Effects of ischemia and reperfusion on isolated ventricular myocytes from young adult and aged Fischer 344 rat hearts

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O’Brien JD, Ferguson JH, Howlett SE. Effects of ischemia and reperfusion on isolated ventricular myocytes from young adult and aged Fischer 344 rat hearts. Am J Physiol Heart Circ Physiol 294: H2174–H2183, 2008. First published March 7, 2008; doi:10.1152/ajpheart.00058.2008.—This study examined the impact of age on contractile function, Ca2+ homeostasis, and cell viability in isolated myocytes exposed to simulated ischemia and reperfusion. Ventricular myocytes were isolated from anesthetized young adult (3 mo) and aged (24 mo) male Fischer 344 rats. Cells were field-stimulated at 4 Hz (37°C), exposed to simulated ischemia, and reperfused with Tyrode solution. Cell shortening and intracellular Ca2+ were measured simultaneously with an edge detector and fura-2. Cell viability was assessed by Trypan blue exclusion. Ischemia (20–45 min) depressed amplitudes of contraction equally in isolated myocytes from young adult and aged animals. The degree of post-ischemic contractile depression (stunning) was comparable in both groups. Ca2+ transient amplitudes were depressed in early reperfusion in young adult and aged cells and then recovered to preischemic levels in both groups. Cell viability also declined equally in reperfusion in both groups. In short, some cellular responses to simulated ischemia and reperfusion were similar in both groups. Even so, aged myocytes exhibited a much greater and more prolonged accumulation of diastolic Ca2+ in ischemia and in early reperfusion compared with myocytes from younger animals. In addition, the degree of mechanical alternans in ischemia increased significantly with age. The observation that there is an age-related increase in accumulation of diastolic Ca2+ in ischemia and early reperfusion may account for the increased sensitivity to ischemia and reperfusion injury in the aging heart. The occurrence of mechanical alternans in ischemia may contribute to contractile dysfunction in ischemia in the aging heart.

Ca2+ transients; cell shortening; senescence

Advanced age is believed to exacerbate deleterious effects of ischemia and reperfusion in the heart (25). Morbidity and mortality rates following myocardial ischemia are higher in older patients than in younger adults (37, 46, 48). Advanced age also increases the risk of death after clinical procedures that involve reperfusion injury, such as coronary angioplasty and coronary artery bypass graft surgery (5, 47). These data suggest that aging exacerbates harmful effects of ischemia and reperfusion in the human heart.

Studies in hearts from senescent animals also suggest that aging aggravates ischemia and reperfusion injury. Contractile function during ischemia is depressed to a greater extent in Langendorff-perfused hearts from aged animals than in hearts from younger animals (12). Furthermore, sustained contractile depression in reperfusion (known as stunning) is more prominent in perfused hearts from aged animals compared with younger hearts (2, 18, 28, 45, 49), although this has not been observed in all studies (27). Aged hearts have been shown to release greater amounts of creatine kinase and lactate dehydrogenase in response to myocardial ischemia than younger hearts (28). In addition, in vitro and in vivo studies have shown that infarct size after ischemia and reperfusion is greater in hearts from aged animals than in younger hearts (3, 38). Therefore, aging exacerbates ischemia and reperfusion injury in intact hearts from various animal models.

Elevated levels of intracellular free Ca2+ in individual cardiac myocytes are believed to play a central role in many of the harmful effects of myocardial ischemia and reperfusion (1, 13, 26, 39). It is possible that age-related alterations in intracellular Ca2+ homeostasis in individual cardiac myocytes may underlie the increased sensitivity to ischemia and reperfusion injury in the aging heart. However, whether aging alters Ca2+ homeostasis in isolated myocytes exposed to ischemia and reperfusion has not been investigated, and whether aging affects responses of isolated cardiac myocytes to ischemia and reperfusion injury is not known.

We have developed a model of simulated ischemia and reperfusion in isolated ventricular myocytes (6, 32, 35) that utilizes an “ischemic” Tyrode solution developed previously (9, 14). This model mimics many features of ischemia, such as hypoxia, acidosis, lactate accumulation, hyperkalemia, hypercapnia, and substrate deprivation. Individual myocytes exposed to simulated ischemia and reperfusion exhibit many characteristics of true ischemia such as depolarization, action potential abbreviation, abolition of contractions, and elevated intracellular Ca2+ levels (6, 32). Cells also exhibit abnormal electrical activity, stunning, and irreversible cell injury in reperfusion (6, 32, 35). The present study utilized this model to determine whether aging alters intracellular Ca2+ homeostasis in the setting of ischemia and reperfusion and to determine whether aging affects responses of individual cardiac myocytes to ischemia and reperfusion injury. In these experiments, we evaluated and compared differences in contractile function, intracellular Ca2+ levels, and cell viability throughout ischemia and reperfusion in ventricular myocytes isolated from the hearts of young adult (3 mo) and aged (24 mo) male Fisher 344 rats.

MATERIALS AND METHODS

Animals. All procedures that involved the use of animals were approved by the Dalhousie University Committee on Animal Care, and experiments were performed according to the guidelines outlined in the books published by the Canadian Council on Animal Care.

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(CCAC, Ottawa, Ontario; Vol. 1, 1980; Vol. 2, 1984). Aged male Fischer 344 rats (~24 mo) were obtained from the National Institute on Aging (Baltimore, MD). Young adult male Fisher 344 rats (~3 mo) were obtained from Charles River Laboratories (St. Constant, QC, Canada). In each facility, rats were housed behind specific pathogen-free barriers and monitored regularly for genetic purity and health status. A detailed health report accompanied each shipment of animals. Animals were housed in microisolator cages in the Carleton Animal Care Facility at Dalhousie University on a 12:12-h light-dark cycle with free access to food and water and were used within 2 wk of arrival.

Cardiac myocyte isolation. Ventricular myocytes were obtained by enzymatic dissociation as described previously (10). Briefly, rats were weighed and injected with heparin (3,000 U/kg ip; Pharmaceutical Partners of Canada, Richmond, ON, Canada) to inhibit blood coagulation. Thirty min later, animals were anesthetized with pentobarbital sodium (220 mg/kg ip; CDMV, Saint-Hyacinthe, QC, Canada). Hearts were removed and anastomosed with 4–0 silk (V. Haer, Bowdoinham, ME, which controlled the stimulation frequency. Unloaded cell shortening was measured with a video edge detector (model 105, Crescend Electronics, Sandy, UT) at 120 Hz. The edge detector was coupled to a video camera (Philips FTM800NH, Philips Canada, Markham, ON, Canada) mounted on the microscope. Cell length and fluorescence were simultaneously recorded from each cell by splitting the microscope light with a dichroic cube. The video edge detector received red light, and remaining light was sent to the photomultiplier tube for fluorescence measurements (described below). Axoscope 8.2 (Molecular Devices, Sunnyvale, CA) was used to collect contraction data. Ten-second recordings were taken every 5 min (with additional record- ings at 1 and 2 min of reperfusion). Analog signals were converted to digital signals through a Digidata 1322A A/D board (Molecular Devices). Trains of 5–10 contractions were averaged and measured with Clampfit 8.2 (Molecular Devices). Contraction amplitude was defined as the difference between systolic and diastolic cell length.

Abnormal contractile activity, such as alternating large and small beats (mechanical alternans), that occurred during ischemia and reperfusion was also recorded. In some experiments, cell length and width were measured with the video edge detector to calculate cell area. Cell viability was evaluated by exposure of myocytes to Trypan blue dye, which has been used previously to assess cell survival in studies of ischemia and reperfusion injury in myocytes (see, e.g., Ref. 21). Cells that become spherical in shape, formed membrane blebs, and exhibited irreversible contracture and Ca2+ overload in reperfusion were considered not viable. These cells typically did not exclude Trypan blue dye.

Intracellular Ca2+ was measured by whole cell photometry (DeltaRam, Photon Technology International). The emission ratio at 510 nm, during alternate excitation at 340 nm and 380 nm was used to determine intracellular Ca2+ concentrations. Fluorescence emission was measured at 200 points/s for each of the excitation wavelengths. Background fluorescence values were determined at each excitation wavelength. These background values were subtracted from the recordings made at each wavelength during each experiment. Emission ratios were converted to intracellular Ca2+ concentrations with an in vitro calibration curve determined experimentally at pH 7.0 as reported previously (32). The Ca2+ concentration measured with this curve is expected to accurately approximate Ca2+ concentration within the pH range used in this study, since the effect of pH on fura-2 within the physiological range is negligible (17). Furthermore, calibration curves determined at pH 6.8, 7.0, or 7.2 in our laboratory were similar at Ca2+ concentrations between 100 nM and 1 μM. Felix32 software (Photon Technology International) was used to record data. Ca2+ transient amplitudes were the difference between systolic and diastolic Ca2+. Ca2+ transients were recorded for ~5 s, and trains of 5–10 Ca2+ transients were averaged and measured with Clampfit 8.2 (Molecular Devices). The occurrence of abnormal Ca2+ transients (alternating large and small transients called Ca2+ alternans) in ischemia and reperfusion also was recorded.

Data analysis. Statistical analyses were performed with SigmaStat 3.1 (Systat Software). Data other than cell viability are presented as 75–80% decrease reported in other cellular models of ischemia and reperfusion (33, 34, 36). In time control experiments, myocytes isolated from young adult and aged hearts were exposed to Tyrode solution for 80 min in the absence of ischemia. Fresh cells were placed in the chamber after each experiment, so that cells were exposed to simulated ischemia and reperfusion only once.

Contractions and Ca2+ transients. Contractions and Ca2+ transients were measured simultaneously in isolated myocytes incubated with the Ca2+-sensitive dye fura-2 AM (5 μM). Cells were incubated for 20 min in the dark at room temperature. Cells were then superfused with Tyrode solution as described above and field-stimulated at 4 Hz with 3-ms pulses delivered via a pair of platinum electrodes. Voltage and pulse duration were controlled with a Grass SD9 stimulator (Grass Technologies, West Warwick, RI) that was triggered by a Pulsar 6i digital stimulator (Frederick Haer, Bowdoinham, ME), which controlled the stimulation frequency. Unloaded cell shortening was measured with a video edge detector (model 105, Crescend Electronics, Sandy, UT) at 120 Hz. The edge detector was coupled to a video camera (Philips FTM800NH, Philips Canada, Markham, ON, Canada) mounted on the microscope. Cell length and fluorescence were simultaneously recorded from each cell by splitting the microscope light with a dichroic cube. The video edge detector received red light, and remaining light was sent to the photomultiplier tube for fluorescence measurements (described below). Axoscope 8.2 (Molecular Devices, Sunnyvale, CA) was used to collect contraction data. Ten-second recordings were taken every 5 min (with additional record- ings at 1 and 2 min of reperfusion). Analog signals were converted to digital signals through a Digidata 1322A A/D board (Molecular Devices). Trains of 5–10 contractions were averaged and measured with Clampfit 8.2 (Molecular Devices). Contraction amplitude was defined as the difference between systolic and diastolic cell length.

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Data analysis. Statistical analyses were performed with SigmaStat 3.1 (Systat Software). Data other than cell viability are presented as.
means ± SE. Cell viability was plotted as a survival curve, which represents the probability of cell survival over time. Differences in survival curves between groups were assessed with a log rank test. All other differences between groups were assessed for significance with either a t-test or a two-way repeated-measures ANOVA. Differences within treatment groups were assessed with one-way repeated-measures ANOVA. Differences were considered significant if P < 0.05.

Chemicals. Fura-2 AM was obtained from Invitrogen (Burlington, ON, Canada), and all other chemicals were obtained from Sigma Aldrich (Oakville, ON, Canada). Fura-2 was prepared as a stock solution in anhydrous DMSO with a final concentration of 0.2% and stored at −20°C until use.

RESULTS

The aged rats used in this study were significantly older than young adult animals (24.2 ± 0.1 vs. 3.1 ± 0.1 mo, n = 47 animals/group; P < 0.05). Body weights were significantly greater in aged rats compared with younger animals (417.9 ± 4 vs. 300.3 ± 4.1 g, n = 47 animals/group; P < 0.05). Cell size was also compared in ventricular myocytes isolated from young adult and aged rat hearts (Table 1). Mean cell length was significantly greater in myocytes isolated from aged hearts than from younger hearts (Table 1). Cell width was not affected by age, but cell area was significantly greater in myocytes isolated from aged hearts compared with cells from younger hearts (Table 1). This characteristic age-related increase in cell size has been described previously in ventricular myocytes isolated from aged rat, mouse, and sheep hearts (7, 8, 11, 16, 29). To compensate for differences in cell size, all contraction data was normalized either to cell length or to control values before ischemia.

Next, we conducted functional experiments in isolated myocytes superfused with Tyrode buffer at 37°C and field-stimulated at 4 Hz as described in MATERIALS AND METHODS. Effects of 30 min of ischemia followed by reperfusion on contraction amplitudes were compared in cells from young adult and aged rats; effects of other durations of ischemia are described below. Representative examples of contractions in young adult and aged cells at selected times during an experiment are shown in Fig. 1, A and B. Before ischemia, contraction amplitudes were similar in cells from young adult and aged rat hearts (3.0 ± 0.5% and 3.4 ± 0.4% cell length; n = 19 young adult and 18 aged cells), as reported in previous studies in rat and mouse myocytes (see, e.g., Refs. 11, 20). Ischemia caused a marked decrease in contraction amplitude in young adult and aged myocytes (Fig. 1, A and B). In the young adult cell, contractions exhibited a brief overshoot in early reperfusion but were depressed later in reperfusion (Fig. 1A). In the aged myocyte, contractions also were depressed later in reperfusion, but there was no overshoot in early reperfusion (Fig. 1B). Mean data are shown in Fig. 1, C and D. Contraction amplitudes were depressed in ischemia in young adult cells compared with time controls (Fig. 1C). Contractions showed a brief overshoot in early reperfusion but then were depressed compared with time controls for the remainder of reperfusion (Fig. 1C). This sustained depression of contraction in reperfusion is known as stunning. Contractions also were reduced in ischemia in aged cells compared with time controls (Fig. 1D). After a brief recovery to preischemic levels in early reperfusion, aged myocytes exhibited stunning throughout the remainder of reperfusion (Fig. 1D). The degree of contractile depression observed in ischemia and reperfusion was similar in young adult and aged cells (Fig. 1E).

We also evaluated effects of ischemia and reperfusion on Ca2+ concentrations in young adult and aged myocytes. Figure 2A shows recordings of Ca2+ transients in a young adult myocyte at selected times during an experiment. Ischemia caused an increase in diastolic Ca2+ (Fig. 2A). Diastolic Ca2+ levels decreased below preischemic values in early reperfusion but recovered later in reperfusion (Fig. 2A). In the aged myocyte, diastolic Ca2+ was markedly elevated throughout ischemia and recovered in early reperfusion (Fig. 2B). Ca2+ transient amplitudes appeared similar during ischemia and reperfusion in young adult and aged myocytes (Fig. 2, A and B). Mean measurements of diastolic and systolic Ca2+ plotted as a function of time are shown in Fig. 2, C and D. In young adult cells, diastolic Ca2+ was elevated early in ischemia compared with preischemic values (at t = 20 min) and then declined as ischemia progressed (Fig. 2C). Systolic and diastolic Ca2+ levels decreased significantly in early reperfusion and then recovered to preischemic values later in reperfusion (Fig. 2C). In aged myocytes, diastolic and systolic Ca2+ increased significantly in ischemia compared with preischemic values (Fig. 2D). In contrast to younger cells, diastolic Ca2+ remained elevated in early reperfusion and then declined to preischemic values as reperfusion progressed (Fig. 2D). Diastolic Ca2+ concentrations appeared lower in aged cells than in younger cells before ischemia, but this difference was not statistically significant. Systolic and diastolic Ca2+ concentrations increased slightly during the first 20 min of the experiment in aged cells, although Ca2+ concentrations remained stable after 20 min in young adult and aged cells used as time controls (data not shown).

Figure 3 shows mean Ca2+ transient amplitudes recorded from young adult and aged myocytes plotted as a function of time. In young adult cells, Ca2+ transient amplitudes were similar to those in time controls before ischemia and declined slightly during ischemia. Ca2+ transients were depressed in early reperfusion and recovered later in reperfusion (Fig. 3A). In aged cells, Ca2+ transients were similar to time controls before and during ischemia but declined in early reperfusion and then partially recovered (Fig. 3B). Ca2+ transient amplitudes in ischemia and reperfusion were similar in young adult and aged cells (Fig. 3C). We also measured the time to 90% decay of the Ca2+ transient at selected times throughout these experiments. The time to 90% decay of the Ca2+ transient was similar in both young adult and aged myocytes before ischemia (160.8 ± 5.4 and 162.5 ± 4.6 ms at t = 20 min; n = 19 young adult and 16 aged cells). Ischemia caused a temporary prolongation of the Ca2+ transient in young adult and aged cells. After 5 min of ischemia, the Ca2+ transient was prolonged to a similar extent in young adult and aged myocytes (188.9 ± 5.4 and 193.5 ± 4.7 ms; n = 18 young adult and 16 aged cells; P < 0.05 compared with preischemic values at t = 20 min).

Table 1. Physical characteristics

<table>
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<th>Parameter</th>
<th>Young Adult</th>
<th>Male</th>
<th>Aged Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell length, μm</td>
<td>105.1 ± 3.6</td>
<td>(25)</td>
<td>125.2 ± 4.9</td>
</tr>
<tr>
<td>Cell width, μm</td>
<td>20.0 ± 1.3</td>
<td>(25)</td>
<td>23.7 ± 1.8</td>
</tr>
<tr>
<td>Cell area, μm²</td>
<td>2.185 ± 157.2</td>
<td>(25)</td>
<td>3.013 ± 268.4</td>
</tr>
</tbody>
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Values are means ± SE for no. of cells in parentheses. *Significantly different from young animal (P < 0.05).
However, this recovered to preischemic levels in reperfusion in both groups.

Figure 4 shows diastolic Ca\textsuperscript{2+} concentrations plotted as a function of time in young adult and aged myocytes. In young adult cells, diastolic Ca\textsuperscript{2+} was significantly elevated on exposure to ischemia compared with time controls. However, diastolic Ca\textsuperscript{2+} levels declined throughout ischemia in younger cells (Fig. 4A). Diastolic Ca\textsuperscript{2+} was depressed relative to time controls in early reperfusion but recovered later in reperfusion (Fig. 4A). In contrast to young adult cells, diastolic Ca\textsuperscript{2+} concentrations were markedly elevated throughout ischemia in aged myocytes (Fig. 4B). Furthermore, diastolic Ca\textsuperscript{2+} levels were elevated in early reperfusion but returned to time control levels as reperfusion progressed (Fig. 4B). Figure 4C shows that the increase in diastolic Ca\textsuperscript{2+} was significantly greater in aged cells than in younger myocytes during the last 15 min of ischemia and in early reperfusion.

In some experiments, contractions and Ca\textsuperscript{2+} transients exhibited a pattern of alternating small- and large-amplitude responses known as mechanical and Ca\textsuperscript{2+} alternans, respec-
This behavior was observed in ischemia but not in reperfusion. The mean mechanical alternans ratio was significantly elevated in aged myocytes compared with young adult cells (Fig. 5B). However, the Ca^{2+} alternans ratio was not significantly different between the two groups (Fig. 5C).

We also determined whether aging affected cell viability during exposure to ischemia and reperfusion by plotting survival curves, which show the probability of cell survival over time. Figure 6, A and B, show survival curves for young adult and aged myocytes exposed to ischemia and reperfusion compared with age-matched time controls. All young adult and aged cells that served as time controls remained viable for the duration of the experiments (Fig. 6, A and B). In contrast, cell survival was significantly reduced in both young adult and aged myocytes exposed to ischemia and reperfusion (Fig. 6, A and B). There was no significant difference in cell survival between young adult and aged myocytes exposed to ischemia and reperfusion (Fig. 6C).

In some experiments, we investigated whether age-related differences in responses to ischemic stress could be observed when the ischemic period was varied from the 30-min period described above. When cells were exposed to very brief periods of ischemia (e.g., <10 min), there was no evidence of ischemic injury. Cells remained viable throughout reperfusion, and there was no evidence of stunning. When we exposed cells to 20 min of ischemia followed by reperfusion, diastolic Ca^{2+} levels at the end of ischemia were significantly greater in aged cells than in younger cells (117.9 ± 5.3% of preischemic levels in younger cells vs. 134.7 ± 8.2% of preischemic values in aged group; P < 0.05). However, the degree of stunning in reperfusion was similar in young adult and aged myocytes [contractions at 10 min of reperfusion were 59.6 ± 15.5% vs. 39.6 ± 11.8% of preischemic values in young adult and aged cells; not significant (NS)]. Cell survival also was similar in the two groups (45.5% of young adult cells and 33.3% of aged cells remained viable at the end of reperfusion, n = 10 or 11 cells/group). When we increased the ischemic period from 30 to 45 min, diastolic Ca^{2+} levels at the end of ischemia were significantly higher in aged cells than in young adult cells (134.4 ± 6.3% of preischemic levels in younger cells vs. 169.0 ± 6.3% of preischemic values in aged group; P < 0.05). Still, a similar level of stunning was observed in young adult and aged myocytes (contractions at 10 min of reperfusion were 56.3 ± 9.7% vs. 42.0 ± 9.8% of preischemic values in young adult and aged cells; NS). When we increased the ischemic period to 60 min, cells rounded up, exhibited membrane blebs and hypercontraction, and did not exclude Trypan blue dye. These results
demonstrate that exposure of myocytes to intermediate periods of ischemia (e.g., 20, 30, and 45 min) caused an increase in intracellular Ca$^{2+}$ levels that was significantly greater in aged myocytes than in young adult cells. Exposure to these intermediate periods of ischemia also promoted stunning and reduced cell viability in reperfusion equally in both young adult and aged myocytes. Our results also show that prolonged ischemia (>60 min) produced irreversible cell injury, while very brief periods of ischemia (<10 min) produced little evidence of ischemic injury.

Fig. 3. Changes in Ca$^{2+}$ transient amplitudes throughout ischemia and reperfusion in myocytes from young adult and aged animals. A and B: Ca$^{2+}$ transient amplitudes declined slightly in ischemia in young adult cells but not in aged cells. Ca$^{2+}$ transient amplitudes were depressed in early reperfusion in both young adult and aged myocytes compared with time controls. C: amplitudes of Ca$^{2+}$ transients were similar in young adult and aged cells. Data were normalized to Ca$^{2+}$ transient amplitudes before ischemia (t = 20 min, dashed lines). n = 19 young adult and 17 aged cells (ischemia and reperfusion) and 11 young adult and 13 aged cells (time controls). *P < 0.05 vs. time controls.

Fig. 4. Ischemia caused a more marked and prolonged increase in diastolic Ca$^{2+}$ in aged cells than in younger cells. A: diastolic Ca$^{2+}$ increased in early ischemia in young adult cells and recovered to time control levels in late ischemia. Diastolic Ca$^{2+}$ was decreased in early reperfusion in young adult cells compared with time controls. B: diastolic Ca$^{2+}$ was elevated for the duration of ischemia in aged myocytes exposed to ischemia and reperfusion compared with time controls. Diastolic Ca$^{2+}$ remained elevated in early reperfusion but returned to levels similar to time controls later in reperfusion. C: the elevation in diastolic Ca$^{2+}$ in ischemia and early reperfusion was more pronounced in aged myocytes than in young adult myocytes. Diastolic Ca$^{2+}$ was normalized to preischemic values (t = 20 min, dashed lines). n = 19 young adult and 17 aged cells (ischemia and reperfusion) and 11 young adult and 13 aged cells (time controls). *P < 0.05 vs. time control (A and B) or young vs. aged cells (C).
DISCUSSION

The objectives of this study were to determine whether aging alters intracellular Ca\(^{2+}\) homeostasis and affects responses of individual cardiac myocytes to ischemia and reperfusion injury. Our results showed that amplitudes of contractions were reduced equally by simulated ischemia in isolated myocytes from young adult and aged hearts. Furthermore, the degree of contractile dysfunction (stunning) in reperfusion was similar in young adult and aged myocytes. However, there was a marked overshoot in contraction amplitude in reperfusion in younger cells that was not observed in cells from aged hearts. Amplitudes of Ca\(^{2+}\) transients measured simultaneously with con-

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Fig. 5. Degree of mechanical alternans in ischemia increased significantly with age. To quantify the magnitude of alternans in young adult and aged myocytes, a ratio was calculated as \(1 - S/L\), where \(S\) is the amplitude of the smaller response and \(L\) is the amplitude of the larger response (51). Ratios near 1 represent a high degree of alternans, while ratios near 0 indicate the absence of this phenomenon. A: contractions and Ca\(^{2+}\) transients recorded from an aged myocyte in ischemia show Ca\(^{2+}\) (top) and mechanical alternans (bottom). B: mean mechanical alternans ratio was higher in aged myocytes than in younger cells. C: average Ca\(^{2+}\) alternans ratio was not significantly difference in young adult and aged myocytes. \(n = 19\) young adult and 17 aged cells. *\(P < 0.05\).

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Fig. 6. Cell survival after ischemia and reperfusion was similar in young adult and aged cells. A and B: cell viability was significantly depressed in young adult (\(n = 34, A\)) and aged (\(n = 29, B\)) myocytes exposed to 30 min of ischemia and reperfusion compared with age-matched time controls (\(n = 13–15\)). C: cell viability was not significantly different between young adult and aged myocytes exposed to ischemia and reperfusion.
contractions decreased in early reperfusion and then recovered later in reperfusion in the two groups. Cell viability declined equally in reperfusion in young adult and aged myocytes. However, aged myocytes exhibited a much greater and more prolonged accumulation of diastolic Ca$^{2+}$ in ischemia and early reperfusion than myocytes from younger animals. In addition, aged myocytes exhibited a higher degree of mechanical alternans during ischemia than cells from younger myocytes. Thus, although some cellular responses to simulated ischemia and reperfusion were similar in both groups, ischemia caused a marked increase in intracellular Ca$^{2+}$ and augmented abnormal contractile activity in aged myocytes compared with younger cells.

To our knowledge, this is the first report of the effects of ischemia and reperfusion injury on intracellular Ca$^{2+}$ homeostasis and functional responses of isolated ventricular myocytes from aged animals. An important finding in the present study was that exposure to simulated ischemia induced a much greater and more sustained increase in diastolic Ca$^{2+}$ levels in aged individual myocytes compared with younger cells. This increase in diastolic Ca$^{2+}$ persisted into early reperfusion in aged myocytes, while diastolic Ca$^{2+}$ levels actually declined in early reperfusion in cells isolated from younger hearts. This observation is consistent with a previous finding that ischemia caused a greater increase in Ca$^{2+}$ levels in intact hearts from aged animals compared with younger hearts (2). Our study extends this observation to demonstrate that intracellular Ca$^{2+}$ levels increase in ischemia and in early reperfusion as a consequence of an increase in intracellular Ca$^{2+}$ at the level of the cardiac myocyte. Elevated levels of free intracellular Ca$^{2+}$ in cardiac myocytes are thought to contribute importantly to many detrimental effects of myocardial ischemia and reperfusion (1, 13, 26, 39). Thus our observation that individual aged myocytes accumulate more diastolic Ca$^{2+}$ in ischemia and early reperfusion than cells from younger hearts may explain, at least in part, the increased sensitivity to ischemia and reperfusion injury in the aging heart (25).

It is possible that age-related changes in proteins involved in sequestration and removal of Ca$^{2+}$ from the myocyte contribute to the elevation in diastolic Ca$^{2+}$ in ischemia and early reperfusion. Indeed, a decrease in the activity and expression of sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase (SERCA) has been reported in the aging heart (4, 41, 42, 44, 52). This would impair the sequestration of Ca$^{2+}$ in the sarcoplasmic reticulum in aging myocytes, in particular under conditions such as ischemia when intracellular Ca$^{2+}$ levels are high. Furthermore, the activity and expression of the Na$^{+}$/Ca$^{2+}$ exchanger also declines with age (19, 30), which would impair Ca$^{2+}$ removal from aged myocytes. However, we found that the time to 90% decay of the Ca$^{2+}$ transient was similar in young adult and aged cells during ischemia, which suggests that differences in SERCA activity in ischemia may not account for our observations. It is possible that the duration of the action potential may shorten more in young adult myocytes during ischemia than in aged myocytes. Abbreviation of action potential duration in ischemia could limit Ca$^{2+}$ influx in ischemia and thereby minimize the rise in intracellular Ca$^{2+}$ in young adult cells.

Our study also demonstrated that aged myocytes exhibited a greater degree of mechanical alternans in ischemia than cells isolated from younger animals. This increased abnormal activity in aged individual myocytes might be linked to the age-related decrease in activity and expression of SERCA (4, 41, 42, 44, 52). In support of this idea, previous studies have shown that inhibition of SERCA in young adult heart can give rise to various types of alternans (50). For example, reduced expression and regulation of SERCA in the failing heart (43) is thought to account for this phenomenon in heart failure (50). Alternatively, age-related differences in the incidence of mechanical alternans may reflect differences in action potential duration in ischemia between young adult and aged cells. If action potential duration shortens more in ischemia in young adult myocytes than in aged cells, this may contribute to the lower incidence of mechanical alternans in young adult cells compared with aged cells. To our knowledge, this is the first report of an increase in the occurrence of mechanical alternans during myocardial ischemia in aging. If this also occurs in vivo, it would be expected to contribute to contractile dysfunction in ischemia in the aging heart.

Results of this study also showed that some cellular responses to simulated ischemia and reperfusion were similar in young adult and aged isolated myocytes. Cell viability declined equally in reperfusion in young adult and aged myocytes. In addition, contractile responses to simulated ischemia and reperfusion were not dramatically different in myocytes isolated from young adult and aged rat hearts. Exposure to ischemia reduced the amplitudes of contraction equally in cells from young adult and aged rats, and the groups showed a similar degree of stunning in reperfusion. However, reperfusion initially induced an overshoot in contraction in young adult cells but not in aged cells. Previous studies in Langendorff-perfused hearts have shown that contractile dysfunction in ischemia and reperfusion is augmented in aging hearts. For example, ischemia depresses contractile function in aged hearts more than in younger hearts (12). In addition, aging promotes stunning in reperfusion in intact hearts (2, 18, 28, 45, 49). It is possible that these differences in results obtained in intact hearts and isolated myocytes arise because of the different experimental models used. Age-related changes in the vasculature may increase susceptibility to ischemia and reperfusion injury in buffer-perfused hearts (24) compared with individual myocytes. In addition, the severity of the ischemic insult in intact heart models may be greater than the 90% decrease in Po$_2$ achieved during ischemia in the present study.

We measured contractions and Ca$^{2+}$ transients simultaneously in ischemia and reperfusion to determine whether contractile changes were linked to changes in Ca$^{2+}$ transients. We found that Ca$^{2+}$ transient amplitudes changed very little in ischemia and reperfusion in myocytes isolated from young adult and aged animals. In early reperfusion, Ca$^{2+}$ transients were reduced equally in young adult and aged cells. This reduction in Ca$^{2+}$ transient amplitude might contribute to contractile dysfunction and stunning in early reperfusion. However, stunning persisted in late reperfusion in cells from both groups, despite recovery of Ca$^{2+}$ transient amplitudes. This suggests that reduced myofilament Ca$^{2+}$ sensitivity in reperfusion, which is believed to contribute importantly to the pathogenesis of stunning (15, 40), occurs equally in individual cells from young adult and aged rat hearts.

The aged rat ventricular myocytes utilized in the present study had many characteristics of aged myocytes reported in previous studies. Cells used in this study exhibited the age-related increase in cell size reported previously in ventricular
myocytes from rat, mouse, and sheep hearts (7, 8, 11, 16, 29). We also found that the amplitudes of contractions, expressed as a percentage of diastolic cell length, were similar in young adult and aged cells under control conditions. Similar results have been reported in previous studies of rat and mouse myocytes subjected to field stimulation (see, e.g., Refs. 11, 20). We also found that Ca\(^{2+}\) transient durations were similar in young adult and aged cells, although ischemia caused a temporary prolongation of Ca\(^{2+}\) transients in both groups. In mouse myocytes investigated under conditions similar to ours, Ca\(^{2+}\) transients are prolonged in aging, at least at rapid pacing rates (>6 Hz; Ref. 31). We examined rat cells paced at 4 Hz, which may be too slow to observe prolongation of Ca\(^{2+}\) transients in aging. It is not clear why Ca\(^{2+}\) transients were prolonged in the initial ischemic period. However, acidosis prolongs action potential duration in rat myocytes (23), which may promote Ca\(^{2+}\) influx and account for the prolongation of Ca\(^{2+}\) transients observed in our study. Activation of ATP-sensitive potassium channels later in ischemia may counteract this effect (22).

There are some limitations to the data presented in this study. Our findings apply to field-stimulated rat ventricular myocytes paced near physiological frequency (e.g., 4 Hz) and investigated at physiological temperature. Whether similar observations would obtain in cells stimulated at lower frequencies and temperatures was not investigated here. In addition, our results apply to cells isolated from the hearts of male rats at 3 and 24 mo of age. However, age-related changes in cardiac function at the level of the individual myocyte have been shown to depend on the sex of the animal (16). Further experiments will be required to determine whether results can be extrapolated to cells isolated from the hearts of female animals at similar ages. An additional limitation of this study, as well as many other studies of isolated myocytes from aged hearts (see, e.g., Refs. 11, 29), is that the yield of viable myocytes is somewhat lower from aged hearts than from younger hearts. However, cells investigated in the present study had morphological and physiological characteristics similar to those previously described in aged ventricular myocytes (7, 8, 11, 16, 20, 29). Thus the cells utilized in this investigation are similar to those investigated in previous studies of the aging cardiac myocyte.

In summary, this study investigated the impact of aging on intracellular Ca\(^{2+}\) homeostasis and responses of individual myocytes to ischemia and reperfusion injury. The effect of ischemia and reperfusion on contractile function and Ca\(^{2+}\) transients was comparable in young adult and aged myocytes. Cell viability also declined equally in reperfusion in both groups. However, myocytes isolated from aged hearts exhibited a large, sustained increase in diastolic Ca\(^{2+}\) in ischemia and early reperfusion compared with cells from younger animals. Furthermore, the degree of mechanical alternans in ischemia increased significantly with age. If this occurs in vivo, it may contribute to contractile dysfunction in ischemia in the aging heart. The observation that aged myocytes accumulate more diastolic Ca\(^{2+}\) in ischemia and early reperfusion than cells from younger hearts may account for the increased sensitivity to ischemia and reperfusion injury in the aging heart.

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REFERENCES

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