Subcellular Electrolyte Alterations During Progressive Hypoxia and Following Reoxygenation in Isolated Neonatal Rat Ventricular Myocytes


This study characterizes the sequential alterations of, and relations between, multiple electrolytes in cytoplasm, mitochondria, and whole cells during hypoxia and on reoxygenation in isolated neonatal rat ventricular myocytes. Subcellular electrolyte content and distribution were measured by electron probe x-ray microanalysis, membrane phospholipid degradation by tritiated arachidonic acid release, and cell morphology by electron microscopy. At 1–2 hours of hypoxia, the myocyte population showed a loss of cytoplasmic potassium, magnesium, and chloride without alteration of cytoplasmic sodium or calcium. Mitochondria showed increased potassium with unchanged magnesium content. There was no morphological evidence of cell injury or tritiated arachidonic acid release. At 3–5 hours of hypoxia, the myocyte population showed a further loss of cytoplasmic potassium and magnesium and an increase in cytoplasmic sodium, chloride, and calcium. At a single-cell level, the increase in cytoplasmic sodium preceded the increase in cytoplasmic calcium. Mitochondria showed increased sodium and chlorine and decreased magnesium before increased calcium content; potassium loss was manifest only at 5 hours of hypoxia. At 3–5 hours of hypoxia, there was also tritiated arachidonic acid release and morphological evidence of cell injury. Reoxygenation for 1 hour after 5 hours of hypoxia partially reversed the mean alterations of all electrolytes, except calcium, in the cytoplasm of the myocyte population, whereas analysis was required at a single-cell level to show a partial reversal in calcium levels in cytoplasm of reoxygenated cells. Reoxygenation for 1 hour after 5 hours of hypoxia partially reversed the mean alterations of all electrolytes, including calcium, in the mitochondria of the myocyte population. Recovery of potassium in the cytoplasm correlated with reduction of mitochondrial calcium content on reoxygenation and best predicted recovery of cellular homeostasis of sodium, chloride, magnesium, and calcium. This study demonstrates that in this experimental model of hypoxia 1) initial losses of cytoplasmic potassium and magnesium occur in the absence of cell injury; 2) increases of sodium, chloride, and calcium occur in association with cell injury, with sodium increasing before calcium; 3) membrane phospholipid degradation and electrolyte derangement, including increased calcium, may contribute to reversible and irreversible phases of cell injury; 4) analysis of calcium at a subcompartmental level and at a single-cell level is required to correlate reduction of calcium on reoxygenation with recovery of cell homeostasis; 5) reduction of calcium content in mitochondria may predict recovery of cell homeostasis; and 6) recovery of potassium on reoxygenation best predicts recovery of cell membrane function and cell homeostasis. (Circulation Research 1992;71:106–119)

KEY WORDS • hypoxia • reoxygenation • elements • x-ray microanalysis • cell injury

One hypothesis for a mechanism of ischemic cell injury is that hypoxia evokes discrete alterations in the energy-dependent membrane-bound ionic pumps and that the subsequent electrolyte derangements cause cell injury.1,2 It has been proposed that inhibition of sarcolemmal Na⁺,K⁺-ATPase results in reduction in the transsarcolemmal Na⁺ gradient, thereby causing elevation of intracellular Ca²⁺ through a Na⁺-Ca²⁺ exchange mechanism.1,2 It has been further postulated that calcium elevation plays a crucial role in mediating the onset of irreversible cell injury.3–5 In considering the above hypotheses, three important aspects are undefined. These are 1) characterization of the sequential alterations of sodium, potassium, magnesium, and calcium in subcellular compartments during the initiation and progression of cardiac myocyte injury; 2) establishment of the relation between the magnitude and nature of the electrolyte derangements with biochemical integrity of cell membranes; and 3) determination of whether alterations in calcium content in cytoplasm or in mitochondria, alone or in conjunction with other electrolyte changes, predicts the ability of a cell to recover membrane function.
We used electron probe x-ray microanalysis to address the above questions since it allows simultaneous measurements of multiple electrolyte levels from subcellular loci of individual cells. The latter technique has been used previously to characterize electrolyte alterations associated with myocardial cell injury produced by metabolic inhibition in cultured myocytes.6–8 hypoxia,9 and ischemia10 in intact myocardial preparations. However, a detailed time course of elemental changes with gradually progressive injury has not been available. The advantage of using the isolated neonatal myocyte preparation with hypoxia as a model is that it allows for a time course slow enough to dissect and to interrelate elemental changes occurring during progressive cell injury. With the data from this model, multiple statistical approaches have been used to define the interrelations among electrolytes during hypoxia and reoxygenation.

Materials and Methods

Experimental Protocol

Hearts were isolated from 2–3-day-old rats, atria were removed, and the ventricles were minced in a HEPESS-buffered balanced salt solution.5–8 The myocardial cells were dispersed by incubation in a pancreatin (60 mg/100 ml) (GIBCO, Lawrence, Mass.) and collagenase type II (6,000–6,400 units/100 ml) (Cooper Biomedical, Freehold, N.J.) solution at 37°C for 20 minutes. The initial supernatant was removed and discarded. The mince was incubated in fresh pancreatin–collagenase for 20 minutes at 37°C. These steps were repeated four times. The cell suspensions from each digestion were combined and centrifuged. The pellets were suspended in culture medium consisting of 68% Dulbecco’s modified Eagle culture medium, 17% medium 199 with Earle’s salts, and 15% serum (10% horse serum and 5% fetal calf serum) with antibiotics (penicillin 10,000 units/ml and streptomycin 10,000 μg/ml). Myocytes were separated from nonmyocytic cells by using a Percoll differential separation gradient.

For biochemical assays, the myocytes were plated directly on 60-mm plastic culture plates. For electron probe microanalysis, the culture dishes were coated with collagen gel (Collagen Corp., Palo Alto, Calif.), 2.5 ml per 60-mm culture plate, forming a layer approximately 2-mm thick.6–8 Addition of a suspension of myocytes resulted in light colonization of the gels. Three days later, medium with unattached myocytes was removed and a second seeding of myocytes was added. After an additional 3–4 days in culture, there were numerous colonies of contracting myocytes. At that time, experiments were undertaken. Experimental cultures were incubated in glucose-free medium 199 (GIBCO) containing 1.8 mM CaCl2 and subjected to either normoxia or hypoxia (1, 2, 3, and 5 hours). The number of experiments undertaken was three to seven, with 10–12 cells analyzed per time point in each experiment. Culture plates were placed in incubators (Forma Scientific, Inc., Marietta, Ohio) gassed with either 93% O2 and 7% CO2 (normoxia) or 93% N2 and 7% CO2 (hypoxia). During the hypoxia, the cultures were placed in a specially constructed plexiglass apparatus containing multiple chambers allowing separate access for removal of plates during the time course of the experiments. Gas samples were obtained from the incubator via a one-way outlet port by suction directly into a syringe. Gas samples were measured through the duration of the hypoxia experiments (over 5 hours) to verify that PO2 concentrations remained less than 10 mm Hg and PCO2 concentrations remained between 35 and 45 mm Hg. PO2, PCO2, and pH measurements were recorded on an acid–base analyzer (model ABL3, Radiometer America, Inc., Westlake, Ohio). Measurements have confirmed that PO2 concentrations of less than 10 mm Hg can be routinely achieved in these incubators. The culture plates were removed from the incubator at the end of the hypoxia period or after 1 hour of reoxygenation.

Electron Probe Microanalysis of Subcellular Elemental Levels

At the end of the experiments, a rectangle of collagen with a cluster of myocytes was cut free and moved onto a piece of Whatman filter paper (Millipore Corp., Bedford, Mass.) to absorb the excess medium. The time period from removal of the myocytes from the hypoxia chamber to freezing was approximately 3–5 minutes. The filter paper–collagen–myocyte preparation was rapidly frozen using a model 669 Cryosnapper (Gatan Corp., Pleasanton, Calif.), which provided freezing with a pair of copper blocks cooled to liquid nitrogen temperature (−196°C).6 Specimens were stored in liquid nitrogen until used for cryoultramicrotomy. Cryosections were cut at a thickness of 150–250 nm with a Dupont Sorvall MT 5000 Ultramicrotome with an FS 1000 cryosectioning attachment (Dupont, Wilmington, Del.) and mounted on 50 mesh copper grids that were previously coated with Formvar and carbon. The sections were transferred to the analytical electron microscope with a Gatan 626 cryotransfer stage device without exposure to the atmosphere. The cryostage was inserted into a JEOL 1200 EX TEMSCAN electron microscope with the stage temperature controlled at −130°C. Cryosections were freeze-dried under vacuum in the electron microscope.

Data were obtained from 10–12 cells from each of three to seven experiments for each group. The JEOL 1200 EX analytical microscope and horizontal entry Kevex 30-mm2 energy-dispersive x-ray detector were used. X-ray spectra were collected and analyzed by using a Tracor Northern 5500 multichannel analyzer. Scan speed was 10 seconds per frame and magnification was ×60,000 to ×200,000 depending on area of interest to be analyzed. Data for the cytoplasm were obtained from regions containing myofibrils as the predominant element; data for the mitochondria were obtained from ovoid, dense structures corresponding to the distribution of mitochondria; data for whole cells were obtained at low magnification to include most of the cells exclusive of the nuclei. Myocytes were analyzed at random from the sections. For each cell, spectra were collected from one or more, usually three, sites in the cytoplasm and mitochondria and the values averaged to provide average values for cytoplasm and mitochondria of each cell. The x-ray spectra were collected over the range of 0–20 keV for 100 seconds at a resolution of 10 eV per channel. Elemental peak intensities were obtained by deconvolution of the spectra by using a multiple least-squares technique.6 A computerized method was used for quantitative analysis of spectra, based on the direct
FIGURE 1. Scanning transmission electron micrographs of control myocytes and myocytes with 1 hour of hypoxia. Top panel: Control myocyte exhibits a nucleus (N), compact cytoplasm, and mitochondria (M) of uniform density. Magnification, ×16,900. Bottom panel: After 1 hour of hypoxia, morphology of myocytes is unchanged compared with control. The mitochondria show generally uniform density without discrete inclusion bodies. Magnification, ×10,200.
FIGURE 2. Scanning transmission electron micrographs of myocytes with 3 hours of hypoxia. Top panel: Many of the mitochondria exhibit small electron-dense inclusions (arrows). Note that the myocytes have one or two nuclei (N). Magnification, ×8,000. Bottom panel: High magnification view showing the electron-dense inclusions (arrows) characteristic of calcium phosphate deposits. Magnification, ×20,650.
relation between the peak-to-continuum ratio and elemental concentration. A series of elemental standards were used to calculate absolute levels from the test spectra. Values obtained were for total electrolyte concentrations, including bound and free (ionized) components. Elemental content was measured and expressed as millimoles per kilogram dry weight, with the denominator based on the local mass density derived from the continuum of the x-ray spectra.

**Tritiated Arachidonic Acid Measurement**

Cultured myocytes were labeled 16 hours before the experiments with [3H]arachidonic acid at 37°C, resulting in approximately 90% incorporation of the [3H]arachidonic acid into the myocytes. The myocytes were subjected to normoxia of 1–5 hours in duration (hypoxia, 0 hours) or hypoxia of 1 to 5 hours in duration and, after the desired time, the medium was aspirated and extracted by the method of Bligh and Dyer. Two aliquots of 50 μl were placed into scintillation vials for measurement of total lipid extract and counted on a beta scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). Twenty-five microliters of the lipid phase was spotted on a silica gel thin-layer chromatography plate under nitrogen and separated using hexane:ether:acetic acid (120:30:1.2 vol/vol/vol) solvent. Plates were dried and developed in iodine vapor so that separations could be visualized. The plates were divided into three sections per column for separation of the free fatty acid band from the other lipid species. The plates were scraped and the sections collected into individually marked scintillation vials and counted on a Beckman beta scintillation counter. Protein content of the myocytes also was measured.

**Statistical Analysis**

Data were expressed as the mean±SEM. Two sets of elemental data were analyzed. One data set provided an evaluation of electrolyte changes over the time course of hypoxia (0–5 hours). The second data set provided an evaluation of the effect of 1 hour of reoxygenation after 5 hours of hypoxia on electrolyte levels. Both data sets were subjected to three types of statistical analysis. First, a comparison was made of overall means for all experiments. Elemental concentrations in each location were compared across treatment groups by using one-way analysis of variance, with Duncan's multiple range tests used to investigate significant pairwise treatment differences (with the findings presented in the text, Table 1, and Figures 5–8). Second, the statistical significance of changes on an experiment-by-experiment basis was made using one-way analysis of variance with Duncan's multiple range test (with the findings presented in the text). Third, cell populations were defined as normal or abnormal for each element. This descriptive characterization of cells was performed using the nearest-neighbor analysis, a nonparametric discrimination technique. Fischer's exact test (two-tailed) then was used to correlate relations between elements for the different cell populations, with p<0.05 as the level of significance (with the findings presented in the text, Table 2, and Figure 9).

All analysis of variance F tests were one-tailed tests and were considered significant when p<0.05; Duncan's multiple range tests were performed at the 0.05 level of significance. Analysis of variance with multiple range testing also was used to evaluate the biochemical data.

**Figure 3.** Scanning transmission electron micrographs of myocytes with 5 hours of hypoxia alone and after 1 hour of reoxygenation. Top panel: After 5 hours of hypoxia, the myocytes exhibit intact nucleus (N); vacuolated cytoplasm, consistent with edema; swollen mitochondria (M); and sub-sarcolemmal blebs (B). Magnification, ×6,800. Bottom panel: After 1 hour of reoxygenation following 5 hours of hypoxia, some myocytes exhibit normal morphology (NM) and other myocytes maintain swollen cytoplasm and mitochondrial inclusions (arrows), indicative of persistent injury (IM). Magnification, ×17,750.

**Figure 4.** Bar graph showing tritiated arachidonic acid release. Myocytes prelabeled overnight with tritiated arachidonic acid were subjected to either normoxia of 1–5 hours in duration (hypoxia, 0 hours) or hypoxia of 1–5 hours in duration. There was no alteration in the tritiated arachidonic acid release (cpm/mg protein) during 1–2 hours of hypoxia, while loss of the radiolabel from the cells was evident at 3–5 hours of hypoxia during cell injury (four to five experiments). *p<0.05 vs. 0 hours of hypoxia. Myocytes subjected to normoxia for up to 5 hours (hypoxia, 0 hours) showed no arachidonic acid release, indicating no cellular death with release of membrane phospholipids.
TABLE 1. Electrolyte Concentrations in Whole Cardiac Myocytes During Progressive Hypoxia

<table>
<thead>
<tr>
<th>Hours of hypoxia</th>
<th>Na</th>
<th>K</th>
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<th>Ca</th>
<th>P</th>
<th>S</th>
<th>Cl</th>
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<td>0 (n=95)</td>
<td>64±6*</td>
<td>643±16*</td>
<td>45±2*</td>
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<td>453±8*</td>
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<td>232±7*</td>
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<td>1 (n=43)</td>
<td>88±10†</td>
<td>627±23†</td>
<td>32±2†</td>
<td>10±3*</td>
<td>426±14*</td>
<td>170±6†</td>
<td>218±14*</td>
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<tr>
<td>2 (n=71)</td>
<td>115±12†</td>
<td>645±22†</td>
<td>33±3†</td>
<td>15±5†</td>
<td>464±14†</td>
<td>181±6†</td>
<td>255±13†</td>
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<tr>
<td>3 (n=92)</td>
<td>273±18†</td>
<td>466±29†</td>
<td>18±2‡</td>
<td>35±13†</td>
<td>441±13*</td>
<td>165±6†</td>
<td>306±15†</td>
</tr>
<tr>
<td>5 (n=81)</td>
<td>470±28‡</td>
<td>227±26‡</td>
<td>2±3§</td>
<td>53±10‡</td>
<td>360±14‡</td>
<td>165±6†</td>
<td>458±28‡</td>
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</table>

Values are mean±SEM (concentrations in millimoles per kilogram dry weight); n is the total number of cells analyzed per group of experiments. For each column, values with different symbols (*, †, ‡, §) are significantly different (p<0.05) from each other by analysis of variance and Duncan’s multiple range test.

Results

Ultrastructure

Unstained, freeze-dried cryosections of the isolated neonatal rat ventricular myocytes were examined by scanning transmission electron microscopy. Sections taken from control cultures illustrated well-preserved ventricular myocytes lacking distortion from freeze-drying or specimen preparation (Figure 1, top panel). Cryosections of myocytes subjected to 1 (Figure 1, bottom panel) to 2 hours of hypoxia showed unchanged morphological appearance as compared with control cultures. Cryosections from myocytes subjected to 3–5 hours of hypoxia illustrated progressive alteration of cell morphology characterized by cytoplasmic fine vacuolation and mitochondrial inclusion bodies (Figure 2). At 5 hours, membrane blebbing occurred in myocytes (Figure 3, top panel). Reoxygenation was associated with a mixed population of structurally normal and abnormal myocytes (Figure 3, bottom panel).

FIGURE 5. Bar graphs showing that within 1 hour of hypoxia, potassium and magnesium loss occurred from cytoplasm, whereas in the mitochondria, potassium levels were increased and magnesium levels were unchanged. There were no alterations in sodium or calcium at this time point. At 3 hours of hypoxia, potassium and magnesium levels were markedly decreased and associated with elevated levels of sodium and calcium in cytoplasm. In the mitochondria, potassium levels had returned to control values, magnesium levels were decreased, and sodium levels were increased; calcium levels were not significantly elevated. Thus, early changes of potassium and magnesium occurred without alterations in sodium or calcium. At 5 hours of hypoxia, changes occurred in all electrolytes in both cytoplasm and mitochondria. Buffering of sodium and calcium occurred by mitochondria during progression of cell injury. The number of experiments at each time point was four to seven; the number of cells analyzed was 95 (control), 43 (1 hour of hypoxia), 71 (2 hours of hypoxia), 92 (3 hours of hypoxia), and 81 (5 hours of hypoxia). For each column, values with different superscripts (A, B, C) are significantly different from each other by analysis of variance and Duncan’s multiple range test.
Biochemical Parameters

In control cultures (hypoxia, 0 hours), tritiated arachidonic acid release from myocytes into perfusion medium was 2,574±394 cpm/mg cell protein; there was no progressive loss of radiolabel from control myocytes. Hypoxia of 1–2 hours in duration did not alter tritiated arachidonic acid release, but from 3–5 hours a progressively increasing release of tritiated arachidonic acid occurred into the perfusion medium (Figure 4).

Electron Probe X-Ray Microanalysis

Quantitative data for sodium, chlorine, potassium, calcium, magnesium, phosphorus, and sulfur for whole cells are shown in Table 1 and for cytoplasm and mitochondria are shown in Figures 5–8. Electrolyte content per kilogram dry weight was lower in the mitochondria than in the cytoplasm because of the greater density of mitochondria than cytoplasm. In control myocytes, the pattern of the electrolyte content with high potassium and magnesium and low sodium and calcium was as described previously in isolated myocytes and myocardium.6–10,17–19 For example, the whole-cell mean sodium and potassium concentrations in the isolated myocytes were 64 and 643 mmol/kg dry wt, respectively (Table 1), values similar to the reported levels obtained by electron probe x-ray microanalysis in heart tissue.10,18,19 The mean Na/K ratio in the whole cell was 0.10, which is in agreement with the measured Na/K ratio in heart tissue.20–22

Electron probe microanalysis does not measure alterations in cell hydration. Because sodium, potassium, and chlorine represent approximately all of the osmotically active elements that are permeable across the cell membrane, the concentrations of these three elements should be relatively constant provided the hydration of the cells does not change. Under control conditions, the sum of whole-cell mean sodium, potassium, and chlorine was 939 mmol/kg dry wt (Table 1). Hypoxia of 1 hour in duration did not alter the concentration of these three elements (933 mmol/kg dry wt). However, hypoxia of 2–5 hours increased their concentration by 11–23%. These overall increases in osmotically active electrolytes can be explained by an increase of fluid content, a loss of mass, and/or a combination of an increase in fluid content and loss of mass by the cells.

Hypoxia of 1 hour in duration (Figures 5 and 6) was associated with a selective loss of potassium, magnesium, chlorine, and sulfur from the cytoplasm. Mitochondria showed an increase in the potassium and reduction in sulfur content. Sodium and calcium in cytoplasm and mitochondria were unchanged. These alterations after 1 hour of hypoxia occurred in the absence of any change in cell hydration. Myocyte beating activity (60–120 beats per minute under control conditions) was variably reduced (20–60 beats per minute) but still maintained within 1–2 hours of hypoxia.

At 2 hours of hypoxia (Figures 5 and 6), elemental analysis in the cytoplasm showed a further loss of magnesium, a continued loss of potassium, a loss of phosphorus, a small, albeit nonsignificant, increase in sodium, unchanged calcium, and a return to control values of chlorine. In addition to analysis of average changes based on all experiments, statistical analysis of alterations also was made on an experiment-by-experiment basis. After 2 hours, the potassium and magnesium levels were decreased in five of five and three of five experiments, respectively, while the sodium and
Calcium levels were increased in one of five experiments in the cytoplasm. After 2 hours, the mitochondrial mean potassium remained elevated and the mitochondria sulfur remained decreased; other elements were not different from control. The mean sodium and calcium levels in the mitochondria were not elevated and were abnormal in one of five and zero of five experiments, respectively.

At 3 hours of hypoxia (Figures 5 and 6), elemental analysis in the cytoplasm showed a further reduction of potassium and magnesium and an increase of sodium and calcium. The potassium and magnesium levels were decreased in six of seven and seven of seven experiments, respectively, while the sodium and calcium levels were elevated in six of seven and two of seven experiments, respectively, in the cytoplasm. The increase in the cytoplasmic calcium content appears to be the result of a large increase in two of seven experiments as opposed to a consistent increase in all experiments. At 3 hours of hypoxia, elemental levels in the mitochondria showed the potassium levels to have returned to control values, magnesium levels to be decreased, sodium and chlorine levels to be elevated, and calcium levels to be not significantly increased. In the mitochondria, the sodium levels were increased in six of seven experiments, whereas the calcium levels were increased in only three of seven experiments. The isolated myocyte preparation was quiescent at 3–5 hours of hypoxia.

At 5 hours of hypoxia (Figures 5 and 6), potassium and magnesium values in the cytoplasm were markedly reduced, and sodium, chloride, and calcium levels in the cytoplasm were greatly elevated; similar changes were observed in the mitochondria. Mean values for calcium were 33±3 mmol/kg dry wt in cytoplasm and 66±17 mmol/kg dry wt in mitochondria. The sulfur content was decreased in cytoplasm and mitochondria, and the phosphorus content was decreased in cytoplasm alone. In individual experiments, cytoplasmic calcium was increased in five of six experiments and mitochondrial calcium in three of six experiments; changes in cytoplasmic and mitochondrial sodium, potassium, and magnesium occurred in five to six of six experiments. The correlation coefficient with reference to potassium and total phosphorus loss from cytoplasm showed a Pearson’s r value of 0.96.

In addition, analysis at a single-cell level was performed to characterize myocytes into population types. At 3 hours of hypoxia, approximately 80–90% of cells had sodium, potassium, and magnesium levels in the cytoplasm dissimilar from control cells, i.e., a single population of predominantly injured cells with respect to the above elements, whereas the distribution of other cellular elements, including calcium, was heterogeneous. At 5 hours of hypoxia, approximately 80–90% of cells had sodium, potassium, magnesium, calcium, chloride, sulfur, and phosphorus levels in the cytoplasm dissimilar from control cells. Thus, analysis at a single-
cell level showed that consistent changes in cytoplasmic potassium, magnesium, and sodium preceded the consistent increase in calcium. Reoxygenation for 1 hour after 5 hours of hypoxia was associated with reversal toward normal of the potassium, magnesium, sulfur, and phosphorus loss and reduction toward normal of the sodium and chlorine elevation in both cytoplasm and mitochondria (Figures 7 and 8). Reoxygenation did not decrease the elevated mean level of calcium in the cytoplasm, but it reduced the elevated mean level of calcium in the mitochondria (Figures 7 and 8).

As previously indicated, single-cell analysis indicated that 80–90% of cells had abnormal elemental levels in cytoplasm and mitochondria after 5 hours of hypoxia. With 1 hour of reoxygenation after 5 hours of hypoxia, analysis at a single-cell level showed 39–82% of reoxygenated cells had sodium (63%), potassium (82%), magnesium (55%), chlorine (55%), phosphorus (69%), and sulfur (39%) levels in the cytoplasm similar to those of control cells and 45% of reoxygenated cells had calcium levels in the cytoplasm similar to those of control cells.

Figure 8. Bar graphs showing that reoxygenation partially reversed the increase of chlorine and loss of sulfur and phosphorus in cytoplasm and mitochondria. The number of experiments at each time point was four to seven; the number of cells analyzed was 44 (control), 51 (5 hours of hypoxia), and 51 (5 hours of hypoxia and 1 hour of reoxygenation). For each column, values with different superscripts (A, B, C) are significantly different from each other by analysis of variance and Duncan's multiple range test.

Figure 9. Characterization of elemental distribution in cytoplasm (left graph) showed that 5 hours of hypoxia induced a predominantly single population type of abnormal cells as compared with control cells. Reoxygenation for 1 hour after 5 hours of hypoxia was associated with two population types of abnormal and normal cells as compared with control cells. Characterization of elemental distribution in mitochondria (right graph) showed that 5 hours of hypoxia induced a predominantly single population type of abnormal cells as compared with control cells. Reoxygenation for 1 hour after 5 hours of hypoxia was associated with two population types of abnormal and normal cells as compared with control cells. The number of experiments at each time point was four to seven; the number of cells analyzed was 44 (control), 51 (5 hours of hypoxia), and 51 (5 hours of hypoxia and 1 hour of reoxygenation).
control cells (Figure 9). With 1 hour of reoxygenation after 5 hours of hypoxia, analysis at a single-cell level showed 35–72% of reoxygenated cells had sodium (50%), potassium (72%), magnesium (65%), chlorine (41%), phosphorus (35%), and sulfur (60%) levels in the mitochondria similar to those of control cells and 66% of reoxygenated cells had calcium levels in the mitochondria similar to those of control cells (Figure 9).

With respect to calcium, reoxygenation was associated with four populations of cells: normal calcium in cytoplasm and in mitochondria (17/51 cells, 33.3%), abnormal calcium in cytoplasm and in mitochondria (11/51 cells, 21.6%), normal calcium in cytoplasm and abnormal calcium in mitochondria (6/51 cells, 11.8%), and abnormal calcium in cytoplasm and normal calcium in mitochondria (17/51 cells, 33.3%). Multivariate analysis was performed to determine the relations between changes in elemental levels after 5 hours of hypoxia alone and with reoxygenation. Recovery of potassium best predicted recovery of other elements (Table 2).

<p>| Table 2. Electrolyte Concentration Recovery Relations in the Cytoplasm and Mitochondria of Cardiac Myocytes After 5 Hours of Hypoxia and 1 Hour of Reoxygenation |
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Nearest three neighbor discriminant analysis led to a classification of each cell that was exposed to reoxygenation as damaged or recovered with respect to each element based on observed electrolyte concentrations in the control cells and in cells exposed to 5 hours of hypoxia only. Simple cross tabulations of the cells for pairs of electrolytes are made based on damage/recovery class. Fischer's exact test (two tailed) was performed, testing whether the rate of recovery with respect to a particular electrolyte is equal (+) or not equal (-) for cells damaged vs. cells recovered with respect to another electrolyte. There were 51 cells for which cytoplasmic and mitochondrial measurements were available.

**Discussion**

The isolated cultured neonatal rat myocyte preparation responds to metabolic inhibition with progressive depletion of ATP, loss of membrane phospholipid content, and impairment of electrolyte homeostasis characterized by loss of potassium and magnesium content and accumulation of sodium and calcium content.\textsuperscript{6–8,23–25} These biochemical changes are associated with morphological alterations of cell injury evident by vacuolation, bleb formation, and electron-dense deposits in the mitochondria.\textsuperscript{6–8,23–25} Thus, the neonatal myocyte preparation displays biochemical, elemental, and morphological responses similar to those of the isolated or in vivo heart preparation subjected to hypoxia.\textsuperscript{6–10,23–25} Although neonatal myocytes may exhibit developmental differences such as higher rates of glycolysis, lower rates of oxidative phosphorylation, and a less well-developed sarcoplasmic reticulum compared with adult myocytes, previous studies have shown that glucose and palmitate metabolism is similar in cultured neonatal myocytes and isolated adult ventricular myocytes\textsuperscript{26} and that important aspects of sarcoplasmic reticulum function are operative in the neonatal myocytes.\textsuperscript{27,28} It is pertinent to note that isolated myocyte preparations perform no pressure work. As a result, their energy utilization and response to metabolic injury is slower than that found in working heart tissue. One major advantage to the preparation, therefore, is the ability to dissect the temporal sequence of electrolyte changes that would not be possible in isolated or in vivo heart preparations in which a more rapid time course of cell injury occurs.

**Early Loss of Potassium and Magnesium During Hypoxia**

Hypoxic cells, within 1–2 hours, exhibit loss of potassium and magnesium from the cytoplasm. These changes occur before the consistent elevation of sodium or calcium in the cytoplasm, loss of membrane phospholipid, or signs of morphological injury. The early loss of potassium occurs independent of inhibition of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase pump since there is increased potassium efflux rather than decreased potassium influx during early hypoxia or ischemia.\textsuperscript{29–31} The myocyte beating activity was maintained during the first 2 hours of hypoxia, an important consideration since potassium efflux may be abolished if the myocyte preparation is quiescent.\textsuperscript{32} The two mechanisms that have been proposed to underlie the loss of potassium are activation of an ATP-sensitive potassium channel and a cotransport of anions to balance transmembrane charge during efflux of cations (lactate and phosphate). Recent evidence favors the concept of activation of an outward potassium current resulting from ATP depletion.\textsuperscript{33–37} Glycolytic ATP has been proposed to be the preferential source\textsuperscript{34} for maintaining the potassium-sensitive ATP channel. In our study, the initial loss of potassium occurred within 2 hours of hypoxia, a time period when ATP content was found to be decreased by only 25%.\textsuperscript{24} It has been argued that activation of only a small percentage (1%) of the maximal ATP-sensitive potassium current at millimolar cytosolic ATP levels may be sufficient to account for cellular potassium loss during early hypoxia or ischemia.\textsuperscript{35,36} Recent evidence shows that inhibition of the ATP-sensitive potassium channel with glibenclamide inhibits the hypoxia-induced shortening of the action potential duration, an alteration linked to loss of potassium.\textsuperscript{37} Another possibility is that increased potassium loss occurs passively with efflux of lactate and inorganic phosphate to balance transsarcolemmal charge movement.\textsuperscript{38–40} In the present study, there is a close correlation (r value of 0.96) between...
cytoplasmic total potassium and total phosphorus loss during cell injury (Figures 5 and 6). Coincident with the early loss of potassium from the cytoplasm, there is an increase in potassium in the mitochondria. A physiological role of a specific K-H antiporter is proposed in mitochondrial volume control.\textsuperscript{10,41} Mitochondrial swelling accompanies mitochondrial potassium accumulation, in vitro, in the presence of phosphate anion.\textsuperscript{42} In this context, rapid phosphate production from ATP breakdown during early cellular hypoxia may provide the basis for the increase in mitochondrial potassium after 1 hour of hypoxia. This argument, however, must be regarded as suggestive since direct measurement of inorganic phosphate was not made.

The decrease in total magnesium during hypoxia is most likely due to dissociation of this divalent cation from its complexes with ATP, ADP, and citrate as energy metabolism is decreased.\textsuperscript{43–45} Although total magnesium may decrease, the cytosolic free magnesium is expected to increase transiently because of less effective chelation by ADP and other adenine nucleotides versus ATP. Indeed, Murphy and colleagues,\textsuperscript{46} using the fluorescent indicator Mg-fura, recently showed an increase in intracellular free magnesium during early ischemia. Because cytosolic free magnesium is the proposed regulatory factor for electrolyte shifts, the early decrease of the total magnesium content is unlikely to mediate the potassium loss. However, our data indicate that depletion of total magnesium content eventually occurs as cell injury progresses.

Late Elevation of Sodium and Calcium During Hypoxia

Hypoxic cells, within 3–5 hours, became quiescent and exhibited a further loss of cytoplasmic potassium and magnesium and an increase of cytoplasmic sodium, chlorine, and calcium. The profile of potassium loss and increased sodium is in accord with inhibition of sarcolemmal Na,K-ATPase activity.\textsuperscript{1,6–10} Elevation of sodium preceded the elevation of calcium in the mitochondria; a similar trend was evident in the cytoplasm. The latter observations are consistent with the operation of the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger in the mode of calcium influx for sodium efflux.\textsuperscript{46} Cardiac myocytes prelabeled with tritiated arachidonic acid showed membrane phospholipid loss in concert with cytoplasmic calcium loading. As observed previously, these changes occur when ATP is severely depleted (by greater than 75%).\textsuperscript{6–8,23} Similar phospholipid changes in ischemic myocardium are associated with a calcium permeability defect in isolated cell membranes.\textsuperscript{47} Indeed, in a recent study, Jones and coworkers\textsuperscript{8} showed that inhibition of phospholipase activity prevents morphological damage, tritiated arachidonic acid loss, and elemental alterations, including calcium accumulation in cardiac myocytes. Our study, therefore, suggests that cytoplasmic calcium may increase by mechanisms involving both Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange and membrane phospholipid loss. In addition to the above factors, earlier studies have indicated that the metabolic status of the cell, especially the magnitude and duration of ATP depletion, determines the extent of calcium loading.\textsuperscript{25} Morris and coworkers\textsuperscript{8} recently showed that in the presence of progressive ATP depletion, the energy-dependent synthetic processes may not be able to compensate for the calcium-medi- ated catabolic processes. Depletion of the total magnesium content occurred at 5 hours of hypoxia. A reduction of cytosolic free magnesium content is more likely to occur at this later time period because of passive leak of this divalent cation out of the cell. A reduction in the free magnesium could predispose to an increase in calcium influx\textsuperscript{43–44}; however, the latter mechanism may be blunted because, at this stage of injury, calcium channels are becoming inactivated because of dephosphorylation from ATP depletion.

Because electron probe microanalysis measures total calcium content, correlation with intracellular Ca\textsuperscript{2+} activity is not possible. It may be questioned whether an early increase in free Ca\textsuperscript{2+} could have occurred in association with potassium and magnesium shifts and with preserved cell morphology. Although recent studies demonstrate an increase in intracellular free Ca\textsuperscript{2+} during contracture produced by hypoxia, metabolic inhibition, or ischemia,\textsuperscript{7,46–53} there is no evidence that calcium accumulates in the cytoplasm during the early period of hypoxia when changes in potassium and magnesium are evident and cell morphology is normal.

Recovery From Cell Injury on Reoxygenation

Despite evidence of cell injury, reoxygenation of a global population of cardiac myocytes partially reverses the mean changes in potassium, magnesium, phosphorus, sulfur, sodium, and chlorine in both cytoplasm and mitochondria and is associated with two population types: 50–82% of cells with control levels of sodium and potassium in the cytoplasm and in mitochondria and 50–18% of cells with sodium and potassium levels similar to hypoxic cells (Figures 7–9). Reoxygenation did not decrease the elevated mean level of calcium in the cytoplasm even though analysis at a single-cell level showed two populations types: 45% of cells with control levels of calcium in the cytoplasm and 55% of cells with calcium levels in cytoplasm similar to that of hypoxic cells. Reoxygenation did, however, reverse the elevated level of calcium in the mitochondria, and this change correlated with reversal of the elevated sodium and reduced potassium content both in the cytoplasm and in the mitochondria (Figures 7–9). The above association implies reestablishment of sodium and potassium gradients across sarcolemmal and mitochondrial membranes and recovery of cell membrane function. Our study, therefore, demonstrates that 1) accurate characterization of the relation between calcium and recovery of cell function requires analysis of calcium at a subcompartmental level and at an individual cell level, 2) accumulation of calcium in the mitochondria does not necessarily imply irreversible myocyte injury as previously suggested,\textsuperscript{54} and 3) a reduction in calcium content in mitochondria (as opposed to cytoplasm) may be a better predictor of recovery of cell function. It is pertinent to note that in a recent study, cytosolic calcium actually fell during early reoxygenation despite the presence of cell hypercontracture.\textsuperscript{55} However, cellular 45Ca uptake increased, indicative of calcium uptake by intracellular organelles (either mitochondria or sarcoplasmic reticulum). This recent report suggests that increases in calcium content in intracellular organelles rather than in cytoplasm may be a better predictor of impairment of cell function.\textsuperscript{55}
Characterization of calcium levels in cytoplasm and mitochondria (within the same cells) showed a population (33.3%) of reoxygenated cells with normal calcium in both compartments, indicative of recovery from cell injury, and populations of cells with elevated calcium in both compartments (21.6%) or in mitochondria alone (11.8%), indicative of persisting injury. In addition, a striking finding was a population (33.3%) of reoxygenated cells with normal calcium in mitochondria but elevated levels in the cytoplasm. A similar finding was reported in isolated ventricular myocytes perfused with zero K⁺ (Na⁺-K⁺ pump inhibition) followed by return to normal K⁺ medium. Persistent elevation of cytosolic calcium on reoxygenation of a global population of myocytes, therefore, does not necessarily imply an inability of a myocyte to recover its functional ability. Previous studies have indeed shown that myocytes can accommodate elevated calcium levels in the cytoplasm without permanent damage. Allshere and coworkers reported that hypoxic rat myocytes tolerate intracellular calcium levels of 2 μM. However, at 5 μM, reoxygenation then produces cell death. Thus, the magnitude and duration of the intracellular calcium elevation during hypoxia appear to influence the ability of a cell to reestablish membrane integrity on reoxygenation. The definition of recovery on reoxygenation after hypoxia or reperfusion after ischemia is extremely difficult with the lack of an early measurement to predict improvement. Specifically, recovery of mechanical function occurs from several hours to days, cytoplasmic enzymes are released on reperfusion, recovery of energy levels may be limited by depleted precursor nucleotides, and cell calcium may increase or decrease. An ideal elemental marker to characterize cardiac myocyte viability should be located intracellularly, present in the free form in the cytoplasm, easily measurable, and not coupled with a calcium exchange process on reperfusion. Potassium appears to be potentially an ideal element as a marker of recovery of cell membrane function since it 1) meets all of the above requirements; 2) best predicts recovery of intracellular magnesium, sodium, chloride, and calcium homeostasis; and 3) correlates with reduction of mitochondrial calcium content. In contrast, approximately 80–90% of intracellular magnesium is in the bound form, sodium is coupled by an exchanger with calcium, and calcium measurement requires subcellular and single-cell analysis, thereby making these elements less ideal markers of cardiac myocyte viability than potassium.

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References


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