Biochimica et Biophysica Acta 1847 (2015) 629–639

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

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

Sandrine Lablanche [a,b,c,*], Cécile Cottet-Rousselle [a,b], Laurent Argaud [d], Camille Laporte [a,b], Frédéric Lamarche [a,b], Marie-Jeanne Richard [e], Thierry Berney [f], Pierre-Yves Benhamou [a,b,c], Eric Fontaine [a,b,c]

[a] University of Grenoble Alpes, LBFA, Grenoble F-38000, France
[b] U1055, INSERM, Grenoble F-38000, France
[c] Department of Endocrinology, Grenoble University Hospital, Grenoble F-38043, France
[d] CarMeN Laboratory, U1060, INSERM, Lyon F-69373, France
[e] Cellular Therapy Unit, EFS Rhône-Alpes, Grenoble University Hospital, Grenoble, France
[f] Cell Isolation and Transplant Center, University of Geneva, Level R, 1 rue Michel Servet, Geneva 4, CH-1211, Switzerland

ARTICLE INFO

Article history:
Received 9 October 2014
Received in revised form 30 March 2015
Accepted 5 April 2015
Available online 11 April 2015

Keywords:
Permeability transition
Apoptosis
Beta cell
Ischemia–reperfusion injury

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.

© 2015 Elsevier B.V. All rights reserved.

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

* Corresponding author at: U1055, INSERM, Bioénergétique Fondamentale et Appliquée, Université Joseph Fourier, BP 53, Grenoble Cedex F-38041, France. Tel.: +33 476 63 56 01; fax: +33 476 51 42 18.
E-mail address: slablanche@chu-grenoble.fr (S. Lablanche).

http://dx.doi.org/10.1016/j.bbabio.2015.04.002
0005-2728/© 2015 Elsevier B.V. All rights reserved.

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

in 100 μl of Annexin V buffer 1X (10 mM HEPES NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$) for 15 min at room temperature in the dark in the presence of 5 μl of Annexin V-FP488. Cells were then transferred in a 5-ml propylene tube containing 900 μl PBS and 10 μl of a 1-mg/ml stock solution of PI and immediately analyzed. Data acquisition (~5,000 cells) was carried out using a BD LSRFortessa™ cell analyzer (Becton-Dickinson Biosciences) equipped with a 150-mW Coherent Compass laser (532 nm) and a 100-mW Coherent Sapphire laser (488 nm), using the BD FACsDiva™ software (Becton-Dickinson Biosciences). Data were plotted as a function of fluorescence intensity on 525/50 nm band-pass filter detector for Annexin V-FluoProbes 488 and 585/15 nm band-pass filter detector for PI. The Annexin V^-/PI^- population was regarded as normal healthy cells.

1.7. Assessment of oxidative stress

INS-1 cells incubated in the absence or presence of 1 μM CsA for 1 h, 5 mM NAC for 1 h or 100 μM metformin overnight were loaded for 15 min with 1 μM MitoSOX™ Red (Molecular Probes™), a cationic probe distributing to the mitochondrial matrix by virtue of the mitochondrial transmembrane potential. The probe becomes highly fluorescent after oxidation by superoxide in the presence of DNA. To follow oxidative stress, MitoSOX™ Red fluorescence images were acquired every 10 min during the procedure. Laser excitation was 514 nm. Fluorescence emission adjusted with AOBS was 550–630.

1.8. Statistics

Results are presented as mean ± SEM. The statistical significance of differences was analyzed by a one-way ANOVA followed by the Tukey–Kramer HSD post hoc test or by the Dunnett test using JMP® (SAS) software. Significance was defined as $p < 0.05$.

2. Results

2.1. Effects of energy substrate withdrawal on INS-1 cell viability

Assuming that oxygen may diffuse better than the energy substrates inside the islets once they have been embolized in a capillary, INS-1 cells were exposed for 1 h in a medium without energy substrates (see Materials and Methods section) with either 20% O_2 (95% air–5% CO_2), 3% O_2 (hypoxia) or no O_2 (anoxia). As shown in Fig. 1, 48 h after such a procedure, INS-1 cells incubated without energy substrates in the presence of either 20% or 3% O_2 exhibited a dramatic alteration in

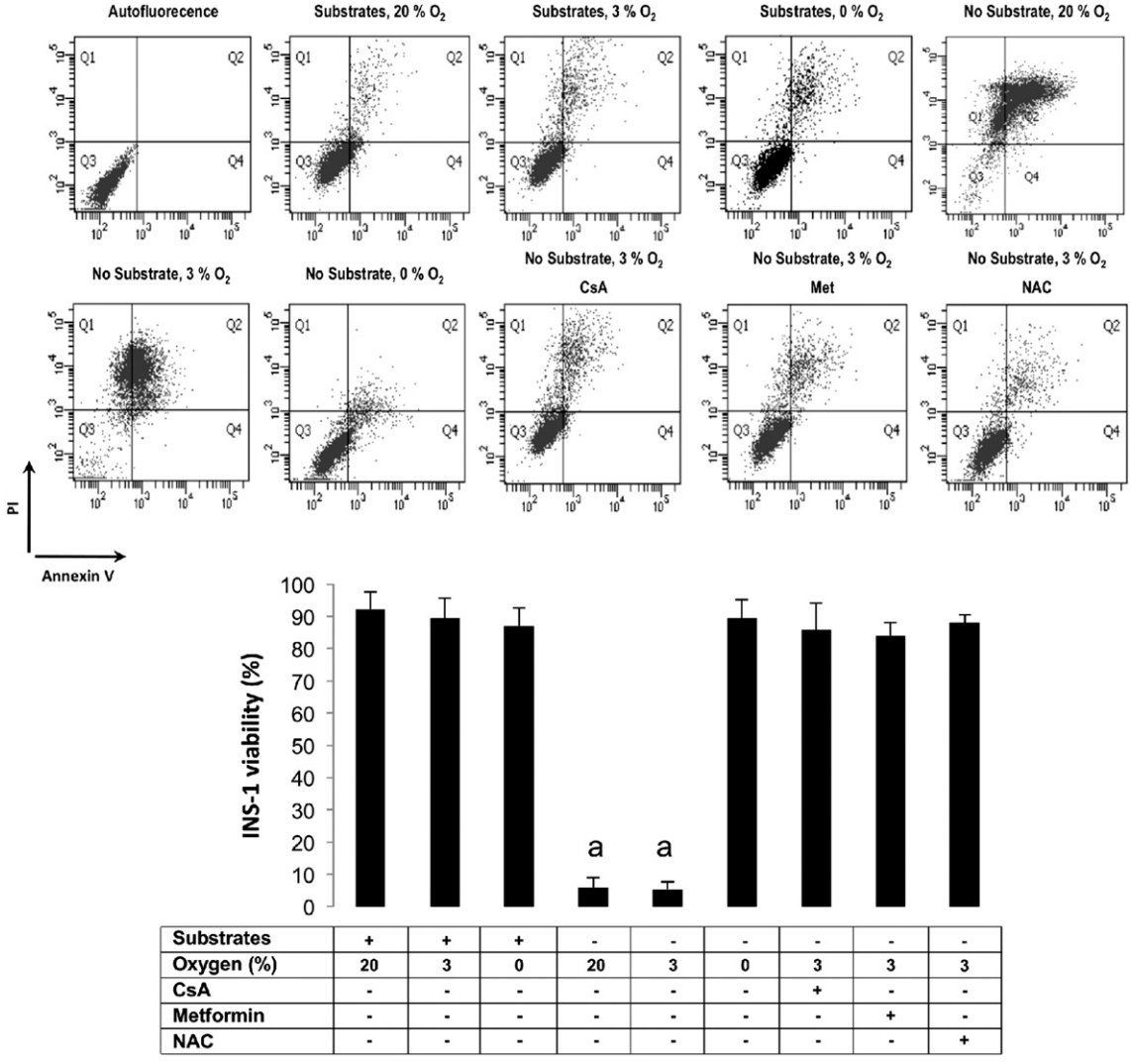

Fig. 1. Respective effects of oxygen and energy substrate deprivation on INS-1 cell viability. INS-1 cells incubated in the absence or presence of either 1 μM CsA for 1 h, 5 mM NAC for 1 h or 100 μM metformin overnight were submitted to the indicated conditions of incubation for 1 h and then incubated in normal conditions for 48 h. Cell viability was assessed by double labeling as described in the Materials and Methods section. Histograms represent the results of three different experiments. Results are presented as mean ± SEM of at least 3 different experiments. Values not connected by the same letter are significantly different (Tukey–Kramer HSD post hoc test, $p < 0.0001$).

viability as compared with control. Surprisingly, the same removal of energy substrates in the absence of O_2 did not induce cell death. Moreover, INS-1 cells exposed for 1 h in a medium with energy substrates but in the absence of O_2 or in the presence of 3% O_2 did not undergo cell death, indicating that INS-1 cells can survive for an hour with no oxygen. Interestingly, cell death induced by a 1-h withdrawal of energy substrates was prevented by either CsA or metformin, which have been shown to inhibit PTP opening in INS-1 cells [31], and by antioxidant NAC.

2.2. Effects of energy substrate withdrawal on PTP state

To test whether the removal of energy substrates led to PTP opening as suggested by the preventive effect of CsA or metformin on cell viability, PTP status was assessed by double channel imaging of NAD(P)H autofluorescence and mitochondrial electrical membrane potential (i.e., TMRM fluorescence) as recently described [40]. Briefly, when PTP is closed, NAD(P)H is mainly localized within mitochondria and colocalizes with TMRM fluorescence (which accumulates in polarized mitochondria). Permanent PTP opening leads to a dramatic collapse in membrane potential associated with an increase in NAD(P)H autofluorescence both in terms of intensity and intracellular distribution.

Using this method, every 10 min, we measured the INS-1 NAD(P)H and TMRM fluorescence submitted to control conditions or to O_2 and energy substrate deprivation procedures. Note that the same microscope settings were kept through the entire procedure. As shown in Fig. 2, under control conditions, NAD(P)H remained stable while TMRM fluorescence slightly decreased due to TMRM dilution caused by medium changes but increased after TMRM reload at the end of reperfusion. On the contrary, after the removal of energy substrates in the presence of 3% O_2, both NAD(P)H area and intensity drastically diminished (most probably due to the consumption of endogenous substrates, see below) then slightly increased (but remained lower than control), while TMRM fluorescence slightly decreased in a way not significantly different from what observed in the control conditions. After the restoration of O_2 and energy substrates, NAD(P)H area and fluorescence continuously increased, reaching values more than twice the control, whereas TMRM fluorescence dramatically decreased, with no recovery after TMRM reload.

In parallel experiments, the adenine nucleotide content was measured at the end of the deprivation procedure and 1 h following the restoration of normal condition of incubation. As shown in Fig. 2, the ATP/ADP ratio remained constant during time in the control experiment. Interestingly, the ATP/ADP ratio slightly decreased 1 h after the removal of energy substrates in the presence of 3% O_2, whereas it collapsed 1 h after the restoration of O_2 and energy substrates, as expected in cells with depolarized mitochondria.

Normally, mitochondrial depolarization is expected to increase NADH consumption by the respiratory chain and therefore to decrease the NAD(P)H fluorescence. In cells in which this prediction was confirmed (HMEC-1 cells, isolated hepatocytes, primary neurons and primary astrocytes [40,42]), the counterintuitive increase in NAD(P)H fluorescence concomitant with mitochondrial depolarization is most probably due to the reduction of the cytosolic NAD^+ pool by the TCA cycle made possible by the diffusion of pyridine nucleotides through the open PTP, a phenomenon reproduced in isolated mitochondria when PTP opening occurs in the presence of NAD^+ [40].

Unexpectedly, as shown in Fig. 3, mitochondrial uncoupling did not collapse NAD(P)H fluorescence in INS-1 cells. Actually, uncoupling only transiently decreased NAD(P)H fluorescence, which eventually increased when mitochondria were fully depolarized. Note that CsA did not prevent this behavior (not shown). Independently of the putative explanation of this observation (see discussion), any increase in NAD(P)H fluorescence concomitant with mitochondrial depolarization cannot be considered as specific to PTP opening in INS-1, unless it is totally prevented by PTP inhibitors.

Therefore, the same assessment of NAD(P)H and TMRM fluorescence by confocal microscopy was performed in all the conditions tested in Fig. 1, including incubation in the presence of PTP inhibitors. Results are presented in Fig. 4 as the overlay of the NAD(P)H and TMRM fluorescence. For an easier comparison the overlays of Fig. 2 are presented in Fig. 4A and B.

As shown in Fig. 4C, when cells were incubated with 3% O_2 in the presence of energy substrates (normal complete medium), no changes in NAD(P)H intensity and area were observed, while TMRM fluorescence was the same as in control conditions after TMRM reload. Exactly the same behavior was observed when the medium without energy substrates (see Materials and Methods section) was supplemented with 11 mM Glucose (data not shown).

On the contrary, the same dramatic increase and decrease in NAD(P)H and TMRM fluorescence respectively was observed when the 1-h removal of energy substrates was performed in normoxia (Fig. 4D). These data suggested that the absence of energy substrates was mandatory to induce such a behavior.

Importantly, this behavior did not occur when PTP opening was prevented by either CsA (Fig. 4E and F) or metformin (Fig. 4G), whereas it was not prevented by verapamil (not shown), which does not affect PTP regulation but inhibits ABC transporter as CsA does [43]. Together, these data strongly suggested that the removal of energy substrates in the presence of O_2 (20% O_2 or 3% O_2) led to permanent PTP opening after energy substrate restoration in INS-1 cells. Interestingly, antioxidant NAC (which does not directly inhibit PTP opening) also prevented mitochondrial depolarization and the increase in NAD(P)H fluorescence after O_2 and energy substrate restoration (Fig. 4H), suggesting that PTP opening was triggered by an oxidative stress during our experimental protocol.

As expected, the NAD(P)H fluorescence dramatically increased when cells were incubated in anoxia in the presence of energy substrates (Fig. 4I). At the same time, TMRM fluorescence decreased. After O_2 restoration, the NAD(P)H fluorescence became normal again while the TMRM fluorescence remained low. This was due to the removal of TMRM outside the cells during the changes of medium preventing mitochondria from taking up TMRM as demonstrated by a large uptake of TMRM after TMRM reloading. The same changes in NAD(P)H fluorescence were observed in the absence of TMRM (data not shown).

When cells were incubated in anoxia deprived of energy substrates (Fig. 4J), NAD(P)H remained stable (due to a lack of O_2 which prevented the consumption of the remaining substrates). After O_2 and energy substrate restoration, the NAD(P)H fluorescence first decreased and then slightly increased but remained lower than what observed when the removal of energy substrates was performed in hypoxia, while TMRM fluorescence was the same as in control conditions after TMRM reload. These data suggested that the presence of O_2 during the removal of energy substrates was mandatory to induce PTP opening.

2.3. Relationship between PTP opening and oxidative stress during O_2 and energy substrate deprivation procedure

To assess superoxide production during O_2 and energy substrate deprivation procedures, INS-1 cells loaded 15 min with MitoSOX™ Red were imaged every 10 min. After the removal of energy substrates in the presence of O_2 (20% O_2 or 3% O_2), a significant increase in oxidative stress was detected as compared with the control conditions (Fig. 5). This first oxidative stress was followed by a burst in ROS production observed after energy substrate restoration. Note, however, that the second oxidative stress was delayed and therefore not related to changes in oxygen concentration as it occurred when cells were continuously incubated in the presence of 20% O_2, while it was not synchronous with the normalization of O_2 concentration when cells had undergone a 1-h exposure to 3% O_2. Note, moreover, that during this second oxidative stress, the fluorescence increased notably in some cells (presumably those that have undergone PTP opening, see below).

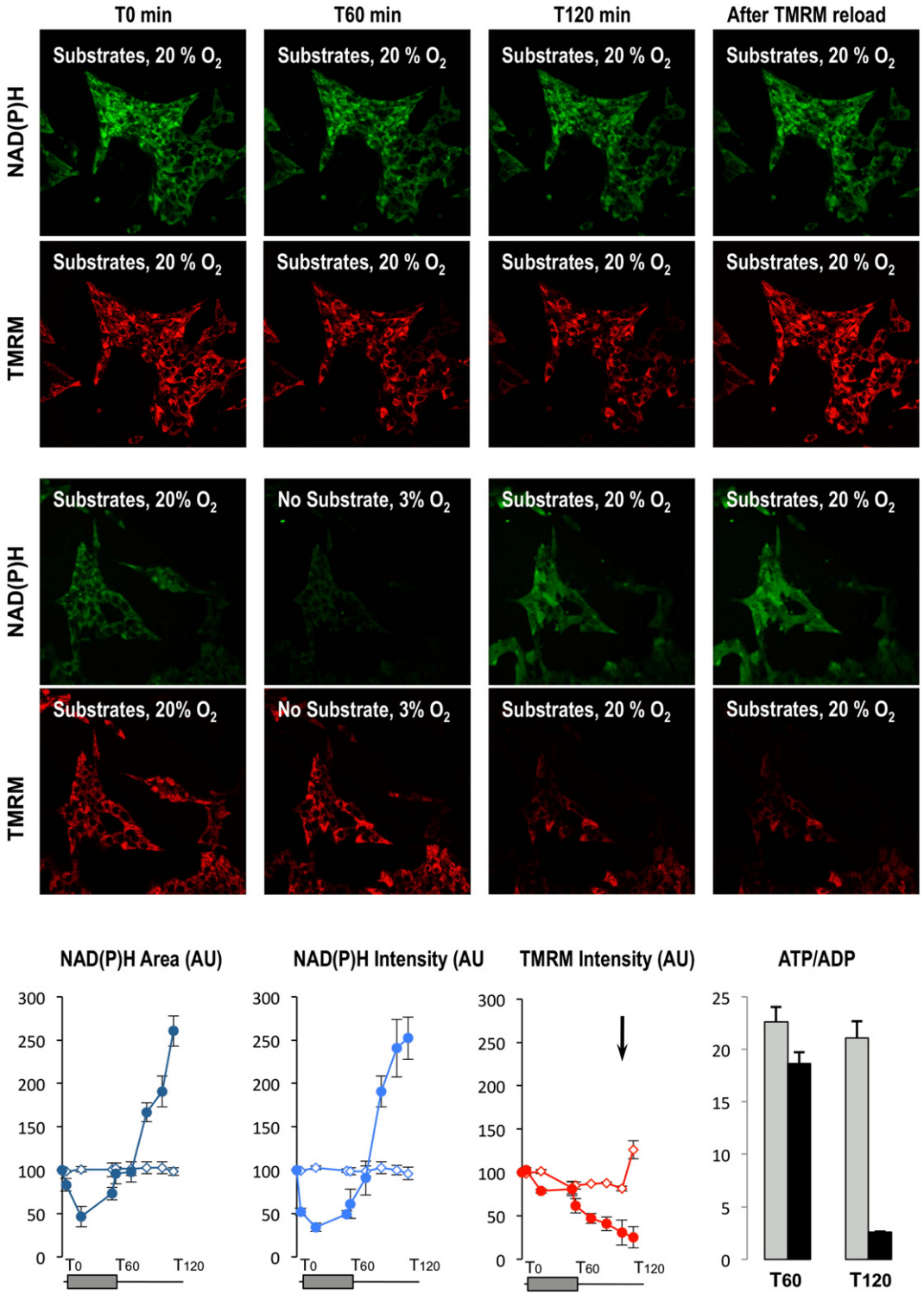

Fig. 2. Respective effects of oxygen and energy substrate deprivation on NAD(P)H autofluorescence, mitochondrial electrical membrane potential and ATP/ADP ratio. INS-1 cells loaded with 10 nM TMRM for 1 h were incubated in normal conditions (substrates and 20% O_2) or submitted to hypoxia (3% O_2) without energy substrates for 60 min, followed by the restoration of normal conditions of incubation for 60 min. The fluorescence of NAD(P)H (pseudo color green) and TMRM (pseudo color red) were imaged every 10 min during the simulated I/R procedure. Image quantification (arbitrary units (AU)) was performed as described in the Materials and Methods section. Curves represent the results of three different experiments. Results are presented as mean ± SEM of at least 3 different experiments. Open symbols, control; closed symbols, simulated I/R; arrow, TMRM reload. The ATP and ADP content were measured in parallel experiments at the end of the deprivation procedure and 1 h following the restoration of normal condition of incubation. Results are presented as mean ± SEM of at least 3 different experiments. Grey, control; black, simulated I/R. Values not connected by the same letter are significantly different (Tukey–Kramer HSD post hoc test, $p < 0.0001$).

In such cells, the fluorescence was clearly observable in the nucleus, suggesting a diffusion of either superoxide or ethidium (the oxidized form of the dihydoethidium moiety of the MitoSOX™) from mitochondria to the nucleus.

As shown in Fig. 5, the second oxidative stress was totally prevented by all the conditions that prevented PTP opening (namely CsA, metformin, NAC, incubation in anoxia without energy substrates, or incubation in the presence of 3% O_2 with energy substrates), indicating that the

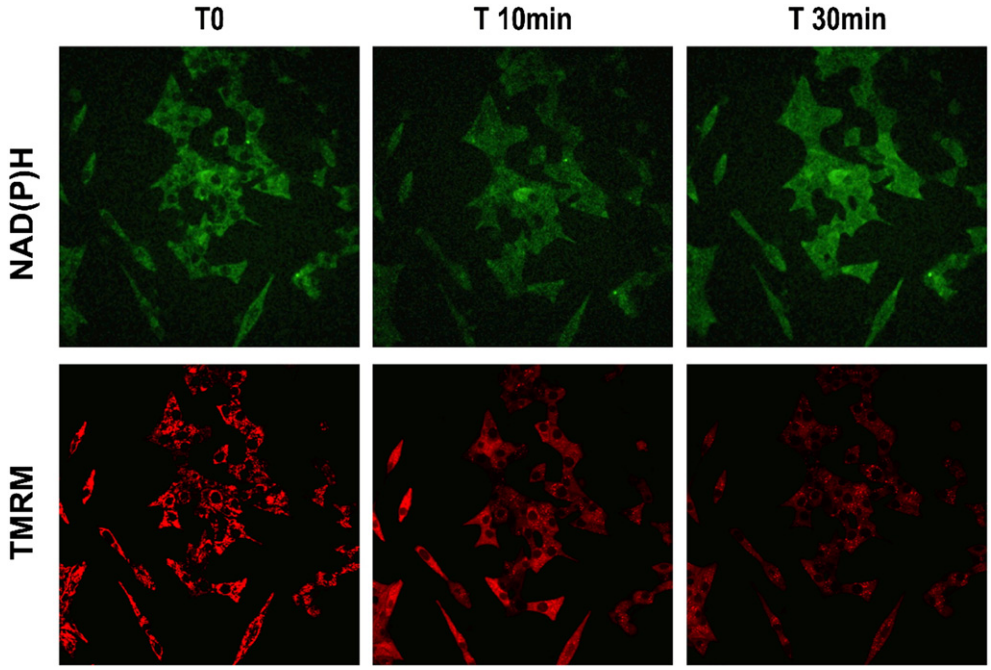

Fig. 3. Effects of FCCP on NAD(P)H autofluorescence and mitochondrial electrical membrane potential. INS-1 cells loaded with 10 nM TMRM for 1 h were incubated in normal conditions (substrates and 20% O_2) and exposed to 50 μM FCCP. The fluorescence of NAD(P)H (pseudo color green) and TMRM (pseudo color red) were imaged every 5 min. Result representative of 3 different experiments.

second oxidative stress was due to PTP opening. As expected, CsA (which is not an antioxidant) did not prevent the first oxidative stress, whereas NAC, anoxia without energy substrates or incubation in the presence of 3% O_2 with energy substrates prevented the first oxidative stress. Interestingly, metformin also prevented the first oxidative stress.

2.4. Effects of energy substrate withdrawal on human islets of Langerhans

Due to the extreme scarcity of human islets of Langerhans, a complete reproduction of the above results was not possible. However, as shown in Fig. 6, a 1-h removal of energy substrates in the presence of 3% O_2 led to a similar CsA-sensitive increase and decrease in NAD(P)H and TMRM fluorescence respectively after the restoration of O_2 and energy substrates.

3. Discussion

In this work, we have reported that a 1-h removal of energy substrates in INS-1 cells led to cell death via PTP opening only if an oxidative stress occurred during the deprivation of energy substrates. In agreement with what is generally reported during I/R injuries, we also observed a robust oxidative stress after the restoration of energy substrates, yet this was not the cause but the consequence of PTP opening.

The involvement of PTP opening in I/R is sustained by a large number of studies that have reported a beneficial effect of PTP inhibition on infarct size. However, none of these studies have directly visualized PTP opening during I/R or analyzed the respective effect of substrate or O_2 deprivation. Here, we show that the deprivation of energy substrates for 1 h—but not the deprivation of O_2 for the same period of time—was responsible for a CsA-sensitive mitochondrial depolarization (i.e., PTP opening) in beta cells.

This may not be a general rule for other types of cells. Note, however, that PTP opening-induced cell death has been reported in brain suffering hypoglycemia [44]. In beta cells, insulin secretion strongly depends on the ATP concentration, which is directly modulated by glucose availability. In order to respond quickly to changes in blood glucose concentration, these cells have no glycogen reserves. The same absence of glycogen reserves can be noted in neurons, which probably explains why these cells are so sensitive to substrate removal.

In normal cells, when anaerobic glycolysis is stimulated, NADH does not accumulate because the lactate dehydrogenase regenerates NAD^+ when pyruvate is converted into lactate; this latter being released via the monocarboxylate transporter. In beta cells, the lactate dehydrogenase activity is very low [45] and the monocarboxylate transporter is not expressed [46]. Although a low amount of monocarboxylate transporter has been reported in INS-1 cells [47], the unusual dramatic increase in NAD(P)H signal observed when INS-1 cells are incubated with substrates in the absence of O_2 suggests that lactate dehydrogenase and the monocarboxylate transporter activities remain lower than the production of NADH by glycolysis. This may also explain the unusual increase in NAD(P)H fluorescence following mitochondrial uncoupling observed in Fig. 3, the NADH produced by glycolysis in the cytosol being unable to enter mitochondria via the electrogenic malate/aspartate shuttle while the PTP remains closed.

On the contrary, complex I can theoretically consume the cytosolic NADH after PTP opening, unless the respiratory chain is inhibited. We have previously demonstrated that PTP opening partly inhibits complex I activity in isolated mitochondria [48] and inhibits oxygen consumption in intact cells [40]. Additional explanation for an inhibition of respiratory chain after PTP opening includes the release of cytochrome *c* and the exhaustion of O_2. However, considering the exchange area between medium and air in our device on the one hand, and the fact that superoxide production increased on the other hand, this latter hypothesis seems unlikely. In addition to the NADH produced by glycolysis, the TCA cycle can produce NADH from the cytosolic NAD^+ pool [40], which can diffuse inside mitochondria after PTP opening.

In our hands, INS-1 cells incubated in the presence of substrates but in the absence of oxygen died after 6 h (data not shown). This does not mean that the cells function properly for 6 h but indicates that they can survive for 6 h thanks to anaerobic glycolysis. Assuming that human islets of Langerhans are totally deprived of monocarboxylate transporter, their survival in the absence of oxygen is probably shorter.

It has been previously shown that the deprivation of O_2 and energy substrates causes a first oxidative stress during deprivation [9,10,49],

followed by a second one after O_2 and energy substrate restoration. However, the origin of these two oxidative stresses remains elusive. Mitochondria are the main source of oxidative stress in cells. Superoxide can be generated both at respiratory chain complexes I and III [50]. Complex I is a reversible proton pump that can generate superoxide during forward and reverse electron flux [51,52]. Complex I inhibitors increase superoxide production driven by a forward electron flux, whereas they decrease superoxide production driven by a reverse electron flux. Metformin, which partly inhibits complex I [53], has been shown to decrease superoxide production driven by a reverse electron flux [16]. Therefore, the observation that metformin dramatically decreased the first oxidative stress (see Fig. 5) suggests that the oxidative stress during the deprivation procedure was due to a reverse electron flux at complex I.

Theoretically, the production of superoxide is impossible in the total absence of O_2, while antioxidants are expected to hamper oxidative

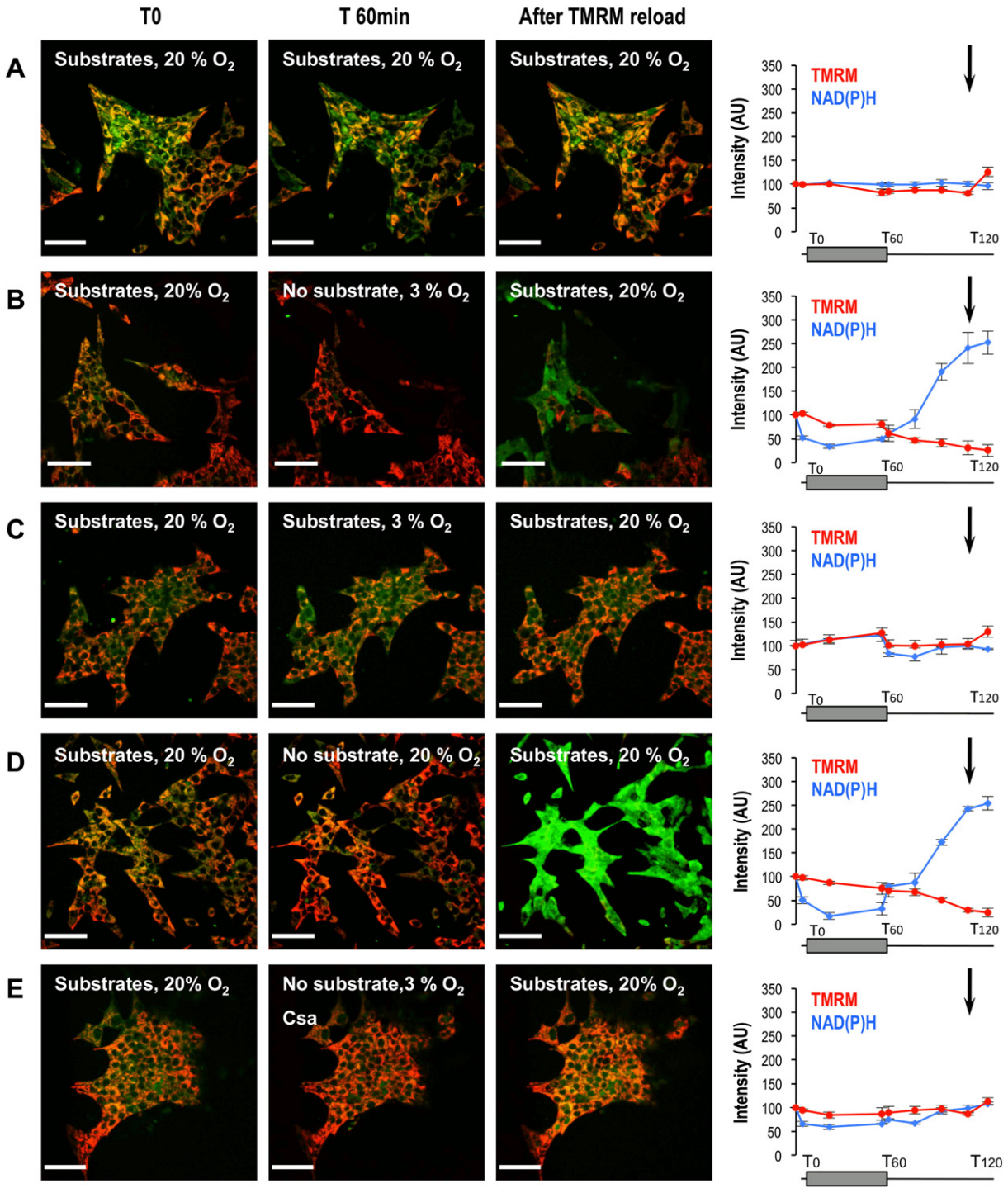

Fig. 4. Effects of CsA, metformin, NAC and anoxia on energy substrate deprivation-induced PTP opening. INS-1 cells incubated in the absence or presence of either 1 μM CsA for 1 h, 5 mM NAC for 1 h or 100 μM metformin overnight loaded with 10 nM TMRM for 1 h were submitted to the indicated conditions for 60 min, followed by the restoration of normal conditions of incubation for 60 min. The fluorescence of NAD(P)H (pseudo color green) and TMRM (pseudo color red) were imaged every 10 min during the procedure, and the two channels were merged. Image quantification was performed as described in the Materials and Methods section. Curves represent the results of three different experiments. Results are presented as mean ± SEM of at least 3 different experiments. Blue symbols, NAD(P)H; red symbols, TMRM; arrow, TMRM reload.

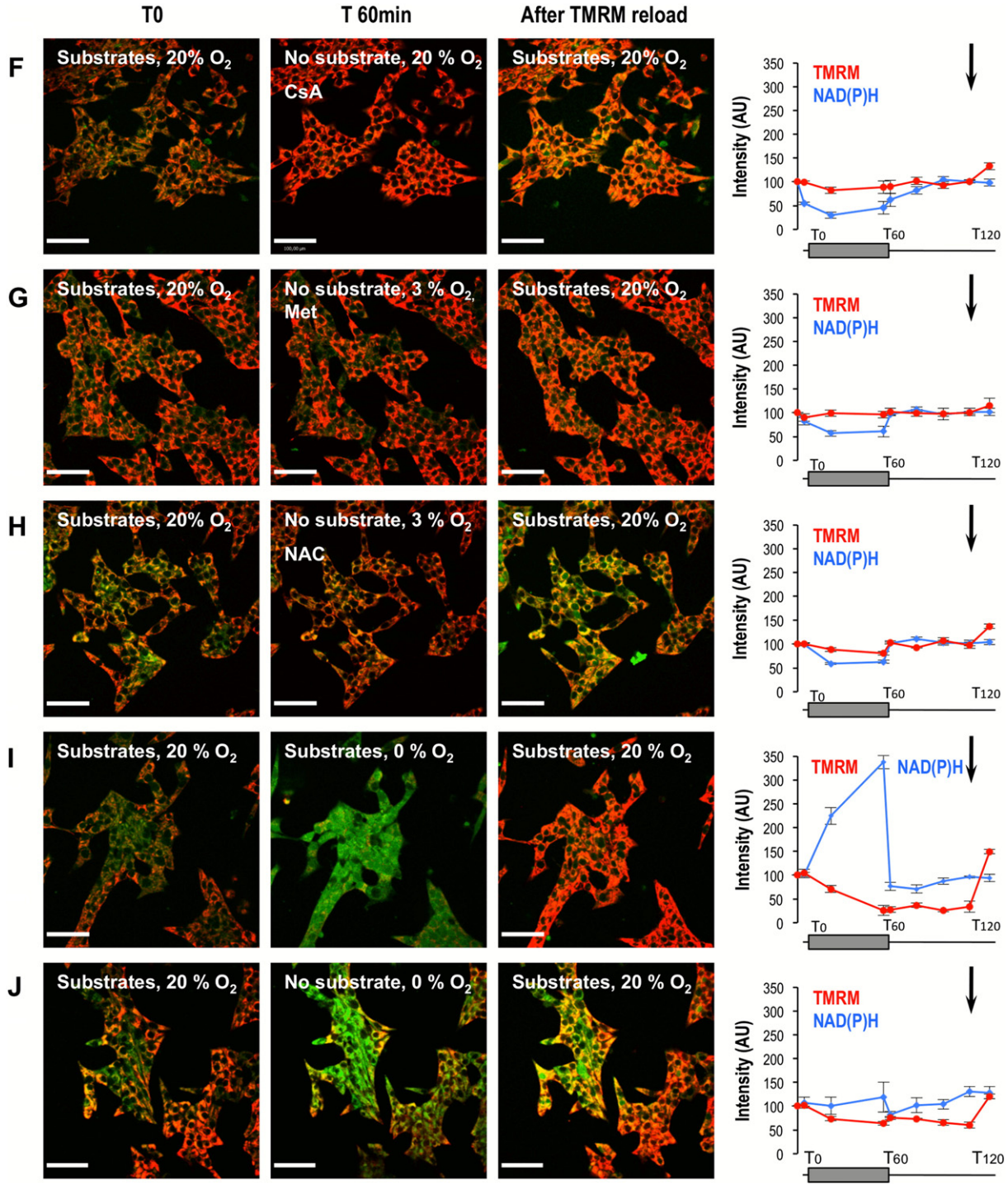

Fig. 4 (*continued*).

stress. As shown in Fig. 5, superoxide production during the removal of energy substrates was prevented by incubating cells either in anoxia or in the presence of NAC. The fact that the prevention of superoxide production during energy substrate removal totally prevented PTP opening and cell death strongly suggests that the oxidative stress during energy substrate deprivation was critical for PTP opening and cell death.

Whether PTP opening occurs during ischemia or only at reperfusion remains debated and may depend on the model used. In the present work, mitochondrial depolarization associated with the increase in NADH fluorescence did not occur during the simulated ischemia (during the deprivation of energy substrates or O_2).

Although oxidative stress is well known to favor PTP opening [15], the exact mechanism by which an oxidative stress during simulated ischemia is a prerequisite for PTP opening after simulated reperfusion remains unknown. Note that mitochondrial depolarization and the increase in NAD(P)H fluorescence (i.e., PTP opening) preceded the second oxidative stress (compare Fig. 4 with Fig. 5). It has been reported that PTP opening stimulates ROS production in intact cells [17], and we have shown in isolated mitochondria that such production occurs at complex I and depends on NADH concentration [48]. Assuming that the same mechanism occurs in intact cells, and taking into account that NAD(P)H concentration was low just after PTP opening but

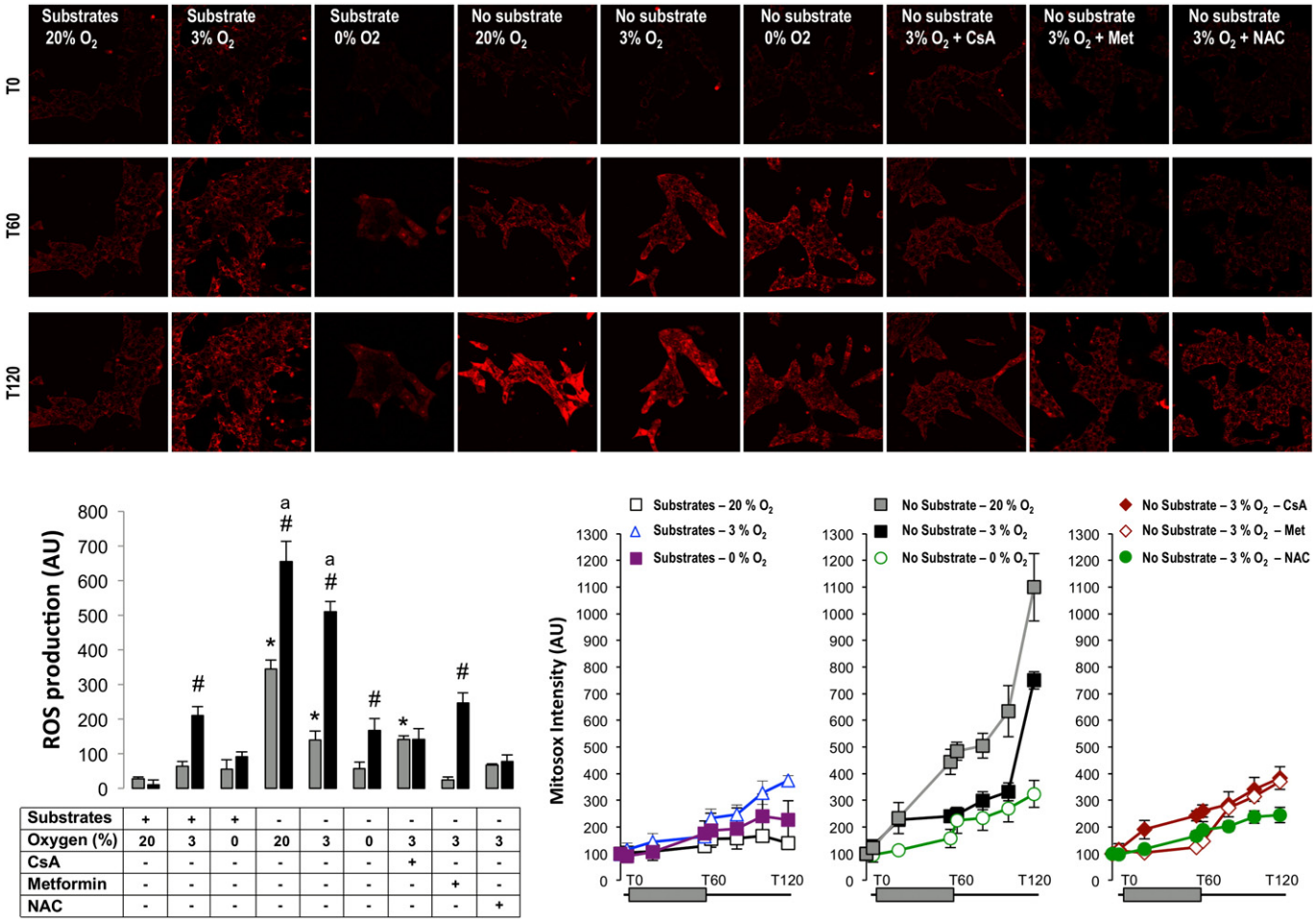

Fig. 5. Effects of oxygen, energy substrates, CsA, metformin and NAC on superoxide production. INS-1 cells incubated in the absence or presence of 1 μM CsA for 1 h, 5 mM NAC for 1 h or 100 μM metformin overnight, loaded with 1 μM MitoSOX™ Red for 15 min, were submitted to the indicated condition for 60 min, followed by the restoration of normal conditions of incubation for 60 min. The fluorescence of MitoSOX™ Red was imaged every 10 min during the procedure. Image quantification was performed as described in the Materials and Methods section. Results are presented as mean ± SEM of at least 3 different experiments. Histograms represent the superoxide production (calculated by fluorescence subtraction: F_{T60}-F_{T0} or F_{T120}-F_{T60}) during the studied condition (grey) and after normal conditions were restored (black). * and # significantly different from control during the same period (* from T0 to T60; # from T60 to T120) (Dunnett's test, $p < 0.05$).Values not connected by the same letter are significantly different (Tukey–Kramer HSD post hoc test, $p < 0.05$).

increased with time (Fig. 4), it is expected that PTP opening does not induce superoxide production until NADH concentration increases. In addition, the second oxidative stress may be exacerbated by the loss of cytochrome *c*, which has been shown to be a powerful superoxide scavenger in its oxidized form [54].

Although substantial progress has been made on islet isolation and immunosuppression protocols [55], obstacles still compromise islet transplant success. A waste of islets occurs during the isolation procedure, while 50% to 70% of islets are estimated to be destroyed in the immediate post transplant period [1], making beta cell death a crucial issue that prevents islet transplantation from spreading. Among several factors, this work suggests that islet viability may be compromised by a lack of energy substrates during isolation procedures or after engraftment. This point should be underlined since most cold storage solutions used for organ conservation are deprived of energy substrate.

It has been shown that immunosuppressive drugs (including CsA) impair insulin secretion [56], partly by inhibiting insulin gene transcription [57]. However, if high concentration of CsA (5 μg/ml, i.e., approximately 4 μM) has been shown to be toxic for pancreatic islets, therapeutic dose of CsA (1 μg/ml, i.e., approximately 0.8 μM) did not affect pancreatic islets viability [58], which is consistent with the results in Fig. 1. If long-term administration of high concentrations of CsA should to be detrimental for islet functionality, the transient use of therapeutic dose of CsA restricted to the early post-transplantation period might be a suitable option to consider in order to preserve islet graft viability during this particular vulnerability period. Since a lack of energy substrates leads to cell death due to PTP opening, preventing PTP opening during the entire islet transplant procedure may enhance beta cells survival and improve islet transplant outcome. Further in vivo studies are necessary to confirm that the prevention of PTP opening does improve islet survival and islet graft success.

Abbreviations

I/R	ischemia–reperfusion
ROS	reactive oxygen species
PTP	permeability transition pore
CsA	cyclosporin A
Met	metformin
NAC	*N*-acetylcysteine
TMRM	tetramethylrhodamine methyl ester
PI	propidium iodide
FCCP	carbonilcyanide *p*-trifluoromethoxyphenylhydrazone

Conflict of interest

The authors confirm that they have no commercial or other associations that might generate a conflict of interest in connection with the submitted article.

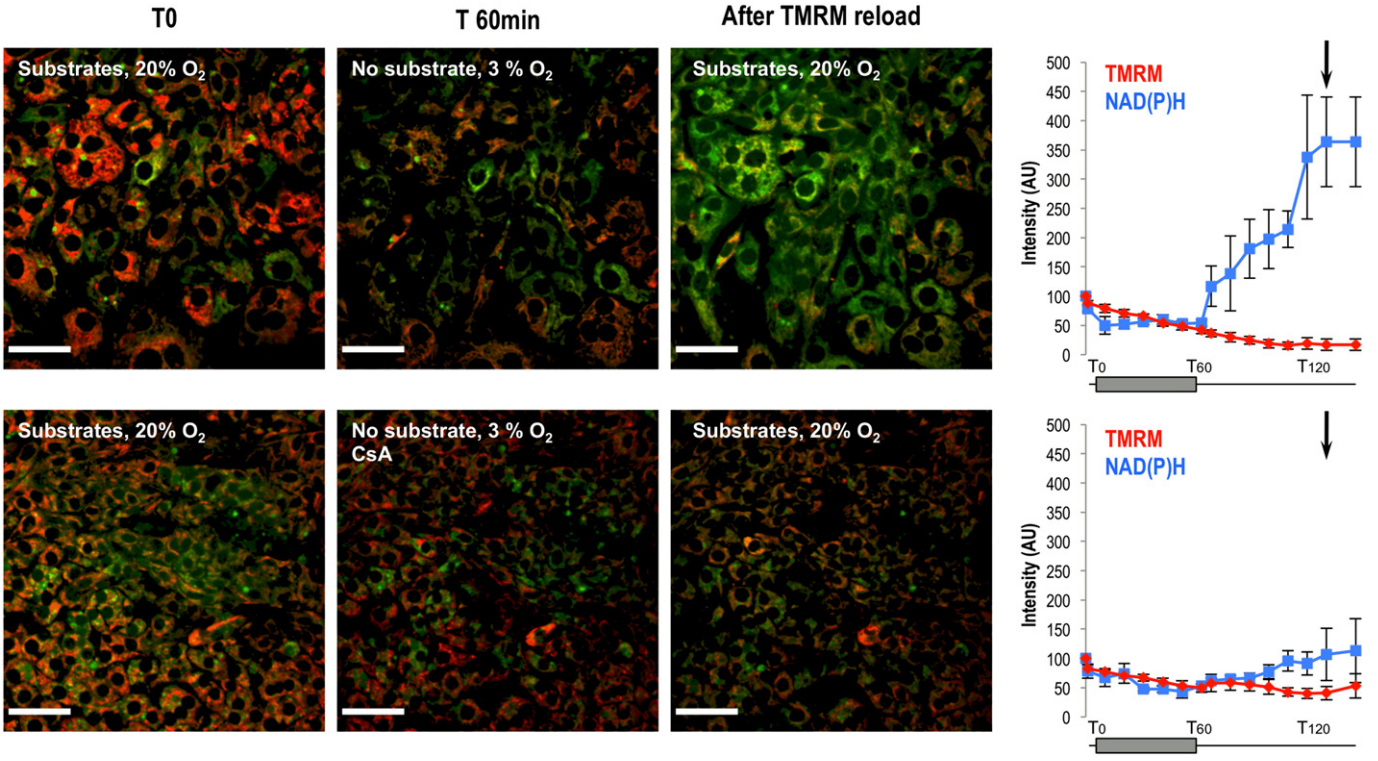

Fig. 6. Effects of simulated hypoxia reperfusion on PTP status in human islets of Langerhans. Human islets of Langerhans incubated in the absence or presence of 1 μM CsA for 1 h loaded with 10 nM TMRM for 1 h were submitted to the indicated conditions for 60 min, followed by the restoration of normal conditions of incubation for 60 min. The fluorescence of NAD(P)H (pseudo color green) and TMRM (pseudo color red) were imaged every 10 min during the procedure and the two channels were merged. Curves represent the results of three different experiments. Results are presented as mean ± SEM of 3 different experiments. Blue symbols, NAD(P)H; red symbols, TMRM; arrow, TMRM reload.

Acknowledgement

S.L. performed energy substrate and oxygen derpivation, flow cytometry and confocal microscopy expirements. C.C-R. performed image analysis. F.L. and C.L. performed cell culture. M.J.R. and T.B. performed human islet isolation and provided human islets. S.L., L.A., P.Y.B. and E.F. participated equally to the writting of the article.

This work was supported by grants from INSERM and the Ministère de l'Enseignement de la Recherche et de la Technologie (MERT). SL was supported and by fellowships from AGIRaDom. Human islets were obtained thanks to grant no. 31-2008-416 from the Juvenile Diabetes Research Foundation. We thank Christophe Cottet for the English corrections to this paper and Stéphane Attias for adenosine nucleotide content determination.

Guarantor's name: Dr Sandrine LABLANCHE.

References

[1] M. Biarnes, M. Montolio, V. Nacher, M. Raurell, J. Soler, E. Montanya, Beta-cell death and mass in syngeneically transplanted islets exposed to short- and long-term hyperglycemia, Diabetes 51 (2002) 66–72.
[2] F.C. Brunicardi, Clinical islet transplantation: a consortium model, Transplant. Proc. 28 (1996) 2138–2140.
[3] L. Jansson, The regulation of pancreatic islet blood flow, Diabetes Metab. Rev. 10 (1994) 407–416.
[4] H.A. Clayton, N.J. London, Survival and function of islets during culture, Cell Transplant. 5 (1996) 1–12 (discussion 13–17, 19).
[5] E.L. Parr, K.M. Bowen, K.J. Lafferty, Cellular changes in cultured mouse thyroid glands and islets of Langerhans, Transplantation 30 (1980) 135–141.
[6] M.D. Menger, S. Jaeger, P. Walter, G. Feifel, F. Hammersen, K. Messmer, Angiogenesis and hemodynamics of microvasculature of transplanted islets of Langerhans, Diabetes 38 (Suppl. 1) (1989) 199–201.
[7] J.O. Sandberg, B. Margulis, L. Jansson, R. Karlsten, O. Korsgren, Transplantation of fetal porcine pancreas to diabetic or normoglycemic nude mice. Evidence of a rapid engraftment process demonstrated by blood flow and heat shock protein 70 measurements, Transplantation 59 (1995) 1665–1669.
[8] R. Bolli, E. Marban, Molecular and cellular mechanisms of myocardial stunning, Physiol. Rev. 79 (1999) 609–634.
[9] G. Loor, J. Kondapalli, H. Iwase, N.S. Chandel, G.B. Waypa, R.D. Guzy, T.L. Vanden Hoek, P.T. Schumacker, Mitochondrial oxidant stress triggers cell death in simulated ischemia–reperfusion, Biochim. Biophys. Acta 1813 (2011) 1382–1394.
[10] E. Robin, R.D. Guzy, G. Loor, H. Iwase, G.B. Waypa, J.D. Marks, T.L. Hoek, P.T. Schumacker, Oxidant stress during simulated ischemia primes cardiomyocytes for cell death during reperfusion, J. Biol. Chem. 282 (2007) 19133–19143.
[11] T.L. Vanden Hoek, C. Li, Z. Shao, P.T. Schumacker, L.B. Becker, Significant levels of oxidants are generated by isolated cardiomyocytes during ischemia prior to reperfusion, J. Mol. Cell. Cardiol. 29 (1997) 2571–2583.
[12] U.M. Fischer, C.S. Cox Jr., S.J. Allen, R.H. Stewart, U. Mehlhorn, G.A. Laine, The antioxidant *N*-acetylcysteine preserves myocardial function and diminishes oxidative stress after cardioplegic arrest, J. Thorac. Cardiovasc. Surg. 126 (2003) 1483–1488.
[13] M.C. McDonald, K. Zacharowski, J. Bowes, S. Cuzzocrea, C. Thiemermann, Tempol reduces infarct size in rodent models of regional myocardial ischemia and reperfusion, Free Radic. Biol. Med. 27 (1999) 493–503.
[14] P. Bernardi, A. Krauskopf, E. Basso, V. Petronilli, E. Blachly-Dyson, F. Di Lisa, M.A. Forte, The mitochondrial permeability transition from in vitro artifact to disease target, FEBS J. 273 (2006) 2077–2099.
[15] M. Zoratti, I. Szabo, The mitochondrial permeability transition, Biochim. Biophys. Acta 1241 (1995) 139–176.
[16] C. Batandier, B. Guigas, D. Detaille, M.Y. El-Mir, E. Fontaine, M. Rigoulet, X.M. Leverve, The ROS production induced by a reverse-electron flux at respiratory-chain complex 1 is hampered by metformin, J. Bioenerg. Biomembr. 38 (2006) 33–42.
[17] D.B. Zorov, C.R. Filburn, L.O. Klotz, J.L. Zweier, S.J. Sollott, Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes, J. Exp. Med. 192 (2000) 1001–1014.
[18] S.P. Kantrow, C.A. Piantadosi, Release of cytochrome *c* from liver mitochondria during permeability transition, Biochem. Biophys. Res. Commun. 232 (1997) 669–671.
[19] S. Desagher, J.C. Martinou, Mitochondria as the central control point of apoptosis, Trends Cell Biol. 10 (2000) 369–377.
[20] X. Saelens, N. Festjens, L. Vande Walle, M. van Gurp, G. van Loo, P. Vandenabeele, Toxic proteins released from mitochondria in cell death, Oncogene 23 (2004) 2861–2874.
[21] E.J. Griffiths, A.P. Halestrap, Mitochondrial non-specific pores remain closed during cardiac ischaemia, but open upon reperfusion, Biochem. J. 307 (Pt 1) (1995) 93–98.
[22] F. Di Lisa, P. Bernardi, Mitochondria and ischemia–reperfusion injury of the heart: fixing a hole, Cardiovasc. Res. 70 (2006) 191–199.
[23] M.G. Perrelli, P. Pagliaro, C. Penna, Ischemia/reperfusion injury and cardioprotective mechanisms: role of mitochondria and reactive oxygen species, World J. Cardiol. 3 (2011) 186–200.

[24] L. Argaud, O. Gateau-Roesch, D. Muntean, L. Chalabreysse, J. Loufouat, D. Robert, M. Ovize, Specific inhibition of the mitochondrial permeability transition prevents lethal reperfusion injury, J. Mol. Cell. Cardiol. 38 (2005) 367–374.
[25] G.S. Bhamra, D.J. Hausenloy, S.M. Davidson, R.D. Carr, M. Paiva, A.M. Wynne, M.M. Mocanu, D.M. Yellon, Metformin protects the ischemic heart by the Akt-mediated inhibition of mitochondrial permeability transition pore opening, Basic Res. Cardiol. 103 (2008) 274–284.
[26] E.J. Griffiths, A.P. Halestrap, Protection by cyclosporin A of ischemia/reperfusion-induced damage in isolated rat hearts, J. Mol. Cell. Cardiol. 25 (1993) 1461–1469.
[27] D.J. Hausenloy, H.L. Maddock, G.F. Baxter, D.M. Yellon, Inhibiting mitochondrial permeability transition pore opening: a new paradigm for myocardial preconditioning? Cardiovasc. Res. 55 (2002) 534–543.
[28] C. Piot, P. Croisille, P. Staat, H. Thibault, G. Rioufol, N. Mewton, R. Elbelghiti, T.T. Cung, E. Bonnefoy, D. Angoulvant, C. Macia, F. Raczka, C. Sportouch, G. Gahide, G. Finet, X. Andre-Fouet, D. Revel, G. Kirkorian, J.P. Monassier, G. Derumeaux, M. Ovize, Effect of cyclosporine on reperfusion injury in acute myocardial infarction, N. Engl. J. Med. 359 (2008) 473–481.
[29] C. Weinbrenner, G.S. Liu, J.M. Downey, M.V. Cohen, Cyclosporine A limits myocardial infarct size even when administered after onset of ischemia, Cardiovasc. Res. 38 (1998) 678–684.
[30] A. Barbu, N. Welsh, J. Saldeen, Cytokine-induced apoptosis and necrosis are preceded by disruption of the mitochondrial membrane potential (Deltapsi(m)) in pancreatic RINm5F cells: prevention by Bcl-2, Mol. Cell. Endocrinol. 190 (2002) 75–82.
[31] S. Lablanche, C. Cottet-Rousselle, F. Lamarche, P.Y. Benhamou, S. Halimi, X. Leverve, E. Fontaine, Protection of pancreatic INS-1 beta-cells from glucose- and fructose-induced cell death by inhibiting mitochondrial permeability transition with cyclosporin A or metformin, Cell Death Dis. 2 (2011) e134.
[32] V. Koshkin, G. Bikopoulos, C.B. Chan, M.B. Wheeler, The characterization of mitochondrial permeability transition in clonal pancreatic beta-cells. Multiple modes and regulation, J. Biol. Chem. 279 (2004) 41368–41376.
[33] L. Marselli, L. Trincavelli, C. Santangelo, R. Lupi, S. Del Guerra, U. Boggi, A. Falleni, V. Gremigni, F. Mosca, C. Martini, F. Dotta, U. Di Mario, S. Del Prato, P. Marchetti, The role of peripheral benzodiazepine receptors on the function and survival of isolated human pancreatic islets, Eur. J. Endocrinol. 151 (2004) 207–214.
[34] K. Fujimoto, Y. Chen, K.S. Polonsky, G.W. Dorn II, Targeting cyclophilin D and the mitochondrial permeability transition enhances beta-cell survival and prevents diabetes in Pdx1 deficiency, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 10214–10219.
[35] T. Linn, J. Schmitz, I. Hauck-Schmalenberger, Y. Lai, R.G. Bretzel, H. Brandhorst, D. Brandhorst, Ischaemia is linked to inflammation and induction of angiogenesis in pancreatic islets, Clin. Exp. Immunol. 144 (2006) 179–187.
[36] Y. Tanioka, B.J. Hering, D.E. Sutherland, J.W. Kronson, Y. Kuroda, T.R. Gilmore, T.C. Aasheim, M.C. Rusten, J.P. Leone, Effect of pancreatic warm ischemia on islet yield and viability in dogs, Transplantation 64 (1997) 1637–1641.
[37] M. Asfari, D. Janjic, P. Meda, G. Li, P.A. Halban, C.B. Wollheim, Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines, Endocrinology 130 (1992) 167–178.
[38] P. Bucher, Z. Mathe, P. Morel, D. Bosco, A. Andres, M. Kurfuest, O. Friedrich, N. Raemsch-Guenther, L.H. Buhler, T. Berney, Assessment of a novel two-component enzyme preparation for human islet isolation and transplantation, Transplantation 79 (2005) 91–97.
[39] C. Ricordi, E.H. Finke, E.S. Dye, C. Socci, P.E. Lacy, Automated isolation of mouse pancreatic islets, Transplantation 46 (1988) 455–457.
[40] J.F. Dumas, L. Argaud, C. Cottet-Rousselle, G. Vial, C. Gonzalez, D. Detaille, X. Leverve, E. Fontaine, Effect of transient and permanent permeability transition pore opening on NAD(P)H localization in intact cells, J. Biol. Chem. 284 (2009) 15117–15125.
[41] D. Argaud, H. Roth, N. Wiernsperger, X.M. Leverve, Metformin decreases gluconeogenesis by enhancing the pyruvate kinase flux in isolated rat hepatocytes, Eur. J. Biochem./FEBS 213 (1993) 1341–1348.
[42] F. Lamarche, C. Carcenac, B. Gonthier, C. Cottet-Rousselle, C. Chauvin, L. Barret, X. Leverve, M. Savasta, E. Fontaine, Mitochondrial permeability transition pore inhibitors prevent ethanol-induced neuronal death in mice, Chem. Res. Toxicol. 26 (2013) 78–88.
[43] R. Perez-Tomas, Multidrug resistance: retrospect and prospects in anti-cancer drug treatment, Curr. Med. Chem. 13 (2006) 1859–1876.
[44] H. Friberg, M. Ferrand-Drake, F. Bengtsson, A.P. Halestrap, T. Wieloch, Cyclosporin A, but not FK 506, protects mitochondria and neurons against hypoglycemic damage and implicates the mitochondrial permeability transition in cell death, J. Neurosci. 18 (1998) 5151–5159.
[45] N. Sekine, V. Cirulli, R. Regazzi, L.J. Brown, E. Gine, J. Tamarit-Rodriguez, M. Girotti, S. Marie, M.J. MacDonald, C.B. Wollheim, et al., Low lactate dehydrogenase and high mitochondrial glycerol phosphate dehydrogenase in pancreatic beta-cells. Potential role in nutrient sensing, J. Biol. Chem. 269 (1994) 4895–4902.
[46] A.P. Halestrap, M.C. Wilson, The monocarboxylate transporter family—role and regulation, IUBMB Life 64 (2012) 109–119.
[47] C. Zhao, M.C. Wilson, F. Schuit, A.P. Halestrap, G.A. Rutter, Expression and distribution of lactate/monocarboxylate transporter isoforms in pancreatic islets and the exocrine pancreas, Diabetes 50 (2001) 361–366.
[48] C. Batandier, X. Leverve, E. Fontaine, Opening of the mitochondrial permeability transition pore induces reactive oxygen species production at the level of the respiratory chain complex I, J. Biol. Chem. 279 (2004) 17197–17204.
[49] S.J. Clarke, I. Khaliulin, M. Das, J.E. Parker, K.J. Heesom, A.P. Halestrap, Inhibition of mitochondrial permeability transition pore opening by ischemic preconditioning is probably mediated by reduction of oxidative stress rather than mitochondrial protein phosphorylation, Circ. Res. 102 (2008) 1082–1090.
[50] E. Cadenas, A. Boveris, C.I. Ragan, A.O. Stoppani, Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome *c* reductase from beef-heart mitochondria, Arch. Biochem. Biophys. 180 (1977) 248–257.
[51] A. Boveris, B. Chance, The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen, Biochem. J. 134 (1973) 707–716.
[52] S.S. Korshunov, V.P. Skulachev, A.A. Starkov, High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria, FEBS Lett. 416 (1997) 15–18.
[53] M.Y. El-Mir, V. Nogueira, E. Fontaine, N. Averet, M. Rigoulet, X. Leverve, Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I, J. Biol. Chem. 275 (2000) 223–228.
[54] P. Pasdois, J.E. Parker, E.J. Griffiths, A.P. Halestrap, The role of oxidized cytochrome *c* in regulating mitochondrial reactive oxygen species production and its perturbation in ischaemia, Biochem. J. 436 (2011) 493–505.
[55] S. Merani, A.M. Shapiro, Current status of pancreatic islet transplantation, Clin. Sci. (Lond.) 110 (2006) 611–625.
[56] B.W. Paty, J.S. Harmon, C.L. Marsh, R.P. Robertson, Inhibitory effects of immunosuppressive drugs on insulin secretion from HIT-T15 cells and Wistar rat islets, Transplantation 73 (2002) 353–357.
[57] E. Oetjen, D. Baun, S. Beimesche, D. Krause, I. Cierny, R. Blume, C. Dickel, S. Wehner, W. Knepel, Inhibition of human insulin gene transcription by the immunosuppressive drugs cyclosporin A and tacrolimus in primary, mature islets of transgenic mice, Mol. Pharmacol. 63 (2003) 1289–1295.
[58] M.A. Ajabnoor, M.M. El-Naggar, A.A. Elayat, A. Abdulrafee, Functional and morphological study of cultured pancreatic islets treated with cyclosporine, Life Sci. 80 (2007) 345–355.