Mitochondrial Oxidative Phosphorylation Injuries Occurring In Situ and In Vitro

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IT IS well known that ischemia/reperfusion (I/R) disrupts the cellular energetics with, among other things, a fall in adenine nucleotide (AdN) concentrations, a rise in cytosolic calcium and inorganic phosphate (Pi) contents, and an oxidative stress.[1] Some critical but not well-defined phenomena form the borderline between recovery and irreversible injury. Mitochondria are proposed to be the site of these determinants of irreversibility preceding the terminal events of necrosis. There are two main ways to study the mitochondrial oxidative phosphorylation (OXPHOS) damages: (1) by isolating mitochondria from organs submitted to various treatments and measuring their bioenergetic parameters and (2) by mimicking in vitro the situations encountered by mitochondria in the cell during ischemia/reperfusion, namely the oxygen depletion and refill, the AdN fall, and the Pi, Ca^{2+}, and peroxide increases.

The first way is the simplest but not the best because (1) the ischemic mitochondria are already reoxygenated during their purification, (2) the isolated mitochondria may be a particularly intact subpopulation, and (3) the mitochondrial respiration is measured in a condition different from the intracellular one: byproducts (Ca^{2+}, peroxides, etc) are eliminated and substrates and ADP are provided. Nevertheless, this type of approach may retain interest if one remembers at least the precise meaning of the bioenergetic parameters.

Basically, we have measured respiration rates of rabbit kidney mitochondria in various standard conditions: (1) In the absence of externally added ADP (V_4): this respiration is sustained by H^+ conductivity through the phospholipid bilayer, ADP recycling, and phosphate uptake; (2) in the presence of oligomycin that inhibits ATPase activity (V_{Olig}): this respiration is sustained by H^+ conductivity and phosphate uptake; (3) in the presence of added ADP (V_3): this active respiration is sustained by the integrated function of coupled OXPHOS, ie, the activity of AdN and Pi carriers and ATP synthase; (4) in the presence of the uncoupler FCCP that permeabilizes the phospholipid membrane to H^+ (V_{FCCP}) and allows the respiratory chain to reach its maximal activity.

From these measurements, two coupling index ratios can be calculated: the respiratory control ($RC = V_3/V_4$) and the uncoupled respiratory control ($URC = V_{FCCP}/V_{Olig}$). A stoichiometric index, the so-called ADP/O ratio, which gives the efficiency of the OXPHOS, is calculated by measuring the amount of oxygen consumed for a given amount of added ADP. From these definitions it appears that it will be possible to discriminate specifically the precise

the respiratory chain, an appearance of a rate-limiting ste outside the respiratory chain (AdN carrier or ATP syntha activities), or an impairing of redox proton pumps stoich ometry (H^+ slip).

Kidneys are submitted to various treatments: (1) th kidney is removed, immediately flushed with 9% NaCl 0°C, and stored on ice 24 or 48 hours (cold ischemia: CI the ischemic kidney is either used or ex vivo reperfuse during 30 minutes at 37°C with a saline oxygenated me dium; (2) the renal artery is clamped and ischemia occurs situ for 45 minutes (warm ischemia: WI), the flushed kidne is then either used immediately or further stored 48 hou at 0°C; (3) the references are obtained by use of a fresh flushed kidney.

After 48 hours of CI we have observed decreases V_{FCCP} (50%), V_3 (50%), and V_4 (30%) that indicate a together an alteration of oxido-reductase activities. There a decrease of the ADP/O ratio (20%) that can only be du to H^+ slip because V_{Olig} does not significantly chang meaning no increase in H^+ permeability. The decrease RC and URC is the result of V_3 and V_{FCCP} decreases ar obviously not an uncoupling. A warm ischemia leads similar results. Warm ischemia followed by 48 hours of C reinforces the decreases and induces a significant increas of V_{Olig}. The latter indicates a partial uncoupling ar therefore the deleterious effect of the WI and CI combin tion. CI followed by 30 minutes reperfusion shows a signi icant decrease of V_{Olig} compared with CI alone. All thes observations together indicate that during CI there is n uncoupling of the mitochondria even if RC, URC, ar ADP/O decrease. In fact, the results may be explained by decrease of the oxido-reductase activities and the occu rence of proton slip at the level of the proton pumps.

We have studied the in vitro recovery of ADP depende respiration and ADP/O ratio induced by successive AD pulses and free fatty acid bovine serum albumin (BSA preincubation of the mitochondria. Four ADP pulses i duce both a 60% increase of V_3 and a 20% increase

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ADP/O ratio after CI only. Three minutes preincubation with 1% BSA induces both a 40% increase of V_3 and a 30% increase of ADP/O ratio after CI only. After the other organ treatments a less extended recovery of V_3 is observed. No changes are observed with control mitochondria. These in vitro recoveries indicate that during CI some factors are produced that impair the oxido-reductase activities and associated proton pumps. These factors can be eliminated by pulsed activation of the oxidative phosphorylation or by extraction from the membrane by free fatty acid BSA.

The second way to study OXPHOS damages may help to determine the starting point of irreversible injury during I/R pathogenesis. I/R leads to metabolic derangements[1] that if reproduced in vitro can induce the opening of a Ca^{2+} dependent permeability transition pore (PTP) in the inner membrane. This is a transient event being reversed by EGTA. When this pore opens, the mitochondrial transmembrane gradients necessary for the ATP synthesis collapse and this fall in the energy supply could be the major cause of cell death. Recently there was been an increasing interest in the possible participation of the AdN carrier in the opening of PTP that occurs when mitochondria are loaded with high Ca^{2+} concentrations. Indeed, some ligands of the mitochondrial AdN carrier are found to influence the pore opening: the inhibitor bongkrekate and ADP decrease the probability of the pore opening while other inhibitors, atractyloside and carboxyatractyloside (CAT), increase it. This has suggested that the AdN carrier is in relationship with the pore or is a part of the pore itself.[2]

Our first experimental protocol consists of two steps: (1) isolated rat liver mitochondria are preincubated 3 minutes at 30°C, without respiratory substrate, in the absence or presence of different Ca^{2+} concentrations; the excess Ca^{2+} is then chelated by EGTA, and (2) after 2 minutes, succinate and ADP are added to determine the V_4 and V_3 respirations, respectively. As CAT is a high affinity inhibitor of the AdN carrier, the titration of ADP-dependent respiration (V_3-V_4) with CAT permits the evaluation of the mitochondrial CAT binding sites (concentration of CAT necessary to block the respiration). Mitochondria incubated as described above present a 40% inhibition of the ADP dependent respiration in the absence of CAT and a decrease of 50% in the amount of CAT binding sites. Because under our experimental conditions the ADP translocation is the limiting step of the ADP stimulated respiration (CAT titration curve is a straight line), and the rate of respiration can be directly related to the amount of active carrier. The effects described here are completely abolished if either EGTA or cyclosporin A (CyA), a known inhibitor of the PTP, are present in the preincubation.

In a second protocol, we have investigated pore opening and closure in rat heart mitochondria in the presence of Ca^{2+}, phosphate, and t-butyl hydroperoxide. The pore opening is checked by the [^{14}C]sucrose permeation into matrix space and its reversibility by CyA. Our results show that ADP uptake by heart mitochondria is affected by pore opening: the rate of uptake is decreased in the presence of pore activators and increased in the presence of CyA, the internal exchangeable ADP concentration being the same. These results directly show that pore opening decreases the activity of the AdN carrier. Thus, by in vitro simulation of in situ conditions encountered by mitochondria after I/R, it is possible to correlate PTP formation and opening with a decrease in the active AdN carrier.

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