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SHORT COMMUNICATIONS

Evaluation of some mitochondrial functions following liver perfusion with perfluorotributylamine emulsions

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The term perfluorocarbons identifies a group of compounds obtained from hydrocarbons, amines or ethers by complete substitution of hydrogens with fluorine [1]. In the last decade renewed attention has been focused to perfluorocarbons because of their ability to dissolve significant quantities of oxygen and carbon dioxide. For this reason emulsions of these compounds have been utilized as blood substitutes both for oxygen supply and circulating volume replacing. Owing to the complete immiscibility with water, the preparation of reasonably stable emulsions of perfluorocarbons requires addition of tensioactives, which adds further problems for the toxicity or side effects of the latter compounds (for a review see Ref. 1).

Experiments on perfused rat liver in which the rate of albumine synthesis, lysine incorporation and bile secretion have been evaluated led to the conclusion that upon prolonged perfusion with perfluorocarbon liver was in a good functional state [2].

In order to evaluate further the use of perfluorocarbon emulsions as oxygen supplying systems and their possible damaging action, we have examined some of the most significant energy-linked processes of mitochondria isolated from rat livers perfused *in situ* for 60 min with a perfluorotributylamine emulsion.

The results reported in the present paper clearly show that perfluorotributylamine emulsions significantly protect mitochondria, isolated from perfused livers, respiring in the presence of NAD-linked substrates.

Materials and methods

The perfusion medium (Krebs–Henseleit [3] + MOPS + BSA)* contained 119 mM NaCl, 2.38 mM KCl, 1.16 mM KH_2PO_4, 1.16 mM $MgSO_4$, 25 mM $NaHCO_3$, 3 mM MOPS, 3.4% BSA (Fraction V, essentially free of fatty acids) (pH 7.4). Perfluorotributylamine was emulsified in the medium by means of Pluronic F-68 (SERVA, Heidelberg/New York) according to the procedure of Lamers and Hulsmann [4] modified as follows. Twenty-two milliliters of perfluorotributylamine were added to 200 ml of the perfusion medium (without albumin) containing 4 g of Pluronic F-68 and the mixture stirred in an ice bath. After sonication (Branson Sonifier mod. B-12) for 4 periods of 5 min each at 4.5 A (keeping the suspension at the temperature of the ice bath) the emulsion was washed twice by centrifugation at 10,000 *g*. The pellets were then resuspended in the perfusion medium with albumin and sonified for 4 periods of 3 min at 4.5 A. After 5 min of centrifugation at 600 *g*, the supernatant was adjusted at pH 7.4, filtered through Whatman 41 paper and used for the perfusion experiment.

Male Wistar rats (24-hr starved) weighing 210–220 g were anesthetized by intraperitoneal administration of Ketalar® (17.5 mg/100 g of body weight) and treated (i.p.) with 500 U.I. of heparine/100 g of body weight. Livers were perfused *in situ* at 37° with the Krebs–Henseleit + MOPS + BSA medium or alternatively with the PFTBA emulsion essentially as described by Mortimore [5]. The medium flow was 8 ml/min; during the initial 5 min the perfusate was discarded and the perfusion was then continued for 60 min by recirculating 120 ml of medium.

The oxygen content of the recirculating perfusion medium was monitored by a Clark's electrode (Yellow Springs Instr. Co., Inc., OH) in a thermostated flow-through oxygraph cell inserted in the perfusion circuit either at the inflow or at the outflow of the liver.

Mitochondria were isolated from perfused livers by differential centrifugation in 0.25 M sucrose, 2.5 mM Na-HEPES (pH 7.4), 0.25 mM EGTA (EGTA was omitted in the final washing). Mitochondrial proteins were assayed according to Gornall *et al.* [6].

The respiratory rates and $\Delta\Psi$ of isolated mitochondria were measured at 25° in the following medium: 100 mM sucrose, 50 mM KCl, 10 mM K-phosphate, 2 mM $MgSO_4$, 1 mM EDTA, 15 mM Tris–HCl (pH 7.4) and either 5 mM glutamate + 1 mM malate or 5 mM succinate + 1.25 μM rotenone. Further additions are indicated in the legends to figures.

Oxygen consumption traces were obtained using a Clark oxygen electrode. Mitochondrial transmembrane electrical potentials were measured by monitoring the TPP^+ distribution across the mitochondrial inner membrane with a TPP^+-selective electrode according to Kamo *et al.* [7]. The electrode potential was linear with respect to log [TPP^+] within the experimental range, with a slope of 59 mV in agreement with Nernst equation. The $\Delta\Psi$ values were calculated from the difference of the electrode potentials measured in the presence and in the absense of mitochondria according to Palmieri and Klingenberg [8].

Results

The oxygen dissolved in the perfusion medium entering the liver was 445 nmoles/ml both for the Krebs–Henseleit + MOPS + BSA medium and for the PFTBA emulsion. In the perfusate leaving the liver the oxygen was reduced to 11–13 nmoles/ml in the perfusion experiments performed with the Krebs–Henseleit + MOPS + BSA medium, and to 51–55 nmoles/ml in the experiments with the PFTBA emulsion. It has to be noted that Clark's electrode is sensitive only to the oxygen dissolved in the aqueous phase, but not to that sequestered within the PFTBA micelles. However, the oxygen enclosed in the perfluorocarbon micelles is in equilibrium with that present in the aqueous

* Abbreviations used: BSA, bovine serum albumine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; KH- or PFTBA-mitochondria, mitochondria prepared from livers perfused respectively with Krebs–Henseleit (+ BSA + MOPS) medium or perfluorotributylamine emulsion; MOPS, 3-(*N*-morpholino)propane sulfonic acid; PFTBA, perfluorotributylamine; RCR, respiratory control ratio; TPP^+, tetraphenylphosphonium; $\Delta\Psi$, electrical transmembrane potential.

phase and is available to the perfused tissue. Implicitly, mitochondria in livers perfused with PFTBA emulsions should be better oxygenated than those in livers perfused with the Krebs–Henseleit + BSA + MOPS medium.

As shown in Fig. 1A mitochondria isolated from livers perfused with Krebs–Henseleit + MOPS + BSA medium (KH-mitochondria) and incubated in the presence of glutamate + malate exhibited a low respiratory rate, which was completely insensitive to ADP or CCCP addition. On the contrary the respiration of mitochondria isolated from livers perfused with the PFTBA emulsion (PFTBA-mitochondria) was significantly higher and significantly increased by addition of either ADP or CCCP.

With succinate as oxidizable substrate the respiration rates in the absence of added ADP and in the presence of 0.15 mM ADP or of 1.6 μM CCCP were almost the same in KH- and PFTBA-mitochondria.

These results have been confirmed by measuring the $\Delta\Psi$ of KH- or PFTBA-mitochondria in the presence of either of the two substrates. As shown in Fig. 2 the maximal value attained by mitochondria energized by glutamate + malate in the absence of added ADP was 170 mV irrespective of whether they had been isolated from PFTBA or Krebs–Henseleit perfused livers. However, the restoration of $\Delta\Psi$ following the drop typical of a phosphorylation pulse [7], induced by ADP addition, was almost suppressed in KH-mitochondria whereas PFTBA-mitochondria were capable of partially restoring $\Delta\Psi$ to values close to those pre-existing the ADP addition. In both types of mitochondrial preparations the complete recovery of initial $\Delta\Psi$ was achieved only following the addition of oligomycin.

When succinate was the substrate, the transmembrane electrical potential was maintained in the normal range [7] either in KH- or in PFTBA-mitochondria and the restoration of $\Delta\Psi$ upon ADP addition occurred promptly in both conditions. Thus where energized by succinate mitochondria, irrespective of the perfusion system of the livers, preserved the intactness of their inner membrane and the efficiency of energy-linked processes.

Discussion

The results here reported clearly indicate that the presence of PFTBA in the perfusion medium exerts a significant protection on mitochondria, as revealed by the efficiency of the energy-linked processes upon isolation from the perfused liver. Very likely the higher amount of oxygen transported by the PFTBA emulsion is relevant in this connection. Evidently PFTBA may provide to the perfused liver sufficient oxygen to prevent the damages of hypoxia occurring when the tissue is perfused under suboptimal conditions of oxygen supply as it seems to happen in the absence of PFTBA.

The results also indicate that the PFTBA emulsion is apparently devoid of noxious effects on *in situ* mitochondria. It has to be noted that these results have been obtained using as oxidizable substrates for isolated mitochondria glutamate + malate, that is NAD-linked substrates. With succinate as the substrate no alteration of the energy-linked functions has been observed in mitochondria isolated from either PFTBA or Krebs–Henseleit perfused livers. Assuming that the main difference between KH- and PFTBA-mitochondria was the amount of oxygen delivered to the organ during the perfusion period (see Results), it would appear that a critical oxygen deprivation might specifically affect the NAD-dependent respiration.

Malis and Bonventre [9] have previously shown that under ischemic conditions alterations of the electron transport chain were not manifest with succinate as substrate. The respiratory and energy-linked alterations occurring with glutamate + malate might be due to a release of NAD(P) from mitochondria. This would indicate that oxygen deprivation makes mitochondrial membranes per-

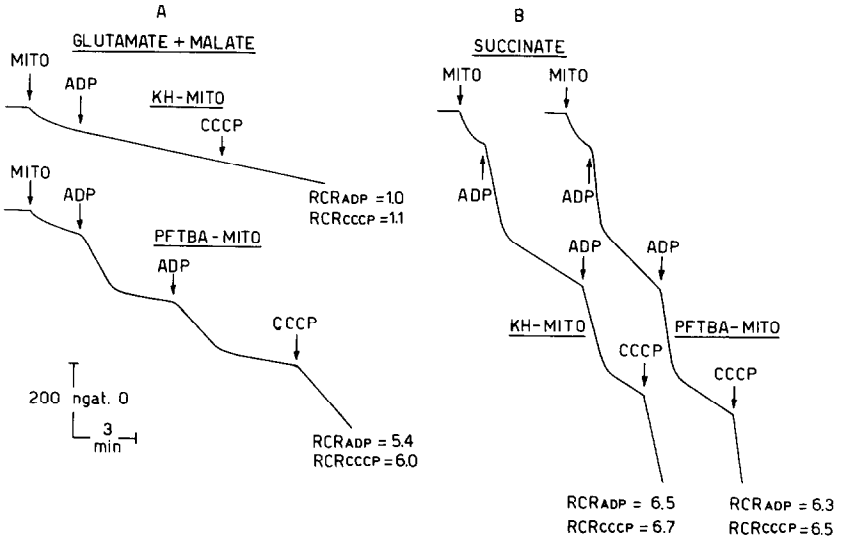

Fig. 1. Oxygen consumption traces and respiratory control ratios of mitochondria obtained from rat livers perfused with control or perfluorotributylamine-containing medium. One milligram of mitochondrial proteins/ml, 150 μM ADP, or 1.6 μM CCCP were added when indicated by the arrows. The oxygen traces represent a typical set of experiments chosen among those obtained with 8 different preparations of mitochondria prepared from Krebs–Henseleit (+ BSA + MOPS) perfused livers and 8 different preparations of mitochondria prepared from livers perfused with perfluorotributylamine-containing medium. In each of the KH- or PFTBA-mitochondrial suspensions tested the respiratory rates in the absence, or in the presence of either ADP or CCCP did not differ from those reported in the figure for more than 4%. RCR_{ADP}: ratio between the oxygen consumption rate in the presence and in the absence of added ADP. RCR_{CCCP}: ratio between the oxygen consumption rate in the presence and in the absence of added ADP and CCCP.

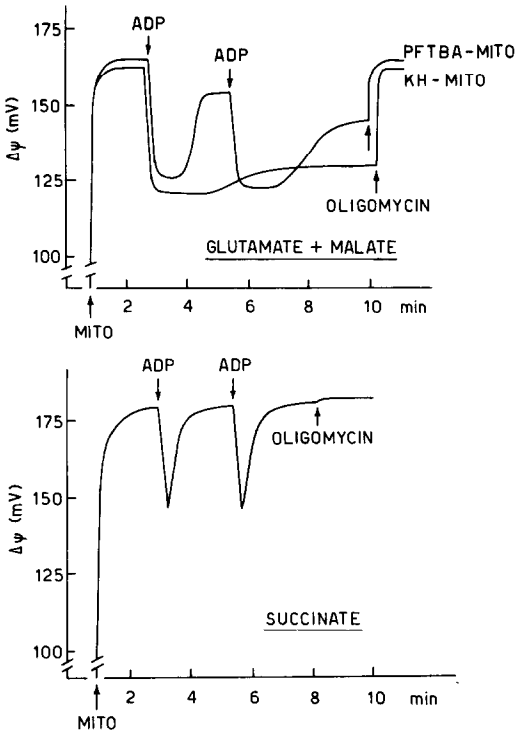

Fig. 2. Transmembrane electrical potential ($\Delta\Psi$) of mitochondria isolated from rat livers perfused with control or perfluorotributylamine-containing medium. One milligram of mitochondrial protein/ml, 150 μM ADP or 1 μg/ml oligomycin were added when indicated by the arrows. The $\Delta\Psi$ traces have been redrawn from the experimental traces using a linear $\Delta\Psi$ scale. The $\Delta\Psi$ traces have been obtained in parallel with the respiratory traces in Fig. 1. In each of the mitochondrial suspensions tested (obtained from livers perfused with Krebs–Hensleit (+ BSA + MOPS) medium or perfluorotributylamine-emulsion) the values measured before and after ADP addition did not differ from those reported in the figure for more than 2%.

meable to intramitochondrial NAD(P), or activates the hydrolase cleaving NAD into nicotinamide plus ADP-ribose [10].

The incapability of ADP to stimulate the respiration supported by malate + glutamate in KH-mitochondria (Fig. 1) is in apparent contrast with the sharp decrease of $\Delta\Psi$ induced by ADP under the same conditions (Fig. 2). An explanation for this discrepancy is that NAD-linked respiration in KH-mitochondria was considerably decreased but still sufficient to create across the mitochondrial membrane a $\Delta\Psi$ of 160–165 mV. Upon addition of ADP $\Delta\Psi$ is utilized for the initial operation of the ATP synthetase, which, however, seems unable to completely phosphorylate added ADP thus preventing the restoration of the original $\Delta\Psi$. $\Delta\Psi$ restoration was possible only upon addition of oligomycin; this action might be due to oligomycin capability to inhibit either the proton reflux into mitochondria or the ATPase activity [11]. The present results add further evidence to the usefulness of PFTBA emulsions in organ perfusion in order to optimize oxygen supply.

In summary, the respiratory control ratio and the transmembrane potential were measured in mitochondrial suspensions prepared from rat livers perfused with either Krebs–Henseleit medium or perfluorotributylamine emulsions. The results indicate that the presence of the perfluorocarbon, a compound capable of dissolving oxygen, in the perfusion medium significantly protects mitochondria against the anoxic damages which become apparent when NAD-linked substrates are oxidized.

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Institute of Biological Chemistry and Centro per eo Studio della Fisiologia Mitocondriale del C.N.R. — DONATA BRANCA, GUIDO SCUTARI, NORIS SILIPRANDI
Institute of Anesthesiology — EZIO VINCENTI
Institute of Industrial Chemistry — GIAMPAOLO GAMBARETTO
University of Padova, 35131 Padova, Italy

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