CMLS, Cell. Mol. Life Sci. 56 (1999) 1061–1069
1420-682X /99/121061-09 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Research Article

Permeability of rat heart myocytes to cytochrome c

P. Sarti[a,*], R. B. Silver[d], L. Paroli[b], I. Nikonorov[b] and T. J. J. Blanck[c,b,d]

[a]Department of Biochemical Sciences, University of Rome 'La Sapienza', P.le Aldo Moro 5, I-00 185 Rome (Italy), Fax + 39 6 4440062, e-mail: sarti@axrma.uniroma1.it
[b]Department of Anesthesiology, Hospital for Special Surgery, 535 East, 70th Street, New York (New York 10021, USA)
[c]Department of Anesthesiology, Joan and Sanford I. Weill College of Medicine of Cornell University, New York (New York 10021, USA)
[d]Department of Physiology and Biophysics, Joan and Sanford I. Weill Graduate School of Medical Sciences of Cornell University, New York (New York 10021, USA)

Received 6 September 1999; accepted 27 October 1999

Abstract. Rat heart myocytes undergoing progressive damage demonstrate morphological changes of shortening and swelling followed by the formation of intracellular vacuoles and plasma membrane blebbing. The damaged myocytes displayed impaired N,N′-tetramethyl-p-phenyldiamine (TMPD) ascorbate-stimulated respiratory activity which was restored by the addition of reduced cytochrome c to the cell culture medium. To clarify the role played by cytochrome c in the impairment of cell respiration, polarographic, spectrophotometric and fluorescence as well as electron microscopy imaging experiments were performed. TMPD/ascorbate-stimulated respiratory activity returned to control levels, at approximately 20 μM cytochrome c, establishing the threshold below which the turnover rate by cytochrome c oxidase in the cell depends on cytochrome concentration. Mildly damaged cardiac myocytes, as indicated by cell shortening, retention of visible striations and free-fluorescein exclusion, together with the absence of lactate dehydrogenase leakage and exclusion of trypan blue, were able to oxidize exogenous cytochrome c and were permeable to fluorescein-conjugated cytochrome c. The results, while consistent with an early cytochrome c release observed at the beginning of cell death, elucidate the role played by cytochrome c in the kinetic control of mitochondrial electron transfer under pathological conditions, particularly those involving the terminal part of the respiratory chain. These data are the first to demonstrate that the sarcolemma of cardiac myocytes, damaged but still viable, is permeable to cytochrome c.

Key words. Cytochromes; mitochondria; myocytes; cell degeneration; electron transfer kinetics.

Introduction

Located in the mitochondrial intermembrane space, cytochrome c is a hydrophilic protein which donates electrons to cytochrome c oxidase (EC 1.9.3.1, CCOX), the terminal acceptor of the respiratory chain [1–3]. Cell respiration is responsible for the formation and maintenance of the mitochondrial proton electrochemical gradient, $\Delta\mu_{H+}$, used by the cell to drive ATP synthesis [4] and to maintain cell viability. In vitro experiments have shown that despite the high rates of CCOX oxidation [$k' = 10^4$, 10^5 s^{-1}; ref. 5], the reduction of CCOX is a slow process with apparent rate constant values ranging from 10^{-1} to maximally 10^2 s^{-1} [6, 7]. The mechanism of control of CCOX reduction is a matter of intensive

* Corresponding author.

study; as proposed, it appears to involve either an intrinsically slow intramolecular electron transfer from the electron entry to the O_2-binding site of CCOX [6] or a slow H^+ diffusion to the O_2-binding site [7, 8]. Regardless of the mechanism, the reduction rate correlates well with the CCOX turnover number under all conditions explored, including purified and phospholipid-reconstituted CCOX [6, 9, 10].

A number of studies based on the specific inhibition of the different respiratory chain components have suggested that the electron flux control coefficient of CCOX can be relatively low [11–13]. In contrast, experiments carried out using either intact cultured human wild-type cells or mitochondrial-DNA-mutation-carrying human cell lines have suggested a tighter in vivo control of respiration by CCOX [14–16]. If this is the case, then availability of cytochrome c and O_2 may become relevant to the mitochondrial respiratory control function over and above that exerted at the level of CCOX. Kinetic spectroscopic experiments [17] with intact myocytes have demonstrated that under normal physiological conditions, the high concentration of reduced cytochrome c in the intermembrane space [18] and the favorable electron transfer efficiency to cytochrome oxidase are both incompatible with the donation of electrons from cytochrome c to oxidase as the rate-limiting step of the oxygen reduction cycle [19]. Furthermore, the diffusion-limited rate of reaction with O_2 excludes the possibility that O_2 supply is rate limiting under normal cell oxygenation conditions, even if the K_M of CCOX for O_2 is higher in vivo than in vitro [20]. Cytochrome c loss from mitochondria and cells occurs in pathologic situations, including activation of the cell apoptotic program [21]. Moreover, early cytochrome c release has been detected in the perfusate of ischemic guinea pig hearts [22], and the cytochrome c stimulation of respiration by skinned muscle fibers isolated from ischemic rat hearts suggests a loss of cytochrome c from mitochondria [23].

With this in mind, we have asked whether electron transfer between cytochrome c and oxidase might become rate limiting early in the cascade of cardiac myocyte damage. We have therefore investigated if isolated rat cardiac myocytes, damaged but still viable, became permeable to exogenous (externally added) cytochrome c and whether their mitochondria were able to oxidize cytochrome c. Primary cultures of rat heart myocytes were employed to examine cell respiration, cell permeability to cytochrome c, and cytochrome c oxidation using spectroscopic and imaging techniques.

Materials and methods

Myocyte isolation. Cardiac myocytes were isolated as previously described [24] with minor modifications. Sprague Dawley rats were anesthetized intraperitoneally with 3% pentobarbital, and heparinized with 0.5 ml heparin (1000 units/ml). During anesthesia, the heart was isolated and cannulated via the aorta and perfused in a retrograde direction for 1 min with Ca^{2+}-free Hepes-buffered minimal essential medium (MEM at 35 °C), gassed with 85% O_2 and 15% N_2 at a flow rate of 8 ml/min. Hepes-MEM pH 7.3 contained 117 mM NaCl, 5.7 mM KCl, 1.5 mM Na_2PO_4, 1.7 mM $MgCl_2$; 20 mM Hepes, amino acids and vitamins; 5.4 mM glucose, 4 mM L-glutamine, 10 mM taurine, and 4 mM $NaHCO_3$ were also added to the medium. Perfusion with Hepes-MEM, containing collagenase 0.05% (w/v) at 35 °C was then carried out for 20–30 min, after which the heart was removed and cut into pieces, in 10 ml Hepes-MEM containing 1 mM $CaCl_2$ and 0.5% (w/v) bovine serum albumin (BSA) to which 0.1% (w/v) collagenase (type 2 from Boehringer Mannheim) had been added. The tissue was then incubated for 10 min in a shaking water bath at 37 °C; the resulting supernatant contained dispersed myocytes and was centrifuged at 35 *g* for 2 min, at room temperature. The pellet was washed three times by centrifugation at 35 *g*. Myocytes were used after fractionation on an isotonic Percoll (Pharmacia, Uppsala, Sweden) gradient containing 0.25% (w/v) BSA at 52 *g* for 10 min, at room temperature. Rod-shaped myocytes were collected in the pellet while damaged cells and subcellular particles layered at the surface. The rod-shaped myocytes were further washed twice using Hepes-MEM (see above), and were diluted to a density ranging between 5 and 1×10^6 cells ml^{-1} (stock suspension).

Myocytes were suspended in Hepes-MEM supplemented with 0.5% BSA or in phosphate-buffered saline (PBS) Dulbecco's, thermostated at the temperature specified in the text. Unless otherwise stated, during the cell degeneration experiments, myocytes were gently shaken in air avoiding evaporation. A total of 17 different myocyte preparations were used for oxygraphic and cytochrome c oxidation experiments. Simultaneous measurements of respiration, cytochrome c oxidation, and morphology (light and fluorescence microscopy) were performed on four preparations; one included cell sampling for electron microscopy and lactate dehydrogenase (LDH) determination.

Microscopic observation. Immediately after preparation or at different stages of aging, myocyte samples were fixed using 10% (w/v) formalin in Na-cacodylate buffer, and processed for electron microscopy. Specimens were observed using a Jeol 100 CX-II (Jeol, Tokyo, Japan) electron microscope operated at 80 KeV. Grids were stained with lead citrate. Light microscopic observations and cell counts were carried out using a Nikon-TMS microscope equipped with a Neubauer hemocytometer chamber (Sigma, St. Louis, MO, USA).

The ability of myocytes to exclude trypan blue (TB) was assessed as described elsewhere [25].

Preparation of fluorescein-conjugated cytochrome c. One milliliter of fluorescein isothiocyanate (1.0 mg/ml in 10% dimethylsulfoxide in 0.1 M carbonate/HCl buffer at pH 9.0) was added dropwise over 10 min to 10 mg/ml of cytochrome c in the same carbonate/HCl buffer. The reaction occurred over 5 h at room temperature. The reacted product was passed through a G-25 column equilibrated with Krebs' solution at pH 7.4. Fluorescein-cytochrome c was separated in the void volume. A spectrum confirmed the presence of fluorescein-cytochrome c at a concentration of 260 μM [26].

Incubation with myocytes. Cardiac myocytes were incubated with 110 μM fluorescein-cytochrome c for 10 min at room temperature. Cells were centrifuged, the supernatant discarded, and the cells resuspended in Krebs' buffer and placed on a glass coverslip.

Fluorescence imaging. As previously described [27, 28], the basic components of the imaging system consist of an inverted epifluorescence microscope (Nikon Diaphot) equipped with a 75 W Xenon lamp, a Nikon CF Fluor 40 × /1.3 oil immersion objective, a fluorescence excitation filter wheel (Metal Tek) coupled to an additional filter wheel (Metal Tek) holding a variety of neutral-density filters, a computer-controllable excitation light shutter, and a cooled CCD camera (Princeton Instruments) with a frame transfer chip (EEV-37) and 12-bit readout. The emitted fluorescence signal is relayed as real-time continuous output to an IBM PC/AT-compatible clone and the image pairs were collected on a Sierra Pinnacle Micro optical disc drive (1.3 GByte). The imaging work station is controlled using the Metafluor/Metamorph software package (Universal Imaging). For disodium fluorescein and fluorescein-conjugated cytochrome c experiments, images were obtained at 490 nm excitation with emission at 520 nm and an exposure time of 500 ms. To minimize photobleaching and photodynamic damage to the cells, a neutral-density filter was coupled to the excitation filter to minimize the transmitted excitation light to 10%. The fluorescence excitation was shuttered off, except during the brief period required to record an image. To correct for intrinsic autofluorescence and background, images were obtained using the experimental acquisition configuration on isolated myocytes not exposed to fluorophore and these counts were subtracted from the fluorescence images. Thirty minutes after isolation, myocytes (~ 75% intact rod and the rest rounded, predominantly TB negative) were exposed to either disodium fluorescein (11 μM) or fluorescein-conjugated cytochrome c (110 μM) for 10 min. An aliquot of these myocytes was placed on a coverslip prepared with Cell Tak and washed with Krebs' buffer. The coverslip was then placed in a plastic chamber which fit on the stage of the inverted epifluorescence microscope. An area consisting of numerous myocytes was identified under transmitted light and recorded on the imaging system prior to obtaining a fluorescence image of the same area.

Polarographic assay. O_2 consumption activity of myocytes was measured using a Clark-type oxygen electrode (Ysi Model 5300,Yellow Springs, Ohio) equipped with a microvolume (600 μl) thermostated cell (Model 600 A, Instech. Lab., Horsham, Pa.). The assay was carried out at 20 °C, to minimize temperature-dependent high oxidation rates of reductants; the baseline was recorded in the presence of air-equilibrated PBS Dulbecco's medium supplemented with 5.4 mM glucose, containing 5 mM Na-ascorbate and 2 mM N,N′-tetramethyl-p-phenylenediamine (TMPD), and 2×10^5 cells ml^{-1}. Cytochrome c at the given concentrations (see text) was added when approximately 25% oxygen was consumed. The ability to oxidize exogenous cytochrome c (see below) as well as the respiratory activity were fully inhibited by the addition of millimolar cyanide.

Spectroscopic assay. Static and kinetic measurements were carried out using a Cary 219 spectrophotometer (Varian, USA). A 2 mM stock solution of reduced cytochrome c was prepared using excess $Na_2S_2O_4$ (sodium dithionite). Excess reductant was removed chromatographically by passage through a Sephadex G25 column (15 × 2.0 cm). This solution was kept refrigerated under nitrogen, for a maximum of 2 weeks. Unless otherwise stated, myocytes to a final density of 1×10^4 cells ml^{-1} were rapidly added to a cuvette containing reduced cytochrome c, 20 μM, in PBS Dulbecco's supplemented with 5.4 mM glucose. The time course of cytochrome c oxidation was followed at 550 nm, where the extinction coefficient of the reduced minus oxidized cytochrome c is 18.5 mM^{-1} cm^{-1} [29]. Initial rates of cytochrome c oxidation (V_o) were measured.

LDH activity of the cell suspension (2×10^5 ml^{-1}) was measured according to Shirhatti and Krishna [30], using the diagnostic kit from Sigma (St. Louis, Mo.) (DG1340-K) and results were expressed as mIU ml^{-1}; 100% LDH activity was determined by treating cells with 0.2% Triton X100 (15 min), and set at 100 mIU ml^{-1}. Horse heart cytochrome c (type VI, Sigma) was used for all experimental purposes, including fluorescein conjugation. All other chemicals were of the purest analytical grade available.

Results

The respiratory activity of intact, freshly prepared myocytes was 166 nmol of O_2 consumed per minute (fig.

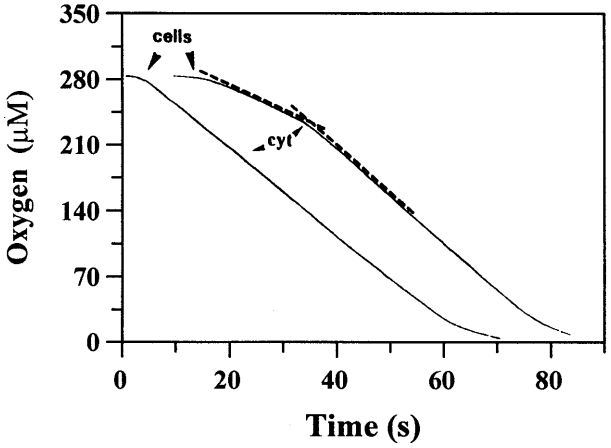

Figure 1. Oxygen consumption by intact (left trace) and damaged (right trace) myocytes (representative data). Cells, 2×10^5 ml^{-1}, were assayed immediately after preparation (~ 100% rod) or after incubation for ~ 300 min at 20 °C in PBS Dulbecco's. Assay medium: Na-ascorbate 5 mM and TMPD 2 mM; cytochrome c 20 μM was added as indicated; 100% air $O_2 \approx 280$ μM. Chamber volume = 0.6 ml, T = 20 °C.

1). The addition of cytochrome c during the reaction had no effect on the rate of O_2 consumption of intact, freshly prepared myocytes (fig. 1). After 300 min of incubation in PBS Dulbecco's (see Materials and methods), the same myocytes consumed 94 nmol O_2 per minute; this value increased to 168 nmol O_2 per minute following the addition of 20 μM cytochrome c (note the change in slope upon the addition of cytochrome c at the arrow; fig. 1).

The dependence of myocyte respiratory activity on the concentration of exogenous cytochrome c was determined after 300 min incubation; as shown in figure 2, saturation was achieved at approximately 20 μM cytochrome c. At 300 min, the cell respiratory index (CRI), i.e., the ratio of the rate observed in the presence and absence of 20 μM cytochrome c, was ~ 1.7 (CRI of freshly prepared intact myocytes set at 1).

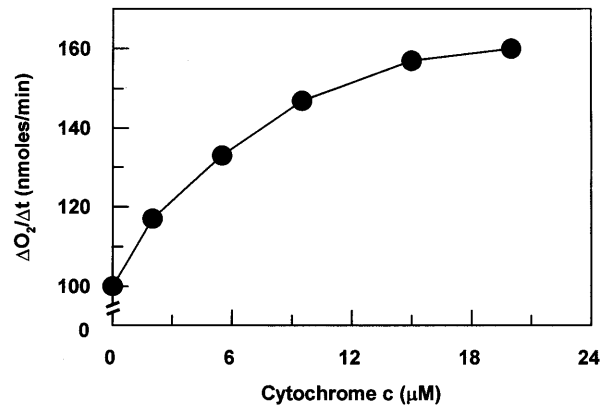

Figure 2. Dependence of the rate of oxygen consumption on the concentration of externally added cytochrome c. Cells, 2×10^5 ml^{-1}, incubated for 300 min at 20 °C in PBS Dulbecco's were supplemented directly in the O_2 electrode vessel with reduced cytochrome c. All other assay conditions as in figure 1 (details in Materials and methods).

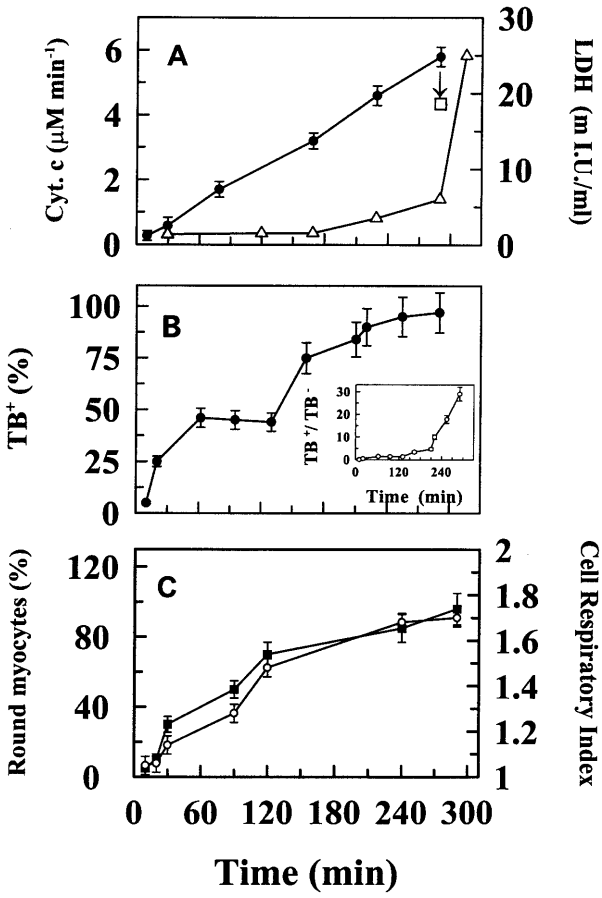

Figure 3. (*A*) Time-dependent development of cytochrome-c-oxidizing ability (●) and leakage of LDH (△). Cells were gently shaken at 20 °C in PBS Dulbecco's and, at increasing aging time after Percoll treatment, cytochrome c oxidation activity was measured at 550 nm (1×10^4 cells ml^{-1}, in the assay). The point indicated (arrow) with an open square refers to myocytes subjected to a second Percoll fractionation (see text). (*B*) Cell viability measured in parallel. The inset shows variation in the TB+/− ratio as a function of time. (*C*) Morphologic (■) and respiratory changes (○). Percoll-treated myocytes were incubated at 20 °C; the microscopic observations and the oxygraphic measurements were carried out in parallel at the given times, using a Neubauer hemocytometer chamber and the polarographic protocol described in Materials and methods.

Exogenous cytochrome c was not oxidized at cell incubation time t = 0, t/2 > 1000 s; however, following 300 min of incubation, cytochrome c was oxidized at a rate of $V_o \cong 6$ μM min^{-1} (fig. 3A). The isolated intact cells (time = 0) had a rod-shaped morphology with well-defined striations by light microscopy and clearly

demonstrated sarcomere structure by electron microscopy (fig. 4A); the supernatant of this cell suspension (2×10^5 ml^{-1}) displayed $\sim$1.5 mIU ml^{-1} LDH activity and cells excluded TB (fig. 3B). At time t = 300 min, 80–90% of the cells were rounded and vacuolated by light microscopy and demonstrated disrupted sarcomere structure and intra- and extracellular aggregates of swollen mitochondria indicative of necrosis (fig. 4C). As shown in figure 3A, LDH activity in the cell supernatant remained constant and low for about 180 min, increasing thereafter by more than a factor of 20 at 320 min incubation; at this time, close to 100% of the cells were TB positive.

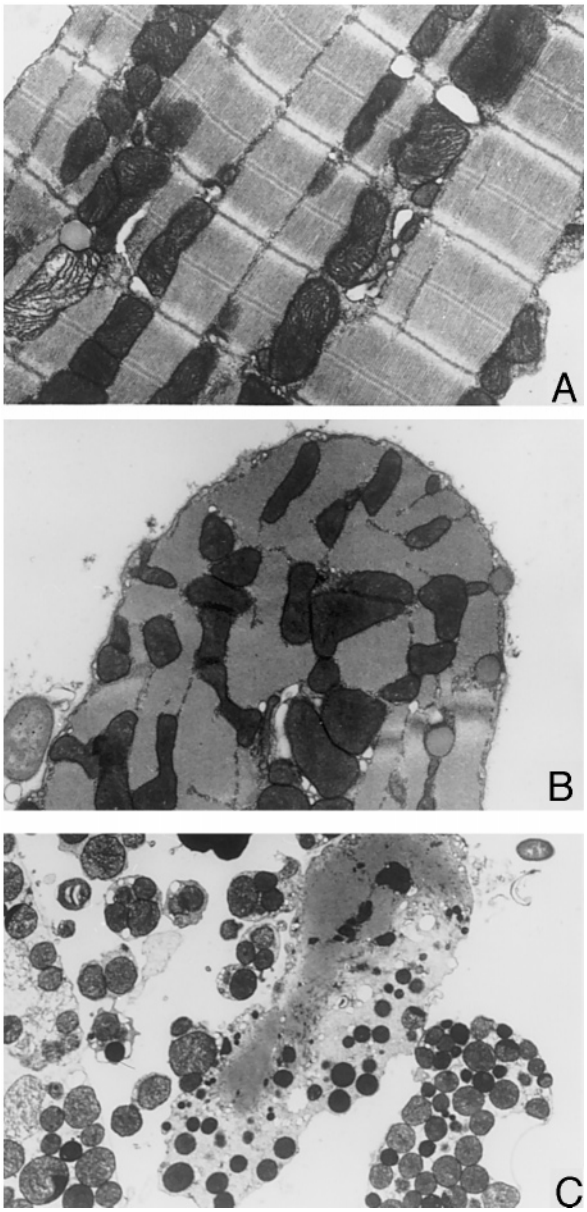

Figure 4. Electron micrographs of cardiomyocytes at different stages of aging. (*A*) Longitudinal section of fully viable rod cells obtained after Percoll treatment, time = 0; $\times$ 15,000. (*B*) Transverse section of rounded myocytes, time = 30 min; $\times$ 15,000. (*C*) Highly damaged myocytes containing mitochondrial aggregates, time = 300 min; $\times$ 7500.

The time dependence of the increase in stimulation of O_2 consumption induced by exogenous cytochrome c was essentially identical to that of the change in cell shape from rod to rounded (fig. 3C) but apparently unrelated either to the onset of positivity to TB (fig. 3B) or to the detection of a significant LDH leakage (fig. 3A). At 30 min following cell isolation, the mitochondria begin to appear swollen without the characteristic cristae observed in intact mitochondria (fig. 4B). The onset and progress of the susceptibility of exogenous cytochrome c to oxidation by the cells (fig. 3A) followed more closely the time sequence observed in CRI and cell morphology (fig. 3C). The initial rate of cytochrome c oxidation (V_o) increased almost linearly with time, from $V_o = 0.5$ to 5.8 μM min^{-1} at 10 and 300 min, respectively. The increase in TB-positive (necrotic) cells as a function of time, as well as the ratio of the TB-positive to TB-negative-rounded (i.e., rod-excluded) cells, is shown in figure 3B and inset. At early stages (up to 30 min), the microscopic observations showed a predominance of rounded cells, still TB negative. Within the following 100 min, the number of TB-positive cells remained constant, increasing abruptly thereafter (fig. 3B and inset). Over the same period of time, i.e., after 120–150 min incubation, LDH release was also constant and low. In contrast, the ability of cells to oxidize cytochrome c increased almost linearly with time; round myocytes which were predominantly TB$^-$ were found to be permeable to fluoresceinated cytochrome c (see below).

As mentioned above, with time the morphology of the cells changed from rod to rounded. Aggregates of damaged mitochondria, mostly within the cell, though later (t > 220 min) also outside the more severely damaged cells, were observed. For this reason, a second Percoll fractionation was carried out at the latest stage of incubation, and aggregate-free cells re-assayed for cytochrome c oxidation (fig. 3A). The aggregate-free cells actively oxidized cytochrome c proving that most of the oxidative activity detected is inside the cells.

Figure 5 confirms the entry of cytochrome c into (round TB$^-$) myocytes $\sim$30 min after their isolation. Cells were exposed to either fluorescein-conjugated cytochrome c (fig. 5A, B) or disodium fluorescein (fig. 5C, D) viewed with a $\times$ 40 oil immersion fluor objective. A correlation between the extent of cell damage, assessed visually, and the fluorescence signal is apparent in the myocytes exposed to fluorescein-conjugated cytochrome c: the more structurally sound the myocyte, the lower the fluorescence signal. This observation is consistent with the hypothesis that damaged myocytes become

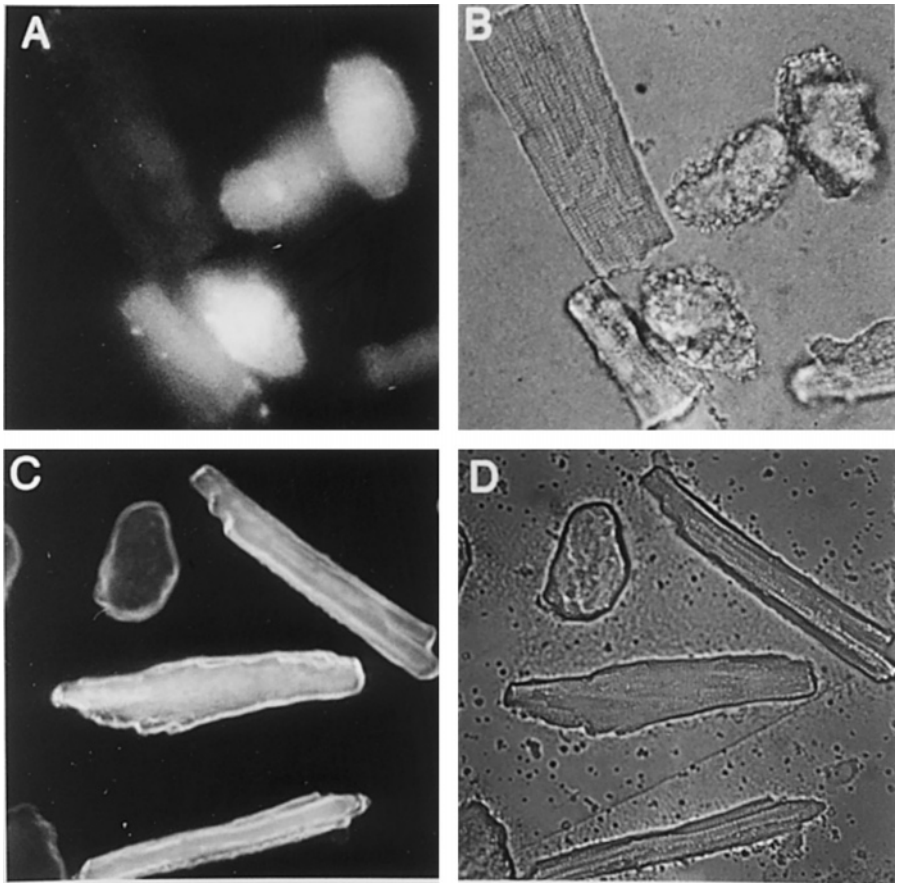

Figure 5. Fluorescence (*A*, *C*) and transmitted-light (*B*, *D*) images of isolated myocytes viewed with a × 40 oil fluor objective. The gray scales are the same in *A* and *C*. (*A*, *B*) Myocytes exposed to fluorescein-conjugated cytochrome c for 10 min: the fluorescence pattern emerging from this procedure is increased fluorescence with decreased cell viability. (*C*, *D*) The opposite pattern is seen with myocytes exposed to free fluorescein.

permeable to cytochrome c quite early (30 min after isolation) as suggested by the onset of stimulation of O_2 consumption which follows the addition of exogenous cytochrome c (fig. 3). In contrast, exposing myocytes to free disodium fluorescein yields the opposite result (fig. 5C vs 5D): the most damaged cell in the field (rounded cell in upper left-hand corner) has the lowest fluorescence, while the intact myocytes have the most fluorescence. This observation is consistent with the interaction of fluorescein compounds with the surface of cells showing varying degrees of viability: the less viable the cell, the less free fluorescein is attached and the lower the fluorescence signal [31].

Discussion

The addition of reduced cytochrome c to the medium of respiring intact myocytes did not alter the observed rate of O_2 consumption. A markedly different behavior was observed in cells with morphological alterations suggestive of cell damage, like round shrunken cells. In a time-dependent manner, these myocytes became sensitive to externally added (reduced) cytochrome c. The progressively damaged cells, predominantly TB negative, and carefully separated from cell fragments, displayed (i) a depression in ascorbate/TMPD-stimulated oxygen consumption, (ii) the ability to oxidize exogenous cytochrome c, (iii) stimulation of respiration by exogenous cytochrome c, and (iv) permeability to fluorescein-conjugated cytochrome c. All these features developed synchronously and correlated with the onset and extent of early cell damage, independently assessed by light/fluorescence and electron microscopy. Over a longer time scale, as cell injury became more severe, leakage of LDH and a response to vital staining appeared.

To focus on the appearance of cell sensitivity to cytochrome c and related kinetic behavior at the level of CCOX, we have carried out polarographic experiments using ascorbate and TMPD, cell-permeable electron

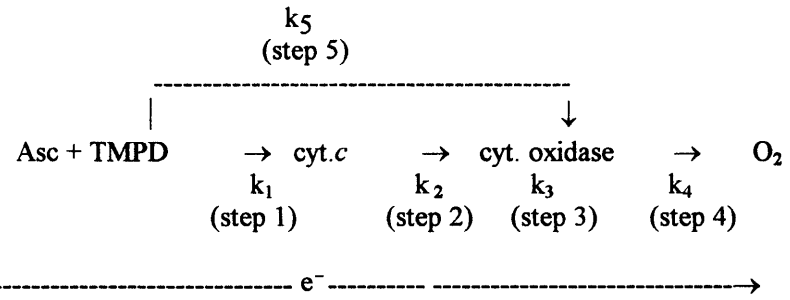

Figure 6. Electron flux through terminal part of respiratory chain. Step 5 is included for completeness though catalytically irrelevant ($k_5 = 2\ s^{-1}$ to be compared with $k_3 = 100\ s^{-1}$; see table 1, and references therein) and accounts for the direct slow electron transfer from TMPD to cytochrome oxidase.

donors [17]. These reductants, bypassing the bc_1 complex, allow one to probe failures of electron flow through the terminal part of the respiratory chain, particularly if they are related to a critical loss of cytochrome c, their primary redox target. In the presence of ascorbate and TMPD, the spontaneous rate of respiration of intact myocytes is maximal and sustained by close to 100% reduced cytochrome c which efficiently transfers electrons to cytochrome c oxidase [17]. When assaying intact myocytes in the presence of excess reductants, because of the abundance of endogenous cytochrome c [18] and in the presence of excess oxygen (air medium), the bimolecular collision with reduced cytochrome c on one side ($k_b = 1 \times 10^7\ M^{-1}\ s^{-1}$) and oxygen on the other ($k_b = 1 \times 10^8\ M^{-1}\ s^{-1}$), will never limit the rate of oxygen reduction [2, 32]. Under these conditions, in fact, the limiting step of O_2 reduction is internal to cytochrome oxidase, proceeding at (maximally) a few tens per second, a rate which has been assigned to either a slow proton diffusion to [7, 8], or an intrinsically slow electron transfer to/within [6, 9] the O_2-reducing site.

Why should the addition of exogenous reduced cytochrome c increase the respiratory rate of damaged myocytes? The description with five irreversible steps of the overall electron flux from the donors to oxygen, via cytochrome c and cytochrome c oxidase, can help to rationalize this finding (fig. 6).

In this simplified kinetic scheme, cytochrome oxidase, which contains four redox-competent sites [including three coppers and two heme irons; ref. 33], is treated as a redox unit through which electrons are transferred to oxygen at k_3 (step 3). According to this minimal scheme and the kinetic rate constant values (table 1), one can predict that the reduction of cytochrome oxidase by cytochrome c may be limiting when the concentration of reduced cytochrome c at the catalytic site of cytochrome oxidase falls below 10 μM [19, 32]. The results in figure 2 reporting a saturation value of 20 μM exogenous cytochrome c observed at the latest stage of damage, i.e., when most of the endogenous cytochrome c is displaced from the intermembrane space of mitochondria, are consistent with this theoretical critical concentration value. Thus, below 10–20 μM reduced cytochrome c, the rate of electron transfer from cytochrome c to cytochrome oxidase (step 2 in the scheme) will proceed even more slowly than the intracytochrome oxidase electron transfer, making CCOX activity rate limited by the electron supply. If the whole respiratory process can also be rate limited by a comparable loss of cytochrome c in the absence of ascorbate and TMPD bypassing electrons to CCOX, this cannot be inferred from our data and needs to be assessed independently. Thus, the appearance of sensitivity to exogenous cytochrome c in damaged (rounded) but viable (minimal LDH leakage, TB-negative) myocytes

Table 1. Kinetic rate constants of the reaction depicted in the scheme in figure 6.

Step	Rate constants		Reference(s)
	bi molecular $k_b\ (M^{-1}\ s^{-1})$	mono molecular $k_m\ (s^{-1})$	
1[a]	$\geq 5.0 \times 10^4$	≥ 100	
2	1.0×10^7	2000[b]	32, 36
3	–	100	19, 32
4	1.0×10^8	30,000	19, 32
5	1.3×10^3	2	37

The number of electron transfer events per time unit, i.e., the monomolecular rate constants are calulated from the bimolecular constants ($k_m = k_b \times [\ ]$) assuming $[O_2] = 280$ μM, [cytochrome c 2+] = 200 μM, and [TMPD] = 2 mM.

[a] Inferred from the (fully reduced) redox level of cytochrome c observed under similar experimental conditions [17].

[b] Notice that when the cytochrome c concentration ≤10 μM, the reduction of cytochrome oxidase by cytochrome c (step 2 in the scheme) occurs at $k_m \leq 100\ s^{-1}$, i.e., a rate comparable or slower than the internal electron transfer rate within cytochrome c oxidase (step 3 in the scheme).

suggests that in these cells, the concentration of cytochrome c available in situ to reduce cytochrome oxidase has fallen below 10–20 μM. The early changes in mitochondria structure such as swelling, leading to dilution and possibly variation in the inner membrane affinity for cytochrome c, together with the permeabilization of the sarcolemma and mitochondrial outer membrane may explain the critical decrease in cytochrome c concentration at the catalytic site.

At which stage of damage does the sensitivity to cytochrome c appear, and how does it correlate with cell viability? A clearcut answer to this question is not provided by our results. However, the observation that the sensitivity to exogenous cytochrome c develops with early changes in myocyte shape, while a significant fraction of cells still do not release LDH significantly and are TB negative, suggests that the cell sarcolemma becomes permeable to cytochrome c quite early in the cascade of cell degeneration events. This finding is consistent with a differential permeation of different molecules at different degrees of plasma membrane damage [34], a condition that in this particular case can be tentatively explained on the basis of the structural properties of cytochrome c which is an extrinsic membrane protein (it sticks on membranes), positively charged at the experimental (physiologic) pH of 7.3 (downhill, electrophoretic cell internalization) [35].

In conclusion, exogenous reduced (by external reductants) cytochrome c can stimulate mitochondrial function in damaged cells and reach cytochrome oxidase in the mitochondrion at an early stage of damage not detectable by vital staining or LDH leakage, restoring to some extent the impairment of cell oxygen consumption. The data are consistent with a theoretical analysis which predicts, at the level of the terminal part of the respiratory chain, the shift of the rate-limiting step from cytochrome oxidase to cytochrome c under pathological circumstances where the concentration of cytochrome c falls below a critical threshold. The results do not identify the stage of myocyte damage at which cytochrome c sensitivity appears, nor do they clarify whether cytochrome c permeability and loss marks an irreversible event leading to cell death. In this respect, it will be of particular interest to verify whether the onset of sensitivity lags behind, antedates, or occurs within the apoptotic cell pathway.

Acknowledgements. We wish to thank Prof. Emilia Chiancone for her critical review and stimulating discussions. This work was partially supported by grants GM30799 from NIGMS-USA and the Charina Foundation, Inc. to T.J.J.B., and from collaborative research grant NATO (CRG 971556) to P.S. and T.J.J.B. Grants from MURST (PRIN Bioenergetica e Trasporto di Membrana) to P.S, and DK45828 from NIDDK-USA, and the Underhill and Wild Wings Foundations to R.B.S. are also gratefully acknowledged.

1 Cooper C. E., Nicholls P. and Freedman J. A. (1991) Cytochrome c oxidase: structure, function and membrane topology of the polypeptide subunits. Biochem. Cell. Biol. **69:** 586–607
2 Ferguson-Miller S. and Babcock G. T. (1996) Heme/copper terminal oxidases. Chem. Rev. **96:** 2889–2907
3 Trumpower B. L. and Gennis R. B. (1994) Energy transduction by cytochrome complexes in mitochondrial and bacterial respiration. Annu. Rev. Biochem. **63:** 675–716
4 Nicholls D. G. (1982) Bioenergetics: an Introduction to the Chemiosmotic Theory, pp. 65–130, Academic Press, New York
5 Einarsdottir O. (1995) Fast reactions of cytochrome c oxidase. Biochim. Biophys. Acta **1229:** 129–147
6 Brunori M., Giuffre' A., D'Itri E. and Sarti P. (1997) Internal electron transfer in Cu-heme oxidases: thermodynamic or kinetic control? J. Biol. Chem. **272:** 19870–19874
7 Verkhovsky M. I., Morgan J. E. and Wikstrom M. (1995) Control of electron delivery to the oxygen reduction site of cytochrome c oxidase: a role for protons. Biochemistry **34:** 7483–7491
8 Verkhovsky M. I., Morgan J. E., Puustinen A. and Wikstrom M (1996) Kinetic trapping of O_2 in cell respiration. Nature **380:** 268–270
9 Malatesta F., Sarti P., Antonini G., Vallone B. and Brunori M. (1990) Electron transfer to the binuclear center in cytochrome oxidase: catalytic significance and evidence for an additional intermediate. Proc. Natl. Acad. Sci. USA **87:** 7410–7413
10 Sarti P., Antonini G., Malatesta F. and Brunori M. (1992) Respiratory control in cytochrome oxidase vesicles is correlated with the rate of internal electron transfer. Biochem. J. **284:** 123–127
11 Groen A. K, Wanders R. J., Westerhoff H. V., Meer R. van der and Tager J. M. (1982) Quantification of the contribution of various steps to the control of mitochondrial respiration. J. Biol. Chem. **257:** 2754–2757
12 Letellier T., Malgat M. and Mazat J. P. (1993) Control of oxidative phosphorylation in rat muscle mitochondria: implications for mitochondrial myopathies. Biochim. Biophys. Acta **1141:** 58–64
13 Letellier T., Malgat M., Rossignol R. and Mazat J. P. (1998) Metabolic control analysis and mitochondrial pathologies. Mol. Cell. Biochem. **184:** 409–417
14 Villani G. and Attardi G. (1997) In vivo control of respiration by cytochrome c oxidase in wild type and mitochondrial DNA mutation carrying human cells. Proc. Natl. Acad. Sci. USA **94:** 1166–1171
15 Villani G., Greco M., Papa S. and Attardi G. (1998) Low reserve of cytochrome c oxidase capacity in vivo in the respiratory chain of a variety of human cell types. J. Biol. Chem. **273:** 31829–31836
16 Attardi G., Bai Y., Villani G., Greco P., Fernandez-Silva C., Ausenda C. et al. (1999) Tight control of gene expression and respiration in mammal mitochondria. Biochimie **81** (Suppl. N6): S58
17 Sarti P., Antonini G., Malatesta F., D'Itri E., Brunori M. and Blanck T. J. J. (1992) Spectral analysis of cytochromes in rat heart myocytes: transient and steady state photodiode array spectrophotometry measurements. Arch. Biochem. Biophys. **299:** 8–14
18 Osheroff N., Koppenol W. H. and Margoliash E. (1978) Effects of onions on the reaction between cytochrome c and cytochrome c oxidase. In: Frontiers in Biological Energetics, Dutton P. L., Leigh J. S. and Scarpa A. (eds.), pp. 439–449, New York. Academic Press
19 Brunori M., Antonini G., Giuffre' A., Malatesta F., Nicoletti F., Sarti P. et al. (1994) Electron transfer and ligand binding in terminal oxidases. Impact of recent structural information. FEBS Lett. **350:** 164–168
20 Brown G. C. (1995) Nitric oxide regulates mitochondrial respiration and cell functions by inhibiting cytochrome oxidase. FEBS Lett. **369:** 136–139

21 Liu X., Kim C. N., Yang J., Jemmerson R. and Wang X. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell **86:** 147–157
22 Naro F., Fazzini A., Grappone C., Citro C., Dini G., Giotti A. et al. (1993) Release of cytochromes from hypoxic and reoxygenated guinea pig heart. Cardioscience **4:** 177–184
23 Borutaite V., Morkuniene R., Budriunaite A., Krasauskaite D., Ryselis S., Toleikis A. et al. (1996) Kinetic analysis of changes in activity of heart mitochondrial oxidative phosphorylation system induced by ischemia. J. Mol. Cell. Cardiol. **28:** 2195–2201
24 White R. L. and Wittenberg B. A. (1993) NADH fluorescence of isolated ventricular myocytes: effects of pacing, myoglobin, and oxygen supply. Biophys. J. **65:** 196–204
25 Howard R. B. and Pesch L. A. (1968) Respiratory activity of intact isolated parenchymal cells from rat liver. J. Biol. Chem. **243:** 3105–3109
26 Goding J. W. (1976) Conjugation of antibodies with fluorochromes: modifications to the standard methods. J. Immunol. Methods **13:** 215–226
27 Cardone M. H., Smith B. L., Mennitt P., Mochly-Rosen D., Silver R. B. and Mostov K. E. (1996) Signal transduction by the polymeric immunoglobulin receptor suggests a role in regulation of receptor transcytosis. J. Cell Biol. **133:** 997–1005
28 Silver R. B. (1998) Ratio imaging: practical considerations for measuring intracellular calcium and pH in living tissue. Methods Cell Biol. **56:** 237–251
29 Margoliash E. and Frohwirt N. (1959) Spectrum of horse heart cytochrome c. Biochem. J. **71:** 570–572
30 Shirhatti V. and Krishna G. (1985) A simple and sensitive method for monitoring drug induced cell injury in culture cells. Anal. Biochem. **147:** 410–418
31 Rotman B. and Papermaster B. W. (1966) Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. Proc. Natl. Acad. Sci. USA **55:** 134–141
32 Brunori M., Antonini G., Malatesta F., Sarti P. and Wilson M. T. (1992) The oxygen reactive species of cytochrome c oxidase: an alternative view. FEBS Lett. **314:** 191–194
33 Tsukihara T., Aoyama H., Yamashita E., Tomizaki T., Yamaguchi H., Shinzawa-Itoh K. et al. (1996) The whole structure of the 13-subunits oxidized cytochrome c oxidase at 2.8 A. Science **272:** 1136–1144
34 Rauen U., Hanssen M., Lauchart W., Becker H. D. and de Groot H. (1993) Energy dependent injury to cultured sinusoidal endothelial cells of the rat liver in UW solution. Transplantation **55:** 469–473
35 Tint H. and Reiss W. (1950) Studies on the purity and specificity of cytochrome c: electrophoretic analyses. J. Biol. Chem. **182:** 385–403
36 Antonini E., Brunori M., Colosimo A., Greenwood C. and Wilson M. T. (1977) Oxygen 'pulsed' cytochrome c oxidase: functional properties and catalytic relevance. Proc. Natl. Acad. Sci. USA **74:** 3128–3134
37 Jones G. D., Jones M. G., Wilson M. T., Brunori M., Colosimo A. and Sarti P. (1983) Reaction of cytochrome c oxidase with sodium dithionite. Biochem. J. **209:** 175–182