Coverslip hypoxia: a novel method for studying cardiac myocyte hypoxia and ischemia in vitro

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Pitts, Kelly R. and Christopher F. Toombs. Coverslip hypoxia: a novel method for studying cardiac myocyte hypoxia and ischemia in vitro. Am J Physiol Heart Circ Physiol 287: H1801–H1812, 2004.—In vitro experimental models designed to study the effects of hypoxia and ischemia typically employ oxygen-depleted media and/or hypoxic chambers. These approaches, however, allow for metabolites to diffuse away into a large volume and may not replicate the high local concentrations that occur in ischemic myocardium in vivo. We describe herein a novel and simple method for creating regional hypoxic and ischemic conditions in neonatal rat cardiac myocyte monolayers. This method consists of creating a localized diffusion barrier by placing a glass coverslip over a portion of the monolayer. The coverslip restricts covered myocytes to a thin film of media while leaving uncovered myocytes free to access the surrounding bulk media volume. Myocytes under the coverslip undergo marked morphology changes over time as assessed by video microscopy. Fluorescence microscopy shows that these changes are accompanied by alterations in mitochondrial membrane potential and plasma membrane dynamics and eventually result in myocyte death. We also show that the metabolic activity of myocytes drives cell necrosis under the coverslip. In addition, the intracellular pH of synchronously contracting myocytes under the coverslip drops rapidly. We conclude that myocyte death in this model is hastened by the combination of hypoxia, metabolites, and acidosis and is facilitated by a reduction in media volume, which may better represent ischemic conditions in vivo.

EVALUATION OF HYPOXIC and ischemic insults on isolated cardiac myocytes typically involves atmospheric chambers or modified culture media. Replicating a hypoxic environment using a nitrogen chamber is a classical manner to deprive a population of myocytes of oxygen, and several metabolic (25, 32, 33), genetic (16, 21, 29), and functional (22, 32) changes have been identified using this method. Reproducing the noxious metabolic environment associated with ischemia, however, is quite difficult. Glucose-depleted media (25, 27), ischemic solutions (2, 6, 22, 31), and low pH values (11) have all been used to mimic environmental changes that occur during ischemia, but these techniques fail to concentrate myocyte metabolic wastes similar to ischemia in vivo.

In this report, we describe a novel and simple method for studying the effects of hypoxia and ischemia in isolated neonatal rat cardiac myocytes. We show that placing a glass coverslip over an existing myocyte culture creates a barrier to diffusion and results in rapid hypoxia and locally concentrated metabolic by-products. Coverslipping myocytes results in dramatic intracellular morphology changes within 15 min that persist over a 3-h duration and lead to cell death. These changes coincide with loss of synchronous contractile activity and mitochondrial membrane potential and appearance of chromatin margination and plasma membrane derangement. These effects are specific to myocytes, as ST3 fibroblasts, H9C2 myoblasts, and isolated primary cardiac fibroblasts show none of these changes over the same duration of time. In addition, myocyte death under the coverslip is prevented when myocytes are cultured on a semipermeable membrane. Furthermore, the intracellular pH of synchronously contracting myocytes under the coverslip decreases rapidly. We conclude that myocyte death in this model is hastened by the combination of hypoxia, metabolites, and acidosis, is facilitated by a reduction in media volume, and may better represent ischemic conditions in vivo.

MATERIALS AND METHODS

Isolation and Culture of Neonatal Cardiac Myocytes and Fibroblasts

Cardiac myocytes were isolated from day 0–2 Sprague-Dawley neonatal rat pup ventricles using the neonatal cardiomyocyte isolation system (Worthington Biochemical; Lakewood, NJ) as described by the manufacturer. Cells were filtered and pelleted by centrifugation at 300 g for 5 min. Myocytes were then additionally purified on a discontinuous Percoll (Amersham Biosciences; Piscataway, NJ) gradient of 1.050, 1.060, and 1.082 g/ml (top to bottom). Cardiac fibroblasts were extracted from the 1.050/1.060 interface and cultured on 35-mm dishes coated with 100 µg/ml Matrigel (BD Biosciences; Bedford, MA) in DMEM (JRH Biosciences; Lenexa, KS) that contained 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.3 mg/ml L-glutamine (Invitrogen; Carlsbad, CA). Extraction of myocytes at the 1.082/1.060 interface routinely produced myocytes >95% pure as judged by confocal fluorescence microscopy with an anti-tropinin I antibody (Santa Cruz Biotechnologies; Santa Cruz, CA) 2 days after plating. Myocytes were plated onto 35-mm dishes coated with 100 µg/ml Matrigel at a density of 1–1.2 million cells per 35-mm dish in DMEM as described above. Myocytes coupled electrically and began synchronously contracting within 48 h, and most monolayers were used for experiments between 3 and 6 days after plating.

For Transwell culture experiments, myocytes were plated onto Matrigel-coated Transwell cell culture inserts that had a pore size of 0.4 µm (Becton-Dickinson; Franklin Lakes, NJ). Inserts were used in conjunction with six-well companion plates (Becton-Dickinson).

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Cell Lines

NIH 3T3 (CRL-1658, American Type Culture Collection; Manassas, VA) and H9c2 (CRL-1446, American Type Culture Collection) cell lines were maintained in DMEM that contained 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) and were regularly passaged. For experiments, cells were plated under the same initial conditions as myocytes (i.e., 1–1.2 million cells per Matrigel-coated, 35-mm dish).

Thin-Film Measurements

Round, glass coverslips 18 mm in diameter (VWR; West Chester, PA) and chambered slides (Naige Nunc International; Naperville, IL) were coated with 100 μg/ml of FITC-labeled collagen I (Sigma; St. Louis, MO) in 0.02 N acetic acid for 3 h at room temperature. Coverslips and slides were then rinsed several times with HBSS, and slide chambers were filled with 2 ml of serum-free DMEM without phenol red (Invitrogen). A coverslip was gently placed in the chamber.

Fluorescence images were acquired using a Zeiss LSM 510 microscope (Carl Zeiss; Thornwood, NY) set in Z-series confocal mode. Optical sections of varying thicknesses were acquired through ×10, ×20, ×40, and ×63 objectives. Measurements were made on three-dimensional reconstructed Z-series images using tools provided within the standard LSM 510 software. The three-dimensional reconstructed Z-series images reported herein are representative of five experiments and were taken with the ×10 (Fig. 1B, inset) and ×63 (Fig. 1B) objectives.

Video Microscopy and Quantitation

A 35-mm dish that contained a synchronously contracting myocyte monolayer was mounted in a Leica DM IRB inverted microscope (Leica Microsystems; Bannockburn, IL) equipped with an environmental stage that consisted of a heating insert (Leica) controlled by a Tempcontrol 37–2 digital controller (Leica). An incubator (Leica) was attached to the heating insert, which maintained a heated and humidified atmosphere of 5% CO2 via a CTI Controller 3700 (Leica). Myocytes were covered using round, 18-mm-diameter coverslips. A single coverslip was gently placed over a portion of the monolayer, and the stage was then positioned to view myocytes directly under the center of the coverslip. Phase-contrast digital video was acquired through a ×10 objective at 30 frames/s using a cooled charge-coupled device (IonOptix; Milton, MA) connected to a FlashBus MV video frame-grabber card (Integral Technologies; Indianapolis, IN). Acquisition was managed using StreamPix software (version 3.6.0, NorPix; Montreal, Canada). Digital frames at specified time points were extracted from the video file, deinterlaced, and processed using Adobe Photoshop (version 7.0.1, Adobe Systems; San Jose, CA). Images in Fig. 2A are representative of seven separate experiments.

High magnification phase-contrast images of nuclei at 0 and 30 min under the center of the coverslip were acquired through a ×63 objective using a cooled charge-coupled device (Diagnostic Instruments; Sterling Heights, MI). Acquisition was managed using SPOT software (version 3.4.5, Diagnostic Instruments), and images were postprocessed using Adobe Photoshop. Images in Fig. 2B are representative of 10 images at each time point collected over 2 separate experiments.

Gross morphological phenotypes were quantitated as follows. Chromatin margination was defined as the time point when all myocytes in the field first displayed a distinct phase-dense ring within the nucleus. Asynchronous beating was defined as the time point when all myocytes in the field first displayed irregular beating patterns compared with those seen in previous frames. Because asynchronous beating progressed to contractile arrest, these two phenotypes were compiled and reported together. Severe vacuolization was defined as the time point when all myocytes in the field exhibited vacuoles in >50% of their cytoplasmic area.

Cell Viability Assay and Quantitation

For a given time point, a portion of a synchronously contracting myocyte monolayer was coverslipped as described (see Video Microscopy and Quantitation). The 35-mm dish that contained the coverslipped area was then returned to a standard tissue-culture incubator (i.e., 37°C and 5% CO2). After the specified duration, the coverslip boundary was marked on the dish, the coverslip was removed, and the monolayer was washed twice with culture media. The monolayer was then poststained with 100 nM tetramethylrhodamine ethyl ester (TMRE, Molecular Probes; Eugene, OR) and 50 μl/ml Alexa Fluor 488-annexin V (Molecular Probes) in serum-free DMEM without phenol red at 37°C for 20 min. Poststained cells were then rinsed twice in serum-free DMEM without phenol red and overlaid with the same for live imaging.

Fluorescence images were acquired using a Zeiss LSM 510 microscope that was prepared to collect 10-μm optical sections through a ×10 objective. Detector gains were adjusted at the border-zone region so that each wavelength was equally represented in the settings. Once adjusted, the gain values were locked for all images acquired in that particular dish. Images were acquired of center, midpoint, border-
zone, slip-edge, adjacent, and far-point regions (described in RESULTS) along the same axis. Images along three separate axes were acquired from each dish at each time point. Each time point was replicated twice using myocytes from separate isolations. Representative images selected from six images at each position and time point are shown (see RESULTS).

Fluorescence intensities for each wavelength were extracted from each image. Pixels binned at 0 and 255 were removed from each image by applying an appropriate threshold. The remaining intensities for a given wavelength were summed and then averaged with the summed intensities from corresponding regions. For a given wavelength, intensity ratios were calculated from intensity values corresponding to adjacent and far-point regions. TMRE ratios are reported in Table 1 as fractions of 1 with standard deviations.

Measurement of pH

The pH values in cultured myocytes were estimated using the fluorescent pH indicator carboxy-seminaphthorhodfluor-4F ace- toxymethyl ester (SNARF-4F, Molecular Probes). When excited at 488 nm, SNARF-4F undergoes a shift in relative intensity at two emission peaks, 640 and 585 nm, in response to a change in pH. Estimates of pH change were obtained by tracking the intensity changes through a 565–615-nm band-pass filter. For loading, a dye solution was prepared by dissolving 50 μg of SNARF-4F in 50 μl of DMSO (Sigma) to create a stock solution, which was diluted into 3 ml of media to obtain a final concentration of 10 μM. Myocytes were then incubated with the dye solution for 40 min at 37°C in 5% CO₂ to obtain SNARF-loaded myocytes. A dish containing SNARF-loaded myocytes was mounted in a Zeiss LSM 510 microscope equipped with the same environmental stage and incubator described (see Video Microscopy and Quantitation). SNARF-loaded myocytes were coverslipped using round, 18-mm-diameter coverslips. A single coverslip was gently placed over a portion of the monolayer, and the program- mable stage was positioned to image myocytes at center, midpoint, border-zone, and adjacent regions as described above. After coverslip placement, images were acquired of each of these regions during a 60-min time course. Experimental images (see Fig. 6) are presented as pseudocolored intensity maps and are representative of three separate experiments.

An approximate pH calibration in SNARF-loaded myocytes was performed as follows: a 35-mm dish that contained SNARF-loaded myocytes was incubated in the presence of a series of HEPES-buffered DMEM solutions of known pH (pH values of 7.0, 6.0, 5.0, and 4.0). After 5 min (to allow equilibration), detector and amplifier gains were optimized and set by the computer using the pH 7.0 dish, and images of 20-μm optical sections at center and adjacent regions were acquired at that pH value. The buffered solution was aspirated, and the steps were performed again (without adjusting detector or amplifier gains) using the next successive buffered solution. The images collected using the 565- to 615-nm band-pass filter and the corresponding pseudocolored intensity maps are presented in Fig. 6A.

RESULTS

Initial studies in which coverslips were placed onto the monolayers for 18 h resulted in universal myocyte necrosis under the coverslipped areas. Surprisingly, regions of the

![Image](http://ajpheart.physiology.org/)

**Fig. 2.** Coverslipped myocytes exhibited time-dependent morphology changes. A portion of a synchronously contracting monolayer of isolated rat neonatal cardiac myocytes was coverslipped, and phase-contrast video microscopy was performed on myocytes under the center of the coverslip. A: frames at 0- and 180-min time points revealed dramatic changes in intracellular morphology, including chromatin margina- tion and cytosolic vacuolization. Sections of frames along the time course (designated by black bar above frame 0) followed the progression of morphology changes. Images are representative of seven separate experiments. B: high-magnification images of separate nuclei at 0- and 30-min time points under the center of the coverslip revealed the striking chromatin margination (arrows) that appeared within 30 min. C: scoring the pheno- types exhibited by cells under the center of the coverslip showed that the onset of chromatin margination was rapid; it appeared in most cultures within 15 min and preceded contractile arrest, whereas severe vacuolization appeared between the 90- and 120-min time points.

**Table 1.** Fluorescence quantitation of tetramethylrhodamine ethyl ester for center, midpoint, and slip-edge regions

<table>
<thead>
<tr>
<th>Time Point, h</th>
<th>Intensity Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Center</td>
</tr>
<tr>
<td>1</td>
<td>0.73 ± 0.15</td>
</tr>
<tr>
<td>2</td>
<td>0.31 ± 0.06</td>
</tr>
<tr>
<td>3</td>
<td>0.27 ± 0.15</td>
</tr>
</tbody>
</table>

Values are intensity ratios derived from six images at each position and time point across two separate experiments (see MATERIALS AND METHODS).
myocyte monolayer not covered by the coverslip were healthy and viable and had retained the ability to synchronously contract. Furthermore, we noted the presence of a transition region within the myocyte monolayer at the coverslip edge. Myocytes in this region contracted more slowly and asynchronously compared with the remaining viable monolayer. Based on these initial observations, we hypothesized that the coverslip had created a regional hypoxic or ischemic event within the myocyte monolayer by drastically reducing the media volume accessible to covered myocytes.

Coverslip Placement Greatly Reduces Media Volume Accessible to Covered Myocytes

To test our hypothesis of coverslip-induced regional hypoxia, we performed confocal microscopy on coverslipped placed in chamber slide wells that contained media. Both the coverslips and the chamber slide floors were coated with FITC-labeled collagen (see Fig. 1A), which allowed us to easily and accurately define the borders of these structures using fluorescence microscopy. Assembling a three-dimensional reconstruction of Z-series optical slices taken with a ×10 objective allowed us to image through the entire chamber slide floor and the coverslip in a single series (see Fig. 1B, inset). Our optical measurements of the chamber slide floor and coverslip thickness were 115 ± 15 and 110 ± 9 μm, respectively. These values are within 15% of the smallest thickness in the range reported by the manufacturer for the coverglass and coverslip (130–160 μm; see DISCUSSION). Collectively, these measurements function as calibrations and confirm our ability to adequately measure thickness in Z series. Reconstruction of Z-series optical slices taken at the coverslip edge (see Fig. 1B) with a ×63 objective revealed that placement of the coverslip created a thin film above the chamber slide floor. The height of this thin film was repeatedly measured and found to be 9.8 ± 2.6 μm. Assuming that these measurements are underestimated by the same margin observed with the coverslip and chamber slide measurements, the height of the thin film could be as great as 14.25 μm. These data suggest that the volume of media available to myocytes beneath the coverslip is drastically reduced compared with the bulk volume that is accessible to myocytes that are not underneath the coverslip.

Coverslipped Myocytes Exhibit Time-Dependent Morphology Changes

Initial studies in which coverslips were placed on monolayers for 18 h resulted in widespread death of myocytes. To more fully characterize this progression, we performed phase-contrast video microscopy of covered myocytes over a shorter time course. Portions of synchronously contracting myocyte monolayers were coverslipped, and coverslipped dishes were then placed in an environmental chamber mounted to a phase-contrast microscope. Digital video of a single region directly under the center of the coverslip was collected at numerous intermittent time points over 3 h (see MATERIALS AND METHODS). Frames from a representative video file were extracted at selected time points and are presented in Fig. 2A. Over the 3-h duration, we observed dramatic and progressive changes in intracellular morphology. In the moments just after coverslip placement (Fig. 2A, frame 0), myocyte morphology and function were unchanged compared with those of uncovered myocytes. As early as 15 min, however, chromatin margination was observed in some cultures and most cultures exhibited this phenotype by 45 min [Fig. 2, A (frames 15 and 45) and C]. The chromatin margination was quite distinct when viewed at higher magnification to compare nuclei at 0 and 30 min after coverslip placement. Nuclear envelopes appeared to be nondescript just after coverslip placement (Fig. 2B, top) but developed a phase-dense boundary with marked thickening by 30 min (Fig. 2B, bottom). Chromatin margination was not observed in uncovered myocytes over the time course.

Interestingly, asynchronous contractions and/or contractile arrest occurred after chromatin margination in most cases (Fig. 2C). Within 30 min, most myocytes under the center of the coverslip exhibited asynchronous and slow sporadic contractions relative to myocytes not beneath the coverslip. Complete contractile arrest under the center of the coverslip was uniformly observed within 60 min (Fig. 2C) and persisted over the 3-h time course. At 3 h, myocytes at the edge of the coverslip continued to contract but with sporadic and reduced frequency compared with myocytes not under the coverslip, which is similar to observations at 18 h in the transition region; this suggests that the development of this region is rapid and sustainable.

Another noticeable phenotype was the appearance of cytoplasmic vacuoles. Some vacuoles could be seen in cultures by 30 min, and severe vacuolization (i.e., vacuoles comprising >50% of the cytoplasm by visual assessment) appeared in most cultures between 90 and 120 min [Fig. 2, A (frames 90 and 120) and C]. At 3 h (Fig. 2A, frame 180), myocytes exhibited complete cytoplasmic vacuolization reminiscent of those seen at 18-h time points. Vacuolization was not observed in uncovered myocytes over the 18-h time course.

Myocyte Viability and Function Are Differentially Compromised Under a Coverslip

The dramatic morphological changes grossly observed suggest that there may be corresponding molecular events driving the loss of myocyte viability and function under the coverslip. One of the first changes observed at the 18-h time point and subsequently at 1-h time points (Fig. 2C) was the cessation of contractile activity under the coverslip. We hypothesized that changes in cellular energy level production or distribution might be affecting contractile activity, and by staining myocytes with the fluorescent marker TMRE we could evaluate changes in mitochondrial membrane potential as an indirect measure of cellular energy changes. In addition, by staining myocytes with Alexa Fluor 488-annexin V, we could observe changes related to cell death. The combination of TMRE and annexin V staining would provide us with a useful viability assay to monitor related changes that occurred under the coverslip.

Portions of synchronously contracting myocyte monolayers were coverslipped for 0, 1, 2, or 3 h. Coverslips were removed, and cells were poststained with TMRE (red) and Alexa Fluor 488-annexin V (green). Confocal images were acquired of five distinct regions of myocytes from the center of the coverslip, past the coverslip edge, and remote regions not beneath the coverslip. Together, these series of images provide regional snapshots of cellular events at a given time point. As shown in Fig. 3A, myocytes in the center, mid-
Fig. 3. Myocyte viability and function were differentially compromised under the coverslip. Portions of synchronously contracting monolayers of isolated rat neonatal cardiac myocytes were coverslipped for varying durations. Coverslips were removed, and cells were poststained with tetramethylrhodamine ethyl ester (TMRE, red) and Alexa Fluor 488-annexin V (green). A: images of myocytes under the center of the coverslip, at the coverslip edge (slip edge), and halfway between these areas (midpoint) taken after 5 min of coverslipping (0 h) show that all myocytes stained positively for TMRE [TMRE(+)] and negatively for annexin V [annexin V(−)] compared with myocytes in images taken either adjacent to the slip edge and out from under the coverslip (adjacent) or at a distant point not beneath the coverslip (far point). At 1-, 2-, and 3-h durations, however, the number of TMRE(+) cells declined, whereas the number of annexin V(+) cells increased. A progressive decrease in TMRE-fluorescence intensity was observed at center regions beginning at 1 h. This progressive decrease was not seen at midpoint regions until 2 h. By 3 h, center- and midpoint-region TMRE fluorescence was similar with little change at the slip edge. B: imaging to within 1,000 μm of the slip edge confirms the presence of a forming border zone that separated TMRE(+) and annexin V(+) cells. Images are representative of six images at each position and time point across two separate experiments.
point, and slip-edge regions stained positive for TMRE (TMRE(+) and negative for annexin V (annexin V(−)) after 5 min of coverslipping (0-h row). No noticeable differences were observed when these regions were compared with the uncovered adjacent and far-point regions, which suggests that simple placement and removal of the coverslip did not alter myocyte viability or function. At 1-, 2-, and 3-h durations, however, generally the number of TMRE(+) cells decreased, whereas the number of annexin V(+) cells increased. Inspection of the fluorescent images revealed that many of the myocytes in the center region had already lost the ability to load the TMRE dye at 1 h (Fig. 3A, 1-h row), which indicates changes in the mitochondrial membrane potential. The lost ability to load TMRE corresponded with annexin V positivity, which suggests a progression to events that drove cell death. At 2 h, only a few TMRE(+) cells were seen; the majority of cells were annexin V(+), and annexin V positivity began increasing in the midpoint region (Fig. 3A, 2-h row). By 3 h, both the center and midpoint regions were devoid of TMRE(+) cells, whereas those cells present were annexin V(+). Also, small changes in the slip-edge region appeared at 3 h (Fig. 3A, 3-h row). Ratiometric quantitation of TMRE fluorescence at regions under the coverslip (see MATERIALS AND METHODS) revealed a progressive decrease in fluorescence intensity (Table 1) at center regions beginning at 1 h. This progressive decrease was not seen at midpoint regions until 2 h. By 3 h, center- and midpoint-region TMRE fluorescence values were similar. Although little change was seen in the slip-edge region at 3 h, imaging to within 1,000 μm of the slip edge confirmed the presence of a forming border zone separating TMRE(+) and annexin V(+) cells (Fig. 3B). These data suggest that myocyte viability and function are progressively and differentially compromised under the coverslip.

Myocyte Death Under Coverslips Is Regulated by a Diffusion Barrier

Based on the above data, we also hypothesized that cell necrosis under the coverslip might be prevented if covered myocytes were given access to a bulk media volume. To test this hypothesis, we employed semipermeable cultureware (i.e., Transwell inserts). Myocytes plated on the semipermeable membrane could be coverslipped from the top, but unlike previous experiments, the cells would have access to a bulk media volume from beneath through pores in the membrane.

Portions of synchronously contracting myocyte monolayers plated on Matrigel-coated culture dishes or Matrigel-coated semipermeable Transwell inserts were coverslipped. After 3 h, coverslips were removed, and cells were poststained with TMRE and Alexa Fluor 488-annexin V. A: images of myocytes on standard-coated cultureware taken at center and midpoint regions showed TMRE(−) and annexin V(−) cells and a forming border zone within 1,000 μm of the slip edge, which are typical for this coverslip duration, whereas adjacent uncovered myocytes remained TMRE(+) and annexin V(−). B: images of myocytes on coated semipermeable Transwell inserts taken at center, midpoint, and border-zone regions revealed no changes in myocyte viability under the coverslips compared with those of adjacent uncovered myocytes. C: changing myocyte viability was due to the ability of covered myocytes to freely exchange O2 and metabolites into a bulk volume that was accessible through the pores (arrows) in the semipermeable insert. Images are representative of six images at each position across three separate experiments.
1,000 μm of the slip edge), and adjacent regions as described above. Myocytes cultured on standard coated cultureware (Fig. 4A) were TMRE(−) and annexin V(+) in the center and midpoint regions, whereas adjacent uncovered myocytes remained TMRE(+) and annexin V(−). In contrast, images of myocytes on coated semipermeable Transwell inserts revealed that myocyte viability under the coverslip was unchanged compared with adjacent uncovered myocytes (Fig. 4B). Also, myocytes in all regions retained the ability to synchronously contract, which indicates that function was not impaired (data not shown). Higher magnification of the center region demonstrated that each contracting myocyte was in close proximity to several pores (Fig. 4C, arrows), which indicates unrestricted access to the bulk media volume under the membrane insert. These data strongly suggest that myocyte death under the coverslip is the result of a diffusion barrier created by placement of the coverslip.

**Cell Death Under Coverslips is Linked to Metabolic Activity**

Thus far we have evaluated only the effects of the diffusion barrier created by the coverslip on cardiac myocyte viability and function. We hypothesized that cell death occurring under the coverslip was related to the metabolic activity of cardiac myocytes and that a less metabolically active cell type might remain viable under the coverslip for the 3-h duration. To test this hypothesis, portions of NIH 3T3 fibroblasts or H9C2 cells [a rat heart myoblast line (15)] plated on Matrigel-coated culture dishes were coverslipped. After 3 h, coverslips were removed and cells were poststained with TMRE and annexin V. Images of 3T3 fibroblasts taken at center, midpoint, and border-zone regions showed TMRE(+) and annexin V(−) cells similar to those observed at uncovered regions adjacent to or at a far point from the slip edge (Fig. 5A); this indicates that covered fibroblasts remain viable over the 3-h duration. Images of H9C2 cells taken at similar regions exhibited a similar level of viability (Fig. 5B) compared with 3T3 fibroblasts. These data suggest that cell death under the coverslip over the 3-h time course was a direct result of the metabolic activity of the cells. Because cell lines might not be as susceptible to environmental insult as primary cells, we coverslipped isolated primary cardiac fibroblasts (see MATERIALS AND METHODS) for 3 h. We observed that cardiac fibroblasts at center, midpoint, and border-zone regions were TMRE(+) and annexin V(−) similar to those observed at uncovered regions adjacent to or at a far point from the slip edge (Fig. 5C). This indicates that covered cardiac fibroblasts remained viable over the 3-h duration. Again, these data suggest that cell death under the coverslip during the 3-h time course is the direct result of the metabolic activity of the cells.

**Intracellular pH Decreases in Myocytes Under Coverslips**

Because cell death under the coverslip is the direct result of the metabolic activity of the cells, we reasoned that the buffering capacity of the thin film under the coverslip might not be sufficient to maintain pH given the high metabolic activity of myocytes. We hypothesized that the intracellular pH of myo-

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**Fig. 5.** Cell death under the coverslip was linked to metabolic activity. Portions of NIH 3T3 cells, H9C2 cells, or isolated primary cardiac fibroblasts (cardiac fibs) plated on Matrigel-coated culture dishes were coverslipped. After 3 h, coverslips were removed and cells were poststained with TMRE and annexin V. Images of 3T3 fibroblasts taken at center, midpoint, and border-zone regions show TMRE(+) and annexin V(−) cells similar to those observed at uncovered regions adjacent to or at a far point from the slip edge (Fig. 5A); this indicates that covered fibroblasts remain viable over the 3-h duration. B: images of H9C2 myoblasts taken at similar regions revealed a similar level of viability compared with 3T3 fibroblasts. C: images of isolated primary cardiac fibroblasts taken at similar regions also exhibited similar levels of viability compared with 3T3 and H9C2 cells. Images are representative of six images at each position across three separate experiments.
cytes under the coverslip decreases over time. To test this hypothesis, a portion of a synchronously contracting myocyte monolayer loaded with SNARF-4F, a fluorescent intracellular dye that undergoes pH-dependent fluorescence intensity changes (see MATERIAL AND METHODS), was coverslipped. With the coverslip in place, images of 20-μm optical sections were acquired at center, midpoint, border-zone, and adjacent regions over 60 min. Pseudocolored intensity maps of these images revealed that intracellular pH decreased in most cells at center and midpoint regions within the first 10 min under the coverslip, whereas no change in pH was observed at border-zone and adjacent regions (Fig. 6A, compare 0- and 10-min rows). Within 30 min, the decrease in intracellular pH value appeared to be uniform for all cells in the field at center and midpoint regions (Fig. 6A, 30-min row). Little change in pH was observed between 30 and 60 min (Fig. 6A, compare 30- and 60-min rows). These data indicate that a rapid and substantial decrease in intracellular pH value occurs in myocytes under the coverslip within the first 60 min. On the basis of an approximate calibration curve (Fig. 6A), we estimate that the intracellular pH of myocytes under the center of the coverslip decreases to 5.0 and possibly lower.

To obtain a better understanding of pH dynamics at 60 min, 15 contiguous images of 100-μm optical sections were acquired in a 3 × 5 rectangular pattern of a region spanning the coverslip edge. Figure 6B shows the corresponding pseudocolored intensity map that reveals a wave of low pH values approaching the coverslip edge at 60 min. Border-zone pH values appeared normal compared with regions more proximal to the center of the coverslip, which suggests that perhaps exchange of metabolites and nutrients to and from the transition region and the surrounding bulk media volume was sufficient to maintain intracellular pH. Taken together, these data suggest that intracellular pH decreases in myocytes under the coverslip and that this decrease is rapid and substantial.

DISCUSSION

We describe a novel method for inducing regional hypoxia/ischemia in isolated neonatal rat cardiac myocytes. One of the strengths of this method is its simplicity including its functional end points. In addition, because the insult is regional, both normal and compromised myocytes within the same electrically coupled and synchronously contracting monolayer can be studied side by side. Furthermore, this model may more accurately represent the effects of accumulating metabolites, ions, and gases (e.g., lactate, K⁺, and CO₂) that are typically associated with ischemia in vivo. This aspect is made possible by restricting a population of myocytes to a thin film of media while preserving the benefits of a bulk media volume for the remaining myocyte monolayer.

Myocyte Life and Death in a Thin Film

Our measurements of the thin film created by the coverslip allowed us to calculate the volume of media to which myocytes under the coverslip are restricted. Using the simple equation for the volume of a cylinder

\[ V = \pi r^2 h \]

where \( V \) is the volume of the thin-film cylinder underneath the coverslip, \( r \) is the radius of the coverslip (9 mm), and \( h \) is the height of the thin film as measured by confocal microscopy (9.8 μm), we calculated the volume under the coverslip to be 2.49 × 10⁻⁶ m³, or 2.49 μl. For myocytes under the coverslip, this represents a 99.9% reduction in the accessible media volume compared with the nominal bulk volume (3 ml) in the culture dish. As noted previously (see RESULTS), however, we typically underestimated the thin-film height as assessed by our measurements of the coverslip and culture-chamber floor compared with reported values. Therefore, we determined the potential impact that this error could have on our calculation of the thin-film cylinder volume. In estimating the potential magnitude of the optical error, we suspected the largest error was observed when the coverslip thickness was measured by our method to be 110 μm, which is in contrast with the manufacturers’ reported range of 130–170 μm. If the manufacturers’ specifications are accepted as relatively accurate, our underestimation of the thin-film thickness is potentially as great as 35.3% [by 1 − (110/170 μm)]. Thus multiplying the observed thin-film thickness by an optical correction factor of 1.545 corrects for the largest possible underestimate. As shown in Table 2, although the difference in either corrected thin-film thickness or volume under the coverslip appears substantial, these differences have no appreciable effect on the reduction in volume experienced by myocytes under the coverslip compared with the surrounding bulk volume.

Apoptotic vs. Necrotic Myocyte Death under Coverslips

The phase-contrast video microscopy data highlight two interesting observations: chromatin margination and vacuolization. The onset of chromatin margination is quite rapid, typically within 15 min in >70% of the cultures, with severe vacuolization being delayed to the 90-min time point (see Fig. 2). Chromatin margination has been associated with early apoptotic events, whereas vacuolization is most often associated with necrotic (or, more appropriately, oncotic) cell death (3, 4, 23, 26, 30). The data suggest that apoptotic events could...
be initiated at early time points but eventually transition to necrotic/oncotic events. Others have reported that such a transition is linked to diminishing energy levels in the cell caused by changes in mitochondrial function (13, 14, 19, 34). A time-dependent change in mitochondrial function was observed as evidenced by the inability of myocytes under the coverslip to load TMRE (see Fig. 3). This suggested that mitochondrial membrane potential and, hence, the production of ATP were dramatically altered. These mitochondrial effects were not observed in myocytes with access to media via diffusion across a porous membrane (see Fig. 4), or in less metabolically active cells (see Fig. 5), all of which survived.
Table 2. Errors in thin-film measurements did not significantly alter volume reduction

<table>
<thead>
<tr>
<th>Observed Film Thickness, μm</th>
<th>Sample-Error Correction</th>
<th>Corrected Film Thickness, μm</th>
<th>Estimated Volume Under Coverslip, μL</th>
<th>Volume Reduction vs. 3-ml Whole Well, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.8 ± 2.6</td>
<td>+2 SD</td>
<td>1.545</td>
<td>23.18</td>
<td>5.89</td>
</tr>
<tr>
<td>9.8 ± 2.6</td>
<td>None</td>
<td>1.514</td>
<td>15.14</td>
<td>3.85</td>
</tr>
<tr>
<td>9.8 ± 2.6</td>
<td>None</td>
<td>None</td>
<td>9.80</td>
<td>99.92</td>
</tr>
<tr>
<td>9.8 ± 2.6</td>
<td>−2 SD</td>
<td>1.545</td>
<td>7.11</td>
<td>1.81</td>
</tr>
</tbody>
</table>

Values for observed film thickness are means ± SD. Represented are four hypothetical scenarios wherein sample error and correction and/or optical correction are applied to observed film thickness.

under the coverslip over the same 3-h duration. It will be interesting to determine whether changes in ATP levels coincide with loss of mitochondrial membrane potential, and more importantly, with the appearance of necrotic markers in this model.

Several studies suggest that apoptosis of cardiac myocytes may play a role in a variety of cardiac ailments, including pacing-induced dilated cardiomyopathy (10, 18, 20), peripartum cardiomyopathy (9, 28, 37), pressure-overload hypertrophy (5, 37, 38), hypertension (7, 8), and ischemia-reperfusion (1, 12; see, however, Ref. 24). Although we imaged dying and/or dead myocytes under the coverslip with fluorescently conjugated annexin V, we have not sufficiently demonstrated in this study that the myocyte death observed under the coverslip is apoptotic in origin. Indeed, at 30 min and 3 h, the annexin V(+) myocytes also label with propidium iodide, which indicates that myocyte death at these time points is necrotic (unpublished data). Again, it is possible that at time points earlier than 30 min, apoptotic events commence but never finish due to energy depletion, and myocytes transition to a necrotic death (see above). In addition, because progression to myocyte death under the coverslip appears to be rapid, it may be difficult to resolve apoptotic and necrotic events. Studies are ongoing to vary the severity of insult and define the apoptotic and necrotic profiles of myocytes in this model.

Is Coverslip Hypoxia Relevant to In Vivo Events?

For in vitro models to be useful, they should adequately and accurately represent in vivo events. Here we report that the coverslip hypoxia model mimics at least three important events associated with ischemia in vivo.

**Capillaries vs. thin film.** Myocytes have access to a continuous flow of blood in vivo via the capillary bed. Myocytes exchange O2 and metabolites with the blood across the myocyte-capillary interface, and the continual flow of blood readily removes metabolites and replaces O2 and nutrients. This exchange is mimicked in vitro by providing myocyte monolayers with a bulk volume of media. Exchange takes place at the myocyte-media interface, and diffusion to and from the bulk volume is relied on to replace O2 from the continual supply available to cultured cells. Although metabolites build up in the media, removal by frequent media changes keeps their concentrations low.

When arterial blood supply to the myocardium is interrupted (i.e., coronary occlusion; Fig. 7B), myocardium in the ischemic zone immediately becomes dependent on whatever remaining blood volume is available in adjacent capillaries to provide O2 and accept metabolic waste products. Because this residual volume is small and static (Fig. 7D), the myocardiun in the ischemic zone is rapidly depleted of O2. In addition, metabolites are concentrated in close proximity to myocytes, and increasing levels of metabolites and fixed acids rapidly become toxic. Ischemic, border, and nonischemic zones in vivo (Fig. 7B) are replicated by distinct zones in the myocyte monolayer (Fig. 7A); the ischemic and border zones are mimicked in the thin film created by the coverslip (Fig. 7C). At 3 h, myocytes under most of the coverslip are annexin V(+) (Fig. 7C, green cells), which indicates they are dead or dying, similar to tissue in the ischemic zone in vivo. Myocytes at the edge of the coverslip, however, are in a transition region where cells are viable yet functionally compromised (Fig. 7C, yellow cells), similar to the border zone in vivo. Finally, myocytes not under the coverslip are TMRE(+) (Fig. 7C, red cells), which indicates they are viable and functional, similar to collaterally perfused tissue in vivo.

**Border-zone dynamics.** The data reported demonstrate that at 3 h, no demarcation between live and dead myocytes is present at the coverslip edge (see Fig. 3). This demarcation appears to be forming with a mixture of nonfunctional and functional myocytes within 1,000 μm of the coverslip edge (see Fig. 3B). By 18 h, however, a distinct demarcation exists within 300 μm of the coverslip edge with a boundary between nonfunctional and functional myocytes. This corroborates our initial observations of a transition region at the coverslip edge where myocytes were viable yet functionally compromised (data not shown). These data suggest the presence of a transition region comprising the border zone that with prolonged insult becomes more defined. Transition regions have been described in vivo (35, 36) and in vitro (11) and present opportunities to study ionic and electrical interactions across cellular regions with differing microenvironments. We are evaluating how these microenvironments give rise to myocyte dysfunction during either ischemia or reperfusion.

**Changes in pHe under coverslips.** One of the hallmarks of ischemia in vivo is acidosis. Ischemic myocardium responds to hypoxia by switching from respiration to glycolysis, which leads to the accumulation of lactic and phosphoric acids and a subsequent decrease in myocardial pH value.

The magnitude of this pH decrease varies depending on several factors including extent of occlusion, changes in cardiac muscle dynamics (e.g., arrhythmias, tachycardia, bradycardia, etc.), and reperfusion. Studies in dogs have shown, however, that intramyocardial pH decreased to 5.4 after 2 h of anoxic arrest (17), which suggests that pH changes can be substantial in vivo. Our observations that intracellular pH under the coverslip drops to 5.0 and perhaps lower (see Fig. 6) are in good agreement with in vivo reports and suggest that this method is capable of reproducing acidosis events as they are generated by the myocytes proper. Interestingly, pH values appear to plateau between 30 and 60 min; this coincides with contractile arrest (see Fig. 2C) and further supports our finding that cell death under the coverslip is the direct result of metabolic activity. Studies are under way to vary the onset of acidosis in this model to gain a better understanding of how myocyte...
survival can be regulated and promoted in ischemic myocardium.

In conclusion, we have presented a novel and simple method to create regional hypoxia and ischemia in neonatal rat cardiac myocytes. This method will allow both normal and compromised myocytes within the same electrically coupled and synchronously contracting monolayer to be studied side by side. Furthermore, this model may more accurately represent the tissue environment that is typically associated with ischemic conditions in vivo and allow for identification of novel pharmacological means to preserve ischemic myocardium at risk of cell death.

ACKNOWLEDGMENTS

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Fig. 7. Coverslip hypoxia is an in vitro model that reflects in vivo hypoxic/ischemic events. A: graphical representation of coverslip hypoxia in a 35-mm dish. Three 10 × 10, tiled confocal images of myocytes on either side of the coverslip edge reveal annexin V(+) cells under the coverslip and TMRE(+) cells out from under the coverslip. Myocytes in the dish are in one of three distinct zones: ischemic zone (cells under the center of the coverslip), border zone (cells at the coverslip edge), or nonischemic zone (cells out from under the coverslip). B: these regions correspond to those observed in vivo, where myocardium distal to the site of coronary occlusion represents the ischemic zone, myocardium at the edge of the area at risk is in the border zone, and myocardium perfused collateral to is in the nonischemic zone. C: cells in the nonischemic zone (red) where diffusion is into a bulk volume are TMRE(+) and healthy. Cells at the border zone (yellow) span a transition region where diffusion is presumed sufficient for viability, yet function may be compromised. Cells in the ischemic zone (green) are dead [i.e., annexin V(+)] as a result of diminishing O2 levels and increasing metabolic waste concentrations in the thin film created by the coverslip. D: rat myocardium was stained with anti-platelet endothelial cell adhesion molecule antibodies to visualize capillaries (arrows). Thin film created by the coverslip in C simulates the residual blood volume remaining in these capillaries at the time of coronary occlusion.
REFERENCES


