Activation of proteases and changes in Na\textsuperscript{+}-K\textsuperscript{+}-ATPase subunits in hearts subjected to ischemia-reperfusion

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Müller AL, Freed D, Dhalla NS. Activation of proteases and changes in Na\textsuperscript{+}-K\textsuperscript{+}-ATPase subunits in hearts subjected to ischemia-reperfusion. J Appl Physiol 114: 351–360, 2013. First published December 6, 2012; doi:10.1152/japplphysiol.01239.2012.—Previous studies have shown that ischemia-reperfusion (I/R) injury is associated with cardiac dysfunction and changes in sarcoplasmic Na\textsuperscript{+}-K\textsuperscript{+}-ATPase subunits and activity. This study was undertaken to evaluate the role of proteases in these alterations by subjecting rat hearts to different times of global ischemia, as well as reperfusion after 45 min of ischemia. Decreases in Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity at 30–60 min of global ischemia were accompanied by augmented activities of both calpain and matrix metalloproteinases (MMPs) and depressed protein content of β1- and β2-subunits, without changes in α1- and α2-subunits of the enzyme. Compared with control values, the activities of both calpain and MMP-2 were increased, whereas the activity and protein content for all subunits of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase were decreased upon reperfusion for 5–40 min, except that α1- and α2-subunit content was not depressed in 5 min I/R hearts. MDL28170, a calpain inhibitor, was more effective in attenuating the I/R-induced alterations in cardiomyocyte function. Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity, and α2-subunit than β2-subunit content was not depressed in 5 min I/R hearts. MDL28170, a calpain inhibitor, was more effective in attenuating the I/R-induced alterations in cardiomyocyte function. Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity, and α2-subunit than β2-subunit content was not depressed in 5 min I/R hearts.

Ischemia-reperfusion injury; Na\textsuperscript{+}-K\textsuperscript{+}-ATPase; proteases; calpain; matrix metalloproteinases

ISCHEMIA-REPERFUSION INJURY (I/R) in the heart, as evident by cardiac cell damage, as well as occurrence of myocardial contracture and depression in contractile activity, is predominantly caused by the development of oxidative stress and intracellular Ca\textsuperscript{2+} overload (7–9). These events have been shown to contribute to cardiac dysfunction by increasing the activities of proteases, such as calpain and matrix metalloproteinases (MMPs) (20, 24–26, 35, 36). Previous studies on I/R-induced cardiac dysfunction have reported a depression in the activities of subcellular organelles, which include sarcoplasmic (SL), sarcoplasmic reticulum (SR), myofilaments, and mitochondria (5, 8, 25, 27, 34); however, it is unknown at what stage of ischemia or I/R the proteolysis of various subcellular organelles occurs and at what stage the activation of proteases may start affecting cardiac dysfunction. It is pointed out that ischemia is known to cause cardiac dysfunction, depress SL Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity, and accumulate extracellular K\textsuperscript{+}, resulting in increases in intracellular Na\textsuperscript{+} (44). Furthermore, it has been shown that this disequilibrium of cations, in addition to a reduction of available ATP, causes a reduction in Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity (12, 19, 24). These changes in the intracellular milieu contribute to the initial damage caused by ischemia; however, what is currently unknown is the relative sensitivity of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase subunits to the ischemic insult. Understanding the complexities of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase abnormality in ischemia is crucial to understand how damage to the myocardium may initially occur because reperfusion is a necessary clinical step to restore nutrients to the ischemic heart. It is also known that the obligatory reentry of nutrients, particularly oxygen, induces additional changes to the heart in the form of oxidative stress and intracellular Ca\textsuperscript{2+} overload (8, 9, 48). In fact, a significant amount of damage elicited in the heart by I/R injury is associated with depressed Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity and impaired cardiac function (7, 10, 28); however, the exact nature of defects in SL Na\textsuperscript{+}-K\textsuperscript{+}-ATPase during the development of I/R injury is not fully understood.

Various proteases, including calpain and MMP-2, are responsible for the degradation of proteins in the cell and have been found to show increased activity in ischemia and I/R injury (27, 34). Calpain, a Ca\textsuperscript{2+}-dependent cysteine protease, is involved in the degradation of various structural proteins and the activation of cell death pathway. Calpain activity is elevated in I/R hearts due to the occurrence of intracellular Ca\textsuperscript{2+} overload (5), whereas its inhibition during I/R injury has been shown to reduce infarct size and improve cardiac function (17, 23), possibly by preventing the degradation of troponin I, a component of the actin-tropomyosin complex (25). Calpain has also been reported to cleave to α-fodrin (20, 22, 45, 47) which may alter the properties of cation channels (22). On the other hand, MMPs utilize a highly conserved zinc-binding catalytic center to cleave proteins and were discovered to be instrumental in tadpole morphogenesis (1, 13). MMPs have originally been thought to exclusively reside in the extracellular matrix, where they are responsible for partial degradation of the interstitial matrix (1), as well as cell adhesion proteins (30). More recently, MMP-2 has been localized in various cellular organelles, including the nucleus and mitochondria, in addition to localizing with sarcomeres (18). MMP-2 colocalizes with troponin I in the thick and thin myofilaments and proteolytically cleaves them to diminish their intracellular integrity (33, 46). It has also been shown to cleave α-actinin and myosin light chain-I, impairing sarcomeric function (33, 41). The activation of MMPs has been reported to contribute to I/R injury in the myocardium (4) and is considered to be a consequence of oxidative stress (1).
This study was carried out to evaluate the sensitivity of Na\(^+\)-K\(^+\)-ATPase subunits and how their pattern of changes affects Na\(^+\)-K\(^+\)-ATPase activity in ischemia and I/R injury. It is important to note that the \(\alpha\)-subunit of the Na\(^+\)-K\(^+\)-ATPase is the catalytic portion, whereas the \(\beta\)-subunit plays a regulatory role by facilitating the localization and assembly of the enzyme into the SL membrane (16). The activities of both calpain and MMP-2 during various stages of ischemia and I/R injury were evaluated to study whether or not the anaerobic environment caused by ischemia would have an effect on protease activity and possibly contribute to the observed decrease of Na\(^+\)-K\(^+\)-ATPase activity. Furthermore, the time course of changes in the activities of calpain and MMP-2 was examined to investigate at which point the proteolytic activities may influence degradation of SL proteins. Inhibition of these proteases during I/R injury was also studied to test its influence on cardiac function, Na\(^+\)-K\(^+\)-ATPase activity, and subunit degradation. To study whether calpain and MMP-2 exert direct or indirect effects on Na\(^+\)-K\(^+\)-ATPase, the effects of both proteolytic enzymes were examined on Na\(^+\)-K\(^+\)-ATPase activity and its subunit composition by incubating SL preparations with active forms of both calpain and MMP-2. This investigation thus can be seen to test the hypothesis that an increase in protease activity induced by ischemia and I/R correlates with a decrease in SL Na\(^+\)-K\(^+\)-ATPase activity, degradation of Na\(^+\)-K\(^+\)-ATPase subunits, and impaired cardiac function.

MATERIALS AND METHODS

Perfusion and experimental protocol. All experimental protocols were approved by the animal care committee of the University of Manitoba, following guidelines established by the Canadian Institutes of Health Research. Male Sprague-Dawley rats weighing 225–275 g were anesthetized with a mixture of xylazine (9 mg/kg) and ketamine (90 mg/kg). Hearts were rapidly removed and cannulated to the Langendorff apparatus for retrograde perfusion with Krebs-Henseleit (K-H) buffer gassed with 95% O\(_2\) and 5% CO\(_2\) at a rate of 10 ml/min. K-H buffer contained the following (in mmol/l): 120 NaCl, 25 NaHCO\(_3\), 11 glucose, 4.8 KCl, 1.2 KH\(_2\)PO\(_4\), 1.25 MgSO\(_4\), and 1.25 CaCl\(_2\). The perfusion medium, pH 7.4, was maintained at a temperature of 37°C. A water-filled latex balloon was inserted in the left ventricle, after removal of both left and right atria, and connected to a pressure transducer (model 1050BP; BioPac System, Goleta, CA) to record left ventricular systolic and diastolic pressures. Left ventricular developed pressure (LVDP) and left ventricular end-diastolic pressure (LVEDP) were calculated using the Acknowledge 3.0.3 software for Windows (BioPac System, Goleta, CA). The method for hemodynamic measurement was the same used by Singh et al. (39).

All hearts were stabilized for a period of 20 min before induction of ischemia and maintained at 37°C for all experimental groups. Global ischemia was induced by stopping the flow of K-H buffer for periods of 15, 30, and 60 min. To induce I/R injury, global ischemia for 45 min was followed by reperfusion for periods of 5, 10, 20, and 40 min. Control hearts were perfused with oxygenated medium for comparable time periods. For studying the effects of calpain or MMP-2 inhibition on I/R injury, the hearts were perfused for 10 min before inducing global ischemia and for 20 min upon initiating reperfusion with 10 μmol/l MDL28170 or 30 μmol/l doxycycline, respectively (11, 40). At the end of the experiments, hearts were freeze-clamped in liquid nitrogen and stored at –80°C for biochemical analysis.

Isolation of SL membrane. SL membranes were isolated from perfused hearts by previously described methods (29, 40). Hearts were homogenized in sucrose (600 mmol/l) and imidazole (10 mmol/l) solution and centrifuged at 12,000 g for 30 min, after which the supernatant was collected, diluted with KCl-MOPS buffer, and centrifuged at 100,000 g for 60 min. The cytosolic fraction was collected as supernatant and used for the analysis of protease activity. The pellet was suspended in KCl-MOPS buffer, which was then layered onto a solution made up of Tris-HCl (100 mmol/l), sodium pyrophosphate (50 mmol/l), KCl (300 mmol/l), and 30% sucrose. This was centrifuged for 90 min at 100,000 g using a swing out rotor, which yielded a three-layer sample where the white middle layer containing the SL membrane was carefully suctioned out and then centrifuged for 30 min at 100,000 g. The pellet of purified SL was suspended in a buffer containing 250 mmol/l sucrose and 10 mmol/l histidine and frozen at –80°C.

Incubation of SL with active proteases. To study the effects of calpain or MMP-2 on Na\(^+\)-K\(^+\)-ATPase, SL preparation was isolated from unperfused hearts and incubated for 1 h at 37°C, with or without active proteases. The experimental preparations were incubated with active proteases (alone or in combination) using specific activity of 25 units (1 unit = 100 pmol/min at 37°C), whereas no active proteases were used for control. Calpain was obtained from Biovios (Milpitas, CA), whereas MMP-2 was purchased from Enzo Life Sciences (Farmingdale, NY).

Measurement of SL Na\(^+\)-K\(^+\)-ATPase activity. Na\(^+\)-K\(^+\)-ATPase activity was measured by incubating SL at 37°C in assay tubes containing 10 mmol/l EGTA, 5 mmol/l Na\(_2\)HPO\(_4\), and 6 mmol/l MgCl\(_2\), with or without 100 mmol/l NaCl plus 10 mmol/l KCl in a total volume of 0.5 ml, as described earlier (40). The supernatant was used for a phosphate assay using the spectrometer (Spectramax Plus, Molecular Devices, Sunnyvale, NY).

Western blot analysis. Protein content of Na\(^+\)-K\(^+\)-ATPase isoforms was determined by separating SL membranes on an 8% SDS-PAGE gel and electroblotting to polyvinylidene difluoride membranes (PVDF), according to the method used by Elmoselli et al. (10). The Na\(^+\)-K\(^+\)-ATPase isoforms were detected using the following primary antibodies obtained from Upstate Biotechnologies (Lake Placid, CA): polyclonal anti-α1 rabbit IgG, polyclonal anti-α2 rabbit IgG, polyclonal anti-β1 rabbit IgG, and polyclonal anti-β2 rabbit IgG. The secondary antibody used to detect all Na\(^+\)-K\(^+\)-ATPase subunits was a biotinylated goat anti-rabbit IgG (Bio-Rad, Hercules, CA). Membranes were incubated for 1 h with streptavidin-conjugated horseradish peroxidase (1:5,000) and then processed for chemiluminescence using a Pierce ECL kit (ThermoFisher Scientific, Waltham, MA). An imaging densitometer (GS800 Calibrated Densitometer, Bio-Rad, Hercules, CA) was used to scan the bands, which were quantified using Quantity-One 4.6.9. To determine relative densities of proteins, blot radiograms were scanned, and the scan values are expressed as a percentage of control taken as 100% in each group. For a loading control, PVDF membranes were incubated with ProSiebe Blue Protein Staining Solution (Lonza, Rockland, ME) for 1 h and then destained two times for 1 h each using 30% ethanol. Similar analysis of the Western blots for SL proteins has also been carried out previously (10, 29, 38). As there is currently no standard available for loading control of SL due to its predominantly phospholipid nature and lack of structural proteins (3), a reading of a nondescript band at 120 kDa was taken as a comparative loading control.

Measurement of calpain activity. Calpain activity was measured in the isolated cytosolic fraction obtained from control and experimental hearts using a kit from Biovios (Milpitas, CA), according to the method employed by Singh et al. (40). The reaction was carried out in a 96-well plate, where samples were read on a fluorescence microplate reader at 400-nm excitation and 505-nm emission (Molecular Devices, Sunnyvale, CA). The results are expressed as relative fluorescent units.

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Table 1. Alterations in cardiac function (LVDP and LVEDP), Na\(^+\)-K\(^+\)-ATPase, calpain, and MMP activity in hearts subjected to global ischemia for different times

<table>
<thead>
<tr>
<th>Time of Ischemia, min</th>
<th>Control</th>
<th>15</th>
<th>30</th>
<th>60</th>
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</thead>
<tbody>
<tr>
<td><strong>Cardiac function</strong></td>
<td></td>
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<tr>
<td>LVDP, mmHg</td>
<td>124 ± 3.9</td>
<td>10.3 ± 5.0*</td>
<td>37 ± 5.4*</td>
<td>26 ± 3.2*</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>4.8 ± 1.0</td>
<td>8.7 ± 4.6*</td>
<td>36 ± 5.4*</td>
<td>25 ± 3.3*</td>
</tr>
<tr>
<td><strong>Enzyme activities</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Na(^+)-K(^+)-ATPase, μmol Pi(\cdot)mg(^{-1})h(^{-1})</td>
<td>18.7 ± 1.5</td>
<td>16.0 ± 0.43</td>
<td>13.2 ± 0.6*</td>
<td>8.9 ± 1.5*</td>
</tr>
<tr>
<td>Calpain, RFU</td>
<td>38.3 ± 4.5</td>
<td>118 ± 4.0*</td>
<td>113 ± 1.3*</td>
<td>102 ± 7.4*</td>
</tr>
<tr>
<td>MMP-2, ratio to control</td>
<td>1.0</td>
<td>1.8 ± 0.2*</td>
<td>3.8 ± 1.0*</td>
<td>10.2 ± 2.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE of 6 hearts in each group. Hearts were subjected to global ischemia for 15, 30, or 60 min. LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; MMP-2, matrix metalloproteinase-2; RFU, relative fluorescent units. *Significantly different (P < 0.05) from control.

**Measurement of MMP-2 activity.** Gelatin zymography was used to determine MMP-2 activity present in the cytosolic fraction of isolated hearts, as described elsewhere (15). Samples were prepared in nonreducing loading buffer and applied to an 8% polyacrylamide gel co-polymerized with 1 mg/ml gelatin. Each lane was loaded with 24 μg of cytosolic protein. Gels were then washed with 2.5% Triton X-100 and shaken slowly at 37°C for 48 h and then stained in Coomassie blue for 1 h. After staining, the gels were destained twice.
for 30 min each using a destaining solution made up of 10% isopropanol and 12% acetic acid. Zymograms were scanned using a GS800 Calibrated Densitometer (Bio-Rad, Hercules, CA), the band intensities were measured using Quantity-One 4.6.9, and the MMP activities are expressed as a ratio to control.

Statistical analysis. The values are expressed as means ± SE, and the differences between multiple groups were statistically evaluated by using ANOVA, followed by the Newman-Keuls test using Prism 5 (Graphpad Software, La Jolla, CA). A level of $P < 0.05$ was considered the threshold for statistical significance between the control and experimental groups, as well as within the groups themselves.

RESULTS

Time course of alterations in ischemia. Changes in cardiac function, Na⁺-K⁺-ATPase, and protease activities were determined at different times (15, 30, and 60 min) of inducing global ischemia, and the results are shown in Table 1. The data show that LVDP was decreased at 15 min, and LVEDP was significantly increased at 30 min upon inducing global ischemia. Na⁺-K⁺-ATPase activity was found to gradually decrease over time and become statistically significant at 30 min of ischemia. The activity of cytosolic protease calpain was increased during ischemia, and became statistically significant at 30 min of ischemia. The activity of cytosolic protease calpain was found to be markedly increased as early as 15 min of ischemia and remained elevated throughout the ischemic period. MMP-2 activity was also increased during ischemia over time and became significant after 15 min. During ischemia, no change was found in the protein content of α₁-subunits and α₂-subunits of Na⁺-K⁺-ATPase; however, the content of β₁-subunits and β₂-subunits was decreased as early as 30 min of ischemia (Fig. 1).

Time course alterations in I/R injury. The data in Table 2 show that a marked depression in LVDP and a marked increase in LVEDP (cardiac contracture) were associated with a marked depression in Na⁺-K⁺-ATPase activity and a marked increase in protease activities at 45 min of ischemia (0 min of reperfusion). Reperfusion of heart subjected to 45 min of global ischemia for 5, 10, 20, and 40 min showed a marked recovery in LVDP, but cardiac contracture was further increased (Table 2). The activity of Na⁺-K⁺-ATPase in ischemic hearts was also further depressed upon reperfusion for 5–40 min, whereas calpain activity remained elevated upon reperfusing the ischemic hearts (Table 2). Although MMP-2 activity in I/R hearts was higher than the control value, reperfusion for 5–20 min decreased the enzyme activity and then increased the MMP-2 activity at 40 min of reperfusion compared with the 45-min ischemic value (Table 2). The results in Fig. 2 show Western blot analysis evaluating the degradation of Na⁺-K⁺-ATPase α₁-, α₂-, β₁-, and β₂-subunits during reperfusion. A significant decrease in the protein content for both Na⁺-K⁺-ATPase α₁- and α₂-subunits was seen as early as 10 min of reperfusion, whereas the content of Na⁺-K⁺-ATPase β₁- and β₂-subunits was decreased at 5 min of reperfusion. It may also be noted from Fig. 2 that the depression in protein content of the α₁-subunit was comparable to that in α₂-subunits during 10–40 min of I/R injury, whereas the depression in β₂-subunit was markedly greater than that in β₁-subunits during 5–40 min of reperfusion. Since the magnitude of alterations in Na⁺-K⁺-ATPase subunits in 30-min ischemic hearts was the same as that in 60-min ischemic hearts (Fig. 1), no measurement of the enzyme subunit content was made in 45-min ischemic hearts.

Effect of calpain inhibition or MMP-2 inhibitor on I/R-induced changes. Treatment of hearts with a calpain inhibitor (MDL28170) or an MMP inhibitor (doxycycline) attenuated the I/R-induced increase in LVEDP without affecting the recovery in LVDP (Table 3). I/R injury was found to cause a decrease in Na⁺-K⁺-ATPase activity, which was significantly attenuated by calpain inhibition with MDL28170, but not by MMP inhibition with doxycycline. On the other hand, cytosolic calpain activity was increased due to I/R injury, and this effect was significantly decreased by MDL28170, unlike doxycycline. MMP-2 activity was also found to be increased in I/R injury, but perfusion of hearts with both MDL28170 and doxycycline significantly attenuated the I/R-induced changes in MMP-2 activity (Table 3). Measurement of Na⁺-K⁺-ATPase subunit content revealed that both MDL28170 and doxycycline were effective in attenuating Na⁺-K⁺-ATPase subunit decrease for α₁-, β₁-, and β₂-subunits due to I/R injury; however, only MDL28170 attenuated the depression of Na⁺-K⁺-ATPase α₂ (Fig. 3). It is pointed out that Na⁺-K⁺-ATPase activity in control hearts in this set of experiments was higher than that in Tables 1 and 2. Although the exact reason for this difference is not clear, seasonal variations as well as differences in the group of animals used in these experiments are a likely explanation. Nonetheless, control and I/R preparations with or without drug treatments were obtained from the same group of animals in each set of experiments.

Effects of active calpain and MMP-2 on Na⁺-K⁺-ATPase. To test whether the depression in Na⁺-K⁺-ATPase activity is caused by calpain and/or MMP-2 directly, isolated SL membrane was incubated with these proteases, and the results are shown in Fig. 4. Active calpain was found to significantly depress Na⁺-K⁺-ATPase activity and decrease protein content

<table>
<thead>
<tr>
<th>Cardiac function</th>
<th>Control</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVDP, mmHg</td>
<td>123 ± 7.5</td>
<td>35.2 ± 1.4*</td>
<td>92 ± 12.8*</td>
<td>95 ± 4.7*</td>
<td>95 ± 9.7*</td>
<td>96 ± 5.1*</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>6.1 ± 1.0</td>
<td>34.4 ± 1.4*</td>
<td>60 ± 13.1*</td>
<td>42 ± 7.5*</td>
<td>53 ± 10.4*</td>
<td>60 ± 9.9*</td>
</tr>
</tbody>
</table>

Enzyme activities

<table>
<thead>
<tr>
<th>Na⁺-K⁺-ATPase, μmol Pⁱ⁻⁻¹-mg⁻¹-h⁻¹</th>
<th>Control</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calpain, RUF</td>
<td>16.8 ± 1.7</td>
<td>11.7 ± 0.5*</td>
<td>9.6 ± 1.0*</td>
<td>9.1 ± 0.6*</td>
<td>10.0 ± 1.2*</td>
<td>10.2 ± 0.9*</td>
</tr>
<tr>
<td>MMP-2, ratio to control</td>
<td>36 ± 5.8</td>
<td>108 ± 10.7*</td>
<td>94 ± 9.5*</td>
<td>108 ± 9.4*</td>
<td>96 ± 11.9*</td>
<td>74 ± 2.8*</td>
</tr>
</tbody>
</table>

Values are means ± SE of 6 hearts in each group. Hearts were subjected to ischemia-reperfusion (I/R) injury, which was induced by subjecting hearts to 45-min global ischemia, followed by 5, 10, 20, and 40 min of reperfusion. The values for 45-min global ischemia are shown as 0 min of reperfusion. *Significantly different ($P < 0.05$) from control.
of Na\(^+-\)K\(^+-\)ATPase \(\alpha_1\)-, \(\alpha_2\)-, and \(\beta_2\)-subunits compared with untreated SL membrane. On the other hand, active MMP-2 was found to have no direct effect on the activity of Na\(^+-\)K\(^+-\)ATPase or any of its subunits. Although incubation of SL membrane with a combination of active calpain and MMP-2 decreased Na\(^+-\)K\(^+-\)ATPase activity as well as Na\(^+-\)K\(^+-\)ATPase \(\alpha_1\)-, \(\alpha_2\)-, and \(\beta_2\)-subunits, the effects of calpain were not amplified by MMP-2. It is pointed out that protein content for the Na\(^+-\)K\(^+-\)ATPase \(\alpha_1\)-subunit or Mg\(^{2+}\)-ATPase activity was not decreased by incubation of SL membrane with either active calpain or active MMP-2 (Fig. 4).

**DISCUSSION**

Cardiac dysfunction due to global ischemia and reperfusion. By employing isolated rat hearts perfused at a constant flow without any electrical stimulation to maintain heart rate, we have observed that LVDP was depressed and LVEDP was increased upon inducing global ischemia. Furthermore, reperfusion of 45-min ischemic hearts for 5–40 min decreased the recovery of LVDP and increased the LVEDP. The isolated perfused hearts without electrical stimulation were used in this study to better understand the endogenous pace-maker mechanism of the heart, which relies heavily on ion transport across the membrane without any potential artifacts that may occur with electrical stimulation. Although information concerning infarct size in the ischemic and I/R hearts can be seen to support the observed changes in the functional parameters, we did not measure infarct size in this study because the entire heart was required for SL isolation. Nonetheless, these observations indicating cardiac dysfunction due to global ischemia and I/R injury are in agreement with different reports from other laboratories (10, 29, 31, 32, 39, 40, 43). However, it is pointed out that the recovery of LVDP in ischemic hearts was much higher and the increase in LVEDP was much greater.
upon reperfusion compared with these previous reports, which employed electrically stimulated heart preparations. Differences in the time of development of cardiac dysfunction due to myocardial ischemia and the start of I/R injury in hearts perfused at constant pressure and in hearts perfused at constant flow have also been observed (40). Furthermore, differences in the I/R-induced changes in endothelial function, Na\(^{+}\)-K\(^{+}\)-ATPase activity, and SR Ca\(^{2+}\)-transport activity have also been observed in hearts

<table>
<thead>
<tr>
<th>Cardiac function</th>
<th>Control</th>
<th>MDL28170</th>
<th>Doxycycline</th>
</tr>
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<tbody>
<tr>
<td>LVDP, mmHg</td>
<td>125 ± 5.6</td>
<td>95 ± 1.7*</td>
<td>104 ± 6.1</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>7.2 ± 1.9</td>
<td>78 ± 8.2*</td>
<td>39 ± 5.9*</td>
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<tr>
<td>Enzyme activities</td>
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<td></td>
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<tr>
<td>Na(^{+})-K(^{+})-ATPase, μmol Pi mg(^{-1}) h(^{-1})</td>
<td>25.6 ± 3.3</td>
<td>15.8 ± 1.7*</td>
<td>23.8 ± 2.9*</td>
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<tr>
<td>Calpain, RFU</td>
<td>48 ± 10.8</td>
<td>119 ± 12.1*</td>
<td>70 ± 11.8*</td>
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<td>MMP-2, ratio to control</td>
<td>1.0</td>
<td>5.4 ± 1.2*</td>
<td>3.2 ± 0.6*</td>
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</table>

Values are means ± SE of 4-6 hearts in each group. I/R injury was induced by subjecting hearts to 45-min global ischemia followed by 30-min reperfusion in the absence (I/R) and presence of MDL28170 (10 μmol/l) or doxycycline (30 μmol/l). *Significantly different (P < 0.05) from control. #Significantly different (P < 0.05) from I/R without drug treatment.

Fig. 3. Protein content of Na\(^{+}\)-K\(^{+}\)-ATPase subunits [α1 (A), α1 (B), β1 (C), and β2 (D)] in SL preparations from hearts subjected to ischemia-reperfusion (I/R) injury in the absence or presence of calpain (CA) inhibitor, MDL28170 (MDL), or metalloproteases (MMP-2) inhibitor, doxycycline (Dox). Representative blots for each Na\(^{+}\)-K\(^{+}\)-ATPase subunit are shown in different panels. Each value is a mean ± SE of 6 experiments in each group. *Significantly different (P < 0.05) from control. #Significantly different (P < 0.05) from I/R without any treatment.
perfused at constant pressure and in hearts perfused at constant flow (40). Thus it appears that alterations in both LVDP and LVEDP during the development of cardiac dysfunction in ischemic hearts, as well as during the process of recovery upon reperfusion may depend on the experimental conditions employed for perfusing the isolated heart preparations.

Depression in Na⁺-K⁺-ATPase activity due to global ischemia and reperfusion. We have observed that SL Na⁺-K⁺-ATPase activity was significantly depressed in hearts subjected to prolonged (30–60 min) global ischemia, as well as in 45-min ischemic hearts upon reperfusion (Table 2); these results are consistent with earlier reports showing depression in Na⁺-K⁺-ATPase activity due to I/R injury (10, 29, 38, 40). These alterations in Na⁺-K⁺-ATPase are not due to any nonspecific changes in the SL membrane, because Mg²⁺-ATPase activity was not depressed upon reperfusion of the ischemic hearts. In view of the well-known function of Na⁺-K⁺-ATPase to serve as a Na⁺ pump, a depression in the SL Na⁺-K⁺-ATPase activity due to I/R injury can be seen to increase the intracellular concentration of Na⁺, which, in turn, would result in the development of intracellular Ca²⁺ overload due to stimulation of the Na⁺/Ca²⁺ exchanger operating in the reverse mode (8, 31, 32). Since the occurrence of intracellular Ca²⁺ overload has been demonstrated to produce cardiac dysfunction in ischemic heart disease (7–9), the observed depression in Na⁺-K⁺-ATPase activity due to I/R injury can be seen to produce cardiac dysfunction indirectly through the induction of intracellular Ca²⁺ overload. However, it should be noted that cardiac dysfunction in hearts subjected to 15 min of ischemia was not associated with any significant depression in SL Na⁺-K⁺-ATPase activity. Thus it is likely that mechanisms other than the inhibition of Na⁺-K⁺-ATPase may also be participating in inducing cardiac dysfunction due to ischemic injury. In this regard, defects in both SR function and myofilibrillar activity have been reported to account for I/R-induced abnormalities in cardiac contractile activity (7, 8).

Fig. 4. Na⁺-K⁺-ATPase activity (A), Mg²⁺-ATPase activity (B), and Na⁺-K⁺-ATPase subunit protein content [α₁ (C), α₂ (D), β₁ (E), and β₂ (F)] in isolated SL membrane treated with or without active CA and/or active MMP-2 (MMP). Representative blots for each Na⁺-K⁺-ATPase subunit are shown in different panels. Each value is a mean ± SE of 4 experiments in each group.

*Significantly different (P < 0.05) from control.
Alterations in Na\(^+\)-K\(^+\)-ATPase subunits due to global ischemia and reperfusion. The results in this study have revealed marked changes in protein content for different subunits of Na\(^+\)-K\(^+\)-ATPase in hearts subjected to global ischemia and reperfusion. Alterations in the subunit composition of Na\(^+\)-K\(^+\)-ATPase due to I/R injury in the heart have also been reported earlier (29). It is noteworthy that degradation of \(\alpha_1\), \(\alpha_2\), \(\beta_1\), and \(\beta_2\)-subunits was associated with a depression in Na\(^+\)-K\(^+\)-ATPase activity upon reperfusion of the ischemic hearts for 5–40 min. However, while both \(\beta_1\)- and \(\beta_2\)-subunits and Na\(^+\)-K\(^+\)-ATPase activity were decreased in ischemic hearts reperfused for 5 min, no changes in \(\alpha_1\)- and \(\alpha_2\)-subunits of Na\(^+\)-K\(^+\)-ATPase were evident. Furthermore, global ischemia for 30–60 min showed depression in Na\(^+\)-K\(^+\)-ATPase activity and \(\beta_1\)- and \(\beta_2\)-subunits without affecting \(\alpha_1\)- and \(\alpha_2\)-subunits of Na\(^+\)-K\(^+\)-ATPase. These observations support the view that \(\beta_1\)- and \(\beta_2\)-subunits of Na\(^+\)-K\(^+\)-ATPase are more sensitive to global ischemia and I/R injury compared with \(\alpha_1\)- and \(\alpha_2\)-subunits. In addition, while alterations in the composition of Na\(^+\)-K\(^+\)-ATPase subunits can be seen to explain the observed depression in Na\(^+\)-K\(^+\)-ATPase activity in I/R hearts, other mechanisms, such as oxidative stress, have been shown to decrease Na\(^+\)-K\(^+\)-ATPase activity due to oxidation of its functional groups (9).

Activation of calpain and MMP-2 due to global ischemia and reperfusion. The observed activation of both calpain and MMP-2 in hearts subjected to global ischemia and reperfusion is in agreement with previous reports showing the effect of I/R injury (5, 34, 35, 38, 40). It may be noted that the activation of calpain was seen during 15–60 min of inducing global ischemia, whereas the activation of MMP-2 became apparent at 30–60 min of ischemia. The sustained activation of calpain in ischemia indicates that calpain does not necessarily require intracellular Ca\(^{2+}\) overload due to excessive entry of extracellular Ca\(^{2+}\) into myocardium caused by reperfusion to become active. It has been found that, although a significant increase in Ca\(^{2+}\) content does occur during the reperfusion period of I/R injury, the increased levels of both cytoplasmic and mitochondrial Ca\(^{2+}\) have been observed within the myocardium, particularly in the early phase of ischemia (14, 42). It has also been shown that the concentration of intracellular free Ca\(^{2+}\) corresponds to mechanical changes induced by ischemia (14). In addition, our results are in agreement with a study by Cheung et al. (4), that increases in the activity of MMP-2 are augmented with the increase in the duration of ischemia. On the other hand, the elevated activity of MMP-2 in the ischemic myocardium is decreased gradually during 5–20 min of reperfusion and then is markedly increased at 40 min. It should be noted that MMP-2 activity in the reperfused hearts was higher than the control value; however, the differential pattern of changes in the activation of calpain and MMP-2 in ischemic as well as reperfused hearts may be due to differences in the mechanisms of activation of these proteases. While activation of calpain in the I/R heart has been suggested to be due to the occurrence of Ca\(^{2+}\) overload (38), the activation of MMP-2 has been indicated to be due to the development of oxidative stress (34). The increase in MMP-2 activity in I/R hearts has also been suggested to be a result of the activation of calpain (40). Nonetheless, activation of both calpain and MMP-2 in the ischemic and I/R hearts can be seen to degrade different subunits of Na\(^+\)-K\(^+\)-ATPase in a time-dependent manner. Since the incubation of SL membrane with calpain, unlike MMP-2, was found to alter Na\(^+\)-K\(^+\)-ATPase activity and its subunit composition, it is likely that the effects of calpain activation are of a direct nature, whereas that of MMP-2 activation may be of an indirect nature. Furthermore, changes in Na\(^+\)-K\(^+\)-ATPase subunit composition in I/R hearts may not only be due to the activation of protease, because I/R has been shown to produce dramatic changes in gene expression for these subunits (28).

Effects of protease inhibition in ischemic and reperfused hearts. It is important to note that the inhibitors used in this study to evaluate the effects of calpain and MMP-2 in I/R injury are nonspecific (2, 40). MDL28170 has been reported to inhibit cathepsin L in monkey kidney epithelial cells when infected with the severe acute respiratory syndrome virus (36). Doxycycline is also a nonspecific inhibitor of MMPs, but it was chosen as it is currently the only FDA-approved MMP inhibitor available for clinical use due to its lack of severe side effects. Doxycycline has been shown to act as an antibacterial agent by inhibiting protein synthesis (6) and has been reported to prevent the I/R-induced leakage of proteins from the myoc--

Fig. 5. Time line diagram of left ventricular developed pressure (LVPDP) and Na\(^+\)-K\(^+\)-ATPase activity (A); Na\(^+\)-K\(^+\)-ATPase subunits \(\alpha_1\), \(\alpha_2\), \(\beta_1\), and \(\beta_2\) (B); and left ventricular end-diastolic pressure (LVEDP), CA activity, and MMP-2 activity (C) during both ischemia and reperfusion. Values expressed as % of control are the averages of data shown in Tables 1 and 2, as well as in Figs. 1 and 2. To obtain 45-min values of Na\(^+\)-K\(^+\)-ATPase subunits, the averages of 30- and 60-min values were used. Control points in each panel are shown as X. \(\uparrow\) represents the point at which reperfusion was started. Time points on the X-axis are not to scale.
cardium (11). Although doxycycline is a broad-spectrum MMP inhibitor, only MMP-2 has been shown to be present inside the cell, whereas the other MMPs are extracellular proteases (46). Since the experiments evaluating MMP activity were performed using isolated cytosol, the influence of extracellular MMP inhibition is highly improbable.

Treatments of hearts with MDL28170 and doxycycline were found to attenuate the I/R-induced increase in LVEDP without further improving the recovery of LVDP in I/R hearts (Table 3). The ineffectiveness of these protease inhibitors to further improve the recovery of LVDP is in contrast to the earlier observation in which electrically stimulated heart preparations were employed and I/R produced a marked depression in LVDP, and both of these agents enhanced the recovery of the heart (40). It is also noteworthy that calpain inhibition by MDL28170 attenuated the I/R-induced depressions in Na⁺-K⁺-ATPase activity and all its subunits. On the other hand, doxycycline reduced the I/R-induced alterations in α₁-, β₁-, and β₂-subunits without affecting the I/R-induced changes in α₂-subunit of Na⁺-K⁺-ATPase, as well as its activity. These observations suggest that the inhibition of Na⁺-K⁺-ATPase activity in I/R hearts may not be entirely due to the activation of calpain and MMP-2, although it has been shown that inhibition of calpain impairs the MMP-2 activity (2, 40). It is likely that activation of other proteases may also be involved in changing the composition of Na⁺-K⁺-ATPase and its activity in the I/R heart (27). Varying degrees of inhibitory effects of MDL28170 and doxycycline on I/R-induced activations of calpain and MMP-2 also indicate the nonspecific nature of these agents. Nonetheless, the overall preventative effects of both MDL28170 and doxycycline in the I/R hearts support the role of calpain and MMP activation in the pathogenesis of cardiac dysfunction due to I/R injury. Such beneficial effects of these agents on the I/R-induced injury to the heart may not be limited to the attenuation of SL changes, because alterations in other subcellular organelles, such as the SR, have been shown to occur as a consequence of protease activation due to I/R injury (5, 38). When comparing the gradual activation of MMP-2 with the relatively immediate activation of calpain, it is possible to consider that calpain activation may be mediating MMP-2 activation further downstream. Thus inhibiting calpain could be more effective in preventing intracellular damage caused by the activation of both calpain and MMP-2 in the heart.

Conclusions. The results described in this study show that subjecting the heart to a prolonged period of ischemia induced cardiac dysfunction, activation of both calpain and MMP-2, as well as depressions in Na⁺-K⁺-ATPase activity and protein content of β₁- and β₂-subunits of Na⁺-K⁺-ATPase. However, the increase in calpain activity and decrease in LVDP at early periods of ischemia were not associated with any significant changes in Na⁺-K⁺-ATPase activity or subunit composition. Reperfusion of the 45-min ischemic heart produced a marked recovery of LVDP, increased in cardiac contracture without any further changes in calpain activity, whereas MMP-2 activity was decreased and then increased without further altering the depressed Na⁺-K⁺-ATPase activity. It is noteworthy that reperfusion of the ischemic heart depressed protein content of α₁-, α₂-, β₁-, and β₂-subunits of Na⁺-K⁺-ATPase, except that protein content of α₁- and α₂-subunits were not affected at an early period of I/R. Time line changes in these parameters during ischemia as well as reperfusion are shown in Fig. 5. Treatment of I/R hearts with a calpain inhibitor (MDL28170) or MMP-2 inhibitor (doxycycline) prevented the I/R-induced changes in LVEDP, Na⁺-K⁺-ATPase activity, and subunit composition, except that I/R-induced depressions in Na⁺-K⁺-ATPase activity and α₂-subunit content were not attenuated by doxycycline. Incubation of SL membranes with calpain, unlike MMP-2, decreased Na⁺-K⁺-ATPase activity and protein content for α₁-, α₂-, and β₂-subunits of Na⁺-K⁺-ATPase. Although activation of both calpain and MMP-2 may alter the subunit composition of Na⁺-K⁺-ATPase due to I/R injury, α₁- and α₂-subunits are more resistant compared with β₁- and β₂-subunits. Alterations in Na⁺-K⁺-ATPase activity and subunit composition may be due to a direct action of calpain on the enzyme, whereas these changes due to the activation of MMP-2 appear to be of an indirect nature.

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AUTHOR CONTRIBUTIONS

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Müller AL et al.


