Molecular and Cellular Biochemistry **195:** 191–197, 1999.

Induction of permeability transition in pancreatic mitochondria by cerulein in rats

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Received 6 April 1998; 24 July 1998

Abstract

Hyperstimulation with cholecystokinin analogue cerulein induces a mild edematous pancreatitis in rats. There is evidence for a diminished energy metabolism of acinar cells in this experimental model. The aim of this study was to demonstrate permeability transition of the mitochondrial inner membrane as an early change in mitochondrial function and morphology. As functional parameters, the respiration and membrane potential of mitochondria isolated from control and cerulein-treated animals were measured, and changes in volume and morphology were investigated by swelling experiments and electron microscopy. Five hours after the first injection of cerulein, the leak respiration was nearly doubled and the resting membrane potential was decreased by about 17 mV. These alterations were reversed by extramitochondrial ADP or did not occur when cyclosporin A was added to the mitochondrial incubation. A considerable portion of the mitochondria isolated from cerulein-treated animals was swollen and showed dramatic changes in morphology such as a wrinkled outer membrane and the loss of a distinct cristae structure. These data provide evidence for the opening of the mitochondrial permeability transition pore at an early stage of cerulein induced pancreatitis. This suggests that the permeability transition is an initiating event for lysis of individual mitochondria and the initiation of apoptosis and/or necrosis, as had been shown to occur in this experimental model. (Mol Cell Biochem **195**: 191–197, 1999)

Key words: experimental pancreatitis, electron microscopy, glutamate dehydrogenase, pancreatic mitochondria, mitochondrial permeability transition pore

Introduction

The opening of the mitochondrial permeability transition pore is widely accepted to be an early event in pathophysiology of cell death [1–4]. It is characterized by swelling of mitochondria, depolarization of the mitochondrial membrane and the loss of the ability for oxidative phosphorylation [5, 6]. Constituents of the mitochondrial compartment may unspecifically leak out and may induce other steps in damaging individual mitochondria and cytosolic constituents of the cell. One of them is cytochrome c which had been shown to be involved in apoptosis by activating caspases [7, 8]. Moreover, the total decline of mitochondrial function is a prerequisite of necrosis which is a different type of cell death [9, 10].

Recently, we have shown that supramaximal cerulein stimulation affects mitochondrial energy metabolism in rat pancreas [11]. In this model of mild and reversible pancreatitis, the activity of the mitochondrial marker enzyme glutamate dehydrogenase decreased to about 50% of the control value within 24 h of cerulein treatment. This was taken as an indication of the breakdown of individual mitochondria. Earlier during the course of cerulein induced pancreatitis, a considerable effect on mitochondrial respiration, indicated by nearly doubled leak respiration (incubation without ADP), was observed. Because of the sensitivity of this elevation to

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extramitochondrial ADP in the presence of oligomycin, an inhibitor of the F_0F_1-ATPase, we speculated about the opening of the permeability transition pore of a subpopulation of the mitochondria [11]. This hypothesis is supported by the finding that the exposure to high concentrations of secretagogues such as cholecystokinin analogue cause elevation of intracellular [Ca^{2+}] [12] which in turn increases the probability for pore opening [5, 6]. Moreover, Kaiser *et al*. [13] had demonstrated that a supramaximally stimulating dose of cerulein induces a mild form of pancreatitis which is characterized by a considerable degree of apoptosis. As outlined above, pore opening is thought to be involved in the cascade of events which are characteristic of apoptosis. Although, there is experimental evidence for pore opening in several pathophysiological situations such as ischemia/reperfusion [14, 15] little is known about the interaction between permeability transition of the mitochondrial inner membrane and damage to individual mitochondria and cells.

The purpose of this study was to demonstrate pore opening in a mitochondrial subpopulation 5 h after cerulein treatment by swelling experiments, electron microscopical analysis of mitochondrial morphology, and by investigating the effect of ADP and cyclosporin A on respiration and membrane potential.

Material and methods

Chemicals

Cerulein was obtained as ceruletide (Takus) from Pharmacia (Erlangen, Germany). Oligomycin and cyclosporin A were products from Sigma (Deisenhofen, Germany). Alamethicin was obtained from Calbiochem (Bad Soden, Germany). All other chemicals were of analytical grade.

Treatment of animals

Female Wistar rats weighing 160–190 g were used after one over-night fast. The animals were injected four times with cerulein (20 µg/kg body wt) at hourly intervals. In the same time sequence, saline injections were performed with control animals. During the experiments the rats had free access to water. Five hours after the first injection, mitochondria were prepared from the pancreatic tissue.

Preparation of mitochondria

Pancreatic mitochondria were isolated according to the method described by Wilson *et al*. [16] using two animals. The purity of the preparation was improved by introducing an additional washing step by centrifuging the suspension at 6,000 g for 5 min. The final pellet was resuspended in about 0.5 ml of medium containing 250 mM sucrose, 22 mM triethanolamine, 22 mM KCl, 11 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EDTA, and 0.5% bovine serum albumin, pH 7.4.

Measurement of respiration and membrane potential

Oxygen uptake of the mitochondria was measured at 30°C in a thermostat controlled chamber equipped with both a Clark-type and a TPP^+-sensitive electrode. For the calibration of the oxygen electrode, the oxygen content of the air-saturated incubation medium was taken to be 217 nmol/ml [17]. The membrane potential was calculated from the extramitochondrial TPP^+ concentration following the procedure described by Kamo *et al*. [18] and was based on the assumption that the matrix volume is 1 µl/mg mitochondrial protein.

Measurement of the glutamate dehydrogenase activity

The activity of the glutamate dehydrogenase activity was measured according to Schmidt [19] in suspensions that were obtained after sonication for 20 sec and treatment with Triton X-100 (0.5%).

Measurement of mitochondrial swelling

Absorption measurements were performed with a Varian spectrophotometer (Cary 1E) at 546 nm. Calcium-depending swelling caused by the opening of the permeability transition pore was induced by adding 50 µM $CaCl_2$ to 1 ml incubation medium containing about 1 mg mitochondrial protein. Afterwards, all pores were opened by adding 3 µM alamethicin as described by Massari [20].

The difference in absorption, related to the glutamate dehydrogenase activity, before and after additions was used as a measure of mitochondrial swelling.

Electron microscopy

For electron microscopy 2 mitochondrial preparations were used for each control and cerulein-treated animals. After sedimentation at 320× g at 4°C, the preparations were fixed with a mixture of freshly prepared 4% paraformaldehyde and 0.4% glutaraldehyde for 1 h at 4°C. Thereafter, the

preparations were thoroughly rinsed with phosphate-buffered saline (PBS, pH 7.4), postfixed with 1% OsO4 for 1 h at 4°C, contrasted with 1% uranyl acetate in 70% ethanol before dehydration in an ethanol series and flat-embedded in Durcupan [21]. Each washing and incubation step was followed by a sedimentation at 320 × g at 4°C to collect the mitochondria. Ultrathin sections were prepared with a MT 7000 ultramicrotome (RMC Tucson, USA) and examined with a Zeiss 900 electron microscope (Oberkochen, Germany).

Statistical analysis of data

Values are given as mean ± S.D. Statistical significance analysis was carried out with Student's *t*-test for independent samples. At a level of $p < 0.05$, differences were considered significant.

Results

Effect of the cerulein treatment on mitochondrial respiration and membrane potential

Recently, we reported that the leak-respiration of mitochondria isolated from cerulein-treated animals was elevated in comparison to controls 5 h after first injection [11]. Here we show that this increase in the rate of respiration is accompanied by a decrease in the membrane potential (Fig. 1 and Table 1). Such changes are characteristic for an increased permeability of the mitochondrial inner membrane. An increase in permeability of protons due to increased concentrations of free fatty acids which result from phospholipid degradation may be responsible for this effect. Alternatively, the opening of the unspecific permeability transition pore may cause this observation. In order to distinguish between both possibilities, the effect of extramitochondrial ADP and cyclosporin A,

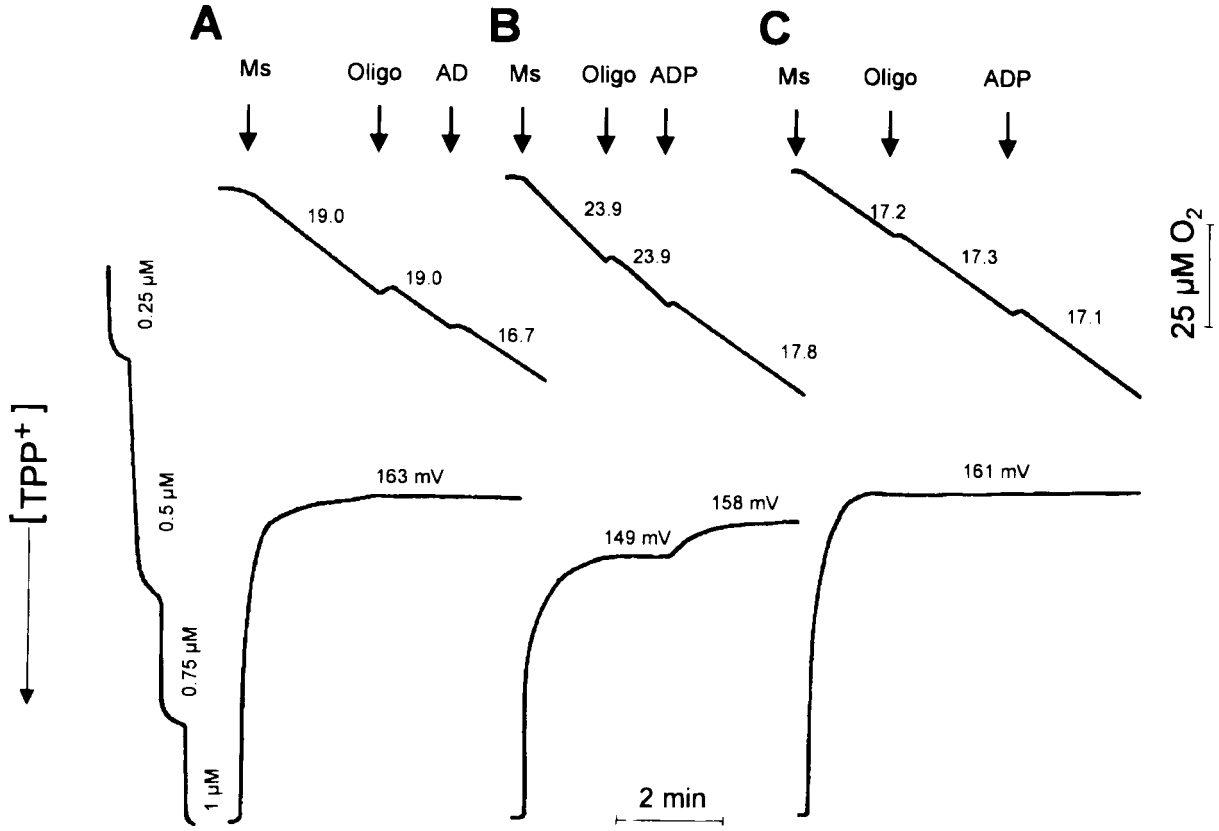

Fig. 1. Leak respiration and membrane potential of pancreatic mitochondria from control and cerulein-treated rats. Incubations were performed in a Mg^{2+}-containing medium with 10 mM succinate and 10 µM rotenone at 30°C. The medium used to produce trace C contained, in addition, 2 µM cyclosporin A. Trace A corresponds to mitochondria isolated 5 h after first saline- (control), traces B and C to mitochondria isolated 5 h after first cerulein injection. Additions: Ms – mitochondrial suspension (typical for at least 4 preparations of mitochondria); Oligo – 2.5 µM oligomycin; ADP – 1 mM ADP. Numbers represent rates of respiration in nmol O_2 min^{-1} ml^{-1} and the membrane potential in mV with glutamate dehydrogenase activities of 1.53 U (A), 1.45 U (B) and 1.47 U (C).

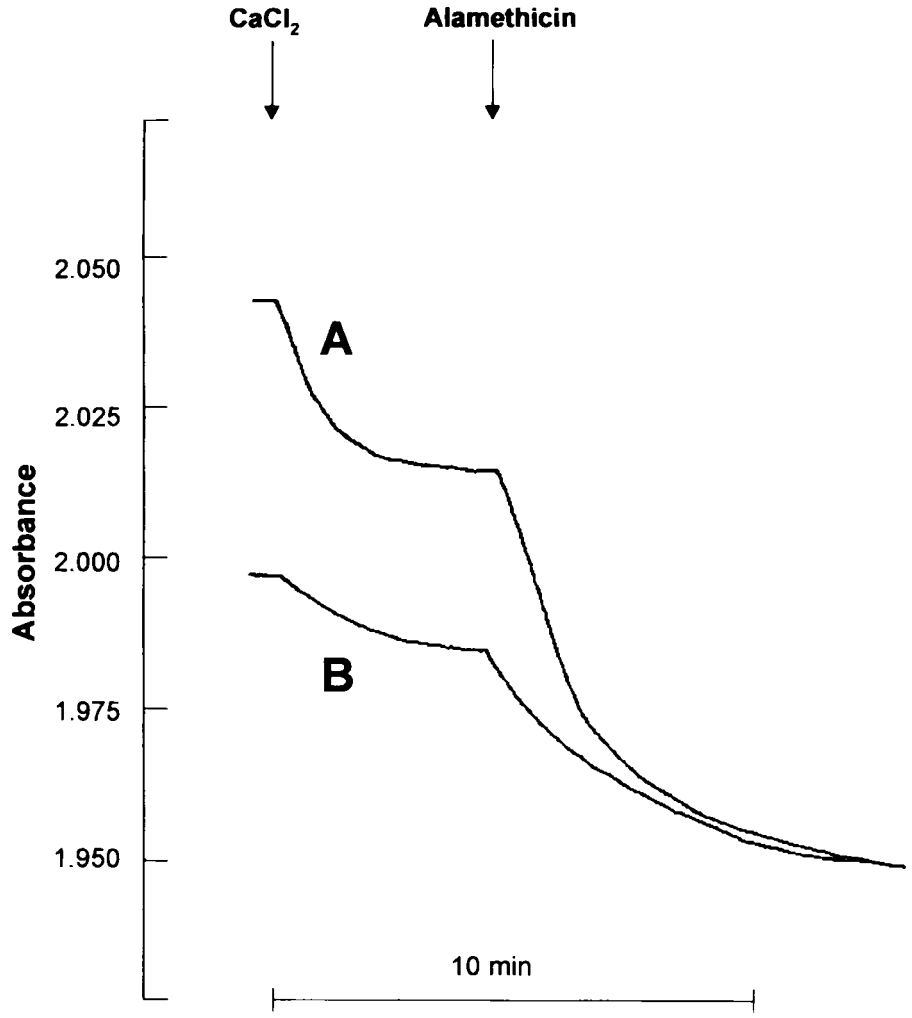

Fig. 2. Mitochondrial swelling in dependence on cerulein treatment. Mitochondria (A: 3.23 U, B: 3.11 U glutamate dehydrogenase activity) were incubated in 1 ml of a Mg^{2+}-containing medium. The additions were: $CaCl_2$, 50 µM $CaCl_2$. Alamethicin, 3 µM alamethicin. Absorption was measured at 546 nm. Trace A represents mitochondria from controls and trace B mitochondria isolated 5 h after first cerulein injection, respectively. The experiment is typical for 4 preparations of mitochondria.

Table 1. Respiration rates, membrane potential and mitochondrial swelling in dependence on cerulein treatment.

Group	Leak respiration (nmol O_2/ (min U GLDH))	Membrane potential (mV)	Change in absorbance ($\Delta A/10^{-2}$ U GLDH)
Control	10.6 ± 0.8	164.3 ± 3.9	3.20 ± 0.59
Cerulein	17.4 ± 1.2*	147.2 ± 5.6**	1.99 ± 0.73*

Incubations were identical to those of the Figs 1 and 2. In order to calculate relevant values of the membrane potential, the data for mitochondrial protein were corrected according to the actual GLDH activity by using its ratio to the mitochondrial protein of the controls. Swelling was presented as the difference in absorptions before and after the additions of calcium and alamethicin. The values are means ± S.D. of 4 preparations of mitochondria. *$p < 0.05$; **$p < 0.01$ compared to controls.

both of which are known to favour pore closure [5, 6], on leak-respiration and membrane potential were investigated. Extramitochondrial ADP added after oligomycin caused a decrease in leak respiration and an increase in the membrane potential to levels of controls (Fig. 1B). Oligomycin was used in order to prevent phosphorylation of ADP by the F_0F_1-ATPase. Moreover, normal levels of respiration and membrane potential of mitochondria from cerulein-treated animals were measured in the presence of cyclosporin A (Fig. 1C). The data clearly demonstrate that cyclosporin A-sensitive permeability transition of the mitochondrial inner membrane is responsible for altered values of leak-respiration and membrane potential.

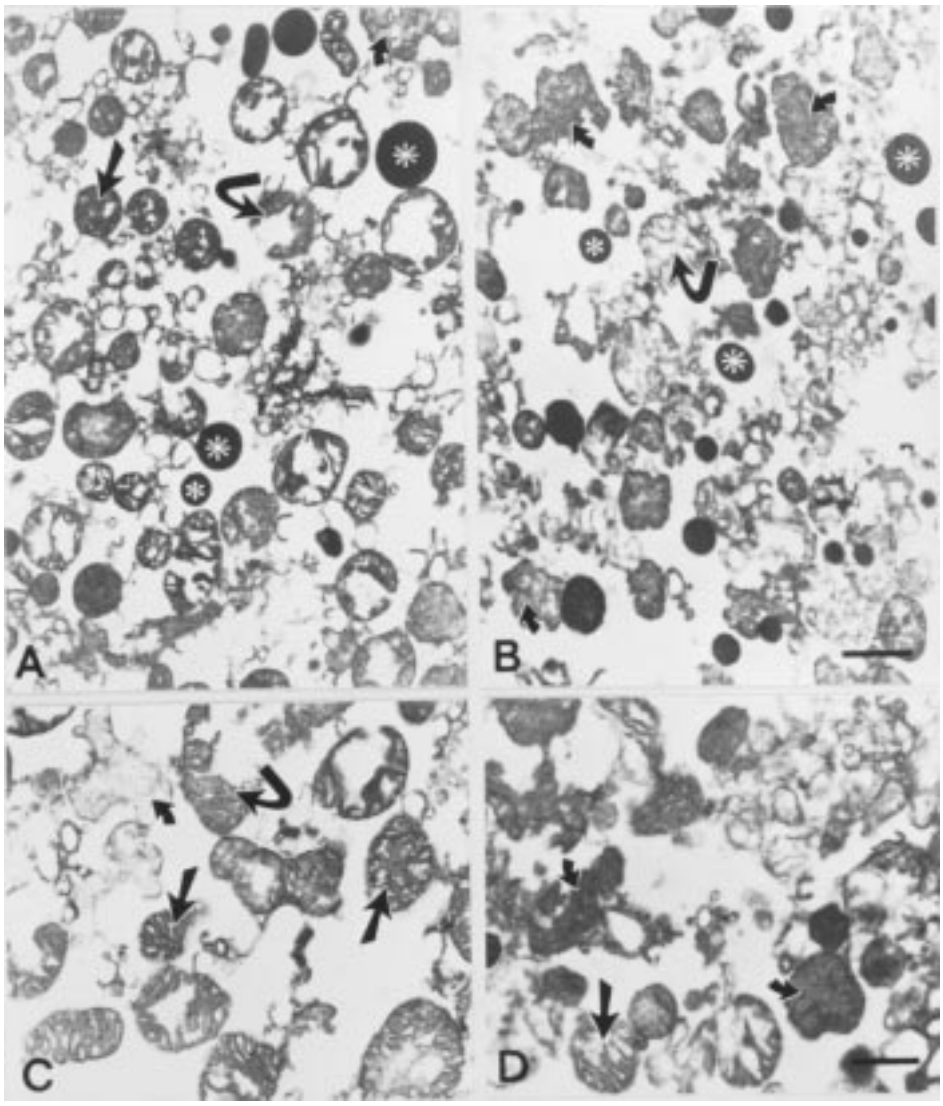

Fig. 3. Electron micrographs of mitochondrial preparations from rat pancreas obtained from normal control (A and C) and cerulein-treated animals (B and D). Note: normal-shaped mitochondria (arrows), shrunken (short curved arrows), ruptured mitochondria (long curved arrows) and zymogen granula (white asterics). Scale bars: A and B, 1. 1 µm; C and D, 0.6 µm.

Effect of cerulein on mitochondrial swelling

In order to support the suggestion of the cerulein-induced permeability transition of the mitochondrial membrane, we performed swelling experiments with mitochondria from controls and cerulein-injected animals. First, pore opening was induced by the addition of 50 µM $CaCl_2$. Afterwards, complete permeability transition was reached by using 3 µM alamethicin as described by Massari [20]. The data presented in Fig. 2 clearly demonstrate that swelling of mitochondria from cerulein-treated animals can be induced by this procedure to a lower extent in comparison with control mitochondria, as is illustrated by the smaller difference in light absorption. Corresponding data from several experiments are presented in Table 1. From these results it may be speculated that the mitochondrial suspension from cerulein-injected animals contained a considerable subpopulation of swollen mitochondria.

Effect of cerulein treatment on mitochondrial morphology

To demonstrate the effect of cerulein on mitochondrial morphology, mitochondria from controls and cerulein-treated

animals were examined by electron microscopy. Both mitochondrial suspensions contained comparable quantities of zymogen granules. Additionally, the preparations were contaminated with vesicles of different origin and debris of membranes of nearly similar amounts (Fig. 3). The mitochondrial suspension of control animals was characterized by about 66 % regular mitochondria with intact outer membrane and clearly visible cristae structure (Fig. 3A). In contrast, the mitochondrial suspension from cerulein-treated animals contained a high amount of mitochondria with irregular shape. The structure of cristae of those organelles nearly disappeared and the outer membrane changed to a wrinkled appearance. Further, partial disruption of mitochondria and swollen mitochondria were found (Fig. 3B). The amount of regular mitochondria in this suspension was about 20%.

Discussion

Supramaximal doses of cerulein induce a mild edematous and reversible form of pancreatitis in the rat [22]. In addition to other parameters, such as edema, increased levels of pancreatic enzymes in serum, inflammatory reaction and focal tissue necrosis, it is characterized by a disappearance of mitochondria [11]. This loss of mitochondria is a time-dependent process and could be detected as early as 5 h after the first cerulein injection. This was demonstrated by decreased activities of the mitochondrial marker enzyme glutamate dehydrogenase, lowered cellular ATP levels, and diminished cellular respiration [11]. Because of the progressive loss of mitochondria within 24 h of the first treatment, we looked for early pathophysiological signs in mitochondrial function. Lüthen *et al*. [23] gave evidence for morphological changes in the mitochondria which were characterized by swelling or rupture of some mitochondria and alterations in the cristae structure. We found an increase in the leak respiration, accompanied by a decrease in the membrane potential of those mitochondria which were isolated from animals 5 h after the first cerulein injection [11]. This led us to speculate a possible permeability transition of the mitochondrial membrane of individual mitochondria. The permeability transition which is influenced by opening of the unspecific pore is believed to play an important role in the pathophysiology of cell death [5].

Our electron microscopical analysis and swelling experiments provide evidence for the opening of this pore within a subpopulation of pancreatic mitochondria after cerulein hyperstimutation. As in the time course of this model of mild pancreatitis, a loss of mitochondria per cell was found [11], the very first consequence seems to be the rupture and lysis of individual mitochondria but not necessarily of the cell. It still remains a matter of speculation how many of the mitochondria have to die in order to initiate apoptosis or necrosis. Our data do not cover this aspect of the cerulein-induced mild form of pancreatitis, because changes in the number of cells which survived following the treatment were not investigated in this study.

In summary, our data provide evidence for the permeability transition of the membrane of individual mitochondria caused by cerulein-induced pancreatitis in the rat. We conclude that this may be an important step within the cascade of events which induce breakdown of mitochondria. Its extent may determine whether the cell will restore or undergo apoptotic or necrotic cell death.

Acknowledgements

This study was supported by the Kultusministerium des Landes Sachsen-Anhalt (Grant 813A/2385). The authors thank Mrs. B. Brauns, Mrs. C. Jechorek and Mrs. U. Schulz for their expert technical assistance.

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