Transpl Int (2000) 13 [Suppl 1]: S580–S582

A. Amberger
H. Weiss
T. Haller
R. Margreiter

Mitochondrial calcium overload is restricted to a few mitochondria in endothelial cells after cold ischemia/reperfusion

A. Amberger · H. Weiss · R. Margreiter (✉)
Department of Transplant Surgery, D. Swarovski Reasearch Laboratory, University Hospital Innsbruck, Anichstrasse 35, A-6020 Innsbruck, Austria
(Tel.: + 43-512-504-2600)

T. Haller
Department of Physiology, University of Innsbruck, Austria

Abstract Changes in cytosolic and mitochondrial calcium content were studied in an endothelial cell model after simulating cold ischemia reperfusion injury. Image analysis demonstrated that only a subpopulation of mitochondria in endothelial cells accumulate calcium. Observations led to the hypothesis that mitochondria which are in close contact with the plasma membrane are mainly affected by the Ca^{2+} efflux across that membrane, while those in other parts of the cell remain unaffected.

Key words Cold ischemia reperfusion injury · Calcium overload · Endothelial cells · Mitochondria

Introduction

Preservation injury is a consequence of a multifactorial process. The mechanisms leading to cold ischemia reperfusion (CIR) injury at the cellular level are still not fully defined. Current evidence demonstrated that calcium can mediate injury to organs exposed to ischemia followed by reperfusion [3, 13]. Recent studies have suggested that calcium induces opening of the permeability transition pore which leads to collapse of the mitochondrial membrane potential (MMP) and the release of cytochrome *c* from the mitochondria [5, 9]. Studies provided evidence that loss of MMP and cytochrome *c* release are the first irreversible events in apoptosis [7, 11]. Most studies have analyzed the physiological function of calcium on mitochondrial function in isolated mitochondria and brain cells [4, 5, 15]. Little is known about the pathophysiological function of calcium on cells and mitochondria in CIR injury. In this study we measured the changes in cytosolic and mitochondral calcium content in an endothelial cell model after simulated CIR injury. With specific image aquisition and analysis, changes of calcium content in individual mitochondria were estimated after reperfusion.

Material and methods

Human umbilical vein endothelial cells (HUVECs) were isolated and cultivated as described previously [6]. Cells after the third subcultivation were seeded onto plastic coverslips and after 3 days in culture they were used for preservation experiments. In experiments of simulated cold ischemia, cells were incubated in University of Wisconsin solution (UW; 4 °C, 12 h, Viaspan; Dupont, USA) and stored in a precooled styrofoam box as described earlier [6]. At the end of the cold storage period, cells were loaded with fura-2 (Molecular Probes, USA) to analyze cytosolic-free calcium concentrations and rhod-2 (Molecular Probes) to analyze mitochondrial calcium content.

Fluorescence measurements were done with a microscope equipped for epifluorescence and photometry as described [8]. The exitation light of 340, 380 nm (fura-2) was directed through a quartz glass fiber and a gray filter into the microscope. The emitted light was directed through a 520-nm cut-off filter to a photomultiplier tube. Each experiment was calibrated with ionomycin and mangan to estimate the cytosolic calcium concentrations. Images were taken with an integrated CCD camera connected to an inverted microscope. Image analysis of mitochondrial calcium content were performed at exitation/emission of 488/520 nm. Image analysis was done with the software FUCAL (Till Photonics).

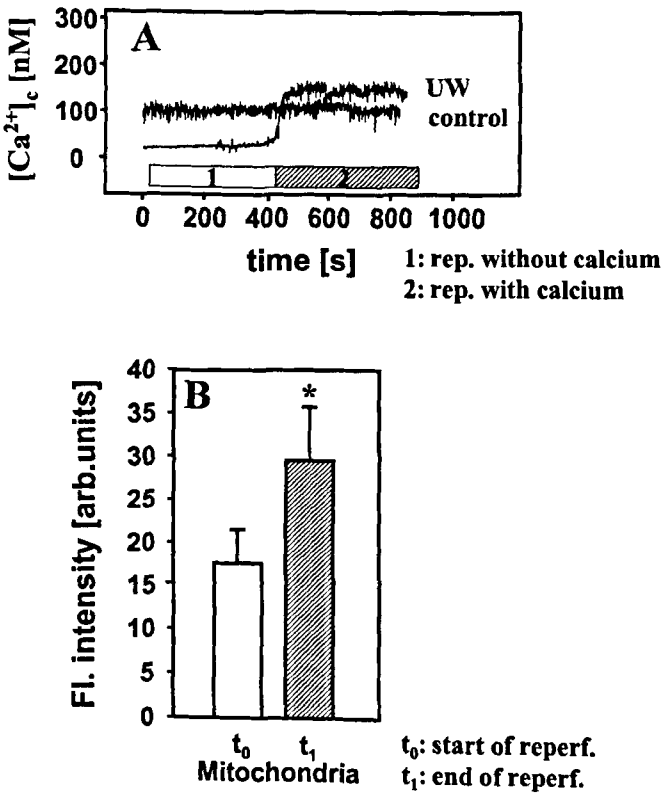

Fig. 1A, B Effect of cold ischemia/reperfusion on cytosolic and mitochondrial calcium content in human endothelial cells. Cytosolic free calcium concentrations $[Ca^{2+}]_c$ decreased during cold ischemia and increased only after reperfusion (*rep.*) with calcium supplemented Ringer solution. Reperfusion had no effect on $[Ca^{2+}]_c$ in control cells (**A**). **B** demonstrates the increase of Rhod-2 fluorescence (*FL.*) in mitochondria during reperfusion (*reperf.*). Data from images at the start and at the end of reperfusion were analyzed with the software FUCAL. Mean and SD from five different experiments are shown. * $P < 0.05$. (*arb.* Arbitrary)

Results

After preserving HUVECs in UW solution at 4 °C for 12 h, coverslips were mounted in the microscope and reperfused with calcium-free Ringer solution (ringer I) for 10 min and subsequently perfused with Ringer solution supplemented with calcium (Ringer II, 0.4 mM) for an additional 10 min (Fig. 1A). The results in Fig. 1A show a significant decrease of $[Ca^{2+}]_c$ after cold storage in UW solution compared to control cells. This low $[Ca^{2+}]_c$ was not changed during reperfusion with Ringer I. Reperfusion with Ringer II lead to a fast increase of $[Ca^{2+}]_c$ to levels not significantly higher than in control cells. This rise in $[Ca^{2+}]_c$ did not change during the perfusion period.

It was shown that mitochondria in a wide variety of cell types undergo large changes in matrix $[Ca^{2+}]$ following elevated cytosolic calcium concentrations [2, 9]. Therefore we estimated mitochondrial Ca^{2+} content with rhod-2, a Ca^{2+}-sensitve dye, which localizes specifically to mitochondria and responds to Ca^{2+} entry [2]. Analysis of rhod-2 fluorescence was performed on images taken at the beginning and at the end of the reperfusion with the software Fucal and Datgraf. Our data revealed a significant increase of mitochondrial Ca^{2+} content during reperfusion with Ringer II medium (Fig. 1B). Image analysis demonstrated that only a subpopulation of mitochondria in endothelial cells accumulate calcium. Rhod-2 fluorescence was not changed in the other areas of the cells including cytoplasm and nucleus.

Discussion

In this study we examined the effect of cold ischemia and reperfusion on cellular and mitochondrial calcium metabolism in isolated human endothelial cells. The results demonstrated a loss of $[Ca^{2+}]_c$ in ischemic calcium-free UW solution which could be due to either reuptake of cytosolic calcium by intracellular organelles or efflux of cytosolic calcium to the extracellular medium. However, reperfusion with Ca^{2+}-free Ringer solution did not increase $[Ca^{2+}]_c$, whereas reperfusion with Ca^{2+}-containing medium increased $[Ca^{2+}]_c$ to levels of controls. Therefore, we suggest an efflux of $[Ca^{2+}]_c$ to the UW solution and a re-uptake of Ca^{2+} during reperfusion. A recent study demonstrated similar observations after hypothermic storage of hepatocytes in UW solution [10]. They showed a significant decrease of $[Ca^{2+}]_c$ and total cellular calcium over a period of 48 h cold storage, but they did not analyze reperfusion-induced changes of cellular and mitochondrial calcium content. It is known that mitochondria can accumulate large quantities of calcium and also participate in cytosolic Ca^{2+} homeostasis [2, 9, 14]. Data presented in this study demonstrated that only a fraction of endothelial mitochondria accumulate Ca^{2+} during reperfusion. Differences between individual mitochondria in the degree of uptake are indicative of the variety of factors that regulate mitochondrial Ca^{2+} uptake. For example, the rate of increase of $[Ca^{2+}]_c$, the level reached, the spatial inhomogeneity, and the duration of high $[Ca^{2+}]_c$ may all influence mitochondrial Ca^{2+} uptake. In the ECV304 HUVEC line it was found that a fraction of mitochondria are closely associated with the cell membrane, which is quite different from HeLa cells, where less than 4% of mitochondria are within 700 nm of the plasma membrane [12]. This observation and our data lead us to hypothesize that mitochondria which are in close contact with the plasma membrane are mainly affected by Ca^{2+} influx across the plasma membrane and that mitochondria in the other parts of the cells remain unaffected.

Hypoxia has a dramatic effect on the ATP concentrations in HUVECs as estimated by Arnould et al. [1].

Their results indicated that endothelial cells actually undergo a siginificant ATP depletion during 2 h hypoxia. This low ATP concentration and the enhanced energy expenditure in handling the elevated $[Ca^{2+}]_c$ after CIR could lead to a reduction of energy supply for processes that are essential for maintainance of cell viability.

References

1. Arnould T, Michiels C, Alexandre I, Remacle J (1992) Effect of hypoxia upon intracellular calcium concentration of human endothelial cells. J Cell Physiol 152: 215–221
2. Babcock DF, Herrington J, Goodwin PC, Park YP, Hille B (1997) Mitochondrial participation in intracellular Ca^{2+} network. J Cell Biol 136: 833–844
3. Benzi RH, Lerch R (1992) Dissociation between contractile function and oxidative metaboliosm in postischemic myocardium. Circ Res 71: 567–576
4. Berridge MJ, Bootman MD, Lipp P (1998) Calcium–a life and death signal. Nature 395: 645–648
5. Borutaite V, Morkuniene R, Brown GF (1999) Release of cytochrome c from heart mitochondria is induced by high Ca^{2+} and peroxynitrite and is responsible for Ca^{2+}-induced inhibition of substrate oxidation. BBA 1453: 41–48
6. Eberl T, Amberger A, Herold M, Hengster P, Steurer W, Hochleitner BW, Gnaiger E, Margreiter R (1999) Expression of stress proteins, adhesion molecules, and interleukin-8 in endothelial cells after preservation and reoxygenation. Cryobiology 38: 106–118
7. Green D, Kroemer G (1998) The central executioner of apoptosis: caspases or mitochondria? Trends Cell Biol 8: 267–271
8. Haller T, Ortmayr J, Friedrich F, Völkl H, Dietl P (1998) Dynamics of surfactant release in alveolar type II cells. Proc Natl Acad Sci USA 95: 1579–1584
9. Ichas F, Mazat JP (1998) From calcium signalling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state. BBA 1366: 33–50
10. Kim JS, Southard JH (1998) Alteration in cellular calcium and mitochondrial functions in the rat liver during cold preservation. Transplantation 65: 369–375
11. Kroemer G, Zamzami N, Susin BA (1997) Mitochondria control of apoptosis. Immunol Today 18: 44–52
12. Lawrie A, Rizzuto R, Pozzan T, Simpson AWM (1996) A role for calcium influx in the regulation of mitochondrial calcium in endothelial cells. J Biol Chem 271: 10753–10759
13. Miyamae M, Camacho A, Weiner MW, Figueredo VM (1996) Attenuation of postischemic reperfusion injury is related to prevention of $[Ca^{2+}]_m$ overload in rat hearts. Am J Physiol 271:H2145–H2153
14. Rutter GA, Fasolato C, Rizzuto R (1998) Calcium and organelles: a two-sided story. Biochem Biophys Res Commun 253: 549–557
15. Trump BF, Berezesky IK (1995) Calcium-mediated cell injury and cell death. FASEB J 9: 219–228