Uncoupling effect of polyunsaturated fatty acid deficiency in isolated rat hepatocytes: effect on glycerol metabolism

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The effects of a 4-week deficiency in polyunsaturated fatty acids (PUFA) in isolated rat hepatocytes have been investigated for oxidative phosphorylation and fatty acid, dihydroxyacetone (DHA) or glycerol metabolism. Oxygen uptake was significantly increased (by 20 %) with or without fatty acid addition (octanoate or oleate) in the PUFA-deficient group compared with controls. The effect persisted after oligomycin addition but not after that of potassium cyanide, leading to the conclusion that, in these intact cells, the mitochondria were uncoupled. The PUFA-deficient group exhibited a significant decrease in the cytosolic ATP/ADP ratio, whereas the mitochondrial ratio was not affected. PUFA deficiency led to a 16 % decrease in DHA metabolism owing to a 34 % decrease in glycerol kinase activity; the significant decrease in the ATP/ADP ratio was accompanied by an increase in the fractional glycolytic flux. In contrast, glycerol metabolism was significantly enhanced in the PUFA-

deficient group. The role of the glycerol 3-phosphate dehydrogenase step in this stimulation was evidenced in hepatocytes perfused with glycerol and octanoate in the presence of increased concentrations of 2,4-dinitrophenol (Dnp): uncoupling with Dnp led to an enhancement of glycerol metabolism, as found in PUFA deficiency, although it was more pronounced than in controls. The matrix/cytosol gradients for redox potential and ATP/ADP ratio were lower in cells from PUFA-deficient rats, suggesting a decreased mitochondrial membrane potential in accordance with the uncoupling effect. Moreover, a doubling of the mitochondrial glycerol 3-phosphate dehydrogenase activity in the PUFA-deficient group compared with controls led us to conclude that the activation of glycerol metabolism is the consequence of two mitochondrial effects: uncoupling and an increase in glycerol 3-phosphate dehydrogenase activity.

INTRODUCTION

Increased metabolic rate accompanied by reduced growth has been reported in polyunsaturated fatty acid (PUFA)-deficient rats [1–3]. The mechanism of this effect is still a matter of debate. Several authors [4,5] have suggested that it is due to a decrease in the efficiency of oxidative phosphorylation, whereas other results with isolated mitochondria did not support this conclusion [6,7]. Moreover, some studies have suggested that changes in oxidative phosphorylation in mitochondria from PUFA-deficient rats might be an artifact, resulting from damage sustained by the deficient mitochondria during their isolation [8,9], the waste of energy being linked to some other energy-consuming process such as transdermal water loss [3]. Studies with isolated mitochondria do not permit a complete answer to be given to the question of the efficiency of ATP synthesis, not only because of the putative artifacts arising from the isolation procedures but also due to the fact that the regulation of oxidative phosphorylation in isolated mitochondria might be different from that in the intact cell.

The purpose of the present work was to investigate the effects of polyunsaturated fatty acid deficiency on isolated rat hepatocytes. As we found a substantial change both in the respiratory rate and in the ATP/ADP ratio in cells from PUFA-deficient rats, we studied the metabolism of dihydroxyacetone (DHA) and glycerol to investigate at a cellular level the consequences of both redox and phosphate potential changes.

MATERIALS AND METHODS

Animals and diets

Male weanling Wistar rats (50–60 g) were fed with a

semi-synthetic diet (21 % casein, 44.07 % maize starch, 23.40 % sucrose, 1.87 % cellulose, 0.12 % DL-methionine, 3.30 % vitamin mixture, 0.94 % mineral mixture) containing either 5.30 % soya oil (control group) or 2.65 % stearic acid and 2.65 % palmitic acid (PUFA-deficient group). After 4–6 weeks, rats starved for 20–24 h were anaesthetized with sodium thiopental intraperitoneally (125 mg/kg). Hepatocytes were isolated by the method of Berry and Friend [10] as modified by Groen et al. [11]. At the time of killing, for the same age, the body masses in the control group were slightly but significantly higher than those of the PUFA-deficient group (270 ± 8 g compared with 234 ± 7 g; results given ± S.E.M.; *n* = 12 in each group, *P* < 0.05).

Oxygen consumption measurements

Hepatocytes (10 mg/ml dry cells) were incubated at 37 °C in 3.5 ml of Krebs bicarbonate buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 24 mM NaHCO₃, pH 7.4) saturated with O₂/CO₂ (19:1) and containing fatty-acid-free BSA (2 %) and calcium (2.4 mM). Experiments were performed either without the addition of exogenous substrate or in the presence of fatty acids: 4 mM octanoate or 2 mM oleate. After 20 min, oxygen uptake (*J*_{O₂}) before and after the addition of 6 µg/ml oligomycin and 4.5 mM potassium cyanide was measured polarographically at 37 °C with a Clark electrode. At the same time, 500 µl aliquots of cell suspensions were removed, quenched in HClO₄ (0.4 g/l final concentration) and neutralized with 2 M KOH/0.3 M Mops for the determination of ketone bodies. Separate aliquots were taken simultaneously for separation into mitochondrial and cytosolic compartments by the digitonin fractionation method as described by Zuurendonk and Tager [12]. ATP, ADP and AMP were determined by HPLC as

Abbreviations used: DHA, dihydroxyacetone; Dnp, 2,4-dinitrophenol; PUFA, polyunsaturated fatty acid.

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described previously [13]. Ketone body production and the 3-hydroxybutyrate/acetoacetate ratio were determined from 3-hydroxybutyrate and acetoacetate accumulation during the 20 min incubation, measured enzymically as described in Bergmeyer [14].

Incubations in closed vials

Hepatocytes (10 mg/ml dry cells) were incubated in 1.5 ml of Krebs bicarbonate buffer with 20 mM glycerol or 20 mM dihydroxyacetone. At 0 and 30 min of incubation, 500 μ l samples of the cell suspension were taken, quenched in HClO_4 and neutralized as described above. Glucose, lactate and pyruvate were measured enzymically as described in Bergmeyer [14]. ATP, ADP and AMP were determined by HPLC.

Glycerol kinase activity

Samples of isolated hepatocytes (60 mg/ml dry cells), stored at -20°C , were sonicated for 3 min, then mechanically crushed (Polytron®) and centrifuged at 12000 *g* for 10 min. Glycerol kinase (EC 2.7.1.30) activity was determined in the supernatant spectrophotometrically by the method of Bergmeyer [14].

Perfusion of hepatocytes

Liver cells (250 mg dry weight of cells in 15 ml) were perfused by the method of van der Meer and Tager [15] as modified by Groen et al. [11,16]. Hepatocytes were perfused at 37°C at a flow rate of 5 ml/min with continuously gassed Krebs bicarbonate buffer, pH 7.4, saturated with O_2/CO_2 (19:1) and containing 1.3 mM calcium. After 40 min, when a steady state had been reached, the cells were titrated with glycerol, as indicated in the Figures. Samples of perfusate were taken at every steady state for subsequent determinations of glucose, lactate, pyruvate, 3-hydroxybutyrate and acetoacetate. The effect of an uncoupler, 2,4-dinitrophenol (Dnp), on the perfused hepatocytes was investigated in the presence of 20 mM glycerol and 0.2 mM octanoate. Because ketone bodies and pyruvate are unstable when frozen, perfusate samples were always kept at 4°C and determinations were performed within 16 h. Proteins in the perfusate were denatured by heating the samples at 80°C for 10 min before centrifugation [17]. During the course of the perfusions, at each steady state, samples of cell suspension were removed from the chambers. Cellular content was separated from the extracellular medium by centrifugation of the cell suspension through a layer of silicone oil as described previously [16]. Dihydroxyacetone phosphate and glycerol 3-phosphate were measured fluorimetrically as described in Bergmeyer [14].

Mitochondrial glycerol 3-phosphate dehydrogenase activity

Mitochondria were isolated as described [18] in a buffer containing 0.25 M sucrose, 20 mM Tris/HCl, 1 mM EGTA, pH 7.2. Glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) activity was determined by measuring the oxygen uptake of isolated uncoupled mitochondria (broken by freezing) incubated with 10 mM glycerol 3-phosphate and 1.25 μM rotenone. Enzyme activity is expressed as n-atom of oxygen per min per mg of protein. Protein content was measured by the Biuret method.

Results are expressed as means $\pm$ S.E.M. for the indicated

numbers of different cell preparations unless otherwise indicated in the legends. Comparisons were made by using Student's *t*-test for unpaired samples.

Materials

Collagenase A and enzymes were purchased from Boehringer, octanoate from Janssen, oleate, dihydroxyacetone and glycerol from Merck, and Dnp from Sigma. Rhodorsil silicone oil was purchased from Rhône-Poulenc.

RESULTS

PUFA deficiency affects cellular oxidative phosphorylation

Table 1 shows the effect of PUFA deficiency on oxygen uptake and fatty acid metabolism in isolated hepatocytes. Hepatocytes oxygen uptake was significantly increased (by 20%) in the PUFA-deficient group compared with controls, both with endogenous substrates and with the addition of fatty acid (octanoate or oleate). The significant increase in oxygen uptake in this group persisted after the addition of oligomycin (30%), whereas it was abolished with potassium cyanide. Hence it seems that the increase in cell respiration is due to a mitochondrial process that is not entirely linked to ATP synthesis. Such an effect could be due to mitochondrial uncoupling. In intact hepatocytes a direct measurement of the rate of ATP synthesis is difficult because there is no accumulation of ATP, as in experiments with isolated mitochondria. The cellular energy status can only be assessed from the steady-state concentrations of adenine nucleotides. These values are shown in Table 2 for both the cytosolic and mitochondrial compartments. The PUFA-deficient group showed a significant decrease in their cytosolic ATP/ADP ratios compared with controls, whereas the sum of adenylic nucleotides was not significantly affected. In contrast, in the mitochondrial matrix no significant change was observed either for the ATP/ADP ratio or for the sum. PUFA deficiency also affected fatty acid metabolism (Table 1). Ketogenesis, which was higher with octanoate than with oleate, was significantly stimulated (by 30%) in the PUFA-deficient group. The 3-hydroxybutyrate/acetoacetate ratio, an indicator of the mitochondrial redox potential [19], was increased with fatty acids, whereas no difference was found between the groups. Furthermore with oleate as substrate the ketone body ratio was significantly higher whereas cellular respiration and ketogenesis were lower than with octanoate. In addition, as previously reported [7,20,21], the increase in oxygen consumption with fatty acids was accompanied by an increase in the mitochondrial ATP/ADP ratio, which in the present work was similar in both groups of cells. Finally, fatty acid metabolism led to an increase in mitochondrial AMP that was larger with octanoate than with oleate. Although the PUFA-deficient group exhibited a lower AMP increase after octanoate addition, both mitochondrial ATP and ADP were increased compared with control cells. The ratio of cytosolic ATP/ADP over mitochondrial ATP/ADP, i.e. the phosphate potential gradient across the mitochondrial membrane, is the consequence of both the electrogenic nature of the adenine nucleotide translocator and the degree of displacement from equilibrium. This ratio was lower in the PUFA-deficient group.

Metabolic consequences of PUFA deficiency in liver cells

The two main consequences of PUFA deficiency on oxidative phosphorylation in intact cells are an increase in the oxidative

Table 1 Effect of PUFA deficiency on oxygen uptake and ketone body production in isolated hepatocytes incubated in closed vials

Hepatocytes from control or PUFA-deficient rats were incubated as described in the Materials and methods section. Experiments were performed either without the addition of exogenous substrate or in the presence of 4 mM octanoate or 2 mM oleate. After 20 min, oxygen uptake (J_{O_2}) before and after the addition of 6 μ g/ml oligomycin and 4.5 mM potassium cyanide was measured. Ketone body production and the 3-hydroxybutyrate/acetoacetate ratio were determined from 3-hydroxybutyrate and acetoacetate accumulation during the 20 min incubation. Results are expressed as means $\pm$ S.E.M., for the indicated numbers of incubations from three to five different cell preparations. * $P < 0.05$ compared with control, † $P < 0.05$ compared with octanoate.

Substrate		J_{O_2} (μ mol/min per g of dry cells)			Production of ketone bodies	
		Basal ($n = 15$)	+ Oligomycin ($n = 15$)	+ Potassium cyanide ($n = 6$)	3-Hydroxybutyrate + acetoacetate (μ mol per 20 min per g of dry cells) ($n = 12$)	3-Hydroxybutyrate/ acetoacetate ($n = 12$)
Endogenous	Control	10.5 $\pm$ 0.3	4.6 $\pm$ 0.2	0.69 $\pm$ 0.06	35 $\pm$ 2	0.09 $\pm$ 0.01
	PUFA-deficient	12.6 $\pm$ 0.4*	5.6 $\pm$ 0.3*	0.59 $\pm$ 0.11	58 $\pm$ 2*	0.08 $\pm$ 0.01
Octanoate	Control	21.1 $\pm$ 0.5	4.6 $\pm$ 0.4	0.81 $\pm$ 0.09	134 $\pm$ 3	0.31 $\pm$ 0.02
	PUFA-deficient	25.9 $\pm$ 0.7*	6.1 $\pm$ 0.4*	0.44 $\pm$ 0.13*	191 $\pm$ 11*	0.27 $\pm$ 0.05
Oleate	Control	14.6 $\pm$ 0.7†	6.8 $\pm$ 0.5†	0.54 $\pm$ 0.17	100 $\pm$ 6†	0.98 $\pm$ 0.15†
	PUFA-deficient	17.6 $\pm$ 0.2*†	8.5 $\pm$ 0.4*†	0.20 $\pm$ 0.09	151 $\pm$ 13*†	0.99 $\pm$ 0.18†

Table 2 Effect of PUFA deficiency on cytosolic and mitochondrial ATP, ADP and AMP concentrations and the ATP/ADP ratio in isolated hepatocytes incubated in closed vials

See legend to Table 1. After 20 min of incubation, 0.3 ml samples were removed for rapid separation of mitochondria and cytosol by the digitonin fractionation procedure. ATP, ADP and AMP concentrations (μ mol/g of dry cells) were measured in each compartment by HPLC. Results are expressed as means $\pm$ S.E.M. for nine different incubations from three different cell preparations in each group. * $P < 0.05$ compared with control; † $P < 0.05$ compared with endogenous; ‡ $P < 0.05$ compared with octanoate.

	Cytosolic					Mitochondrial					
Substrate	ATP	ADP	AMP	ATP + ADP + AMP	ATP/ADP	ATP	ADP	AMP	ATP + ADP + AMP	ATP/ADP	(Cyto. ATP/ADP)/ (Mito. ATP/ADP)
Endogenous											
Control	6.84 ± 0.32	1.31 ± 0.15	0.25 ± 0.06	8.40 ± 0.49	5.69 ± 0.56	2.41 ± 0.17	1.49 ± 0.08	0.51 ± 0.02	4.41 ± 0.21	1.66 ± 0.14	3.48 ± 0.21
PUFA-deficient	7.14 ± 0.23	1.98 ± 0.18*	0.37 ± 0.09	9.49 ± 0.23	3.94 ± 0.46*	2.56 ± 0.25	1.57 ± 0.08	0.59 ± 0.04	4.71 ± 0.33	1.63 ± 0.14	2.53 ± 0.34*
Octanoate											
Control	7.15 ± 0.10	1.15 ± 0.08	0.31 ± 0.06	8.61 ± 0.18	6.42 ± 0.37	1.36 ± 0.11†	0.66 ± 0.06†	3.03 ± 0.16†	5.06 ± 0.24	2.12 ± 0.13†	2.89 ± 0.51
PUFA-deficient	6.54 ± 0.13*†	1.53 ± 0.20	0.36 ± 0.05	0.43 ± 0.21	4.48 ± 0.51*	1.98 ± 0.08*†	1.02 ± 0.06*†	2.63 ± 0.09*†	5.63 ± 0.15	1.99 ± 0.13	2.70 ± 0.54
Oleate											
Control	7.01 ± 0.25	1.56 ± 0.11‡	0.26 ± 0.04	8.83 ± 0.24	4.72 ± 0.44‡	1.99 ± 0.16‡	0.94 ± 0.09‡	1.75 ± 0.19‡	4.68 ± 0.17	2.18 ± 0.13‡	2.15 ± 0.13‡
PUFA-deficient	6.47 ± 0.41	2.34 ± 0.35*‡	0.31 ± 0.06	9.11 ± 0.16	3.32 ± 0.49*	2.17 ± 0.15	1.16 ± 0.08†	1.72 ± 0.23†‡	5.05 ± 0.22†	1.91 ± 0.12	1.84 ± 0.32

flux associated with a decrease in the cytosolic ATP/ADP ratio. Because our purpose was to investigate the metabolic consequences of PUFA deficiency we studied DHA metabolism, which is mainly controlled by the cytosolic phosphate potential [13,20], and glycerol metabolism, whose pathway is very similar to that of DHA although it is controlled by the redox state [22]. These results are presented in Table 3. The rate of DHA metabolism expressed as three-carbon equivalents (i.e. twice the sum of glucose plus lactate plus pyruvate) was significantly lower in the PUFA-deficient group, as was glucose production (-20%), whereas the slight increase in lactate plus pyruvate flux did not reach significance. These changes were accompanied by a significant decrease in the cellular ATP/ADP ratio in the PUFA-deficient group while the sum of adenine nucleotides was slightly but significantly increased. Although we found the expected increase in the fractional flux through pyruvate kinase (30% compared with 20% of the total flux respectively for the PUFA-deficient group and controls) due to the decrease in the ATP/ADP ratio [13,20],

we also observed a significant (16%) decline in total DHA metabolism in contrast with our previous work [20]. Because, as reported, the first step of DHA metabolism entirely controls its flux [20], the decrease reported here must be related to some change at this level. This led us to determine the activity of glycerol kinase, which was shown to be significantly lower in the PUFA-deficient group than in controls (1.70 ± 0.19 compared with 2.58 ± 0.28 μ mol of glycerol per min per g of dry cells respectively; $P < 0.02$).

Glycerol metabolism shows a different picture from that of dihydroxyacetone (Table 3): total glycerol metabolism as well as glucose production were both significantly increased (by 15%) in the PUFA-deficient group compared with controls. Additionally, in the presence of glycerol, the cellular adenine nucleotide content was significantly higher (by 50%) in the PUFA-deficient group. Hence in this group the adenine nucleotide pool was less affected by glycerol. Moreover the ATP/ADP ratio in the presence of glycerol was slightly, although significantly, higher in the PUFA-deficient group. The mechanism by which PUFA deficiency

Table 3 Effect of PUFA deficiency on gluconeogenesis and lactate plus pyruvate production from dihydroxyacetone and glycerol, in isolated hepatocytes incubated in closed vials

Hepatocytes isolated from control or PUFA-deficient rats were incubated as described in the Materials and methods section with 20 mM dihydroxyacetone or 20 mM glycerol. Glucose and lactate plus pyruvate production were determined from glucose, lactate and pyruvate accumulation during the 30 min incubation. The lactate/pyruvate ratio and substrate utilization expressed as three-carbon equivalents by the sum of $2 \times$ glucose plus lactate plus pyruvate ($2 \times \text{Glu} + \text{Lac} + \text{Pyr}$) were calculated. After 30 min of incubation, total cellular ATP, ADP and AMP concentrations were measured. Results are expressed as mean $\pm$ S.E.M.; $n = 15$ incubations from five different cell preparations in each group for glucose, lactate and pyruvate measurements (μmol per 30 min per g of dry cells), and $n = 6$ incubations from three different cell preparations in each group for ATP, ADP and AMP determinations (μmol per g of dry cells). * $P < 0.05$ compared with control.

Product	Dihydroxyacetone		Glycerol	
	Control	PUFA-deficient	Control	PUFA-deficient
$2 \times \text{Glu} + \text{Lac} + \text{Pyr}$	712 ± 27	$596 \pm 12^*$	282 ± 12	$328 \pm 11^*$
Glucose	276 ± 8	$212 \pm 6^*$	134 ± 8	$154 \pm 4^*$
Lactate + pyruvate	159 ± 13	173 ± 18	15 ± 1	20 ± 3
Lactate/pyruvate	8.4 ± 0.7	8.5 ± 0.7	20.4 ± 3.7	15.7 ± 1.9
ATP	9.99 ± 0.16	$10.63 \pm 0.19^*$	7.01 ± 0.08	$10.6 \pm 0.08^*$
ADP	1.79 ± 0.03	$2.18 \pm 0.05^*$	1.77 ± 0.03	$2.54 \pm 0.02^*$
ATP + ADP + AMP	12.16 ± 0.19	$13.13 \pm 0.26^*$	9.10 ± 0.11	$13.65 \pm 0.12^*$
ATP/ADP	5.57 ± 0.04	$4.87 \pm 0.04^*$	3.97 ± 0.08	$4.18 \pm 0.04^*$

increased glycerol metabolism and glucose production was investigated with a different experimental approach that allowed us to study this pathway in steady-state sub-saturating conditions, as is possible with the perfusion of hepatocytes.

Glycerol metabolism in perfused hepatocytes from PUFA-deficient rats

As found in the closed-vial experiments, glycerol metabolism was clearly enhanced in hepatocytes from PUFA-deficient rats (Figure 1A). This effect was largely due to a marked activation of glycolytic flux, which was increased 3-fold (Figure 1C). In contrast with the results obtained in closed-vials experiments, glucose production (Figure 1B) was not affected.

Because glycerol metabolism is controlled by redox state [22], activation of its flux in cells from PUFA-deficient rats must be due to an effect located at the glycerol 3-phosphate dehydrogenase step. This is in good agreement with the results presented in Figure 1(D), which shows the different relationships between glycerol 3-phosphate and J_{glycerol} for control and PUFA-deficient hepatocytes. The lower concentration of glycerol 3-phosphate in this latter group together with a clear enhancement of the flux argues in favour of an activation of the dehydrogenase step. This activation is also clear when the production of lactate plus pyruvate is considered (Figure 1F). The determination of dihydroxyacetone phosphate concentrations in such steady-state conditions permits the investigation of the pathway beyond the glycerol 3-phosphate dehydrogenase step. From the different relationships observed in the two groups of hepatocytes, it seemed that the sum of glucose and lactate plus pyruvate production (Figure 1G) was inhibited in PUFA-deficient cells, owing to an inhibition of the gluconeogenic pathway (Figure 1H). Indeed, the unique relationship found for the glycolytic pathway in the two groups of cells (Figure 1I) led us to conclude that this part of the pathway was not affected by PUFA deficiency. Hence the large increase in lactate plus pyruvate formation in this group was due to an increase in the

Figure 1 Effect of PUFA deficiency on glycerol metabolism

Hepatocytes (250 mg of dry cells in 15 ml), isolated from 24 h-starved control (■) or PUFA-deficient (△) Wistar rats, were titrated with glycerol as indicated in the Materials and methods section. The rate of glycerol metabolism ($J_{\text{glycerol}} = 2 \times [\text{glucose}] + [\text{lactate}] + [\text{pyruvate}]$) was calculated from the glucose, lactate and pyruvate concentrations in the perfusate. Intracellular dihydroxyacetone phosphate and glycerol 3-phosphate concentrations were measured in the neutralized cell fractions. Results are expressed as means $\pm$ S.E.M.; $n = 3$ in each group.

Figure 2 Effect of PUFA deficiency on glycerol 3-phosphate and dihydroxyacetone phosphate accumulation and on glycerol 3-phosphate/dihydroxyacetone phosphate and lactate/pyruvate ratios in isolated hepatocytes perfused with glycerol

Hepatocytes (250 mg of dry cells in 15 ml), isolated from 24 h-starved control (■) or PUFA-deficient (△) Wistar rats, were perfused as indicated in Figure 1 and in the Materials and methods section. Intracellular dihydroxyacetone phosphate and glycerol 3-phosphate concentrations were measured in the neutralized cell fractions. Results are expressed as means $\pm$ S.E.M.; $n = 3$ in each group.

dihydroxyacetone phosphate concentration, a consequence of the activation of the glycerol 3-phosphate dehydrogenase step. These results show that PUFA deficiency led to an inhibition of the gluconeogenic part of the glycerol pathway downstream of dihydroxyacetone phosphate, although the main effect was a clear enhancement of the overall metabolism by an activation of the flux through the glycerol 3-phosphate dehydrogenase step.

This effect of PUFA deficiency on the glycerol 3-phosphate dehydrogenase step and the cytosolic redox state is presented in Figure 2. The marked change in redox state in cells from PUFA-deficient rats was demonstrated by the 65% decrease in glycerol 3-phosphate concentration (Figure 2A) together with a doubling in dihydroxyacetone phosphate (Figure 2B). Figures 2(C) and 2(D) show the relationships between glycerol metabolism and cytosolic redox state assessed either by the glycerol 3-phosphate/dihydroxyacetone phosphate ratio (Figure 2C) or by the lactate/pyruvate ratio (Figure 2D). From these Figures it is clear that there was a different relationship in the two groups of liver cells. Whichever indicator of cytosolic redox state was used (i.e. glycerol 3-phosphate/dihydroxyacetone phosphate ratio or lactate/pyruvate ratio) the curves were shifted to the left for the PUFA-deficient group, indicating that the cytosolic redox potential was always more oxidized in this case and that, for a given value of cytosolic redox potential, glycerol metabolism was higher, i.e. the rates of NADH transport into the matrix and reoxidation were higher. This finding is in agreement with the uncoupling effect of PUFA deficiency in intact hepatocytes shown above (see Table 1).

Similarities between the Dnp-uncoupling effect and PUFA deficiency on glycerol metabolism in perfused hepatocytes

To test the role of such an uncoupling effect as the main explanation for the activation of glycerol metabolism in cells from PUFA-deficient rats we studied the effects of Dnp in hepatocytes from control or PUFA-deficient rats, perfused with 20 mM glycerol and 0.2 mM octanoate. Fatty acids were added to increase the control of glycerol metabolism by the redox state and also to provide sufficient amounts of substrate to the respiratory chain for a fully Dnp-uncoupled state [21]. Our results show that glycerol metabolism (Figure 3A) and glucose production (Figure 3B) were higher in cells from PUFA-deficient rats. As expected, glycerol metabolism and glucose production were strongly inhibited by the addition of octanoate (see Figures 1A, 1B, 3A and 3B). Cytosolic and mitochondrial redox potentials were more reduced when glycerol was present (Figures 3C and 3D). Uncoupling with Dnp led to an enhancement of glycerol metabolism and glucose production that was more pronounced in the PUFA-deficient group. At the same time, cytosolic and mitochondrial redox potentials became more oxidized owing to uncoupling by Dnp. At the highest uncoupled state, glucose production declined in cells from PUFA-deficient rats whereas glycerol metabolism was further stimulated (Figures 3A and 3B). This was due to the very large increase in glycolytic flux in this group. The ratio of mitochondrial to cytosolic NADH/NAD⁺ is linked to the mitochondrial membrane potential because the glutamate carrier is electrogenic [23]. From

Figure 3 Effect of Dnp on perfused hepatocytes from control or PUFA-deficient rats

Hepatocytes (200 mg of dry cells in 15 ml), isolated from 24h-starved control (■) or PUFA-deficient (△) Wistar rats, were perfused without exogenous substrate. When a steady state had been reached, 20 mM glycerol was added and cells were titrated with 12.5, 25 and 50 μ M Dnp. The rate of glycerol metabolism ($J_{\text{glycerol}} = 2 \times [\text{glucose}] + [\text{lactate}] + [\text{pyruvate}]$) was calculated from the glucose, lactate and pyruvate concentrations in the perfusate. Cytosolic and mitochondrial NADH/NAD⁺ were calculated from lactate, pyruvate, 3-hydroxybutyrate and acetoacetate concentrations in the perfusate. This Figure represents a typical experiment; similar results were obtained in another. Abbreviation: Dnp, Dnp.

Figure 3(E) it seems that this gradient was lower in the cells from the PUFA-deficient rats than in the controls, in accordance with a decreased mitochondrial membrane potential, already suggested by the decline of the cytosolic to mitochondrial ATP/ADP ratio in cells from PUFA-deficient rats (Table 2).

As well as the activation of mitochondrial NADH oxidation due to the uncoupling effect of PUFA deficiency, the enhancement of glycerol metabolism must be accompanied by an activation of cytosolic reducing equivalents transferred across the inner membrane. This could be achieved either via the malate-aspartate shuttle or via mitochondrial glycerol 3-phosphate dehydrogenase. The flux through the malate-aspartate shuttle is probably decreased because of the uncoupling effect, as the glutamate carrier is electrogenic. Therefore we investigated the role of mitochondrial glycerol 3-phosphate dehydrogenase by measuring its activity in mitochondria from both groups. It was found to have a significantly increased activity in the PUFA-deficient group compared with controls: 3.13 ± 0.22 compared with 1.49 ± 0.14 n-atoms of oxygen per min per mg protein; $P < 0.001$; $n = 20$ and $n = 16$ respectively for PUFA-deficient group and controls. This result was confirmed by the fact that the inhibition of the malate-aspartate shuttle by the transaminase inhibitor amino-oxyacetate (0.3 mM) in the presence of 20 mM glycerol led to a more pronounced inhibition of oxygen consumption (18% compared with 7%), as well as glucose

production (40% compared with 30%), in controls than in the PUFA-deficient group respectively.

DISCUSSION

This model of PUFA deficiency *in vivo* (i.e. a diet free of unsaturated fatty acid and containing only palmitic and stearic acids) is a classical model when given to rats immediately after weaning and for at least 4 weeks [5,24,25]: it leads to marked changes in the lipid composition of the plasma, liver [3] and mitochondrial [5] membranes. The increase in energy consumption related to this model of PUFA deficiency has been well established *in vivo* [2] as well as *in vitro*, in isolated mitochondria from various cell types (hepatocytes, brown adipose tissue) where oxygen uptake was increased [5,26]. However, the exact mechanism is still a matter of debate. Among the different controversies, the quality of isolated mitochondria has been questioned because mitochondria from PUFA-deficient animals seem to be more fragile and thus subject to leaks as artifacts. From our results, where mitochondria were not isolated, it clearly appears that the significant increase in cellular respiratory rate was indeed due to a mitochondrial process that is not entirely linked to phosphorylation. Hence an artifact due to a greater fragility of mitochondria from PUFA-deficient rats might not account for the reported differences. This effect is probably

explained by uncoupling as already suggested [4,24]. An increased cell fragility due to PUFA deficiency is unlikely because (1) more than 90% of an isolated cell preparation routinely excluded Trypan Blue, and (2) no difference in cellular total nucleotide content was found between both groups of cells. The much larger increase in oxygen uptake in the PUFA-deficient group in the absence rather than in the presence of oligomycin suggests that as well as the uncoupling effect there was an enhancement of oxidative phosphorylation. Moreover, the decline in the cytosolic ATP/ADP ratio indicates that this increased rate of oxidative phosphorylation is not sufficient to compensate for the lack of efficiency due to uncoupling and the probable increase in energy consumption. The significant decrease in the cytosolic ATP/ADP ratio observed was the consequence of a decrease in ATP together with an increase in ADP concentration. In contrast, there was no difference in the mitochondrial ATP/ADP ratio between the PUFA-deficient group and controls. The discrepancy between the effect on the cytosolic ATP/ADP ratio and the lack of change in mitochondrial ATP/ADP ratio is most probably due to a change in translocase activity. This may be due to a decrease in the mitochondrial membrane potential related to the uncoupling in the PUFA-deficient group and/or to a change in some kinetic property of this enzyme linked to the changes in the phospholipid environment. The addition of exogenous fatty acid affected the matrix nucleotides: ATP and ADP decreased whereas AMP increased, this being the consequence of fatty acid activation. This effect was larger with octanoate than with oleate. Although PUFA deficiency did not modify the effect of oleate addition on mitochondrial nucleotides, the effect of octanoate was not the same in both groups of cells: changes in ATP, ADP and AMP due to octanoate addition were significantly smaller in the PUFA-deficient group. This cannot be explained by a lower metabolism of octanoate in the PUFA-deficient group because oxygen uptake and total ketone body production were significantly higher in this group. The uncoupling effect of PUFA deficiency might be an alternative explanation because, as we have reported recently [21], a similar change occurs after cellular uncoupling by Dnp related to an activation of mitochondrial AMP metabolism. Indeed, on one hand, transphosphorylation of GTP plus AMP into GDP plus ADP is the main mitochondrial pathway involved in AMP metabolism; on the other hand, uncoupling might activate GTP production by increasing mitochondrial ADP, a well-known allosteric activator of 2-oxoglutarate dehydrogenase (EC 1.4.1.3) [27].

Although a similar mitochondrial ATP/ADP ratio was found with either octanoate or oleate, oleate led to a greater 3-hydroxybutyrate/acetoacetate ratio, whereas oxygen uptake and ketone body production were lower. This is in accordance with the proposed inhibitory effect of oleate on the respiratory chain [28].

The balance between the gluconeogenic and glycolytic fractional fluxes in DHA metabolism is very sensitive to the effect of the cytosolic ATP/ADP ratio on pyruvate kinase [13,20]. Hence it is not surprising that PUFA deficiency affects the relative rates of glucose and lactate plus pyruvate production because it decreases the cytosolic ATP/ADP ratio. However, in the present study total DHA metabolism was decreased, whereas it was not affected in our previous study [20]. From the literature it is not completely clear which enzyme is responsible for DHA phosphorylation. It is usually stated that glycerol kinase is the main enzyme implicated [29], whereas triokinase (EC 2.7.1.28) is also suspected to catalyse the reaction [30]. The decrease in glycerol kinase activity in hepatocytes from PUFA-deficient rats reported here might explain the decreased rate of DHA phosphorylation. Although glycerol kinase is involved in both

DHA and glycerol phosphorylation, the discrepancy between the effects of PUFA deficiency on these two pathways can be explained by the difference in their control, i.e. glycerol kinase or glycerol 3-phosphate dehydrogenase (see below).

The activation of glycerol metabolism due to the uncoupling effect of PUFA deficiency is similar to the uncoupling effect of Dnp. Activation of glycerol metabolism, together with lactate plus pyruvate formation, is the consequence of activation of the glycerol 3-phosphate dehydrogenase step (Figure 1), whereas the gluconeogenic pathway is inhibited downstream of dihydroxyacetone phosphate (Figure 1H). Hence, despite the increase in dihydroxyacetone phosphate concentration in the PUFA-deficient group (Figure 2B), glucose production was not activated (see Figure 1B). It is not possible from these results to draw conclusions about the gluconeogenic step(s) involved in this inhibitory effect of PUFA deficiency, although the fructose 6-phosphate/fructose 1,6-bisphosphate or glucose 6-phosphate/glucose cycles are probable targets because they are known as possible controlling steps in gluconeogenesis [31,32]. There is an apparent discrepancy in the gluconeogenic flux values presented (Table 3 and Figures 1–3): glucose production in the PUFA-deficient group increased both in closed-vial experiments (Table 3) and during perfusion performed with octanoate (Figure 3), whereas this flux showed no difference when the perfusions were done in the absence of octanoate (Figures 1 and 2). As far as the closed-vial experiments are concerned, glucose production is overestimated because part of the lactate and pyruvate that accumulates over time is reutilized in the gluconeogenic pathway. Indeed it is clear when comparing fluxes from Table 3 and Figure 1 that, although total glycerol metabolism results are comparable for the two groups of cells in the two different models (vials or perfusions), the respective fluxes of gluconeogenesis and glycolysis are different. The increase in glycolysis in the PUFA-deficient group is clearer under perfusion conditions because perfusate is continuously rinsed out, so neither lactate nor pyruvate can be reutilized in the gluconeogenic pathway. From the results in Figure 3, glucose production was higher in the PUFA-deficient cells because of the addition of octanoate, which led to a stronger inhibition of glycerol metabolism in controls than in the PUFA-deficient group, owing to uncoupling in this latter group.

In the presence of glycerol (Table 3), cells from PUFA-deficient rats exhibited a lower response to glycerol in the decrease of total adenine nucleotide content as well as in the decrease in ATP/ADP ratio. Although the lower level of glycerol 3-phosphate accumulation in the PUFA-deficient condition may explain the smaller decrease of the ATP/ADP ratio and ATP plus ADP, the lack of a compensatory AMP increase in control cells remains to be explained.

The increase in glycerol metabolism must be accompanied by an increase in the rate of reducing equivalents transferred into mitochondria. Both the malate-aspartate shuttle and mitochondrial glycerol 3-phosphate dehydrogenase are reported to be involved in this transfer during glycerol metabolism [17,22,33–35]. From our results and from the data in the literature it seems that the uncoupling effect of PUFA deficiency leads to a decrease in the proton-motive force as either measured directly [26] or deduced from the ratios of the cytosolic to mitochondrial ATP/ADP ratio (Table 2) or NADH/NAD⁺ ratio (Figure 3). The increased rate of reducing equivalents transported across the mitochondrial membrane must be accompanied by an increased gradient owing to a change in intermediate concentration and/or to a change in carrier activity. The consequence of PUFA deficiency on the role of the malate-aspartate shuttle is probably to decrease rather than to increase it. In conditions

of glycerol metabolism, the role of mitochondrial glycerol 3-phosphate dehydrogenase is probably as important as the malate-aspartate shuttle [22,33,34]. This is confirmed by the present results, which show that the doubling in mitochondrial glycerol 3-phosphate dehydrogenase activity in cells from PUFA-deficient rats is accompanied by an increase in the rate of reducing equivalents transferred into the matrix, as demonstrated by the enhancement in glycerol metabolism. Nevertheless such an increase in mitochondrial enzyme activity does not explain the concomitant increase in flux associated with a decreased proton-motive force.

In conclusion, in hepatocyte metabolism PUFA deficiency leads to a mitochondrial uncoupling with an increased respiratory rate and a decreased cytosolic ATP/ADP ratio. The effects on DHA or on glycerol pathways are due to the resultant uncoupling of oxidative phosphorylation as well as to changes in enzyme activity (glycerol kinase and glycerol 3-phosphate dehydrogenase). The mechanism by which polyunsaturated fatty acid deficiency might interfere with the efficiency of oxidative phosphorylation and enzyme activities remains to be studied. In partly uncoupled hepatocytes the changes in enzyme activities described in this paper might be an adaptative advantage.

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