Adenine nucleotide compartmentation in foetal rat hepatocytes

Effects of atractyloside, oligomycin, calcium ionophore and adrenergic agonists

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In the presence of lactate plus pyruvate, or glucose or alanine as substrates, ATP/ADP ratios in the cytosol were higher than in mitochondria in isolated rat foetal hepatocytes. The cytosolic ATP/ADP ratios were dependent on substrate (lactate + pyruvate > glucose > alanine). Oleate increased the cytosolic ATP/ADP ratios in the presence of the other substrates studied. Atractyloside decreased the cytosolic ATP/ADP ratios, oligomycin decreasing these values in both compartments. Isoproterenol, phenylephrine and Ca^{2+} ionophore decreased the cytosolic ATP/ADP ratio, without altering this value in mitochondria.

Adenine nucleotide *(Rat fetal hepatocyte)* *Compartmentation*

1. INTRODUCTION

During the perinatal period rat liver mitochondria undergo many morphological and metabolic changes [1]. However, it seems that the energy-conservation system of rat liver mitochondria becomes fully functional only in the immediate post-natal period. Information concerning the phosphorylation state of adenine nucleotides has been reported [2–7].

Nevertheless, intracellular [ATP]/[ADP] ratios from approximately 1.5 to 5.7 were found using the freeze-clamp technique. Moreover, results reported in [7] using the digitonin fractionation procedure described by Zuurendonk and Tager [8] were obtained with foetal rat hepatocytes isolated by dispersion with lysozyme [7]. However, lysozyme has been proved to be disadvantageous for cell isolation, as pointed out by Seglen [9]. In addition, no molar concentrations of adenine nucleotides in both compartments were given [7].

The uncertainty about the phosphorylation state of adenine nucleotides in foetal rat liver, the availability of a non-perfusion method for the production of isolated parenchymal liver cells, well characterized, from late foetal rats, based on a collagenase incubation technique [10], prompted us to undertake a systematic study of the intracellular adenine nucleotide distribution in foetal rat hepatocytes. We also used digitonin to separate mitochondrial and cytosolic fractions from the cells.

This paper reports, for foetal rat hepatocytes obtained 22 days after conception, the adenine nucleotide contents and the [ATP]/[ADP] ratio in the cytosolic and mitochondrial compartments under several conditions.

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2. MATERIALS AND METHODS

Albino Wistar rats on day 22 of gestation (200–300 g body wt), fed on a stock laboratory diet, were killed for experiments between 09:00 and 10:00 h.

Isolated foetal rat hepatocytes were obtained and the cells (10–12 × 10^6 cells/ml) incubated as in [10]. This experimental procedure uses a collagenase-dispersion technique, which produces routinely about 1.5 × 10^7 cells/g foetal liver, representing approx. 15% recovery. Of these cells, at least 96% exclude trypan blue.

The method for cell fractionation was that described in [8]. For this technique we used silicone oil (Wacker Chemmie, type AR 200).

ATP and ADP were measured by standard enzymatic methods [11]. When the inulin-permeable space of the digitonin-treated cells was determined, [^{14}C]inulin (0.25 μCi/ml) and ^3H_2O (2.5 μCi/ml) were present in the digitonin fractionation medium.

Collagenase was purchased from Boehringer, Mannheim. Radioactive compounds were obtained from The Radiochemical Centre, Amersham, Bucks. Atractyloside, oligomycin, phenylephrine, isoproterenol and calcium ionophore A23187 were purchased from Sigma, St. Louis, MO. All other chemicals were of the highest purity available.

3. RESULTS AND DISCUSSION

3.1. *Viability of foetal rat liver cells submitted to the digitonin fractionation technique*

The foetal liver cell preparation isolated by the collagenase method contains at least 95% of viable cells as judged by trypan blue exclusion, a higher percentage than that reported by Van Lelyveld and Hommes [7]. The relative number of haematopoietic cells in the foetal liver cell preparation was < 5% at day 22 of gestation. When the isolated cells were fractionated with digitonin > 95% of the total lactate dehydrogenase was found in the supernatant (cytosolic fraction) in the presence of 1.6 mM digitonin. The supernatant contained maximally 9 and 0% of the total amount of glutamate dehydrogenase and cytochrome *c* oxidase, respectively. The high percentage of total lactate dehydrogenase found in the supernatant of centrifugation, digitonin-treated foetal hepatocytes, shows that a complete rupture of the plasma membrane occurs. On the other hand, digitonin fractionation did not damage the mitochondria, as shown by the low percentages of

Table 1

Adenine nucleotide compartmentation in digitonin-treated foetal rat hepatocytes

Substrates	Cytosol			Mitochondria		
	[ATP]	[ADP]	[ATP]/[ADP]	[ATP]	[ADP]	[ATP]/[ADP]
10 mM lactate + 1 mM pyruvate	3.08 ± 0.12	0.82 ± 0.08	3.76 ± 0.15	1.00 ± 0.07	1.00 ± 0.06	1.00 ± 0.06
5 mM glucose	2.96 ± 0.04	1.45 ± 0.05	2.04 ± 0.06***	0.88 ± 0.06	0.84 ± 0.09	1.05 ± 0.08
10 mM alanine	2.01 ± 0.06	1.76 ± 0.12	1.14 ± 0.08***	0.72 ± 0.04	1.21 ± 0.20	0.59 ± 0.08***
10 mM lactate + 1 mM pyruvate + 1 mM oleate	2.94 ± 0.15	0.63 ± 0.09	4.67 ± 0.20^{+++}	1.14 ± 0.06	1.00 ± 0.02	1.14 ± 0.07^{+}
5 mM glucose + 1 mM oleate	2.75 ± 0.06	1.06 ± 0.08	2.59 ± 0.09^{+++}	0.93 ± 0.01	0.79 ± 0.06	1.18 ± 0.07
10 mM alanine + 1 mM oleate	2.24 ± 0.06	1.48 ± 0.17	1.51 ± 0.21^{+}	0.95 ± 0.07	0.93 ± 0.04	1.02 ± 0.10^{++}

22 day foetal liver cells were fractionated as described in section 2 after incubations with the substrates shown for 20 min. Results (in mM) are expressed as means ± SE (n = 5–9). Values significantly different from those for 10 mM lactate + 1 mM pyruvate as substrates*, or from values for the corresponding substrates in the absence of oleate^{+}: $^{+}P<0.05$; $^{++}P<0.01$; ***,$^{+++}P<0.001$

glutamate dehydrogenase and cytochrome *c* oxidase found in the supernatant.

Table 1 shows that the intracellular ATP concentration in foetal rat hepatocytes, as obtained by summation of the cytosolic and mitochondrial contents, is about 4 mM (using 10 mM lactate and 1 mM pyruvate as substrates). This agrees with the level found in brown fat cells from foetus [12], but is much lower than that found in adult rat hepatocytes (about 13 mM) [13].

3.2. *Volume of the mitochondrial and cytosolic water space in foetal rat liver cells*

The presence of [^{14}C]inulin and $^{3}H_2O$ in the digitonin fraction allows one to calculate, after centrifugation, the relative distribution of the radioactivity in both compartments, viz. cytosol and mitochondria. Appropriate calculations yielded a cytosolic volume of 3.12 + 0.33 [5] μl/10^6 cells, and a mitochondrial volume of 0.43 + 0.05 [5] μl/10^6 cells. Anderson and Jones [14] obtained a value of 4.8 μl/10^6 cells for the cell volume in adult hepatocytes, which is of the same magnitude as those calculated in our studies. Moreover, subcellular morphometric studies using electron microscopy, and performed in foetal liver cells in the same gestational period [15], show similar values for the mitochondrial matrix space as compared with that obtained in our studies.

3.3. *ATP and ADP concentrations, and [ATP]/[ADP] ratios in the cytosolic and mitochondrial compartments of isolated foetal rat liver cells*

As shown in table 1, the cytosolic [ATP]/[ADP] ratio obtained in the presence of lactate (10 mM) and pyruvate (1 mM) as substrates was higher than that found using glucose (5 mM) or alanine (10 mM). With alanine, the [ATP]/[ADP] ratio in mitochondria was lower than with lactate plus pyruvate or glucose as substrates (table 1). These results indicate that lactate is the best oxidative substrate compared with glucose or alanine.

In the presence of oleate, the [ATP]/[ADP] ratio increases in cytosol with all substrates and in mitochondria with alanine (table 1). However, the values for cytosolic [ATP]/[ADP] in the foetal hepatocytes were about 1.5–5 mM under all conditions studied and in adult hepatocytes about 9 mM [13], suggesting that at the foetal stage oxidative phosphorylation did not occur at state 3 rates.

Table 2

Effect of atractyloside, oligomycin, phenylephrine, isoproterenol and ionophore A23187 on cytosolic and mitochondrial [ATP]/[ADP] ratios in digitonin-treated foetal rat hepatocytes

Effectors	Cytosol			Mitochondria		
	[ATP]	[ADP]	[ATP]/[ADP]	[ATP]	[ADP]	[ATP]/[ADP]
-	3.08 ± 0.12	0.82 ± 0.08	3.75 ± 0.15	1.00 ± 0.07	1.00 ± 0.06	1.00 ± 0.06
Atractyloside (100 μM)	2.47 ± 0.06	1.78 ± 0.08	1.39 ± 0.12[a]	0.95 ± 0.11	0.70 ± 0.13	1.36 ± 0.22
Oligomycin (1 μg/ml)	1.73 ± 0.09	2.03 ± 0.08	0.85 ± 0.05[a]	0.46 ± 0.02	1.39 ± 0.16	0.33 ± 0.10[a]
Phenylephrine (10 μM)	2.66 ± 0.10	1.48 ± 0.42	1.80 ± 0.18[a]	1.04 ± 0.17	1.00 ± 0.13	1.04 ± 0.09
Isoproterenol (1 μM)	2.70 ± 0.14	1.40 ± 0.10	1.93 ± 0.10[a]	0.95 ± 0.07	0.95 ± 0.15	1.00 ± 0.10
Calcium ionophore A23187 (5 μM)	2.43 ± 0.10	1.33 ± 0.12	1.83 ± 0.15[a]	0.84 ± 0.05	0.79 ± 0.07	1.06 ± 0.09

22 day foetal liver cells were incubated for 20 min in the presence of 10 mM lactate + 1 mM pyruvate, and then incubated for a further 10 min in the presence of effectors. The cells were then fractionated as described in section 2. Results (in mM) are expressed as means ± SE (n = 5–9). Values significantly different from those for no effector added: [a]$P < 0.001$

3.4. *Effects of inhibition of adenine nucleotide translocation, inhibition of oxidative phosphorylation, adrenergic stimulation and calcium ionophore action, on adenine nucleotide compartmentation in isolated foetal hepatocytes*

As shown in table 2, inhibition of adenine nucleotide translocation by atractyloside (100 μM) leads to a marked decrease in the cytosolic [ATP]/[ADP] ratio. Such inhibition is in agreement with the concept that during the perinatal period, the inner mitochondrial membrane undergoes a maturation process which changes a relatively permeable membrane into the osmotically active membrane present in adult rat liver mitochondria [16]. The foetus would reach this status of mitochondrial differentiation at about the time of birth.

Inhibition of oxidative phosphorylation by oligomycin (1 μg/ml) (table 2) decreased the cytosolic and mitochondrial [ATP]/[ADP] ratios. This marked decrease demonstrates the sensitivity of mitochondrial phosphorylation activity to oligomycin at this time of foetal development.

Phenylephrine, isoproterenol or ionophore A23187 decreased the cytosolic [ATP]/[ADP] ratio, without altering the value in mitochondria (table 2). In addition, phenylephrine and isoproterenol stimulate glycogen breakdown and glucose release in foetal hepatocytes [17]. Then, the α-agonist phenylephrine (or ionophore A23187) and the β-agonist isoproterenol may stimulate glycogen breakdown in foetal hepatocytes, which may account for the decrease in [ATP]/[ADP] ratios observed in the cytosolic compartment (table 2).

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