The Effect of an Isovolumic Left Ventricle on the Coronary Vascular Competence during Reflow after Global Ischemia in the Rat Heart

STUART M. HUMPHREY, ROBERT W. THOMSON, AND JOHN B. GAVIN

SUMMARY During global ischemia in isolated rat hearts, the development of contracture, due to irreversible myofilament sliding, causes reduction of left ventricle luminal volume. Also, a considerable area of the myocardium cannot be reperfused after 1 hour's global ischemia. The purpose of this study was to reduce myofilament sliding by placing a fluid-filled isovolumic balloon in the left ventricular cavity of isolated rat hearts and assess the extent of reflow, after 60 minutes' ischemia, by perfusion of a 1% fluorescein tracer solution. Light and electron microscopy was used to determine the state of the vasculature and myofibrillar apparatus. In hearts without the left ventricular balloon (control) the ischemia produced a no-reflow zone comprising 46% of the myocardial wall. In contrast, if an isovolumic balloon was in place during the ischemic period, only 6% of the wall was involved. The volume of the capillary bed in the subendocardium of the control hearts was about 80% of that in the isovolumic hearts. In the isovolumic ("isometric") mode, ischemic contracture was associated with more severe myocardial cell injury than in the corresponding control ("isotonic") mode. Our results support the concept that intramyocardial pressure generated by ischemic contracture plays a major role in the production of the no-reflow phenomenon in globally ischemic rat hearts, and indicate that it is the series elastic component of cardiac muscle which imparts the stiffness necessary to prevent reopening of coronary vessels after a severe ischemic insult. Circ Res 48: 784-791, 1981

REDUCED myocardial perfusion on resumption of flow after periods of ischemia in the dog heart has been observed by several authors (Kloner et al., 1974; Willerson et al., 1975). More recently, the development of a no-reflow phenomenon has been demonstrated in the isolated rat heart (Alanen et al., 1980; Humphrey et al., 1980). In these latter studies, approximately 60% of the ventricle was resistant to reperfusion after 60 minutes of global ischemia. A mechanism involving the tension generated by contracture has been suggested to explain this severe vascular shutdown (Humphrey et al., 1980). By means of this tension, the development of ischemic contracture could result in the exertion of pressure on all of the interstitial elements, including arterioles and capillaries, particularly in the endocardium where pressure is greatest (Baird and Ameli, 1971). Subsequent development of rigor would then prevent re-opening of the compressed vascular bed.

If the perfusion defect is related to myofilament movement, as assessed by measurement of Z band widths, the maintenance of an isovolumic left ventricle should considerably reduce the shortening of the left ventricular muscle and thereby reduce this defect.

We therefore undertook the present study to ascertain, by means of the distribution of a fluorescein tracer and electron microscopy, whether 60 minutes of ischemia in the presence of an incompressible balloon in the left ventricle could prevent no-reflow. Our results support the concept that shortened fiber length resulting from contracture and rigor plays a major role in the production of a no-reflow phenomenon.

Methods

Twenty-four male albino Wistar Rats (270-330 g) each were lightly anesthetized with diethyl ether and injected with 200 I.U. heparin via the femoral vein. One minute later, the heart was carefully excised, arrested in ice-cold perfusion medium, quickly mounted on a stainless steel cannula, and subjected to a non-recirculating Langendorff perfusion. The perfusion medium was Krebs-Henseleit bicarbonate solution, pH 7.4, gassed with O$_2$:CO$_2$ (95:5 mixture). All hearts were perfused at a pressure of 100 cm H$_2$O and at a temperature of 37°C. The hearts were kept at 37°C during ischemia by placing them inside a water-jacketed glass chamber. Twelve of the 24 hearts used had a latex balloon catheter introduced into the left ventricle via the pulmonary vein and left atrium (isovolumic group). The balloon was tied in place and connected to a pressure transducer (Bell and Howell 4-422) for...
monitoring intraventricular pressure. The balloon was inflated with water until the ventricular lumen was just filled, indicated by a sudden increase in resistance to filling pressure. At this point the balloon volume and, hence, left ventricular volume, was between 0.22 and 0.25 ml.

All 24 hearts (12 isovolumic and 12 control) were perfused for a 10-minute equilibration period, during which the coronary flow and heart rates were monitored as a check for normal function.

At the end of the equilibration period, the aortic cannulas were clamped, and the resultant global ischemia was maintained for 60 minutes. In the isovolumic group, the balloons were deflated and removed after 55 minutes of ischemia. At the end of the ischemic period, hearts were perfused, via the 3rd arm of a 3-way tap connected to the aortic cannula, with one of the following three solutions: (1) fluorescein for perfusion-defect analysis (reflow), (2) glutaraldehyde for study by transmission electron microscopy (T.E.M.), or (3) methyl methacrylate for study by scanning electron microscopy (S.E.M.).

The details are as follows:

Reflow (6 Control, 6 Isovolumic)

Perfusion medium supplemented with fluorescein to a final concentration of 1% was allowed to perfuse the hearts at a pressure of 100 cm H2O for approximately 20 seconds, by which time the yellow-colored fluorescein solution could be seen draining from the pulmonary artery. The hearts then were carefully removed from the perfusion rig and suspended in the freezer for up to 48 hours. The frozen hearts were bisected at the first branch of the left anterior descending coronary artery to produce apical and basal halves which were photographed under U.V. light (250 nm). The resultant 35-mm slides were projected on to a white paper background to give an image approximately 20 cm in diameter. These outlines were traced and areas of flow and no-reflow calculated, using a planimeter (Tamaya & Co. Ltd. no. 12808).

T.E.M. (3 Control, 3 Isovolumic)

Ice-cold 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 (460 mOsmol) was injected at a rate of 10 ml/min for 2 minutes. The heart was removed from the apparatus, cut in half, and samples were quickly dissected from the areas of flow and no-reflow, cut into 1-mm cubes, and fixed overnight in glutaraldehyde at 4°C. Perfused tissue had a light reflow, cut into 1-mm cubes, and fixed overnight in glutaraldehyde at 4°C. Perfused tissue had a light freeze fixed. The tissue was washed in 0.1 M phosphate buffer prior to postfixation in 1% osmium tetroxide. Following dehydration and epoxy-resin embedding, thick and thin sections were cut. Toluidine blue-stained sections, 1 µm thick, were examined by light microscopy. Thin sections were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with a Philips E.M. 300 electron microscope operating at 60 kV.

Six blocks were examined from each area of each heart and 10 photomicrographs of suitable sections from all blocks at calibrated magnifications of 4000× or 8000× were used to measure sarcomere length. The measurements were taken from Z band to Z band at their mid-points, and results expressed as average values obtained from 60 electron micrographs. Only intact sarcomeres were measured, and no allowance was made for tissue shrinkage. Capillary density was measured by first projecting slides with 1-µm thick transverse sections on to a screen to give a final magnification of 3000× (3 mm − 1 µm). The total number of capillaries (lumen diameter <15 µm) within a 290-µm diameter circular field were counted, and for each vessel the polar (a) and equatorial (b) diameters were recorded so that the percentage dilation could be calculated from the formula (100a/½ (a + b)). A value for the overall volume of capillary bed (V), was obtained by multiplying the capillary density per mm² by the percent dilation. Ten such measurements were carried out for each condition and the mean values recorded.

S.E.M. (3 Control, 3 Isovolumic)

Approximately 1 ml of methyl methacrylate mixture, prepared according to the method of Murakami (1971) was injected until it began to discharge from the pulmonary artery. The polymer within the hearts then was allowed to polymerize fully at room temperature for several hours and subsequently was kept at 4°C for 1–4 days. Each heart was divided transversely through the ventricle (as for the flow study). We mounted the half hearts on coverslips and removed tissue by sodium hydroxide treatment before mounting the coverslips on aluminum stubs, vacuum-coating with carbon and gold, and examining in a Cambridge II A scanning electron microscope, operating at 20 kV. Further details of the methodology are given in another paper (submitted for publication).

Results

The coronary flow in all hearts was 10–16 ml/min, the average value for the isovolumic series being slightly less than the average for the control hearts.

The intracavitary pressure of the isovolumic hearts fell to approximately 10 mm Hg within 2 minutes of cessation of flow. This fall at the beginning of the ischemic period was followed by the progressive development of contracture pressure starting after approximately 5 minutes and reaching a maximum of 30–60 mm Hg within 20–30 minutes of the onset of ischemia. Subsequently, the pressure fell by 30–40% of the peak contracture tension at the time of removal of the balloon. Intracavitary pressure throughout the ischemic period of the nonisovolumic hearts was not monitored.
**Reflow**

Figure 1A shows a black and white plate of the fluorescein distribution in the bisected heart after 60 minutes of ischemia. The subendocardial and mid-myocardial layers of the left ventricular wall (EN) surrounding the lumen are dark, demonstrating absence of flow, whereas the peripheral subepicardial zone (EP) is lighter, indicating the presence of fluorescein and, hence, reflow. This lighter zone appears yellow under U.V. light.

Analysis of six hearts treated in this way gave a mean value for the percentage of reflow of 45 ± 5% (Table 1).

Figure 1B shows a black and white plate of the fluorescein distribution in the bisected heart subjected to 60 minutes of ischemia with an isovolumic left ventricle. The flow area is greater than in Figure 1A. Analysis of six hearts treated in this way gave a mean value for the percentage reflow of 94 ± 4% (Table 1). Four hearts in this group showed fluorescence throughout the myocardial walls, whereas two hearts showed small non-fluorescent areas. However, these areas occurred primarily distributed around the compressible right ventricular lumen.

**T.E.M.**

In the control ischemic hearts, tissue from both the areas with fluorescein (EP) and without fluorescein (EN) showed features usually associated with severe ischemic injury, including moderate swelling of mitochondria and formation of typical intra-mitochondrial amorphous dense inclusions (Fig. 2A, and B). The EP tissue generally was less severely affected and often showed persistence of glycogen granules, which were always absent in EN.

Careful analysis of the Z band widths in these hearts gave an average value of 1.56 ± 0.7 μm (Table 1) (range 1.18-2.15 μm). Both contracted and relaxed sarcomeres were evident in the EP and the EN. The appearance of the capillary network by light microscopy is shown in Figure 3. Average tissue capillary density was 2720/mm² in the EP flow area and 1634/mm² in the EN no-flow area. Percentage dilations were 80 ± 2% in the former and 70 ± 3% in the latter, giving a capillary bed volume (V) of 2163 for control EP and 1144 for the EN (Table 1).

The subepicardial layer of the isovolumic hearts was ultrastructurally similar to that of the control subepicardial layer (Fig. 2A and C). However, the remainder showed more severe injury than in the control series, with greater evidence of myofibrillar tearing (Fig. 2D) and disruption at the intercalated discs (Fig. 2F). The EP and EN region had very similar Z to Z band widths, giving an average value for the total myocardium of 1.79 ± .03 μm (range 1.50-2.25 μm). There was a significant difference between the control and isovolumic groups in the

<table>
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<th>Table 1</th>
<th>60-Min control ischemia</th>
<th>60-Min isovolumic ischemia</th>
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<tbody>
<tr>
<td>Extent of myocardial wall reflow</td>
<td>45 ± 5%</td>
<td>94 ± 4%</td>
</tr>
<tr>
<td>Average sarcomere width</td>
<td>1.56 ± 0.07 μm</td>
<td>1.79 ± 0.03 μm</td>
</tr>
<tr>
<td>Capillary-bed volume (V)</td>
<td>EP 2163 (100%)</td>
<td>2545 (118%)</td>
</tr>
<tr>
<td></td>
<td>EN 1144 (53%)</td>
<td>1936 (90%)</td>
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Note: Capillary-bed volume has no units—see text for details. EP subepicardial ventricular wall, EN subendocardial and mid-myocardial ventricular wall.
Figure 2  Electron micrographs of the flow and no-flow areas of heart tissue after 60 minutes of ischemia. A: EP flow region of control ischemic heart; B: EP no-flow region of control ischemic heart; C: EP flow region of isovolumic heart; D, E, F: EN flow region of isovolumic heart. Some mitochondria (M) contain electron-dense inclusions (arrows). Sarcomeres vary in length but appear more stretched and torn (T) in D. Note glycogen granules (G) in A. Z lines (Z) appear wavy in E and F. There is severe disruption at the intercalated disc (I). Scale bar = 2 μm except for E = 1 μm.
average sarcomere length (Table 1). The Z lines consistently showed a wavy appearance (Fig. 2E) not observed in the control ischemic tissue (Fig. 2B).

The capillary network appeared extremely open and widespread (Fig. 3, A and C). The EP had 2860 capillaries/mm² and the EN 2450/mm². EP vessels were 89 ± 2% of maximum opening, whereas the EN region had an average of 79 ± 6% of maximum dilation, to give values for (V) of 2545 and 1936, respectively.

S.E.M.

The perfusion pattern obtained by the polymer in control hearts after 60 minutes of ischemia has been well documented in another paper (submitted for publication). This consists of a normal vasculature with the subepicardial layer of the myocardium, a marginal zone of only large vessels, 15-30 μm, and complete absence of vessels in the subendocardium (Fig. 4, A and C). In contrast, the cast of the isovolumic ischemic heart showed a picture similar to that of non-ischemic heart. A dense plexus of capillaries (5-10 μm) with many loops extended throughout the endocardium (Fig. 4, B and D).

Discussion

Our results demonstrate that if the muscle fibers of the left ventricle of the rat heart are prevented from shortening during the contracture induced by 60 minutes of ischemia, then the concomitant reperfusion defect is virtually abolished. An earlier study (Humphrey et al., 1980) also implicated ventricular contracture in the genesis of no-reflow by demonstrating that depletion of extracellular calcium reduces both the force of contracture and the extent of vascular incompetence.

Ultrastructurally, the isovolumic hearts appeared less altered in the EP (Fig. 2C) than in the EN (Fig. 2, D, E, and F). The greater damage, in the area of greatest intramyocardial pressure (Baird et al., 1970 and Stein et al., 1980) was not unexpected. Bing and Fishbein (1979) have demonstrated fiber disruption with contracture under isometric conditions (equivalent to our isovolumic ventricle), and they attributed this to the direct effect of mechanical stress. It did not occur extensively when the sarcomeres were allowed to undergo isotonic contracture (equivalent to the empty ventricle in our study). The distorted "wavy" appearance of the Z lines seen in subendocardium of isovolumic ventricles (Fig. 2, E and F) was not
observed in the control ischemic hearts. Such distortions of the Z lines were demonstrated in the hypoxic isometric papillary muscle experiments of Bing and Fishbein, and we believe they support the concept that the myofilaments are subjected to excessive stress due to the tension generated during isometric contracture.

The beneficial effect of the balloon on reflow in the subendocardium can easily be seen by a comparison of Figure 3, B and C. Only about half of the capillary-bed volume observed in the control EP was seen in the control EN (Table 1). However, the EN of isovolumic hearts had approximately 90% of the capillary-bed volume with respect to control EP. Similarly, the EP of isovolumic hearts had significantly increased vasculature volume compared to the control hearts (Table 1). The decrease in (V) seen in both groups from epicardium to endocardium presumably would reflect the prevailing increasing intramyocardial pressure gradient (Baird and Ameli, 1971).

The polymer-injected hearts were used to determine whether the increased reflow in balloon-hearts might not simply be due to the greater myocardial damage observed under electron microscopy. If the vasculature in these hearts were non-patent and

FIGURE 4 Appearance of myocardium after 60 minutes of ischemia followed by methyl methacrylate injection. A: control ischemia; note white appearance of methyl methacrylate in the outer 1/3 of myocardium. The inner portion of heart looking dark by comparison. B: isovolumic ischemic heart. White color of methyl methacrylate extends from the epicardium to the endocardial surface. C: scanning electron microscopy of border zone of control ischemic heart (arrow in A) showing only large 20-μm vessels penetrating into the subendocardium. D: scanning electron microscopy of endocardial surface of isovolumic heart (arrow in B) showing extensive array of small (5-10 μm) capillaries. Scale bar = 1 mm for A + B, 200 μm for C, and 20 μm for D.
fluorescein were merely infusing via the ruptured muscle, then the cast would reflect this by presenting a confluent appearance. In fact, the capillary network was clearly seen, indicating that the microvasculature remained intact and patent. Since the duration of ischemia was identical in both groups, these results suggest that ischemic-induced injury to the endothelium of the capillary network cannot account for the reperfusion defect in the isolated rat heart. In dog hearts, in which the no-reflow phenomenon develops much later, after 60–90 minutes of ischemia, considerable capillary damage has been observed and undoubtedly contributes to impaired reflow (Kloner et al., 1974; Willerson et al., 1975; Powell et al., 1976). The relative absence of vascular damage that we observed could be attributed to the shorter duration of ischemia, to species differences, or to perfusion with a crystalloidal solution rather than blood.

Also, release of norepinephrine, which has been implicated in hypoxic contraction of coronary arteries (Borda et al., 1980), would be expected to exert an equal effect in the two experimental groups. Therefore, norepinephrine release would not appear to contribute to the no-reflow phenomenon. However, exocytosis of norepinephrine may depend upon some coronary flow and may thus play no role in this totally ischemic situation.

Removal of the balloon, prior to fluorescein perfusion, caused an observable collapse of the heart. Since we would consider at least the subendocardial myofilaments to be in rigor by 60 minutes, this collapse must reflect a relaxation of the elastic elements of the cardiac muscle. This being so, the observed protection against occlusion of the microvasculature could be explained as follows:

In the normal sequence of events, complete cessation of coronary flow results in the development of ischemic contracture during the ensuing 5–30 minutes (Hearse et al., 1977; Jarmakani et al., 1978, Bing and Fishbein, 1979). This phenomenon is caused by a sliding of myofilaments, to give a shortening of muscle length which reduces the size of the ventricular lumen. It is generally accepted (Hearse et al., 1977; Apstein and Ogilby, 1980) that at or soon after peak contracture, when sarcoplasmic ATP has declined to near zero levels, the actin/myosin moieties become locked in rigor complexes and no relaxation can take place unless ATP can be regenerated or infused into the myofibrillar complex. If the ventricular lumen is made incompressible by insertion of a non-compressible balloon, then the resulting ischemic contracture will be manifested mainly by increased tension generation but at a constant “diastolic” fiber length. Since sliding is reduced, the tension exerted against the balloon must be relayed by the series elastic (SE) component of the myofibrillar apparatus. Fluorescein perfused at this time is unable to penetrate even into the subepicardial region of the heart (unpublished results). However, once the balloon is removed, the stretched series elastic element can presumably “give” sufficiently for the capillaries to be re-opened when subjected to perfusion at 100 cm H2O pressure. (Maruyama et al., 1980) have recently demonstrated that the stiffness of the series elastic element is elevated significantly in ischemic muscle fibers. These ischemic fibers were immersed in “relaxation medium” prior to “stiffness” analysis, thus presumably releasing any rigor complexes present in the ischemic fibers. Their results therefore show an impairment of muscle fibers, observed as an increased stiffness, that is, not associated with Ca2+-activated or rigor-activated contracture.

Our results support the concept that intramyocardial pressure generated by ischemic contracture plays a major role in the development of the no-reflow phenomenon in the globally ischemic rat heart. Thus, utilizing the Hill model of cardiac muscle, the contractile element (CE) will irreversibly shorten during ischemia. The SE element will be unaltered in isotonic (control group) ischemia and stretched in isometric (isovolumic group) ischemia. If the SE element stiffens in the isotonic mode, the incompressibility of the muscle may act to prevent re-opening of the vasculature, once vessels are closed by sufficient extra-luminal pressure. However, if the SE element is stretched and then stiffens, as in isometric contracture, then once the force is removed, (i.e., balloon removal) the incompressibility of the muscle is reduced. Previous observations (Alanen et al., 1980; Humphrey et al., 1980) that the extent but not the time course of contracture was related to the no-reflow phenomenon could be explained if we assume that the incompressibility (rigor) of ischemic muscle is imparted mostly by the SE element and that earlier contracture stiffness is due mostly to the CE. The SE element would be compressible during and some time after contracture completion and would then stiffen to produce muscle rigor. The extent to which this interpretation of our results on globally ischemic hearts can be applied in vivo, where regionally ischemic tissue is stretched with each systole, has yet to be determined. Recent studies have described how cyclic loading of cardiac muscle can reduce or abolish stiffness of ischemic (Apstein and Ogilby, 1980) and hypoxic (Lewis et al., 1980) muscle. These results support the theory that it is the SE element that confers the state of rigor in cardiac muscle. Our results, unfortunately, do not help in identifying the structural component of muscle to which each of these various elements allude. Both the CE and SE may well be associated with the actin/myosin cross-bridges.

This study may have significance in the currently debated practice of venting of the left ventricle during cardiac surgery (Buckberg, 1975). The occurrence of myocardial contracture in an unvented rather than a vented left ventricle may allow for a
greater degree of reperfusion once surgery has been completed.

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