Differences in ischemia-reperfusion-induced endothelial changes in hearts perfused at constant flow and constant pressure

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Singh RB, Elimban V, Dhalla NS. Differences in ischemia-reperfusion-induced endothelial changes in hearts subjected to I/R injury are poorly defined. The present study was therefore undertaken to test the hypothesis that alterations in endothelial function play a critical role in I/R-induced defects in cardiac contractile and subcellular activities.

Several cardiac abnormalities due to I/R have been identified using different experimental models including isolated rat heart preparations perfused either at constant flow (CF) or at constant pressure (CP) (6, 16, 33, 35, 37, 40, 41). Studies on I/R injury in hearts perfused at CF and CP are dependent on the duration of ischemia and reperfusion (40). Although the activity of SL Na+/K+-ATPase was depressed in CF-perfused hearts upon subjecting to I/R (27), no such information is available for CP-perfused hearts. In view of the role of endothelium in the regulation of cardiac performance, these experimental models of I/R injury were employed to test if differences in I/R-induced changes in cardiac function as well as SL ATPase activities in CF-perfused and CP-perfused hearts are due to differential alterations in the endothelial function with respect to NO production. Because the duration of ischemia has been shown to determine the extent of I/R-induced injury (4, 32), some experiments were carried out to investigate if the I/R-induced changes in cardiac performance in hearts perfused at CF and CP are dependent on the duration of ischemia. The I/R-induced alterations in SR Ca2+-uptake and SL Na+/K+-ATPase activities as well as the activity of calpain, a proteolytic enzyme (35), in both CF-perfused and CP-perfused hearts were examined to study if subcellular changes in these preparations are associated with the activation of proteolysis. It should be pointed out that a marked increase in calpain activity has been shown to occur in I/R hearts (35). The role of endothelium in eliciting I/R-induced changes in cardiac function and subcellular activities was studied on treatment of CF-perfused hearts and CP-perfused hearts with l-arginine (l-NAME), a substrate for NO synthase (6) and N\textsuperscript{G}-nitro-l-arginine methyl ester (l-NAME; abbreviated as LN), an inhibitor of NO synthase [endothelial NO synthase (eNOS)] inhibitor (6), re-

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spectively. Furthermore, the status of NO production was monitored by measuring the concentration of nitrate/nitrite in perfusate as well as protein content for eNOS in the myocardium. In addition, the CF-perfused and CP-perfused hearts were used for studying changes in coronary pressure due to some agents that are known to affect the coronary vessels through their actions on the endothelium (12, 14, 23, 44, 45).

**MATERIALS AND METHODS**

The experimental protocol was approved by the Animal Care and Ethics Committee of the University of Manitoba and conforms to the Guidelines of the Canadian Council on Animal Care.

**Perfusion and experimental protocol.** Male Sprague-Dawley rats weighing 225–275 g were anesthetized with a mixture of ketamine (90 mg/kg) and xylazine (9 mg/kg). Hearts were rapidly excised and cannulated to the Langendorff apparatus for retrograde perfusion with a modified Krebs-Henseleit (K-H) buffer as described earlier (6, 35). Left ventricular developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP), and the rate of ventricular pressure development (+dP/dt) and rate of ventricular pressure decay (-dP/dt) were measured with a water-filled latex balloon inserted into the left ventricle and attached to a pressure transducer. Data were recorded using model MP100 analog-to-digital interface and processed using the AcqKnowledge 3.5.3 software for Windows (BIOPAC Systems, Goleta, CA). Hearts were perfused at a CF of 10 ml/min or at a CP of 80 mmHg and stabilized with K-H buffer for 20 min; these parameters of perfusion in CF-perfused and CP-perfused hearts were selected to match the coronary flow in both experimental preparations. All experimental hearts were exposed to 30 min global no-flow ischemia, unless otherwise indicated in the text, followed by 60 min of reperfusion. Control hearts perfused at CF or CP for corresponding duration were not subjected to IR. Hearts perfused at CF and exposed to 30 min ischemia and 60 min reperfusion were treated with and without LA (3 mM) a substrate for NO synthase, (6), or a combination of LA and a NO synthase inhibitor, LN (200 μM) (6). On the other hand, hearts perfused at CP and exposed to 30 min ischemia and 60 min reperfusion were treated with and without LN (200 μM) or LA (3 mM). Treatments with these agents were carried out for 10 min before inducing ischemia as well as for 20 min during reperfusion (6). It should be mentioned that these treatments of CF-perfused hearts did not alter the control coronary pressure (76.1 ± 4.7 mmHg) in CP-perfused hearts or control coronary flow (12.5 ± 1.1 ml/min) in CP-perfused hearts.

**Isolation of SL membrane and SR vesicles.** SL membranes were isolated from perfused hearts by a method as described earlier (11, 26, 29). Briefly, hearts were homogenized and centrifuged at 12,000 g for 30 min, and the supernatant was collected and centrifuged at 100,000 g for 60 min. The resultant pellet was suspended in MOPS-KCl buffer and layered onto a solution containing Tris- HCl, sodium pyrophosphate, KCl, and sucrose, and centrifuged at 100,000 g. This yielded three layers; the middle white layer was carefully suctioned out and centrifuged at 100,000 g for 30 min. The resultant pellet containing purified SL was suspended in 250 mM sucrose-10 mM histidine buffer and frozen in liquid nitrogen and stored at −80°C until further use. In another set of experiments, SR vesicles were isolated using an established technique described earlier (6, 35, 42). Briefly, ventricular tissue was homogenized and centrifuged for 20 min at 10,000 g. The supernatant was centrifuged for 45 min at 43,666 g. The cytosolic fraction from the resultant supernatant was frozen in liquid nitrogen before storage at −80°C for determining the calpain activity. The pellet was resuspended in a buffer containing 0.6 M KCl and 20 mM Tris-HCl (pH 6.8) and centrifuged for 45 min at 43,666 g. The final pellet representing SR fraction was suspended in a buffer containing 250 mM sucrose and 10 mM histidine (pH 7.0) and frozen in liquid nitrogen before storage at −80°C. All buffers used for the isolation of SL and SR fractions contained a cocktail of protease inhibitors to prevent protein degradation during the isolation procedure (6, 26).

**Measurement of SL Na⁺-K⁺-ATPase activity and SR Ca²⁺ uptake.** Na⁺-K⁺-ATPase activity was measured in SL preparations by a technique described earlier (11, 20, 26). SL was incubated at 37°C in assay tubes containing 1.0 mM EGTA, 5 mM NaCl, 6 mM MgCl₂, 100 mM NaCl, and 10 mM KCl in a total volume of 0.5 ml. Reaction was started by the addition of 25 μl of 80 mM Tris-ATP, pH 7.4, and terminated after 10 min with 0.5 ml ice-cold 12% trichloroacetic acid. The tubes were centrifuged at 3,000 rpm for 10 min, and the supernatant was used for Pi assay. Na⁺-K⁺-ATPase activity was calculated as the difference between activities with and without Na⁺ plus K⁺. SR Ca²⁺ uptake was measured by a procedure described earlier (6, 35). Ruthenium red was added to the reaction mixture as an inhibitor of the Ca²⁺-release channel (6, 35). Ca²⁺ uptake was initiated by adding SR vesicles (20 μg protein) to the reaction mixture at 37°C and terminated after 1 min by filtration; the filters were washed, dried, and counted in a beta scintillation counter (Beckman Coulter, Fullerton, CA).

**Determination of NO formation and eNOS protein content.** The concentration of nitrate/nitrite, an index of NO formation, was determined in the coronary perfusate by modifying a previously described method (6). Coronary perfusate from the CF-perfused and CP-perfused hearts during the 60 min period of reperfusion was collected, and its volume in both cases was adjusted to 750 ml with K-H buffer but no correction was made for these adjustments for reporting the concentration of nitrate/nitrite. The assay detects NO photometrically by measuring its oxidation products, nitrate and nitrite; nitrate is converted to nitrite, which reacts with sulfanilamide to form a red diazo dye that is read at 550 nm. Using the standard solution provided (potassium nitrate), calibration curves were made and samples were read using a spectrophotometer. On the other hand, eNOS protein content was determined in SL membrane by Western immunoblotting (6). SL preparation (20–25 μg) was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. The membranes were blocked in milk-TBS overnight and probed with eNOS antibody (Affinity BioReagents Golden, CO) in a dilution of 1:1,500. The antibody detects eNOS at ~140 kDa. A molecular weight marker from Sigma-Aldrich (Oakville, Ontario, Canada) was used to determine the band corresponding to eNOS. Equal protein loading was checked in every experiment by staining the membrane with Porcine red before immunoblotting as well as with Coomasie Brilliant Blue at the end of the experiment. It should be mentioned that inducible NO synthase (iNOS), unlike eNOS, is mainly expressed in vascular smooth muscle, and thus changes in inOS content were not measured while studying alterations in endothelial function due to IR injury.

**Measurement of coronary perfusion pressure and coronary flow.** Coronary perfusion pressure was measured in CF-perfused hearts to examine the endothelial function/responsiveness in the presence and absence of acetylcholine (ACh; Sigma-Aldrich) and U-46619 (10 nM) were infused in the perfusion stream for a period of 10 min (12, 14, 23, 44, 45). To measure coronary perfusion pressure in CP-perfused preparations, the hearts were switched to CF system for 3 min before infusing ACh or U-6619. For the measurement of coronary perfusion pressure, a pressure transducer was connected to the aortic cannula and the pressure was recorded using model MP100 analog-to-digital interface and processed using the AcqKnowledge 3.5.3 software for Windows (BIOPAC Systems, Goleta, CA). Coronary flow was measured by collecting the perfusate coming out of the left ventricular opening.

**Estimation of calpain activity.** Calpain activity was measured in the cytosolic fraction obtained from above hearts as described earlier (35) using a kit from Biovision. The reaction was carried out in a 96-well plate, and the samples were read in a fluorescence microplate reader at 400 nm excitation and 505 nm emission (Molecular Devices, Sunnyvale, CA). The results were expressed in relative fluorescent units (RFU). It is pointed out that no protease inhibitor was used in the buffer when the cytosolic fraction was obtained for the determination of calpain activity.
To examine if the recovery of cardiac function in I/R perfused at CF were subjected to ischemia for 15 and 30 min, hearts was dependent on the duration of ischemia, hearts as well as between CF-perfused and CP-perfused hearts. Statistical significance between the control and experimental groups as comparison. A level of \( t \)-test. Differences among more than two groups were evaluated statistically by the Statistical analysis. The data were expressed as means ± SE, and the differences between two groups were evaluated statistically by the Student’s \( t \)-test. Differences among more than two groups were evaluated by one-way ANOVA followed by the Newman-Keuls test. The data for the I/R-induced changes in CF-perfused and CP-perfused heart were also analyzed by two-way ANOVA test for the purpose of comparison. A level of \( P < 0.05 \) was considered the threshold for statistical significance between the control and experimental groups as well as between CF-perfused and CP-perfused hearts.

RESULTS

Cardiac performance in CF-perfused and CP-perfused hearts. To examine if the recovery of cardiac function in I/R hearts was dependent on the duration of ischemia, hearts perfused at CF were subjected to ischemia for 15 and 30 min, whereas hearts perfused at CP were subjected to 30 and 60 min of ischemia before initiating reperfusion for 60 min. The results in Table 1 indicate that values for the recovery of LVDP, \( +\text{dP/dt} \), and \( -\text{dP/dt} \) were progressively depressed whereas those for LVEDP were progressively increased on reperfusion in 15 and 30 min ischemic CF-perfused hearts as well as in 30 and 60 min ischemic CP-perfused hearts. Furthermore, data in Table 1 show that I/R-induced depressions in LVDP, \( +\text{dP/dt} \), and \( -\text{dP/dt} \) as well as increase in LVEDP in 15 and 30 min ischemic hearts perfused at CF were comparable to the respective values for these parameters in 30 min and 60 min ischemic hearts perfused at CP, respectively. Thus it is apparent that hearts perfused at CP required longer duration of ischemic insult for inducing cardiac dysfunction similar to that in hearts perfused at CF. For all other experiments, hearts perfused at CF and CP were made ischemic for

<table>
<thead>
<tr>
<th>LVDP, mmHg</th>
<th>15 min Ischemia</th>
<th>30 min Ischemia</th>
<th>60 min Ischemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>116 ± 7.2</td>
<td>73.2 ± 5.0</td>
<td>20.1 ± 3.2*</td>
</tr>
<tr>
<td>CF Perfused</td>
<td>4.4 ± 0.13</td>
<td>24.1 ± 7.1</td>
<td>94.9 ± 8.0*</td>
</tr>
<tr>
<td>+dP/dt, mmHg/s</td>
<td>2,456 ± 540</td>
<td>1,386 ± 111</td>
<td>200 ± 69*</td>
</tr>
<tr>
<td>-dP/dt, mmHg/s</td>
<td>1,804 ± 412</td>
<td>1,208 ± 140</td>
<td>178 ± 55*</td>
</tr>
<tr>
<td>Control</td>
<td>7.2 ± 0.1</td>
<td>5.0 ± 0.1</td>
<td>140 ± 11</td>
</tr>
<tr>
<td>CP Perfused</td>
<td>66.2 ± 5.1</td>
<td>40 ± 7.2</td>
<td>89.4 ± 8.0#</td>
</tr>
<tr>
<td>15 min</td>
<td>1,960 ± 386</td>
<td>1,486 ± 110</td>
<td>145 ± 67#</td>
</tr>
<tr>
<td>30 min</td>
<td>1,426 ± 307</td>
<td>1,436 ± 119</td>
<td>195 ± 56#</td>
</tr>
</tbody>
</table>

Values are means ± SE of 5 hearts for each group. Since the control values at 15 min and 30 min for constant flow (CF)-perfused as well as at 30 min and 60 min for constant-pressure (CP)-perfused hearts were overlapping, the respective values were grouped together. LVDP, left ventricular developed pressure, LVEDP, left ventricular end-diastolic pressure, \( +\text{dP/dt} \), rate of pressure development; \( -\text{dP/dt} \), rate of pressure decay. \(* P < 0.05 \) compared with 15-min ischemia values in CF-perfused hearts. \# \( P < 0.05 \) compared with 30-min ischemia values in CP-perfused hearts. Statistical evaluation of data for 15 min and 30 min ischemia groups in CF-perfused and CP-perfused hearts as well as for 30 min and 60 min ischemia groups in CF-perfused and CP-perfused hearts did not show any significant \( (P > 0.05) \) differences, respectively.

Table 1. Cardiac performance of hearts subjected to varying duration of ischemia followed by reperfusion for 60 min

Constant Flow Perfusion (30 min Ischemia)

![Graph A: LVDP](image1)

![Graph B: LVEDP](image2)

![Graph C: dP/dt](image3)

![Graph D: -dP/dt](image4)

Fig. 1. Cardiac performance of the isolated rat hearts exposed to 30 min of ischemia and 60 min of reperfusion at constant flow (CF) (10 ml/min) treated with and without a substrate of nitric oxide (NO) synthase, \( t \)-arginine (LA; 3 mM), and an NOS inhibitor, \( L \)-arginine methyl ester (\( L \)-NAME; abbreviated as LN; 200 \( \mu \)M). A: left ventricular developed pressure (LVDP). B: left ventricular end-diastolic pressure (LVEDP). C: rate of pressure development \( (+\text{dP/dt}) \). D: rate of pressure decay \( (-\text{dP/dt}) \). Values are means ± SE of 6 hearts for each group. \(* P < 0.05 \) compared with control. \# \( P < 0.05 \) compared with ischemia-reperfusion (I/R). ** \( P < 0.05 \) compared with LA.
30 min before inducing reperfusion to study differences in the effects of I/R in these preparations.

Another set of experiments was undertaken to investigate the role of NO production in determining the differential effects of I/R in CF-perfused and CP-perfused hearts. The data in Fig. 1 indicate that the I/R-induced depressions in LVDP, +dP/dt, and −dP/dt as well as increase in LVEDP were significantly attenuated by treatment of CF-perfused hearts with 3 mM LA, which is known to serve as a substrate for NO synthase in the myocardium (6). This beneficial effect of LA was not apparent when the hearts were treated with LA in the presence of 200 μM LN (Fig. 1); this indicated that the effects of LA in improving the recovery of cardiac function are associated with the generation of NO. On the other hand, it can be seen from Fig. 2 that the I/R-induced depressions in LVDP, +dP/dt, and −dP/dt as well as increase in LVEDP were augmented by treatment of CP-perfused hearts with 200 μM LN, which is known to inhibit the production of NO (6). Comparison of data in Figs. 1 and 2 indicated that the I/R-induced changes in cardiac function were greater than those in CP-perfused hearts (P < 0.05). Furthermore, treatment of CP-perfused hearts (n = 3) with 3 mM LA was found to prevent the I/R-induced depressions in LVDP, +dP/dt, and −dP/dt as well as increase in LVEDP by 85–95% (data not shown).

SL Na⁺-K⁺-ATPase, SR Ca²⁺-uptake, and calpain activities. To examine the effects of I/R on subcellular function in hearts perfused at CF and CP, SL Na⁺-K⁺-ATPase and SR Ca²⁺-uptake activities were determined and the results are shown in Fig. 3. Although I/R was found to decrease SL Na⁺-K⁺-ATPase and SR Ca²⁺-uptake activities in both CF-perfused and CP-perfused hearts, statistical analysis of results in Fig. 3 revealed that the I/R-induced depressions in both SL Na⁺-K⁺-ATPase and SR Ca²⁺-uptake activities in CP-perfused hearts were greater than those in CP-perfused hearts (P < 0.05). Furthermore, the treatment of CF-perfused hearts with 3 mM LA attenuated the I/R-induced depressions whereas the treatment of CP-perfused hearts with 200 μM LN augmented the I/R-induced depressions in both SL Na⁺-K⁺-ATPase and SR Ca²⁺-uptake activities (Fig. 3).

To determine if the differences in I/R induced changes in subcellular activities in CF-perfused and CP-perfused hearts are associated with differential alterations in the proteolysis in these preparations, the activity of a proteolytic enzyme, calpain, was measured in the cytosolic fraction. The results in Table 2 show that I/R induced an increase in calpain activity in both CF-perfused and CP-perfused hearts; however, the increase in calpain activity due to I/R in CF-perfused hearts was greater than that in CP-perfused hearts (P < 0.05). Treatment of CP-perfused hearts with 3 mM LA attenuated whereas treatment of CP-perfused hearts with 200 μM LN augmented the I/R-induced increase in calpain activity (Table 2).

Formation of NO and eNOS protein content. In a separate set of experiments, the status of endothelial function in CF-perfused and CP-perfused hearts was examined by monitoring the concentration of nitrate/nitrite, an index of NO formation, in the coronary perfusate as well as protein content for eNOS in the myocardium. It is pointed out that the total volume of SL Na⁺-K⁺-ATPase, SR Ca²⁺-uptake, and calpain activities.

Constant Pressure Perfusion (30 min Ischemia)

Fig. 2. Cardiac performance of isolated rat hearts exposed to 30 min of ischemia and 60 min of reperfusion at constant pressure (CP) (80 mmHg) treated with and without an NO synthase inhibitor, LN (200 μM). A: LVDP. B: LVEDP. C: +dP/dt. D: −dP/dt. Values are means ± SE of 6 hearts for each group. *P < 0.05 compared with control. #P < 0.05 compared with I/R group.
perfusate in CF-perfused and CP-perfused heart was about 600 ml and 735 ml, respectively during the 60 min of reperfusion, and thus this volume in both cases was adjusted to an equal amount of 750 ml before monitoring the concentration of nitrate/nitrite. The data in Fig. 4 reveal that I/R decreased the concentration of nitrate/nitrite in both CF-perfused and CP-perfused hearts; however, the depression in CF-perfused hearts was greater than that in CP-perfused hearts (P < 0.05).

Furthermore, treatment of CF-perfused hearts with 3 mM LA not only prevented the I/R-induced decrease in nitrate/nitrite levels but in fact also increased its level in the perfusate above the control levels. Treatment of CP-perfused hearts with 200 μM LN augmented the I/R-induced depression in the nitrate/nitrite level in the perfusate (Fig. 4). It can also be seen from Fig. 4 that I/R decreased the eNOS protein content in both CF-perfused and CP-perfused hearts; however, the I/R-induced depression in eNOS protein content in CF-perfused hearts was significantly greater (P < 0.05) than that in CP-perfused hearts (P < 0.05). The I/R-induced decrease in eNOS protein content was attenuated by treatment of CF-perfused hearts with 3 mM LA. On the other hand, the I/R-induced eNOS protein content slightly but significantly decreased in CP-perfused hearts on treatment with 200 μM LN (Fig. 4).

### Table 2. Cytosolic calpain activity in hearts subjected to 30 min ischemia and reperfused for 60 min

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Calpain Activity, RFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant flow perfusion</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100±6</td>
</tr>
<tr>
<td>I/R</td>
<td>396±5*</td>
</tr>
<tr>
<td>I/R + LA</td>
<td>200±13#</td>
</tr>
<tr>
<td>Constant pressure perfusion</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>83±4</td>
</tr>
<tr>
<td>I/R</td>
<td>236±17*</td>
</tr>
<tr>
<td>I/R + LN</td>
<td>387±13#</td>
</tr>
</tbody>
</table>

All values are mean ± SE of 4 hearts in each group. RFU, relative fluorescence units; I/R, ischemia-reperfusion; LA, L-arginine (3 mM); LN, N\textsuperscript{G}-nitro-L-arginine methyl ester (200 μM). *P < 0.05 compared with respective control. #P < 0.05 compared with respective I/R.
perfusion ischemic hearts was increased during the first 5 min of starting reperfusion but thereafter started declining toward the control levels during the reperfusion period.

At the end of coronary pressure and coronary flow measurements in CF-perfused and CP-perfused hearts, respectively, responses of CF-perfused and CP-perfused hearts to 1 μM ACh and 10 nM U-46619 infusions were examined. For this purpose, CF-perfused hearts were further perfused in the same mode for 3 min whereas the CP-perfused hearts were switched to CF perfusion mode, and the changes in coronary perfusion pressure were monitored to determine changes due to ACh and U-46619 infusion. It should be noted from the results shown in Fig. 5 that infusion of ACh decreased the coronary perfusion pressure in both CF-perfused and CP-perfused hearts; however, the ACh-induced decrease in coronary perfusion pressure was greater in CP-perfused hearts than that in CF-perfused hearts \( (P < 0.05) \). On the other hand, infusion of U-46619 did not increase the coronary perfusion pressure in CF-perfused hearts significantly \( (P > 0.05) \) whereas this agent produced an increase in coronary perfusion pressure in CP-perfused hearts significantly (Fig. 5).

**DISCUSSION**

In this study we have shown that I/R produced cardiac dysfunction as reflected by depressed LVDP, \(+dP/dt\), and \(-dP/dt\), as well as increased LVEDP in isolated rat hearts. Although I/R-induced impaired recovery of cardiac function is in agreement with our previous reports \((32, 40–42)\), the observed alterations in all parameters of cardiac performance due to I/R were of greater magnitude in hearts perfused at CF than those at CP. These contractile changes in CF-perfused and CP-perfused I/R hearts were found to be dependent on the duration of ischemia because 15 min and 30 min of ischemia in CF-perfused hearts produced changes comparable to those seen when the CP-perfused hearts were exposed to 30 min and 60 min of ischemia, respectively. Since oxidative stress in the I/R hearts is considered to play a critical role in depressing cardiac function \((10, 32)\), it is likely that the differences of changes in cardiac function due to I/R in CF-perfused and CP-perfused hearts may be due to differences in the magnitude of oxidative stress in these preparations. Although I/R was found to depress the activities of both SL Na\(^{+}\)-K\(^{+}\)-ATPase and SR Ca\(^{2+}\) uptake, which observations are in agreement with our previous work \((26, 42)\), the I/R-induced depressions in these membrane activities in CF-perfused hearts were greater than those in CP-perfused hearts. Since the depressed SL Na\(^{+}\)-K\(^{+}\)-ATPase and SR Ca\(^{2+}\)-uptake activity in the I/R hearts are considered to induce Ca\(^{2+}\)-handling defects in cardiomyocytes and cardiac dysfunction \((10, 26, 42)\), the observed differences with respect to these biochemical parameters in CF-perfused and CP-perfused hearts can be seen to contribute in explaining the I/R-induced differential changes in cardiac function in these preparations. In view of the fact that the I/R-induced increase in calpain activity \((18, 25, 38)\) in CF-perfused hearts was much higher than that in CP-perfused hearts, it is evident that the greater depressions in both SL Na\(^{+}\)-K\(^{+}\)-ATPase and SR Ca\(^{2+}\)-uptake activities in CF-perfused hearts compared with CP-

![Image](http://jap.physiology.org/)

**Table 3. Changes in perfusion pressure and coronary flow in I/R hearts perfused at constant flow and constant pressure**

<table>
<thead>
<tr>
<th>Time</th>
<th>Coronary Pressure (CF Perfusion), mmHg</th>
<th>Coronary Flow (CP Perfusion), ml/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before ischemia</td>
<td>76.1 ± 4.7</td>
<td>12.5 ± 1.1</td>
</tr>
<tr>
<td>During ischemia</td>
<td>1.1 ± 0.1</td>
<td>0</td>
</tr>
<tr>
<td>Reperfusion at minute:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>51.0 ± 1.2</td>
<td>15.2 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>41.5 ± 0.9</td>
<td>16.3 ± 0.3</td>
</tr>
<tr>
<td>5</td>
<td>45.9 ± 1.2</td>
<td>20.2 ± 0.7</td>
</tr>
<tr>
<td>10</td>
<td>48.4 ± 1.3</td>
<td>18.4 ± 1.0</td>
</tr>
<tr>
<td>20</td>
<td>51.6 ± 1.1</td>
<td>13.3 ± 0.6</td>
</tr>
<tr>
<td>30</td>
<td>54.0 ± 1.2</td>
<td>11.2 ± 0.4</td>
</tr>
<tr>
<td>60</td>
<td>57.0 ± 1.3</td>
<td>9.9 ± 0.1</td>
</tr>
</tbody>
</table>

All values are means ± SE for \( n = 4–5 \) hearts in each group. Isolated hearts perfused at constant flow (CF) or constant pressure (CP) were made ischemic for 30 min followed by reperfusion for 60 min.
perfused hearts may be due to differences in degree of proteolysis in these preparations.

The results in this study indicate that endothelial function with respect to NO formation in I/R hearts may be impaired, and this defect may be of a greater magnitude in CF-perfused hearts than that in CP-perfused hearts. This view is based on our observations that the concentration of nitrate/nitrite in the perfusate as well as protein content for eNOS in the myocardium were lower in CF-perfused hearts compared with those in CP-perfused hearts. In fact, the concentration of nitrate/nitrite in the effluent as well as the volume of perfusate from control hearts perfused at CF were also lower than those from control hearts perfused at CP. It should be pointed out that endothelium is now well known to control the coronary flow as well as cardiac function through the release of several substances including NO (6, 19, 21, 23, 44). Furthermore, coronary flow in the heart is determined by coronary perfusion pressure (12), which remained below the control level, while the coronary flow is maintained during reperfusion of CF-perfused hearts. On the other hand, coronary flow is increased above the control level during early periods while the coronary pressure is maintained during reperfusion of CP-perfused hearts. Thus it is likely that the CP-perfused and CF-perfused preparations are subjected to varying degrees of shear and stress, and differences in the characteristics of coronary flow and coronary pressure may explain the differences in their responses to I/R. In this regard, it should be noted that shear and stress have been shown to cause endothelial dysfunction due to the development of intracellular Ca\(^{2+}\) overload in the endothelial cells (17, 22, 34, 43). However, this may not serve as a mechanism for observed changes because shear stress is likely to be higher in CP-perfused hearts due to greater blood flow, but the endothelial dysfunction was found to be less compared with the CF-perfused hearts. Since we did not measure shear stress in both CF-perfused and CP-perfused heart preparations, some caution should be exercised while explaining differential response of these preparations in terms of differences in the degree of shear stress. Nonetheless, differences in the degree of defects in the endothelium in CF-perfused and CP-perfused hearts were evident from the differences in responses of these preparations to ACh, which is known to produce coronary vasodilation (44), and U-46619, a thromboxane A\(_2\) mimetic, which is known to produce coronary constriction (12, 14, 23, 45). This point is substantiated by our observations that the depression in coronary perfusion pressure due to ACh in control CP-perfused hearts was greater than that in control CF-perfused hearts. On the other hand, the increase in coronary perfusion pressure due to U-46619 was more in CP-perfused hearts compared with that in CF-perfused hearts.

This study has revealed that the I/R-induced changes in cardiac function (as seen by depressed LVDP, +dP/dt, and −dP/dt as well as elevated LVEDP) were attenuated in both CF-perfused and CP-perfused hearts on treatment with LA, whereas these alterations were augmented in CP-perfused hearts on treatment with LN. Furthermore, the beneficial effects of LA on I/R-induced changes in cardiac performance in
CF-perfused hearts were ameliorated by LN. These observations suggest the possibility of substrate deficiency for the conversion of endogenous l-arginine to NO under conditions of I/R. Depressions in the SL Na\(^+\)-K\(^-\)-ATPase and SR Ca\(^{2+}\)-uptake activities as well as increase in calpain activity due to I/R were also attenuated in CF-perfused hearts by treatment with LA and augmented in CP-perfused hearts with LN. In view of the role of LA as a substrate for NO synthase and LN as an inhibitor of eNOS (6), it is apparent that the observed differences for changes in cardiac function, subcellular activities, and calpain activity due to I/R in CF-perfused and CP-perfused hearts may be a consequence of differential alterations in the endothelium with respect to NO production. These results therefore indicate that differences in the I/R-induced changes in cardiac function as well as subcellular activities in CF-perfused and CP-perfused hearts may partly be due to differential alterations in endothelial function with respect to NO production. Whether these differences between CF-perfused and CP-perfused hearts are also mediated by differential development of intracellular Ca\(^{2+}\) overload, which is known to be a major mechanism of I/R injury (10), remain to be investigated.

In conclusion, the results described in this study indicate that alterations in cardiac performance due to I/R injury in both CF-perfused and CP-perfused hearts were dependent on the time of ischemic insult. These I/R-induced changes in cardiac performance were not only greater in CF-perfused hearts compared with those in CP-perfused hearts but were also associated with corresponding alterations in SL Na\(^+\)-K\(^-\)-ATPase, SR Ca\(^{2+}\)-uptake, and calpain activities. There was also a greater decrease in nitrate/nitrite concentration in perfusate as well as eNOS protein content in CF-perfused hearts compared with that in CP-perfused hearts. The beneficial effects of LA and adverse actions of LN indicate that I/R-induced changes in cardiac function, subcellular, and biochemical activities may be due to alterations in endothelial function and availability of substrate for NO bioavailability. In addition, the difference with respect to endothelial function between CF-perfused and CP-perfused preparations was apparent on studying responses to ACh or U-46619. Since maintaining the coronary flow in the CF-perfused hearts by using a pump was found to produce greater damage to endothelial function compared with the CP-perfused hearts, it would be prudent to exercise some caution for the use of a pump, which is commonly employed during cardiac surgery involving a heart-lung bypass machine or during organ transplant procedure. Furthermore, agents such as LA, which was found to prevent I/R-induced injury to endothelium, should be used in the perfusion fluid during cardiac bypass surgery for improving recovery of the heart.

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