The effect of delayed reperfusion following infarction in the rat on structural changes in viable myocardium

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Abstract

Objective: Evidence indicates that patency of the infarct related artery following the completion of myocardial necrosis can attenuate ventricular remodeling. Data have also demonstrated that inhibition of infarct expansion contributes to the anti-remodeling effect of delayed reperfusion. However, the influence of a patent artery on components of the remodeling process in the viable myocardium is poorly understood.

Methods: Myocyte morphometrics (isolated cell technique) and collagen content (hydroxyproline analysis) were assessed 28 days following experimental myocardial infarction from rats with permanently ligated left coronary vessels (NRP; n = 10) compared with rats who underwent reperfusion 150 minutes after ligation (RP; n = 11) and a sham-operated group (n = 10).

Results: Analysis of infarct size planimetry in a separate group of rats demonstrated that reperfusion at this late time point did not reduce infarct size (NRP: 33 ± 3 vs. RP: 35 ± 5%). Myocyte length in RP rats was less than in NRP rats in viable, non-infarcted left ventricular tissue (155 ± 3 vs. 167 ± 4 µm, p = 0.02), in the right ventricle (154 ± 4 vs. 167 ± 3 µm, p = 0.02) and in the septum (158 ± 4 vs. 169 ± 4 µm, p = 0.05). Reperfusion also attenuated the expected increase in cell volume compared with NRP rats (left ventricle 39.4 ± 1.7 × 10³ vs. 44.1 ± 1.6 × 10³ µm³, p = 0.06; right ventricle 36.7 ± 1.6 × 10³ vs. 42.7 ± 2.0 × 10³ µm³, p = 0.02; septum 41.0 ± 1.6 × 10³ vs. 44.2 ± 1.8 × 10³ µm³, p = 0.19). Hydroxyproline content increased in the viable left ventricular tissue in both the reperfused and non-reperfused groups.

Conclusion: Reperfusion without myocardial salvage attenuates the increase in myocyte length and volume that occurs in remodeling myocardium following infarction in the rat, with no effect on the increase in collagen content. These data indicate that patency of the infarct vessel, which is known to have an inhibitory effect on infarct expansion, also has an anti-remodeling effect remote from the area perfused by this artery. © 1997 Elsevier Science B.V.

Keywords: Myocyte hypertrophy; Rat myocardial infarction; Reperfusion; Collagen; Ventricular remodeling

1. Introduction

Left ventricular chamber enlargement following myocardial infarction has a detrimental effect on long-term prognosis [1]. This structural change occurs as a result of different pathologic processes including infarct expansion [2], myocyte hypertrophy and slippage of surviving myocytes [3,4] and growth of the interstitium as well as replacement fibrosis in the myocardium [5]. Pharmacologic interdiction, such as converting enzyme inhibitor therapy [6–8] and nitrates [9–11], have been successful in attenuating remodeling. Recent data have suggested that patency of the infarct artery, even when achieved beyond the time for myocardial salvage, may also be effective in attenuating chamber dilation following infarction [12–16]. Delayed reperfusion limits infarct expansion, one of the early contributors to left ventricular remodeling [17]. Whether reperfusion also influences structural changes in viable, non-infarcted myocardium remote from the infarction has not been extensively studied. Data from Erlebacher and colleagues [18] indicate that attenuation of infarct expansion limits dilation of non-infarcted zones, suggesting that a...
The investigation conforms with the National Institutes of Health NIH Publication No. 85-23, ligature procedure thoracotomy with no ligature placement were sacrificed for analysis of myocyte size NRP:

of infarct size. The remaining rats who survived to 28 days reperfused groups were sacrificed to allow for estimation eight pre-assigned rats from both the reperfused and non-reperfused groups were analyzed for myocardial collagen content in non-infarcted left and right ventricular myocardium from reperfused hearts with both non-reperfused and sham-operated hearts.

2. Methods

One hundred and thirty-five rats (Sprague–Dawley; 250–300 g) were studied. One hundred and twenty-five rats underwent left coronary ligation. Thirty-five of the surviving 63 rats were randomly assigned to reperfusion (RP) 150 minutes following ligature placement. Twenty-five rats survived this procedure. The left coronary artery in the other 28 rats remained ligated (NRP). It has been shown by other laboratories that 120 minutes of coronary ligation is sufficient to complete a transmural infarction in this model [12,19,21]. Seven days following infarction eight pre-assigned rats from both the reperfused and non-reperfused groups were sacrificed to allow for estimation of infarct size. The remaining rats who survived to 28 days were sacrificed for analysis of myocyte size (NRP; n = 11; RP; n = 10) and estimation of hydroxyproline concentration (n = 5 both NRP and RP). Ten rats underwent a sham ligation procedure (thoracotomy with no ligature placement) and were followed for 28 days before sacrifice for myocyte sizing (n = 5) and hydroxyproline analysis (n = 5). The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 8523, Revised 1985). The protocol was approved by the University of Minnesota Animal Care Committee.

2.1. Rat myocardial infarction and reperfusion

Under general ether anesthesia a left thoracotomy was performed, and a loose purse string stitch of 2-0 sterile suture was placed to facilitate rapid closing and prevention of pneumothorax. The heart was exteriorized, and the left coronary artery was ligated using 4-0 ethibond. The heart was then returned to the chest cavity, and as the lungs were re-expanded, the purse-string suture was tied. The skin was closed using sterile suture. Operative procedures in sham-operated rats were identical, with the exception of the placement of the coronary ligature. The perioperative mortality in our laboratory for this experiment was 50%, with a subsequent mortality of approximately 10% over the first thirty days.

Reperfusion was achieved as described by Hochman and Choo [12] with the exception that ligature removal was performed two and one-half hours after ligation rather than at two hours as previously described. At reperfusion the rat was reanesthetized with ether. The skin sutures were cut and a new purse string suture placed. The original purse string was cut, and the heart exteriorized as described above. The left coronary artery and the coronary ligature were exposed. The ligature was cut using a #11 surgical blade, and the suture pulled out of the myocardium. The heart was returned to the chest cavity, and the chest wall and skin sutured closed as described above. Reperfusion was associated with a 30% procedural mortality in our laboratory and has been shown by microsphere studies to result in return of blood flow to the infarcted zone [39].

2.2. Estimation of infarct size

Eight NRP and eight RP rats were sacrificed 7 days following infarction for estimation of infarct size. At the time of sacrifice hearts were removed from the chest and the atria were dissected free from the ventricular tissue. The ventricular tissue was then placed in phosphate-buffered formalin. Subsequently the heart was imbedded in paraffin and sectioned serially from apex to base into four transverse slices and stained with trichrome. The image of each slice was projected onto a digitization board and using a Sigma-Scan System (Tardel Scientific, Corte Madera, CA) infarcted and noninfarcted areas were planimetered. The infarct size, expressed as a percentage of the left ventricle, was calculated by dividing the area of the infarct by the total area of the left ventricle including the septum.

2.3. Myocyte isolation and morphometry

Cardiac myocytes were isolated by modified Langendorff retrograde perfusion [22]. Animals were anesthetized with an intraperitoneal injection of sodium phenobarbital (30 mg/kg). The heart was quickly removed and put in ice-cold saline and then blotted and weighed. The aorta was cannulated and connected to Langendorff apparatus. The heart was cleared of blood by brief perfusion with calcium-free solution (having the following composition: 135 mM NaCl; 2 mM KCl; 100 µM MgCl2; 1 mM HEPES; 360 µM NaH2PO4; and 10 mM dextrose warmed to 37°C and gased with 95% O2 and 5% CO2). To separate cardiac myocytes from the tissue, enzyme perfusate containing 120 U/ml collagenase, type II (Worthington Biochemical Co.), 0.04% bovine serum albumin, fraction V (ICN Immuno Biologicals) and 1.3 U/ml protease, type XIV (Sigma) was recirculated for approximately 20 min-
Fig. 1. Diagrammatic representation of zones of analysis for myocyte morphometry and hydroxyproline analysis.

utes. Isolated myocytes were collected from the right ventricle, septum, and non-infarcted left ventricle. Cells from the right ventricle were obtained from the free wall of this chamber. The interventricular septum was not involved in the infarction and the non-infarcted left ventricle refers to that portion of the free wall of the left ventricle that remains free of infarction (see Fig. 1). Cells were immediately fixed in 25% glutaraldehyde (final concentration is 2%). The yield of rod-shaped cells was 70–90%.

Cell length was measured under light microscopy using a micrometer. Fifty cells (rod-shaped, with clear sarcomere striation in the absence of membrane blebs) were used from each sample. Cell length was defined as the longest length parallel to the longitudinal axis of cells. Cell volume was determined using a Coulter Channelizer (Model 256) connected to a Coulter Counter (Model ZM). Cell volume was assessed as a change in electrical resistance resulting from the displacement of electrolyte as cells moved through the aperture using a shape factor of 1.05. Cell cross-sectional area (CSA) was calculated as cell volume divided by cell length.

2.3.1. Nuclear patterns determination
Myocyte nuclei were stained by placing one drop of 4',6-diamidino-2-phenylindole (DAPI) solution onto a glass slide having one drop of glutaraldehyde fixed cells. DAPI solution was prepared as follows: 100 ml aqueous solution containing 1 mg of DAPI, 138 mg of Na₂PO₄ · 12H₂O, 0.9 g of NaCl, 11 mg of CaCl₂, 12 mg of MgSO₄ · 7H₂O, 0.6 ml of Igepal CA-630. Nuclear numbers were counted under Fluorescent Microscope (Olympus BX70). A wide ultraviolet filter was used and Images were collected with Metamorph software in Gateway 2000 P5-90 Computer. The final image processing was performed using Adobe Photoshop and printed using a Fuji Pictrography 3000 digital printer (Tokyo, Japan). One hundred cells were counted randomly from each sample.

2.3.2. Sarcomere length measurement
Glutaraldehyde fixed cells were examined with a Bio-Rad MRC 1000 Confocal Microscope equipped with an argon laser (Bio-Rad Life Science Group, Hercules, CA). 60 × oil lens and zoom of 4 were used. Sarcomere lengths were determined using Bio-Rad CoMOS software. The image was then imported to Adobe Photoshop for enhancement and coloration. The final image was printed using a Fuji Pictrography 3000 digital printer. Ten sarcomeres were measured from one cell, and ten cardiac cells were examined from each individual sample.

2.4. Tissue hydroxyproline assay
Tissue was harvested from the non-infarcted left ventricle, the interventricular septum and the right ventricle. For the extraction of hydroxyproline the frozen tissue was dehydrated at 40–45°C for 48 hours and then weighed. The dried tissue was hydrolyzed in 6 N HCl at 107°C for 16–18 hours. The hydrolysate was then dried under vacuum and reconstituted with 2.0 ml distilled H₂O. Hydroxyproline concentration was quantitated in an 50 μl aliquot of aqueous extract [38]. The hydroxyproline imino acid was oxidized with 0.02 M chloramine T (Sigma Chemicals, St. Louis, MO) in acetate–citrate buffer pH 6.0 at room temperature for 20 minutes. The oxidized product
was then reacted with 0.25 M p-dimethylaminobenzaldehyde (Sigma Chemicals, St. Louis, MO) in perchloric acid:1-propanol (1:4) at 60°C for 15 minutes and the resulting chromophore quantitated spectrophotometrically at 560 nm against a hydroxyproline standard curve. Hydroxyproline concentration was calculated by linear regression analysis and expressed as mg/g dry wt.

2.5. Statistics

Infarct size between the two groups was compared using an unpaired student’s ‘t’ test. Differences in myocyte length, volume, and cross-sectional area and tissue hydroxyproline content between the sham-operated, nonreperfused and reperfused groups were assessed using analysis of variance. Subsequent inter-group comparison was performed with unpaired student’s ‘t’ tests with adjustment of level of significance to a p value ≤ 0.025 because of 2 between group comparisons. All data are expressed as mean ± standard error of the mean.

3. Results

One hundred and thirty five rats underwent coronary ligation and subsequent randomization to either permanent ligation or reperfusion. Two reperfused and four nonreperfused rats died during the 28 day observation period. There were no deaths among the ten sham-operated animals. Body weight increased by a similar degree in all three groups (Sham: +25 ± 6 g; Reperfused 30 ± 7 g; Non-reperfused 31 ± 6 g). At sacrifice, heart weight:body weight ratios were significantly greater in both the reperfused (4.01 ± 0.18) and permanently ligated groups (4.28 ± 0.1) compared with sham-operated rats (3.58 ± 0.23, both p < 0.02). While there was a trend towards a smaller heart weight:body weight ratio in the reperfused group compared with the permanently ligated group, this did not reach statistical significance.

3.1. Infarct size in reperfused and nonreperfused infarcts

Infarct size was similar in both the reperfused and nonreperfused infarct groups (35 ± 5 vs. 33 ± 3%). These data demonstrate that reperfusion 150 minutes following left coronary ligation did not alter infarct size.

3.2. Myocyte morphometrics

3.2.1. Reperfused versus nonreperfused rats

Delayed reperfusion resulted in an attenuation of the increase in myocyte length compared with the nonreperfused group in the viable left ventricle (155 ± 3 vs. 167 ± 4 μm, p = 0.02) and the right ventricle (154 ± 4 vs. 167 ± 3 μm, p = 0.02) with a similar trend in the septal area (158 ± 4 vs. 169 ± 4 μm, p = 0.05) (Fig. 2). Reperfusion also reduced the increase in myocyte volume in the right ventricle (36.7 ± 1.6 × 10³ vs. 42.7 ± 2.0 × 10³ μm³, p = 0.02) with a similar trend in both the viable left ventricle (39.4 ± 1.7 × 10³ vs. 44.1 ± 1.6 × 10³ μm³, p = 0.06) and the septal zone (41.0 ± 1.6 × 10³ vs. 44.2 ± 1.8 × 10³ μm³, p = 0.19) (Fig. 3). Reperfusion appeared to have little influence on the change in myocyte cross sectional area (left ventricle 255 ± 11 vs. 265 ± 7 μm², p = 0.44; septal 259 ± 8 vs. 262 ± 9 μm², p = 0.81; right ventricle 238 ± 8 vs. 255 ± 8 μm², p = 0.13).

3.2.2. Reperfused versus sham operated rats

Despite the attenuation of myocyte remodeling by reperfusion, myocytes from the viable left ventricle, the septum and the right ventricle still demonstrated a significant increase in length in reperfused hearts compared with myocytes from sham-operated animals. The increase in length was similar in magnitude in all three zones (left ventricle, 155 ± 3 vs. 135 ± 7 μm, p = 0.001; right ventricle, 154 ± 4 vs. 133 ± 8 μm, p = 0.003; septum, 158 ± 4 vs. 134 ± 7 μm, p = 0.004) (Fig. 2). A similar observation was made with respect to myocyte volume (Fig. 3) (left
ventricle, 39.4 ± 1.7 × 10^3 vs. 33.0 ± 1.9 × 10^3 μm³, p = 0.001; right ventricle 36.7 ± 1.6 × 10^3 vs. 30.1 ± 2.1 × 10^3 μm³, p = 0.01; septum 41.0 ± 1.6 × 10^3 vs. 32.1 ± 1.5 × 10^3 μm³, p = 0.001). Myocyte cross-sectional area demonstrated a similar trend in all three areas (left ventricle 265 ± 7 vs. 243 ± 11 μm², p = 0.11; septum 262 ± 9 vs. 238 ± 9 μm², p = 0.06 and right ventricle 255 ± 8 vs. 225 ± 13 μm², p = 0.12).

3.2.3. Nuclear pattern and sarcomere length

Nuclear pattern was not altered by infarction or reperfusion. The preponderance of cells in the sham group (90%), NRP (94%) and RP groups (93%) were binucleated with the remainder being mononucleated (9%, 5%, 6%, respectively) or polynucleated (all groups = 1%).

Similarly, sarcomere length was not altered by infarction or reperfusion. [Sham: 1.96 ± 0.1 μm; Permanently ligated: 1.95 ± 0.12 μm; Reperfused group: 1.95 ± 0.12 μm].

3.3. Hydroxyproline content

There was an increase in hydroxyproline concentration in the viable left ventricular tissue in NRP rats compared with sham-operated rats (Table 1). No change in hydroxyproline was observed in either the septum or the right ventricle. Reperfusion did not attenuate the increase in hydroxyproline concentration in the left ventricle observed in the NRP group (Table 1). Right ventricular hydroxyproline concentration increased in the right ventricle in the RP group.

Table 1
Hydroxyproline content (mg/g dry weight: mean ± SE) in right ventricle (RV), septum (S) and noninfarcted left ventricle (LV) in sham-operated (SO) nonreperfused (NRP) and reperfused (RP) hearts

<table>
<thead>
<tr>
<th></th>
<th>LV</th>
<th>RV</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO</td>
<td>2.7 ± 0.3</td>
<td>4.1 ± 0.9</td>
<td>2.9 ± 0.4</td>
</tr>
<tr>
<td>NRP</td>
<td>5.0 ± 0.9*</td>
<td>4.2 ± 0.4</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td>RP</td>
<td>5.2 ± 0.9*</td>
<td>6.4 ± 0.5*</td>
<td>3.7 ± 0.4</td>
</tr>
</tbody>
</table>

*p < 0.025 with SO.

4. Discussion

Patency of the infarct artery has prognostic benefit [14,16,17,23–25]. Several mechanisms have been put forward to explain this observation including the association between patency and inhibition of remodeling [15,23,25,26]. Studies have demonstrated that delayed reperfusion, beyond the time for myocardial salvage, attenuates infarct expansion thereby reducing ventricular dilation following infarction [12,13]. In human studies late reperfusion also reduces infarct zone thinning and progressive chamber enlargement [18,27]. It remains unclear whether a patent artery mediates its anti-remodeling effects solely through attenuation of infarct expansion or whether there may also be an effect on changes in the viable myocardium. To date, no study has analyzed the effect of delayed reperfusion on either myocyte remodeling or growth of the interstitium in viable myocardium following infarction.

The results of this and other studies [3–5,28–32] demonstrate that significant myocyte hypertrophy, predominantly eccentric, occurs in the viable myocardium following infarction. The degree of eccentric hypertrophy observed in this study is similar in all three measured areas, a finding consistent with the data of Zimmer et al. [30]. Others have found hypertrophy most marked in the peri-infarct zone [31].

Delayed reperfusion attenuated myocyte remodeling, manifest predominantly by a reduction in eccentric growth of the cell. This effect was noted in all three zones, albeit to a lesser extent in the septum. The increase in myocyte volume was also attenuated by reperfusion. This effect was most marked in the right ventricle but a similar trend was also observed in the free wall of the left ventricle and in the septum. Available data on the influence of delayed reperfusion on remote myocardium are few an have been contradictory [18–21]. Moreover, these studies have focused on gross structural changes and have not analyzed the impact of reperfusion on myocyte morphometry or the cardiac interstitium.

In agreement with other laboratories our data demonstrate that nonreperfused infarction resulted in an increase in hydroxyproline concentration in the viable left ventricular myocardium [33–35]. However, no change was noted in collagen content in the septum or in the free wall of the right ventricle in this study. This is in contrast to other studies where modest increases in collagen content have been noted in these areas [33,35]. Reperfusion had no effect on the increase in hydroxyproline noted in the viable left ventricular tissue suggesting that absolute collagen content is not altered by this intervention. This analysis does not provide information on collagen subtypes or potential realignment in the interstitial infrastructure. Therefore, it is possible that reperfusion could alter collagen crosslinks and the relative expression of the various forms of collagen without altering hydroxyproline content [36]. The noted increase in right ventricular collagen content in the reperfused group is unexplained but may relate to the effect of repeated surgeries in this group. This observation will require further investigation.

The mechanism explaining the effect of delayed reperfusion on myocyte remodeling remains unclear. It is not accounted for by reduced infarct size as many laboratories, including our own, have shown that reperfusion two hours or later following coronary ligation does not influence infarct size in the rat [19–21]. Delayed reperfusion attenuates infarct expansion [12,13,19] which could decrease...
wall stress especially in the peri-infarct zone. This would reduce the stimulus for remodeling in the viable myocardium. Reperfusion may alter the balance between growth-promoting and growth-inhibiting substances in the surviving myocardium. Whereas there are no data to support this hypothesis, it would be important to study the influence of reperfusion on known proliferative or anti-proliferative substances.

While delayed reperfusion may attenuate cardiomyocyte remodeling, many issues remain unresolved regarding this intervention. Is the timing of reperfusion of importance? Boyle and Weisman [21] showed a benefit 6–8 hours after ligation compared with 150 minutes in our study. Nidorf and colleagues [27] demonstrated in a clinical study that reperfusion towards the end of the first week following infarction appeared to attenuate remodeling. Knowledge of the mechanisms of benefit of reperfusion with help clarify this issue. Furthermore, the potential for an additive benefit between delayed reperfusion and pharmacologic inhibition of remodeling needs to be explored. It is clear that the attenuation of myocyte remodeling by reperfusion was not complete. The degree of inhibition of myocyte lengthening was similar to that observed with converting enzyme inhibitor therapy in nonreperfused infarcts [29]. To our knowledge the question of an additive benefit has been addressed by only two studies neither of which provided information on the impact on myocyte or interstitial remodeling [20,11].

Certain aspects of the design of this study need to be considered in the interpretation of the presented results. The major methodological issue in this study concerns the use of different groups of rats for the different analyses within this experiment. The impact of delayed reperfusion could be more precisely analyzed if confirmation of reperfusion, estimation of infarct size and morphometric studies were performed on each rat. However, because of preparation techniques required for the morphometric studies, pathologic examination of infarct size was not possible in the same rats. If the mortality of the reperfusion procedure was concentrated among rats with larger infarcts, the study would be biased toward a positive influence of reperfusion on remodeling as a result of smaller infarct size in the reperfused group. To address this concern we have demonstrated in a separate group of rats reported in this study that reperfusion does not alter infarct size, a finding supported by other laboratories [12,19–21]. We did not measure the transmural extent of the infarct but several laboratories have shown that delayed reperfusion results in a similar degree of transmural extension of necrosis compared with non-reperfused infarction [12,13,19,21]. Likewise delayed reperfusion does not appear to salvage myocytes within the infarct zone [12,13]. It should also be noted that infarct size in this study was moderate and the effect of reperfusion in the setting of larger or smaller infarcts may be different.

While there is a debate as to the best means of performing morphometric studies on myocytes, the techniques used in this study are well established [3,32]. The varied techniques used for myocyte analysis do produce different absolute values but the trends noted following interventions such as infarction are very similar [3,28,29].

Finally, this study did not assess the influence of reperfusion on left ventricular mass, chamber size, or wall thickness. The major aim of the study was to assess the influence of reperfusion on changes of myocyte shape and collagen content. A trend towards a reduced heart weight/body weight ratio in the reperfused group is consistent with the attenuation of myocyte hypertrophy with reperfusion. Future work will address the important question of the correlation between these observed structural changes and overall chamber volume.

5. Conclusion

Delayed reperfusion of an infarct artery has prognostic benefit potentially mediated by its impact on ventricular remodeling. This study demonstrates that cardiomyocyte remodeling is attenuated after reperfusion of the infarct artery without apparent effect on interstitial growth. Further work is needed to clarify the mechanism of this effect, and to determine whether its benefit is additive to the already proven anti-remodeling effect of pharmacologic therapy.

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