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CYCLOSPORIN A PROTECTS HEPATOCYTES AGAINST PROOXIDANT-INDUCED CELL KILLING

A STUDY ON THE ROLE OF MITOCHONDRIAL Ca^{2+} CYCLING IN CYTOTOXICITY

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Abstract—Cyclosporin A (CsA) is a potent inhibitor of the prooxidant-induced release of Ca^{2+} from isolated mitochondria. In this investigation, pretreatment of hepatocytes with CsA before exposure to the prooxidants *tert*-butyl hydroperoxide (tBH), cumene hydroperoxide or 3,5-dimethyl-*N*-acetyl-*p*-benzoquinone imine (3,5-Me_2-NAPQI) prevented the loss of cell viability. HPLC analysis of adenine and pyridine nucleotide concentrations in hepatocytes treated with 3,5-Me_2-NAPQI showed a rapid depletion of ATP prior to the loss of cell viability versus the maintenance of near control levels of ATP in hepatocytes treated with CsA before 3,5-Me_2-NAPQI. In 3,5-Me_2-NAPQI-exposed hepatocytes there was also a rapid loss of cellular NAD^+ which could be accounted for initially by a transient increase in $NADP^+$. Measurement of the intracellular Ca^{2+} pools showed an early depletion of the mitochondrial Ca^{2+} pool in hepatocytes exposed to 3,5-Me_2-NAPQI, tBH or cumene hydroperoxide; this loss was prevented by CsA. In conclusion, these results show that CsA protected hepatocytes from prooxidant injury by preventing mitochondrial Ca^{2+} cycling and subsequent mitochondrial dysfunction. This suggests that in prooxidant injury, excessive Ca^{2+} cycling is an early and important event leading to mitochondrial damage and subsequently to cell death.

Oxidative stress as a result of exposure to prooxidants such as H_2O_2, organic hydroperoxides and redox cycling xenobiotics, or during the reoxygenation of hypoxic tissue often leads to acute cell injury [1, 2]. Over the past decade, work from our laboratory and others has pointed to a causative link between perturbation of intracellular Ca^{2+} homeostasis and cell killing during oxidative stress [2–4]. The perturbation of intracellular Ca^{2+} homeostasis is a consequence of the oxidative inactivation of Ca^{2+} translocation systems located at the plasma [5, 6] and endoplasmic reticular [7] membranes, as well as the inhibition of Ca^{2+} sequestration by mitochondria, and is reflected in a sustained increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$†) [6, 8–10]. The prooxidant-induced elevation of $[Ca^{2+}]_i$ has been associated with the activation of a number of catabolic processes involving proteases, phospholipases and endonucleases which are thought to be responsible for cell killing [2].

Recently, a great deal of attention has been focused on mitochondrial Ca^{2+} transport during oxidative stress. The mitochondrial inner membrane contains separate routes for Ca^{2+} uptake and release [11]. Ca^{2+} is taken up via a Ruthenium red-sensitive uniporter in response to the membrane potential generated by the electron transport chain. The mitochondrial Ca^{2+} release pathways involve antiporters that allow Ca^{2+} efflux in exchange for H^+ or Na^+, the relative contribution of H^+ versus Na^+ to Ca^{2+} efflux depending on the tissue of origin of the mitochondria. Although mitochondria do not contain a large pool of Ca^{2+} able to be mobilized under physiological conditions [12], their capacity to sequester Ca^{2+} is extremely large [11]. This raises the possibility that mitochondria may act as intracellular buffers during toxic insult thereby counteracting the toxic increase in $[Ca^{2+}]_i$. However, under conditions of oxidative stress, isolated mitochondria (see Ref. 13 for a recent review) or mitochondria *in situ* [14, 15] rapidly lose their Ca^{2+} pool. It is now well established that the initial prooxidant-stimulated Ca^{2+} release occurs from intact mitochondria (i.e. in the absence of inner membrane damage) [13]. This release does not appear to result from the reversal of the uniporter Ca^{2+} uptake system but involves the activation of a distinct efflux pathway which may be regulated by protein mono(ADP-ribosylation) [13]. Recently, the immunosuppressant drug cyclosporin A (CsA) has been shown to be a potent inhibitor of Ca^{2+} release from mitochondria by prooxidants [16–18].

The presence of separate routes for Ca^{2+} uptake and release, with the stimulation of the latter by prooxidants, will result in the stimulation of Ca^{2+} cycling during oxidative stress. If excessive, this cycling may lead to a loss of the transmembrane potential, inhibition of ATP synthesis and damage to the mitochondria which would compromise cell survival [13, 19–22]. Therefore, the present study

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† Abbreviations: $[Ca^{2+}]_i$, cytosolic free calcium concentration; CsA, cyclosporin A; 3,5-Me_2-NAPQI, 3,5-dimethyl-*N*-acetyl-*p*-benzoquinone imine; tBH, *tert*-butyl hydroperoxide; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid.

was designed to investigate the relative contribution of mitochondrial Ca^{2+} cycling to the mechanism of prooxidant-induced injury in isolated hepatocytes. We report that CsA protected against the loss of cell viability during prooxidant injury, and this was linked to the ability of CsA to prevent the prooxidant-induced Ca^{2+} release from mitochondria.

MATERIALS AND METHODS

Chemicals. Arsenazo III (98% pure) and *tert*-butylhydroperoxide (tBH) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Cumene hydroperoxide was obtained from Fluka (Buchs, Switzerland) and carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) from the Aldrich Chemical Co. (Steinheim, F.R.G.). The Ca^{2+} ionophores, ionomycin and A23187, were purchased from Calbiochem (La Jolla, CA, U.S.A.). 3,5-Dimethyl-*N*-acetyl-*p*-benzoquinone imine (3,5-Me_2-NAPQI) was obtained from Dalton Chemical Laboratories (Toronto, Ontario, Canada). Collagenase (grade II) and bovine serum albumin were purchased from Boehringer (Mannheim, F.R.G.). CsA was a generous gift from Sandoz. All other chemicals were of the highest purity grade commercially available.

Hepatocyte isolation and incubation. Hepatocytes were isolated from male Wistar rats (170–210 g, fed *ad lib.*) by collagenase perfusion as described previously [23]; approximately 90–95% of the freshly isolated hepatocytes routinely excluded Trypan blue. Immediately after isolation the cells were incubated at a concentration of 3×10^6 cells/mL in Krebs–Henseleit buffer, pH 7.4, supplemented with 12.5 mM Hepes, 1% (w/v) bovine serum albumin and 15 mM glucose at 37° in rotating round-bottomed flasks in an atmosphere of 93.5% O_2, 6.5% CO_2. After 30 min the hepatocytes were washed and incubated at 1×10^6 cells/mL as above except that bovine serum albumin and glucose were omitted from the incubation buffer, and following a 20-min preincubation period in the absence or presence of CsA, the cells were exposed to the prooxidants. Cell viability was determined by measuring Trypan blue [0.12% (w/v)] exclusion. Plasma membrane blebbing was measured by counting the number of Trypan blue-excluding cells which had bubble-like protrusions (blebs) on the cell surface.

HPLC analysis of adenine and pyridine nucleotides. The procedure for the analysis of adenine and pyridine nucleotides was adapted from the method described by Jones [24]. For the analysis of oxidized pyridine nucleotides and adenine nucleotides, 200 μL aliquots from the incubation mixture were added to 50 μL of 3 M perchloric acid on ice, vortexed and stored at −70° until analysed by HPLC (within 48 hr after the experiment). For the analysis of reduced pyridine nucleotides, 250 μL aliquots of the cell suspension were mixed on ice with 25 μL of 0.5 M KOH containing 50% (v/v) ethanol and 35% (w/v) cesium chloride and immediately frozen at −70°. HPLC analysis of the reduced pyridine nucleotides was performed within 24 hr of sample generation. The separation and detection of the nucleotides by HPLC was carried out as reported previously [25] except that a CT-sil 5 μm ODS column (Chromtech AB, Norsborg, Sweden) was used with a flow rate of 1 mL/min, and the reduced pyridine nucleotides were separated isocratically using 0.1 M potassium phosphate buffer containing 5% (v/v) methanol, pH 5.93.

Measurement of the mitochondrial Ca^{2+} *pool in intact hepatocytes.* The size of the mitochondrial Ca^{2+} pool was estimated as the FCCP-releasable Ca^{2+} pool after separation of the hepatocytes from the incubation mixture by centrifugation through Percoll and after resuspension of the cells in Ca^{2+}- and Mg^{2+}-free Hanks' solution at a concentration of 3×10^6 cells/mL [15, 26]. The final concentrations of FCCP and ionomycin (or A23187 in some experiments) were 20 and 10 μM, respectively.

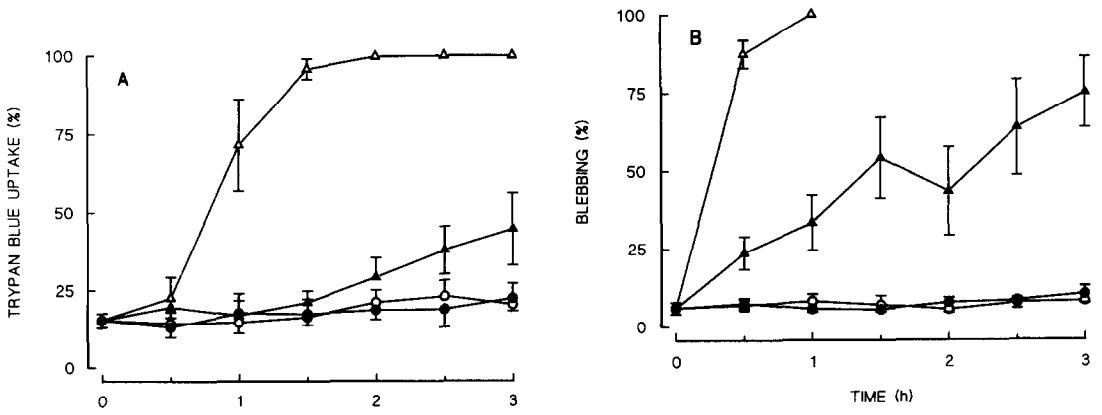

Fig. 1. Cyclosporin A protects hepatocytes from 3,5-Me_2-NAPQI-induced cell killing. Following the pretreatment of hepatocytes with CsA (300 nM) (closed symbols) or carrier solvent (ethanol) (open symbols) for 20 min, the prooxidant or carrier solvent (Me_2SO) was added. The final concentration of ethanol and Me_2SO did not exceed 0.15 and 0.2%, respectively. Cytotoxicity was scored by examining the cells for the appearance of plasma membrane blebs (expressed as % of Trypan blue-excluding cells with plasma membrane blebs) and Trypan blue uptake (expressed as % of the cells which had taken up the dye). For further details, see Materials and Methods. Each point is the mean ± SE of four separate experiments conducted on different hepatocyte preparations. Symbols and concentrations used are: (○) control; (△) 3,5-Me_2-NAPQI (225 μM).

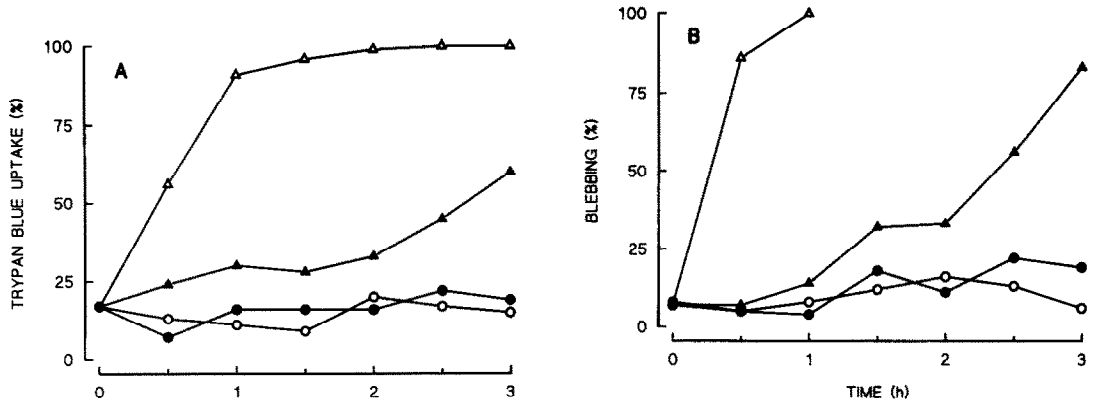

Fig. 2. Cyclosporin A protects hepatocytes from tBH-induced cell killing. Details are as reported in the legend to Fig. 1, except that the concentration of cyclosporin A was 500 nM. The data shown here are from one experiment representative of four. Symbols and concentrations used are: (○) control; (△) tBH (200 μM).

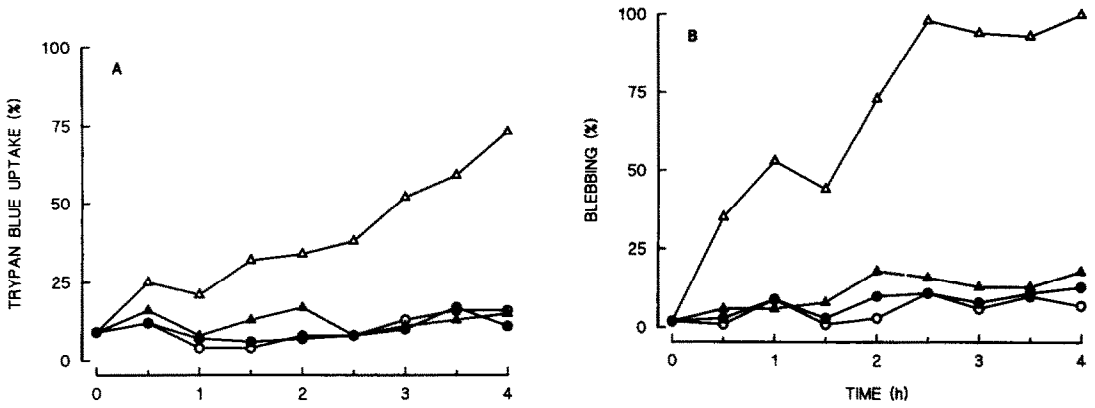

Fig. 3. Cyclosporin A protects hepatocytes from cumene hydroperoxide-induced cell killing. For details, see legend to Fig. 1. The data shown here are from one experiment representative of four. Symbols and concentrations used are: (○) control; (△) cumene hydroperoxide (100 μM).

Cellular protein content (after centrifugation through Percoll) was estimated using the biuret method [27]. The Ca^{2+} pool titration method has been re-examined recently and it has been confirmed that treatment with mitochondrial uncouplers does not mobilize the endoplasmic reticular Ca^{2+} pool [28].

Statistical analysis. Duncan's multiple range test was performed to determine the significance of differences among individual group means. When appropriate, primary data were log-transformed prior to statistical evaluation in order to achieve homogeneity of variances. The significance level chosen for all statistical analyses was $P < 0.05$. The statistical analyses were carried out with the SPSS/PC$^+$ statistical package.

RESULTS

Cyclosporin A protects hepatocytes from prooxidant-induced cell killing

The prooxidants tBH (200 μM), cumene hydroperoxide (100 μM) and 3,5-Me_2-NAPQI (225 μM) caused a rapid loss of hepatocyte viability as estimated by Trypan blue uptake (Figs 1A–3A). The decrease in cell viability by the prooxidants was preceded by the appearance of plasma membrane blebs, in agreement with previous reports [29, 30] (Figs 1B–3B). The loss of cell viability induced by the prooxidants 3,5-Me_2-NAPQI, tBH and cumene hydroperoxide was largely prevented by pre-incubation with CsA (300 nM). CsA also completely blocked plasma membrane blebbing induced by cumene hydroperoxide (Fig. 3B) versus partial protection from 3,5-Me_2-NAPQI- (Fig. 1B) or tBH-induced blebbing (Fig. 2B). Additional experiments were conducted to investigate the molecular mechanisms of the CsA protective effect. In particular, the influence of CsA on mitochondrial Ca^{2+} homeostasis during prooxidant injury was explored to assess whether the protection provided by CsA against prooxidant-induced cell injury could be rationalized by an effect on mitochondria *in situ*.

CsA prevents the 3,5-Me_2-NAPQI-induced decrease in cellular energy content

Mitochondria are the main source of energy production in hepatocytes and damage to the mitochondrial inner membrane through excessive Ca^{2+} cycling would be expected to result in a loss of cellular ATP content. As shown in Fig. 4A,

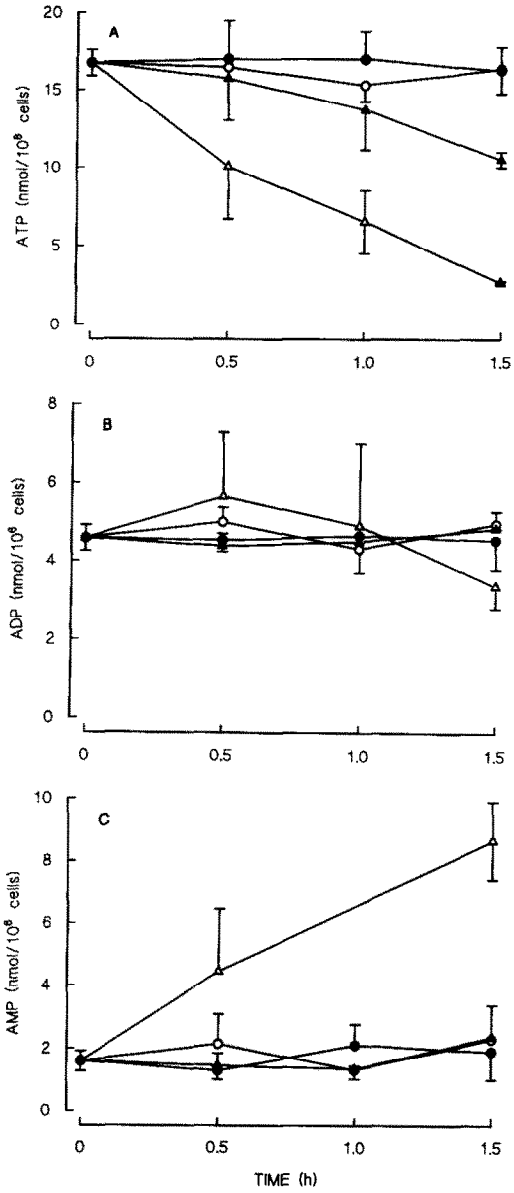

Fig. 4. Effect of 3,5-Me_2-NAPQI on intracellular adenine nucleotides. Hepatocytes, preincubated in the absence (open symbols) or presence (closed symbols) of cyclosporin A (300 nM), were processed at the indicated time points for the analysis of adenine nucleotides by HPLC as described in Materials and Methods. Each point is the mean ± SE of four separate experiments conducted on different hepatocyte preparations. Symbols and concentrations used are: (○) control; (△) 3,5-Me_2-NAPQI (225 μM).

incubation of hepatocytes with 3,5-Me_2-NAPQI resulted in a rapid decrease in cytoplasmic ATP content. The ATP level was reduced from 16.7 ± 0.8 nmol/10^6 cells (mean ± SE, N = 4 separate experiments) at time zero to 10.0 ± 3.3 nmol/10^6 cells at 30 min without any significant loss of cell viability (Fig. 1A). The decrease in cellular ATP content was to a great extent accounted for by a parallel increase in cellular AMP (Fig. 4C), whereas only a minor alteration in ADP concentration was observed (Fig. 4B). CsA totally prevented the loss of ATP caused by 3,5-Me_2-NAPQI over a 1 hr incubation period. At 1.5 hr a 37% decrease of ATP was observed that was not accounted for by conversion to ADP or AMP (Fig. 4B and C).

Alterations in pyridine nucleotide content during 3,5-Me_2-NAPQI-induced oxidative stress

In a number of cell types, cellular injury by prooxidants leads to a rapid decrease in intracellular NAD^+ content as a result of the stimulation of poly(ADP-ribose) synthesis following DNA damage by the prooxidant [31–33]. Inhibitors of poly(ADP-ribose) polymerase such as 3-aminobenzamide and nicotinamide have been reported to protect from the cytotoxicity of prooxidants by preventing

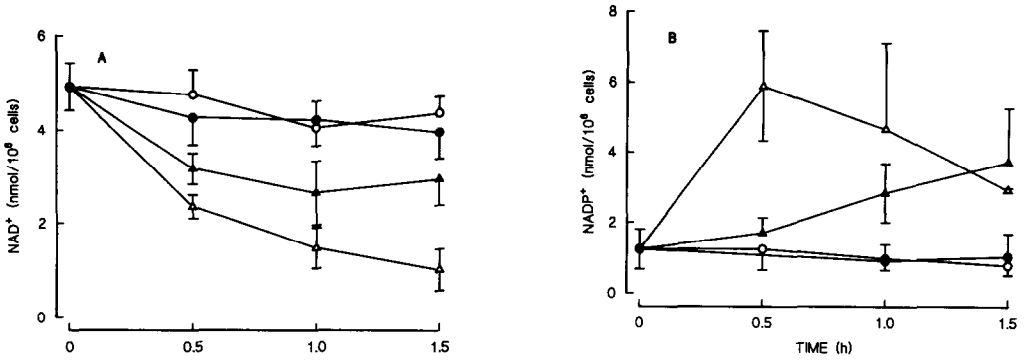

Fig. 5. Effect of 3,5-Me_2-NAPQI on oxidized pyridine nucleotides. Hepatocytes, preincubated in the absence (open symbols) or presence (closed symbols) of cyclosporin A (300 nM), were processed at the indicated time points for the analysis of oxidized pyridine nucleotides by HPLC as described in Materials and Methods. Each point is the mean ± SE of four separate experiments conducted on different hepatocyte preparations. Symbols and concentrations used are: (○) control; (△) 3,5-Me_2-NAPQI (225 μM).

pyridine nucleotide hydrolysis by poly(ADP-ribose) polymerase [33–36]. Since work from our laboratory [25] and others [18] has shown that CsA prevented Ca^{2+} release from mitochondria by inhibiting pyridine nucleotide hydrolysis, the possibility that CsA exerted its protective effect by preventing NAD^+ consumption by the polymerase was explored.

Incubating hepatocytes with 3,5-Me_2-NAPQI (225 μM) led to a rapid decrease in NAD^+ content which preceded cell killing (Fig. 5A). In contrast to cells responding to oxidants by a massive net consumption of NAD^+ via activation of poly(ADP-ribose) polymerase, the decrease in NAD^+ content following exposure of the hepatocytes to 3,5-Me_2-NAPQI occurred simultaneously with a transient elevation of $NADP^+$ (Fig. 5B). This suggests that the lost NAD^+ was recovered as $NADP^+$ as a result of an interconversion of NAD^+ to $NADP^+$. CsA slightly decreased the extent of the initial NAD^+ loss and delayed, but did not prevent, the elevation of $NADP^+$ (Fig. 5A and B), suggesting that CsA did not substantially alter pyridine nucleotide metabolism in hepatocytes during 3,5-Me_2-NAPQI-induced oxidative stress. The effect of 3,5-Me_2-NAPQI or 3,5-Me_2-NAPQI in combination with CsA on the cellular concentration of reduced pyridine nucleotides was also studied. We found that NADH and NADPH levels accounted for 3 and 29% of total NAD(H) and NADP(H) content, respectively. These levels did not appreciably change during the exposure to 3,5-Me_2-NAPQI (except for a transient elevation in NADPH from 0.52 ± 0.25 to 1.0 ± 0.48 nmol/10^6 cells (mean ± SE, N = 4) at 1 hr) but were maintained at a level marginally higher than control (data not shown). Hence, the overall contribution of the change in reduced pyridine nucleotide content to the total change in pyridine nucleotide content induced by the prooxidant was minor. Furthermore, no significant effect of CsA was observed (data not shown).

Additional evidence dissociating the involvement of pyridine nucleotide consumption by poly(ADP-ribose) polymerase from the protection of prooxidant-induced cell killing by CsA was obtained by examining the effect of the polymerase inhibitor, 3-aminobenzamide, on 3,5-Me_2-NAPQI-mediated cytotoxicity. Neither 2.5 nor 20 mM 3-aminobenzamide protected the hepatocytes from plasma membrane blebbing or loss of viability caused by the prooxidant; in fact, at both concentrations of the inhibitor, a significant enhancement of the cytotoxicity occurred (data not shown).

Effects of prooxidants and CsA on the mitochondrial Ca^{2+} *pool*

In hepatocytes exposed to 3,5-Me_2-NAPQI (225 μM), mitochondrial Ca^{2+} content (estimated from the FCCP-releasable Ca^{2+} store) transiently increased at 15 min of incubation, after which time a complete loss of Ca^{2+} from this organelle occurred prior to the onset of cell death (Table 1). The presence of CsA (which in the absence of prooxidant stimulated a net accumulation of Ca^{2+} in mitochondria) essentially prevented the loss of the mitochondrial Ca^{2+} pool with 3,5-Me_2-NAPQI (Table 1).

The addition of tBH or cumene hydroperoxide to hepatocytes also resulted in the loss of mitochondrial Ca^{2+} (Tables 2 and 3). The loss of this pool of Ca^{2+} was followed by a depletion of cellular ATP content and a loss of cell viability. CsA prevented the loss of the mitochondrial Ca^{2+} pool and also protected the hepatocytes from cell death. The ability of the cells to maintain the mitochondrial Ca^{2+} pool correlated well with the subsequent effect of the prooxidants on cell viability. For instance, CsA did not prevent the loss of the mitochondrial Ca^{2+} pool with concentrations of prooxidants that were higher than those shown in the figures and tables of this paper, and also did not prevent or delay their cytotoxicity (data not shown). Similarly, concentrations of the prooxidants that did not completely deplete or only produced a transient loss of mitochondrial Ca^{2+}, were also not toxic to the cells.

Table 1. Cyclosporin A prevents the loss of the mitochondrial (FCCP-releasable) Ca^{2+} pool in hepatocytes by 3,5-Me_2-NAPQI

Treatment	Mitochondrial Ca^{2+} pool (nmol/10^6 cells)	
	at 15 min	at 30 min
Control	0.28 ± 0.04	0.46 ± 0.03
CsA	1.30 ± 0.67*	1.98 ± 0.65†
3,5-Me_2-NAPQI	0.73 ± 0.15	0.09 ± 0.03†
3,5-Me_2-NAPQI + CsA	0.61 ± 0.15	0.67 ± 0.05

Following the pretreatment of hepatocytes in the absence or presence of CsA (300 nM) for 20 min, 3,5-Me_2-NAPQI (225 μM) was added as indicated. The size of the mitochondrial Ca^{2+} pool was measured as described in Materials and Methods. Each value is the mean ± SE of four separate experiments conducted on different hepatocyte preparations.

* Significantly different from control at 15 min ($P < 0.05$).

† Significantly different from control at 30 min ($P < 0.05$).

In contrast with the data obtained with 3,5-Me_2-NAPQI, CsA did not affect the loss of cellular ATP following the treatment of the hepatocytes with tBH or cumene hydroperoxide. Hence, there was no correlation between the loss of ATP and the cytotoxicity of the latter two prooxidants in hepatocytes.

DISCUSSION

The presence of CsA (at a concentration reported previously to prevent prooxidant-induced Ca^{2+} release from isolated mitochondria [16–18]) protected hepatocytes from the loss of viability caused by the prooxidants, tBH, cumene hydroperoxide and 3,5-Me_2-NAPQI. The concentration of prooxidant added to the hepatocyte suspension greatly modulated the ability of CsA to prevent cell death. For instance, protection by CsA only occurred at concentrations of tBH that killed the cells 1–1.5 hr after the addition of the prooxidant. Under these conditions, CsA maintained the mitochondrial Ca^{2+} pool. Similarly, with concentrations of tBH that killed all the cells within 15–30 min, CsA was without effect on the depletion of Ca^{2+} from mitochondria and the loss of cell viability. CsA also only slightly delayed the loss of cell viability with concentrations of cumene hydroperoxide which induced cell killing before 1–1.5 hr (as opposed to the results shown in Fig. 3). The protection by CsA against cumene hydroperoxide-induced cell death again correlated with the ability of the immunosuppressant to maintain the mitochondrial Ca^{2+} pool in the presence of the prooxidant.

Several conclusions can be drawn from these findings. Firstly, the depletion of the mitochondrial Ca^{2+} pool by the prooxidants precedes and appears to be causally related to the loss of cell viability. Secondly, the ability of CsA to protect hepatocytes from the loss of cell viability correlates with its protective effect on the mitochondrial Ca^{2+} pool. Thirdly, the range of prooxidant concentrations at which CsA protected against the loss of cell viability was limited, and the degree of CsA protection from cell killing varied with the nature of the prooxidant. Thus, it appears from the latter conclusion that additional cellular targets and toxic pathways may be differentially recruited depending not only on the concentration but also on the type of prooxidant used. CsA only protects hepatocytes against the toxicity of those prooxidants whose primary target (at a specific concentration) is mitochondrial Ca^{2+} homeostasis. These conclusions are further illustrated

Table 2. Comparison between the effects of tBH on the mitochondrial Ca^{2+} pool, ATP content and hepatocyte viability

Experiment	Concentration of tBH (μM)	CsA	Mitochondrial Ca^{2+} pool* (nmol/10^6 cells)		ATP content† (%)	Viability (%)	
			at 15 min	at 30 min	at 30 min	at 30 min	at 60 min
I	250	−	0.27	0.06	54	80	47
		+	0.30	0.38	45	74	66
II	200	−	<0.03	0.13	ND	78	57
		+	0.76	1.79	ND	84	80
III	200	−	<0.03	<0.03	76	70	40‡
		+	2.90	1.90	75	74	63‡

Following the pretreatment of hepatocytes in the absence or presence of CsA (300 nM) for 20 min, tBH was added as indicated. The size of the mitochondrial Ca^{2+} pool, cellular ATP content and viability were measured as described in Materials and Methods.

* Control mitochondria contained 0.46 (range 0.32–0.65) and 1.85 (range 1.39–2.30) nmol Ca^{2+}/10^6 cells in the absence and presence of CsA, respectively.

† Expressed as % of control value of each individual experiment; average control ATP level was 17.2 nmol/10^6 cells (range 13.8–21.5).

‡ Viability at 90 min; average control viability at the indicated times was 80% (range 75–84).

ND, not determined.

Table 3. Comparison between the effects of cumene hydroperoxide on the mitochondrial Ca^{2+} pool, ATP content and hepatocyte viability

Experiment	Concentration of cumene hydroperoxide (μM)	CsA	Mitochondrial Ca^{2+} pool* (nmol/10^6 cells) at 1 hr	Mitochondrial Ca^{2+} pool* (nmol/10^6 cells) at 1.5 hr	ATP content† (%) at 1.5 hr	Viability (%) at 1.5 hr	Viability (%) at 3 hr
I	150	−	0.90	0.09	93	67	35
		+	0.79	0.67	66	85	78
II	100	−	<0.03	<0.03	67	60	12
		+	0.88	0.71	66	71	68

Following the pretreatment of hepatocytes in the absence or presence of CsA (300 nM) for 20 min, cumene hydroperoxide was added as indicated. The size of the mitochondrial Ca^{2+} pool, cellular ATP content and viability was measured as described in Materials and Methods

* Control mitochondria contained 0.26 (range 0.20–0.34) and 1.50 (range 0.96–1.95) nmol $Ca^{2+}/10^6$ cells in the absence and presence of CsA, respectively.

† Expressed as % of control value of each individual experiment; average control ATP level was 15.1 nmol/10^6 cells (range 11.8–18.3).

by the inability of CsA to protect against the cytotoxicity caused by the prooxidants 2-methyl-1,4-naphthoquinone (menadione) (50–100 μM), diamide (0.5–1.5 mM), cystamine (5–15 mM) and dibenzoyl peroxide (0.25–2 mM).* It is likely that damage to the plasma membrane may be of primary importance in the case of some prooxidants such as cystamine [6]. Similarly, Bellomo *et al.* [37] have demonstrated recently that in isolated hepatocytes menadione causes early Ca^{2+}-independent damage to mitochondria. Hence, with the latter prooxidants, no protection by CsA would be expected.

Although plasma membrane blebbing is often regarded as an early morphological marker preceding the loss of cell viability [29], the degree of protection by CsA against cell killing did not always correlate with the degree of protection against plasma membrane blebbing. Prooxidant-induced blebbing results from a disruption of the cytoskeletal network [38, 39]; this is owed to the combination of a Ca^{2+}-dependent disorganization of the cytoskeleton and the oxidation of critical protein thiol groups by the prooxidants (see Ref. 40 for a recent review). Since the latter event is unlikely to be affected (at least during the initial stages of toxicity) by the presence of CsA, the lack of complete protection by CsA against plasma membrane blebbing is not surprising.

Inhibition of nuclear NAD^+ hydrolysis by 3-aminobenzamide, which has been shown in a number of experimental systems to delay the onset of cell death induced by prooxidants [33–36], did not prevent hepatocyte killing by 3,5-Me_2-NAPQI. Our findings corroborate a recent report by Stubberfield and Cohen [41] who observed no protective effect of 3-aminobenzamide against menadione toxicity and only a brief delay in the onset of the toxicity produced by glucose/glucose oxidase.

The initial loss of cellular NAD^+ content following exposure to 3,5-Me_2-NAPQI could be accounted for essentially by conversion to $NADP^+$. A similar phenomenon has been reported previously with hepatocytes exposed to the redox-cycling naphthoquinones, menadione and 2,3-dimethoxy-1,4-naphthoquinone [42]. Although a number of routes could account for this interconversion phenomenon, the simplest explanation involves the phosphorylation of NAD^+ to form $NADP^+$ by the action of NAD^+ kinase. This enzyme which is calmodulin-dependent for its activity [43, 44] would consequently have become activated by the increase in the cytosolic Ca^{2+} concentration reported previously to occur following exposure of hepatocytes to 3,5-Me_2-NAPQI [30]. The significance of this prooxidant-mediated interconversion of NAD^+ to $NADP^+$ is presently not known. It may, however, be important in providing an additional cofactor for the pentose phosphate pathway thereby supplying the cell with more NADPH for detoxification via the glutathione peroxidase–glutathione reductase system, and the biosynthetic pathways involved in cellular repair mechanisms.

Several investigators have linked the loss of cellular ATP during prooxidant challenge to the hydrolysis of NAD^+ by poly(ADP-ribose) polymerase and subsequent inhibition of glycolysis (and consequently oxidative phosphorylation) through the lack of cofactor availability [20, 32, 33, 45]. In contrast, our present findings of ATP depletion during the prooxidant challenge of hepatocytes by 3,5-Me_2-NAPQI and the marked protection by CsA of the energy content of the cell did not correlate with the change in total NAD^+ levels. However, there was one major difference between 3,5-Me_2-NAPQI and the two organic hydroperoxides, tBH and cumene hydroperoxide: the decrease in ATP content with the latter two prooxidants occurred independently of the presence of CsA. Whether this effect of the organic hydroperoxides, but not of 3,5-Me_2-NAPQI, was due to additional inhibition of the glycolytic pathway [20], possibly through the inhibition of glyceraldehyde-3-phosphate dehydrogenase [20, 46, 47], requires further investigation.

A striking observation was that CsA stimulated a rapid and time-dependent accumulation of Ca^{2+} within the mitochondrial pool. A similar observation has been made previously [48], although these investigators found a marked accumulation of Ca^{2+}

* Juedes MJ and Kass GEN, unpublished observations.

also in the non-mitochondrial (A23187-releasable) Ca^{2+} pool. In the present study, we failed to see any significant change in the size of the latter pool; however, it should be pointed out that the concentration of CsA used here was much lower than the concentration reported by Nicchitta *et al.* [48] (0.3 versus 8.3 μM). Additionally, at a similarly high concentration (10 μM), severe toxicity to the hepatocytes was observed,* possibly as a consequence of the Ca^{2+} ionophoretic properties displayed by CsA at such high concentrations [49]. The long-term accumulation of Ca^{2+} by mitochondria may play an important role in the mechanism of nephro- and hepatotoxicity of CsA. This hypothesis is substantiated by the recent findings that calcium channel antagonists can counteract CsA-mediated nephrotoxicity in both humans [50] and rats [51, 52].

In conclusion, it is reported that CsA prevents the cytotoxicity induced by a number of prooxidants by preventing Ca^{2+} cycling by mitochondria thereby maintaining intact mitochondrial functioning. Our results support the possibility of the clinical use of CsA to protect against certain forms of oxidative injury such as that occurring following the reperfusion of ischemic tissues [53, 54].

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