Mechanisms of Reoxygenation Injury in Cultured Ventricular Myocytes

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To investigate factors contributing to reperfusion and reoxygenation myocardial injury, we exposed layers of cultured chick ventricular myocytes to severe hypoxia for up to 3 hours in the presence of 20 mM 2-deoxyglucose, zero glucose, and 5 mM pyruvate, and then exposed the myocytes to reoxygenation. Lactate dehydrogenase (LDH) release was moderately increased during 3 hours of hypoxia but was increased markedly during reoxygenation. Coincident changes in intracellular calcium concentration ([Ca^{2+}]_{i}) and cell motion were also measured during hypoxia and reoxygenation. During hypoxia, [Ca^{2+}]_{i} increased to more than 1 μM, and with reoxygenation, [Ca^{2+}]_{i} abruptly decreased slightly but remained elevated more than 1 μM. Cells developed a stable rigor after 30 minutes of hypoxia. Reoxygenation caused a marked hypercontracture within 5 minutes. Pretreatment of myocytes with either 2,3-butanedione monoxime, which inhibits Ca^{2+}-dependent force development, or cyanide inhibited reoxygenation hypercontracture. LDH release after reoxygenation was also significantly reduced in the presence of 2,3-butanedione monoxime. Treatment of myocytes with superoxide dismutase and catalase during hypoxia also resulted in a decrease in LDH release during reoxygenation. We conclude that an abrupt increase in [Ca^{2+}]_{i} during reoxygenation does not account for reoxygenation injury. However, in the presence of elevated [Ca^{2+}]_{i}, reoxygenation and the resulting probable resynthesis of ATP causes [Ca^{2+}]-dependent myofilament crossbridge cycling, and the resulting hypercontracture contributes to myocyte damage. The generation of oxygen free radicals after reoxygenation also appears to contribute to cell injury in this system. (Circulation 1991;83:566–577)

Myocardial ischemia is associated with multiple metabolic and physiological processes that combine to produce myocyte injury. As recently summarized by Reimer and Jennings,1 oxygen deprivation and associated high-energy phosphate depletion results in loss of cellular sodium and potassium gradients, calcium overload, and activation of endogenous phospholipases and proteases, which, in turn, can damage the cytoskeleton and sarcolemma. During ischemia, potentially toxic metabolites, including lactate, H^{+}, and NADH, also accumulate and products of lipid degradation may cause damage to cell membranes by detergent action. In addition, free radicals, possibly produced by metabolism of adenine nucleosides and bases, may contribute to ischemic myocyte injury. Reperfusion of myocardium after prolonged ischemia may result in an acceleration of myocyte injury and is associated with cell swelling, sarcolemmal bleb formation, and hypercontraction of sarcomeres with disruption of myofibrils.1 A similar increase in myocyte injury can be produced by reoxygenation of severely hypoxic myocardium, a process termed the “oxygen paradox,”2 suggesting that resupply of oxygen during reperfusion may be an important contributor to reperfusion injury.

At least three major mechanisms have been proposed to explain the increased myocyte damage that occurs after reperfusion or reoxygenation. An abrupt increase in total calcium content3 and in calcium-45 uptake in reperfused myocardium4 has led investigators to suggest that reperfusion causes an increase in intracellular calcium concentration ([Ca^{2+}]_{i}), with resulting injury due to [Ca^{2+}]_{i}-dependent processes.1 Ganote and Kaltenbach5 suggested that the phenomenon of reperfusion or reoxygenation hypercontracture contributes to cell rupture by causing sarcolemmal stress. In addition, the resupply of molecular oxygen to ischemic or hypoxic myocytes may result in enhanced production of oxygen free radical species, which, in turn, may damage the sarcolemma and
further inhibit cellular metabolism. However, the identity of mechanisms by which reperfusion or reoxygenation myocyte injury occurs, and the relative importance of the mechanisms, is still controversial and the subject of intense investigation.

It is difficult to directly study the cellular mechanisms of reperfusion injury in intact tissue because of the presence of multiple cell types and the complexity of the vascular bed. On the other hand, it is difficult to accurately model ischemia in isolated cell systems because of the lack of diffusion barriers. We have attempted to partially model ischemia by producing severe hypoxia and inhibition of glycolysis, which are major components of ischemia, in cultured chick embryo ventricular myocytes, and we have studied the mechanisms by which reoxygenation induces cell injury in this model.

Methods

Tissue Culture

Spontaneously contracting layers of chick embryo ventricular cells were prepared according to previous methods. Briefly, ventricles from 10-day-old embryos were removed and digested in calcium–magnesium–free 0.025% trypsin solution at 37°C. Four dissociation cycles of 7 minutes in duration were performed, and the cells in the supernatant removed from each cycle were plated in cold trypsin inhibitor and centrifuged at 1,400 rpm for 10 minutes. Cells were resuspended in culture media consisting of 6% heat inactivated fetal calf serum, 40% medium 199 (Gibco, Grand Island, N.Y.), 0.1% penicillin streptomycin antibiotic solution, and 54% balanced salt solution containing (mM): 116 NaCl, 1.0 NaH2PO4, 0.8 MgSO4, 1.18 KCl, 26.2 NaHCO3, 0.87 CaCl2, and 5 glucose. Cells were plated on 25-mm glass coverslips or in glass Leighton culture tubes at a density of 7.5×10^5 cells/ml. Cultures were incubated in a 95% room air and 5% CO2 atmosphere at 37°C, and studies were performed on cells after 3–4 days of culture.

Production of Hypoxia and Reoxygenation

For physiological studies, cells were superfused with bicarbonate-buffered Hanks’ solution, pH 7.35. Hypoxia was produced by gassing cells in Hanks’ solution with 95% nitrogen and 5% CO2, bubbled through a 5% alkaline pyrogallol solution to maintain partial pressure of oxygen less than 1 mm Hg. Samples of gas and buffer solution from representative experiments were analyzed for oxygen content on an automated blood gas analyzer (ABL-300, Radiometer, Copenhagen). An oxygen-sensitive electrode was calibrated to the hypoxic gas (Po2 <1 mm Hg), inserted in the sealed chamber, and used to document the Po2 level in the solution superfusing the cells. During a typical experiment, the Po2 decreased to approximately 1 mm Hg within 20 minutes after initiation of superfusion with hypoxic solution. For cell motion and [Ca2+] measurements, a coverslip of cultured cells was placed in a round aluminum chamber with a sealed screw lid on the stage of the microscope. Two holes, one on the lid and one at the base, were fitted with 25-mm glass coverslips to allow observation of the cells. The chamber was gassed with hypoxic gas, and a constant gas flow was maintained throughout the study with minimal positive pressure. At the time of reoxygenation, the gassing mixture was changed to 95% room air and 5% CO2. Control cells were incubated in 95% room air and 5% CO2 atmosphere for the duration of the experiment. For lactate dehydrogenase (LDH) release experiments, cells were subjected to hypoxia and reoxygenation in Leighton tubes. A manifold of copper tubing was constructed that allowed gassing of cells cultured in four Leighton tubes simultaneously. All studies were performed at 37°C and pH 7.35.

Measurement of Cell Motion

Cell motion was measured as reported by Barry et al. Microspheres were added to layers of cultured myocytes attached to glass coverslips. After placement of a coverslip in the sealed chamber (described above) on the stage of a Nikon Diaphot inverted microscope (S&M Microscopes, Colorado Springs, Colo.), the motion signal was quantified with a video motion detector. Contraction was measured by tracking the motion of a plastic microsphere attached to the surface of a layer of cultured cells, along a raster line segment of a video image. The video image of the microsphere was adjusted by rotating the video camera so that the analog voltage output of the motion detector increased during systole. The motion signal thus recorded reflected contraction of a segment of the cell layer. These signals were recorded on a strip chart recorder and on magnetic tape.

Measurement of Changes in [Ca2+]

[Ca2+], was measured simultaneously with cell motion by means of the fluorescent Ca2+-probe indo 1 as described by Peeters et al. After 30 minutes of incubation in 5 μM indo 1-AM loading solution, coverslips were washed in normal culture medium for 10 minutes and then were placed in the sealed chamber on the stage of the microscope. Indo 1 loaded cells were excited by a mercury–arc lamp system at a 360-nm wavelength through an epifluorescence attachment and a ×40 Fluor (Nikon) objective lens. Fluorescent light was collected by the objective lens and then was divided with a dichroic mirror to permit simultaneous measurement of the intensity of both 410- and 480-nm wavelengths by means of two separate photomultiplier tubes (model 1869 AH, Hamatusu, Toyooka, Japan). The intensity of the fluorescence at the 410-nm wavelength increases, whereas that at 480-nm decreases, with increases in [Ca2+]. Therefore, the ratio of 410- to 480-nm fluorescence intensities was used as an indicator of [Ca2+]. The average [Ca2+] was measured from a 300-μm-diameter segment of the objective field. Output from the two photomultiplier tubes and the ratio values were recorded on a strip chart.
45Ca Uptake

These experiments were performed as described by Barry and Smith.11 Briefly, coverslips with attached layers of myocytes were incubated for 24 hours in an l-(4,5-3H,N)-leucine (2 μCi/ml) solution. The 3H-leucine was incorporated into the cell protein and was used to normalize 45Ca uptake relative to cell protein concentration. After equilibration in Hanks’ balanced salt solution at 37°C and in a 5% CO2 atmosphere for 30 minutes, cells were exposed to balanced salt solution with 20 mM 2-deoxyglucose and 5 mM pyruvate in a bell jar. Hypoxic gas was introduced into the jar for 3 hours at 37°C. After 3 hours of hypoxia, cells were immediately placed in a room air HEPES-buffered 1.8-mM Ca2+ solution containing 5 μCi/ml 45Ca maintained at 37°C. Coverslips were removed from 45Ca-uptake solutions after 30 seconds, 1 minute, or 5 minutes and were washed in ice-cold HEPES buffer containing 1.8 mM Ca three times for 15 seconds each. After washing, cells were scraped off the coverslips, placed in a solution containing 1% sodium dodecyl sulfate and 10 mM Na2B407, were allowed to dissolve for 2 hours, and then were placed in 10 ml Aquasol for liquid scintillation counting. Samples were counted for 3H and 45Ca. Subsequently, the ratio of 3H counts to protein, determined by the Lowry method12 and the 45Ca-uptake value for each coverslip were calculated as nanomoles of calcium per milligram of protein. In some experiments, cyanide (1 mM) was added to hypoxic cells 5 minutes before reoxygenation and was added to the 45Ca-uptake solution as well. Control cells were exposed to room air and 5% CO2 for 3 hours, and then 45Ca-uptake was measured as described above.

Lactate Dehydrogenase Release

LDH release was quantified as previously described.13 Ventricular myocytes previously cultured in Leighton tubes were incubated in 2 ml Hanks’ balanced salt solution and were gassed for 3 hours with hypoxic gas (PO2 < 1 mm Hg). Aliquots (200 μl) of cell supernatant were removed during hypoxia, and LDH release was measured. Subsequently, the cells were reoxygenated with 95% room air and 5% CO2, and aliquots of supernatant for measuring LDH release were obtained after 5, 10, and 15 minutes of reoxygenation. After 15 minutes of reoxygenation, the remaining cells were resuspended in 1 ml buffer and were sonicated to disrupt cell membranes, and the concentration of LDH was determined. The percentage of LDH released for each time point was then calculated (aliquot LDH concentration multiplied by solution volume and divided by the total LDH content, which was the sum of LDH in aliquots and LDH in medium after cell disruption). Control cells were incubated in Hanks’ solution in 95% air and 5% CO2 atmosphere, and aliquots were obtained and processed in the same manner.

ATP Content

Cellular ATP content was measured during hypoxia and reoxygenation by means of a luciferase assay (ATP Bioluminescence CLS, Boehringer Mannheim, New York) as previously described.13 Coverslips of cultured myocytes were incubated in Hanks’ balanced salt solution containing 2-deoxyglucose, and hypoxia was produced for 3 hours. Control cells, cells subjected to 3 hours of hypoxia, or cells reoxygenated for 1, 2, 3, 4, 5, or 15 minutes were frozen in liquid nitrogen and were scraped into 1 ml ice-cold 0.7N HClO4. After homogenization and addition of 1 ml ice-cold H2O, the samples were divided equally for ATP assay and for protein assay. To the ATP sample, 60 μl 1.5 M K2HPO4 was added, followed by centrifugation at 3,000 rpm and 4°C for 20 minutes. After removal of the supernatant, a 10-μl sample was added to 990 μl distilled water and analyzed in a liquid scintillation counter using the ATP CLS kit. A standard curve with known ATP concentrations was prepared to determine the ATP concentration. Protein was assayed by the Lowry method,12 and the final ATP concentration was expressed as nanomoles per milligram of protein for each coverslip.

Solutions

The Hanks’ bicarbonate-buffered balanced salt used contained (mM): 113 NaCl, 1.0 NaH2PO4, 0.81 MgSO4, 4.0 KCl, 26 NaHCO3, 1.8 CaCl2, and 5 glucose. In hypoxia experiments, Hanks’ solution contained zero glucose, 20 mM 2-deoxyglucose (to inhibit glycolysis), and 5 mM pyruvate (to serve as metabolic substrate after reoxygenation). The physiological HEPES solutions used contained (mM): 137 NaCl, 3.7 KCl, 0.5 MgCl2, 1.8 CaCl2, 5.6 glucose, and 5.0 HEPES. Choline chloride was substituted for sodium chloride in zero-sodium–HEPES solutions to examine the effects of 2,3-butanedione monoxime (BDM) on zero-sodium contracture. BDM was directly dissolved in either Hanks’ or HEPES salt solutions. To examine the effect of free radical scavengers, 100 units/ml each of superoxide dismutase and catalase were directly dissolved in Hanks’ solution.

Statistical Analysis

Means±SEM were obtained for each group. Intergroup comparisons were interpreted with the t test for two groups, and multiple treatments were analyzed by the unpaired analysis of variance. Statistical significance was identified as the 95% confidence level.

Results

We first sought to demonstrate that reoxygenation of hypoxic myocytes results in injury in this model. LDH enzyme release was used as a marker of
myocyte injury. The cells were exposed to severe hypoxia (Po₂ <1 mm Hg) alone or to hypoxia followed by reoxygenation, and the supernatant was analyzed for LDH activity (expressed as percentage of total LDH). As shown in Figure 1, LDH release during hypoxia (23.7±2.8%, n=8) was substantially greater than during control conditions (7.28±1.0%, p<0.001). However, 15 minutes after resupply of oxygen, a much greater cumulative release of LDH occurred in reoxygenated cells (63.2±4.6%) than in hypoxic cells (32.3±1.4%, p<0.05). In reoxygenated cells, most of the increase in LDH release occurred within the first 5 minutes of reoxygenation. Thus, these cells exhibit an “oxygen paradox” in which LDH release is increased by reoxygenation after prolonged hypoxia. This indicates that this preparation is a suitable model for the study of the mechanisms involved in reoxygenation injury.

We next examined the effects of severe hypoxia, followed by reoxygenation, on [Ca²⁺] and cultured myocyte motion. Simultaneous [Ca²⁺] and cell layer motion during spontaneous contractions were measured in 10 separate hypoxia–reoxygenation experiments with tracings during control, after exposure to hypoxic solution containing 20 mM 2-deoxyglucose and 5 mM pyruvate, at 1 and 3 hours of hypoxia, and then during reoxygenation. Examples of effects of hypoxia and reoxygenation on [Ca²⁺] and motion are

**Figure 1.** Plot showing effects of hypoxia and reoxygenation on lactate dehydrogenase (LDH) release. Cells were made hypoxic for 3 hours and then were reoxygenated for 15 minutes. Samples were obtained during hypoxia (0 minutes) and at 5, 10, and 15 minutes after reoxygenation for the hypoxia–reoxygenation group (top line). A second group of cells (middle line) was made hypoxic for 3 hours and 15 minutes, and LDH release was measured at 3 hours and at 3 hours 15 minutes of hypoxia. LDH release at 3 hours 15 minutes was significantly greater in the hypoxia–reoxygenation group than in either the hypoxia–hypoxia group (p<0.05) or the control group (bottom line, p<0.001).

**Figure 2.** Tracings showing initial effects of hypoxia in the presence of zero glucose, 20 mM 2-deoxyglucose, and 5 mM pyruvate on [Ca²⁺], and motion. Ultraviolet light source was shut off during the center of the trace to reduce bleaching of indol 1. Note that the cells initially stopped beating after initiation of hypoxia in the presence of 2-deoxyglucose and pyruvate (arrow), then developed rigor (upward displacement of the motion signal) after 20 minutes. At this time, [Ca²⁺], was elevated and slowly increasing.
shown in Figures 2, 3, and 4. Figure 2 shows an example of the initial effects of exposure to hypoxia and 2-deoxyglucose plus pyruvate. Contractions ceased within 5 minutes, and the cultured cells initially relaxed. After approximately 20 minutes of hypoxia, the motion trace moved upward to a point above the position reached during normal contraction, indicating development of rigor. During rigor development, [Ca\textsuperscript{2+}]i was higher than the diastolic levels during normal beating. These effects of hypoxia plus 2-deoxyglucose are similar to those produced by cyanide plus 2-deoxyglucose in these cultured myocytes.9

Examples of the subsequent time course of changes in [Ca\textsuperscript{2+}]i and motion are shown in Figure 3. By 1 hour, the [Ca\textsuperscript{2+}]i in hypoxic cultured cells had risen much higher than the level achieved at peak systole during normal beating before hypoxia. The [Ca\textsuperscript{2+}]i remained at this level after 3 hours of hypoxia. The motion trace displayed a stable “rigor position” after 1 and 3 hours of hypoxia. Reoxygenation was then produced by exposing cells to warm, humidified 95% room air and 5% CO\textsubscript{2}. Within 1–2 minutes after reoxygenation, [Ca\textsuperscript{2+}]i decreased, and hypercontracture occurred, defined as an upward displacement of the motion trace from the previously stable rigor position. To estimate the actual values of [Ca\textsuperscript{2+}]i, achieved after 3 hours of hypoxia, and during reoxygenation, we attempted to calibrate the [Ca\textsuperscript{2+}]i, corresponding to the 410- to 480-nm ratio by exposing cells to the nonfluorescent Ca\textsuperscript{2+} ionophore BromoA23187 in the presence of Ca\textsuperscript{2+}-buffered solutions.10 This is difficult under these conditions because after the initial decline, the [Ca\textsuperscript{2+}]i signal usually deteriorates because of indo 1 loss from damaged cells. Also, because of the fragility of the cells, they were easily dislodged from the coverslips during solution changes. However, calibrations were obtained in two experiments, one of which is shown in Figure 4. As expected from our previous work,9 the [Ca\textsuperscript{2+}]i, after 3 hours of hypoxia plus glycolytic inhibition was much higher than the normal peak systolic values, and although [Ca\textsuperscript{2+}]i declined slightly with reoxygenation, it remained greater than 1 μM, higher than the normal peak systolic level.10

The above-mentioned finding of a slight, but consistent, initial decrease in [Ca\textsuperscript{2+}]i on reoxygenation appeared to contrast with previous reports of an increase in \textsuperscript{45}Ca uptake with reperfusion of ischemic tissue.4 Therefore, we investigated effects of reoxygenation on \textsuperscript{45}Ca uptake in these cultured myocytes. Cultured myocytes were exposed to 3 hours of hypoxia with 20 mM 2-deoxyglucose and 5 mM pyruvate and then reoxygenated in the presence of \textsuperscript{45}Ca as described in “Methods.” \textsuperscript{45}Ca uptake was also measured in normoxic cells and in hypoxic cells reoxygen-
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Figure 4. Tracings showing effects of hypoxia and reoxygenation on $[Ca^{2+}]_i$ and motion. $[Ca^{2+}]_i$ and motion traces were stable between 1 and 3 hours of hypoxia. Note again the decrease in $[Ca^{2+}]_i$ and the increase in motion trace above the rigor position after reoxygenation (arrow). On the right are the calibrated $[Ca^{2+}]_i$ concentrations determined by the bromo-A23187 method. Again, because of noise in $[Ca^{2+}]_i$ signals at 1 and 3 hours, the signal was filtered at 1 Hz during reoxygenation.

Figure 5. Plot showing effects of reoxygenation on $^{45}$Ca uptake. Cells were made hypoxic for 3 hours, then $^{45}$Ca uptake during reoxygenation was measured (upper curve, hypoxia–reoxygenation). $^{45}$Ca uptake was also measured during reoxygenation in the presence of 1 mM cyanide (N, middle curve) and in control cells (lower curve). Note that resupply of oxygen significantly increased $^{45}$Ca uptake compared with control values at each time point. Presence of 1 mM cyanide reduced $^{45}$Ca uptake, although uptake was still greater than control. Points indicate mean±SEM, n=8.

ated in the presence of cyanide. The results are shown in Figure 5. $^{45}$Ca uptake after reoxygenation was significantly increased relative to control uptake. $^{45}$Ca uptake on reoxygenation was significantly reduced by the presence of cyanide, suggesting that ATP production by oxidative phosphorylation is involved in stimulation of calcium uptake with reoxygenation. Because $[Ca^{2+}]_i$ is declining under these conditions (see above), these data suggest also that the increase in calcium uptake is largely due to sequestration of $Ca^{2+}$ within intracellular organelles, such as sarcoplasmic reticulum and mitochondria.

Within 5 minutes after reoxygenation as described above, cell position increased above the previously stable rigor position. Reoxygenation may have been producing hypercontracture by initiating ATP resynthesis in the presence of an increased $[Ca^{2+}]_i$, thus inducing $Ca^{2+}$-dependent myofilament crossbridge cycling. This hypothesis was investigated by studying the effects on reoxygenation hypercontracture of
either BDM, a reported inhibitor of Ca\textsuperscript{2+}-dependent crossbridge cycling\textsuperscript{14,15} or cyanide, an inhibitor of oxidative phosphorylation. Figure 6 shows the effect of BDM on [Ca\textsuperscript{2+}], and motion in cultured ventricular myocytes. In panel A, 20 mM BDM decreased the amplitude of [Ca\textsuperscript{2+}], transients slightly, but it totally eliminated motion. Subsequent exposure to zero Na\textsuperscript{+}, which increases [Ca\textsuperscript{2+}], by sodium–calcium exchange,\textsuperscript{16} produced an increase in [Ca\textsuperscript{2+}], above peak systolic values, but no upward deflection of the motion trace occurred. This result should be contrasted with that shown in panel B, in which exposure to zero Na\textsuperscript{+} in the absence of BDM caused both an increase in [Ca\textsuperscript{2+}], and a marked upward displacement of the motion signal. Thus, exposure to 20 mM BDM markedly decreased the sensitivity of the contractile elements to Ca\textsuperscript{2+} in these cultured ventricular myocytes.

Figure 7 shows the effects of pretreatment with 20 mM BDM on rigor development and reoxygenation hypercontracture. These myocytes were made hypoxic for 2 hours. BDM significantly prolonged the time to rigor development (p < 0.01) and reduced the magnitude of rigor as shown in Table 1. On reoxygenation in the presence of BDM, monocytes relaxed within 5 minutes, whereas reoxygenated myocytes from the same cultures not exposed to BDM consistently developed hypercontracture. The motion of the microscope was calibrated in micrometers and arbitrarily given a + sign for upward displacement from the rigor position and a − sign for downward displacement from the rigor position. After 2 hours of hypoxia, cells that were reoxygenated in the absence of BDM hypercontracted +11.5±7.3 μm, whereas cells in the presence of BDM relaxed −19.1±5.9 μm (n=5) (p<0.005). After 3 hours of hypoxia, cells that were reoxygenated in the absence of BDM hypercontracted +6.1±1.9 μm, and cells in the presence of BDM relaxed −4.2±1.3 μm (p<0.001).

These results suggest that resupply of oxygen allows resynthesis of ATP, thus sensitizing the myofilaments to Ca\textsuperscript{2+} and causing hypercontracture. To investigate further the possible role of ATP resynthesis by oxidative phosphorylation, we studied the effects of cyanide on reoxygenation hypercontracture. Cells that were reoxygenated in the presence of cyanide after 2 hours of hypoxia relaxed −6.9±1.5 μm, and cells reoxygenated in the absence of cyanide hypercontracted +11.5±7.3 μm. We next sought to determine whether resupply of oxygen results in resynthesis of ATP. Under normoxic control conditions, ATP content was 29.4±2.6 nmol/mg protein. As shown in Figure 8, ATP content decreased to 2.6±0.7 nmol/mg protein after 3 hours of hypoxia and slowly recovered after reoxygenation to approximately one fourth of the normal values by 15 minutes (p<0.01). Thus, the total ATP content of myocytes increased slightly early after resupply of oxygen.

Hypercontracture during reoxygenation has been suggested to contribute to cell injury by producing mechanical stress on a fragile sarcolemma.\textsuperscript{5,17} Because the above-mentioned results indicate that
BDM inhibits reoxygenation hypercontracture, we investigated the effects of BDM on reoxygenation-induced cell injury. In these experiments, Leighton tubes with cells from the same culture with and without BDM were simultaneously reoxygenated. The results are shown in Table 2. LDH release after 2 or 3 hours of hypoxia followed by 15 minutes of reoxygenation was reduced in the presence of BDM ($p<0.001$). Thus, BDM appears to reduce cell injury during reoxygenation, and this result is consistent with the idea that a component of reoxygenation injury results from hypercontracture and may be due to resynthesis of ATP in the presence of high $[Ca^{2+}]_{i}$.

As previously mentioned, considerable work has suggested that enhanced production of free radicals during reoxygenation or reperfusion may cause myocyte injury. To investigate the possible contribution of free radical production to reoxygenation injury in these cultured ventricular myocytes, we also studied the effects of the free radical scavengers superoxide dismutase and catalase on LDH release induced by reoxygenation. LDH release during 3 hours of hypoxia was not significantly different between cells in the presence and absence of free radical scavengers (19.9±1.9% versus 23.1±2.7%). In contrast, LDH release after reoxygenation was significantly less (26.6±2.2%) in cells reoxygenated in the presence than in the absence of free radical scavengers (43.8±3.1%, $p<0.001$). This effect of free radical scavengers in reducing reoxygenation-induced LDH release suggests that free radicals also contribute to reoxygenation-induced cell injury in this model.

**Discussion**

**Reperfusion and Reoxygenation Injury**

Complete interruption of coronary arterial flow (ischemia) for more than 40–60 minutes results in

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Values are mean±SEM. $n=8–12$.  
BDM, 2,3-butanedione monoxime.
the onset of myocardial cell necrosis, and myocardial damage is complete after 3–6 hours of ischemia. The institution of reperfusion before 3–6 hours is necessary for partial salvage of ischemic myocardium, although restoration of coronary flow may accelerate myocardial injury.16,19 The concept of "reperfusion injury" requires that a portion of ischemic myocardium becomes irreversibly injured as a result of processes initiated on restoration of arterial blood flow.19 The success of pharmacological and mechanical interventions, such as thrombolysis or percutaneous transluminal coronary angioplasty, in preserving myocardium during myocardial infarction may be limited by this phenomenon, and therefore, an understanding of the mechanisms involved is important.

These mechanisms can be much more readily studied in cultured or isolated myocardial cells, but as mentioned previously, modeling of ischemia is experimentally difficult. We have simulated two major components of ischemia by producing hypoxia and inhibiting glycolysis in cultured ventricular myocytes. We chose a 3-hour period of hypoxia because previous studies in our laboratory showed that this time period causes both reversible and irreversible injury of these cultured myocytes.13 After 3 hours of hypoxia, we observed almost a threefold increase in LDH release after reoxygenation. This is consistent with previous findings in isolated cultured mouse hearts20 and in perfused rat hearts17 that a significant abrupt release of LDH and creatine kinase occurs after resupply of oxygen to myocardium previously made severely hypoxic. Enzyme release on reexposure to oxygen has been temporally correlated with ultrastructural changes such as separation of sarcomere attachments and contraction-band formation.5 As mentioned previously, this phenomenon, the oxygen paradox,2 may account for an important component of reperfusion injury in myocardium. However, other factors including washout of extracellular metabolites with abrupt cell swelling and injury to the microvasculature may be also important in intact tissue.

**Role of Changes in \([Ca^{2+}]\) in Reoxygenation Injury**

Several investigators have suggested that calcium overload after reoxygenation may be a major cause of reoxygenation-induced cellular injury.4,21,22 Shen and Jennings23 proposed that ischemia may result in membrane alterations, including a calcium leak, and Reimer and Jennings1 suggested that this uncontrolled calcium influx on reperfusion activates phospholipases and proteases that may contribute to reperfusion-induced myocardial damage.1 Consistent with these hypotheses, other studies have noted that \(^{45}Ca\) uptake is increased after resupply of oxygen in perfused rabbit septum,4 and that total calcium content may increase after reoxygenation in isolated perfused hearts22 and in cultured ventricular myocytes.24 This calcium gain on reperfusion of reoxygenation may be due to a nonspecific "leak," to enhanced calcium entry by the slow calcium channel,4 or to sodium–calcium exchange.24 However, Nakanishi et al25 found that in the arterially perfused hypoxic rabbit intraventricular septum preparation, reoxygenation caused increased uptake of isotopic Ca\(^{2+}\) and Sr\(^{2+}\), but not of Ba\(^{2+}\), although these divalent cations can enter by the slow Ca\(^{2+}\) channel. Because at the experimental temperature used (27°C), Ca\(^{2+}\) and Sr\(^{2+}\) were taken up by mitochondria and sarcoplasmic reticulum but Ba\(^{2+}\) was not, these investigators25 concluded that increased Ca\(^{2+}\) uptake on reoxygenation of myocardium reflected Ca\(^{2+}\) uptake by sarcoplasmic reticulum or mitochondria and that this was dependent on resumption of ATP synthesis.

Our data are consistent with this interpretation in that we show that \(^{45}Ca\) uptake is increased with reoxygenation and that this increase in \(^{45}Ca\) uptake may be dependent, in part, on the resynthesis of ATP.
by oxidative phosphorylation. Moreover, direct measurement of \([\text{Ca}^{2+}]\), with indo 1 in our experiments demonstrates that although \([\text{Ca}^{2+}]\) is markedly increased during prolonged hypoxia in the presence of glycolytic inhibition, with reoxygenation there is not an initial further increase in intracellular \([\text{Ca}^{2+}]\), but rather a decline. This finding is similar to that recently reported by Allshire and associates\(^{26}\) in isolated rat ventricular myocytes loaded with aequorin and subjected to 60 minutes of hypoxia. In these cells, reoxygenation resulted in a decrease of \([\text{Ca}^{2+}]\), and this effect was inhibited by 5 mM caffeine. In addition, Smith and Allen\(^{27}\) recently reported an abrupt decrease in \([\text{Ca}^{2+}]\), after washout of metabolic inhibitors after a 20-minute exposure in ferret papillary muscles. Thus, resynthesis of ATP after reoxygenation may activate \(\text{Ca}^{2+}\) uptake by sarcoplasmic reticulum and perhaps mitochondria, resulting in a decrease in \([\text{Ca}^{2+}]\), coincident with increased cellular calcium uptake. Despite a fall in \([\text{Ca}^{2+}]\) on reoxygenation, it is important to note that \([\text{Ca}^{2+}]\), remained considerably elevated in our experiments, and in those of Smith and Allen,\(^{27}\) at a \([\text{Ca}^{2+}]\), that is sufficient to activate contractile element force development. However, our results argue that an abrupt increase in \([\text{Ca}^{2+}]\), with reoxygenation is not the initial cause of cultured myocyte injury during the oxygen paradox, although it must be recognized that differences in factors contributing to reoxygenation injury may differ in adult and fetal ventricular myocytes.

There is probably some heterogeneity of cell injury during hypoxia and reoxygenation in this model system, and consideration should be given to how this may influence interpretation of the results. During hypoxia, there is increased LDH release reflecting an increase in sarcolemmal permeability in some cells (Figure 1). This increase in permeability would be expected to result in loss of indo 1 and intracellular proteins from those cells; thus, the 410- to 480-nm fluorescence ratio after 2 and 3 hours of hypoxia is likely being recorded primarily from those cells in the microscope field from which LDH has not yet been released. However, because the 410- to 480-nm fluorescence ratio used to estimate \([\text{Ca}^{2+}]\), is relatively independent of intracellular indo 1 concentration\(^{10}\) and because the indo 1 released from damaged cells (or transported out of intact cells) diffuses away in the superfusate, the changes in \([\text{Ca}^{2+}]\), that we have estimated from the indo 1 signals in these experiments should continue to be a reasonably valid estimate of \([\text{Ca}^{2+}]\), in intact cells before and immediately after reoxygenation. Likewise, changes in ATP contents and \(^8\)Ca uptake are normalized to cell protein and, thus, are contributed to primarily by cells that are still intact and attached to the coverslip or Leighton tube surface. Thus, the results reported should be valid for cells that are intact before and immediately after reoxygenation.

Role of \([\text{Ca}^{2+}]\), in Reoxygenation Hypercontracture

The cause of reoxygenation hypercontracture has been investigated for some time. Hohl et al\(^{28}\) showed that hypoxia plus glucose deprivation caused rod-shaped adult rat ventricular myocytes to shorten to a square rigor shape. Reoxygenation of these hypoxic cells resulted in hypercontracture manifest by a change in morphology from a square to a round form. This occurred even in the presence of 0.1 mM EGTA, which brought into question the importance of \(\text{Ca}^{2+}\) in this phenomenon. In addition, Nichols and Lederer\(^{29}\) demonstrated that in saponin-skinned rat ventricular myocytes, slight shortening beyond a stable rigor length can occur during resupply of ATP at very low \([\text{Ca}^{2+}]\). Other experiments\(^{30}\) in digitonin-permeabilized myocytes showed that at low concentrations of ATP (1-10 \(\mu\)M), hypercontracture can occur at very low concentrations of \(\text{Ca}^{2+}\), which is consistent with a marked leftward shift in the \(\text{Ca}^{2+}\)-force relation at low ATP levels noted in skinned myocardial fibers by McClellan and Winegrad.\(^{31}\) More recently, however, Li et al\(^{32}\) reported that in intact adult rat ventricular myocytes, only when \([\text{Ca}^{2+}]\) increases greater than 500 nM during deenergization with amyal and carbonyl cyanide \(m\)-chlorophenylhydrazone (CCCP) does hypercontracture from a square to round form occur with reenergization. This suggests that an increased \([\text{Ca}^{2+}]\), is important in reoxygenation hypercontracture in intact cells. Our data also indicate that reoxygenation hypercontracture is dependent on calcium and probably ATP because this process could be inhibited by either BDM, an agent that inhibits calcium-induced force development by myofilaments,\(^{14}\) or cyanide, an inhibitor of oxidative phosphorylation. It is true that ATP content only increased slowly during reoxygenation, requiring 15 minutes to reach significance. However, ATP content reflects the balance between utilization and production. During resupply of oxygen, changes in total ATP content probably do not accurately reflect the time course of increased ATP production because of a high ATP utilization rate. Thus, we speculate that hypercontrac- ture results, at least in part, from myofilament crossbridge cycling induced by ATP synthesis in the presence of an elevated \([\text{Ca}^{2+}]\).

Cell Injury Produced by Hypercontracture

During severe hypoxia, alterations in the cytoskeletal system within myocytes have been suggested to occur, which include weakening of actin filament attachments to the sarcolemma at the fascia adherens of the intercalated disks and at the lateral costamere junctions.\(^{17}\) During reoxygenation-induced hypercontracture, increased stress is applied to the altered cytoskeleton and weakened sarcolemma, which has been proposed to contribute to cell rupture and loss of cell enzymes.\(^{5,17}\) Our data are consistent with this hypothesis in that BDM inhibited hypercontracture on reoxygenation and significantly reduced LDH release on reoxygenation. This result is also consistent with studies showing that the addition
of oxidative phosphorylation uncouplers and inhibitors, such as CCCP, cyanide, and 2,4-dinitrophenol, can reduce reoxygenation-induced enzyme release\textsuperscript{33,34} and hypercontracture (see above). Of note, in our studies, the magnitude of hypercontracture on reoxygenation was greater after 2 hours than after 3 hours of hypoxia, whereas the magnitude of LDH release on reoxygenation was less. This finding is consistent with the hypothesis that hypercontracture is not the only factor involved in reoxygenation injury and that the fragility of the sarcolemma is also important.

Our results regarding the protective effects of BDM are also consistent with the work of Nayler et al\textsuperscript{35} who found that 30 mM BDM increased functional recovery after reoxygenation of adult rat hearts subjected to glucose-free hypoxic perfusion for 30 minutes, and with the work of Mulieri et al\textsuperscript{36} who found that 30 mM BDM protected ventricular myocardium from “cutting injury.” Nayler et al\textsuperscript{35} found that BDM attenuated, but did not prevent, rigor in adult rat hearts subjected to 2 hours of hypoxia. Nichols and Lederer\textsuperscript{29} reported that ATP-depletion shortening (rigor) of permeabilized myocytes could be inhibited by BDM during an experimental observation time of 1–3 minutes. In our experiments, BDM reduced the magnitude and delayed the onset of rigor, but it did not prevent the development of rigor when intact cells were subjected to ATP depletion for 60–90 minutes. Thus, BDM may delay, but does not appear to completely inhibit, crossbridge rigor bond development.

**Role of Oxygen Free Radicals**

Increasing evidence supports the hypothesis that reperfusion or reoxygenation injury is partially a result of the production of oxygen free radicals.\textsuperscript{6} These include superoxide anions, hydroxyl radicals, and hydrogen peroxide produced within myocytes or by surrounding endothelial cells.\textsuperscript{37} Superoxide radicals are most often implicated as etiologic agents in primary and secondary free radical damage.\textsuperscript{38,39} In Langendorff perfused rabbit hearts, Zweier and coworkers\textsuperscript{40} found a burst of free radical production within 10 seconds after reperfusion of ischemic myocardium. The presence of recombinant superoxide dismutase reduced the oxygen free radical signal and preserved function in rabbit hearts throughout the reperfusion period. In addition, a beneficial effect of long-acting polyethylene glycol superoxide dismutase was recently reported by Chi and coworkers\textsuperscript{41} with limitation of infarct size after 6 hours of coronary artery occlusion followed by 24 hours of reperfusion in a canine model. Free radicals may induce sarcolemmal and cytoskeletal damage\textsuperscript{42} and, thus, contribute to increased myocyte fragility. Our results showing reduced cell injury with either free radical scavengers or BDM suggest that both increased myocyte fragility and increased sarcolemmal stress during hypercontracture contribute to reoxygenation-induced LDH release after prolonged hypoxia.

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