Oxygen sensitivity of mitochondrial metabolic state in isolated skeletal and cardiac myocytes

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Richmond, Keith Neu, Steven Burnite, and Ronald M. Lynch. Oxygen sensitivity of mitochondrial metabolic state in isolated skeletal and cardiac myocytes. Am. J. Physiol. 273 (Cell Physiol. 42): C1613–C1622, 1997.—In striated muscle the coupling of blood flow to changes in tissue metabolism is hypothesized to be dependent in part on release of vasodilating metabolic by-products generated when mitochondrial metabolism becomes O2 limited. Cytochrome oxidase, the terminal step in oxidative phosphorylation, is half-maximally saturated at <1 mmHg PO2 in isolated mitochondria. However, blood flow is regulated at tissue PO2 of ~20 mmHg. If the affinity of mitochondrial respiration for O2 were higher in vivo than in vitro, O2 limitation of mitochondrial metabolism near mean tissue levels could occur. In the present study the PO2 at which mitochondrial metabolism becomes inhibited (critical PO2) was measured for cardiac myocytes in suspension (1.1 ± 0.15 mmHg) and single cells (1.0 ± 0.22 and 1.25 ± 0.22 mmHg in cardiac myocytes and rat spinotrapezius cells, respectively). These measurements are consistent with those from isolated mitochondria, indicating that vasodilators produced when oxidative phosphorylation becomes inhibited may be important for regulating blood flow only in highly glycolytic muscles or under conditions of severe O2 limitation.

blood flow regulation; nicotinamide adenine dinucleotide fluorescence; striated muscle; oxidative metabolism; spectral imaging

THE WORK OUTPUT AND ENERGY requirements of striated muscle can vary over a wide range. As muscle work increases, stores of high-energy phosphates, primarily ATP and phosphocreatine, are depleted. In oxidative fibers, these stores are replenished when mitochondrial metabolism is elevated in proportion to the energy used and, therefore, the level of work performed. During these periods of increased demand for O2 and substrates, blood flow and, thereby, O2 delivery to the muscle increase (9).

One hypothesis that has been proposed to explain the coupling of blood flow and metabolism suggests that mitochondrial oxidative phosphorylation becomes O2 limited in working muscle, leading to production of vasodilator end products (9) such as adenosine (from ATP breakdown). The dilution that is produced ultimately results in the required increase in delivery of O2 and substrates to the perfused tissue. The O2 level at which mitochondrial metabolism becomes limited has been estimated from in vitro experiments carried out with isolated mitochondria. The majority of these measurements have monitored changes in O2 uptake or the oxidation state of cytochrome oxidase, the terminal step in oxidative phosphorylation. The concentration of O2 at which cytochrome oxidase is reduced by one-half from the maximally oxidized state (Km) ranges from 0.05 to 1.0 µM in isolated mitochondria, depending on the experimental conditions (8, 15). However, on the basis of measurements of mean tissue PO2 in skeletal muscle, which range from 16.7 mmHg (~22 µM) (23) to 22.8 mmHg (~32 µM) (1), metabolic feedback for blood flow regulation would have to occur at PO2 levels far above the Km measured in vitro. In an attempt to reconcile the observed differences between the Km of cytochrome oxidase in vitro and the high (by comparison) mean tissue PO2 measurements with the metabolic theory of vasodilator production, it has been suggested that the Km of cytochrome oxidase for O2 in mitochondria in vivo may be higher than that measured for mitochondria in vitro (4).

In the present study the hypothesis that there are differences between the O2 sensitivity of isolated mitochondria and that of mitochondria in intact cells was tested by following changes in the fluorescence of pyridine nucleotides (NADH and NADPH), as an indicator of mitochondrial metabolic state, during reductions in PO2. NADH and NADPH are metabolic cofactors that fluoresce in their reduced but not in their oxidized form, and therefore NADH fluorescence is indicative of the redox state of these cofactors and activity of the metabolic pathways in which they are involved (4, 7). NADH fluorescence has been utilized to monitor the redox state of isolated mitochondria, cells, and intact tissue (4–6). To confirm that changes in cell NADH fluorescence provide a good indicator of changes in mitochondrial redox state for the present study, single cell imaging was used to identify the subcellular origin of the change in NADH fluorescence associated with inhibition of mitochondrial metabolism.

Changes in NADH fluorescence and the phosphorescence of an O2-sensitive metalloporphyrin probe (22) also were monitored simultaneously at the single cell level using spectral imaging microscopy and spectrofluorometry for cells in suspension. PO2 of the perfusion medium was reduced, and the PO2 at which mitochondrial metabolism became O2 limited was determined, using the spectroscopy approaches. The relationship between the fall in O2 concentration and inhibition of mitochondrial respiration, estimated from the rise in NADH fluorescence, was used to calculate a critical PO2. The critical PO2 for these studies is defined empirically as the O2 concentration at which NADH fluorescence first begins to increase above control levels.

MATERIALS AND METHODS
Cardiac Myocyte Dissociation and Cell Preparation

Isolated cardiac myocytes were prepared using minor modifications from previously described procedures (12).
Briefly, nonfasted male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing $\geq 150$ g were anesthetized with CO$_2$ inhalation and euthanized by cervical dislocation. Hearts were removed and cannulated through the aorta on a modified Langendorff perfusion apparatus. Reverse perfusion provided flow to the coronary vessels and the left ventricle, ensuring rapid distribution of the perfusing medium throughout the coronary microvasculature. A physiological saline solution (PSS; composition in mM: 5.0 KCl, 0.3 KH$_2$PO$_4$, 138.0 NaCl, 4.0 NaHCO$_3$, 0.3 Na$_2$HPO$_4$, 10.0 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 55.0 glucose, 1.0 creatine, 1.0 taurine) containing bovine serum albumin (BSA, 1 mg/ml; Sigma Chemical) was perfused through the heart to remove blood. The solutions used for cell isolation and subsequent storage contained no added calcium. This rinse solution was replaced with PSS containing BSA and type II collagenase (1 mg/ml; Sigma Chemical). Hearts were pumped by manual compression to ensure adequate perfusion of the coronary circulation. When hearts became soft and began to lose their distensibility, the collagenase solution was replaced with enzyme-free PSS. Aliquots of rinse medium containing cells were stored at room temperature (25°C). For suspension experiments, cells were allowed to settle in the collection tubes, and two or three of the subsequent cell pellets were combined and resuspended in PSS to a final volume of 3 ml, resulting in a cell volume-to-suspension fluid volume ratio of $\sim 1:30$. For single cell experiments, cells were selected on the basis of morphological criteria: the appearance of an intact membrane and elongated shape. In addition, a spectral image was obtained to verify cell viability, since nonviable cells typically exhibit green fluorescence due to oxidized flavoproteins (4), and to ensure that the blue NADH fluorescence signal was present.

Striated Muscle Cell Preparation

Nonfasted male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing $\leq 125$ g were anesthetized with CO$_2$ inhalation and euthanized by cervical dislocation. An incision was made along the left dorsal side, and the skin was separated from the underlying tissue by blunt dissection. The spinotrapezius muscle was isolated from the surrounding muscle using blunt dissection with scissors. This muscle is thin, with an average thickness of $\sim 200$ µm, $\sim 2.5$ cm long, and 1 cm wide. The muscle was placed in a 1.5-ml culture tube containing 1 ml of PSS and type II collagenase (1 mg/ml PSS) and cut into smaller pieces along its long axis. After incubation for 10 min at room temperature (25°C), medium containing the cells was diluted 10-fold in a 15-ml conical tube, and the cells were resuspended in the tube by gentle shaking. About 15 min were allowed for cells to settle, then excess medium was removed. Aliquots of the cell-containing medium were subsequently diluted 20-fold into the microscope incubation chamber for experiments.

Cell Viability and Spectral Characteristics

Our ability to estimate a critical P$_O_2$ from single cells is dependent on the isolation of viable, metabolically functioning myocytes. One of the criteria used to determine viability was morphology. Figure 1 is a phase-contrast photomicrograph of two spinotrapezius cells. The distinct striated pattern is visible as light and dark bands of striping perpendicular to the axis in both cells. These cells were typically 50–60 µm in diameter with variable length. In addition to morphology, the spectral shape of the output emission (360-nm excitation) was used to determine viability of cells for imaging. Figure 2 shows the spectrum of...
fluorescence emission (360-nm excitation) from a spinotrapezius myocyte incubated with the phosphorescent O2 probe palladium meso-tetra-[4-carboxyphenyl]porphine (TCPP) phosphorescence. Apparent blue shift in TCPP phosphorescence is due to transmission characteristics of dichroic mirror and low wavelength of excitation (365 nm). Note increase in TCPP signal without change in NADH fluorescence when O2 decreased from 43 to 2.5 mmHg. Top spectrum was recorded at 0.5 mmHg PO2 after NADH fluorescence reached a maximal value.

Metabolic Changes/ NADH Fluorescence

Changes in the redox state of mitochondria in myocytes were estimated by monitoring changes in pyridine nucleotide fluorescence. Previous studies carried out in vivo and in vitro indicate that the primary contribution to 450-nm emission is from pyridine nucleotides, of which NADH is the primary component (4). The use of NADH fluorescence to estimate the mitochondrial redox state in cells is based on the property of pyridine nucleotides to fluoresce in their reduced state but not in their oxidized state. Because the redox state (reduced/oxidized) of these compounds is an indicator of the activity of the metabolic pathways in which they act as coenzymes, measurement of changes in NADH fluorescence may be used as an estimate of the mitochondrial metabolic state of cells (4).

Three-Dimensional Imaging and Analysis

Cells were incubated at 37°C on the stage of an Olympus IMT-2 inverted microscope equipped for epifluorescence using a 200-W mercury lamp as the illuminator. Images were acquired using a liquid-cooled CCD camera (model AT200, Photometrics, Tucson, AZ) with a Tecnorix 512 chip. The focusing mechanism of the microscope is motorized and computer controlled to provide rapid changes in focus. An eddy current sensor (Kaman Instruments, Boulder, CO) provides precise stage position information (0.1-µm resolution) to ensure accurate spacing of images acquired along the optical axis. Mathematical deconvolution of three-dimensional image sets was performed on a Silicon Graphics INDIGO-2 to reduce blurring inherent in wide-field microscopy (2, 3). Use of this approach is required for analysis of NADH fluorescence distribution, since NADH fluorescence is inherently low and can bleach rapidly under conditions required for imaging (10). For imaging experiments, cells were loaded with 4-[4-(dimethylamino)styryl]-N-methylpyridinium (DASPMI, 1 µg/ml; Molecular Probes, Eugene OR), which labels mitochondria. NADH fluorescence was imaged using 360-nm (10-nm band pass) excitation and emission isolated with a 30-nm band-pass filter centered at 450 nm coupled with an appropriate dichroic mirror (405-nm long pass). DASPMI fluorescence was imaged using 460-nm (10-nm band pass) excitation, and emission was isolated with a 570-nm long-pass filter coupled with an appropriate dichroic mirror (480-nm long pass). The DASPMI three-dimensional image set was acquired first, then a control NADH set was obtained, followed by acquisition of an image set 1–3 min after addition of 0.5 mM cyanide. Time-course experiments demonstrated that a maximal response to cyanide was attained by this time. To analyze the relationship between the spatial distribution of mitochondria and the regions where cyanide elicted a change in NADH fluorescence, several basic image-processing steps were undertaken. The two three-dimensional NADH image sets were first aligned along the z-axis using a cell nucleus as a spatial marker. The control image was then subtracted from the image acquired after cyanide treatment to obtain an image that shows where the change in NADH occurred. Next, a binary mask of the DASPMI image (mitochondrial distribution) was made and compared with the subtraction image to determine the degree of colocalization between the two images. A concern is the possibility that mitochondria may move within the myocytes between collection of independent image sets. To test this, two image sets of DASPMI distribution were acquired 10 min apart, and dye distributions were compared. No substantial changes in mitochondrial distribution were apparent from these comparisons (n = 3).

Measurement of O2

Changes in PO2 were monitored using the phosphorescent probe TCPP (Porphyrin Products, Logan, UT). The use of phosphorescence intensity of this probe to monitor O2 was described by Runsey et al. (17), and the characterization of the albumin-bound probe for use as an O2 sensor was described by Shonat et al. (18, 19). The probe has two excitation peaks in the visible spectrum (maxima at 420 and 525 nm) and a single broad emission peak centered at 700 nm. The excitation peak at 420 nm is sufficiently broad to provide significant excitation at 360 nm, allowing use of a single light source to excite the O2 probe and NADH simultaneously. Use of this low-wavelength excitation is likely to underlie the apparent blue shift in the TCPP emission spectrum seen in Fig. 2. After excitation, the excited triplet state of TCPP returns to the ground state by light emission or by energy transfer to other molecules (quenching), with the principal quenching agent being O2. The quenching effect of
O$_2$ on phosphorescence is described by the Stern-Volmer relation

$$I_0/I = \tau_0/\tau = 1 + \tau_0 k_0 [O_2]$$

(1)

where $I_0$ and $\tau_0$ are the phosphorescence intensity and lifetime, respectively, in the absence of O$_2$, I and $\tau$ are the intensity and lifetime, respectively, at a given O$_2$ concentration ([O$_2$]), and $k_0$ is the quenching rate constant. Once $\tau_0$ and $k_0$ are determined for a given set of experimental conditions (temperature and pH), they are assumed to remain constant throughout the experiment, and O$_2$ concentration can be calculated from the intensity of the phosphorescence or the lifetime of the triplet state. For the pH range of 7.3–7.5 and the temperature range of 25–35°C, errors in PO$_2$ estimates are <5% for this probe (19). For this study, changes in intensity of phosphorescence were followed rather than changes in the lifetime of the phosphorescence (17). The value of $I_0$ for each individual experiment was estimated as the highest intensity reached during the plateau phase at the end of the experimental protocol. Intensity measurements are easier to implement, and the primary variables that complicate intensity measurements in vivo (e.g., concentration of probe and sample thickness) can be controlled in vitro.

Instrumentation

Suspension measurements. Cells were placed in a sealed quartz cuvette (1-cm pathlength) in a PSS solution at 25°C. The cuvette was placed directly over a magnetic stir plate, and the cells were held in suspension by stirring constantly with a magnetic stir bar inside the cuvette. This also provided mixing of the cuvette contents.

Excitation of NADH fluorescence and Pd-porphyrin phosphorescence was provided by two separate light sources. To excite the Pd-porphyrin probe, a spectrofluorometer equipped with a 200-W xenon bulb (FluoroPlex III, Tracor Northern, Middleton, WI) was modified to provide monochromatic light at 525 nm by running a 1-cm-diameter fiber-optic light guide from the output of the monochromator to the quartz cuvette containing the cell suspension. Phosphorescence was detected by a phototube placed on the side opposite the light source with a 650-nm long-pass filter placed between the cuvette and the phototube. This arrangement provided an adequate signal-to-noise ratio at the emission peak of 700 nm by preventing excitation light as well as scattered light from reaching the phototube.

Excitation for NADH fluorescence and Pd-porphyrin phosphorescence was provided by direct-long column of light (2 mm) from a 100-W mercury lamp to the sample chamber using a telescopic illuminator (Olympus). A 360-nm (10-nm band-pass) filter between the cuvette and the collimating lens provided excitation of NADH fluorescence. A second phototube (model HC-120, Hamamatsu) was placed at the edge of the cuvette 90° from the light path. A 450-nm (10-nm band-pass) filter was placed between the cuvette and the phototube to collect the NADH fluorescence. Both signals were amplified and sent to a custom analog-to-digital converter and displayed on a computer monitor using software written in-house. A record of the signal from each phototube was written to a computer file for off-line analysis. This record included the time when each data point was collected and time markers to indicate the addition of O$_2$ probe or other experimental perturbations. This system provided continuous measurement of both parameters with a temporal resolution of 1 s.

The O$_2$ probe was added to the cuvette containing cells and allowed to equilibrate for several seconds. Glucose oxidase was subsequently added to the cuvette to decrease the time necessary to bring PO$_2$ to zero. This was necessary for two reasons: 1) the cells were relatively metabolically quiescent (not contracting) and, therefore, consumed O$_2$ at a relatively slow rate, and 2) in order for the excitation light to sufficiently penetrate the cuvette, the density of the cell suspension was low, with the previously stated cell volume-to-medium volume ratio of 1:30. Addition of glucose oxidase increased the rate of O$_2$ consumption in the cuvette but did not affect the emission signal for NADH, as demonstrated by control experiments.

The parameter of interest was the time at which NADH fluorescence became elevated above baseline. The baseline NADH was determined by fitting the signal output to a line, and a 95% confidence interval about the line was calculated. For example, in Fig. 7 a straight line (A) was drawn between the first NADH data point to fall outside the confidence interval and the previous data point. The intersection of this line and the upper 95% confidence interval line (B) was used to define the time point for the initial change in NADH fluorescence. A vertical line (C) was drawn from the intersection of lines A and B to the x-axis. Finally, a line (D) was drawn between the two O$_2$ data points that corresponded in time with the two NADH fluorescence data points. The intersection of lines C and D was used to define the PO$_2$ at which NADH fluorescence first begins to change (the critical PO$_2$). Occasionally, the NADH signal fluctuated before the increase, and for this reason 100 points before the onset of the NADH fluorescence increase were used to fit the "steady-state" NADH data to a line. The first of two consecutive data points to fall outside the 95% confidence interval established the time at which the PO$_2$ had reached the critical level.

Single cell spectral measurements. An Olympus IMT-2 inverted microscope equipped with a 200-W mercury lamp as the illumination source was used for single cell spectral imaging, as described in detail previously (13). Briefly, excitation for NADH fluorescence and TCPP phosphorescence was accomplished by passing the light from the mercury lamp through a 365-nm (10-nm band-pass) filter. An electronic shutter in the excitation light path could be triggered manually and by computer control. Imaging optics included an Olympus ×60 1.4 NA oil immersion objective and a 6.7-pH photographic eyepiece to focus the image onto the input slit of a grating monochrometer (Aries 250iS/SM spectograph, Chromex, Albuquerque, NM). A grating blazed at 150 lines/mm provided a spectral bandwidth of 350 nm. The spectral output was imaged onto a liquid-cooled CCD camera (model CH250, Photometrics, Tucson, AZ) equipped with a 512 × 512-element (27° µm/pixel) back-illuminated imaging chip (Tectronix). The output image contains spectral information from the cell along the x-axis (13). The y-axis contains information on the position of the cell relative to the imaging slit. The image size was reduced in the y-axis, resulting in images that were typically 512 × 200 pixels. Each pixel in the image has a numerical value associated with it that represents the intensity of light at that location. These values are used to plot the fluorescence intensity at each position along the serial register (wavelength) per image line. The Tectronix CCD chip has ~70% quantum efficiency at the peak wavelengths for endogenous NADH fluorescence (450 nm) and TCPP (700 nm), providing a good signal-to-noise ratio. The large dynamic range of the cooled CCD camera allows concurrent imaging of the relatively weak NADH signal and of the O$_2$ probe. An exposure time of 500 ms was used to limit photobleaching of the fluorophores, particularly the relatively weak pyridine nucleotide fluorescence. Digitized output from the CCD camera was stored as an image file on a personal computer. Pd-porphyrin phosphorescence and NADH fluores-
cence were measured simultaneously every 15 s by spectral imaging microscopy. Image analysis was performed on a Silicon Graphics Personal IRIS computer, and the subsequent analysis of spectra was carried out on a 486 Gateway computer using Microsoft Excel (Microsoft, Redmond, WA) and SigmaPlot for Windows (Jandel Scientific, San Rafael, CA).

Each spectral image was viewed to determine the position of cell and out-of-cell areas, which varied depending on the orientation of the cell to the imaging slit. Because the O₂ probe surrounds the cell and because there is blurring of light from the cell into the out-of-cell area, fluorescence emission from both regions exhibits two peaks. The dominant peak in the cellular region is from NADH fluorescence (NADH peak at 450 nm), whereas the dominant peak in the noncellular region is from the O₂ probe (TCPP peak emission at 700 nm). Spectral information from the region of the image identified as a cellular area was used to determine NADH intensity changes. The spatial dimensions of the cell (or out-of-cell area) cover a finite region along the serial register (y-axis) of the CCD chip, which is read out as image lines along this register (0.3 μm/line). The region identified as cellular was segmented from the image, and the fluorescence intensity at all spatial positions within the cell boundary was averaged at each wavelength (0.65 nm/pixel along x-axis). Spectral information from the region of the image identified as out of cell was used to determine changes in the phosphorescence intensity of the TCPP probe. The peak intensity for NADH or TCPP was computed by averaging intensity values centered (±20 nm) around the two peaks of interest at each time point, with each image in the data set representing an individual time point. The highest value recorded for the TCPP peak was assumed to be the intensity at zero O₂, and this value was used to compute the Po₂ using the Stern-Volmer relation (Eq. 1). With an image acquisition rate of 4/min, occasionally substantial Po₂ and NADH fluorescence changes occurred between acquisition of sequential images. The approach described above for the suspension data analysis was used to calculate the slope for steady-state NADH and to determine the critical Po₂.

RESULTS

Effect of the O₂ Probe (TCPP) and Glucose Oxidase on NADH Fluorescence

Potential interactions of TCPP and glucose oxidase with the NADH fluorescence signal were tested in a cell-free medium (PSS) containing NADH (2 μM). TCPP and glucose oxidase were added to this medium, and changes in the NADH fluorescence signal were followed using a fluorometer. Glucose oxidase had no effect on the NADH fluorescence signal. However, addition of TCPP (5 μg) resulted in a 20% decrease in the NADH fluorescence signal. This decrease was also noted in experiments carried out in the presence of cells. On the basis of the absorption spectrum of TCPP, this decrease in NADH fluorescence is likely due to absorption at the NADH excitation wavelength (360 nm) as well as other factors such as absorption of the 450-nm NADH fluorescence by the porphyrin probe. Importantly, the magnitude of the decrease in NADH fluorescence was not influenced by changes in Po₂. Moreover, sequential addition of the stock NADH to the medium elicited linear increases in the fluorescence signal intensity at 450 nm, as would be expected if the attenuation of NADH fluorescence by TCPP was due to signal absorp-

tion. Therefore, the effect of TCPP on the NADH signal was stable during an experimental period and did not affect our ability to monitor the time course of change in NADH fluorescence or, specifically, increases in NADH fluorescence after the addition of cyanide and glucose oxidase.

Substrate Dependence of NADH Signal Response

The NADH signal declined throughout the sampling period until the critical limiting Po₂ was reached, at which point NADH fluorescence began to increase. The decline in NADH fluorescence is likely due to photobleaching and possibly decreased availability of metabolic substrates. The second possibility was tested by adding metabolic substrate during the measurement period. Addition of succinate (a citric acid cycle intermediate; n = 3) to the suspension attenuated the rate of decline in NADH fluorescence (see Fig. 5), suggesting that substrate limitation may partially explain the decline in signal. The remaining rate of decline after substrate addition is likely due to photobleaching (10).

Single Cell NADH Fluorescence Distribution

To verify the use of NADH fluorescence as an indicator for change in mitochondrial metabolic state, the subcellular origin of the change in NADH fluorescence during periods of metabolic limitation was examined using three-dimensional imaging coupled with mathematical deconvolution (2). Cyanide was used to mimic O₂ limitation, because the time course of its effect on NADH fluorescence could be more precisely controlled than O₂ removal. Cyanide is an electron transport inhibitor that blocks oxidative phosphorylation at cytochrome aₐₐ₃, eliciting a maximal reduction in the NADH/NAD⁺ couple. After cyanide addition, fluorescence of NADH increased to a maximum within 1 min and remained stable for at least 5 min for cardiac and spinotrapezius myocytes. Figure 3 shows NADH fluorescence images from a skeletal myocyte before (A) and after (B) the addition of cyanide. Figure 3C is the result of subtracting image A from image B. The change in NADH fluorescence appears limited to regions that run parallel with the myofibrils. This pattern is consistent with mitochondrial distribution in these cells. The possibility that this apparent distribution is due to inner filtering by the myofibrils was tested by loading cells with a fluorescent probe that is known to be trapped within the cell cytosol (dichlorofluorocarbocyanine) (10). The distribution of this probe was uniform without an obvious localized pattern (not shown), as demonstrated previously for cardiac myocytes (10). Thus inner filter effects of myofibrils cannot explain the spatial pattern associated with cyanide-induced changes in NADH fluorescence.

To obtain higher-resolution images of regions where the change in NADH fluorescence occurs, three-dimensional image sets of single myocytes were acquired before and 1 min after addition of cyanide. By subtracting the precyanide image from the cyanide image, the areas where NADH fluorescence increased could be identified. Before cyanide addition, cells were
labeled with a mitochondrial membrane-specific dye (DASPMI), and images of mitochondrial distribution in the cell were used to determine colocalization of the NADH fluorescence increase with mitochondria. Figure 4 shows a subtraction image prepared from deconvolved images (A) and a mitochondrial mask prepared from a DASPMI image of the same skeletal myocyte (B). Note the longitudinal pattern of DASPMI and NADH fluorescence. The relatively large diameter of the myocytes (40–50 µm) made quantitative analysis of the data by cross-correlation analysis difficult. However, the regions where the change in NADH fluorescence was the greatest during metabolic perturbations clearly were aligned with regions identified as mitochondria. This observation is in agreement with previous studies for cardiac myocytes (10, 14).

Critical Po2 in Cardiac Myocyte Suspensions

Po2 and NADH fluorescence were measured in suspensions of cardiac myocytes to determine the Po2 at which mitochondrial metabolism became O2 limited. Figure 5 shows the result from one experiment, in which the calculated critical Po2 was 1.4 mmHg. The critical Po2 measured for cardiac myocytes in suspension ranged from 0.5 to 1.9 mmHg with a mean of 1.1 ± 0.15 mmHg for 9 trials.

Cellular Response to Metabolic Inhibitors and Uncouplers

The metabolic response of isolated cardiac myocytes in suspension to cyanide and the protonophore carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) was examined. An assumption that is implicit for calculating the critical Po2 is that the NADH/NAD+ couple will become maximally reduced when O2 becomes limiting. To test this assumption, cyanide was added to cell suspensions at the end of an experiment, when the NADH fluorescence signal was at its peak due to removal of O2. Figure 6 shows the effect of cyanide on the NADH fluorescence signal after treatment with glucose oxidase, i.e., after NADH fluorescence increased due to O2 limitation. In all cases when cyanide was added at the peak of the glucose oxidase-induced NADH response, no further increase in NADH fluorescence was observed (n = 4). In cell suspensions where O2 was not limited (glucose oxidase free), the response to cyanide was a rapid increase (complete within 15 s) in NADH fluorescence. To ensure that the cells were still metabolically responsive, FCCP (1 µM) was added to the cell suspension 60 s after the cyanide. FCCP eliminates respiratory control and maximally oxidizes the NADH/NAD+ redox couple. After FCCP addition,
the NADH fluorescence intensity declined to values observed before O2 limitation within 45 s, with 76% of the decline occurring in the first 15 s.

Critical Po2 in Single Striated Muscle Cells

The spectral imaging microscope was used to monitor medium Po2 (TCPP phosphorescence) and mitochondrial metabolic state (NADH fluorescence) simultaneously at the single cell level. The data from each of the spectral images were analyzed for emission intensities at 450 (cell NADH) and 700 (medium TCPP) nm at 15-s intervals.

Figure 7 shows representative critical Po2 data from a rat cardiac myocyte. The decrease in NADH fluorescence that occurs in the first 80 s is typical for these measurements and, as discussed earlier, is likely due to a combination of photobleaching and substrate limitation. NADH fluorescence intensity falls slowly through the sampling period, then rises above the 95% confidence interval at a Po2 of 0.7 mmHg. The critical Po2 from single cardiac myocytes ranged from 0.00 to 1.68 mmHg with a mean of 1.0 ± 0.22 mmHg for 7 experiments. This value is not different from that measured for cardiac myocytes in suspension.
Figure 8 shows representative data used to estimate the critical PO$_2$ from measurements made on a single rat spinotrapezius muscle fiber. The critical PO$_2$ for this experiment as estimated by the method described above was 0.96 mmHg. The critical PO$_2$ for spinotrapezius cells ranged from 0.73 to 1.95 mmHg with a mean of $1.25 \pm 0.22$ mmHg for six experiments.

DISCUSSION

The mechanisms by which changes in vessel diameter are linked to the metabolic state of a tissue are incompletely understood. Production and release of vasodilating substances (e.g., adenosine) during periods of O$_2$ limitation could provide this link. However, mitochondrial oxidative metabolism does not become limited ($K_m = 0.5$–0.05 mmHg) until tissue PO$_2$ is at least an order of magnitude below the normal operating level (20 mmHg). To explain this discrepancy, we tested the hypothesis that the affinity of mitochondrial respiration for O$_2$ was lower in intact cells in isolated mitochondria (20) by examining the O$_2$ sensitivity of single isolated cardiac and skeletal muscle cells as well as cardiac cells in suspension.

The critical PO$_2$ reported here for cardiac cells in suspension is $1.1 \pm 0.15$ mmHg ($\sim 1.5$ µM). There are no reports in the literature of critical PO$_2$ measurements for cardiac myocytes in suspension in terms of the $K_m$ of cytochrome oxidase for O$_2$. However, the phosphorescence technique has been used in studies of mitochondria isolated from liver to estimate the $K_m$ of cytochrome oxidase for O$_2$ (24). The $K_m$ for well-coupled mitochondria measured using this method was $\sim 0.7$ µM O$_2$ and ranged from 0.5 to 0.9 µM, depending on medium pH (7.2–7.4). There are other reports in the literature of a much lower $K_m$. Oshino et al. (15) reported a $K_m$ of cytochrome oxidase in well-coupled isolated mitochondria of $<0.05$ µM O$_2$. O$_2$ microelectrodes have been used to measure the $K_m$ of cytochrome oxidase for O$_2$ in suspensions of cultured kidney cells (25), neuroblastoma, and sarcoma 180 ascites tumor cells.
cells (24), and these values consistently fell within 0.5–1.0 µM (0.3–0.7 mmHg). Rumsey et al. (16) reported an O2 half-saturation pressure of hemoglobin (P50) for O2 consumption in isolated quiescent cardiac myocytes in suspension of 2.23 µM (~1.5 mmHg). They also pointed out that the P50 is dependent on the metabolic state of the cells, which is not likely to be constant. However, the P50 rose only to 3.47 µM (~2.3 mmHg) under their experimental conditions, except under the extreme conditions of respiratory uncoupling, when it rose to 9.5 µM. Although experimental differences make direct comparisons difficult, our measurement of critical PO2 compared with the P50 measured by Rumsey et al. and the \( K_m \) of cytochrome oxidase for O2 are consistent. Erecinska and Wilson (11) posited that these differences in reported \( K_m \) values provide evidence that the \( K_m \) can change as a result of variations in metabolic parameters and the consequent alteration in the baseline redox state of cytochrome oxidase. As an example of these variations in metabolic parameters, they cite the difference between the steady-state NAD\(^+\)/NADH concentration in isolated mitochondria (range 0.01–0.1 mmHg) and in cells in suspension (range 1–100 mmHg). Nevertheless, our measurements with single muscle cells, the thickness and morphology of which differ significantly from the other cell types, are only slightly higher and generally consistent with these other cell types. The small difference may be due to the relatively large size (60 µm diameter) of the myocytes compared with the other cell types listed above (5–10 µm diameter) and, therefore, may be dependent on diffusion of O2 from the cell membrane to the more centrally located mitochondria (Fig. 4).

By use of the value for the mean \( K_m \) of cytochrome oxidase for O2 reported by Wilson et al. (26) of 0.5 mmHg and a mean critical PO2 of 1.1 ± 0.15 mmHg for intact myocytes, the mean PO2 difference between the extracellular space and the mitochondria of cardiac cells would be 0.6 ± 0.15 mmHg. Rumsey et al. (16) calculated a decrease in O2 concentration from the plasma membrane to mitochondria for cardiac myocytes from 0.18 to 1.4 mmHg. The variation in the gradient reflects differences in assumptions regarding the distribution of mitochondria relative to the plasma membrane and mitochondrial density. Our data yield a range for the calculated O2 gradient of ~0.2 to 1.2 mmHg, which is consistent with the calculations of Rumsey et al.

There was no significant difference between the critical PO2 measured at the level of a single cardiac myocyte (1.0 ± 0.22 mmHg) and that measured in a suspension of cardiac cells (1.1 ± 0.15 mmHg). This observation validates the use of the spectral imaging approach to monitor critical PO2 in single isolated cells. The mean critical PO2 for the cardiac cells was 1.0 ± 0.22 mmHg. The mean critical PO2 for the spinotrapezius cells measured by spectral imaging microscopy was 1.3 ± 0.22 mmHg. These data are not significantly different from those for cardiac myocytes. This is an interesting finding, since there are several factors that, if considered separately, might be expected to result in differences in the critical PO2 between the two cell types, including differences in myoglobin content, mitochondrial density, and cell size.

Measurement-Induced Error in Estimating Critical PO2

Our ability to estimate the critical PO2 requires that we can determine the PO2 at which NADH fluorescence first begins to change. Because the spectral images are collected at 15-s intervals, we have interpolated between points to determine the critical PO2. It is possible that in some cases we have overestimated and in others underestimated the critical PO2. However, these random errors average out over a series of experiments. Moreover, the standard error for the cardiac cell critical PO2 measurement in this study (0.22 mmHg) is only 7% higher than the standard error of the measurement made in cardiac cell suspensions (0.15 mmHg), where the sampling rate was 1/s. Thus the sensitivity of the measurement system is such that a twofold difference in critical PO2 between different cell types should be observable.
In summary, we have measured critical $P_{O_2}$ in a suspension of rat cardiac myocytes and from individual cardiac myocytes and spinotrapezius cells. The critical $P_{O_2}$ in single cardiac cells is the same as that for single spinotrapezius cells and for suspensions of cardiac cells. If we assume a minimal $O_2$ gradient between the extracellular space and the mitochondria, our results are consistent with estimates of the $K_m$ of cytochrome oxidase for $O_2$ in isolated mitochondria, using the same $O_2$-sensitive phosphorescent probe. The results observed here validate the use of endogenous NADH fluorescence changes and exogenous phosphorescent probe changes to estimate the critical $P_{O_2}$.

It is clear that the critical $P_{O_2}$ in intact rat skeletal muscle and cardiac cells is similar to the $K_m$ of cytochrome oxidase for $O_2$ measured in isolated mitochondria. For blood flow to be regulated by the release of metabolites produced during $O_2$ limitation of oxidative phosphorylation, some proportion of the tissue would be required to be at or near the critical $P_{O_2}$. Boegehold and Johnson (1) report a range of tissue $O_2$ concentrations from 4 to 40 mmHg in the cat sartorius muscle measured by microelectrode. Even the lowest value observed was 3 mmHg higher than our estimate of the critical $P_{O_2}$. Toth et al. (2), in resting cat sartorius muscle, found that $O_2$ supply appeared sufficient to support oxidative metabolism in >95% of the tissue monitored. This suggests that the great majority of tissue is well above a hypoxic level and suggests that regulation of blood flow, at least during periods of moderate mismatch between substrate delivery and substrate consumption, is likely controlled by mechanisms that are not directly related to the $O_2$ limitation of oxidative phosphorylation.

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