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# Cyclosporin A protects hepatocytes subjected to high $Ca^{2+}$ and oxidative stress

Kimberly M. Broekemeier[a], Laurie Carpenter-Deyo[b], Donald J. Reed[b] and Douglas R. Pfeiffer[a]

[a]*The Hormel Institute, University of Minnesota, Austin, MN 55912, USA* and [b]*The Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331, USA*

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Hepatocytes incubated with 0.8 mM *t*-butylhydroperoxide are protected by cyclosporin A when the medium $Ca^{2+}$ concentration is 10 mM, but not when it is 2.5 mM. The highest $Ca^{2+}$ level is associated with an inhibition of *t*-butylhydroperoxide-dependent malondialdehyde accumulation and with mitochondrial $Ca^{2+}$ loading within the cells. These findings are new evidence that *t*-butylhydroperoxide can kill cells by peroxidation-dependent and -independent mechanisms, and suggest that the mitochondrial permeability transition and the resultant de-energization are components of the peroxidation-independent mechanism. Cyclosporin A may have considerable utility for the protection of cells subjected to oxidative stress.

Oxidative stress; Permeability transition; Cyclosporin A; Cell injury; Lipid peroxidation; Hepatocyte

## 1. INTRODUCTION

*t*-Butylhydroperoxide (TBH) is widely utilized to investigate the mechanism of cell injury initiated by oxidative stress. While the mechanism remains unknown lipid peroxidation and altered cellular thiol status are implicated as components (see [1–3] for reviews).

TBH and other prooxidants induce the permeability transition in $Ca^{2+}$-loaded mitochondria (see [4] for review). The phenomenon, once referred to as $Ca^{2+}$-induced damage, creates a high-permeability state of the inner membrane resulting in loss of coupled function and $Ca^{2+}$ release [4]. Such effects should greatly diminish the effectiveness of mitochondria in defending cells against the loss of cytosolic $Ca^{2+}$ regulation associated with oxidative stress, and may be components of the cell injury mechanism per se. It has been difficult to test involvement of the transition in cell injury because it is not known how to manipulate it in vivo, and because known inhibitors were of low potencies and known to possess other pharmacological activities. Recently, however, the immunosuppressive cyclic peptide, cyclosporin A (CsA), has been shown to inhibit the transition with high potency and selectivity [4]. This finding suggests CsA could be used to evaluate the role of the transition in cell injury and to augment the defensive role of mitochondria. An initial examination of these possibilities is presented here.

*Abbreviations:* TBH, *t*-butylhydroperoxide; CsA, cyclosporin A; LDH, lactate dehydrogenase; MDA, malondialdehyde; DBH, dibutylphthalate; PCA, perchloric acid; CCP, carbonyl cyanide *p*-chlorophenylhydrazone.

*Correspondence address:* D.R. Pfeiffer, The Hormel Institute, University of Minnesota, 801 16th Avenue NE, Austin, MN 55912, USA. Fax: (1) (507) 437-9606.

## 2. MATERIALS AND METHODS

### 2.1. *Preparation and treatment of hepatocytes*

Parenchymal cells were isolated from the livers of male, Sprague–Dawley rats (175–200 g) as previously described [5]. They were resuspended at $2 \times 10^6$ cells/ml in modified Fisher's medium containing 2.5 mM $Ca^{2+}$. Initial viability of cells was >92%. Aliquots of cell suspension (20–22 ml) were placed in 125-ml culture flasks and treated with CsA (0.5 $\mu$M) or an equivalent volume of the ethanol vehicle (<0.3% by volume). Flasks were rotated under an atmosphere of humidified 95% $O_2$/5% $CO_2$ for 20 min (37°C). Following this, additional calcium was added to give a final concentration of 10 mM in some of the flasks. After a further 10 min, TBH (0.8 mM) was added where appropriate. Aliquots of cell suspension were taken from each flask prior to and at various times after calcium and TBH addition for analysis of lactate dehydrogenase (LDH) release, lipid peroxidation, or intracellular $Ca^{2+}$ content.

### 2.2. *Biochemical analyses*

Lipid peroxidation was assessed by assaying for malondialdehyde (MDA). Prior to determination of intracellular $Ca^{2+}$ content, separation of live from dead cells was achieved by centrifuging an aliquot of the cell suspension (0.5 or 1 ml) through dibutylphthalate (DBH), into either 36% buffered Percoll or 10% perchloric acid (PCA), as previously described [5]. LDH activity in the medium above the DBH layer was measured using a Sigma assay kit. It is expressed as percent of maximal activity elicited by treatment of cells with Triton X-100 (0.1%). The concentration of $Ca^{2+}$ in the PCA extract was determined by atomic absorption spectrometry [5]. In some experiments the intracellular $Ca^{2+}$ distribution was also examined. Cell pellets obtained by centrifugation through DBH and into Percoll (described above) were washed twice with, and rapidly resuspended in, $Ca^{2+}$- and $Mg^{2+}$-free Hank's balanced salt solution. Arsenazo III (80 $\mu$M) was then added to the cell suspension (2.5 ml) and the difference absorbance at 654–675 nm was monitored using an Aminco DW2a dual-wavelength spec-

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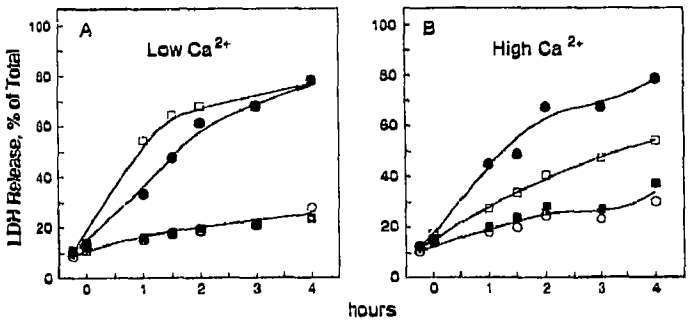


Fig. 1. Effect of CsA on the viability of hepatocytes subjected to oxidative stress. Incubations and the determination of LDH release were conducted as described in Materials and Methods. In panels A and B, the medium $Ca^{2+}$ concentration was 2.5 and 10 mM, respectively. ●, TBH present, CsA absent; □, TBH present and CsA present; ■, TBH absent, CsA present; ○, TBH absent, CsA absent.

trometer. The absorbance changes observed after sequential addition of uncoupler (carbonyl cyanide *p*-chlorophenylhydrazone, CCP, final conc. 10 $\mu$M) and calcium ionophore (A23187, final conc. 6 $\mu$M) were taken to represent $Ca^{2+}$ release from the mitochondrial and non-mitochondrial pools, respectively [11]. Intracellular $Ca^{2+}$ values were expressed per number of viable cells as determined by the DNA content of the cell pellet or from the distribution of LDH activity.

## 3. RESULTS

Fig. 1 shows the effects of 0.5 $\mu$M CsA on the viability of hepatocytes incubated in the presence of TBH. When the medium $Ca^{2+}$ concentration was 2.5 mM (Fig. 1A) the peptide produced a modest enhancement of TBH toxicity. However, at 10 mM extracellular $Ca^{2+}$, CsA markedly improved the retention of viability. Throughout the course of seven replicate experiments, like the one shown in Fig. 1 when 2.5 mM $Ca^{2+}$ was employed, the effect of CsA ranged from a small potentiation to a small protective action. At the higher $Ca^{2+}$ concentration a strong protective effect of CsA was always observed. In the most favorable cases loss of viability proceeded at the same rate as in the control incubation (CsA and 10 mM $Ca^{2+}$ present, TBH absent), while the minimum effect observed was a shift in the cell death curve of ~1 h to the right.

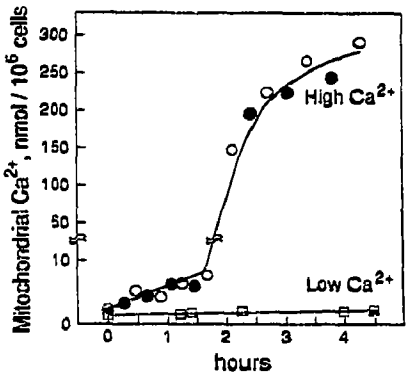


Fig. 3. Effects of medium $Ca^{2+}$ concentration and CsA on the $Ca^{2+}$ content of mitochondria in hepatocytes. Cells were incubated and the mitochondrial $Ca^{2+}$ content was determined as described in Materials and Methods. ■ and □, the medium $Ca^{2+}$ concentration was 2.5 mM with 0.5 $\mu$M CsA absent or present, respectively. ● and ○, the medium $Ca^{2+}$ concentration was 10 mM with 0.5 $\mu$M CsA absent or present, respectively.

The medium $Ca^{2+}$ concentration markedly altered the extent of lipid peroxidation as indicated by formation of MDA. At the low value (Fig. 2A) peroxidation was extensive and sustained, whereas with high $Ca^{2+}$ (Fig. 2B) little MDA was formed after the first hour. CsA decreased MDA formation slightly, however, this effect was negligible compared to the effect of $Ca^{2+}$ concentration. When Figs. 1 and 2 are viewed together they suggest that toxicity of TBH arises from peroxidation-dependent and -independent mechanisms, and that CsA is primarily effective against the independent process.

The medium $Ca^{2+}$ concentration also markedly affected the cell $Ca^{2+}$ content and the distribution of this cation between subcellular compartments. At 2.5 mM external $Ca^{2+}$, live cells contained 2.4 ± 0.6 nmol $Ca^{2+}$/$10^6$ cells ($n$ = 6), and 75% of this amount was released following addition of CCP, suggesting a mitochondrial location [6,7]. The size of this pool was constant during 4 h of incubation and was not affected by CsA (Fig. 3). At 10 mM external $Ca^{2+}$, the mitochondrial $Ca^{2+}$ pool increased by 3–4-fold during the first 2 h and then increased markedly as the experiment continued. Again,

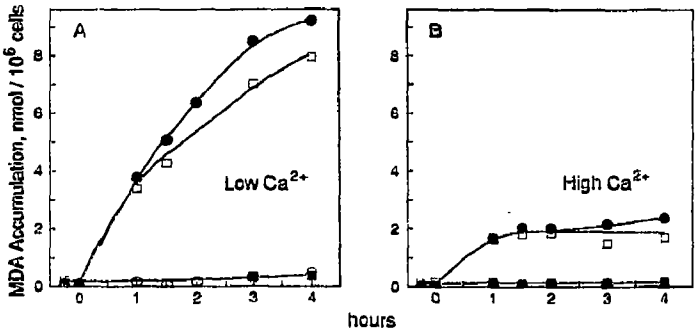


Fig. 2. Effect of CsA on MDA accumulation in hepatocytes subjected to oxidative stress. Samples were taken from the same incubations used to generate Fig. 1, and MDA was determined as described in Materials and Methods. In panels A and B, the medium $Ca^{2+}$ concentration was 2.5 and 10 mM, respectively. The symbols are as described in the legend to Fig. 1.

this behavior was little affected by CsA (Fig. 3). Once the marked rise in $Ca^{2+}$ content had begun, substantial $Ca^{2+}$ was released from live cells recovered from the incubation medium without addition of CCP or A23187 (data not shown). Thus, it is uncertain what fraction of $Ca^{2+}$ was contained within mitochondria after the first 2 h of incubation. At either low or high external $Ca^{2+}$, the A23187-releasable $Ca^{2+}$, which remained after release of mitochondrial $Ca^{2+}$, did not increase beyond 1.1 nmol/$10^6$ cells during the first 2 h (not shown).

## 4. DISCUSSION

The present data support the hypothesis that the mitochondrial permeability transition can occur under conditions of oxidative stress and contribute to the mechanism of cell death. This is indicated by the fact that CsA, a high-activity inhibitor of the transition [4], prolongs survival of hepatocytes treated with TBH under conditions where extensive lipid peroxidation is prevented (Figs. 1 and 2).

Hepatocytes contain CsA binding sites of non-mitochondrial origin [8] and, therefore, the possibility that protection arises from an action independent of the permeability transition cannot be excluded. However, using literature information [9–11] one can calculate that CsA was available to hepatocyte mitochondria at ~0.5 nmol/mg protein under the present conditions. This level is typical of that used to block the transition in isolated mitochondria [4]. One can further calculate from literature information [4,9,10], and from Fig. 3, that the mitochondrial $Ca^{2+}$ content and the TBH concentration were sufficient to induce the transition if it occurs in situ, as with isolated mitochondria. It is thus reasonable to attribute the protective action of CsA to its known activity as a transition inhibitor.

The condition used here to inhibit lipid peroxidation (a high medium $Ca^{2+}$ concentration) is effective (Fig. 2) and appropriate for the present purposes because the role of $Ca^{2+}$ in the permeability transition is reasonably well understood [4]. However, the mechanism by which inhibition occurs is not clear. TBH-dependent peroxidation in hepatocytes is sensitive to deferoxamine, indicating a requirement for $Fe^{3+}$ [12]. $Ca^{2+}$ loading may displace intracellular iron or influence its transport, and thereby alter iron availability for the initiation of peroxidation. Because the toxicity of TBH in hepatocytes is diminished by superoxide dismutase [13] the ability of high $Ca^{2+}$ to inhibit $O_2^-$-dependent lipid peroxidation [14] could also be a factor (but see [15]). Finally, elevated medium $Ca^{2+}$ causes isolated hepatocytes to maintain a normal content of vitamin E, and the antioxidant properties of that compound could be suppressing peroxidation (see [16] for review). Regardless of why $Ca^{2+}$ loading inhibits peroxidation the present results are new evidence that TBH can kill hepatocytes by peroxidation-dependent and -independent mechanisms [12,17], while implicating the transition as a component of the independent process. The mitochondrial de-energization associated with cell killing by TBH, when lipid peroxidation is allowed [12,17], may not represent the transition, because cyclosporin A is without effect under those conditions (Figs. 1 and 2). Alternatively, peroxidation may simply kill cells regardless of the mitochondrial state and further work will be required to distinguish between these possibilities.

Preliminary experiments indicate that CsA protects hepatocytes against TBH toxicity when peroxidation is inhibited by antioxidants instead of high $Ca^{2+}$ and that it is protective against the actions of menadione. Furthermore, it appears that mitochondrial de-energization, per se, correlates closely with cell death induced by several agents which influence the transition (e.g. [18]). Thus, the protective action of CsA demonstrated here may be of wider utility, and studies to test this possibility are in progress.

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