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Alteration of mitochondrial bioenergetics due to intravenous injection of a perfluorocarbon emulsion

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Abstract. Wistar albino rats were intravenously injected with 1 ml of an oxyphoretic emulsion of perfluorobutylfurane and killed 3, 7 or 30 days later. Mitochondria isolated from the liver and kidneys of treated rats showed a small decrease in the transmembrane electrical potential and a substantial depression of the rates of both ATP synthesis and ADP-stimulated respiration. These alterations in mitochondrial oxidative phosphorylation appear to be induced by perfluorocarbon and/or tensioactive molecules interacting with hydrophobic cell structures.
Key words. Mitochondria; oxidative phosphorylation; perfluorocompound injection; rat kidney; rat liver.

In the last few years considerable attention has been paid to the development of oxygen-transporting solutions and emulsions to be used systemically or for maintenance of isolated organs. At present the most feasible appear to be stroma-free hemoglobin solutions[1] and perfluorochemical emulsions[2–5]. Stroma-free hemoglobin preparations have some advantages, such as low viscosity and the ability to transport adequate volumes of oxygen at environmental oxygen tension. However, these preparations have the drawbacks of the potential toxicity of large-scale commercial products and a short intravascular half-life. An alternative is the use of perfluorochemicals (PFC), perfluorinated molecules of various size and nature which act as biochemically inert solvents for large amounts of oxygen. PFC are largely hydrophobic molecules, and their biological use therefore requires the preparation of fine emulsions by means of tensioactives and ultrasonic or mechanical procedures. The main disadvantage of many PFC emulsions is that they are able to contribute significantly to tissue oxygenation only if high oxygen concentrations are available. This problem can be overcome when PFC emulsions are used for the maintenance of isolated organs in the presence of high-oxygen gaseous mixtures. Relevant advantages of PFC emulsions include the ability of the particles to act as gas stores; the possibility of long-term storage; the lack of a need for blood typing; the absence of disease transmission; low viscosity and good oncotic activity.

We have studied the bioenergetic behaviour of mitochondria isolated from livers perfused with emulsions of either perfluorotributylamine[6] or a mixture of perfluorodecalin and N,N-perfluorodiethylcyclohexylamine[7], both emulsified with Pluronic F-68. The results indicate that the PFC emulsions preserved both mitochondrial oxidative phosphorylation and transmembrane electrical potential production and maintenance. We also examined the histological appearance of livers perfused with PFC emulsions[8] and found evidence that some particles were taken up by hepatocytes; this amount increased significantly when the removal of blood from the perfused tissue was even slightly delayed.

In this paper we report and discuss the efficiency of oxidative phosphorylation in mitochondria isolated from the liver and kidneys of rats injected with a PFC emulsion.

Materials and methods

The PFC mixture used was RM-101 (Enichem Synthesis, Italy) containing 82.1% (w/w) perfluorobutylfuran, 7.5% perfluropropylpyran and 7.4% isomers of perfluorobutylfuran differing in their alkyl substituents (each isomer represented less than 1% of the mixture, as assayed by gas-chromatography). To obtain the emulsion, 10 ml of RM-101 were added under vigorous stirring to 80 ml of Krebs-Henseleit + 3-(N-morpholino)propanesulfonic acid (MOPS) medium (110 mM NaCl, 2.38 mM KCl, 1.16mM KH_2PO_4, 1.16 mM $MgSO_4$, 25 mM $NaHCO_3$, 3 mM MOPS (pH 7.4)) supplemented with 1 g of the fluorinated tensioactive agent RM 507 (Enichem Synthesis, Italy). After cooling to 3–4 °C, the mixture was sonicated, keeping it in an ice-bath, for 3 periods of 3 min (with 10 min intervals) with a Labsonic Sonifier mod. 1510 (B. Braun Melsungen AG) set at 350 W. Four grams of bovine serum albumin (BSA, Fraction V, Boehringer Mannheim GmbH) were then dissolved in the mixture, the volume was adjusted to 100 ml and the emulsion sonicated for 3 min as before. In order to minimize the presence of fluoride ions and free tensioactive agent, which might be released during the sonication steps, the emulsion was centrifuged at 300 × *g* and the sedimented

PFC particles were mechanically resuspended (to a final volume of 100 ml) in Krebs-Henseleit + MOPS medium supplemented with 4% (w/v) BSA and previously sterilized by filtration through 0.22 μm Millipore filters; the emulsion was finally filtered through Whatman paper n. 42 (particles retained >2.5 μm). The reconstituted emulsion was layered in Petri dishes, exposed overnight to UV radiations in laminar flow hood with constant gentle shaking, and finally stored in sterile sealed vials. The final volume percentage occupied by the packed PFC particles after centrifugation in hematocrit capillaries was about 10% and remained constant for at least 3 weeks at 4 °C. The light sediment appearing upon prolonged storage was easily resuspended by short sonication.

Male Albino Wistar rats weighing about 250 g were lightly anesthetized by i.p. administration of ketamine (70 mg/kg of b. wt). Rats were injected with either 1 ml of PFC emulsion (PFC-treated) or 1 ml of Krebs-Henseleit + MOPS medium supplemented with 4% (w/v) BSA (control) by venipuncture in a caudal vein. Rats had free access to water and meal and were sacrificed by decapitation 3, 7 or 30 days after the injection. The livers and kidneys of 2 rats were pooled at each time, and control and PFC-treated mitochondria were isolated and examined in parallel.

Mitochondria were isolated from the pooled livers and kidneys by differential centrifugation in 0.25 M sucrose, 5 mM Na-(N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) (pH 7.4), 0.25 mM ethylene glycol bis (β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA). EGTA was omitted in the final washing. The protein content of mitochondrial suspensions was assayed according to Gornall et al.[9]

The respiratory rates and transmembrane electrical potential ($\Delta\psi$) of isolated mitochondria were measured at 25 °C in the following medium: 100 mM sucrose, 50 mM KCl, 10 mM KH_2PO_4, 2 mM $MgSO_4$, 1 mM ethylenediaminetetraacetic acid, 15 mM Tris-HCl (pH 7.4), 5 mM succinate, 1.25 μM rotenone, 10 μM tetraphenylphosphonium (TPP^+) chloride, 1 mg of mitochondrial protein/ml and, when present, 150 μM ADP. Oxygen consumption was followed using a Clark oxygen electrode and $\Delta\psi$ by monitoring the distribution of TPP^+ across the inner mitochondrial membrane with a TPP^+-selective electrode according to Kamo et al.[10]

The rates of ATP synthesis were evaluated following the rates of proton consumption in the basal medium from which Tris-HCl had been omitted, by means of a combined pH electrode (Ingold Messtechnik AG)[11]. The rate of ATP synthesis was taken as the difference between the rates obtained in the absence and in the presence of 1 μg oligomycin/ml.

Four rats (2 controls + 2 PFC-injected) were used for each experiment. Each reported value is the mean of the experimental values obtained with 9 different mitochondrial preparations.

Results

Tables 1 and 2 summarize some bioenergetic parameters measured in liver and kidney mitochondria from rats injected with either the PFC emulsion or the control medium. Since both liver and kidney mitochondria isolated from control rats killed at 3, 7 or 30 days after the

Table 1. Bioenergetic parameters of mitochondria isolated from livers of control rats and of rats injected with PFC emulsion.

	Steady-state $\Delta\psi$	Oxygen consumption rate −ADP	+ADP	RCR	ATP synthesis rate	P/O
Control	175 ± 5	13.9 ± 1.6	87.0 ± 3.1	6.2 ± 0.8	157.0 ± 6.7	1.8 ± 0.08
PFC-3 days	166 ± 4*	18.8 ± 1.9*	87.8 ± 4.0	4.6 ± 0.6*	122.3 ± 6.0*	1.4 ± 0.10*
PFC-7 days	165 ± 4*	13.9 ± 1.2	67.9 ± 2.9*	4.9 ± 0.7*	99.2 ± 4.2*	1.5 ± 0.06*
PFC-30 days	163 ± 3*	15.4 ± 2.4	63.4 ± 3.0*	3.5 ± 0.6*	95.7 ± 4.1*	1.5 ± 0.05*

The values are expressed as mV for transmembrane electrical potential ($\Delta\psi$), ngatom O/min/mg of mitochondrial protein for oxygen consumption rates, nmol ATP/min/mg of mitochondrial protein for ATP synthesis rates. RCR: the ratio between the oxygen consumption rates in the presence and in the absence of 150 μM exogenous ADP. P/O: the ratio between the rates of ATP synthesis and oxygen consumption in the presence of 150 μM ADP. Succinate was the oxidizable substrate. Except for control values, each value is the mean of 9 experimental results (on 9 different mitochondrial preparations) ± standard deviation. For control values see 'Methods'. *Significantly different from control (Student's, t test, $p < 0.01$).

Table 2. Bioenergetic parameters of mitochondria isolated from kidneys of control rats and of rats injected with PFC emulsion.

	Steady-state $\Delta\psi$	Oxygen consumption rate −ADP	+ADP	RCR	ATP synthesis rate	P/O
Control	173 ± 4	72.1 ± 5.3	271 ± 15	3.7 ± 0.5	435 ± 21	1.6 ± 0.06
PFC-3 days	177 ± 7	63.6 ± 3.4*	214 ± 18*	3.4 ± 0.5	358 ± 17*	1.7 ± 0.08
PFC-7 days	178 ± 6	55.8 ± 3.7*	207 ± 14*	3.7 ± 0.6	360 ± 16*	1.7 ± 0.06
PFC-30 days	170 ± 6	60.0 ± 3.5*	201 ± 14*	3.3 ± 0.7	336 ± 16*	1.7 ± 0.06

For details see legend to table 1

*Significantly different from control (Student's, t test, $p < 0.01$).

injection behaved identically, control values were pooled for statistical analysis and are reported only once in each table. This is consistent with the fact that injection of 1 ml of Krebs-Henseleit + MOPS + BSA medium had no effect on mitochondrial oxidative phosphorylation. No tensioactive agent was added to this control mixture since in the PFC emulsions the tensioactive was associated only with the emulsion particles (see methods).

In liver mitochondria (table 1) from PFC-injected rats the transmembrane electrical potentials were approximately 10 mV lower than in control mitochondria, regardless of the time interval from the injection. Conversely, kidney mitochondria (table 2) isolated from PFC-injected rats maintained a steady-state $\Delta\psi$ of the same magnitude as control mitochondria.

In liver mitochondria the oxygen consumption rates measured in the absence of added ADP (resting respiration) were approximately the same for control and PFC-injected rats, while those measured following addition of ADP decreased by about 20% in 7 and 30-days PFC-injected rats in comparison with control rats. As expected from the decrease of ADP-stimulated respiration, both the rate of ATP synthesis and the P/O ratio (i.e. the ratio between the ADP phosphorylation and oxygen consumption rates) were significantly depressed in liver mitochondria isolated from PFC-treated rats. The respiratory control ratios (RCR), i.e. the ratios between the oxygen consumption rates measured in the presence and in the absence of added ADP, were also apparently lower in liver mitochondria isolated from PFC-injected rats than in liver mitochondria from control rats.

As shown in table 2, a marked decrease in the ADP-stimulated respiration was also observed in kidney mitochondria isolated from PFC-injected rats. However the resting respiration of kidney mitochondria, unlike that of liver mitochondria, was significantly inhibited; as a consequence the RCR was comparable to that of control mitochondria. The rates of ATP synthesis in kidney mitochondria isolated from PFC-injected rats were significantly slower than in control mitochondria, but the P/O ratio values appeared unmodified due to the parallel inhibition of the ADP-stimulated oxygen consumption rate.

It may be observed (table 1 and 2) that the oxygen consumption rates, measured either in the presence or in the absence of added ADP, were markedly faster in kidney than in liver mitochondria. In addition, the RCR values were lower and the rates of ATP synthesis faster for kidney mitochondria. These are common features of isolated kidney mitochondria, but whether they represented an artifact is beyond the scope of this research.

Discussion

Although some bioenergetic parameters such as the rate of ADP-stimulated respiration and ATP synthesis were depressed, the overall bioenergetic efficiency of the mitochondria obtained from PFC-treated rats appeared sufficient to meet the energetic demand of the cell. In particular, ATP synthesis, although decreased if compared to control values, was maintained at an acceptable rate, and P/O ratios (i.e. the number of ADP molecules phosphorylated per oxygen atom reduced by the respiratory chain) were similar to the values reported for normal succinate-oxidising miltochondria (the theoretical value is 2).

In previous papers[6,7] we evaluated the oxygen consumption rates and $\Delta\psi$ in mitochondria isolated from rat livers perfused with various PFC emulsions. The presence of PFC particles in the perfusion medium was shown to improve the efficiency of mitochondrial energy-linked processes by increasing the oxygen availability. Note that the experimental conditions described in the present paper are substantially different from those of our previous perfusion experiments: then, the oxygenation of control livers, which were perfused with an oxygenated aqueous medium having the same composition as the aqueous phase of PFC emulsions, was significantly restricted due to the limited amount of oxygen dissolved in the perfusion medium. In the experiments reported in this paper the oxygen availability to the liver and kidneys of control rats was normal since control animals were only injected with 1 ml of Krebs-Henseleit + MOPS medium supplemented with albumin (as described in the 'Methods' section). In PFC-treated rats 1 ml of injected emulsion, with a 10% fluorocrit was rapidly diluted by approximately 17 ml of circulating blood with a mean hematocrit of 42%. Thus the level of PFC particles in blood was, at atmospheric oxygen levels, not enough to increase significantly the oxygenation provided by haemoglobin. Therefore the differences observed between the mitochondria isolated from control and PFC-injected rats cannot be ascribed to differences in tissue oxygenation.

Histological examination of rat liver sections obtained a few days following injection of the PFC-emulsion used in this work[8] or other PFC-emulsions[12–16] revealed the presence of PFC particles within a relatively small proportion of hepatocytes and, to a lesser extent, Kupffer cells. The presence of intracellular PFC particles decreased with the time elapsed from PFC administration and was scarcely detectable in 30-day samples. PFC particles were not detected in kidney specimens. The fact that a less efficient ATP synthesis and ADP-stimulated respiration were observed both in kidney and in 30-day liver mitochondria from PFC-treated rats (tables 1 and 2), regardless of the barely detectable level of PFC emulsion particles within the cells, suggests that the observed mitochondrial alterations were not elicited by structural damages to the cell architecture caused by the particle size.

It appears likely that following the disappearance of PFC particles the highly hydrophobic PFC molecules

remain dissolved in the lipid phase of subcellular structures. The fact that ATP synthesis decreased both in liver and in kidney mitochondria while $\Delta\psi$ decreased only in liver mitochondria may reflect the different degree of PFC accumulation by different tissues. In kidneys, where uptake was undetectable, the very low amount of PFC released by the particles is likely to interact with a small number of hydrophobic sites in the inner mitochondrial membrane, affecting specific functions such as the ATP synthetase activity. In liver, due to the large number of particles taken up, the amount of released perfluorocarbon might be sufficient to cause an overall disturbance of the mitochondrial membrane, resulting in an increase in its permeability to protons. This might explain the observation of a limited decrease of $\Delta\psi$ in liver but not in kidney mitochondria.

We also tested the direct effects of the tensioactive agent used to obtain the PFC emulsion, on isolated liver and kidney mitochondria. Considering that the PFC-particles contain less than 25% of the original amount of tensioactive agent (as estimated gravimetrically on the dried aqueous phase after particle removal) and assuming that this tensioactive agent would be completely free, its concentration in the blood of PFC-treated rats should be lower than 0.15 mg/ml (approximately 7.8×10^{-5} mol/l). When tested on mitochondria isolated from the livers or kidneys of untreated rats this concentration of tensioactive agent induced a slight increase (6 to 8%) of the resting respiration rate, while leaving the ADP-stimulated respiration unaffected (data not shown). These results strongly suggest that, under the experimental conditions tested, the tensioactive agent did not interfere with either the respiratory chain or the ATP synthetase.

It has to be emphasised that the in vitro interaction between the tensioactive agent and isolated mitochondria may be substantially different from that occurring in animals. PFC-particles diffuse through the whole organism and liver cells take up only a small, although significant, fraction of them; thus the released tensioactive agent is probably at a much lower concentration than that tested in vitro. Moreover, since the metabolic fate of this compound is still unknown, additional interactions may occur in the cell which cannot be revealed by in vitro studies.

Finally, it has to be noted that, due to its hydrophobicity, the pure perfluorocarbon could not be directly tested on isolated mitochondria.

A PFC emulsion, Fluosol DA, is commercially available in the USA for clinical use, but many adverse effects have been reported after its use in patients[5, 17–19]. Although the observations reported in this paper refer only to PFC-treated animals, and we do not have clinical experience with PFC, these reports indicate that new perfluoro- or fluoro-compounds are required for generalised clinical use.

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