Visualization of Myoglobin-Facilitated Mitochondrial O_2 Delivery in a Single Isolated Cardiomyocyte

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ABSTRACT The purpose of the present study was to visualize myoglobin-facilitated oxygen delivery to mitochondria at a critical mitochondrial oxygen supply in single isolated cardiomyocytes of rats. Using the autofluorescence of mitochondrial reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H), the mitochondrial oxygen supply was imaged from ~1.4 μm inside the cell surface at a subcellular spatial resolution. Significant radial gradients of intracellular oxygenation were produced by superfusing the cell suspension with a mixed gas containing 2–4% oxygen while stimulating mitochondrial respiration with an uncoupler of oxidative phosphorylation. Augmentation of the NAD(P)H fluorescence started from the core of the cell (anoxic core) and progressively expanded toward the plasma membrane, as the extracellular Po_2 was lowered. Inactivation of cytosolic myoglobin by 5 mM $NaNO_2$ significantly enlarged such anoxic regions. Nitrite affected neither mitochondrial respiration in uncoupled cells nor the relationship between Po_2 and the NAD(P)H fluorescence in coupled cells. Thus we conclude that myoglobin significantly facilitates intracellular oxygen transport at a critical level of mitochondrial oxygen supply in single cardiomyocytes.

INTRODUCTION

Myoglobin occurs relatively abundantly in the cytosol of oxidative muscles such as cardiac and skeletal muscles. In situ ferrous myoglobin reversibly binds with oxygen molecule. Thus myoglobin may carry O_2 along O_2 concentration gradients if gradients of myoglobin oxygenation are produced within a cell (myoglobin-facilitated O_2 diffusion) (Wittenberg, 1970; Wittenberg and Wittenberg, 1989). The function of myoglobin as a physiological O_2 carrier depends on the following three factors: 1) diffusivity in the cytoplasm, 2) concentration in the cytoplasm, and 3) gradients of myoglobin O_2 saturation (S_{Mb}) within the cytoplasmic space. Recent studies appear to challenge the effectiveness of myoglobin-facilitated O_2 diffusion in the in vivo myocardium. First, myoglobin diffusion may be significantly slower in situ than in diluted myoglobin solutions (Jürgens et al., 1994; Papadopoulos et al., 1995). Second, hearts isolated from gene knockout mice in which myocardial myoglobin concentration is virtually zero respond to hypoxia in a manner similar to that of the response of normal hearts (Garry et al., 1998). Finally, myocardial capillary Po_2 (20–30 Torr), the pressure head for O_2 diffusion from the capillary blood to mitochondria, far exceeds the P_{50} of myoglobin, whereas intracellular gradients of Po_2 seem negligible in myocardial cell at a normal resting metabolic rate (Gayeski and Honig, 1991; Rumsey et al., 1990; Wittenberg and Wittenberg, 1985). Thus sufficient intracellular gradients of S_{Mb} might not be produced in the in vivo heart (Arai et al., 1999).

We undertook the present study to demonstrate that myoglobin facilitates O_2 transport in single cardiac myocytes isolated from the rat ventricle. Significant gradients of intracellular oxygenation that favor myoglobin-facilitated O_2 diffusion were produced by increasing the intracellular O_2 flux, using an uncoupler of mitochondrial oxidative phosphorylation while extracellular Po_2 was carefully regulated.

Mitochondrial reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) may indicate the balance between the supply of reducing equivalents and that of O_2 at cytochrome *c* oxidase. If the electron supply to the respiratory chain is not limiting, the O_2 supply to mitochondria can be assessed by mitochondrial NAD(P)H (Chance, 1976; Esumi et al., 1991; White and Wittenberg, 1993). In ventricular myocytes, mitochondria discretely occur in parallel with myofibrils, but the spatial distribution within a cell is relatively uniform. Thus measurement of mitochondrial NAD(P)H autofluorescence (NADH fluorescence) with a subcellular spatial resolution allows quantitation of intracellular heterogeneities of mitochondrial O_2 supply (intracellular Po_2 gradients) (Takahashi et al., 1999). Using this technique, we visualized the intracellular O_2 supply with and without functional myoglobin.

MATERIALS AND METHODS

Isolation of ventricular myocytes

Male Sprague-Dawley rats weighing 250–300 g were anesthetized with 50 mg/kg sodium pentobarbital i.p. Single ventricular myocytes were isolated from the heart by the collagenase (type 2; Worthington, Freehold, NJ) digestion method as reported previously (Takahashi and Doi, 1995) and subsequently suspended in HEPES buffer solution (150 mM NaCl, 3.8 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 10 mM glucose, and 10 mM HEPES). The medium also contained 0.1% bovine serum albumin, and the

Received for publication 8 December 1999 and in final form 22 February 2000.

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0006-3495/00/06/3252/08 $2.00

pH was adjusted to 7.35 at room temperature. The cell suspension was kept in the dark until use.

Imaging of the intracellular O_2 supply by mitochondrial NAD(P)H autofluorescence

We assessed intracellular oxygenation from the mitochondrial NAD(P)H autofluorescence upon UV excitation (Takahashi et al., 1999). An aliquot of a 20-μl cell suspension containing ~2000 cells was placed in an airtight measuring cuvette on the stage of an inverted microscope (IX70; Olympus, Tokyo, Japan). Cells were excited with a U-MWU filter set (excitation 330–385 nm, fluorescence > 420 nm), and the cellular autofluorescence was captured and digitized using a 10-bit digital cooled CCD camera (C4742; Hamamatsu Photonics, Hamamatsu, Japan). Digitized images were processed and quantitated using IPLab image-processing software (Scanalytics, Fairfax, VA) and finally represented in pseudocolors by using Noesys Transform software (Fortner Software, Sterling, VA). With an LC Plan Fl40× object lens (×40 magnification, numerical aperture 0.6; Olympus), one pixel on the computer monitor corresponded to 0.2 μm. All of the fluorescence images were analyzed after background fluorescence subtraction.

Visualization of the plasma membrane of an isolated single cardiomyocyte

The plasma membrane of an individual cardiomyocyte was visualized using a lipophilic styril fluorescence dye, *N*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styril)pyridinium dibromide (FM 1–43; Molecular Probes, Eugene, OR). Cells were incubated with 2 μM FM 1–43 for 5 min at room temperature, and the fluorescence for excitation at 470–490 nm was captured. To more accurately locate the plasma membrane, hazes in the captured image were removed using a mathematical deconvolution software (HazeBuster, VayTek, Fairfield, IA).

Measurement of cellular O_2 consumption

Oxygen consumption of the cell suspension (containing ~10^5 cells/ml) was determined using a polarographic O_2 electrode (model 17026; Instrumentation Laboratory, Lexington, MA) as described previously (Takahashi et al., 1998). The data were normalized to the total number of cells.

Inactivation of cytosolic myoglobin by $NaNO_2$

Nitrite converts myoglobin to the ferric myoglobin that no longer reversibly binds with O_2. First, we determined the concentration of $NaNO_2$ required for complete oxidation of intracellular myoglobin. This was judged from changes in the optical density of a single individual cardiomyocyte at 434 nm (an absorption peak of deoxy myoglobin) with an anaerobic to an aerobic transition, with an assumption that light absorption by intracellular pigments other than myoglobin is not significantly affected by NO_2^- at this wavelength. Measurements of optical density were conducted in the presence of 1 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; see below) at 26°C. In control cells, aerobic optical density divided by the corresponding anaerobic optical density was 0.73 ± 0.04 (mean ± SD, $n = 7$). In cells incubated with 2 mM $NaNO_2$ for 15 min, the value was 0.90 ± 0.04 ($n = 7$), still significantly different from 1. Finally, the change in the optical density was abolished by either 5 mM or 10 mM $NaNO_2$; ratios of aerobic to anaerobic optical density were 1.02 ± 0.12 ($n = 5$) and 0.98 ± 0.14 ($n = 5$), respectively. Therefore, in the present study, inactivation of cytosolic myoglobin was conducted by incubating cells with 5 mM $NaNO_2$ for >15 min.

EXPERIMENTAL PROCEDURES

We demonstrated in our previous studies (Takahashi et al., 1998, 1999) that incubation of single isolated cardiomyocytes with an uncoupler of oxidative phosphorylation, 1 μM CCCP, at room temperature produces considerable intracellular gradients of myoglobin oxygenation where the core of single cardiomyocytes may be completely depleted of O_2 (anoxic core). The present experiments were carried out in exactly the same conditions as these studies. To quantitate the intracellular O_2 supply by the NADH fluorescence, the cell suspension in the measuring cuvette was superfused with a humidified gas containing <0.001% O_2 while the NADH fluorescence was imaged (anaerobic NADH fluorescence). Then the cell suspension was superfused with a mixed gas containing 2.09%, 3.14%, 4.18%, or 20.2% O_2, while the NADH fluorescence was imaged again (hypoxic NADH fluorescence). The oxygen concentration in the mixed gas was regulated with computer-controlled mass flow controllers (STEC320; STEC, Kyoto, Japan). Finally, NAD(P)H oxidation was quantitated by dividing the hypoxic NADH fluorescence image by the corresponding anaerobic NADH fluorescence image. These procedures were repeated in cardiomyocytes treated with 5 mM $NaNO_2$. Photobleaching of the autofluorescence was not compensated for. All of the experiments were conducted at 27–28°C. We discarded the data if cellular morphological changes (i.e., hypercontracture) were observed during the experiment.

Statistics

Data are represented as the mean ± SD. The difference between two means was judged by Student's *t*-test, where $p < 0.05$ was considered significant.

RESULTS

NADH fluorometry

Intracellular heterogeneities of mitochondrial O_2 supply were reconstructed using the NADH fluorescence. Fig. 1 exemplifies quantitation of the NADH fluorescence in a single cardiomyocyte treated with 1 μM CCCP. Radial changes in the NADH fluorescence at an arbitrary cross section of the cell were indicated. When O_2 concentration of the superfusion gas was switched from <0.001% to 2.1% (15 Torr), the NADH fluorescence intensities were decreased (*dashed line*), but intensities were decreased more near the plasma membrane than at the core of the cell, reflecting intracellular gradients of mitochondrial NADH oxidation (Fig. 1 *A, closed circles*). In contrast, in the 5 mM $NaNO_2$-treated cell (Fig. 1 *B*), mitochondrial NADH appeared to be almost maximally reduced in most regions of the cell for the same extracellular Po_2.

Fig. 2 shows three-dimensional displays of the NADH fluorescence for the control and 5 mM $NaNO_2$-treated cardiomyocytes at various superfusion gas O_2 concentrations. At the superfusion gas O_2 concentration of 20.2% (Po_2 = 144 Torr), mitochondrial NAD(P)H was fully oxidized, and no heterogeneity in the NADH fluorescence was observed. As O_2 concentration of the superfusion gas was decreased, reduction of mitochondrial NAD(P)H started from the cell core that progressively expanded toward the plasma membrane, suggesting enlargement of the anoxic core. In 5 mM

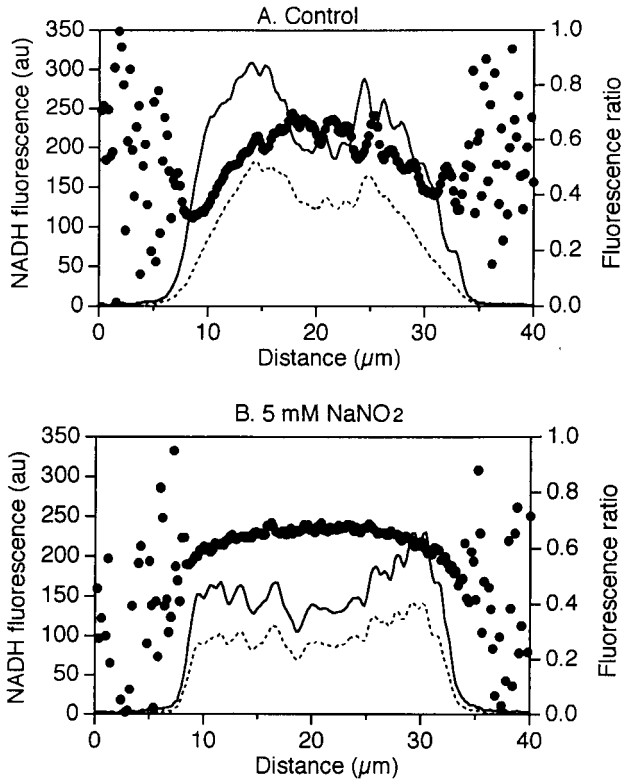

FIGURE 1 Representative data demonstrating reconstruction of radial profiles of the NADH fluorescence in a single cardiomyocyte. Raw mitochondrial NAD(P)H fluorescence intensities within a region of interest were averaged for anaerobic (——) and then hypoxic (– – –) states, and the latter was divided by the former (●). (*A*) Results for the control cardiomyocyte. (*B*) Results for the 5 mM $NaNO_2$-treated cell. Oxygen concentration of the superfusion gas was 2.09%. Cellular respiration was stimulated by 1 μM CCCP. Photo bleaching was not compensated for.

$NaNO_2$-treated cells, such reductions of mitochondrial NAD(P)H were always observed at higher O_2 levels.

Fig. 3 shows radial intracellular profiles of the NADH fluorescence for individual cardiomyocytes. As described above, treatment with 5 mM $NaNO_2$ relatively elevated the NADH fluorescence for a given superfusion gas Po_2, except for Po_2 = 144 Torr.

Cellular O_2 consumption

According to Fick's law of diffusion, oxygen flux determines the magnitude of the extracellular (from the surface of the medium to the plasma membrane) and intracellular (from the plasma membrane to mitochondrial membranes) Po_2 gradients. Therefore, alterations of cellular O_2 consumption that might be associated with nitrite treatment (Doeller and Wittenberg, 1991; Glabe et al., 1998) should be investigated before we discuss the effects of myoglobin inactivation on intracellular O_2 diffusion. In coupled cardiomyocytes, treatment with 5 mM $NaNO_2$ significantly increased cellular O_2 consumption from 52 ± 9 nmol/min/10^6 cells (n = 10) to 63 ± 5 nmol/min/10^6 cells (n = 10). Contrastingly, in uncoupled cardiomyocytes, O_2 consumption at Po_2 = 10–30 Torr (Po_2 at which the NADH fluorometry was conducted) was 353 ± 50 nmol/min/10^6 cells (n = 10), which was not different from the 5 mM $NaNO_2$-treated uncoupled cells (352 ± 59 nmol/min/10^6 cells, n = 10). Five millimolar $NaNO_2$ did not affect the calibration of the Po_2 electrode.

Direct effects of 5 mM $NaNO_2$ on NADH fluorescence

A possibility that treatment with $NaNO_2$ might affect the NADH/NAD^+ equilibrium at a given Po_2 through direct effects on the respiratory chain (Chung et al., 1996; Glabe et al., 1998) was examined. We used coupled cardiomyocytes in which O_2 flux is low and intracellular gradients of myoglobin oxygenation should be negligible. This means that, in coupled cells, one can ignore effects of nitrite on myoglobin-facilitated O_2 diffusion and specifically address direct effects of the compound on the NADH/NAD^+ equilibrium. Five millimolar $NaNO_2$ did not affect the absolute fluorescence intensity of anoxic cardiomyocytes, while it decreased NADH fluorescence by ~22% at Po_2 = 144 Torr. However, values for the normalized NADH fluorescence were similar in control and 5 mM $NaNO_2$-treated cardiomyocytes for a Po_2 range of 1.8–23 Torr (Fig. 4).

Spatial resolution of the NADH fluorescence measurement near the plasma membrane

Because the present assessment of intracellular oxygenation relies upon mitochondrial autofluorescence, spatial resolution of the measurement is determined in part by the intracellular localization of mitochondria. It has been predicted that intracellular Po_2 may drop quite quickly just below the plasma membrane (Federspiel, 1986; Groebe, 1995). Thus it is important to specifically define the spatial resolution of the NADH fluorescence measurement near the plasma membrane. As shown in Fig. 5, the plasma membrane of an individual cardiomyocyte was clearly visualized by FM 1–43 fluorescence (Fig. 5 *A*). As demonstrated previously (Eng et al., 1989; Richmond et al., 1997), a longitudinal stripe pattern in the NADH fluorescence image (Fig. 5 *B*) originates in mitochondria. This pattern corresponds to small peaks in the radial NADH fluorescence intensity profile (*bottom panel, dashed line*). Thus the distance from the plasma membrane to the nearest mitochondrion can be quantitated by superimposing the FM 1–43 fluorescence image on the NADH fluorescence image. The average distance from the plasma membrane to the closest mitochondrion thus calculated was 1.4 ± 0.6 μm (n = 39).

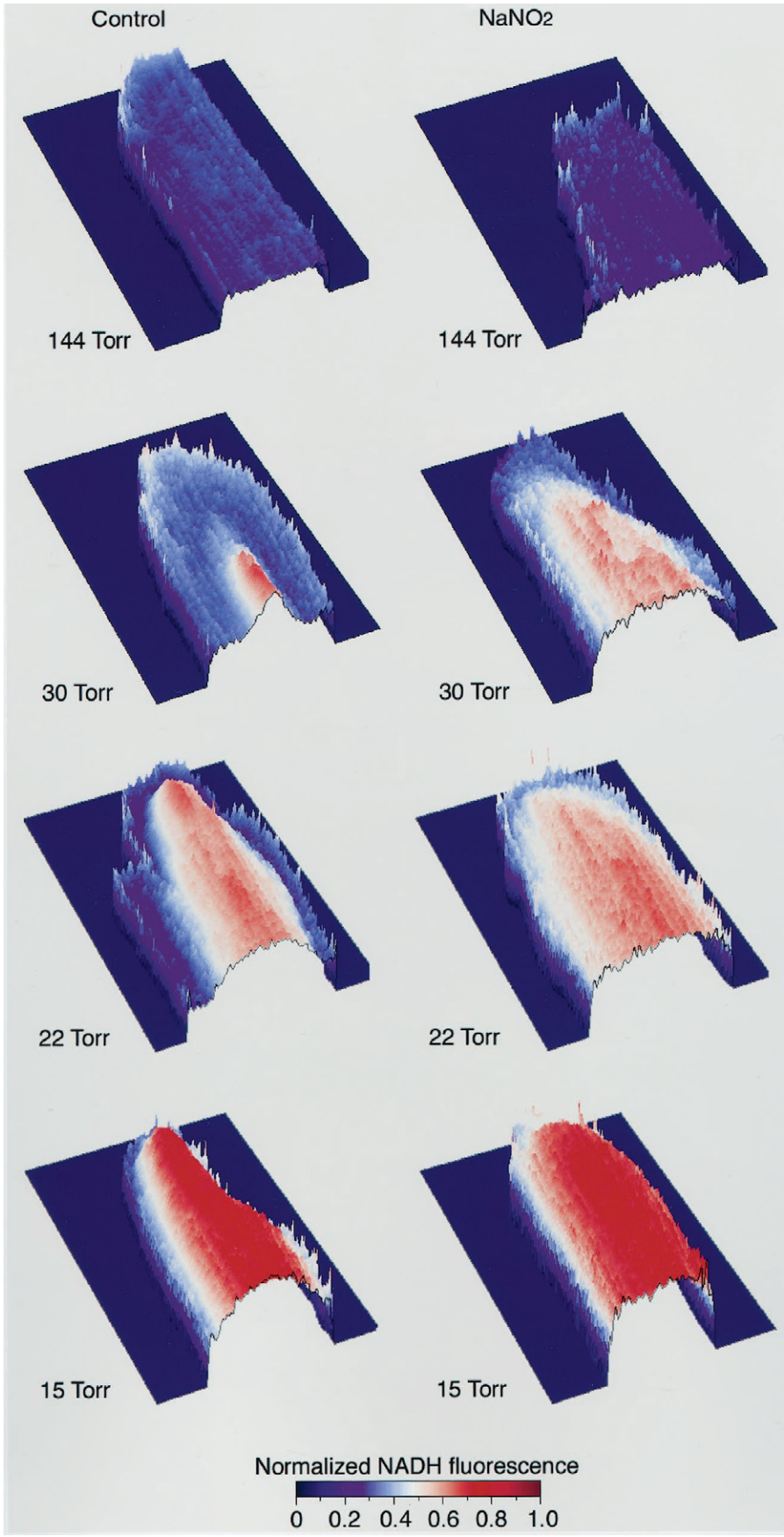

FIGURE 2 Three-dimensional displays of mitochondrial NAD(P)H fluorescence intensities for superfusion gas O_2 concentrations of 20.2% (144 Torr), 4.08% (30 Torr), 3.14% (22 Torr), and 2.09% (15 Torr). Fluorescences for the control and $NaNO_2$-treated cells were compared for identical superfusion gas O_2 concentrations. The NADH fluorescence normalized as described in Fig. 1 is represented in pseudocolors. Cell contours were manually traced and the data outside the cell boundaries were set to 0, because there was no significant NADH fluorescence outside the cell and the ratio cannot be calculated. The image was cross-sectioned near the center of the cell so that the radial NADH fluorescence profile can be clearly seen. The short axis of the base plane corresponds to 54 μm. Cellular respiration was stimulated by 1 μM CCCP. Photobleaching was not compensated for. Individual images are from different cells.

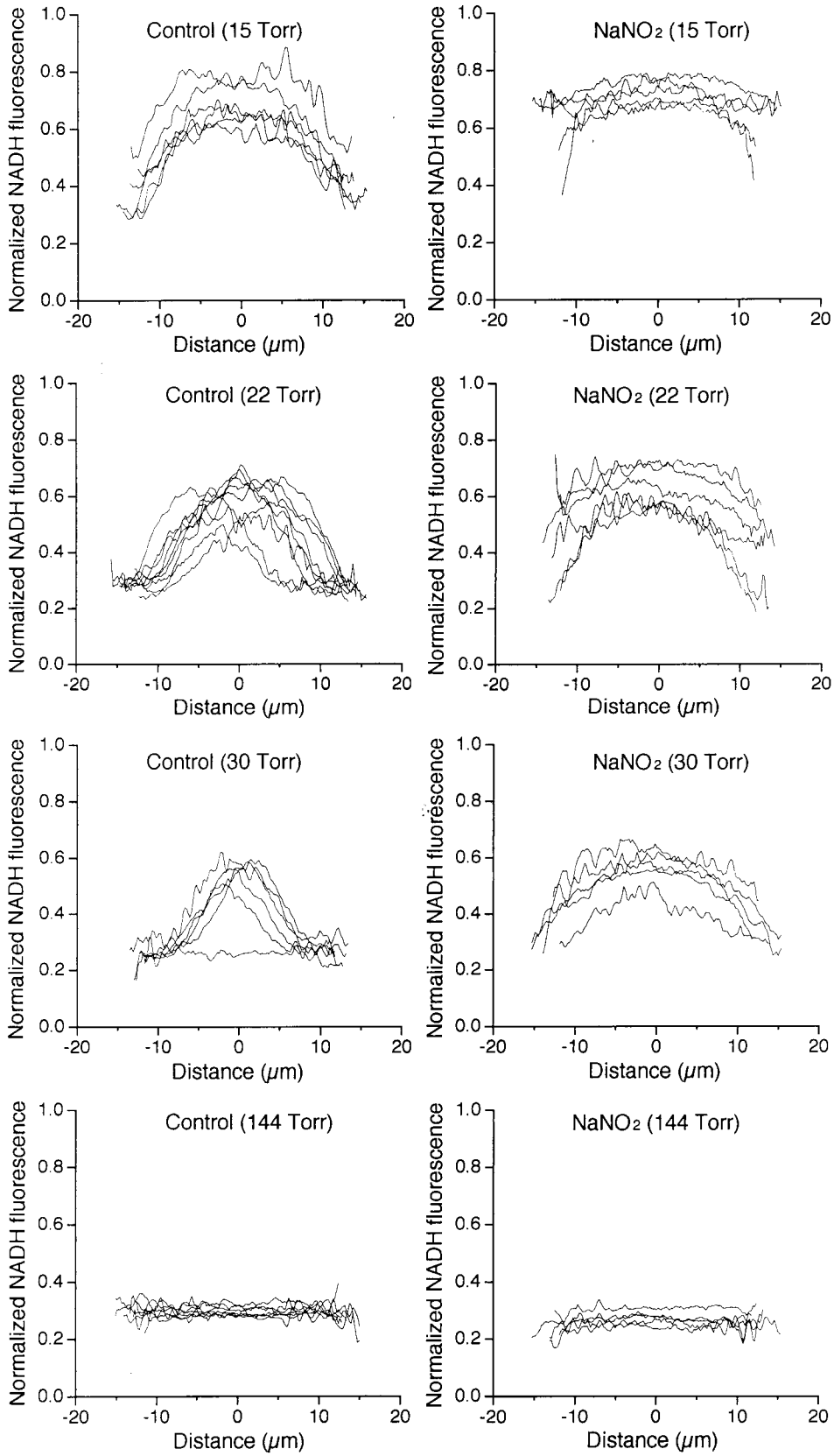

FIGURE 3 Radial profiles of the NADH fluorescence intensity for individual cardiomyocytes. Superfusion gas O_2 concentrations were 20.2% (144 Torr), 4.08% (30 Torr), 3.14% (22 Torr), and 2.09% (15 Torr). Distance = 0 corresponds to the center of the cell. Data from cells in which the width was >32 μm or <24 μm were not included. Cellular respiration was stimulated by 1 μM CCCP. Photobleaching was not compensated for.

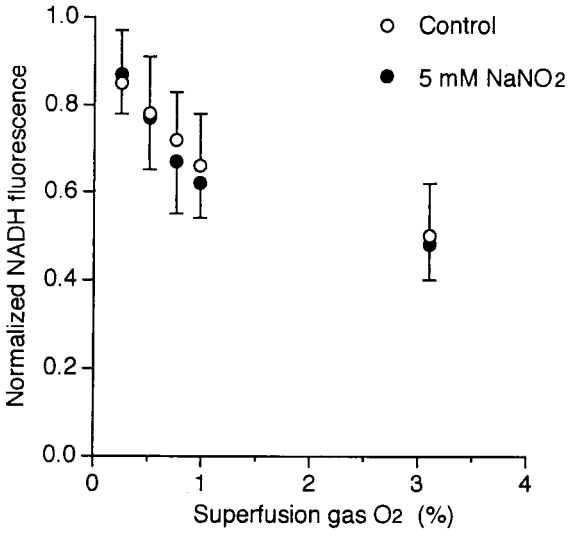

FIGURE 4 Relationship between the extracellular O_2 concentration and the NADH fluorescence for the control and 5 mM $NaNO_2$-treated cardiomyocytes. The data were collected from coupled cells. Data are means ± SD.

DISCUSSION

Myoglobin in the heart tissue can enhance intracellular O_2 transport by adding an extra flux of O_2 to mitochondria (Wittenberg, 1970; Wittenberg and Wittenberg, 1989). Theoretical study (Groebe, 1995) has predicted that myoglobin-facilitated O_2 diffusion may increase the effective O_2 conductivity by ~2.3 times at the P_{50} of myoglobin (5.3 Torr) and by ~6 times at Po_2 close to zero. Therefore, myoglobin-facilitated O_2 diffusion would be more important in hypoxia and/or for increased cardiac work where intracellular Po_2 is low. However, studies have indicated that in situ myoglobin diffusion in muscles is substantially slower than that in solutions (diffusion coefficient is only ~30% of the value used for the model study; Jürgens et al., 1994), thus significantly discounting the role of myoglobin as a physiological intracellular O_2 carrier. Along the same lines as these findings, hearts isolated from mice that genetically lack myoglobin can normally increase stroke work to increases in preload, even in hypoxia (Garry et al., 1998), although the absence of myoglobin in vivo may have stimulated the expression of other compensatory mechanisms (Gödecke et al., 1999).

Gradients of S_{Mb} within the cytoplasm are a prerequisite for myoglobin-facilitated O_2 diffusion. In previous studies (Takahashi and Doi, 1995; Takahashi et al., 1998) we demonstrated that S_{Mb} in a single individual cardiomyocyte can be spectrophotometrically imaged at a subcellular spatial resolution, thus allowing quantitation of intracellular gradients of S_{Mb}. Subsequently, we showed that the mitochondrial O_2 supply can also be imaged by NADH fluorometry at the same subcellular spatial resolution in a single cardiomyocyte. We further demonstrated that stimulation of mitochondrial respiration by 1 μM CCCP while adjusting the O_2 concentration of the superfusion gas to 2–4% produces significant radial intracellular gradients of myoglobin oxygenation with radial NADH fluorescence gradients that mirror image the S_{Mb} gradients (Takahashi et al., 1999). Light absorption by myoglobin attenuates the NADH fluorescence, depending on the oxygenation level. Thus radial S_{Mb} gradients may also produce gradients of the NADH fluorescence. Deoxymyoglobin locating near the cell core should attenuate the NADH fluorescence (peak fluorescence at ~450 nm; Eng et al., 1989) more than oxymyoglobin locating near the peripheries of the cell (absorption

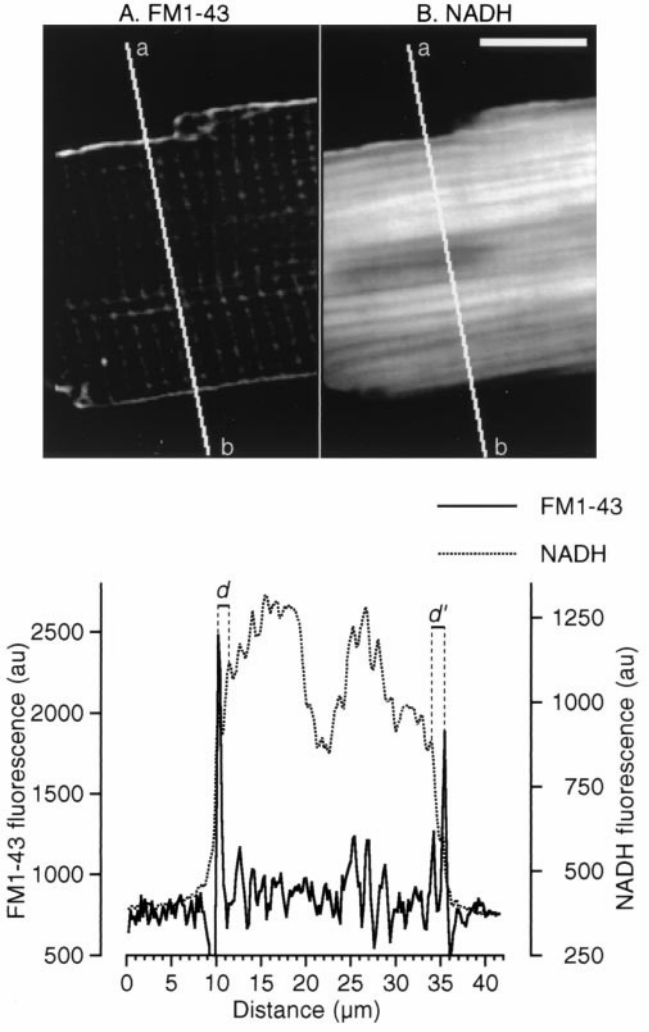

FIGURE 5 Representative data demonstrating simultaneous imaging of the plasma membrane (*A*) and the NADH fluorescence (*B*) in a single cardiomyocyte. A 10-μm scale bar for *A* and *B* appears in *B*. Radial profiles of FM 1–43 and NADH fluorescence along lines *a* and *b* are indicated in the bottom panel. Because small peaks in the NADH fluorescence correspond to individual mitochondria, distances from the plasma membrane to the closest mitochondria were determined as *d* and *d*′. The FM 1–43 fluorescence image was deconvoluted to accurately localize the plasma membrane.

peaks in the Soret band are at 434 nm and 418 nm for deoxy- and oxymyoglobin, respectively; Antonini and Brunori, 1971). In contrast, we found that the NADH fluorescence was gradually increased toward the cell core. Thus our finding strongly suggests that the core of a cardiomyocyte may become anoxic, whereas myoglobin near the plasma membrane is sufficiently oxygenated. These conditions certainly favor myoglobin-facilitated O_2 transport.

The present study, carried out under the same experimental conditions, demonstrated that 5 mM $NaNO_2$ significantly suppresses the oxidation of mitochondrial NAD(P)H for extracellular Po_2 values that would produce significant intracellular gradients of S_{Mb}. In addition to the abolition of myoglobin-facilitated oxygen diffusion, nitrite might directly affect NADH/NAD^+ equilibrium (Chung et al., 1996; Doeller and Wittenberg, 1991; Glabe et al., 1998; Wittenberg and Wittenberg, 1989). We examined the latter possibility in coupled cardiomyocytes in which intracellular gradients of myoglobin oxygenation and myoglobin-facilitated oxygen diffusion should be negligible. At higher ambient Po_2 (144 Torr), we certainly found a significant reduction of the NADH fluorescence after treatment with 5 mM $NaNO_2$. This effect, however, was not demonstrated in the anoxic condition. Moreover, in the Po_2 range in which imaging experiments were performed (1.8–23 Torr), levels of the normalized NADH fluorescence were similar for control and nitrite-treated coupled cardiomyocytes. Direct nitrite effects on the NADH fluorescence can be assessed in CCCP-uncoupled cells if the cell is exposed to higher Po_2 (144 Torr), where cytosolic myoglobin is fully oxygenated and no longer facilitates intracellular oxygen diffusion. As shown in the bottom two panels of Fig. 3, nitrite appeared to decrease the normalized NADH fluorescence of uncoupled aerobic cardiomyocytes (superfusion gas $Po_2 = 144$ Torr), but the magnitude was small (0.29 ± 0.02 and 0.27 ± 0.03 for control and nitrite-treated cells, respectively; $p = 0.054$). These results together indicate that the direct nitrite effect was insignificant in the present NADH fluorescence imaging experiments. Furthermore, nitrite did not affect cellular oxygen consumption of uncoupled cardiomyocytes. Hence we conclude that elevations of the normalized NADH fluorescence that reflect reductions of oxygen delivery to mitochondria arise from the inhibition of myoglobin-facilitated O_2 diffusion by nitrite.

In the present study, NADH fluorescence intensities near the peripheries of a cell appeared to be elevated in NO_2^--treated cells compared to corresponding control cells (Fig. 3), although Po_2 at the plasma membrane should be the same for control and NO_2^--treated cells. Previous theoretical model studies (Federspiel, 1986; Groebe, 1995) have predicted that radial changes in Po_2 in the cytosol of muscle cells at near-maximum performance would be steepest just below the plasma membrane. In the present case where cytosolic myoglobin was not functioning and O_2 diffusion conductance was low, the magnitude of intracellular Po_2 gradients should expand and may approach that of the extracellular carrier free region (~7 Torr/μm; Honig and Gayeski, 1993). On the other hand, the spatial resolution near the plasma membrane was 1.4 μm in the present optical measurement. It is possible that the Po_2 gradient in NO_2^--treated uncoupled cells was too steep to be detected with the present technique, in which case the present result is not incompatible with the particularly steep Po_2 drop near the plasma membrane at increased O_2 flux.

By direct visualization of intracellular oxygenation in individual cardiomyocytes, the present study strongly supports the role of myoglobin as an intracellular O_2 carrier at a critical level of mitochondrial O_2 supply. Then, whether myoglobin significantly facilitates intracellular O_2 transport in the in vivo heart depends on the presence of gradients of S_{Mb} within the cytoplasm. Previous measurements of S_{Mb} in the in vivo heart indicated that cytoplasmic myoglobin is nearly half-saturated with O_2, and the level of S_{Mb} is resistant to changes in cardiac work and arterial O_2 supply (Coburn et al., 1973; Gayeski and Honig, 1991). We therefore precisely regulated the extracellular Po_2 so that S_{Mb} averaged over the cell was ~50%, while significantly increasing the O_2 flux (Takahashi et al., 1998). However, more recent measurements of reflectance spectra on the surface of in vivo pig hearts demonstrated that myocardial myoglobin was almost fully saturated with O_2, even at moderately elevated workloads (Arai et al., 1999). Along the same lines as this finding are the studies using ^{1}H NMR, which detects deoxymyoglobin in blood-perfused in vivo heart tissue; they reported that myoglobin in cardiac tissue was almost fully saturated with O_2, even at moderately high myocardial O_2 consumption (Bache et al., 1999) or at hypoxia (Tran et al., 1998). At present, it is not clear whether the bulk measurement of deoxy myoglobin by the NMR method is able to detect gradients of S_{Mb} along a radial diffusion length of less than 8.5 μm in normal ventricular myocytes (Opie, 1991).

In the cardiac tissue, capillary blood Po_2 that serves as the pressure head for movement of O_2 from red blood cells is 20–30 Torr, far exceeding the K_m of cytochrome *c* oxidase. However, recent studies have pointed out the importance of surprisingly large extracellular Po_2 gradients (Gnaiger et al., 1995). Furthermore, intracellular Po_2 gradients may increase at increased O_2 flux, so that they affect mitochondrial respiration as a consequence of decreased cytoplasmic O_2 concentration surrounding mitochondria in the "anoxic core" (Takahashi et al., 1998, 1999). Taken together, significant gradients of myoglobin oxygenation could be produced in the cytoplasm of the in vivo heart. Now that the present study has demonstrated myoglobin-facilitated O_2 transport to mitochondria for a critical mitochondrial O_2 supply, then efforts should be made to prove or disprove intracellular S_{Mb} gradients in the in vivo myocardium, so that physiological relevances of myoglobin are specified.

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