Mitochondrial dysfunction in myocardium obtained from clinically normal dogs, clinically normal anesthetized dogs, and dogs with dilated cardiomyopathy

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Objective—To compare mitochondrial complex I and complex IV activity in myocardial mitochondria of clinically normal dogs, clinically normal dogs exposed to inhalation anesthesia, and dogs affected with dilated cardiomyopathy.

Sample—Myocardial samples obtained from 21 euthanized dogs (6 clinically normal [control] dogs, 5 clinically normal dogs subjected to inhalation anesthesia with isoflurane prior to euthanasia, 5 dogs with juvenile-onset dilated cardiomyopathy, and 5 dogs with adult-onset dilated cardiomyopathy).

Procedures—Activity of mitochondrial complex I and complex IV was assayed spectrophotometrically in isolated mitochondria from left ventricular tissue obtained from the 4 groups of dogs.

Results—Activity of complex I and complex IV was significantly decreased in anesthetized dogs, compared with activities in the control dogs and dogs with juvenile-onset or adult-onset dilated cardiomyopathy.

Conclusions and Clinical Relevance—Inhalation anesthesia disrupted the electron transport chain in the dogs, which potentially led to an outburst of reactive oxygen species that caused mitochondrial dysfunction. Inhalation anesthesia depressed mitochondrial function in dogs, similar to results reported in other species. This effect is important to consider when anesthetizing animals with myocardial disease and suggested that antioxidant treatments may be beneficial in some animals. Additionally, this effect should be considered when designing studies in which mitochondrial enzyme activity will be measured. Additional studies that include a larger number of animals are warranted. (Am J Vet Res 2012;73:1759–1764)

Mitochondria generate most of the cellular energy, primarily through oxidative phosphorylation. Within the mitochondrial inner membrane, electrons pass through a series of carrier molecules known as the ETC.1-3 The ETC together with H+ transporting ATP synthase constitutes the process of oxidative phosphorylation.1,1 The ETC consists of NADH-ubiquinone oxidoreductase (complex I), succinate-ubiquinone oxidoreductase (complex II), ubiquinone-cytochrome c reductase (complex III), and CcO (complex IV).3 Electrons derived from metabolism enter the ETC via NADH + H+ or succinate. Complex I oxidizes NADH + H+, and the electrons are conveyed to ubiquinone. Complex II oxidizes succinate and ubiquinone; the electrons transfer to complex III, which passes the electrons to cytochrome c. From there, they are shifted to complex IV and finally to atomic oxygen to ultimately produce H2O. The energy generated is used to pump protons from inside the mitochondrial matrix into the intermembrane space, thus creating an electrochemical gradient, which is used as a source of potential energy for ATP synthase to synthesize ATP for the cell.2
Mitochondrial diseases have been described in the human literature, with most causing dysfunction of the multiple organs that rely particularly heavily on mitochondrial energy production. Therefore, cardiomyopathy secondary to mitochondrial dysfunction may be a heart-specific condition or associated with other abnormalities (neurologic or skeletal muscle). As humans age, they accumulate mitochondrial mutations in postmitotic tissues, which hampers the energy capacity of those tissues. Eventually, the capacity of those tissues decreases below the minimum mitochondrial energy requirement, which results in organ failure and disease. If a person starts with a high mitochondrial energy capacity, then these thresholds are not attained until old age and the process is called aging. If a person inherits low energy capacity as a result of a mitochondrial mutation, then that person would cross the energetic threshold at a comparatively younger age and the process is termed mitochondrial disease. Thus, mitochondrial mutations that are present at birth often lead to cardiac abnormalities in young people, but cumulative mitochondrial damage with age may predispose to acquired disease in high-energy tissues such as the heart. These mitochondrial deletions result in impaired respiratory chain function, which leads to formation of oxygen-free radicals and oxidative damage.

The process of mitochondrial injury is accelerated in humans with ischemic heart disease, and many of the abnormally regulated genes on microarray data sets for heart failure are involved with mitochondrial dysfunction and oxidative phosphorylation. Moreover, mitochondrial-derived ROS are upregulated in the myocardium of human patients with DCM. In a study that included a large cohort of human patients with DCM, >22% had mitochondrial mutations; this suggests that in a subset of human patients with idiopathic DCM, defects in mitochondrial DNA contribute to development of the disease. However, independent of its origin, mitochondrial dysfunction may contribute to the worsening of heart failure. In such situations, an ETC dysfunction could be a consequence of oxidative damage and in turn could promote additional oxidative damage. In a study that involved the use of explanted human hearts (ischemic and nonischemic), investigators detected reduced activity of complexes III and IV in both groups of heart disease, which suggests that these deficiencies may contribute to the development of heart failure irrespective of the apparent cause. Moreover, the reduced activity was correlated with the ejection fraction in those patients in which the ejection fraction data were available. Thus, mitochondrial abnormalities appear to play an important role in heart disease in humans.

Mitochondrial mutations and dysfunction have also been recognized as causes of cardiac pathological changes in mice with several types of experimentally induced cardiomyopathy. In contrast, pathological changes were reduced by increases in antioxidant defenses. Although little is known about mitochondrial diseases in dogs, major alterations in the expression of cardiac mitochondrial protein concentrations related to oxidative phosphorylation dysfunction were more prevalent in left ventricular myocardial samples from dogs with naturally and experimentally induced DCM than in clinically normal control dogs. These data suggest that mitochondrial abnormalities play a role in heart disease of dogs, similar to the role in heart disease of humans.

Dilated cardiomyopathy is a primary myocardial disease characterized by left ventricular or biventricular dilation, impaired myocardial contractility, and progressive systolic dysfunction, and it is the second most common form of acquired heart disease in dogs. The result of this condition is typically congestive heart failure. Cardiac arrhythmias also frequently develop in this population of dogs, and the disease generally carries a poor long-term prognosis. Dilated cardiomyopathy develops most frequently as an acquired disease in adult dogs; however, an inherited, juvenile-onset, rapidly progressive form of DCM has been described in Portuguese Water Dogs. The pathophysiological aspects of JDCM have been evaluated by our laboratory group. In that study, we identified reductions in complex I and complex IV activity in affected dogs that had been subjected to functional cardiac evaluations while anesthetized with isoflurane. The alteration initially was thought to represent a molecular defect in these dogs. However, a similar enzyme pattern was recognized in myocardial samples from clinically normal control dogs anesthetized immediately prior to euthanasia. Therefore, we hypothesized that this alteration in mitochondrial complex I and complex IV activity in these dogs was secondary to exposure to the inhalation anesthetic (isoflurane), rather than to DCM.

Inhalation anesthetic agents depress mitochondrial function in several species. Depression of myocardial function is the most important cardiac effect of the commonly used inhalation anesthetics (eg, isoflurane and sevoflurane), and studies of cardiac mitochondria exposed to inhalation anesthetics have suggested that complex I of the respiratory chain is a common site of anesthetic action. Specifically, investigators in a study detected a dose-dependent selective inhibition of complex I (NADH:ubiquinone oxireductase) in guinea pig ventricular myocytes after anesthetic exposure. Other studies with isolated mitochondria have revealed that anesthesia induced by the administration of halothane inhibits the ETC at complex I, and an increase in NADH fluorescence caused by halothane (which is consistent with inhibition of complex I) has also been observed after exposure to isoflurane.

However, on the basis of studies in the human literature, it would not be unexpected for mitochondrial enzyme activity to be affected by myocardial disease such as DCM, and such enzyme activity could directly and negatively impact the course of the disease. The objective of the study reported here was to compare mitochondrial complex I and complex IV activity in left ventricular myocardium samples obtained from clinically normal dogs that had been exposed to an inhalation anesthetic (isoflurane), clinically normal dogs that had not been exposed to the inhalation anesthetic, and 2 groups of dogs affected with DCM (one group consisting of dogs with adult-onset DCM and the other group consisting of young Portuguese Water Dogs with JDCM). Our hypothesis was that cardiac samples from
dogs affected with dilated cardiomyopathy, whether adult onset or juvenile, or from dogs exposed to isoflurane would have reduced complex I and IV activity.

**Materials and Methods**

**Sample**—Myocardial samples were obtained from 21 euthanized dogs. Client-owned dogs were euthanized because of severe cardiac disease (ie, DCM) or extracardiac disease, whereas research dogs were clinically normal dogs euthanized after anesthesia as part of that research protocol. All owners consented to inclusion of their dogs in the study, and all animal care procedures conformed to guidelines established by the University of Pennsylvania Institutional Animal Care and Use Committee.

The dogs comprised 4 groups. One group consisted of 6 clinically normal (control) dogs (mean age, 5.5 years). Dogs in the control group were free of heart disease as determined on the basis of results of echocardiography or postmortem examination. Two groups consisted of dogs with DCM; there were 5 dogs with adult-onset DCM (mean age, 6 years) and 5 Portuguese Water Dogs with JDCM (mean age, 3 months). The fourth group consisted of 5 clinically normal dogs that had been anesthetized prior to euthanasia (mean age, 6 years). All dogs in the anesthetized group had been anesthetized with isoflurane for at least 120 minutes prior to euthanasia. All dogs were euthanized with an overdose of pentobarbital sodium administered IV.

**Isolation of mitochondria**—Myocardial tissue samples (approx 1 to 3 g of tissue/sample) were collected from the left ventricular free wall of each dog immediately after dogs were euthanized. Samples were stored at –80°C until analysis. Mitochondria were prepared in accordace with a differential centrifugation procedure described elsewhere. Briefly, 1 g of tissue was washed with PBS solution and homogenized in 10 volumes of sucrose-mannitol buffer (70 mM sucrose, 210 mM mannitol, 2 mM EDTA, and 2 mM HEPES [pH 7.4]) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride and each of the following at 1 µM: leupeptin, pepstatin, aprotinin, chymostatin, and antipain). The homogenate was centrifuged twice at 600 g and antipain). The homogenate was centrifuged twice for 10 minutes at 4°C, and the supernatant then was centrifuged at 10,000 × g for 20 minutes at 4°C to obtain the mitochondrial pellet. The postmitochondrial supernatant was centrifuged at 100,000 × g for 1 hour at 4°C to obtain the cytosolic and microsomal fractions. The pellet (microsomal fraction) was suspended in 50 mM Tris-HCl (pH 7.4) containing 50 mM KCl, 10% glycerol, 0.1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride and then was stored at –80°C. The mitochondrial pellet was washed twice and suspended in the sucrose-mannitol buffer, and mitochondria were passed through 0.8 M sucrose by centrifugation at 14,000 × g for 20 minutes. The resultant pellet was washed twice with sucrose-mannitol buffer, resuspended in that same buffer, and then used for further analysis.

**Assay of complex I activity**—The activity of complex I (NADH:ubiquinone oxidoreductase) was assayed as described elsewhere. Briefly, complex I activity (NADH:ubiquinone oxidoreductase) was measured by incubating 10 µg of freeze-thawed mitochondrial extract from the canine myocardial samples in 1 mL of assay medium (25 mM potassium phosphate, 5 mM MgCl2, 2 mM NaCN, 2.5 µg of bovine serum albumin/mL, 15 mM NADH, 63 µM ubiquinone, and 2 µg of antimycin A/mL [pH 7.4]) and measuring the decrease in absorbance at 340 nm attributable to NADH oxidation (slope 1). Rotenone-sensitive complex I activity was measured at 340 nm after the addition of 40 µM of rotenone/mL (slope 2). The resulting slope was determined by slope 1 minus slope 2 and was used to calculate the activity of NADH oxidized via the following equation: (resulting slope × 1,000)/(6.2 × micrograms of mitochondrial protein). The experiment was performed in triplicate for all samples. The resulting activities were used to calculate a mean final activity value.

**Assay of complex IV activity**—The activity of complex IV (ie, CcO) was assayed spectrophotometrically via a modified method described elsewhere. The rate of oxidation of CcO was measured as the decrease in absorbance at 550 nm for 1 minute. Assays were performed in 1 mL of reaction volume containing 950 µL of PBS solution, 10% lauryl maltoside, and 10 µg of mitochondrial protein. The reaction was initiated by the addition of 50 µL of reduced CcO. Complex IV activity was determined by the slope for results obtained at 550 nm. The slope was used to calculate the activity of CcO oxidized via the following equation: (slope × 1,000)/(21.1 × micrograms of mitochondrial protein). The experiment was performed in triplicate for all samples. The activities were used to calculate a mean final activity value.

**Statistical analysis**—Statistical differences were determined via unpaired 2-tailed Student t tests. Results for the canine myocardial mitochondrial samples were reported as mean ± SD. Data for complex IV activity of 2 anesthetized dogs were removed before statistical analysis because the results were widely disparate from those of other dogs in that group. Values of P < 0.05 were considered significant.

**Results**

Mean ± SD complex I activity was decreased in the DCM groups, compared with the value for the control group. However, the values did not differ significantly between the control (1.87 ± 0.19 µmol of NADH oxidized/min/mg of protein) and the adult-onset DCM (1.44 ± 0.34 µmol of NADH oxidized/min/mg of protein [P = 0.12]) or JDCM (1.56 ± 0.41 µmol of NADH oxidized/min/mg of protein [P = 0.18]) groups. Mean ± SD complex I activity in the anesthetized group (1.06 ± 0.16 µmol of NADH oxidized/min/mg of protein) was significantly (P = 0.015) decreased, compared with the mean value for the control group (Figure 1).

For the CcO activity, there was no significant difference in mean ± SD activity between the control group (1.24 ± 0.02 µmol of CcO oxidized/min/mg of protein) and the adult-onset (1.32 ± 0.02 µmol of CcO oxidized/min/mg of protein [P = 0.34]) or JDCM (1.45 ± 0.01 µmol of CcO oxidized/min/mg of protein [P = 0.18]) group (Figure 2). However, the mean ± SD CcO
Deficits in mitochondrial function are often associated with cardiomyopathies in humans, and it has been reported that mitochondrial protein expression of complexes I to V are altered in naturally occurring and induced forms of DCM in dogs. However, to our knowledge, the study reported here is the first in which the function of specific respiratory chain enzymes in canine myocardial samples from clinically normal dogs or dogs with naturally occurring disease has been evaluated. Analysis revealed reduced activity of mitochondrial complexes I and IV in clinically normal dogs with no evidence of cardiac disease that had been anesthetized with isoflurane for at least 120 minutes. However, although both groups of DCM dogs had reduced activity of complex I, compared with results for the control dogs, the values did not differ significantly from those for the control dogs. It is possible that including more dogs in the present study would have enabled us to detect significant differences. In addition, it is important to remember that aging plays a crucial role in causing mitochondrial dysfunction. Therefore, a specific control group matched on the basis of age with the JDCM group would have been optimal and should be considered for future studies. From a broader perspective, results for the present study align with those of another study in which mitochondrial enzyme activity was reduced in mice with experimentally induced DCM.

Several IV administered anesthetic drugs can act as ROS scavengers; however, inhalation anesthetics have been found to generate ROS in the heart (probably because of their inhibitory effects on the cardiac mitochondrial). Alteration of protein structure or lipid peroxidation attributable to the effects of free radicals can lead to altered function and enhanced susceptibility to proteolysis. In fact, progressive congestive heart failure has been linked to chronic myocardial exposure to ROS. Deleterious effects of free radicals have been known for decades. In addition, free radicals are known to act as second messengers in a cell-signaling pathway that includes control of apoptotic pathways and triggering of cell proliferation and hypertrophy. Moreover, brief exposure (approx 15 minutes) of the heart to an inhalation anesthetic can induce a state of protection against the effects of ischemia-reperfusion injury in animals. This fact, coupled with other findings, suggests that partial blockage of the ETC may be a common method by which some drugs, including isoflurane, induce pharmacological preconditioning in the heart of mammals.

Analysis of results of the present study suggested that exposure to inhalation anesthetics disrupts the ETC at complex I, which potentially leads to an outburst of ROS. Complex I is the first stage of electron donation for NADH and therefore a potential point of electron escape from the ETC, which can lead to the formation of free radicals. Additionally, in accordance with results of the present study, CcO (which is the terminal oxidase of the ETC) may also be disrupted. In a healthy individual subjected to anesthesia, this alteration in mitochondrial function may be tolerated and even protective of future ischemic insult. However,
with cumulative injury (as in an individual with DCM), the suppression of function may be substantial. Moreover, these findings suggest that antioxidant treatments may be beneficial in dogs with DCM. In mice engineered to overexpress catalase targeted to the mitochondria, age-related cardiac pathological changes were reduced and life spans (median and maximum) were increased, which suggested treatment via gene delivery is also possible.15

It is unclear whether ROS exposure led to the inhibition of myocardial mitochondrial complex I activity in the isoflurane-exposed dogs of the present study. Isoflurane can directly inhibit cardiac mitochondrial complex I activity.24 However, although brief anesthetic preconditioning with isoflurane appears to be a cardio-protective procedure, longer exposure to isoflurane, as for the dogs of the present study, appears to significantly inhibit complex I and IV activity and may negatively impact myocardial function. There are 2 major direct negative inotropic actions of inhalation anesthetics, neither of which is associated with impaired oxidative metabolism.31 The negative inotropic action of halothane and isoflurane has been associated with reduced availability of intracellular calcium ions and decreased responsiveness of the cardiac contractile apparatus to calcium ions, rather than to inhibitory mitochondrial effects.27 Therefore, there are at least 2 mechanisms by which inhalation anesthetics could decrease cardiac reserve: partial inhibition of the respiratory chain and a direct negative inotropic effect.25 Although these effects are probably not critical for most patients subjected to anesthesia, the effect may be more important in patients with pathological conditions.24 This may be of particular concern in dogs with DCM, given the dysfunction in mitochondrial complex I that exists (as determined on the basis of results of the present study). It is likely that a study with more dogs would be useful to better assess alterations in mitochondrial enzyme activity in these populations. For a power of 0.8, α of 0.05, and the means and SDs of the control and DCM groups (data for complex IV activity) determined in the study reported here, 16 dogs/group would be needed to detect differences. As previously mentioned, a control group that is matched on the basis of age with the JDCM group would be optimal. Additionally, although our analysis indicated that canine mitochondrial transcription and therefore protein concentrations are affected by inhalation anesthesia and DCM, additional studies should be performed to evaluate mitochondrial gene expression. However, despite these limitations, it is clear that activity of mitochondrial complexes I and IV in samples obtained from the left ventricle of dogs was significantly reduced following exposure to isoflurane. This novel finding from the present study must be considered when designing experiments that involve myocardial tissues obtained after animals have been anesthetized, particularly if mitochondrial function is to be assessed. This novel concept of alteration of mitochondrial function in DCM promises to be an area for future research and could help in generating mitochondrial ROS scavengers as a therapeutic tool for dogs and humans.

The activity of complexes I and IV were significantly decreased in the anesthetized group, compared with values for the control and DCM groups. These results suggest that anesthesia with an inhalation anesthetic disrupts the ETC in dogs, which can potentially lead to an increase in ROS. Larger scale studies are warranted to evaluate these findings.

References


