Chronic skeletal muscle ischemia preserves coronary flow in the ischemic rat heart


Abstract

The purpose of the present study was fourfold. First, we sought to investigate whether the decreased myocardial necrosis, observed previously in limb ischemic animals after myocardial ischemia-reperfusion (I/R), is translated into improved left ventricular (LV) function during the immediate reperfusion period. Second, we examined the functional capacity of neovascularization by measuring coronary flow after regional myocardial ischemia. Third, we aimed to confirm the proangiogenic effects of chronic skeletal muscle ischemia in the rat model. Finally, we further investigated the potential underlying beneficial mechanisms of chronic limb ischemia by examining the previously proposed hypothesis (4, 8, 21, 30, 31), namely, that chronic skeletal muscle ischemia decreases myocardial necrosis by ameliorating reperfusion injury. To this end, we measured infarct size after coronary artery ligation without reperfusion in animals with and without chronic limb ischemia; comparable infarct sizes in the two groups would validate this hypothesis, whereas decreased myocardial necrosis have been demonstrated after ischemia in a variety of remote ischemic preconditioning (2, 14). Cytoprotection in the ischemic episodes of peripheral tissues, a process termed ischemic myocardial preconditioning describes the situation where the ventricular myocardium is rendered more resistant to ischemia during infarction, but the underlying mechanisms remain unclear. Although neovascularization in the left ventricular myocardium has been proposed as a possible mechanism, the functional capacity of such vessels has not been studied. We examined the effects of chronic limb ischemia on infarct size, coronary blood flow, and left ventricular function after ischemia-reperfusion. Hindlimb ischemia was induced in 65 Wistar rats by excision of the left femoral artery, whereas 65 rats were sham operated. After 4 wk, myocardial infarction was generated by permanent coronary artery ligation. Infarct size was measured 24 h postligation. Left ventricular function was evaluated in isolated hearts after ischemia-reperfusion, 4 wk after limb ischemia. Neovascularization was assessed by immunohistochemistry, and coronary flow was measured under maximum vasodilatation at different perfusion pressures before and after coronary ligation. Infarct size was smaller after limb ischemia compared with controls (24.4 ± 8.1% vs. 46.2 ± 9.5% of the ventricle and 47.6 ± 8.7% vs. 80.1 ± 9.3% of the ischemic area, respectively). Indexes of left ventricular function at the end of reperfusion (divided by baseline values) were improved after limb ischemia (developed pressure: 0.68 ± 0.06 vs. 0.59 ± 0.05, P = 0.008; maximum +dp/dt: 0.70 ± 0.08 vs. 0.59 ± 0.04, P = 0.004; and maximum –dp/dt: 0.86 ± 0.14 vs. 0.72 ± 0.10, P = 0.041). Coronary vessel density was markedly higher (P = 0.00021) in limb ischemic rats. In contrast to controls (F = 5.65, P = 0.00182), where coronary flow decreased, it remained unchanged (F = 1.36, P = 0.28) after ligation in limb ischemic rats. In conclusion, chronic hindlimb ischemia decreases infarct size and attenuates left ventricular dysfunction by increasing coronary collateral vessel density and blood flow. Chronic limb ischemia; infarct size; left ventricular function

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crosis after chronic skeletal muscle ischemia would implic-
ate additional beneficial mechanisms.

MATERIALS AND METHODS

The initial experimental animal cohort comprised 130 Wistar rats (252 ± 23 g). All animals were housed in individual cages in a climate-controlled environment with an artificial 12:12-h light-dark cycle. Access to food and water was unrestricted. All animals received humane care, and the procedures were in accordance with the recommen-
dations of the Declaration of Helsinki. This investigation con-
formed with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996). The experimental protocol was approved by the Department of Veterinary Medicine and Animal Welfare (Athens, Greece).

Induction of femoral ischemia. Chronic ischemia of the left hindlimb was surgically induced in 65 rats (256 ± 24 g), whereas the remaining 65 rats (255 ± 27 g) were sham operated and served as controls. The induction of hindlimb ischemia was performed as previously described (31) and is briefly summarized below.

Animals were intubated and mechanically ventilated using a rodent ventilator (model 7025, Ugo Basile, Comerio Varese, Italy). Anesthe-
sia was maintained with a mixture of oxygen and 2% isoflurane. After a longitudinal incision in the left thigh, the femoral artery was dissected free along its entire length, including all major branches, i.e., the inferior epigastric, deep femoral, lateral circumflex, and superficial epigastric arteries. After the popliteal and saphenous arteries were further dissected distally, the external iliac artery and all major branches of the femoral artery were ligated. Finally, the femoral artery was completely excised from its proximal origin to its distal bifurcation. After the procedure, animals were left to recover under close supervision and care. Prophylactic antibiotics were administered subcutaneously for a total of 5 days postoperatively. Analgesia was administered subcutaneously as required, by evidence of discomfort throughout the study duration. Sham-operated animals received identi-
tical anesthesia and antibiotic regimens. At the end of the fourth week, all animals were macroscopically examined for skin necrosis and limb atrophy.

Four weeks after the induction of ischemia (or sham operation), animals were divided into four series of experiments. The first series (n = 20) consisted of the assessment of LV function during I/R in isolated heart preparations. The second series (n = 20) consisted of coronary flow measurements during I/R in isolated heart preparations. In the third series (n = 20) of experiments, histological assessment of coronary neovascularization was performed. Finally, the fourth series (n = 70) consisted of infarct size measurement after the induction of myocardial infarction without reperfusion.

Myocardial infarction generation. Myocardial infarction was gen-
erated in vivo as previously described (1, 9). In brief, animals were intubated and mechanically ventilated as outlined above. Through a left thoracotomy, the pectoralis muscles were dissected, and the thoracic cage was opened with a blunt curved forceps. The pericar-
dium was carefully dissected, the heart was exteriorized, and the left coronary artery was encircled and ligated near its origin with a 6-0 suture placed between the pulmonary artery cone and the left atrial appendage. Following these anatomic landmarks ensures the induc-
tion of myocardial infarction of comparable size in all experiments. The incision was closed in three layers, and the remaining air was aspirated from the thorax, allowing the resumption of spontaneous respiration. A six-lead ECG was obtained, and ST elevation in two or more leads was considered proof of induced infarction.

Infarct size measurement. Twenty-four hours after ligation, animals were killed with a lethal dose of potassium chloride (arresting the heart in diastole), and infarct size was determined as previously described (33). Briefly, the heart was excised and frozen at −20°C for 1 h; subsequently, it was hand cut into 2-mm slices and incubated in triphenyltetrazolium chloride at a temperature of 37°C for 15 min. Once the color had been established, slices were fixed in 10% formalin for 20 min. With this method, living tissue is colored red, whereas infarcted tissue retains its pale tan color. Slices were placed on a Plexiglas holder and scanned with a high-resolution scanner (Scanjet 4570c, Hewlett-Packard). Areas of infarcted and non-
infarcted myocardium were determined by planimetry of the magnified scanned images using a previously validated software program (Image Tool, University of Texas, San Antonio, TX). Infarct size, expressed as a percentage of the LV cross-sectional area, was defined as the ratio between the infarcted area divided by the total LV area, as follows: infarct size/LV mass (in %) = Σ (infarct weight in each slice/total LV weight) × 100.

To exclude differences in infarct size secondary to disparate ischemic areas at risk, we measured ischemic and necrotic areas in a separate set of experiments using a previously utilized method in our laboratory (19). After anesthesia with ketamine and xylazine, the heart was harvested and mounted on a perfusion apparatus. The coronary ligature was released, and the heart was perfused via the aorta with normal saline at room temperature for 2 min. When all residual blood had been removed from the coronary arteries, the coronary ligature was retightened at the same site, and 5 ml of green fluorescent microspheres (Duke Scientific, Palo Alto, CA), 2–5 μm in diameter, suspended in saline, were infused over 5 min. After removal of the right ventricle, hearts were frozen at −20°C for 24 h and sliced into 2-mm sections from the apex to the base. Slices were incubated in 1% triphenyltetrazolium chloride in isotonic phosphate buffer solution (at 37°C, pH 7.4) for 20 min and immersed in 10% formaldehyde solution for 24 h. Slices were placed between glass plates, and the risk zone, infarcted area, and normal myocardium were identified under ultraviolet light (wavelength: 366 nm) and measured with the use of Image Tool. Volumes of the infarct area and area at risk were expressed in centimeters cubed, and the percent ratio of infarct area/area at risk was calculated.

I/R ex vivo. After animals were anesthesized with a mixture of ketamine and xylazine, a left lateral thoracotomy was performed. The heart was quickly dissected, canulated through the ascending aorta, and perfused with oxygenated (95% O2-5% CO2) Krebs-Henseleit buffer (supplemented with 0.001% adenosine) on a Langendorff apparatus (ML870B2 system, AD Instruments, Oxfordshire, UK), as previously delineated (15). The isolated heart preparation was paced at 300 beats/min and perfused at a constant coronary perfusion pressure of 70 mmHg. A water-filled transducer balloon connected to a pressure transducer was placed into the LV through a left atrial incision. The balloon volume was adjusted to maintain a stable LV end-diastolic pressure of 6–7 mmHg. The LV pressure signal was continuously recorded and analyzed online by a dedicated software program (Chart 5, version 5.4.2, AD Instruments). Zero-flow global ischemia was induced for 20 min followed by a 45-min reperfusion period.

Assessment of LV function. The following variables were recorded at baseline (steady state for a minimum of 10 min) and at the end of reperfusion: peak systolic pressure, end-diastolic pressure, LV devel-
oped pressure (LVPD; defined as peak systolic pressure minus end-
diastolic pressure), and the first derivative of LV pressure (maximum +dP/dt and −dP/dt). They were expressed as the ratio of values at the end of reperfusion divided by baseline values.

Ischemic contracture and hypercontracture were recorded as addi-
tional measures of LV function during I/R. We recorded the time point of ischemic contracture, which was defined as a marked, abrupt increase in the minimal value of LV pressure during ischemia. Hypercontracture was expressed as the peak value of LV diastolic pressure during the first 5 min of reperfusion.

Coronary flow in isolated heart preparations. Coronary flow was assessed on a Langendorff apparatus as previously described (10). Briefly, isolated heart preparations were perfused with oxygenated (95% O2-5% CO2) Krebs-Henseleit buffer (supplemented with 0.001% adenosine to induce maximal coronary vasodilatation). After
RESULTS

All animals recovered well after the induction of left hindlimb ischemia. Four weeks after the procedure, signs of severe limb ischemia, namely, skin necrosis and limb atrophy, were present in all animals.

Infarct size after myocardial infarction without reperfusion in vivo. Myocardial infarction was generated in 25 rats with limb ischemia and in 25 control rats. Of these, four animals (2 animals with limb ischemia and 2 sham-operated animals) died during surgical manipulations and were excluded. During the 24-h observation period, five limb ischemic animals (21.7%) and seven sham-operated animals (30.4%) died due to myocardial infarction complications, but this difference failed to reach statistical significance \((P = 0.73)\). Infarct size was significantly \((P < 0.0001)\) smaller in rats subjected to chronic limb ischemia \((25.4 \pm 8.1\%)\) than in sham-operated animals \((46.2 \pm 9.5\%)\). Representative examples are shown in Fig. 1A, and values are shown in Fig. 1B.

In a separate set of experiments of 10 limb ischemic animals and 10 sham-operated animals, the necrotic area was expressed as a percentage of the area at risk. Four animals (3 sham-operated animals and 1 limb ischemic animal, \(P = 0.58)\) died during the 24-h postinfarction period and were excluded. The necrotic area was smaller \((P < 0.0001)\) in limb ischemic animals \((47.6 \pm 8.7\%)\) than in sham-operated animals \((80.1 \pm 9.3\%)\). The area at risk (expressed as a percentage of the LV) did not differ between groups \((58.9 \pm 4.6\% vs. 59.4 \pm 7.1\%, respectively, P = 0.61)\). Values are shown in Fig. 1B.

LV function after I/R. Ischemic contracture occurred \(11.92 \pm 3.85\) min after ischemia in control hearts and \(15.18 \pm 1.66\) min after ischemia in hearts with prior limb ischemia \((P = 0.045;\) Fig. 2). In contrast, the maximal value of diastolic pressure during reperfusion (hypercontracture) did not differ \((P = 0.61)\) between the two groups (limb ischemia: \(66.5 \pm 13.5\) mmHg and control: \(69.8 \pm 11.8\) mmHg).

LV peak systolic pressure at the end of reperfusion exhibited improved \((P = 0.018)\) recovery in the limb ischemia group.

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Fig. 1. Infarct size. A: examples of three heart sections (S1, S2, and S3) from a sham-operated rat (left) and a limb ischemic rat (right). B: infarct size as plots of the infarct area/total left ventricular (LV) area (I/LV) and of the infarcted area (I)/area at risk (R) for sham-operated and limb ischemic rats.
than in the control group (0.96 ± 0.11 vs. 0.83 ± 0.09 of baseline values). Similarly, the ratio of LVDP at the end of reperfusion divided by baseline values was higher ($P = 0.0081$) in the limb ischemia group (0.68 ± 0.06) compared with the control group (0.59 ± 0.05). Finally, the recovery of $+dP/dt$ was superior ($P = 0.0044$) in the limb ischemia group (0.70 ± 0.08) than in the control group (0.59 ± 0.04).

In contrast to systolic LV function, differences in diastolic function were less pronounced. The recovery of $-dP/dt$ was 0.86 ± 0.14 in the limb ischemia group versus 0.72 ± 0.10 in...
the control group ($P = 0.041$). However, end-diastolic pressure remained elevated after reperfusion in both groups, displaying a $5.5 \pm 2.59$- and $4.62 \pm 1.11$-fold increase of baseline values in the control and limb ischemia groups, respectively ($P = 0.39$). All values are shown in Fig. 3.

**Coronary flow in isolated heart preparations.** Compared with baseline, coronary flow under regional ischemia decreased significantly ($F = 5.65, P = 0.00182$) in control rats. This decrease was more pronounced under perfusion pressures of 120 and 140 mmHg compared with 60 mmHg. In contrast, coronary flow was maintained ($F = 1.36, P = 0.28$) in rats with chronic limb ischemia (Fig. 4). No statistically significant difference was observed in coronary flow baseline values between the two groups at various perfusion pressures. In contrast, under regional ischemia, coronary blood flow was higher ($P < 0.05$) at all perfusion pressures examined (Table 1).

**Table 1. Coronary flow at baseline and under regional ischemia**

<table>
<thead>
<tr>
<th>Perfusion Pressure</th>
<th>Sham-operated group</th>
<th>Limb ischemic group</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline 60 mmHg</td>
<td>8.87 ± 2.02</td>
<td>8.83 ± 1.00</td>
<td>0.96</td>
</tr>
<tr>
<td>80 mmHg</td>
<td>12.45 ± 2.99</td>
<td>11.53 ± 1.73</td>
<td>0.51</td>
</tr>
<tr>
<td>100 mmHg</td>
<td>15.53 ± 3.83</td>
<td>15.03 ± 2.41</td>
<td>0.78</td>
</tr>
<tr>
<td>120 mmHg</td>
<td>18.18 ± 3.22</td>
<td>18.36 ± 3.29</td>
<td>0.92</td>
</tr>
<tr>
<td>140 mmHg</td>
<td>21.11 ± 3.96</td>
<td>21.02 ± 3.76</td>
<td>0.96</td>
</tr>
<tr>
<td>Regional ischemia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 mmHg</td>
<td>5.38 ± 1.23</td>
<td>7.09 ± 1.24</td>
<td>0.025</td>
</tr>
<tr>
<td>80 mmHg</td>
<td>8.09 ± 1.17</td>
<td>10.16 ± 1.56</td>
<td>0.015</td>
</tr>
<tr>
<td>100 mmHg</td>
<td>10.59 ± 1.65</td>
<td>13.70 ± 1.82</td>
<td>0.006</td>
</tr>
<tr>
<td>120 mmHg</td>
<td>12.67 ± 2.22</td>
<td>15.91 ± 1.75</td>
<td>0.012</td>
</tr>
<tr>
<td>140 mmHg</td>
<td>14.38 ± 2.19</td>
<td>17.84 ± 2.30</td>
<td>0.014</td>
</tr>
</tbody>
</table>

Values are means ± SD.

**Coronary vessel counts.** The total number of arterioles was higher ($P = 0.000034$) in limb ischemic rats ($78 \pm 18.33$) compared with control rats ($34.88 \pm 12.41$). Similarly, the vessel density was higher ($P = 0.000212$) in limb ischemic rats ($1.9 \pm 0.463$) than in control rats ($0.97 \pm 0.328$; Fig. 5). The areas of the tissue examined were comparable ($P = 0.24$) in the sham-operated and limb ischemic groups ($37.5 \pm 12.5$ vs. $45.3 \pm 14.8$ mm$^2$, respectively).

**DISCUSSION**

The present study demonstrates that chronic (4 wk) severe ischemia in the rat hindlimb results in cytoprotection of the ischemic myocardium, confirming prior observations in the rabbit (31). Therefore, the beneficial effects of chronic skeletal muscle ischemia on the ventricular myocardium are not specific for a particular species. Although the cardioprotective effects of short-term skeletal muscle ischemia have been previously evaluated in experimental (7, 14, 16–18, 20) and clinical (4, 8, 21, 30) studies, our previous (31) and present studies are the first to examine the effects of chronic skeletal muscle ischemia.

The findings of the present study indicate that chronic hindlimb ischemia 1) decreases infarct size after myocardial infarction without reperfusion (expressed either as a percentage of the total LV mass or as a percentage of the ischemic area at risk), 2) ameliorates LV systolic dysfunction after global myocardial ischemia followed by reperfusion, and 3) increases the density of noncapillary coronary arterioles and thereby 4) maintains coronary blood flow during regional myocardial ischemia.

**Infarct size after permanent coronary ligation.** Previous studies (4, 7, 8, 14, 16–18, 20, 21, 30, 31) have demonstrated that ischemia of a skeletal muscle reduces infarct size after myocardial ischemia followed by reperfusion. The explanation for these findings is not well defined, and several suggestions have been put forward, such as inflammatory gene suppression (12), modulation of ATP-sensitive K$^+$ channels (32), nuclear factor-κB-p105 or inducible nitric oxide synthase (13), and free radical pathways (6). A common denominator of these potential mechanisms is the reduction of reperfusion injury after I/R. However, previous studies have not provided a clear picture as to whether the beneficial effects of chronic skeletal muscle ischemia also apply to myocardial infarction without reperfusion. Should this be the case, additional mechanisms of cytoprotection are bound to be operative during myocardial ischemia, irrespective of subsequent reperfusion.

In the present study, we reported a marked decrease in the necrotic area by $\sim 45\%$ after permanent coronary ligation. Thus, our results demonstrate that chronic limb ischemia affords cytoprotection after myocardial ischemia with or without subsequent reperfusion and imply that additional mechanisms to the amelioration of reperfusion injury are operative. As such, neovascularization appears to be capable of maintaining coronary blood flow through functional collateral vessels, thereby limiting myocardial necrosis, as discussed in detail below.

**LV dysfunction after I/R.** An important finding in our study is the beneficial effects of chronic limb ischemia on LV function after global ischemia followed by reperfusion. Indices of LV systolic function at the end of reperfusion, such as LV peak systolic pressure and LVDP as well as the first derivative of LV pressure, were improved in the limb ischemic state compared with control rats. This observation suggests that chronic limb ischemia limits myocardial necrosis, as discussed in detail below.
group compared with the control group. During the ischemic phase, diastolic dysfunction was lower in hearts from limb ischemic animals, as indicated by the profile of ischemic contracture. Finally, diastolic function after reperfusion was also improved, although end-diastolic pressure remained elevated. Examined collectively, these findings indicate that the decrease in necrotic tissue after myocardial infarction is translated into the preservation of LV systolic and, to a lesser extent, diastolic function during I/R. The finding of the disproportionate improvement in LV diastolic function in our experiments is hard to explain but may be attributed to the lack of a significant effect of chronic skeletal muscle ischemia on reperfusion injury. Indeed, this process is likely mediated by excessive Ca\(^{2+}\) influx during reperfusion (29), resulting initially in diastolic dysfunction followed by cell death. Thus, the disparate improvement in diastolic function in our experiments reinforces our conclusion on the minor role of reperfusion injury amelioration as part of the explanation of the benefits observed after chronic hindlimb ischemia.

Preservation of coronary blood flow. We (31) have previously demonstrated that chronic limb ischemia, induced with a surgical protocol identical to that used in the present study, increases the number of noncapillary, nonlymphatic blood vessels in the normal rabbit heart. However, the functional significance of these vessels, in terms of the maintenance of coronary blood flow and LV function, was not examined in our previous study. Here, we report a significant decrease in coronary blood flow after left coronary artery ligation in control rats but preserved flow in rats subjected to chronic limb ischemia. These results indicate a functioning collateral circulation network between the right and left coronary arteries capable of maintaining coronary blood flow during regional ischemia under conditions of maximal vasodilatation (attained by the addition of adenosine in the perfusion solution). Thus, our present and previous findings indicate that neovascularization in the ventricular myocardium, secondary to chronic skeletal muscle ischemia, is a prominent mechanism for the decreased necrotic area after coronary artery ligation in the presence or absence of reperfusion.

Coronary vessel density. We found a marked increase in neovascularization in the ventricular myocardium after chronic limb ischemia, resulting in almost double coronary vessel density compared with control animals. These changes, observed mostly in small arterioles, confirm our previous findings (31) in the rabbit model and indicate that this process is not species specific.

During chronic limb ischemia, blood supply is maintained, which is mediated by the development of collateral circulation. The main stimulus that initiates arteriogenesis is the increased shear stress inside preexisting collateral vessels and small arteries proximal to a point of severe stenosis or total occlusion (10). Neovascularization is supported by growth factors, chemokines, proteases, and inflammatory cells, which are excited locally during ischemia, but they circulate and may act in remote tissues, such as in the ventricular myocardium (13).

Previous research has tried to identify the stimuli for local or remote neovascularization process; in these studies, vascular endothelial growth factor, hypoxia inducible factor-1, and fibroblast growth factor have been proposed as the main stimuli for angiogenesis and capillary formation (3). However, angiogenesis is regulated by complex molecular mechanisms involving the participation of multiple factors. Recent studies (25, 26) on the interaction between skeletal muscle and the myocardium have implicated follistatin-like 1, an extracellular glycoprotein secreted by skeletal muscles in response to ischemic insult. Follistatin-like 1 was found to stimulate vascularization through its ability to activate Akt-endothelial nitric oxide synthase signaling. In this study (26), ischemic hindlimb surgery in the mouse model, with a protocol almost identical to our experiments, led to the induction of follistatin-like 1 in skeletal muscle and increased circulating levels of this protein. It has been proposed that skeletal muscle secretes factors, referred to as “myokines,” that can influence the function of remote tissues, such as the intact ventricular myocardium (28). These findings reinforce our hypothesis, namely, that chronic skeletal muscle ischemia increases the production of proangiogenic factors that circulate and may act in other vascular beds, such as the coronary circulation. Moreover, recent evidence has indicated that follistatin-like 1 has multiple actions, exerting not only proangiogenic actions but also cardioprotective actions, by exerting antiapoptotic actions in cardiomyocytes (24).
Conclusions. The animal model of chronic hindlimb ischemia provides evidence of increased collateral blood flow in the ventricular myocardium. Neovascularization preserved coronary blood flow during regional ischemia, which, in turn, decreased myocardial necrosis and improved LV function. Further studies on this model are warranted, aiming at the identification of potentially important mediators for neovascularization.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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