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Loss of plasma membrane integrity, complement response and formation of reactive oxygen species during early myocardial ischemia/reperfusion

Pradeepkumar Charlagorla[a,1], Junying Liu[a,1], Monaliben Patel[a], Julie I. Rushbrook[a], Ming Zhang[a,b,*]

[a] Department of Anesthesiology, SUNY-Downstate Medical Center, Brooklyn, NY 11203, United States
[b] Department of Cell Biology, SUNY-Downstate Medical Center, Brooklyn, NY 11203, United States

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ABSTRACT

Loss of plasma membrane integrity (LPMI) is a hallmark of necrotic cell death. The involvement of complement and ROS in the development of LPMI during the early stages of murine myocardial ischemia–reperfusion injury was investigated. LPMI developed within 1 h of reperfusion to a level that was sustained through 24 h. C3 deposition became significant at 3-h reperfusion and thus contributed little to LPMI prior to this time. SOD1 transgenic mice had significantly less LPMI compared with WT mice at 1 h of reperfusion but not at later time points. Catalase transgenic mice were not protected from LPMI at 1-h reperfusion compared with WT mice, but had 69% less LPMI at 3-h reperfusion. This protection was transient. At 24-h reperfusion the LPMI of catalase transgenic mice was identical to that of WT mice. The delayed benefits of over-expressed catalase compared with SOD1 are consistent with its antioxidant action downstream of SOD1. The onset of LPMI occurs within 1 h of reperfusion at a level that is maintained through 24 h. ROS contribute significantly to LPMI during the first 3 h of reperfusion, while complement deposition, which becomes significant after 3-h reperfusion, may contribute thereafter.

1. Introduction

Loss of plasma membrane integrity (LPMI), a hallmark of necrotic cell death, has been studied extensively in cell culture. However, *in vivo* studies are relatively few due to the lack of effective methods for LPMI identification *in vivo*. Necrosis of cardiomyocytes occurs in myocardial ischemia, and typically, myocardial necrosis (infarction) is determined histologically using 2,3,5-triphenyltetrazolium chloride (TTC). TTC enters all cells and is reduced to red-color 1,3,5-triphenylformazan by endogenous dehydrogenases/cofactors when cells are intact. In necrotic cells, when reperfusion following ischemia is extensive, the enzymes/cofactors have been washed out and TTC remains colorless, defining infarction. Since inadequate reperfusion underestimates the extent of infarction (Birnbaum et al., 1997; Ito et al., 1997), effective TTC staining requires at least 3-h reperfusion in the coronary artery occlusion model (Birnbaum et al., 1997). The need for adequate reperfusion limits the use of TTC when focusing on events early in reperfusion. In addition, TTC-stained tissue is not suitable for detection of other markers of injury, obviating a valuable experimental option and requiring study of additional animals.

Propidium iodide (PI), described in three studies using *in vivo* rodent myocardial ischemia/reperfusion (I/R) models (Ito et al., 1997; Weinbrenner et al., 2004; Wolff et al., 2000), provides an alternative approach for identifying LPMI. PI enters necrotic, but not intact, cells after compromise of plasma membrane integrity, intercalating with DNA to produce red fluorescence. PI can be introduced *in vivo* and does not require extensive reperfusion. In addition, we recognized that PI-stained tissues can be analyzed for concurrent pathological events.

In the present study, we extend previous PI-based assessments of LPMI occurring during I/R injury (Ito et al., 1997; Weinbrenner et al., 2004; Wolff et al., 2000) by (i) evaluating the early temporal development of LPMI in a murine myocardial I/R model, (ii) working with PI-stained tissue, documenting the innate inflammatory response as denoted by deposition of complement C3, the central molecule in complement pathways associated with myocardial infarction in clinical and animal studies (Weisman et al., 1990), and (iii) using superoxide dismutase 1 (SOD1) and catalase transgenic mice, evaluating the time-dependent contribution of reactive oxygen species (ROS) to necrosis following myocardial I/R.

The ability of PI to assess LPMI early in reperfusion provided an opportunity to re-examine conflicting results using TCC to study the effects of over-expressing or administering SOD1 on reperfusion

* Corresponding author at: Department of Anesthesiology, Box 6, SUNY Downstate Medical Center, 450 Clarkson Avenue, Brooklyn, NY 11203, United States. Tel.: +1 718 270 1413; fax: +1 718 270 3928.
E-mail address: ming.zhang@downstate.edu (M. Zhang).
[1] These authors contributed equally to this paper.

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injury. While several animal studies found that SOD1 could reduce infarction in both regional (Gross et al., 1986; Hangaishi et al., 2001; Kanamasa et al., 2001) and global heart ischemia (Ambrosio and Flaherty, 1992; Nishikawa et al., 1991; Otani et al., 1986; Wang et al., 1998), others found that it failed to protect against infarction in various ischemia models (Gallagher et al., 1986; Jones et al., 2003; Klein et al., 1988; Matsuda et al., 1991; Nejima et al., 1989; Ooiwa et al., 1991; Patel et al., 1990; Przyklenk and Kloner, 1989; Richard et al., 1988; Uraizee et al., 1987; Watanabe et al., 1993). Downey et al. (1991) suggested that SOD1 might interfere with the ability of TTC to differentiate between living and dead cells, with the result that TTC produced an artifactually reduced infarct size in SOD1-treated animals (Shirato et al., 1989).

2. Methods

2.1. Mouse model of myocardial I/R injury

The WT (C57BL/6) mouse strain was obtained from the Jackson Laboratory (Bar Harbor, ME). Transgenic mice over-expressing human SOD1 or catalase and their WT littermates were generously provided by Dr. Arlan Richardson's group (Chen et al., 2003). All mice were maintained at the Division of Laboratory Animal Resources of SUNY Downstate Medical Center. Genotyping was provided by GeneTyper (New York, NY) using established PCR protocols (Chen et al., 2003; Wessels et al., 1995). All mice were used in compliance with the requirements of the NIH and the SUNY Downstate Institutional Animal Care and Use Committee.

A murine myocardial I/R injury model was used (Zhang et al., 2006). Briefly, adult male mice (10–12 weeks old, average body weight 25–30 g) were anesthetized with pentobarbital (60 mg/kg), intubated and ventilated. Heart rate, core body temperature and ECG were monitored. The left anterior descending artery (LAD) was ligated and occlusion was confirmed by the lack of color of the anterior wall of the left ventricle and the ST elevation on ECG. Following 1 h of LAD occlusion, the ligature was removed and reperfusion confirmed by return of color to the left ventricle anterior wall and the appropriate ECG changes. After the surgery, analgesia (buprenorphine) was given; the mouse was weaned off the ventilator and allowed to recover under a heat lamp with oxygen *via* a mask.

To evaluate the early temporal development of LPMI and the concomitant deposition of complement C3, seven groups of WT mice were used. The controls, group (i), were sham-operated, with hearts being harvested 1 h after sternotomy. Six groups (ii)–(vii) were subjected to 1 h LAD occlusion followed by the following reperfusion times: 0, 1, 2, 3, 6 and 24 h. $n = 5$ mice/group/time point, except for the 24-h group where $n = 10$.

To investigate the contribution of ROS to LPMI during the early stages of reperfusion injury, SOD1 tg mice ($n = 4$/group/time point) and WT littermates ($n = 3$/group/time point) were subjected to 1 h LAD occlusion followed by the following reperfusion times: 1, 3 and 24 h. Catalase tg mice ($n = 5$/group/time point) and WT littermates ($n = 5$/group/time point, except for the 24-h group where $n = 3$) were subjected to 1 h LAD occlusion followed by the following reperfusion times: 1, 3 and 24 h.

Ninety percent of all experimental animals survived the heart surgery (non-survivors were excluded from the final data analyses), the most common cause of surgery-related death being accidental bleeding during the sternotomy and LAD occlusion.

2.2. Evaluation of tissue with LPMI and of tissue at risk for LPMI using two fluorescent probes delivered in vivo

LPMI was evaluated using modification of a previously described PI-fluorescent method (Ito et al., 1997; Weinbrenner et al., 2004; Wolff et al., 2000). Fifteen minutes before harvesting, mice were re-anesthetized, intubated and injected with 60 μl PI (5 mg/ml) through the retro-orbital sinus to stain cells with LPMI. The chest was re-opened and the LAD re-occluded 1 min before harvesting. Blue fluorescent microspheres (BFMs, 2 μm, ThermoFisher, PA), 100 μl of a 1% solution (in deionized water with trace amount of surfactant supplied by the vendor), were injected through the aortic arch to delineate non-ischemic tissue. Their absence located ischemic tissue. The heart was harvested, held at −20 °C for 15 min to facilitate sectioning, then cut horizontally into four slices, the first cut at the site of LAD occlusion, the remaining three through the ventricles. Each slice was weighed and imaged on each side under a fluorescent microscope (2× objective lens) using the red fluorescent channel for the PI fluorescent complex, the blue for BFMs.

Weight of tissue with LPMI

$$= (A_1 \times \mathrm{wt}_1) + (A_2 \times \mathrm{wt}_2) + (A_3 \times \mathrm{wt}_3) + (A_4 \times \mathrm{wt}_4),$$

where A is the percent of the area of a slice exhibiting red fluorescence (average of both sides of a slice) and wt is the weight of that slice.

Weight of tissue lacking blood flow which is at risk for LPMI

$$(\text{weight of at} - \text{risk tissue}) = (R_1 \times \mathrm{wt}_1) + (R_2 \times \mathrm{wt}_2) + (R_3 \times \mathrm{wt}_3) + (R_4 \times \mathrm{wt}_4)$$

where R is percent of the area of a slice lacking blue fluorescence (average of both sides).

The percentage of tissue which is at risk for LPMI and which stains for LPMI (% of at-risk tissue with LPMI) = (weight of tissue with LPMI/weight of at risk tissue) × 100.

2.3. Immunohistochemical analysis

Frozen sections were cut from the slices described above, fixed in acetone and stained with an FITC-labeled anti-C3 antibody (Dako, CA). Each section was imaged (2× objective lens) using channel 4 (for all fluorescence) to give total area. The C3 positive area was imaged (10× objective lens) and quantified by Image J software. The percentage of the total area that was C3 positive was determined.

2.4. Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). Levene's test was used to determine the homogeneity of variances. Statistical significance was assessed by a one-way ANOVA test followed by a *post hoc* Bonferroni's test (when the equality of variances assumption held) or Dunnett T3 test (when the equality of variances was not met). $P < 0.05$ was considered significant.

3. Results

3.1. LPMI occurred early in myocardial reperfusion

LPMI was studied in 7 groups of WT (C57BL/6) mice (Fig. 1a). Group (i) was sham-operated; groups (ii)–(vii) were subjected to 1 h of LAD occlusion followed by 0, 1, 2, 3, 6 or 24 h of reperfusion, respectively ($n = 5$ mice/group/time point, except for the 24-h group where $n = 10$).

Since the one-way ANOVA test for % of at-risk tissue with LPMI of all groups showed statistical significance ($P < 0.01$), Levene's test of the homogeneity of variances was applied and indicated $P < 0.01$. Therefore, the Dunnett T3 test was used for *post hoc* analyses. A small but non-significant % of at-risk tissue with LPMI was