Functional Coronary Microvascular Injury Evident As Increased Permeability Due to Brief Ischemia and Reperfusion

Ira M. Dauber, Karyl M. VanBenthuysen, Ivan F. McMurtry, Gretchen S. Wheeler, Edward J. Lesnefsky, Lawrence D. Horwitz, and John V. Weil

Although morphological studies suggest that coronary vascular injury is a result of prolonged ischemia and subsequent reperfusion, whether functional coronary microvascular injury develops during brief in vivo ischemia is unclear. In other organs, permeability is a sensitive indicator of functional vascular injury. Therefore, a new double-indicator method of assessing vascular protein permeability, a method that is both sensitive and specific for vascular injury, was used to investigate the effects of ischemia of graded duration followed by reperfusion on coronary microvascular function. To help confirm functional coronary vascular injury, endothelium-dependent vasodilation of isolated coronary vascular rings also was examined. Microvascular permeability was quantitatively assessed as a protein leak index by measuring the rate of extravascular accumulation of radiolabeled protein (indium 113m transferrin) normalized for vascular surface area (technetium 99m erythrocytes). Anesthetized dogs underwent 0 (control), 15, 30, or 60 minutes of left anterior descending coronary artery occlusion followed by 60 minutes of reperfusion. Even 15 minutes of ischemia increased the protein leak index by 50% (3.16±0.30 ischemic vs. 2.09±0.11 control). Longer periods of ischemia increased the protein leak index in proportion to the duration of ischemia. The protein leak index increased threefold (6.51±0.60) after 60 minutes of ischemia. At each duration of ischemia, there was significant regional variation in the protein leak index that correlated with the severity of ischemic blood flow to that region measured with microspheres. Endothelial injury also was evident after 15 and 30 minutes of ischemia as impaired vasodilation of isolated coronary rings in response to the endothelium-dependent vasodilators acetylcholine and the calcium ionophore A23187. Electron microscopy and in vitro direct immunofluorescence revealed evidence of vascular injury after 60 minutes but not after 15 minutes of ischemia. We conclude that even brief ischemia and reperfusion cause functional coronary vascular injury evident as increased microvascular permeability and impaired endothelium-dependent vasodilation and that regional differences in the degree of microvascular injury correlate with differences in the severity of ischemia. (Circulation Research 1990;66:986–998)

Whether functional coronary microvascular injury occurs after brief in vivo ischemia and reperfusion is unclear.1–4 Based on morphological studies, coronary vascular injury appears to be a late sequel of ischemia and reperfusion because ultrastructural evidence of vascular injury is seen only after prolonged ischemia, when significant myocardial injury has occurred.5–11 However, the sensitivity of morphological techniques for assessing either reversible or irreversible vascular injury is unknown, and vascular injury or dysfunction could occur without morphologically evident abnormalities.12–14 Indirect assessments of coronary vascular function in vivo such as impaired tissue perfusion (“no-reflow”),7,13,16 increased coronary vascular
resistance,1,17,18 leakage of macromolecules,1,11,19,20 myocardial hemorrhage,2 and changes in myocardial protein content8,21 suggest that functional vascular injury occurs after prolonged ischemia and reperfusion. However, these changes have not been investigated after brief ischemia. In contrast, in vitro studies of myocardial ischemia and reperfusion using permeability to directly assess vascular function suggest that functional coronary vascular injury can occur after even brief ischemia followed by reperfusion.1,22-24 Thus, it is unclear whether coronary vascular injury occurs after brief ischemia and reperfusion in vivo or whether it is a result of prolonged ischemia, possibly due to accompanying myocardial injury or infarction.

In other organs, permeability appears to be a sensitive and specific indicator of both reversible and irreversible functional vascular injury.12,13,25-27 We previously developed a double-radioisotope indicator method for the assessment of pulmonary vascular permeability in vivo.27,28 We measured the rate of extravascular accumulation of radiolabeled protein after intravascular administration and normalized the result for perfused surface area and intravascular contributions by simultaneously injecting radiolabeled erythrocytes. This method was specific for protein accumulation due to vascular injury and considerably more sensitive than tissue water for assessing vascular injury.27 It was unaffected by increased hydrostatic pressure or alterations in perfused vascular surface area. Modification of this method has made it possible to measure permeability in other organs and on a regional basis within organs.29,30

Although several investigators have demonstrated increased permeability after in vitro myocardial ischemia and reperfusion,1,22-24 in vivo studies have been limited. After in vivo ischemia and reperfusion, Kloner et al,19 Camilleri et al,20 and others9,11,18 described increased myocardial accumulation of macromolecules, which provided qualitative evidence of altered vascular permeability. Using first-pass tracer-exchange methodology, McDonagh and Lake31 and Harris et al32 found abnormalities that suggested increased permeability. However, interpretation of these tracer-exchange studies has been hampered by regional variability in vascular injury and alterations in myocardial blood flow with the consequent changes in perfused vascular surface area that can accompany ischemia and reperfusion and may affect measurements.13,31-33 After brief ischemia and reperfusion, Jennings et al8 and Go et al21 found small increases in the albumin content of canine myocardium by using radiolabeled albumin. However, they did not distinguish increases in extravascular albumin content, which may have been due to increased permeability, from differences due to changes in intravascular albumin content.

Therefore, whether in vivo ischemia and reperfusion caused functional coronary microvascular injury was investigated, and the effects of regional variability in the severity of ischemia on microvascular injury were examined. Using our sensitive double-indicator radioisotope technique, protein permeability as an index of coronary microvascular function was assessed, and the effects of graded durations of ischemia (0, 15, 30, and 60 minutes) followed by reperfusion on regional coronary microvascular function were measured. In addition, as an independent indicator of coronary vascular dysfunction, endothelium-dependent vasodilation of isolated coronary vascular rings34-36 after 15 and 30 minutes of ischemia was assessed. These functional assessments of vascular injury were compared with ultrastructural changes detected by electron microscopy and protein immunofluorescence.

Methods

Experimental Protocol

After preanesthesia with sodium thiopental (20 mg/kg i.v.), dogs were anesthetized with α-chloralose (100 mg/kg i.v., 5% solution), intubated, and ventilated. Additional chloralose was given as needed during the experiment. Catheters were inserted in the femoral artery and vein for pressure monitoring and drug administration, and electrocardiographic monitoring leads were attached. The catheters were periodically flushed with heparinized saline (100 units/l). A thoracotomy was performed in the fourth left intercostal space, the pericardium was opened, the heart was suspended in a pericardial cradle, and a snare occluder was placed around the left anterior descending coronary artery (LAD) distal to the first diagonal branch. A catheter was placed in the left atrial appendage for the administration of microspheres. After baseline hemodynamic and arterial blood gas measurements, microspheres were injected into the left atrium to measure resting myocardial blood flow.

Then, animals underwent 15 (n=7), 30 (n=8), or 60 (n=8) minutes of LAD occlusion by tightening the snare occluder. Control animals (n=11) underwent no occlusion. During LAD occlusion, ischemia was confirmed by noting typical electrocardiographic changes, the presence of paradoxical wall motion, and a dusky color of the ischemic area. The epicardial borders of the ischemic zone also were marked by direct application of a colored dye to aid in visual determination of the ischemic region. Ten minutes before the end of the LAD occlusion, a second set of microspheres was injected into the left atrium to measure ischemic (collateral) blood flow. Reperfusion was initiated by opening the LAD occluder and it was maintained for 60 minutes. After 5 minutes of reperfusion, autologous radiolabeled protein (indium 115m transferrin) and radiolabeled erythrocytes (technetium 99m) were injected intravenously for measurement of microvascular permeability by the protein leak method (see below). Ten minutes before the end of reperfusion, myocardial blood flow was measured again with a third set of microspheres. Ventricular tachycardia was not treated, and animals
that developed ventricular fibrillation during reperfusion were excluded from the study.

After reperfusion, the animals were killed with an overdose of thiamylal, and the heart was removed. The heart was cleaned of excess blood, sealed in a watertight bag, and frozen in liquid nitrogen to facilitate cutting. In half of the animals in each group, the left ventricle was divided into 40–60 tissue sections for regional determination of protein leak and myocardial blood flow. The ventricles were sectioned into four or five rings cut perpendicular to the long axis of the heart. Each ring was sectioned into quadrants, and each quadrant was cut in half. Final tissue samples were 0.5–2.0 g in size. The tissue samples were counted for radiolabeled protein, erythrocytes, and microsphere activity in a Packard 8000 well-counter (Packard Instrument Co, Downers Grove, Illinois). In the remaining animals within each group, three tissue sections were taken from the visually determined ischemic LAD zone and three from the nonischemic circumflex zone. Each section was divided in half and counted in the well-counter. The remaining LAD and circumflex myocardial tissues were cleaned carefully of residual blood and used for the determination of the gravimetric ratio of wet weight to dry weight.

For group comparisons, myocardial tissue was considered ischemic if measured blood flow during LAD occlusion was 20 ml/min/100 g myocardium or less (myocardial blood flow in unoccluded control animals averaged more than 100 ml/min/100 g myocardium). Animals in which LAD occlusion did not decrease regional blood flow to less than 20 ml/min/100 g myocardium were excluded from analysis due to insufficient ischemia. Anatomic studies were performed on tissue visually chosen from within the central portion of the ischemic LAD region and compared with tissue from the nonischemic circumflex zone. In studies in which the entire heart was divided into rings, quadrants from rings corresponding to the LAD and circumflex zones were chosen for the anatomic comparisons.

Isolated coronary vascular rings for in vitro measurements of vascular reactivity were prepared from animals undergoing 15 or 30 minutes of ischemia (see below). An additional group of animals underwent either control or 15- or 60-minute occlusions and were used for histological studies (see below).

**Determination of Coronary Microvascular Permeability**

**Rationale.** Radioisotope measurement of coronary vascular permeability relies on several basic concepts, which are reviewed in detail elsewhere.²⁷–³⁰ First, the rate at which radiolabeled intravascular protein moves from the intravascular (blood) space to the extravascular space is a function of its concentration in the blood, of vascular protein permeability, and of perfused vascular surface area. Second, the radiolabeled protein activity within a tissue sample can be partitioned into intravascular and extravascular components by using radiolabeled erythrocytes to assess the intravascular component. Third, even poorly perfused vasculature can be assessed if sufficient time is allowed (60 minutes in our studies) for intravascular-to-extravascular protein movement to occur, thus minimizing the effects of limited blood flow on delivery of the radiolabeled tracers. The radiolabeled erythrocyte and protein activities of a peripheral blood sample are used as a measure of the erythrocyte and protein activity of intramyocardial blood (and thus no correction is made for the Fahraeus effect). Using these concepts, measurement of the amount of extravascular radiolabeled protein that accumulates over time after intravascular injection of the radiolabeled protein can be measured. To minimize the effects of changes in surface area, extravascular protein accumulation is normalized for vascular surface area using the radiolabeled erythrocyte content of each tissue sample as a measure of the perfused vascular surface area within that tissue region. However, use of erythrocytes as a measure of perfused surface area could be limited in small regions of interest due to nonuniform distribution of cells within the microvasculature.¹⁵,¹⁶ Further normalization of the extravascular protein accumulation for the concentration of radiolabeled protein in the blood allows a quantitative assessment of vascular permeability.

**Calculation of protein leak index.** Vascular permeability was assessed by modification of methods previously described.²⁷–³⁰ Radiolabeled protein and erythrocytes were injected intravenously at the beginning of reperfusion. Sixty minutes after injection, myocardial tissue biopsies were taken from both the ischemic and nonischemic areas of the heart, and a reference blood sample was drawn from the aorta. The radiolabeled protein and erythrocyte activity of each tissue sample and of the reference blood sample were then determined. Measured activity was corrected for decay, Compton scatter, and background activity. Samples were counted to achieve a statistical counting error of less than 1%.

The amount of radiolabeled extravascular protein that accumulated during the 60 minutes after intravascular injection was calculated as follows:

$$\text{Extravascular protein activity} = \text{Total tissue protein activity} - \text{Intravascular protein activity}$$

where

$$\text{Intravascular protein activity} = \text{Tissue blood weight} \times \text{Blood protein activity}$$

Also,

$$\text{Tissue blood weight} = \frac{\text{Tissue RBC activity}}{\text{RBC activity per gram blood}}$$

and

$$\text{Blood protein activity} = \frac{\text{Protein counts per gram blood}}{\text{Volume of blood}}$$
where RBC is red blood cell. The calculated extravascular protein activity is the amount of extravascular radiolabeled protein that accumulated during the 60 minutes after intravascular injection. This amount was normalized for the concentration of radiolabeled protein in the blood (blood protein activity) and for vascular surface area (represented by the weight of blood in the tissue) and expressed as a protein leak index assessment of permeability where:

\[ \text{Protein leak index} = \frac{\text{Extravascular protein activity}}{\text{Blood protein activity}} \times \frac{\text{Tissue weight}}{\text{Blood weight}} \]

**Isolated Coronary Vascular Rings**

After removal of the heart (and before sectioning for protein leak measurements), a segment of the LAD just distal to the occlusion ligature (ischemic and reperfused) and of the proximal portion of the circumflex (control) were excised. Rings of 3-mm width were cut from each arterial segment, and isolated coronary vascular rings were prepared as described previously. The rings were placed in a muscle bath containing physiological salt solution that was aerated at 37°C with 21% oxygen, 5% CO₂, and 74% nitrogen. The rings were mounted on hooks suspended from Grass FT03 force-displacement transducers. Resting tension was adjusted to a previously determined optimum of 4g. Output from the transducers was displayed on a multichannel recorder. The rings were equilibrated for 60 minutes before administration of vasoactive drugs.

The rings were precontracted with 10⁻⁵ M U46619 (a thromboxane analogue). Vasodilator responses of ischemic LAD rings and nonischemic circumflex rings to the endothelium-dependent vasodilators acetylcholine and calcium ionophore A23187 and to the endothelium-independent vasodilator sodium nitroprusside were measured. Relaxation concentration-response curves for acetylcholine (10⁻¹⁰ to 10⁻⁵ M), A23187 (10⁻⁹ to 10⁻⁶ M), and sodium nitroprusside (10⁻⁹ to 10⁻⁶ M) were plotted using the ratio of drug-induced relaxation to the precontracted tension. Graphical determinations of the concentration of drug required to elicit 50% of maximal response (EC₅₀) of the relaxation response curves were used as a measure of vasodilator responsiveness.

**Calculation of Myocardial Blood Flow**

Regional myocardial blood flow was measured using radiolabeled microspheres. Radiolabeled microspheres (15±3-μm diameter, Dupont/New England Nuclear, Boston, Massachusetts) in Tween 80 and dextran (Sigma Chemical, St. Louis, Missouri) were diluted in normal saline. Before injection, the total amount of injected microsphere activity was determined. After vigorous mechanical agitation to prevent clumping, 1.5–2.0 million spheres were injected into the left atrium over 15 seconds, and the catheter was then rapidly flushed with normal saline. The injection syringe was later counted for residual radioactivity. More than 98% of the premeasured microsphere aliquot was usually injected.

Beginning 30 seconds before and continuing for 2 minutes after the injection of microspheres, blood was withdrawn from the aorta at a constant rate of 2 ml/min. After death of the animals, myocardial tissue samples were obtained as described in the protocol. Tissue and blood samples were counted for microsphere content. The blood flow of each tissue sample was determined using a modification of the reference sample method as previously reported. Specific microsphere radiolabels were chosen to minimize peak radioactivity overlap between spheres as well as with the tracers used for protein leak determinations.

**Preparation of Radiopharmaceuticals**

Autologous canine plasma was labeled in vitro with 113mIn hydrochloride (half-life, 110 minutes), which is avidly bound to transferrin (MW, 76,000). 113mIn was freshly eluted from a 113mSn/113mIn generator using 0.05N HCl. A heparinized aortic blood sample was drawn from the animal under study and centrifuged, and the plasma was separated. Approximately 20 μCi/kg 113mIn was added to the plasma sample (10:1 plasma to indium volume), which was then gently agitated for 10 minutes at room temperature. Autologous erythrocytes were labeled in vitro with 99mTc (half-life, 6 hours) using Brookhaven National Laboratory erythrocyte-labeling kits (Cadeema Medical Products, Inc, Middletown, New York).

Both in vitro and in vivo assessments of the quality of autologous labeling were performed. After in vitro labeling of plasma with 113mIn, an excess of ammonium sulfate was added to an aliquot of plasma to precipitate all protein. More than 97% of the 113mIn was protein bound. Erythrocyte labeling was assessed by centrifuging an aliquot of 99mTc-labeled cells. The free 99mTc in the supernatant was counted and compared with the activity of the centrifuged erythrocytes. More than 98% of the 99mTc was associated with the erythrocytes. Using the same methods on plasma and erythrocyte samples from experimental animals after injection of the radiolabeled indicators confirmed that comparable binding was maintained in vivo.

**Myocardial Water and Blood Content**

Gravimetric ratios of wet weight to dry weight of myocardial tissue were determined and corrected for residual blood content as previously described. After death of the animals, myocardial tissue from the ischemic (LAD) and nonischemic (circumflex) zones was obtained, and the wet weights were measured. A blood sample was obtained at the time of death, and its wet weight was also determined. Samples of the myocardial tissue and of the blood were counted for radiolabeled erythrocyte activity (99mTc). Then, tissue and blood samples were dried to constant weight in a 55°C oven. The 99mTc activity of the myocardial tissue compared with the 99mTc activity per gram of blood was used to determine the weight.
of blood present in the myocardial tissue. Blood weight was subtracted from both the wet weight and dry weight of the myocardial tissue, and the corrected ratio of wet weight to dry weight was calculated.

**Histology**

*Electron microscopy.* The heart was isolated and perfused retrogradely at 100 mm Hg via the aorta with 1.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.3. After 10 minutes of perfusion, tissue biopsies were taken from the central portion of the ischemic (LAD) and nonischemic (circumflex) zones. The biopsies were submerged in cacodylate-buffered glutaraldehyde for 24 hours at 4°C. The tissue was subdivided into 1-mm³ pieces and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.3) at 4°C for 1 hour. En bloc 1% aqueous uranyl acetate staining was performed, and then the tissues were dehydrated in acetone, cleared in propylene oxide, and infiltrated overnight with Embed/Araldite. The embedded tissues were cured 2 days at 70°C. Sections were cut on an ultramicrotome (LKB Instruments, Gaithersburg, Maryland) and stained with 3% aqueous uranyl acetate and Reynold’s lead stain. Up to four thick sections from the Embed/Araldite–infiltrated blocks were examined, after which thin sections from each block were examined on an electron microscope (model 400T, Philips Electronic Instruments, Mahwah, New Jersey) at an accelerating voltage of 60 kV.

*Immunofluorescence studies.* The heart was isolated and perfused retrogradely at 100 mm Hg via the aorta with normal saline. After 10 minutes of perfusion, tissue samples were taken from the central portion of the ischemic (LAD) and nonischemic (circumflex) zones. Tissue samples were carefully cleaned of excess blood, cut into 3-mm sections, infiltrated with OCT, and snap-frozen at 4°C on alloy metal chux platforms. Four-micron sections were cut on a cryostat and placed on microscope slides. Sections were incubated for 1 hour in a 1:100 dilution of fluorescein-conjugated rabbit anti-dog albumin immunoglobulin G (IgG, Sigma Chemical). The tissue sections were rinsed with phosphate-buffered saline and examined under an epifluorescence microscope. Immunofluorescence intensity of five fields of each of three to five sections per sample was assessed by measuring the exposure time necessary to trigger an autoexposure camera with a preset light intensity threshold.

**Statistics**

Comparisons among groups were made with a one-way analysis of variance (ANOVA) with multiple comparisons and within groups with a paired t test. Correlations between the protein leak index and myocardial blood flow within groups were made by linear regression analysis and tested by ANOVA. Among groups, comparisons for relations between protein leak index and myocardial blood flow were made by ANOVA. Comparisons of percent relaxation and EC₅₀ of isolated vascular rings were performed using paired t tests between ischemic and nonischemic vessels. A value of p≤0.05 was considered significant. All values are expressed as mean±SEM.

**Results**

*In Vivo Microvascular Protein Leak*

Ischemia and reperfusion increased the myocardial protein leak index. The increase was proportional to the duration and the severity of ischemia. The data are summarized in Table 1. The myocardial protein leak index increased in graded fashion with ischemia of progressively longer duration (Figure 1). In ischemic myocardium (blood flow, ≤20 ml/min/100 g), 15 minutes of ischemia increased the protein leak index by approximately 50% (3.16±0.30 ischemic vs. 2.09±0.17 control). Sixty minutes of ischemia produced a nearly threefold increase in the protein leak index of ischemic (LAD) zone tissue compared with either LAD zone tissue from control animals or with nonischemic (blood flow, 100 ml/min/100 g) circumflex zone tissue (6.51±0.60 ischemic LAD zone vs. 2.09±0.11 control LAD zone or vs. 2.35±0.13 nonischemic circumflex zone). As shown in Table 1, protein leak indexes of tissue samples grouped based on their anatomic location within the ischemic LAD zone or distance from it (circumflex zone) were comparable to those of samples grouped as ischemic or nonischemic using blood flow criteria (anatomic location used to select tissue for ratios of wet weight to dry weight and for histological comparisons).

There was considerable regional variation in the protein leak index of tissue samples from a single ischemic zone. Data from a representative animal are shown in Figure 2. This regional variation was proportional to the severity of regional ischemia measured as myocardial blood flow during LAD occlusion. Group data are shown in Figure 3. Longer durations of ischemia produced a steeper relation between the protein leak index and ischemic blood flow (Figure 3).

Because reperfusion may contribute to vascular injury and because impaired radioisotope delivery at low flow rates may affect the assessment of injury
using the protein leak method, the relation between the protein leak index and myocardial blood flow during reperfusion was examined. Although the protein leak index varied inversely with regional blood flow during ischemia, there was no relation between the protein leak and reperfusion myocardial blood flow after 15, 30, or 60 minutes of ischemia. Data from the 60-minute group are shown in Figure 4. Only in the 60-minute ischemia group was there evidence of impaired reperfusion flow (<50 ml/min/100 g).

To assess reperfusion flow, radiolabeled blood weight per gram of myocardium, a measure of perfused surface area, did not vary

<table>
<thead>
<tr>
<th>TABLE 1. Protein Leak Index Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Control (n=11)</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>9</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>11</td>
</tr>
<tr>
<td>Mean±SEM</td>
</tr>
<tr>
<td>15-Min ischemia (n=7)</td>
</tr>
<tr>
<td>12</td>
</tr>
<tr>
<td>13</td>
</tr>
<tr>
<td>14</td>
</tr>
<tr>
<td>15</td>
</tr>
<tr>
<td>16</td>
</tr>
<tr>
<td>17</td>
</tr>
<tr>
<td>18</td>
</tr>
<tr>
<td>Mean±SEM</td>
</tr>
<tr>
<td>30-Min ischemia (n=8)</td>
</tr>
<tr>
<td>19</td>
</tr>
<tr>
<td>20</td>
</tr>
<tr>
<td>21</td>
</tr>
<tr>
<td>22</td>
</tr>
<tr>
<td>23</td>
</tr>
<tr>
<td>24</td>
</tr>
<tr>
<td>25</td>
</tr>
<tr>
<td>26</td>
</tr>
<tr>
<td>Mean±SEM</td>
</tr>
<tr>
<td>60-Min ischemia (n=8)</td>
</tr>
<tr>
<td>27</td>
</tr>
<tr>
<td>28</td>
</tr>
<tr>
<td>29</td>
</tr>
<tr>
<td>30</td>
</tr>
<tr>
<td>31</td>
</tr>
<tr>
<td>32</td>
</tr>
<tr>
<td>33</td>
</tr>
<tr>
<td>34</td>
</tr>
<tr>
<td>Mean±SEM</td>
</tr>
</tbody>
</table>

LAD, left anterior descending coronary artery (occluded zone); Cx, circumflex artery (nonoccluded zone); x, no blood flow data available; . . . , no tissue samples with indicated level of blood flow.

All ischemia times include 60 minutes of reperfusion.
with blood flow during the ischemic period in any of the groups, nor was myocardial blood content different among groups. Myocardial water content was increased after 60 minutes of ischemia (4.86±0.15 vs. 4.32±0.22 g/g bloodless dry myocardium in ischemic vs. control LAD) but not after 15 or 30 minutes of ischemia (4.36±0.08 and 4.30±0.19, respectively).

**Hemodynamics**

Mean aortic pressure was not different among 0 (control), 15-, 30-, or 60-minute ischemia groups at baseline (105±7, 99±4, 103±5, and 108±9 mm Hg, respectively) or among ischemia and reperfusion groups during LAD occlusion (98±5, 101±6, and 102±8 mm Hg), at 30 minutes (104±5, 101±6, and 102±6 mm Hg), or at 60 minutes of reperfusion (100±4, 103±6, and 107±8 mm Hg, respectively). Control animals had higher pressures at times corresponding to LAD occlusion and to 30 and 60 minutes of reperfusion (107±9, 110±6, and 116±6 mm Hg, respectively).

**In Vitro Endothelium-Dependent Vasodilation**

Endothelium-dependent relaxation of precontracted isolated coronary vascular rings was impaired after 15 and 30 minutes of ischemia and reperfusion (Figure 5). Maximal relaxation (as a percent of precontracted tension) was diminished, and the EC50 was increased for the endothelium-dependent vasodilators acetylcholine and calcium ionophore A23187 in the ischemic-reperfused LAD rings compared with the nonischemic circumflex rings from the same animals. Responses to the endothelium-independent vasodilator sodium nitroprusside were not different among the groups.

**Histological Studies of Microvascular Injury**

Electron microscopy of the microvasculature revealed marked endothelial injury after 60 minutes of ischemia followed by reperfusion compared with control (Figure 6). Severe diffuse injury was present with intracellular vacuolization and loss of tight junctions. There were intercellular gap formation, perivascular edema, and surrounding myocardial cellular injury. In contrast, after 15 minutes of ischemia, the endothelium appeared normal, and there was no perivascular edema or surrounding myocardial injury.

**Immunofluorescence Studies**

To help confirm that increased tissue radioactivity represented increased tissue protein accumulation, immunofluorescence studies to assess the presence of albumin were done. The uptake of fluorescein-labeled anti-albumin antibody by tissue sections from animals undergoing 60 minutes of ischemia and reperfusion was measured. The length of exposure necessary to trigger a preset autoexposure camera is inversely proportional to the intensity of tissue fluorescence. Ischemic tissue sections required less time for autoexposure (9±6 seconds) than did nonischemic tissue (29±4 seconds). The increased fluorescence intensity of ischemic-reperfused myocardial
tissue after 60 minutes compared with nonischemic tissue was consistent with an increased albumin content of the ischemic tissue.

**Discussion**

These studies showed that myocardial ischemia and reperfusion caused functional coronary microvascular injury that was evident as increased protein permeability. The increases in permeability were proportional to both the duration and severity of ischemia. Ischemia as brief as 15 minutes caused permeability to increase by nearly 50%. Permeability was increased approximately threefold after 60 minutes of ischemia and reperfusion. There was significant regional variation in the degree of microvascular injury, which was proportional to regional differences in the severity of ischemia. Vascular injury was also evident as impaired endothelium-dependent dilation of isolated coronary vascular rings after brief (15 or 30 minutes) ischemia and reperfusion. Functional assessment of vascular injury using either permeability or endothelium-dependent relaxation was more sensitive than analysis of either ultrastructure or myocardial water content. Electron microscopic and direct immunofluorescence studies confirmed the presence of microvascular and endothelial injury after 60 minutes of ischemia. However, after 15 minutes of ischemia, there was no ultrastructural evidence of injury.

In previous studies of in vivo myocardial ischemia and reperfusion, vascular injury was found only after prolonged ischemia, and it appeared to develop after myocardial injury or infarction had occurred.Using morphological techniques, Kloner et al. found that after coronary occlusion, vascular injury was not present until after 60 minutes of ischemia, nor was it present until after myocardial injury was apparent. After brief (15 minutes) ischemia plus reperfusion, Jennings et al. found capillary endothelium to be morphologically indistinguishable from control. Microvascular obstruction, as occurs with the "no-reflow" phenomenon and which may be due to vascular injury, was not apparent until after significant morphological myocardial injury was observed. Other findings suggesting vascular injury such as changes in myocardial albumin content, altered blood flow distribution, and increased coronary vascular resistance also are not evident until after prolonged ischemia. Thus, vascular injury appeared to be a late result of ischemia and reperfusion. However, the apparent late development of coronary vascular injury in these studies may reflect the relative insensitivity of the methods used to assess vascular injury.

In contrast, in the present study, vascular injury was evident after even brief ischemia and reperfusion. This may reflect the use of permeability as a functional index of vascular injury since in other organs, permeability is a sensitive indicator of vascular injury. This is shown by previous studies of pulmonary vascular injury in which vascular injury evident as increased permeability occurred without accompanying changes in morphology or tissue water. We have previously described a double-radioisotope indicator method for assessing pulmonary vascular protein permeability in vivo. Using this method to assess permeability as a protein leak index, it has been shown to be a sensitive and specific indicator of vascular injury. After administration of small amounts of the lung toxin thiourea (which causes vascular injury), the protein leak index nearly doubled, whereas gravimetric tissue water was unchanged. Graded doses of thiourea produced graded increases in the protein leak index, whereas tissue water did not increase until after high doses of thiourea were administered. In addition, elevation of microvascular hydrostatic pressure to more than threefold that of baseline (produced by left atrial balloon inflation), which resulted in a significant increase in tissue water, did not significantly increase the protein leak index. Modification of this method has made it possible to measure permeability in other organs and on a regional basis within organs.

Radiolabeled erythrocytes were important in our previous studies for the assessment of permeability using the protein leak method. The use of labeled erythrocytes allows the separation of tissue protein content into intravascular and extravascular contributions. In addition, the labeled erythrocytes can be used to normalize the measured protein accumulation for perfused vascular surface area. However, in these calculations, peripheral blood erythrocyte and protein activity was used as a measure of intramyocardial blood erythrocyte and pro-
FIGURE 6. Photomicrographs of microvascular ultrastructure appear normal after 15 minutes but not after 60 minutes of ischemia plus reperfusion. Electron micrographs (original magnification, ×9,000) of myocardial microvasculature after 15 or 60 minutes of ischemia plus 60 minutes of reperfusion. Microvascular endothelium (End) from both the nonischemic (circumflex) region (panel A) and the 15-minute ischemic (LAD) region (panel B) appear normal with intact intercellular junctions (arrows) and no surrounding perivascular edema or myocardial injury. In contrast, after 60 minutes of ischemia, significant endothelial injury is present (panel C) with intracellular vacuolization, formation of intercellular gaps (*), and perivascular edema as well as surrounding myocardial injury. PMN, polymorphonuclear leukocyte; RBC, red blood cell.
tein activity. Thus no correction was made for the Fahraeus effect by which small-vessel (e.g., intramyocardial) hematocrit may differ from large-vessel (peripheral blood) hematocrit. Empirically, in the lung, the use of radiolabeled erythrocytes to normalize for changes in surface area improved the sensitivity and specificity of permeability assessment using the protein leak method. The greatest improvement was under conditions of elevated capillary hydrostatic pressure with an accompanying large change in perfused surface area. Whether there is a similar improvement in assessing coronary permeability has not been tested.

To test the efficacy of the use of radiolabeled erythrocytes on the measurement of myocardial protein leak, an alternate “uncorrected” protein leak index was calculated without the erythrocyte indicator ([tissue radiolabeled protein/blood radiolabeled protein specific activity]/tissue sample weight). The uncorrected protein leak index of ischemic myocardium (<20 ml/100 g/min) was increased 25%, 90%, and 175% after 15, 30, and 60 minutes, respectively, of ischemia and reperfusion compared with the uncorrected protein leak index of nonischemic (>100 ml/100 g/min) tissue. Thus, use of the erythrocyte indicator affected the magnitude of the calculated increases in the protein leak index, but the observed increases after ischemia and reperfusion are not due to changes in radiolabeled erythrocyte content alone.

Some potential problems exist with these studies. The use of radiolabeled erythrocytes to assess perfused vascular surface area may not fully reflect changes in surface area since they measure blood volume rather than surface area. Furthermore, since erythrocytes are not uniformly distributed within the microvasculature, their usefulness may be limited when used at a microscopic rather than a regional level, as in our studies. In addition, the erythrocyte indicator may not be important unless erythrocyte-perfused vascular surface area changes. However, McDonagh and Roberts observed a decrease in myocardial red blood cell capillarity after ischemia and reperfusion, suggesting that changes in red blood cell surface area can occur with ischemia and reperfusion. Hemorrhage would alter both the blood volume–surface area relation and the assumption that erythrocytes are an intravascular indicator. In our studies, there was no evidence of hemorrhage either by histological assessment or by measured blood content of ischemic tissue, although hemorrhage could occur with more severe injury.

Another potential problem is that vascular injury in areas of impaired reperfusion may not be adequately assessed by this perfusion-oriented method. Sixty minutes of reperfusion was used to allow additional time for delivery of the radiolabeled protein indicator to even poorly perfused regions. Furthermore, there was no correlation between the protein leak index and reperfusion myocardial blood flow, suggesting that the protein leak index was not flow or delivery limited over the range of flows in these studies. However, nonperfused vasculature, even if significantly injured, would not be represented due to lack of adequate protein-tracer delivery. The relatively brief periods of ischemia used in our studies.
were chosen to minimize the occurrence of “no-reflow” accompanying longer periods of ischemia.\textsuperscript{7,16}

Assessment of permeability using a protein leak method does not appear to be significantly affected by changes in capillary hydrostatic pressure. Although increased capillary pressure will increase transvascular protein and fluid flux, the effect on protein and fluid accumulation is less clear.\textsuperscript{12,13} Furthermore, permeability is not increased except at very high levels of pressure.\textsuperscript{12,13,21} Therefore, a method for assessing permeability should be relatively insensitive to changes in pressure. In our previous studies,\textsuperscript{27} elevation of left atrial pressure to more than threefold that of baseline in an uninjured lung produced only a 20\% increase in the protein leak index despite a large increase in tissue water content. In these studies, elevated hydrostatic pressure increased the “uncorrected” protein leak index more than twofold.

Assessment of protein leak in the injured coronary microvasculature may be more sensitive to alterations in hydrostatic pressure than in the intact pulmonary circulation. However, the studies of McDonagh and Roberts\textsuperscript{23} suggest that protein leak measurements in the heart after ischemia-reperfusion are relatively insensitive to changes in hydrostatic pressure. Using fluorescence microscopy of the epicardial microvasculature of isolated, perfused rat hearts, they studied leakage of fluorescein-labeled albumin as well as perfused red blood cell capillarity in hearts subjected to ischemia and reperfusion. Albumin leakage was increased to 48\% more than baseline during early (10-minute) reperfusion and further increased to 68\% more than baseline during late (20-minute) reperfusion, whereas red blood cell capillarity was decreased by 25\% at each time. Coronary perfusion pressure during early reperfusion was 48 mm Hg during early reperfusion (compared with 95 mm Hg at baseline) and more than doubled to 102 mm Hg during late reperfusion. Thus, although coronary perfusion pressure more than doubled from early to late reperfusion, protein leakage increased by less than 50\%. Some of the observed increase in protein leakage was likely due to further vascular injury as a result of the longer period of reperfusion. Also, despite a significantly lower perfusion pressure during early reperfusion than at baseline, the protein leakage was substantially increased.

These findings suggest that protein leak assessment of permeability in the injured coronary microvasculature is not markedly affected by changes in pressure.

Although in vivo studies of coronary vascular function after brief ischemia and reperfusion are limited, several previous investigators have found evidence of functional vascular injury after longer periods of ischemia and reperfusion. In addition to the studies previously mentioned\textsuperscript{2,7,9,11,18–21} that indirectly assessed coronary vascular function after ischemia and reperfusion, McDonagh and Laks\textsuperscript{31} and Harris et al\textsuperscript{32} used first-pass tracer-exchange techniques and Feola and Glick\textsuperscript{42} used a lymph flow preparation to more directly assess vascular function and suggested that vascular permeability was increased after prolonged (60 minutes or longer) ischemia and reperfusion. Interpretation of these studies has been complicated by alterations in myocardial blood flow, vascular surface area, and, potentially, regional heterogeneity of vascular injury (as occurs with ischemic myocardial injury). Ko\textsuperscript{33} and VanBenthuyzen et al\textsuperscript{36} found impaired endothelium-dependent vasodilation, suggesting endothelial dysfunction after ischemia and reperfusion. Bush et al\textsuperscript{44} found abnormalities of in vivo coronary vascular reactivity present as cyclic flow variations, which may be due to functional vascular injury, after low-flow ischemia.

In contrast, in vitro studies of myocardial ischemia and reperfusion have suggested that vascular injury can occur after brief ischemia. Tilton et al\textsuperscript{22} found increased myocardial albumin accumulation after ischemia brief enough to produce reversible myocardial dysfunction in isolated, perfused rabbit hearts. Sunnergren and Rovetto\textsuperscript{24} demonstrated accumulation of radiolabeled albumin in perfused rat hearts after 20 minutes of ischemia. McDonagh and Roberts\textsuperscript{29} demonstrated increased epicardial leakage of fluorescein-labeled protein in isolated, perfused rat hearts after ischemia and reperfusion as brief as 30 minutes.

Further evidence that our observed changes in microvascular permeability reflect endothelial dysfunction is provided by our findings of impaired endothelium-dependent vasodilation. The importance of intact functional endothelium in the response to certain vasodilators (endothelium dependent) has been demonstrated by several investigators.\textsuperscript{34–36} Endothelium-dependent vasodilation is impaired by atherosclerosis, diabetes, and balloon angioplasty\textsuperscript{34,43} as well as by ischemia and reperfusion.\textsuperscript{35,36} Additionally, impaired endothelium-dependent vasodilation of epicardial coronary arterial rings after 15 and 30 minutes of ischemia suggests that functional endothelial injury is not limited to the microvasculature after brief ischemia and reperfusion.

Functional assessment of endothelial or vascular injury using permeability was a more sensitive index of injury than ultrastructural analysis in our studies. Evidence of vascular injury was present by both electron microscopic ultrastructural analysis and protein leak permeability assessment after 60 minutes of ischemia. However, after 15 minutes of ischemia, electron microscopic analysis showed intact endothelium and intercellular junctions that were indistinguishable from control endothelium. In contrast, not only did permeability increase after 15 minutes of ischemia but also endothelium-dependent vasodilation was impaired. Ultrastructural evidence of microvascular injury may have been missed due to sampling error resulting from the limited number of samples used for analyses in our studies. However, Kloner et al\textsuperscript{14} analyzed a large number of samples and did not find ultrastructural evidence of microvascular
injury in myocardium exposed to up to 40 minutes of ischemia. Jennings et al also found no evidence after 15 minutes ischemia followed by reperfusion. In our studies, direct tissue immunofluorescence confirmed the presence of additional protein in ischemic myocardial tissue.

Coronary vascular injury after ischemia and reperfusion, with resultant endothelial dysfunction, may have important functional consequences. 1, 2, 4, 7, 36 Endothelial injury can directly contribute to postischemic myocardial dysfunction, 1, 2, 22 and may promote recurrent ischemic episodes. 1, 18, 35, 36, 44 Thus, it may play a role in the transition from stable to unstable angina. 30, 46 Tissue edema as a result of increased permeability due to endothelial injury can affect myocardial function by impairing the exchange of metabolites. 1, 2, 32, 43 Both tissue nutrient delivery and washout of metabolic by-products can be affected by tissue edema. In addition, tissue edema can impair myocardial contractile function, 22, 47, 48 and it may lead to vascular compression. 1, 2, 7, 18 Altered vasoreactivity due to endothelial dysfunction can impair myocardial blood flow due to spasm or impaired vasodilation in response to metabolic demands. 34, 36, 45 Loss of the normal vascular surface due to endothelial injury can lead to adherence of formed blood elements, 1, 14, 16, 26 such as neutrophils and platelets, and to thrombosis 1, 46 with resulting vascular obstruction. This obstruction can further compromise myocardial nutrient delivery and lead to further tissue injury, possibly by the release of toxic mediators. 3, 4, 14, 26 In addition, edema, spasm, and vascular obstruction due to endothelial injury induced by ischemia and reperfusion could lead to increased and/or recurrent ischemia resulting in further vascular injury that may contribute to the transition of stable to unstable angina. 44, 46

In summary, even brief myocardial ischemia and reperfusion caused functional coronary vascular injury evident as increased permeability and impaired endothelium-dependent vasodilation. Microvascular injury was proportional to both the duration and severity of ischemia. There was significant regional variability in the degree of microvascular injury that correlated with regional differences in the severity of ischemia. Functional assessment of vascular injury was more sensitive than ultrastructural assessment of injury. We conclude that functional coronary microvascular injury occurs after even brief ischemia and reperfusion and may contribute to postischemic myocardial dysfunction and the development of unstable ischemic syndromes.

Acknowledgments

We gratefully acknowledge the expert technical assistance of Ms. Holly Coller and Ms. Julie Peach and the patient secretarial skills of Ms. Susan Hansen. We thank Ms. Sheryl Campbell and Ms. Jan Henson of the National Jewish Center for Respiratory Medicine and Immunology for their advice and support in the preparation of electron microscopic studies. We also thank Dr. Sylvia Brice and Dr. Clark Huff of the Department of Dermatology, University of Colorado Health Sciences Center, for their guidance in the preparation of immunofluorescence studies.

References


Key Words • microvascular injury • ischemia and reperfusion • permeability • endothelium-dependent relaxation • endothelial dysfunction
Functional coronary microvascular injury evident as increased permeability due to brief ischemia and reperfusion.

doi: 10.1161/01.RES.66.4.986

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/66/4/986