Contribution of the mitochondrial permeability transition to lethal injury after exposure of hepatocytes to *t*-butylhydroperoxide

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We have developed a novel method for monitoring the mitochondrial permeability transition in single intact hepatocytes during injury with *t*-butylhydroperoxide (*t*-BuOOH). Cultured hepatocytes were loaded with the fluorescence probes, calcein and tetramethylrhodamine methyl ester (TMRM). Depending on loading conditions, calcein labelled the cytosolic space exclusively and did not enter mitochondria or it stained both cytosol and mitochondria. TMRM labelled mitochondria as an indicator of mitochondrial polarization. Fluorescence of two probes was imaged simultaneously using laser-scanning confocal microscopy. During normal incubations, TMRM labelled mitochondria indefinitely (longer than 63 min), and calcein did not redistribute between cytosol and mitochondria. These findings indicate that the mitochondrial permeability transition pore ('megachannel') remained closed continuously. After addition of 100 μM *t*-BuOOH, mitochondria filled quickly with calcein, indicating the onset of mitochondrial permeability transition. This event was accompanied by mitochondrial depolarization, as shown by loss of TMRM. Subsequently, the concentration of ATP declined and cells lost viability. Trifluoperazine, a phospholipase inhibitor that inhibits the permeability transition in isolated mitochondria, prevented calcein redistribution into mitochondria, mitochondrial depolarization, ATP depletion and cell death. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a mitochondrial uncoupler, also rapidly depolarized mitochondria of intact hepatocytes but did not alone induce a permeability transition. Trifluoperazine did not prevent ATP depletion and cell death after the addition of CCCP. In conclusion, the permeability transition pore does not 'flicker' open during normal incubation of hepatocytes but remains continuously closed. Moreover, mitochondrial depolarization *per se* does not cause the permeability transition in intact cells. During oxidative stress, however, a permeability transition occurs quickly which leads to mitochondrial depolarization and cell death.

INTRODUCTION

In isolated mitochondria, a wide variety of chemicals induce increased permeability of the inner membrane to ions and solutes of M_r less than about 1200. This mitochondrial permeability transition (MPT) causes mitochondrial swelling and uncoupling of oxidative phosphorylation (reviewed in ref. [1]). The opening of an unselective pore (minimum diameter 2.8 nm) of very high conductance, a so-called megachannel, causes the MPT [2–4]. Cyclosporin A, an immunosuppressive agent, blocks the megachannel and inhibits the MPT [5–9]. Activation of Ca^{2+}-dependent matrix phospholipases may also contribute to the MPT, as products of phospholipid hydrolysis promote the transition and phospholipase inhibitors, such as trifluoperazine, inhibit it [10].

Cellular ATP depletion is a common feature of hypoxic and toxic injury. In hepatocellular injury induced by various oxidant chemicals, disruption of mitochondrial ATP formation appears to be a common mechanism leading to lethal cell injury [11]. Several groups have recently investigated the possible role of the MPT in oxidant-induced cell injury. Protection by cyclosporin A against reoxygenation injury to cardiac myocytes suggests pore opening during the oxidative stress of ischaemia/reperfusion [12,13]. Cyclosporin A also protects against lethal oxidative stress associated with incubation of hepatocytes in high-Ca^{2+} medium [14]. The megachannel may also be involved in continuous cycling of Ca^{2+} across the mitochondrial inner membrane of hepatocytes, and it was suggested that pro-oxidant chemicals perturb this normal cycling, causing release of mitochondrial Ca^{2+} into the cytosol before the onset of lethal injury [15]. Mitochondrial Ca^{2+} release under these conditions may result from nicotinamide nucleotide hydrolysis and protein mono-ADP-ribosylation [16].

We have also investigated the possible role of the MPT in oxidative injury to hepatocytes [17,18]. Cyclosporin A and trifluoperazine protected against toxicity by *t*-butylhydroperoxide (*t*-BuOOH) and iodoacetate. With *t*-BuOOH, lower concentrations ($\leq$ 50 μM) inhibited but did not uncouple mitochondrial ATP formation. Higher concentrations ($\geq$ 100 μM) uncoupled mitochondria, an event apparently associated with an MPT as trifluoperazine and cyclosporin A protected against both mitochondrial depolarization and cell death [18].

The purpose of the present study was to develop a method for monitoring the MPT in single intact hepatocytes in order to test the hypothesis that the MPT occurs after *t*-BuOOH exposure and contributes to lethal cell injury. Specifically, we wanted to evaluate megachannel activity *in situ* during normal incubations and during oxidative stress with *t*-BuOOH. Our results indicate that the megachannel does not open during incubations under normal conditions, but remains continuously closed. In contrast, during oxidative stress with *t*-BuOOH, a permeability transition develops in mitochondria of individual cells. This MPT leads to mitochondrial depolarization, ATP depletion and cell death.

EXPERIMENTAL

Hepatocyte isolation and culture

Hepatocytes were isolated by collagenase perfusion of livers

Abbreviations used: *t*-BuOOH, *t*-butylhydroperoxide; calcein AM, acetoxymethyl ester of calcein; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; KRH, Krebs–Ringer/Hepes buffer containing 115 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM Hepes buffer (sodium salt), pH 7.4; MPT, mitochondrial permeability transition; TMRM, tetramethylrhodamine methyl ester; $\Delta\Psi$, mitochondrial membrane potential.

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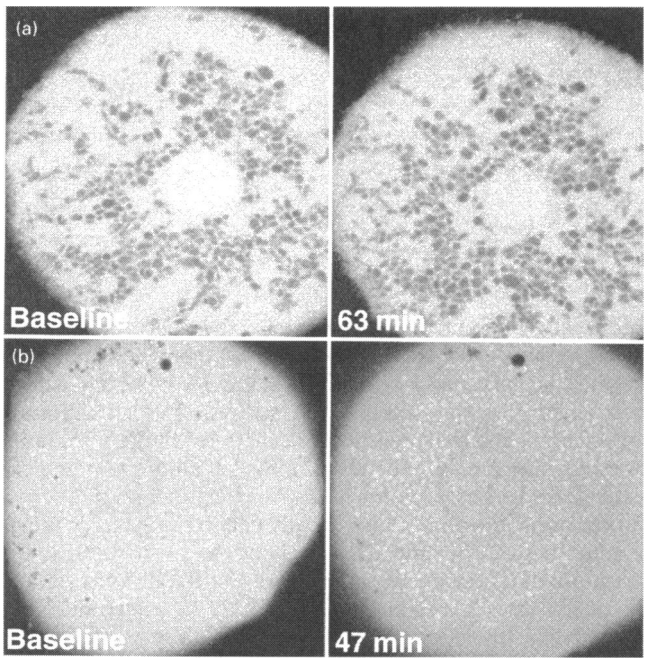

Figure 1 Temperature-dependence of calcein loading

Cultured hepatocytes were loaded with 1 μM calcein AM for 15 min at 37 °C (**a**) or 2 h at 4 °C (**b**). Green fluorescence was imaged by laser-scanning confocal microscopy as described in the Experimental section. After baseline images had been collected (left), subsequent images were collected at the time points indicated (right). Note dark round voids of fluorescence in (**a**) representing mitochondria.

from fed male Sprague–Dawley rats (200–250 g), as described previously [19]. Cell viability routinely exceeded 90%, as determined by Trypan Blue exclusion. Aliquots (4 ml) of cell suspension (100000 cells/ml) in Waymouth's MB-742/1 medium containing 26.7 mM $NaHCO_3$, 2 mM L-glutamine, 10% fetal calf serum, 100 nM insulin and 10 nM dexamethasone were plated on collagen-coated 40 mm round glass coverslips in 60 mm Petri dishes. Cells were cultured for 18–24 h in 5% CO_2/air at 37 °C.

Laser-scanning confocal microscopy

The fluorescence of calcein, tetramethylrhodamine methyl ester (TMRM) and propidium iodide was monitored using an Olympus LSM-GB 200 laser-scanning confocal microscope (Lake Success, NY, U.S.A.) or a Bio-Rad MRC-600 laser-scanning attachment (Hercules, CA, U.S.A.) mounted on a Nikon Diaphot inverted microscope (Garden City, NY, U.S.A.). With the Olympus system, the 488 nm and 543 nm lines of argon and helium–neon lasers were directed to the sample by a double-dichroic mirror. Fluoresced light was passed back through a double-dichroic reflector and divided by a second dichroic mirror into light greater or less than 575 nm. Green fluorescence of calcein continued through a 515 nm (25 nm band pass) barrier filter to a variable pinhole photodetector. Red fluorescence of TMRM and propidium iodide was passed through a 590 nm (long-pass) barrier filter. Pinhole settings of 2 were used for both the red and green channels. In the Bio-Rad system, samples were excited with the 488 and 568 nm lines of an argon–krypton laser. Green and red fluorescence was divided by a 560 nm dichroic mirror and detected through 522 nm (35 nm band pass) and 585 nm (long-pass) barrier filters respectively using pinhole settings of 3. In both systems, 60X N.A. 1.4 planapochromat objective lenses were used, and laser intensity was attenuated 0.1–1% to minimize photobleaching. Confocal images were transferred to a Silicon Graphics 310 VGX Imaging WorkStation (Mountain View, CA, U.S.A.) for processing.

Cell loading and incubation

Cultured hepatocytes were loaded in culture medium with 500 nM TMRM for 15 min followed by TMRM plus 1 μM acetoxymethyl ester of calcein (calcein AM) for 15 min at 37 °C, or with 1 μM calcein AM for 2 h at 4 °C and then by TMRM for 30 min at 37 °C. After three washes with Krebs–Ringer/Hepes (KRH) buffer, cells were mounted on the microscope stage and incubated at 35 °C in KRH containing 100 nM TMRM and 3 μM propidium iodide. Propidium iodide fluorescence did not interfere with TMRM fluorescence, because propidium iodide entry into non-viable cells occurs only after depolarization of mitochondria [20]. Moreover, nuclear fluorescence can be clearly distinguished from mitochondrial fluorescence in the confocal images. In some experiments, hepatocytes were loaded with TMRM and calcein at 37 °C and then imaged in medium containing 300 μM calcein free acid.

Cytotoxity assay

Cell viability was determined by propidium iodide fluorescence [20]. Briefly, hepatocytes (500000 cells/well) were cultured on six-well plates for 18–24 h. Cells were preincubated with 20 mM fructose and 5 μM trifluoperazine or no addition for 30 min in 4 ml of KRH buffer containing 30 μM propidium iodide at 37 °C. After addition of 100 μM *t*-BuOOH or 10 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), cell viability in each well was monitored by measuring propidium iodide fluorescence with a Millipore CytoFluor 2300 fluorescence scanner (Bedford, MA, U.S.A.) using 560 nm (40 nm band pass) excitation and 645 nm (50 nm band pass) emission filters. In some experiments, cell killing was determined from leakage of lactate dehydrogenase into the medium [21].

Measurement of ATP concentration

Hepatocytes (500000 cells/well) were cultured for 18–24 h in six-well plates in the culture medium described above. At various time points, the culture medium was aspirated, 1 ml of cold 1 M $HClO_4$ was added, and the cells were scraped off with a rubber policeman. After centrifugation (9000 ***g***) in a microcentrifuge (Fisher Scientific, Pittsburgh, PA, U.S.A.), the supernatants were neutralized with 5 M KOH and 0.4 M imidazole. After further centrifugation, the supernatants were diluted 200-fold with deionized distilled water. ATP was determined with a commercial luciferin–luciferase kit (Promega Enliten, Madison, WI, U.S.A.) using an MGM Instruments Optocomp I luminometer (Hamden, CT, U.S.A.).

Materials

Calcein, calcein AM and TMRM were purchased from Molecular Probes (Eugene, OR, U.S.A.); propidium iodide, *t*-BuOOH, type-I collagen and CCCP were from Sigma (St. Louis, MO, U.S.A.); Hepes and type-II collagenase came from Boehringer-Mannheim Biochemicals (Indianapolis, IN, U.S.A); Waymouth's medium MB-252/1 was obtained from Gibco Laboratories (Grand Island, NY, U.S.A.); insulin was from Squibb-Novo (Princeton, NJ, U.S.A.); and dexamethasone sodium phosphate was from LyphoMed (Rosemont, IL,

U.S.A.). Other chemicals were of analytical grade obtained from the usual commercial sources.

RESULTS

Stability of calcein compartmentation during normal incubation of cultured hepatocytes

TMRM, a cationic fluorophore, accumulated in the mitochondria of cultured hepatocytes in response to the negative mitochondrial membrane potential ($\Delta\Psi$) [22]. Consequently individual mitochondria were imaged as small bright fluorescent spheres (see Figure 2a). In contrast, calcein diffusely labelled the cytosol of cultured hepatocytes loaded with calcein AM at 37 °C (Figures 1a and 2a). Areas corresponding to TMRM-labelled mitochondria were dark round voids in the calcein fluorescence. Compartmentation of calcein was quite stable, with no redistribution from cytosol into mitochondria even after more than an hour of incubation (Figure 1a, right). Similarly, TMRM labelling of mitochondria was constant for more than 2 h (results not shown).

When calcein was loaded at 4 °C rather than 37 °C, the fluorophore entered both the cytosolic and mitochondrial compartments (Figure 1b, left). In many cells, calcein was somewhat concentrated in the mitochondria. Again, the compartmental distribution of calcein was quite stable, and images collected an hour apart were almost identical except for slight photobleaching (Figure 1b, compare left with right).

Redistribution of calcein into mitochondria after addition of *t*-BuOOH

Oxidant chemicals cause a permeability transition in isolated mitochondria [1]. We therefore investigated whether an MPT would occur in intact hepatocytes after exposure to *t*-BuOOH. Cultured hepatocytes were co-loaded with calcein AM and TMRM at 37 °C, so that TMRM labelled polarized mitochondria and calcein labelled the cytosol (Figure 2a). After 7 min of exposure to 100 μM *t*-BuOOH, no change in TMRM or calcein fluorescence was evident (Figure 2b). However, after 9 min, mitochondria began to lose TMRM fluorescence and filled with calcein (Figure 2c). Cell-surface blebbing, an early indication of cell injury, also became evident. Subsequently, mitochondria depolarized completely, and the cell lost viability as indicated by nuclear staining with propidium iodide and leakage of all cytosolic calcein (Figure 2d). In some experiments, faint mitochondrial calcein labelling remained after cell death (results not shown).

After exposure to *t*-BuOOH, an overall 42 % decrease in pixel intensity for calcein fluorescence occurred in cytosolic and nuclear regions (compare Figure 2c with Figure 2a). To determine whether this decrease in fluorescence was due to gradual leakage of calcein across the plasma membrane, hepatocytes were loaded with TMRM and calcein at 37 °C and then incubated with calcein free acid in the extracellular medium. Under these conditions, extracellular calcein fluorescence was very bright (Figure 3a). Labelling of the cytosol was less intense (average pixel intensity 113), and mitochondrial regions were dark. After

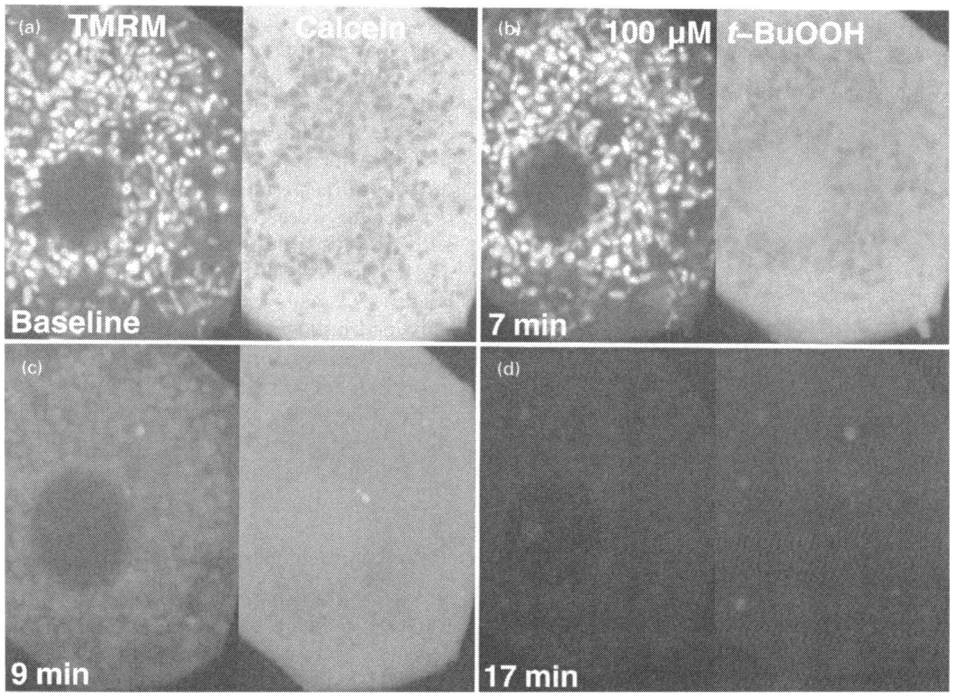

Figure 2 *t*-BuOOH-induced increase in mitochondrial membrane permeability in cultured hepatocytes

Cultured hepatocytes were loaded with 500 nM TMRM for 30 min and calcein AM for 15 min at 37 °C and incubated in KRH buffer containing 3 μM propidium iodide. Red (TMRM and propidium iodide) and green (calcein) fluorescence was imaged as described in the Experimental section. After the baseline image had been collected (**a**), 100 μM *t*-BuOOH was added. Images were collected 7 (**b**), 9 (**c**) and 17 (**d**) min later. In (**d**) note the faint staining of nuclear chromatin by propidium iodide.

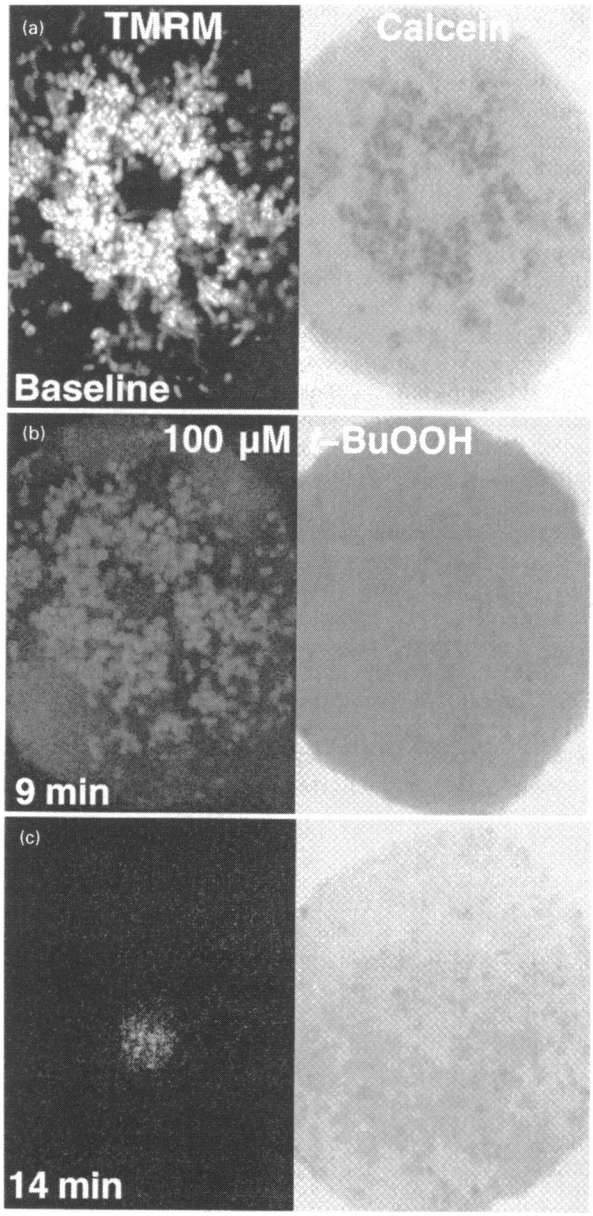

Figure 3 Effect of *t*-BuOOH on the leakage of calcein across the plasma membrane

Cultured hepatocytes were loaded with TMRM and calcein AM and then incubated with 300 μM calcein free acid and 3 μM propidium iodide in KRH buffer; fluorescence was imaged as described in Figure 2. After collection of a baseline image (**a**), 100 μM *t*-BuOOH was added, and images were collected 9 (**b**) and 14 (**c**) min later.

exposure to *t*-BuOOH, fluorescence of the cytosol decreased moderately (average pixel intensity 76) as mitochondria depolarized and the mitochondrial voids filled with fluorescence (Figure 3b). Several minutes later, calcein suddenly entered the cytoplasm (average pixel intensity 146) and, simultaneously, extracellular propidium iodide began to label the nucleus (Figure 3c). These last events were the consequence of bleb rupture and breakdown of the plasma-membrane permeability barrier at the onset of cell death. After calcein entry at cell death, darker mitochondrial voids were again visible. The observation that intracellular calcein fluorescence did not increase after exposure to *t*-BuOOH in medium containing high extracellular calcein indicates that *t*-BuOOH did not cause calcein to leak across the plasma membrane before loss of cell viability. Instead, the gradual decrease in calcein fluorescence after the addition of *t*-BuOOH was due to some other mechanism, such as photobleaching and fluorophore dilution as cells swelled and calcein redistributed into mitochondria.

Lack of effect of CCCP, a mitochondrial uncoupler, on mitochondrial calcein permeability

To determine whether mitochondrial depolarization *per se* increases the permeability of mitochondria to calcein, we exposed cultured hepatocytes to 10 μM CCCP, a mitochondrial uncoupler. After addition of CCCP, mitochondria began to lose TMRM fluorescence within 20 s (Figure 4b), virtually all of it having disappeared after 3 min (Figure 4c). After uncoupling, calcein only slowly redistributed into mitochondria, if at all. Even after 19 min of uncoupling, when the hepatocyte was blebbed, dark voids representing mitochondria were readily evident in the calcein fluorescence (Figure 4d). These results indicate that mitochondrial depolarization alone is not sufficient to explain increased mitochondrial permeability to calcein after exposure to *t*-BuOOH. We obtained similar results after adding CCCP, 2.5 mM KCN and 1 μg/ml oligomycin in the presence of 20 mM fructose (results not shown). The latter conditions caused mitochondrial depolarization but not cellular ATP depletion, blebbing or cell death [20].

Blockade by trifluoperazine of the MPT in intact hepatocytes induced by *t*-BuOOH

Trifluoperazine, a phospholipase inhibitor, blocks the permeability transition in isolated mitochondria [10]. We previously showed that trifluoperazine in the presence of fructose strongly protected hepatocytes exposed to *t*-BuOOH, and we hypothesized that the protection was due to blockade of the MPT [18]. We therefore investigated whether trifluoperazine prevents increased mitochondria permeability to calcein after *t*-BuOOH. Cultured hepatocytes were loaded with calcein and TMRM and incubated with 10 μM trifluoperazine plus 20 mM fructose. As before, TMRM localized to mitochondria and calcein labelled the cytosol (Figure 5a). After exposure to 100 μM *t*-BuOOH, mitochondria remained polarized and did not fill with calcein, even after 1 h and 55 min (Figures 5b–5d). Blebbing, an early indication of injury to hepatocytes, was also prevented by trifluoperazine. Thus trifluoperazine prevented oxidant-induced MPT and mitochondrial depolarization in intact hepatocytes, as well as loss of cell viability.

Prevention by trifluoperazine of ATP depletion caused by *t*-BuOOH

To determine whether protection by trifluoperazine against *t*-BuOOH toxicity was related to increased cellular ATP, we measured ATP in relation to cell viability after exposure to *t*-BuOOH. *t*-BuOOH caused rapid ATP depletion that slightly preceded but otherwise paralleled cell death (Figure 6). Trifluoperazine preserved ATP levels in the presence of *t*-BuOOH and prevented loss of cell viability. In contrast, trifluoperazine did not prevent ATP depletion of cell death caused by CCCP (Figure 7). In the experiments of Figures 6 and 7, cell viability was determined by propidium iodide fluorimetry, but essentially

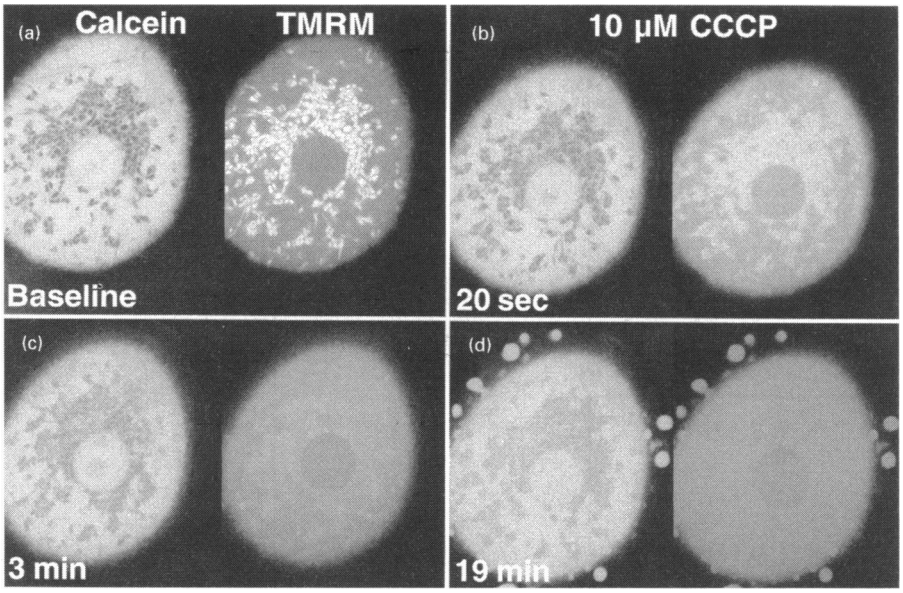

Figure 4 Effect of uncoupling on mitochondrial membrane permeability in cultured hepatocytes

Cultured hepatocytes were loaded with TMRM and calcein and fluorescence was imaged as described in Figure 2. After the baseline image had been collected (**a**), 10 μM CCCP was added. Images were then collected after 20 s (**b**), 3 min (**c**) and 19 min (**d**).

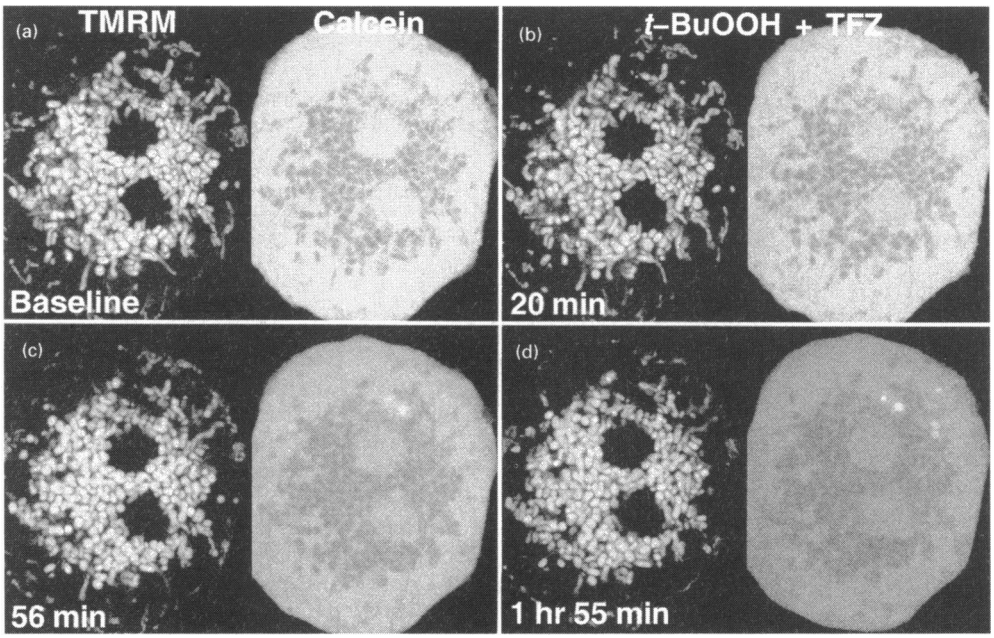

Figure 5 Protection by trifluoperazine against MPT and cell death in cultured hepatocytes after exposure to *t*-BuOOH

Cultured hepatocytes were preincubated for 30 min with 10 μM trifluoperazine and 20 mM fructose, loaded with TMRM and calcein, and fluorescence was imaged as described in Figure 2. After the baseline image had been collected (**a**), 100 μM *t*-BuOOH was added. Images were then collected at 20 min (**b**), 56 min (**c**) and 1 h 55 min (**d**).

identical results were obtained when cell damage was assessed by measuring lactate dehydrogenase release (results not shown). Taken together, these results support the hypothesis that trifluoperazine prevents the *t*-BuOOH-induced MPT that, in turn, causes uncoupling of oxidative phosphorylation, ATP depletion and cell death.

DISCUSSION

In Mitchell's [23] chemiosmotic hypothesis, impermeability of the inner mitochondrial membrane to ions is critical for maintaining the proton electrochemical gradient and mitochondrial function. Thus opening of the MPT pore is an event that

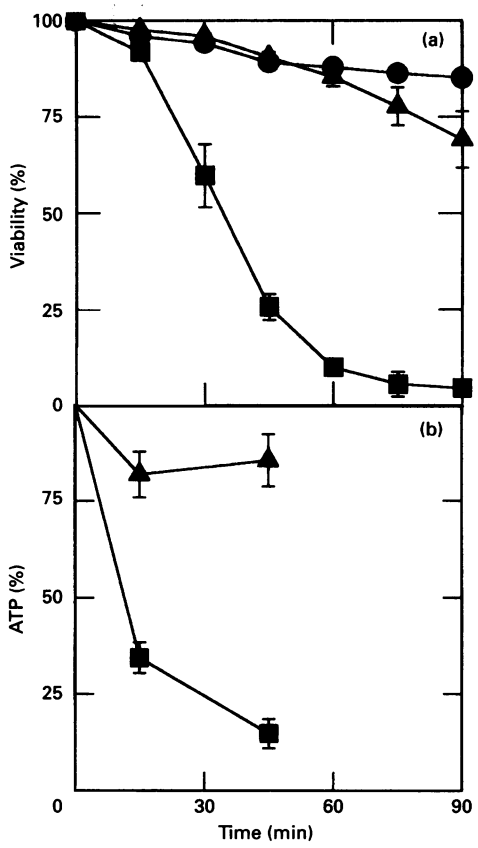

Figure 6 Effect of trifluoperazine on cell viability and ATP levels during exposure to *t*-BuOOH

Hepatocytes were cultured overnight in six-well plates (500 000 cells/well) and then preincubated for 30 min with 20 mM fructose in the presence (▲) or absence (■) of 5 μM trifluoperazine in KRH buffer containing 30 μM propidium iodide at 37 °C (●, control). At zero time, cells were exposed to 100 μM *t*-BuOOH. Cell viability (**a**) was assessed by measuring propidium iodide fluorescence and ATP (**b**) was measured by the luciferase bioluminescent assay. Points are means ± S.E.M. (if larger than symbol). ATP measurements were performed in triplicate on hepatocytes from three different isolations. Viabilities were determined in duplicate or triplicate in hepatocytes from two different isolations.

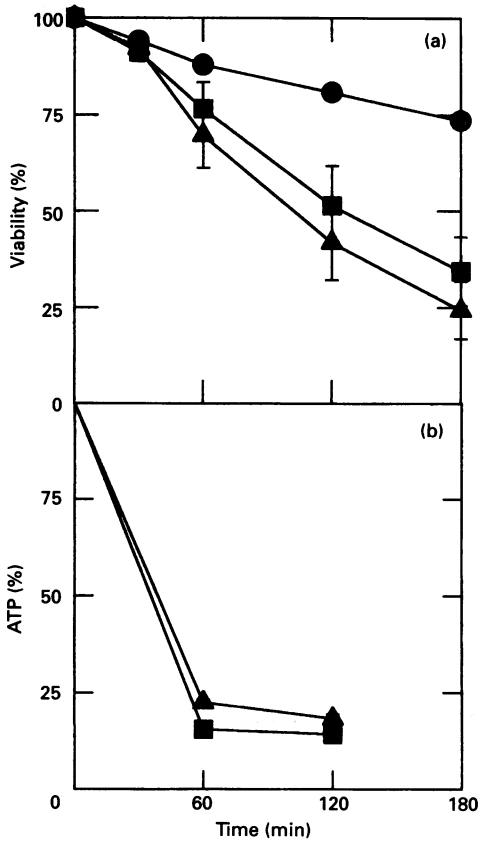

Figure 7 Effect of trifluoperazine on cell viability and ATP levels during exposure to CCCP

Hepatocytes were cultured overnight in six-well plates (500 000 cells/well), and then preincubated for 30 min with 20 mM fructose in the presence (▲) or absence (■) of 5 μM trifluoperazine in KRH buffer containing 30 μM propidium iodide at 37 °C (●, control). At zero time, cells were exposed to 10 μM CCCP. Cell viability (**a**) and ATP (**b**) were measured as described in Figure 6. Points are means ± S.E.M. from the number of experiments in Figure 6.

uncouples oxidative phosphorylation and stimulates ATP hydrolysis by the mitochondrial F_1F_o–ATPase. In isolated mitochondria, many agents induce onset of the MPT, including oxidant chemicals such as phenylarsine oxide, benzoquinone and *t*-BuOOH [15,24,25]. Other agents, such as cyclosporin A and trifluoperazine, block the transition. In patch-clamped mitochondria, the permeability transition pore has been identified as an unselective cyclosporin A-sensitive channel of very high conductance ('megachannel') with a minimum diameter of 2.8 nm in inner mitochondrial membranes [4]. However, an MPT has never been demonstrated in intact cells. Our goal was therefore to develop a method to monitor mitochondrial membrane permeability *in situ* to determine whether an MPT occurs after oxidative injury to intact cells and to assess whether the mitochondrial megachannel has activity during normal cellular incubations.

Temperature-dependence of calcein loading

Temperature greatly affected the intracellular distribution of calcein loaded as its acetoxymethyl ester. When loaded at 37 °C, calcein distributed predominantly into the cytosol. In confocal-microscopic images of calcein fluorescence, mitochondria were dark voids. Co-loading with TMRM, a cationic fluorophore that accumulates in mitochondria in response to mitochondrial $\Delta\Psi$ [22], clearly showed that voids in these calcein images represented individual mitochondria (Figure 1a). In contrast, after loading at 4 °C, calcein entered both cytosol and mitochondria. At the lower temperature, the activity of intracellular esterases may be decreased, allowing calcein acetoxymethyl ester to diffuse through the cytosol into mitochondria before ester hydrolysis occurs. Once inside the mitochondria, calcein AM is hydrolysed and impermeant calcein free acid is trapped in the matrix space. Calcein is a fluorescein derivative with fluorescence intensity that is independent of pH in the range 6.5–9.5. Its M_r (623) is small enough that it can be readily transported across the mitochondrial inner membrane when the permeability transition pore is open. Previously, Bernardi et al. [26] showed in isolated mitochondria that 2′,7′-bis-(2-carboxyethyl)-5-(and -6-) carboxyfluorescein, another fluorescein derivative of M_r 520, permeates the inner membrane at the onset of the MPT.

MPT does not occur in intact hepoatocytes during normal conditions

The permeability transition pore is regulated by several factors,

including matrix pH, $\Delta\Psi$ and bivalent cations. Matrix pH less than 7 favours pore closing, whereas depolarization promotes pore opening [26,27]. Elevated matrix Ca^{2+} concentration also favours the open state, whereas high Mg^{2+} closes the pore [26,28]. Other physiological factors, including adenine nucleotides, inorganic phosphate and fatty acids, also influence pore opening (reviewed in refs. [1] and [29]). Thus regulation of pore activity is a complex phenomenon. In general, experiments with isolated mitochondria are performed under relatively unphysiological conditions of low Mg^{2+} and high matrix Ca^{2+}. Thus a relevant question to ask was does the transition pore have any activity *in situ*. Here, we showed that calcein does not redistribute across the mitochondrial membrane even after long incubation periods under normal conditions (Figure 1). This finding indicates that the permeability transition pore remains closed continuously in the normal metabolic state.

It has been suggested that Ca^{2+} continuously cycles across the mitochondrial membrane via pathways that include the cyclosporin A-sensitive permeability transition pore [15]. In the present study, even if the permeability pore was flickering open for only brief instants, calcein would nonetheless redistribute between cytosol and matrix. This was not observed. Thus the permeability transition pore seems not to contribute to Ca^{2+} cycling.

MPT is induced by *t*-BuOOH and blocked by trifluoperazine in intact hepatocytes

Recently we showed that the oxidant chemical, *t*-BuOOH, causes mitochondrial depolarization followed by cell death in glycogen-depleted hepatocytes isolated from starved rats [18]. Fructose, a potent substrate for glycolytic ATP formation in hepatocytes, protects against lethal cell injury from low concentrations of *t*-BuOOH ($\leqslant 100\ \mu M$), consistent with *t*-BuOOH-induced inhibition of mitochondrial ATP formation. Similarly, fructose protects against anoxia and respiratory inhibitors such as cyanide [11,20,30]. At higher concentrations of *t*-BuOOH (100–300 μM), however, fructose alone fails to protect. In contrast, fructose in combination with cyclosporin A or trifluoperazine, two inhibitors of the MPT, does delay mitochondrial depolarization and cell death. It should be noted that cyclosporin A and trifluoperazine are ineffective without fructose. Our hypothesis based on these observations is that *t*-BuOOH causes an MPT that uncouples mitochondria and stimulates the F_1F_o-ATPase. As a consequence, ATP generated by glycolysis of fructose is hydrolysed and can no longer rescue cells from lethal injury. Similarly, we have shown that the uncoupler, CCCP, reverses fructose protection against cyanide toxicity, an effect mediated by accelerated mitochondrial ATP hydrolysis [11,20]. Here, we have demonstrated directly for the first time that the MPT indeed occurs in intact hepatocytes during *t*-BuOOH toxicity. This transition is accompanied by mitochondrial depolarization and subsequently by cell death. The interval between onset of mitochondrial permeability to calcein and mitochondrial depolarization is always short, and often these two events cannot be temporally dissociated (compare Figures 2b and 2c). We never observed a situation in which depolarization preceded the MPT.

In isolated mitochondria, trifluoperazine, a phospholipase inhibitor, blocks the MPT, possibly by preventing the accumulation of the end products of phospholipase activity, such as lysophospholipids and fatty acids, that can induce the MPT [10,25]. Alternatively, trifluoperazine may have a direct inhibitory effect on the permeability transition pore [28]. As our earlier results demonstrated that trifluoperazine protected strongly against *t*-BuOOH toxicity, we investigated whether this protection was due to inhibition of the MPT. Indeed, trifluoperazine strikingly prevented the *t*-BuOOH-induced MPT in intact hepatocytes (Figure 5). It also prevented *t*-BuOOH-induced ATP depletion (Figure 6). However, it did not confer unspecific cytoprotection, as it did not prevent ATP depletion or cell death after exposure to the mitochondrial uncoupler, CCCP (Figure 7). Thus the present findings indicate that protection by trifluoperazine is related specifically to prevention of the MPT and preservation of mitochondrial function. In isolated mitochondria, cyclosporin A also inhibits the MPT [5–9]. In hepatocytes, however, cyclosporin A is weakly effective compared with trifluoperazine for prevention of *t*-BuOOH-induced cytotoxicity [17,18]. In the present work using cultured rather than suspended hepatocytes, cyclosporin A (0.5–5 μM) was again poorly effective in preventing *t*-BuOOH-induced cell death (results not shown). In intact cells, the effect of cyclosporin A on the MPT may be transient, as has been reported in isolated mitochondria [25] (see also ref. [17]).

Mitochondrial uncoupling is not sufficient to cause the MPT in intact hepatocytes

In isolated mitochondria, membrane depolarization favours opening of the permeability transition pore. To determine if mitochondrial depolarization alone was sufficient to induce the MPT in intact cells, cultured hepatocytes were exposed to CCCP, a protonophoric uncoupler. CCCP produced ATP depletion and cell death but alone did not induce MPT, demonstrating that depolarization *per se* is not sufficient to induce MPT (Figures 4 and 7).

t-BuOOH does not cause a non-specific increase in plasma-membrane permeability to calcein until the onset of cell death

After exposure to *t*-BuOOH, an overall 42% decrease in calcein fluorescence intensity occurred as injury progressed. To determine whether loss of fluorescence intensity was due to non-specific leakage of calcein across the plasma membrane, hepatocytes were loaded with calcein and then incubated in calcein-containing medium during exposure to *t*-BuOOH. During incubation in calcein-containing medium, *t*-BuOOH still produced a decrease in cytosolic calcein fluorescence (Figures 3a and 3b). The decrease in intensity of cytosolic calcein fluorescence is probably due, in part, to the dilution effects of cell swelling and calcein redistribution into mitochondria. The cytosol including the nucleus constitutes about 60% of total cell volume and mitochondria about 20% [31,32]. Thus equilibration of calcein between mitochondria and cytosol would alone account for a 25% decrease in cytosolic pixel intensity. In addition, total cell volume increases by about a third during ATP depletion [33], which can account for another 25% decrease in fluorescence intensity. Another mechanism contributing to the decrease in calcein fluorescence may be accelerated photobleaching fostered by oxygen radical generation in response to the added *t*-BuOOH.

At the onset of cell death, the plasma-membrane permeability barrier fails. Extracellular markers rush in and intracellular markers disappear. Interestingly, when extracellular calcein entered at the onset of cell death, it did not penetrate mitochondria (Figure 3c). Conversely, in cells labelled with calcein AM, mitochondrial calcein was sometimes not fully released at cell death. Such findings seem inconsistent with increased permeability of the mitochondrial inner membrane. However, although high intramitochondrial Ca^{2+} opens the permeability transition pore, high extracellular Ca^{2+} closes it [28]. Thus the influx of extracellular Ca^{2+} at cell death probably closes the permeability pore and prevents movement of calcein in and out of the mitochondria.

MPT and cell injury

Despite recent advances in understanding regulation of the MPT, the physiological and pathological implications of MPT *in vivo* remain unclear. The results shown here, however, indicate that MPT can contribute to lethal oxidant-induced injury to hepatocytes. As shown recently by Petronelli et al. [34], *t*-BuOOH and other pro-oxidant chemicals oxidize vicinal thiols in cysteine residues of the putative permeability transition pore, leading to an increased probability of the pore being open in isolated mitochondria. *t*-BuOOH also sensitizes pore opening by Ca^{2+} [35]. Recently in preliminary experiments using u.v.–visible laser-scanning confocal microscopy, we observed rapid oxidation of nicotinamide nucleotides and a rise in mitochondrial Ca^{2+} concentration after exposure to *t*-BuOOH, consistent with a role for both mitochondrial oxidation and increased mitochondrial Ca^{2+} in the MPT occurring in intact hepatocytes [36].

The MPT may also have a role in the oxidative stress that occurs in ischaemia/reperfusion injury. In heart, post-ischaemic reperfusion leads to mitochondrial Ca^{2+} overload and subsequent mitochondrial dysfunction [37,38]. As high matrix Ca^{2+} levels induce opening of the permeability transition pore in isolated mitochondria, mitochondrial Ca^{2+} overload during reperfusion may trigger the MPT, leading to mitochondrial depolarization, ATP depletion and cell death. Recent reports that cyclosporin A preserves mitochondrial function and reduces lethal cell injury after reperfusion of isolated rat hearts and isolated cardiac myocytes support this hypothesis [12,13,39]. Reperfusion at acid pH (6.5) also protects against reperfusion injury after ischaemia in liver and heart [40,41]. As acid pH (< 7) conditions strongly inhibit opening of the permeability transition pore, protection in such conditions may be related to pore closing [42].

Conclusion

Using a novel method to monitor the MPT in intact hepatocytes by laser-scanning confocal microscopy, we have demonstrated that the permeability transition pore does not 'flicker' open under normal conditions. During exposure to *t*-BuOOH, however, MPT occurs quickly and leads to collapse of the mitochondrial $\Delta\Psi$, ATP depletion and cell death. Trifluoperazine inhibits the *t*-BuOOH-induced MPT and prevents mitochondrial depolarization, ATP depletion and loss of cell viability. Mitochondrial depolarization *per se* is not sufficient to cause an MPT in intact hepatocytes. Induction of the MPT may be a common feature in oxidative injury by toxic chemicals and after ischaemia/reperfusion. Future studies will address the role of pH, Ca^{2+} and other factors involved in the initiation of the MPT in intact cells under these conditions.

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