Biochemical evaluation of mitochondrial respiratory chain enzymes in canine skeletal muscle

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Objective—To perform respiratory chain enzymatic activity assays on canine skeletal muscle biopsy specimens and establish reference range values of skeletal muscle enzyme activities for dogs.

Sample Population—Biopsy specimens from the vastus lateralis muscle were obtained from 24 dogs (8 sexually intact males and 14 sexually intact females) ranging from 15 months to 6 years of age.

Procedure—Mean values of citrate synthase, cytochrome-*c* oxidase, succinate dehydrogenase, succinate dehydrogenase-cytochrome-*c* reductase, nicotinamide adenine dinucleotide (NADH) dehydrogenase, and NADH dehydrogenase-cytochrome-*c* reductase activities were established by use of 6 standard spectrophotometric assays for respiratory chain enzyme analysis.

Results—Compared with published data for skeletal muscle enzyme activities in humans, skeletal muscle enzyme activities in dogs were 2- to 4-fold higher. Additionally, citrate synthase activity, a marker for mitochondrial volume, was positively correlated with age in dogs, suggesting that mitochondrial volume increases with age, although no apparent change in respiratory chain enzymatic activity with an increase in age was found.

Conclusions and Clinical Relevance—Reference range values for skeletal muscle enzyme activities of dogs are needed to accurately interpret results of respiratory chain enzymatic activity assays. During investigation of metabolic myopathies, if skeletal muscle biopsy specimens are evaluated for respiratory chain enzyme kinetics, they should be performed and evaluated in concert with skeletal muscle biopsy specimens from clinically normal animals of the same species. (*Am J Vet Res* 2004;65:480–484)

Over the past 15 years, there have been numerous mitochondrial encephalomyopathies identified in human medicine that are directly linked to mutations of **mitochondrial DNA (mtDNA)**. Mitochondrial DNA contains the genetic code for 13 subunits of the enzyme complexes I to V in the respiratory chain. Of the 5 major complexes in the respiratory chain, only complex II (succinate dehydrogenase) does not contain subunits that are formed by mtDNA transcription and translation. In the approximately 16,000-bp circular mtDNA molecule, there are also 22 transfer RNAs that are directly involved in the production of the 13 subunits of the respiratory chain.[1,2] In the event of a mutation in mtDNA, it is likely that there will be loss of complex I (**nicotinamide adenine dinucleotide [NADH]** dehydrogenase), complex III (cytochrome-*c* reductase), complex IV (cytochrome-*c* oxidase), and complex V (ATP synthase) activity depending on whether the mutation is substantial enough to alter subunit function or the transfer RNA reading frame.[1-6] Unfortunately, investigation into genetic mechanisms and clinical implications of such genetic mutations cannot be performed on an experimental basis because the technologic advances for producing transgenic mtDNA knock-out rodents has yet to be developed.

In searching for animal models of mtDNA encephalomyopathies, it has been suggested that companion animals, in particular dogs, may be useful as a result of their high activity level, their often extensive pedigree information, and the ability to detect exercise intolerance as a myopathic sign.[7] The diagnosis of mitochondrial disease, whether mtDNA derived or nuclear DNA derived, can be difficult; thus, many diagnostic tests are routinely performed to evaluate patients. Abnormalities in serum concentrations of metabolic parameters such as lactate, pyruvate, and organic acids can often be a rewarding first step in identifying metabolic compromise.[3-5] Histologic examination of muscle biopsy specimens to confirm ragged-red muscle fiber formation and biochemical staining for cytochrome-*c* oxidase and succinate dehydrogenase are often of diagnostic value in mitochondrial disorders in humans, but can be difficult in dogs as a result of a larger mitochondrial volume. Considering the ease of obtaining muscle biopsy specimens, biochemical analysis for respiratory chain enzymes I to IV has been used as a primary screen for patients with suspect mitochondrial encephalomyopathies to help clarify the deficits in the respiratory chain activity and rule in or out suspect enzymes.[3-5]

Of the suspected reports[7-9,a,b] of mitochondrial myopathy in dogs, only 1 dog[8] had confirmation of true mitochondrial disease through the use of electron microscopy and the measurement of biochemical respiratory chain enzyme deficits or western blot analysis of respiratory chain enzyme subunits. The confirmed report of mitochondrial myopathy in that dog revealed that the disease was autosomal recessive in nature.[8] In many instances, such as that of the X-linked myopathy in Irish Terriers, mitochondrial proliferation is a nonspecific secondary change, making the testing of respi-

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ratory chain enzymatic activity critical to the eventual diagnosis of the myopathic condition.[10] Unfortunately, the clinical manifestations of such diseases probably go undiagnosed as a result of a lack of awareness in the veterinary medical community and a lack of institutions that have the facilities necessary to diagnose such diseases. Thus, when such data are required for dogs, laboratories for human testing are used. Unfortunately, the ability to interpret information gained from assays for human testing is confounded by the lack of control values for dogs. The objective of the study reported here was to perform respiratory chain enzymatic activity assays on muscle biopsy specimens from dogs within a limited age group to establish reference range values for dogs.

Materials and Methods

Skeletal muscle biopsy specimens—Fresh canine skeletal muscle was obtained from mature Beagles (15 dogs) and mixed-breed dogs (9) immediately after euthanasia that was induced by IV administration of sodium pentobarbital. Dogs varied from 15 months to 6 years old. The dogs used in this study were part of another terminal study, and the methods implored for tissue collection had no effect on the analyses performed. Approximately 1 g of muscle was taken from the mid region of the contractile portion of the vastus lateralis muscle. The muscle specimens were immediately snap frozen in liquid nitrogen and stored at –80°C until further analysis.

Homogenates—Homogenates were prepared from 50 mg of muscle tissue in 500 µL of homogenizing medium by use of a tissue homogenizer[c] at 4°C. Homogenizing medium consisted of 150mM sucrose, 2mM EDTA, and 100mM Tris-HCl (pH, 7.45). The homogenate was then centrifuged at 1,450 $\times$ *g* at 4°C for 20 minutes, and the supernatant was collected for analysis. Before respiratory chain enzyme analysis was performed, each homogenate was assessed for protein content by use of the Bradford technique.[11] During experimentation, each homogenate was run in triplicate for each enzymatic analysis. The mean value from 3 readings for each dog was taken as the true value for each of the enzymatic analyses; the repeated measurements allowed for an intra-assay coefficient of variation to be determined for each of the assays. Enzymatic activity determinations by use of all 6 assays were performed on the same muscle homogenate on the same day.

Respiratory chain analysis—Spectrophotometric analysis of respiratory chain enzyme activity was assessed by use of previously published techniques from the Columbia College of Physicians and Surgeons Neuromuscular Disease Laboratory[5] with a spectrophotometer.[d] Cytochrome-*c* oxidase (complex IV) activity was measured by use of methods of Rieske.[12] Succinate dehydrogenase (complex II) activity was measured by use of the method of King.[13] Nicotinamide adenine dinucleotide dehydrogenase (complex I) and NADH dehydrogenase-cytochrome-*c* reductase (complex I-III) activities were measured by use of the methods of Hatefi and Rieske.[14,15] Succinate dehydrogenase-cytochrome-*c* reductase (complex II-III) activity was measured by use of the methods of Tisdale.[16] Citrate synthase activity was measured by use of methods adapted from Srere.[17] These techniques have been implemented as a rapid enzymatic activity screening procedure to determine reference range values of respiratory chain enzyme activities for humans.[5] To date, no available spectrophotometric assay exists to assess the activity of ATP synthase (complex V).

Statistical analysis—Extrapolation of values from humans for mitochondrial respiratory chain enzymatic activities was done by use of previously published data.[5] Values for humans were compared with results for dogs (obtained by identical methods) by use of a 2-tailed *t* test. Alpha was set at a level of 0.05. A value of $P < 0.05$ was considered significant. All enzyme activities were further analyzed by use of correlation coefficients to assess whether age was correlated with enzyme activity.

Results

Canine skeletal muscle respiratory chain activity—Activity for each of the enzymes tested was reported as micromole of substrate oxidation per gram of tissue (**Table 1**), as well as micromole of activity per minute per milligram of protein. The former measurement allowed comparison of values for dogs to those published for humans, whereas the later measurement

Table 1—Mean (± SD) amount of substrate oxidized per gram of tissue versus per milligram of protein in homogenates of vastus lateralis muscle biopsy specimens from 24 dogs

Homogenates (n = 24)	Oxidized substrates					
	Cit synth	COX	SDH	SCCR	NCCR	NADH dehydrogenase
Tissue (µM/g)	32.8 ± 10.7	7.69 ± 1.78	2.08 ± 0.49	1.31 ± 0.46	5.07 ± 1.61	75.3 ± 22.9
Protein (µM/mg)	559.9 ± 217.3	33.31 ± 9.49	8.71 ± 2.25	4.42 ± 1.76	4.39 ± 1.72	1308.6 ± 492.0

Cit synth = Citrate synthase. COX = cytochrome-*c* oxidase (complex IV). SDH = Succinate dehydrogenase (complex II). SCCR = Succinate dehydrogenase-cytochrome-*c* reductase (complex II-III). NCCR = Nicotinamide adenine dinucleotide dehydrogenase-cytochrome-*c* reductase (complex I-III). NADH dehydrogenase = Nicotinamide adenine dinucleotide dehydrogenase (complex I).

Table 2—Interassay coefficients of variation and correlation coefficients of age versus enzymatic assays results for homogenates of vastus lateralis muscle biopsy specimens from 24 dogs

Variables	Oxidized substrates					
	Cit synth	COX	SDH	SCCR	NCCR	NADH dehydrogenase
Interassay coefficient of variation (%)	4.9	10.1	6.2	6.6	5.6	5.1
Correlation coefficient (age vs assay results)	0.67	0.20	0.15	0.10	0.28	0.09

See Table 1 for key.

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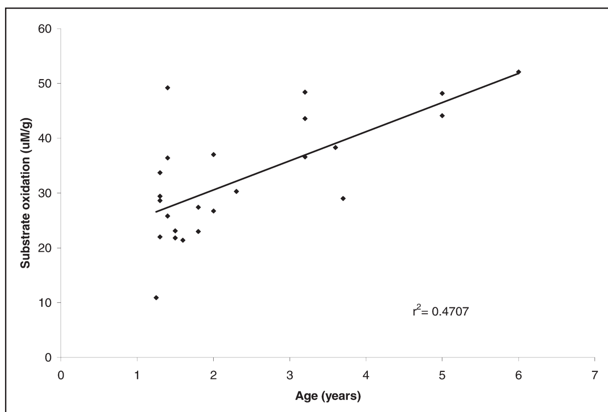

Figure 1—Linear regression results comparing age versus citrate synthase activity for homogenates of vastus lateralis muscle biopsy specimens from 24 dogs. A significant ($P < 0.001$; $r = 0.69$) correlation was found between the age of dogs and citrate synthase activity.

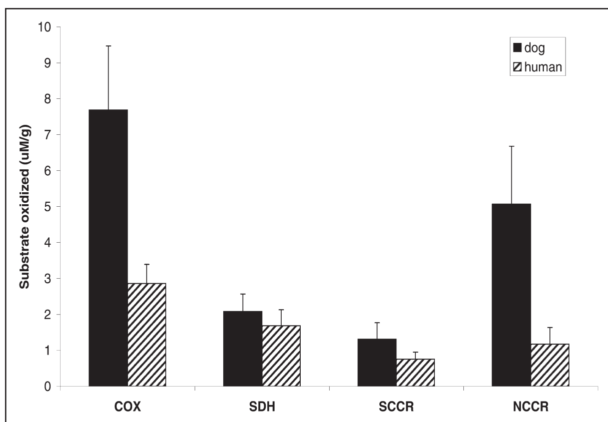

Figure 2—Comparison of mean (± SD) activity (µM of substrate oxidized/g of skeletal muscle) of respiratory chain enzyme complexes in muscle biopsy specimens from clinically normal dogs and humans.[5] For all enzymes, significant ($P < 0.01$) differences between values for dogs and humans were found. COX = Cytochrome-*c* oxidase. SDH = Succinate dehydrogenase. SCCR = Succinate dehydrogenase-cytochrome-*c* reductase. NCCR = Nicotinamide adenine dinucleotide dehydrogenase-cytochrome-*c* reductase.

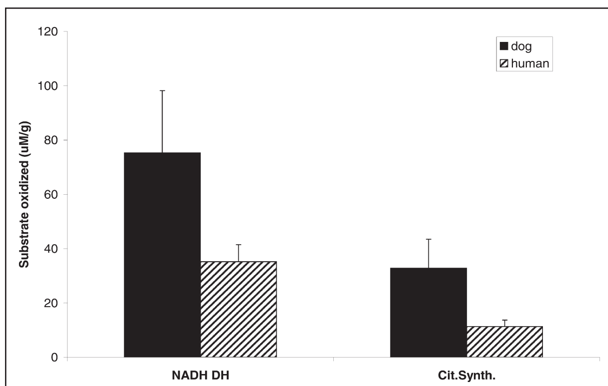

Figure 3—Comparison of mean (± SD) activity (µM of substrate oxidized/g of skeletal muscle) of citrate synthase and nicotinamide adenine dinucleotide dehydrogenase complexes in muscle biopsy specimens from clinically normal dogs and humans.[5] For all enzymes, significant ($P < 0.01$) differences between values for dogs and humans were found. Cit synth = Citrate synthase. NADH DH = Nicotinamide adenine dinucleotide dehydrogenase.

enabled us to quantify the enzymatic activity as a fraction of the protein concentration in the homogenate.

Interassay coefficients of variation were determined for each of the assays, and correlation coefficients were generated to determine whether age was correlated to any of the enzymatic analysis results (**Table 2**). A significant correlation ($P < 0.001$) was found between the age of dogs and citrate synthase activity ($r = 0.67$), suggesting that as dogs got older, the citrate synthase activity increased (**Fig 1**).

Comparison to skeletal muscle respiratory chain activity in humans—Comparison of values obtained from canine skeletal muscle to values for humans by DiMauro et al[4] revealed dramatic differences between the mean respiratory chain enzymatic activity of dogs, compared with that of humans (**Fig 2 and 3**). A significant ($P < 0.001$) 2- to 4-fold increase was found in activities of all enzymes of canine skeletal muscle, compared with enzyme activities of human skeletal muscle.

Discussion

Skeletal muscle biopsy should be the first procedure performed for histologic and biochemical analysis if a patient is believed to have a myopathy of metabolic origins. Often, lactate and pyruvate values may be helpful in diagnosis of a metabolic myopathy, but muscle biochemistry values will be the first clue as to the origins of the metabolic disorder whether it be a glycolytic, Krebs cycle, or respiratory chain abnormality. Once a pathway has been implicated, the focus can be on a further genetic diagnosis of the disease. Considering that the entire mitochondrial genome for dogs has been published through genetic sequencing of mitochondrial genes extracted from mtDNA, a definitive diagnosis can be made.[18]

It has been recognized for years that canine skeletal muscle mitochondrial mass is nearly 3 times greater than that of humans, resulting in higher oxygen consumption values and increased mitochondrial mass as seen through electron microscopy.[19-21] Data from our study suggest that the canine skeletal muscle respiratory chain enzyme (complexes I to IV) activities are between 2- to 4-fold higher than in humans. It has been routine practice by many veterinary clinicians to collect skeletal muscle specimens from patients and send these specimens to laboratories used for human testing to evaluate enzyme activities. Our data suggest that as a result of the difference in mitochondrial volume and respiratory chain activity, interpretation of results on the basis of control data for humans may not be ideal for evaluating enzyme activity in canine skeletal muscle.

When evaluating skeletal muscle homogenates in our laboratory, we simultaneously measured protein concentrations in an effort to assess enzymatic activity in relation to total protein in the homogenate; this was done to reduce the error in calculation resulting from excess fascia and adipose tissue. It was expected that standardizing our measurements on the basis of protein concentrations would decrease the SD value across dogs when evaluating each enzyme, thus producing more consistent measurements among dogs, compared

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with assessment based on micromoles of substrate oxidized per gram of tissue. Our results (Table 1 and 2) indicated that little to no difference exists in SD values between the 2 methods of assessing enzyme activity. The large SD values found in both the human data as well as our data may be related to variable percentages of type I versus type II muscle fibers collected in each biopsy specimen and the activity level of the patient being evaluated. In our study, we evaluated the vastus lateralis muscle, which contains about 54% type I and 46% type II muscle fibers; depending on the ratio of fiber type in the biopsy specimen evaluated, there may be slight changes in enzymatic activity.[22]

Enzymatic activity in skeletal muscle homogenates will decrease dramatically if frozen and thawed before analysis. Considering this problem with freezing and thawing of specimens and inherent variability in muscle biopsy specimens as a result of muscle fiber type variation among biopsy specimens, the interassay coefficient of variation cannot be accurately determined for these assays. Variation in mitochondrial activity among biopsy specimens of dogs, much like that in biopsy specimens of humans, is likely the result of age-related changes, differing activity levels of the patient, and genetic influences. With age, there will be increased numbers of mtDNA mutations that may result in decreased respiratory chain activity in tissues, which has been confirmed in multiple species including dogs.[23,24] In our evaluation, we chose skeletally mature dogs between 15 months and 6 years of age, well before geriatric changes begin to negatively influence respiratory chain activity. By determining correlation coefficients between age and the various mitochondrial enzymatic activities, we ensured that no interaction existed between values obtained in the assays that could be attributed to geriatric changes ($r < 0.30$), but interestingly a modest correlation of increased citrate synthase activity was found with increases in age. Citrate synthase is a marker of mitochondrial volume in skeletal muscle and not a direct reflection of the respiratory chain enzyme kinetics. Considering these findings in this population of dogs, we speculate that mitochondrial volume increases with age with no concurrent change in respiratory chain dynamics, therefore providing the possibility that cellular compensation may be taking place to maintain normal oxidative function by increasing mitochondrial volume.

There are multiple protocols for the assessment of respiratory chain enzyme activity in various organs.[5,25-27] Biochemical evaluation on frozen specimens may not measure maximal activity of the respiratory complexes, particularly NADH dehydrogenase and cytochrome oxidase, and evidence supports that fresh specimens may provide more accurate results.[28,29] In our study, chosen methods were used because of their widespread use in a major medical facility, their ease of preparation, the small amount of tissue needed, the ease of tissue collection for analysis, and the ability to perform many tests if necessary. Often in metabolic myopathies, the biochemical deficiency leaves no question as to the origins of the myopathic compromise because little to no enzymatic activity is remaining, thereby giving conclusive evidence that a deficiency exists in a specific respiratory enzyme.

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[c]Tissue homogenizer, Brinkman Inc, Westbury, NY.
[d]DU-640 spectrophotometer, Beckman Inc, Fullerton, Calif.

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