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Mitochondrial Defect in Endothelial Cold Ischemia/Reperfusion Injury

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ENDOTHELIAL ischemia/reperfusion injury is critical for organ preservation, yet the mechanisms of intracellular damage are little understood. Permeabilization of the cell membrane disrupts ion homeostasis, leading to secondary mitochondrial defects by influx of high extracellular Ca^{2+}.[1] In contrast, primary mitochondrial damage is due to short-term (4 to 8-hour) cold ischemia/reoxygenation (CIR) of endothelial cells in University of Wisconsin (UW) or histidine-tryptophane-ketoglutarate (HTK) solution, while the cell membrane remains intact.[2] This study was designed to further characterize mitochondrial defects in CIR injury of endothelial cells.

MATERIALS AND METHODS

Human umbilical vein endothelial cells (HUVEC) were grown in primary culture in 75 cm^2 fibronectin- or gelatin-coated culture flasks in endothelial cell growth medium (EGM; PromoCell, Heidelberg, Germany), containing 2% fetal calf serum, supplemented with endothelial growth factor, gentamicin, and amphotericin B.[2] Third-passage cells were exposed to cold hypoxia (4°C; 8 hours) in UW solution. Subsequently, cells were suspended by mild trypsinization in extracellular EGM (1 mmol/L free Ca^{2+}) or in intracellular mitochondrial medium (mitomedium: 200 mmol/L sucrose, 20 mmol/L HEPES, 20 mmol/L taurine, 10 mmol/L KH_2PO_4, 3 mmol/L $MgCl_2$, 1 g/L bovine serum albumin [BSA], 0.5 mmol/L EGTA, and zero free Ca^{2+}).[1] After rewarming to 37°C and reoxygenation, cellular respiration was recorded in these media by high-resolution respirometry (OROBOROS Oxygraph and DatLab software, Innsbruck, Austria).[3,4]

RESULTS

Routine cellular respiration, reflecting the aerobic metabolic activity of cells under physiologic conditions in culture, was significantly inhibited after simulated CIR, as was trypan blue exclusion as an index of cell viability (Fig 1). Routine respiration in EGM was reduced in controls with a lower index of viability (90%), compared to an independent data set with superior viability (96%; Fig 1). Whereas succinate had no stimulatory effect on respiration in EGM when viability of controls was high,[2] a significant stimulation by 16% and 14% was observed in HUVEC with lower viability after addition of succinate to EGM in controls and simulated CIR, respectively. This and high levels of rotenone-inhibited respiration indicated a partial membrane permeability for succinate, whereas CIR-induced loss of viability was not accompanied by further respiratory stimulation by succinate.[1,2]

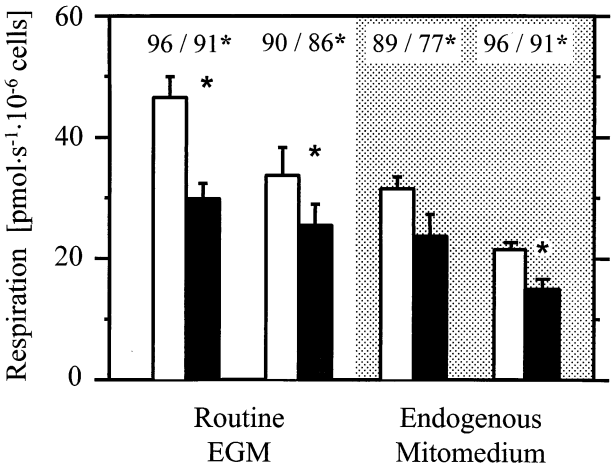

Fig 1. Respiration of HUVECs suspended in extracellular culture medium, EGM, or intracellular mitomedium before (controls: open bars) and after 8 hours of cold hypoxic storage in UW (simulated CIR: closed bars). Trypan blue exclusion (%) is shown by numbers for controls and simulated CIR, which were always performed as parallel tests (n = 3, 5, and 7 independent cultures for the first,[2] second/third, and fourth pair of columns, respectively). Routine respiration in EGM and endogenous respiration in mitomedium were measured in parallel tests (middle bars, with lower trypan blue exclusion in controls), showing no difference between the two reoxygenation media. Cultures were grown on fibronectin, except for the last bars, where cells were grown on gelatin.* P bars ± SE.

After permeabilization of the cell membrane by digitonin (10 $\mu g \cdot 10^{-6}$ cells) in mitomedium, passive respiration (without adenosine diphosphate [ADP] or adenosine triphosphate [ATP]) was identical in control and CIR cells (27 and 37% of endogenous respiration), but maximum capacity of ADP-stimulated respiration was significantly reduced after simulated CIR (complex I, NADH-CoQ reductase with pyruvate and malate; complex II, succinate-

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CoQ reductase with rotenone and succinate). The respiratory control ratio (flux with excess ADP divided by flux in the absence of ADP and ATP) was 8.4 ± 1.8 SD in controls and 7.0 ± 2.6 SD after CIR ($n = 5$; not different $P = .05$), indicating well-coupled mitochondria.

Paired experiments in EGM and mitomedium showed (1) no difference between routine and endogenous respiration in controls, 32.1 $pmol \cdot s^{-1} \cdot 10^{-6}$ cells (±10.4 SD; EGM) and 31.4 $pmol \cdot s^{-1} \cdot 10^{-6}$ cells (±4.1 SD; mitomedium, $n = 5$; Fig 1; two open bars in middle); (2) no difference between routine and endogenous respiration after cold hypoxia and reoxygenation at high Ca^{2+} in EGM vs low Ca^{2+} in mitomedium (Fig 1; two dark bars in middle; 81% and 75% of control respiration); (3) a lower trypan blue exclusion after CIR in mitomedium (77.4% ± 1.7% SD) compared with EGM (86.1% ± 1.0% SD); (4) a higher uncoupler-stimulated respiration in unpermeabilized cells compared to ADP-stimulated respiration in permeabilized cells; and (5) conserved coupling after simulated CIR, indicated by (a) a significant increase of the uncoupling control ratio (FCCP-stimulated respiration divided by routine respiration in EGM) from 2.99 ± 0.18 SD (controls) to 3.65 ± 0.44SD (CIR), (b) a constant ADP activation ratio (ADP-stimulated respiration divided by endogenous respiration in mitomedium) of 2.19 ± 0.08 SD (control) and 2.4 ± 0.43 SD (CIR; $n = 5$), and (c) a constant respiratory control ratio (see above).

To improve the compromised viability of control cells (possibly due to poor fibronectin), culture conditions were changed to gelatin. In the following respiratory experiments in mitomedium, (1) trypan blue exclusion was identical to results obtained in EGM with high variability of controls (Fig 1; data sets on far left and far right); (2) significant reduction of respiration after CIR to 64% and 70% of controls was comparable in the EGM and mitomedium; (3) endogenous respiration of control cells (21.9 $pmol \cdot s^{-1} \cdot 10^{-6}$ cells ± 3.1 SD; $n = 7$), however, was reduced to one half the routine rate when comparing the data sets with high trypan blue exclusion (Fig 1; open bars on far left and far right). These results illustrate the significance of strictly parallel experiments with cultured cells and the importance of quality control of experimental cell cultures.

DISCUSSION

In our study of endothelial cold preservation, reoxygenation injury occurred in "extracellular" EGM and "intracellular" mitomedium. Comparable levels of respiration in EGM and mitomedium after CIR indicate that (1) the availability of endogenous substrates was not limiting even after 8 hours of cold storage in UW and (2) cell membrane permeability for external Ca^{2+} does not represent an early event in CIR injury. Further evidence[1,2] is provided that reduction of mitochondrial capacity is an early event in CIR injury, independent of external Ca^{2+} during reoxygenation. Endothelial CIR injury was characterized by a reduced routine cellular respiration and a defect of complex I and II respiration, leading to a correspondingly lower rate of ATP production at a preserved mitochondrial coupling of electron transport to phosphorylation of ADP to ATP. In contrast, loss of intracellular Ca^{2+} after long-term storage of liver in UW (24 to 48 hours) reduces the mitochondrial uncoupling control ratio and respiratory control ratio.[5] Respiratory indices of preservation injury of the cell membrane were not simply related to trypan blue exclusion.[2] Similarly, viability tests based on the release of various cellular enzymes yield different sensitivities.[6] Mitochondrial localization of early CIR injury provides an important basis for optimization of site-directed intracellular intervention strategies in endothelial cell and organ preservation.

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