The mitochondrial consequences of uncoupling intact cells depend on the nature of the exogenous substrate

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In isolated mitochondria the consequences of oxidative phosphorylation uncoupling are well defined, whereas in intact cells various effects have been described. Uncoupling liver cells with 2,4-dinitrophenol (DNP) in the presence of dihydroxyacetone (DHA) and ethanol results in a marked decrease in mitochondrial transmembrane electrical potential ($\Delta\Psi$), ATP/ADP ratios and gluconeogenesis (as an ATP-utilizing process), whereas the increased oxidation rate is limited and transient. Conversely, when DHA is associated with octanoate or proline, DNP addition results in a very large and sustained increase in oxidation rate, whereas the decreases in $\Delta\Psi$, ATP/ADP ratios and gluconeogenesis are significantly less when compared with DHA and ethanol. Hence significant energy wastage (high oxidation rate) by uncoupling is achieved only with substrates that are directly oxidized in the mitochondrial matrix. Conversely in the presence of substrates that are first oxidized in the cytosol, uncoupling results in a profound decrease in mitochondrial $\Delta\Psi$ and ATP synthesis, whereas energy wastage is very limited.

Key words: 2,4-dinitrophenol, hepatocytes, octanoate, proline, respiration.

INTRODUCTION

The discovery of the function of brown adipose tissue in mammals, which is related to an uncoupling protein (UCP), has opened a new era in our understanding of the regulation of oxidative phosphorylation by describing a physiological role for energy wastage [1]. Recently several other uncoupling proteins have been described, belonging to the UCP family [2,3]. In addition to brown adipose tissue (UCP1), some of these uncoupling proteins (UCP2 or UCP3) have been found in most tissues, including white adipose tissue, muscle, macrophages, spleen, thymus, Kupffer cells etc. [3–7]. Recently the cDNA encoding a mouse or human brain mitochondrial carrier protein (BMCP) has been cloned. This protein (BMCP1), which is homologous to the UCPs, is a potent uncoupler when expressed in yeast and could be a new member of the UCP family [8]. These findings raise the question as to what is the physiological role of mitochondrial uncoupling in these various tissues. It has been suggested that a general modulation of oxidative phosphorylation efficiency by proton leak might be involved in the evolution of energy metabolism [9], whereas the physiological function of uncoupling in brown fat is well recognized as a heat producing mechanism. The presence of uncoupling proteins in white adipose tissue and in muscle could be related to the regulation of the whole body energy balance by finely tuning the yield of energy produced by metabolism. Therefore this mechanism might play a role in the pathogenesis of obesity, although some results do not favour this hypothesis. For example, the striking rise in muscle UCP3 expression during fasting in animals and in humans [4,10,11], and the lack of effect of UCP3 knock-out on adipose tissue in mice [12,13], do not favour this hypothesis. However, this question remains debatable in the light of the metabolic effects observed in mice exhibiting overexpression of human UCP3 [14]. Recently, it was proposed that mitochondrial uncoupling could also represent a mechanism for the regulation of mitochondrial production of reactive oxygen species [15–17]. Moreover it is clear that the mitochondrial transmembrane electrical potential ($\Delta\Psi_m$) exerts a central role in the regulation of many major cellular functions, such as calcium signalling, permeability of the mitochondrial permeability transition pore, apoptosis etc. [18]. Therefore it is probable that mitochondrial uncoupling plays a role in cellular homoeostasis, which is certainly not limited to the regulation of substrate wastage and heat production.

We have reported that the 2,4-dinitrophenol (DNP)-related increase in glycolysis, accompanied by a decrease in gluconeogenesis from dihydroxyacetone (DHA), was partially prevented by addition of octanoate or oleate [19]. Furthermore, the increase in the respiratory rate due to DNP uncoupling was only transient with DHA, although it was stable and of a much higher extent with fatty acid addition [19]. Under these conditions the decrease in $\Delta\Psi_m$ induced by DNP addition was not transient in the presence of DHA, whereas fatty acid addition was responsible for a lessening of the effect of the uncoupler on $\Delta\Psi_m$ [20]. In the present study we have compared the consequences of uncoupling when liver cells are in the presence of different mitochondria-oxidized substrates (octanoate or proline) with the consequences in the presence of substrates that are first oxidized in the cytosol (DHA or ethanol). Pharmacological uncoupling by DNP is a classical tool for investigating the consequences of pure protonophoric uncoupling, and the metabolic consequences of such uncoupling can be classified into one of three categories: (1) those related to changes in oxidation rate and redox state, (2) those related to changes in mitochondrial protonmotive force and (3) those related to changes in ATP synthesis and phosphate potentials [21,22]. We found that uncoupling resulted in a profound decrease in $\Delta\Psi_m$, as well as in cytosolic and mitochondrial ATP/ADP ratios, whereas the change in oxidation

Abbreviations used: BMCP, brain mitochondrial carrier protein; DHA, dihydroxyacetone; DNP, 2,4-dinitrophenol; $\Delta\Psi_m$, mitochondrial transmembrane electrical potential; UCP, uncoupling protein.

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rate was very limited, with exogenous substrates that were first oxidized in the cytosol. Conversely, a large increase in oxidation rate associated with a limited effect on $\Delta\Psi_m$ and ATP/ADP ratios was found with substrates that were directly oxidized in the mitochondrial matrix.

MATERIALS AND METHODS

Hepatocytes were isolated from fasted (24 h) male Wistar rats (weighing 200–300 g) as previously described [23,24]. Liver cells (100–120 mg dry weight of cells) were perifused by the method of van der Meer and Tager [25] as previously described [19,20]. Isolated hepatocytes were placed into a 15 ml perifusion chamber continuously flushed with Krebs-bicarbonate buffer [120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24 mM $NaHCO_3$ and 1.3 mM $CaCl_2$ (pH 7.4)] saturated with O_2/CO_2 (19:1). DHA, as exogenous carbohydrate source, was always continuously infused to a final concentration of 10 mM. When the initial steady state had been reached (45 min), the uncoupler DNP (100 μM) was infused for 35 min. Subsequently octanoate (0.4 mM), proline (10 mM) or ethanol (10 mM) was added and a new steady state was obtained. A Clark electrode continuously monitored oxygen consumption. Glucose, lactate, pyruvate and ketone body concentrations were determined enzymically [26] in the perifusate after protein denaturation by heat (80 °C for 10 min) [27]. Cytosolic and mitochondrial $NADH/NAD^+$ ratios were calculated from lactate/pyruvate ($K_{eq} = 1.11 \times 10^{-4}$) and 3-hydroxybutyrate/acetoacetate ($K_{eq} = 4.93 \times 10^{-2}$) ratios respectively [28]. During the perifusions, samples of cell suspensions were taken from the chambers without interrupting the flow as previously described [19]. Mitochondrial and cytosolic spaces were separated by the digitonin fractionation method [29], and adenine nucleotides were determinated by HPLC [30]. $\Delta\Psi_m$ was assessed on intact hepatocytes (5×10^6 cells/ml) stained in the dark with the fluorescent probe, rhodamine 123 (200 ng/ml) [24]. The liver cells ($1.3 \pm 0.2 \times 10^6$ cells/ml) were then incubated for 30 min in the presence of DHA (20 mM), with or without DNP (100 μM), and proline (20 mM), octanoate (2 mM) or ethanol (20 mM) as indicated. The rhodamine 123-associated fluorescence (488 nm excitation and 530 ± 10 nm emission) was analysed using a FACScan cytometer (Becton Dickinson, Le Pont de Claix, France) and cell analysis was performed using Lysis II software® (Becton Dickinson) [31,32]. Since the staining efficiency was variable between experiments, the results are expressed as a percentage of change from the initial values (time = 0) obtained without DNP addition. Rhodamine 123 (laser dye purity), DNP, oligomycin, myxothiazol, proline, octanoate and propidium iodide were purchased from Sigma (L'Isle d'Abeau, France), and collagenase A was obtained from Roche (Meylan, France). Results are expressed as means ± S.E.M., and comparisons were performed by ANOVA followed by a Fisher's protected least-squares difference test.

RESULTS

Effect of uncoupling on oxygen consumption rate and glucose production

Liver cells perifused with DHA (10 mM) exhibited an increase in oxygen consumption rate after uncoupling with DNP (100 μM) as compared with coupled basal respiration (18 μmol/g dry weight of cells per min compared with 10 μmol/g dry weight of cells per min respectively) (Figure 1A). This effect on respiration was only transient and 30 min after DNP addition the respiratory rate was no different from its initial value. When either octanoate (0.4 mM) or proline (10 mM) was then added to these hepatocytes, the respiration was stimulated again, but to a much higher rate (28 μmol/g dry weight of cells per min and 25 μmol/g dry weight of cells per min respectively) compared with the first transient increase observed with DNP. Furthermore, the activation of respiration after addition of octanoate or proline was sustained during 30 min of further perifusion. Conversely to the addition of octanoate or proline, ethanol addition (10 mM) hardly affected the respiratory rate. Gluconeogenesis from DHA is an ATP-utilizing pathway representing metabolic work. As shown in Figure 1(B), glucose production from DHA (5.9 ± 0.3 μmol/g dry weight of cells per min) was abolished by DNP addition (0.03 ± 0.03 μmol/g dry weight of cells per min) and this effect was not transient. In contrast with ethanol, addition of either octanoate or proline was followed by the restoration of some glucose production from DHA (3.3 ± 0.3 μmol/g dry weight of cells per min and 3.7 ± 0.3 μmol/g dry weight of cells per min respectively). Hence from these results it seemed that, when judged from the respiratory rate, DNP was only responsible for a transient uncoupling effect with DHA alone, whereas a large and sustained uncoupling resulted from the addition of either octanoate or proline. When judged by glucose production, the uncoupling effect of DNP (i.e. abolition of gluconeogenesis) was sustained with DHA, with or without ethanol, and addition of either octanoate or proline was responsible for a substantial maintenance of the glucose flux (i.e. 50–60 % of the production in the absence of uncoupling).

Effects of DNP uncoupling on $\Delta\Psi_m$, redox states and ATP/ADP ratios

Since the uncoupling effects of DNP seemed to depend on the experimental conditions, as well as on the metabolic parameters considered, we determined $\Delta\Psi_m$ across the inner mitochondrial membrane in intact liver cells as being a more direct reflection of DNP uncoupling. As shown in Table 1, DNP addition decreased $\Delta\Psi_m$ compared with the value obtained with DHA alone. Conversely to the increase in oxygen uptake, which was transient and returned to the initial value after 30 min, the decrease in $\Delta\Psi_m$ was present after 30 min. Uncoupling in the presence of DHA + octanoate or DHA + proline also lowered $\Delta\Psi_m$ when compared with DHA in the absence of DNP, but this effect was significantly lessened when compared with uncoupling with DHA or DHA + ethanol.

Since the main consequence of uncoupling is a decreased efficiency of oxidative phosphorylation, it should increase the oxidation rate while decreasing ATP synthesis. Hence under our conditions, DNP is expected to affect redox states and ATP/ADP ratios. As shown in Table 1, uncoupling in the presence of DHA alone was responsible for a significant increase in the cytosolic $NADH/NAD^+$ ratio, while this ratio decreased in the mitochondrial matrix. The increase in the cytosolic $NADH/NAD^+$ ratio with DNP was abolished when either octanoate or proline was added, the ratio being no different from that obtained with DHA alone. These values are in agreement with values previously found in isolated liver cells under similar conditions [19,20,33–36], and initially described in rat liver by Williamson et al. [28]. DNP uncoupling with DHA + ethanol was responsible for a very large increase in the cytosolic $NADH/NAD^+$ ratio. In the mitochondrial matrix, DNP was responsible for a decrease in the $NADH/NAD^+$ ratio with DHA alone. Addition of either octanoate or ethanol seemed to further decrease this, although these later changes were not significant. It was not possible to assess this ratio in the presence of proline since 3-hydroxybutyrate was

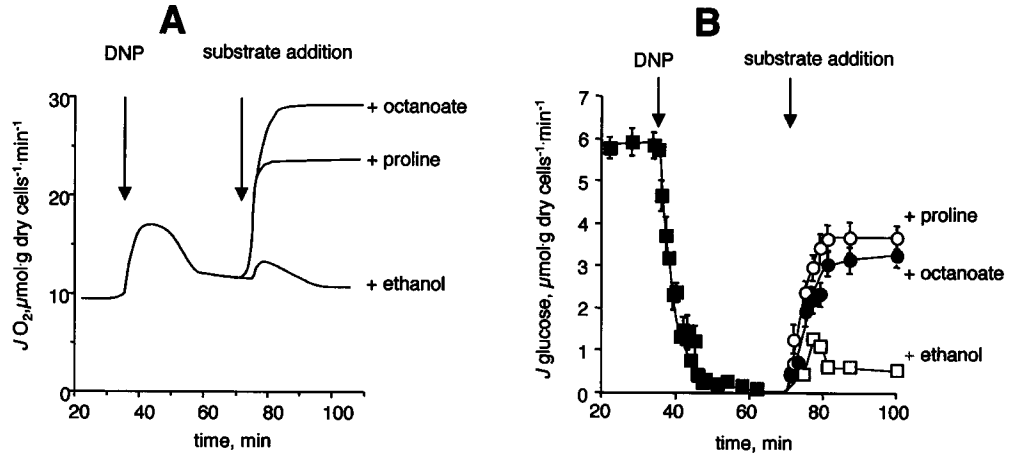

Figure 1 Oxygen consumption and glucose production following DNP uncoupling in the presence of various substrates in perifused hepatocytes

Hepatocytes (100–120 mg dry weight of cells in 15 ml) were perifused (5 ml/min) with continuously gassed [O_2/CO_2 (19:1)] Krebs-bicarbonate buffer (pH 7.42) containing 10 mM DHA. When a steady state had been reached 100 mM DNP was infused in the chamber and after 35 min octanoate (0.4 mM), proline (10 mM) or ethanol (10 mM) were infused. A Clark electrode continuously monitored oxygen consumption of the cell suspension and glucose production rate was determined from the concentration of glucose in the perifusate. (**A**) Represents oxygen consumption from a typical experiment. (**B**) Represents the means ± S.E.M. of glucose production; $n = 8$ for octanoate, $n = 3$ for proline and $n = 3$ for ethanol. *J*, metabolic flux.

Table 1 Effect of various substrates on $\Delta\Psi_m$, and cytosolic and mitochondrial ATP/ADP and NADH/NAD$^+$ ratios in hepatocytes uncoupled with DNP

$\Delta\Psi_m$ was determined in hepatocytes (5×10^6 cells/ml) pre-stained with rhodamine 123 (200 ng/ml) in Krebs-bicarbonate buffer. Cells were then washed in dye-free medium and incubated [$(1.3 \pm 0.2) \times 10^6$ cells/ml] in the presence of DHA (20 mM) and DNP (100 μM) with or without octanoate (4 mM), proline (20 mM) or ethanol (20 mM). Cellular fluorescence was analysed by flow cytometry. Values (means ± S.E.M.) are expressed as percentages of the value obtained at time zero in the absence of DNP. Lactate, pyruvate, 3-hydroxybutyrate and acetoacetate concentrations were determined in perifused hepatocytes as described in the legend to Figure 1 (also see the Materials and methods section) for subsequent calculation of mitochondrial and cytosolic NADH/NAD$^+$ ratios [33]. ATP and ADP were determined by HPLC in the acid-soluble fraction of cellular extracts after digitonin fractionation. Results are expressed as means ± S.E.M. Statistical differences were assessed by ANOVA followed by Fisher's protected least-squares difference test. cyto, cytosolic; mito, mitochondrial; n.d., not determined.

	Substrate addition				
Parameter	DHA	DHA + DNP	DHA + DNP + octanoate	DHA + DNP + proline	DHA + DNP + ethanol
$\Delta\Psi$ (% fluorescence)	100	60.92 ± 5.13 ($n = 11$)*	90.37 ± 6.79 ($n = 11$)*†	79.80 ± 1.07 ($n = 5$)*†	64.33 ± 5.46 ($n = 6$)*
NADH/NAD$^+$ cyto (10^{-4})	5.42 ± 0.61 ($n = 11$)	8.54 ± 0.59 ($n = 9$)*	5.97 ± 0.52 ($n = 8$)†	4.49 ± 0.57 ($n = 3$)†	29.75 ± 0.74 ($n = 3$)*†
NADH/NAD$^+$ mito (10^{-4})	521.1 ± 74.04 ($n = 10$)	219.1 ± 82.36 ($n = 9$)*	136.6 ± 53.3 ($n = 7$)*	n.d.	88.7 ± 14.2 ($n = 3$)*
ATP/ADP cyto	5.40 ± 0.44 ($n = 6$)	0.74 ± 0.10 ($n = 6$)*	2.68 ± 0.66 ($n = 4$)*†	3.37 ± 0.14 ($n = 3$)*†	0.65 ± 0.04 ($n = 3$)*†
ATP/ADP mito	2.32 ± 0.33 ($n = 6$)	0.19 ± 0.14 ($n = 6$)*	0.70 ± 0.28 ($n = 4$)	0.20 ± 0.02 ($n = 3$)	0.13 ± 0.01 ($n = 3$)

* Indicates a significant difference ($P < 0.05$) compared with DHA; † indicates a significant difference ($P < 0.05$) compared with the DHA + DNP.

not detectable in this model. As expected, with DHA alone, DNP was responsible for a dramatic decrease in both cytosolic and mitochondrial ATP/ADP ratios. Uncoupling in the presence of DHA and either octanoate or proline resulted in a higher cytosolic ATP/ADP ratio when compared with DNP + DHA. This ratio was also significantly higher in the matrix with DHA + octanoate when compared with DHA. The ATP/ADP ratios were not affected by ethanol addition compared with DHA + DNP.

DISCUSSION

The effects of protonophoric uncouplers are well characterized in isolated mitochondria. The increased permeability of the mitochondrial inner membrane to protons leads to a drop in protonmotive force and ATP synthesis while it activates respiration. Hence the net measurable effects are: (1) an increased oxygen consumption, (2) a drop in protonmotive force and (3) a drop in the ATP/oxygen ratio. However, various consequences have been reported in intact cells and the origin of such discrepancy is a matter of debate [19,20,37,38]. Whereas some of these differences could be tissue-related, we report in the present study different effects of protonophoric uncoupling according to the nature of the exogenous substrate in one cell type, i.e. rat liver cells.

In the presence of DHA, with or without ethanol addition, DNP uncoupling is responsible for a decrease in $\Delta\Psi_m$ and in cytosolic and mitochondrial ATP/ADP ratios. The increased oxygen uptake, resulting from the drop in $\Delta\Psi_m$, is transient because of the exhaustion of the substrate supply to the respiratory chain, as attested by the decrease in the mitochondrial NADH/NAD$^+$ ratio. This is probably the consequence of the drop in $\Delta\Psi_m$, as was previously proposed [19,20], since the malate/aspartate shuttle depends on $\Delta\Psi_m$ due to the electrogenic nature of the glutamate carrier [39,40]. This is in accordance with the drop in $\Delta\Psi_m$ seen with DHA and DHA + ethanol: the cytosolic NADH/NAD$^+$ ratio increases and the matrix NADH/NAD$^+$ ratio decreases. It is of interest to note that both DHA

and ethanol metabolism involve cytosolic NAD^+-linked dehydrogenases.

The consequences of uncoupling are rather different in the presence of octanoate or proline, which are both directly oxidized in the matrix. DNP addition results in a large and sustained increase in oxidation rate, whereas the decrease in cytosolic and mitochondrial ATP/ADP ratios is significantly less when compared with DHA or DHA + ethanol. Furthermore, ATP synthesis and utilization (e.g. gluconeogenesis) is partly preserved. The sustained increase of respiratory chain activity in uncoupled hepatocytes in the presence of octanoate or proline could be the consequence of the direct supply of reducing equivalents from the matrix without the need for the malate/aspartate shuttle, in contrast with DHA and ethanol metabolism. Hence the large increase in respiration, by compensating, at least partly, for the DNP-protonophoric effect, permits the maintenance of higher $\Delta\Psi_m$ with a persistence of ATP synthesis. We failed to find an increase in the mitochondrial $NADH/NAD^+$ ratio with octanoate when compared with DHA + ethanol. This could indicate that under our conditions 3-hydroxybutyrate dehydrogenase is not in equilibrium with the mitochondrial redox state and complex 1 of the respiratory chain. In addition, the increase in respiration could be the consequence of a high rate of $FADH_2$ supply to the respiratory chain since both β-oxidation and proline oxidation result in a 1:1 stoichiometry of NADH and $FADH_2$ production.

Hence, uncoupling liver cells in the presence of substrates, which are oxidized in the cytosol, such as carbohydrates, decreases (1) mitochondrial $\Delta\Psi_m$, (2) ATP/ADP ratios and (3) ATP-utilizing processes (e.g. gluconeogenesis); the increase in respiratory rate is only transient. Conversely, uncoupling liver cells in the presence of substrates oxidized in the mitochondrial matrix, such as fatty acids, results in a large and sustained increase in oxidation rate while the decrease in $\Delta\Psi$, ATP/ADP ratios and ATP synthesis and utilizing processes (e.g. gluconeogenesis) are limited.

Several uncoupling proteins have been described in various tissues [3–6,10]. In brown adipose tissue triacylglycerol storage, associated with active fatty acid transport and β-oxidation rate in mitochondria, permits very efficient fatty acid oxidation. Conversely, glycolysis is the main pathway for ATP synthesis in immune cells, whereas in muscle cells both carbohydrate and fatty acids can be substrates for ATP synthesis depending on the metabolic surroundings (fed or fasted state, insulin etc.). Hence, to achieve very efficient heat production, fatty acids represent the most suitable substrates and can limit the drop in $\Delta\Psi$ and ATP/ADP ratios. Conversely in cells which are mainly glycolytic, such as immune-competent cells, the result of uncoupling will be a decrease in $\Delta\Psi$ with its various consequences rather than a large change in the rate of energy wastage.

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