Anoxic cell core can promote necrotic cell death in cardiomyocytes at physiological extracellular \( \text{PO}_2 \)

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Takahashi E. Anoxic cell core can promote necrotic cell death in cardiomyocytes at physiological extracellular \( \text{PO}_2 \). Am J Physiol Heart Circ Physiol 294: H2507–H2515, 2008. First published April 18, 2008; doi:10.1152/ajpheart.00168.2008.—The physical law of diffusion imposes \( \text{O}_2 \) concentration gradients from the plasma membrane to the center of the cell. The present study was undertaken to determine how such intracellular radial gradients of \( \text{O}_2 \) affect the fate of isolated single cardiomyocytes. In single rat cardiomyocytes, mitochondrial respiration was moderately elevated by an oxidative phosphorylation uncoupler to augment the intracellular \( \text{O}_2 \) gradient. At physiological extracellular \( \text{O}_2 \) levels (2–5%), decreases in myoglobin \( \text{O}_2 \) saturation and increases in NADH fluorescence at the center of the cell were imaged (anoxic cell core) while the mitochondrial membrane potential (\( \Delta \Psi_m \)) and ATP levels at the anoxic cell core were relatively sustained. In contrast, treatment with 0.5 mM iodoacetamide (IA) to inhibit creatine kinase (CK) resulted in depletion of both \( \Delta \Psi_m \) and ATP at the anoxic cell core. Even at normal extracellular \( \text{PO}_2 \), actively respiring cardiomyocytes developed rigor contracture followed by necrotic cell death. Furthermore, such rigor was remarkably accelerated by IA, whereas cell injury was perfectly rescued by mitochondrial \( \text{F}_1\text{F}_0 \) inhibition by oligomycin. These results suggest that increases in radial gradients of \( \text{O}_2 \) potentially promote cell death through the reverse action of \( \text{F}_1\text{F}_0 \) in mitochondria located at the anoxic cell core. However, in the intact cardiomyocyte, the CK-mediated energy flux from the subsarcolemmal space may sustain \( \Delta \Psi_m \) at the cell core, thus avoiding uncontrolled consumption of ATP that can lead to necrotic cell death. Mitochondria at the anoxic core can cause necrotic cell death in cardiomyocytes at physiological extracellular \( \text{PO}_2 \).

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1 μM CCCP-treated cardiomyocytes represents a level that is well below the maximal O2 consumption in vivo.

Cell Survival Experiments

An aliquot (8 μl) of the cell suspension (~10⁶ cells/ml) was placed in an airtight cuvette with gas inlet/outlet ports on the stage of an inverted microscope (IX71, Olympus). The suspension was superfused with humidified gas (2 ml/min) with O2 concentrations ranging from ~0.001% to 10%. To facilitate a PO2 equilibrium between gas and liquid phases, the thickness of the medium was reduced to ~230 μm. The transmitted light image at 435 nm was captured by a charge-coupled device (CCD) camera (SV512, PixelVision, Tigard, OR) every 15 s (unless otherwise noted), and data were stored in the computer. Drugs [CCCP, iodoacetamide (IA), or vehicle (DMSO)] were added to the suspension immediately before measurements. Rigor development was defined as >30% shortening of the cell length. The fraction of cardiomyocytes that developed rigor contracture is indicated in the Kaplan-Meier plot.

Using phosphorescence quenching techniques, Rumsey et al. (19) generated a high-resolution O2 map in the beating heart in the anesthetized piglet ventilated with room air. Intravascular PO2, presumably representing that of capillaries and venules, ranged 18–26 mmHg. In the in vivo heart, PO2 at the sarcolemma should be lower than the capillary PO2 of ~20 mmHg (19, 31). In the present study, superfusion of the medium in the measuring cuvette with 10% (or/H11011) humidified gas (2 ml/min) with O2 concentrations ranging from ~0.001% to 10% was arbitrary defined perpendicular to the long axis of the cell image. Because the PO2 drop between the superfusion gas and sarcolemma was not determined, “hypoxia” merely means that the cell is less oxygenated compared with the “normoxia.”

Measurements of Intracellular Ca2⁺ Concentration

Changes in intracellular Ca2⁺ concentration ([Ca2⁺]i) were assessed by calculating the fluorescence ratio at 340 and 380 nm (F340/F380) of the Ca2⁺ indicator dye fura-2 (Invitrogen/Molecular Probes, Eugene, OR). Cardiomyocytes were incubated with 5 μM fura-2 AM for 30 min at 35°C. Fluorescence images of cells at 510 nm were serially captured by a CCD camera for excitation at both 340 nm (F340) and 380 nm (F380). After the background fluorescence had been subtracted, F340/F380 was calculated using image-processing software (IPLab, Scanalytics/BD Biosciences, Rockville, MD). Transmitted cell images at 435 nm were captured simultaneously.

Imaging of Smb

Smb was imaged at a subcellular spatial resolution from light absorption of individual cells at 435 nm (an absorption peak of deoxy-Mb). Transmitted light images of cells at 435 nm were captured by a CCD camera every 10 s. In a separate experiment using a Mb solution (with the addition of Na2S2O4), oxy-Mb was found to be converted to the deoxy-Mb form within 150 s in the present experimental setup (data not shown). A rectangular region of interest (ROI) was arbitrary defined perpendicular to the long axis of the cell image (see Fig. 5A). Following low-pass filtering, optical density (OD) was calculated along the arrow (see Fig. 5A) using IPLab software. Finally, Smb was determined using the following formula: Smb = (OD – ODanaerobic)/(ODaerobic – ODanaerobic). Detailed methods have been published elsewhere (25, 28).

Imaging of Mitochondrial NADH Oxidation

Mitochondrial oxidative metabolism was assessed by autofluorescence of mitochondrial NADH at a subcellular spatial resolution. Single cardiomyocytes were first superfused with 18% O2. Fluorescence images were captured using a CCD camera (excitation at 365 nm and emission at 450 nm). Subsequently, the O2 concentration of the superfusion gas was reduced to 2%. After 5 min, subsequent fluorescence images were taken. Changes in NADH fluorescence were determined, after subtraction of the nonspecific background fluorescence, by dividing the second image by the first image. Image processing was conducted using IPLab software. Data are represented in pseudocolors. Detailed methods have been published elsewhere (27).

Imaging of Mitochondrial Membrane Potential

The fluorescent dyes JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide, Invitrogen/Molecular Probes) or tetramethylrhodamine methyl ester (TMRM; Invitrogen/Molecular Probes) were used to assess changes in mitochondrial membrane potential (ΔΨm) in isolated single cardiomyocytes.

Cells were incubated with 2 μM JC-1 for 30 min at 35°C and washed twice with HEPES-Tyrode buffer. ΔΨm was assessed from the ratio of the J-aggregate fluorescence (red fluorescence, excitation at 525 nm and emission at 595 nm) and monomer fluorescence (green fluorescence, excitation at 490 nm and emission at 535 nm). A CCD camera captured fluorescence images. Intensities of the fluorescence images were averaged over the cells, and the fluorescence ratios (red/green fluorescence) were calculated.

Single cardiomyocytes were incubated with 0.5 μM TMRM for 15 min at 35°C and washed twice with HEPES-Tyrode solution. TMRM was excited at 525 nm, and fluorescence images at 595 nm were captured by a CCD camera every 7.5 s. ROIs of 20 pixel width were defined near the periphery and at the center of the fluorescence images. Average fluorescence intensities in the respective ROIs were calculated. Radial gradients of TMRM fluorescence were determined by dividing the average fluorescence at the center by that at the periphery.

Indirect Imaging of ATP Changes

There is no convenient technique currently available that allows for repeated measurements of ATP at a subcellular spatial resolution in living cells. In the present study, ATP heterogeneities were indirectly assessed by imaging the intracellular free Mg2⁺ concentration ([Mg2⁺]i) (14). This technique is based on the assumption that, at a physiological pH, Mg2⁺ is liberated upon hydrolysis of ATP and taken up during ADP phosphorylation. Thus, transient reductions of ATP may be reflected by increases in [Mg2⁺]. [Mg2⁺], was imaged at a subcellular spatial resolution using the Mg2⁺-sensitive ratiometric fluorescent dye mag-fura-2 (Invitrogen/Molecular Probes). The affinity of mag-fura-2 to Ca2⁺ is low (Kd = 5 μM at 22°C) (11) compared with resting [Ca2⁺]i, in rat ventricular myocytes (0.09 μM) (23). Mag-fura-2 fluorescence has been demonstrated to behave independently of [Ca2⁺], under a variety of physiological and pathophysiological conditions (14).

Cells were incubated with 5 μM mag-fura-2 for 30 min at 35°C. Fluorescence images of cells at 510 nm were serially captured by a CCD camera for excitation at both 340 nm (F340) and 380 nm (F380). After the background fluorescence had been subtracted, the fluorescence ratio (F340/F380) was calculated.

RESULTS

The present study consisted of cell survival experiments and the imaging of intracellular parameters relevant to oxidative energy metabolism.

Cell Survival Experiments

Cell survival experiment 1: definition of the end point. In isolated single cardiomyocytes treated with 1 μM CCCP, cell death at lowered extracellular O2 concentrations followed a typical time course (Fig. 1): I) in the early phase, there was the development of rigor contracture (represented by a longitudinal shortening) with slight increases in [Ca2⁺]; 2) over the following 2 h,
Necrotic cell death in single cardiomyocytes at lowered extracellular O2 concentrations. Single cardiomyocytes were treated with 1 μM CCCP and 0.5 mM iodoacetamide (IA) and exposed to 2% O2. Changes in the intracellular Ca2+ concentration ([Ca2+]i) were assessed by the fluorescence ratio of fura-2 at 340 and 380 nm (F340/F380). Note the abrupt shortening of the cell (rigor contracture) with a slight elevation in F340/F380. At 120 min, the formation of blebs on the cell surface was seen, as indicated by the arrowheads in the cell image at 150 min. Because fura-2 fluorescence was almost undetectable due to the escape of the dye from the intracellular space, F340/F380 was not calculated at 150 min. Representative data from 1 of 5 experiments are shown.

Cell survival experiment 2: irreversible cell injury at physiological extracellular O2 concentrations. The induction of rigor contracture was examined in single cardiomyocytes. In control (untreated) cardiomyocytes, no alteration in cellular morphology was detected at either 2% (n = 32) or 5% (n = 18) extracellular O2 levels during the 20-min observation period. These cells were quiescent and mitochondrial respiration was slow, with an insignificant intracellular O2 gradient (28).

Next, the effects of an increased magnitude of radial O2 gradients on cellular viability were tested. In quiescent cardiomyocytes, intracellular radial O2 gradients were augmented by elevating O2 flux to the mitochondria using an uncoupler of oxidative phosphorylation. CCCP (1 μM) produced a sixfold increase in O2 consumption in quiescent single cardiomyocytes (see MATERIALS AND METHODS). Figure 2 (thick curves) shows the development of rigor contracture under conditions of reduced O2 concentration. In both hyperoxia (10% O2) and normoxia (5% O2), most cells retained a normal morphology at the end of the 20-min observation period. In contrast, under conditions of both hypoxia (2% O2) and anoxia (<0.001% O2), cells deteriorated quickly. The fraction of cells in which rigor development was detected at the end of the observation period was 0% (n = 18, superfused 10% O2), 7.1% (n = 42, superfused 5% O2), 45.7% (n = 35, superfused 2% O2), and 100% (n = 17, superfused <0.001% O2).

Cell survival experiment 3: effects of IA on the cell injury at physiological extracellular O2 concentrations. Next, the role of the PCr-CK system in such cell injuries was examined. This question arose from the prominent physiological functions of this enzymatic system in cardiac muscle: 1) the primary energy reserve that comprises a temporal buffer of energy and 2) the primary intracellular energy transfer system between the ATP source (mitochondrial ATP synthase) and the ATP sink (ATPases) (21).

IA was used to nonspecifically inhibit CK (5, 9, 10, 29). Strikingly, as shown in Fig. 2 (thin curves), 0.5 mM IA considerably accelerated cell injury in both normoxic (5% O2) and hypoxic (2% O2) cardiomyocytes, whereas hyperoxic (10% O2) cardiomyocyte viability was unaffected. Treatment with 0.5 mM IA in normoxic (5% O2) cardiomyocytes with low O2 flux (i.e., without CCCP treatment) did not affect viability (0% rigor contracture at 20 min, n = 11; data not shown).

Fig. 1. Necrotic cell death in single cardiomyocytes at lowered extracellular O2 concentrations. Single cardiomyocytes were treated with 1 μM CCCP and 0.5 mM iodoacetamide (IA) and exposed to 2% O2. Changes in the intracellular Ca2+ concentration ([Ca2+]i) were assessed by the fluorescence ratio of fura-2 at 340 and 380 nm (F340/F380). Note the abrupt shortening of the cell (rigor contracture) with a slight elevation in F340/F380. At 120 min, the formation of blebs on the cell surface was seen, as indicated by the arrowheads in the cell image at 150 min. Because fura-2 fluorescence was almost undetectable due to the escape of the dye from the intracellular space, F340/F380 was not calculated at 150 min. Representative data from 1 of 5 experiments are shown.

Fig. 2. Cell injury in single cardiomyocytes with a moderate elevation of mitochondrial O2 consumption. In hyperoxia (10% O2; top left), cell viability was maintained irrespective of IA treatment. In normoxic (5% O2; top right) and hypoxic (2% O2; bottom left) cells, the development of rigor contracture was dependent on the O2 concentration. Treatment with 0.5 mM IA (thin curves) remarkably accelerated rigor development in these cells (P < 0.05 by log-rank test). In anoxic cardiomyocytes (<0.001% O2; bottom right), the effect of IA was not clear because the cells deteriorated quickly. IA(−), cardiomyocytes treated with 1 μM CCCP and vehicle for IA; IA(+), cardiomyocytes treated with 1 μM CCCP and 0.5 mM IA. Each curve consists of 2–6 individual experimental runs.
Cell survival experiment 4: effects of IA on survival of energy-depleted cardiomyocytes. First, the role of glycolytic ATP in the survival of cardiomyocytes was determined. Oxidative ATP production in mitochondria was inhibited by anoxia (<0.001% O₂) along with inhibition of mitochondrial ATP synthase by oligomycin (15 μg/ml). Cardiomyocytes were not treated with the uncoupler. Thus, mitochondrial respiration was predicted to be slow and radial O₂ gradients negligible (28).

Cardiomyocytes incubated with a normal concentration of glucose successfully survived without oxidative ATP for at least for 40 min [99% survival; Fig. 3, IA(−)/glucose]. In contrast, cardiomyocytes in which glycolysis was inhibited (in addition to oxidative ATP inhibition) by 2-deoxyglucose (2-DG; 10 mM) deteriorated quickly [49% survival at 20 min and 19% survival at 40 min; Fig. 3, IA(−)/2-DG]. These results may indicate that glycolytic ATP alone is sufficient for maintaining basic (noncontracting) cellular functions in cardiomyocytes.

IA affects not only CK-mediated energy flux but also glycolytic ATP production through inhibition of the glycolytic enzyme GAPDH (5, 9, 10, 29). Thirteen percent of cardiomyocytes with 0.5 mM IA in the normal glucose concentration medium developed rigor contracture at 40 min [P < 0.05 by log-rank test; Fig. 3, IA(+)/*glucose]. This result appears to favor an assumption that survival of oligomycin-treated cardiomyocytes (with a slow mitochondrial respiration) relies upon glycolytic ATP and that 0.5 mM IA inhibits glycolytic ATP production. However, at 20 min, the effect of IA on rigor development was not significant (Fig. 3, dashed curves). Thus, at least at 20 min, inhibition of glycolysis by IA did not fully account for the accelerated rigor development demonstrated in these cells.

In cardiomyocytes with arrested ATP productions, inhibition of CK by 0.5 mM IA did not significantly affect rigor contracture at 20 min [log-rank test; Fig. 3; comparisons between IA(−)/2-DG and IA(+)/*2-DG], indicating that PCr, as a source of high-energy phosphate, does not appear to contribute to cardiomyocyte survival in this time frame.

Cell survival experiment 5: role of cyclosporin A-sensitive permeability transition pore in cell injury at elevated O₂ flux. In cardiomyocytes, cell death may be preceded by opening of the mitochondrial permeability transition pore (PTP). The role of cyclosporin A (CsA)-sensitive mitochondrial permeability transition (mPT) opening of the mitochondrial permeability transition pore (PTP). The role of cyclosporin A (CsA)-sensitive mitochondrial permeability transition (mPT). The role of cyclosporin A (CsA)-sensitive mitochondrial permeability transition (mPT). The role of cyclosporin A (CsA)-sensitive mitochondrial permeability transition (mPT) opening of the mitochondrial permeability transition pore (PTP).

Imaging Experiments

Imaging experiment 1: intracellular gradients of O₂ and NADH oxidation at elevated O₂ flux. Previously, we have reported that in 1 μM CCCP-treated cardiomyocytes, significant radial gradients of S_Mb have been demonstrated at a constant extracellular O₂ ranging from 2% to 4% (see Fig. 7 in Ref. 28 and Figs. 2 and 4 in Ref. 25). Also, we have demonstrated similar radial S_Mb gradients in electrically paced (CCCP untreated) cardiomyocytes (see Figs. 3 and 4 in Ref. 25). In addition to these steady-state measurements, dynamic changes in S_Mb heterogeneities during a transition from aerobic to anaerobic conditions were demonstrated in the present study (Fig. 5B). Thus, radial gradients of S_Mb in actively respiring single cardiomyocytes were demonstrated in both steady and transient states.

For technical reasons, the assessment of mitochondrial oxidative metabolism by NADH autofluorescence was conducted separately. In 1 μM CCCP-treated cardiomyocytes, localized increases in NADH autofluorescence were demonstrated at physiological extracellular O₂ (Fig. 5C) (also see Fig. 3 in Ref. 27 and Figs. 5 and 6 in Ref. 25). As discussed elsewhere (27), these increases in NADH fluorescence may reflect compromised NADH oxidation due to lack of O₂ in mitochondria. Thus, under the present experimental conditions, the magnitude of radial O₂ gradients appears large enough to produce the anoxic cell core in single cardiomyocytes exposed to physiological extracellular O₂.

Imaging experiment 2: intracellular ΔΨ_m gradients at elevated O₂ flux. Suppression of mitochondrial NADH oxidation in the O₂-deficient cell core (Fig. 5C) should affect ΔΨ_m. Depolarization of the mitochondrial inner membrane may affect cell fate in two distinct ways: 1) suppression of oxidative ATP production and 2) opening of the mitochondrial PTP (22).

To seek a possible linkage between the formation of an anoxic core and accelerated cell injury in IA-treated cardiomyo-
cytes, changes in $\Delta \Psi_m$ were imaged at a subcellular spatial resolution. As shown in Fig. 6A, top, a decrease in the extracellular O$_2$ concentration from 10% to 2% in 1 mM CCCP-treated cardiomyocytes mildly reduced TMRM fluorescence, indicating a depolarization of the mitochondria. This reduction in fluorescence appeared relatively uniform in these control cardiomyocytes. These findings appear inconsistent with the suppression of NADH oxidation due to a lack of O$_2$ at the anoxic cell core (Fig. 5C).

In contrast, significant heterogeneities in TMRM fluorescence were demonstrated in 0.5 mM IA-treated cardiomyocytes with significant reductions in fluorescence specifically at the cell core (Fig. 6A, bottom). Figure 6B shows heterogeneities in TMRM fluorescence in individual cardiomyocytes. After levels of super-

Fig. 4. Effects of inhibition of cyclosporin A (CsA)-sensitive mitochondrial permeability transition (mPT) on rigor development in actively respiring cardiomyocytes treated with 0.5 mM IA. Single cardiomyocytes were pre-treated with 0.5 μM CsA. Under conditions of 2% extracellular O$_2$, the rate of rigor development was significantly lower ($P < 0.05$ by log-rank test) in CsA-treated actively respiring cardiomyocytes (1 μM CCCP + 0.5 mM IA). However, rigor developed within 15 min irrespective of mPT inhibition. Each curve consists of 4 individual experimental runs.

Fig. 5. Radial gradients of the fractional O$_2$ saturation of myoglobin ($S_{Mb}$) and mitochondrial NADH autofluorescence in 1 μM CCCP-treated single cardiomyocytes. A: transmitted light image of a single ventricular myocyte. A rectangular region of interest (ROI) is shown (box). B: calculated radial profiles of $S_{Mb}$ at 10, 40, 50, and 120 s after the superfusion gas was switched from 10% O$_2$ to <0.001% O$_2$. $S_{Mb}$ decreased with time, whereas Mb located near the cell core was desaturated more quickly, indicating the radial gradients of $S_{Mb}$. C: mitochondrial O$_2$ metabolism as assessed by the autofluorescence of mitochondrial NADH at 2% extracellular O$_2$ levels. Radial gradients of mitochondrial NADH fluorescence were not observed in single cardiomyocytes with a low O$_2$ flux [CCCP($-$)]. In contrast, in 1 μM CCCP-treated actively respiring cardiomyocytes, NADH fluorescence was significantly elevated, specifically at the cell core. This indicates impaired NADH oxidation to NAD, presumably due to lack of O$_2$ at the cell core.
fused O2 declined from 10% to 2%, TMRM fluorescence in the cell core was lower than that at the periphery. In IA-treated cardiomyocytes (red), such heterogeneities were significantly larger, and rigor developed in all cells within 12 min.

**Imaging experiment 3: intracellular ATP gradients at elevated O2 flux.** At the anoxic core, it is reasonable to assume the existence of regional depletions of ATP since intracellular diffusion of ATP in muscles (0.5 \( \times \) 10\(^{-9}\) m\(^2\)/s) (3) is much slower than that of O2 (2.4 \( \times \) 10\(^{-9}\) m\(^2\)/s) (1). It is important, therefore, to clarify the role, if any, of the ATP-depleted cell core in the overall cell fate.

Intracellular ATP heterogeneities were indirectly assessed by imaging \([\text{Mg}^{2+}]\)_i. Figure 7A shows serial imaging of \([\text{Mg}^{2+}]\)_i in 1 \( \mu \)M CCCP-treated cardiomyocytes. The percentage of superfused gas was lowered from 10% O2 to <0.001% O2. The mag-fura-2 fluorescence ratio \((F'_{340}/F'_{380})\) increased over time, presumably reflecting gradual ATP decreases, whereas radial gradients of \(F'_{340}/F'_{380}\) were not observed (Fig. 7C). The effects of 0.5 mM IA were then examined. As shown in Fig. 7B, as the concentration of O2 gradually decreased, \(F'_{340}/F'_{380}\) increased first in the cell core, producing a radial gradient of \(F'_{340}/F'_{380}\) (Fig. 7D). These findings were consistent with the \(\Delta\text{Ψ}_m\) imaging experiments shown in Fig. 6. In cardiomyocytes with moderately elevated O2 flux, IAsensitive mechanisms may sustain \(\Delta\text{Ψ}_m\) and ATP levels in the cell core where the mitochondrial O2 supply is limited.

**DISCUSSION**

Diffusion of O2 in tissues depends on its concentration gradient. Thus, in addition to capillary Po2, intracellular O2...
uncouplers, such as CCCP, are H⁺ carriers. The oxidative phosphorylation process is disrupted at low O₂ concentrations (2% O₂ or lower), which may be an important factor that regulates oxidative metabolism in mitochondria and is responsible for mitochondrial O₂ consumption and inhibition of CK, reducing ATP production, which would lead to the depletion of cellular ATP stores.

Recently, we (25, 28) visualized radial SMₘ gradients in isolated single cardiomyocytes with elevated O₂ consumptions. In separate experiments, we (27) demonstrated radial gradients of NADH oxidation that were consistent with the SMₘ gradients. These results indicate that the intracellular diffusion of O₂ may be an important factor that regulates oxidative metabolism in mitochondria, particularly those located in the center of the cell.

The question then arises as to whether the heterogeneities of intracellular O₂ and mitochondrial oxidative metabolism, particularly the anoxic cell core, affect the overall cell fate. To answer this question, imaging of predominant parameters of O₂ metabolism (SMₘ, mitochondrial NADH oxidation, ΔΨₘ, and Mg²⁺/ATP), along with cell survival experiments, was carried out in single cardiomyocytes with elevated O₂ flux.

Increased O₂ flux induced irreversible cell injury at physiological extracellular O₂ levels. Importantly, cell injury developed at O₂ concentrations (for example, 2% O₂ or ~14 mmHg) that are substantially higher than the apparent Kₘ for cytochrome c oxidase determined in isolated mitochondria (~0.1 mmHg) (6). Augmented intracellular radial O₂ gradients in actively respiring cardiomyocytes may, in fact, decrease PO₂ averaged over the cell. However, despite such O₂ concentration gradients, most mitochondria, particularly those located in the subsarcolemmal space, should still enjoy abundant O₂ supply due to their very high affinity for O₂. Therefore, a decrease in diffusional O₂ supply to the mitochondria does not simply explain such cell injuries. Another important finding is that the administration of 0.5 mM IA, aimed at nonspecifically inhibiting CK, significantly accelerated the cell injury. In cardiomyocytes, contrary to expectations, significant heterogeneity was not found in either ATP levels or ΔΨₘ despite the radial gradients of SMₘ and mitochondrial NADH oxidation. In contrast, IA treatment in these cells revealed ATP- and ΔΨₘ-depleted cell cores. How could the findings regarding cell survival and intracellular gradients of O₂ metabolism be connected?

**Pharmacological Interventions**

In the present study, two drugs, CCCP and IA, were used to increase mitochondrial O₂ consumption and inhibit CK, respectively. Before the present findings are interpreted, the effects of these drugs should be carefully examined.

Cell injuries after treatment with the uncoupler are not surprising, at least qualitatively. Oxidative phosphorylation uncouplers, such as CCCP, are H⁺ ionophores and reduce H⁺ gradients across the mitochondrial inner membrane. Thus, these uncouplers decrease ΔΨₘ. It is possible that 1 μM CCCP might have eliminated ΔΨₘ, the driving force for ATP synthesis, which would lead to the depletion of cellular ATP stores. Then, to determine whether 1 μM CCCP completely abolishes ΔΨₘ, a semiquantitative measurement of ΔΨₘ was carried out in single cardiomyocytes loaded with JC-1 (Fig. 8). At 5% O₂, the JC-1 fluorescence ratio (red/green fluorescence) was 86% of controls, whereas the ratio declined to 53% at <0.001% O₂ (n = 5). These results are consistent with the hypothesis that the magnitude of ΔΨₘ is a function of both H⁺ leaks across the mitochondrial inner membrane and the rate of electron transport in the respiratory chain, the latter being limited by O₂ availability. It is concluded that in the present CCCP-treated cardiomyocytes, ΔΨₘ was not abolished, at least under conditions of normal extracellular O₂ (5%), whereas the equilibrium between the H⁺ leak and the electron transport-coupled H⁺ pumping was shifted.

Another concern that should be addressed is the use of IA as an inhibitor of CK. IA has been used by many investigators to inhibit CK (5, 9, 10, 29) despite its lack of specificity. In fact, previous studies have demonstrated that 0.4 mM IA inhibited 70–90% of the activity of the glycolytic enzyme GAPDH in the isolated perfused rat heart (9, 10), whereas activities of adenylate kinase (9, 10), hexokinase, and phosphofructokinase (5) were unaffected. It may be noteworthy that Tian et al. (29) demonstrated by direct estimation of the glycolytic rate in the perfused rat heart that an 80% decrease in the GAPDH activity by IA did not affect the rate of glycolysis, suggesting a large reserve of the enzymatic activity.

Reductions of ΔΨₘ in the anoxic core after the administration of IA would be consistent with the inhibition of glycolysis by this alkylation agent, if ΔΨₘ in the anoxic core is sustained exclusively by glycolytically produced ATP through the reverse action of F₁Fo (see below). However, the results of the cell survival experiments are not fully compatible with this hypothesis. In the present study, it was demonstrated that survival of oligomycin-treated cardiomyocytes (with a slow mitochondrial respiration) relies on glycolytic ATP [Fig. 3, IA(−)/glucose] certainly suggests an effect of this drug on glycolytic ATP production. However, at 20 min, the effect of IA on rigor development was not significant (Fig. 3, dashed curves). Additionally, in 1 μM CCCP-treated cardiomyocytes...
in which oxidative phosphorylation was suppressed by anoxic superfusion, rigor development was identical in control and IA-treated cardiomyocytes (Fig. 2, bottom right). This result is inconsistent to the hypothesis that the inhibition of glycolysis by IA causes accelerated cell injury. Thus, in the time frame in which cell survival experiments were conducted, inhibition of glycolysis by IA did not fully account for the accelerated rigor development in cardiomyocytes with elevated O$_2$ consumption (Fig. 2, thin curves). Why, then, does IA treatment accelerate rigor development in cardiomyocytes with elevated intracellular O$_2$ flux?

**Mechanisms for Accelerated Cell Death in CCCP-Treated Cardiomyocytes Without Functional CK**

It is assumed that complex V (F$_1$F$_0$-ATP synthase/ATPase) in mitochondria located at the anoxic core might be the causative factor. At the anoxic core, corruption of ΔΨ$_m$ due to a lack of O$_2$ may turn mitochondrial F$_1$F$_0$-ATP synthase to a potent ATPase, thus partially restoring ΔΨ$_m$ through the reverse mode action of this enzyme (18, 30). At the same time, rapid and uncontrolled ATP consumption by F$_1$F$_0$ in mitochondria at the anoxic core may produce a flux of ATP from the cytoplasm to the mitochondrial matrix with a reverse action of adenine nucleotide translocase in the inner membrane. Finally, if intracellular diffusion of ATP from the O$_2$-abundant subsarcolemmal space is not fast enough to replenish the anoxic core with ATP, cellular ATP levels would reduce at normal extracellular O$_2$ levels. A decline in ATP in myofibrils results in rigor development (12). If cellular ATP levels continue to decline, ionic homeostasis, particularly that of Ca$^{2+}$, is lost. Plasma membrane integrity is disturbed, the final step in necrotic cell death (16, 32).

Additionally, at the moment when ATP (including that produced by anaerobic glycolysis) is completely consumed at the anoxic core, complex V no longer sustains ΔΨ$_m$. Dissipation of ΔΨ$_m$ at the anoxic core could open the PTP (22). This would be followed by a massive release of Ca$^{2+}$ from the mitochondrial matrix to the cytosol under pathophysiological conditions. In the present study, mPT is likely to partially account for cell injury (Fig. 4). These distinct mechanisms with different time courses would result in Ca$^{2+}$ overload and eventual necrotic cell death. Thus, mitochondria at the anoxic core can lead to overall cell injury in cardiomyocytes exposed to normal extracellular P$_O_2$.

In the intact cardiomyocyte, there may be an intrinsic mechanism by which such cell injuries at increased O$_2$ consumption is avoided. In cardiac muscle, intracellular transfer of energy predominantly relies on CK-mediated PCr/Cr flux rather than direct diffusion of adenine nucleotides (3, 21). Therefore, it is likely that CK-mediated energy flux from the O$_2$-abundant subsarcolemmal space to the O$_2$-depleted cell core might stabilize ΔΨ$_m$ at the anoxic core by substantially accelerating diffusional ATP transport to F$_1$F$_0$. Thus, CK-mediated flux of energy to the anoxic core delays overall cell death.

**Stabilization of ΔΨ$_m$ Blocks Cell Injury in Cardiomyocytes With Elevated O$_2$ Flux**

Untreated cardiomyocytes can survive without oxidatively produced ATP for a prolonged period of time (Fig. 3). As outlined above, cell injury in actively respiring cardiomyocytes may be caused by the extra consumption of ATP through the reverse operation of F$_1$F$_0$, following ΔΨ$_m$ dissipations (18, 30) in the anoxic core. Theoretically, such catastrophic wasting of ATP may be interrupted by stabilizing ΔΨ$_m$ through the supply of ATP to F$_1$F$_0$ in the anoxic core or by inhibiting this enzyme.

Based on these assumptions, the ability of inhibition of this enzyme to prevent irreversible injury in rapidly respiring cardiomyocytes lacking a CK-mediated ATP supply to the anoxic core was examined. As shown in Fig. 9, within the 20-min observation period, rigor developed in 94% of actively respiring IA-treated cells (presumably due to ATP wasting in the anoxic cell core). This was perfectly reversed by the inhibition of mitochondrial F$_1$F$_0$ by 15 μg/ml oligomycin.

Consumption of ATP by F$_1$F$_0$ may be inhibited by the subunit IF$_1$ (17). The amount and activity of IF$_1$ in cardiac mitochondria vary among animal species. Fast heart rate species, including the rat in the present study, show very little ATPase inhibition during ischemia due to small amounts of higher-affinity IF$_1$ subunits (17, 24). Instead, pharmacological inhibition that targeted F$_1$F$_0$ in the present study (Fig. 9) unveiled the pivotal role of this enzyme in determining the fate of actively respiring cardiomyocytes.

**Expansion of the PCr Energy Shuttle Theory**

In cardiomyocytes, the PCr-CK system is the predominant energy transfer system (the energy shuttle) between the site of production and those of consumptions (21). Intermyofibrillar mitochondria in the mammalian ventricular myocyte are uniformly distributed, with a highly ordered pattern such that strands of mitochondria run along the corresponding myofibrils, in which the average distance between adjacent mitochondria is ~1.8 μm (2). Thus, it is tempting to assume that the transfer of energy between mitochondria and myofibrils...
ATPases is confined to a small, spatially restricted compartment. Saks et al. (20) proposed the intracellular energy unit (ICEU), in which mitochondria form a functional complex with adjacent myofibrils and sarcoplasmic reticulum ATPases. However, if the energy shuttling takes place predominantly within the ICEU, the benefit of the CK-mediated and -facilitated energy transfer may be somewhat discounted because both mitochondria and myofibrils in the ICEU are arranged closely. The direct diffusion of adenine nucleotides may suffice for energy transfer over such a short diffusion length (<2 µm) (3).

The present findings appear to expand the energy shuttle theory of the PCr-CK system. Here, the CK-mediated energy shuttling along the radial axis of the cell, with a maximum diffusion distance of ~10 µm in the rat ventricular myocyte, is proposed to provide an important step in the intracellular energy transport. Consequently, at elevated O₂ flux, not only intra-ICEU energy transfer but also inter-ICEU energy transfer may be supported by the PCr-CK system. A radial flux of energy may be required for the equilibration of the supply of energy under inherent heterogeneities of intracellular O₂ supply within actively respiring cardiomyocytes.

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