Albumin Microbubble Persistence During Myocardial Contrast Echocardiography Is Associated With Microvascular Endothelial Glycocalyx Damage

Jonathan R. Lindner, MD; Suad Ismail, MD; William D. Spotnitz, MD; Danny M. Skyba, PhD; Ananda R. Jayaweera, PhD; Sanjiv Kaul, MD

Background—We hypothesized that the persistence of albumin microbubbles within the myocardium during crystalloid cardioplegia (CP) infusion and ischemia-reperfusion (I-R) occurs because of endothelial injury.

Methods and Results—The myocardial transit rate of albumin microbubbles was measured in 18 dogs perfused with different CP solutions and in 12 dogs undergoing I-R. Electron microscopy with cationized ferritin labeling of the glycocalyx was performed in 9 additional dogs after CP perfusion and in 3 additional dogs undergoing I-R. Microbubble transit was markedly prolonged during crystalloid CP perfusion. The addition of whole blood to the CP solution accelerated the transit rate in a dose-dependent fashion ($P<0.05$), which was greater with venous than with arterial blood ($P<0.05$). The addition of plasma or red blood cells to CP solutions was less effective in improving transit rate than addition of whole blood ($P<0.05$). Microbubble transit rate was independent of the temperature, K$^+$ content, pH, P_{O_2}, osmolality, viscosity, and flow rate of the perfusate. Similarly, a proportion of microbubbles persisted in the myocardium after I-R, which was related to the duration of ischemia ($P<0.01$) but not of reflow. Crystalloid CP perfusion and I-R resulted in extensive loss of the endothelial glycocalyx without other ultrastructural changes. This effect was partially reversed in the case of crystalloid CP when it was followed by blood CP.

Conclusions—Sonicated albumin microbubbles persist within the myocardium in situations in which the endothelial glycocalyx is damaged. The measurement of the myocardial transit rate of albumin microbubbles may provide an in vivo assessment of endothelial glycocalyx damage. (*Circulation*. 1998;98:2187-2194.)

Key Words: microspheres ■ echocardiography ■ endothelium

In a normal blood-perfused heart, the microvascular rheology of air-filled albumin microbubbles is similar to that of red blood cells (RBCs). Consequently, the mean myocardial transit rates of these microbubbles measured with myocardial contrast echocardiography (MCE) correlate closely with those of $^{99m}$Tc-labeled RBCs. The behavior of these bubbles, however, is markedly different during crystalloid cardioplegia (CP) perfusion, in which they are noted to persist within the myocardium. The mechanism(s) underlying abnormal microbubble behavior during crystalloid CP perfusion is unknown. We had previously hypothesized that reversible endothelial injury caused by crystalloid CP may be responsible for this effect. This hypothesis was supported by our more recent observation that the mean transit rate of albumin microbubbles is faster when the human myocardium is reperfused after CP arrest with venous than with arterial blood. Venous blood may have resulted in less production of O$_2$-derived free radicals, which have been implicated in endothelial injury after reperfusion. In the present study, we performed MCE and electron microscopy during CP delivery and after ischemia-reperfusion (I-R) to gain insights into possible mechanisms of microbubble-endothelium interactions.

Methods

Animal Preparations

Forty-two adult mongrel dogs (mean weight, 28±3 kg) served as the study animals, of which 27 were used for the CP and 15 for the I-R experiments. Another 18 dogs served as blood donors for the CP experiments. Dogs were anesthetized with 30 mg · kg$^{-1}$ sodium pentobarbital, intubated, and mechanically ventilated with a respirator pump (Harvard Apparatus). Additional anesthesia was given as needed. Catheters were placed in the right femoral artery for pressure measurement and in the right femoral vein for administration of fluids and drugs.

For the CP experiments, a median sternotomy was performed. Heparin (500 U · kg$^{-1}$) was administered intravenously and supple-

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mented (100 U · kg⁻¹) every 90 minutes. Both venae cavae were cannulated transatrially, and veins were placed in the right and left ventricles. The left femoral artery was cannulated, and cardiopulmonary bypass was initiated with a roller pump (6002, Sarns) and a membrane oxygenator (American Bentely Corp). Blood flow through the bypass system was held constant for each dog and varied from 2.0 to 3.0 L · min⁻¹ in individual dogs. Body temperature was held at 37°C by means of a blood warmer (Blanketrol, Subzero Products).

An infusion catheter (DLP) was placed in the ascending aorta, and its side arm was used to monitor aortic pressure. The main port was connected, via 1 arm of a Y-connector, to a power injector (Angiomat 3000, Liebel-Flarsheim) for microbubble injection. The other arm was used for CP delivery, which was varied from 125 to 250 mL · min⁻¹ between dogs but was held constant for each dog. The CP delivery rate was controlled by means of a roller pump (S10K II, Sarns) connected to a mixing reservoir (CPS4000, Gish Biomedical). This chamber allowed for the compositions of different CP solutions and contained a heating/cooling coil to maintain constant temperature. Arterial or venous blood was added to the mixing chamber, which had a port in its bottom for sample collection. All pressure lines were connected to a multichannel recorder (4568C, Hewlett Packard) via fluid-filled transducers. The ECG and core body temperature were also monitored and recorded.

For the I-R experiments, a left lateral thoracotomy was performed, and the proximal left anterior descending coronary artery (LAD) was dissected free from surrounding tissue. Its basal flow was measured with a flow probe (series SB, Transonics) connected to a flowmeter (T206, Transonics). After exposure of the right carotid artery, a 12F plastic cannula (C.R. Bard) was inserted into its lumen and secured in place with a tie. This cannula was attached to plastic tubing, the other end of which was connected to a custom-designed metal cannula. The tubing was placed in a roller pump (2501 Harvard Apparatus), and flow through it was measured with an extracorporeal flow probe (12C, Transonics). After the tubing was primed with blood, the LAD was ligated, and the cannula was inserted distal to the site of ligation and held in place by a silk tie. Flow to the LAD was adjusted to the basal flow rate. A side port on the tubing just proximal to the metal cannula was connected to a power injector (MV1-AT, Medrad) for administration of microbubbles.

**Myocardial Contrast Echocardiography**

MCE was performed with phased-array systems equipped with 5-MHz transducers. For the CP experiments, a Sonos 1500 system (Hewlett Packard) was used, and for the I-R experiments, an RT5000 system (General Electric Medical Systems) was used. A saline bath served as an acoustic interface between the transducer and the heart, and images were acquired at the short-axis mid–papillary muscle level. Power output, which was intentionally kept low, and gain settings were optimized at the beginning of each experiment and were then held constant throughout. The maximal dynamic range was used. Images were recorded on S-VHS videotape.

Sonicated albumin microbubbles (Albunex, Molecular Biosystems) with a mean size of 4.3 μm and a concentration of 0.5×10⁹ · mL⁻¹ were used for MCE. The microbubbles have 15-nm-thick shells composed of insoluble denatured human albumin with intramolecular disulfide bonds and carry a negative charge (~3 mV). For the CP experiments, 1 to 1.5 mL of Albunex, diluted to a total volume of 2 mL with 0.9% NaCl, was injected into the aortic root at a rate of 3 mL · s⁻¹. For the I-R experiments, 0.5 to 0.8 mL of Albunex was diluted to a total volume of 1.0 mL with 0.9% NaCl and injected into the LAD at a rate of 0.75 mL · s⁻¹. Three separate injections were made at each stage. The dose of Albunex was held constant in each dog.

MCE data were analyzed as previously described. Images encompassing the period from just before contrast injection until its disappearance from the myocardium were transferred from videotape to the computer memory. A region of interest (>2000 pixels) was defined over the anterior myocardium, and the average video intensity in this region was measured in every end-diastolic frame in the I-R experiments and in every fifth frame of the nonbeating heart in the CP experiments. Time-intensity data were background-subtracted and, in the CP experiments, fit to a γ-variate function (see Appendix). In the I-R experiments, time-intensity data were fit to a model that accounts for persistence of microbubbles in the microcirculation (see Appendix).

**Radiolabeled RBC Transit**

At the beginning of each CP experiment, 50 mL of blood was withdrawn from each dog and centrifuged. The separated RBCs were labeled ex vivo with ⁹⁹mTc. Aliquots of radiolabeled RBCs equivalent to ~100 μCi of radioactivity were diluted with 0.9% normal saline to a total volume of 2 mL and injected into the aortic root at a constant rate of 3 mL · s⁻¹ so that their input function was identical to that of microbubbles. Data were acquired with a custom-designed miniature CsI₂ probe (Oxford Instruments) equipped with a converging collimator placed 3 cm from the anterior surface of the heart. The probe was connected to a preamplifier/amplifier unit designed to detect the ⁹⁹mTc photo peak and was interfaced with a personal computer. Time-activity plots were generated and fitted to a γ-variate function (see Appendix).

**Electron Microscopy**

In dogs undergoing glyocalyx staining, human serum albumin (25 mg · mL⁻¹) was infused at 150 mL · min⁻¹ into the aortic root in the CP experiments or at the baseline flow rate directly into the LAD in the I-R experiments. This step was undertaken to flush cellular components from the coronary circulation without causing glyocalyx disruption. Electron-dense staining of the glyocalyx was then performed by perfusing the heart for 30 seconds at the same flow rate with a 5 mg · mL⁻¹ solution of cationized ferritin (Sigma) in 0.9% NaCl, followed by fixation.

A small portion of the anterior myocardium was removed, and several 1-mm² tissue sections were cut. These sections were immersion-fixed in a 4°C glutaraldehyde paraformaldehyde solution for 4 hours, followed by staining with 1% osmium tetroxide. Randomly selected sections were dehydrated in ethanol and embedded in epoxy resin. Thin (40-nm) sections were cut with a microtome and stained with saturated aqueous uranyl acetate and lead citrate. Electron microscopic examination (100CX, Jeol) was made at various magnifications.

Assessment of ferritin labeling of the microvascular glyocalyx was made from photographic prints with a final magnification of ×150 000. Glyocalyx thickness was determined at 1-cm intervals along the endothelial luminal surface by measuring the distance from the cell membrane to the farthest cationized ferritin molecule. A value of 0 was recorded if no ferritin was seen or if there was marked separation of the glyocalyx from the cell surface. The proportion of the luminal surface labeled by cationized ferritin was measured by a wheel caliper.

**Protocols**

The first 18 dogs from the CP experiments were used to determine the effect of different CP perfusates on myocardial microbubble behavior. A randomly assigned CP solution was administered through the cross-clamped aortic root until cardiac arrest occurred. After 1 minute, MCE and ⁹⁹mTc-labeled RBC data were acquired, and the perfusate was sampled for analysis of K⁺, total protein, osmolality, pH, Po₂, viscosity, and temperature. This procedure was sequentially repeated with 7 to 10 other CP solutions in random order, including crystalloid CP (Plegisol, Abbott Laboratories), venous and arterial blood, various dilutions of arterial and venous blood with crystalloid CP, and 5% human serum albumin. Crystalloid CP was activated before use by addition of 20 mL · L⁻¹ of 8.4% NaHCO₃. Supplemental K⁺ was added to the solution as necessary. Each stage lasted for no more than 8 minutes without reperfusion between stages.

In 6 dogs, additional stages were performed during perfusion with CP solutions containing either plasma or washed RBCs obtained from venous blood of a donor dog. Stages were performed in a random fashion with infusions of pure component and after 1:1
dilutions of each component with crystalloid CP. Similar infusions were repeated with washed RBCs containing carboxyhemoglobin (Hb) formed by bubbling CO at 1 L·min⁻¹ through 150 to 200 mL of RBCs.

The last 9 dogs from the CP experiments were used for assessing the endothelium: 1 received no CP and served as control, and 2 each received arterial blood alone, arterial blood followed by crystalloid CP, or arterial blood followed by crystalloid CP with subsequent arterial or venous blood reperfusion. A constant infusion rate of 150 mL·min⁻¹ for 3 minutes was used. At the end of the infusions, staining of the endothelial glycocalyx was performed in 1 dog from each group. In the remaining dogs, the heart was fixed by perfusion with 2.5% glutaraldehyde and 4% paraformaldehyde polymer in 0.1 mol/L phosphate buffer at 150 mL·min⁻¹ for 1 minute.

In 12 dogs from the I-R experiments, MCE was performed at baseline, after which LAD flow was interrupted for either 15, 30, 45, or 60 minutes (n=3 for each duration). Immediately before reflow, blood within the tubing was removed so as to minimize introduction of activated blood elements, and reperfusion was achieved with fresh blood from the carotid artery delivered at the basal LAD flow rate. MCE was repeated at 5, 15, 30, and 60 minutes after reflow. At the end of the experiment, the heart slice corresponding to the MCE images was stained with triphenyltetrazolium chloride to exclude the presence of infarction.

Three additional dogs from the I-R experiments were used for histological analysis. In 1 control dog, glycocalyx staining and fixation were performed 45 minutes after LAD cannulation without an intervening ischemic period. In the other 2 dogs, LAD flow was interrupted for 30 minutes, and glycocalyx staining and fixation were performed at either 15 or 45 minutes after reflow.

**Statistical Methods**

Data are expressed as mean±SD. Interval comparisons were made by 1-way repeated-measures ANOVA. Comparisons between perfusate variables and transit rate data from the CP experiments were made by multiple regression analysis. Differences were considered significant at P<0.05 (2-sided).

**Results**

**Effect of CP Solution Composition on Microbubble Transit Rate**

A total of 144 different infusions were analyzed from 18 dogs in which MCE was performed. Figure 1A illustrates time-intensity curves obtained from a single dog during perfusion with crystalloid and arterial blood solutions at identical flow rates. Compared with the curve obtained during initial perfusion with arterial blood, the curve during perfusion with crystalloid CP perfusion was much wider. Prolongation of microbubble transit was almost completely reversed by subsequent perfusion with arterial blood. Figure 1B shows time-activity curves obtained after injection of ⁹⁹mTc-labeled RBCs during the same stages as the MCE data in Figure 1A. RBC transit was rapid and was not affected by the perfusate used.

Figure 2 depicts the microbubble and RBC transit rates during perfusion with crystalloid CP and arterial blood in all dogs. Similar to the example shown in Figure 1, the mean microbubble transit rate was markedly reduced during crystalloid CP compared with that obtained during initial arterial blood perfusion, whereas the RBC transit rate remained constant. Subsequent perfusion with arterial blood significantly improved the microbubble transit rate, although not to the same rate as during initial arterial blood perfusion. Table 1 depicts the flow rate and composition of the perfusates. The total protein, viscosity, pH, and PO₂ of blood solutions were significantly greater than for crystalloid CP.

**TABLE 1. Ranges of Cardioplegic Perfusate Variables**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate, mL·min⁻¹</td>
<td>125–250</td>
</tr>
<tr>
<td>K⁺, mEq·L⁻¹</td>
<td>16.2–25.4</td>
</tr>
<tr>
<td>Temperature, °C</td>
<td>9–37</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0–54</td>
</tr>
<tr>
<td>pH</td>
<td>6.90–7.68</td>
</tr>
<tr>
<td>PO₂, mm Hg</td>
<td>16–300</td>
</tr>
<tr>
<td>Total protein, mg·dL⁻¹</td>
<td>0–6.7</td>
</tr>
<tr>
<td>Osmolality, mOsm</td>
<td>294–358</td>
</tr>
<tr>
<td>Viscosity, cp</td>
<td>0.9–2.4</td>
</tr>
</tbody>
</table>
The mean microbubble transit rate also correlated with both the viscosity and the total protein of the perfusate, which, like the hematocrit, reflect the amount of whole blood in the CP solution. Neither the arterial pressure nor the pH, PO2, K+, temperature, or osmolality of the CP solution, the ranges for which are listed in Table 1, significantly influenced microbubble transit rate. By multivariate analysis, the only perfusate variable that independently correlated with the microbubble transit rate was the hematocrit (F=11.5, P=0.015).

The effect of RBCs on microbubble rheology is depicted in dogs undergoing perfusion with whole blood and separated RBCs is depicted in Table 2. Commensurate with the findings just described, a 1:1 dilution of whole blood with crystalloid CP, which reduced the mean hematocrit by half, resulted in a moderate (38%) decrease in the mean microbubble transit rate. Whereas the hematocrit of pure RBC perfusates was more than twice that of whole blood, the mean microbubble transit rate was significantly lower. A 1:1 dilution of RBCs with crystalloid CP resulted in a hematocrit similar to that measured from whole blood, yet the mean microbubble transit rate was slow, indicating that the improvement in microbubble transit rate afforded by blood CP solutions is not entirely due to RBCs. Nonetheless, the marked (74%) decrease in the transit rate when pure RBCs were diluted with crystalloid CP indicates that the RBCs do influence microbubble rheology. The microbubble transit rates were not significantly altered when washed RBCs were treated with CO and administered whole (0.64±0.24 s−1) or when they were diluted 1:1 with crystalloid CP (0.23±0.11 s−1).

The effect of plasma proteins on microbubble transit rate is illustrated in Figure 4. It was significantly greater during delivery of solutions containing pure plasma compared with crystalloid CP, whereas an intermediate value was obtained when plasma was diluted 1:1 with crystalloid CP. The microbubble transit rate during infusion of solutions containing 5% serum albumin was also significantly greater than during crystalloid CP. The microbubble transit rate during pure plasma and 5% serum albumin perfusion was considerably lower than during perfusion with whole blood.

**Effect of I-R on Microbubble Transit**

Apart from a small region localized to the anterolateral papillary muscle in 1 dog undergoing 60 minutes of ischemia, no infarction was noted on tissue staining. Illustrated in Figure 5 are MCE-derived time-intensity curves from 2 dogs that underwent LAD flow interruption for 15 (A) and 60 (B) minutes. Before ischemia, the transit rate of microbubbles was rapid, and myocardial persistence of microbubbles was not seen. At 30 minutes after reflow, a proportion of micro-
bubbles was retained as indicated by persistent myocardial opacification after the initial bolus effect. The fraction of microbubbles that persisted \( (f) \) was greater with longer ischemia time (B versus A in Figure 5) and correlated with the duration of ischemia \( (P<0.01, \text{Figure 6A}) \) but not of reflow \( (P=0.39, \text{Figure 6B}) \).

The microbubble transit rate derived from the initial bolus effect was slower after 5 and 15 minutes of reflow compared with baseline (Figure 6B). Because mean transit rate \( = \text{flow/volume} \) and because coronary blood flow was constant at all stages, these results indicate that the changes in transit rate of the main bolus were due to changes in blood volume induced by reactive hyperemia. Because this phenomenon is transient, the transit rates of the main bolus were not prolonged at 30 to 60 minutes after reflow (Figure 6B).

### Ultrastructural Changes After CP Arrest

In the control dog, myocellular and endothelial ultrastructure, as well as the cationized ferritin–labeled glycocalyx, were normal (Figure 7A). On higher magnification, a fairly continuous layer of 4 to 7 ferritin molecules, representing the glycocalyx, was seen on the endothelial surface that spanned intercellular clefts (Figure 7B). Glycocalyx labeling was uniform and the thickness was consistent with that found in previous studies. \(^{15,16} \) (Table 3).

In dogs that were perfused with arterial blood CP alone, discontinuity of the glycocalyx was observed (Figure 8A), with lower glycocalyx thickness and percentage of endothelial surface labeled with cationized ferritin (Table 3). In dogs that underwent perfusion with arterial blood followed by crystalloid CP, the most striking finding was the flocculent appearance of the glycocalyx and complete absence of cationized staining in large expanses of the endothelial cell surface, and the regions with cationized ferritin staining had markedly reduced thickness (Figure 8B, Table 3). Partial restitution of the glycocalyx was noted in animals undergoing reperfusion with blood (Table 3), which was more marked with venous than with arterial blood (Figure 8C and 8D).

### Ultrastructural Changes After I-R

In the control dog, endothelial cell appearance was normal and glycocalyx staining was uniform and almost continuous (Figure 9A). After 30 minutes of ischemia and either 15 or 45

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**Figure 5.** Background-subtracted MCE time-intensity curves from anterior myocardium in 2 dogs at baseline and after (A) 15 minutes and (B) 60 minutes of reflow following 30 minutes of ischemia. See text for details. \( f \) indicates calculated fraction of microbubbles persisting in microcirculation (see Appendix).

**Figure 6.** A, Mean \( \pm \text{SEM} \) fraction of microbubbles persisting within myocardium \( (f) \) after various durations of LAD flow interruption (using all time points after reflow). B, Mean \( \pm \text{SEM} \) microbubble transit rate of main bolus at baseline and after reflow. See text for details. \( *P<0.05 \) vs baseline.

**Figure 7.** Electron micrographs at magnifications of \( \times6600 \) (A) and \( \times50\,000 \) (B) of small arterioles from left ventricle of a control dog that did not undergo CP arrest. A, Bar=200 nm and B, bar=50 nm.
minutes of reflow, endothelial cell appearance was normal, but the glycocalyx was thinned, nonuniform, and absent in patches (Figure 9B and 9C and Table 3). The duration of reflow did not have a significant effect on these measurements.

**Discussion**

The glycocalyx is a network of proteoglycans lining the luminal surface of the endothelium and plays an important role in local flow regulation and solute flux. Our observations show that the persistence of sonicated albumin microbubbles in the myocardium is associated with injury to the microvascular glycocalyx as measured by cationized ferritin binding. Thus, partial removal of or damage to the glycocalyx may be responsible for the myocardial adherence of microbubbles during crystalloid CP perfusion observed in previous studies.

The sonicated albumin microbubbles used in this study have a mean size of 4.3 μm and a negative charge. In a blood-perfused heart, their transit rate is similar to that of RBCs, which also have a negative charge from the coat of sialylglycoproteins around them. This negative charge plays an important role in preventing RBCs from adhering to the vessel wall, which itself carries a negative charge imparted by the endothelial glycocalyx. Removal of the glycocalyx or a change in its charge can conceivably result in adherence of the negatively charged microbubbles to the endothelial cells. The greater persistence of microbubbles during crystalloid CP perfusion compared with I-R experiments may be due to the more generalized glycocalyx disruption seen in the former situation.

There are other potential reasons for microbubble persistence during crystalloid CP infusion, such as exposure of underlying interstitial matrix after endothelial cell injury. In the present study, electron microscopy did not reveal significant ultrastructural changes in the endothelium. Upregulation of adhesion molecules may also occur during I-R. In a previous study using intravital microscopy, we noted white cell migration and adhesion to venular surfaces after prolonged CP delivery followed by reperfusion, which is consistent with expression of adhesion molecules. In our present study, each CP solution was infused for no more than 8 minutes of reperfusion after 30 minutes of ischemia. Bars = 50 nm.

**Figure 8.** Electron micrographs at a magnification of ×50,000 from left ventricle of dogs that underwent perfusion with (A) arterial blood alone, (B) arterial blood followed by crystalloid CP, (C) arterial blood followed by crystalloid CP with subsequent perfusion by venous blood CP, and (D) arterial blood followed by crystalloid with subsequent perfusion with arterial blood. Bars = 50 nm.

**Figure 9.** Electron micrographs at a magnification of ×50,000 from anterior myocardium with (A) no ischemia, (B) 15 minutes of reperfusion after 30 minutes of ischemia, and (C) 45 minutes of reperfusion after 30 minutes of ischemia. Bars = 50 nm.
Neither pure RBCs nor pure plasma with the same concentrations as that in whole blood produced the same effects as whole blood itself. This effect may be related in part to the independent abilities of both RBCs and plasma to scavenge O2-derived free radicals.23,24 It has been postulated that the greater O2 delivery by blood is a major reason for preventing endothelial ischemia and that this mechanism plays a major role in preserving endothelial function during blood compared with crystalloid CP delivery.25 In the presence of a high pH and hypothermia, however, O2-Hb dissociation curve shifts to the left, thereby limiting O2 available to tissue.26 Nonetheless, to completely refute the argument that our observations regarding microbubble transit rates could still be explained at least in part by Hb-bound O2, we treated RBCs with CO, and we found that RBCs containing carboxy-Hb had an effect on microbubble transit similar to that of RBCs containing oxy-Hb. There was also no effect of the Po2 of the CP solutions, reflecting their concentration of dissolved O2, on the mean microbubble transit rate. The Po2 of crystalloid CP solutions was sometimes higher than that of arterial blood solutions and always greater than those of venous blood solutions.

The high K+ content of CP required for cardiac arrest has been associated with ultrastructural changes of the endothelium.27 In our study, in which ultrastructural changes were absent, no relation was noted between a wide range of K+ concentrations and microbubble transit rate. The perfusate temperature has likewise been implicated in CP-induced endothelial dysfunction.28 No effect of temperature on the mean microbubble transit rate was noted over a wide range of perfusate temperatures in our study.

The major determinant of microbubble adherence in the I-R experiments was the duration of ischemia and not that of reperfusion, which is consistent with previous findings that the degree of glycocalyx disruption is related to the period of ischemia29 but not reflow.30 Microbubble persistence immediately after reflow may be related to the early production of O2-derived free radicals the magnitude of which is dependent on the duration of ischemia.20,31 In contradistinction, the duration of reflow determines the degree of cellular inflammation, which may not have an association with microbubble adherence.

Study Limitations
Cationized ferritin binds primarily to the negatively charged glycosaminoglycan residues of the glycocalyx,16,17 and therefore, sparse or absent binding could also represent alterations in charge. Regardless, any loss of negative charge of the glycocalyx should also be accompanied by the loss of an electrostatic repelling force on the microbubbles.

Unlike microbubble persistence, we did not observe any persistence of 99mTc-labeled RBCs in hearts undergoing CP delivery. Lack of any detectable RBC persistence may have been due to loss of their negative charge18 from their extensive washing in protein-free solutions during their preparation for ex vivo labeling. It is possible that microbubbles, being more buoyant, could adhere to the microvasculature during crystalloid CP delivery and become dislodged by RBCs when solutions containing whole blood or pure RBCs are perfused. We did not observe such effects in a previous study in which microbubble rheology was observed in the microcirculation during crystalloid CP delivery by intravital microscopy.5

Although we have provided correlative data implying endothelial glycocalyx disruption as a possible cause of myocardial microbubble persistence in a limited number of animals, we have not provided a definitive mechanism of glycocalyx-microbubble interaction. Future studies are necessary to address these issues. Finally, although other independent parameters of endothelial function have been shown to be abnormal in the models studied in these experiments, these need to be correlated with the magnitude of microbubble adherence. A model of endothelial dysfunction with intact glycocalyx would be useful to determine the role of MCE in assessing endothelial function, in which alterations in endothelial charge may not be the only abnormality.

Conclusions
Our results indicate that the mean transit rate of sonicated albumin microbubbles measured on MCE may provide an assessment of the integrity of the microvascular endothelial glycocalyx. By detecting glycocalyx disruption, which may precede ultrastructural changes in the endothelium, it may be possible to quantify microvascular endothelial injury early and to evaluate strategies aimed at attenuating this injury. Further studies are needed to ascertain the clinical utility of MCE for the assessment of microvascular endothelial dysfunction.

Appendix
When microbubbles behave as free-flowing tracers, their concentration within the myocardium (and hence video intensity) after a direct coronary bolus injection can be described by a y-variate function:

\[ y = A e^{-\alpha t} \]

where A is a scaling factor, t is time, and \( \alpha \) is the mean transit rate.18 If all microbubbles injected were to, instead, persist within the myocardial microcirculation and remain stable, then their concentration can be described by the integral of the equation above:

\[ y = \frac{A}{\alpha} [1 - (1 + \alpha t) e^{-\alpha t}] \]

If only a fraction ( \( f \) ) of the microbubbles persist and the remaining (1 - \( f \)) pass through unencumbered, then the concentration of microbubbles within the myocardium can be described by the function

\[ y = f \frac{A}{\alpha} [1 - (1 + \alpha t) e^{-\alpha t}] + (1 - f) A e^{-\alpha t} \]

which can be simplified to

\[ y = A \left( \frac{f}{\alpha} (1 - e^{-\alpha t}) + \left[ 1 - f \left( \frac{1 + \frac{1}{\alpha}}{\alpha} \right) \right] e^{-\alpha t} \right) \]

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