Microdialysis separately monitors myocardial interstitial myoglobin during ischemia and reperfusion

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Microdialysis separately monitors myocardial interstitial myoglobin during ischemia and reperfusion. Am J Physiol Heart Circ Physiol 289: H924–H930, 2005. First published April 15, 2005; doi:10.1152/ajpheart.01207.2004—Direct monitoring of myoglobin efflux during ischemia and reperfusion has been limited because of inherent sample collection problems in the ischemic region. Recently, the cardiac dialysis technique has offered a powerful method for monitoring myocardial interstitial levels of low-molecular-weight compounds in the cardiac ischemic region. In the present study, we extended the molecular target to high-molecular-weight compounds by use of microdialysis probes with a high-molecular-mass cutoff and monitored myocardial interstitial myoglobin levels. A dialysis probe was implanted in the left ventricular free wall in anesthetized rabbits. The main coronary artery was occluded for 60 or 120 min. We examined the effects of myocardial ischemia and reperfusion on myocardial interstitial myoglobin levels. Interstitial myoglobin increased within 15 min of ischemia and continued to increase during 120 min of ischemia, whereas blood myoglobin increased at 45 min of ischemia. Lactate and myoglobin in the interstitial space increased during the same period. At 60 min of ischemia, reperfusion markedly accelerated interstitial myoglobin release. The interstitial myoglobin level was fivefold higher at 0–15 min of reperfusion than at 60–75 min of coronary occlusion. The dialysis technique permits earlier detection of myoglobin release and separately monitors myoglobin release during ischemia and reperfusion. Myocardial interstitial myoglobin levels can serve as an index of myocardial injury evoked by ischemia or reperfusion.

infarction; interstitial space; membrane permeability

It is well known that certain proteins, including myoglobin, called serum cardiac markers, are released into the bloodstream in large quantities from necrotic cardiac muscle cells after myocardial infarction (20, 26, 43). However, because direct samples from the ischemic region are not readily obtainable, in situ studies on efflux of these proteins in the cardiac ischemic region have been limited (22). This problem of sample collection from the ischemic region remains unresolved. First, it is uncertain exactly when cardiac markers appear in injured myocardium. The appearance of cardiac markers indicates the turning point from reversible injury to irreversible damage (43). However, the first appearance of cardiac markers in the bloodstream is influenced by the slow transport of cardiac markers from the interstitial space into the bloodstream (20). Thus the detection of this appearance is of great value in understanding the pathophysiological events induced by myocardial ischemia. Second, recent experimental and clinical findings suggest that reperfusion itself seems to accelerate the release of cardiac markers (18, 37, 38). However, the extent to which reperfusion contributes to relative changes in their release is unclear. To determine myocardial injury evoked by reperfusion, more information is needed about the extent to which ischemia and reperfusion affect changes in the release of cardiac markers. Third, present methods used to measure infarct size require tissue analysis several hours after the ischemic event (8). Furthermore, histochemical analysis depends on the times of ischemia and reperfusion (23, 33). Concise, dissociated assessments of ischemia and reperfusion injury have been a frequent object of research.

In general, mobilization of cardiac markers from ischemic myocardium to the bloodstream has been divided into two different sequences: release from the myocardial cell to the interstitial space and transport from the interstitial space into the bloodstream (20). Therefore, if we examine the first process in situ myocardium, we can discuss the pathophysiological changes during development of ischemic myocardial necrosis. However, little information is available on interstitial protein kinetics in the ischemic region (15). Examination of protein kinetics in the ischemic region has been limited to assessment of protein kinetics in the isolated Langendorff-perfused heart (28, 39). Recently, a cardiac dialysis technique has provided a powerful method for monitoring myocardial interstitial levels of low-molecular-weight compounds in the cardiac ischemic region (2, 6, 14, 31). Furthermore, this method is suitable for distinguishing between ischemia and reperfusion responses (32). By improving the microdialysis probes with a high-molecular-mass cutoff membrane, we have extended the molecular target to high-molecular-weight peptides and proteins and monitored myocardial interstitial protein levels.

In the present study, we chose myoglobin as one of the earliest biochemical markers in myocardial injury (4, 34). We applied the dialysis technique to the heart of anesthetized rabbits and investigated myocardial interstitial myoglobin levels during coronary occlusion and reperfusion. To address the above-mentioned issues, we compared the first appearance of myocardial interstitial myoglobin levels with that of low-molecular-weight metabolites (lactate and glycerol). Further-
more, we compared the time course of myocardial interstitial myoglobin during reperfusion after ischemia with that of sustained ischemia and examined the changes in myoglobin release evoked by reperfusion. The results of the present study indicate that microdialysis is suitable for distinguishing between ischemia and reperfusion injury.

MATERIALS AND METHODS

Animal Preparation

The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996). All protocols were approved by the Animal Subjects Committee of the National Cardiovascular Center. Thirty adult male Japanese White rabbits (2.5–3.2 kg) were anesthetized with pentobarbital sodium (30–35 mg/kg iv). The level of anesthesia was maintained with a continuous intravenous infusion of pentobarbital sodium (1–2 mg·kg\(^{-1}\)·h\(^{-1}\)). The rabbits were intubated and ventilated with room air mixed with oxygen. Body temperature was maintained at \(-39^\circ\text{C}\) with a heating pad and lamp. Heart rate (HR), mean arterial pressure (MAP), and electrocardiogram were monitored and recorded continuously. Heparin sodium (200 IU/kg) was first administered intravenously and then maintained with a continuous infusion (5–10 IU·kg\(^{-1}\)·h\(^{-1}\)) to prevent blood coagulation. With the animal in the lateral position, the fifth or sixth rib on the left side was partially removed to expose the heart. A small incision was made in the pericardium, and the dialysis probe was implanted in the region perfused by the left circumflex coronary artery (LCX) of the left ventricular wall. A snare was placed around the main branch of the LCX to act as the occluder for later coronary occlusion. To ensure that the sampling area was in the ischemic region, we examined the color and motion of the ventricular wall during a brief occlusion and confirmed that the dialysis probe was correctly located. To avoid a preconditioning effect, the duration of occlusion was limited to a few seconds.

Dialysis Technique

We designed a handmade long transverse dialysis probe (1). One end of a polyethylene tube (25 cm long, 0.5 mm OD, 0.2 mm ID) was diluted with a 27-gauge needle (0.4 mm OD). Each end of the dialysis fiber (8 mm long, 0.215 mm OD, 0.175 mm ID, 300 Å pore size; Evaflex type 5A, Kuraray Medical) was inserted into the polyethylene tube and glued. A fine guiding needle (25 mm long, 0.51 mm OD, 0.25 mm ID) was used for implantation of the dialysis probes. A guiding needle was connected to the dialysis probe with a stainless steel rod (5 mm long, 0.25 mm OD). At a perfusion speed of 5 μl/min, the in vitro recovery rate (RR) of myoglobin was 15 ± 0.6% (number of dialysis probes = 3). In vitro RR was defined as follows: RR = (C_{in} - C_{out})/C_{in} where C_{in} and C_{out} are the concentrations of myoglobin in the perfusate and in the dialysate, respectively (19). For monitoring myocardial interstitial lactate and glycerol levels, we used a conventional dialysis fiber (PAN-1200, Asahi Chemical Japan) to detect low-molecular-weight compounds (1).

Dialysis probes were perfused with Ringer solution (in mM: 147.0 NaCl, 4.0 KCl, and 2.25 CaCl\(_2\)) at 5 μl/min using a microinjection pump (model CMA/100, Carnegie Medicine). Figure 1 shows the time course of dialysate myoglobin levels collected at 1-h intervals over a 4-h period after probe implantation. Dialysate myoglobin rapidly decreased to 261 ± 56 ng/ml at 2 h after probe implantation. Thereafter, it gradually decreased, reaching an almost steady level of 222 ± 37 ng/ml 4 h after probe implantation. On the basis of the results of this experiment, in the subsequent protocol, we discarded the first 120-min collections of dialysate and measured the dialysate myoglobin level twice at 30-min intervals. When dialysate myoglobin levels reached the steady level, we started the experimental protocol.

Sampling periods were 15 min (1 sampling volume = 75 μl) in control and during occlusion and reperfusion. Taking into consideration the dead space between the dialysis fiber and sample tube, we sampled the dialysate.

Dialysate myoglobin concentrations were measured as an index of myocardial interstitial myoglobin levels. Blood samples were obtained from the femoral artery. Using immunochemistry (Cardiac Reader, Roche Diagnostics), we measured the myoglobin levels (7). The detection limit of myoglobin was 30 ng/ml. The dialysate lactate and glycerol levels were measured by kinetic enzymatic analysis (CMA 600 analyzer, Carnegie Medicine) (30).

Experimental Protocols

After control sampling, we occluded the main branch of the LCX for 60 min and then released the occluder. We continuously sampled the dialysate from the ischemic region during 60 min of coronary occlusion and reperfusion.

Time course of dialysate lactate, glycerol, and myoglobin levels during myocardial ischemia. We compared the dialysate myoglobin levels with the blood myoglobin levels. After control sampling, we observed the time course of dialysate and blood myoglobin levels during 60 min of coronary occlusion. In addition, we measured simultaneously dialysate lactate and glycerol levels from the ischemic region in separate rabbits.

Time course of dialysate myoglobin levels during 60 min of reperfusion following 60 and 120 min of ischemia. Reperfusion modulates myocardial membrane damage and may accelerate dialysate myoglobin levels (18, 21, 37). We compared the time course of dialysate myoglobin during 60 min of reperfusion following 60 min of ischemia with that of 120 min of ischemia.

Time course of dialysate myoglobin levels during local administration of cyanide. To confirm whether the dialysate myoglobin level reflects myocardial damage evoked by ischemia or hypoxia, we tested the effect of local sodium cyanide (NaCN) administration on dialysate myoglobin levels. We collected a control dialysis sample and then replaced the perfusate with Ringer solution containing NaCN (30 mM), thereby locally administering NaCN for 60 min. We obtained four consecutive dialysate samples and measured the dialysate myoglobin levels.

At the end of each experiment, the rabbits were killed with an overdose of pentobarbital sodium, and the implant regions were checked to confirm that the dialysis probes had been implanted within the cardiac muscle.

Statistical Analysis

Dialysate lactate, glycerol, and myoglobin responses to coronary occlusion were statistically analyzed by one-way analysis of variance with repeated measures. When a statistically significant effect of
coronary occlusion was detected as a whole, the Newman-Keuls test was applied to determine which mean values differed significantly from each other (40). Statistical significance was defined as $P < 0.05$.

Values are means ± SE. Data were obtained during control (C), after 5, 15, 30, 45, 60, 75, 90, and 105 min of coronary artery occlusion, and after 5, 15, 30, and 45 min of reperfusion (R). *$P < 0.05$ vs. control. †$P < 0.05$ vs. 45 min occlusion.

RESULTS

Time Course of HR and MAP

Table 1 shows the time courses of HR and MAP during coronary occlusion and reperfusion. Coronary occlusion decreased HR and MAP. Reperfusion did not alter HR but temporarily decreased MAP.

Time Course of Dialysate Lactate, Glycerol, and Myoglobin Levels During Myocardial Ischemia

Coronary occlusion significantly altered dialysate myoglobin levels (Fig. 2). Dialysate myoglobin levels increased significantly from 168 ± 32 ng/ml in the control to 570 ± 107 ng/ml at 0–15 min of occlusion. During 60 min of coronary occlusion, dialysate myoglobin levels progressively increased and reached 2,583 ± 208 ng/ml at 45–60 min of occlusion. A significant increase in blood myoglobin occurred at 45–60 min of coronary occlusion. Dialysate lactate levels were 1.00 ± 0.21 mmol/l in the control and increased after coronary occlusion (Fig. 3). During 60 min of coronary occlusion, dialysate lactate levels markedly increased and reached 3.34 ± 0.50 mmol/l at 45–60 min of occlusion. During 60 min of coronary occlusion, dialysate glycerol levels also increased and reached 232 ± 33 μmol/l at 45–60 min of occlusion.

Time Course of Dialysate Myoglobin Levels During 60 min of Reperfusion Following 60 and 120 Minutes of Ischemia

There were no significant differences in the control dialysate myoglobin levels between the two groups (Fig. 4). During ischemia, the dialysate myoglobin levels progressively increased and reached 4,054 ± 659 ng/ml at 105–120 min of coronary occlusion. During 60 min of coronary occlusion, there were no statistically significant differences in the dialysate myoglobin levels between the two groups. After release of the occluder, the dialysate myoglobin levels markedly increased to 12,569 ± 2,347 ng/ml at 0–15 min of reperfusion. The dialysate myoglobin levels at 0–15 min of reperfusion were fivefold higher than those at 60–75 min of 120 min of coronary occlusion. Furthermore, these values were higher than peak levels during 120 min of coronary occlusion.

![Fig. 2. Time courses of dialysate and blood myoglobin levels during 60 min of ischemia. Values are means ± SE. *$P < 0.05$ vs. control.](http://ajpheart.physiology.org/)

![Fig. 3. Time courses of dialysate lactate (top) and glycerol (bottom) levels during 60 min of ischemia. Values are means ± SE. *$P < 0.05$ vs. control.](http://ajpheart.physiology.org/)
dialysate myoglobin levels gradually decreased and reached 4,391 ± 879 ng/ml at 45–60 of reperfusion. At 0–15 min of reperfusion, dialysate lactate and glycerol levels were 3.27 ± 0.61 mmol/l and 242 ± 37.7 μmol/l, respectively. Dialysate lactate and glycerol levels remained unchanged at 0–15 min of reperfusion.

Time Course of Dialysate Myoglobin Levels During Local Administration of NaCN

Local administration of NaCN increased the dialysate myoglobin levels (Fig. 5). This increase was statistically significant compared with the control level at all collection periods during NaCN administration, except at 0–15 min. The maximum myoglobin level was comparable to that observed during 60 min of ischemia.

DISCUSSION

Using the dialysis technique in the in vivo rabbit heart, we observed myocardial interstitial myoglobin levels during myocardial ischemia and reperfusion. Our data demonstrated myoglobin release in the early stage of cardiac ischemia and its enhancement by reperfusion. We discuss here the time course of myocardial myoglobin release during coronary occlusion and after reperfusion.

We show for the first time that myoglobin release increases within 15 min of ischemia and continues to increase during 60 min of ischemia. However, significant changes in the blood myoglobin level occurred at 45–60 min of coronary occlusion. Our data suggest a contrast between blood and dialysate
myoglobin levels during ischemia. The delay of the first appearance of myoglobin in the bloodstream is mainly due to the slow transport of myoglobin from the interstitial space into the bloodstream (20). Therefore, myoglobin concentration measured by cardiac microdialysis provides information regarding early release of cytosol protein into the interstitial space. Within the 15-min time resolution, this increase in myoglobin release was accompanied by increases in interstitial lactate. Dead space volume between the dialysis fiber and the sample microtube was identical for lactate, glycerol, and myoglobin. The currently accepted concept (20) is that leakage of anaerobic metabolites precedes macromolecular protein release during ischemia. Anaerobic metabolites accumulate and leak from the ischemic region within minutes via diffusion or transport (6, 12, 41). In contrast to low-molecular-weight metabolites, macromolecular proteins could be released into the interstitial space without cytosol accumulation of myoglobin, probably via bleb or altered permeability. Although sampling periods of 15 min are too long to enable us to distinguish the rate of release of lactate vs. myoglobin, our data at least suggest that cellular metabolic derangement is involved in membrane disruption for myoglobin release.

Myocardial injury caused by ischemia-reperfusion is associated with membrane phospholipid degradation, which is thought to underlie disruption of the cell membrane (27). Glycerol is an end product of membrane phospholipid degradation and has been used to study membrane phospholipid degradation after cerebral ischemia and seizures (12). In the present study, dialysate glycerol was examined as a potential marker for membrane phospholipid degradation in myocardial ischemia and reperfusion. We observed increases in dialysate glycerol levels during 15–60 min of ischemia but not during reperfusion. In general, phospholipid degradation is accentuated during reperfusion (27). Therefore, dialysate glycerol is not suitable as an index of membrane phospholipid degradation, and the release of glycerol from membrane phospholipid degradation might be too small to allow detection in blood-perfused heart.

Early change of cytosol myoglobin was detected by immunofluorescence after occlusion of the coronary artery (16, 25). Histochemical studies demonstrated that intracellular diffusion of cytosol myoglobin into the nuclei and mitochondria was evident as early as 0.5 h after coronary artery occlusion (17, 25). Our data demonstrate early loss of cytosol myoglobin into the interstitial space. Release of cytosol protein is caused by membrane damage via alteration of permeability or bleb formation. Blebs appeared on the cell surfaces, and the cell began to swell within 10–20 min of ATP depletion in a glia cell line or hepatocytes (13, 24). Furthermore, NMR spectroscopy suggested that sarcolemmal membranes are gradually permeabilized to large molecules by ischemia (3). These alterations of sarcolemmal membranes might be involved in early release of myoglobin during the myocardial ischemia. Our method offers extremely fast and sensitive analysis of membrane injury in myocardial ischemia that is not evident by histological or blood analysis. Quantitative assessment of interstitial myoglobin levels could be performed independently of reperfusion cell injury and could be helpful in devising various myocardial preservation treatments.

We show that reperfusion markedly accelerates myoglobin release in the ischemic region. The interstitial myoglobin levels at 0–15 min of reperfusion were fivefold higher than those at 60–75 min of 120 min of coronary occlusion. During the reperfusion period, interstitial accumulated myoglobin might be washed out into the bloodstream (37). Therefore, the amount of released myoglobin at reperfusion could be markedly greater than the changes in interstitial myoglobin concentrations at reperfusion. Release of cytosolic protein resulted from a disruption of a sarcolemmal bleb or an enhancement of membrane permeability (5, 29, 35). Either condition may gain relevance during the reperfusion period. Thus the release of myoglobin during the reperfusion seems to serve as an index of disrupted sarcolemmal membrane.

Although the exact mechanisms of accelerated myoglobin release cannot be determined from the present study, our data suggest that substances induced during reperfusion differ from those induced during ischemia. Reperfusion enhanced myoglobin release but did not accelerate lactate or glycerol release in the interstitial space, whereas ischemia accompanied macromolecular myoglobin release as well as anaerobic metabolite release. Furthermore, in the previous studies, neither catecholamine nor acetylcholine release was accelerated by reperfusion in ischemic cardiac sympathetic and parasympathetic nerve endings (2, 14). During reperfusion, surviving myocardial cells and nerve terminals quickly recover aerobic metabolism and take up these accumulated substances, whereas myocardial cells have no capability of myoglobin uptake via the sarcolemmal membrane, leading to continued myoglobin release via the disrupted membrane. Reperfusion may enhance membrane permeability (5). Further disruption of membrane blebs may cause rupture of the membrane (29, 35). Alternatively, in isolated perfused rats, leakage of cytoplasmic enzymes during reoxygenation is accelerated by cardiac revived beating, because the cell membrane becomes fragile during the preceding anoxia (36). In either condition, reperfusion-induced breakdown of membrane phospholipids contributes to an alteration of permeability or bleb formation (27). Disruption of the membrane phospholipid bilayer is likely to play a role in myoglobin release from the cytosol into the interstitial spaces.

In the present study, we demonstrate that loss of cytosol myoglobin occurs during myocardial ischemia and reperfusion and might be involved in the outcome and pathophysiology of the ischemic heart. Loss of cytosol myoglobin may precede, at least in part, histological evidence of necrosis and occur in the remaining viable myocardium that is not necrotic (11). In vertebrate heart, myoglobin is involved in the transport of oxygen from the sarcolemma to the mitochondria (42). Recent studies from myoglobin knockout mice indicate that myoglobin contributes to the scavenging of bioactive nitric oxide (NO) or oxygen radicals during ischemia-reperfusion (9, 10). NO production and/or oxidant injury occur during the reperfusion period. In hearts lacking myoglobin, changes in NO and oxidative stress have a much larger impact on the maintenance of vascular tone and cardiac function (44). Similarly, in myoglobin knockout mice, loss of cytosol myoglobin may be involved in the delayed restoration of cardiac contractility in the postischemic region.

There are several limitations to the present study. First, with application of the dialysis technique to the heart, we had to perform this experiment as an acute surgical preparation. Probe implantation and/or surgical preparation might affect the con-
centration of myocardial interstitial myoglobin. To examine the effect of probe implantation and/or surgery, we performed the preliminary experiment on brief occlusion (3 min). Three minutes of coronary occlusion did not alter dialysate myoglobin levels. Furthermore, to confirm whether the dialysate myoglobin level reflects myocardial damage evoked by ischemia or hypoxia, we tested the effect of local NaCN administration on dialysate myoglobin levels: with NaCN, we found increases in dialysate myoglobin levels similar to the increase evoked by myocardial ischemia. Therefore, we believe that dialysate myoglobin levels reflect the release of myoglobin evoked by ischemia as well as by chemical hypoxia. The absolute myoglobin level might be affected by implantation and/or surgical preparation. However, it is possible to estimate myoglobin release from relative changes in myoglobin levels.

Second, in the present study, myocardial interstitial myoglobin levels during coronary occlusion and reperfusion were determined regionally. We implanted the dialysis probe in the midwall of the left ventricle. When the dialysis probe was implanted in the subendocardial zone, it is likely that subendo- cardial ischemia was much more severe than in the midwall, where the sampling was performed. Actually, subendocardial lactate was significantly greater than epicardial lactate during severe ischemia in the anesthetized dogs (6). Further studies are warranted concerning the influence of the ischemic area (subendocardial or marginal zone) on its myocardial interstitial myoglobin levels.

In summary, this microdialysis study in an ischemic animal model shows that coronary occlusion induced myoglobin release in minutes. Micromolecular metabolite (lactate) and macromolecular protein (myoglobin) increased during the first 15 min of ischemia. Reperfusion markedly enhanced myoglobin release without increases in lactate or glycerol levels. Elevation of myoglobin release represents an increase in sarcolemmal permeability or bleb formation during ischemia and reperfu- sion. Massive disruption of myocardial membrane occurs immedi- ately after ischemia and is markedly accelerated by reper- fusion. The dialysis technique permits more concise in vivo monitoring of myocardial membrane disruption during ische- mia and reperfusion separately.

GRANTS

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