BRIEF COMMUNICATIONS

Release of Unassembled Rat Cardiac Myosin Light Chain 1 Following the Calcium Paradox

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SUMMARY. To determine the intracellular source and release kinetics of myosin light chain 1 immediately following irreversible myocytic injury, we perfused rat hearts in a Langendorff apparatus under control conditions (20 minutes), or during global cellular injury produced by oxygenated, calcium-free perfusion (5 minutes), followed by reperfusion with buffer containing 2.5 mM calcium (15 minutes). Light chain 1 concentration (double antibody radioimmunoassay) and creatine kinase activity were measured in both the coronary effluent and a 140,000 g supernatant extract of perfused ventricular tissue (after homogenization and ultracentrifugation). Calcium reperfusion caused the rapid release of both light chain 1 and creatine kinase activity (peak light chain 1 = 1.09 ± 0.19 µg/g; peak creatine kinase = 74.9 ± 10.7 IU/g at 1 minute, mean ± SD, n = 3); 28.5 ± 13.5% of total light chain 1 and 86.5 ± 0.6% of total creatine kinase activity were depleted from the tissue extract during the 15-minute reperfusion. No light chain 1 or creatine kinase was detected in the effluents of control-perfused hearts. Dodecyl sulfate polyacrylamide gel electrophoresis and immunodetection with specific antibody to myosin heavy chain and light chain 1 showed that the effluent light chain 1 was of similar molecular weight (mol wt = 27,000) to the subunit bound to myofibrils. In addition, light chain 1 was released in the absence of myosin heavy chain. Thus, a small soluble pool of unassembled myosin light chain 1 subunits exists in the cytoplasm of cardiac myocytes that is released from irreversibly injured cells. This pool demonstrates initial washout kinetics similar to creatine kinase. (Circ Res 58:166-171, 1986)

CARDIAC myosin light chains are low molecular weight polypeptide subunits of the myosin molecule that can be detected by radioimmunoassay in the serum of patients and experimental animals following acute myocardial infarction (Khaw et al., 1978; Gere et al., 1978, Nagai et al., 1979, Katus et al., 1982). The appearance and disappearance of these structural proteins in the serum following irreversible ischemic cellular injury differ from that of cytosolic creatine kinase MB and myoglobin in that serum levels of both cardiac myosin light chain 1 (LC1) and light chain 2 (LC2) rise early after the onset of infarction and remain elevated for several days after injury (Trahern et al., 1978; Nagai et al., 1980; Katus et al., 1984). The cellular mechanisms responsible for both the early and sustained release of myosin light chains from irreversibly injured myocardium are not known with certainty. Nagai et al. (1979, 1980, 1981), Gere et al. (1978), and Khaw et al. (1978) all have suggested that myosin light chain subunits are initially released after irreversible ischemic injury from an unassembled intracellular light chain pool. The earlier observations of Horvath and Gaetjens (1972), Morkin et al. (1973), and Zak et al. (1977) lend support to the existence of a sarcoplasmic pool of myosin light chains in both rabbit skeletal muscle and rat heart myocytes. Presumably, sarcolemmal damage produced early in the course of ischemic injury leads to the release into the circulation of these unassembled light chains, along with other cytosolic components of the myocyte (CK-MB, LDH, myoglobin, etc.). Subsequent continuous release of myosin light chains (noncovalently bound to myosin heavy chain within myofibrils) occurs because of acidic dissociation (Smitherman et al., 1980) or because of proteolytic degradation of myofibrils (Nagai et al., 1980).

With the availability of specific antisera for both LC1 and myosin heavy chain, and experience with a well-characterized model for the rapid production of global myocardic cellular injury (i.e., the calcium paradox in isolated perfused rat heart), we undertook the present investigation to provide additional proof for the existence of a soluble pool of unassembled LC1 within the sarcoplasm. Furthermore, we demonstrate that this soluble LC1 pool is rapidly released from perfused rat heart following sarcolemmal disruption produced by the calcium paradox.
Methods

Reagents

Sodium [125I]iodide was obtained from Amersham, goat anti-guinea pig IgG and horseradish peroxidase-conjugated rabbit anti-guinea pig IgG were obtained from Cappel, and nitrocellulose sheets (0.45 μm) were a product of Biorad. All other reagents were of the highest grade commercially available and were obtained from Sigma Chemical Co., and Scientific Products.

Experimental Animals

Male Sprague-Dawley rats (250–300 g) were obtained from Harlan Industries. Male English short-haired guinea pigs (700–800 g) were obtained from Scientific Animals.

Heart Perfusion

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital. Hearts were removed and cannulated via the aorta to a double-reservoir Langendorff apparatus, as previously described (Ganote et al., 1984). Perfusion was at 37°C at a pressure of 8–10 kPa. Coronary flows were measured by timed collections of effluents, and aliquots were collected for subsequent analysis of CK activity and LCI content by radioimmunoassay. Control perfusion medium was a standard Krebs-Henseleit-bicarbonate buffer consisting of NaCl, 118; NaHCO3, 25; KCl, 4.7; KH2PO4, 1.2; CaCl2, 2.5; and glucose, 11 mM. Calcium-free medium was prepared by exclusion of calcium without ionic substitution but with inclusion of 100 μM ethylenediaminetetraacetic acid (EDTA) to ensure removal of residual calcium contaminants. All solutions were continuously gassed with 95% O2, 5% CO2. All hearts were perfused with control medium for 15 minutes before the experiments were initiated.

Tissue Homogenization and Extraction

Hearts were immersed in 150 mM NaCl (4°C), and the ventricular muscle was trimmed of blood vessels, valvular structures, and atria. The tissue then was homogenized (4 bursts of 15 seconds, with a Polytron homogenizer) in 25 volumes of 10 mM Tris-HCl buffer 7.4, containing 250 mM sucrose, 10 mM ethyleneglycol-bis-(β-aminoethyl ether)-N,N’-tetraacetic acid (EGTA), and 1 mM 2-mercaptoethanol. The homogenate was subsequently centrifuged at 140,000 g for 60 minutes. The supernatant extract then was removed for subsequent analysis.

Protein and Creatine Kinase Assays

Creatine kinase activity in aliquots of perfusion effluents and rat heart extracts was assayed with a Gilford/model 3402 automatic analyzer and Worthington reagents. Protein concentration was determined by the method of Lowry et al. (1951).

Purification of Myosin Light Chain 1

In preparing standards and tracer for LCI radioimmunoassay, we purified myosin from human ventricular muscle (obtained at autopsy) by the method of Wikman-Coffelt et al. (1973). Light chains were dissociated from myosin heavy chain by denaturation in 5 M guanidinium-HCl at 25°C, followed by ethanol:water (4:1) precipitation at 4°C to remove myosin heavy chain (Khaw et al., 1978). The supernatant solution containing light chains as well as trace protein contaminants was dialyzed against water, concentrated by ultrafiltration, and freeze-dried. LCI then was completely separated from LC2 and other trace protein contaminants by molecular exclusion high-pressure liquid chromatography (HPLC), using a Spherogel-TSK 2000 SW column (7.5 X 30 mm) equilibrated with 10 mM sodium phosphate buffer, pH 6.8, containing 150 mM NaCl. Flow rate was 0.4 ml/min at a pressure of 800 psi.

Radioiodination of Light Chain 1

Highly purified LCI (2.5 μg) was radioiodinated by the chloramine T method of Greenwood et al. (1963) using 1 μCi of sodium [125I]iodide. Labeled protein was separated from unincorporated isotope, as previously described (Samarel et al., 1981). Specific radioactivities of the tracer ranged from 60–120 μCi/μg protein.

Preparation of Antisera to Cardiac Myosin Light Chain 1 and Myosin Heavy Chain

In preparing antisera to LCI, LCI was completely separated from LC2 (and other minor protein contaminants in the freeze-dried light chain preparation) by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Laemmli, 1970) using 12.5% vertical slab gels. Proteins were stained with Coomassie brilliant blue. Gel bands containing LCI (mol wt = 27,000) were cut from the slabs, emulsified with complete Freund's adjuvant, and injected intramuscularly and subcutaneously into guinea pigs. The animals received injections on days 0, 7, 28, and 59 of 400–500 μg of purified LCI. The antibody response was monitored by enzyme-linked immunosorbent assay (ELISA) in which purified LCI was “dot-blotted” or electrophoretically transferred from SDS-polyacrylamide gels to nitrocellulose sheets (Towbin et al., 1979) and exposed to diluted antiserum from test bleeds. Primary antibody was detected following incubation with peroxidase-conjugated rabbit anti-guinea pig IgG. 4-Chloro-l-naphthol was used for color development in the sandwich assay.

Antiserum to myosin heavy chain was prepared in a similar fashion. Myosin was purified from rabbit ventricular muscle, and the heavy chain (mol wt = 200,000) was separated from the light chains by SDS-polyacrylamide gel electrophoresis on 7% vertical slab gels. After staining, gel bands containing heavy chain were excised and emulsified in complete Freund’s adjuvant. Guinea pigs received injections on days 0, 7, 28, and 38 of 400–500 μg of purified protein. The antibody response was monitored as described above.

Radioimmunoassay of Light Chain 1

A double-antibody radioimmunoassay was employed to measure the concentration of LCI in rat heart extracts and perfusion effluents. Guinea pig antisera to LCI was first titrated by incubating serial dilutions of antiserum (diluted in 50 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and 1% (wt/vol) of bovine serum albumin) with a constant amount (5000 counts/min) of 125I-labeled LCI. The dilution capable of approximately 50% maximum binding (after precipitation of tracer-antibody complexes with goat anti-guinea pig IgG) was used in the radioimmunoassay.

In the final radioimmunoassay mixture, 100 μl of diluted antiserum, 50 μl of 125I-labeled LCI (5000 counts/min), and 500 μl of standard (6000 ng/ml of the highly purified LCI, prepared following HPLC) or unknown (perfusion effluent or rat ventricular
muscle extract) were incubated in duplicate at 4°C for 24 hours. Antibody-bound LCI was separated from free antigen by adding goat anti-guinea pig IgG (50 μl) and nonimmune guinea pig serum (5 μl). After incubation at 4°C for an additional 24 hours, the immunoprecipitates were removed by centrifugation (1500 g, 15 minutes) and were counted for 125I radioactivity in an LKB model 1275 Minigamma Counter. Nonspecific binding of labeled tracer (determined by incubating 125I-labeled LCI and standard or unknown in the absence of primary antibody) was determined, and subtracted from each value of counts bound. LCI concentration of unknowns was read from a standard competitive binding curve of log LCI concentration vs. B:BO (where B:BO is the ratio of precipitated radioactivity for the standard solutions of LCI to the 0 concentration standard). Data were expressed as nanograms of LCI per milliliter of unknown solution.

Imrnunoel ectrophoretic Detection of Myosin Light Chain 1 and Myosin Heavy Chain

Tissue homogenates, resuspended myofibrillar sediments, and 140,000 g supernatant extracts (50-100 μg protein) of control hearts and hearts subjected to the calcium paradox were analyzed by SDS-polyacrylamide gel electrophoresis. In addition, an ultrafiltration concentrate of the peak fraction of LCI release following the calcium paradox (50 μg protein) was applied to 7-17% vertical gradient slab gels. After electrophoresis, the separated proteins were either stained with Coomassie brilliant blue, or were electrophoretically transferred to nitrocellulose sheets (Towbin et al., 1979). LCI and myosin heavy chain then were detected by incubating the electroblots (1 hour, 25°C) with specific antisera to either LCI or myosin heavy chain. Primary antibody binding then was demonstrated by incubation (1 hour, 25°C) with peroxidase-conjugated rabbit anti-guinea pig IgG. 4-Chloro-1-naphthol was used for color development for peroxidase.

Results and Discussion

Purification of Myosin Light Chain 1 Used for Radioiodination and Standards in the Radioimmunoassay

Molecular exclusion HPLC of the freeze-dried human myosin light chain preparation yielded multiple peaks, all containing LCI (Fig. 1). Protein eluting with a retention time of 15-17 minutes (fraction II) demonstrated a single band (mol wt = 27,000) free of LC2 (mol wt = 20,000) and other higher and lower molecular weight protein contaminants when analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1). The purified LCI from several HPLC separations was pooled and concentrated by ultrafiltration for subsequent use in radioiodination and as standards in the radioimmunoassay.

Specificity of Antisera to Myosin Light Chain 1 and Myosin Heavy Chain

Immunization of guinea pigs with human cardiac LCI (extracted from SDS-polyacrylamide gels) produced a nonprecipitating IgG antibody highly specific for LCI. This antibody was completely cross-reactive with LC1 isolated from human skeletal muscle, as well as LC1 isolated from rat, rabbit, and dog myocardia. In addition, the antibody failed to cross-react with myosin heavy chain, troponin subunits, or tropomyosin isolated from cardiac and skeletal muscle of all species examined. Slight cross-reactivity was noted between this antiserum and rat cardiac actin, as well as human and canine cardiac LC2, when analyzed by SDS-polyacrylamide gel electrophoresis and electroblotting (Towbin et al., 1979).
Immunization of guinea pigs with rabbit cardiac myosin heavy chain (similarly extracted from SDS-polyacrylamide gels) produced a nonprecipitating IgG antibody highly specific for myosin heavy chain isolated from ventricular muscle of several species, including rabbit, rat, dog, and human myocardia. This antibody did not cross-react with any other myocardial proteins of all species examined, when analyzed by SDS-polyacrylamide gel electrophoresis and electroblotting.

**Radioimmunoassay of Myosin Light Chain 1**

The LC1 antiserum displayed typical equilibrium-binding characteristics with $^{125}$I-labeled human cardiac LC1 (Fig. 2A). An 800-fold dilution of the LC1 antiserum yielded half-maximal binding of tracer, and this dilution was utilized in the radioimmunoassay. Assay sensitivity was 3 ng/ml with a working range of 12.5–400 ng/ml (Fig. 2B). Within-assay variation was less than 10% near the middle of the dose-response curve. Perfusion effluents and rat heart extracts were diluted 2-fold (in 50 mm of a sodium phosphate buffer, pH 7.4, containing 150 mm NaCl and 1% bovine serum albumin) to provide LC1 concentrations near the middle of the standard curve.

We took advantage of the cross-species reactivity of our LC1 antiserum to confirm the presence of LC1 in the 140,000 g supernatant extract of nonperfused rat ventricular myocardium, in a fashion analogous to experiments performed by Horvath and Gaetjens (1972) using chicken skeletal muscle. LC1 was readily detected in this cytosolic fraction, at a concentration of 3.40 ± 0.21 μg/g wet weight (mean ± SD, n = 10). Based upon previous radioimmunoassay measurements of the myosin content of rabbit ventricular muscle (Everett et al., 1983), this cytosolic pool of LC1 accounted for approximately 0.1% of the total amount of LC1 present in ventricular myocardium.

**Release of Unassembled Myosin Light Chain 1 Following the Calcium Paradox**

To determine whether the cytosolic pool of LC1 was the source of rapidly released LC1 following irreversible cellular injury, we employed the radioimmunoassay to measure LC1 in the coronary effluent of rat hearts subjected to the calcium paradox. This form of massive cellular injury occurs when hearts briefly perfused with calcium-free buffer are reexposed to a calcium-containing solution (Zimmerman and Hulsmann, 1966). Calcium reperfusion results in irreversible contracture, massive cellular disruption, and rapid loss of cytosolic components (CK, LDH, myoglobin) (Zimmerman and Hulsmann, 1966).

Continuous perfusion of rat hearts for 35 minutes with control medium (2.5 mM Ca$^{++}$) did not cause the release of either creatine kinase (CK), LC1, or other cellular protein into the coronary effluent. However, sequential perfusion with calcium-free medium (5 minutes) followed by 2.5 mM Ca$^{++}$ reperfusion (15 minutes) led to the rapid release of both CK and LC1 (Fig. 3). Peak CK activity and LC1 release at 1 minute after calcium reperfusion were 74.9 ± 10.7 IU/g wet weight, and 1.09 ± 0.19 μg LC1/g wet weight, respectively (mean ± SD, three experiments). Homogenization and centrifugation of ventricular tissue from calcium paradox-perfused hearts (with subsequent CK assay and LC1 radioimmunoassay of the 140,000 g supernatant fraction) demonstrated that the cytosolic fraction was depleted of 86.5 ± 0.6% of total CK activity, and 28.5 ± 13.5% of immunoreactive LC1 (mean ± SD, three experiments), compared to extracts prepared from hearts continuously perfused with medium contain-
ing 2.5 mM Ca\textsuperscript{2+}. Thus, LCI was rapidly released from irreversibly injured myocardium, with initial washout kinetics similar to that of CK. The LCI released immediately after calcium reperfusion appeared to originate from the cytosolic pool.

Additional experiments were performed to characterize further the cytosolic pool of LCI, and to determine whether the LCI released after calcium reperfusion was derived from assembled myosin. Protein samples (50–100 \mu g) from the total homogenates, resuspended myofibrillar sediments, and 140,000 g supernatant extracts of control and calcium paradox-perfused hearts, as well as a concentrate (50 \mu g protein) of perfusate effluent collected during the first minute following calcium reperfusion, were separated by SDS-polyacrylamide gel electrophoresis on 7–17% vertical gradient slab gels. The presence or absence of myosin heavy chain and LCI was determined by protein staining or electroblotting and exposure to specific antisera.

**Figure 3.** Release of creatine kinase (CK) and rat cardiac myosin light chain 1 (LCI) following the calcium paradox. After 15 minutes of retrograde perfusion with Krebs-Henseleit-bicarbonate buffer containing 2.5 mM Ca\textsuperscript{2+}, a rat heart was perfused for 5 minutes with calcium-free buffer, followed by reperfusion (15 minutes) with medium containing 2.5 mM Ca\textsuperscript{2+}. Coronary effluents (1-minute collections) were assayed for CK (open circles) and LCI (triangles). Data are expressed as units or nanograms released per minute per gram of tissue wet weight (duplicate determinations).

**Figure 4.** Immunoelectrophoretic detection of myosin heavy chain (MHC) and myosin light chain 1 (LCI) in fractions of perfused rat heart. Protein samples (50–100 \mu g) were of the total homogenates, resuspended myofibrillar sediments and 140,000 g supernatant fractions of hearts perfused under control conditions (C) or after calcium-free perfusion and reperfusion with 2.5 mM Ca\textsuperscript{2+} (P). In addition, 50 \mu g of perfusion effluent protein (effluent) from the peak fraction of LCI release after the calcium paradox were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 7–17% vertical slab gels. Separated proteins were either stained with Coomassie brilliant blue (left panel) or were transferred electrophotographically to nitrocellulose sheets. MHC (middle panel) and LCI (right panel) were identified by immunodetection with specific antibody. Molecular weight standards (STD’s) were as in Figure 1.
As seen in Figure 4, myosin heavy chain was detected (by protein staining and immunodetection with specific antibody) in the total homogenates and myofibrillar sediments of both control and calcium paradox-perfused hearts. However, myosin heavy chain was not detected in either of the two supernatant fractions, or in the concentrated effluent following calcium reperfusion. As expected from the radioimmunoassay results, immunoreactive LCI was present in all tissue fractions, as well as in the coronary effluent following the calcium paradox. Furthermore, the immunoreactive LCI released during the first minute after calcium reperfusion was of identical apparent molecular weight (mol wt = 27,000) to the LCI present in the total homogenates, 140,000 g supernatant fractions, and myofibrillar sediments of both control and calcium-reperfused hearts (Fig. 4). These results indicate that the LCI present in the cytosolic fraction (and released from the heart following the calcium paradox) was not derived from assembled myosin.

In summary, calcium-free perfusion followed by reperfusion with buffer containing 2.5 mM Ca++ led to the rapid release of cardiac LCI from an intracellular pool of myosin light chains that was separable by homogenization and centrifugation from the bulk of LC1 bound to myofibrils. This LCI pool demonstrated initial wash-out kinetics similar to that of CK, a sarcomplasmic enzyme, suggesting that the LCI originated from a cytosolic pool of unassembled myosin light chain subunits. Release of LCI after irreversible myocytic injury appears unrelated to the initial type of damage produced, because rapid release of LCI occurs after ischemic injury, as well as after global cellular injury caused by the calcium paradox. This cytoplasmic pool of myosin light chain 1 may be important in the assembly and turnover of myofibrils.

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