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Respective effects of oxygen and energy substrate deprivation on beta cell viability

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ABSTRACT

Deficit in oxygen and energetic substrates delivery is a key factor in islet loss during islet transplantation. Permeability transition pore (PTP) is a mitochondrial channel involved in cell death. We have studied the respective effects of oxygen and energy substrate deprivation on beta cell viability as well as the involvement of oxidative stress and PTP opening. Energy substrate deprivation for 1 h followed by incubation in normal conditions led to a cyclosporin A (CsA)-sensitive-PTP-opening in INS-1 cells and human islets. Such a procedure dramatically decreased INS-1 cells viability except when transient removal of energy substrates was performed in anoxia, in the presence of antioxidant *N*-acetylcysteine (NAC) or when CsA or metformin inhibited PTP opening. Superoxide production increased during removal of energy substrates and increased again when normal energy substrates were restored. NAC, anoxia or metformin prevented the two phases of oxidative stress while CsA prevented the second one only. Hypoxia or anoxia alone did not induce oxidative stress, PTP opening or cell death. In conclusion, energy substrate deprivation leads to an oxidative stress followed by PTP opening, triggering beta cell death. Pharmacological prevention of PTP opening during islet transplantation may be a suitable option to improve islet survival and graft success.

Islet transplantation is a treatment to be considered for selected type 1 diabetic patients. The amount of engrafted islets is essential for islet transplantation success, but unfortunately, 50–70% of the transplanted islets are lost in the early post transplant period [1]. Among factors responsible for poor islet graft, islet exposition to the ischemia–reperfusion (I/R)[7] phenomenon is important to consider. Normally, pancreatic islets have a dense glomerular-like capillary network that is ideal for the delivery of oxygen and nutrients [2,3]. During the isolation process and in vitro culture, islets vasculature dedifferentiates or degenerates [4,5]. Immediately after transplantation into the portal vein (i.e., after embolization in a capillary), islets are supplied with oxygen and nutrients only by diffusion from the surrounding tissues. The revascularization process is initiated within a few days, and islets are generally thought to be fully revascularized by 15 days post transplant [6,7]. Meanwhile, islets inevitably endure some restrictions in oxygen and nutrients.

Tissue ischemia is characterized by severe hypoxia, acidosis, energy depletion and cell death. Excessive oxidative stress is well accepted as an important component of I/R injury [8]: Reactive oxygen species (ROS) production begins early in ischemia and is followed by a large burst of oxidative stress during the first few minutes of reperfusion [9–11]. Although many details regarding the sources and targets of oxidant stress during I/R injury are not known, a consensus as regards the importance of ROS in I/R injury has emerged, based on studies showing cell death protection during I/R by pretreatment with antioxidants [12,13].

Other studies involved the mitochondrial permeability transition pore (PTP) in I/R-induced cell death. The PTP is a Ca^{2+}-sensitive mitochondrial inner membrane channel, which, on opening, causes cell death [14,15]. Normally closed in order to allow ATP synthesis, permanent PTP opening leads to a drastic ATP synthesis inhibition through the collapse of the proton-motive force, a dramatic increase in ROS production [16,17] and a release of mitochondrial pro-apoptotic proteins (cytochrome *c* or AIF) [18], which results in cell death [19,20]. It has been proposed that PTP remains closed during ischemia and only opens with reperfusion [21] when the conditions for its opening are present: ROS production, high mitochondrial [Ca^{2+}], adenine

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nucleotide depletion and accumulation of inorganic phosphate [22,23]. Supporting a central role of PTP in I/R injury, several PTP inhibitors (i.e., cyclosporin A (CsA), NIM811 or metformin) have been shown to reduce cardiomyocytes, I/R-induced cell death or infarct size both in animal and human models [24–29].

As regards pancreatic-derived cells, PTP opening has been shown to be involved in cytokine [30], as well as high glucose and fructose-induced apoptosis [31]. CsA has been shown to inhibit Ca^{2+}-induced PTP opening in INS-1 [31] and MIN-6 cells [32], to prevent PK11195-induced cell death in isolated human pancreatic islets [33] and to protect MIN-6 cells against Pdx1 insufficiency-induced cell death [34], while genetic ablation of the endogenous PTP-inducers Cyclophilin D prevents diabetes in Pdx1$^{+/-}$ mice [34]. The negative impact of I/R injury on human or dog islet viability is well described [35,36]. However, the involvement of oxidative stress and PTP opening in pancreatic I/R-induced beta cell death has never been studied.

In the present study, we tested the effects of an O_2 and energy substrate deprivation on INS-1 cell viability. We next examined whether PTP opening was involved in O_2 and substrate deprivation-induced cell death and whether CsA and metformin prevented INS-1 cell viability after an O_2 and energy substrate deprivation. Finally, we clarified the relationship between oxidative stress and PTP opening during O_2 and energy substrate deprivation and reproduced the effect of substrate deprivation on PTP opening in human islets of Langerhans.

1. Materials and methods

1.1. Cell culture conditions

INS-1 cell lines were maintained in RPMI 1640 medium supplemented with 10 mM HEPES, 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and 50 mM 2-mercaptoethanol. Cells were incubated at 37 °C in a humidified atmosphere (95% air–5% CO_2) [37].

1.2. Islets isolation and culture

Islets from pancreas of brain-dead multiorgan donors were isolated as previously described according to a modified Ricordi method [38,39]. Briefly, the pancreatic duct was catheterized and the pancreas was distended by infusion of a cold collagenase solution (Collagenase NB1, Serva Gmbh Heidelberg, Germany). After digestion (37 °C), a purification of cell suspension was performed in a continuous Biocoll gradient (Biochrom AG, Berlin, Germany) using a refrigerated cell separator (Cobe 2991 cell processor, Caridian BCT, France). In our experiments, we handled islet preparations that could not be used for clinical islet transplantation. Islets were provided by Geneva (Switzerland) or Grenoble–Saint Ismier (France) cellular therapy centers and exhibited a purity ranging from 30% to 70% as determined by dithizone staining. After isolation, human islets were cultured at 37 °C, in a 5% CO_2 atmosphere, in Connaught's Medical Research Labs (CMRL 1066-based medium; Sigma Aldrich) supplemented with 10% decomplemented Fetal Calf Serum, 25 mmol/L HEPES, 1 mM sodium pyruvate, 100 UI/ml penicillin and 100 μg/ml streptomycin.

1.3. O_2 and energy substrate deprivation procedure

O_2 and energy substrate deprivation was achieved in a perfusion chamber (POC chamber, LaCom®, Erbach, Germany) coupled with an incubation system (O_2-CO_2-°C, PeCom®, Erbach, Germany) allowing a variation of O_2 from 0% to 21% thanks to N_2 bubbling. The chamber was mounted on a Leica TCS SP2 AOBS inverted laser scanning confocal microscope. Under baseline conditions, cells previously incubated in the absence or presence of 1 μM CsA for 1 h, 5 mM *N*-acetylcysteine (NAC) for 1 h or 100 μM metformin overnight were incubated in the standard complete RPMI 1640 for INS-1 cells (Glucose = 11 mmol/L) or CMRL1066 for human islets of Langerhans (Glucose = 5.5 mmol/L) under controlled O_2 and CO_2 conditions (95% air–5% CO_2). Energy substrate deprivation was achieved by replacing the normal complete medium by a medium-deprived substrate (102.7 mM NaCl, 5.36 mM KCl, 23 mM $NaHCO_3$, 0.83 mM $MgSO_4$, 5.65 mM Na_2HPO_4, 0.42 mM $Ca(NO_3)_2$, 10 mM HEPES). O_2 deprivation was achieved by equilibrating the incubation media with either 3% O_2, 5% CO_2 and 92% N_2 (hypoxia) or 5% CO_2 and 95% N_2 (anoxia). After 1 h, basal conditions were restored by replacing the tested medium by the normal complete medium equilibrated with 95% air–5% CO_2. Cells undergoing two changes of complete RPMI 1640 medium equilibrated with 95% air–5% CO_2 were used as control.

1.4. Determination of PTP state by confocal microscopy

The open/closed PTP state was assessed by double channel imaging of NAD(P)H autofluorescence and mitochondrial electrical membrane potential (i.e., TMRM fluorescence) as recently described [40]. INS-1 cells set on collagen I-coated cover slips were studied by time-lapse laser confocal microscopy at 37 °C in a humidified atmosphere (95% air, 5% CO_2) using a microscope equipped with a perfusion chamber (POC chamber, LaCom®, Erbach, Germany) and an incubation system (O_2-CO_2-°C, PeCom®, Erbach, Germany). Images were collected with a Leica TCS SP2 AOBS inverted laser scanning confocal microscope equipped with a Coherent 351–364 UV laser using a 63× oil immersion objective (HCX PL APO 63.0 X 1.40). Laser excitation was 351–364 nm for NAD(P)H and 543 nm for TMRM. Fluorescence emission adjusted with AOBS was 390–486 nm for NAD(P)H and 565–645 nm for TMRM. In order to allow the overlay of NAD(P)H and TMRM signals, image acquisition was set with the same pinhole aperture (Airy 3.55), necessarily increased because of the low signal of NAD(P)H autofluorescence. To follow PTP status, NAD(P)H and TMRM fluorescence images were acquired every 10 min during the simulated ischemia–reperfusion procedure. Note that the changes of medium led to the removal of TMRM outside the cells. This led to a release of TMRM from mitochondria even when the electrical membrane potential remained constant. To discriminate between this normal decrease in TMRM fluorescence and a real decrease in mitochondrial electrical membrane potential, cells were reloaded with 10 nM TMRM, 15 min before the end of the experiment. Experiments were performed on a randomly chosen field containing 15–25 cells. The background noise of NAD(P)H autofluorescence was removed by fine filter (Kernel 3x3) using Volocity® (Improvision) software, while the other images (TMRM and MitoSOX) were not electronically manipulated. Image quantification was performed using ImageJ (NIH images) and Volocity® (Improvision) software as described in [40].

1.5. Determination of adenine nucleotide content

INS-1 cells set on 100 mm Petri dishes were exposed to O_2 and energy substrate deprivation as described above using an O_2 control cabinet for in vitro studies (CoyLab®). At the end of the simulated ischemia and 1 h after the simulated reperfusion, samples of INS-1 cells were withheld and lysed in ice-cold PCA (2.5%)-EDTA (6.25 mM) for 5 min. The insoluble material was eliminated by centrifugation at 12,000 g for 5 min, and the supernatant fraction was immediately neutralized with KOH/MOPS. After removal of the formed $KClO_4$ by quick spin, the final extract was analyzed by HPLC as described in [41].

1.6. Quantification of cell death by flow cytometry

Cell viability analysis was performed with a double-stain system using Annexin V (Interchim) combined with FluoProbes 488 and propidium iodide (PI) (Sigma Aldrich). Forty-eight hours after a 1-h removal of O_2 and/or energy substrate, collected supernatant and cells detached by trypsination were centrifuged. The cells were then incubated