Volume 224, number 2, 331–336 November 1987

2,5-Di(*tert*-butyl)-1,4-benzohydroquinone – a novel inhibitor of liver microsomal Ca^{2+} sequestration

Gregory A. Moore, David J. McConkey, Georges E.N. Kass, Peter J. O'Brien and Sten Orrenius

Department of Toxicology, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden

Received 18 August 1987; revised version received 9 October 1987

Treatment of rat liver microsomes with 2,5-di(*tert*-butyl)-1,4-benzohydroquinone caused a dose-related inhibition ($K_i \simeq 1$ μM) of ATP-dependent Ca^{2+} sequestration. This was paralleled by a similar impairment of the microsomal Ca^{2+}-stimulated ATPase activity. In contrast, the hydroquinone failed to induce Ca^{2+} release from Ca^{2+}-loaded liver mitochondria (supplied with ATP), and inhibited neither the mitochondrial F_1F_0-ATPase nor the Ca^{2+}-stimulated ATPase activity of the hepatic plasma membrane fraction. The inhibition of microsomal Ca^{2+} sequestration was not associated with any apparent alteration of membrane permeability or loss of other microsomal enzyme activities or modification of microsomal protein thiols. These findings suggest that 2,5-di(*tert*-butyl)-1,4-benzohydroquinone is a potent and selective inhibitor of liver microsomal Ca^{2+} sequestration which may be a useful tool in studies of Ca^{2+} fluxes in intact cells and tissues.

Microsome; Ca^{2+} sequestration; ($Ca^{2+} + Mg^{2+}$)-ATPase; 2,5-Di(*tert*-butyl)-1,4-benzohydroquinone; (Rat liver)

1. INTRODUCTION

The regulation of intracellular Ca^{2+} homeostasis has been extensively studied in recent years because of the important role that Ca^{2+} plays in mediating various cellular functions [1,2]. Particularly important in the modulation of cytosolic free Ca^{2+} concentration is the ATP-dependent Ca^{2+}-sequestration system of the hepatic endoplasmic reticulum [3]. There is now convincing evidence that the endoplasmic reticular Ca^{2+} pool is the major intracellular source of the Ca^{2+} release into the cytosol when hepatocytes are treated with various Ca^{2+}-mobilizing agents [4,5]. Inositol 1,4,5-trisphosphate appears to be a specific mediator of this Ca^{2+} release but other endogenous compounds, such as GTP and certain arachidonic acid metabolites, can also cause Ca^{2+} release from liver microsomes [5–9].

It is well known that various drugs and hepatotoxins can inhibit ATP-dependent Ca^{2+} sequestration by liver microsomes. This may be the result of altered membrane permeability or the modification of protein thiol groups critical for microsomal Ca^{2+}-stimulated ATPase activity [3,10–15]. However, few xenobiotics have been documented to perturb selectively the endoplasmic reticular Ca^{2+} pool. For example, vanadate, a potent inhibitor of microsomal Ca^{2+} sequestration and Ca^{2+}-stimulated ATPase activity, also inhibits other ATPase systems and is involved in various nonspecific cellular reactions [16–18].

Recently, our laboratory has been engaged in determining the mechanism(s) of quinone toxicity in hepatocytes and subcellular organelles [19,20]. During these investigations we found that 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (tBuBHQ) impaired microsomal Ca^{2+} sequestration. Here, we present data demonstrating that tBuBHQ

Correspondence address: S. Orrenius, Department of Toxicology, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

inhibits ATP-dependent Ca^{2+} sequestration in liver microsomes without affecting liver mitochondrial Ca^{2+} fluxes or the Ca^{2+}-ATPase activity of the hepatic membrane fraction.

2. MATERIALS AND METHODS

Livers from male Sprague-Dawley rats (180–200 g, fed ad libitum) were used in all experiments. Hepatocytes were isolated by the collagenase perfusion method in [21]. The hepatic plasma membrane fraction was prepared as in [22]. Mitochondria were isolated by differential centrifugation according to [23]. Microsomes were prepared essentially as described in [24], except that 150 mM KCl-20 mM Hepes, pH 7.4, was used as the homogenizing and washing medium. Protein concentration was measured according to Lowry et al. [25].

Ca^{2+} sequestration by liver microsomes was measured by two independent methods. Firstly, $^{45}Ca^{2+}$ sequestration was monitored as described by Moore et al. [3], with the modification that the microsomes were incubated in the absence of oxalate in 150 mM KCl-20 mM Hepes, pH 7.1, subsequently referred to as incubation buffer, supplemented with 1 mM $MgCl_2$ and 20 μM $^{45}Ca^{2+}$ (0.5 μCi/ml) at 37°C. Secondly, for continuous monitoring of Ca^{2+} fluxes, a Ca^{2+}-selective electrode was used [15]; the microsomes (1 mg protein/ml) were incubated in incubation buffer supplemented with ATP-Mg^{2+} (1.0–4.0 mM) and 20 μM Ca^{2+} at 37°C. Investigation of the reversibility of tBuBHQ-induced inhibition of Ca^{2+} sequestration was performed as follows. Microsomes (0.25 mg protein/ml) were incubated in the absence or presence of tBuBHQ (10 μM) at 4 and 37°C in 20 ml incubation buffer for 10 min. The microsomes were then sedimented by centrifugation, washed once, and $^{45}Ca^{2+}$ sequestration was monitored as described above. Mitochondrial Ca^{2+} fluxes were measured using the Ca^{2+} electrode in a medium containing 120 mM sucrose, 60 mM KCl, and 3 mM Hepes, pH 7.1, supplemented with ATP-Mg^{2+} (3–4 mM) and 15 μM Ca^{2+}, at 25°C.

The Mg^{2+}-dependent, Ca^{2+}-stimulated ATPase activity of the plasma membrane fraction was measured as in [26]. Mitochondrial F_1F_0-ATPase activity was determined as in [27]. In brief, mitochondria (1 mg protein/ml) were preincubated in the absence or presence of tBuBHQ for 2.5 min at 25°C. The reaction was then initiated by the addition of 5 mM ATP, and maximal activity obtained by the addition of 1 μM CCCP (carbonyl cyanide *m*-chlorophenylhydrazone). Microsomal ATPase activity was determined essentially according to Dawson and Fulton [17], except that incubation buffer was used and [P_i] was determined as in [28].

Reduced protein sulfhydryl groups were measured using Ellman's reagent (dithiobisdinitrobenzoic acid) as described [15]. Lipid peroxidation was assayed as in [29].

The effects of tBuBHQ on microsomal enzyme activities and cytochrome P-450 content were studied by incubating microsomes (1 mg/ml) in incubation buffer in the absence or presence of tBuBHQ (20 or 200 μM) for 15 min at 30°C. Glucose-6-phosphatase, NADPH-cytochrome *c* reductase, and cytochrome P-450 content were then assayed [30–32].

All chemicals were of the highest purity commercially available. tBuBHQ (97% pure) was obtained from Aldrich. 2,5-Di(*tert*-butyl)-1,4-benzoquinone was synthesized by oxidation of tBuBHQ with Ag_2O (5 equiv.) in dry diethyl ether until completion. The quinone was then crystallized from petroleum ether (b.p. 30–40°C).

3. RESULTS AND DISCUSSION

The experiment shown in fig.1 demonstrates the inhibition of ATP-dependent Ca^{2+} sequestration in liver microsomes by tBuBHQ. Half-maximal and maximal effects occurred at about 1×10^{-6} and 1×10^{-5} M tBuBHQ, respectively. Preincubation of microsomes with tBuBHQ (10 μM) at either 4 or 37°C followed by washing as described in section 2 did not reverse tBuBHQ-induced inhibition (not shown). The hydroquinone also inhibited the microsomal Ca^{2+}-stimulated ATPase activity in a dose-dependent manner (fig.2); half-maximal and maximal inhibition was obtained with about 5×10^{-7} and 2×10^{-6} M tBuBHQ, respectively. In contrast, the Ca^{2+}-independent Mg^{2+}-ATPase activity was not affected by tBuBHQ.

Possible mechanisms of tBuBHQ inhibition of microsomal Ca^{2+} sequestration include the modification of essential protein thiols (cf. [3,15])

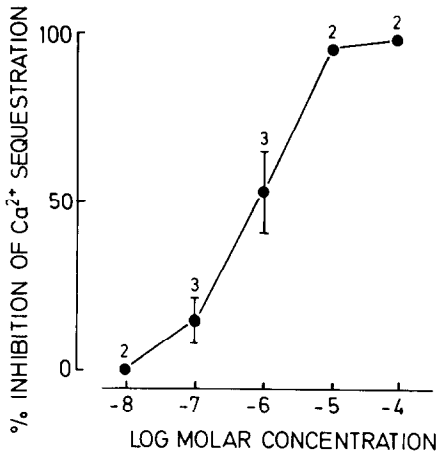

Fig.1. tBuBHQ-induced inhibition of Ca^{2+} sequestration by liver microsomes. Microsomes were incubated for 15 min at 37°C with tBuBHQ at the indicated concentrations before rapid filtration (see section 2 for details). The incubation medium for measuring Ca^{2+} sequestration contained in a final volume of 1 ml: 1 mM $MgCl_2$, 20 μM Ca^{2+} (0.5 μCi/ml $^{45}Ca^{2+}$), and 150 mM KCl-20 mM Hepes, pH 7.1. The reaction was initiated by the addition of 5 mM ATP plus microsomes (0.25 mg protein/ml), final concentrations. Under these conditions 6.2 ± 0.5 nmol Ca^{2+}/mg protein ($\bar{x}$ ± SD, n = 5) were sequestered within 5 min and could be retained for up to 30 min. Each point either represents the mean of two or mean ± SD of three determinations.

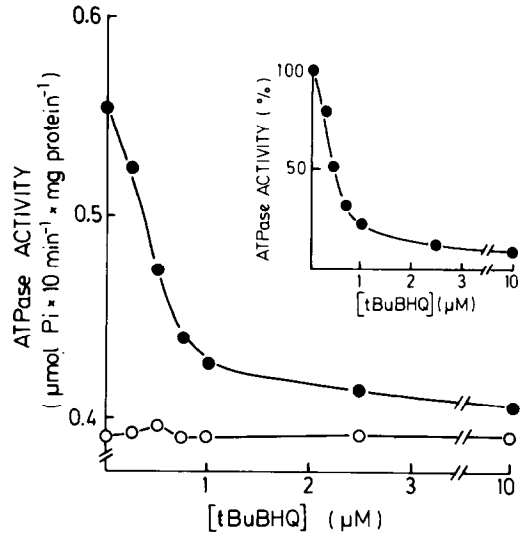

Fig.2. Effects of tBuBHQ on liver microsomal ATPase activity. The ATPase assay is described in section 2. Microsomes were preincubated with the indicated concentrations of tBuBHQ for 2 min before addition of ATP. (○) 1 mM EGTA and 28 μM Ca^{2+} (free [Ca^{2+}] = 16 nM); (●) 0.1 mM EGTA and 77 μM Ca^{2+} (free [Ca^{2+}] = 1.76 μM). (Inset) Effect of tBuBHQ on (Ca^{2+} + Mg^{2+})-ATPase activity.

and alteration of microsomal membrane permeability. However, incubation of liver microsomes in the presence of tBuBHQ, or its quinone (up to 100 μM), for 1 h did not result in a measurable loss of protein thiols. Furthermore, the presence of either dithiothreitol (5 mM) or reduced glutathione (2 mM) did not prevent tBuBHQ-induced inhibition of Ca^{2+} sequestration (not shown). It seems unlikely that nonspecific membrane effects would be responsible for the tBuBHQ inhibition since the structurally related *tert*-butyl analogs, 3,5-di(*tert*-butyl)-4-hydroxytoluene (BHT) and 3(2)-*tert*-butyl-4-hydroxyanisole (BHA), were not inhibitory even at concentrations up to 1 mM. In addition, microsomal lipid peroxidation was not observed (not shown) and the activities of glucose-6-phosphatase and NADPH-cytochrome *c* reductase were not altered, whereas the cytochrome P-450 content was only slightly decreased by treatment of the microsomes with 200 μM tBuBHQ (table 1).

Since tBuBHQ potently inhibits both microsomal Ca^{2+} sequestration and Ca^{2+}-stimulated ATPase activity, further experiments were conducted to investigate possible effects of tBuBHQ on other hepatic Ca^{2+}-transport systems. As shown in fig.3, isolated rat liver microsomes and mitochondria, which had accumulated Ca^{2+} in the presence of ATP, responded differently to the addition of the benzohydroquinone. Microsomes exhibited dose-dependent Ca^{2+} release between 2.5 and 10 μM tBuBHQ (fig.3A). At 20 μM the rate of Ca^{2+} release was maximal. Interestingly, at 10 and 20 μM tBuBHQ, Ca^{2+} release was biphasic. These results were independently confirmed by the measurement of $^{45}Ca^{2+}$ release using the same conditions as in fig.1 (not shown). In contrast, Ca^{2+}-loaded mitochondria, energized by ATP-Mg^{2+} (3–4 mM), did not release Ca^{2+} even at concentrations up to 400 μM tBuBHQ (fig.3B). Furthermore, neither the mitochondrial F_1F_0-ATPase

Table 1

Effect of tBuBHQ on microsomal enzyme activities and cytochrome P-450 content

Additions	Glucose-6-phosphatase[a]	NADPH-cytochrome *c* reductase[b]	P-450[c]
None	170 ± 12	69 ± 13	0.53 ± 0.04
tBuBHQ (20 μM)	184 ± 11	72 ± 10	N.D.
(200 μM)	183 ± 10	79 ± 11	0.41 ± 0.04

Microsomes (1 mg/ml) were preincubated with tBuBHQ before determination of enzyme activities and cytochrome P-450 content as described in section 2. [a] nmol $P_i \cdot min^{-1} \cdot mg^{-1}$ protein; [b] nmol cytochrome *c* reduced $\cdot min^{-1} \cdot mg^{-1}$ protein; [c] nmol $\cdot mg^{-1}$ protein. N.D., not determined. Values represent means ± SD of 3–4 determinations

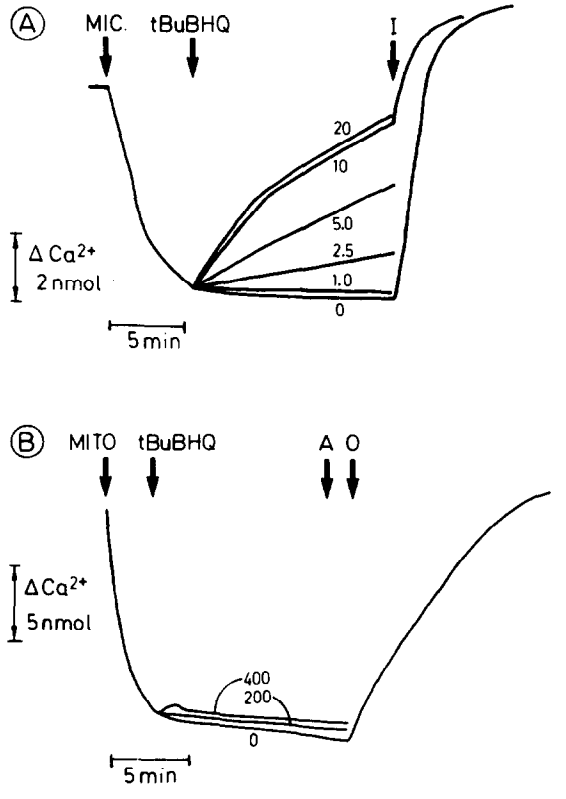

Fig.3. tBuBHQ-induced Ca^{2+} release from Ca^{2+}-loaded microsomes (A) and mitochondria (B). Ca^{2+} loading was initiated by the addition of either microsomes (1 mg protein/ml) or mitochondria (1 mg protein/ml). When a steady Ca^{2+} level was reached, the benzohydroquinone was added at the concentrations indicated (in μM). Other additions: (I) A23187 (2 μM); (A), antimycin A (2 μM); and (O), oligomycin (0.5 μM). Downward and upward deflections correspond to uptake and release of Ca^{2+}, respectively.

(not shown) nor the Ca^{2+}-stimulated ATPase activity of the hepatic plasma membrane fraction was inhibited by tBuBHQ (fig.4).

Several lines of evidence suggest that the effects of tBuBHQ are selective for the microsomal Ca^{2+}-sequestration system. Firstly, alterations of the microsomal membrane integrity seem unlikely to occur, since no lipid peroxidation was observed and relatively high concentrations of structurally

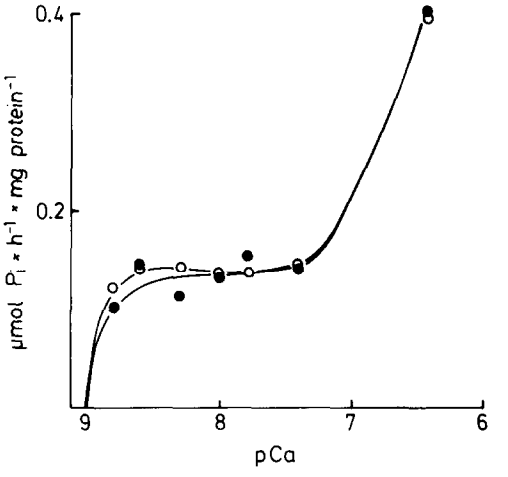

Fig.4. Lack of effect of tBuBHQ on plasma membrane Ca^{2+}-ATPase activity. A suspension of polyacrylamide beads with attached plasma membrane fragments (0.2–0.25 mg protein/ml), isolated from untreated hepatocytes, was incubated in the absence (○) or presence (●) of tBuBHQ (20 μM) for 5 min at 37°C before adding ATP (1 mM). After 60 min the reaction was stopped and [P_i] measured as described in section 2.

similar analogs (BHT and BHA) did not impair Ca^{2+} sequestration. Secondly, the lack of both alteration of microsomal protein sulfhydryls and NADPH-cytochrome *c* reductase activity, an enzyme critically dependent on thiols for activity, demonstrates that neither arylation nor oxidation of these groups occurred. Previously, we have shown that 2,5-dimethylbenzoquinone rapidly arylates GSH [20]. However, incubation of either tBuBHQ or its quinone with GSH did not result in loss of GSH (not shown), suggesting that the *tert*-butyl groups sterically hinder Michael-type addition reactions on the ring. The possibility remains, however, that tBuBHQ may alkylate other critical protein groups. Thirdly, competition of tBuBHQ with Ca^{2+} or Mg^{2+} is ruled out because the effects of tBuBHQ were not reversed by washing following preincubation of microsomes with tBuBHQ at either 4 or 37°C. Finally, inhibition of the $(Ca^{2+} + Mg^{2+})$-stimulated ATPase activity and impairment of Ca^{2+} sequestration occurred within a similar concentration range suggesting that these two events may be associated. That maximal inhibition of the $(Ca^{2+} + Mg^{2+})$-ATPase activity by tBuBHQ occurred at 2×10^{-6} M compared with the 1×10^{-5} M required to impair Ca^{2+} sequestration may depend on the different assay conditions used. Measurement of Ca^{2+} sequestration in the absence of the Ca^{2+} ionophore A23187 [cf. $(Ca^{2+} + Mg^{2+})$-ATPase activity], results in a relatively higher intramicrosomal Ca^{2+} concentration which may partially reverse tBuBHQ inhibition. Similar effects have been observed with vanadate [17].

In conclusion, our findings demonstrate that tBuBHQ is a potent inhibitor of liver microsomal Ca^{2+}-stimulated ATPase activity and Ca^{2+} sequestration, without apparent modification of protein thiols or the integrity of the microsomal membrane. Mitochondrial Ca^{2+} sequestration and F_1F_0-ATPase activity, as well as the Ca^{2+}-stimulated ATPase activity of the plasma membrane fraction, are not affected by the hydroquinone. It appears therefore that tBuBHQ is a selective inhibitor of microsomal Ca^{2+} sequestration, and that this is associated with the loss of Ca^{2+}-stimulated ATPase activity. It is suggested that the hydroquinone may be a useful tool in the further elucidation of the contribution of the endoplasmic reticulum to the maintenance of Ca^{2+} homeostasis in hepatocytes and, possibly, other cells.

REFERENCES

[1] Cheung, W.Y. (1982) Calcium and Cell Function, vol.2, Academic Press, New York.
[2] Williamson, J.R., Cooper, R.H. and Hoek, J.B. (1981) Biochim. Biophys. Acta 639, 243–295.
[3] Moore, L., Chen, T., Knapp, H.R. and Landon, E.J. (1975) J. Biol. Chem. 250, 4562–4568.
[4] Rasmussen, H. and Barrett, P.Q. (1984) Physiol. Rev. 64, 938–984.
[5] Berridge, M.J. (1986) Biol. Chem. Hoppe-Seyler 367, 447–456.
[6] Streb, H., Irvine, R.F., Berridge, M.J. and Schulz, I. (1983) Nature 306, 67–68.
[7] Joseph, S.K., Thomas, A.P., Williams, R.J., Irvine, R.F. and Williamson, J.R. (1984) J. Biol. Chem. 259, 3077–3081.
[8] Dawson, A.P. (1985) FEBS Lett. 185, 147–150.
[9] Kutsky, P., Falck, J.R., Weiss, G.B., Manna, S., Chacos, N. and Capdevila, J. (1983) Prostaglandins 26, 13–21.
[10] Moore, L. (1982) Biochem. Pharmacol. 31, 1465–1467.
[11] Long, R.M. and Moore, L. (1986) Biochem. Pharmacol. 35, 4131–4137.
[12] Younes, M., Albrecht, M. and Siegers, C.P. (1983) Res. Commun. Chem. Pathol. Pharmacol. 40, 405–415.
[13] Luthra, R., Kyle, G.M., Mehta, P.S. and Bruckner, J.V. (1984) Biochem. Pharmacol. 33, 3295–3298.
[14] Benedetti, A., Fulceri, R. and Comporti, M. (1984) Biochim. Biophys. Acta 793, 489–493.
[15] Thor, H., Hartzell, P., Svensson, S.-Å., Orrenius, S., Mirabelli, F., Marinoni, V. and Bellomo, G. (1985) Biochem. Pharmacol. 34, 3717–3723.
[16] Epping, R.J. and Bygrave, F.L. (1984) Membrane Biochem. 5, 167–180.
[17] Dawson, A.P. and Fulton, D.V. (1983) Biochem. J. 210, 405–410.
[18] Macara, I.G. (1980) Trends Biochem. Sci. 5, 92–94.
[19] Thor, H., Smith, M.T., Hartzell, P., Bellomo, G., Jewell, S.A. and Orrenius, S. (1982) J. Biol. Chem. 257, 12419–12425.
[20] Rossi, L., Moore, G.A., Orrenius, S. and O'Brien, P.J. (1986) Arch. Biochem. Biophys. 251, 25–35.
[21] Moldéus, P., Högberg, J. and Orrenius, S. (1978) Methods Enzymol. 52, 60–71.

[22] Nicotera, P., Moore, M., Bellomo, G., Mirabelli, F. and Orrenius, S. (1985) J. Biol. Chem. 260, 1999–2002.
[23] Moore, G.A., O'Brien, P.J. and Orrenius, S. (1986) Xenobiotica 16, 873–882.
[24] Prasad, J.S., Erickson, R.R., Crankshaw, D.L. and Holtzman, J.L. (1986) Arch. Biochem. Biophys. 248, 639–645.
[25] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[26] Nicotera, P., Moore, M., Mirabelli, F., Bellomo, G. and Orrenius, S. (1985) FEBS Lett. 181, 149–153.
[27] Carafoli, E. and Semenza, G. (1979) Membrane Biochemistry: A Laboratory Manual on Transport and Bioenergetics, p.72, Springer, Berlin.
[28] Carter, S.G. and Karl, D.W. (1982) J. Biochem. Biophys. Methods 7, 7–13.
[29] Smith, M.T., Thor, H., Hartzell, P. and Orrenius, S. (1982) Biochem. Pharmacol. 31, 19–26.
[30] Swanson, M.A. (1955) Methods Enzymol. 2, 541–543.
[31] Williams, C.H. and Kamin, H. (1962) J. Biol. Chem. 237, 587–595.
[32] Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2370–2378.