IN VITRO AND IN VIVO EFFECT OF *ESCHERICHIA COLI* ENDOTOXIN ON MITOCHONDRIAL PHOSPHOLIPASE A_2 ACTIVITY

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1. Introduction

Endotoxemia causes mitochondrial alterations that have been characterized by both morphologic and enzymatic changes [1–9]. Endotoxin elicits a clear diminuition in respiratory control (RCR and ADP/O ratio) and succinate–cytochrome *c* reductase activity of liver mitochondria from shocked rats [10], in agreement with the correlation between shock onset and loss of mitochondrial ability to maintain membrane-bound Mg^{2+} [5–9]. Effectively, an intact mitochondrial membrane is required for oxidative phosphorylation and active cation transport [11,12] and this integrity depends largely upon phospholipids [13].

The content in arachidonic acid is highly diminished in both phosphatidylcholine and phosphatidylethanolamine from shocked mitochondria; the released arachidonic acid contributes to the pool of mitochondrial free fatty acids [10].

To study further the molecular mechanisms of endotoxic shock, we have investigated the relation between the main mitochondrial phospholipids, and free fatty acids and calcium translocation in mitochondria.

2. Experimental

Male Wistar rats (200–250 g body wt) were used in all experiments. They were fasted overnight and allowed water until the experiments were begun.

Endotoxin shock was induced in unanesthetized rats by an intravenous (i.v.) injection of *Escherichia coli* lipopolysaccharide W (Difco Labs.) at 5 mg/kg body wt in 5 mg/ml saline solution. Shocked rats were classified in groups exhibiting either a reversible state of shock (E_1) or an irreversible state with circulatory collapse (E_2) [14]. Livers were quickly excised from the rats from which tissue homogenates or mitochondrial preparations were obtained [15].

In the in vitro experiments 25 μCi/animal of [2-^{14}C]-ethanolamine hydrochloride (spec. act. 44 mCi/mmol; Amersham) was i.v. administered 1 h before sacrifice.

Total lipids [16] were fractionated into lipid classes by thin-layer chromatography as in [17]. Phospholipid classes were evaluated according to [18]. Fatty acid methyl esters [19] were quantitated by the internal patron method [20]. Positional fatty acid distribution in phosphoglycerides was done as in [21].

Phospholipase A_2 activity in mitochondria was measured as in [22] using 75 mM sucrose, 225 mM manitol, 20 mM Tris (pH 7.4), 2 mM $CaCl_2$, 0.06 mg lipopolysaccharide/mg mitochondrial protein in 0.5 ml mitochondrial suspension (30 mg protein/ml) at 18°C for 30 min. Incubation was stopped with chloroform–methanol (2:1,v/v).

Calcium concentration in mitochondrial preparations was determined through the absorbance changes at 540–510 nm in a dual wavelength Aminco DW-2a spectrophotometer using murexide as indicator, according to [23]. The assay medium was that described for the phospholipase A_2 assay.

Radioactivity measurements were carried out in a Packard 3255 liquid scintillation spectrometer using as scintillation mixture PPO 4 g, POPOP 100 mg and toluene to 1 liter.

All biochemical reagents were from Sigma Chemical Co. (St Louis MO). Solvents were of analytical grade.

Table 1
Percentages of lysophosphatidylethanolamine in mitochondria in different incubation systems

System	Incubation time (min)		
	0	30	60
Control (n = 7)	1.34 ± 0.28	1.57 ± 0.28	1.94 ± 0.36
Ca^{2+} (n = 10)		3.00 ± 0.70	2.32 ± 0.32
Ca^{2+}, LPS (n = 10)		2.56 ± 0.58	1.88 ± 0.43
Ca^{2+}, A-23187 (n = 6)		3.23 ± 0.34	–
LPS (n = 6)		1.79 ± 0.11	1.32 ± 0.28
A-23187 (n = 6)		1.36 ± 0.25	1.43 ± 0.13

Values are referred to the total ethanolamine phosphoglycerides

3. Results and discussion

Rat liver mitochondria were incubated in vitro in the presence of endotoxin, Ca^{2+}, ionophore A-23187 and their mixtures and the levels of lysophosphatidylethanolamine were evaluated in all cases (table 1). The precise determination of this lyso-derivative was done by [^{14}C]ethanolamine injection into the rats before sacrifice; thus, the levels of phosphatidylethanolamine and its lyso-form were carefully evaluated by radioactivity measurement after two-dimensional thin-layer chromatography. As can be seen in table 1, the presence of lipopolysaccharide is only accompanied by a small alteration in the relative content of lysophosphatidylethanolamine when mitochondria were incubated for as long as 30 min, but it is accompanied by a notable increase in the mitochondrial content of some free fatty acids (table 2). However, a clear increase in the amount of mitochondrial lysophosphatidylethanolamine is achieved in response to Ca^{2+} alone or plus either lipopolysaccharide or the ionophore; thus, the presence of Ca^{2+} results in a notable alteration of the phospholipid composition. The presence of the ionophore A-23187 does not modify the relative composition of mitochondrial ethanolamine phosphoglycerides. In the light of a positional analysis of phospholipid acyl moieties, these data suggest that 1-acyl-lysophosphatidylethanolamine accumulates in mitochondria in response to Ca^{2+} loading.

The free fatty acid accumulation correlates extensively with the increase of the lyso-derivative. Table 2 gives the relative release of two groups of fatty acids, saturated and monounsaturated fatty acids acylating carbon 1 of the glycerol moiety, and the polyunsaturated acids occurring at carbon 2. These values demonstrate the high extent to which the positional segregation of individual fatty acid species affects position 2. Thus, the absence of release of fatty acids by the ionophore correlates with the constancy of mitochondrial lysophosphatidylethanolamine. Further, Ca^{2+} induces the accumulation in mitochondria

Table 2
Ratios of μg free fatty acid/μg free fatty acid in control mitochondria

Fatty acid	Incubation system				
	Ca^{2+}	Ca^{2+} + LPS	A-23187 + Ca^{2+}	LPS	A-23187
16:0	1.08 ± 0.12	1.03 ± 0.06	1.04 ± 0.12	0.98 ± 0.07	1.00 ± 0.20
18:0	1.48 ± 0.34	1.53 ± 0.37	1.18 ± 0.32	1.64 ± 0.16	0.93 ± 0.19
18:1	1.85 ± 0.49	1.38 ± 0.23	1.18 ± 0.23	0.97 ± 0.02	1.15 ± 0.32
18:2	6.72 ± 1.75	4.42 ± 0.80	4.57 ± 0.44	1.40 ± 0.55	1.06 ± 0.10
20:4[a]	1.54 ± 0.35	1.99 ± 0.68	1.19 ± 0.15	2.24 ± 0.20	1.20 ± 0.42

[a] Including 20:3

Mitochondria were incubated for 30 min in different systems (n as in table 1)

of linoleic acid, that is partially counteracted by the simultaneous presence of endotoxin. However, the release of fatty acids from phosphatidylethanolamine attained under mitochondrial membrane loading conditions with lipopolysaccharide results in a selective increase in the level of arachidonic acid; supported by [10] where a high correlation of the arachidonic acid content in mitochondria with the shock process was established. The specific release of arachidonic acid stimulated by lipopolysaccharide is also in agreement with the low increase in the levels of the lysoderivative produced by the endotoxin. Since both polyunsaturated fatty acids, 18:2 and 20:4, are mainly located at position 2 of phosphatidylethanolamine, the preferential accumulation of either linoleic acid or arachidonic acid, induced by Ca^{2+} or lipopolysaccharide, respectively, could result from a degree of enzyme specificity of phospholipase A_2 towards classes of substrate.

Fig.1 shows the in vitro variation of concentrations of extramitochondrial calcium in a series of conditions, measured through the absorbance changes at 540–510 nm in a dual wavelength spectrophotometer using murexide as indicator. The addition of $CaCl_2$ is followed by an initial decrease of Ca^{2+} and the corresponding translocation into the mitochondria. The initial slope of Ca^{2+} translocation gives an indication of the uptake rate of a certain amount of calcium. The total amount of cation translocated varies according to the experiment and afterwards calcium is released from the mitochondria into the medium. The presence of 1.2 mg lipopolysaccharide/ml clearly accelerates the uptake that achieves its maximum value at ~4 min after the addition of the Ca^{2+} to the system; the higher the concentration of lipopolysaccharide, the higher the rate of calcium uptake by the mitochondria. Thus, in the concentration range studied, the presence of lipopolysaccharide correlates closely with the rate of Ca^{2+} uptake; also, the higher the levels of lipopolysaccharide the more rapid is Ca^{2+} release from the mitochondria.

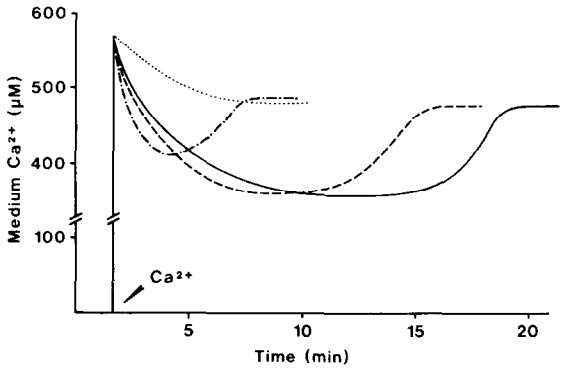

Fig.1. Changes of extramitochondrial calcium concentration using 30 μM murexide as indicator. Rat liver mitochondrial protein concentration 2 mg/ml. Control (——); 0.2 lipopolysaccharide/ml (– – –); 1.2 mg lipopolysaccharide/ml (–·–·–); 4 μM ionophore A-23187 (. . .).

These data support the proposal that lipopolysaccharide modifies the Ca^{2+} uptake and the Ca^{2+}-activation of the mitochondrial phospholipase A_2 with resulting hydrolysis of inner membrane phospholipids and increase in the permeability.

Comparison of data from table 2 and fig.1 indicates, nevertheless, that the effect of lipopolysaccharide on the mitochondria does not follow a fatty acid pattern identical to that of Ca^{2+} added to the incubation medium, although the endotoxin effect involves a

Table 3
Mitochondrial phosphoglycerides (μg P/mg mitochondrial protein) in different shock conditions

State	Phosphoglyceride			
	PE	LPE	PC	LPC
Control	1.54 ± 0.45	0.062 ± 0.013 (3.8)[a]	2.11 ± 0.30	0.016 ± 0.009 (0.7)[b]
E_1	1.74 ± 0.34	0.061 ± 0.027 (3.4)	2.18 ± 0.25	0.020 ± 0.012 (0.9)
E_2	1.34 ± 0.22	0.120 ± 0.028 (8.2)	2.33 ± 0.30	0.150 ± 0.029 (6.0)

[a] Percentages of the total ethanolamine phosphoglycerides
[b] Percentages of the total choline phosphoglycerides

E_1, Reversible endotoxic state; E_2, Irreversible endotoxic state

Table 4
Levels of free fatty acids in serum (μg/ml), liver (μg/mg protein) and liver mitochondria (μg/mg protein) in different shock conditions

Fatty acid	Serum			Liver			Mitochondria		
	Control	E_1	E_2	Control	E_1	E_2	Control	E_1	E_2
Total	121 ± 30	155 ± 40	207 ± 50	2.03 ± 0.22	2.16 ± 0.24	2.70 ± 0.62	1.24 ± 0.38	1.43 ± 0.26	4.83 ± 0.49
16:0	39.0 ± 3.5	58.7 ± 6.1	82.8 ± 14.4	1.08 ± 0.02	1.20 ± 0.19	1.27 ± 0.12	0.49 ± 0.16	0.59 ± 0.19	1.55 ± 0.30
18:1	31.4 ± 14.2	38.8 ± 11.6	29.4 ± 6.8	0.42 ± 0.10	0.46 ± 0.11	0.78 ± 0.20	0.35 ± 0.09	0.41 ± 0.10	0.92 ± 0.17
18:2	21.2 ± 10.9	13.6 ± 6.6	2.0 ± 0.4	0.16 ± 0.05	0.11 ± 0.05	0.39 ± 0.14	0.04 ± 0.02	0.04 ± 0.01	0.22 ± 0.07
20:4	1.4 ± 0.5	1.1 ± 0.3	1.7 ± 0.1	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.01 ± 0.005	0.02 ± 0.01	0.20 ± 0.02

E_1, Reversible endotoxic state; E_2, Irreversible endotoxic state

significant uptake and mobilization of the cation. The difference is mainly concerned with the nature of fatty acid release from the phospholipids, either 18:2 or 20:4, according to the mitochondrial treatment. In both cases, either lipopolysaccharide or Ca^{2+} treatment, the large scale hydrolysis of endogenous phospholipids is likely due to the involvement of a mitochondrial phospholipase; on the other hand, the preferential release of polyunsaturated fatty acids could be interpreted to indicate that different phospholipids are selectively distributed in the mitochondrial membranes. It is clear, then, that lipopolysaccharide stimulates Ca^{2+} uptake by the mitochondria, possibly through stimulation of Ca^{2+} binding. The endotoxin-mediated stimulation of Ca^{2+} uptake shows concentration dependency and provokes simultaneously a decrease in the variation ratio 18:2/20:4 of the released fatty acids, that becomes <1 when lipopolysaccharide acts in the absence of Ca^{2+} (table 2).

Phospholipids from the outer membrane, mainly phosphatidylethanolamine and phosphatidylcholine, contain high percentages of 20:4 while 18:2 is specially abundant in phospholipids, including cardiolipins, from the inner mitochondrial membrane. It is also known that calcium influences the structure of membranes containing acidic phospholipids [24–32] through fusion [24,25], lateral phase separation [26–29] and permeability changes; the release of bound calcium accompanies a phospholipid structural reorganization [30] mainly dependent upon calcium concentration, ionic strength and phospholipid molecular species [29–32,33]. In spite of these observations it is not possible to predict the structural or organizational impact of calcium on any native biological membrane [34]. These data could support the proposal that intramitochondrial phospholipase A_2 is activated by either Ca^{2+} or lipopolysaccharide at locations with different phospholipid environments from which the different polyunsaturated fatty acids are released. Also, the Ca^{2+}-dependent mobilization of arachidonic acid from an endogenous phospholipid pool lends support to the idea that Ca^{2+}-mediated activation of phospholipase A_2 participates in the control of mitochondrial activity.

Ionophore A-23187 alone does not result in any significant influence on the level of lyso-derivative (table 1) nor in the modification of the fatty acid release (table 2); also, the presence of the ionophore in the Ca^{2+}-system does not affect the variations mediated through Ca^{2+} alone.

Table 3 gives the induced changes in the levels of phosphatidylethanolamine, phosphatidylcholine and their lyso-derivatives by endotoxin given in vivo at two different clinical states of shock. Thus, a reversible endotoxic state does not induce any modification on these levels whereas in an irreversible state the levels of both lyso-derivatives increase markedly. However, the constancy in the levels of the diacylated phosphoglycerides may be explained through transacylation reactions in agreement with other results [35] on the increase of palmitoyl transferase in dog heart mitochondria by endotoxin given in vivo or in vitro. The net results of this situation would be an activation of the lipolytic system with a generalized increase of mitochondrial free fatty acids that influence, although to a lesser extent, the fatty acid content of liver and serum (table 4) at the irreversible shock state. Concerning the nature of the fatty acids released in vivo, the relative increase follows the order 20:4 > 18:2 > 18:1 = 16:0 in both liver mitochondria and liver; this variation does not affect significantly the levels of free fatty acids in the serum. Interestingly, the level of 18:2 in the serum free fatty acids at the irreversible shock condition is extremely low, suggesting an in vivo specific activity of lipopolysaccharide on either lipolytic activity of adipose tissue or fatty acid reutilization; it is difficult, however, to evaluate the physiopathological relevance of this result at this time.

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