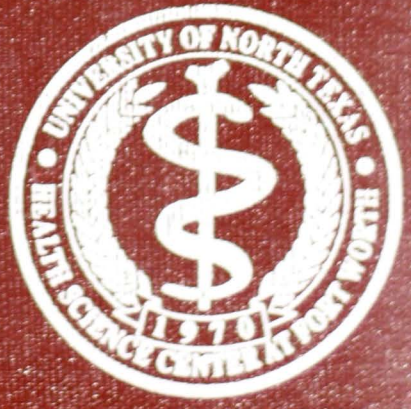
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Aerobic exercise training evokes adaptations in the myocardial contractile machinery that enhance cardiac functional capabilities, and the myocardium's capacity to consume energy. Despite considerable investigative effort, the effects of exercise training on myocardial intermediary metabolism, the source of energy for cardiac function, have not been defined. The investigations described herein were undertaken to delineate the effects of aerobic exercise training on key rate-controlling enzymes of myocardial intermediary metabolism and energy transport, and to characterize the effects of acute exercise on cardiac messenger RNA transcripts encoding metabolic enzymes. To address these questions, dogs were conditioned by a 9 wk treadmill running program or cage rested for 4 wk. Exercise conditioning was documented by a significant decrease in heart rate at rest and during submaximal exercise. A panel of glycolytic and oxidative enzymes was measured in myocardial extracts. It was demonstrated that aerobic exercise training of dogs selectively increased capacities of key rate-controlling enzymes of each of the major pathways of intermediary metabolism in ventricular myocardium. In addition, it appeared that the training-evoked increases in enzyme activities were due to increased enzyme contents, not to changes in substrate affinity.

The same training program was implemented to investigate the effects of aerobic exercise training on the myocardium's energy shuttling system. Total creatine kinase (CK) activity and content of the CK_{MB} isoenzyme were measured in canine myocardial extracts. It was demonstrated that aerobic exercise training increased total myocardial CK activity and CK_{MB} content, although the CK_{MB} isoenzyme remained a minor component of the myocardial CK system.

A third investigation was conducted to examine the effects of aerobic exercise on the abundance of messenger RNA (mRNA) encoding key enzymes involved in myocardial energy production and transport. Left ventricular myocardium was sampled 30 min after an exercise bout, and messenger RNA transcripts were analyzed by reverse transcriptase polymerase chain reaction. Exercise increased the myocardial abundance of mRNA transcripts encoding glyceraldehyde 3-phosphate dehydrogenase, citrate synthase, and the CK-M subunit. These mRNA enhancements could be responsible, at least in part, for the exercise-evoked adaptations in myocardial metabolic enzymes demonstrated in the first two investigations.

EXERCISE-EVOKED METABOLIC ADAPTATIONS

IN CANINE MYOCARDIUM

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EXERCISE-EVOKED METABOLIC ADAPTATIONS IN CANINE MYOCARDIUM

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

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By

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Original Articles

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Abstracts

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LIST OF ABBREVIATIONS

αKGDH	alpha-ketogluterate dehydrogenase
β-ox	beta oxidation pathway
CS	citrate synthase
cDNA	complimentary DNA
Cr	creatine
CK	creatine kinase
CK-B	creatine kinase brain subunit
CK_{BB}	creatine kinase isoenzyme containing two brain subunits
CK-M	creatine kinase muscle subunit
CK_{MM}	creatine kinase isoenzyme containing two muscle subunits
CK_{mito}	creatine kinase mitochondrial isoenzyme
sCK-mito	creatine kinase: sarcomeric mitochondrial subunit
ETC	electron transport chain
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
G-6-P	glucose 6-phosphate
HADH	3-hydroxyacyl CoA-dehydrogenase
HK	hexokinase
K_m	Michaelis constant
LDH	lactate dehydrogenase

LIST OF ABBREVIATIONS, continued

MDH	malate dehydrogenase
mRNA	messenger RNA
PCr	phosphocreatine
PFK	phosphofructokinase
PK	pyruvate kinase
RT-PCR	reverse transcriptase polymerase chain reaction
SR	sarcoplasmic reticulum
TBS	tris-buffered saline
TTBS	tween-20 tris-buffered saline
V_{max}	maximal reaction rate

CHAPTER I

INTRODUCTION

The investigations described in this dissertation were performed to address the paucity of current information in the literature regarding myocardial metabolic adaptations evoked by aerobic exercise training in large mammals. Most previous investigations of these metabolic adaptations have been conducted in small mammals, primarily rats. In these species, changes in the activities of intermediary metabolic enzymes due to exercise training have proven to be inconsistent. Two possible reasons for these equivocal findings are i) the intrinsically high resting heart rates of small mammals dictate that the myocardial metabolic rate also be high, even at rest, thus limiting exercise-evoked myocardial metabolic adaptations in these animals, and ii) the effects of endurance training on myocardial enzyme activities varies with the training paradigm.

The objective of this dissertation is to delineate potential biochemical mechanisms that could contribute to the cardioprotective adaptations elicited by aerobic exercise training in a large animal model. I examined the effects of aerobic exercise training on myocardial energy metabolism in dogs. By analyzing a panel of several different glycolytic and oxidative metabolic enzymes, I have been able to characterize the

myocardial metabolic adaptations evoked by an intensive training regimen in canine myocardium.

REVIEW OF RELATED LITERATURE

Cardioprotective Effects of Exercise Training

Nations have passed away and left no traces. History gives us the naked cause of it. One single reason in all causes – they fell because their people were not fit.
Rudyard Kipling

Although incidence of coronary heart disease and stroke have progressively declined since the late 1960's, these cardiovascular diseases still remain the major causes of death, disability, and health care expenditures in the United States (20). In the U.S. alone, more than 860,000 deaths were attributed to heart disease and stroke in 1992 (53), and high blood pressure, one of the major risk factors for cardiovascular disease, is estimated to affect about 50 million Americans (53). The prevalence of these diseases, not only in the U.S., but in the global community, has spurred extensive research aimed at delineating measures which serve to protect cardiovascular function and improve quality of life and longevity. The 1996 Surgeon General's Report on physical activity and health (53) reviewed several studies suggesting that regular physical activity and higher levels of cardiorespiratory fitness decrease overall mortality rates from cardiovascular disease. It is also worth noting that morbidity and mortality due to coronary artery disease was decreased (35) in individuals who were physically active but nevertheless developed coronary artery disease. Thus, aerobic exercise training exerts

salutary effects which serve to lessen the incidence and severity of cardiovascular disease.

Exercise-evoked adaptations protect the myocardium against a variety of pathophysiological stresses. It is well recognized that carefully applied training regimens can be effective in ameliorating the detrimental effects of cardiovascular disease, advanced age, and diabetes. A comprehensive review of this literature is beyond the scope of this dissertation. Instead, selected issues and experimental findings related to the cardioprotective effects of exercise training will be addressed in detail.

Myocardial ischemia occurs when coronary blood flow is reduced to the extent that the myocardium's oxygen and fuel supplies are insufficient to meet the energy demands of the tissue. Cardiac contractile function declines rapidly after ischemic onset, and severe and/or prolonged ischemia kills cardiomyocytes resulting in an infarct. However, if coronary flow is restored before irreversible cell damage occurs, the ischemic myocardium can be salvaged (26). Many clinical scenarios of coronary occlusion and reperfusion, such as thrombolytic therapy, coronary artery bypass surgery, and angioplasty, are associated with protracted systolic and diastolic contractile abnormalities following revascularization despite survival of the temporarily ischemic tissue (14, 26). One of salient characteristics of the trained myocardium is that it is more resistant to the deleterious effects of acute ischemic episodes and reperfusion injury (4, 9, 10, 25, 30, 37, 45, 48). Exercise training has also proven effective in ameliorating the

diastolic dysfunction associated with hypertension (5, 12, 13, 41-44). Although the underlying mechanisms responsible for the cardioprotective effects of exercise training are not well understood, it is evident that a program of regular physical exercise could protect the myocardium from the harmful effects of ischemia that occur in numerous clinical settings.

Adaptations to Aerobic Exercise Training

The human body is designed to respond to any physical task through a series of integrated responses that involve most, if not all, of its physiological systems. During dynamic exercise, the metabolic demand of the skeletal muscle greatly increases to generate sufficient energy to perform work. The increased metabolic demand is met by an increase in blood flow to the exercising muscles which augments oxygen delivery and removal of metabolic waste products. In order to adequately increase blood flow to the exercising muscle, cardiac output must be increased in proportion to exercise intensity. Repeated bouts of exercise evoke adaptive responses that increase the body's capacity for sustained physical activity. These adaptations are dependent upon several factors including initial fitness level, the mode, intensity, duration, and frequency of the exercise bouts, and the length of the training regimen. Thus, it is evident that repetitive bouts of dynamic exercise evoke adaptive changes in the body, which allow it to efficiently respond to increased levels of work.

The trained phenotype is characterized by several cardiovascular adaptations which result in a general improvement in the ability to perform physical activity. Systemically, training evokes an increase in the maximal oxygen consumption rate (VO_2max), bradycardia at rest and during low intensity exercise, decreased arterial blood pressure at rest and during submaximal exercise, and an increase in plasma volume. The increase in VO_2max results from increased maximal stroke volume and maximal arteriovenous O_2 content difference, i.e. O_2 extraction. The resting and submaximal exercise bradycardia is believed to be caused by decreased sympathetic and increased parasympathetic drive (3, 46), and possibly intrinsic adaptations of the heart which remain to be characterized. The mechanism(s) responsible for the training-induced reduction in arterial blood pressure at rest and during submaximal exercise are not well understood. However, peripheral vascular modifications in response to exercise training may be responsible for the reductions in arterial blood pressure demonstrated in trained individuals (18). Terjung *et al.* (51) observed that exercise training increased the number of capillaries in trained skeletal muscle, and this increased vascularity would tend to decrease total peripheral resistance. Convertino *et al.* (17) proposed that the increase in plasma volume is achieved by two mechanisms: 1) plasma renin and vasopressin concentrations are elevated during exercise facilitating the retention of sodium and water, and 2) chronically increased levels of total plasma protein content (mainly albumin) in response to exercise training serves to retain water in the vascular compartment.

Aerobic exercise training also evokes several adaptations in the heart which serve to augment the myocardium's ability to generate force and pump blood. A hallmark characteristic of the trained heart is increased stroke volume at all levels of submaximal work. Several factors contribute to this adaptation. First, cardiac dimensions are expanded. End-diastolic volumes at rest and during exercise are greater in trained subjects attributable, in part, to increased diastolic filling time secondary to the training-induced bradycardia. Also, the absolute mass of the heart is increased by synthesis of myofilaments and other proteins in the cardiomyocytes (7, 8, 11, 16, 32). Other factors contributing to the increase in stroke volume include increased preload, due in part to the increase in plasma volume, and a decrease in afterload resulting from decreased systemic vascular resistance. These myocardial adaptations to exercise training culminate in an increased mechanical efficiency of the heart at all submaximal workloads. Also, considerable evidence in the literature suggests that the coronary vasculature adapts to exercise training in order to preserve adequate regional coronary blood flow during periods of increased cardiac work (28, 29). Thus, the heart is capable of adapting by a combination of several mechanisms to meet the increased demands imposed on it by chronic exercise.

Skeletal muscle also adapts to endurance exercise training in several ways which are briefly summarized here. For a comprehensive review of this topic, the reader is referred to the work of Abernathy *et al.* (1). Training increases the cross sectional area of slow-twitch fibers and the density of capillaries in the trained skeletal muscle (51). As

noted above, increased capillarization serves to increase the capacity for blood flow to the exercising muscle and contributes to the reduction in systemic vascular resistance observed in the trained state. Significant metabolic adaptations are also observed in trained skeletal muscle. These adaptations are exemplified by increased size and number of mitochondria, increased activity of oxidative enzymes, increased capability to mobilize and oxidize fat, and increased glycogen deposition. As a consequence of these metabolic adaptations, the capacity for aerobic metabolism is increased.

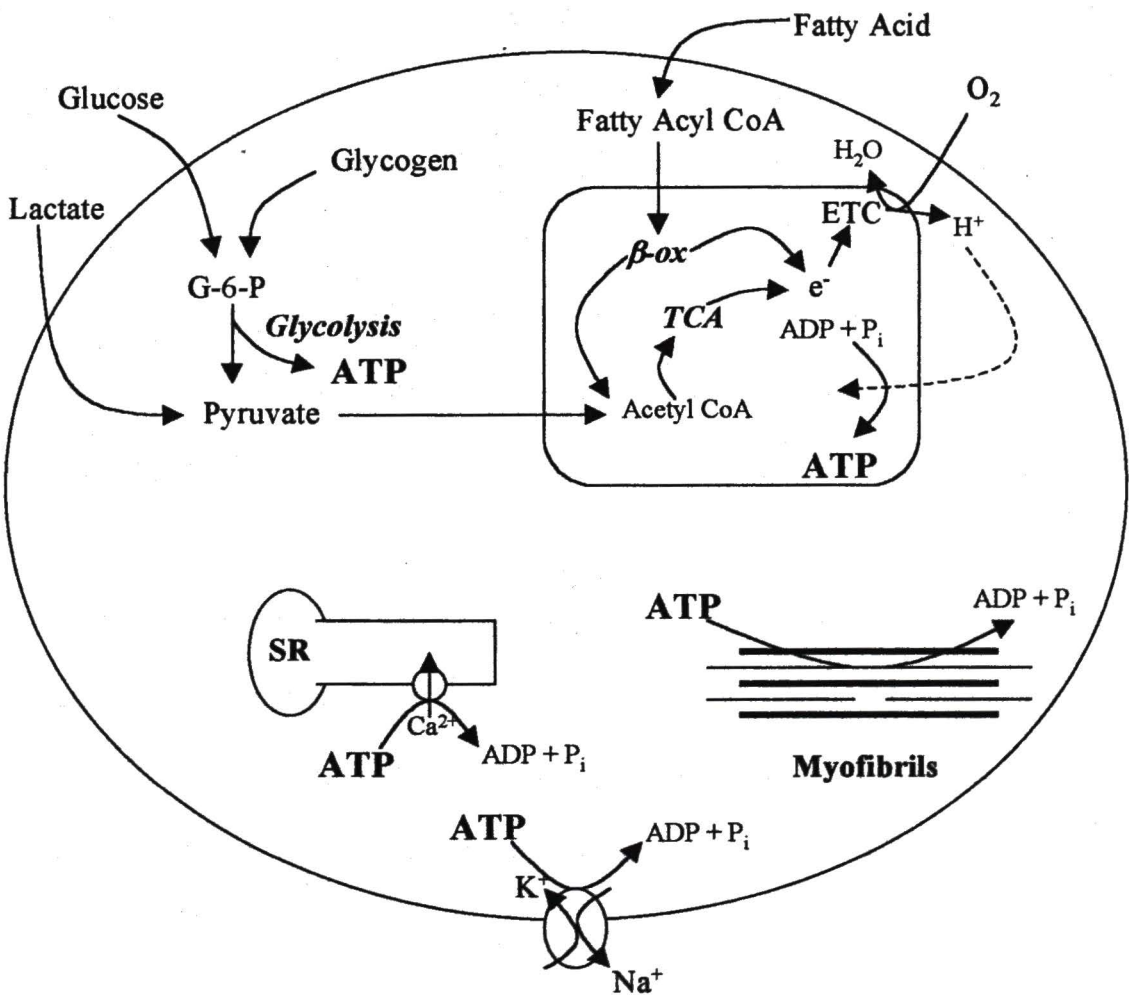

Figure 1. *Pathways of energy production and utilization in cardiomyocytes.* Abbreviations: G-6-P; glucose-6-phosphate, β-ox; β-oxidation pathway, TCA; tricarboxylic acid cycle, ETC; electron transport chain, SR; sarcoplasmic reticulum.

Effects of Aerobic Exercise Training on Myocardial Energy Metabolism

Myocardial contractile performance results from the coordinated activities of several energy consuming processes. Actin-myosin crossbridge cycling and sarcolemmal and sarcoplasmic reticular ion transport, the major processes involved in myocardial contractile activity, require chemical energy liberated from the hydrolysis of ATP (Figure 1.). The myocardium has limited energy reserves, and is heavily dependent upon a continuous supply of ATP generated by oxidative metabolism of exogenous fuels. These fuels, primarily glucose, fatty acids, and (in exercise) lactate, are extracted from the coronary circulation and oxidized to generate reducing equivalents for oxidative phosphorylation of ATP. Unlike skeletal muscle, the heart cannot function under an oxygen debt; anaerobic glycolysis does not provide enough energy to sustain cardiac function and the heart is continuously beating and unable to suspend its activity to replete energy reserves. Therefore, it is imperative that the heart maintain tight coupling between energy supply and demand.

Myocardial energy consumption increases concomitantly with increases in workload. Coupling between myocardial energy supply and demand is well maintained unless cardiac workload is increased sufficiently to exceed the myocardium's energy producing capabilities. When this occurs, cellular energy reserves fall, and myocardial

contractile function is impaired. Dynamic exercise increases demand for delivery of oxygen and fuels to exercising skeletal muscle which is met by marked increases in the myocardium's mechanical and metabolic activity. It is well accepted that the myocardium adapts to repeated bouts of exercise by synthesizing myofilaments and other proteins (7, 8, 11, 16, 31, 32). Although these adaptations increase the myocardium's capacity to function at high workloads, they also increase energy demand. If this increase in the myocardium's capacity to consume energy is not met by an increase in its ability to produce energy, myocardial energy reserves could be compromised at high workloads, and cardiac function must decline to restore energy supply:demand balance. At present, a clear unequivocal consensus regarding the effects of chronic exercise on myocardial energy metabolism has not been reached. In order to ascertain the cardiovascular adjustments to exercise training, it is critical that we understand the alterations in cardiac enzymology and energetics which are elicited by aerobic exercise training.

A variety of animal models using varying intensities, durations, and modalities of exercise training have been utilized to examine the effects of exercise training on myocardial energy metabolism. Therefore, it is not surprising that consensus conclusions regarding the training-induced adaptations of these cellular systems are often difficult, if not impossible to draw. With these limitations in mind, the following section summarizes information in the current literature with reference to myocardial metabolic adaptations evoked by exercise training.

In general, exercise training appears to affect myocardial energy metabolism more subtly than skeletal muscle metabolism. However, it should be mentioned that most previous investigations of cardiac metabolic adaptations to exercise training have used rats as the animal model, and in this species the myocardium might not adapt metabolically to training. The equivocal response of rat myocardium to training may be explained in part by the intrinsically high resting heart rate of the rat (~300 beats/min) which requires robust oxidative metabolism to match energy supply with demand, even at rest. Therefore, the pathways of oxidative metabolism are already well adapted in rat myocardium to support the high energy demands imposed by daily bouts of dynamic exercise.

Exercise-evoked adaptations of myocardial glycolytic and TCA cycle enzymes have proven inconsistent in training studies in rats. For instance, the activities of the glycolytic enzymes hexokinase (HK) (52), phosphofructokinase (PFK) (2, 24, 56), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (24, 30), and lactate dehydrogenase (LDH) (23, 24), were unaltered following exercise training. Although several reports concluded that chronic exercise does not affect the activities of glycolytic enzymes, it should be recognized that not all studies report negative findings. For example, training significantly increased the activities of pyruvate kinase (PK) (24, 52, 56), HK (22), LDH (21, 37, 56), GAPDH (30), and PFK (37) in rat myocardium. These contradictory findings exemplify the discrepancies between different investigations even when the same animal model is utilized. With regard to the activities of TCA cycle

enzymes, citrate synthase (CS) (2, 23, 24, 36, 37) and malate dehydrogenase (MDH) (36) activities were unaltered by training.

The inconsistencies in myocardial metabolic adaptations to chronic exercise are further demonstrated by comparing results of different training paradigms. In the swim-trained rat myocardium, the activities of PFK (2, 24, 56), GAPDH (24), LDH (23, 24), CS (36), and MDH (36) were unaltered. However, in some studies swim-training did evoke significant increases in the activities of PK (24, 56) and LDH (21, 37, 56). The same discrepancies are also observed when comparing training studies that utilized treadmill running. Endurance treadmill training increased the activities of PK (52), PFK (2, 37), and LDH (37), but had no effect on HK (52), CS (2, 37), and GAPDH (30) in rat myocardium. In contrast, sprint/interval-training increased rat myocardial GAPDH activity (30), but did not alter rat myocardial PFK or CS (2). These studies demonstrate that even when the same animal model is utilized, the effects of endurance training on myocardial enzyme activities vary depending on the training modality and even the intensity of the exercise regimen.

Surprisingly few studies have examined the cardiac metabolic adaptations to exercise training in larger mammals. Laughlin and coworkers (27) investigated the effects of treadmill training on cardiac enzyme activities in miniature pigs. These workers reported no changes in the activities of phosphofructokinase, citrate synthase, or 3-hydroxyacyl-CoA dehydrogenase in myocardium of trained pigs when compared to

sedentary controls. They concluded that aerobic exercise training does not impose sufficient stress on the heart of a large mammal to induce changes in the pathways involved in intermediary metabolism. Sordahl *et al.* (47) examined the effects of exercise training on mitochondrial function in dog hearts. These investigators found no training effect on energy producing capacity of mitochondria from ventricular myocardium. They concluded that exercise training does not alter the energy-producing capabilities of the mitochondria in canine myocardium. However, these workers did not report measurements of intermediary metabolic enzymes. This is important because adaptive increases in intermediary metabolic enzymes would have the effect of generating more reducing equivalents, thereby increasing the energy-producing capability of the mitochondria without obligatorily altering the capacity of the mitochondrial respiratory chain.

The creatine kinase (CK) energy shuttle (Figure 2) links myocardial energy production and utilization. This mechanism channels high-energy phosphate bonds, generated by oxidative phosphorylation in the mitochondria, to sites of energy utilization at contracting myofibrils and sarcolemmal and sarcoplamic reticular ion pump ATPases. Creatine kinase isoenzymes are strategically located in the mitochondrial intermembrane space (34) and in close association with the myofibrillar and membrane ATPases (6, 39, 54). Mitochondrial creatine kinase (CK_{mito}), located on the outer surface of the inner mitochondrial membrane, is spatially associated with the adenine nucleotide translocase (40) and transfers the terminal phosphate from mitochondrial ATP to creatine forming

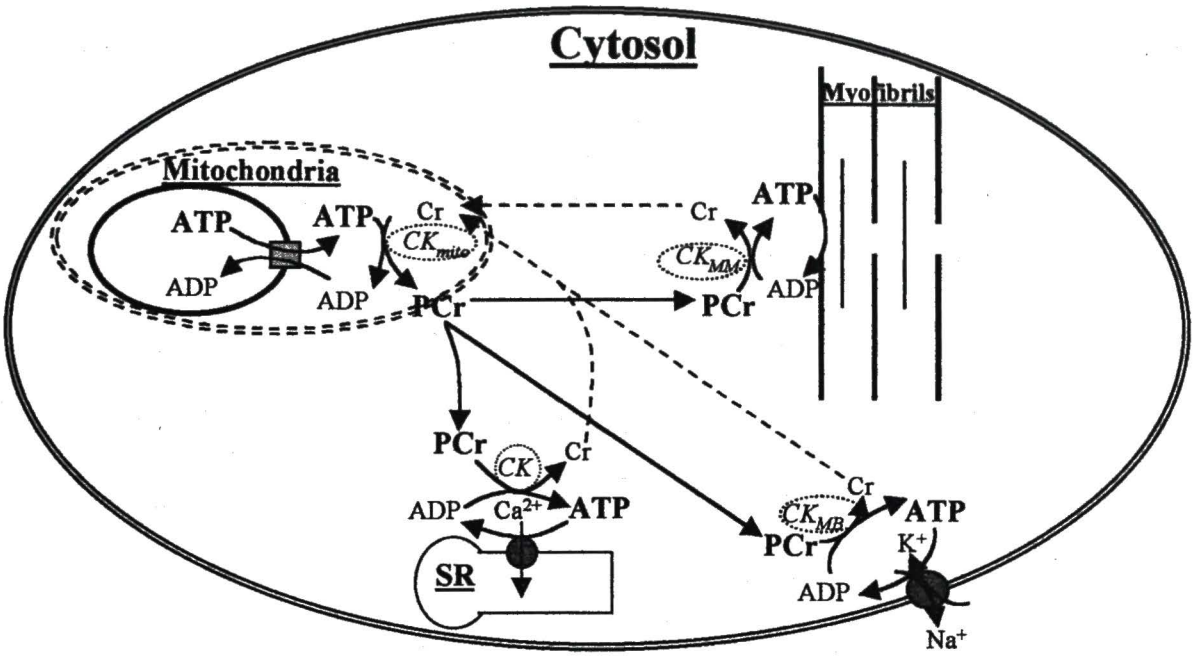

Figure 2. *Creatine kinase energy shuttle.* Abbreviations: Cr, creatine, PCr, phosphocreatine, CK_{MM}, CK_{MB}, CK_{mito}; creatine kinase isoenzymes, SR; sarcoplasmic reticulum. The shaded square box is the adenine nucleotide translocase, and the two shaded circles located in the sarcoplasmic reticular and sarcolemmal membranes are the Ca^{2+}-ATPase and the Na^+/K^+-ATPase, respectively.

creatine phosphate (PCr), which rapidly diffuses into the cytosol. ADP generated by this reaction reenters the mitochondria to provide substrate for oxidative phosphorylation. The cytosolic CK isoenzymes, CK_{MB} and CK_{MM}, transfer the high energy phosphate from PCr to ADP, generating ATP in close proximity to sites of energy consumption at the myofibrils and membrane pump ATPases.

The effects of exercise training on the myocardial CK system have been examined in several animal models. Rockstein and coworkers (38) demonstrated that swim training in rats attenuated the age-related decline observed in myocardial CK activity. Chesky *et al.* (15) also found that CK activity was increased in rats that were swim-trained for 3 months beginning at 6 months of age. In mice, wheel running increased myocardial CK activity only in young animals (50). Taken together, these three studies indicate that, at least in small mammals, myocardial CK activity is increased by exercise training only in young animals. The effect of exercise training on total myocardial CK activity has not been examined in large mammals. However, it has been demonstrated that endurance exercise training does not alter the myocardial CK isoenzyme profile in dogs (33).

From these data, it should be appreciated that the task of establishing a clear, incontrovertible consensus regarding the effects of exercise training on the pathways of intermediary metabolism in the myocardium is not an easy one. The considerable variability in training models and modalities used in these studies only serves to cloud the issue further. Therefore, caution is advised when comparing exercise-evoked myocardial enzyme adaptations between different species, training modalities, intensities, and durations.

SPECIFIC AIMS

The dog is a well accepted and widely used model of human cardiovascular physiology, and, conceivably, myocardial metabolic adaptations to training could provide valuable insights into human myocardial responses to exercise training. However, the myocardial metabolic adaptations to exercise training in this species have not been delineated. With this in mind, two primary objectives were developed for this dissertation: 1) to determine the effects of an intense exercise training regimen on the myocardium's capacity for oxidative energy production and intracellular energy transport; and 2) to define the effects of an acute bout of exercise on myocardial gene expression of selected enzymes. Specifically, I proposed that aerobic exercise training evokes adaptive increases in the myocardium's oxidative metabolic capacity by selectively increasing activities of specific rate-controlling enzymes of intermediary metabolism and energy transport. Further, I anticipated that increases in enzyme activities would reflect increased content of enzyme proteins. In addition, I postulated that training enhances the myocardial creatine kinase energy shuttle, thereby supporting more efficient coupling of energy supply and demand. Lastly, I hypothesized that exercise induced increases in myocardial enzyme protein content are mediated by increased mRNA abundance. In order to investigate these hypotheses, the following specific aims were addressed:

I. To test the hypothesis that aerobic exercise training increases the activities of key rate-controlling enzymes of the glycolytic, TCA cycle, and β-oxidation pathways in canine myocardium.

II. To test the hypothesis that the myocardium's creatine kinase energy shuttle is increased in response to exercise training as a result of an increase in total creatine kinase content including the normally scarce CM_{MB} isoenzyme.

III. To test the hypothesis that exercise induces increases in myocardial contents of glycolytic and oxidative enzymes, as well as creatine kinase, by increasing the abundance of mRNA encoding these enzymes.

EXPERIMENTAL DESIGN

Two different training protocols were employed to investigate specific aims I, II, and III. These experiments are discussed in detail in the following chapters; however, a brief description of the rationale and experimental design for each of the aims follows:

Exercise training enhancement of myocardial activities of glycolytic and oxidative enzymes. It is well accepted that the myocardium adapts to repeated bouts of exercise by synthesizing myofilaments and other proteins (7, 8, 11, 16, 31, 32). Although these adaptations increase the myocardium's ability to function at high workloads, they

also increase its energy demand. If this increase in the capacity to consume energy is not matched by an increase in the ability to produce energy, myocardial energy reserves and function could be compromised at high workloads. To examine the effects of exercise training on the myocardium's energy-generating systems, we studied dogs conditioned by a combination of high intensity sprint and endurance running. Dogs were conditioned by a 9 wk treadmill running program, and sedentary control dogs were cage-rested for 4 wk. The activities and Michaelis-Menten kinetics of several rate-controlling enzymes of intermediary metabolism were determined in myocardium of trained and sedentary control dogs. Myocardial enzyme protein contents were also examined by immunoblot analysis. I anticipated that aerobic exercise training would increase the myocardium's capacity to produce energy by increasing its content of enzyme protein.

Exercise training enhancement of the myocardial creatine kinase energy shuttle. The creatine kinase energy shuttle is active in tissues, e.g. striated muscle, with high and variable energy demand. In cardiac muscle, this mechanism efficiently channels energy generated by the mitochondria to energy consuming processes elsewhere in the cell. Because of the importance of the creatine kinase energy shuttle, changes in total CK activity and the distribution of CK isoenzymes could have important physiological implications. For example, an increase in the capacity of this energy shuttling mechanism could support more efficient energy transfer in the myocytes, thus sustaining cardiac function during metabolically stressful conditions such as coronary hypoperfusion and maximal exercise. The same exercise training regimen employed in

the first investigation was utilized in these experiments. Creatine kinase enzyme activity was measured in myocardium from exercise-trained and sedentary dogs. The creatine kinase isoenzyme, CK_{MB}, normally a very small fraction of total CK, was isolated by anion exchange chromatography and quantitated using agarose gel electrophoresis. I anticipated that aerobic exercise training would increase the capacity of the CK energy shuttle due, in part, to an increase in CK_{MB} activity.

Aerobic exercise training increases myocardial contents of intermediary metabolic enzymes by increasing the abundance of mRNA encoding these enzymes. Control of cardiac gene expression is a complex process, subject to regulation at several steps. In mammalian myocardium, one mechanism for increased protein synthesis is increased transcription of messenger RNA, which provides more templates for protein synthesis. Messenger RNA contents are likely to be transiently increased in response to an exercise bout, unlike protein products which accumulate progressively over several weeks. Accordingly, an abbreviated (10 day) training regimen was utilized to determine whether exercise increases the abundance of mRNA encoding the enzyme proteins that were increased by aerobic exercise training. Since it was anticipated that any increases in mRNA levels would be detectable in tissue samples obtained within a short time after completion of an exercise bout, ventricular myocardium was harvested at 30 min following exercise.

METHODS

Although the experimental design and methods for each investigation are described in the following chapters, it is appropriate at this point to discuss special considerations taken into account in choosing the techniques and methods of analyses utilized in our experiments. One of the hallmarks of the exercise-trained phenotype is a resting and submaximal exercise bradycardia. Accordingly, cardiovascular conditioning of the dogs was monitored by measuring heart rates at rest and during submaximal exercise. When resting and submaximal heart rates of the training dogs were significantly lower than the respective baseline values prior to the start of the training regimen, it was judged that the dogs had achieved a conditioned state. Independent confirmation of exercise training in these animals was obtained from measurements in *vastus lateralis* of citrate synthase activity, a biochemical marker of exercise conditioning in skeletal muscle (49, 55). Michaelis-Menten enzyme kinetics were analyzed by measuring enzymes activities over wide ranges of reaction substrate concentrations. The Michaelis constant (K_m) and maximal activity (V_{max}) for each enzyme were determined from relationships between the reciprocals of reaction rate (*i.e.* 1/[V]) and substrate concentration (*i.e.* 1/[S]) according to Lineweaver-Burk. Because of statistical concerns associated with the Lineweaver-Burk procedure (19), K_m and V_{max} were also determined from Hanes plots (*i.e.* V/[S] *vs.* [S]).

With regard to the immunoblot analyses, it was imperative that each lane of the SDS polyacrylamide gels be loaded with the same amount of total protein. Therefore,

left ventricular homogenates from four trained and four sedentary dogs were identically loaded on two gels and simultaneously electrophoresed. One gel was stained with Coomassie brilliant blue to ensure that protein loading was similar in all lanes; protein in the other gel was then transferred to nitrocellulose membrane for immunoblotting.

Several potential methodological concerns were recognized and obviated in order to conduct mRNA measurements in ventricular biopsies. First, it was realized that the dogs must be habituated to the environment in which the exercise bout was to take place to limit the amount of non-exercise stress on the animal. Habituation was achieved by running the dogs on the treadmill nine separate days prior to the day on which the tissue samples were collected. Also, we anticipated that mRNA would likely be transiently increased in response to an exercise bout. Therefore, tissue samples from each of the exercised dogs were collected within 30 min following the final exercise bout. Lastly, because mRNA is very susceptible to degradation by RNAses, all of the tissue samples were collected under sterile conditions to limit RNAse contamination.

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CHAPTER II

EXERCISE TRAINING ENHANCES GLYCOLYTIC AND OXIDATIVE ENZYMES IN CANINE VENTRICULAR MYOCARDIUM

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ABSTRACT

Aerobic exercise training evokes adaptations in the myocardial contractile machinery that enhance cardiac functional capacity; in comparison, the effects of training on the myocardium's energy generating pathways are less well characterized. This study tested the hypothesis that aerobic exercise training can increase the capacities of the major pathways of intermediary metabolism in canine myocardium. Mongrel dogs were conditioned by a 9 wk treadmill running program or cage rested for 4 wk. Exercise conditioning was evidenced by 26 and 22% decreases ($P < 0.05$) in respective heart rates at rest and during submaximal exercise and by a 40% increase ($P < 0.05$) in citrate synthase (CS) activity of the *vastus lateralis*. Glycolytic, TCA cycle, and β-oxidative enzymes were assayed in myocardial extracts at 37°C. Relative to sedentary controls, training increased glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity by 49% in left and 33% in right ventricle, and pyruvate kinase, CS, and 3-hydroxyacyl CoA dehydrogenase (HADH) activities by 74%, 91%, and 77%, respectively, in left ventricle ($P < 0.05$). Immunoblotting further confirmed that training increased left ventricular contents of CS and GAPDH.Other measured enzymes (hexokinase, phosphofructokinase, lactate dehydrogenase, α-ketoglutarate dehydrogenase, malate dehydrogenase) were not altered by training in either ventricle. Kinetic analyses revealed increased maximum rates but unaltered substrate affinities of GAPDH, CS and HADH following training. Thus, aerobic exercise training augments the intermediary metabolic capacity of canine myocardium by selectively increasing contents of regulatory enzymes of glycolysis and oxidative metabolism.

INDEX TERMS: glycolysis, β-oxidation, TCA cycle, Michaelis-Menten kinetics, immunoblotting

ABBREVIATIONS: CS: citrate synthase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; HK: hexokinase; HADH: 3-hydroxyacyl CoA-dehydrogenase; KGDH: α-ketoglutarate dehydrogenase; K_m: Michaelis constant; LDH: lactate dehydrogenase; MDH: malate dehydrogenase; PFK: phosphofructokinase; PK: pyruvate kinase; TBS: Tris-buffered saline; TTBS: Tween-20 Tris buffered saline; V_{max}: maximal reaction rate

ENZYMES: citrate synthase (4.1.3.7), glyceraldehyde 3-phosphate dehydrogenase (1.2.1.12), hexokinase (2.7.1.1), 3-hydroxyacyl CoA-dehydrogenase (1.1.1.35), α-ketoglutarate dehydrogenase (1.2.4.2), lactate dehydrogenase (1.1.1.27), malate dehydrogenase (1.1.1.37), phosphofructokinase (2.7.1.11), pyruvate kinase (2.7.1.40)

INTRODUCTION

During aerobic exercise, cardiac output increases to meet the metabolic demands of the exercising skeletal muscle, and myocardial energy demand increases concomitantly. In exercise conditioning programs, repeated bouts of aerobic exercise stimulate synthesis of new sarcomeres in the ventricular myocardium, which serves to further increase cardiac energy consumption (24, 25, 31). The working myocardium has limited energy reserves and is heavily dependent on oxidative metabolism of exogenous fuels to support its contractile activity. Myocardium cannot sustain an oxygen debt, so metabolic energy production in the working myocardium must increase with cardiac workload to maintain tight coupling between energy supply and demand during exercise. This increase in energy production is sustained by increased flux through pathways of intermediary metabolism. Therefore, during exercise training it could be expected that the myocardium's energy-generating metabolic capacity would increase to match the increased energy consuming capacity.

Despite considerable investigative effort, the effects of exercise training on myocardial intermediary metabolism have not been conclusively defined. Differences among animal species and training paradigms could influence metabolic adaptations evoked by training. In rats, for example, results of training studies of myocardial glycolytic and TCA cycle enzymes have been equivocal. Several studies have reported increases in the glycolytic enzymes pyruvate kinase (16, 37, 43), hexokinase (14), lactate

dehydrogenase (10, 29, 43), glyceraldehyde-3-phosphate dehydrogenase (20), and phosphofructokinase (29), but in other studies, hexokinase (37), phosphofructokinase ,(2, 16, 43), glyceraldehyde-3-phosphate dehydrogenase (16, 20), and lactate dehydrogenase (15, 16), were unchanged post-training. Fewer studies have investigated the response of the citric acid cycle enzymes to training; however, citrate synthase (2, 15, 16, 28, 29), and malate dehydrogenase (28) were unaltered in swimming (15, 16, 28) and running conditioned rats (2, 29).

Relative to rats, few studies have examined myocardial metabolic adaptations to aerobic exercise training in larger mammals. Laughlin *et al.* (19) demonstrated that the activities of phosphofructokinase, citrate synthase, and 3-hydroxyacyl CoA dehydrogenase in left ventricular myocardium were unaltered in running conditioned pigs. In dogs, although training did not augment myocardial calcium transport systems and respiratory chain components (32, 34), the responses of intermediary metabolic enzymes to aerobic training are unknown. Accordingly, this study was conducted to determine the effects of aerobic exercise training on rate-controlling enzymes of the glycolytic, TCA cycle, and β-oxidative pathways in canine myocardium. Enzyme activities and Michaelis-Menten kinetics were evaluated in myocardium and hindlimb skeletal muscle from running-conditioned and sedentary control dogs. Enzyme protein contents in myocardium of trained and sedentary dogs were further examined by immunoblotting. Selective increases in key rate-controlling enzymes of glycolysis and oxidative metabolism were demonstrated in myocardium of running conditioned dogs.

METHODS

Animals and training program. Animal experimentation was approved by the Animal Care and Use Committee of the University of North Texas Health Science Center and conformed to the *Guide for the Care and Use of Laboratory Animals* (NIH publication 85-23, revised 1996). Twelve healthy mongrel dogs were randomly assigned to the training group or sedentary control group. Each group consisted of 3 males and 3 females. All dogs were provided food and water *ad libitum*, and were housed in 4 x 8 ft. enclosures that permitted quiet ambulatory activity. Body mass (mean ± SE) of the exercising dogs was 23.7 ± 0.8 kg, and that of the sedentary dogs was 21.2 ± 1.4 kg (P = NS).

Sedentary dogs were cage-rested for 4 wk. Dogs in the training group were conditioned by a 9 wk treadmill running program described by Tipton *et al.* (35) as modified by Gwirtz (11). This program consisted of sprint or endurance running sessions on alternate days for 5 days per wk. The intensity and duration of the sessions were increased each week during the first 8 wk of the training program, with a total running time of 35 min per session in wk 1 and 75 min in wk 8. In wk 1, endurance bouts consisted of running at 4.8 kph on level grade for 5 min (warmup), at 9.7 kph on level grade for 10 min (speed), at 4.8 - 8.0 kph on 16% grade for 15 min (endurance), and at 4.8 kph on level grade for 5 min (warm-down). Sprint sessions consisted of the same warmup and speed runs, followed by ten 1 min sprints, in which the treadmill was

accelerated from 6.4 to 12.9 kph over 30 s, then decelerated to 6.4 kph over 30 s. The sprints were followed by 5 min endurance running at 4.8 - 8.0 kph on 16% grade, and 5 min warm-down. Endurance bouts in wk 8 consisted of 5 min warmup followed by 15 min running at 11.3 kph on 8% grade, 50 min running at 11.3 kph on 16% grade, and 5 min warm-down. Sprint bouts in wk 8 included 5 min warmup, 15 min running at 11.3 kph on 8% grade, 50 min of sprints at 9.7 – 19.3 kph on 4% grade, and 5 min warm-down.

The degree of cardiovascular conditioning effected by the training regimen was assessed by subjecting dogs to treadmill performance tests as described by Tipton *et al.* (35). These tests were conducted in the week preceding the training program and at weeks 4-9 of the program. Each test lasted 18 min and consisted of six 3 min running stages: stage 1: 4.8 kph on level grade; stage 2: 6.4 kph on level grade; stage 3: 6.4 kph on 4% grade; stage 4: 6.4 kph on 8% grade; stage 5: 6.4 kph on 12% grade; stage 6: 6.4 kph on 16% grade. Heart rate was monitored throughout the test by electrocardiography.

Sampling of myocardium and skeletal muscle. Tissue was harvested for biochemical analyses on the day following the final running session. Dogs were fasted overnight, then anesthetized with sodium pentobarbital (30 mg/kg *iv*), intubated, and ventilated with room air (0.2 liter $\cdot$ kg^{-1} $\cdot$ min^{-1}). The heart was exposed via a left thoracotomy in the fifth intercostal space and quickly excised. Transmural samples (3-4 g) of left and right ventricular myocardium were quickly freeze clamped with

Wollenberger tongs pre-cooled to constant temperature in liquid N_2. Samples (3-4 g) of *vastus lateralis* were collected through an incision in the left hindlimb and were also quickly freeze clamped. Frozen tissues were stored at -90°C prior to extractions.

Extraction and measurement of metabolic enzymes. Enzymes were extracted from frozen tissue essentially as described by Braasch *et al.* (5). Tissue was pulverized to a fine powder in a precooled mortar under liquid N_2, then homogenized in 6-8 vol ice-cold phosphate buffer (0.1 M KH_2PO_4, 10 mM reduced glutathione, 10 mM EDTA, pH 7.2) using a precooled, motor-driven teflon piston (700 rpm). The suspension was centrifuged at 100 000 · g_{max} for 20 min at 4°C in a Dupont Sorvall Ultra 80 preparative ultracentrifuge. The pellet was washed twice in 4 vol phosphate buffer and the three supernatant fractions were combined, divided into 1 ml aliquots, and stored at -90°C. Protein concentrations in the extracts were determined colorimetrically as described by Bradford *et al.* (6), and ranged from 1.93 - 3.69 mg · ml^{-1}.

Protein yield from the extraction procedure was determined. The amount of protein extracted from left ventricular myocardium (mg protein · g wet^{-1}; n=6) was 70±18 in sedentary and 62±16 in trained dogs (P = NS). Total myocardial protein was also determined by measuring the amount of protein remaining in the pellet after extraction and adding this amount to the extracted protein. Total protein content (mg protein · g wet^{-1}) was 115±12 and 114±13 in left ventricular myocardium of sedentary and trained dogs, respectively. Fractional protein extraction was 60.5±2.3% in sedentary and

54.4±0.9% in exercise trained left ventricle ($P < 0.05$). Because fractional protein extraction differed by only about 10% between the two groups, measured enzyme activities were not corrected for fractional extraction.

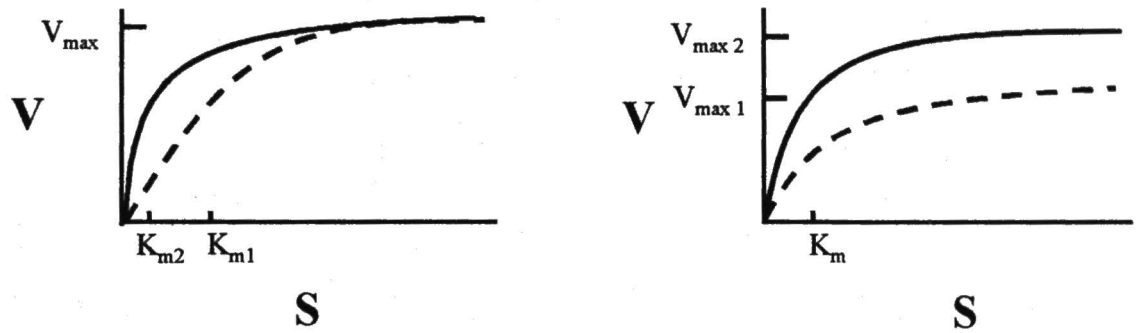
Activities of glycolytic (hexokinase, phosphofructokinase, glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase, lactate dehydrogenase), β-oxidative (3-hydroxyacyl CoA dehydrogenase), and TCA cycle (citrate synthase, α-ketoglutarate dehydrogenase, malate dehydrogenase) enzymes were assayed at 37°C in a Perkin-Elmer *uv*/vis spectrophotometer. 3-Hydroxyacyl CoA dehydrogenase activity was measured as described by Bass *et al.* (3); all other enzymes were assayed according to procedures described by Bergmeyer (4). Activities were determined from rates of formation or disappearance of NAD(P)H monitored at 337 nm wavelength ($\varepsilon = 6.24\ mM^{-1} \cdot cm^{-1}$). Enzyme activities were expressed as units (U) per mg protein, where one unit equals one μmol substrate converted to product per min.

Enzyme kinetics. Michaelis-Menten kinetics of lactate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), citrate synthase (CS), and 3-hydroxyacyl CoA dehydrogenase in left ventricular myocardial extracts were analyzed by measuring enzyme activities as concentrations of the respective reaction substrates were varied. The Michaelis constant (K_m) and maximum activity (V_{max}) for each enzyme were determined from relationships between the reciprocals of reaction rate (*i.e.*, 1/V) and substrate concentration (*i.e.*, 1/[S]) according to Lineweaver-Burk. K_m and V_{max} were

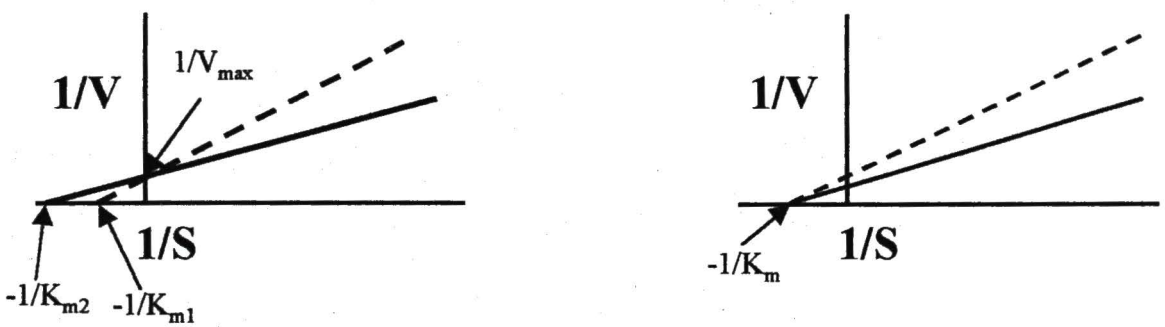
Increased Substrate Affinity

Increased Capacity

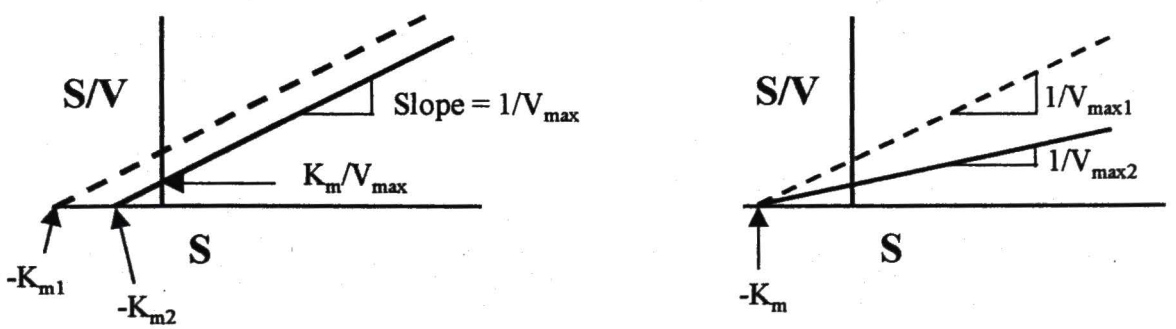
Substrate *vs.* Velocity Plot



Lineweaver-Burk Plot



Hanes Plot



Graphic analysis of Michaelis-Menten kinetics. Increases in enzyme activities could be due to increased substrate affinities or increased catalytic capacity. An increase in substrate affinity would result in a lower K_m with a similar V_{max}. Alternatively, an increased capacity would result in a higher V_{max} but unchanged K_m. To determine K_m and V_{max}, reaction rates (**V**) are measured over a wide range of substrate concentrations (**S**), and enzyme kinetics are analyzed by Lineweaver –Burk and Hanes plots as depicted in this figure.

also determined from Hanes plots (*i.e.*, V/[S] *vs.*[S]) to confirm results of the Lineweaver-Burk analyses. The figure presented above depicts the changes in these plots due to changes in substrate affinity at a constant enzyme content (left side of the figure: altered K_m; V_{max} unchanged) or to changes in enzyme content at constant substrate affinity (right side: altered V_{max}; K_m unchanged). In Lineweaver-Burk plots, the *y* intercept equals $1/V_{max}$ and the *x* intercept equals $-1/K_m$; in Hanes plots, these intercepts equal K_m/V_{max} and $-K_m$ respectively, and the slope equals $1/V_{max}$. The Lineweaver-Burk analysis of Michaelis-Menten kinetics can be criticised because the double reciprocal plot may actually conceal a poor fit between the data and a straight line. In Lineweaver-Burk plots, experimental error is grossly unsymmetrical among the points; those nearest the ordinate have small errors, while those furthest from the ordinate have much larger errors. Thus, the points that influence the slope of the line the most (those points furthest away from the ordinate) have the greatest uncertainty. In the Hanes plot uncertainty (i.e. experimental errors) is much more symmetrically distributed among the points than in the Lineweaver-Burk plot.

Immunoblot analyses. The contents of citrate synthase (CS) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein in left ventricular myocardium of sedentary and exercise trained dogs were determined by immunoblotting (36). Powdered left ventricular myocardium (*c.* 5 mg) was homogenized in 0.5 ml lysis buffer (18) for 1 min using a hand-held tissue homogenizer (Biospec Products, model 985-370). Homogenates from four trained and four sedentary dogs were identically loaded (7.5 μg

protein/lane) on two 10% SDS polyacrylamide gels (36) and simultaneously electrophoresed for 2 h at 100 V. One gel was stained with Coomassie brilliant blue to assess protein loading; protein in the other gel was electrophoretically transferred to nitrocellulose membrane (36). Protein transferral was confirmed by reversibly staining the membrane with Ponceau red S, which revealed distinct bands in a pattern identical to that of the Coomassie blue-stained gel. To verify that protein transferral to the membrane was complete, the source gel was also stained with Coomassie blue, and was found to be free of any discernable bands.

The nitrocellulose membrane was washed in H_2O to remove Ponceau red S, incubated for 3 hr in 5% nonfat dry milk in Tween-20 Tris buffered saline (TTBS) to block nonspecific immunoreactivity, and subsequently washed in TTBS to remove the milk. Next, the membrane was incubated with primary antibody to CS or GAPDH diluted 1: 1000 in TTBS and gently agitated for 2 h. Primary antibodies were rabbit anti-pig heart CS IgG (7) and mouse anti-rabbit brain GAPDH IgG (9). Blots exposed to primary antibody were then washed with Tris buffered saline (TBS; 20 mM Tris, 500 mM NaCl, pH 7.5) for 5 min and twice in TTBS for 10 min, and incubated with gentle agitation for 1 h in a solution containing the appropriate horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG for CS and goat anti-mouse IgG for GAPDH; Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted 1:2000 in TTBS. After incubation, the blot was washed as before with TBS and TTBS. Immune complexes were visualized by incubating blots in 20 ml peroxidase substrate (50 mM

phosphate, pH 5.7, 30% hydrogen peroxide, 8% NiCl, 0.5% diaminobenzidine) for 5 min. Bands corresponding to GAPDH and CS were identified by immunoreactivity and by comparison of electrophoretic mobilities with those of molecular weight standards run on the same gel. For densitometry, the blots were scanned and digitized by a UMAX MagicScan scanner interfaced with a MacIntosh computer. Densities were quantified using the Image program, version 1.4, developed at the National Institutes of Health.

Statistical analyses. Heart rate data from the treadmill performance test were analyzed by One-Way ANOVA in combination with *post hoc* Student-Newman-Keuls procedure. Enzyme activities in left and right ventricular myocardium and skeletal muscle in trained *vs.* sedentary dogs were compared by one way analysis of variance in combination with *post hoc* Student-Newman-Keuls procedure. The kinetic variables K_m and V_{max} were determined, assuming first-order Michaelis-Menten enzymes kinetics, from least squares regressions analyses of Lineweaver-Burk and Hanes plots of data from individual experiments. Kinetic variables and densitometry results were compared between trained and sedentary groups by unpaired student's t tests. Values are reported as means ± SE. P values < 0.05 were considered statistically significant. Statistical analyses were performed with SigmaStat software, version 2.0 (Jandel Scientific, San Rafael, CA).

RESULTS

Heart rates. Heart rate was monitored during treadmill running performance tests to asses the level of cardiovascular conditioning in dogs during the 9 wk running program. Figure 1 presents heart rates measured at each stage of the performance test. Resting heart rate fell 26% during the training program, from 105 ± 9 to 77 ± 3 min^{-1} ($P < 0.05$). Similarly, heart rate fell at the 3 lower intensity stages of the performance test. At stage 2, for example, heart rate was 164 ± 9 min^{-1} pre-training and 127 ± 4 min^{-1} at 9 wk training ($P < 0.05$). Exercise training did not significantly alter heart rate during high intensity exercise, as expected (38).

Enzyme activities. Glycolytic, β-oxidative, and TCA cycle enzyme activities were measured in both left and right ventricular myocardium and in exercising skeletal muscle (*vastus lateralis*) of running conditioned and sedentary control dogs (Table 1). Two of the glycolytic enzymes increased in myocardium in response to the training regimen. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity increased by 49% in left and 33% in right ventricular myocardium following 9 wk training, relative to activities in sedentary control dogs cage-rested for 4 wk ($P < 0.05$). Pyruvate kinase activity also increased ($P < 0.05$) in the left but not in the right ventricles of the trained dogs. On the other hand, activities of the other glycolytic enzymes (hexokinase, phosphofructokinase, lactate dehydrogenase) did not change appreciably in either ventricle following training. None of these glycolytic enzymes were altered in *vastus lateralis* in response to training. Thus, the running program increased myocardial

activities of certain specific glycolytic enzymes but did not elicit a nonselective increase in all enzymes of the glycolytic pathway.

Metabolically regulated, rate-controlling enzymes of the TCA and β-oxidative cycles were also measured. Citrate synthase (CS) activity increased 91% ($P < 0.05$) in the left ventricular myocardium of the trained *vs.* sedentary dogs, but CS activity in right ventricle was unaltered by training. Although the activities of α-ketoglutarate dehydrogenase and malate dehydrogenase tended to increase in response to training in both ventricles, these changes were insignificant. Activity of 3-hydroxyacyl-CoA dehydrogenase (HADH), an allosterically regulated, rate controlling enzyme of the fatty acid β-oxidation pathway, increased 77% ($P < 0.05$) in left ventricular myocardium of the trained dogs. Overall, training-induced increases in enzyme activity were more substantial in left ventricular myocardium than in right ventricular myocardium. In the *vastus lateralis*, CS activity was increased 40% ($P < 0.05$) in trained *vs.* sedentary dogs. Other measured TCA cycle enzyme activities in *vastus lateralis* were not significantly increased by training, although tendencies toward increases were noted.

Enzyme kinetics. Training-evoked increases in enzyme activity could have resulted from increased substrate affinity, or from augmented myocardial enzyme content. To differentiate between these two mechanisms, the Michaelis-Menten kinetics of three training enhanced enzymes (GAPDH, CS, HADH) and that of unaltered lactate dehydrogenase were evaluated in left ventricular myocardium of trained and sedentary

control dogs. Enzyme activities were measured over broad ranges of substrate concentration, and Lineweaver-Burk and Hanes plots were constructed from these measurements (Figures 2-5). Kinetic variables determined by analyses of these plots are reported in Table 2: substrate affinities were indexed by Michaelis constants (*i.e.*, K_m values), and enzyme contents were judged from V_{max} values. None of the K_m values were decreased in trained *vs.* sedentary myocardium, indicating that substrate affinities of these enzymes were not increased after training. In contrast, V_{max} of GAPDH (Figure 2), CS (Figure 3), and HADH (Figure 4) increased markedly in the left ventricle in response to training. As expected, lactate dehydrogenase kinetics were unaltered by training (Figure 5).

Immunoblot analyses of enzyme protein. The kinetic analyses described above indicated that exercise training increased contents of key glycolytic, TCA cycle, and β-oxidative enzymes in left ventricular myocardium. To confirm and extend these findings, CS and GAPDH contents in left ventricular myocardium were examined by immunoblotting. Bands corresponding to CS and GAPDH monomers were readily detected by peroxidase (Figure 6, panel A). By comparison of electrophoretic mobilities with standards, estimated molecular weights of CS and GAPDH were 46 and 39 kD, respectively, in excellent agreement with the respective molecular weights deduced from the amino acid sequences of these proteins (7, 9). Densitometry revealed marked increases in both proteins following training: CS and GAPDH contents were 2.2 ± 0.4-fold ($P = 0.03$) and 3.2 ± 0.4 fold ($P = 0.003$) the respective contents in left ventricular

myocardium of sedentary dogs (Figure 6, panel B). These immunoblots confirm that aerobic exercise training increased contents of these regulatory enzymes of intermediary metabolism in canine left ventricular myocardium.

DISCUSSION

This study demonstrated that aerobic exercise training of dogs selectively increases capacities of rate controlling enzymes of each of the major intermediary metabolic pathways in ventricular myocardium. The Michaelis constants of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 3-hydroxyacyl CoA dehydrogenase (HADH), and citrate synthase (CS) were unchanged in the left ventricle of exercising dogs, indicating that substrate affinities of these enzymes had not changed. On the other hand, the catalytic capacities of these enzymes as judged from V_{max} increased appreciably in the left ventricle of the exercise conditioned dogs. Thus, it appears that training evoked increases in metabolic enzyme activities resulted from increased protein contents but not increased substrate affinities. Immunoblot analyses further confirmed that enhancement of GAPDH and CS was due, at least in part, to increased content of these proteins in left ventricular myocardium of exercise trained dogs. Taken together, these findings indicate that a 9 wk running program is sufficient to induce marked, albeit selective enhancements of regulated, rate-controlling components of the myocardium's metabolic machinery.

It should be noted that enzyme activities were measured in standard buffers that differed markedly from the chemical milieu to which the enzymes are normally exposed in the intact cell. Free ion activities, ionic strength, pH, concentrations of allosteric regulatory metabolites, and subcellular compartmentation can influence the catalytic activities of enzymes. These intracellular factors could not be duplicated in the assay

systems, and, consequently, the measured enzyme activities may have either exceeded or underestimated the respective *in vivo* activities. It should be recognized, however, that enzyme measurements in the trained and sedentary groups were performed under identical conditions, and thus could be taken to reliably indicate training-induced enzyme adaptations.

Conditioning effect of exercise program. In this investigation, exercise conditioning was documented by two signature adaptations. Heart rate at rest and during low-intensity exercise fell appreciably during the 9 wk running program. Such declines in heart rate are recognized hallmarks of cardiac conditioning and physiological hypertrophy in response to aerobic conditioning (11, 35). In addition, citrate synthase activity, a biochemical marker of exercise conditioning in skeletal muscle (33, 42), increased 40% in the *vastus lateralis* of trained vs. sedentary dogs.

Comparison of exercising dogs with other animal models of training. Numerous animal models have been subjected to varying intensities, durations, and modalities of exercise training to examine its effects on myocardial energy metabolism. Not surprisingly, a clear consensus regarding these effects has not emerged from these studies. In rats, for example, exercise-evoked adaptations of myocardial glycolytic and citric acid cycle enzymes have proven equivocal. Activities of various glycolytic enzymes either increased (10, 14, 16, 20, 29, 37, 43), or did not change (2, 15, 16, 20, 37, 43) following exercise training in rats. The training paradigm appears to influence the

metabolic adaptations. In swimming-conditioned rats, myocardial activities of phosphofructokinase (2, 16, 43), GAPDH (16), CS (28), and malate dehydrogenase (28) were unaltered, although swim-training did increase pyruvate kinase activity (16, 43). Also, lactate dehydrogenase activity was increased in some (10, 29, 43) but not all (15, 16) swim-training studies. Similar inconsistencies were observed in running-conditioned rats. Endurance running increased pyruvate kinase (37), phosphofructokinse (2, 29), and lactate dehydrogenase (29) activities in rat left ventricle, but did not affect hexokinase (37), CS (2, 29), or GAPDH (20). Unlike endurance running, interval training in rats did not alter phosphofructokinase activity (2), and sprint-training increased myocardial GAPDH activity (20). These studies in rats demonstrate that even within the same species, exercise training effects on myocardial enzymes vary with training paradigm and exercise intensity.

Fewer studies have examined the effects of exercise training on myocardial intermediary metabolism in larger mammals. Laughlin *et al.* (19) demonstrated that treadmill running failed to increase phosphofructokinase, CS, and HADH activities in porcine myocardium. In agreement with Laughlin *et al.* (19), we found that phosphofructokinase activity did not change in trained canine myocardium. However, in contrast to the findings in pigs, CS and HADH activities did increase appreciably in left ventricle of running conditioned dogs. The differences between the present study and that of Laughlin *et al.* (19) may reflect differences in the training protocols. The sprint portion of the training regimen utilized by Laughlin *et al.* (19) was comparable to the

speed portion of the training protocol in this study, while sprint running in this study was conducted as an interval running stimulus. Laughlin and coworkers (19) proposed that different training paradigms may evoke effects on the myocardium which were not elicited by the training protocol utilized in their study. The results of the present investigation appear to confirm this proposal.

Possible mechanisms for increased myocardial enzyme capacity. The chronic increases in myocardial enzyme content evoked by running could only have resulted from increased synthesis and/or decreased degradation of enzymes protein. Thus, it seems possible that the observed adaptations were elicited by activating cardiac gene expression, translational machinery, or both. In myocardium, the regulation of genes encoding metabolic enzymes in response to exercise is poorly understood. Several factors could potentially stimulate expression of metabolic enzyme genes, including mechanical stretch, calcium, hypoxia, cytosolic redox state (1), metabolites including oxygen, glucose, and fatty acids (39), and adrenoceptor stimulation (8, 41). Studies reporting enhancement of gene expression by these factors have been conducted in many different cell and tissue preparations which were subjected to stimuli other than exercise (1, 8, 39, 41). Whether the mechanisms of gene expression defined in these studies operates in intact working myocardium during exercise, is unclear at present.

Physiological and cardioprotective implications of exercise-evoked metabolic adaptations. Energy consumption of canine left ventricular myocardium approaches

90% of its ATP-generating capacity during intense exercise (26), leaving only a small metabolic reserve under these conditions. During exercise-evoked cardiac hypertrophy, myocardial energy-generating capacity must increase in lockstep with sarcomere formation to maintain this small metabolic reserve. Although it seems unlikely that cytosolic energetics are enhanced in myocardium of trained *vs.* sedentary dogs when the animals are at rest, an increased metabolic capacity could ameliorate energy depletion in hypertrophied myocardium challenged by high workload (22, 44) and/or ischemia.

The training-evoked increases in myocardial GAPDH capacity could have important cardioprotective implications. Glyceraldehyde 3-phosphate dehydrogenase activity is the principal determinant of glycolytic flux when phosphofructokinase is maximally activated at high levels of cardiac work (17) and, importantly, during ischemia (23, 30). Although glycolysis provides only a small fraction of the total myocardial ATP requirement, it is the only significant source of ATP in severely ischemic myocardium (12). This glycolytic ATP could be crucial for sustaining myocytes during ischemic episodes (27, 40) by maintaining Ca^{2+} homeostasis (13), and, thus, delaying ischemic contracture (21). Thus, an increased glycolytic flux capacity in response to aerobic exercise training could ameliorate myocardial ischemic injury and contractile dysfunction. This proposal is supported by the findings of Libonati *et al.* (20), who recently demonstrated that hearts isolated from rats conditioned by high intensity sprint training, in which GAPDH activity was increased 63%, exhibited improved post-ischemic contractile recovery *vs.* hearts of sedentary rats or rats trained by low intensity

endurance running, where GAPDH was not significantly increased. It remains to be determined whether the training-evoked enhancements of glycolytic and oxidative enzyme activities demonstrated in the present study are sufficient to improve the energetics and function of ischemic canine myocardium.

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Table 1. *Activities of metabolic enzymes in myocardium and skeletal muscle of trained and sedentary dogs.*

	Left Ventricle		Right Ventricle		*Vastus lateralis*	
	Sedentary	Trained	Sedentary	Trained	Sedentary	Trained
HK	0.12±0.03	0.14±0.05	0.10±0.02	0.13±0.04	0.26±0.08	0.40±0.06
PFK	0.66±0.06	0.77±0.16	0.50±0.08	0.50±0.09	0.61±0.12	0.60±0.10
GAPDH	4.2±0.3	6.3±0.5 *	4.5±0.4	6.0±0.4 *	9.6±1.2	11.3±0.8
PK	2.7±0.5	4.7±0.8 *	2.6±0.4	3.3±0.3	6.2±1.4	8.8±1.3
LDH	8.3±0.5	9.6±0.5	7.0±0.4	8.6±0.7	11.4±1.6	10.3±1.9
CS	0.43±0.05	0.82±0.10 *	0.49±0.09	0.57±0.10	0.24±0.03	0.34±0.03 *
α KGDH	0.022±0.001	0.026±0.006	0.017±0.003	0.030±0.006	0.004±0.002	0.008±0.002
MDH	6.2±0.7	8.3±1.8	5.6±0.5	6.3±0.8	3.2±0.6	9.9±4.4
HADH	0.60±0.05	1.06±0.12 *	0.53±0.08	0.87±0.13	0.23±0.06	0.26±0.05

Enzymes (HK: hexokinase; PFK: phosphofructokinase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; PK: pyruvate kinase; LDH: lactate dehydrogenase; CS: citrate synthase; KGDH: α-ketoglutarate dehydrogenase; MDH: malate dehydrogenase; HADH: 3-hydroxyacyl CoA dehydrogenase) were assayed at 37° C. Enzyme activities are reported in U · mg protein^{-1}. Data are means ± SE, n=6. *: $P<0.05$ *vs.* sedentary.

Table 2. *Kinetics of left ventricular metabolic enzymes.*

		Lineweaver-Burk		Hanes	
Enzyme		K_m	V_{max}	K_m	V_{max}
CS	Trn	0.100±0.021	1.57±0.22*	0.108±0.018*	1.53±0.22*
	Sed	0.060±0.005	0.80±0.06	0.058±0.005	0.77±0.06
GAPDH	Trn	0.501±0.057	8.32±0.94*	0.701±0.107	8.59±0.93*
	Sed	0.392±0.049	4.99±0.47	0.409±0.067	4.92±0.55
HADH	Trn	0.007±0.001	2.18±0.15*	0.010±0.003	2.36±0.24*
	Sed	0.007±0.001	1.64±0.10	0.009±0.002	1.70±0.14
LDH	Trn	0.109±0.002	14.8±0.8	0.092±0.004	13.6±0.8
	Sed	0.105±0.003	12.2±1.0	0.086±0.005	11.1±1.0

Enzymes (GAPDH: glyceraldehyde 3-phosphate dehydrogenase; CS: citrate synthase; HADH: 3-hydroxyacyl CoA dehydrogenase; LDH: lactate dehydrogenase) were measured in left ventricular myocardium from 6 exercise trained (Trn) and 6 sedentary control (Sed) dogs. Kinetic variables (means ± SE) were determined by measuring reaction rates at different concentrations of the following substrates: 3-phosphoglycerate for GAPDH, acetyl CoA for CS, acetoacetyl CoA for HADH, and pyruvate for LDH. K_m values are in mM; V_{max} values are in $\mu mol \cdot min^{-1} \cdot$ mg protein^{-1}. *: $P<0.05$ *vs.* sedentary.

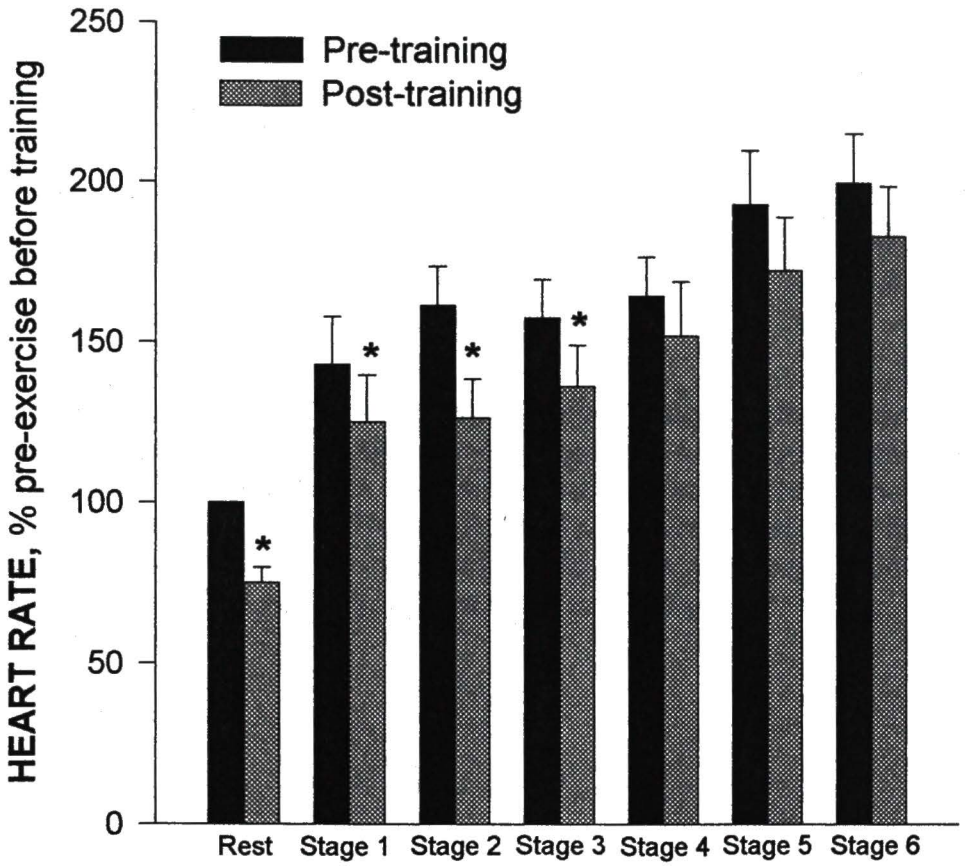

Figure 1. *Heart rate response to exercise training.* Cardiac conditioning was assessed by subjecting dogs to treadmill performance tests as described in methods. Tests were conducted one week prior to training (solid bars) and at weeks 4-9 of the training program, data from week 9 (shaded bars) are shown. Resting heart rate prior to training was 105 ± 9 min^{-1}. *: $P < 0.05$ vs. pre-training at the same exercise intensity.

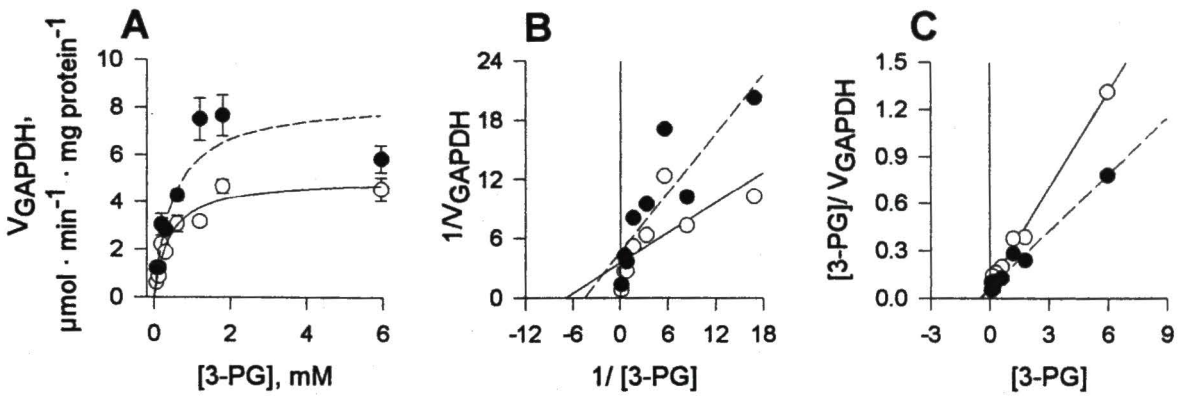

Figure 2. *Kinetic analysis of glyceraldehyde 3-phosphate dehydrogenase in left ventricular myocardium.* In this figure and in Figures 3-5, enzyme activities were measured as concentrations of the respective substrates were varied (panel A). Lineweaver-Burk (panel B) and Hanes (panel C) plots were constructed from these data to obtain the K_m and V_{max} values reported in Table 2. Open symbols, solid lines: sedentary controls; filled symbols, broken lines: exercise trained.

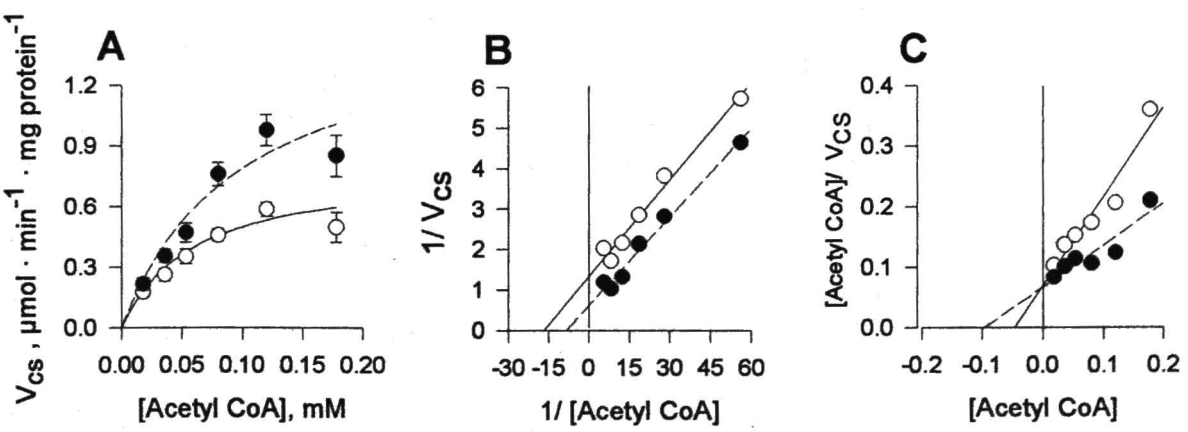

Figure 3. *Kinetic analysis of citrate synthase in left ventricular myocardium*

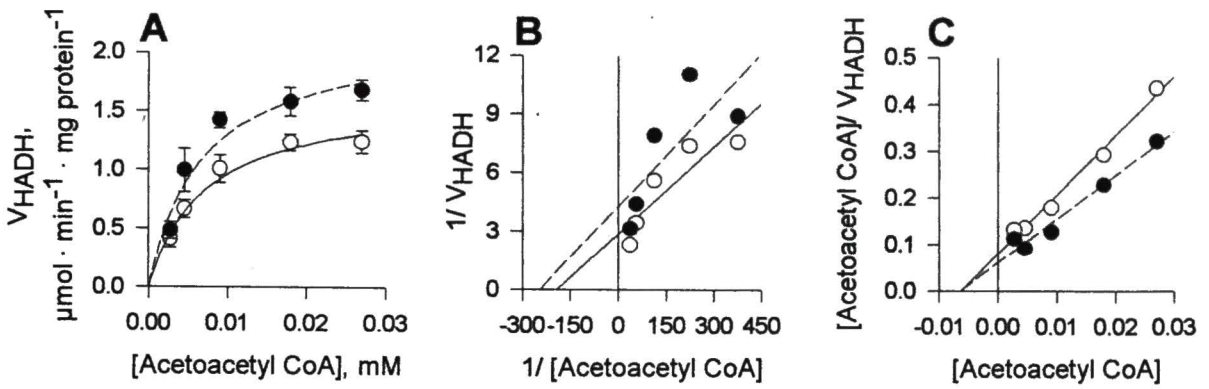

Figure 4. *Kinetic analysis of 3-hydroxyacyl CoA dehydrogenase in left ventricular myocardium.*

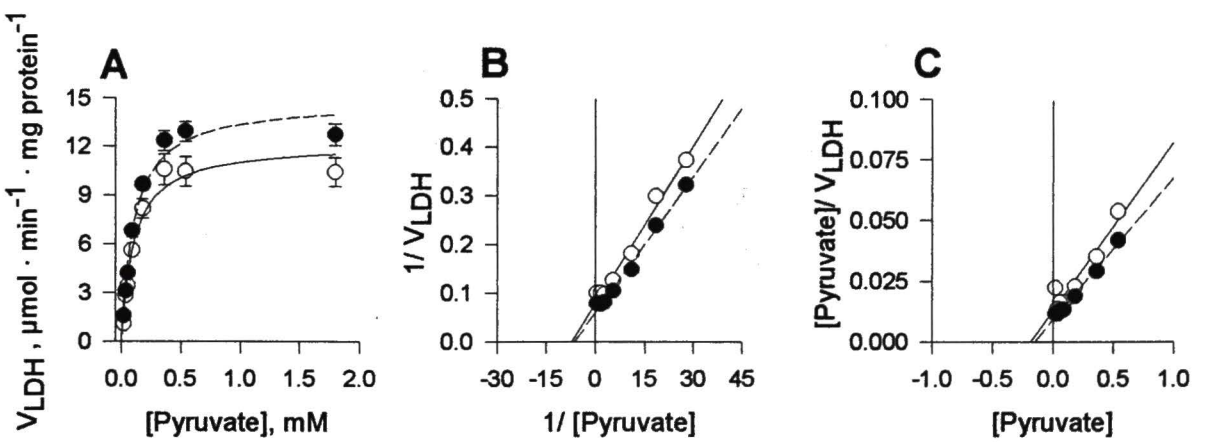

Figure 5. *Kinetic analysis of lactate dehydrogenase in left ventricular myocardium.*

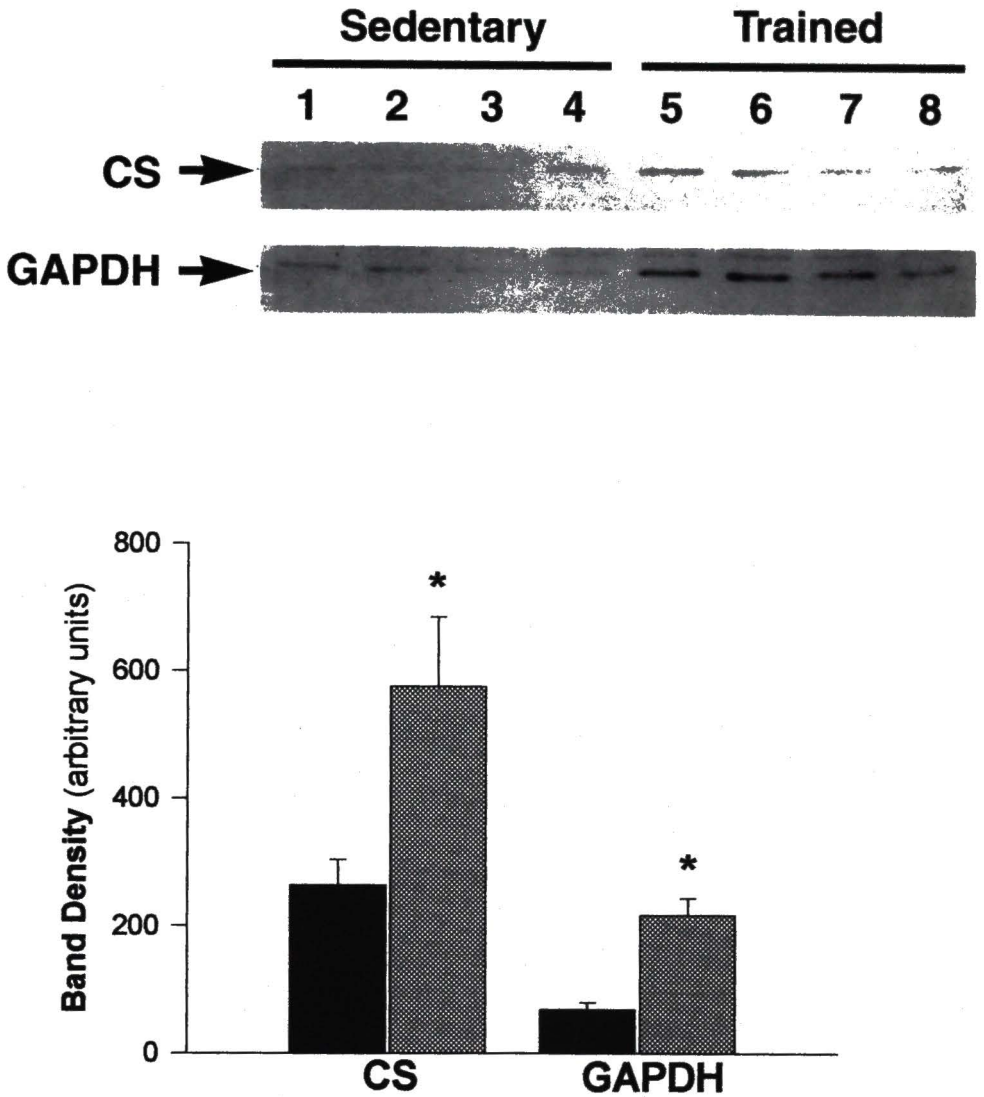

Figure 6. *Immunoblots of citrate synthase (CS) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).* Immunoblots of left ventricular homogenates were prepared as described in methods. 7.5 μg protein were loaded in each lane. Blots were probed for CS and GAPDH with specific antibodies and visualized by peroxidase reaction. Lanes 1-4 are from sedentary control dogs; lanes 5-8 are from trained dogs (panel A). Electrophoretic mobilities of CS and GAPDH correspond to approximate molecular weights of 46 and 39 kD, respectively. CS and GAPDH contents were 2.2 ± 0.4-fold (P = 0.03) and 3.2 ± 0.4 fold (P = 0.003) the respective contents in left ventricular myocardium of sedentary dogs (panel B). Blots for CS and GAPDH were scanned and analyzed separately, so density data in panel B do not reflect the comparative densities of the two proteins apparent in panel A.

PREFACE TO CHAPTER III

The results of the previous investigation demonstrated that aerobic exercise training selectively increases the capacity of rate controlling enzymes of each of the major intermediary metabolic pathways in canine ventricular myocardium. Additionally, it appears that training-evoked increases in metabolic enzyme activities resulted from increased protein contents but not increased substrate affinities of the enzymes. These results indicated that the exercise training paradigm utilized in the previous investigation evoked marked enhancements of regulated, rate-controlling components of the myocardium's metabolic machinery. These adaptations served to increase the myocardium's capacity to generate chemical energy. However, the the possibility that exercise training also augmented the cellular mechanisms for energy transport and delivery to energy-consuming systems, remained to be tested. The creatine kinase (CK) energy shuttle of cardiomyocytes channels metabolic energy from the mitochondria to sites of energy utilization at contracting myofibrils and sarcolemmal and sarcoplasmic reticular ion pumps. The plasticity of the myocardial CK system in response to hemodynamic overload has been repeatedly demonstrated. However, the effects of exercise training on myocardial CK are less well understood. Accordingly, the second investigation was conducted to test the hypothesis that aerobic exercise training increases the capacity of the CK system in canine myocardium.

CHAPTER III

EXERCISE TRAINING INCREASES CREATINE KINASE CAPACITY IN CANINE MYOCARDIUM

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ABSTRACT

The creatine kinase (CK) energy shuttle of cardiomyocytes channels metabolic energy from the mitochondria to sites of energy utilization at contracting myofibrils and sarcolemmal and sarcoplasmic reticular ion pumps. Although plasticity of the myocardial CK system in response to hemodynamic overload has been repeatedly demonstrated, the effects of aerobic exercise training on myocardial CK are less well understood. This investigation tested the hypothesis that aerobic exercise training increases the capacity of the CK system in canine myocardium. Mongrel dogs were conditioned by a 9 wk treadmill running program or cage-rested for 4 wk. Total CK activity was measured spectrophotometrically; CK_{MB} was isolated and measured by electrophoresis. Relative to sedentary controls, training increased left ventricular total CK activity 46% ($P<0.05$), but did not alter total CK activity in right ventricular myocardium. Activity of the CK_{MB} isoenzyme increased fourfold in left ventricular myocardium of trained *vs.* sedentary dogs ($P<0.05$). The CK_{MB} fraction of total CK activity in left ventricular myocardium increased 3-fold in response to training, from 1.1 ± 0.4 to $3.4 \pm 0.8\%$, ($P<0.05$). In contrast to left ventricle, CK_{MB} activity and its fraction of total CK activity were not altered by training in right ventricular myocardium. Thus, aerobic exercise training increases total myocardial CK activity and the amount of CK_{MB} present in canine left ventricular myocardium, although CK_{MB} remains a minor component of the myocardial CK system.

KEY WORDS: creatine kinase-MB isoform, conditioning, energy metabolism

ABBREVIATIONS: CK: creatine kinase; CK_{BB}, CK_{MB}, CK_{MM}, CK_{mito}: creatine kinase isoenzymes; EDTA: ethylene diamine tetracetic acid

ENZYMES: citrate synthase (EC 4.1.3.7), creatine kinase (EC 2.3.7.2), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12), hexokinase (EC 2.7.1.1)

INTRODUCTION

Creatine kinase (CK) catalyzes the reversible transfer of a high energy phosphate bond between creatine and ADP. This enzyme is abundant in tissues with high and/or highly variable energy demands, especially brain, skeletal muscle and myocardium. The CK energy shuttle efficiently couples energy production to utilization in these tissues. The shuttle effects this coupling by channeling high energy phosphate bonds, generated by oxidative phosphorylation in the mitochondria, to energy consuming ATPases elsewhere in the cell. In myocardium these ATPases catalyze crossbridge cycling of contracting myofibrils and sarcolemmal and sarcoplasmic reticular ion transport (24). Thus, the CK energy shuttle plays a pivotal role in cardiac contractile function.

Plasticity of myocardial CK activity and isoenzyme distribution has been demonstrated in response to chronically altered hemodynamic load and energy demand. Cardiac hypertrophy induced by pressure and volume overload is consistently accompanied by an increase in the cardiac-specific CK_{MB} isoform (6, 9, 14, 21, 26, 28, 31, 34), although alterations in total myocardial CK activity have been equivocal (6, 9, 14, 21, 26, 28, 31, 34). Indeed, it has been proposed that the increase in CK_{MB} activity is an adaptive response in compensated cardiac hypertrophy resulting in more efficient ATP synthesis from phosphocreatine (10, 26).

The effects of aerobic exercise training on the myocardial CK system have been examined in several animal models. In rats, swim-conditioning delayed or prevented the

aging-related decline in total CK activity in left ventricular myocardium (22), although myocardial CK activity in the swimming rats was not increased above the peak activity observed at 3 months of age. Chesky *et al.* (5) also found that CK activity was increased in rats that were swim-trained beginning at 6 months of age. In mice, running conditioning increased myocardial CK activity, albeit only in young animals (29). The effects of aerobic exercise training on total myocardial CK activity in large mammals have not been defined. Miller *et al.* (15) demonstrated that endurance exercise training did not alter myocardial CK_{MB} in dogs. However, the effects of more intense running exercise on the myocardial CK system of large mammals are unknown.

This investigation was conducted to determine if an intensive, 9 wk exercise training regimen combining sprint and endurance running evokes changes in the myocardial creatine kinase system in dogs. Total CK activity and the CK_{MB} fraction were measured in left and right ventricular myocardium from trained and sedentary control dogs. It was demonstrated that the sprint/endurance running paradigm increased both total CK activity and CK_{MB} content in left ventricular myocardium, but did not alter the right ventricular CK system.

METHODS

Animals and training program. Animal experimentation was approved by the Animal Care and Use Committee of the University of North Texas Health Science Center and conformed to the *Guide for the Care and Use of Laboratory Animals* (NIH publication 85-23, revised 1996). Sixteen healthy mongrel dogs were randomly assigned to the training or sedentary control groups (8 dogs in each group). Dogs were provided food and water *ad libitum* throughout the study, and were housed in 4 x 8 ft. enclosures that permitted quiet ambulatory activity. Body mass (mean ± SE) of the exercising dogs was 22.4 ± 1.3 kg, and that of the sedentary control dogs was 23.4 ± 0.6 kg.

Sedentary control dogs were cage-rested for 4 wk. Dogs in the training group were conditioned by a 9 wk treadmill running program described by Tipton *et al.* (30) as modified by Gwirtz (7). The training program consisted of endurance and sprint running sessions on alternate days, 5 days per wk. The intensity and duration of the running sessions were increased each week during the first 8 wk of the program, with a total running time of 35 min per session in wk 1 and 75 min in wk 8. In wk 1, endurance running sessions consisted of running at 4.8 kph on level grade for 5 min (warm-up), at 9.7 kph on level grade for 10 min (speed), between 4.8 and 8.0 kph on 16% grade for 15 min (endurance), and at 4.8 kph on level grade for 5 min (warm-down). Sprint running sessions consisted of the same warm-up and speed runs, followed by ten 1 min sprints, during which the treadmill was accelerated from 6.4 to 12.9 kph over 30 s, then decelerated to 6.4 kph over 30 s. The sprints were followed by 5 min of endurance

running at 4.8 - 8.0 kph on 16% grade, and 5 min warm-down. Endurance bouts in wk 8 consisted of 5 min warmup followed by 15 min running at 11.3 kph on 8% grade, 50 min running at 11.3 kph on 16% grade, and 5 min warm-down. Sprint bouts in wk 8 included 5 min warmup, 15 min running at 11.3 kph on 8% grade, 50 min of sprints (30 s acceleration from 9.7 to 19.3 kph, 30 s deceleration to 9.7 kph) on 4% grade, and 5 min warm-down. Speed and grade for warmup and warm-down periods in wk 8 were the same as in wk 1.

Cardiovascular conditioning effects of the training regimen were assessed by treadmill performance tests (30). These tests were conducted in the week preceding the training program and at weeks 4-9 of the program. Each test lasted 18 min and consisted of six 3 min running stages: stage 1: 4.8 kph on level grade; stage 2: 6.4 kph on level grade; stage 3: 6.4 kph on 4% grade; stage 4: 6.4 kph on 8% grade; stage 5: 6.4 kph on 12% grade; stage 6: 6.4 kph on 16% grade. Heart rate was monitored throughout the test by electrocardiography.

Sampling of myocardium and skeletal muscle. Tissue was harvested for biochemical analyses on the day following the final running session. Dogs were fasted overnight, then anesthetized with sodium pentobarbital (30 mg/kg *iv*), intubated, and ventilated with room air (0.2 liter $\cdot$ kg^{-1} $\cdot$ min^{-1}). The heart was exposed via a left thoracotomy in the fifth intercostal space and quickly excised. Transmural samples (3-4 g) of left and right ventricular myocardium were quickly freeze clamped with

Wollenberger tongs pre-cooled in liquid N_2. Samples (3-4 g) of *vastus lateralis* were collected through an incision in the left hindlimb and were also quickly freeze clamped. Frozen tissues were stored at -90°C prior to extractions. Citrate synthase activity in *vastus lateralis*, a biochemical marker of exercise conditioning (32), was extracted as described by Braasch *et al.* (3) and assayed spectrophotometrically (2).

Extraction and measurement of total CK activity. Creatine kinase was extracted from frozen tissue essentially as described by Sharkey *et al.* (27). Tissue was pulverized to a fine powder in a pre-cooled mortar under liquid N_2. Powdered tissue (5 mg) was added to 2 ml ice-cold buffer (100 mM KH_2PO_4, 5mM EDTA, 5 mM β-mercaptoethanol, 50% glycerol (w/v), pH 7.2), homogenized with an Omni GHL tissue homogenizer (ING10-195 probe; 15 s at setting 3), and then maintained at room temperature for 1 hr. The suspension was centrifuged at 40,000 · g_{max} for 30 min at 4°C in a Dupont Sorvall Ultra 80 preparative ultracentrifuge. Protein concentrations in the extracts were determined colorimetrically as described by Bradford *et al.* (4).

Creatine kinase activity was assayed at 37°C in a Perkin-Elmer *uv*/vis spectrophotometer (23). In this assay CK-catalyzed formation of ATP from phosphocreatine and ADP was linked via hexokinase and glucose 6-phosphate dehydrogenase to formation of NADPH, which was monitored at 337 nm wavelength (extinction coefficient = 6.24 $mM^{-1} \cdot cm^{-1}$). Enzyme activities ($U \cdot mg\ protein^{-1}$) were

computed from differences in the rates of absorbance change before and immediately following addition of reaction substrate to the cuvette.

Isolation and analysis of CK_{MB}. The CK_{MB} isoform was extracted essentially as described by Younes *et al.* (34). Powdered tissue (1 g) was homogenized in 10 vol ice-cold Buffer 1 (1 mM EDTA, 1 mM β-mercaptoethanol, 1% (w:v) sodium deoxycholate, 50 mM Tris-Maleate, pH 8.0) using an Omni GHL tissue homogenizer (ING10-195 probe) at setting 4 for two 15 s bursts separated by a 30 s interval. After cooling on ice, each sample was further homogenized for 1 min with a pre-cooled, motor-driven teflon piston (700 rpm). The samples were then centrifuged at 45,000 · g_{max} for 20 min at 4°C in a Dupont Sorvall Ultra 80 preparative ultracentrifuge. The pellet was resuspended in Buffer 1, extracted again, and the two supernatants were combined. Protein concentrations in the extracts were determined colorimetrically (4).

The CK_{MB} isoform was isolated by anion exchange chromatography as described by Younes *et al.* (34) with modifications. Crude extract (1 ml) was added to a 2 ml column of DEAE-Sepharose CL-6B (Pharmacia) equilibrated with 10 ml of 50 mM Tris-Maleate, pH 7.5 (Buffer 2). The CK_{MM} and CK_{mito} isoforms co-eluted with 6 ml of Buffer 2. The CK_{MB} isoform was then eluted with 3 ml of 180 mM NaCl. Eluent was collected in 0.5 ml fractions. Creatine kinase activity in the eluent fractions was assayed (23) and fractions containing peak CK activity were combined, lyophilized and

resuspended in 100 µl of water. Protein concentrations in these samples were determined colorimetrically (4).

Measurement of CK_{MB} activity. Activity of the CK_{MB} isoform was determined in anion exchange eluent by agarose gel electrophoresis (Sigma Diagnostics Kit, Sigma, St. Louis, MO). Gels loaded with 2 µg protein per well were then electrophoresed at 120 V for 45 min. After electrophoresis and development of color, the gels were scanned (Howtek Scanner, Model No. SM3, Hudson, MN) and quantitated using Diversity One software (Protein + DNA ImageWare Systems, Huntington Station, NY). The CK_{MB} isoform was identified by comigration with 2.25 mU purified human CK_{MB} (Biodesign, Catalog No. A32250H, Kennebunk, ME). CK_{MB} as a fraction of total CK activity was calculated using the activities measured by densitometry.

Statistical analyses. Comparisons of total CK activities and CK_{MB} fractions of total CK in left and right ventricular myocardium and skeletal muscle in trained *vs.* sedentary dogs were accomplished by one way analysis of variance in combination with *post hoc* Student-Newman-Keuls procedure. Densitometry data from exercise trained and sedentary groups were compared by unpaired student's t tests. All data are reported as means ± SE. Values of $P < 0.05$ were taken to indicate statistical significance. Statistical analyses were performed with SigmaStat software, version 2.0 (Jandel Scientific, San Rafael, CA).

RESULTS

Physiological and biochemical markers of exercise training. Heart rate was monitored during treadmill running performance tests to evaluate the level of cardiovascular conditioning in dogs subjected to the 9 wk running program. At wk 9, heart rate (min^{-1}) was 77 ± 4.0 at rest and 130 ± 9.0 during moderate intensity exercise; these rates were 25 and 21% lower ($P < 0.05$) than the respective pre-training values of 102 ± 7.0 and 164 ± 7.0. Citrate synthase activity in exercising skeletal muscle was also measured to assess exercise conditioning (32). Citrate synthase activity was increased 38% ($P < 0.05$) in *vastus lateralis* of trained *vs.* sedentary dogs, which provided biochemical evidence that the exercising dogs were conditioned by the training program.

Total myocardial creatine kinase. Total creatine kinase activity was measured in left and right ventricular myocardium of exercise-trained and sedentary control dogs (Figure 1). Creatine kinase activity in left ventricular myocardium of exercise-trained dogs was increased 46% relative to sedentary control dogs (50.8 ± 3.9 *vs.* 34.7 ± 5.9 U · mg $protein^{-1}$, respectively; $P < 0.05$). On the other hand, total creatine kinase activity was unaltered by 9 wk of training in right ventricular myocardium.

Creatine kinase MB isoform. The CK_{MB} isoform was separated from CK_{MM} and CK_{mito} by anion exchange chromatography and its activity measured by agarose gel electrophoresis in left and right ventricular myocardium. Bands corresponding to the CK_{MB} isoform were readily detectable upon colorimetric development of the agarose gel,

and by comparison of electrophoretic mobility with human CK_{MB} standard. Densitometry measurements were used to calculate the activity of CK_{MB} and its fraction of total creatine kinase activity. CK_{MB} activity in left ventricular myocardium of trained dogs (1.8 ± 0.4 U · mg protein^{-1}) was increased 4.5-fold ($P < 0.05$) relative to that of sedentary dogs (0.4 ± 0.1 U · mg protein^{-1}; Figure 2). These measurements also revealed a 3-fold ($P < 0.05$) increase in CK_{MB} as a fraction of total creatine kinase activity in trained *vs.* sedentary left ventricular myocardium ($3.4 \pm 0.8\%$ and $1.1 \pm 0.4\%$, respectively; Figure 3). Training did not increase CK_{MB} activity or its fraction of total CK in right ventricular myocardium (Figures 2, 3). The CK_{BB} could not be detected in any of the myocardial samples of either group.

DISCUSSION

This study demonstrated that aerobic exercise training increases total creatine kinase activity in left ventricular myocardium of dogs. The increase in total CK activity was accompanied by a marked increase in the CK_{MB} isoenzyme fraction, although CK_{MB} remained only a minor component of total myocardial CK. These findings indicate that a 9 wk running program is sufficient to increase the capacity of the CK energy transport system in the left ventricular myocardium, due in part to increased CK_{MB} content. However, the CK system was not enhanced by exercise training in right ventricular myocardium.

Exercise training and the myocardial creatine kinase system. Most investigations of training-evoked adaptations of the myocardial creatine kinase system have been conducted in small mammals. Rockstein *et al.* (22) observed that rats conditioned by lifelong physical exercise exhibited higher myocardial CK activities than age-matched sedentary controls. In a similar study, it was observed that running conditioning prevented the aging-associated decrease in myocardial CK activity in mice (29). Chesky *et al.* (5) demonstrated that myocardium of rats exercise trained between 6 and 9 months of age had higher CK activity than age-matched sedentary rats. It is also interesting to note that aerobic exercise training partially restored total CK activity in diabetic rat hearts (17).

The only study that examined the effects of exercise training on the myocardial creatine kinase system in larger mammals did not demonstrate a significant training effect on CK_{MB} in canine myocardium (15). However, it should be recognized that the training program of the present study combined endurance and high-intensity sprint-interval running. The combined sprint/endurance training program may have provided a stronger stimulus of CK adaptations than endurance running alone. In accordance with this proposal, Libonati *et al.* (13) demonstrated that exercise-evoked myocardial metabolic adaptations depend upon the specific training paradigm, and that sprint interval running increased myocardial glyceraldehyde 3-phosphate dehydrogenase activity, but endurance running alone did not. Thus, endurance running alone may not be sufficient to elicit the the CK adaptations observed in this study.

Physiological implications of increased creatine kinase capacity. The myocardial creatine kinase system channels high energy phosphate bonds, generated by oxidative phosphorylation in the mitochondria, to sites of energy utilization at contracting myofibrils and sarcolemmal and sarcoplasmic reticular ATPases (24). The possible benefits of increasing the capacity of the cardiac creatine kinase system have not been directly examined in normal myocardium. The pivotal role of this system for supporting increased cardiac function has been demonstrated by manipulating CK flux either by use of creatine kinase inhibitors or by decreasing the amount of available creatine substrate. In hearts depleted of creatine by the non-physiological creatine analogues β-guanidinopropionate or β-guanidinobutyrate (11, 35), or in which creatine kinase is

inhibited with iodoacetamide (8), the myocardium's ability to increase its contractile performance in response to pressure and/or volume overload, adrenergic stimulation, and hypercalcemic perfusion, is compromised. Also, both creatine content and CK activity are reduced in experimental models of chronic heart failure (12, 19) and in the failing human myocardium (20). These alterations in CK energy shuttle components decrease myocardial energy reserve sufficiently to limit cardiac function and inotropic reserve. Thus, reductions in the myocardium's creatine kinase system effectively limit the capacity of the heart muscle to respond to increased contractile and energetic challenges. By inference, it seems possible that training-induced enhancement of the myocardial creatine kinase system could have the opposite effect, to augment energy supply to the contractile machinery when cardiac workload is increased.

The creatine kinase system is instrumental in providing ATP to the sarcoplasmic reticulum to power Ca^{2+} sequestration (16). Indeed, CK appears to be functionally coupled to the sarcoplasmic reticular Ca^{2+}-ATPase, and binding of CK to the sarcoplasmic reticular membrane optimizes Ca^{2+} transport by the ATPase. By extension, it could be proposed that an increase in total CK activity may ensure adequate Ca^{2+} sequestration by the sarcoplasmic reticulum when cardiac workload and heart rate are increased, as during exercise.

Repeated bouts of aerobic exercise stimulate synthesis of new sarcomeres in ventricular myocardium (18). Sarcomere synthesis increases the myocardium's energy-

consuming capacity and could threaten energy reserves unless the myocardium's energy production and delivery systems increase coincidently. Training-evoked enhancements of the myocardial CK system may better couple myocardial energy production to energy utilization to better maintain myocardial energy reserve during intense exercise.

Creatine kinase MB isoform. Alterations in the isoenzyme profile of the myocardial creatine kinase system have been observed in a variety of experimental models. In particular, it has been demonstrated repeatedly that chronically increased hemodynamic load increases in the amount of CK_{MB} in myocardium. This adaptation occurs in response to aortic banding and renal hypertension in the dog (9) and rat (6, 14, 21, 28, 34), volume overload produced by aortocaval fistula (9) or mitral regurgitation in the dog (26), and pacing–induced heart failure in the dog (31). The failing human myocardium exhibits similar changes in myocardial CK isoenzyme profile (20). These changes in CK isoenzymes in response to hemodynamic overload are thought to represent an adaptive change enabling the myocardium to utilize phosphocreatine more efficiently (10, 26). This study demonstrates a similar adaptive increase CK_{MB} in response to the increased hemodynamic demands imposed on the myocardium by chronic exercise.

An increase in the amount of CK_{MB} present in myocardium may confer physiological advantages to the heart. Creatine kinase isoenzymes containing B subunits have higher affinities for ADP substrate (25, 33). Thus, increased CK_{MB} would favor the transferral of high-energy phosphate bonds from phosphocreatine to ADP, and, thus,

more efficiently generate ATP for myofibrillar and membrane-bound ATPases. Aerobically trained skeletal muscle contains higher amounts of the CK_{MB} isoform, which could contribute to increased oxidative capacity of these muscle fibers (1). Thus, the increased CK_{MB} in left ventricular myocardium of trained animals demonstrated in the present study may constitute an energetically favorable adaptive response.

Limitations and alternative hypotheses. The CK_{MM} and CK_{mito} isoforms were not separated and individually measured in this study. However, the overall increase in CK activity in left ventricle greatly exceeded the increase in CK_{MB} alone, and CK_{BB} was undetectable, so left ventricular CK_{MM} and/or CK_{mito} must have increased following training. Indeed, the sum of the CK_{MM} and CK_{mito} isoforms increased from 34.4 ± 5.9 to 49.0 ± 3.7 U · mg protein^{-1} in response to training.

The exercise-evoked adaptations in the creatine kinase system were only observed in left ventricular myocardium. It is unclear why the left ventricle responded to training and the right ventricle did not. In steady state, the stroke volume of the two ventricles are equal, so hemodynamic workloads normalized to the respective baselines must have increased to similar extents in the two ventricles. Nevertheless, absolute workload of the left ventricle is much greater than that of the right ventricle. It is possible that a critical level of absolute work must be achieved to elicit the responses demonstrated in the present study. This proposal remains to be tested.

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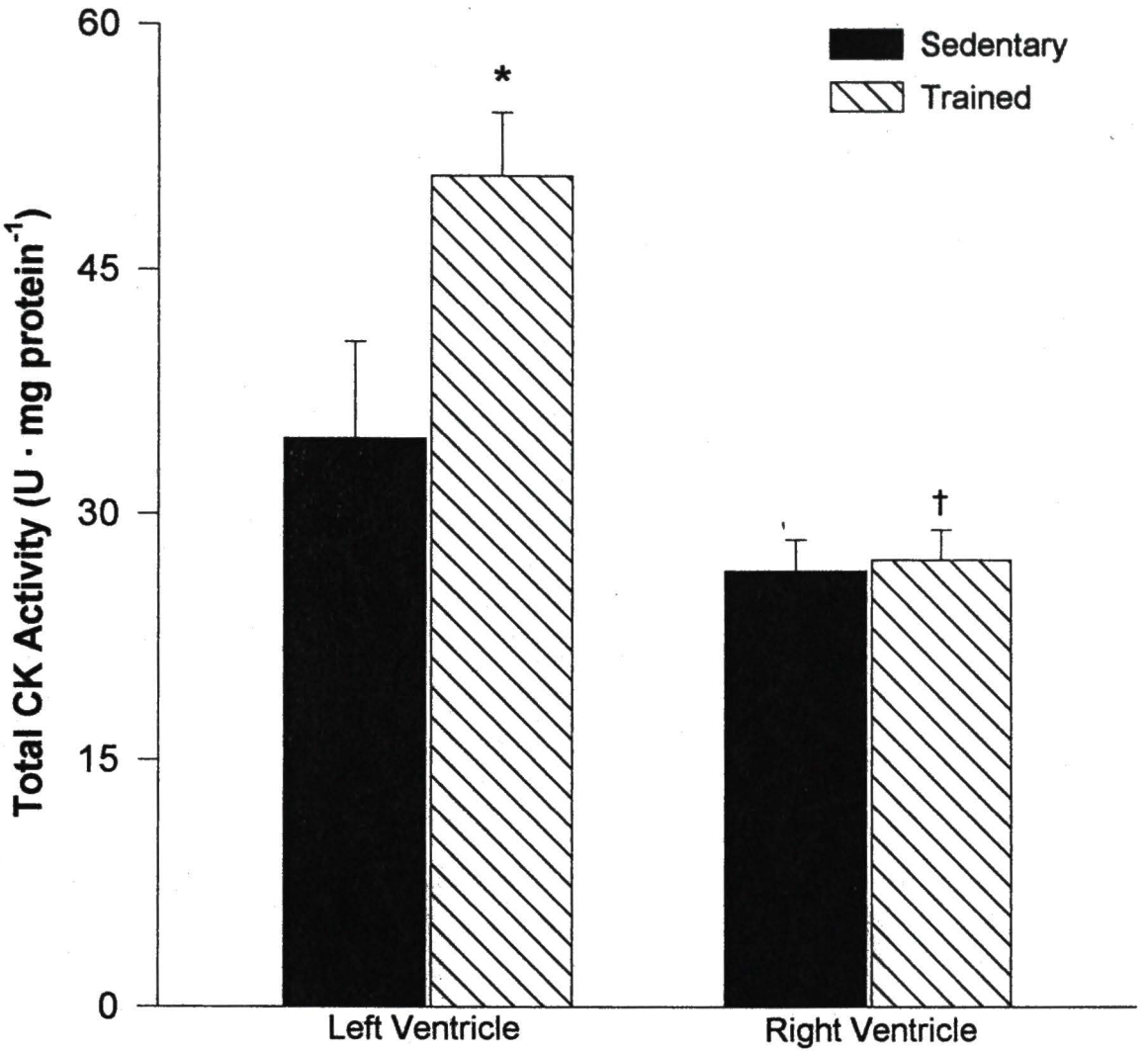

Figure 1. *Total creatine kinase activity in response to exercise.* Total creatine kinase (CK) activity was measured in left and right ventricular myocardium. In left ventricle, CK activity was increased from 34.7 ± 5.9 to 50.8 ± 3.9 U · mg protein^{-1} by training. Training did not alter CK activity in right ventricle. Solid bars: sedentary controls; striped bars: exercise trained. *: $P < 0.05$ *vs.* sedentary control in the same ventricle. †: $P < 0.05$ *vs.* left ventricle of the same group.

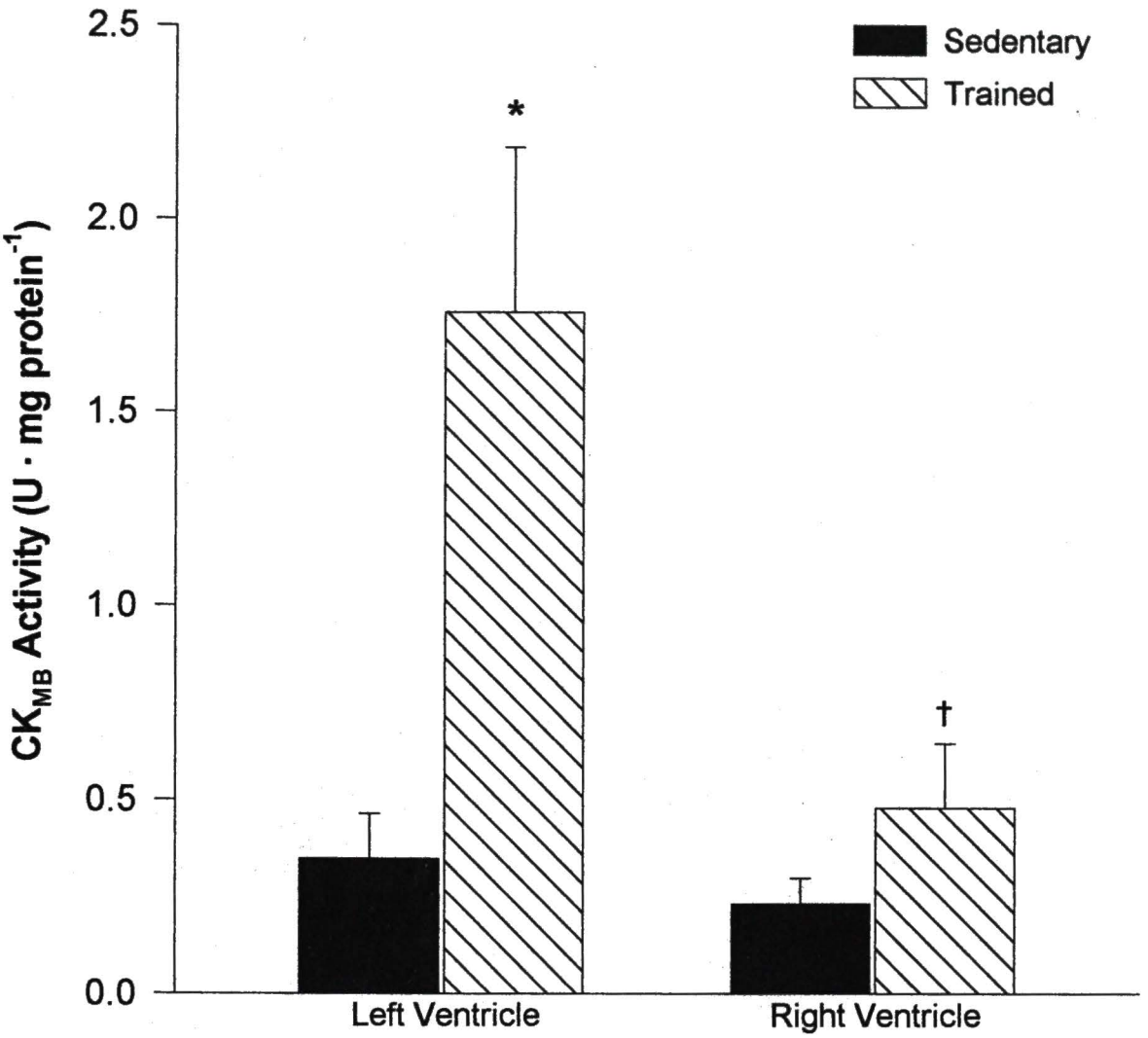

Figure 2. *CK_{MB} activity in response to exercise training.* CK_{MB} activity was determined in agarose gels following chromatographic and electrophoretic separation of CK_{MB} from the other CK isoenzymes. The activity of the CK_{MB} isoenzyme was increased significantly in trained left ventricular myocardium. Training did not alter the activity of the CK_{MB} isoenzyme in right ventricular myocardium. Solid bars: sedentary controls; striped bars: exercise trained. *: $P < 0.05$ *vs.* sedentary control in the same ventricle. †: $P < 0.05$ *vs.* left ventricle of the same group.

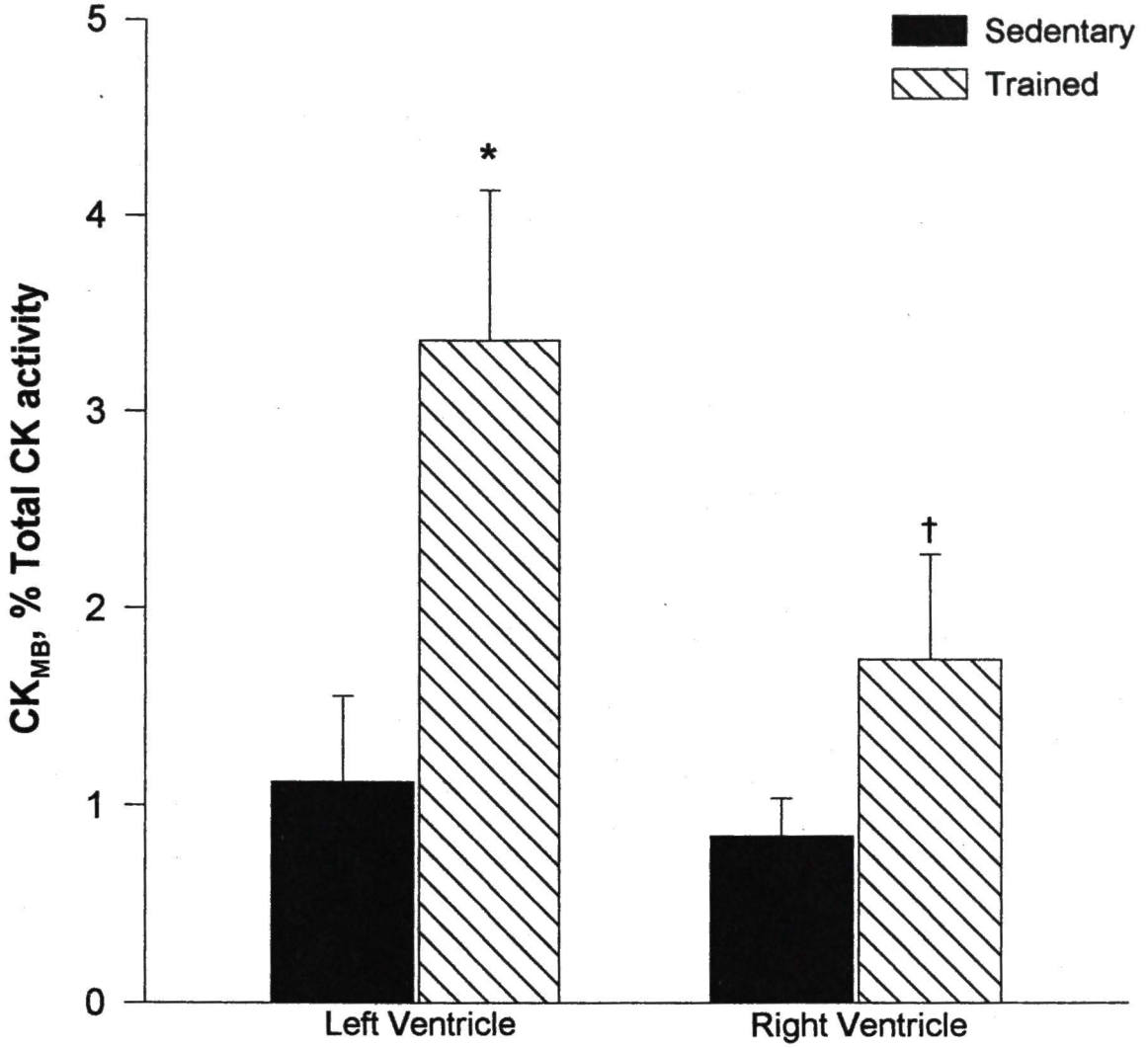

Figure 3. *CK_{MB} as a fraction of total CK activity.* The CK_{MB} fraction of total CK increased 3-fold in left ventricular myocardium of trained *vs.* sedentary control dogs (3.4 ± 0.8% and 1.1 ± 0.4%, respectively). Training did not increase the fraction of CK_{MB} in right ventricular myocardium. Solid bars: sedentary controls; striped bars: exercise trained. *: $P < 0.05$ *vs.* sedentary control in the same ventricle. †: $P < 0.05$ *vs.* left ventricle of the same group.

PREFACE TO CHAPTER IV

The results of the two previous investigations suggested that the capacities of the myocardium's energy generating and transporting systems are augmented by aerobic exercise training in canine myocardium. This is result of exercise-evoked increases in contents of several key, rate-controlling enzymes that mediate myocardial energy production and transport. The exercise-evoked adaptations result from the cumulative effects of repeated bouts of exercise. The acute biochemical and biophysical events and signaling mechanisms responsible for these adaptations would be expected to occur with each exercise bout. Little is known about the cellular and molecular events initiated by an acute bout of exercise that are responsible for these exercise-evoked myocardial adaptations. In addition, it has been demonstrated that exercise is capable of increasing the abundance of specific mRNA transcripts in myocardium. However, the effects of exercise on the abundance of myocardial mRNA transcripts encoding metabolic enzymes are unknown. Accordingly, the third investigation was conducted to delineate the effects of running exercise on the abundance of mRNAs encoding citrate synthase, glyceraldehyde 3-phosphate dehydrogenase, and creatine kinase muscle, brain and sarcomeric mitochondrial subunits in canine myocardium. These mRNAs were selected for study because each of the enzymes was augmented by exercise training.

CHAPTER IV

EXERCISE INCREASES ABUNDANCES OF mRNAS ENCODING METABOLIC ENZYMES IN CANINE MYOCARDIUM

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ABSTRACT

The functional and trophic adaptations of the myocardium to exercise training have been well characterized; in contrast, the acute cellular, metabolic, and molecular mechanisms responsible for these chronic adaptations are not as well understood. This study tested the hypothesis that exercise increases the abundance of messenger RNA (mRNA) encoding key enzymes involved in myocardial energy production and transport in canine left ventricular myocardium. Samples of left ventricular myocardium were collected from the dogs 30 min after 45 min treadmill running bout. Messenger RNA (mRNA) transcripts encoding rate-controlling intermediary metabolic enzymes of glycolysis (glyceradlehyde 3-phosphate dehydrogenase) and the tricarboxylic acid cycle (citrate synthase) were amplified and measured by use of reverse transcriptase polymerase chain reaction. Also measured were mRNAs encoding the creatine kinase (CK) brain (CK-B), muscle (CK-M), and sarcomeric mitochondrial (sCK-mito) subunits. Exercise increased the abundances of left ventricular myocardial glyceraldehyde 3-phosphate dehydrogenase and citrate synthase mRNAs threefold in comparison with those of sedentary control dogs ($P < 0.05$). The abundance of mRNA encoding CK-M subunits was also increased threefold ($P < 0.05$) in left ventricular myocardium of exercised *vs.* sedentary dogs. In contrast, the abundances of CK-B and sCK-mito mRNA transcripts were unaltered by exercise. The selective increases in messenger RNA transcripts evoked by aerobic exercise could contribute to the recently reported increases in encoded enzyme activities in left ventricular myocardium of exercise trained dogs.

INDEX TERMS: reverse transcriptase polymerase chain reaction, citrate synthase, glyceraldehyde 3-phosphate dehydrogenase, creatine kinase, conditioning

ABBREVIATIONS: CS: citrate synthase; GAPDH: glyceraldehyde 3-phosphate dehydrgenase; CK-M, CK-B: creatine kinase muscle (M) and brain (B) subunits; sCK-mito: sarcomeric mitochondrial creatine kinase subunit; mRNA: messenger RNA; cDNA: complimentary DNA; RT-PCR: reverse transcriptase polymerase chain reaction: HSP-70; 70-kDa heat shock protein

ENZYMES: citrate synthse (EC 4.1.3.7), glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12), hexokinase (EC 2.7.1.1), creatine kinase (EC 2.3.7.2)

INTRODUCTION

The physiological challenge of exercise impacts many biochemical and biophysical aspects of cellular function in cardiomyocytes. During exercise, cardiac mechanical activity increases, which imposes heavier demand on the enzymatic processes that generate and transport energy within the cardiomyocyte. The myocardium adapts to an exercise training program through structural changes that serve to augment stroke volume and maximum cardiac output. Although these adaptations result from the cumulative effects of repeated bouts of exercise, the initial biochemical and biophysical responses that culminate in these adaptations could be activated by each individual exercise bout. However, little is known about the acute cellular and molecular mechanisms responsible for these exercise-evoked myocardial adaptations.

Only limited information is available regarding the effects of acute exercise bouts on the myocardial abundance of specific messenger RNA (mRNA) transcripts. Locke *et al.* (10) found that 40 min of treadmill running was sufficient to cause accumulation of heat shock protein (HSP)-70 mRNA in rat myocardium. It has also been demonstrated that treadmill running to exhaustion increases rat myocardial HSP-70 mRNA immediately following exercise (17). Maeda *et al.* (11) found that treadmill running in rats markedly increased myocardial prepro-endothelin-1 mRNA. Thus, it is evident that exercise is capable of increasing the abundance of specific mRNA species in myocardium. However, the effect of exercise on the contents of mRNA transcripts encoding metabolic enzymes remains to be determined.

Recently, we demonstrated that a 9 wk program of aerobic exercise training evokes increases in the activities of several key enzymes involved in energy production and transport in canine left ventricular myocardium (21, 22). This investigation was conducted to delineate the effects of running exercise on the abundance of mRNAs encoding citrate synthase (CS), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the creatine kinase muscle (M), brain (B) and sarcomeric mitochondrial (mito) subunits in canine myocardium. Total RNA was extracted from left ventricular myocardium of sedentary and exercised dogs and analyzed using reverse transcriptase polymerase chain reaction (RT-PCR). It was demonstrated that exercise increased abundance of mRNAs encoding CS, GAPDH, and the CK-M subunit. To our knowledge, this is the first investigation to demonstrate that exercise increases the amount of mRNA encoding key enzymes involved in intermediary metabolism and energy transport in mammalian myocardium.

METHODS

Animals and protocol. Animal experimentation was approved by the Animal Care and Use Committee of the University of North Texas Health Science Center and conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1996). Eleven healthy mongrel dogs were randomly assigned to the exercised or sedentary control groups (5 dogs in the exercised group, and 6 dogs in the sedentary group). Dogs were provided food and water *ad libitum* throughout the study, and were housed in 4 x 8 ft. enclosures that permitted quiet ambulatory activity. Body mass at the time of tissue collection (mean ± SE) of the exercised dogs was 29.1 ± 1.4 kg, and that of the sedentary dogs was 28.9 ± 2.2 kg.

Sedentary dogs were cage-rested for 4 wk. Over a 2 wk period, dogs in the exercised group were habituated to running on a motor driven treadmill. On the day of the experiment, exercised dogs ran for 45 min on the treadmill. The running bout consisted of running at 4.8 kph on level grade for 5 min, at 9.7 kph on level grade for 15 min, at 8 kph at 16 % grade for 20 min, and at 4.8 kph level grade for 5 min.

Sampling of left ventricular myocardium. Immediately after completing the run, dogs in the exercised group were anesthetized with sodium pentobarbital (30 mg/kg iv), intubated, and ventilated with room air (0.2 liter · kg^{-1} · min^{-1}). The heart was exposed under sterile conditions via a left thoracotomy in the fifth intercostal space and quickly excised. Transmural samples (3-4 g) of the left ventricle were placed in sterile

polypropylene tubes and quickly frozen in liquid N_2. Frozen tissues were stored at -90°C prior to RNA extractions.

RNA isolation. Total cellular RNA from left ventricular myocardium was isolated as described by Chirgwin *et al.* (3) with modifications. Briefly, 200 mg of tissue was homogenized in 9 ml buffer (4 M guanidine thiocyanate, 10 mM EDTA, 50 mM sodium acetate, pH 5.2, 40% sodium lauryl sarcosine, and 1 M β-mercaptoethanol) using an Omni 5000 homogenizer at setting 4. Homogenates were then centrifuged for 5 min at 3,000 rpm (Beckman TJ-6, Beckman Instruments, Palo Alto, CA) at room temperature, to pellet the genomic DNA. The supernatant was then layered on a 2.5 ml cushion of 5.7 M cesium chloride, 50 mM sodium acetate, pH 5.2, and centrifuged at 35,000 rpm, 20°C for 18 hrs (Beckman L8-80 untracentrifuge, SW41 rotor, Beckman Instruments, Palo Alto, CA). The pellets were resuspended at 65°C in 400 µl of 0.2 M lithium chloride, 0.3% sodium lauryl sulfate. One milliliter of ice-cold ethanol was added and the suspension was stored at -20°C overnight for RNA precipitation. The suspension was centrifuged at 12,000 rpm for 5 min (Eppendorf 5415, Brinkmann Instruments, Westbury, NY). The pellets were resuspended in 600 µl of RNAse free dH_2O and stored at -80°C.

Reverse transcriptase - polymerase chain reaction (RT-PCR). Messenger RNA transcripts encoding CS, GAPDH, and creatine kinase M, B and sarcomeric mitochondrial (sCK-mito) subunits in canine left ventricular myocardium were analyzed by RT-PCR. Abundance of these transcripts were normalized to β-actin mRNA content.

Total RNA (5 μg) of each sample was used to synthesize complimentary DNA (cDNA) in a total reaction volume of 50 μl according to standard protocols (18). Briefly, 2 μl of random primer (Promega Corp., Madison, WI) were added to each RNA sample, which were then incubated at 85°C for 3 min, then chilled on ice for 3 min. Complimentary DNA was synthesized using 10 units of AMV Reverse Transcriptase (Promega Corp., Madison, WI) at 42°C for 30 min. The cDNA samples were then stored at -20°C until analysis. Each cDNA sample (2.5 μl) was used for PCR analysis carried out using sense and anti-sense primers (Genosys, The Woodlands, TX) in a master mix containing all of the components of the PCR reaction. The number of cycles, expected PCR product sizes and nucleotide sequences of PCR primers for each mRNA analyzed are presented in Table 1. PCR reactions were run using the Clontech hot start method which employs a monoclonal antibody to Taq polymerase. The PCR amplifications were performed using a programmable thermocycler (Perkin Elmer, Norwalk, CT) as follows: initial denaturation cycle for 5 min at 94°C, 5 min at 60°C, followed by a series of amplification cycles (2 min at 72°C, 1 min at 94°C, and 1 min at 60°C) and a final extension phase of one 10 min cycle at 72°C. Control reactions for each pair of primers in the absence of cDNA template were conducted with each amplification.

After completion of PCR amplification, 20 μl of product were electrophoretically separated in a 1% agarose gel at 80 V for 1.5 hrs. The gels were visualized using a UV lamp and photographed. Negatives of the gels were scanned (Howtek Scanner, Model No. SM3, Hudson, MN) and quantitated using Diversity One software (Protein + DNA

ImageWare Systems, Huntington Station, NY). Southern blot hybridization of the PCR products with ^{32}P-labeled antisense oligonucleotides which hybridize to a portion of the amplified sequence were used to confirm specificity of the amplified sequences.

Statistical analyses. Comparisons of densitometry data from sedentary and exercised groups were accomplished by unpaired student's t-test. All data are reported as means ± SE. Values of $P < 0.05$ were taken to indicate statistical significance. Statistical analyses were performed with SigmaStat software, version 2.0 (Jandel Scientific, San Rafael, CA).

RESULTS

Myocardial CS and GAPDH mRNA content following exercise. Total RNA was extracted from left ventricular myocardium of sedentary and exercised dogs and analyzed using RT-PCR. The amounts of each of the PCR products, normalized to β-actin abundance, are presented in Table 2. Analyses of the PCR products revealed that the abundance of mRNA encoding the glycolytic enzyme, GAPDH (Figure 1) increased nearly threefold in left ventricular myocardium following exercise from 3.44 ± 0.37 to 9.04 ± 2.62 ($P < 0.05$). The abundance of mRNA encoding the TCA cycle enzyme CS (Figure 2) also increased threefold ($P < 0.05$) following exercise (2.46 ± 0.43) relative to its abundance in left ventricular myocardium of sedentary dogs (0.82 ± 0.11).

Myocardial mRNA contents of the creatine kinase M, B, and sarcomeric mitochondrial subunits. Analyses of the PCR products revealed that mRNA content of the CK-M subunit (Figure 3) in left ventricular myocardium of exercised dogs was increased threefold ($P < 0.05$) relative to sedentary dogs (5.11 ± 1.02 vs. 1.72 ± 0.13, respectively). On the other hand, contents of CK-B (Figure 4) and sCK-mito (Figure 5) mRNAs were unaltered by exercise in left ventricular myocardium.

DISCUSSION

This study demonstrated that an acute bout of treadmill running in dogs increased the myocardial contents of three messenger RNA transcripts encoding metabolic enzymes. By providing more template for protein translation, the increased abundance of citrate synthase, glyceraldehyde 3-phosphate dehydrogenase, and CK-M subunit mRNAs could support increased synthesis and accumulation of the respective protein products in response to repeated exercise bouts. Thus, the increased activities of these metabolic enzymes in left ventricular myocardium of exercise trained dogs (21, 22) could be due, at least in part, to increased abundance of the respective mRNA transcripts.

The regulation of myocardial metabolic gene expression is poorly understood, and even less is known about responses of these genes to an exercise stimulus. However, a few recent studies have examined the effects of exercise on metabolic gene expression in skeletal muscle. Studies in rats (14) and human subjects (8) reported increased adundance of hexokinase II mRNA in skeletal muscle following exercise. Moreover, it has been demonstrated that exercise increases the abundance of glucose transporter (GLUT4) mRNA in skeletal muscle (9). These studies demonstrate that exercise is capable of increasing mRNA abundance of proteins involved in intermediary metabolism in skeletal muscle. It has also been reported that exercise increases the abundance of mRNAs encoding HSP-70 (10, 17) and endothelin-1 (11) in rat heart. However, the effects of aerobic exercise on the abundance of mRNAs encoding metabolic enzymes in myocardium have not been reported. This is the first investigation to demonstrate

exercise-evoked increases in abundance of mRNAs encoding metabolic enzymes in mammalian myocardium.

Relationship between messenger RNA abundance and protein content. Several mechanisms, alone or in combination, could increase the abundance of mRNA transcripts, including increased RNA transcription rate, accelerated post-transcriptional processing and transport of nascent mRNA, and increased mRNA stability due to decreased degradation. However, the specific mechanisms that increased mRNA transcripts in this study have not been delineated. The increases in abundance of CS, GAPDH, and the CK-M subunit mRNAs evoked by exercise in this study could contribute to the recently reported increases in myocardial contents of the proteins encoded by these mRNA species (21, 22). However, it has not been clearly established whether the abundance of a particular mRNA in myocardium can modulate the synthesis of its translation product (23). Steady state protein content is determined by the rates of translation of available mRNA and degradation of the protein. It is uncertain whether the increases in mRNAs demonstrated in this study did, indeed, increase the translation of encoded protein. The changes in mRNA abundance reported herein may constitute an early adaptive response of the cardiomyocytes to exercise, culminating after several weeks of training in increased left ventricular citrate synthase, glyceraldehyde 3-phosphate dehydrogenase, and creatine kinase protein contents (21, 22).

Creatine kinase B and sarcomeric mitochondrial subunit mRNA contents. Abundances of CK-B and sCK-mito subunit mRNA transcripts were not altered 30 min after a running bout in canine left ventricular myocardium. Although these mRNA species might not respond to running exercise, the apparent lack of an exercise response of these two mRNA species may reflect a different time course for the increase in these transcripts relative to those of the other mRNA species examined. The abundances of mRNA transcripts are likely to be only transiently altered following a stimulus (1, 6, 15, 19), and the time point for tissue sampling (30 min post-exercise) may have missed increases in CK-B and sCK-mito transcripts. It should also be recognized that genes are differentially regulated, and exercise related factors that increase expression of certain genes might not activate all metabolic enzyme genes. The time courses of CK subunit accumulation following physiological stimuli such as exercise are unknown. To address these issues, studies must be conducted to measure the abundance of mRNAs encoding the different creatine kinase subunits and other enzymes at several time points following the exercise bout.

Possible mechanism for increased abundance of mRNAs encoding metabolic enzymes. During exercise, several biochemical and biophysical changes occur in cardiomyocytes that could potentially alter the abundance of mRNA transcripts. The task of elucidating the mechanisms contributing to the responses of the myocardium to exercise is complicated by the complexity of this physiological stimulus. Exercise increases circulating concentrations of catecholamines (4) and angiotensin II (5, 20), and

increases mechanical stretch on the ventricular wall. In addition, the increased mechanical activity of the myocardium during exercise transiently increases intracellular [Ca^{2+}] which could serve as an important signal to activate transcription (2, 12). Each of these factors have been shown to modulate cardiac gene expression (2, 7, 13, 16), and, thus, could potentially activate expression of metabolic genes. However, the specific mechanisms of exercise-evoked increases in myocardial abundance of mRNA transcripts encoding metabolic enzymes remain to be delineated.

Summary. This investigation demonstrated that aerobic exercise increases the abundance of mRNAs encoding key rate-controlling enzymes of energy production and transport in canine left ventricular myocardium. The increases in abundance of these mRNA species may constitute an early adaptive response of cardiomyocytes to exercise that culminates in the previously demonstrated increases in contents of metabolic enzymes of myocardial intermediary metabolism and energy transport (21, 22).

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Table 1. *Primers and conditions for polymerase chain reaction amplification of messenger RNA transcripts.*

Gene	Primer Sequence 5'-3'	Number of Cycles	Expected Product Size (bp)
GAPDH	**(A)**ATGGACGGTGGTCATGAGG	25	110
	(S)TCAAGATTGTCAGCAATGCC		
CS	**(A)**TCCTCTGTTGGGATCTGTCC	30	192
	(S)CATGAGAGGCATGAAGGGAT		
CK-M	**(A)**TGGTAGGACTCCTCGTCACC	30	204
	(S)GGAGTACCCTGACCTCACCA		
CK-B	**(A)**ATGGGCAGGTGAGGATGTAG	30	112
	(S)GTGTTCACTCGGTTCTGCAA		
sCK-mito	**(A)**TCTTTTCACAATCCACCAGG	30	352
	(S)GTATTTGAGCGATTCTGTCG		
β-actin	**(A)**TTTGATGTCACGCACGATTTCC	25	500
	(S)TGTGATGGTGGGAATGGGTCAG		

Oligonucleotide sequences of primers, number of cycles, and expected product sizes for polymerase chain reaction amplification are indicated. (A) denotes antisense primer, (S) denotes sense primer.

Table 2. *Optical densities of PCR products from left ventricular samples normalized to optical density of β-actin PCR product.*

PCR Product	Sedentary	Exercised
CS	0.82 ± 0.11	2.46 ± 0.43*
GAPDH	3.44 ± 0.37	9.04 ± 2.62*
CK-M	1.72 ± 0.13	5.11 ± 1.02*
CK-B	0.68 ± 0.11	1.03 ± 0.14
sCK-mito	1.56 ± 0.32	2.96 ± 1.15

Means ± SEM for 6 sedentary and 5 exercise experiments. In each case, a value of 1 was assigned to the optical density of the β-actin PCR product.

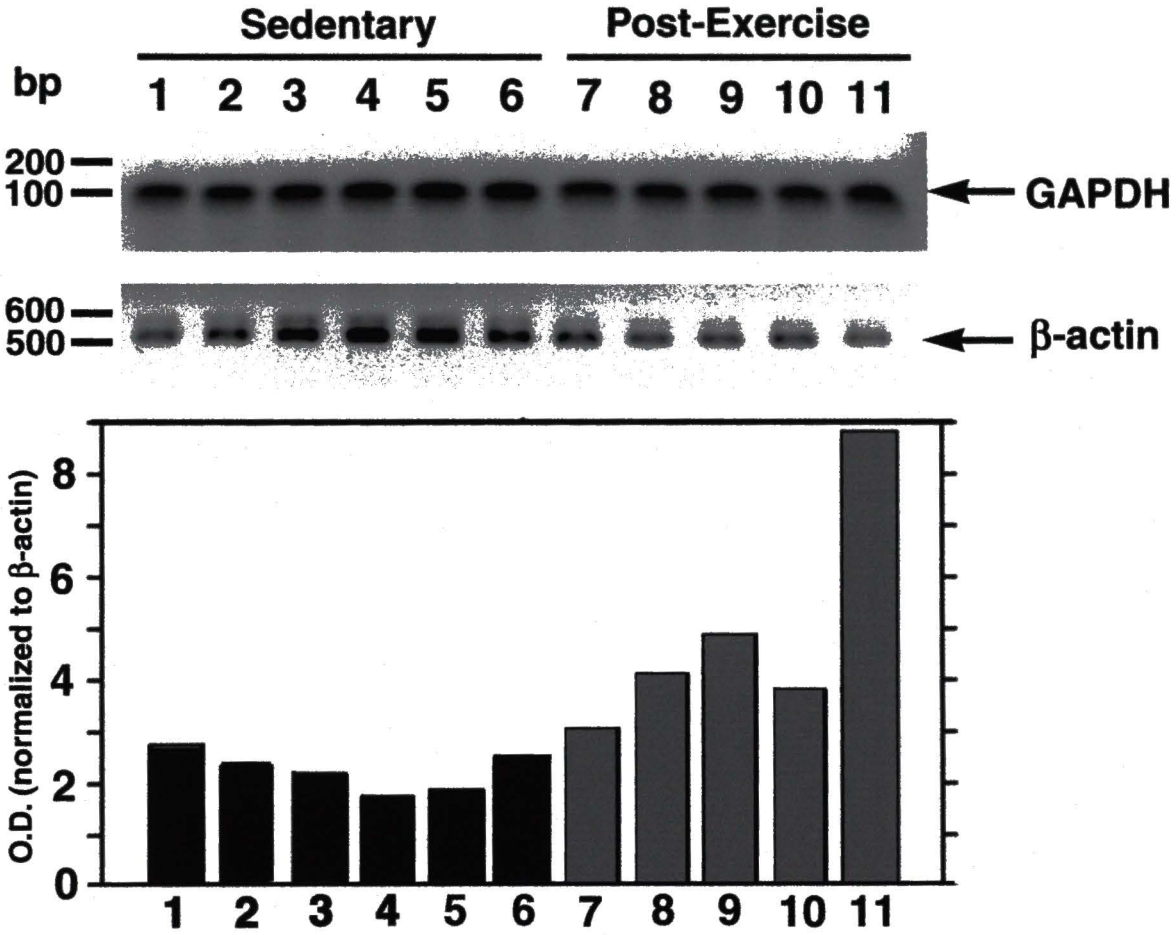

Figure 1. *Reverse transcription polymerase chain reaction (RT-PCR) analysis of Glyceraldehyde 3-phosphate dehydrogenase mRNA in left ventricular myocardium.* In this figure and in Figures 2-5, total RNA was extracted from left ventricular myocardium of sedentary and post-exercise dogs. The RT-PCR analysis was performed as described in. The top panel presents gels from the PCR product being analyzed and β-actin used to normalize the samples. Data mormalized to β-actin are presented in the bottom panel. Lanes 1-6 are from sedentary dogs; lanes 7-11 are from exercised dogs.

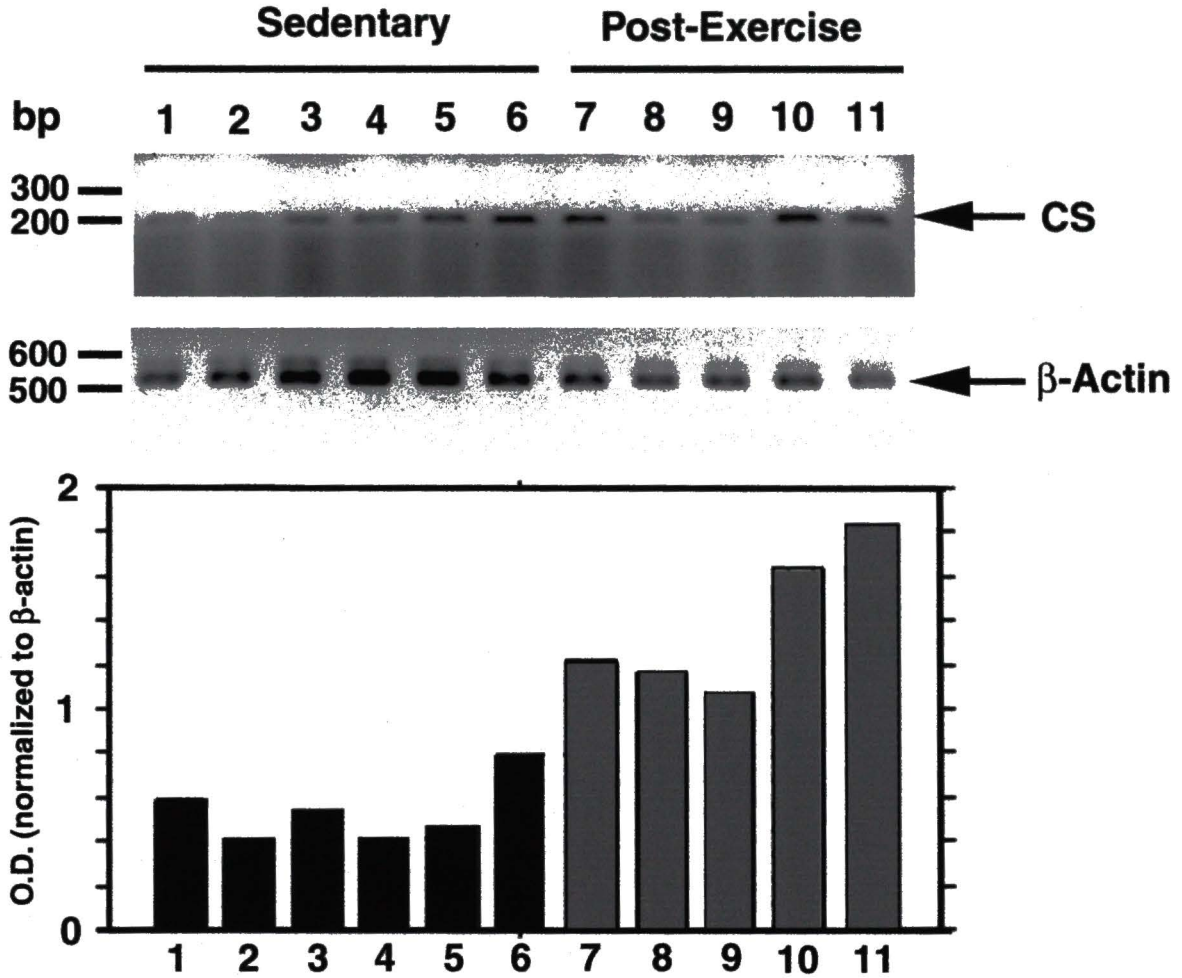

Figure 2. *Citrate synthase mRNA.* Polymerase chain reactions and data analysis were conducted as described in Figure 1 and Table 1.

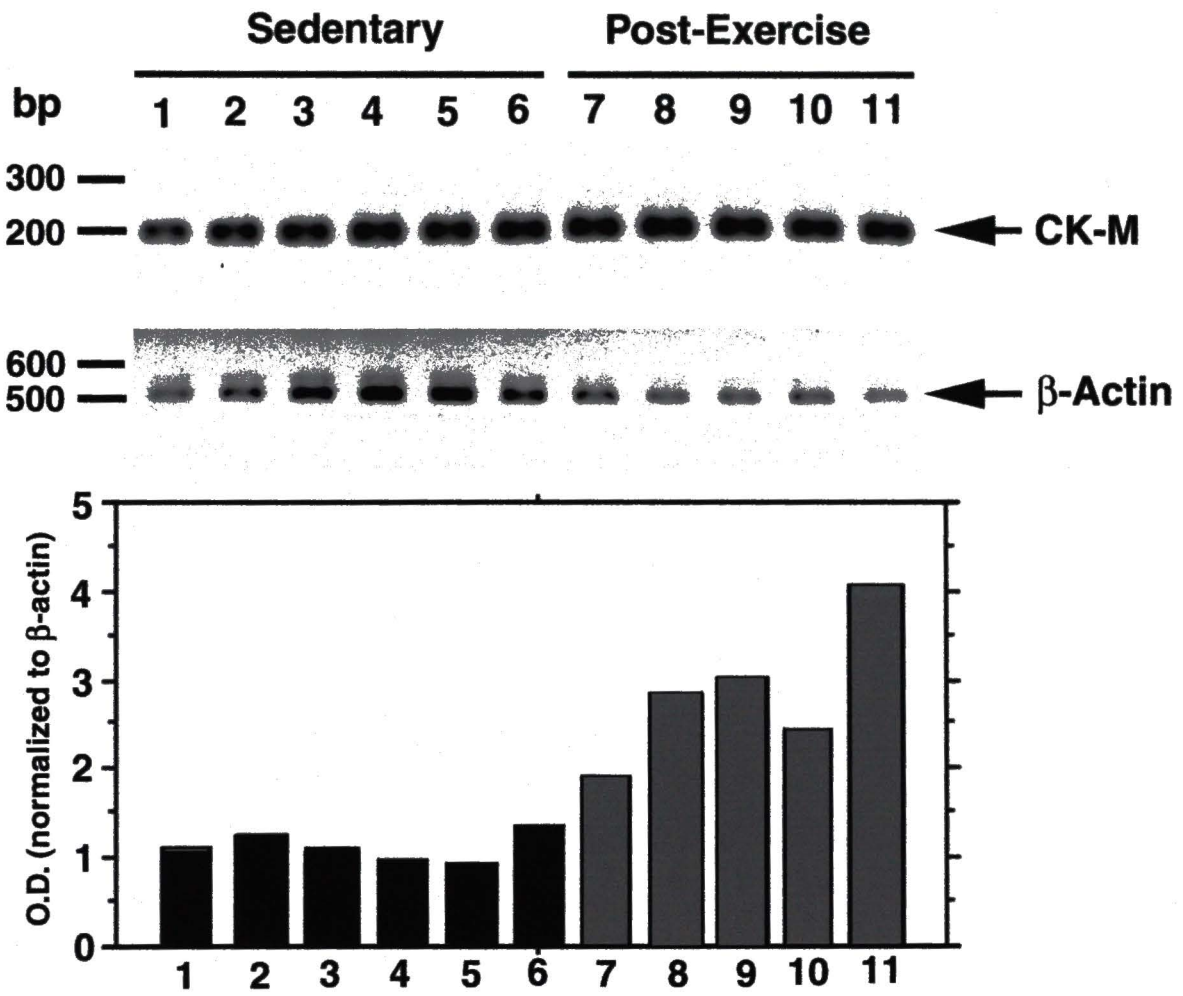

Figure 3. *Creatine kinase M subunit mRNA.* Polymerase chain reactions and data analysis were conducted described in Figure 1 and Table 1.

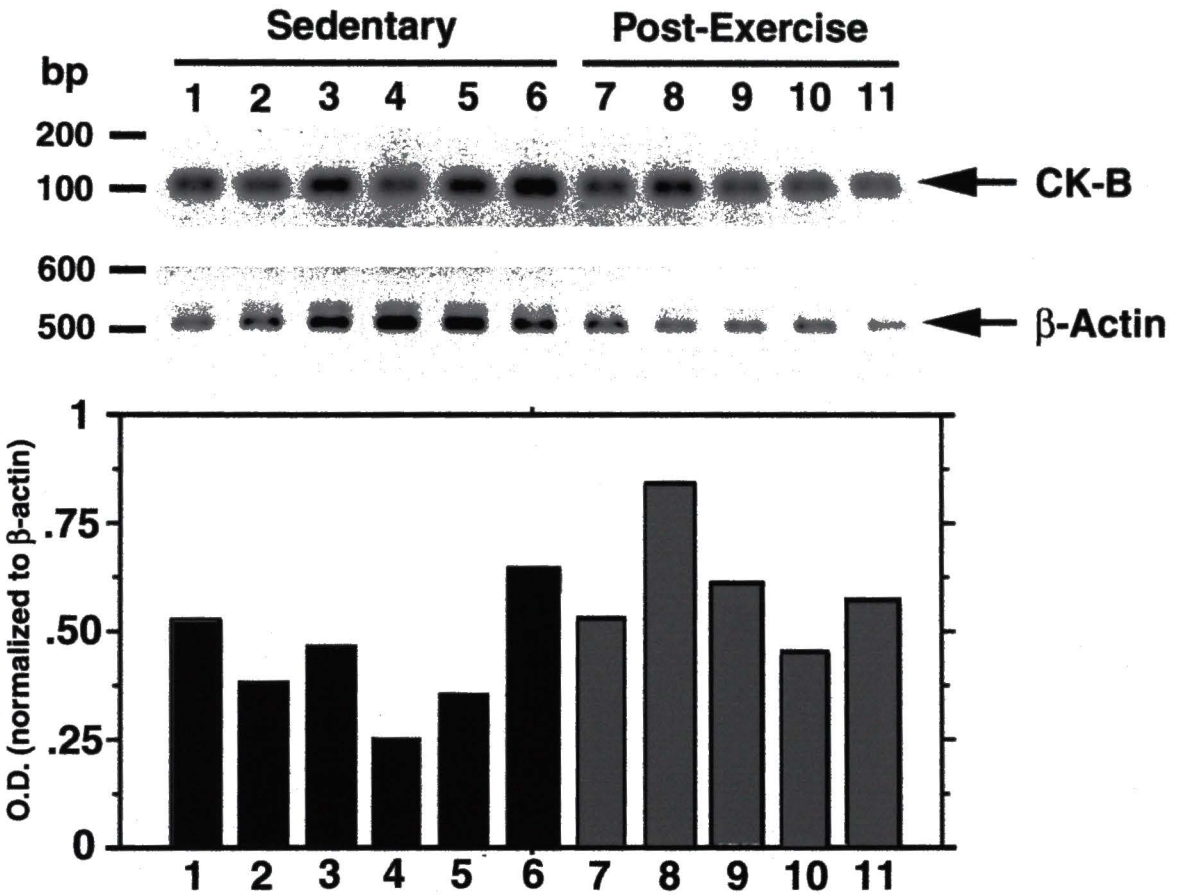

Figure 4. *Creatine kinase B subunit mRNA.* Details of PCR reaction conditions and data analysis are presented in Figure 1 and Table 1.

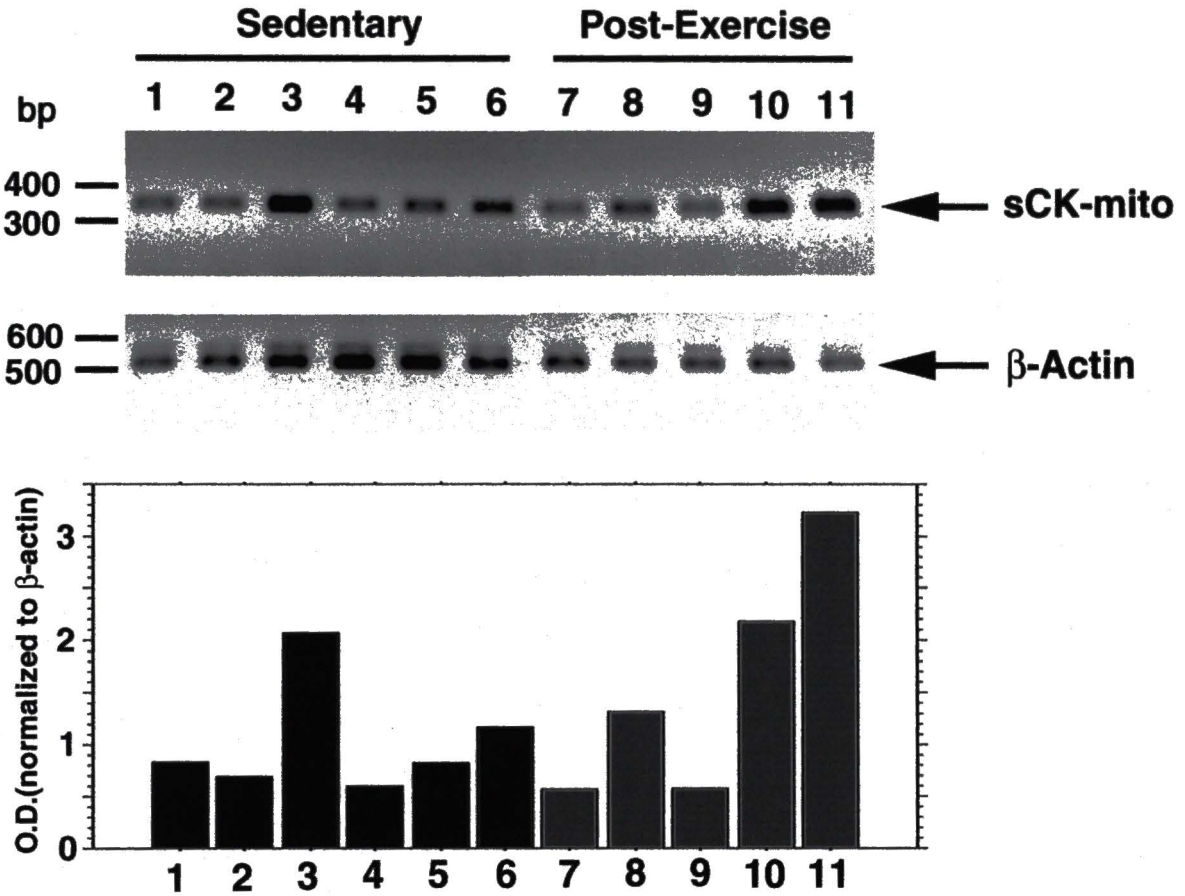

Figure 5. *Sarcomeric mitochondrial creatine kinase subunit mRNA.* Analytical procedures were conducted as described in Figure 1 and Table 1.

CHAPTER V

CONCLUSIONS

The results of the three investigations described herein support the concept that a running training program is capable of evoking significant adaptations in the metabolic systems involved in energy production and transport in canine myocardium. The first investigation demonstrated that aerobic exercise training selectively increases the capacity of rate controlling enzymes of each of the major intermediary metabolic pathways in canine left ventricular myocardium. Additionally, it was shown that the training-evoked increases in metabolic enzyme activities were due to increased contents of the enzymes, not increased substrate affinities. These metabolic adaptations likely increase the myocardium's capacity to consume metabolic fuels to support the demands of intense exercise.

The second investigation demonstrated that aerobic exercise training increases the capacity of the creatine kinase (CK) energy shuttling system in canine left ventricular myocardium. In addition, it was demonstrated that exercise training significantly increased CK_{MB} activity and its fraction of total CK activity in left ventricular myocardium. However, the CK_{MB} isoenzyme remained a minor component of the myocardial CK system. The increase of cardiomycytic energy transport capacity of

trained dogs could, in conjunction with the metabolic adaptations characterized in the first investigation, provide more energy to the ATP-dependent processes required for contractile activity.

The third investigation demonstrated that aerobic exercise increases the abundance of mRNAs encoding enzymes involved in energy production and transport in canine left ventricular myocardium. The increases in abundance of these mRNA transcripts may represent an early adaptive response of cardiomyocytes to exercise that culminates in the increased contents of metabolic enzymes of myocardial intermediary and energy metabolism reported in the first two investigations. Together the metabolic adaptations delineated in this dissertation would increase the myocardium's capacity to generate and transport energy-enriched ATP and phosphocreatine. This increased metabolic capacity could maintain myocardial energy supply:demand balances in the face of increased energy-consuming capacity in trained myocardium.

CHAPTER VI

SUGGESTIONS FOR FUTURE RESEARCH

Although this dissertation research generated novel information regarding exercise-evoked metabolic adaptations in canine myocardium, many questions remain to be addressed. For example, the findings of the first two investigations suggest that the myocardium's capacity to produce and transport energy is increased in response to training. These adaptations could enable the myocardium to better maintain its cytosolic energy reserves during times of increased energy demand. However, this hypothesis remains to be tested. Several proposed investigations, designed to extend the research presented and explore the possible mechanisms involved in the adaptations evoked by exercise training are presented below. These proposed investigations are intended: 1) to examine the role of β-adrenoceptor stimulation in the myocardial metabolic adaptations evoked by exercise training; 2) to determine if these adaptations preserve function and cytosolic energetics in ischemic myocardium; and 3) to further characterize the molecular response of the myocardium to an acute bout of exercise.

I. To test the hypothesis that stimulation of β-adrenoceptors contributes to the myocardial metabolic adaptations evoked by exercise training, a set of experiments could

be designed with protocols similar to that described in Chapters II and Chapter III. In these experiments, β-adrenoceptors would be selectively blocked during exercise with timolol, a short-acting β-adrenoceptor antagonist. Administration of timolol during exercise should effectively block β-adrenoceptor stimulation, thereby blunting the contribution of β-adrenergic signaling pathways to the adaptations evoked by exercise training. Based on preliminary studies, I anticipate that the myocardial metabolic adaptations elicited by aerobic exercise training would be attenuated by administration of timolol throughout the training program. Such a finding would substantiate the hypothesis that the myocardial metabolic adaptations evoked by aerobic exercise training in dogs are mediated, at least in part, by β-adrenoceptor stimulation.

II. In ischemic myocardium, depletion of cellular energy reserves produces contractile dysfunction and, ultimately, cellular injury and death. Studies in rats indicate that training improves function and cytosolic energetics of ischemic myocardium. However, it has yet to be determined whether the exercise-evoked myocardial metabolic adaptations characterized in this dissertation could augment energy production and transport in the cardiomyocyte to better sustain cardiac function and cellular energetics during ischemia. To test this hypothesis, experiments could be conducted in which sedentary and trained dogs are subjected to a level of coronary hypoperfusion severe enough to elicit myocardial ischemia. Cardiac contractile function and energy metabolites could be measured to determine if the myocardial metabolic adaptations evoked by exercise training are, indeed, protective against an ischemic insult. Based on

the current literature, I predict that the myocardium of exercise trained dogs would be more resistant to ischemic metabolic stress than that of sedentary control dogs.

III. Control of cardiac gene expression is a complex process, subject to regulation at several steps. In mammalian myocardium, increasing the abundance of templates required for protein synthesis could increase the protein content. The investigation described in Chapter IV indicates that aerobic exercise can increase the abundance of specific messenger RNA (mRNA) transcripts encoding myocardial metabolic enzymes in left ventricular myocardium. However, several questions remain regarding the time course of the response, the stimulus/stimuli that evoke it, and whether similar responses occur in right ventricular myocardium. To test the hypotheses that i) the time course for other mRNA transcripts is different from that of the mRNA transcripts already examined, ii) β-adrenoceptor stimulation is responsible for the increase in abundance of myocardial mRNA transcripts encoding metabolic enzymes following exercise, and iii) right ventricular mRNA transcripts encoding metabolic enzymes are increased in response to exercise, a set of experiments could be designed incorporating the techniques described in Chapter IV in combination with β-adrenoceptor antagonists.