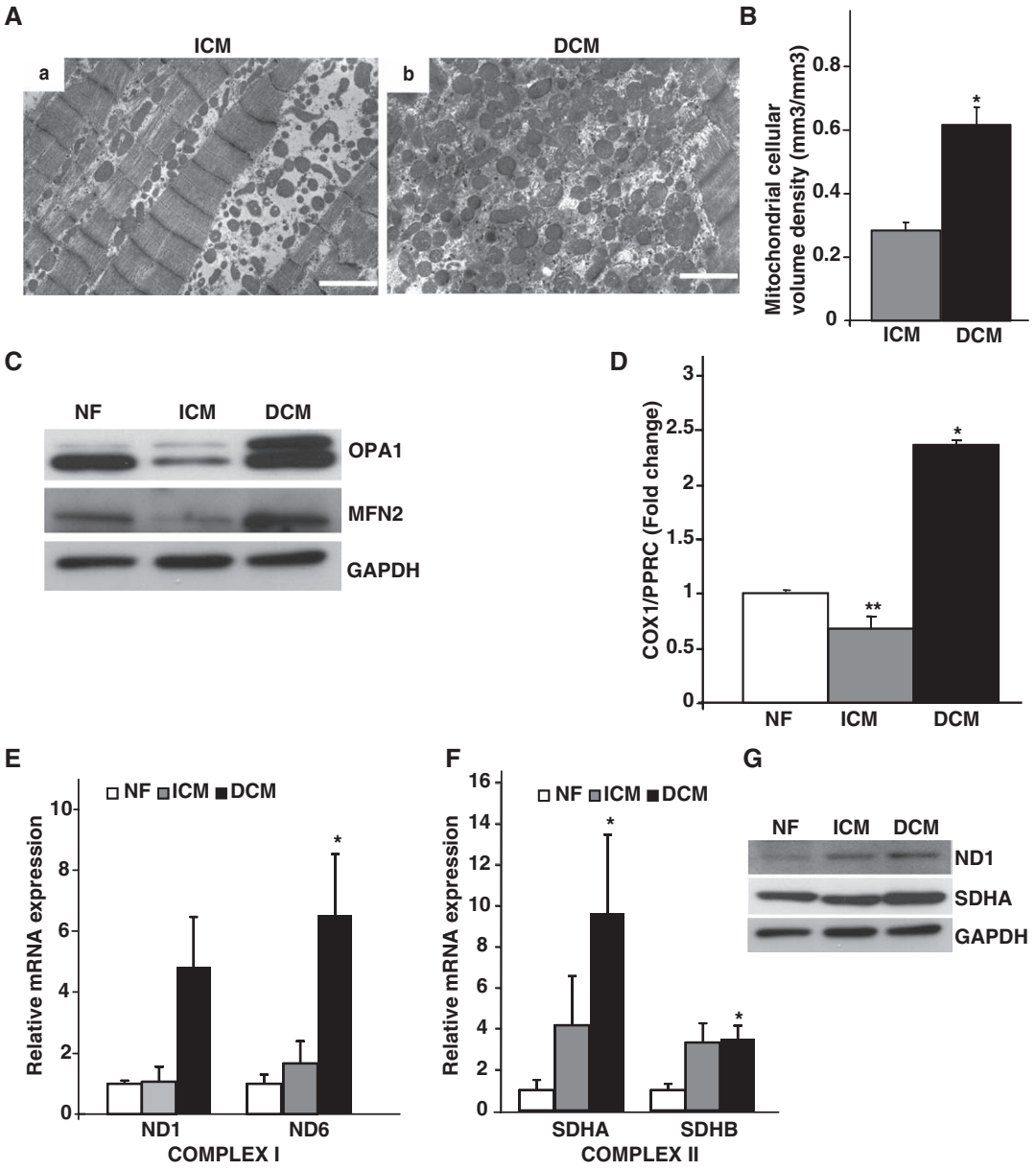

Figure 2. Mitochondrial (mt) morphology and biogenesis in failing hearts. **A**, Transmission electron microscopy performed on heart sections from ischemic (ICM; **a**) or dilated (DCM, **b**) cardiomyopathic myocardium demonstrating abnormal mt biogenesis in failing hearts. Scale bar, 1μm. **B**, Quantitative morphometric measurement of mt cellular volume density ($\mu m^3/\mu m^3$) based on analysis of electron micrographs from ICM and DCM ventricles (*P<0.001 vs NF; n=4/group). **C**, Total ventricular protein lysates from indicated phenotypes were probed with antibodies against mt fusion proteins (OPA1 and MFN2) along with GAPDH as a loading control (n=4/group). **D**, Quantitative real-time PCR on the mt gene *COX1*, along with the nuclear gene *PPRC* as an internal control from nonfailing (NF), ICM, and DCM hearts showing increase in mtDNA content in DCM hearts. (*P<0.001, **P<0.05 vs NF; n=8/group). **E**, Quantification of mt encoded Complex I ND1 and ND6 genes on NF, ICM, and DCM hearts. (n=8/group, *P<0.05 vs NF). **F**, Quantification of nuclear encoded Complex II SDHA and SDHB genes on NF, ICM, and DCM hearts. (*P<0.05 vs NF). **G**, Total ventricular protein lysates from indicated phenotypes probed with antibodies against electron transport Complex I and II proteins (ND1 and SDHA) along with GAPDH as a loading control (n=4/group).

To investigate the basis for the mt dysfunction (Figure 1), we assessed expression levels of a panel of genes encoded by mtDNA (Complex I genes ND1 and ND6) and nuclear DNA (nDNA) encoded genes (Complex II genes SDHA and SDHB) in failing hearts. As shown in Figure 2E, DCM hearts demonstrated increased expression of both mtDNA encoded subunits ND1 and ND6 (Figure 2E; 4.8 and 6.5-fold respectively; P<0.05 versus NF hearts), whereas ICM hearts had no significant change in expression of both mtDNA encoded genes (Figure 2E). Interestingly, expression levels of nDNA encoded genes SDHA and SDHB were upregulated in both HF models compared with NF hearts (Figure 2F). These changes in gene expression were associated with a corresponding increase in expression of mt encoded Complex I protein, ND1 and nuclear encoded Complex II protein, SDHA in DCM hearts (Figure 2G). Taken together, these results demonstrate that mtDNA content and mtDNA-encoded genes are upregulated in DCM hearts and, conversely, not significantly changed in ICM failing hearts.

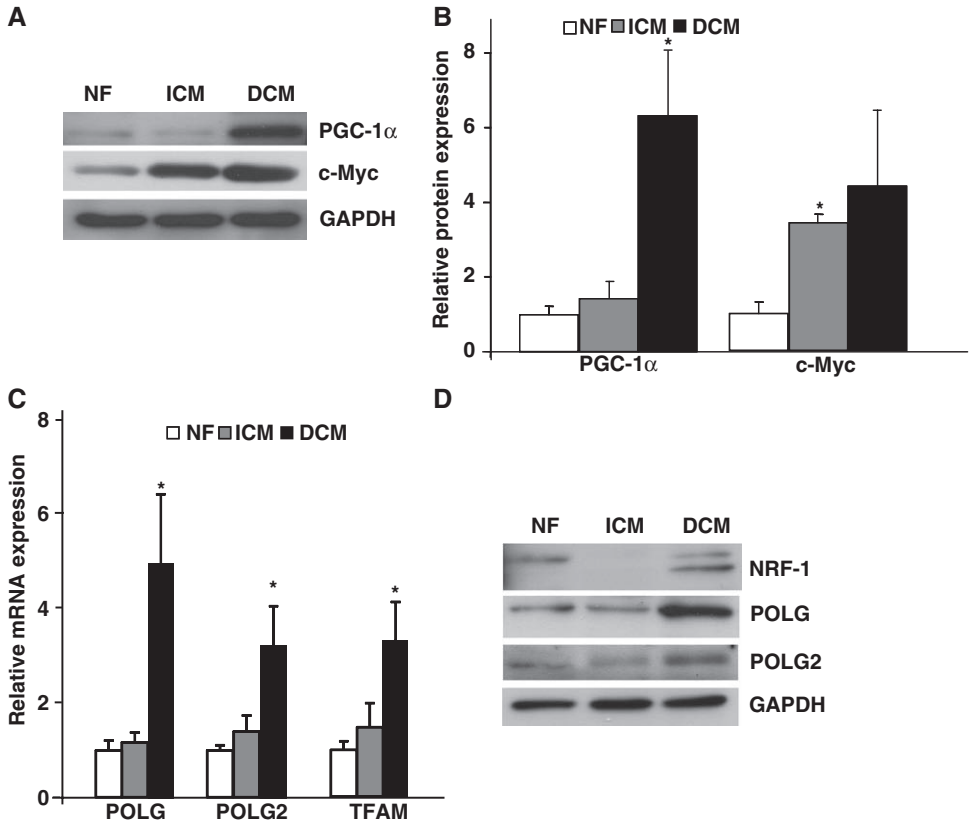

Figure 3. Mitochondrial (mt) biogenesis in failing hearts. Total ventricular protein from nonfailing (NF), ischemic (ICM), or dilated (DCM) cardiomyopathic ventricles assayed by (**A**) immunoblot and (**B**) quantified for mt biogenesis regulators PGC-1α and c-Myc. (*P<0.05 vs NF; (n=4/group). **C**, Real-time PCR from NF and failing ventricles for mt biogenesis genes POLG, POLG2, and TFAM. (*P<0.05, **P<0.05 vs NF; n=8/group). **D**, Total ventricular lysates from NF, ICM, and DCM probed with antibodies against NRF-1, POLG, and POLG2 along with GAPDH as a loading control (n=4/group).

Impaired Mitochondrial Biogenesis in Heart Failure

To determine the mechanisms underlying abnormal mt biogenesis in failing hearts, we evaluated the expression levels of key transcriptional regulators of mt biogenesis in heart, PGC-1α,[26] and c-Myc[8] in NF, ICM, and DCM hearts (Figure 3A and 3B). As shown in Figure 3A, DCM hearts showed a 6-fold increase in protein levels (Figure 3B; P<0.05 versus NF hearts), whereas protein levels of PGC-1α did not change significantly in ICM failing hearts. Protein levels of c-Myc were increased in both ICM and DCM (Figure 3B), suggesting the important factor mediating the difference in mt biogenesis in the two etiologies was PGC-1α.

We analyzed total ventricular RNA from failing hearts for downstream targets of PGC-1α, such as nuclear respiratory factor 1 (NRF-1), mtDNA polymerase catalytic subunits POLG and POLG2, and mt transcription factor A (TFAM). As shown in Figure 3C, there was a 4.9-fold increase in POLG (P<0.05), 3.25-fold (P<0.05) in POLG2, and 3.34-fold increase in TFAM (P<0.05) compared with NF hearts, whereas ICM hearts demonstrated no significant changes in the expression of these genes. These changes in expression of mt biogenesis regulatory genes were associated with a corresponding increase in protein expression of NRF-1, POLG, and POLG2 in DCM hearts with no significant changes in ICM hearts when compared with NF hearts (Figure 3D). Thus, our results suggest that there is PGC-1α–associated abnormal mt biogenesis in DCM failing hearts in contrast to no significant change of mtDNA replication and biogenesis in ICM failing hearts.

Mitochondrial DNA Damage in Heart Failure

To determine whether increase in mtDNA in DCM failing hearts is associated with mtDNA damage, we analyzed mtDNA for deletion mutations. The prevalence of mtDNA deletions is inversely correlated with mt function in heart and skeletal muscle.[21] mtDNA deletion frequencies in the common deletion site in the major arc of the mt genome were determined using the random mutation capture assay.[21] As shown in Figure 4A, DCM failing hearts showed a 4.1-fold increase in frequency of mtDNA deletions (P<0.05 versus NF hearts). The trend for increased mtDNA deletions in ICM hearts was not significant when compared with NF hearts. To determine whether the deletion mutations in DCM failing hearts were acquired during development of heart failure, we analyzed heart samples from DCM subjects who had been supported with left ventricular assist devices (LVAD) for an average of 2 months (Figure 4A). LVAD support is associated with reverse remodeling and correction of mt defects in end-stage failing hearts (for review see[27]). mtDNA deletions were significantly reduced in LVAD-supported DCM hearts when compared with DCM failing hearts (Figure 4A; P<0.05 versus DCM). These data suggest that deletion mutations observed in mtDNA of

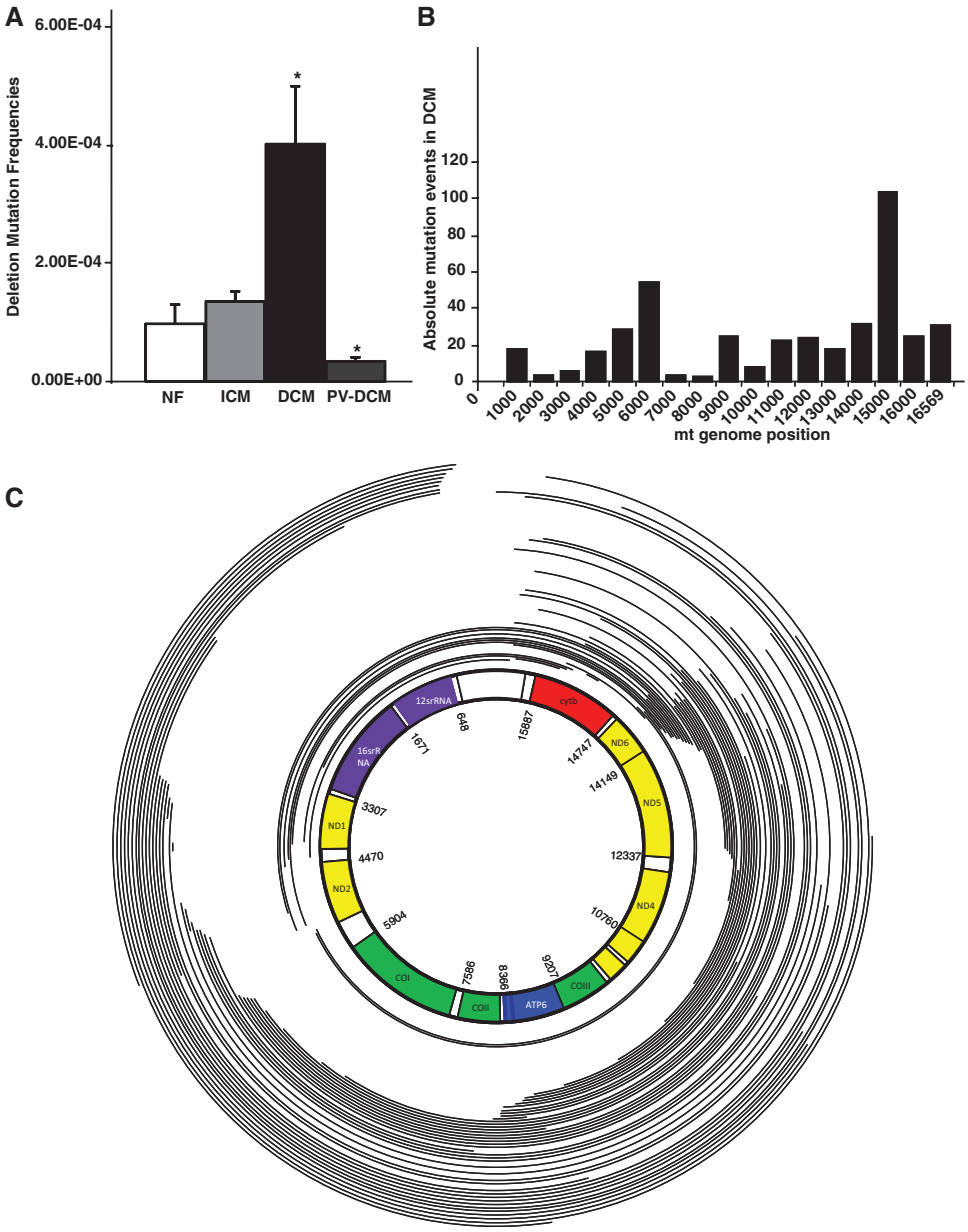

Figure 4. Mitochondrial (mt) DNA deletion mutations in failing and in reverse remodeled hearts. **A**, Deletions were PCR-amplified and quantified using random mutation capture assay on DNA obtained from nonfailing (NF), ischemic (ICM), or dilated (DCM) cardiomyopathic and post–ventricular assist device (VAD) supported hearts demonstrating increased frequency of mtDNA mutations in DCM hearts (*P<0.05, vs normal; n=8/group). **B**, Histogram showing frequency of deletion mutations observed across the mt genome in 1 of the DCM failing heart. **C**, Schematic representation of the human mtDNA-deletion mutations obtained from an end-stage DCM failing heart. mtDNA deletion mutations were determined by Solexa sequencing of mtDNA, and breakpoints were determined by DNA sequence analysis. Arcs represent the deleted regions of the genome.

DCM failing hearts accumulate with the pathophysiologic signals in the failing ventricle.

To confirm and better characterize the increased mtDNA deletions in an unbiased manner, we performed next generation sequencing of mtDNA from NF and DCM failing hearts. For each sample, we obtained 5×10^5 to 1.1×10^6 mtDNA reads spanning 75 base pairs resulting in a 3500- to 4000-fold mt genome coverage. Both ends of each sequence read were mapped onto the mtDNA genome, and sequences with ends that mapped to different locations were identified as deletions. To minimize the chances of artifactual deletions, we only considered deletions that were identified by ≥4 independent sequence reads (Table 2). Two hundred thirteen distinct deletions were identified by ≥4 independent sequence reads in DCM hearts representing deletions in 0.05% of the mt genomes. Although all DCM hearts exhibited mtDNA deletions, we observed deletions in only 1 NF heart, representing a deletion rate of 0.003% in NF hearts (Table 2). Figure III in the online-only Data Supplement depicts positions, sizes, frequencies, and affected genes for each deletion for all 6 DCM subjects used in the study. We did not observe a correlation between the age of the subject and the frequency of mtDNA deletions; however, the sample size was small and the stimulus for these mutations in failing myocardium may be much greater than that occurring with normal aging (Table 2). The histogram for 1 representative end-stage DCM failing heart is shown in Figure 4B. It demonstrates that most of the mtDNA deletions were concentrated in the mt major arc. Figure 4C is a schematic representation of the spectrum of deletion mutations observed in the same DCM failing heart, demonstrating that most of the mtDNA deletion mutations were in the major arc of mt genome. These data suggest that the mt dysfunction is associated with mtDNA damage and deletions specifically in DCM hearts, which could be

Table 2. Deletions of mtDNA Found in Nonfailing and Dilated Cardiomyopathy Patients by High-Throughput Sequencing

Myocardial Sample	Age and Sex of the Patient	Distinct Deletions*	Independent Reads	Deletion Frequency (%)
NF 1	54, male	90	448	0.01
NF 2	86, male	n.d.	n.d.	n.d.
NF 3	84, male	n.d.	n.d.	n.d.
DCM 24	31, male	73	353	0.03
DCM 33	63, male	11	45	0.01
DCM 28	74, male	55	246	0.02
DCM 15	62, male	61	265	0.03
DCM 46	49, male	4	16	0.003
DCM 44	51, male	9	132	0.2

*Distinct deletions is column A, independent reads is sum of column E and deletion frequency is percentage of sum of column H over column I in Figure III in the online-only Data Supplement.

DCM indicatesdialted cardiomyopathy; and NF, nonfailing.

a consequence of mutating stress coupled with a PGC-1α–dependent stimulus for mt biogenesis.

Mitochondrial DNA Repair in Failing Hearts

To evaluate the status of antioxidants and DNA repair mechanisms, we examined ventricular RNA. Base excision repair (BER) genes, such as human endonuclease III (NTHL11) and 8-oxoG DNA glycosylase (OGG1), were upregulated in both HF causes. There was an increase in NTHL11 expression by 1.62- and 2.38-fold, and OGG1 by 2.75- and 5.3-fold in ICM and DCM hearts, respectively, (Figure 5A; $P<0.05$ versus NF hearts). Expression levels of a panel of antioxidant genes, such as manganese superoxide dismutase (SOD2), glutathione peroxidase-1 (GPX1), γ-glutamyl-cysteine synthetase light subunit (GCLM), and γ-glutamyl-cysteine synthetase heavy subunit (GCLC) in failing hearts were also elevated. Significant increases in mRNA levels for SOD2 (2.83- and 3.64-fold), GPX1 (1.5- and 2.69-fold), GCLM (2.65- and 3.8-fold), and GCLC (2.74- and 5.2-fold) were found in ICM and DCM failing hearts (Figure 5C; $P<0.05$ versus NF hearts). A small but significant increase in activity of MnSOD was also observed in mitochondria isolated from NF, ICM, and DCM hearts (Figure 5D). Interestingly, despite elevations of BER gene expression, protein levels for NTHL1 were reduced in failing hearts (Figure 5B).

Discussion

Although the underlying injuries and stressors that lead to DCM or ICM are diverse, it has been assumed the pathophysiology, particularly with respect to cardiac mt dysfunction, is similar between these etiologies. This may be related to the fact that the mechanisms responsible for the mt dysfunction in human hearts are poorly understood, and the animal models used to elucidate mechanisms may not reflect the true pathophysiology of human DCM. In the current study we performed a detailed morphological and molecular analysis of end-stage failing human hearts to characterize mt morphological integrity, proliferation, mtDNA content, and damage in ICM and DCM. Our data demonstrate that DCM is associated with an increase in the number of fragmented mitochondria, mtDNA copy number, and expression of mtDNA-encoded genes. In contrast, end-stage ICM hearts show a reduction in the number of mt, with corresponding decrease in mtDNA. This abnormal mt proliferation in DCM hearts appears to be PGC-1α–dependent and, interestingly, is associated with an increase in mtDNA deletions. We speculate that this increase in mutated mtDNA in DCM hearts is a consequence of a stimulus for mt biogenesis in the setting of increased oxidative stress and a defective mtDNA repair system. Although certain limitations of the current study, such as low sample size with a male predominance and increase in metabolic factors such as diabetes mellitus and hypertension in ICM, cannot be disregarded; however, this study was intended to be a pilot study to provide important insight into the mechanisms underlying human HF.

The electron transport chain is an essential component of ATP production, and is embedded in the inner mt membrane. Defects in the individual complexes have been documented in HF (for review, see).[28] Decreased electron transport chain enzyme activity occurs in all HF patients independent of the cause.[29] Consistent with this, both DCM and ICM were associated with a decrease in activity of COX and SDH, subunits of Complex IV and II, respectively, indicative of mt respiratory chain dysfunction in HF, which was also corroborated with demonstration of increased oxidation of protein in these failing hearts. The impairment of electron transport chain was accompanied by defects in the mt dynamics in failing hearts. Although mt were more numerous with smaller and fragmented morphology in both DCM and ICM hearts, suggesting a predominance of mt fission, in DCM there was concomitant mt biogenesis leading to an increase in total mt volume in contrast to ICM hearts, which displayed a significant reduction in mt volume. Defects in mt organization and the presence of abnormally small and fragmented mitochondria have been previously observed in end-stage DCM, myocardial hibernation, and ventricular associated congenital heart disease.[30] There was also an increase in mt fusion proteins such as OPA1 and MFN2 in DCM hearts; however, both were decreased in ICM hearts. Decreased protein levels of OPA1 have also been previously reported in human ICM, however with increased MFN2. In contrast, in DCM OPA1 protein levels were unchanged, but MFN2 was increased.[31] The significance and contribution of abnormal mt dynamics in HF remains to be determined.

Alterations in intracellular levels of reactive oxygen species are often associated with changes in mt abundance, mtDNA copy number, and expression of respiratory genes (for review[16]). Our data demonstrated increased mt proliferation in DCM hearts associated with increased mtDNA copy number and expression of mtDNA-encoded genes, whereas ICM hearts displayed a reduction in mtDNA. Our observations in ICM are similar to those made in mouse models of pathological remodeling after myocardial infarction, where the decline of mt function was associated with a reduction in mtDNA copy number.[32] Our findings in human DCM hearts parallel those reported in aging human tissues[33,34] and in response to DNA-damaging agents,[35] where increased mtDNA is associated with

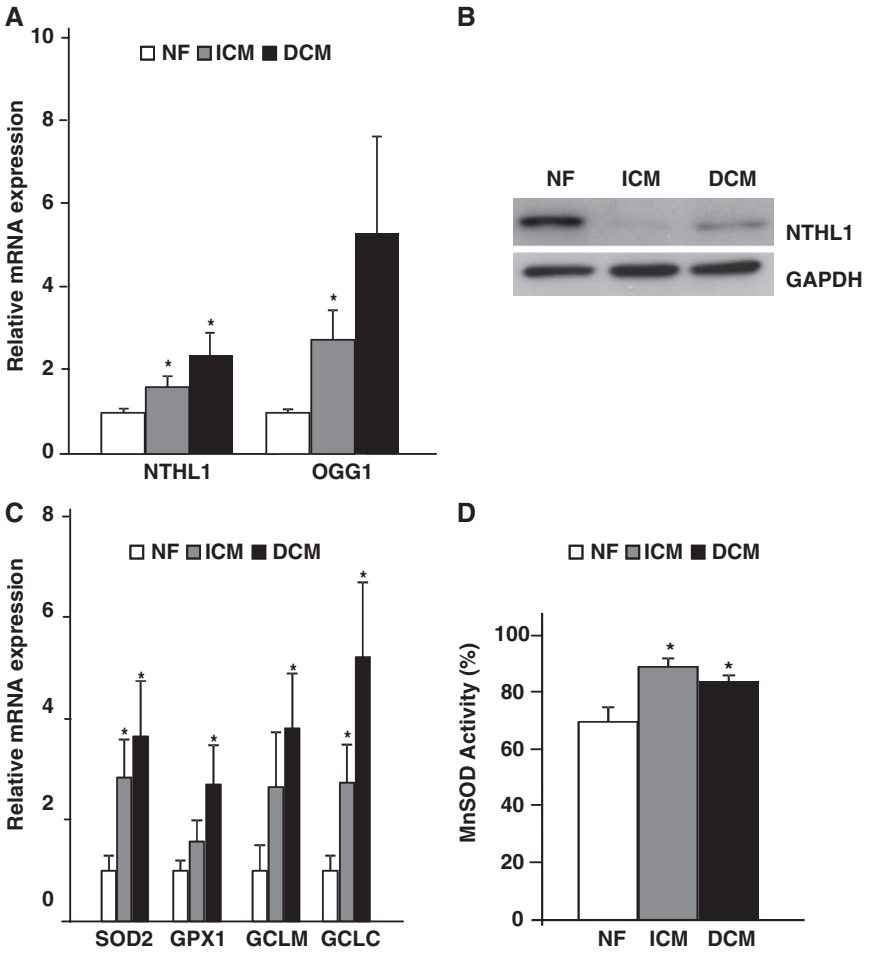

Figure 5. Mitochondrial DNA repair in failing hearts. Total ventricular RNA (**A**) and protein (**B**) from nonfailing (NF), ischemic (ICM), or dilated (DCM) cardiomyopathic ventricles assayed by real-time PCR and immunoblot for mt DNA repair genes NTHL1 and OGG1 (*P<0.05 vs NF; n=8/group). **C**, Real-time PCR from NF and failing ventricles for mitochondrial antioxidant genes SOD2, GPX1, GCLM, and GCLC (*P<0.05 vs NF; n=8/group. **D**, Total ventricular protein lysates from NF, ICM, and DCM determined for MnSOD activity (n=8/group).

oxidative damage. The increase in mt mass and mtDNA in these studies has been proposed to be a compensatory mechanism against oxidative damage to mtDNA and respiratory chain components.[16] However, it should be noted that mtDNA estimations done in HF patients in the current study were compared with age-matched NF hearts, thus implying that age cannot account for the increase in mtDNA content in DCM hearts. Interestingly, Karamanlidis et al[13] recently showed a decrease in mtDNA, with an increase in PGC-1α, in failing hearts. This unexpected finding might be attributable to use of ventricular samples from mixed causes in their study. Our data from DCM hearts suggest that mt biogenesis was PGC-1α–associated because Myc levels were similar in DCM and ICM and PGC-1α targets, NRF-1, mtDNA transcription factor, TFAM, and mtDNA polymerase catalytic subunits POLG and POLG2 required for mtDNA replication were induced in DCM. Thus, under oxidative stress, there is a coordinated nuclear and mt genome activation to induce mt biogenesis presumably to compensate for mt dysfunction. In skeletal muscle, mt proliferation has been shown to partly compensate for the respiratory dysfunction by maintaining overall ATP production.[36] However, in cardiac muscle, induction of mt biogenesis has been proposed to be a maladaptive response. In fact, cardiac-specific induction of PGC-1α in mice results in cardiac dysfunction with morphological features of myocyte mt proliferation and myofibrillar disorganization and loss.[37]

In the current study, we found that mt respiratory dysfunction, abnormal mt proliferation, and biogenesis in failing DCM hearts were associated with an increased frequency of mtDNA deletion mutations. Because the frequency of mtDNA deletions was found to be significantly reduced with LVAD-support in DCM failing hearts, these data suggest that the accumulation of mtDNA deletions are acquired during the development of heart failure as opposed to part of a more global mt syndrome. Although the exact cause of accumulation of mtDNA deletions in DCM hearts is unknown, mtDNA deletion mutations and mt abnormalities have been previously described in idiopathic DCM in humans.[38,39] It has been proposed, based on the size of mtDNA deletion mutations, that deletion-containing mt genomes have a replicative advantage.[40] Support for the replicative advantage of smaller genomes comes from repopulation kinetic studies, where it was shown that partially deleted mtDNA molecules repopulate mitochondria faster than full-length genomes.[41] Although the deletion mutation frequency observed in DCM failing hearts was quite low to affect the mt function, it is interesting to note that it has been shown that deletion mutations in cardiac myocytes accumulate clonally in aging heart.[42] mtDNA deletions were shown to accumulate to high fractions in certain cells within the cardiac tissue while most cells remain deletion free, which unfortunately could not be demonstrated in the our study because the 4-fold mutation rate observed was in total ground up LV tissues. However, focal

accumulation of mtDNA deletions beyond a certain threshold could lead to drop out of cardiac myocytes, causing respiratory chain abnormalities and further production of free radicals exacerbating the HF syndrome. Whether the rates of mtDNA deletions we observed in DCM hearts have pathophysiologic consequences or are simply a marker for the enhance mtDNA synthesis remains to be determined. DCM is the most challenging form of heart failure, because it lacks any molecular diagnostic assays or effective therapies. In fact, accumulation of mtDNA deletions could potentially serve as a marker for the enhance mtDNA synthesis, mitochondrial genomic instability, or mtDNA damage in DCM failing hearts and are areas of mitochondrial biology that should be looked in detail in future.

In our study, mtDNA damage in both models of HF was not associated with induction of antioxidant enzymes such as SOD2, GPX1, and GCL or BER enzymes, NTHL1 and OGG1, despite a marked upregulation of these genes at the RNA level. Similar results have been previously reported in human HF,[43] leading us to speculate that end-stage HF is unable to respond to oxidative stress by adequately increasing the antioxidant response and mtDNA repair mechanisms. The best-characterized DNA repair pathway in mitochondria has been BER.[44] However, accumulation of mtDNA deletions has been linked to decline in BER activities. The accumulation of mtDNA deletions in aging cells is associated with decreased mitochondrial BER glycosylases expression levels.[45] Additionally, mice deficient for NEIL1, a BER glyocsylase, accumulate mtDNA deletions compared with wild-type mice,[46] suggesting an important role of mitochondrial BER in prevention of mtDNA damage and deletions.

In summary, our findings suggest that although HF of both causes is associated with mt dysfunction, they exhibit divergent phenotypes with respect to mt biogenesis. DCM is associated with enhanced mt biogenesis and mtDNA deletions. mtDNA deletions can cause mt dysfunction, resulting in an inability of the heart muscle to maintain adequate energy production and continuing to cause oxidative stress. Although we cannot resolve whether the rate of mtDNA deletion we observed contributed to the mt dysfunction, it does suggest a unique pathophysiology for the development of DCM. A better understanding of mechanisms underlying human HF could lead to etiology specific therapies in HF, something that up to now has been lacking.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Although the underlying injuries and stressors that lead to dilated cardiomyopathy (DCM) or ischemic cardiomyopathy (ICM) are diverse, it has been assumed that the pathophysiology, particularly with respect to cardiac mitochondrial (mt) dysfunction, is similar between these causes. This may be related to the fact that the mechanisms responsible for the mt dysfunction in human hearts are poorly understood, and the animal models used to elucidate mechanisms may not reflect the true pathophysiology of human DCM. In the current study we performed a detailed morphological and molecular analysis of end-stage failing human hearts to characterize mt morphological integrity, proliferation, mtDNA content, and damage. Our data demonstrates that DCM is associated with an increase in the number of fragmented mitochondria, mtDNA copy number, and expression of mtDNA-encoded genes. This abnormal mt proliferation in DCM hearts appears to be proliferator-activated receptor γ coactivator 1α–dependent and interestingly, is associated with an increase in mtDNA deletions. We speculate that this increase in mutated mtDNA in DCM hearts is a consequence of a stimulus for mt biogenesis in the setting of increased oxidative stress and a defective mtDNA repair system. In summary, our findings suggest that DCM is associated with more mtDNA deletions than ICM, which can cause mt dysfunction, resulting in an inability of the heart muscle to maintain adequate energy production and continuing to cause oxidative stress. This suggests a unique pathophysiology for the development of DCM and thus a potential novel target for cause-specific therapies in heart failure, something that up to now has been lacking.