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On isolated hepatocytes mitochondrial swelling induced in hypoosmotic medium does not affect the respiration rate

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Abstract

In isolated hepatocytes incubated in hypoosmotic media, a large increase in the mitochondrial volume is not directly involved in the activation of respiration. Moreover, results of the quantification of the various bioenergetic parameters are not in accordance with an activation of the respiratory chain as previously proposed (Halestrap, A.P. (1989) Biochim. Biophys. Acta, 973, 355–382), but point more to an inhibition of respiration. The same respiration rate is obtained in hypoosmolar incubation media in vitro and in situ for a higher overall thermodynamic driving force over the electron transport chain.

Keywords: Hepatocyte; Volume; Osmolarity; Energetics; Cellular respiration

1. Introduction

It is now well known that an increase in cell volume, both in perfused liver and isolated hepatocytes, affects fluxes through some metabolic pathways, acts like an anabolic signal ([1]; for review), and stimulates and inhibits the biosynthesis and degradation pathways, respectively. Cell volume increase can be a consequence of either a decrease in extracellular osmolarity or an incubation with Na^+-cotransported amino acids [2]. Thus, cell swelling induced by either a hypoosmotic medium, exposure to amino acids or insulin (i) inhibits glycogenolysis, glycolysis [3,4], proteolysis [1,5–7], glutamine synthesis and urea synthesis from NH_4^+ [5,8] (ii) stimulates glycogen synthesis [2], protein synthesis [5], amino acid uptake [5,8,9], glutaminase [5] and urea synthesis from amino acids [5,8].

While the intermediary metabolism has been extensively studied and some mechanisms linking cell volume changes and the alterations of metabolic function have been proposed [10–12], little is known in this field regarding mitochondria in situ and the bioenergetic parameters (in hypoosmotic conditions the β-hydroxybutyrate/acetoacetate ratio decreases; Haüssinger's unpublished results, but see [1]).

On isolated mitochondria, the matrix volume influences mitochondrial metabolism in various ways. Hypotonic conditions [[13,14], for review] or hormone-induced swelling [15] increase the mitochondrial ATP/ADP ratio at the same time as stimulating pyruvate carboxylation, citrulline synthesis, succinate oxidation [16] and respiratory chain activity. It has been proposed that changes in mitochondrial matrix volume can be considered as a mechanism of hormone signal transduction across the liver mitochondrial membrane [14]. Glucagon, cAMP, and vasopressin all induce mitochondrial swelling and cell shrinkage, while phenylephrin induces both mitochondrial and cell swelling [14].

In this paper, we investigate the bioenergetic parameters of hypoosmotic cell swelling. As cell volume changes in hepatocytes are the consequence of the modification in extracellular osmolarity, this may greatly affect the mitochondrial compartment. Until now, no systematic studies concerning the energy state of the cell have been described in these conditions.

Abbreviations: ΔpH, mitochondrial transmembrane difference in pH; $\Delta\psi_m$, mitochondrial transmembrane difference in electrical potential; Δp, protonmotive force; $\Delta E'_h$, Gibbs free-energy difference in oxidation reaction, $\Delta G'_p$, Gibbs free-energy of ATP hydrolysis reaction; $\Delta\psi_p$, cellular transmembrane difference in electrical potential; J_{O2}, oxygen consumption flux; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TPMP, tetraphenyl methyl phosphonium; DMO, 5,5′-dimethyloxazolidine-2,4-dione.

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2. Materials and methods

2.1. Preparation and incubation of hepatocytes

Hepatocytes from 20–24 h starved male Wistar rats (200–250 g) were isolated by the collagenase method of Berry and Friend [17]; as modified by Groen et al. [18].

Hepatocytes (8–10 mg dry weight/ml) were incubated in 20 ml stopped plastic or glass vials in a shaking waterbath. The basic incubation medium was a Krebs-Henseleit-bicarbonate buffer (144 mM Na^+) at pH 7.4 [19] containing 2.5 mM Ca^{2+}, 2% defatted bovine serum albumin, a substrate (either 20 mM glucose or 4 mM octanoate as indicated), 0.1 μM $TPMP^+$, 0.5 mM mannitol, and 0.2 mg/ml inulin. Hypoosmotic, Na^+-depleted media were obtained by decreasing the Na^+ concentration of the buffer as indicated. All media were in equilibrium with a gas phase containing O_2/CO_2 (95:5). The temperature was 37° C.

2.2. Mitochondria preparation

The rat liver was quickly removed and put into an ice-cold isolation medium containing 225 mM sucrose, 1 mM EGTA and 10 mM Tris-HCl (pH 7.2). Mitochondria were isolated in the same medium according to Klingenberg and Slenzka [20]. The standard incubation medium was the isolation medium supplemented by 6 mM glutamate, 6 mM malate, 1 mM ADP, 5 μM $TPMP^+$ and 5 mM P_i-Tris.

2.3. Measurement of respiration rate

After 20 min incubation, the cell suspension was transferred to a thermostatically controlled oxygraph vessel equipped with a Clark-type electrode for determination of the respiration rate. Myxothiazol was used at 2 μg/ml in order to completely abolish mitochondrial respiration activity. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was used at 15 μM, which is the concentration inducing the maximal respiratory rate (not shown).

2.4. Measurement of cell and mitochondria volumes in situ

Hepatocytes were incubated in the presence of (i) either 2.5 μCi/ml ^3H_2O and 0.1 μCi/ml [^{14}C]carboxymethylinulin (added 1 min before sampling) for measurement of cell volume (ii) or 2.5 μCi/ml ^3H_2O and 0.1 μCi/ml [^{14}C]mannitol for measurement of mitochondrial volume [21]. After 25 min incubation, 1 ml of cell suspension was transferred to an Eppendorf™ tube and centrifuged for 5 s at 7600 × *g*. Within 10 s, 100 μl of the supernatant was removed into a scintillation vial containing 5 ml of a liquid scintillation cocktail (Beckman™), and the remainder was aspired. The pellet was resuspended with 300 μl $HClO_4$ 10%, centrifuged, and 200 μl of the supernatant treated as before. The volume of each isotope in the pellet was $(\mathrm{dpm}_c \cdot 3/2)/(\mathrm{dpm}_s/100)$ in μl.

2.5. Measurement of mitochondrial and cytosolic membrane potential in situ

Hepatocytes were incubated in the presence of (i) either 1 μCi/ml [^{3}H]$TPMP^+$ for measurement of $TPMP^+$ accumulation (ii) or 0.1 μCi/ml $^{36}Cl^-$ for measurement of Cl^- distribution [22]. Cell samples were treated as above. The [^{3}H]$TPMP^+$ accumulation ratio and the $^{36}Cl^-$ distribution were calculated as in [23]. Thus, the mitochondrial membrane potential was,

$$\Delta\Psi_{m(i-e)} = -60\log\frac{V_c a_m}{V_m a_{cy}} \times \left(\frac{[TPMP^+]_i}{[TPMP^+]_e}\,\frac{[Cl^-]_i}{[Cl^-]_e}\,\frac{(V_m+V_c)a_{cy}}{V_c a_e} - 1 \right) \tag{1}$$

(see also [24–26]). V_c, V_m, a_e, a_{cy} and a_m refer respectively to cellular and mitochondrial volumes, and apparent activity coefficients for $TPMP^+$ in the extracellular medium, the cytoplasm and the mitochondrial matrix. We redetermined a_e (because of our varying extracellular medium), as well as a_{cy} and a_m (see [23,27], for experimental procedures). We will take $a_e = 0.85$, $a_{cy} = 0.43$, $a_m = 0.38$.

2.6. Measurement of mitochondrial ΔpH in situ

The mitochondrial $\Delta pH_{(i-e)}$ was calculated from the [P_i] gradient across the mitochondrial inner membrane (the phosphate carrier is thought to operate close to equilibrium, see [28,29]). If the anion is translocated in an electroneutral manner associated with an equivalent amount of proton [30], and $[P_{i,tot}]_m = [P_i^{2-}]_m + [P_i^-]_m$

$$\log\frac{[P_i^-]_m}{[P_i^-]_{cy}} = pH_m - pH_{cy} \tag{2}$$

$$pH_m = pK_{a,m} + \log\frac{[P_i^{2-}]_m}{[P_i^-]_m} \tag{3}$$

Eq. (3) is re-arranged:

$$[P_i^-]_m = \frac{[P_{i,tot}]_m \cdot 10^{pK_{a,m}}}{10^{pH_m} + 10^{pK_{a,m}}} \tag{4}$$

Substituting into (2) gives:

$$10^{-13.7} \cdot (10^{pH_m})^2 + 10^{-7} \cdot 10^{pH_m} - \frac{[P_{i,tot}]_m}{[P_i^-]_{cy}} = 0 \tag{5}$$

We assumed that: pH_{cy} remained constant at 7, $pK_{a,m} = 6.7$ and $pK_{a,cy} = 6.9$ [31]. Thus, $[P_i^-]_{cy} = [P_{i,tot}]_{cy} \cdot 0.44$.

Subscripts m, cy, tot, refer to mitochondrial, cytosolic and total amounts, respectively.

2.7. Measurement of matrix volume, ΔpH and $\Delta\psi$ in isolated mitochondria

The mitochondrial suspension (4 mg protein ml^{-1}) was incubated in two different osmotic media (either 80 mM sucrose or 225 mM sucrose) supplemented with 6 mM glutamate, 6 mM malate, 1 mM ADP, 5 μM $TPMP^+$ and 5 mM P_i-Tris. In each condition, the mitochondrial volume, the electrical potential ($\Delta\psi_{m,i-e}$) and the pH difference (ΔpH_{i-e}) across the inner mitochondrial membrane were routinely determined in the presence of (i) 2.25 μCi ml^{-1} ^3H_2O and 0.45 μCi ml^{-1} [^{14}C]mannitol (ii) 2.25 μCi ml^{-1} [^{3}H]$TPMP^+$ and 0.45 μCi ml^{-1} [^{14}C]mannitol (iii) 2.25 μCi ml^{-1} [^{3}H]acetate and 0.45 μCi ml^{-1} [^{14}C]mannitol, respectively. After equilibration (3 min), mitochondria were separated from the medium by rapid centrifugation (10 s, 8000 × g). Within 10 s, 100 μl of the supernatant was removed into a scintillation vial containing 5 ml of a liquid scintillation cocktail, and the remainder was aspired. The pellet was resuspended with 300 μl $HClO_4$ 10%, centrifuged, and 200 μl of the supernatant treated as before. $\Delta\psi_{i-e}$ was measured by distribution of 0.05 μCi ml^{-1} $^{86}Rb^+$ (in the presence of 0.1 μg ml^{-1} valinomycin) and ΔpH_{i-e} was also measured by distribution of 0.45 μCi ml^{-1} [^{14}C]DMO. These two latter methods gave the same results as those routinely used (see above).

2.8. Compartmentation of ATP, ADP and P_i

The mitochondrial and cytosolic distribution of ATP, ADP and P_i were studied by using the digitonin fractionation method as described by Zuurendonk and Tager [32] (see also [33]), with a slight modification concerning the volumes used.

2.9. Measurement of intramitochondrial redox potential

The intramitochondrial $NADH/NAD^+$ ratio was determined by the metabolite indicator method [33] and by assuming the β-hydroxybutyrate dehydrogenase reaction was in near equilibrium (K_{app} = [AcAc] [NADH]/ [βOHBu] [NAD^+] i.e. 4.93 10^{-2}; [34]).

2.10. Assays

β-Hydroxybutyrate and acetoacetate were measured fluorometrically in neutralised $HClO_4$ extracts [35]. ATP, ADP were measured by HPLC using a reverse phase (Spherisorb, ODS II, 5 μm) column (0.46 × 25 cm) at 30° C. Elution was performed with a 25 mM sodium pyrophosphate/pyrophosphoric acid (pH 5.75) buffer at a flow rate of 1.2 ml/min. ATP, ADP were eluted after 3.97 and 4.92 minutes, respectively. Adenine nucleotide detection was performed at 254 nm; the determination was linear in a range of 3 to 3000 pmol. P_i was measured according to the method of Berenblum and Chain [36].

3. Results and discussion

Hepatocytes incubated in various hypoosmotic Na^+-depleted media (Table 1) swell whatever the substrate: on glucose as previously shown [2] and on octanoate. Cell swelling is related to a large increase in the mitochondrial volume. Both cellular and mitochondrial swelling are proportional to the decrease in extracellular medium osmolarity. It is worth noting that the increase in mitochondrial volume represents (at $[Na^+]_{ext}$ = 90 mM) 80% and 90% of the total increase in cell volume, with glucose and octanoate, respectively. This indicates that the cytosolic volume per se does not vary significantly during hypotonic exposure. While cellular response to a hypotonic stress is mainly due to ionic movements which limit water uptake, it may be that the mitochondrial response, in spite of some well known regulatory volume mechanisms (for example the K^+/H^+ exchanger [37, for review]), is essentially due to water uptake. Therefore, in Na^+-depleted media and whatever the substrate used, mitochondria behave like an osmometer and swell to balance the drop in cytosolic osmolarity.

The respiration rate was estimated as the oxygen consumption sensitive to myxothiazol at a concentration that completely inhibited the electron transfer through the respiratory chain. Myxothiazol-insensitive oxygen consumption did not vary when the extracellular medium osmolarity was decreased; it was measured at 7.5 ± 1.7 nat/min/mg dw ($n = 3$, each condition) and 5.3 ± 1.5 ($n = 2$, each condition) on glucose and octanoate, respectively. Fatty acids compared to glucose maintained a higher respiration rate by increasing the $NADH/NAD^+$ ratio, which in turn increased the supply of electrons to the respiratory chain (see [23,38]). This increased respiration rate correlated with an increased protonmotive force (Δp) as previously described [23]. Moreover, if we consider the relationship between J_{O2} and Δp, at the same protonmotive force the respiration rate was higher with octanoate than with glucose. This indicates that Δp-consuming processes are also stimulated by fatty acids [39,40].

When the extracellular medium osmolarity decreased either with glucose or octanoate, the respiration rate was maintained, whereas the $NADH/NAD^+$ ratio and the protonmotive force were decreased (Δp mainly followed the variations in $\Delta\psi_m$ although the ΔpH was greatly affected). From a thermodynamic point of view, the respiration rate is controlled by two associated forces: the span of the redox potential over the respiratory chain ($\Delta E'_h$) and the protonmotive force (Δp). This overall thermodynamic driving force over the electron transport chain is expressed

Table 1
Effects of hypoosmolar media on different cytosolic and mitochondrial parameters

	V_c µl/mg TP	V_m µl/mg MP	J_{O2} [a] nat/min/mg dw	J_{O2} [a] +CCCP nat/min/mg dw	NADH/NAD^{+} [b] ($\times 10^{-3}$)	NADH/NAD^{+} [b] +CCCP ($\times 10^{-3}$)	$-\Delta\Psi m_{(i-e)}$ (mV)	$\Delta pH_{(i-e)}$	$-\Delta p_{(i-e)}$ (mV)
Glucose; control	2.75 ± 0.15 (10)	0.98 ± 0.12 (5)	18.6 ± 1.3 (10)	29.0 ± 2.5	15.3 ± 1.5 (6)	4.1 ± 0.6	152.4 ± 5.8 (10)	0.34 ± 0.02	172.8
120 mM Na$^+$	3.22 ± 0.21 [e]	2.16 ± 0.24 [e]	21.6 ± 1.5	28.4 ± 1.9	11.3 ± 2.4 [e]	3.1 ± 0.4	136.34 ± 4.5 [e]	0.14 ± 0.02 [e]	144.7
90 mM Na$^+$	3.68 ± 0.18 [e]	2.97 ± 0.14 [e]	20.6 ± 1.1	30.1 ± 1.7	8.9 ± 1.9 [e]	2.8 ± 0.5	119.9 ± 6.3 [e]	0.06 ± 0.03 [e]	123.5
Octanoate; control	2.89 ± 0.14 (12)	0.95 ± 0.05 (8)	34.6 ± 1.1 (9)	37.0 ± 2.5	31.6 ± 2.3 (5)	22.5 ± 1.9	160.3 ± 4.3 (9)	0.44 ± 0.03	186.7
120 mM Na$^+$	3.34 ± 0.19 [e]	1.71 ± 0.18 [e]	33.4 ± 1.5	36.2 ± 1.8	27.6 ± 2.1 [e]	19.7 ± 2.2 [e]	142.8 ± 5.7 [e]	0.33 ± 0.02 [e]	162.6
90 mM Na$^+$	3.58 ± 0.11 [e]	2.66 ± 0.08 [e]	33.7 ± 1.5	39.5 ± 2.1	18.7 ± 2.9 [e]	14.9 ± 0.8 [e]	128.5 ± 3.4 [e]	0.14 ± 0.02 [e]	136.9
	$2\Delta E'_h - n\Delta p$ (d) (Volt)	$-\Delta\Psi_{p(i-e)}$ (mV)	ATP/ADP (b)	ATP/ADP (c)	$\Delta G'p$ (b) ($kJ \cdot mol^{-1}$)	$\Delta G'p$ (c) ($kJ \cdot mol^{-1}$)	ATP (b) nmol/mg dw	ATP (c) nmol/mg dw	
Glucose; control	−0.42	16.5 ± 1.2 (8)	2.61 ± 0.11	9.03 ± 0.09	40.34	51.33	4.42 ± 0.19 (9)	11.08 ± 0.47 (9)	
120 mM Na$^+$	−0.71	20.2 ± 1.7 (4) [e]	2.45 ± 0.09 [e]	11.83 ± 0.11 [e]	44.88	51.83	4.16 ± 0.22 (4) [e]	11.10 ± 0.39 (4)	
90 mM Na$^+$	−0.92	27.4 ± 2.3 (5) [e]	2.21 ± 0.12 [e]	10.02 ± 0.08 [e]	46.85	51.59	3.66 ± 0.25 (4) [e]	11.52 ± 0.38 [e]	
Octanoate; control	−0.29	14.7 ± 1.2 (9)	2.71 ± 0.24	6.92 ± 0.10	38.60	51.43	1.76 ± 0.24 (9)	9.43 ± 0.45 (8)	
120 mM Na$^+$	−0.59	19.7 ± 1.4 (5) [e]	2.52 ± 0.20 [e]	10.94 ± 0.11 [e]	41.89	53.21	1.45 ± 0.17 (4) [e]	10.05 ± 0.42	
90 mM Na$^+$	−0.80	29.3 ± 2.5 (4) [e]	2.19 ± 0.27 [e]	10.66 ± 0.14 [e]	45.81	52.72	1.22 ± 0.18 (4) [e]	10.48 ± 0.40 (4) [e]	

The basic incubation medium and the experimental conditions are described in the text. Hypoosmotic, Na$^+$-depleted media were obtained by decreasing the Na$^+$ concentration of the buffer from 144 mM to 120 or 90 mM as indicated. Values are means ± SD for (n) or at least 3 cellular preparations. [e] Value significantly different to the control ($P \leq 0,05$; ANOVA).

V_c = cellular volume; V_m = mitochondrial volume; MP = mitochondrial protein; TP = total protein (1 mg TP = 0.366 MP); [a] myxothiazol-sensitive; [b] mitochondrial; [c] cytosolic; [d] $\Delta E'_h$ is the difference in redox potential across the electron transport chain and n is the H$^+$/O stoichiometry of the electron transport chain ($n = 10$).

as $2\Delta E'_h - 10\Delta p$. It is worth noting that in spite of the net increase (more negative) in this thermodynamic force, the respiration rate remained stable. This result is inconsistent with the explanation proposed by Halestrap [14], for whom the increase in the respiration rate observed in vitro, when the mitochondrial volume increases, is mainly the consequence of a kinetic activation of the respiratory chain [16]. Such an explanation, based on the fact that when mitochondria swell the NADH level and the redox state of the flavoproteins decrease, is acceptable only if the protonmotive force measured in these conditions is not significantly changed. In fact, it is well known that when Δp decreases, the NADH level and the redox state of the cytochromes decrease as J_{O2} increases.

From our data, when the extramitochondrial osmolarity decreases, the increase in the overall thermodynamic force would be sufficient to cause an increase in the respiration rate, without any change in the kinetic constraints. It is therefore very surprising that the respiration rate in our conditions remained unchanged, whatever the extracellular osmolarity. Two explanations may be proposed: (i) a kinetic constraint may intervene at the level of the respiratory chain, thus drastically limiting the increase in the electron flux in response to the decrease in the protonmotive force (but this hypothesis is not in accordance with an increase in the oxidized state of the first complex) (ii) as the activity of the membrane enzymes is strongly dependent on their physical environment, it may be that changes in the mitochondrial volume induce a modification of the membrane organization that changes the response of the respiratory flux to the associated forces.

To test this latter hypothesis, we determined in isolated mitochondria the relationship between J_{O2} and Δp at two different extracellular sucrose concentrations: 80 mM and 225 mM. The mitochondrial volumes were 1.50 and 0.76 μl mg protein^{-1}, respectively. Fig. 1 shows the titration of the respiratory flux at different rates of ATP synthesis obtained by different quantities of oligomycin. We observed two distinct relationships between these two parameters. At the same respiration rate, the protonmotive force maintained in a hypoosmotic medium was always lower than that measured in an isoosmotic medium. This confirms the latter hypothesis.

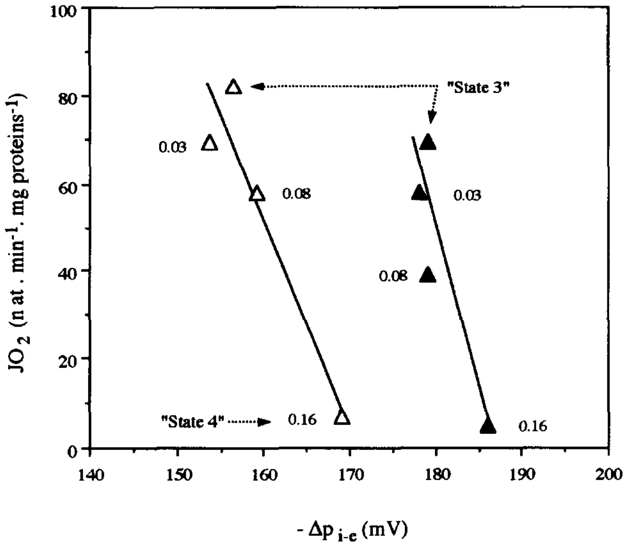

Fig. 1. Relationships between the respiration rate and the proton motive force obtained in isolated rat liver mitochondria when J_{O2} is titrated with various oligomycin quantities. (△) hypoosmolar medium: 80 mM sucrose; (▲) isoosmolar medium: 225 mM sucrose. The amount of oligomycin used (in μg mg prot^{-1}) is mentioned on the graph. For experimental conditions, see Materials and methods.

We also measured the respiration rate and the mitochondrial NADH/NAD$^+$ ratio in hepatocytes in the presence of a protonophoric uncoupler. The use of CCCP made it possible to estimate to what extent the reduced equivalents were delivered to the respiratory chain, and hence to determine the maximal respiration capacity. In each experimental condition, the addition of CCCP induced (i) a stimulation of the respiration rate, which was greater with glucose than with octanoate and (ii) a decrease in the NADH/NAD$^+$ ratio, which was also greater with glucose than with octanoate. These CCCP effects showed that whatever the external medium osmolarity, a drop in both Δp and NADH/NAD$^+$ ratio is linked to an increase in respiratory rate, as expected. However, in hypoosmotic conditions, and in spite of a significant decrease in both Δp and NADH/NAD$^+$ ratio, the respiratory rate was not enhanced. Considering our results obtained on both isolated and in situ mitochondria, this can be explained by the fact that an increase in mitochondrial volume induced a modification in the response of the respiratory chain to its associated forces. This change is such that the response of the respiratory rate to its associated forces is qualitatively maintained at each osmolarity; but quantitatively, when the mitochondrial volume increases, the overall thermodynamic driving force over the electron transport chain must be higher ($2\Delta E'_h - 10\Delta p$ more negative) to maintain the same respiratory rate.

When the incubations performed in the hypoosmolar medium were compared to their respective control, with both glucose or octanoate the maximal respiration rate (in the presence of CCCP) was unchanged. Moreover, while the NADH/NAD$^+$ ratio did not change with glucose, it decreased with octanoate (while respiration was maintained). In isolated mitochondria it is generally observed that the decrease in extramitochondrial medium osmolarity leads to an increase in the uncoupled respiration rate [41]. Unlike the situation observed in vitro, the availability in situ of the respiratory substrates is strictly dependent on some ATP-consuming processes involved in either glycolysis or fatty acid activation. In the presence of CCCP, the decreased ATP content could limit to the same extent the respiration rate in iso- or hypoosmotic media.

Beyond the availability of the reduced equivalents, the Δp-consuming pathways, through the availability of ADP and P_i, exert a significant control over the respiration rate.

Therefore, we measured the mitochondrial and cytosolic ATP/ADP ratios. Both with glucose and octanoate, an increased mitochondrial volume was related to a slight but significant decrease in the mitochondrial ATP/ADP ratio and a decrease in the mitochondrial ATP content. Our results obtained in situ seem in disagreement with those observed on isolated mitochondria by Armston et al. [13] (see also [14]): in vitro, in state 3, increasing the matrix volume by incubating mitochondria in a hypotonic medium increases the mitochondrial ATP/ADP ratio and also the mitochondrial ATP content. In situ, both with glucose and octanoate, when the extracellular medium osmolarity decreased, the mitochondrial P_i concentration decreased drastically (not shown) and led to a net increase in the intramitochondrial phosphate potential ($\Delta G'p$). Hence, the thermodynamic force involved in ATP synthesis ($\Delta G'p$) increases both (i) in situ, as a consequence of the decreased P_i concentration and (ii) in vitro, as a consequence of the increased mitochondrial ATP/ADP ratio.

In cytosol, the situation is different: (i) both with glucose and octanoate the ATP/ADP ratio was enhanced; (ii) the phosphate potential was stable on glucose and slightly increased on octanoate. In both cases the cytosolic ATP content was slightly enhanced. The rise in the cytosolic ATP/ADP ratio and the fact that the respiration rate remained stable indicate that the pathways of the intermediary metabolism that were activated during cell swelling did not represent a large source of energy consumption. Moreover, both with glucose and octanoate, increasing the cellular volume in the hypoosmotic medium increased the cellular transmembrane electrical potential ($\Delta\psi_p$), in accordance with the results obtained by Wondergem and Castillo [42] and Wang and Wondergem [43]. It has been demonstrated by these authors that hepatocytes exhibit a regulatory volume decrease during hypotonic shock, which comprises loss of intracellular K^+ and Cl^- accompanied by hyperpolarization of the transmembrane potential due to an increase in membrane K^+ conductance. They have also shown that ouabain has no effect on the change of $\Delta\psi_p$ either during or after hypotonic stress, thus suggesting that active pumping of K^+ by the Na^+-K^+-ATPase plays no role in compensating changes in cell K^+ during an increase in cell water volume. In these conditions, the ATP consumption linked to the functioning of the Na^+-K^+-ATPase was not enhanced. Moreover, the greater the decrease in extracellular osmolarity, the more the Na^+ gradient ($\Delta\mu Na^+$) on both sides of the membrane is decreased. This implies a lesser energy consumption to maintain the Na^+ gradient.

At present, the value of the driving force $2\Delta E'_h - 10\Delta p$ measured in situ seems to indicate that the rate of ATP synthesis decreases as external osmolarity decreases. In fact, the adenine nucleotide transporter is an electrogenic carrier and there is a relationship between the $\Delta\psi_m$ and the $(ATP/ADP)_{cyto}/(ATP/ADP)_{mito}$ ratio. It is well known that the adenine nucleotide translocator is a controlling step of ATP synthesis [44], and the observed $(ATP/ADP)_{cyto}/(ATP/ADP)_{mito}$ ratio does not reflect the thermodynamic equilibrium. Classically this ratio decreases when $|\Delta\psi_m|$ decreases. Here, it increased as the $\Delta\psi_m$ decreased, indicating that the adenine nucleotide carrier tends towards near its thermodynamic equilibrium. Moreover, in non-phosphorylating conditions (state 4), the proton flux generated by the respiratory chain strictly balances the ionic movements linked to the leak. In isolated mitochondria (Fig. 1), when the extramitochondrial medium osmolarity was decreased, the respiration was slightly increased while $-\Delta p_{i-e}$ was decreased. This indicates a higher proton or cation leak as the extramitochondrial medium osmolarity decreases. In situ, when the extracellular medium osmolarity decreased, the cytosolic phosphate potential was unchanged and the mitochondrial phosphate potential was increased. This result is in accordance with a lower energy consumption and therefore with a lower ATP synthesis. Moreover, the fact that the term $2\Delta E'_h - 10\Delta p$ was decreased in situ is not in favor of an increase in ATP synthesis.

In situ, the respiration rate is maintained in spite of the net decrease in ATP synthesis; this can be explained by the rise or the triggering of another source of energy consumption: for example, ion movements across the inner membrane that could dissipate $\Delta\psi m$ and/or ΔpH. Unlike the situation observed in vitro, in the extramitochondrial compartment, ions are largely involved in the maintenance of osmotic pressure. Moreover, changes in mitochondrial matrix volume strongly exhibit energy-dependent salt movements [37].

It is now well documented that cell swelling induced either by a hypoosmotic medium or insulin exposure stimulates glycogen and urea synthesis [2,5,8]. This implies a higher energy consumption through these metabolic pathways. From the results previously published by Gustafson et al. [45], in the presence of 20 mM glucose, the ATP flux associated to these pathways was 2.56 $\mu mol\ min^{-1}\ g^{-1}$ at 70 mM $[Na^+]_{ext}$, compared to the value of 1.34 in the control condition (isoosmotic medium). If we consider the respiratory rate measured in our experiments (Table 1) and an ATP/O ratio of 2.5, the increase in ATP consumption due to these pathways represents only 2 or 3% of the calculated ATP flux produced by oxidative phosphorylations. This leads to the conclusion that under these particular conditions (low level of substrates for these anabolic pathways), the increase in ATP consumption was very small compared to ATP production. The lack of significant increase in J_{O2} under hypoosmotic conditions was not in contradiction with the stimulation of some anabolic pathways.

In the literature, an increase in mitochondrial volume is often associated with an activation of respiration (i) in situ as a consequence of hormonal action, (ii) in vitro in hypoosmolar media. Halestrap [14,15] has proposed that the hormone-induced increase in the matrix volume could be directly involved in situ in respiratory chain activation. On isolated mitochondria Armston et al. [13] have shown that hypotonic incubations stimulate respiratory chain activity. In accordance with Halestrap, Quinlan et al. [21] also showed that on isolated mitochondria, an increase in the mitochondrial volume caused by valinomycin (a K^+ ionophore) is accompanied by an increase in the respiration rate. It should be noticed that in their experiments performed with hepatocytes, the increase in respiratory rate was always linked to an ion uptake in an unchanged external medium osmolarity. Thus, the respiration rate increase could be a consequence of a matrix ion accumulation rather than a rise in the volume per se (in our experiments, it is clear that water movements are the main processes involved in mitochondrial osmotic pressure adjustment).

4. Conclusion

In this paper we show that in isolated hepatocytes incubated in hypoosmotic media, a large increase in the mitochondrial volume is not directly involved in the activation of respiration. Moreover, results of the quantification of the various bioenergetic parameters are not in accordance with an activation of the respiratory chain as previously proposed, but point more to an inhibition of respiration. The same respiration rate is obtained in hypoosmolar incubation media in vitro and in situ for a higher overall thermodynamic driving force over the electron transport chain ($2\Delta E'_h - 10\Delta p$). From a strictly thermodynamic point of view, the decrease in $2\Delta E'_h - 10\Delta p$ could be associated with an increase in the respiration rate. However, since this is not so, there would seem to be greater kinetic constraints at the level of the respiratory chain. Moreover, when extracellular medium osmolarity decreases, it seems that the control exerted by the translocase decreases. This could be in accordance with an increase in the kinetic control at the level of the respiratory chain (or at the level of the supply of reduced equivalents). Such an increase in the kinetic control, at this level, could explain the lack of response of the respiratory flux to the overall thermodynamic force ($2\Delta E'_h - 10\Delta p$) (see [46]).

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References

[1] Häussinger, D. and Lang, F. (1991) Biochim. Biophys. Acta 1071, 331–350.
[2] Baquet, A., Hue, L., Meijer, A.J., Van Woerkom, G.M. and Plomb P.J.A.M. (1990) J. Biol. Chem. 265, 955–959.
[3] Graf, J., Haddad, P., Häussinger, D. and Lang, F. (1988) Renal Physiol. Biochem. 11, 202–220.
[4] Lang, F., Stehle, T. and Häussinger, D. (1989) Pflügers Arch. 413, 209–216.
[5] Hässinger, D., Hallbrucker, C., Vom Dahl, S., Lang, F. and Gerok, W. (1990) Biochem. J. 272, 239–242.
[6] Häussinger, D., Hallbrucker, C., Vom Dahl, S., Decker, S., Schweizer, U., Lang, F. and Gerok, W. (1991) FEBS Lett. 283, 70–72.
[7] Hallbrucker, C., vom Dahl, S., Lang, F. and Häussinger D. (1991) Eur. J. Biochem. 197, 717–724.
[8] Häussinger, D. and Lang, F. (1990) J. Cell. Biochem. 43, 355–361.
[9] Bode, B. and Kilberg, M. (1991) J. Biol. Chem. 266, 7376–7381.
[10] Meijer, A.J., Bacquet, A., Gustafson, L., Van Woerkom, G.M. and Hue, L. (1992) J. Biol. Chem. 267, 5823–5828.
[11] Baquet, A., Gaussin, V., Bollen, M., Stalmans, W. and Hue, L. (1993) Eur. J. Biochem. 217, 1083–1089.
[12] Völkl, H., Busch, G.L., Haüssinger, D. and Lang, F. (1994) FEBS Lett. 338, 27–30.
[13] Armston, A.E., Halestrap, A.P. and Scott R.D. (1982) Biochim. Biophys. Acta 681, 429–439.
[14] Halestrap, A.P. (1989) Biochim. Biophys. Act, 973, 355–382.
[15] Halestrap, A.P., Davidson, A.M. and Potter W.D. (1990) Biochim. Biophys. Acta 1018, 278–281.
[16] Halestrap, A.P. and Dunlop, J.L. (1986) Biochem. J. 239, 559–565.
[17] Berry, N.M. and Friend, D.S. (1969) J. Cell. Biol. 43, 506–520.
[18] Groen, A.K., Sips, H.J., Vervoorn, R.C. and Tager, J.M. (1982) Eur. J. Biochem. 122, 87–93.
[19] Krebs, H.A. and Henseleit, K. (1932) Hoppe-Seyler's Z. Physiol. Chem. 210, 33–66.
[20] Klingenberg, M. and Slenczka, W. (1959) Biochem. Z. 331, 486–495.
[21] Quinlan, P.T., Thomas, A.P., Armston, A.E. and Halestrap, A.P. (1983) Biochem. J. 214, 395–404.
[22] Nobes, C.D. and Brand, M.D. (1989) Biochim. Biophys. Acta 987, 115–123.
[23] Nobes, C.D., Hay, W.W. and Brand, M.D. (1990) J. Biol. Chem. 265, 12910–12915.
[24] Scott, I.D. and Nicholls, D.G. (1980) Biochem. J. 186, 21–33.
[25] Berry, M.N., Gregory, R.B., Grivell, A.R., Henly, D.C., Nobes, C.D., Phillips, J.W. and Wallace P.C. (1988) Biochim. Biophys. Acta 936, 294–306.
[26] Hoek, J.B., Nicholls, D.G. and Williamson J.R. (1980) J. Biol. Chem. 255, 1458-1464.
[27] Murphy, M.P. and Brand, M.D. (1987) Biochem. J. 243, 499–505.
[28] Van Dam, K., Westerhoff, H.V., Krab, K., Van der Meer, R. and Arents, J.C. (1980) Biochim. Biophys. Acta 591, 240–250.
[29] Ligeti, E., Brandolin, G., Dupont, Y. and Vignais, P. (1985) Biochemistry 24, 4423–4428.
[30] Palmieri, F., Quagliariello, E. and Klingenberg, M. (1970) Eur. J. Biochem. 17, 230–238.
[31] Ogawa, S., Rottenberg, H., Brown, T.R., Shulman, R.G., Castillo, C.L. and Glynn, P. (1978) Proc. Natl. Acad. Sci. USA 75, 1796–1800.
[32] Zuurendonck, P.F. and Tager, J.M. (1974) Biochim. Biophys. Acta 333, 393–399.
[33] Akerboom, T.P.M., Van der Meer, R. and Tager, J.M. (1979) Techniques in Metabolic Research, B205, 1–33.
[34] Krebs, H.A. and Veech, R.L. (1969) Pyridine nucleotide interrelations (S. Papa, J.M. Tager, E. Quagliariello and E.C. Slater, eds.), Adriatica Editrice, Bari, pp. 329–382.
[35] Bergmeyer, H.U. (Ed.) (1970) Methods of Enzymatic Analysis, 2nd ed., Verlag Chemie, Academic Press, London.
[36] Berenblum, I. and Chain, E. (1938) Biochem. J. 32, 286–294.
[37] Beavis, A.D. (1992) J. Bioenerg. Biomembr. 24 77–90.

[38] Berry, M.N., Clark, D.G., Grivell, A.R. and Wallace, P.G. (1985) Metabolism 34, 141–147.

[39] Debeer, L.J., Mannaerts, G. and DeSchepper, P.J. (1974) Eur. J. Biochem. 47, 591–600.

[40] Plomp, P.J.A.M., Van Roermund, C.W.T., Groen, A.K., Meijer, A.J. and Tager, J.M. (1985) FEBS Lett. 193, 243–246.

[41] Sitaramam, V., Sambasivarao, D. and Mathai, J.C. (1989) Biochim. Biophys. Acta 975, 252–266.

[42] Wondergerm, R. and Castillo, L.B. (1988) Am. J. Physiol. 254, G795–G801.

[43] Wang, K. and Wondergem, R. (1993) J. Membr. Biol. 135, 237–244.

[44] Groen, A.K., Wanders, R.J.A., Westerhoff, H.V., Van der Meer, R. and Tager, J.M. (1982) J. Biol. Chem. 257, 2754–2757.

[45] Gustafson, L.A., Romp, N., Van Woerkom, G.M. and Meijer, A.J. (1994) Eur. J. Biochem. 223, 553–556.

[46] Rigoulet, M., Guerin, B. and Denis, M. (1987) Eur. J. Biochem. 168, 275–279.