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ENERGETIC BEHAVIOUR OF MITOCHONDRIA ISOLATED FROM RAT LIVERS PERFUSED WITH A PERFLUORODECALIN + *N,N*-PERFLUORODIETHYLCYCLOHEXYLAMINE EMULSION

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Abstract—Rat livers have been perfused with a saline control medium or with a perfluorocarbon emulsion containing perfluorodecalin and *N,N*-perfluorodiethylcyclohexylamine, and the respiratory rates and transmembrane electrical potentials of mitochondria isolated following perfusion have been evaluated. The results indicate that the perfluorocarbon emulsion used, by providing a good oxygen supply to the perfused liver, allowed to preserve the efficiency of mitochondrial oxidative phosphorylation.

Perfluorocompounds are a class of structurally heterogeneous molecules whose common features are the complete substitution of hydrogen with fluorine and an excellent capacity to physically dissolve oxygen without binding it. Thus perfluorocompounds may be exploited either as oxygen carriers in isolated organs or biological samples, or as blood substitutes; however, due to their water-insolubility, care has to be taken to obtain suitable emulsions [1–4].

We have previously reported that perfusion of rat livers with a perfluorotributylamine emulsion increased the efficiency of some energy-linked processes in the mitochondria thereby isolated [5]. This effect was apparent when the respiratory rate and transmembrane electrical potential were measured in mitochondria oxidizing NAD-linked substrates and was ascribed to the protection of mitochondria against the damages of hypoxia exerted by the perfluorocarbon emulsion during perfusion.

The present paper reports the behaviour of mitochondria isolated from rat livers perfused with an emulsion containing perfluorodecalin (PFD)‖ and *N,N*-perfluorodiethylcyclohexylamine (PFECEA). PFD is perhaps the best known among perfluorocompounds being one of the components of Fluosol-DA, the most widely tested of blood substitutes; on the other hand the use of PFECEA emulsions as oxygen carriers *in vivo* is relatively recent. A relevant feature of both PFD and PFECEA are their retention times in the tissues, which are substantially shorter than that of perfluorotributylamine [6, 7].

MATERIALS AND METHODS

All perfusion experiments were performed using a basal medium (Krebs–Henseleit + MOPS) containing 110 mM NaCl, 2.38 mM KCl, 1.16 mM KH_2PO_4, 1.16 mM $MgSO_4$, 25 mM $NaHCO_3$, 3 mM MOPS (pH 7.4). In control perfusions this medium was supplemented with 1 mM $CaCl_2$ and 4% (w/v) BSA (Fraction V, essentially free of fatty acids, Boehringer Mannheim GmbH, F.R.G.).

The perfluorocompound emulsions were prepared as follows. Twenty milliliters of a 1:1 (v/v) PFD:PFECEA mixture were added to 200 ml of Krebs–Henseleit + MOPS medium supplemented with 5% (w/v) Pluronic F-68 (Serva) while stirring vigorously. After cooling to 3–5° the mixture was sonified, keeping it in an ice bath, for 4 periods of 1 min (with 1 min intervals) with a Branson Sonifier model B-12 at 80 W. The emulsion was then centrifuged at 400 *g* (2°) for 10 min, the pellet discarded and the supernatant centrifuged at 5000 *g* (2°) for 10 min. The 5000 *g* pellet was resuspended in 200 ml of ice-cold Krebs–Henseleit+MOPS medium, centrifuged at 5000 *g* as before and finally resuspended in 200 ml of Krebs–Henseleit+MOPS medium supplemented with 4% BSA and 1 mM $CaCl_2$. After gentle homogenization with a Potter homogenizer (75 μm clearance) the emulsion was filtered through Whatman paper no. 42 (particles retained >2.5 μm) and used for liver perfusion. The emulsions so obtained had a fluorocrit (i.e. the per-

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‖ Abbreviations used; BSA, bovine serum albumin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; KH-mitochondria, mitochondria isolated from livers perfused with control medium; MOPS, 3-(*N*-morpholino) propanesulfonic acid; PFD, perfluorodecalin; PFECEA, *N,N*-perfluorodiethylcyclohexylamine; PFD+PFECEA-mitochondria, mitochondria isolated from livers perfused with PFD + PFECEA emulsions; RCR, respiratory control ratio; TPP^+, tetraphenylphosphonium; $\Delta\psi$, transmembrane electrical potential.

Table 1. Oxygen contents of control medium and perfluorocompounds emulsions during liver perfusion

	ngatoms O/ml of perfusion medium	
	Inflow	Outflow
Control medium	450 ± 8.0	12 ± 0.2
PFD + PFECEA emulsion	458 ± 10.0	397 ± 6.0

The oxygen dissolved in the aqueous phase of the perfusion media remained approximately constant, after an initial equilibration phase of 10–15 min, both at the inlet and at the outlet of the liver.

The reported values are the mean ± SD of 6 perfusion experiments.

centage volume occupied by the packed perfluorocarbon particles after centrifugation in hematocrit capillaries) of approximately 7% and were stable at 4° for one week; the light sediment appearing upon longer storage could be easily resuspended by a short sonication.

The oxygen capacity of PFD and PFECEA at 37° and were evaluated by measuring in a closed apparatus the pressure decrease following the equilibration of a known volume of oxygen at 1 atm with a known amount of the perfluorocompound. With this method the oxygen dissolving capacities of pure PFD and PFECEA were 50.0 and 35.5 ml of oxygen/100 ml respectively.

The techniques of recirculating liver perfusion, oxygen measurement in the perfusion media and liver mitochondria isolation have been described in detail elsewhere [5]. Isolated mitochondria were incubated at 25° in a medium containing 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. The oxygen consumption and $\Delta\psi$ traces were obtained as previously [5] using a Clark oxygen electrode and a TPP^+-selective electrode. The mitochondrial respiratory states are those defined by Chance and Williams [8].

Each set of experimental curves was repeated 6 times in duplicate, and the results obtained never differed from those shown in the figures for more than 2.5%.

RESULTS

The data reported in Table 1 indicate that the oxygen contents measured during perfusion in the control medium and in the PFD+PFECEA emulsion, although almost identical at the inflow of the liver, were markedly different at the outflow. In control perfusions the oxygen concentration in the medium was decreased of approximately 97% by the passage through the liver, while in the case of PFD + PFECEA emulsions the decrease did not reach 15%. Since the oxygen contents measured in the perfluorocarbon emulsions refer only to their aqueous phases [5] these results indicate that the oxygen amount carried by the PFD + PFECEA micelles was able, by equilibrating with that of the aqueous phase, to largely compensate for the oxygen uptake of the perfused tissue.

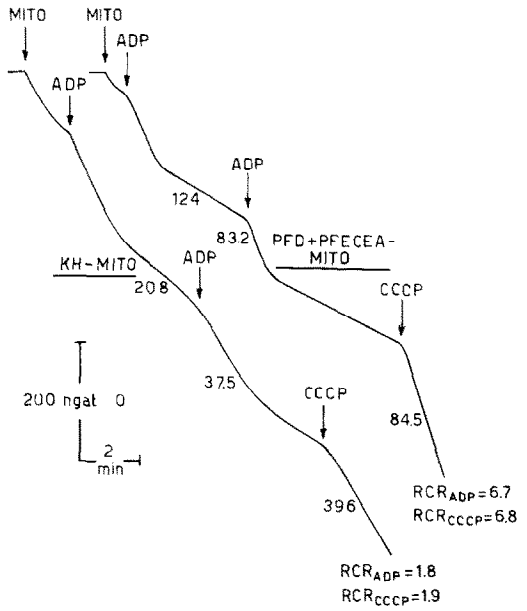

Fig. 1. Oxygen consumption of mitochondria isolated from rat livers perfused with either control medium or PFD +PFECEA emulsion. One milligram of mitochondrial protein/ml (MITO), 150 μM ADP or 1.6 μM CCCP were added when indicated by the arrows. The figures beside the experimental traces are the respiratory rates expressed as ngatoms O/min/mg of mitochondrial protein. RCR_{ADP} is the ratio between the oxygen consumption rates in State 3 and in State 4; RCR_{CCCP} is the ratio between the oxygen consumption rates in the presence of CCCP and in State 4.

Figure 1 shows the respiratory rates of mitochondria isolated from rat livers perfused for 1 hr with either control medium (KH-mitochondria) or perfluorocompound emulsions (PFD+PFECEA-mitochondria). Although both mitochondrial preparations were able to actively oxidize the NAD-dependent substrates glutamate+malate, the increase of the respiratory rate induced by the addition of either ADP or CCCP was significantly greater in PFD+PFECEA- than in KH-mitochondria, as evidenced by the respiratory control ratios. The lower RCR of KH-mitochondria, with respect to those of PFD+PFECEA-mitochondria, resulted both from an increase of State 4 respiration and from a decrease of the respiratory rates in State 3 and in the presence of CCCP.

The measurement of $\Delta\psi$ under the same experimental conditions used to monitor mitochondrial respiration (Fig. 2) shows that KH- and PFD+PFECEA-mitochondria attained the same $\Delta\psi$ value in State 4 conditions and responded to the addition of 150 μM ADP with the transient $\Delta\psi$ drop typical of ATP-synthesizing mitochondria [9]. However, if a second ADP pulse was added to the incubation mixture, PFD+PFECEA-mitochondria were still able to substantially restore the steady-state $\Delta\psi$, while in KH-mitochondria the second recovery of $\Delta\psi$ was abolished. On the other hand in both types of mitochondrial preparations the subsequent addition of oligomycin fully restored $\Delta\psi$ to the value preexisting the addition of ADP.

When succinate was used as the oxidizable sub-

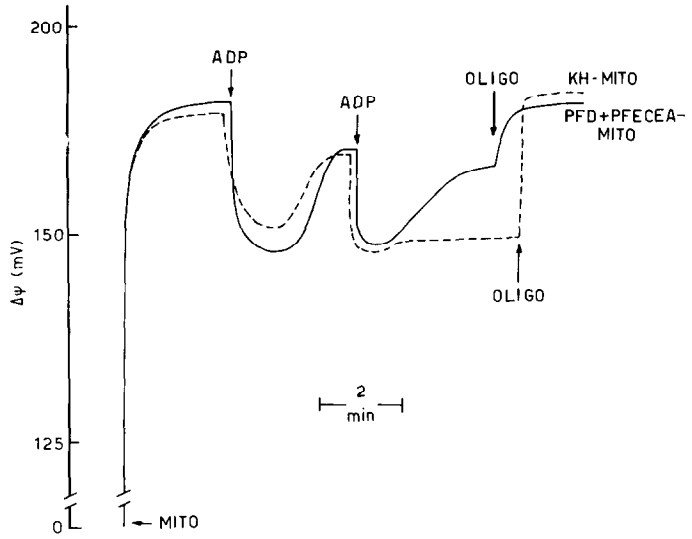

Fig. 2. Transmembrane electrical potential ($\Delta\psi$) of mitochondria isolated from rat livers perfused with either control medium or PFD+PFECEA emulsion. The $\Delta\psi$ traces have been obtained in parallel with the oxygen consumption traces of Fig. 1. One milligram of mitochondrial protein/ml (MITO), 150 μM ADP or 1 μg/ml oligomycin were added when indicated by the arrows. The $\Delta\psi$ curves have been redrawn from the experimental traces using a linear $\Delta\psi$ scale.

strate (in the presence of rotenone) mitochondria isolated from control or PFD+PFECEA-perfused livers had good respiratory control ratios and $\Delta\psi$ restoring efficiencies (data not shown) although the respiratory rate in state 4 was slightly faster in control mitochondria (32.7 ± 2.9 vs 26.2 ± 2.8 ngatoms of oxygen/min/mg of mitochondrial protein in control and PFD+PFECEA-mitochondria respectively).

DISCUSSION

The PFD+PFECEA emulsion described in the present paper showed several improved features if compared with the perfluorotributylamine emulsion previously employed by us in liver perfusion [5]. The use of glycerol, in addition to the tensioactive Pluronic F-68, in the emulsification procedure allowed to easily obtain PFD+PFECEA particles having diameters smaller than 2.5 μm. It should be noted that both glycerol and Pluronic F-68, which could be noxious to the liver, were present at high concentrations only during the formation of the perfluorocarbon-containing micelles, since they were extensively removed from the aqueous phase of the emulsions to be used in liver perfusion in the subsequent centrifugations.

The oxygen capacity at 37° of the PFD + PFECEA emulsion (calculated from the oxygen dissolving capacity of PFD, PFECEA and control medium, and the emulsion fluorocrit [1]) was 4.6 μgatoms of oxygen/ml, while that of the control medium was 2.1 μgatoms oxygen/ml [10]. Although under the experimental conditions used the recirculating perfusion media were not fully saturated with oxygen, it may reasonably assumed that the oxygen amount carried by the perfluorocarbon emulsion was at least twice that dissolved in the control medium. The measurement of the oxygen content of the aqueous phase of the perfusion media at the outlet of the livers (Table 1) clearly shows that the PFD+PFECEA emulsion was able to maintain the oxygen available to the perfused tissue at a high level, while in control medium the oxygen supply was reduced during perfusion to levels capable to significantly affect the energy metabolism and redox state of hepatocytes [11, 12].

The assessment of the functional state of liver mitochondria following perfusion with the control medium or the PFD + PFECEA emulsion strongly suggests that the higher oxygen availability provided by the perfluorocarbon emulsion is relevant in the maintenance of a good liver function during perfusion. In fact the efficiency of oxidative phosphorylation in the presence of NAD-linked substrates, as judged by the respiratory rates (Fig. 1) and transmembrane potentials (Fig. 2), appeared substantially higher in PFD + PFECEA- than in KH-mitochondria.

In particular the utilization of glutamate + malate in KH-mitochondria was significantly inhibited, as revealed by the low respiratory rate measured in the presence of the uncoupler CCCP (Fig. 1), i.e. when substrate oxidation should proceed at maximal speed. This inhibition was probably the cause of the decreased ability of KH-mitochondria to restore $\Delta\psi$ when it had been decreased by ATP synthesis (Fig. 2), i.e. when the backflow of protons through the ATP synthetase should be compensated by an increased proton pumping by the respiratory chain. This view is supported by the finding that the addition of oligomycin, which inhibits both the proton backflow through the ATP synthetase and the ATP synthetase activity [13], allowed the complete recovery of the original steady-state $\Delta\psi$ (Fig. 2). By contrast when succinate was used as the respiratory substrate the efficiency of its utilization (as judged by the oxygen consumption rates and $\Delta\psi$ values) appeared

substantially unmodified in KH-mitochondria with respect to PFD+PFECEA-mitochondria.

The susceptibility of the NAD-linked mitochondrial respiration to conditions of cellular oxygen shortage was further confirmed by the observation that when glutamate+malate were used as respiratory substrates the maintenance of a steady-state $\Delta\psi$ very close to that of PFD+PFECEA-mitochondria (Fig. 2) required in KH-mitochondria a markedly faster respiratory rate (Fig. 1), while only a slight increase was detected when mitochondria were energized with succinate. The stimulation of state 4 respiratory rate in glutamate+malate-supplemented KH-mitochondria can not be explained solely by an overall increase of the proton permeability of the inner mitochondrial membrane (which would affect state 4 respiration with both glutamate+malate and succinate), but has to be ascribed to a damage involving only the functionality of the complex I of the respiratory chain. The observed discrepancy between the behaviour of KH-mitochondria oxidizing either glutamate + malate or succinate (in the presence of rotenone) might then be explained by an intrinsic uncoupling of the redox-driven proton pump of complex I which would transfer some electrons without moving protons across the inner membrane [14]. This kind of "localized uncoupling" would increase the oxygen consumption rates of mitochondria oxidizing glutamate + malate, but would be abolished by the presence of the site I blocking agent rotenone [15]. Thus the mitochondrial injuries induced by a suboptimal oxygen availability appear to specifically affect the NAD-dependent respiration leaving succinate oxidation substantially unmodified, as previously indicated in the case of both kidney mitochondria *in vitro* [16] and of liver mitochondria isolated following persusion with a perfluorotributylamine emulsion [5].

We have previously reported that in mitochondria isolated from rat livers perfused with Krebs–Henseleit medium, under the same experimental conditions used in the present work, the oxidation of glutamate+malate was so strongly inhibited that mitochondria were totally unable to respond to the addition of either ADP or CCCP [5]. By contrast in the present work KH-mitochondria have been shown to respond, although to a limited extent, to the addition of both ADP and CCCP (Fig. 1). This discrepancy can not be ascribed to a difference in tissue oxygenation during perfusion since the oxygen content of control medium was the same in the present and in the previous work both at the inlet and at the outlet of the liver, but is probably a consequence of the addition of 1 mM $CaCl_2$ to the Krebs–Henseleit + MOPS medium in the present work. Thus the presence of physiological calcium concentrations in the perfusion medium seems essential in order to preserve the efficiency of liver mitochondria also under conditions of suboptimal oxygen supply. This observation is confirmed by the higher steady-state $\Delta\psi$ reported for KH-mitochondria in the present work (180 mV vs 165 mV of KH-mitochondria isolated following perfusion without added calcium [5]) and by their increased capacity to restore $\Delta\psi$ following a phosphorylation pulse.

The reported results confirm the usefulness of perfluorocarbon emulsions as oxygen carriers in tissue perfusion. The perfluorocompound mixture used in the present work represents an improved tool for organ perfusion, with respect to the perfluorotributylamine emulsion described previously [5], since the retention times in the tissues of both PFD (7 days) and PFECEA (62 days) are substantially shorter than that of perfluorotributylamine (approximately 500 days) [6, 7].

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