On the relationship between Ca^{2+} efflux and membrane damage during *t*-butylhydroperoxide metabolism by liver mitochondria

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The release of Ca^{2+} from rat liver mitochondria induced by *t*-butylhydroperoxide metabolism is caused by pyridine nucleotide oxidation and precedes alterations in membrane permeability which appear to result from, rather than cause, Ca^{2+} release.

Mitochondria *Calcium efflux* t-*Butylhydroperoxide* *Swelling* *Ruthenium red*

1. INTRODUCTION

The multiple effects of several hormones are now known to be mediated by alterations of intracellular Ca^{2+} homeostasis (see [1]). Various toxic agents, including *t*-butylhydroperoxide (tBH) have been found to cause perturbation of intracellular Ca^{2+} compartmentation in isolated hepatocytes prior to other indications of toxicity [2]. The metabolism of tBH by the glutathione peroxidase–glutathione reductase system results in the oxidation of glutathione and NAD(P)H. Simultaneously, Ca^{2+} is depleted from the mitochondrial and endoplasmic reticular compartments by pyridine nucleotide- and thiol-dependent processes [3]. The mechanisms controlling intracellular Ca^{2+} homeostasis, and the consequences of its alterations, are thus of both physiological and toxicological interest.

The mitochondria sequester a large portion of the cellular Ca^{2+} and play a major role in the regulation of the Ca^{2+} concentration in the cytosol [4]. Ca^{2+} cycling in liver mitochondria occurs by two distinct transport routes (see [5]), an electrogenic, ruthenium red-sensitive uptake carrier and an electroneutral efflux pathway which is not yet well characterized. The latter has been proposed to involve a $Ca^{2+}/2\,H^+$ antiport carrier regulated by the intramitochondrial pyridine nucleotide redox state [6]. Several studies with tBH have correlated NAD(P)H oxidation and Ca^{2+} release [7,8] supporting this idea. However, others have proposed a less specific release mechanism involving the stimulated formation of free fatty acids and lysophospholipids by phospholipase A_2 [9,10]. This would result in a non-specific membrane permeability increase, swelling, loss of transmembrane potential and Ca^{2+} release, which would occur independently of NAD(P)H oxidation.

The aim of this study was to determine the relative importance of NAD(P)H oxidation and membrane permeability changes during tBH-induced Ca^{2+} release by liver mitochondria. Under our conditions the pyridine nucleotide redox state and Ca^{2+} fluxes are closely linked. Permeability changes can be dissociated from these events and seem to result from, rather than cause, Ca^{2+} release.

2. MATERIALS AND METHODS

Rat liver mitochondria were isolated from male Sprague–Dawley rats (200–280 g) as in [11]. Protein was determined by the Lowry assay [12].

The standard incubation mixture (1 ml) contained 1 mg mitochondrial protein, 210 mM mannitol, 70 mM sucrose, 5 mM succinate, 3 μM rotenone, 0.15% bovine serum albumin (BSA), free fatty

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acid, 10 mM Tris–HCl (pH 7.4) and (for Ca^{2+} measurements only) 60 μM arsenazo III. All measurements were conducted at 25°C. Where indicated, incubations were performed in this medium without BSA.

Measurements of mitochondrial calcium fluxes, swelling and pyridine nucleotide oxidation state were performed spectrophotometrically at 654–685 nm, 540 nm and 340–370 nm, respectively. To correlate these events, Ca^{2+} fluxes and swelling or NAD(P)H changes were measured simultaneously with two spectrophotometers (Aminco DW-2 and Sigma ZWS-II). Additions to the incubations were made as indicated in the legends to figures. All concentrations given are final concentrations.

Chemicals were obtained commercially and were of analytical reagent grade, except ruthenium red (45% pure), which was used without further purification.

3. RESULTS AND DISCUSSION

Bovine serum albumin (0.1–1.0%) is routinely used in mitochondrial incubations to bind free fatty acids and lysophospholipids and to prevent non-specific membrane effects [13,14]. Fatty acids are known to disrupt mitochondrial functions by interfering with substrate transporters, increasing membrane permeability and uncoupling respiration [13]. Fig.1 shows a comparison of the effects of tBH on Ca^{2+} release by mitochondria incubated in the presence or absence of BSA. In both cases the pattern of Ca^{2+} release was dependent on the concentration of tBH. In the presence of BSA the mitochondria could maintain high Ca^{2+} loads ($\leq$ 100 nmol/mg protein) for at least 15 min and released Ca^{2+} steadily when exposed to fairly high concentrations of tBH. When BSA was not present, the mitochondria were far more sensitive to both Ca^{2+} and tBH. Ca^{2+} could be taken up but was released spontaneously within several minutes. Addition of very low amounts of tBH ($\leq$ 10 μM) caused a partial release of Ca^{2+} which was followed by reuptake. At higher tBH concentrations very rapid and complete Ca^{2+} release was induced. In both incubation systems an increased sensitization towards tBH was observed as the Ca^{2+} load was increased (not shown) in agreement with [8].

To determine the chronological order of events

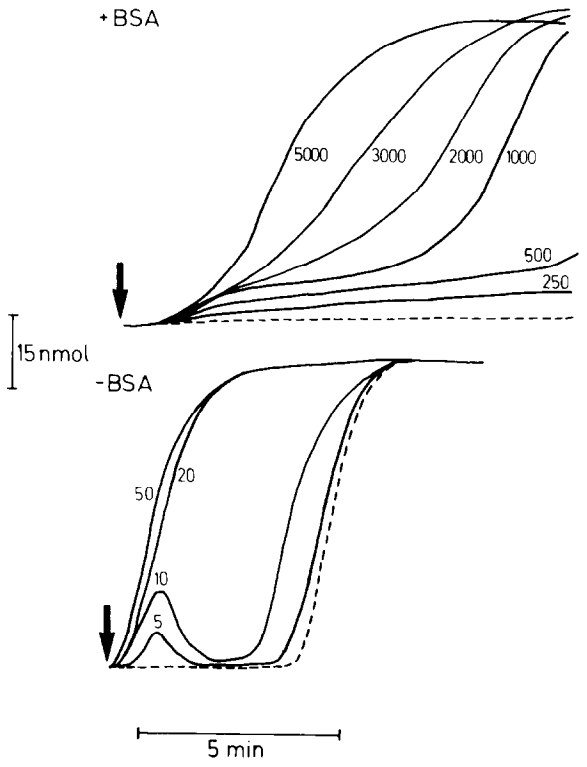

Fig.1. Mitochondrial Ca^{2+} release induced by tBH in the presence and absence of 0.15% BSA. Mitochondria were preloaded with 70 nmol Ca^{2+}/mg protein: (– – –) control traces, for which no further additions were made; (——) tBH was added (⟶) at the μM levels shown.

associated with tBH-induced Ca^{2+} release, simultaneous measurements of Ca^{2+} release, NAD(P)H oxidation and mitochondrial swelling were performed. Results from incubations in the presence of BSA are shown in fig.2. The sequence occurred as follows: NAD(P)H oxidation, Ca^{2+} release, swelling. Complete NAD(P)H oxidation occurred within 4 min after tBH addition. Swelling began after 15% of the total Ca^{2+} had been released and did not reach a maximum rate until release was 80% complete, indicating that it is not permeability changes which initiate Ca^{2+} efflux. In the absence of BSA, the commencement of these events occurred too rapidly for determination of their sequence (not shown).

Several lines of evidence indicate that mitochondrial Ca^{2+} release induced by tBH is linked to the metabolism of the hydroperoxide. We had shown

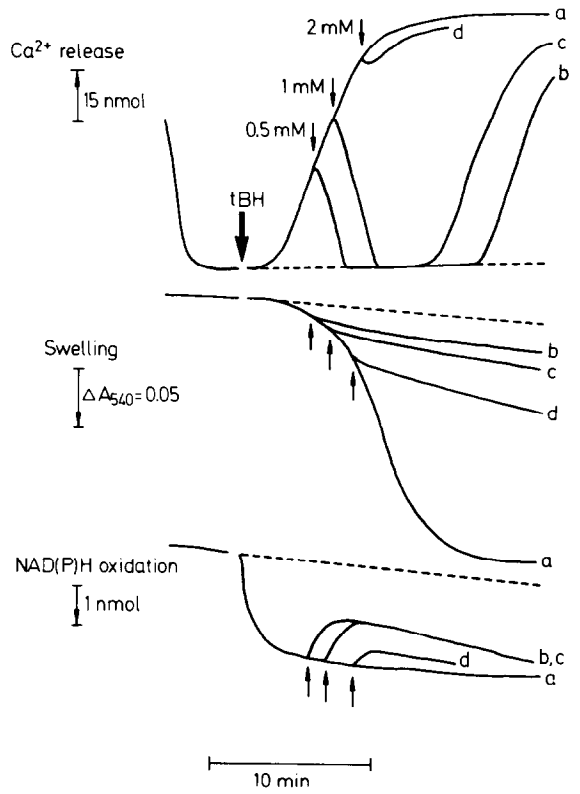

Fig.2. Sequence and reversibility of tBH-induced Ca^{2+} release, mitochondrial swelling and NAD(P)H oxidation. Mitochondria were preloaded with 105 nmol Ca^{2+}/mg protein in an incubation medium containing BSA: (– – –) control traces, for which no further additions were made; (——) tBH (3 mM) was added where indicated by the large arrow. Subsequent additions (at small arrows) were as follows: (a) none; (b) 0.5 mM isocitrate; (c) 1 mM isocitrate; (d) 2 mM isocitrate.

this by diethylmaleate pretreatment of the rats, which prevents both tBH metabolism (by depletion of GSH required for glutathione peroxidase activity) and Ca^{2+} release [11]. Similar results have been obtained when glutathione peroxidase activity has been reduced by keeping rats on a selenium-deficient diet [7]. Further evidence that tBH-induced effects are metabolically linked is shown in fig.2. Addition of the $NAD(P)^+$-reducing agents isocitrate or β-hydroxybutyrate caused complete Ca^{2+} reuptake if added before 75% of total Ca^{2+} had been released. Beyond this point reversibility was poor. Both the extent of reuptake and duration of retention were dependent upon the amount of reducing agent added, indicative of a metabolically dependent process. Pyridine nucleotides were reduced to 40% of their original value during Ca^{2+} reuptake. When Ca^{2+} reuptake could not be induced, little $NAD(P)^+$ reduction was observed. This loss of reversibility may be associated with Ca^{2+}-dependent hydrolysis of $NAD(P)^+$ as in [8]. In contrast, mitochondrial swelling was permanently inhibited by these agents regardless of the state of reversibility.

Results very similar to those observed with isocitrate could be obtained with the thiol reductant dithiothreitol (DTT) (not shown). This compound causes non-enzymatic regeneration of reduced glutathione, independent of glutathione reductase and NADPH consumption. With DTT, Ca^{2+} release induced by tBH could be reversed in a dose-dependent manner, which was accompanied by decrease of NADPH oxidation and a lasting inhibition of swelling. Once the restorative effects of isocitrate or DTT had been exhausted, release of Ca^{2+} occurred at the same rate and to the same extent as in the absence of these agents (fig.2). During re-release, no occurrence of swelling or NAD(P)H oxidation was observed. However, in the latter case, hydrolysis of $NAD(P)^+$ may have decreased pyridine nucleotides to below detectability.

Further experiments were performed to compare tBH-induced mitochondrial swelling and Ca^{2+} release. Under appropriate conditions, these two events could be completely dissociated (fig.3). The addition of ruthenium red together with tBH caused a more rapid (although slightly less extensive) release of Ca^{2+} than with tBH alone, yet swelling was virtually eliminated by the presence of ruthenium red. Swelling was also prevented by the inclusion of EGTA in the medium during Ca^{2+} release. These observations, together with the sequence and reversibility patterns in fig.2, strongly suggest that the mitochondria are intact during all but the last stages of Ca^{2+} release, and that swelling is a consequence of this release. This agrees with results obtained from measurements of mitochondrial membrane potential during tBH metabolism in the presence and absence of EGTA [15]. Our results with ruthenium red indicate that the binding of released Ca^{2+} to the uniport carrier may be a key event in initiating swelling. This had

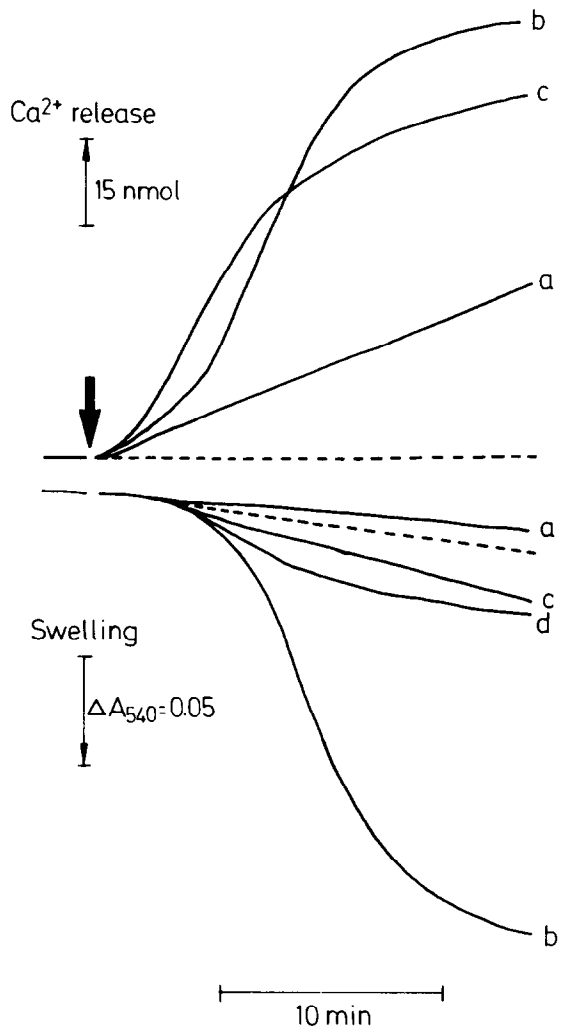

Fig.3. Dissociation of tBH-induced Ca^{2+} release from mitochondrial swelling. Mitochondria were preloaded with 65 nmol Ca^{2+}/mg protein in the presence of BSA: (---) control traces recorded without further additions; (——) additions were made (at large arrow) as follows: (a) 0.6 nmol ruthenium red; (b) 3 mM tBH; (c) 0.6 nmol ruthenium red plus 3 mM tBH; (d) 0.1 mM EGTA plus 3 mM tBH. Ca^{2+} fluxes cannot be measured by our method in the presence of EGTA.

been suggested in studies on the Ca^{2+}-induced membrane transition of beef heart mitochondria [16].

Our results support the idea that tBH-induced Ca^{2+} release is not due to an increase in membrane permeability, but to a specific efflux process which is sensitive to the NAD(P)H and thiol redox states. These results are also in agreement with results obtained from studies of tBH toxicity in intact hepatocytes [3].

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