Ischemic-reperfused isolated working mouse hearts: membrane damage and type IIA phospholipase A\textsubscript{2}

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De Windt, Leon J., Jodil Willems, Theo H. M. Roemen, Will A. Coumans, Robert S. Reneman, Ger J. Van Der Vusse, and Marc Van Bilsen. Ischemic-reperfused isolated working mouse hearts: membrane damage and type IIA phospholipase A\textsubscript{2}. Am J Physiol Heart Circ Physiol 280: H2572–H2580, 2001.—For the murine heart the relationships between ischemia-reperfusion-induced loss of cardiac function, enzyme release, high-energy phosphate (HEP), and membrane phospholipid metabolism are ill-defined. Accordingly, isolated ejecting murine hearts were subjected to varying periods of ischemia, whether or not followed by reperfusion. On reperfusion, hemodynamic function was almost completely restored after 10 min of ischemia [83 ± 14% recovery of cardiac output (CO)], but was severely depressed after 15 and 20 min of ischemia (40 ± 24 and 31 ± 24% recovery of CO, respectively). Reperfusion was associated with partial recovery of HEP stores and enhanced degradation of phospholipids as indicated by the accumulation of fatty acids (FA). Myocardial FA content and enzyme release during reperfusion were correlated ($r = 0.70$), suggesting that membrane phospholipid degradation and cellular damage are closely related phenomena. To investigate the role of type IIA secretory phospholipase A\textsubscript{2} (sPLA\textsubscript{2}) in this process, hearts from wild-type and sPLA\textsubscript{2}-deficient mice were subjected to ischemia-reperfusion. Posts ischemic functional recovery, ATP depletion, enzyme release, and FA accumulation were not significantly different between wild-type and sPLA\textsubscript{2}-deficient hearts. These findings argue against a prominent role of type IIA sPLA\textsubscript{2} in the development of irreversible cell damage in the ischemic-reperfused murine myocardium.

Duration of flow-deprivation required to induce marked functional impairment in the isolated mouse heart ranged from 6 min (3) to 50 min (17). Furthermore, in the studies (17, 20, 21, 26, 36) on isolated murine hearts reported so far, attempts to correlate posts ischemic functional recovery with parameters other than cell necrosis as estimated by enzyme release into the coronary effluent are virtually lacking. Accordingly, the present study was designed to obtain insight into the effect of ischemia-reperfusion on hemodynamic recovery, irreversible cell damage, and high-energy phosphate depletion using a left ventricular ejectioning isolated mouse heart preparation (9). Because previous studies indicated that loss of cellular integrity relates to, among others, impaired membrane phospholipid (PL) homeostasis (5, 8, 29, 32), special attention was paid to the relationship between these parameters and the accumulation of PL-derived fatty acid (FA) moieties.

It is generally acknowledged that ischemia-reperfusion-induced degradation of membrane PL is associated with enhanced phospholipase A\textsubscript{2} (PLA\textsubscript{2}) activity (5, 8, 11, 13, 27, 29). At least four types of PLA\textsubscript{2}, differing in substrate preference and calcium dependency have been identified in myocardial tissue (4, 6, 10, 11, 31). The type of PLA\textsubscript{2} playing a dominant role in mediating membrane PL degradation in the ischemic-reperfused heart remains a matter of continuous speculation (31–33). The low-molecular-weight, calcium-dependent type IIA secretory phospholipase A\textsubscript{2} (sPLA\textsubscript{2}) has been detected in a wide variety of cell types, including cardiac myocytes (10) and has been implicated in ischemia-reperfusion injury in both brain and intestine (16, 25). The pathophysiological role of type IIA sPLA\textsubscript{2} in ischemia-reperfusion-induced cellular damage in cardiac tissue, if any, has not been well defined. In this light, the recent finding of inbred mouse strains that display a frameshift mutation in the pl\textsubscript{a2}ga gene, resulting in the absence of a functional type IIA sPLA\textsubscript{2} enzyme (14, 19), provides a valuable tool to investigate the specific role of this enzyme in the ischemia-reperfused heart.

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To explore the relationship among hemodynamic function, cell necrosis, energy status, and membrane damage in the murine heart in more detail, the severity of the ischemic insult was modulated by varying the duration of flow deprivation before reinstallation of flow. For these studies we used isolated hearts from Swiss mice, the functional characteristics of which were explored under normoxic conditions in a previous study (9). The isolated hearts from the wild-type C57BL/Ks substrain and the type IIA sPLA2-deficient C57BL/6 substrain were then subjected to ischemia-reperfusion to assess the potential contribution of this enzyme in ischemia-reperfusion-induced cardiac injury and loss of function.

MATERIALS AND METHODS

Chemicals. Except for d(+)-glucose and pyruvate (Sigma; St. Louis, MO), all chemicals used for the Krebs-Henseleit solution were of the highest grade available and purchased from Merck (Darmstadt, Germany). Insulin was purchased from Novo Nordisk ( Bagsvaerd, Denmark).

Animals and surgery. Adult female Swiss mice (Iffa Credo; Lyon, France) 3–4 mo of age were used for studies in which the duration of ischemia was varied. To assess the role of type IIA sPLA2 in ischemia-reperfusion-induced cardiac injury adult male mice of the two closely related inbred C57/B1 substrains, namely C57BL/Ks (wild-type pla2gα-gene; M&B, Ry, Denmark), and C57BL/6 (mutated pla2gα gene; B&K, Hull, UK), were used (15, 18, 24). The animals were allowed to adjust to the new housing conditions for 2 wk before experimental use. All mice were kept under standard housing conditions with an artificial 12-h light cycle with free access to standard rodent food (Diet SRM-A, Hope Farms; Woerden, The Netherlands) and tap water. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Maastricht University.

PCR genotyping. To confirm thymidine insertional mutation in exon 3 of the pla2gα gene in the inbred C57BL/6 strain and its absence in the inbred C57BL/Ks strain, exon 3 was amplified from a number of individual mice by PCR as described previously (14, 15, 19). Because the thymidine insertion disrupts a BamHI site in exon 3 of the Pla2αa gene, PCR products were digested with BamHI before gel electrophoresis. Briefly, genomic DNA was isolated from tails using a Qiamp genomic DNA prep kit (Qiagen; Leusden, The Netherlands). PCR analysis of type IIA sPLA2 exon 3 was performed using the following primers: 5′-primers (5′-CTGGCT TTTCCTTCTGTACGCCTGG-3′, 3′-primers (5′-GGAAAC CACTGGGACACTGAGGTAGTG-3′). Full-length genomic PCR products from both strains were of the expected length of 500 bp. The C57BL/6 exon 3 PCR fragments could not be digested by BamHI, indicating disruption of the wild-type BamHI site, whereas digestion of the C57BL/Ks PCR products resulted in two cleavage signals of 300 and 200 bp, respectively (data not shown). Later in this paper C57BL/Ks and C57BL/6 mice are referred to as sPLA2α(+/+) and sPLA2α(−/−) mice, respectively.

Isolated ejecting mouse heart preparation. The isolated ejecting mouse heart preparation used in the present study has been described in detail before (9). Briefly, animals were anesthetized with 50 mg/kg ip pentobarbital sodium (Nembutal, Sanofi-Sante; Maassluis, The Netherlands). After thoracotomy, the hearts were quickly excised and transferred to ice-chilled perfusion buffer (for composition see below). The ascending aorta was cannulated with a cannula matching the hemodynamic impedance characteristics of the murine ascending aorta (9). Retrograde perfusion at a perfusion pressure of 50 mmHg was started immediately after which the hearts resumed spontaneous beating. The left atrium was cannulated with a cannula through one of the lung veins. The recirculating modified Krebs-Henseleit perfusion buffer, prefiltered by a microfilter (0.45 μm diameter, Millipore), had the following composition: 118 mM NaCl, 4.7 mM KCl, 3.0 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3, 0.5 mM Na-EDTA, 10 mM d(+)-glucose, 1.5 mM sodium pyruvate, and 5 U/I insulin. The buffer was continuously gassed with 95% O2-5% CO2. Care was taken to maintain the temperature of the perfusate, and thus the heart, at 38.5°C.

Hemodynamic data. All hemodynamic variables were continuously recorded on a personal computer, using specialized software (Hemodynamic Data Acquisition System, Technical Department, Maastricht University), allowing the on-line acquisition, presentation, and calculation of left atrial filling flow, aortic flow (AOF), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), diastolic aortic pressure, and the first maximal and minimal derivatives of left ventricular pressure (LV dP/dtmax and LV dP/dtmin). Left ventricular developed pressure (LVPD) was defined as the difference between LVSP and LVEDP. Cardiac output (CO) was defined as the sum of AOF and coronary flow (CF). CF was determined from the difference between AOF, as measured by an 1 N in-line aortic flow probe and left atrial filling flow, as measured by a 2 N in-line flow probe placed in the left atrial inflow tract. Calculated CF data were periodically checked by timed collection of the coronary perfusate. CF data were used to calculate coronary resistance, which was defined as aortic pressure divided by CF normalized for individual heart weights.

Experimental protocol. After an initial 10-min retrograde stabilization period (perfusion pressure 50 mmHg), antegrade perfusion was started by opening the left atrial conduit. Left atrial filling pressure was set at 10 mmHg, whereas diastolic aortic pressure was kept at 50 mmHg. Except for the ischemic period and the first 5 min of reperfusion, the hearts were paced artificially throughout the whole experiment at a frequency of 380 beats/min, a rate slightly higher than the intrinsic rate of the isolated hearts of the mouse strains tested.

The hearts were normoxically perfused for 20 min (preischemic period). Just before the ischemic period, the water-jacketed chamber was filled with warm perfusate solution (38.5°C) until the heart was completely submerged, pacing was stopped, and the aortic and atrial lines were clamped to create normothermic, global ischemia. After the ischemic period, the water-jacketed chamber was emptied, and hearts were reperfused retrogradely at a perfusion pressure of 50 mmHg for 10 min. Subsequently, the left atrial conduit was reopened, and the hearts were allowed to work in the antegrade mode at a preload pressure of 10 mmHg and a diastolic aortic pressure of 50 mmHg for an additional 50 min.

In the first series of experiments, the hearts of the Swiss mice were subjected to either 10, 15, or 20 min of ischemia, whether or not followed by reperfusion. As potential strain differences have to be appreciated, the critical time duration whether or not followed by reperfusion. As potential strain differences have to be appreciated, the critical time duration was determined in a series of pilot experiments first. It was found that 17.5 min of ischemia resulted in 30–50% postischemic recovery of CO. Accordingly, it was decided to subject the hearts of the C57/BL substrains to 17.5 min of ischemia.

In this way it was possible to establish potential improve-
Membrane damage in ischemic-reperfused mouse hearts

Table 1. Preischemic hemodynamic values measured in isolated, left ventricular ejecting murine hearts from Swiss, C57BL/Ks [sPLA2 (+/+)] and C57BL/6 [sPLA2 (−/−)] mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>Swiss C57BL/Ks sPLA2 (+/+)</th>
<th>C57BL/6 sPLA2 (−/−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HW/BW, mg/g</td>
<td>7.9 ± 0.6</td>
<td>8.6 ± 0.3</td>
</tr>
<tr>
<td>CO, ml·min⁻¹·g⁻¹</td>
<td>50.0 ± 2.0</td>
<td>40.0 ± 2.0</td>
</tr>
<tr>
<td>CF, ml·min⁻¹·g⁻¹</td>
<td>8.3 ± 0.3</td>
<td>4.9 ± 1.3</td>
</tr>
<tr>
<td>LVSP, mmHg</td>
<td>86 ± 2</td>
<td>85 ± 2</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>7 ± 2</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>LVDP, mmHg</td>
<td>77 ± 2</td>
<td>78 ± 2</td>
</tr>
<tr>
<td>LV dp/dt max, mmHg/s</td>
<td>5.652 ± 831</td>
<td>6.461 ± 1386</td>
</tr>
<tr>
<td>LV dp/dt min, mmHg/s</td>
<td>-3.054 ± 415</td>
<td>-3.435 ± 434</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD (n = 9–13 hearts). Isolated hearts were perfused at a pre- and afterload pressure of 10 and 50 mmHg, respectively. Hearts were paced at 380 beats/min. HW, heart weight; BW, body weight; CO, cardiac output; CF, coronary flow; SV, stroke volume; LVDP, left ventricular developed pressure; LVSP, left ventricular systolic pressure, LVEDP, left ventricular end-diastolic pressure; LV dp/dt max, positive maximal first derivative of left ventricular pressure; LV dp/dt min, negative maximal first derivative of left ventricular pressure. *P < 0.05 vs. Swiss mice, †P < 0.05 vs. preischemic value.

Results

Preischemic cardiac function. Hemodynamic function of the normoxically perfused isolated hearts of the Swiss outbred strain and the two C57BL substrains are presented in Table 1. Heart weight-to-body weight ratios and CO, normalized to heart weight, were comparable for each (sub)strain (Table 1). In hearts from C57BL/Ks mice, CF was significantly lower than in hearts from Swiss mice. CF and LVDP tended to be higher in the hearts from C57BL/6 mice compared with C57BL/Ks mice, but the differences were not significant (Table 1). LV dp/dt max and LV dp/dt min, however, were significantly higher in C57BL/6 than in C57BL/Ks hearts.

Functional recovery. To test the ischemic tolerance of the hearts of Swiss mice the duration of the ischemic episode was varied. Following 10 min of global ischemia and 60 min of reperfusion, functional recovery was almost complete (Fig. 1). CO recovered to 83 ± 14% of its preischemic value (P < 0.05 vs. preischemia), and CF returned to preischemic values (Fig. 2). The recovery of LVDP, LV dp/dt max, and LV dp/dt min, ranged from 80 to 90% of their preischemic values (Fig. 2).

Extending the ischemic duration from 10 to 15 min resulted in a markedly depressed recovery of CO (Figs. 1 and 2). Postischemic CO and LVDP amounted to 40 ± 24 and 62 ± 18% of their respective preischemic values (P < 0.05 vs. preischemia). Recovery of LV dp/dt max and LV dp/dt min was also more severely depressed after 15 min of ischemia. Functional recovery was severely impaired after 20 min of ischemia. CO recovered to only 31 ± 24% of its preischemic value (Figs. 1 and 2). Additionally, CF remained seriously depressed during the entire reperfusion phase. Because of a marked rise in end-diastolic pressure and a reduction in systolic function, LVDP recovered to only 28 ± 21% of its preischemic value (Fig. 2).

Enzyme and lactate release. Preischemic LDH release was low in all groups and amounted to 166 ± 9 mU·min⁻¹·g⁻¹. Following 10 min of global ischemia, a small additional release of LDH was observed during

Fig. 1. Recovery of cardiac output (CO) of Swiss murine hearts after 10 (●), 15 (○), and 20 min (△) of normothermic no-flow ischemia. During the first 5 min of reperfusion, the hearts were perfused in Langendorff mode. Thereafter, left atrial inflow was reinstalled. Data are expressed as means ± SD (n = 10–11 per group). *P < 0.05 vs. preischemic value, †P < 0.05 vs. hearts subjected to 10 min of ischemia plus 60 min of reperfusion.
products, i.e., adenosine, inosine, hypoxanthine, and xanthine, was very low before ischemia (Table 2).

During 10 and 15 min of ischemia, tissue ATP content progressively decreased, whereas ADP and AMP levels increased (Fig. 3). Extending the ischemic duration to 20 min was associated with further reductions in ATP, a decrease in ADP content and a marked increase in tissue AMP levels. Adenine nucleotide degradation products, in particular adenosine and inosine, increased as a function of the duration of ischemia (Table 2).

Restoration of flow for 60 min resulted in an almost complete recovery of tissue ATP levels after 10 min of ischemia. In contrast, after an ischemic insult of 15 or 20 min only partial restoration of tissue ATP content was observed (Fig. 3). Tissue ADP and AMP levels returned to preischemic values irrespective of the duration of the preceding ischemic episode (Fig. 3). Restoration of coronary flow after 10 as well as 15 min of ischemia resulted in a removal of adenine degradation products that had accumulated in the flow-deprived tissue. However, in hearts subjected to 20 min of ischemia and 60 min of reperfusion, the tissue content of (oxy)purines remained elevated (Table 2). The tissue content of lactate in reperfused hearts was found to be 15.4 ± 11.3, 21.0 ± 9.6, and 41.1 ± 24.8 μmol/g dry wt after 10, 15, and 20 min of ischemia, respectively. The finding of markedly enhanced tissue levels of lactate and adenine degradation products in hearts subjected to 20 min of ischemia plus reperfusion corroborates the lack of adequate recovery of coronary flow during reperfusion as observed in this experimental group.

Tissue FA, TG, and PL. The preischemic tissue content of FA, PL, and TG in the hearts of Swiss mice and their relative FA composition are shown in Table 3. The total amount of FA esterified to the glycerol backbone via an ether bond (plasmalogens) averaged 3.6% of the total PL pool. Palmitic (16:0), stearic (18:0), linoleic (18:1), and docosahexaenoic acid (22:6) were the most abundant fatty acyl moieties in the FA as well as in the PL pool, each species accounting for 10–20% of total FA present (Table 3). The polyunsaturated FA arachidonic acid (20:4) accounted for 6.7 ± 0.7 and 4.0 ± 0.4% of total fatty acyl moieties in the PL and FA pool, respectively (Table 3). Cardiac TG mainly consisted of 16:0, 18:1, and 18:2, each representing about 30% of all TG fatty acyl moieties. In contrast to the FA and PL pool, 18:0, 20:4, and 22:6 accounted for only a small percentage of FA esterified in the TG pool (Table 3).

Irrespective of the duration of the ischemic insult, the tissue content of TG and PL did not change significantly during ischemia or the subsequent period of reperfusion (data not shown). The total cardiac (unesterified) FA content, however, transiently declined during ischemia. This initial reduction in total FA content was associated with marked changes in the percent mole of individual FA species (Table 4). In general, the percent mole of saturated FA species increased, whereas the percent mole of polyunsaturated FAs declined. In this respect, the shift in the percent mole of

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**Fig. 2.** Percentage recovery of CO, coronary flow (CF), left ventricular developed pressure (LVDP), and first maximal and minimal derivative of left ventricular pressure (LV dP/dt max and LV dP/dt min, respectively) after 60 min of reperfusion after 10 (open bars), 15 (hatched bars), and 20 min (solid bars) of ischemia. Data are expressed as percentage of corresponding values measured before induction of ischemia and means ± SD (n = 10–11 per group). *P < 0.05 vs. preischemic value. †P < 0.05 vs. hearts subjected to 10 min of ischemia plus 60 min of reperfusion.

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the initial 20-min reperfusion. During 60 min of reperfusion, the cumulative release of LDH into the coronary effluent amounted to 13.1 ± 3.4 U/g in this group. The cumulative release of LDH increased as a function of the duration of the preceding ischemic period and amounted to 23.0 ± 7.7 and 27.5 ± 12.1 U/g after 15 and 20 min of ischemia, respectively. To estimate the percentage of irreversibly damaged cells, the cumulative release of LDH was normalized to the total LDH content of the Swiss mouse heart (273 U/g murine ventricular tissue). Accordingly, the percentage of necrotic cells was calculated to be 5, 8, and 10% after 10, 15, and 20 min of ischemia, respectively.

Before the onset of ischemia cardiac lactate release into the coronary effluent was found to be 3.6 ± 0.9 μmol·min⁻¹·g⁻¹. Following 10 and 15 min of ischemia a significantly higher lactate release rate was observed during the first 5 min of reperfusion (7.2 ± 0.5 and 7.2 ± 2.7 μmol·min⁻¹·g⁻¹, respectively; P < 0.05 vs. preischemia) reflecting the washout of lactate accumulated during the preceding ischemic period. Thereafter, lactate release returned to preischemic values. In contrast, after 20 min of ischemia no significant washout of lactate was observed in the initial reperfusion phase. Lactate release in this group amounted to 4.5 ± 1.4 μmol·min⁻¹·g⁻¹ and remained constant during the remainder of the reperfusion phase.

Tissue adenine nucleotides and degradation products. In the Swiss mice, the preischemic ventricular ATP, ADP, and AMP contents were 18.9 ± 2.0, 4.3 ± 0.5, and 0.6 ± 0.1 μmol/g dry wt, respectively (Fig. 3). The tissue content of adenine nucleotide degradation
16:0 and 22:6 was most pronounced. Reperfusion itself was associated with a marked increase in tissue FA content (Table 4). Accumulation of FA was most pronounced in reperfused hearts previously subjected to 20 min of ischemia. Irrespective of the duration of the preceding ischemic episode, reperfusion was accompanied by a substantial rise in the tissue content of 18:2, 20:4, and 22:6, both in absolute amounts and in the percentage of contribution to the total FA pool (Table 4).

Involvement of type IIA sPLA₂. To test whether endogenous cardiac type IIA sPLA₂ might play a role in ischemia-reperfusion-induced cardiac damage, a functional and biochemical analysis was performed on ischemia-reperfused isolated ejecting hearts of sPLA₂(+/+) and sPLA₂(−/−) C57/BL substrains. Following 17.5 min of global, normothermic ischemia and 60 min of reperfusion, CO recovered to 39 ± 25% and 37 ± 23% of the corresponding preischemic values in sPLA₂(+/+) and sPLA₂(−/−) hearts, respectively (Fig. 4). CF recovered to preischemic values in both groups. In contrast, postischemic LVDP, LV dP/dt max, and LV dP/dt min were substantially reduced, but to similar extents in sPLA₂(+/+) and sPLA₂(+/−) hearts (Fig. 4).

No significant differences in postischemic irreversible cell injury were observed in sPLA₂(+/+) and sPLA₂(+/−) hearts. Preischemic LDH release was low and comparable in both sPLA₂(+/+) and sPLA₂(+/−) hearts (0.15 ± 0.05 and 0.16 ± 0.11 U·min⁻¹·g⁻¹, respectively; not significant, NS). Cumulative LDH re-

Table 2. Tissue content of adenine nucleotide degradation products in preischemic, ischemic, and ischemic-reperfused Swiss mouse hearts

<table>
<thead>
<tr>
<th></th>
<th>Pre-I</th>
<th>10 min I</th>
<th>10 min I + 60 min R</th>
<th>15 min I</th>
<th>15 min I + 60 min R</th>
<th>20 min I</th>
<th>20 min I + 60 min R</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(n = 6)</td>
<td>(n = 10)</td>
<td>(n = 6)</td>
<td>(n = 10)</td>
<td>(n = 5)</td>
<td>(n = 11)</td>
</tr>
<tr>
<td>A</td>
<td>0.07 ± 0.02</td>
<td>0.82 ± 0.35</td>
<td>0.18 ± 0.22</td>
<td>1.07 ± 0.57</td>
<td>0.06 ± 0.09</td>
<td>1.87 ± 0.45</td>
<td>0.35 ± 0.20</td>
</tr>
<tr>
<td>I</td>
<td>ND</td>
<td>0.71 ± 0.26</td>
<td>0.01 ± 0.03</td>
<td>1.27 ± 0.65</td>
<td>0.29 ± 0.44</td>
<td>1.85 ± 0.38</td>
<td>1.09 ± 1.29</td>
</tr>
<tr>
<td>HX</td>
<td>0.04 ± 0.02</td>
<td>0.21 ± 0.04</td>
<td>0.03 ± 0.05</td>
<td>0.34 ± 0.08</td>
<td>0.11 ± 0.23</td>
<td>0.48 ± 0.14</td>
<td>0.40 ± 0.43</td>
</tr>
<tr>
<td>X</td>
<td>ND</td>
<td>0.02 ± 0.02</td>
<td>0.08 ± 0.16</td>
<td>0.08 ± 0.07</td>
<td>0.04 ± 0.10</td>
<td>0.21 ± 0.12</td>
<td>0.15 ± 0.28</td>
</tr>
<tr>
<td>Total</td>
<td>0.11 ± 0.04</td>
<td>1.76 ± 0.51</td>
<td>0.30 ± 0.41</td>
<td>2.76 ± 1.34</td>
<td>0.50 ± 0.77</td>
<td>4.41 ± 0.72</td>
<td>1.98 ± 1.90</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD. Presented are individual purines in μmol/g dry wt ventricular tissue. n indicates number of hearts analyzed. Pre-I, Preischemia; I, ischemia; R, reperfusion. Total refers to the sum of adenosine (A), inosine (I), hypoxanthine (HX), and xanthine (X) (expressed as μmol/g dry wt). *P < 0.05 vs. preischemic value, †P < 0.05 vs. corresponding end-ischemic value. ND, not detectable.
Table 3. Tissue content and relative fatty acid composition of phospholipid, triacylglycerol, and unesterified fatty acid pool in preischemic Swiss mouse hearts

<table>
<thead>
<tr>
<th></th>
<th>Phospholipids</th>
<th>Triacylglycerols</th>
<th>Fatty Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>198.4 ± 10.3</td>
<td>40.8 ± 5.3</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Mol%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>19.0 ± 0.5</td>
<td>30.9 ± 6.2</td>
<td>18.0 ± 1.6</td>
</tr>
<tr>
<td>16:1 PM</td>
<td>0.2 ± 0.0</td>
<td>4.5 ± 1.2</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>18:0</td>
<td>18.6 ± 0.8</td>
<td>5.4 ± 1.1</td>
<td>17.0 ± 2.5</td>
</tr>
<tr>
<td>18:0 PM</td>
<td>0.5 ± 0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1</td>
<td>11.4 ± 0.6</td>
<td>29.2 ± 4.0</td>
<td>13.2 ± 0.6</td>
</tr>
<tr>
<td>18:1 PM</td>
<td>1.1 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2</td>
<td>15.9 ± 0.5</td>
<td>27.3 ± 5.9</td>
<td>12.5 ± 0.8</td>
</tr>
<tr>
<td>20:0</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>20:4</td>
<td>6.7 ± 0.7</td>
<td>0.9 ± 0.1</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>22:4</td>
<td>0.2 ± 0.0</td>
<td>0.3 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>22:6</td>
<td>22.7 ± 1.4</td>
<td>4.3 ± 1.0</td>
<td>28.7 ± 3.5</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD, n = 4 hearts. Total refers to sum of fatty acyl equivalents in each lipid pool (expressed as μmol/g dry wt). Mol%, percentage contribution of individual fatty acids to total phospholipid, triacylglycerol, and fatty acid pool. PM, plasmalogen form of fatty acyl chain. Fatty acids are denoted by their chemical notation.

lease over 60 min of reperfusion amounted to 11.3 ± 3.5 and 10.5 ± 3.6 U/g wet wt for sPLA2(−/−) and sPLA2(+/-) hearts, respectively (NS).

Tissue adenine nucleotide levels in preischemic hearts did not differ between the two experimental groups. Preischemic ATP content amounted to 22.0 ± 1.9 and 20.0 ± 2.8 μmol/g dry wt tissue in sPLA2(−/−) and sPLA2(+/-) hearts (NS), respectively. Postischemic values of ventricular ATP content in sPLA2(−/−) and sPLA2(+/-) hearts were reduced to 10.3 ± 3.5 and 8.2 ± 2.3 μmol/g dry wt tissue, respectively. Tissue content of ADP, AMP, or (oxy)purines in reperfused hearts did not differ from the corresponding preischemic values in each of the substrains (data not shown).

The preischemic PL content amounted to 180 ± 21 and 171 ± 23 mmol/g dry wt in sPLA2(−/−) and sPLA2(+/-) hearts, respectively. The contribution of plasmalogens was low in both strains and amounted to 4.5 ± 3.6 and 4.0 ± 3.2% of the fatty acyl moieties present in the PL pool of sPLA2(−/−) and sPLA2(+/-) hearts, respectively. The preischemic TG content amounted to 31 ± 7 and 36 ± 10 μmol/g dry wt for sPLA2(−/−) and sPLA2(+/-) hearts, respectively. The tissue content as well as the fatty acyl composition of both esterified lipid pools did not differ between preischemic hearts from both strains, nor did they change during ischemia-reperfusion (data not shown).

Preischemic unesterified FA content in hearts of sPLA2(−/−) and sPLA2(+/-) mice amounted to 334 ± 127 and 198 ± 80 nmol/g dry wt, respectively (NS). In the reperfused hearts of both sPLA2(−/−) and sPLA2(+/-) mice, significant amounts of FA accumulated. However, between the two groups, significant differences in total FA content were not observed (Fig. 5). Significant differences in the tissue content of 20:4, a sensitive marker reflecting membrane PL degradation, were also not found (Fig. 5).

DISCUSSION

In the present study, the ischemia tolerance of the murine heart defined as functional and biochemical changes was first investigated. It is demonstrated that hearts from the Swiss strain tolerate 10 min of ischemia well, as evidenced by a near-complete recovery of

Table 4. Ventricular fatty acid content and relative composition of the unesterified fatty acid pool of preischemic, ischemic, and ischemic-reperfused Swiss mouse hearts

<table>
<thead>
<tr>
<th></th>
<th>Pre-I</th>
<th>10 min I</th>
<th>10 min I + 60 min R</th>
<th>15 min I</th>
<th>15 min I + 60 min R</th>
<th>20 min I</th>
<th>20 min I + 60 min R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>0.9 ± 0.1</td>
<td>0.5 ± 0.2</td>
<td>2.2 ± 1.3†</td>
<td>0.4 ± 0.2</td>
<td>2.5 ± 0.3†</td>
<td>1.0 ± 0.7</td>
<td>3.6 ± 1.3†</td>
</tr>
<tr>
<td>Mol%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>18.0 ± 1.6</td>
<td>32.7 ± 5.7†</td>
<td>21.8 ± 1.6†</td>
<td>31.9 ± 3.5†</td>
<td>22.1 ± 2.7†</td>
<td>31.3 ± 6.1†</td>
<td>22.2 ± 1.9†</td>
</tr>
<tr>
<td>18:0</td>
<td>17.0 ± 2.5</td>
<td>22.3 ± 5.0</td>
<td>27.1 ± 3.9†</td>
<td>27.0 ± 5.4†</td>
<td>27.7 ± 3.5†</td>
<td>21.6 ± 3.4</td>
<td>21.5 ± 2.3†</td>
</tr>
<tr>
<td>18:1</td>
<td>13.2 ± 0.6</td>
<td>14.0 ± 1.9</td>
<td>15.9 ± 1.4</td>
<td>12.5 ± 2.7</td>
<td>16.3 ± 2.3†</td>
<td>13.5 ± 2.0</td>
<td>15.8 ± 1.5†</td>
</tr>
<tr>
<td>18:2</td>
<td>12.5 ± 0.8</td>
<td>11.2 ± 2.1</td>
<td>15.9 ± 1.4†</td>
<td>9.8 ± 3.2</td>
<td>12.1 ± 3.1</td>
<td>14.1 ± 2.3</td>
<td>14.9 ± 2.5</td>
</tr>
<tr>
<td>20:4</td>
<td>4.0 ± 0.4</td>
<td>1.8 ± 1.7</td>
<td>2.9 ± 0.2†</td>
<td>0.8 ± 0.9</td>
<td>3.0 ± 0.8</td>
<td>2.2 ± 1.4</td>
<td>4.4 ± 1.0†</td>
</tr>
<tr>
<td>22:6</td>
<td>28.7 ± 3.5</td>
<td>7.2 ± 2.7†</td>
<td>15.8 ± 2.2†</td>
<td>8.0 ± 3.2†</td>
<td>15.0 ± 3.8†</td>
<td>9.3 ± 5.0</td>
<td>14.7 ± 1.5†</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SD, n = 4–6 heart in each group. Presented are percentages of total tissue (unesterified) fatty acids. I, ischemia; R, reperfusion; Total, sum of individual ventricular fatty acids (expressed as μmol/g dry wt). *P < 0.05 vs. corresponding end-ischemic value; †P < 0.05 vs. preischemic value.
between groups were not observed.

Factors likely to play a role in the spread in the observations are variations in nominally free Ca\(^{2+}\) concentrations in the perfusate, choice of substrates, temperature of the isolated hearts during global ischemia, differences in workload (perfusion pressure, retrograde versus antegrade perfusion), and strain differences. The present findings are in line with the earlier expressed notion of a higher susceptibility of the mouse heart to ischemia-reperfusion-induced damage compared with other species, such as the rat (29).

Energy metabolism and lipid homeostasis. Prolonged duration of ischemia results in a substantial reduction of ATP (<25% of preischemic value) and accumulation of AMP and (oxy)purines, mainly adenosine and inosine in the myocardium. Comparable findings were obtained in the isolated working rat heart subjected to varying periods of ischemia-reperfusion (30). The increase of ATP levels after restoration of flow indicates that mitochondrial ATP production is not seriously compromised in the surviving cells of the postischemic heart. The data suggest that the reduced total adenine nucleotide pool (sum of ATP, ADP, and AMP) at the end of reperfusion results mainly from washout of its degradation products, which prevents fast resynthesis of the parent adenine nucleotides.

The decrease in tissue FA levels during initial ischemia might point to subtle changes in the turnover of PL and TG in the initial ischemic phase. This finding is in accordance with previous observations in the ischemic rat heart, in which the transient decline in tissue FA content was associated with the accumulation of glycerol, which indicates increased TG-FA cycling (29, 32). The observation that the arachidonoyl content of the myocardial (unesterified) FA pool increased when the duration of ischemia is prolonged, is in favor of a switch from increased TG cycling to enhanced deacylation and/or decreased reacylation of the PL pool during more prolonged ischemia (Table 4) (5, 29). The substantial contribution of polyunsaturated FA such as arachidonic acid and docosahexaenoic acid relative to saturated FA is in agreement with an accelerated net degradation of the cardiac PL pool during the reperfusion phase (5, 8, 27, 29) and is indicative for phospholipase A\(_2\)-mediated membrane PL degradation.

**Fatty acid accumulation, sPLA\(_2\), and irreversible cell damage.** The release of the cytosolic enzyme LDH during reperfusion was taken as a measure of the loss of cellular integrity. The strong positive correlation (\(r = 0.78\)) between the amount of LDH released during reperfusion and ventricular arachidonic acid accumulation during reperfusion supports the notion of a causative relation between accelerated PL degradation during ischemia-reperfusion and irreversible cell damage (13, 29, 32, 33).

To test whether type IIA sPLA\(_2\) known to be present in cardiac myocytes (10) plays a prominent role in postischemic cardiac PL degradation, the ischemia-reperfusion tolerance of type IIA sPLA\(_2\)-deficient mice was investigated. This mouse model already enabled...
investigators to explore the role of this sPLA₂ isoform in prostaglandin synthesis (22, 28), in intestinal tumorigenesis (7) and the gastric epithelial apoptotic response to Helicobacter infection (35). We hypothesized that the absence of type IIA sPLA₂ activity would attenuate myocardial ischemia-reperfusion-induced membrane PL degradation and thus irreversible cell damage and improve postischemic functional recovery.

Type IIA sPLA₂ has been implicated as a candidate for cardiac ischemia-reperfusion-induced membrane PL degradation because of its Ca²⁺ dependency and nonspecific hydrolytic action toward the acylglycerol bonds of PL (23, 29, 31). Previous studies (8, 27, 32) have demonstrated that antibodies that recognize type II sPLA₂ and pharmacological inhibition of type IIA sPLA₂ resulted in a reduction of both ischemia-reperfusion-induced membrane degradation and cellular damage of the rat heart.

A potential experimental caveat might be that some differences in preischemic hemodynamic function were apparent between the experimental groups (see Table 1). The two inbred substrains were specifically selected for their high degree of genetic similarity (15, 18, 24). It cannot be fully excluded, however, that the remaining genetic differences might account for undetermined phenotypical differences affecting hemodynamic function and/or cardiac ischemia-reperfusion susceptibility between the two substrains.

The present study clearly shows that postischemic cardiac accumulation of total unesterified FA in general and arachidonic acid in particular did not differ between the two substrains, indicating that a crucial role of type IIA sPLA₂ in ischemia-reperfusion-induced myocardial cell damage is less likely. One implication of the current observation is that PLA₂₅₈ other than type IIA sPLA₂ must be responsible for the enhanced PL degradation in the transiently ischemic heart. Indeed, previous studies employing synthetic PLA₂ inhibitors strongly suggest that multiple PLA₂ types are likely to be involved (13). In this context, the recent identification of another low-molecular-weight, Ca²⁺-dependent sPLA₂ isoform (type V) with limited substrate preference but abundantly present in the heart is of interest (4, 23). It is feasible that type IIA and type V sPLA₂ fulfill redundant functions in the heart as far as ischemia-reperfusion-induced PL degradation is concerned.

In conclusion, the isolated, antegradeperfused, ejecting mouse heart model as described in the present study allows detailed evaluation of the functional and metabolic consequences of transient ischemia. Furthermore, the findings in the present study provide evidence against a decisive role of type IIA sPLA₂ in ischemia-reperfusion-induced PL degradation, cellular injury and cardiac dysfunction.

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REFERENCES


