Increase in cytosolic Ca^{2+} concentration during *t*-butyl hydroperoxide metabolism by isolated hepatocytes involves NADPH oxidation and mobilization of intracellular Ca^{2+} stores

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Activation of phosphorylase *a* in hepatocytes incubated with *t*-butyl hydroperoxide indicates that hydroperoxide metabolism is associated with an increase in cytosolic free Ca^{2+} concentration which appears to be mediated by NADPH oxidation and to involve mobilization of intracellular Ca^{2+} stores.

Isolated hepatocyte *Cytosolic Ca^{2+}* *Phosphorylase* a t-*Butyl hydroperoxide* *NADPH*

1. INTRODUCTION

In isolated hepatocytes, *t*-butyl hydroperoxide (*t*-BH) is metabolized by the glutathione peroxidase–glutathione reductase enzyme system present in the cytosolic and mitochondrial compartments [1]. Depending on the concentration of the hydroperoxide, its metabolism may result in a decrease in both the glutathione (GSH)/glutathione disulfide (GSSG) and NADPH/$NADP^+$ redox ratios, and in a diminished intracellular concentration of exchangeable Ca^{2+} [2]. The latter effect is due to loss of Ca^{2+} sequestered in both the mitochondria and endoplasmic reticulum which appears to be associated with NAD(P)H oxidation and GSH depletion, respectively [2]. Studies with isolated liver mitochondria [3–5] and microsomes [6] have confirmed the observations made with the intact hepatocyte system.

Moreover, we have recently reported that the ATP-dependent Ca^{2+} translocase of the liver plasma membrane fraction is dependent on free sulfhydryl groups for activity, and strongly inhibited by *t*-BH [7]. It appeared likely, therefore, that exposure of isolated hepatocytes to *t*-BH should result in, at least a transient, increase in cytosolic free Ca^{2+} concentration. However, for technical reasons previous attempts to monitor fluctuations in cytosolic Ca^{2+} concentration during *t*-BH metabolism by isolated hepatocytes have been unsuccessful. Thus, use of the intracellular Ca^{2+} indicator quin-2 [8] for this purpose has not been possible because of fluorescence interference due to the oxidation of NADPH associated with *t*-BH metabolism, and the 'null-point' titration procedure in [9] has not given consistent results in our hands.

We here have overcome this problem by using phosphorylase *a* activity to monitor alterations in cytosolic free Ca^{2+} concentration during *t*-BH metabolism by isolated hepatocytes. Phosphorylase *a* has previously been demonstrated to be a valid indicator of fluctuations in cytosolic Ca^{2+} level, since, under appropriate experimental conditions, its activation is strictly dependent on a Ca^{2+}-requiring phosphorylase kinase [10]. Using this approach, we can now report that *t*-BH metabolism by isolated hepatocytes is associated with an increase in cytosolic free Ca^{2+} concentration which appears to be mediated by pyridine nucleotide oxidation and to involve mobilization of intracellularly sequestered Ca^{2+}.

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2. MATERIALS AND METHODS

Collagenase (grade II) was obtained from Boehringer-Mannheim. The cation ionophore A23187 was purchased from Calbiochem-Behring. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), *t*-butyl hydroperoxide, glucose 1-phosphate, glycogen and EGTA were purchased from Sigma. All other reagents were of highest available grade of purity, and obtained from local commercial sources.

Hepatocytes were isolated from male, phenobarbital-treated Sprague-Dawley rats (200–250 g), allowed food and water ad libitum, by collagenase perfusion of the liver as described in [11], and incubated at 37°C in a Krebs-Henseleit medium supplemented with 20 mM Hepes (pH 7.4) at 1–1.3 × 10^6 cells/ml.

Phosphorylase *a* activity was assayed as in [12]. Briefly, hepatocytes were homogenized for 4–5 s in a Polytron homogenizer, setting at 5, in a medium containing 100 mM NaF, 20 mM EDTA, 0.5% (w/v) glycogen and 50 mM glycylglycine (pH 7.4). A portion (0.1 ml) of the homogenate was incubated with an equal volume of a medium containing 100 mM glucose 1-phosphate, 2% (w/v) glycogen, 0.3 M NaF and 1 mM caffeine (pH 6.1). The reaction was stopped at 0, 30 or 60 min with 0.5 ml of 20% trichloroacetic acid followed by 3.6 ml of water. Inorganic phosphate released from glucose 1-phosphate was measured as in [13]. Glutathione level was determined by the colorimetric method in [14] and $NADP^+$ and NADPH concentrations were measured using the spectrophotometric method in [15]. ATP content was measured as in [16].

The results reported in the figures are typical of 2–6 experiments.

3. RESULTS

Addition of the cation ionophore A23187 to a suspension of hepatocytes incubated in Krebs-Henseleit medium results in equilibration of the concentrations of Ca^{2+} in the extracellular and various intracellular compartments, and hence in a dramatic rise in cytosolic free Ca^{2+} concentration (from ~10^{-7} to ~10^{-3} M). As shown in fig.1, this treatment was associated with activation of phosphorylase *a* which was maximal at 10 μM

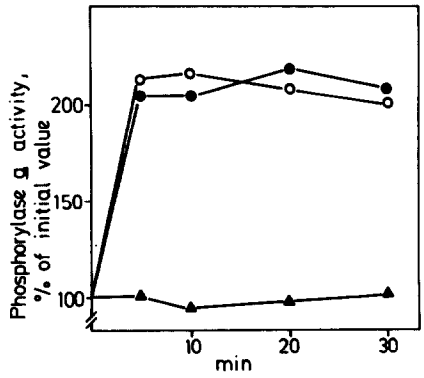

Fig.1. Phosphorylase *a* activation induced by addition of ionophore A23187 and uncoupler CCCP to isolated hepatocytes. A23187 or CCCP [dissolved in dimethyl sulfoxide (DMSO)] was added to the cell suspension and samples taken, when indicated, for phosphorylase *a* assay; DMSO volume did not exceed 0.1% of total volume. Control, no additions (▲); 10 μM A23187 (●); 10 μM CCCP (○). Phosphorylase *a* activity at 0 min was 0.112 units/10^6 cells.

A23187 under our experimental conditions. The uncoupler CCCP, which has previously been found to cause rapid release of Ca^{2+} sequestered within the mitochondria of isolated hepatocytes [2], had a similar effect on phosphorylase *a* activity (fig.1). Both treatments resulted in approximately a doubling of the non-stimulated phosphorylase *a* activity.

As illustrated in fig.2, incubation of hepatocytes

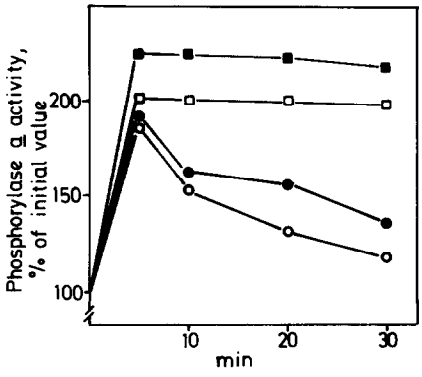

Fig.2. Phosphorylase *a* activation by *t*-butyl hydroxyperoxide does not involve influx of extracellular Ca^{2+}. *t*-BH, 0.5 mM (●, ○) or 1 mM (■, □) was added to a hepatocyte suspension in the absence (●, ■) or presence (○, □) of 3 mM EGTA added 1 min before *t*-BH. Phosphorylase *a* activity at 0 min was 0.13 units/10^6 cells.

with *t*-BH, under conditions which have previously been found to result in a decrease in Ca^{2+} sequestered in the mitochondria and, to a lesser extent, endoplasmic reticulum [2], was also associated with activation of phosphorylase *a*. This effect of *t*-BH was dose-dependent; the lower concentration employed (0.5 mM) caused a submaximal and transient activation of the enzyme whereas the higher concentration (1 mM) resulted in maximal stimulation of phosphorylase *a* activity which lasted during the entire (30 min) incubation period. There was no further stimulation of phosphorylase *a* activity at *t*-BH concentrations above 1 mM. In addition, concentrations of *t*-BH above 1 mM caused dose-dependent cytotoxicity during the incubation period, and were therefore not further used.

To exclude the possibility that influx of extracellular Ca^{2+} was responsible for the activation of phosphorylase *a* observed during *t*-BH metabolism by isolated hepatocytes, the Ca^{2+} concentration in the medium was decreased to below 1 μM by addition of the Ca^{2+} chelator EGTA. As shown in fig.2, also under these conditions incubation with *t*-BH resulted in phosphorylase *a* activation to a similar extent as in the absence of EGTA. This observation strongly suggests that *t*-BH-induced phosphorylase activation is mediated by mobilization of intracellularly sequestered Ca^{2+}, rather than by an influx of Ca^{2+} from the medium.

Fig.3 illustrates the relationship between phosphorylase *a* activation, NADPH oxidation and GSH depletion in hepatocytes incubated with 0.5 mM (A) or 1 mM (B) *t*-BH. Whereas there was no apparent decrease in cellular ATP level during incubation with either *t*-BH concentration employed, there was a progressive depletion of GSH which was more extensive at the higher substrate concentration. Further, at 1 mM *t*-BH there was also rapid oxidation of NADPH and persisting activation of phosphorylase *a*, whereas the alterations in both the NADPH/$NADP^+$ redox ratio and phosphorylase activity were less extensive, and transient, at the lower *t*-BH level. Taken together with our previous observation that NAD(P)H oxidation is associated with release of Ca^{2+} sequestered in the mitochondria during *t*-BH metabolism in hepatocytes, our results strongly suggest that the observed activation of phosphorylase *a* is caused by release of Ca^{2+} from the mitochondria into the cytosol during metabolism of the hydroperoxide.

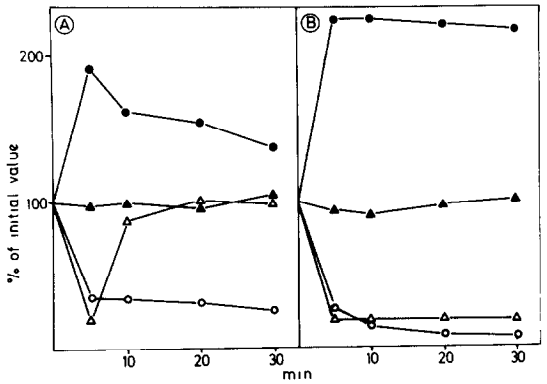

Fig.3. Relationship between phosphorylase *a* activity (●), NADPH/($NADP^+$ + NADPH) redox ratio (△), glutathione concentration (○), and ATP level (▲) during *t*-butyl hydroperoxide metabolism in isolated hepatocytes. Hepatocytes were incubated with 0.5 mM (A) or 1 mM (B) *t*-BH, and samples taken for assays as indicated. The absolute values (per 10^6 cells) were at 0 min: Phosphorylase *a*, 0.103 units; NADPH/($NADP^+$ + NADPH), 0.65; GSH, 58 nmol; ATP, 11 3 nmol.

4. DISCUSSION

It is now well established that an increase in cytosolic Ca^{2+} level in hepatocytes, above the normal concentration of 0.1–0.2 μM, is invariably associated with stimulation of phosphorylase activity. Thus, the α-adrenergic agents epinephrine and phenylephrine have been found to cause a 2–3-fold increase in cytosolic Ca^{2+} concentration, and a parallel stimulation of phosphorylase *a* activity from 12 to 21 units/g tissue [17,18]. In addition, we have recently found that inhibition of Ca^{2+} efflux from isolated hepatocytes results in an increase in cellular Ca^{2+} content and phosphorylase *a* activation [19]. Taken together with our present observation that both mobilization of Ca^{2+} from intracellular stores and stimulated influx of extracellular Ca^{2+} result in phosphorylase *a* activation (cf. fig.1), these findings strongly suggest that phosphorylase *a* activity can be used as a valid and sensitive indicator of alterations in cytosolic free Ca^{2+} concentration in hepatocytes.

We have previously reported that *t*-BH metabolism by isolated hepatocytes can be associated with the mobilization of Ca^{2+} from intracellular stores [2,20], and this study has shown that this mobilization results in an increase in cytosolic Ca^{2+} concentration which is sufficient to cause phosphorylase activation. The present findings, in particular the experiments with EGTA (cf. fig.2), strongly suggest that this increase in cytosolic Ca^{2+} level is not due to plasma membrane damage and influx of extracellular Ca^{2+}, but is of intracellular origin.

Several lines of evidence support the assumption that the mitochondria represent the compartment from which Ca^{2+} is mobilized during *t*-BH metabolism by hepatocytes.

(i) The mitochondrial Ca^{2+} pool is decreased during incubation of hepatocytes with *t*-BH [2,20].

(ii) The apparent relationship between NADPH oxidation and phosphorylase activation (cf. fig.3) suggests that the Ca^{2+} responsible for phosphorylase activation is of mitochondrial origin. Although, some controversy does exist [21], a large body of evidence indicates that, at least in the case of *t*-BH, oxidation of pyridine nucleotides is the factor triggering Ca^{2+} release from mitochondria [3–5,22,23]. Moreover, inclusion of β-hydroxybutyrate in the incubation medium to maintain pyridine nucleotides in the reduced form, delays the mitochondrial Ca^{2+} depletion induced by *t*-BH [19] and prevents the full stimulation of phosphorylase *a* (unpublished).

(iii) Low concentrations of *t*-BH (0.5 mM), which are still able to stimulate phosphorylase *a*, induce release of Ca^{2+} from mitochondria without affecting the endoplasmic reticular pool [20].

In conclusion, this study has shown that metabolism of *t*-BH by isolated hepatocytes, under the experimental conditions employed, is associated with phosphorylase *a* stimulation. We propose that this effect is due to an increased cytosolic free Ca^{2+} concentration caused by release of Ca^{2+} from the mitochondria into the cytosol as a consequence of oxidation of mitochondrial pyridine nucleotides during *t*-BH metabolism. It is possible that inhibition of the plasma membrane Ca^{2+} translocase, which can also result from exposure to *t*-BH (cf. [7]), may contribute to this increase in cytosolic free Ca^{2+} concentration.

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