Biochimica et Biophysica Acta, 777 (1984) 216–220
Elsevier

BBA 72273

EFFECT OF *t*-BUTYL HYDROPEROXIDE ON LIVER MICROSOMAL MEMBRANES AND MICROSOMAL CALCIUM SEQUESTRATION

LEON MOORE

Department of Pharmacology, Uniformed Services University, Bethesda, MD 20814 (U.S.A.)

(Received January 25th, 1984)
(Revised manuscript received June 27th, 1984)

Key words: tert-Butyl hydroperoxide; Ca^{2+} sequestration; (Liver microsome membrane)

In vitro exposure of hepatocytes or liver microsomes to *t*-butyl hydroperoxide resulted in a marked decrease of liver microsomal calcium pump activity. Decreased calcium pump activity was dependent upon both concentration and time. Liver microsomes could be protected from this effect by glutathione or dithiothreitol. In addition to decreased calcium pump activity, exposure of liver microsomes to *t*-butyl hydroperoxide produced a concentration-dependent aggregation of microsomal membrane protein as determined by polyacrylamide gel electrophoresis. Inhibition of microsomal calcium pump activity was observed when intact hepatocytes were incubated, in vitro, with *t*-butyl hydroperoxide. However, aggregation of microsomal membrane protein was not observed when hepatocytes were incubated with *t*-butyl hydroperoxide. The effects produced by exposure of liver microsomes to this compound do not appear to be a complete model of actions of the compound on intact cells.

Introduction

Recent studies have shown that exposure of isolated hepatocytes to *t*-butyl hydroperoxide disrupted cellular calcium homeostasis and decreased both mitochondrial and extramitochondrial pools of calcium [1–3]. The extramitochondrial pool of calcium may represent calcium sequestered in the endoplasmic reticulum [4] by a calcium pump activity found in the microsomal fraction [4,5]. Jones et al. [4] have reported that exposure of liver microsomes to *t*-butyl hydroperoxide, in vitro, inhibited liver microsome calcium pump activity. These workers suggested that calcium pump activity may be regulated by the glutathione redox state and that oxidative stress imposed by *t*-butyl hydroperoxide may be responsible for *t*-butyl hydroperoxide-produced damage to liver microsomal (endoplasmic reticulum) calcium pump activity. Reexamination of the effect of *t*-butyl hydroperoxide on liver microsomes in vitro confirms that exposure to this agent dramatically decreased calcium pump activity and that microsomal pump activity was protected by glutathione and dithiothreitol. Exposure of microsomes to *t*-butyl hydroperoxide produced marked aggregation of microsomal proteins. It is possible that *t*-butyl hydroperoxide-induced protein aggregation was responsible for *t*-butyl hydroperoxide-induced loss of calcium pump activity in liver microsomes exposed to this agent in vitro. The effect of *t*-butyl hydroperoxide on microsomes in vitro may not accurately model the effect of the compound on intact hepatocytes.

Experimental procedures

Liver microsomes were prepared from male Sprague-Dawley rats (175–250 g) in 250 mM sucrose/3 mM EDTA, as previously described [6],

except that the animals were not pretreated with phenobarbital. Microsomal pellets were resuspended in a solution comprising 125 mM sucrose/50 mM KCl, transferred to glass tubes, quickly frozen by immersion in liquid N_2 and stored at −70°C for up to 30 days. Calcium pump activity was determined with [$^{45}Ca^{2+}$] as previously described [5,6] in a medium comprising 100 mM KCl/30 mM imidazole-histidine buffer (pH 6.8)/5 mM sodium azide/5 mM ammonium oxalate/5 mM $MgCl_2$/5 mM ATP/20 μM $CaCl_2$ ([$^{45}Ca^{2+}$] 0.1 μCi/ml) and 20–40 μg/ml microsomal protein. Protein was determined by the Coomassie brilliant blue binding method described by Bradford [7]. Release of sequestered calcium was determined as follows: microsomes (0.2–0.3 mg/ml) were incubated at 37°C in a medium comprising 100 mM KCl/30 mM imidazole-histidine buffer (pH 6.8)/5 mM sodium azide/5 mM $MgCl_2$/5 mM ATP/20 μM $CaCl_2$ ([$^{45}Ca^{2+}$] 1 μCi/ml) until uptake equilibrium was reached (10–20 min). An aliquot of the reaction mixture was diluted 10-fold into medium containing 100 mM KCl, 30 mM imidazole-histidine buffer (pH 6.8), 0.1% DMSO with or without *t*-butyl hydroperoxide (1 μl/ml). Samples were taken at timed intervals and processed as described above. Hepatocytes were isolated from rats, anesthesized with pentobarbital, by the method of Berry and Friend [8] as modified by Crisp and Pogson [9].

Samples were collected for polyacrylamide gel electrophoresis at timed intervals. SDS-polyacrylamide gel electrophoresis was conducted in a 10% separating, 5% stacking gel system as previously described [10]. Proteins employed as molecular weight standards were obtained from Sigma (St. Louis, MO, USA): myosin (205 kDa), beta-galactosidase (116 kDa), phosphorylase *B* (97.4 kDa), albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa).

Results and Discussion

As reported by Jones and co-workers [4], incubation of liver microsomes in the presence of *t*-butyl hydroperoxide produced inhibition of liver microsomal calcium pump activity (Fig. 1). Inhibition of pump activity depended upon the concentration of *t*-butyl hydroperoxide and upon the length of exposure of microsomes to the hydroperoxide. Within 10 min of incubation with the hydroperoxide, microsomal calcium pump activity was inhibited at all concentrations tested. When microsomes were exposed to 0.3 μl/ml (approx. 210 μM, Fig. 1) or 1 μl/ml (data not shown), calcium pump activity was completely inhibited within 3 min.

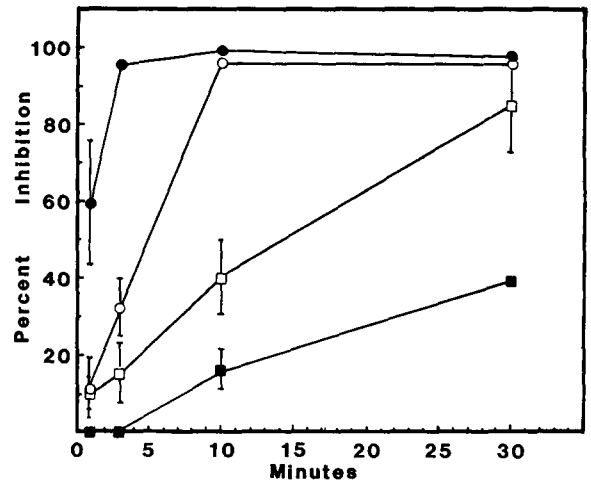

Fig. 1. Effect of *t*-butyl hydroperoxide on liver microsomal calcium pump activity. Liver microsomes (2–3 mg/ml) were preincubated with 0.1% DMSO (control) or DMSO and *t*-butyl hydroperoxide (0.1–0.3 μl/ml) in a medium comprising 50 mM KCl/125 mM sucrose at 37°C for periods of time up to 30 min. At timed intervals, aliquots were diluted 10-fold into additional KCl-sucrose at 4°C. An additional 10-fold dilution of protein, DMSO and *t*-butyl hydroperoxide was achieved as part of determination of liver endoplasmic reticulum calcium pump activity [5,6]. Each point represents the mean of percent inhibition ± S.E. for three or four experiments. Symbols used for preincubation concentration of *t*-butyl hydroperoxide are: ■, 0.01; □, 0.03; ○, 0.1, and ●, 0.3 μl/ml. Calcium pump activity declined during the preincubation. Microsomal calcium pump activity of control incubations was 230 ± 40 nmol Ca^{2+}/mg protein per 30 min after 1 min of preincubation, 201 ± 15 after 3 min, 176 ± 23 after 10 min and 133 ± 9.0 after 30 min.

Hydroperoxides have been shown to be metabolized by two routes in hepatocytes. Hydroperoxides are substrates for glutathione peroxidase and thus oxidize glutathione and pyridine nucleotides [11]. Hydroperoxides are also substrates for the cytochrome *P*-450 system. Metabolism by this system generates a free radical and induces lipid peroxidation [13]. As Jones and co-workers re-

ported [4], either dithiothreitol or glutathione would protect the liver microsomal calcium pump from the effect produced by *t*-butyl hydroperoxide (Table I). $MnCl_2$ has been shown to protect against hydroperoxide-induced lipid peroxidation [4,13]. Jones and co-workers [4] reported that pretreatment with $MnCl_2$ would not protect against *t*-butyl hydroperoxide-induced loss of microsomal calcium pump activity. This has also been confirmed (data not shown).

Others have reported that prolonged treatment of intact hepatocytes with cumene hydroperoxide produced lipid peroxidation and generated high-molecular-weight protein material that only slightly entered the separating gel upon polyacrylamide gel electrophoresis analysis [12]. When liver microsomes are incubated with *t*-butyl hydroperoxide for a shorter period of time, a similar effect was produced (Fig. 2). Incubation of microsomes with the hydroperoxide appeared to have produced aggregation of membrane proteins which was seen as high-molecular-weight material that only slightly entered the separating gel. As high-molecular-weight material appeared at the top of the polyacrylamide gel electrophoresis gel, several lower molecular weight protein bands were depleted. This may be an effect of the hydroperoxide independent of lipid peroxidation. CCl_4, a hepatotoxin that is metabolized to a free radical, is known to induce lipid peroxidation. CCl_4 has been shown to inhibit the liver microsomal calcium pump when incubated with microsomes and a NADPH-generating system [6,14,15]. CCl_4 did not induce formation of this high-molecular-weight material (Fig. 2).

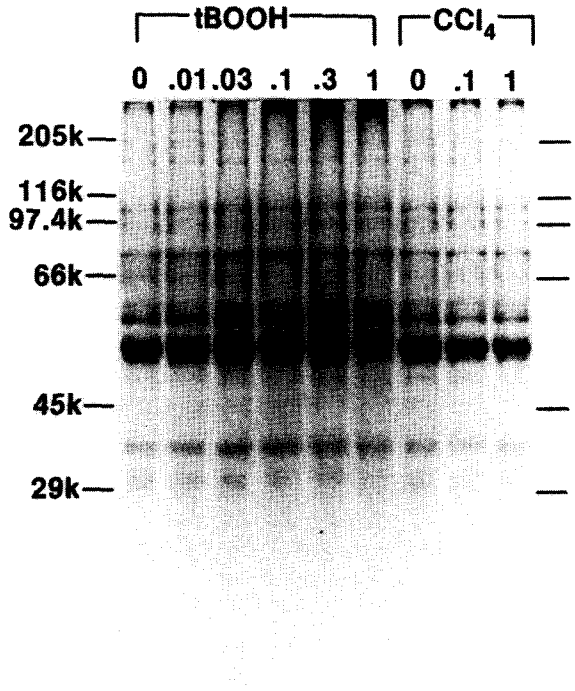

Fig. 2. SDS-polyacrylamide gel electrophoresis of liver microsomes incubated with *t*-butyl hydroperoxide or CCl_4. Liver microsomes were preincubated with 0.1% DMSO or DMSO and *t*-butyl hydroperoxide (0.01–1 μl/ml) as described in the legend for Fig. 1. Liver microsomes were also incubated with CCl_4 (0.1 and 1 μl/ml) and a NADPH-generating system, as previously described [6]. After incubation for 30 min, an aliquot was prepared for SDS-polyacrylamide gel elctrophoresis. Each lane was loaded with 20 μg protein and polyacrylamide gel electrophoresis was conducted as described in Experimental procedures. Samples are as follows (left to right). Lanes 1–6, *t*-butyl hydroperoxide at 0, 0.01, 0.03, 0.1, 0.3 and 1 μl/ml; lanes 7–9, CCl_4 at 0, 0.1 and 1 μl/ml.

Treatment of intact hepatocytes with *t*-butyl hydroperoxide (1 μl/ml, approx. 7 mM) produced 57 ± 8.2% inhibition of liver endoplasmic reticulum calcium pump activity within 1 min of incubation of the cells with the hydroperoxide. After 3 and 10 min of incubation, 74 ± 5.8 and 88 ± 5.8%

TABLE I

EFFECT OF GLUTATHIONE AND DITHIOTHREITOL ON *t*-BUTYL HYDROPEROXIDE-INDUCED LOSS OF CALCIUM PUMP ACTIVITY

Liver microsomes were preincubated with glutathione (GSH) or dithiothreitol (DTT) for 1 min at 37 °C and either *t*-butyl hydroperoxide (*t*-BOOH) in DMSO or DMSO was added. The samples were then incubated for an additional 3 min. Samples were taken and calcium pump activity was determined as previously described [5,6]. Calcium pump activity is expressed as nmol Ca^{2+}/mg protein/30 min. Each point is representative of three or more experiments.

t-BOOH (μl/ml)	GSH (mM)	Calcium pump activity	*t*-BOOH (μl/ml)	DTT (mM)	Calcium pump activity
0.3	0	3	0.3	0	3
0.3	0.3	9	0.3	0.3	13
0.3	1	14	0.3	1	21
0.3	3	48	0.3	3	63
0	0	124	0	0	142
0	3	150	0	3	132

inhibition of pump activity was observed. SDS-polyacrylamide gel electrophoresis of homogenates or microsomal preparations from these cells did not demonstrate an effect of *t*-butyl hydroperoxide to produce protein aggregation (data not shown). This suggested that hydroperoxide-produced aggregation of microsomal protein occurred only when microsomes were directly exposed to the hydroperoxide. This marked change to microsomal protein material implied that large changes in membrane structure had occurred during incubation of microsomes with *t*-butyl hydroperoxide. This suggested that changes of passive permeability properties of the microsomal membrane may have occurred. In fact, microsomal membrane permeability to calcium was increased within minutes after exposure to the hydroperoxide. If liver microsomes accumulated calcium in the absence of ammonium oxalate, calcium release could be studied when the reaction mixture was diluted sufficiently to minimize calcium pump activity [5]. In the experiments depicted in Fig. 3, liver microsomes accumulated calcium in the absence of oxalate until an apparent equilibrium was reached. Upon dilution, calcium release was observed. Within minutes after addition of *t*-butyl hydroperoxide, calcium release from the vesicles was substantially enhanced. Within 8 min, a statistically

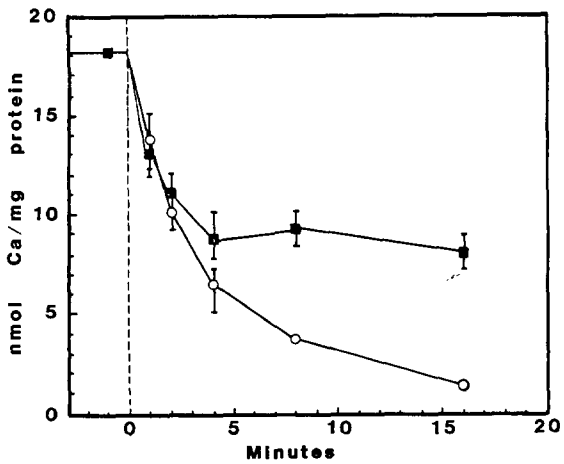

Fig. 3. Effect of *t*-butyl hydroperoxide on release of calcium from liver microsomes. Liver microsomes were incubated and calcium release was determined as described in Experimental procedures. Each point represents the mean ± S.E. for four experiments.

significant increase in the rate of calcium release was apparent. These experiments did not exclude inhibition of calcium pump activity as the major effect of *t*-butyl hydroperoxide. But, these experiments did suggest that increased permeability may have contributed to the observed effect of the hydroperoxide on calcium sequestration by liver microsomes.

Inhibition of liver endoplasmic reticulum calcium pump activity by hepatotoxins may contribute to expression of hepatotoxin action [16]. However, inhibition of pump activity by these compounds is complex. Pump activity is inhibited by chlorinated hydrocarbons, such as CCl_4 that induce lipid peroxidation but that do not deplete glutathione, and by compounds such as 1,1-dichloroethylene that do not induce lipid peroxidation but do deplete glutathione. 1,1-Dichloroethylene is a potent inhibitor of calcium pump activity in vivo [18] and in vitro [17]. However, neither lipid peroxidation [15] nor glutathione depletion [17] appears to be required for inhibition of endoplasmic reticulum calcium pump activity by hepatotoxins. Likewise, depletion of liver glutathione is not sufficient for loss of endoplasmic reticulum calcium pump activity [17]. In addition, hepatotoxins that are not chlorinated hydrocarbons, e.g., carbon disulfide [19] and thioacetamide [20] have been shown to promptly inhibit liver endoplasmic reticulum calcium pump activity. The results presented in this report are consistant with the findings of Jones, Orrenius and co-workers [1–4] that *t*-butyl hydroperoxide, another type of agent toxic to hepatocytes in vitro, intereferes with calcium homeostasis in hepatocytes and that the compound promptly inhibits liver endoplasmic reticulum calcium pump activity. However, the results presented in this report suggest that exposure of liver microsomes to the compound in vitro is not an adequate model of the effects of *t*-butyl hydroperoxide on intact cells.

Acknowledgements

The excellent technical assistance of Mr. Michael Fraley and Ms. Beth Leary-DiGiulian is acknowledged. The opinions or assertions contained herein are the author's and are not to be construed as official or reflecting the views of the

Department of Defense or the Uniformed Services University of the Health Sciences. The experiments reported herein were conducted according to the principles set forth in the 'Guide for the Care and Use of Laboratory Animals', Institute of Animal Resources, National Research Council, DHEW Pub. No. (NIH) 78-23. This study was supported in part by a grant from the U.S. Public Health Services (ESO2691).

References

1 Bellomo, G., Jewell, S.A., Thor, H. and Orrenius, S. (1982) Proc. Natl. Acad. Sci. USA 79, 6842–6846
2 Jewell, S.A., Bellomo, G., Thor, H., Orrenius, S. and Smith, M.T. (1982) Science (Wash. D C) 217, 1257–1259
3 Thor, H., Smith, M.T., Hartzell, P., Bellomo, G., Jewell, S.A. and Orrenius, S. (1982) J. Biol. Chem. 257, 12419–12425
4 Jones, D.P., Thor, H., Smith, M.T., Jewell, S.A. and Orrenius, S. (1983) J. Biol. Chem. 258, 6390–6393
5 Moore, L., Chen, T., Knapp, H.R., Jr. and Landon, E.J. (1975) J. Biol. Chem. 250, 4562–4568
6 Ray, P. and Moore, L. (1982) Arch. Biochem. Biophys. 218, 26–30
7 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254
8 Berry, M.N. and Friend, D.S. (1969) J. Cell Biol. 43, 506–520
9 Crisp, D.M. and Pogson, C.I. (1972) Biochem. J. 126, 1009–1023
10 Laemmli, U.K. (1970) Nature 227, 680–685
11 Lotscher, H.R., Winterhalter, K.H., Carafoli, E. and Richter, C. (1979) Proc. Natl. Acad. Sci. USA 76, 4340–4344
12 Koster, J.F., Slee, R.G. and Van Berkel, Th.J.C. (1982) Biochim. Biophys. Acta 710, 230–235
13 O'Brien, P.J. and Rahimtula, A. (1975) J. Agric. Food Chem. 23, 154–158
14 Lowrey, K., Glende, E.A., Jr. and Recknagel, R.O. (1981) Biochem. Pharmacol. 30, 135–140
15 Waller, R.L., Glende, E.A., Jr. and Recknagel, R.O. (1983) Biochem. Pharmacol. 32, 1613–1617
16 Recknagel, R.O. (1983) Life Sci. 33, 401–408
17 Moore, L. (1982) Biochem. Pharmacol. 31, 1463–1465
18 Moore, L. (1980) Biochem. Pharmacol. 29, 2505–2511
19 Moore, L. (1982) Biochem. Pharmacol. 31, 1465–1467
20 Younes, M.M. Albrecht, M. and Siegers C.-P. (1983) Res. Commun. Chem. Path. Pharmacol. 40, 405–415