Changes in $[\text{Na}^+]_i$, compartmental $[\text{Ca}^{2+}]_i$, and NADH with dysfunction after global ischemia in intact hearts

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Varadarajan, Srinivasan G., Jianzhong An, Enis Novalli, Steven C. Smart, and David F. Stowe. Changes in $[\text{Na}^+]_i$, compartmental $[\text{Ca}^{2+}]_i$, and NADH with dysfunction after global ischemia in intact hearts. Am J Physiol Heart Circ Physiol 280: H280–H293, 2001.—We measured the effects of global ischemia and reperfusion on intracellular $\text{Na}^+$, NADH, cytosolic and mitochondrial (subscript mito) $\text{Ca}^{2+}$, relaxation, metabolism, contractility, and $\text{Ca}^{2+}$ sensitivity in the intact heart. Langendorff-prepared guinea pig hearts were crystalloid perfused, and the left ventricular (LV) pressure (LVP), first derivative of LVP (LV dP/dt), coronary flow, and $O_2$ extraction and consumption were measured before, during, and after 30-min global ischemia and 60-min reperfusion. $\text{Ca}^{2+}$, $\text{Na}^+$, and NADH were measured by luminescence spectrophotometry at the LV free wall using indo 1 and sodium benzofuran isothalate, respectively, after subtracting changes in tissue autofluorescence (NADH). Mitochondrial $\text{Ca}^{2+}$ was assessed by quenching cytosolic indo 1 with MnCl$_2$. Mechanical responses to changes in cytosolic-systolic (subscript sys), diastolic (subscript dia), and mitochondrial $\text{Ca}^{2+}$ were tested over a range of extracellular $[\text{Ca}^{2+}]_o$ before and after ischemia-reperfusion. Both $[\text{Ca}^{2+}]_o$sys and $[\text{Ca}^{2+}]_o$dia doubled at 1-min reperfusion but returned to preischemia values within 10 min, whereas $[\text{Ca}^{2+}]_omito$ was elevated over 60-min reperfusion. Reperfusion dissociated $[\text{Ca}^{2+}]_omito$ and $[\text{Ca}^{2+}]_o$ from contractile function as LVP$_{sys-dia}$, and the rise in LV dP/dt (LV dP/dt$_{max}$) was depressed by one-third and the fall in LV dP/dt (LV dP/dt$_{min}$) was depressed by one-half at 30-min reperfusion, whereas LVP$_{dia}$ remained markedly elevated. $[\text{Ca}^{2+}]_o$sys-dia sensitivity at 100% LV dP/dt$_{max}$ was not altered after reperfusion, but $[\text{Ca}^{2+}]_o$dia at 100% LV dP/dt$_{min}$ and $[\text{Ca}^{2+}]_omito$ at 100% LV dP/dt$_{max}$ were markedly shifted right on reperfusion (ED$_{50}$ +36 and +125 nM [Ca$^{2+}$]), respectively, with no change in slope. NADH doubled during ischemia but returned to normal on initial reperfusion. The intracellular $[\text{Na}^+]_i$ increased minimally during ischemia but doubled on reperfusion and remained elevated at 60-min reperfusion. Thus $\text{Na}^+$ and $\text{Ca}^{2+}$ temporally accumulate during initial reperfusion, and cytosolic $\text{Ca}^{2+}$ returns toward normal, whereas $[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_omito$ remain elevated on later reperfusion. Na$^+$ loading likely contributes to $\text{Ca}^{2+}$ overload and contractile dysfunction during reperfusion.

Cardiac injury; contractility and relaxation; cytosolic $\text{Ca}^{2+}$; mitochondrial $\text{Ca}^{2+}$; myocardium; intracellular sodium concentration

Reperfusion after cardiac ischemia causes mechanical dysfunction because of cell injury. This has been demonstrated in isolated myocyte models with simulated ischemia and in intact isolated hearts and whole animal models. Depending upon the duration and/or magnitude of the ischemic insult, the cellular injury may be reversible (stunned) or irreversible (infarcted) (14). Various interrelated mechanisms have been implicated in reperfusion cellular injury. Among these are 1) cytosolic Na$^+$ (17, 26, 28) and Ca$^{2+}$ (1) overload resulting in myofibrillar hypercontracture, cytoskeletal damage, and cell disruption during reperfusion; 2) mitochondrial Ca$^{2+}$ overload (3, 13, 24) causing inefficient ATP synthesis and utilization as a result of NADH accumulation, 3) reduced maximal Ca$^{2+}$ activated force and/or sensitivity of myofilaments to Ca$^{2+}$; and 4) impaired myocardial vascular perfusion.

Of these several mechanisms, cytosolic and/or mitochondrial Ca$^{2+}$ overload, along with free radical damage, ultimately accounts for much of the cellular damage during reperfusion after ischemia. Free radicals damage sarcolemmal and intracellular membranes and impair ATP-dependent Na$^+$ and Ca$^{2+}$ reuptake mechanisms. Intracellular acidosis promotes Na$^+$/H$^+$ exchange, and this enhances reverse Na$^+$/Ca$^{2+}$ exchange (28, 37) during reperfusion (21), as suggested by Ca$^{2+}$ loading on reperfusion (22). Myofilament Ca$^{2+}$ sensitivity may also be reduced because of free radical damage and Ca$^{2+}$ overload (14).

Time-dependent interrelationships of intracellular free Na$^+$ and compartmental Ca$^{2+}$ concentrations, reduction-oxidation potential, cardiac metabolism, contractility, and relaxation during ischemia and reperfusion have not been examined in the beating, intact heart. It is critically important to know whether Na$^+$

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overloading occurs simultaneously with cytosolic Ca\(^{2+}\) overloading, whether the mitochondria also accumulate Ca\(^{2+}\), and whether NADH/NAD\(^+\); primarily a measure of mitochondrial energy state, is restored on reperfusion. The hypothesis tested was that NADH increases incrementally during ischemia and that Na\(^+\) and compartmental Ca\(^{2+}\) rise together early during reperfusion and remain elevated during later reperfusion with continued myocardial dysfunction. To test this, we measured sequential changes in intracellular Na\(^+\), NADH, and cytosolic and mitochondrial Ca\(^{2+}\) with myocardial contractility, relaxation, metabolism, and coronary perfusion. The aim was to gain a more complete understanding of the dissociation among these variables at distinct time points during global ischemia and reperfusion. From this knowledge, treatments directed toward improving ionic homeostasis and function during reperfusion injury can be rationally designed and applied.

METHODS

Langendorff Isolated Heart Preparation and Measurements

The investigation conformed to the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health (NIH No. 85-23, Revised 1996). Prior approval was obtained from the Medical College of Wisconsin Animal Studies Committee. A portion of our methods have been described in detail previously (27, 34, 35, 36). Ketamine (30 mg) and heparin (1,000 units) were injected intraperitoneally into 85 albino English short-haired guinea pigs (250–300 g) 15 min before the animals were decapitated when unresponsive to noxious stimulation. After thoracotomy, we cut the inferior and superior venae cavae, and the aorta was cannulated distal to the aortic valve. Each heart was immediately perfused retrograde through the aorta with a cold, oxygenated, modified Krebs-Ringer (KR) solution (equilibrated with 97% \(\text{O}_2\) and 3% \(\text{CO}_2\)). The perfusate, a modified KR solution (pH 7.39 ± 0.01, \(\text{PO}_2\), 560 ± 10 mmHg), was filtered (5-μm pore size) in line and had the following calculated composition (in mM; non-ionicized): 137 \(\text{Na}^+\), 5 \(\text{K}^+\), 1.2 \(\text{Mg}^{2+}\), 2.5 \(\text{Ca}^{2+}\), 134 \(\text{Cl}^−\), 15.5 \(\text{HCO}_3^−\), 1.2 \(\text{H}_2\text{PO}_4^-\), 11.5 glucose, 2 pyruvate, 16 mannitol, 0.05 EDTA, and 0.1 probenecid and 5 U/l insulin. Perfusion and bath temperatures were maintained at 37.2 ± 0.1°C with the use of a thermostatically controlled water circulator.

Left ventricular (LV) pressure (LVP) was measured isovolumetrically with a transducer connected to a thin, saline-filled latex balloon inserted into the LV through the mitral valve from a cut in the left atrium. Balloon volume was adjusted to maintain a diastolic LVP (LVP\(_{\text{diast}}\)) of 0 mmHg during the initial control period, so that any increase in LVP\(_{\text{diast}}\) reflected an increase in LV wall stiffness or diastolic contracture. Pairs of bipolar electrodes were placed in the right atrial appendage and right ventricular free wall to monitor spontaneous heart rate and atrial-ventricular conduction time. Coronary flow (aortic inflow) was measured at constant temperature and constant perfusion pressure (55 mmHg) by a self-calibrating, in-line, ultrasonic flowmeter (Transonic T106X, Ithaca, NY) placed directly into the aortic inflow line.

Coronary inflow and coronary effluent Na\(^+\), K\(^+\), Ca\(^{2+}\), \(\text{PO}_4^−\), \(\text{PCO}_2\), and pH were measured off-line with an intermittently self-calibrating analyzer system (Radiometer Copenhagen ABL 505, Copenhagen, Denmark). Coronary sinus effluent was collected by placing a small catheter into the right ventricle through the pulmonary artery after ligating both venae cavae. Coronary sinus venous \(\text{PO}_2\) tension was also measured continuously on-line with an \(\text{O}_2\) Clark-type electrode (model 203B, Instech, Plymouth Meeting, PA). The percent \(\text{O}_2\) extraction was calculated as 100 × arterial \(\text{PO}_2\) – (venous \(\text{PO}_2\)/arterial \(\text{PO}_2\)); myocardial \(\text{O}_2\) consumption (MVO\(_2\)) was calculated as (coronary flow/g) × (arterial \(\text{PO}_2\) – venous \(\text{PO}_2\)) × 24 μL/μg at 760 mmHg; and cardiac work efficiency was calculated as LVP systolic-diastolic (LVP\(_{\text{sys-diast}}\) × heart rate/MVO\(_2\)).

Measurement of Cytosolic and Noncytosolic Free Ca\(^{2+}\) in Intact Hearts

Calculation of dissociation constant. Eight hearts (1.5 ± 0.1 g) were perfused with standard KR perfusate (see Langendorff Isolated Heart Preparation and Measurements) for 20 min to wash out the blood. Hearts were removed from the perfusion apparatus, immersed in 15 ml of a mixture of 5 mM HEPES buffer (pH adjusted to 7.0) with 20 mM NaCl and 115 mM KCl, and homogenized with the use of a polytron blender. The soluble protein fraction was collected after centrifugation at 5,000 g for 20 min and ultracentrifugation at 100,000 g for 1 h at 25°C. With all liquid retained, final soluble heart protein concentration was 0.4 g/ml. Fluorescence (F; subscripted numbers indicate fluorescence wavelength) was measured with a modified luminescence spectrofluorometer (SLM Aminco-Bowman II, Spectronic Instruments, Urbana, IL). Peak fluorescence activity of paired test homogenates, 75 μM free indo 1 and an indo 1 free blank, was measured in a quartz cuvette at 37°C for a ratio (R) of Ca\(^{2+}\) fluorescence (F) at 456 and 385 nm for minimal Ca\(^{2+}\) (R\(_{\text{min}}\), 20 μM EGTA to chelate Ca\(^{2+}\)) and from 0.7 to 160 μM (R\(_{\text{max}}\)).

Emission scans were conducted over a range of excitation wavelengths from 370 to 550 nm in 1-nm increments. The excitation wavelength was changed every 5 s. The monochromator of the emission photomultiplier tube alternated between 385 and 456 nm every 2.5 s. Total emission scan duration was 15 min. Homogenate [Ca\(^{2+}\)] was measured with a Ca\(^{2+}\) selective electrode (Orion Research, Cambridge, MA). The emission scans done at zero, intermediate, and maximal [Ca\(^{2+}\)] revealed that the dissociation constant (K\(_d\)) of indo 1 was 249 ± 8 nM at 37°C. R\(_{\text{max}}\) was calculated as 5,986, and R\(_{\text{min}}\) was calculated as 0.059.

Loading fluorescent probe indo 1 and recording Ca\(^{2+}\) transients. Experiments were carried out in a light-shielded Faraday cage. The heart was partially immobilized by hanging it from the aortic cannula, the pulmonary artery catheter, and the LV balloon catheter. The heart was immersed in a bath. The distal end of a trifurcated fiber silica fiber-optic cable (optical surface area 3.85 mm\(^2\)) was placed against the LV epicardial surface through a hole in the bath, and a rubber O-ring was placed between the ferrule and the heart to reduce cardiac motion at the contact point of the fiber-optic tip. Background autofluorescence was determined for each heart after initial perfusion and equilibration at 37°C. Thereafter, hearts were loaded with indo 1-acetoxymethyl ester (AM) at room temperature (25 ± 0.6°C) for 20–30 min with 165 ml of a recirculated, modified KR solution containing 6 μM indo 1-AM (Sigma Chemical, St. Louis, MO). Indo 1-AM was initially dissolved in 1 ml of dimethyl sulfoxide containing 16% (wt/vol) pluronic I-127 (Sigma Chemical) and diluted to 165 ml with the modified KR solution. Loading was stopped when the F\(_{485}\) intensity was increased 10-fold. Residual indo
1-AM was washed out by perfusing the heart with standard perfusate for at least another 20 min, and each heart was then rewarmed to 37.5 ± 0.2°C before initiating the study. The perfusate contained probenecid (100 μM) to retard leakage of indo 1. Loading and washout of indo 1 in its vehicle reduced LVP from 96 ± 3 mmHg before loading to 72 ± 4 mmHg after washout. In the hearts given the vehicle alone to assess changes in tissue autofluorescence, LVP decreased from 92 ± 2 mmHg prevehicle to 70 ± 2 mmHg postvehicle; this showed that reduced contractility is mostly due to the vehicle rather than to intracellular Ca2+ buffering by indo 1 per se. Emission F385 and F456 declined over time after washout of extracellular indo 1-AM but remained at least fivefold greater than background for at least 3 h at 37°C; however, the F385-to-F456 ratio did not change over time.

Emissions at F385 and F456 were recorded with the use of a modified luminescence spectrophotometer. The LV region of the heart was excited with light arising from a 150-W xenon arc lamp and filtered through a 360-nm monochromator with a bandwidth of 16 nm. The beam was focused onto the ingrowth fibers through the optic bundle. The excitation light penetrated transmurally (5 mm). To avoid blanching of indo 1, the arc lamp shutter was only opened for 2.5-s recording intervals. Emission fluorescence was collected by fibers of the remaining two limbs of the cable and filtered by square interference filters (Corion, Franklin, MA) at 385 nm (390 ± 5 nm) and 456 nm (460 ± 5 nm).

On the basis of calibration studies, photomultiplier tube output settings for F385 and F456 were set at 525 and 385 mV, respectively, to optimize recordings of physiological concentrations of Ca2+. At each sampling interval, F385/F456, F385/F456, and LVP were recorded digitally over eight to nine cardiac cycles every 10 ms for 2.5 s (1,000 data points). Each experiment comprised 40–50 recordings. Data were computer stored (software OS/2, version 4, IBM, Armonk, NY) for background correction and conversion of fluorescence data to [Ca2+] off-line (Excel, Microsoft, Redmond, WA).

Calculation of compartmental Ca2+ concentration from Ca2+ transients. Background fluorescence, but not indo 1 dye fluorescence, is influenced by the tissue oxygenation state at these two isobestic wavelengths (6, 7). Thus it was necessary to measure any change in tissue fluorescence over the course of the study, especially during ischemia and reperfusion, both with and without infusion of MnCl2 to quench indo 1 in the cytosol. In eight hearts, only the indo 1 vehicle was washed in and out, after which autofluorescence was measured during the time course of ischemia and reperfusion; in eight additional hearts, this protocol was repeated in the absence of ischemia and reperfusion (cytosolic Ca2+ time control). In nine hearts, the vehicle was washed out just before continuous infusion of MnCl2 and followed by ischemia and reperfusion; in six hearts, this protocol was repeated in the absence of ischemia and reperfusion (noncytosolic Ca2+ time control). In all experiments, the mean F385 and F456 background values were subtracted from the corresponding indo 1 F385 and F456 values at the same time point and for the same experimental condition.

The Ca2+ transient obtained from the fluorescence ratio of F385 to F456 in nonlinearly proportional to [Ca2+]. Calibration curves were derived according to previously published protocols by Brandes et al. (6, 7) with the use of modifications of a standard equation for fluorescent indicators (15). Total \((\text{Ca}^{2+})_{\text{tot}}\) intracellular \([\text{Ca}^{2+}]\) was calculated from the total F385-to-total F456 ratio \((R_{\text{tot}})\), \(R_{\text{max}}\) [the ratio of light intensities (I) at the same wavelength (S) ratio for minimum and maximum Ca2+ (S_s) and slope (b) of total F385 as a function of total F456 (H) for >100 μM Ca2+)], \(R_{\text{min}}\) \([R_{\text{max}} \times S_{\text{S85}}/S_{\text{S456}}]\) (for 0 Ca2+)], \(S_{\text{S85}}/S_{\text{S456}}\) (at minimum/maximum Ca2+ = 0.05), \(S_{\text{S456}}\) \([I_{\text{S456}}/I_{\text{S456}}]\) (at maximum/minimum Ca2+ = 2.4), and \(K_d\) according to the equation
\[
[\text{Ca}^{2+}]_{\text{tot}} = S_{\text{S85}} \times K_d \left( R_{\text{tot}} - R_{\text{min}} \right) / \left( R_{\text{max}} - R_{\text{min}} \right) \] (1)
where \(S_x = (1 - S_{\text{S456}})/(1 - S_{\text{S85}})\) = -1.48.

The time-related effect of changes in indo 1 fluorescence units and contractility were examined at 2.1 mM extracellular ionized [Ca2+] over 3 h. Systolic and diastolic F385, respectively, decreased from 5.2 ± 0.4 and 4.8 ± 0.4 U initially to 3.2 ± 0.3 and 1.9 ± 0.2 U after 2 h; the units for F356 decreased from 4.8 ± 0.4 and 3.9 ± 0.4 to 1.6 ± 0.2 and 1.4 ± 0.2 U after 2 h. However, the F385-to-F456 ratio, initially 1.6 ± 0.3 and 1.1 ± 0.3, was unchanged at 1.9 ± 0.1 and 1.3 ± 0.2 after 2 h, indicating no change in effective [Ca2+] occurred over that time. Developed LVP after indo 1 loading, initially 68 ± 4 mmHg and then 76 ± 4 mmHg after 2 h, also was not significantly altered over time.

Measurement of Mitochondrial (Nocytosolic) Ca2+ in Intact Hearts

Nocytosolic (primarily mitochondrial) \([\text{Ca}^{2+}]_{\text{mito}}\) was calculated similarly
\[
[\text{Ca}^{2+}]_{\text{mito}} = S_{\text{S456}} \times K_d \left( R_{\text{mito}} - R_{\text{min}} \right) / \left( R_{\text{max}} - R_{\text{mito}} \right) \] (2)
where \(R_{\text{mito}}\) was calculated as the ratio of the noncytosolic fluorescence, mitochondrial F385, and mitochondrial F456, respectively. In one group (n = 12), noncytosolic fluorescence was measured at the end of each experiment by perfusing hearts with 100 μM MnCl2 to quench fluorescence derived from the cytosolic compartment (6, 32). Mitochondrial F385 and mitochondrial F456 were calculated at each time point by multiplying the residual mitochondrial fluorescence fractions (f385 and f456) by total end-diastolic fluorescence so that \(R_{\text{mito}} = (\text{f}_{\text{mito}} \times \text{end-diastolic total F}_{\text{385}}) + (\text{f}_{\text{mito}} \times \text{end-diastolic total F}_{\text{456}})\) (3)

Similar to Eqs. 1 and 2, cytosolic \([\text{Ca}^{2+}]_{\text{cyto}}\) was calculated as
\[
[\text{Ca}^{2+}]_{\text{cyto}} = S_{\text{S456}} \times K_d \left( R_{\text{cyto}} - R_{\text{min}} \right) / \left( R_{\text{max}} - R_{\text{cyto}} \right) \] (4)
where \(R_{\text{cyto}}\) was derived from the ratio of the cytosolic fluorescence, cytosolic F385, and cytosolic F456, respectively, calculated at each time point by effectively subtracting \([\text{Ca}^{2+}]_{\text{mito}}\) from \([\text{Ca}^{2+}]_{\text{tot}}\) and multiplying the remainder by total end-diastolic fluorescence (as in Eq. 3) so that \(R_{\text{cyto}} = \left( \left[ \text{total F}_{\text{385}} - \left( \text{f}_{\text{mito}} \times \text{end-diastolic total F}_{\text{385}} \right) \right] / \left[ \text{total F}_{\text{456}} - \left( \text{f}_{\text{mito}} \times \text{end-diastolic total F}_{\text{456}} \right) \right] \) (5)

In another group (n = 9), MnCl2 was infused immediately after indo 1 loading and washout to measure nocytosolic Ca2+ throughout ischemia and reperfusion. It was important to know if Mn2+ quenches indo 1 over time in the noncytosolic as well as in the cytosolic compartment. In nine anillary isolated heart experiments, Mn2+ quenching reduced average F385 and F455 signals, respectively, to values 3.9 and 10.7 times the indo 1-unloaded baseline (0 h), to 2.8 and 6.6 times baseline after 1 h, and to 2.5 and 5.4 times baseline after 2 h. Accordingly, the F356-to-F456 ratio was 0.36 (0 h), 0.42 (1 h), and 0.46 (2 h) of the baseline autofluorescence value. This indicates the cellular indo 1 fluorescence ratio is not altered appreciably over time so that nocytosolic Ca2+ does not also become quenched. Nonstimulated endothelium does not contribute significantly to \([\text{Ca}^{2+}]_{\text{tot}}\) (6, 24).
Measurement of Intracellular NADH in Intact Hearts

Tissue autofluorescence was measured by the same methods in eight hearts as described in Calculation of compartmental Ca$^{2+}$ concentration from Ca$^{2+}$ transients. These hearts were loaded with only the indo 1 vehicle. This background fluorescence, insensitive to Mg$^{2+}$ and Ca$^{2+}$, could arise from unknown intracellular constituents or instrumental autofluorescence, but the vast majority of the signal arises from NADH (but not NAD$^+$) fluorescence (5, 6, 8). The ratio of $R_{f456} / R_{f380}$ is interpreted as a measure of NADH/NAD$^+$, where NADH redox state is calculated as $R_{(R_{control} - R_{min})/R_{max} - R_{min}}$ (5). NADH was not calibrated in this study, but calibrated NADH differs by only ~20% from the noncalibrated signals. These indo 1 vehicle autofluorescence signals ($F_{380}$ and $F_{456}$) were also subtracted from the signals generated by indo 1 in the Ca$^{2+}$ transient studies as noted above.

Measurement of Intracellular Free Na$^+$ in Intact Hearts

Loading of sodium benzofuran isophthalate fluorescent indicator. The Na$^+$ binding fluorescent dye sodium benzofuran isophthalate (SBFI) was used to follow changes in myocyte Na$^+$ concentration in the isolated heart (28, 38). The ratio-ometric technique for Na$^+$ is similar to that for indo 1 except that the fluorescence ratio is obtained by alternating the excitation wave length from 340 to 380 nm and collecting and dividing the emitted light at 530 nm (10, 18, 20, 28, 38). The same trifurcated fiber-optic system was used as for indo 1, but Na$^+$ and Ca$^{2+}$ measurements were done in separate hearts because a change in filters and software was necessary. Each of the eight hearts were loaded for 30 min with 6 μM SBFI at 25°C until the emitted signal after loading was between 6 to 10 times the baseline signal. SBFI fluorescence gradually declined over time but the $F_{f456} / F_{f380}$ ratio remained relatively stable. To assure that the fluorescence ratio was being calculated only from the component of heart fluorescence produced by SBFI, in 11 additional hearts only the fluorescence produced by SBFI, in 11 additional hearts only the same trifurcated fiber-optic system was used as for indo 1, but Na$^+$ and Ca$^{2+}$ measurements were done in separate hearts because a change in filters and software was necessary. Each of the eight hearts were loaded for ~30 min with 6 μM SBFI at 25°C until the emitted signal after loading was between 6 to 10 times the baseline signal. SBFI fluorescence gradually declined over time but the $F_{f456} / F_{f380}$ ratio remained relatively stable. To assure that the fluorescence ratio was being calculated only from the component of heart fluorescence produced by SBFI, in 11 additional hearts only the SBFI vehicle was loaded and washed out before initiating the same ischaemia-reperfusion protocol. Corrections were made for the average basal autofluorescence (AF), measured before SBFI loading, and for the change in autofluorescence that occurred during ischemia and reperfusion at each measurement point (time $t$). The ratio $R$ was calculated from the formula

$$R = (F_{f456} - AF_{f456})/(F_{f380} - AF_{f380})$$

Calibration of SBFI in isolated hearts. Cardiac cell surface membranes were made permeable to Na$^+$ by adding the Na$^+$ ionophores gramicidin D and monensin to clamp [Na$^+$]pc (and strophantidin to poison Na$^+$-K$^+$-ATPase) and by removing divalent ions (primarily Ca$^{2+}$ and Mg$^{2+}$) from the perfusate solution (38). The pore-forming antibiotics allow extracellular Na$^+$ to pass through the sarcolemmal L-type Ca$^{2+}$ channels to equilibrate with the internal milieu. Under these conditions, intracellular [Na$^+$]i ([Na$^+$]i) approaches extracellular [Na$^+$]e ([Na$^+$]e); however, because SBFI is also sensitive to [K$^+$], it was necessary to calibrate at a constant [Na$^+$]e and [K$^+$]. Hence, calibration solutions were made that contained unequal proportions (0, 10, 70, and 140 mM) of NaCl or 140 mM KCl in a solution containing 10 mM HEPES, 1 mM EGTA 1, 10 mM glucose, 0.2 μM gramicidin D, and 40 μM monensin; pH was then adjusted to 7.4 through either NaOH or KOH. In additional experiments, 5 and 70 mM [Na$^+$]e were also used. From the calibration curves of six hearts, the $K_d$ for SBFI was calculated as 9.1 ± 0.9 mM at 37°C.

$[\text{Na}^+]_e$ was calculated from $R$ (Eq. 6) and the in vivo calibration values of $R_{\text{min}}$, the ratio at 0 mM [Na$^+$], $R_{\text{max}}$ (the maximum ratio observed after fitting the ratio values to Na$^+$), $S_\text{p}$ (the 380 nm signal at 0 mM [Na$^+$]), $S_\text{c}$ (the 380 signal at 140 mM [Na$^+$]), and $K_d$ (the apparent dissociation constant when $[\text{Na}^+]_e + [\text{K}^+] = 140$ mM) with the use of the following relationship

$$[\text{Na}^+]_e = K_d (S_p/S_c) (R_{\text{max}} - R)/(R_{\text{min}} - R)$$

where $R_{\text{min}} = 0.83 ± 0.11$, $R_{\text{max}} = 1.89 ± 0.20$, $S_p/S_c = 1.55 ± 0.17$, and $K_d = 9.1 ± 0.9$ mM (as derived from Eq. 7 with all values known except $K_d$). Because of increased intracellular acidosis during ischemia, our measure of $[\text{Na}^+]_e$ during ischemia may be underestimated by more than 50% (28).

Protocol

There were four primary study groups, each with identical protocols to measure mechanical and metabolic function during changes in extracellular ionized Ca$^{2+}$ ($[\text{Ca}^{2+}]_e$), 30-min no-flow, global ischemia and 60-min reperfusion: $[\text{Ca}^{2+}]_e$ (noncytotoxic) ($[\text{Ca}^{2+}]_\text{letho}$, intracellular NADH, and $[\text{Na}^+]_e$). Each of these groups was backed by a number of calibration studies, time controls, and autofluorescence controls, as detailed above. Initial control measurements were obtained 30 min after fluorescent dye or vehicle washout. Recordings were obtained every 5 min at a nominal 2.1 mM ionized Ca$^{2+}$ group. In the two Ca$^{2+}$ groups, $[\text{Ca}^{2+}]_e$ was then increased incrementally from 0.4 to 4.8 mM over 18 min by infusing a concentrated CaCl$_2$ solution into the perfusate initially lacking CaCl$_2$. During the increase in $[\text{Ca}^{2+}]_e$, metabolic, functional, $F_{f385}$, and $F_{f566}$ measurements were scanned at 1-min intervals until LVP reached a stable maximum. Each heart underwent a change in $[\text{Ca}^{2+}]_e$, twice, once before and then after ischemia (preischemia, reperfusion), so that each heart served as its own control. In the companion, time control nonischemia studies ($n = 25$), $[\text{Ca}^{2+}]_e$ was again changed 1 h after the first change. Unlike reperfusion, increasing $[\text{Ca}^{2+}]_e$ over 20 min does not greatly increase mitochondrial compartment Ca$^{2+}$, as we have preliminarily reported (39). In ischemia and time control groups, 100 μM MnCl$_2$ was infused at the end (cytosolic Ca$^{2+}$ group) or beginning (noncytosolic Ca$^{2+}$ group) to quench cytosolic indo 1 so that cytosolic Ca$^{2+}$ transients became no longer visible.

Data Presentation and Interpretation

Cytosolic-systolic Ca$^{2+}$ and diastolic Ca$^{2+}$. $F_{f385}$, $F_{f566}$, and $F_{f385}/F_{f566}$ Ca$^{2+}$ transient signals, LVP, and the first derivative of LVP (LV dP/dt) were displayed simultaneously on a computer screen and stored digitally using proprietary software on an IBM OS/2 system. After correcting for tissue autofluorescence over time, with or without ischemia and reperfusion, and quenching of the cytosolic Ca$^{2+}$ compartment, we assessed the mitochondrial Ca$^{2+}$ compartment, we calibrated the signals to nanomolar [Ca$^{2+}$] with the use of algorithms developed by our group. LVP and raw metabolic data were recorded (MacLab, AD Instruments, Castle Hills, Australia) and, together with the Ca$^{2+}$ transient data, were later analyzed together (Excel Microsoft). Various characteristics of $[\text{Ca}^{2+}]_e$ were analyzed: peak systolic, peak diastolic, and phasic systolic-diastolic $[\text{Ca}^{2+}]_e$, i.e., released $[\text{Ca}^{2+}]_e$; $[\text{Ca}^{2+}]_\text{mito}$ is phasic. The characteristics of isovolumetric LVP analysis were followed in the following LVP systolic, systolic-dia, the rise in LV dP/dt (LV dP/dt max), and the fall in LV dP/dt (LV dP/dt min).
Statistical Analysis

All data were expressed as means ± SE. Within group data for a given variable were compared with a preischemia control period (at 75 min) by Duncan’s comparison of means tests whenever univariate ANOVA for repeated measures were significant (Super ANOVA 1.11 software for Macintosh, Abacus Concepts, Berkeley, CA). Peak LVP-indo 1 Ca\(^{2+}\) transients relationships over the range of \([\text{Ca}^{2+}]_e\) were determined by nonlinear regression with slope comparisons by parallelism tests with the use of the Boltzmann equation (Prism, version 2.1a, Graph Pad Software; San Diego, CA). This equation (linear × axis) fits intact heart data better than the Hill equation (log × axis) commonly used to assess contractility as a function of \([\text{Ca}^{2+}]_e\) in skinned muscle preparations. Different groups were not compared. Differences among means were considered statistically significant when \(P < 0.05\).

RESULTS

Figures 1A–5A show data obtained from the cytosolic Ca\(^{2+}\) group; Figs. 5B and 6B show data from the mitochondrial Ca\(^{2+}\) group; Fig. 7A shows data from the NADH group; and Fig. 7B shows data from the Na\(^{+}\) group. Mechanical and metabolic data obtained before ischemia and after reperfusion were similar among these groups; these intergroup comparisons were not conducted. Variables unchanged from before ischemia to 60-min reperfusion, respectively, for all groups were heart rate (252 ± 4 and 252 ± 3 beats/min), atrioventricular conduction time (76 ± 3 and 72 ± 2 ms), and percent O\(_2\) extraction (75 ± 2 and 73 ± 3%). Figure 1A displays calibrated Ca\(^{2+}\) transients and LVP from one experiment before ischemia and during

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Fig. 1. A: beat-to-beat changes in cytosolic \([\text{Ca}^{2+}]_c\) and left ventricular (LV) pressure (LVP) before global ischemia and after 2- and 60-min reperfusion in 1 heart. Note the peaked Ca\(^{2+}\) transient before the rise in LVP transient, the elevated systolic and diastolic \([\text{Ca}^{2+}]_c\) during early reperfusion, the depressed systolic LVP, and the elevated diastolic LVP during early reperfusion. B: individual coordinates of LVP and cytosolic \([\text{Ca}^{2+}]_c\) over 7 cardiac cycles before and after ischemia in 1 heart. Again, note that \([\text{Ca}^{2+}]_c\) peaks before LVP rises and that the coordinates are shifted rightward during reperfusion.
reperfusion. During early reperfusion, both $[\text{Ca}^{2+}]_{\text{sys}}$ and $[\text{Ca}^{2+}]_{\text{dia}}$ rose, whereas LVP dia increased and LVP sys decreased; during late reperfusion, $[\text{Ca}^{2+}]_{\text{sys}}$ and $[\text{Ca}^{2+}]_{\text{dia}}$ approached the preischemia controls, whereas LVP dia remained elevated, and LVP sys remained depressed. Figure 1B shows three 2.5-s recordings of 250 LVP/[Ca$^{2+}$] coordinates at the same time periods shown in Fig. 1A. The relationship shifted downward and to the right during early reperfusion and leftward during late reperfusion, whereas (developed) LVP$\text{sys-dia}$ remained depressed.

Figure 2A shows $[\text{Ca}^{2+}]_{\text{sys}}$ plotted against LVP$\text{sys}$ and LV $dP/dt_{\text{max}}$ during 60-min reperfusion after global ischemia. Compared with before ischemia, the relationship was shifted downward and far to the right at 1-min reperfusion and gradually shifted upward and to the left during later reperfusion. At 60-min reperfusion, the relationship remained shifted downward. Figure 2B shows $[\text{Ca}^{2+}]_{\text{dia}}$ plotted against LVP$\text{dia}$ and LV $dP/dt_{\text{min}}$ during 60-min reperfusion after global ischemia. Compared with before ischemia, the relationship was shifted upward and far to the right at 1-min reperfusion and gradually shifted downward and to the left during later reperfusion. At 60-min reperfusion, the relationship remained shifted upward.

Figure 3A shows that $[\text{Ca}^{2+}]_{\text{sys}}$ fell from 564 ± 63 to 365 ± 22 nM at 30-min ischemia and increased twofold to 1,236 ± 114 nM at 1-min reperfusion; $[\text{Ca}^{2+}]_{\text{sys}}$ returned to control levels by 10-min reperfusion. At 60-min reperfusion, $[\text{Ca}^{2+}]_{\text{sys}}$ was 598 ± 75 nM, which was not different from the preischemia value. Both LVP$\text{sys}$ and LV $dP/dt_{\text{max}}$ remained depressed throughout 60-min reperfusion. Figure 3B shows that $[\text{Ca}^{2+}]_{\text{dia}}$ progressively increased during global ischemia from
152 ± 14 nM before ischemia to 290 ± 16 nM at 30-min ischemia; [Ca\textsuperscript{2+}]\textsubscript{dia} peaked at 394 ± 44 nM at 1-min reperfusion and was 175 ± 18 nM at 60-min reperfusion, which was not different from the preischemia value. [Ca\textsuperscript{2+}]\textsubscript{dia} was associated with a marked increase in LVP\textsubscript{dia} and a marked decrease in LV dP/dt\textsubscript{min} throughout 60-min reperfusion.

Figure 4A displays LV dP/dt\textsubscript{max} as a function of [Ca\textsuperscript{2+}]\textsubscript{sys-dia} during incremental increases in [Ca\textsuperscript{2+}]\textsubscript{e} from 0.39 ± 0.03 to 4.81 ± 0.21 mM. These changes were conducted 30 min before and 30 min after ischemia and were repeated in the time control group, which was not exposed to ischemia. The peak value for LV dP/dt\textsubscript{max} was decreased by 56 ± 3% after ischemia; there was no significant change in this relationship in the time control group. Figure 4B displays the same data after all values for LV dP/dt\textsubscript{max} were normalized to 100%. There were no significant differences in the slopes, indicating no apparent change in contractile sensitivity to Ca\textsuperscript{2+}. In data not displayed, peak LV dP/dt\textsubscript{max} and dP/dt\textsubscript{min} as a function of maximal [Ca\textsuperscript{2+}]\textsubscript{dia} during the change in [Ca\textsuperscript{2+}]\textsubscript{e} was markedly reduced after ischemia, and there were significant rightward shifts in the 100% normalized LV dP/dt\textsubscript{max} and dP/dt\textsubscript{min} slopes after ischemia of 30 ± 9 and 36 ± 7 nM [Ca\textsuperscript{2+}], respectively, compared with before ischemia.

Figure 5A shows that phasic [Ca\textsuperscript{2+}] gradually fell from a preischemia value of 412 ± 53 to <25 ± 10 nM at 30-min ischemia. On reperfusion, phasic [Ca\textsuperscript{2+}] increased twofold to 842 ± 110 nM at 1 min and then declined to within the preischemia value, 446 ± 66 nM,
at 10-min reperfusion. As shown, MV\(\dot{O}_2\), coronary flow, and cardiac efficiency remained depressed throughout reperfusion, but cardiac efficiency rose from 5 \(\pm\) 1 to 14 \(\pm\) 1 mmHg \cdot \text{beat}^{-1} \cdot \mu\text{L} \cdot \text{O}_2^{-1} \cdot \text{g}^{-1} \text{ between 1-}
\text{and 60-min reperfusion. For data not displayed, percent O}_2\text{ extraction was 81 \(\pm\) 2, 63 \(\pm\) 3, 61 \(\pm\) 2, 62 \(\pm\) 2, 61 \(\pm\) 2, and 63 \(\pm\) 3% at 10 min before ischemia and at 1-, 5-, 10-, 30-, and 60-min reperfusion, respectively. Figure 5B shows that noncytosolic, primarily [Ca\(^{2+}\)]\(_{\text{mito}}\) increased during ischemia from a preischemia value of 234 \(\pm\) 30 to 427 \(\pm\) 74 nM at 30-min ischemia; on initial reperfusion, [Ca\(^{2+}\)]\(_{\text{mito}}\) increased nearly 2.5-fold and remained significantly elevated at 60-min reperfusion. LVP\(_{\text{sys}}\) remained depressed, and LVP\(_{\text{dia}}\) remained elevated, throughout 60-min reperfusion.

Figure 6A displays LV dP/dt\(_{\text{max}}\) as a function of [Ca\(^{2+}\)]\(_{\text{mito}}\) during the induced 0.3 to 4.8 mM increase in [Ca\(^{2+}\)]\(_e\) conducted 30 min before and 30 min after reperfusion. The peak value for LV dP/dt\(_{\text{max}}\) was decreased by 32 \(\pm\) 4\% after reperfusion. [Ca\(^{2+}\)]\(_{\text{mito}}\) did not rise significantly to an increase in [Ca\(^{2+}\)]\(_e\) alone before or after ischemia and reperfusion. Figure 6B displays the same data after LV dP/dt\(_{\text{max}}\) was normalized to 100\%. There was a marked rightward shift of 125 \(\pm\) 33 nM in the curve but no significant difference between the slopes, indicating similar contractile responses with elevated [Ca\(^{2+}\)]\(_{\text{mito}}\) despite Ca\(^{2+}\) loading after reperfusion. Results were nearly identical for 100\%-normalized LV dP/dt\(_{\text{min}}\) (data not displayed). In the time control group (\(n = 5\)), there was no shift in LV dP/dt\(_{\text{max}}\) and dP/dt\(_{\text{min}}\) as function of [Ca\(^{2+}\)]\(_{\text{mito}}\).
Figure 7A shows that NADH (in arbitrary units) increased up to 2.5-fold during ischemia but there was a tendency for NADH to decrease during the last 10 min of ischemia. On reperfusion, NADH immediately decreased to the preischemia value, indicating rapid removal of the excess protons. Note that mitochondrial free Ca^{2+} more than doubled during early reperfusion and remained elevated at 60-min reperfusion. The decrease in systolic LVP and the increase in diastolic LVP were similar to that of the cytosolic free Ca^{2+} group (Fig. 7B).

DISCUSSION

Cardiac ischemia and reperfusion cause a multitude of derangements in cardiac function and metabolism. This is the first report in which the time course and interrelationship of a number of ionic and metabolic factors that underlie contractile, relaxant, and metabolic dysfunction were measured repetitively during global ischemia and early and late reperfusion in intact hearts. We found that NADH rapidly accumulated on initial ischemia and persisted during ischemia; this was accompanied by slow increases in myoplasmic free Ca^{2+} and especially free Ca^{2+}mito but little change in free Na^{+}. Phasic free Ca^{2+} transients remained, but phasic contractility was absent during initial ischemia. On initial reperfusion, there was a more than doubling of free Ca^{2+}dia, free Ca^{2+}sys, free Ca^{2+}mito and free Na^{+}. Each of these variables peaked at 1- to 2-min initial reperfusion; however, NADH returned toward preischemia values within the first minute of reperfusion. More-
over, by 10-min reperfusion, \([\text{Ca}^{2+}]_{\text{dia}}, [\text{Ca}^{2+}]_{\text{sys}}, \) and phasic \([\text{Ca}^{2+}]_{\text{cyto}}, \) like NADH, had returned to the preischemia levels, but \([\text{Na}^{+}]_{\text{i}}, [\text{Ca}^{2+}]_{\text{mito}}\) remained elevated for up to 60-min reperfusion.

These ionic changes were associated with a depression of developed LVP, LV \(dP/dt_{\text{max}}, dP/dt_{\text{min}}, \) coronary flow, \(MV_{O_2}, \) and cardiac efficiency for up to 60 min; much of this results from a reduction of viable cells (about a 45% LV infarct size with this protocol). The loop of LVP as a function of transient \([\text{Ca}^{2+}]_{\text{cyto}}\) over the cardiac cycle at normal \([\text{Ca}^{2+}]_{\text{cyto}}, \) was shifted downward and right at 2-min reperfusion; by 60-min reperfusion, this loop remained truncated, indicating depressed contractility and relaxation at a given \([\text{Ca}^{2+}]_{\text{cyto}}, \) As a function of external \([\text{Ca}^{2+}]_{\text{cyto}}\)-induced increases in phasic \([\text{Ca}^{2+}]_{\text{cyto}}, \) peak LV \(dP/dt_{\text{max}}\) at 30-min reperfusion was markedly reduced, but when peak LV \(dP/dt_{\text{max}}\) was normalized to 100% of the preischemia value, the curve was neither significantly shifted nor the slope altered. Thus, over the range of external \([\text{Ca}^{2+}]_{\text{cyto}}, \) functional myocardial sensitivity to cytosolic \([\text{Ca}^{2+}]_{\text{cyto}}, \) was not significantly altered after reperfusion. In contrast to \([\text{Ca}^{2+}]_{\text{cyto}}, [\text{Ca}^{2+}]_{\text{mito}}\) remained persistently elevated during reperfusion but was insensitive to external \([\text{Ca}^{2+}]_{\text{cyto}}, [\text{Ca}^{2+}]_{\text{mito}}\) was markedly right shifted after 30-min reperfusion. These experiments demonstrate interesting links between excess intracellular \([\text{Na}^{+}]_{\text{i}}, [\text{Ca}^{2+}]_{\text{cyto}}, [\text{Ca}^{2+}]_{\text{mito}}\), and NADH. The data support the hypothesis that \([\text{Na}^{+}]_{\text{i}}, [\text{Ca}^{2+}]_{\text{cyto}}, [\text{Ca}^{2+}]_{\text{mito}}\) overload is an essential cause of \([\text{Ca}^{2+}]_{\text{cyto}}\) overload and cardiac dysfunction during early reperfusion and suggest that the rapid return of NADH is related to the elevation in \([\text{Ca}^{2+}]_{\text{mito}}, \) A limitation of this study is that

![Figure 6](http://ajpheart.physiology.org/)
the contribution of oxygen-reactive species to reperfusion contractile dysfunction was not assessed. Moreover, the intracellular measurements apply only to cells surviving reperfusion because nonviable cells are permeable to large molecules.

There are many reports in a variety of models showing that Ca$^{2+}$ is elevated on reperfusion, but there are no known studies in which beat-to-beat changes in diastolic, systolic, and mitochondrial Ca$^{2+}$ were measured repetitively in intact hearts during the critical periods of ischemia-reperfusion injury. We found that cytosolic Ca$^{2+}$ transients were not immediately abolished on ischemia so energy consumption during the anaerobic period might contribute to impaired relaxation and contractility on reperfusion. Moreover, cytosolic Ca$^{2+}$ overload was reversed after 10-min reperfusion, whereas mitochondrial Ca$^{2+}$ remained elevated over 60-min reperfusion. Dissociation between mitochondrial Ca$^{2+}$ and contractility and relaxation were also evidenced by decreased maximal contractile effort as a function of maximum [Ca$^{2+}$] and by the rightward shift in mitochondrial Ca$^{2+}$ contractility and relaxation curves after reperfusion. Excess cytosolic Ca$^{2+}$ inhibits breaking of the troponin C-Ca$^{2+}$ complex so that relaxation, and consequently contraction, becomes impaired. Improved cardiac efficiency during reperfusion may reflect cellular respiration increasingly directed toward contractile function.

Activation of contraction by Ca$^{2+}$ ultimately derives from increased availability of myosin-binding sites on actin. The number of sites relates to the experimental determination of maximal Ca$^{2+}$-activated force (11,
23). The equivalent of this force, contractility, for a given [Ca\textsuperscript{2+}] was depressed by more than 30% after ischemia in isolated hearts; a large part of this effect must be due to nonviable tissue no longer contributing to contractile function. Ca\textsuperscript{2+} also regulates actin-myosin interactions via changes in myofilament Ca\textsuperscript{2+} sensitivity, which can be induced theoretically by a change in the Ca\textsuperscript{2+} affinity of troponin C or by a change in cross-bridge kinetics. Inotropic mechanisms can be defined as “upstream” if they primarily affect the amplitude or time course of the Ca\textsuperscript{2+} transient, “central” if they change the Ca\textsuperscript{2+} binding to regulatory proteins such as troponin C, and “downstream” if they change the response of myofilaments, per se, to a given level of occupancy of troponin C by Ca\textsuperscript{2+} (4, 23, 41). Although central and downstream Ca\textsuperscript{2+} sensitivity in terms of phasic cytosolic Ca\textsuperscript{2+} was not affected after reperfusion, central Ca\textsuperscript{2+} sensitivity was affected in terms of diastolic or mitochondrial Ca\textsuperscript{2+}, so this suggests diastolic and or mitochondrial Ca\textsuperscript{2+} loading does contribute in some way to the Ca\textsuperscript{2+}-myofilament interaction.

Normal functions of myocyte mitochondria are the generation of ATP via the proton pump and Ca\textsuperscript{2+} homeostasis. Both processes are driven by the proton gradient (ΔpH) generated by electron transport in the inner mitochondrial membrane. A small increase in [Ca\textsuperscript{2+}]\textsubscript{mito} during increased work demand activates pyruvate and other dehydrogenases to produce NADH (5, 8, 16, 40). Excess NADH inhibits these dehydrogenases, so entry of pyruvate into the tricarboxylic acid cycle is blocked and oxidative phosphorylation ceases (40). As proton pumps fail, matrix pH decreases as H\textsuperscript{+} is increased. During ischemia, cytosolic ATP is generated by glycolysis and may be used in reverse fashion by F\textsubscript{0}F\textsubscript{1}-ATPase to attempt to maintain ΔpH (11). A very large acute increase in [Ca\textsuperscript{2+}]\textsubscript{mito} is thought to be a marker for irreversible cellular injury (33). Indeed, persistently elevated [Ca\textsuperscript{2+}]\textsubscript{cyto} may be associated with nonreversible myocyte injury (24). Reperfusion after prolonged ischemia may result in mitochondrial Ca\textsuperscript{2+} overload severe enough to decrease ΔpH and the proton force for ATP synthesis (11) so that damage to mitochondrial membranes leads to cell death (1, 3).

ATP synthesis is dependent on the proton motive force generated by NADH, so mitochondrial NADH/ NAD\textsuperscript{+} is a measure of mitochondrial energy state. Reduced NADH is generated from NAD\textsuperscript{+} in the cytosol and matrix from substrates via pyruvate and fatty acids (31). Energy stored by the electrochemical ΔpH in the inner membrane drives ATP synthesis by the F\textsubscript{0}F\textsubscript{1}-ATP synthase complex. The electron transfer from NADH (and FADH\textsubscript{2}) to O\textsubscript{2} via cytochrome oxidase generates the motive force ΔpH to drive H\textsuperscript{+} through the complex to power ATP synthesis (9). With adequate substrate and O\textsubscript{2}, this system is in equilibrium to maintain ΔpH at about −200 mV at a pH of ≤ 8.2. Thus oxidation of NADH and phosphorylation are normally matched. During ischemia, as the supply of [1/2]O\textsubscript{2} to accept electrons from NADH diminishes, electron flux through the electron transport chain falters and NADH accumulates (12). Normalization of NADH early during reperfusion may reflect energy expenditure away from myofilament relaxation via actin-myosin-ATPase, resulting in systolic contractile dysfunction and toward noncontractile repair processes, such as restoration of transmembrane ion transport.

Excess mitochondrial Ca\textsuperscript{2+} can impair cellular respiration and reduce ATP synthesis (25, 29). ATP is required to break the actin-myosin bond, reaccumulate Ca\textsuperscript{2+} into the sarcoplasmic reticulum, extrude Ca\textsuperscript{2+} from the myocyte, and reestablish the sarcolemmal membrane potential, primarily by restoring Na\textsuperscript{+} equilibrium. But it is unknown if a moderate increase in mitochondrial Ca\textsuperscript{2+} directly contributes to contractile dysfunction. On the contrary, because NADH rapidly returned to normal in the present study, ATP levels may have also been restored. The elevation in mitochondrial Ca\textsuperscript{2+} might actually help to reestablish NADH during reperfusion. In this way, the very quickly renormalized mitochondrial electrochemical gradient (ΔpH ≈ NADH/NAD\textsuperscript{+}) could restore synthesis of ATP, which is hydrolyzed by Ca\textsuperscript{2+}-ATPase and Na\textsuperscript{+}/K\textsuperscript{+}-ATPase to reestablish ion pump activities, perhaps at the expense of actin-myosin-ATPase required for relaxation contraction cycling. It is assumed that only viable myocytes contribute to the measure of NADH autofluorescence, as suggested by the small decline in NADH during late ischemia.

A major cause of myocardial damage during reperfusion is likely excess Na\textsuperscript{+} accumulation during reperfusion leading to Ca\textsuperscript{2+} loading. During ischemia, metabolic acidosis is believed to cause excessive Na\textsuperscript{+} entry via Na\textsuperscript{+}/H\textsuperscript{+} exchange, which in turn raises intracellular pH. The resting membrane potential becomes depolarized during ischemia as Na\textsuperscript{+}-K\textsuperscript{+}-ATPase pump activity decreases; this increase in [Na\textsuperscript{+}] shifts the reversal potential for Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange toward more negative membrane potentials. This, in turn, promotes a greater Ca\textsuperscript{2+} influx by the exchanger to increase [Ca\textsuperscript{2+}], i.e., by enhanced “reversed” mode operation (18, 37). Because repolarization is slowed during reperfusion, probably because the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase pump is incapable of rapidly reversing the Na\textsuperscript{+} load (19), the increase in Na\textsuperscript{+} entry, via Na\textsuperscript{+}/H\textsuperscript{+} exchange, increases [Ca\textsuperscript{2+}] as a result of reduced Ca\textsuperscript{2+} efflux or increased Ca\textsuperscript{2+} influx via reversed Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange (30). Consistent with this idea, we observed a small, but significant, increase in [Na\textsuperscript{+}] during ischemia but a more than doubling of [Na\textsuperscript{+}] immediately on reperfusion. Na\textsuperscript{+} remained elevated along with mitochondrial Ca\textsuperscript{2+} during later reperfusion, so it is possible that cytosolic Ca\textsuperscript{2+} was removed at the expense of excess cytosolic Na\textsuperscript{+} to reduce excess mitochondrial Ca\textsuperscript{2+}. The above mechanism is supported by a preliminary study in the same model. We found that a Na\textsuperscript{+}/H\textsuperscript{+} exchange inhibitor, BIIB-513, given for 10 min before 30-min global ischemia, improved return of cardiac function (2); importantly, this drug normalized [Na\textsuperscript{+}] during ischemia and at 60-min reperfusion and reduced peak [Na\textsuperscript{+}] on initial reperfusion by 50%.
In summary, Na\(^+\) accumulation, during ischemia and particularly on reperfusion, likely accounts for the initial increase in cytosolic Ca\(^{2+}\) that leads to mitochondrial Ca\(^{2+}\) excess and cardiac dysfunction. Elevated mitochondrial Ca\(^{2+}\) may help to normalize NADH early on reperfusion while buffering excess cytosolic Ca\(^{2+}\) during later reperfusion. Therapeutic interventions that reduce Na\(^+\) and Ca\(^{2+}\) loading, e.g., Na\(^+\)/H\(^+\) exchange inhibitors, should be quite beneficial to attenuate ischemia-reperfusion injury, particularly if effective immediately on reperfusion. Lastly, it is important to recognize that other factors, such as free radical formation, leukocyte migration, and complement activation, are also involved in reperfusion injury. The role of the endothelium and vasculature in mediating reperfusion injury must also be acknowledged.

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