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Cyclosporine A protects mitochondria in an in vitro model of hypoxia/reperfusion injury

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Hypoxia/reperfusion injury is a major clinical problem. One of its hallmarks is an increased cytosolic Ca^{2+} content and an increased generation of reactive oxygen species in the cytosol and in mitochondria. In the present study of an in vitro model of hypoxia/reperfusion injury, mitochondria are exposed to Ca^{2+} in combination with extra- and intramitochondrially acting prooxidants. In this model mitochondria are damaged in a Ca^{2+}-dependent manner. The extent and the site(s) of damage depend on both the kind of respiratory substrate and prooxidant used. The major damage occurs specifically at site I of the respiratory chain, and is due to hydrolysis of oxidized pyridine nucleotides and Ca^{2+} release followed by Ca^{2+} re-uptake (Ca^{2+} 'cycling'). Cyclosporine A completely protects against this damage. The protection is due to inhibition of pyridine nucleotide hydrolysis, an obligatory step in the sequence of events that links prooxidants to Ca^{2+} release from intact mitochondria.

Reactive oxygen; Calcium; Pyridine nucleotide; Rat liver

1. INTRODUCTION

A major problem in organ infarct or transplantation is hypoxia/reperfusion injury (HRI), where an initial, rather innoxious deprivation of oxygen and energy is followed upon re-oxygenation by major damage. A hallmark of HRI is an increased cytosolic Ca^{2+} content caused by hypoxia, followed by an increased generation of reactive oxygen species in the cytosol and in mitochondria subsequent to re-oxygenation [1,2]. These organelles have a pivotal role in HRI: on the one hand, they are a safety device against a toxic increase of the cytosolic Ca^{2+} concentration, because they can take up and transiently store large amounts of Ca^{2+} with impunity; on the other hand, an ongoing release of Ca^{2+}, which can be induced by prooxidants, followed by Ca^{2+} re-uptake (Ca^{2+} 'cycling') damages mitochondria and can lead to cell death [3–5].

In the present in vitro study we investigated the response of mitochondria to conditions that prevail in HRI, i.e. relatively high Ca^{2+} concentrations in combination with extra- and intramitochondrially acting prooxidants. We found that under these conditions (i) mitochondria are damaged in a Ca^{2+}-dependent manner, and that the extent and site(s) of damage depend on both the kind of respiratory substrate and of prooxidants used; (ii) the major damage is due to hydrolysis of oxidized pyridine nucleotides followed by Ca^{2+} cycling; (iii) this damage is effectively prevented by a therapeutically relevant concentration of cyclosporine A (CSA).

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Abbreviations: CSA, cyclosporine A; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; HRI, hypoxia/reperfusion injury; RCI, respiratory control index; tbh, *t*-butylhydroperoxide; XO/HX/Fe, xanthine oxidase/hypoxanthine/iron ions.

2. MATERIALS AND METHODS

2.1. *Materials*

CSA was a gift of Sandoz Pharma Preclinical Research, Basel, Switzerland. It was stored in solid form at −20°C and dissolved in ethanol immediately prior to use. All other chemicals were purchased from standard suppliers, and were of the highest purity commercially available.

2.2. *Isolation of mitochondria*

The isolation of rat liver mitochondria was performed by differential centrifugation. Briefly, livers were homogenized in ice-cold buffer (210 mM mannitol, 70 mM sucrose, and 5 mM 4-(2-hydroxyethyl)-1-piperazinesulphonic acid containing 1 mM of the chelator, ethylenediaminetetraacetic acid. The mitochondrial pellet obtained after the high-speed centrifugation was washed once in the same buffer without the chelator. The protein content was determined by the Biuret method with BSA as standard.

2.3. *Labelling of mitochondrial pyridine nucleotides in vivo*

Overnight-fasted rats were injected intravenously with [*carboxyl*-^{14}C]nicotinic acid (12.5 μCi, 0.223 μmol) [6] in phosphate-buffered saline. After 3 h, the animals were killed, and liver mitochondria were isolated.

2.4. *Standard incubation procedure*

Mitochondria were incubated at 25°C with continuous stirring, and in the case of nicotinamide release measurements with oxygenation, in 100 mM KCl, 100 mM sucrose, 5 mM Tris, pH 7.4, containing 1

mM KH_2PO_4 with 5 mM β-hydroxybutyrate or 5 mM succinate as respiratory substrates.

2.5. *Mitochondrial respiration*

This was measured with a Clark-type electrode. Mitochondria were incubated according to the standard procedure with a protein content of 1.5 mg/ml. The respiratory control index (RCI) was calculated as the ratio of the rate of oxygen consumption during (state 3) and after (state 4) ADP-stimulated respiration [7]. The statistical analysis was done by a Student's *t*-test.

2.6. *Pyridine nucleotide hydrolysis*

Mitochondrial pyridine nuleotides were labelled in vivo (see above), mitochondria were isolated, and incubated at 1 mg of protein/ml according to the standard procedure. Nicotinamide release was analyzed by Millipore filtration as described [6].

3. RESULTS

Fig. 1 reports mitochondrial oxygen consumption supported by the site I respiratory substrate β-hydroxybutyrate. Mitochondria in the presence of EGTA are tightly coupled (Fig. 1A, trace a). Exposure of these mitochondria to the prooxidant *t*-butylhydroperoxide (tbh) (Fig. 1A, trace b), which is metabolized intramitochondrially [8], or to Ca^{2+} (Fig. 1A, trace c) leaves their degree of coupling and maximal respiration essentially unaffected. In contrast, tbh and Ca^{2+} together uncouple mitochondria and decrease their maximal respiration (Fig. 1A, trace d). CSA prevents the mitochondrial deterioration induced by the combined presence of tbh and Ca^{2+} (Fig. 1A, trace e). Similar results are obtained when tbh is replaced by the extramitochondrial generator of oxygen radicals and hydrogen peroxide, xanthine oxidase/hypoxanthine/iron ions (XO/HX/Fe) (Fig. 1B), except that the damage observed under these conditions is more pronounced. Fig. 2 compiles the respiratory rates and RCI values obtained under the various conditions.

With respiration supported by the site II substrate, succinate, mitochondria had a respiratory control index (RCI) of 3.5. tbh and Ca^{2+} alone or in combination neither affected the degree of coupling nor the maximal respiration (results not shown).

Prooxidant-induced Ca^{2+} release from mitochondria, followed by Ca^{2+} cycling, causes selective damage of site I of the respiratory chain (Fig. 3). Thus, when tbh is

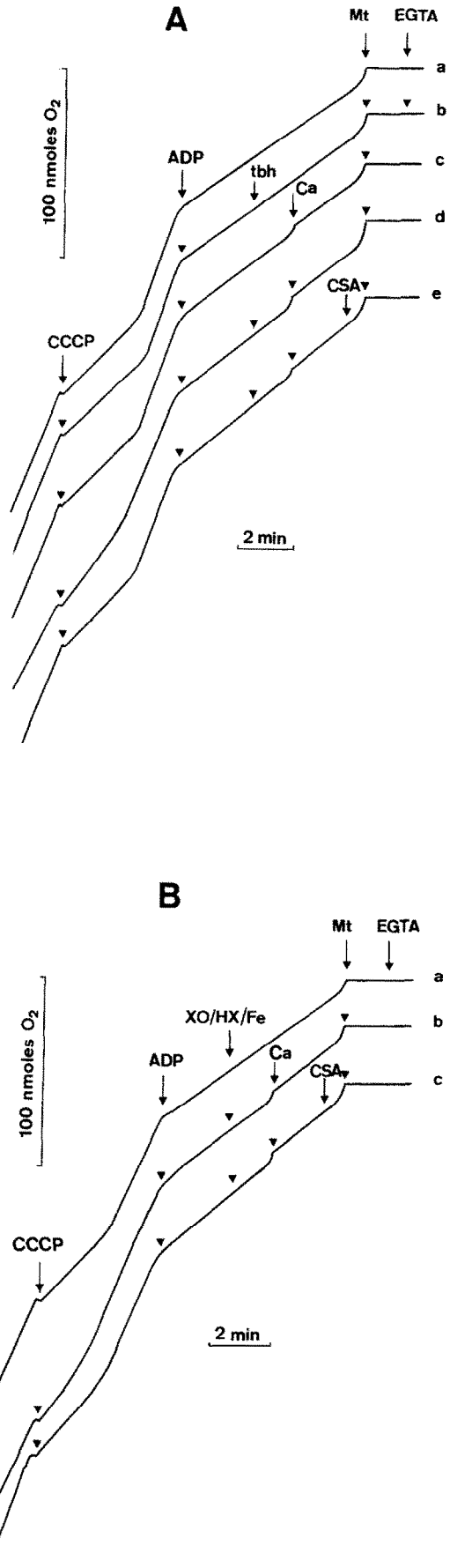

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Fig. 1. Mitochondrial respiration supported by 5 mM β-hydroxybutyrate in the presence of prooxidants. Mitochondria (Mt) were incubated according to the standard procedure. Respiration was measured with a Clark-type electrode (see section 2). EGTA (5 mM), ADP (150 μM), and the uncoupler CCCP were added as indicated by the arrows and triangles. (A) tbh as prooxidant; trace a, control; trace b, as trace a + tbh; trace c, as trace a + Ca^{2+} (20 nmol/mg of protein); trace d, as trace a + tbh and Ca^{2+}; trace e, as trace d + CSA. (B) XO/HX/Fe as prooxidant; trace a, XO/HX/Fe; trace b, as trace a + Ca^{2+} (20 nmol/ mg of protein); trace c, as trace b + CSA. XO/HX/Fe, 0.02 U xanthine oxidase/ml, 15 μM hypoxanthine, 15 μM Fe_2SO_4; tbh, 30 μM; CCCP, 0.5 μM; CSA, 1 μM; EGTA, 5 mM.

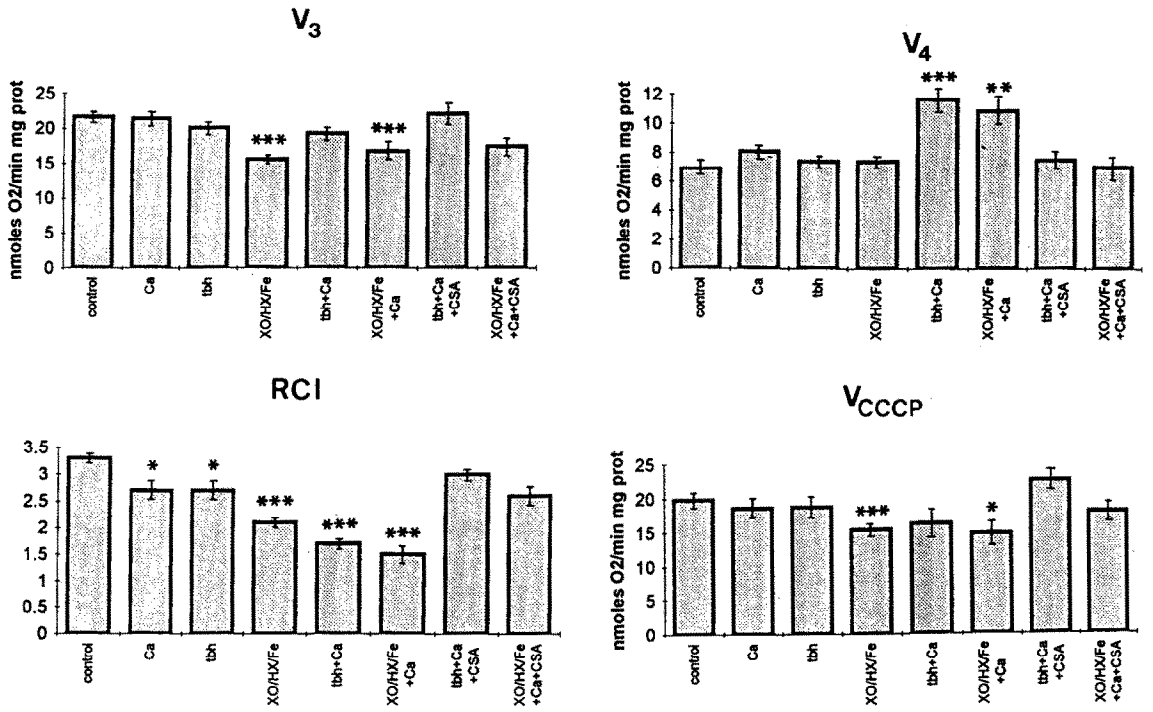

Fig. 2. Respiration rate and respiratory control index. Mitochondrial respiration supported by 5 mM β-hydroxybutyrate in the presence of the prooxidants, tbh or XO/HX/Fe, was measured as in Fig. 1. Respiratory control indices were calculated as described in section 2. Data are expressed as mean ± S.E.M. (n = 4–6); $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$. V_3, respiration rate in state 3; V_4, respiration rate in state 4; V_{CCCP}, respiration rate in the presence of uncoupler; RCI, respiratory control index.

added to Ca^{2+}-loaded mitochondria, β-hydroxybutyrate-supported respiration initially increases, then decreases and becomes unresponsive to the uncoupler; however, these mitochondria remain fully responsive to succinate (Fig. 3, trace a). CSA (Fig. 3, trace b) or EGTA (Fig. 3, trace c) protect mitochondria from the prooxidant + Ca^{2+}-induced, site I-specific damage. The same results were obtained with XO/HX/Fe instead of tbh (not shown).

In Ca^{2+}-loaded mitochondria respiring on β-hydroxybutyrate, extensive hydrolysis of pyridine nucleotides occurs, as judged from nicotinamide release (Fig. 4A, traces a–c). Even without added prooxidant hydrolysis takes place (Fig. 4A, trace a) because in β-hydroxybutyrate-energized mitochondria Ca^{2+} uptake causes extensive pyridine nucleotide oxidation (result not shown), a prerequisit for their hydrolysis. Hydrolysis is stimulated by prooxidants, either generated extramitochondrially (Fig. 4A, trace b), or being metabolized intramitochondrially (Fig. 4A, trace c). CSA completely prevents pyridine nucleotide hydrolysis induced by either prooxidant (Fig. 4A, traces d and e). No hydrolysis takes place in Ca^{2+}-depleted mitochondria exposed to the prooxidants (Fig. 4A, traces f and g). With succinate as the respiratory substrate (Fig. 4B) pyridine nucleotide hydrolysis is very limited despite the joint presence of prooxidant and Ca^{2+}, because succinate keeps pyridine nucleotides largely reduced (result not shown).

4. DISCUSSION

To mimick in an in vitro model the conditions prevailing upon reperfusion after hypoxia, in the present study isolated rat liver mitochondria were energized and exposed to Ca^{2+} and prooxidants which act intra- or extramitochondrially. Several conclusions can be drawn from the results obtained. (i) When mitochondria contain oxidized pyridine nucleotides and Ca^{2+} they experience specific damage at site I of the respiratory chain. (ii) Not simply the exposure to prooxidants and Ca^{2+} is deleterious to mitochondria, but the prooxidant-induced stimulation of the Ca^{2+} release pathway, which requires pyridine nucleotide hydrolysis, followed by Ca^{2+} cycling. This is evident from the transient increase in β-hydroxybutyrate-supported respiration due to increased Ca^{2+} cycling followed by inhibition of respiration, and from the protection by CSA and EGTA, compounds which prevent pyridine nucleotide hydrolysis and/or Ca^{2+} cycling, respectively. (iii) CSA, at a therapeutically relevant concentration [9], completely protects mitochondria in this in vitro model of HRI.

CSA protects mitochondria by inhibiting the hydrolysis of oxidized pyridine nucleotides, an obligatory step in the sequence of events leading to Ca^{2+} release and subsequent Ca^{2+} cycling [4], whereas succinate protects by keeping mitochondrial pyridine nucleotides largely reduced. Some of the radioactivity lost from mitochondria may also be attributed to intact pyridine nucleo-

tides since, in the presence of CSA or EGTA, the inner mitochondrial membrane may become non-specifically permeable [10,17]. The production by succinate is due to reversed electron flow, as will be described in detail elsewhere. Pyridine nucleotide hydrolysis is also the cause of the site I-specific inhibition of respiration in the presence of prooxidants and Ca^{2+}, as is evident from protection by CSA or succinate.

Our findings confirm and extend previous reports. Thus, CSA prevents prooxidant-induced Ca^{2+} release from isolated heart [10], liver [11,12], and kidney [13] mitochondria. The drug also protects hepatocytes against prooxidant-induced mitochondrial Ca^{2+} cycling and cell killing [14]. Specific loss of site I activity of kidney mitochondria exposed to the oxygen radical generator XO/HX/Fe and Ca^{2+} has been reported [15], but the underlying mechanism was not elucidated. It has also been shown that CSA restores ATP synthesis and coupling in mitochondria not before but after reperfusion of previously hypoxic rat liver [16]. It therefore appears that CSA, in addition to its immunosuppressive properties, is useful to mitigate HRI-related organelle, cell, and organ damage caused by prooxidant-induced mitochondrial Ca^{2+} cycling.

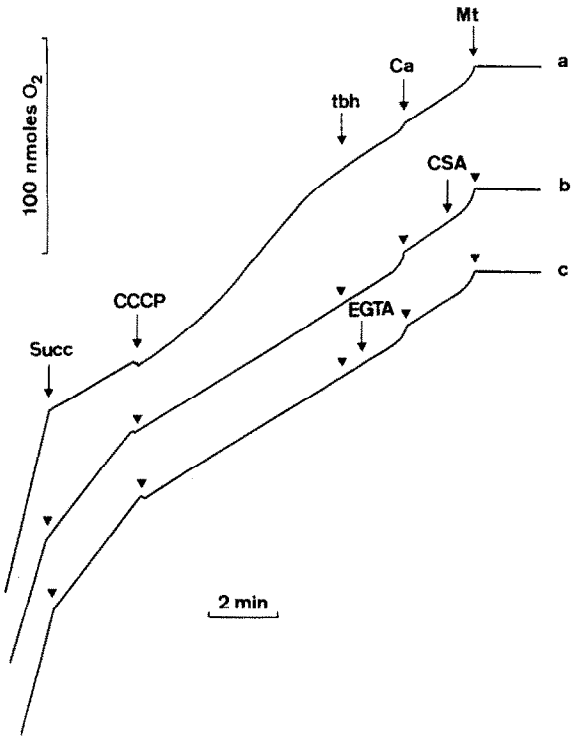

Fig. 3. *t*-Butylhydroperoxide-induced uncoupling and inhibition of mitochondrial respiration. Mitochondria were incubated according to the standard procedure in the presence of 5 mM β-hydroxybutyrate. Respiration was measured with a Clark-type electrode (see section 2). Ca^{2+} (20 nmol/mg of protein), tbh, the uncoupler CCCP, and succinate were added as indicated by the arrows and triangles. Trace a, control; trace b, as trace a + CSA; trace c, as trace a + EGTA. Succ, 5 mM; tbh, 30 μM; CCCP, 0.5 μM; CSA, 1 μM; EGTA, 5 mM.

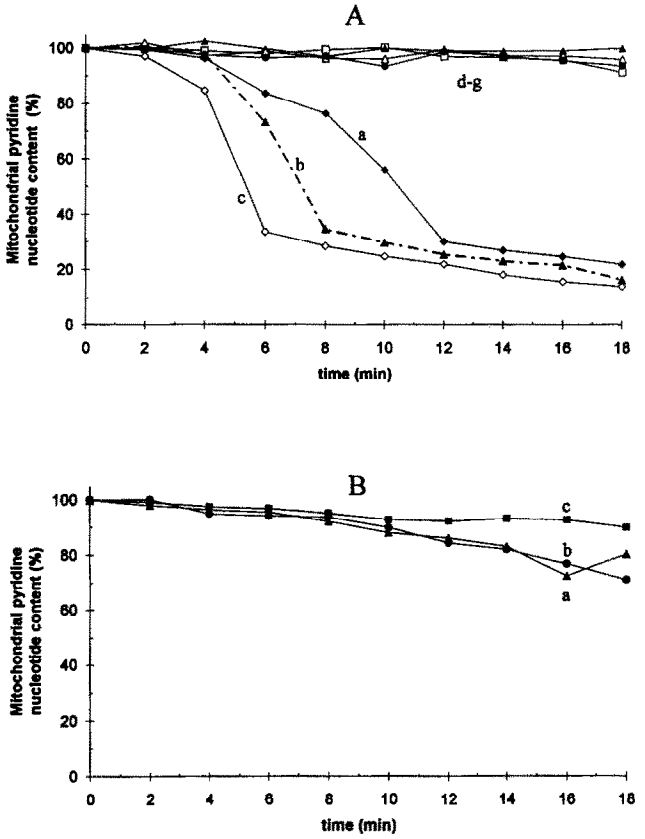

Fig. 4. Hydrolysis of mitochondrial pyridine nucleotides. Mitochondria labelled at the nicotinamide moiety were incubated (time 0 min) according to the standard procedure. After 30 s they were exposed to 20 nmol of Ca^{2+}/mg of protein (lanes a–e), and 2 min later the prooxidant was added as indicated below. Pyridine nucleotide hydrolysis was analyzed by determination of nicotinamide release with Millipore filtration. (A) 5 mM β-hydroxybutyrate as respiratory substrate; trace a, Ca^{2+} (20 nmol/mg of protein); trace b, as trace a + XO/HX/Fe; trace c, as trace a *plus* tbh; trace d, as trace b + CSA; trace e, as trace c+CSA; traces f and g, mitochondria were depleted of Ca^{2+} with 5 mM EGTA prior to addition β-hydroxybutyrate and at time 2 min 15 sec exposed to XO/HX/Fe (trace f) or tbh (trace g). (B) 5 mM succinate as respiratory substrate; trace a, Ca^{2+} (20 nmol/mg of protein); trace b, as trace a + XO/HX/Fe; trace c, as trace a + tbh. XO/HX/Fe, 0.02 U xanthine oxidase/ml, 15 μM hypoxanthine, 15 μM Fe_2SO_4; tbh, 30 μM; CSA, 1 μM; EGTA, 5 mM.

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REFERENCES

[1] Braunwald, E. and Kloner, R.A. (1985) J. Clin. Invest. 76, 1713–1719.
[2] Bolli, R. (1991) Cardiovasc. Drugs Ther. 5, 249–268.
[3] Nicotera, P., Bellomo, G. and Orrenius, S. (1990) Chem. Res. Toxicol. 3, 484–494.
[4] Richter, C. and Kass, G.E.N. (1991) Chem. Biol. Interact. 77, 1–23.
[5] Hennet, T., Richter, C. and Peterhans, E. (1993) Biochem. J. 289, 587–592.

[6] Lötscher, H.R., Winterhalter, K.H., Carafoli, E. and Richter, C. (1980) J. Biol. Chem. 255, 9325–9330.
[7] Chance, B. and Williams, G.R. (1955) J. Biol. Chem. 217, 409–427.
[8] Lötscher, H.R., Winterhalter, K.H., Carafoli, E. and Richter, C. (1979) Proc. Natl. Acad. Sci. USA 76, 4340–4344.
[9] Kahan, B.D. (1993) Transplant. Proc. 25, 508–510.
[10] Crompton, M. and Costi, A. (1988) Eur. J. Biochem. 178, 489–501.
[11] Richter, C., Theus, M. and Schlegel, J. (1990) Biochem. Pharmacol. 40, 779–782.
[12] Schweizer, M., Schlegel, J., Baumgartner, D. and Richter, C. (1993) Biochem. Pharmacol. 45, 641–646.
[13] Schlegel, J., Meier, P., Kass, G.E.N. and Richter, C. (1991) Biochem. Pharmacol. 42, 2193–2197.
[14] Kass, G.E.N., Juedes, M.J. and Orrenius, S. (1992) Biochem. Pharmacol. 44, 1995–2003.
[15] Malis, C.D. and Bonventre, J.V. (1986) J. Biol. Chem. 261, 14201–14208.
[16] Kurokawa, T., Kobayashi, H., Harada, A., Nakao, A., Sugiyama, S., Ozawa, T. and Takagi, H. (1992) Transplantation 53, 308–311.