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Mitochondrial defects by intracellular calcium overload versus endothelial cold ischemia/reperfusion injury

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Abstract Questions as to the critical stress factor and primary targets of cold ischemia/reperfusion (CIR) injury were addressed by comparing mitochondrial defects caused by (1) CIR injury and (2) intracellular Ca^{2+} overload. CIR was simulated in transformed human umbilical vein endothelial cell cultures (tEC) by 8 h cold anoxia in University of Wisconsin solution and reoxygenation at 37 °C. Intracellular Ca^{2+} concentrations were changed by permeabilization of suspended cells with digitonin in culture medium (RPMI, 0.4 mM Ca^{2+}). Binding of free Ca^{2+} by ethylene glycol-bis(*β*-aminoethylether)-*N,N,N',N'*-tetraacetic acid in RPMI or mitochondrial incubation medium served as controls. Extracellular Ca^{2+} protected the cell membrane against permeabilization. Mitochondrial functions were determined before and after permeabilization of the cell membrane. After CIR, mitochondrial respiratory capacity declined, but oxygen consumption remained coupled to adenosine triphosphate (ATP) production. In contrast, Ca^{2+} overload caused uncoupling of mitochondrial respiration. High intracellular Ca^{2+} overload, therefore, does not reproduce cold ischemia/reperfusion injury in endothelial cells.

Key words Ischemia/reperfusion injury · Endothelial cells · Plasma membrane · Intracellular Ca^{2+} · Mitochondrial respiratory chain · Oxidative phosphorylation

Introduction

Endothelial cell damage plays a key role in cold ischemia-reperfusion (CIR) injury as a consequence of organ preservation [2]. Several organ preservation solutions do not contain Ca^{2+}, although lack of extracellular Ca^{2+} is known to induce oxidative stress, with the consequence of glutathione and vitamin E loss and a decline of cell viability [11]. The sensitivity to external calcium in chemically stressed cells is modulated by antioxidants added to the medium [3]. Since oxidative stress by exposure of endothelial cells to hydrogen peroxide [8] and plasma membrane permeabilization [4] were only partially able to reproduce mitochondrial defects caused by CIR injury, the present study was designed to compare the consequences of Ca^{2+} overload and CIR in an endothelial cell culture model.

Materials and methods

Cell culture, cold ischemia/reperfusion, and modification of intracellular Ca^{2+}

Transformed human umbilical vein endothelial cells (tEC, lung carcinoma, EA.hyb 926) were grown in culture medium RPMI 1640 (PAA Laboratories) containing 2 mM glutamine. For simulation of CIR, confluent cell cultures were exposed to anoxia at 4 °C for 8 h in University of Wisconsin (UW) solution supplemented with 3 mM reduced glutathione [10]. CIR experiments were designed as parallel tests against control groups without cold anoxia, tested at the onset of preservation treatment of the experimental

groups [6, 10]. Cell viability and bioenergetic parameters were measured in cells suspended after mild trypsinization in mitochondrial medium (MitoMedium: 200 mM sucrose, 20 mM HEPES, 20 mM taurine, 10 mM KH_2PO_4, 3 mM $MgCl_2$, 1 g/l BSA, 0.5 mM ethylene glycol bis-(β-aminoethylether)-*N,N,N',N'*-tertra-acetic acid EGTA) [6]. Digitonin (Sigma; stock solutions prepared in dimethylsulfoxide (DMSO) served for selective permeabilization of the plasma membrane. Intracellular calcium overload was induced by permeabilization of cells in original RPMI with 0.4 mM free Ca^{2+}. In control experiments, free calcium in RPMI was bound by 4 mM EGTA. Plasma membrane permeability was quantified by trypan blue staining (microscopic cell count), LDH leakage (spectrophotometric enzyme assay), and propidium iodide uptake (FACS analysis, Becton Dickinson).

Cellular respiration and substrate/inhibitor titrations

Respiration of intact and permeabilized cells was measured at 37 °C in suspended cells (2 ml) by high-resolution respirometry (Oroboros Oxygraph, Innsbruck, Austria) [5, 9]. Air-saturated medium without cells was used for oxygen calibration of the polarographic oxygen sensor. After adding the suspended cells, taking samples for cell count determinations and closing the chambers of the respirometer, 5–10 min were required for stabilization of the oxygen flux recorded on-line as the time derivative of oxygen concentration. Cellular respiration was recorded for an additional period of 10 min. Then digitonin was added in proportion to cell density ($1 \cdot 10^6$ to $3 \cdot 10^6$ cells per ml). Pyruvate 10 mM plus 5 mM malate were added simultaneously to prevent the mitochondria from losing respiratory substrates. After complete permeabilization of cells, oxygen uptake declined sharply to resting levels (state 2) during a 10-min period, owing to the release of adenylates. Subsequent addition of 1 mM adenosine diphosphate (ADP) stimulated complex I respiration to the maximum coupled rate (state 3), which was inhibited to 1.5–2 % by 0.5 μM rotenone. Addition of 10 mM succinate supported complex II respiration, which was inhibited by 5 μM antimycin A to the same extent as rotenone inhibition. The instrumental background, measured in the absence of cells, was supplied by linear functions of oxygen concentration and was subtracted accordingly for the calculation of mitochondrial oxygen consumption [5, 9]. The medium was re-aerated periodically to prevent oxygen limitation of respiration [9]. Finally, cellular respiration was continued until full depletion of oxygen in the chamber for zero oxygen calibration of the polarographic oxygen sensor.

Results

Ca^{2+} and permeabilization of the cell membrane

Trypan blue staining remained low and unchanged at 1.5 % ± 1.1 % before and after 8 h cold storage in UW and reoxygenation in both RPMI and MitoMedium. Permeabilization of the endothelial cell membrane was further tested by respirometry, and a significant stimulation of oxygen uptake by succinate added to MitoMedium after CIR was observed (no effect in controls). Succinate added to RPMI had no effect on respiration in the control and CIR groups. This indicates a modest plasma membrane CIR injury when tEC are reoxygenated in MitoMedium, and a membrane-stabilizing effect of extracellular Ca^{2+} during reoxygenation.

In agreement with observations on primary cultures of human umbilical vein endothelial cells (HUVEC) [10], the intact plasma membrane of tEC provides an effective barrier against the entry of external succinate and ADP, as shown by the sharp decline of respiration below resting levels after inhibition of complex I by rotenone, in the presence of 10 mM succinate and 1 mM ADP in MitoMedium. Respiration through complex II was then stimulated by stepwise titration of digitonin, indicating the increasing permeabilization of the cell membrane for succinate and ADP. Maximum respiration was observed at a digitonin concentration of $10\ \mu g \cdot 10^{-6}$ cells, with cell concentrations ranging from $1 \cdot 10^6$ to $5 \cdot 10^6$ cells/ml. Full permeabilization of the plasma membrane was confirmed by > 95 % trypan blue staining and 100 % LDH leakage at a digitonin concentration of $10\ \mu g \cdot 10^{-6}$ cells in Ca^{2+}-free MitoMedium. In contrast, the same digitonin concentration resulted in merely 18.8 % ± 7.8 % (SD; $n = 6$) trypan blue staining in RPMI with 0.4 mM Ca^{2+}. Similarly, trypan blue positive cells were reduced at a digitonin concentration of $10\ \mu g \cdot 10^{-6}$ cells in MitoMedium when the free calcium concentration was increased. A digitonin concentration of $30\ \mu g \cdot 10^{-6}$ cells was required for full permeabilization of tEC in RPMI, indicating the stabilizing effect of external calcium for the cell membrane against damage caused by the mild detergent digitonin.

Mitochondrial respiratory capacity and respiratory control

Endogenous respiration of tEC in MitoMedium (without external respiratory substrates) was 27.2 ± 3.1 $pmol \cdot s^{-1} \cdot 10^{-6}$ cells (SD; $n = 8$), whereas routine respiration in RPMI (containing the substrates required for mitochondrial respiration and cell growth) was significantly higher (30.7 ± 2.2 $pmol \cdot s^{-1} \cdot 10^{-6}$ cells; SD; $n = 6$; $P < 0.05$). This difference of respiration in the two media was amplified after 8 h of cold anoxia in UW solution and reperfusion, when endogenous respiration dropped to 22.5 ± 2.0 $pmol \cdot s^{-1} \cdot 10^{-6}$ cells (SD; $n = 6$) in MitoMedium and to 27.1 ± 1.8 $pmol \cdot s^{-1} \cdot 10^{-6}$ cells (SD; $n = 8$) in RPMI. Stimulation of respiration by succinate in MitoMedium but not in RPMI (see above) reduced the difference in cellular respiration in the two media.

To characterize the mitochondrial CIR injury leading to reduced respiratory flux in tEC, cells were permeabilization by digitonin ($10\ \mu g \cdot 10^{-6}$ cells) in MitoMedium. This treatment yields full accessibility of the mitochondria to external substrates and ADP, without affecting mitochondrial integrity. The respiratory adenylate control ratio, RCR, was calculated as the maximum com-

plex I respiration in the presence of ADP (state 3) divided by the minimum respiration measured before ADP addition (state 2). RCR of control and CIR cells was not significantly different at 5.9 ± 0.4 SD and 5.1 ± 0.6 SD ($n = 4$; each independent experiment with 2 or 3 determinations). This indicates well-coupled mitochondria, with coupling preserved after CIR. In contrast, Ca^{2+} overload induced by permeabilization of cells in RPMI resulted immediately in uncoupling of oxidative phosphorylation and in strong inhibition of mitochondrial respiration.

Discussion

Cold ischemia/reperfusion injury in tEC was characterized by a significant reduction of respiratory capacity without a compromise in the degree of coupling between respiration and phosphorylation of ADP to ATP. This agrees with previous results for HUVEC [4, 10], although tEC were more resistant than HUVEC against trypan blue staining following 8 h of anoxic cold storage [10]. Even control cells of tEC had a significantly lower trypan blue staining than HUVEC (1.5% versus 5%). A modest cell membrane injury of tEC was detected after CIR upon reoxygenation in the absence of extracellular Ca^{2+}, whereas extracellular calcium stabilized the cellular membrane without eliminating the mitochondrial CIR injury.

The pattern of mitochondrial CIR injury observed in tEC differs from the consequences of Ca^{2+} overload and of oxidative stress induced by hydrogen peroxide [8], although a reduction of respiratory capacity is observed in all cases. Reduction of the capacity of complex I respiration is a common phenomenon. The uncoupling control ratio remains unchanged after CIR but is reduced after exposure to H_2O_2 [8] and high intracellular Ca^{2+}. In agreement with these results obtained after cold anoxia, the intracellular Ca^{2+} increase in endothelial cells after warm hypoxia is not responsible for the mortality [1]. Exclusion of Ca^{2+} from the preservation/reperfusion medium adds a stress factor for the cell membrane [11], but protects critical mitochondrial functions. Addition of more effective antioxidants to the flush medium for reoxygenation could further protect from CIR injury. Maximal Ca^{2+} uptake capacity into mitochondria of digitonin-permeabilized neural cells is increased by Bcl-2, concomitant with an increased resistance to Ca^{2+}-induced respiratory inhibition [7]. Bcl-2 overexpression, therefore, offers a further possibility to analyze the potential role played by mild Ca^{2+} overload in cellular CIR injury.

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