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CYTOPLASMIC CELLULAR STRUCTURES CONTROL PERMEABILITY OF OUTER MITOCHONDRIAL MEMBRANE FOR ADP AND OXIDATIVE PHOSPHORYLATION IN RAT LIVER CELLS

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The kinetics of regulation mitochondrial respiration by external ADP in permeabilized hepatocytes was studied further. In digitonin-permeabilized hepatocytes, the apparent Km for ADP in regulation of respiration was decreased from 275 ± 35 μM in control to 48 ± 8 μM by a treatment with trypsin (15 min, 0.125 mg/ml). In liver tissue homogenates, trypsin treatment similarly decreased the Km value for ADP. These results show that ADP diffusion in hepatocytes may be retarded due to some unknown cytoplasmic trypsin-sensitive protein factor(s) which may be lost during isolation of mitochondria. Since we have previously reported a limited permeability of the outer mitochondrial membrane in isolated hepatocytes (Saks *et al.* 1995, Biochem. Biophys. Res. Commun., 208, 919-926), we conclude that an important site of control of respiration in liver cells *in vivo* is located at the porin channels of the outer mitochondrial membrane.

The outer membrane of mitochondria is considered to be permeable for compounds with molecular mass less than 6 kDa due to large diameter (about 2.5 nm) of its porin pores (1, 2). However, it has recently been shown that in permeabilized isolated cardiomyocytes, skinned cardiac fibers, slow twich skeletal muscle fibers and permeabilized hepatocytes the permeability of the outer membrane of mitochondria for ADP is very low. In these cells, the apparent Km for ADP in regulation of oxidative phosphorylation was shown to be higher by more than order of magnitude when compared to isolated mitochondria (3-8). Mitochondrial swelling leading to disrupture of the outer membrane (3, 6-8) resulted in a low Km for ADP, comparable to that of isolated mitochondria. The purpose of the current work was to investigate the cellular factors which may influence the characteristics of regulation of respiration by ADP in permeabilized hepatocytes or liver tissue homogenates by using short

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treatment with trypsin. The results show that mitochondrial outer membrane permeability to external ADP is controlled by some cytoplasmic protein structure(s).

MATERIALS AND METHODS

Preparations. Hepatocytes were isolated from rat liver by the method of Groen *et al.* (9) and permeabilized by using a digitonin treatment (6 µg/mg dw, 2 min; 4°C). After washing, cells were resuspended in an incubation medium containing 250 mM sucrose (or 125 mM KCl as indicated), 1 mM EGTA, 20 mM Tris-HCl, pH 7.2. Cell membrane permeabilization was always evidenced by the lack of Trypan blue exclusion. Liver mitochondria were prepared according to Klingenberg and Slenczka (10) in the incubation medium. For homogenization of liver tissue liver samples (approx 2 g wet mass) were minced and rinsed with the ice-cold incubation medium to remove residual blood cells. The liver mash was then placed in a glass homogenizer tube. After 3 passages with a loose Teflon pestle, the homogenate was passed though a 0.8 mm filter and centrifuged (50 g, 2 min). The supernatant was removed and the pellet resuspended in the incubation medium.

Trypsin treatment. Permeabilized hepatocytes, isolated liver mitochondria or liver homogenates were treated with trypsin (0.125 mg/ml) at 4°C. After 15 min, trypsin action was stopped with trypsin inhibitor (0.5 mg/ml).

Electron microscopy. For electron microscopy study, cell preparation was initially fixed in 2.5 % glutaraldehyde in 0.05 M cacodylate buffer pH 7.2, then post-fixed in 1 % osmic acid buffered by 0.05 M cacodylate, dehydrated in ethanol and propylen oxid, and embedded in Epon. Semi-thin and thin sections were prepared according to the standard procedure.

Analytical procedures. Oxygen consumption rates were measured polarographically at 25°C using a Clark electrode in a 2 ml closed vessel and in the incubation medium. The basal respiratory rate was obtained with addition of 5 mM tris-succinate and 5 mM tris-Pi in the presence of 1.25 µM rotenone. Increasing concentrations of ADP were added (from 5 to 1200 µM) and oxygen consumption rates were recorded. Cytochrome aa3 determination was performed using a double beam spectrophotometer (941 plus Uvikon - Kontron). Difference spectra between suspensions in the incubation medium supplemented with 1.25 µM rotenone and in the same medium supplemented with rotenone plus 5 mM tris-succinate and 5 mM KCN were recorded. Cytochrome aa3 concentrations were calculated using the extinction coefficient at 605 nm equal to 24/mM.cm (11).

RESULTS

Figure 1 shows permeabilized hepatocytes before (A-B) and after trypsin treatment (C-D). After trypsin treatment of permeabilized hepatocytes, mitochondria preserve their normal appearance but vacuolization of endoplasmic reticulum with some loss of ribosomes seems to be apparent. Figure. 2 shows the recordings of respiration of hepatocytes. In intact cells (Fig. 2, H), addition of ADP (1 mM) had no effect on respiration rate demonstrating the integrity of the plasma membrane of our preparation, since it is impermeable to ADP. Conversely, after permeabilization of the cells with digitonin (Fig. 2, PH), ADP addition stimulated respiration. Since addition of cytochrome c had no effect on the respiration rate in permeabilized hepatocytes, it indicates the intactness of the outer mitochondrial membrane. It must be noted that respiration rate was dependent upon cytochrome c addition when outer membrane was disrupted by osmotic shock (8). Trypsin treatment of permeabilized hepatocytes (Fig. 2, TPH) did not alter the maximal respiration rate in the presence of ADP, and the respiration was not dependent on cytochrome c addition. Thus, after trypsin

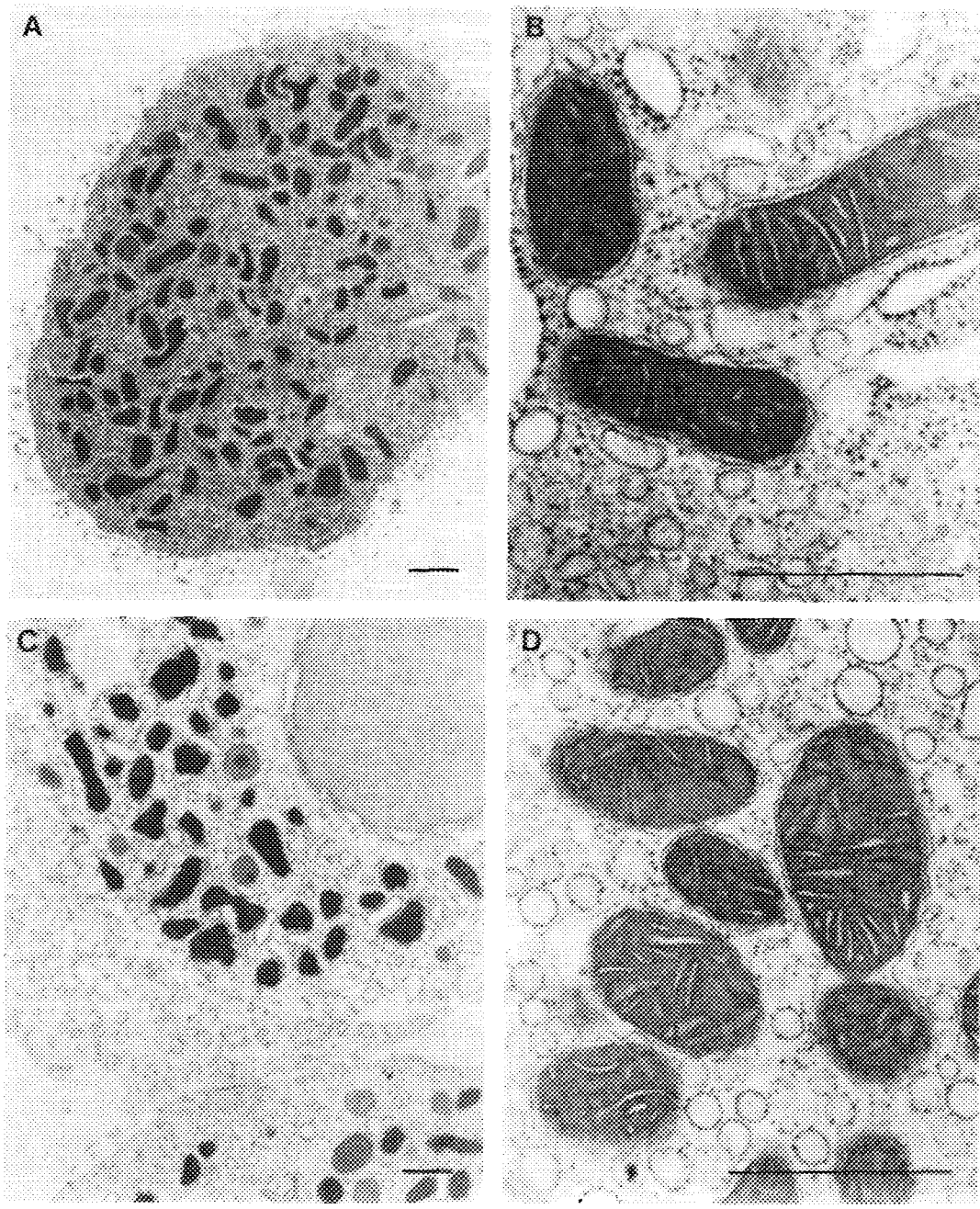

Fig. 1. Electron microscopy photographs of permeabilized hepatocytes before (A, B) and after (C, D) trypsin treatment.
In trypsin-treated permeabilized hepatocytes, only slight morphological modifications appear, including loss of some ribosomes. The bar length represents 1 μm.

treatment, the outer mitochondrial membrane remained intact. Figure. 3 A-B shows the kinetics of respiration regulation by ADP in permeabilized hepatocytes with or without trypsin treatment and in isolated mitochondria. In isolated liver mitochondria, in accordance

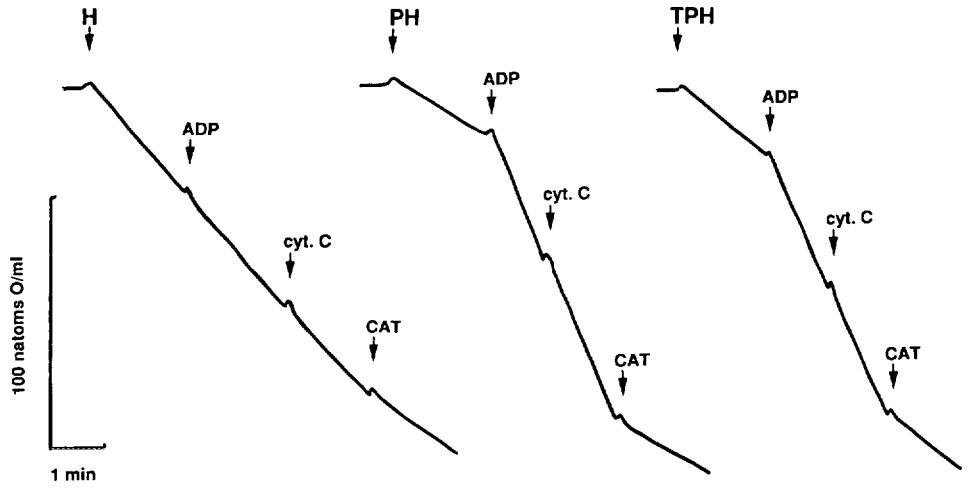

Fig. 2. Recording of respiration of isolated hepatocytes (H), permeabilized hepatocytes (PH) and trypsin-treated permeabilized hepatocytes (TPH).
The plasmic membrane permeabilization is demonstrated by the fact that oxygen uptake increased with ADP addition. The intactness of outer mitochondrial membrane is demonstrated by the absence of effet of cytochrome c in a 125 mM KCl medium.

with classical data by Chance and Williams (12) and Lardy and Wellman (13), respiration was maximally activated in the presence of several tens of micromoles of ADP, with apparent Km value of 22 ± 3 μM (Fig. 3 and Table I). In permeabilized hepatocytes, the respiration rate increased slowly with the increase of ADP concentration in the medium and for maximal respiration activation, almost millimolar ADP concentration was necessary and, in accordance with our recent data (8), the apparent Km was 275 ± 35 μM (Fig. 3, Table I). Figure 3 A-B shows that short treatment with trypsin had a drastic effect on the value of apparent Km for ADP in the permeabilized hepatocytes which was shifted to a low value equal to 48 ± 8 μM (Table I), close to that in isolated mitochondria. However, in isolated mitochondria trypsin did not alter this parameter (Table I). It should be emphasized that the dramatic change in Km observed in Figure 3 was not accompanied by a significant change in the Vmax when expressed as natom O_2 per nmol cyt.aa3. Thus, the large Km decrease observed after trypsin treatment was not linked to a change in Vmax.

Potentially important aspect for respiration regulation may be the influence of mitochondrial localization in the cell, and more precisely, the influence of mitochondrial clustering, proposed by Jones *et al.* for explanation of altered oxygen concentration dependence of respiration of liver cells (14,15). To study this question, we decided to investigate the respiration regulation of mitochondria in liver tissue homogenates, in which cellular structures are significantly destroyed by homogenization procedure. Figure 3 C-D shows the results which were obtained in studies of the dependence of respiration on ADP concentration in tissue homogenates before and after trypsin treatment. The results were similar to those obtained in permeabilized liver cells. Before trypsin treatment, the respiration rate in tissue homogenates increased slowly with elevation of extracellular ADP concentration, and the Km value was high, as already described for permeabilized

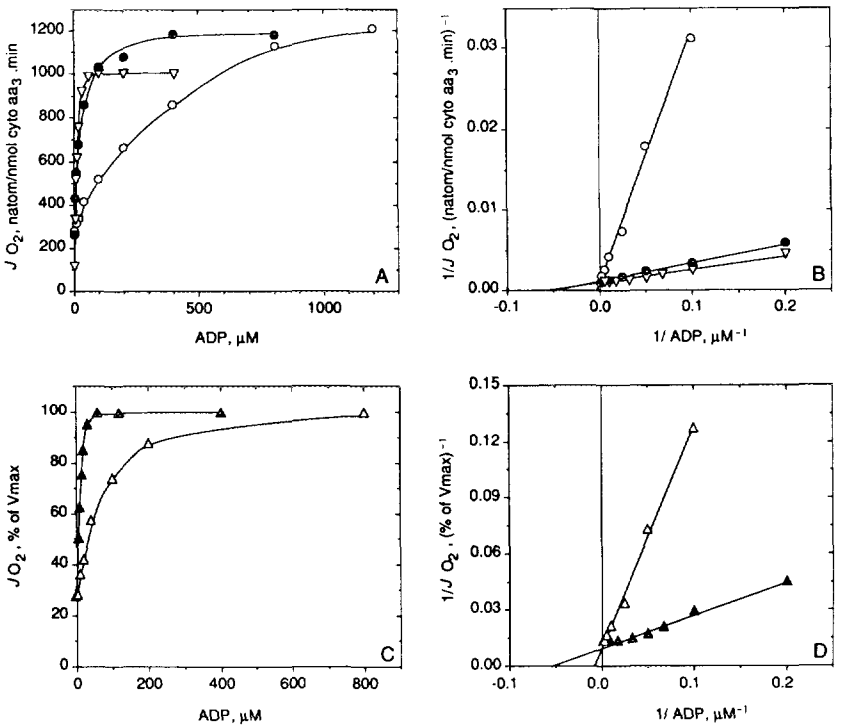

Fig. 3. A.B. The dependence of respiration rates of isolated mitochondria (▽) and of permeabilized hepatocyte before (○) or after (●) trypsin treatment on external ADP concentration (A) and their linearization in double-reciprocal plots (B).

C.D. The dependence of respiration rates of rat liver homogenates before (Δ) and after (▲) trypsin treatment on external ADP concentration (C) and their linearization in double-reciprocal plots (D).

hepatocytes. However, after trypsin treatment, the respiration rate increased very rapidly with elevation of ADP concentration, decreasing apparent Km value to that found in isolated mitochondria. These results show that the trypsin-sensitive cytoplasmic factor controlling the mitochondria outer membrane permeability for ADP is still present in tissue homogenates. The various data obtained under all experimental conditions and the relevant statistical analysis are summarized in Table I.

DISCUSSION

The mechanism of mitochondrial oxidative phosphorylation and its regulation are thought to be well understood (16,17), and one of the most interesting and basic problems of modern bioenergetics is development and application of methods of Metabolic Control Analysis for quantitative description of flux - rate of oxygen consumption and oxidative phosphorylation - control by various steps of the process (16-21). For this purpose, usually the experimental data obtained in the *in vitro* experiments with isolated mitochondria from liver (less often from heart) are used. Generally, flux control is considered to be distributed over various steps of the process, such as adenine nucleotide translocase, respiratory chain

Table I : Apparent Km of oxidative phosphorylation for ADP, in different preparations of permeabilized hepatocytes, liver homogenate and liver mitochondria

Preparation	n	apparent Km (ADP), μM	
Permeabilized hepatocytes	9	275 ± 35	
Permeabilized hepatocytes treated with trypsin	11	48 ± 8	*
Liver homogenate	7	120 ± 20	*
Liver homogenate treated with trypsin	7	24 ± 6	$
Mitochondria	10	22 ± 3	
Mitochondria treated with trypsin	7	20 ± 2	

Results are expressed as mean ± standard error. Comparisons were made by using unpaired t test, $p < 0.01$ denoting significance (* : *vs* permeabilized hepatocytes, $: *vs* liver homogenates).

or Krebs cycle enzymes, substrate and phosphate carriers, ATP synthase, ... etc (18-25). In accordance with many earlier experimental data, we have previously reported (8) that in the liver cells an important site of control of oxidative phosphorylation *in vivo* is in fact located at the outer mitochondrial membrane which significantly retards the intracellular diffusion of ADP. The present data strongly suggest that the permeability of the outer mitochondrial membrane for ADP may be controlled by cytoplasmic protein structure(s). This mechanism seems to be general for several types of cells, since it has been recently shown to exist in cardiomyocytes and in slow skeletal muscle cells (3-7).

The results of the current study are in line with those reported by Jones *et al.* (14, 15) who have demonstrated the existence of concentration gradients for ATP and oxygen in liver cells. The explanation proposed by the authors was mitochondrial clustering (15). That may work well for explanation of oxygen concentration gradients (14, 15); but in the process of intracellular diffusion of adenine nucleotides, such an explanation may be only of minor importance. Indeed, firmly based on the data reported in this work, it appears that the high apparent Km for ADP in regulation of the rate of respiration, showing its retarded diffusion, is decreased by order of magnitude by short treatment with trypsin, without any change in mitochondrial clustering (see Fig. 1). Furthermore, a high apparent value of Km for ADP in regulation of respiration is also seen in liver tissue homogenates, in which the clustering effect may be expected to decrease in the process of mechanical destruction of the cells.

The possibility of control of ADP movement across the outer mitochondrial membrane has been discussed already rather intensively in detailed studies of porin channels of the outer mitochondrial membrane (1, 2, 26-36). In addition, in mitochondrial preparations the permeability of porin channels may be changed by dextran or other polymers which increase the oncotic pressure (26, 27, 34). Colombini *et al.* have reported the presence of conserved protein in mitochondrial preparations which may modulate the activity of the porin channel (35, 36). In accordance with these data, we report here that the low permeability of the outer mitochondrial membrane for ADP in permeabilized liver cells (8) seems to be controlled by cytoplasmic protein structures since trypsin eliminates the

kinetic difference between isolated mitochondria and mitochondria in their intact surrounding.

The alternative explanation that the apparent Km for exogenous ADP may be increased in permeabilized cells due to ADP-utilizing or binding enzymes is strongly invalidated by the experimental findings that inhibition of adenylate kinase in liver (8) and other type of permeabilized cells (3, 4) and complete extraction of myosin from muscle cells (3) did not change the high value of this parameter. According to the data described in this paper and previous reports, there are three ways of decreasing the apparent Km for ADP in these cells: mitochondrial outer membrane rupture by hypoosmotic treatement, trypsin treatment and mitochondrial isolation. The first procedure also does not change mitochondrial clustering (See Fig 2d in ref. 8) but opens the direct way for ADP to translocase. The two other procedures do not change the properties of the outer membrane itself as shown by cytochrome c test but increase the permeability of the porin pores in this membrane. Tryspsin most probably destroys some cytoplasmic proteins attached to membrane and controling the permeability of porin, and mitochondrial isolation may detach this (these) protein(s) from mitochondria. The nature of the cytoplasmic protein factor(s) which seem(s) to control cellular respiration is unknown (6). However, it is interesting to note that there is a rapidly increasing number of publications describing the connection of mitochondrial outer membrane to the cytoskeleton elements. Leterrier *et al.* (37) have reported for brain mitochondria that there are specific porin-containing domains in outer membrane which bind microtubule-associated proteins and several other still unidentified cytoskeleton or cytoplasmic elements. This mitochondrial - cytoskeleton interaction was influenced by ATP hydrolysis (37). Bereiter-Hahn and Voth (38) demonstrated recently the dynamics of mitochondria in living cells (shape changes, dislocation, fusion and fission) that may result from interaction of mitochondria with elements of the cytoskeleton. Lazzarino *et al.* (39) demonstrated the binding of yeast mitochondria to phalloidin-stabilized F-actin, that was ATP-sensitive, reversible, and blocked after protease digestion of mitochondrial outer membrane proteins. The results reported by us in this work are in line with these studies and show that the interaction of mitochondria with cytoplasmic proteins or elements of cytoskeleton may be important for *in vivo* regulation of respiration. The existence of cytoplasmic (cytoskeletal) controlling factor(s) described in this work should result in further compartmentation of adenine nucleotides in the intermembrane space of mitochondria, beside their compartmentation in mitochondrial matrix and in cytoplasm (4). Understanding the importance of the compartmentation of adenine nucleotides in the intermembrane space of mitochondria for regulation of liver cells energy metabolism requires further studies.

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