REGULATION OF THE MITOCHONDRIAL OXIDATION-REDUCTION STATE IN INTACT HEPATOCYTES BY CALCIUM IONS

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SUMMARY: The mitochondrial NADH:NAD ratio of isolated intact liver cells incubated in calcium-free Hanks solution, in the endogenous state or with lactate, alanine, α-ketoglutarate, glutamate, fumarate, malate, or albumin-bound palmitate, was elevated by 1 mM $CaCl_2$. The chloride salts of Ba^{+2}, Cd^{+2}, Cu^{+2}, Mn^{+2}, Sr^{+2}, Zn^{+2}, Al^{+3}, Ce^{+3} and La^{+3} caused no such change. In contrast, calcium decreased the mitochondrial NADH:NAD ratio of hepatocytes incubated with succinate. Calcium did not affect the NADH:NAD ratio in the liver cell cytosol or the energy charge. The calcium-induced elevation in the mitochondrial NADH:NAD ratio was reversed by the uncoupler 1799. These observations demonstrate a specific effect of calcium ions in the regulation of the mitochondrial oxidation-reduction state in intact liver cells.

INTRODUCTION

In liver mitochondria acetoacetate is produced in the matrix compartment (1) and undergoes reduction to β-hydroxybutyrate, catalyzed by β-hydroxybutyrate dehydrogenase (EC 1.1.1.30, equation 1), an integral protein (2) of the inner membrane (3). Equilibrium concentrations of acetoacetate and β-hydroxybutyrate are achieved, which reflect the NADH:NAD ratio (NAD oxidation-reduction state) in the mitochondrial matrix (4).

$$\text{acetoacetate} + \text{NADH} + H^+ \rightleftharpoons \text{D-}\beta\text{-hydroxybutyrate} + NAD^+ \quad \text{(equation 1)}$$

The production ratio of β-hydroxybutyrate:acetoacetate in isolated intact hepatocytes from fasting rats was 0.4-0.5 (5,6). However, we have observed that the ratio in blood and liver in these rats ranges from 2 to 3, in agreement with other observations (7). In the study of factors responsible for this difference, it was found that calcium ions elevate the mitochondrial NADH:NAD ratio during the oxidation of NAD-linked substrates in intact hepato-

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cytes as described herein. This is in contrast to the decrease in the NADH:NAD ratio when calcium is added to isolated mitochondria in the presence of phosphate and various substrates, demonstrated by pyridine nucleotide fluorescence and analysis of respiratory states (8,9). The mitochondrial NAD oxidation-reduction state is of major significance in energy production and in the control of cell metabolism. The regulatory influence of calcium on the mitochondrial NADH:NAD ratio in intact hepatocytes was therefore investigated.

MATERIALS AND METHODS

Male Holtzman rats (200-300 g) were given water and Rockland rat diet ad libitum. Food was removed between 9 and 11 a.m. and 24 hours later the liver cells were isolated by the procedure of Berry and Friend (10) as previously described (5). The liver cells were incubated at 37° C in a total volume of 2.0 ml in calcium- and glucose-free Hanks medium containing 10 mM sodium phosphate buffer, pH 7.4. This medium is designated in the text as suspension medium. The incubations were terminated by the addition of 0.5 ml of cold 30% perchloric acid and after centrifugation, the extracts were neutralized with 0.24 ml of 3 M K_2CO_3. The pH was adjusted to 6.0-6.5 with small increments of 3 M K_2CO_3. Acetoacetate, β-hydroxybutyrate and liver cell dry weight were determined as described earlier (5). ATP, ADP and AMP were measured by fluorometric procedures (11). Lactate (12) and pyruvate (13) were also measured. Palmitate was bound to fatty acid-free bovine serum albumin as previously described (5). Substrate solutions were all adjusted to pH 7.4 with NaOH or HCl.

TABLE I

β-HYDROXYBUTYRATE:ACETOACETATE RATIO IN LIVER CELLS: EFFECT OF CALCIUM

Incubation Medium	β-Hydroxybutyrate: Acetoacetate Ratio
(a) 2.25% albumin - 1.5 mM palmitate	.32 ± .04
(b) 50% blood serum	1.66 ± .06*
(c) 2.25% albumin - 1.5 mM palmitate + calcium chloride (1.25 mM)	1.72 ± .02*

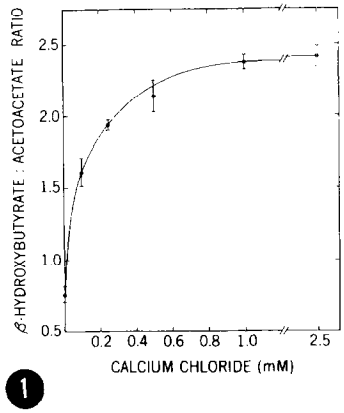
*P < .001 relative to group (a); Mean values ± S.E.M. are given.

All flasks contained 1.0 ml of isolated liver cells (14.5 mg dry wt) in suspension medium. The three groups below contained, in addition (a) 1.0 ml of 4.5% albumin - 3 mM palmitate in suspension medium, (b) 1.0 ml of blood serum, from rats fasted 24 hours, fortified with palmitate to a total free fatty acid concentration of 3.0 mM, and (c) 1.0 ml of 4.5% albumin - 3.0 mM palmitate containing 2.5 mM $CaCl_2$. The flasks were incubated 30 min at 37°C. The blood serum contained 190 nmoles of acetoacetate and 930 nmoles of β-hydroxybutyrate. These quantities of ketone bodies were added to the flasks in groups a and c prior to incubation.

RESULTS AND DISCUSSION

The β-hydroxybutyrate:acetoacetate production ratio of 0.35, exhibited by isolated liver cells incubated with albumin-bound palmitate, was markedly elevated by replacement of the albumin-palmitate substrate with blood serum (Table I). This elevation was equalled by the addition of calcium chloride, 1.25 mM, to the albumin-palmitate medium. Repeated demonstration of this observation has led to the conclusion that calcium ions exert a regulatory influence on the oxidation-reduction state of the pyridine nucleotides in the mitochondria of intact hepatocytes. This effect of calcium ions is opposite to that observed in isolated mitochondrial systems (8,9).

A half-maximal increase in the β-hydroxybutyrate:acetoacetate ratio was exhibited at a calcium chloride concentration of 100 μM and this elevation reached a maximum at a concentration of 1 mM (Fig. 1). Higher levels of calcium in the medium did not cause reversal. An observable effect of calcium on the hepatocyte β-hydroxybutyrate:acetoacetate ratio was detected after 8 minutes of incubation and a steady state was achieved after 20 minutes (Fig. 2).



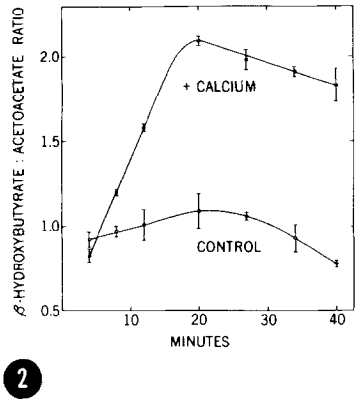


Figure 1: Effect of $CaCl_2$ level on the β-hydroxybutyrate:acetoacetate ratio in liver cells. The cells (14.3 mg dry wt) were incubated with 1.5% albumin - 0.75 mM palmitate for 20 min. Total ketogenesis at 0, 0.1, 0.25, 0.5, 1.0 and 2.5 mM added $CaCl_2$ was 49, 63, 58, 59, 65 and 60 nmoles/mg dry wt. Ranges of duplicate values are shown.

Figure 2: Rate of elevation of the β-hydroxybutyrate:acetoacetate ratio in liver cells by $CaCl_2$. The cells (11.1 mg dry wt) were incubated with 1.5% albumin - 0.75 mM palmitate. Total ketogenesis at 4, 8, 12, 20, 27, 34 and 40 min in the control was 11, 23, 29, 50, 67, 82 and 89 and with 1 mM $CaCl_2$ was 12, 22, 31, 58, 78, 106 and 118 nmoles/mg dry wt. Ranges of duplicate values are shown.

TABLE II

EFFECTS OF CALCIUM ON THE β-HYDROXYBUTYRATE:ACETOACETATE RATIO IN ISOLATED HEPATOCYTES INCUBATED WITH DIFFERENT SUBSTRATES

		Total Ketone Body Production (nmoles/mg dry wt)		β-Hydroxybutyrate: Acetoacetate Ratio	
	Substrate	Control	+ 1mM $CaCl_2$	Control	+ 1mM $CaCl_2$
1.	endogenous	32.1± .3	37.1± .7†	0.02±.00	0.07±.01*
	fumarate (10 mM)	28.7± .1	36.2±1.1†	0.20±.01	0.27±.01†
	L-malate (10 mM)	31.8± .3	35.6± .4†	0.19±.00	0.34±.01*
	L-glutamate (10 mM)	28.9±1.0	37.5± .5†	0.10±.00	0.22±.01*
2.	endogenous	24.3± .3	27.4± .3†	0.06±.01	0.18±.00*
	α-ketoglutarate (10 mM)	15.3± .4	24.2± .4*	0.19±.00	0.30±.01*
	DL-β-hydroxybutyrate (20 mM)	——	——	1.51±.06	3.73±.04*
3.	endogenous[¶]	22.3±1.1	28.4± .3[§]	0.13±.01	0.20±.01†
	palmitate (.75 mM)-albumin (1.5%)	37.2±1.1	45.5± .5†	0.22±.01	0.75±.01*
	L-lactate (10 mM)	22.4± .6	23.5± .5	0.36±.01	0.62±.01*
	succinate (10 mM)	20.3± .4	32.1±1.1*	1.07±.00	0.80±.05*

Isolated hepatocytes were incubated 30 min at 37°C in suspension medium with various substrates at the final concentrations shown. The total volume was 2.0 ml. The dry cell weights/flask in the 3 experiments above were: 1) 9.5 mg 2) 17.5 mg and 3) 9.2 mg.

[§]$P < .01$; $^{\dagger}P < .005$; *$P < .001$ relative to control. Mean ± S.E.M.

[¶]Endogenous ± 1.5% fatty acid free albumin gave identical results.

The chloride salts of Ba^{+2}, Cd^{+2}, Cu^{+2}, Mn^{+2}, Sr^{+2}, Zn^{+2}, Al^{+3}, Ce^{+3} and La^{+3} exerted no such effect, indicative of a high degree of specificity in the calcium-induced elevation of the mitochondrial NAD oxidation-reduction state.

In the foregoing experiments, effects of calcium were observed in cells incubated with palmitate. Calcium also increased the β-hydroxybutyrate:acetoacetate ratio in hepatocytes incubated in the absence of added substrates and in the presence of fumarate, L-malate, L-glutamate, α-ketoglutarate, β-hydroxybutyrate, or L-lactate (Table II). In contrast, when the cells were incubated with succinate, calcium decreased the β-hydroxybutyrate:acetoacetate ratio.

Calcium also increased ketogenesis in the endogenous state and in the presence of nearly all substrates examined. In addition, all substrates elevated the β-hydroxybutyrate:acetoacetate ratio relative to the endogenous state and fumarate, L-glutamate, α-ketoglutarate, and succinate decreased ketogenesis.

The effect of calcium on the lactate:pyruvate ratio and the energy charge was then investigated (Table III). L-alanine (2.5 mM) was added to produce sufficient pyruvate to accurately measure, in the same extracts used for all other assays. Alanine itself only slightly increases the β-hydroxybutyrate: acetoacetate ratio and calcium exerts an effect on this ratio of similar magnitude to that observed in the endogenous state. Succinate alone elevates the lactate + pyruvate pool size to levels accurately measurable. This effect of succinate may be caused by inhibition of pyruvate dehydrogenase.

Whereas calcium markedly altered the β-hydroxybutyrate:acetoacetate ratio

TABLE III

β-HYDROXYBUTYRATE:ACETOACETATE RATIO, LACTATE:PYRUVATE RATIO AND ENERGY CHARGE IN ISOLATED LIVER CELLS INCUBATED WITH DIFFERENT SUBSTRATES

Addition‡	β-Hydroxybutyrate: Acetoacetate Ratio		Lactate:Pyruvate Ratio		Energy Charge§	
	Control	+ $CaCl_2$	Control	+ $CaCl_2$	Control	+ $CaCl_2$
1. alanine	.18±.01	.24±.01†	4.3± .6	3.3±.4	.86±.00	.87±.01
succinate + alanine	.65±.02	.43±.02*	6.3± .4	5.1±.5	.85±.01	.85±.01
palmitate-albumin + alanine	.63±.04	1.05±.03*	7.2± .2	7.5±.9	.82±.01	.83±.00
2. succinate	1.16±.06	.55±.02*	9.2±1.2	8.3±.6	.86±.01	.85±.01
3. endogenous	.11±.01	.17±.01†	——	——	.86±.01	.85±.01

The isolated liver cells were incubated for 30 min at 37°C in a final volume of 2.0 ml with and without $CaCl_2$, 1 mM. The dry cell weights per flask in the 3 experiments above were 1) 34.0 mg 2) 24.3 mg and 3) 28.1 mg.

§Energy charge = [ATP] + 1/2 [ADP] ÷ [ATP] + [ADP] + [AMP]

‡Final concentrations were: 2.5 mM L-alanine; 10 mM succinate; 0.75 mM palmitate-1.5% albumin.

†$P < .005$; *$P < .001$ relative to the control value. Mean values ± S.E.M. are given.

in liver cells, the lactate:pyruvate ratio was unaffected (Table III). The calcium-induced alteration in the mitochondrial NAD oxidation-reduction state therefore appears to be mediated by direct interaction of calcium in the mitochondria and not via primary effects on the NADH:NAD ratio in the cytosol. Improved definition of the mitochondrial cristae, when calcium was added to the medium during the isolation of liver cells, reported by Howard, Lee and Pesch (14), may be a morphological correlate of the present findings. Palmitate and succinate, with or without added calcium, elevated the NADH:NAD ratio in both compartments (Table III). Despite the wide range in the mitochondrial NADH:NAD ratio, in response to the different substrates employed, the energy charge (15) remained in the normal range.

In other experiments it was observed that the β-hydroxybutyrate:acetoacetate ratio, in the presence of 0.75 mM palmitate-1.5% albumin was: control 0.31±.01; + 1 mM $CaCl_2$ 1.95±.04; + 5 μM 1799* .12±.04 and + 1 mM $CaCl_2$ + 5 μM 1799 .09±.01. The uncoupler 1799 reversed the effect of calcium.

Numerous studies with isolated mitochondria have shown that in the presence of phosphate and various oxidizable substrates, calcium decreases pyridine nucleotide fluorescence and increases oxygen consumption, indicative of NADH:NAD ratio depression concurrent with calcium accumulation (8,9). The opposite effect of calcium on the mitochondrial NADH:NAD ratio observed in the present study indicates the importance of the plasma membrane and/or the cell cytosol in modulating the interaction of calcium with mitochondria in liver cells.

The elevation of the mitochondrial NADH:NAD ratio by calcium in the endogenous state and in the presence of palmitate and numerous other substrates (Table II) could result from either enhanced NADH production, via accelerated substrate oxidation, or restricted NADH utilization. That calcium increased the β-hydroxybutyrate:acetoacetate ratio from all NAD-linked substrates (Table II) favors the hypothesis that calcium restricts NADH utilization. Calcium-dependent control of the flow of electrons from NADH to oxidized flavin, and

* 1,1,1,7,7,7-hexafluoro-2,6-dihydroxy-2,6-bis(trifluoromethyl)-hepta-4-one

possibly also at subsequent sites in the respiratory chain, might thereby influence the formation of the intermediate form of energy utilized for the phosphorylation of ADP. Depletion of this energy by the uncoupler 1799 reversed the effect of calcium, indicating that calcium regulates the steady state redox level of the pyridine nucleotides only in the coupled system. Calcium-controlled restriction of electron flow would be expected to restrict reverse electron flow (16). The fact that calcium decreased the mitochondrial NADH:NAD ratio when the hepatocytes were incubated with succinate, demonstrative of decreased reverse electron transport, thereby supports this concept.

Wilson et al. (17) have reported near-equilibrium between the mitochondrial respiratory chain and the extramitochondrial phosphorylation potential in liver cells. Calcium may therefore affect the oxidation-reduction state of other segments of the respiratory chain, in addition to the NAD couple, and provide a greater oxidation-reduction gradient between sequential isopotential groups of electron carriers.

Although it is not clear how calcium exerts the defined effects on the NADH:NAD ratio in liver cells, evidence detailed herein suggests interaction of calcium with one or more respiratory carriers of the inner mitochondrial membrane. The influence of calcium on the mitochondrial NAD oxidation-reduction state may be of major importance in the regulation of cell metabolism, via several pyridine nucleotide-linked reactions, and in the function of the respiratory chain in maintaining the phosphorylation state of the adenine nucleotide system.

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