Mitochondrial Abnormalities in Tumor Necrosis Factor-α–Induced Heart Failure Are Associated With Impaired DNA Repair Activity

Yun You Li, MD, PhD; Dexi Chen, PhD; Simon C. Watkins, PhD; Arthur M. Feldman, MD, PhD

Background—Recent studies suggest that mutations in cardiac mitochondrial DNA (mtDNA) may contribute to the development of dilated cardiomyopathy. The mechanisms that regulate those mutations, however, remain undefined. Thus, we studied cardiac mtDNA repair mechanisms, mtDNA damage, and mitochondrial structure and function in mice with heart failure secondary to overexpression of TNF-α (TNF1.6 mice).

Methods and Results—We studied mtDNA repair by measuring the uracil DNA glycosylase (mtUDG) and base excision repair activities. mtDNA damage was assessed by Southern blot of Fpg protein–digested mtDNA. Mitochondrial ultrastructural changes were examined by electron microscopy, and function by cytochrome c oxidase and succinate dehydrogenase activity assays. The results showed that both mtUDG and base excision repair activities were significantly reduced in TNF1.6 mouse heart. Fpg-sensitive sites were markedly increased in TNF1.6 mouse cardiac mtDNA, suggesting increased mtDNA damage. Mitochondrial function as demonstrated by cardiac cytochrome c oxidase activity was also markedly reduced. Cardiac ATP content was not changed, however, suggesting a shift from oxidative phosphorylation to glycolysis, as shown by increased LDH and ALT activities and lactate/pyruvate ratio. Ultrastructurally, the TNF1.6 mouse cardiac mitochondria became irregular in shape and smaller, and the cristae were decreased and appeared disorganized, with breaks.

Conclusions—These results suggest that mtDNA mutations and mitochondrial structural and functional alterations in TNF-α–induced heart failure may be associated with reduced mtDNA repair activity, and the pathophysiological effects of TNF-α on the heart may be mediated, at least in part, through these changes in mitochondria. (Circulation. 2001;104: 2492-2497.)

Key Words: heart failure • molecular biology • metabolism • structure

Dilated cardiomyopathy is a heart muscle disease of unknown origin characterized by progressive ventricular dilation, deterioration of cardiac function, and eventually heart failure.1 Recent studies suggest that mutations in the mitochondrial DNA (mtDNA) may contribute to the development of dilated cardiomyopathy.2 For example, significant amounts of mtDNA mutations have been found in cardiomyopathic myocardium.3–5 When mutated mtDNA from cultured cells is introduced into mice, it results in dilated cardiomyopathy.6 In addition, mtDNA mutations have also been implicated in the myocardial dysfunction associated with aging and coronary atherosclerotic heart disease.7 The mechanisms that regulate mtDNA mutations in the myocardium, however, are poorly defined.

Human mtDNA exists as a double-stranded closed circular 16 569–bp DNA molecule within the mitochondria.8 mtDNA encodes 13 components of the respiratory chain and 22 tRNA and 2 rRNA species. Because of proximity to the electron transport system, the lack of protective proteins such as histones, and the presence of reactive oxygen species, high levels of oxidative damage to the cardiac mtDNA occur.9,10 The DNA adduct 8-oxo-deoxyguanosine (8-oxodG) occurs endogenously at high frequency and was shown to be premutagenic.11 The reaction of reactive oxygen species with DNA yields a wide variety of DNA damages, including strand breaks and adducts. As a result, mtDNA has a significantly higher (3– to 9-fold) 8-oxodG/dG value than nuclear DNA in the heart. Conversely, reduced repair activity may also result in increased mtDNA lesions, because the steady-state level of mtDNA damage is a function of both adduct formation and adduct removal by DNA repair. The accumulation of oxidative mtDNA damage must be repaired to maintain proper function of the mitochondria. Recent studies have found that the mitochondria have a DNA repair system similar to that of the nucleus.12,13 Oxidative mtDNA damages are repaired mainly via the base excision repair...
(BER) pathway. BER is initiated by the removal of the damaged base by a glycosylase. The resulting abasic site is further processed and repaired by apurinic/apyrimidinic endonucleases (AP lyases), DNA polymerase, and DNA ligase. The latter often replaces just 1 nucleotide and fills the gap.\textsuperscript{14} The role of this system in the development of mitochondrial dysfunction and heart diseases is not defined. Because of the association between mitochondrial mutations and left ventricular dysfunction, we hypothesized that the high mutation rate of the cardiac mtDNA is due to insufficient DNA repair activity in addition to elevated oxidative damage, which may in turn result in dysfunction of the respiratory system, damage of the mitochondrial structure and function, and eventually heart failure.

Tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) is implicated in the development of heart failure. Indeed, transgenic mice with cardiac-specific overexpression of TNF-\(\alpha\) develop heart failure (TNF1.6).\textsuperscript{15} Thus, the TNF-\(\alpha\)-overexpressing mouse model would provide an ideal platform to study the mechanism of mtDNA mutations and test the hypothesis that changes in myocardial mtDNA mutations are associated with the development of cardiomyopathy.

**Methods**

**TNF-\(\alpha\) Transgenic Mouse Cardiac Sample Preparation**

Transgenic mice with cardiac-specific overexpression of TNF-\(\alpha\) (TNF1.6 mice) were created in our laboratory.\textsuperscript{15} Mice were euthanized under deep anesthesia, and the heart was excised and rinsed in ice-cold PBS. Ventricular tissues were weighed, used fresh or snap-frozen in liquid nitrogen for protein and enzyme analyses, fixed in 4\% paraformaldehyde for cryostat section and cytochemical analysis, or fixed in 2.5\% glutaraldehyde for electron microscopy. This study was performed according to the NIH guidelines as outlined in the Guide for the Care and Use of Laboratory Animals, and the protocols were approved by the Institutional Animal Care and Use Committee, University of Pittsburgh.

**In Vitro mtDNA Repair Activity Assay**

Cardiac mitochondria from 12-week-old mice were isolated as described previously\textsuperscript{16} and homogenized in mitochondrial lysis buffer (400 mmol/L KCl, 1 mmol/L EDTA, 5\% glycerol, 0.05\% Triton X-100, 2 mmol/L DTT, 0.5 mmol/L PMSF, 20 mmol/L HEPES [pH 7.4], and protease inhibitor cocktail). Two separate experiments were performed to measure mitochondrial uracil DNA glycosylase (mtUDG) and DNA polymerase and DNA ligase activities in mitochondrial extracts.

For the measurement of the overall BER activity, a 50-bp oligonucleotide containing a U:G mismatch at the 26th position, as described previously\textsuperscript{16} and homogenized in mitochondrial lysis buffer (400 mmol/L KCl, 1 mmol/L EDTA, 5\% glycerol, 0.05\% Triton X-100, 2 mmol/L DTT, 0.5 mmol/L PMSF, 20 mmol/L HEPES [pH 7.4], and protease inhibitor cocktail). Two separate experiments were performed to measure mitochondrial uracil DNA glycosylase (mtUDG) and DNA polymerase and DNA ligase activities in mitochondrial extracts. The measurement of the overall BER activity, a 50-bp oligonucleotide containing a U:G mismatch at the 26th position, as shown in Figure 1A, was used as the DNA repair substrate. The mismatch can be identified by mtUDG and excised. The gap can then be filled by DNA polymerase and ligase present in the mitochondrial extracts with the incorporation of [\(\alpha\)-\(\text{\(^{32}\text{P}\)}\)]dCTP. The mitochondrial extracts were incubated with this mismatched oligonucleotide substrate (0.3 \(\mu\)g at 30\(^\circ\)C in 50 \(\mu\)L) containing 45 mmol/L HEPES-KOH [pH 7.5], 1 mmol/L EDTA, 1 mmol/L DTT, 75 mmol/L KCl, 50 \(\mu\)g/mL BSA, 90 fmol of end-labeled oligonucleotide containing a U:G mismatch, and 25 \(\mu\)g of mitochondrial protein were incubated at 32\(^\circ\)C for 1 hour. The DNA was processed and separated as described above for BER assay. Incubation of mitochondrial extracts with end-labeled oligonucleotide results in the removal of the mismatched U by mtUDG. The resulting fragments were separated in a denaturing gel. The intensity of the smaller fragment reflects the activity of mtUDG.

**mtDNA Damage Assay**

Cardiac mtDNA was purified from myocardial mitochondria isolated from 22-week-old mice with a Qiagen plasmid isolation column. The purified mtDNA was dissolved in 0.2 mmol/L EDTA/10 mmol/L Tris-HCl (pH 8.0) buffer and subjected to digestion with formamidopyrimidine DNA glycosylase (Fpg) protein, which recognizes 8-oxodG sites and cleaves \(\text{\(^{N}\)}\)-glycosylc bonds between the modified DNA and 20 mmol/L EDTA, and the DNA was phenol-extracted and precipitated with the addition of 5 \(\mu\)g of carrier tRNA. The precipitates were separated in 15\% polyacrylamide gel containing 10 mmol/L Tris-HCl, 75 mmol/L urea at 10 W for 3 hours. The gel was dried and exposed to x-ray films. Incorporation of [\(\alpha\)-\(\text{\(^{32}\text{P}\)}\)]dCTP at the U:G mismatch site generated 2 different-sized bands, which were quantified with ImageQuant software.

mtUDG assay was performed with mitochondrial extracts as described.\textsuperscript{17} The end-labeled hairpin-looped oligonucleotide was cut with S1 nuclease and used for mtUDG assay (Figure 1C). Reactions (20 \(\mu\)L) containing 45 mmol/L HEPES-KOH [pH 7.5], 1 mmol/L EDTA, 1 mmol/L DTT, 75 mmol/L KCl, 50 \(\mu\)g/mL BSA, 90 fmol of end-labeled oligonucleotide containing a U:G mismatch, and 25 \(\mu\)g of mitochondrial protein were incubated at 32\(^\circ\)C for 1 hour. The DNA was processed and separated as described above for BER assay. Incubation of mitochondrial extracts with end-labeled oligonucleotide results in the removal of the mismatched U by mtUDG. The resulting fragments were separated in a denaturing gel. The intensity of the smaller fragment reflects the activity of mtUDG.
base and deoxyribose, and phosphate backbone. The reaction was stopped by the addition of alkaline loading buffer (40 mmol/L NaOH, 1 mmol/L EDTA, 2.5% Ficol-400, 0.025% bromophenol blue). The mixture was separated in a denaturing alkaline agarose gel and transferred to Genescreen Plus nylon membrane. Southern hybridization with random prime–labeled probes from full-length mtDNA was performed. The results were visualized by exposure to a Phosphomager screen.

Mitochondrial Function Assay
The cytochrome c oxidase (COX; EC 1.9.3.1, complex IV) and succinate dehydrogenase (SDH; EC 1.3.5.1, complex II) activities were assessed by cytochemical methods as described. COX consists of 13 polypeptide subunits, of which 3 are encoded by mtDNA. COX was therefore used as an indicator of mtDNA-induced changes in mitochondrial function. Because the 4 subunits of SDH are encoded by nuclear genes, SDH was used to monitor mitochondrial function that is affected by nuclear-encoded genes. Cryostat sections incubated at 37°C for 1 hour in medium containing 0.1% 3,3'-diaminobenzidine, 0.1% cytochrome c from horse heart, and 0.02% catalase in 5 mmol/L phosphate buffer (pH 7.4). For control sections, 10 mmol/L KCN was added to the incubation medium. The sections were rinsed 3 times with water for 5 minutes each. The slides were mounted with warm glycerin gel and were observed with a light microscope.

For the measurement of SDH activity, the same procedure as for COX was performed, except the incubation medium contained (in mmol/L) EDTA 5, KCN 1, phenazine methosulfate 0.2, succinic acid 50, and nitro blue tetrazolium 1.5 in 5 mmol/L phosphate buffer, pH 7.4. The control sections were incubated with the same medium with the addition of 10 mmol/L sodium malonate.

Lactate and Pyruvate Determinations
Weighed cardiac samples were homogenized in ice-cold PBS. The homogenates were centrifuged at 600g for 8 minutes at 4°C, and the supernatant was used for the measurement of ALT, LDH, and lactate levels with an automatic chemistry system (opera Chemistry System, Bayer). Pyruvate content in the supernatant was measured spectrophotometrically as described previously.

Transmission Electron Microscopy:
Mitochondrial Ultrastructure
Transmission electron microscopy was performed with a routine protocol at our laboratories. The fixed myocardial tissue blocks from 42-week-old mice were rinsed in PBS, soaked in 1% OsO₂, dehydrated in 30% to 100% ethanol, and incubated in propylene oxide, then in epoxy resin before embedding and cutting. The ultrathin sections were imaged with a Joel 1210 transmission electron microscope. Mitochondrial morphology was interpreted by an experienced microscopist who was blinded to sample identity. Mitochondria were considered normal if they contained electron-dense matrix and showed intact, orderly, and tightly packed cristae. A mild degree of injury was considered normal if they contained electron-dense matrix and showed intact, orderly, and tightly packed cristae. A mild degree of injury was defined when the cristae appeared disorganized, with breaks, and/or other structural abnormalities. A moderate degree of injury was defined as diffuse clearance of the matrix without any other structural abnormalities. A moderate degree of injury was considered when the cristae appeared disorganized, with breaks, and/or decreased in number. Severely injured mitochondria were characterized by overt membrane damage such as rupture, extrusions or invaginations of outer membrane, or incorporation of myelin figures. The electron densities of the mitochondria and myofibrils were quantified by ImageQuant software.

Statistical Analysis
Independent t test was applied to compare changes in different experimental groups with a statistical analysis software (SPSS). The quantitative data are presented as mean±SEM. Statistical significance was considered at a value of P<0.05.
bands until reaching a saturating concentration, suggesting that Fpg-sensitive sites are recognized and cut. mtDNAs from both wild-type and TNF1.6 mouse heart were treated with saturating concentrations of Fpg. As seen in Figure 2B, a markedly decreased intensity of larger bands was observed in TNF1.6 mtDNA, suggesting increased mtDNA damage in TNF1.6 mouse heart.

Mitochondrial Dysfunction and Shift From Oxidative Phosphorylation to Glycolysis

Measurement of SDH was used to monitor mitochondrial function that is affected by nuclear-encoded genes, whereas measurement of COX was used as an indicator of changes in mitochondrial function induced by both nuclear DNA and mtDNA. The mitochondrial COX activity was markedly reduced, whereas there was no change in SDH activity in the TNF1.6 mouse heart (Figure 3).

Although there was significant reduction in COX activity, no change in total ATP content was observed in TNF1.6 mouse myocardium compared with that of the wild-type (904.1±177.3 versus 808.4±85.6 pg/mg myocardium). To determine whether this was due to a shift in metabolic pathways, we measured cardiac ALT, LDH, lactate, and pyruvate levels. Dysfunction of the respiratory chain would lead to inadequate removal of lactate and pyruvate, as seen in mitochondrial cytopathies. Therefore, the lactate/pyruvate ratio is widely used as a denominator of mitochondrial dysfunction. Indeed, glycolysis measures of LDH, lactate, pyruvate, and lactate/pyruvate ratio were significantly elevated in TNF1.6 mouse heart (Table), suggesting a shift from oxidative phosphorylation to glycolysis as the major source of ATP generation.

Mitochondrial Structural Damage

To learn whether the changes in mtDNA and mitochondrial function have any impact on the structure of myocardial mitochondria, we studied the myocardial ultrastructure by transmission electron microscopy. The results showed marked myofibril disarray and breakdown in TNF1.6 mouse heart. The TNF1.6 mouse cardiac mitochondria were moderately to severely injured. The matrix was diffusely cleared, with the ratio of electron density of the mitochondrial matrix

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Values are mean±SEM.
*P<0.05 vs wild-type.

Figure 3. Mitochondrial enzyme cytochemical assays. Cardiac cytochrome c oxidase activity was normal in wild-type mice (A, C) and reduced in TNF1.6 mice (B, D), but cardiac succinate dehydrogenase activity was same in wild-type (E) and TNF1.6 (F) mice.
to that of the myofibril being significantly reduced (wild-type 3.47 ± 0.14, TNF1.6 1.23 ± 0.06, P < 0.01). Mitochondria became irregular in shape and smaller, the cristae appeared disorganized, with breaks and decreased number, and the outer membrane extrusion and invagination were apparent in some mitochondria (Figure 4).

**Discussion**

mtDNA mutations arise more frequently in somatic cells and may accumulate with age. The may also become fixed in the population as a low-frequency variant and can predispose carriers to late-onset diseases, such as dilated cardiomyopathy. The reactive oxygen species–induced DNA adducts may be the initial mechanism of those mutations. There are >100 types of oxidative DNA lesions, of which 8-oxodG is one of the most abundant. 8-oxodG is considered to be a premutagenic lesion, because it can mispair with residue A during DNA replication, causing a G-to-T transversion. These mutations may be repaired by DNA repair pathways, such as the BER pathway, initiated by mtUDG or oxoguanine DNA glycosylase in the mitochondria. The mtDNA repair systems have only recently been identified but have not been evaluated in the development of heart failure.

We found in the present study that the DNA repair activity was significantly reduced in the cardiac mitochondria of mice with dilated cardiomyopathy secondary to TNF-α overexpression. This alteration in DNA repair was associated with increased Fpg-sensitive sites in cardiac mtDNA. Furthermore, the mtDNA changes were associated with dysfunction and structural damage of the mitochondria as demonstrated by decreased COX activity and mitochondrial ultrastructural changes. The proposition that mitochondrial dysfunction results from mtDNA damage is supported by the finding that only COX activity was affected but SDH activity was not in TNF1.6 mouse heart. The 3 mtDNA encoded proteins (COI, COII, COIII) in the COX are the actual catalytic subunits that carry out the electron transport function.

Several clinically heterogeneous varieties of neuromuscular and nonneuromuscular disorders are associated with COX subunit mutations and COX deficiency (Online Mendelian Inheritance in Man, OMIM). Johns Hopkins University, Baltimore, Md. MIM Number 220110: 7/10/2000. http://www.ncbi.nlm.nih.gov/omim/). Reduced mtDNA-encoded COI and COII was observed in patients with histochemically defined COX deficiency in skeletal muscle, suggesting the presence of mtDNA defects.

The mitochondrial ultrastructural changes in the present study are consistent with previous reports that assessed specimens obtained from patients with dilated cardiomyopathy. In addition, significant reduction in mitochondrial matrix density, which may be a result of chronic release or reduced expression of mitochondrial content, such as cytochrome c, was similar to that seen in human dilated cardiomyopathy. TNF-α has been shown previously to induce the opening of mitochondrial permeability transition pores and the release of cytochrome c into the cytosol as a trigger of apoptosis. Although cardiomyocyte apoptosis was rarely seen in TNF1.6 mouse because of the upregulation of antiapoptotic and other protective pathways, the opening of mitochondrial permeability transition pores would contribute to the structural damage and dysfunction of mitochondria.

Although significant changes in mitochondrial structure and function were observed in the TNF1.6 mouse heart, the ATP content did not change, which may be explained by the adaptive changes in the energy-generation mechanism. When the reduced oxidative ability caused by mtDNA damage could not meet the cellular requirement for energy, upregulation of glycolysis may occur, as demonstrated previously in aged rats. Indeed, there is a significant shift from oxidative phosphorylation to glycolysis in the TNF1.6 mouse heart, as demonstrated by significantly increased LDH and ALT activities and lactate/pyruvate ratio (Table). The increased lactate and lactate/pyruvate ratio are also indicators of mitochondrial dysfunction.

In summary, these results demonstrated that mtDNA mutations and abnormalities in mitochondrial structure and function during the development of TNF-α–induced heart failure may be explained by reduced mtDNA repair activity. Furthermore, these results suggest that the pathophysiological effects of TNF-α on the heart may be mediated, at least in part, through alterations of the mitochondrial structure and function.

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**References**


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