Canine Myocardial Creatine Kinase Isoenzymes After Chronic Coronary Artery Occlusion

Scott W. Sharkey, MD; MaryAnn M. Murakami, BA; Stephen A. Smith, MD; and Fred S. Apple, PhD

Background. Creatine kinase (CK) exists as three cytosolic isoenzymes, CK-MM, CK-MB, and CK-BB, and one mitochondrial isoenzyme. Animal and human observations suggest that the CK-MB content of myocardium is dynamic and may increase in response to ischemia, but the response of the myocardial CK system to chronic coronary artery occlusion is not well-defined.

Methods and Results. We measured serial changes in myocardial total CK, percent CK-MB, and percent CK-BB before and 3 weeks after coronary artery occlusion in 17 pentobarbital-anesthetized dogs. Tissue biopsies were obtained from the left anterior descending (LAD) coronary artery myocardium, the right coronary artery (RCA) myocardium, and the circumflex coronary artery myocardium at baseline and 3 weeks after LAD occlusion (n=6), RCA occlusion (n=5), and no coronary artery occlusion (n=6). Tissue samples were assayed for total CK, percent CK-MB, and percent CK-BB. Samples were also examined by electron microscopy for evidence of ischemic myopathy. Total myocardial CK activity did not change over 3 weeks. Percent CK-MB increased significantly in the tissue supplied by the occluded artery (4.1-fold in dogs with LAD occlusion and 6.7-fold in dogs with RCA occlusion). Percent CK-BB did not change. Dogs with LAD occlusion had ultrastructural evidence of myopathic fibers interspersed with normal fibers in the LAD myocardium. Dogs with RCA occlusion had no ultrastructural evidence of myopathic fibers in the RCA myocardium.

Conclusions. Chronic coronary artery occlusion causes a pronounced change in the canine myocardial CK system that is limited to the tissue supplied by the occluded coronary artery. These biochemical alterations do not correlate with any cellular ultrastructural changes. Myocardial CK-MB content is dynamic, varies geographically within the heart, and increases rapidly after coronary artery occlusion. (Circulation 1991;84:333–340)

Creatine kinase (CK) catalyzes the reversible transfer of phosphate groups between ADP and creatine phosphate.1 This enzyme system likely plays a key role in supplying the cardiac myofibrils with the ATP required for contraction.1,2 CK exists as three cytosolic isoenzymes, CK-MM, CK-MB, and CK-BB, and one mitochondrial isoenzyme, mitochondrial-CK.3 In many mammals, the myocardium contains substantial amounts of CK-MM and CK-MB but little or no CK-BB. Pressure overload stimulates an increase in myocardial CK-MB in both the dog and rat model.4,5 Abrupt coronary artery occlusion also stimulates an acute increase in canine myocardial CK-MB in both ischemic and nonischemic tissue.6 In addition, Ingwall et al7 have recently reported that CK-MB comprised only 1.1% of total CK activity in normal human myocardium; however, in patients with coronary artery disease, CK-MB comprised 18% of total myocardial CK activity.

These animal and human observations suggest that the CK-MB content of myocardium is dynamic and may increase in response to ischemia. If myocardial CK-MB content increases after coronary artery occlusion, then the current use of the serum CK-MB to diagnose and assess the extent of acute myocardial infarction may need to be reevaluated. Further, an increase of myocardial CK-MB could have functional consequences with respect to myocardial ATP production. The CK isoenzyme response to chronic
artery occlusion has not been explored. We hypothesized that chronic coronary artery occlusion would stimulate an increase in CK-MB in myocardium supplied by the occluded coronary artery. We also hypothesized that changes in myocardial CK-MB could vary within the heart and might be associated with ultrastructural changes. Therefore, we designed this study to 1) quantitate myocardial CK-MM, CK-MB, and CK-BB at baseline and 3 weeks after coronary artery occlusion, 2) compare the effects of right coronary artery (RCA) occlusion with those of left anterior descending (LAD) coronary artery occlusion, and 3) compare the myocardial biochemical changes with those of the ultrastructural changes observed by electron microscopy. The canine model is well suited for this study because normal canine myocardium contains less than 2% CK-MB and no CK-BB; thus, an increase in either of these isoenzymes would be readily detectable.5

Methods

Surgical Procedure

Seventeen mongrel dogs of either sex weighing 17–26 kg were anesthetized with intravenous pentobarbital sodium (30 mg/kg), intubated, and ventilated with a volume respirator. Two polyethylene catheters were introduced into the right femoral artery and vein to monitor arterial blood gases and to measure left and right ventricular pressures. Supplemental oxygen was delivered as needed to maintain physiological arterial blood gas values. The heart was exposed by a left lateral thoracotomy and was suspended in a pericardial cradle. A short segment of either the proximal RCA or the mid-LAD was dissected free of the surrounding epicardium and coronary vein. Lidocaine was administered as prophylaxis against ventricular arrhythmias (2.7 mg/kg bolus, followed after 10 minutes by a 1.3 mg/kg bolus and then an infusion of 0.7 mg/min). After these preliminary procedures, the dogs were assigned to one of three treatment groups: LAD occlusion (n=6; the mid-LAD was occluded with a silk ligature), RCA occlusion (n=5; the proximal RCA was occluded with a silk ligature), and control (n=6; no coronary artery was occluded). The coronary artery occlusion precipitated ventricular fibrillation in 12 additional dogs, and they were excluded from the study. Visible cyanosis and dyskinesis of the LAD or RCA myocardium occurred in the dogs subjected to LAD or RCA occlusion, respectively.

Biopsy

In all dogs, myocardial punch biopsies were obtained from the LAD vascular bed, the RCA vascular bed, and the circumflex (CX) vascular beds at baseline (before coronary occlusion). Each biopsy specimen was split into two equal parts, one for biochemical analysis and one for morphological analysis by electron microscopy. The specimen was split randomly. (In an earlier study the specimen was split into epicardial and endocardial halves, but we noted no differences with respect to creatine kinase isoenzymes.6) The specimen designated for biochemical analysis was immediately frozen in liquid nitrogen and stored at ~45°C. The specimen designated for electron microscopic analysis was placed in a solution of phosphate-buffered glutaraldehyde fixative and was subsequently processed into plastic.

Hemodynamics

Left and right ventricular systolic and end-diastolic pressures were measured through two fluid-filled 7F pigtail catheters. Intracardiac pressures and heart rate were recorded on a six-channel physiological recorder (model 2600 S, Gould, Cleveland, Ohio). Measurements were made at baseline with the chest and pericardium open and at 15 minutes after coronary artery occlusion (for control dogs, 15 minutes after the baseline biopsy procedure). The catheters were removed, the thoracotomy was closed, and the dogs were allowed to recover. Antibiotics (1 g ticarcillin and 40 mg gentamicin) were administered daily for 3 days after surgery.

Three-Week Measurements

At 3 weeks, the dogs were reanesthetized and the chest was reopened. Pigtail catheters were again advanced into the left and right ventricles from the right femoral artery and vein, respectively. Intracardiac pressures and heart rate were recorded as described above. Repeat myocardial biopsies were obtained as described above. Care was taken to obtain the biopsy specimen from an area of grossly normal myocardium. Areas of dense scar were not sampled.

Biochemical Analysis

Muscle homogenates were prepared in ice-cold phosphate buffer (200 mmol/l potassium phosphate, pH 7.4, containing 5 mmol/l EGTA, 5 mmol/l β-mercaptoethanol, and 10% glycerol [wt/vol]) to release both cytoplasmic and mitochondrial enzymes. Total CK activities in serum and muscle homogenates were measured at 37°C on a kinetic enzyme analyzer with N-acetylcysteine–activated reagents.8 Homogenate activities in excess of 1,000 units/l were diluted with buffer so that the total activity did not exceed the linearity of the system. Protein concentrations in the muscle homogenates were determined by the procedure of Lowry et al.9 Total CK activity was expressed per milligram of total protein (units/mg protein).

CK isoenzymes were separated by electrophoresis on agarose and were visually inspected after reaction with CK reagents as described by the manufacturer (Corning ACI, Ciba Corning Diagnostic, Medfield, Mass.). The agarose system was linear to 1,000 units/l. Muscle homogenates were electrophoresed undiluted if the total CK activity was less than 1,000 units/l. Any sample in which the total CK activity exceeded linearity was diluted with buffer to give an activity of 880±100 units/l. Also, duplicate samples were electrophoresed and incubated with and with-
out substrate (creatine phosphate) to rule out non-CK artifacts. CK-MB purified from human heart was used as a standard, and a human CK isoenzyme control (MM, MB, BB, Beckman Instruments, Brea, Calif.) was used to identify the isoenzyme migration. Within-run precision (coefficient of variation) for CK-MB was 7.5% (n=18), and between-run precision was 8.8% (n=38). Percentages of CK-MM, CK-MB, and CK-BB (%CK-MM, %CK-MB, and %CK-BB, respectively) were quantified by scanning densitometry.

Since the total CK activity of the myocardial homogenates was diluted to 800±200 units/l before CK-MB and CK-BB determination and since the electrophoretic system's lower limit of sensitivity was 3 units/l, myocardial %CK-MB and %CK-BB could not be detected at 0.4% or less of total CK activity. Muscle samples with 0.4% or less CK-MB or CK-BB activity were assigned a value of 0.4%.

**Electron Microscopic Analysis**

Plastic-embedded thick sections were stained with toluidine blue, and representative areas were selected for thin sectioning and ultrastructural examination. Photographs were taken at various magnifications and examined for evidence of ischemic myopathic changes. The electron microscopist (S.A.S.) was blinded to the identify of the dog as well as to the location and timing of the samples examined. Electron microscopic analysis was performed on 15 dogs (five each from the control group, the LAD occlusion group, and the RCA occlusion group).

**Data Analysis**

At baseline, data on all 17 dogs were analyzed together, since no coronary artery had yet been occluded in any dog. At 3 weeks, data were analyzed by treatment group (described above). A two-way analysis of variance (ANOVA) was used to make comparisons between the control dogs and the dogs subjected to LAD or RCA occlusion. Within each treatment group, comparisons were also made among the LAD myocardium, the CX myocardium, and the RCA myocardium using a one-way ANOVA. If the ANOVA showed significant differences, then the various pairs of data were compared using Fisher's protected least significant difference method for multiple comparisons. Significant differences (p<0.05) are indicated on the figures and tables.

**Results**

**Hemodynamics**

The intracardiac pressures at baseline, at 15 minutes after coronary artery occlusion, and at 3 weeks are summarized in Figure 1. The left ventricular end-diastolic pressure showed no significant change, even in dogs subjected to LAD occlusion. Similarly, right ventricular end-diastolic pressure did not change significantly, even after RCA occlusion. At all three sampling points, both left ventricular and right ventricular systolic pressures were relatively constant. Heart rate was not significantly different among the three groups.

**Myocardial Total CK, %CK-MB, and %CK-BB**

Myocardial total CK activity at baseline (n=17) was as follows: LAD, 13.5±12.7 units/mg; CX, 12.1±7.4 units/mg; RCA, 8.2±10.3 units/mg. No significant differences were present among the LAD, the CX, or the RCA myocardium at baseline. The change in myocardial total CK activity from baseline to 3 weeks is presented in Figure 2. No significant changes in total CK activity occurred over this 3-week period.

Myocardial %CK-MB at baseline (n=17) was 2.7% or less and was uniformly distributed within the heart (Table 1). At 3 weeks, %CK-MB of the control dogs had increased slightly in the LAD, CX, and RCA myocardium, but this was not statistically significant (Figure 2 and Table 1). In the control dogs, %CK-MB remained uniformly distributed among the three vascular distributions (Figure 2 and Table 1). In contrast, when dogs were subjected to a 3-week coronary artery occlusion, %CK-MB was no longer uniformly distributed within the heart (Figure 2). This was due to an increase in %CK-MB of the myocardium supplied by the occluded coronary artery. Dogs subjected to LAD occlusion exhibited a
4.1-fold increase in %CK-MB of the LAD myocardium, which was statistically significant when compared with control dogs (Figure 2 and Table 1). Similarly, dogs subjected to RCA occlusion exhibited a 6.7-fold increase in %CK-MB of the RCA myocardium, which was statistically significant when compared with the control dogs (Figure 2 and Table 1). Percent CK-MB of the nonischemic tissue of dogs subjected to either LAD or RCA occlusion did not change significantly over 3 weeks when compared with the control dogs (Figure 2 and Table 1).

Myocardial %CK-BB at baseline was usually undetectable (Table 1). No significant changes in myocardial %CK-BB were measurable over this 3-week period.

**Electron Microscopy**

Control dogs showed no evidence for ischemic myocardial injury at 3 weeks. At three weeks, all dogs subjected to LAD occlusion showed some degree of ischemic injury in the LAD myocardium, with interspersed areas of normal-appearing fibers (Figure 3, top panel). RCA and CX myocardium were normal in these dogs. Dogs subjected to RCA occlusion did not show any ultrastructural abnormalities in RCA myocardium at 3 weeks (Figure 3, bottom panel). LAD and CX myocardium were also normal in these dogs.

**Discussion**

A 3-week occlusion of either the LAD or RCA stimulated a significant increase in %CK-MB of the myocardium supplied by the occluded coronary artery. The stimulus for the increased %CK-MB observed in our study is unclear. It is not possible to know whether the myocytes sampled in this study were chronically ischemic. The enzyme changes observed could have been a response to the insult of the acute ischemia occurring at the time of the coronary artery occlusion. Other mechanisms might also be operating. For example, the CK-MB changes observed might be part of a hypertrophic response of viable myocytes adjacent to an area of necrosis. Altered ventricular end-diastolic pressures do not appear to trigger the increased %CK-MB, since we did not observe any significant differences in these values between control dogs and dogs subjected to a

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<th>Table 1. Percent Creatine Kinase–MB and Percent Creatine Kinase–BB in Myocardium at Baseline and After 3 Weeks</th>
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<td><strong>LAD myocardium</strong></td>
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Values are mean±SD. LAD, left anterior descending coronary artery; CX, left circumflex artery; RCA, right coronary artery; %CK-MB, percent MB isoenzyme of creatine kinase (CK); %CK-BB, percent BB isoenzyme of CK; n, number of dogs.

*<p value=0.02 vs. control LAD myocardium at 3 weeks; †p<0.003 vs. control RCA myocardium at 3 weeks.
Figure 3. Top panel: Representative electron micrograph from myocardium in the distribution of the left anterior descending coronary artery obtained 3 weeks after occlusion. Percent creatine kinase-MB in this biopsy was 14.2%. Myofibril (MF) loss is evident, and the Z lines (arrowhead) are abnormally dense. An intercalated disc (arrow) is disrupted. Mitochondria (M) and glycogen (G) are normal. Magnification, \( \times 7,600 \); bar, 1 \( \mu \text{m} \). Bottom panel: Representative electron micrograph from myocardium in the distribution of the right coronary artery obtained 3 weeks after occlusion. Percent creatine kinase-MB in this biopsy was 21.4%. The myofibrils (MF) and Z lines (arrowhead) are normal. Mitochondria (M) are abundant, and glycogen (G) is plentiful within the sarcoplasm. An intercalated disc (arrow) is well preserved. Magnification, \( \times 7,600 \); bar, 1 \( \mu \text{m} \).
coronary artery occlusion. Furthermore, a change in a hemodynamic parameter, such as an increase in the left ventricular end-diastolic pressure after LAD occlusion would be expected to have an impact on the entire left ventricle and not just the myocardium supplied by the occluded coronary artery. Thus, it appears that local factors in some way modulate the signal to increase CK-MB synthesis.

Human coronary artery occlusion is also associated with an increase in %CK-MB of myocardium. Ingwall et al have reported that normal human myocardium contains 1.1% CK-MB but that myocardium from patients with coronary artery disease contains 18% CK-MB. The M and B subunits of CK are under separate genetic control and assemble to form three dimeric isoenzymes: CK-MM, CK-M, and CK-BB. CK gene expression is tissue specific, with the B subunit expressed in adult brain, smooth muscle, and heart and the M subunit expressed in differentiated skeletal and cardiac muscle. CK gene expression is developmentally regulated. Proliferating myoblasts express the B subunit, whereas differentiated muscle cells express the M subunit. Acute myocardial ischemia induces a significant increase in the myocardial CK-B subunit mRNA and a concomitant decline in the CK-M subunit mRNA. Similar changes in the synthesis rates of the CK-B and CK-M subunits would result in an increase in %CK-MB isoenzyme.

In a previous study in the canine model, using the same biopsy sites, we demonstrated that LAD occlusion stimulated an acute increase in myocardial %CK-MB. At baseline, the myocardial CK-MB was 1.3%, which increased to 3.6% at 5 hours after the LAD occlusion. In contrast to the current study, the early increase in %CK-MB occurred in both the occluded LAD myocardium and the nonoccluded CX myocardium. Acute occlusion of a canine coronary artery places an immediate stress on the normal myocardium. This stress involves both an increase in the end-diastolic fiber length and an increase in the systolic fiber shortening. These mechanical changes in the nonischemic tissue are proportional to the size of the ischemic zone and usually persist chronically. If such mechanical factors were stimulating an increase in %CK-MB, one would expect the observed acute increase in nonischemic myocardium %CK-MB to persist at 3 weeks. This disparate acute and chronic behavior of the nonischemic tissue with respect to %CK-MB cannot be explained by this study.

Whether the increase in myocardial %CK-MB has any functional importance is unknown. CK-MB significantly exceeds CK-MM in its affinity for the substrate ADP during the reaction that forms ATP. Also, CK-MB exhibits greater enzyme activity than CK-MM in an acid environment, although this difference is small. Myocardial ischemia also induces an acute increase in the level of mRNA coding for stress protein 71 in the dog heart and an increase in heat shock protein 70 in the rabbit heart. Stress proteins are postulated to afford protection to cells from a variety of insults, including hypoxia. Thus, the increase in CK-MB may be a part of a general myocyte response to the stress of ischemia.

The biochemical changes observed in this study were not associated with any identifiable ultrastructural changes. Occlusion of the LAD produced a grossly visible heterogeneous infarction, with areas of scar interspersed with areas of viable-appearing myocardium. This correlated well with the electron microscopic observation that tissue from the area of LAD occlusion contained both normal-appearing muscle cells and myopathic cells. Presumably, the normal-appearing cells were the source of increased %CK-MB in this sample, although specific staining for CK-MB was not performed. Immunohistochemical staining in a dog model of LAD occlusion has shown markedly decreased CK-M and CK-B staining from the area of scar but normal staining of the interspersed viable cells. Occlusion of the RCA did not result in a grossly visible area of right ventricular infarction. This is presumably due to the fact that the right ventricular myocardial biopsies were obtained from the right ventricular outflow tract. This myocardium is likely protected from necrosis by collateral flow from overlapping vessels from the LAD. The electron microscopic analysis of the RCA myocardium after RCA ligation revealed ultrastructurally normal-appearing cells in the presence of a striking increase in %CK-MB. Other investigators have also found that significant biochemical alterations can occur within the myocardial cell in the absence of any change in myocyte ultrastructure.

Certain limitations of this study deserve comment. First, we measured CK-MB as a percent of total CK and did not quantitate the actual mass of the CK-MB or the synthesis rates of the CK-M or CK-B subunits. Selective loss of CK-MM from ischemic or necrotic cells would result in an increase in the percent myocardial CK-MB. However, immunohistochemical techniques have shown that selective loss of CK-MM from cells surrounding an area of myocardial necrosis does not occur. A decrease in the synthesis rate of the CK-M subunit might also cause an increase in %CK-MB but would also be expected to cause a decrease in total myocardial CK activity. Second, if the increase in myocardial %CK-MB was due to an increase in the synthesis rate of the CK-B subunit, then an increase in the myocardial %CK-BB should also occur. However, we could not demonstrate any increase in %CK-BB. Electrophoresis is a rather insensitive method for the measurement of CK-BB, and small increases might not be detected. Third, we did not observe a significant change in total CK activity even in the myocardium supplied by the occluded coronary artery. Other investigators have observed that coronary artery occlusion in the rabbit or the dog is associated with depression of total CK activity in the infarcted myocardium. These studies differ from ours in that these investigators mea-
(bufurred CK at 24 hours and took biopsy specimens from the most intensely necrotic region as indicated by electrocardiographic mapping. Our biopsies were made at 3 weeks and intentionally avoided the areas with intense scar formation. This may explain why we found total CK activity to be unchanged. A final limitation deserving comment concerns the changes in myocardial %CK-MB observed in control dogs (Figure 2 and Table 1). Between baseline and 3 weeks, %CK-MB increased (approximately 1.8-fold) in the LAD, RCA, and CX myocardium. This increase, while not statistically significant, suggests that the procedure itself (anesthesia, surgery, or biopsy) may stimulate a slight increase in myocardial CK-MB. Even so, the increases stimulated by coronary occlusion were greater and statistically different from those observed in control dogs.

The diagnostic hallmark of acute myocardial infarction is a rise in the serum total CK-MB.32 It is widely accepted that human myocardium contains a significant quantity of CK-MB, yet the myocardial samples on which these observations were based were obtained either at the time of open heart surgery or autopsy.33 Therefore, these samples are not necessarily representative of normal human myocardium. As stated earlier, Ingwall et al7 have reported that normal human myocardium contains only 1.1% CK-MB but that ischemic human myocardium contains 18% CK-MB. The data in our study further suggest that myocardial CK-MB content is dynamic, varies geographically within the heart, and may increase rapidly in response to coronary artery occlusion. If human myocardial CK-MB also behaves in this way, then the use of serum CK-MB to diagnose and assess the extent of acute myocardial infarction may be more complicated than originally anticipated.

Acknowledgments

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