Myosin-Binding Protein C Phosphorylation, Myofibril Structure, and Contractile Function During Low-Flow Ischemia

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Background—Contractile dysfunction develops in the chronically instrumented canine myocardium after bouts of low-flow ischemia and persists after reperfusion. The objective of this study is to identify whether changes in the phosphorylation state of myosin-binding protein C (MyBP-C) are a potential cause of dysfunction.

Methods and Results—During low-flow ischemia, MyBP-C is dephosphorylated, and the number of actomyosin cross-bridges in the central core of the sarcomere decreases as thick filaments dissemble from the periphery of the myofibril. During reperfusion, MyBP-C remains dephosphorylated, and its degradation is accelerated.

Conclusions—Dephosphorylation of MyBP-C may initiate changes in myofibril thick filament structure that decrease the interaction of myosin heads with actin thin filaments. Limiting the formation of actomyosin cross-bridges may contribute to the contractile dysfunction that is apparent after low-flow ischemia. Breakdown of MyBP-C during reperfusion may prolong myocardial stunning. (Circulation. 2005;111:906-912.)

Key Words: myocytes ■ myosins ■ phosphorylation ■ contractility ■ ischemia

Transient episodes of low-flow ischemia provoke varying degrees of reversible contractile dysfunction in canine myocardium in the absence of either ischemic necrosis or apoptosis. Although the subcellular mechanism(s) that regulate(s) contractile function remains unresolved, changes in calcium handling and the properties of myofilament proteins likely are involved in evolving mechanical dysfunction. In large-animal models, contractile dysfunction displayed by ischemically stunned canine and porcine myocardium has been attributed to intrinsic changes in myofilament sensitivity to calcium that may develop as a consequence of the dephosphorylation of phospholamban and troponin I after bouts of low-flow ischemia. Because proteolysis of troponin I has not been observed consistently in larger animals, mechanisms that appear to mediate contractile dysfunction in rodents may not modulate myocardial function in larger animals during and after low-flow ischemia. Alterations in other cytoskeletal and myofibrillar proteins also are likely to influence contractile function.

Disassembly of myofibrillar thick filaments parallels the reduction of coronary flow and contractile function in a canine model of low-flow ischemia. How individual thick filament disruption evolves and whether it contributes to contractile dysfunction in this model is unclear, but recent experiments implicate myosin-binding protein C (MyBP-C) in regulating both thick filament structure and function and imply that changes in the phosphorylation of MyBP-C may affect contractility. Phosphorylation of a unique amino-terminal sequence in cardiac MyBP-C is believed to have a role in modulating contractile function in the heart. Decreasing extracellular calcium has been reported to dephosphorylate MyBP-C, increase the packing density of myosin rods, and decrease the orientation of myosin heads in thick filaments, thereby reducing calcium-activated force generation. Conversely, protein kinase A–mediated phosphorylation of MyBP-C reduces myosin packing, increases the order of myosin heads, and promotes force production.

Such observations suggest that reductions in coronary flow may alter the phosphorylation state of MyBP-C, modify myofibril thick filament structure, and contribute to contractile dysfunction. This study identifies changes in the phosphorylation pattern of MyBP-C and the structure of the thick filament that are compatible with this hypothesis.

Methods

Experimental Animal Preparation

Mongrel dogs of both sexes weighing 23 to 36 kg were studied with the use of protocols that conform to the principles outlined in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication 85-23, 1996) and were approved by the Institutional Animal Care and Use Committee of Northwestern University. Twenty-three animals were instrumented after an overnight fast and a 3-week period of on-site conditioning, as described previously. One to 2 weeks later, left circumflex (LC) coronary flow was reduced under continuous...
hemodynamic monitoring while the animal was lightly sedated and resting in a sling to which it had previously been acclimated. The LC was occluded sufficiently to reduce LC segment shortening by 50% for 2 hours (n=7) or 75% for 5 hours (n=13). Three dogs in the 2-hour group and 5 dogs in the 5-hour group were euthanized (pentobarbital and potassium chloride) immediately after the partial occlusion. Four animals in the 2-hour group were reperfused for 2 hours before euthanasia, and 1 animal was reperfused for 48 hours. Four dogs in the 5-hour group were reperfused for 24 hours before euthanasia; 4 other animals in the 5-hour group were reperfused until segment shortening returned to baseline, preocclusion values. Two instrumented animals and 1 uninstrumented animal were euthanized as control preparations. Coronary blood flow was monitored with ultrasonic flow probes and periodically by infusing fluorescently labeled microspheres. Samples for fine structure and biochemical analyses were obtained in situ immediately after euthanasia or after reperfusion and included “test” (LC) and “remote” (left anterior descending [LAD]), normally perfused myocardium. Hemodynamic and segmental function measurements were made periodically during low-flow ischemia/reperfusion and just before euthanasia.

**Isoelectric Focusing and SDS-PAGE**

**Gel Electrophoresis**

Frozen tissue derived from test and remote sites was lysed in extraction buffer and electrophoresed in either 1- or 2-dimensional isoelectric focusing (IEF) gels to separate the 4 possible phosphorylated forms of cardiac MyBP-C, as described in detail previously. The gels were transferred to nitrocellulose and Western blotted with a polyclonal antibody specific for the unique COC1 region of cardiac MyBP-C. Paired aliquots of tissue extract also were Western blotted after separation on 4% to 15% SDS-PAGE linear gradient slab gels to identify any potential degradation fragments of MyBP-C. The relative amount of MyBP-C and each of its phosphorylated forms was determined with the use of laser densitometry and was expressed as a percentage of the total MyBP-C. The relative distribution of phosphorylated MyBP-C content in test and remote tissue extracts was compared with sham samples with the use of a 1-way ANOVA followed by post hoc testing with the Student-Newman-Keuls test; P<0.05 was considered statistically significant.

**Immunofluorescence Microscopy**

The distribution of MyBP-C and fibrillar actin was monitored by double-label laser scanning confocal microscopy. Five-micrometer frozen sections were preserved for 30 minutes in 4% paraformaldehyde-borohydride-periodate fixative at 4°C. Sections were rinsed 3 times in PBS plus 10% normal goat serum and incubated for 1 hour at room temperature in a 1:200 dilution of a polyclonal antibody that recognizes the COC1 epitope of MyBP-C. Sections were rinsed x3 in PBS plus 10% normal goat serum for 1 hour and then incubated in a 1:300 dilution of goat anti-rabbit IgG labeled with FITC and a 1:200 dilution of rhodamine phalloidin to stain MyBP-C and fibrillar actin, respectively. Sections were washed in PBS and mounted with DAPI-labeled medium and viewed in a Zeiss 510 confocal microscope.

**Electron Microscopy**

Hearts from euthanized animals were bisected in situ, and 0.5-μm-thick test and remote tissue specimens were fixed in cacodylate-buffered 2% glutaraldehyde (pH 7.4) and processed for transmission electron microscopy. Other bisected samples were rapidly frozen in isopentane cooled to liquid nitrogen temperature and then freeze-substituted in 2% osmium tetroxide in absolute acetone at ~80°C, dehydrated, and embedded in epoxy resin. The bisected samples were preserved in contracture so that the number of activated cross-bridges could be estimated from high-resolution electron microscopy negatives of longitudinally imaged myofilaments. To quantify the number of cross-bridges that spanned adjacent thick and thin filaments, an electronic raster was inserted parallel and adjacent to the long axis of a centrally located thick filament in the C-zone of the A-band. The C-zone was digitally scanned, and then the raster was placed next to a neighboring thin filament in the same region of the A-band and rescaned. The density scans derived from adjacent thick and thin filament pairs were superimposed over one another. Those densities that overlapped in both scans represent cross-bridges, whereas those densities adjacent to the thick filament that failed to span the distance between the thick and thin filaments were considered partially elevated myosin heads. The potential number of cross-bridges of a thick/thin filament pair was calculated by summing the cross-bridges and myosin heads tabulated from both digital scans. Mean cross-bridge value for each animal was derived from...
Figure 1 illustrates hemodynamic recordings obtained before occlusion (baseline) and during and after 5 hours of low-flow ischemia. When coronary flow in the LC was reduced 75%, segment shortening decreased in parallel with coronary flow (compare Figure 1a with 1b). The normally perfused LAD segment showed no change in its segment shortening profile (compare Figure 1a with 1b). The normally perfused LAD segment function, % shortening 15.5±3.2 4.3±0.6** 8.6±2.5** 15.8±4.6
Thick filament disassembly, +/+ -- ++ ++ --
Dephosphorylated MyBP-C, % of total 1.5±0.4 20.2±2.7†† 27.8±2.6†† 2.0±0.6
Potential cross-bridges,¶ No. per 100 nm of A-band 4.17±0.14 4.09±0.08 4.21±0.11 4.12±0.06
Measured cross-bridges,§ No. per 100 nm of A-band 3.27±0.06 2.44±0.05** 2.13±0.07** 3.18±0.33

LFI indicates low-flow ischemia; n, number of animals.
*Sham-instrumented animals.
†LC shortening reduced 75% for 5 hours (LFI).
‡LC shortening reduced 75% for 5 hours and reperfused for 24 hours (LFI).
§Animals reperfused for 6 and 21 days after LFI.
¶Percentage of total MyBP-C ±SEM.
††Potential (cross-bridgesþmyosin heads) cross-bridges per 100 nm of A-band.
#Measured cross-bridges engaged per 100 nm of A-band.
**P<0.05 vs sham; ††P<0.01 vs sham.

Results

Hemodynamics and Phosphorylation State of MyBP-C
Figure 1 illustrates hemodynamic recordings obtained before occlusion (baseline) and during and after 5 hours of low-flow ischemia. When coronary flow in the LC was reduced 75%, segment shortening decreased in parallel with coronary flow (compare Figure 1a with 1b). The normally perfused LAD segment showed no change in its segment shortening profile after partial coronary occlusion (Figure 1a, 1b). After the coronary occluder was deflated when aortic and coronary pressures were once again equal (Figure 1a, 1c), circumflex segment shortening remained depressed ~60%, whereas coronary flow returned to normal (Figure 1c). Baseline microsphere blood flows in test and remote subendocardium averaged 0.75±0.09 and 0.77±0.07 mL/min per gram, respectively. The test/remote flow ratios in individual animals averaged 0.99±0.09 at baseline, 0.35±0.12 at the time of euthanasia in nonreperfused animals, and 0.89±0.12 at the time of euthanasia in reperfused animals. In most instances, segmental function returned to within 10% of preocclusion values 4 days after partial occlusion (Figure 1e). In the experiment illustrated in Figure 1, 2 weeks of reperfusion was required to fully restore segment shortening to preocclusion, baseline values (Figure 1d, 1e; Table). During that 2-week period, the remote, LAD segment displayed no significant changes in its segment shortening profile (Figure 1a to 1d). In contrast, reducing coronary flow 50% for 2 hours decreased segment shortening proportionately (from a baseline value of 17.5±0.9% to 8.7±2.1%; n=3). After 2 hours of reperfusion, both coronary flow and contractile function improved to ~85% of baseline, indicative of short-term hibernation.

Sample extracts prepared from hearts in which coronary flow was reduced for 5 hours displayed significant increases in the amount of unphosphorylated MyBP-C compared with either remote or sham-instrumented myocardial samples (Figure 2; Table). A small amount of triphosphorylated MyBP-C (<5% of total) also was observed in test extracts. In the remote, normally perfused left ventricle, there was a more pronounced increase in triphosphorylated MyBP-C (~20%), with only a small rise (<5% of total) in the dephosphorylated form compared with sham extracts that disclosed only monophosphorylated and diphosphorylated MyBP-C (Figure 2). No significant decline in segment function developed until the amount of dephosphorylated MyBP-C exceeded 8% of total MyBP-C; thereafter, contractile function decreased precipitously with increasing amounts of unphosphorylated MyBP-C (Figure 3a). In contrast, remote, normally perfused myocardium displayed insiginificant changes in MyBP-C dephosphorylation during and after severe

![Figure 2](image_url)

Figure 2. Western-blotted, 1-dimensional IEF gel separates the 4 forms of MyBP-C on the basis of their phosphate content: 0, 1, 2, 3 phosphates. Tissue extracts were prepared from a sham-instrumented dog heart and from test and remote regions of the left ventricle of an animal subjected to severe low-flow ischemia for 5 hours.
low-flow ischemia (Figure 3a). In reperfused heart, dephosphorylated and triphosphorylated forms of MyBP-C remained elevated in the stunned myocardium (Figures 3a, 7b; Table). When coronary flow was reduced only moderately (ie, 50% for 2 hours), dephosphorylated MyBP-C increased to 14.9 ± 2.3% (n = 3) of total MyBP-C; after 2 hours of reperfusion, <6% of MyBP-C remained unphosphorylated in the short-term hibernating hearts.

Myofibril and Cross-Bridge Structure
Myofibril disorder developed during low-flow ischemia and persisted after reperfusion (Figure 4). Thick filaments and, to a lesser extent, thin filaments appeared to disassemble from the periphery of the myofibril (Figure 4a, 4b). In many instances, the length of the A-band also decreased, especially after reperfusion, suggesting a partial disassembly of thick filaments from their ends to approximately the edge of the C-zone of the A-band (Figure 4b). The degree of disorder also appeared correlated with the duration and severity of low-flow ischemia and the amount of unphosphorylated MyBP-C (Table).

Because changes in the MyBP-C phosphorylation state have been reported to affect the structure of the thick filament and the orientation of its myosin heads, the number of actomyosin cross-bridges was estimated from rapidly frozen myocardium. In the hearts of sham-operated animals, the density of detectable myosin heads was 4.17 ± 0.14 per 100 nm of thick filament length (Figure 5; Table). The frequency of myosin heads within the C-zone was not altered significantly regardless of the duration of ischemia or the length of reperfusion; however, the number of actomyosin cross-bridges (ie, a myosin head attached to an actin binding site) was substantially decreased after 5 hours of low-flow ischemia (Table). Furthermore, the relative frequency of cross-bridges appeared directly correlated with the degree of segment shortening (Figure 3b) and inversely related to the percentage of dephosphorylated MyBP-C (Figure 3c). When flow was reduced moderately (ie, 50% for 2 hours), cross-bridge numbers declined somewhat (2.89 ± 0.23 per 100 nm of A-band; n = 3). Restoration of segmental function to baseline values after reperfusion was paralleled by the rephosphorylation of MyBP-C, an increase in cross-bridge density, and the reappearance of intact myofibril structure (Figure 3b, 3c;
Table). Myocardial samples obtained from the remote, nonischemic zone of the left ventricle displayed only small changes in the amount of dephosphorylated MyBP-C (i.e., $<5\%$ of total) and negligible changes in actomyosin cross-bridge numbers ($3.11\pm0.05; n=7; P<0.2$ versus sham).

**Distribution and Degradation of MyBP-C**

Because thick filament disruption and a reduction in actomyosin cross-bridge numbers accompanied low-flow ischemia and persisted after reperfusion, the subcellular distribution of MyBP-C was examined by immunofluorescence confocal microscopy. An antibody that recognized the N-terminus (C0C1 epitope, 13, 14) of MyBP-C revealed changes in the distribution of MyBP-C during low-flow ischemia and after reperfusion. In sham as well as remote myocardium, MyBP-C was localized in the A-band of the myofibril, encroaching on neither the I-band nor the M-line of the sarcomere (Figure 6a to 6c). After 5 hours of low-flow ischemia, the distribution of MyBP-C remained unchanged, but the intensity of staining seemed reduced in most sarcomeres and missing altogether in others (Figure 6e, 6f). The C0C1 staining anomalies only developed in the presence of dephosphorylated MyBP-C (Figure 6j). The distribution of the N-terminus of MyBP-C was markedly altered after 24 hours of reperfusion (Figure 6b). Myofibrillar A-band staining was faint, and small aggregates of antibody stained-C0C1 were observed in stunned cardiomyocytes (Figure 6h, 6i). When double-labeled for actin, the depletion of the N-terminal epitope appeared to have little influence on the distribution of actin in I-bands (Figure 6a, 6d, 6g). The decline in MyBP-C staining after reperfusion suggested that the N-terminus was cleaved off the intact molecule after the restoration of coronary flow. Western blots of myofibrillar fractions disclosed significant degradation of the N-terminus of MyBP-C in reperfused, stunned myocardium but not in remote tissue (Figure 7a). Several proteolytic fragments were identified, including a pair of 40-kDa peptides. When these peptides were subjected to 2-dimensional IEF and blotted with the C0C1 antibody, all 4 phosphorylated forms of MyBP-C were apparent (Figure 7b); moreover, significant amounts of the dephosphorylated and triphosphorylated forms of the peptide appeared during reperfusion. In those animals reperfused until flow and function were restored to preocclusion values, no evidence of MyBP-C redistribution or degradation was apparent in the recovered myocardium (data not shown). In animals subjected to moderate low-flow ischemia, MyBP-C staining was not altered significantly; moreover, after reperfusion, no degradation fragments of MyBP-C were evident (data not shown) in the hibernating myocardium.

**Discussion**

Regional contractile dysfunction develops in chronically instrumented canine myocardium after episodes of low-flow ischemia and can persist for extended periods after reperfusion1,2 (Figure 1). MyBP-C becomes dephosphorylated and the interaction of the C0C1 antibody with the N-terminus of MyBP-C decreases during low-flow ischemia. The number of actomyosin cross-bridges also decreases with dephosphorylation of MyBP-C and the decline in segmental function. The reduction in cross-bridge numbers parallels the redistribution of the MyBP-C C0C1 epitope but appears to precede any significant MyBP-C degradation. Prolonged reperfusion restores contractile function, MyBP-C phosphorylation state,
and myofibril structure. These parameters were not altered appreciably in the remote, normally perfused myocardium. The results support the hypothesis that the dephosphorylation of MyBP-C initiates changes in myofibril thick filament structure that decrease the interaction of myosin heads with actin thin filaments and limit the formation of actomyosin cross-bridges. The degree of dephosphorylation and the extent of MyBP-C degradation that develops during reperfusion may prolong and perhaps exacerbate myocardial stunning.

The relationship between actomyosin cross-bridge numbers, segmental function, and MyBP-C dephosphorylation (Figure 3) identifies another myofibrillar protein that has the potential to modulate contractile function after low-flow ischemia. There is no change in the frequency of myosin heads in intact myofibrils from hearts of sham-operated animals and intact regions of thick filaments from coronary-flow obstructed dogs. Nevertheless, there is a consistent difference in these 2 populations with respect to the frequency of myosin heads that extends to the thin filament in the form of cross-bridges (Table). Because myofibril scalloping (Figure 4) typical of rigor was rarely observed and cross-bridges were oriented at variable angles to the thick filament (Figure 5), myofibrils appear to be preserved in an activated state of contraction. Therefore, the relative frequency of cross-bridges appears correlated with the percentage of unphosphorylated MyBP-C and the degree of myocardial segment shortening (Figure 3). The average periodicity for cross-bridges in the myofibrils of sham-operated and ischemic hearts was 34 and 42 nm, respectively. In view of the variability in each measurement, these values are similar to the actin periodicity of 37 nm and the myosin periodicity of 43 nm derived by optical diffraction. These observations imply that MyBP-C phosphorylation allows the myosin heads to rotate in a manner to accommodate the actin periodicity in the thin filament and form a larger number of non–force-generating weak interactions between actin and myosin. The number of weakly interacting myosin heads may limit the formation of strongly bound heads that generate force. In the absence of this flexibility, the mobility of the myosin head, presumably at the S1-S2 joint, is limited, resulting in the myosin heads attaching to the thin filament.
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References


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