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2,5-Di(TERT-BUTYL)-1,4-BENZOHYDROQUINONE — A NOVEL MOBILIZER OF THE INOSITOL 1,4,5-TRISPHOSPHATE-SENSITIVE Ca^{2+} POOL

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Isolated hepatocytes and the isolated perfused rat liver have been used to study the alterations of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) produced by 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (tBuBHQ), a potent inhibitor of hepatic microsomal Ca^{2+} sequestration (Moore, G.A., McConkey, D.J., Kass, G.E.N., O'Brien, P.J. and Orrenius, S. FEBS Lett., **224,** 331-336), (1987). Addition of tBuBHQ to isolated hepatocytes caused a rapid increase in $[Ca^{2+}]_i$ which was similar in magnitude to the $[Ca^{2+}]_i$ elevation induced by the Ca^{2+} mobilizing hormone, vasopressin. In contrast with vasopressin which caused a Ca^{2+} transient, tBuBHQ elevated $[Ca^{2+}]_i$ to a new steady state that was maintained for up to 15–20 min. When vasopressin was administered during the tBuBHQ-induced period of elevated $[Ca^{2+}]_i$, $[Ca^{2+}]_i$ rapidly returned to basal levels. Similarly, if vasopressin was administered just prior to tBuBHQ, the resultant tBuBHQ-dependent change in $[Ca^{2+}]_i$ was transient, and not sustained. The hydroquinone mobilized the same intracellular Ca^{2+} pool as inositol 1,4,5-trisphosphate, but tBuBHQ did not produce any detectable inositol polyphosphate accumulation. tBuBHQ stimulated glucose release from perifused hepatocytes, mimicking the effect of vasopressin. In the perfused liver, tBuBHQ infusion produced a single, slow and prolonged release of Ca^{2+} into the perfusate and inhibition of subsequent vasopressin-induced Ca^{2+} effluxes. Inhibition of the response to vasopressin was reversed over time, and closely correlated with the extent of inhibition of both Ca^{2+} sequestration and (Ca^{2+}-Mg^{2+})-ATPase activity in microsomes isolated from the isolated perfused liver. The present results are consistent with tBuBHQ inhibiting ATP-dependent Ca^{2+} sequestration by a direct effect on the endoplasmic reticular Ca^{2+} pump, which results in net Ca^{2+} release and elevation of $[Ca^{2+}]_i$. Furthermore, vasopressin appears to stimulate active removal of increased $[Ca^{2+}]$ from the hepatocyte cytosol by a mechanism which does not depend on reuptake of Ca^{2+} into the endoplasmic reticulum.

KEY WORDS: 2,5-Di(*tert*-butyl)-1,4-benzohydroquinone, calcium, hepatocytes, perfused liver, endoplasmic reticulum.

INTRODUCTION

For well over 4,000 years man has used quinones for various purposes including control of fungi, bacteria, viruses, cancer and inflamation as well as vitamin supplements and as respiratory chain substrates to circumvent inborn errors of mitochondrial metabolism.[1,2] The use of quinones in pharmacological preparations and indu-

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strial processes, and their occurence in primary and secondary metabolism has recently led to the recognition and investigation of their toxicological properties towards man and his environment.

The cytotoxicity of many quinones has been attributed to their pronounced alkylating and/or redox–cycling properties.[2,3] We have recently shown that quinone–induced toxicity to isolated hepatocytes results from a marked disturbance of the intracellular Ca^{2+} homeostasis as evidenced by a sustained elevation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). This in turn will activate a number of metabolic processes such as increased proteolytic degradation, phospholipase activation and DNA fragmentation, which can cause cell death.[4,5] Studies on the subcellular Ca^{2+} compartmentation have shown that cytotoxic quinones, such as p-benzoquinone or menadione increase $[Ca^{2+}]_i$ by means of the concerted release of mitochondrial Ca^{2+}, inhibition of microsomal Ca^{2+} sequestration and inhibition of the plasma membrane Ca^{2+} extrusion system.[6,7] Unfortunately, the very wide spectrum of cellular effects produced by cytotoxic quinones precludes identification of the contribution of each Ca^{2+} handling organelle to the development of toxicity.

In this paper we describe the effects of 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (tBuBHQ), a potent and specific inhibitor of the liver microsomal Ca^{2+} translocase[8] on intracellular Ca^{2+} homeostasis in isolated hepatocytes and in the isolated perfused rat liver.

MATERIALS AND METHODS

Materials

[Arg[8]]-Vasopressin, saponin, inositol 1,4,5-trisphosphate, fura-2 AM, and quin-2 were from Sigma Chemical Co. (St. Louis, MO, USA). 2,5-Di(*tert*-butyl)-1,4-benzohydroquinone was from EGA-Chemie (Steinheim, FRG) and ionomycin was supplied by Calbiochem (San Diego, CA, USA). $^{45}CaCl_2$ (20 Ci/g) and D-[6-^{3}H(N)] glucose (30.2 Ci/mmol) were from New England Nuclear (UK) and *myo*-[2-^{3}H] inositol (17 Ci/mmol) was purchased from Amersham International (UK). All other chemicals were of the highest purity commercially available.

Isolation of Rat Liver Microsomes

Male Wistar rats (200–300 g) having free access to food and water were used in this study. Liver microsomes were isolated and $^{45}Ca^{2+}$-sequestration and (Ca^{2+}–Mg^{2+})-ATPase activity were measured as previously described.[8]

Measurement of $[Ca^{2+}]_i$

Cytosolic free calcium concentration was measured in freshly isolated hepatocytes using the fluorescent Ca^{2+}-indicator, fura-2. Details of the measurement procedure have been reported elsewhere.[9]

Measurement of Ca^{2+} Fluxes in the Isolated Perfused Rat Liver

Isolated rat livers were perfused in single–pass mode with modified Krebs–Henseleit buffer (pH 7.4, 37°C, 1.3 mM Ca^{2+}) at a rate of 5 ml/min per g liver.[10] The $[Ca^{2+}]$ of

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the effluent perfusate was continuously recorded using a Ca^{2+} -selective electrode as previously reported.[10,11] Test chemicals were infused (0.05% of perfusate flow) by means of a syringe-driven infusion pump (Carnegie Medicin AB, Stockholm, Sweden).

RESULTS AND DISCUSSION

Effects of tBuBHQ on Hepatic Subcellular Fractions

We have previously reported[8] that tBuBHQ is a potent inhibitor (K_i = 0.35 μM) of ATP-dependent Ca^{2+} sequestration in microsomes (Figure 1). Inhibition was associated with a similar impairment of the microsomal (Ca^{2+}–Mg^{2+})-ATPase activity. In contrast, the hydroquinone failed to induce Ca^{2+} release from Ca^{2+} -loaded liver mitochondria, it did not inhibit ATP-dependent Ca^{2+} sequestration by isolated liver nuclei, and it inhibited neither the mitochondrial $F_1 \cdot F_0$-ATPase nor the Ca^{2+}–stimulated ATPase activities of the hepatic plasma membrane fraction (Table 1).

Alterations of $[Ca^{2+}]_i$ induced by tBuBHQ in Isolated Hepatocytes

Addition of tBuBHQ to intact hepatocytes caused a rapid concentration dependent increase in $[Ca^{2+}]_i$ from 0.2 μM to around 0.4 μM (Figure 2B). Half-maximum and maximum $[Ca^{2+}]_i$ elevation occurred with 1-2 μM and 10 μM tBuBHQ, respectively (Figure 3). The amplitude of tBuBHQ-induced $[Ca^{2+}]_i$ increase was the same as that produced by vasopressin. In contrast with the effects of vasopressin (Figure 2A), the increase in $[Ca^{2+}]_i$ following tBuBHQ addition was not transient but was maintained

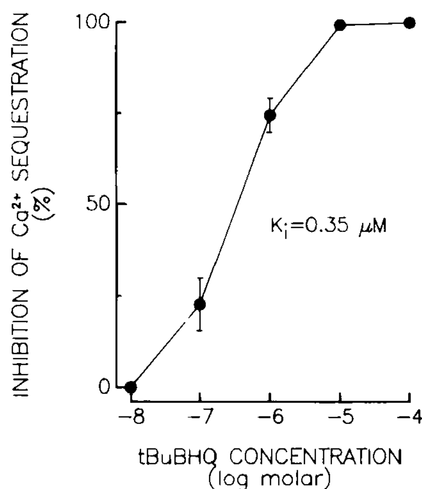

FIGURE 1 tBuBHQ-induced inhibition of Ca^{2+} sequestration by liver microsomes. Microsomes were incubated with increasing concentrations of tBuBHQ for 2 min before addition of ATP (for further details, see Ref.[8]). The microsomes were allowed to accumulate $^{45}Ca^{2+}$ for 5 min before their $^{45}Ca^{2+}$ content was determined by rapid filtration. Each point represents the mean ± S.E. of 4 separate microsomal preparations. Control microsomes had accumulated 18.6 ± 0.08 nmol $^{45}Ca^{2+}$ per mg protein at 5 min (= 0% inhibition).

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TABLE 1
The Effects of tBuBHQ on Ca^{2+} Fluxes and ATPase Activity of Hepatic Subcellular Fractions

Subcellular fraction	Ca^{2+} fluxes	ATPase activity	[Ref.]
microsomes	sequestration inhibited	$(Ca^{2+}$-$Mg^{2+})$-ATPase inhibited	8
mitochondria	no effect	no effect on $F_1 \cdot F_0$-ATPase	8
nuclei	no effect	N.D.[a]	12
plasma membrane	no effect[b]	no effect on $(Ca^{2+}$-$Mg^{2+})$-ATPase	8

[a]N.D., not determined
[b]G.E.N.K. and S.K.D., unpublished observation

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for up to 15 to 20 min (shown in part in Figure 2B and C). Thus, it appears that intracellular Ca^{2+} released by tBuBHQ was not resequestered by the endoplasmic reticulum and mitochondria, and was only slowly pumped out of the cell via the plasma membrane. Addition of vasopressin after tBuBHQ (when $[Ca^{2+}]_i$ elevation was still maintained), caused a rapid decrease of $[Ca^{2+}]_i$ to near basal levels (Figure 2C). With concentrations of tBuBHQ that did not maximally increase $[Ca^{2+}]_i$, subsequent addition of vasopressin first caused a transient elevation of $[Ca^{2+}]_i$ before returning to basal levels. A transient Ca^{2+} elevation was also observed when tBuBHQ was added after prior addition of vasopressin (Figure 2D). Addition of a second Ca^{2+}-mobilizing hormone, such as angiotensin II (100 nM) or ATP (50 μM), when $[Ca^{2+}]_i$ had returned to basal levels following stimulation with tBuBHQ plus vasopressin, failed to cause an increase in $[Ca^{2+}]_i$ (not shown). Similarly, no change in $[Ca^{2+}]_i$ was inducted by vasopressin in cells that had been exposed to tBuBHQ for

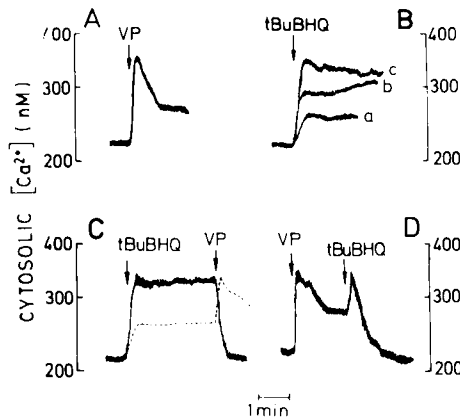

FIGURE 2 Effect of tBuBHQ on $[Ca^{2+}]_i$ in isolated hepatocytes. Changes in $[Ca^{2+}]_i$ were measured using the fluorescent Ca^{2+}-indicator, fura-2. A: effect of 50 nM vasopressin (VP). B: effect of increasing concentrations of tBuBHQ on $[Ca^{2+}]_i$; the concentrations shown were 0.5 μM (a), 1 μM (b), and 10 μM (c). In C, 50 nM vasopressin was added after tBuBHQ (25 μM, solid trace; 0.5 μM, dashed line). In D, the hepatocytes were first stimulated with vasopressin (50 nM) before addition of tBuBHQ (25 μM). (Modified from Ref.[9]).

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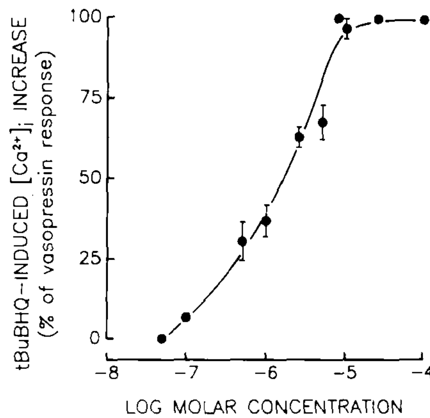

FIGURE 3 Dose-response for tBuBHQ-induced increase in $[Ca^{2+}]_i$ in hepatocytes. Cells were incubated with tBuBHQ at the indicated concentrations, followed 3–4 min later by 50 nM vasopressin. Each point represents the mean ± S.E. of 4–7 cell preparations. (Taken from Ref.[9]).

1 h.[9] These results suggest that tBuBHQ inhibits hormone-stimulated Ca^{2+} mobilization in hepatocytes by depleting the inositol 1,4,5-trisphosphate–sensitive Ca^{2+} pool. This Ca^{2+} pool is located within the endoplasmic reticulum.[13,14]

Origin of tBuBHQ-Mobilized Ca^{2+}

To confirm that tBuBHQ affects the same intracellular Ca^{2+} pool as Ca^{2+} mobilizing hormones, the effects of tBuBHQ were compared with inositol 1,4,5-trisphosphate (IP_3) in saponin-permeabilized hepatocytes in the presence of ATP and a mitochondrial uncoupler, carbonylcyanide *m*-chlorophenylhydrazone (CCCP). Addition of IP_3 to permeabilized cells caused a rapid and transient release of Ca^{2+} into the medium

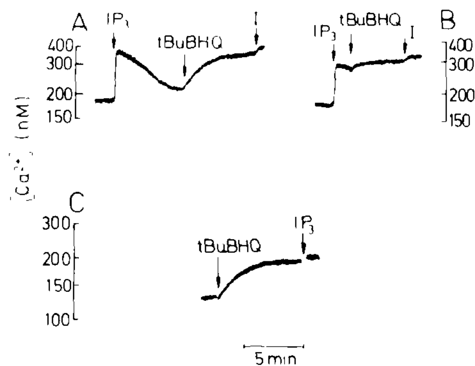

FIGURE 4 Effect of tBuBHQ and inositol 1,4,5-trisphosphate on the Ca^{2+} status of saponin permeabilized hepatocytes. Cells were permeabilized with saponin (75 μg/ml) and were allowed to accumulate Ca^{2+} in the presence of ATP and CCCP. Abbreviations and concentrations used are: IP_3, inositol 1,4,5-trisphosphate (5 μM); tBuBHQ, 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (20 μM for A and C; 40 μM for B); I, ionomycin (5 μM). (Adapted from Ref.[9]).

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TABLE II
Effect of tBuBHQ and vasopressin on the formation of [^{3}H] inositol phosphates

Treatments	n	Inositol trisphosphates	Total Inositol phosphates
		dpm/mg protein	
Control (Me_2SO)	8	213 ± 23	1680 ± 215
tBuBHQ (10 μM)	3	257 ± 54	2244 ± 611
Vasopressin (50 nM)	8	3919 ± 342	9357 ± 651

Hepatocytes were equilibrated with *myo*-[^{3}H] inositol for 2 h, washed and incubated for 10 min in the presence of Li^+ (10 mM) before addition of the agents or dimethyl sulfoxide (Me_2SO) (10 μl/ml). After 5 min, the reactions were terminated and the amounts of [^{3}H]inositol phosphates analysed by ion-exchange chromatography. Me_2SO had no significant effect on any of the parameters measured. Data are given as the mean ± S.E. of (n = 3 or 8) experiments (Modified from Ref.[9])

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(Figure 4A). As with intact hepatocytes, tBuBHQ caused a sustained release of Ca^{2+}. Addition of tBuBHQ shortly after IP_3 did not cause an additional Ca^{2+} release and *vice-versa* (Figure 4B and C). Furthermore, neomycin, a cationic aminoglycoside antibiotic that binds inositol polyphosphates, completely abolished IP_3-induced Ca^{2+} release, but was without effect on tBuBHQ-mediated Ca^{2+} mobilization (not shown). These results indicate that tBuBHQ increased cytosolic Ca^{2+} by mobilizing the same intracellular Ca^{2+} pool that is released by IP_3, but through a mechanism that is independent of IP_3 formation. This conclusion was supported by the further finding that in intact hepatocytes, tBuBHQ did not mobilize inositol phosphates (Table 2).

Stimulation of Glycogenolysis by tBuBHQ

There is convincing evidence that the endoplasmic reticulum Ca^{2+} pool is the major intracellular source of Ca^{2+} release into the cytosol when hepatocytes are treated with various Ca^{2+}-mobilizing hormones. Because tBuBHQ releases the same intracellular Ca^{2+} pool, it should elicit similar intracellular responses such as glycogen breakdown and release of glucose from hepatocytes.[15]

Using a cell perifusion system, we found that tBuBHQ did indeed cause glucose

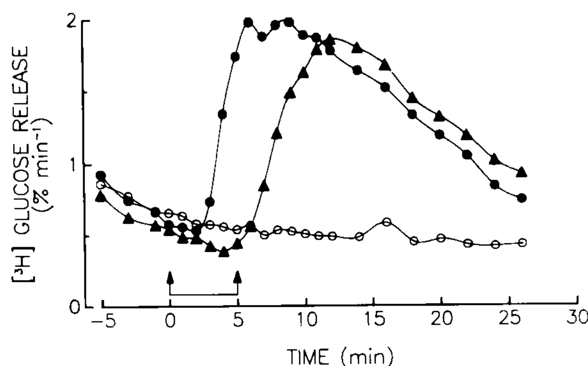

FIGURE 5 Vasopressin- and tBuBHQ-induced [^{3}H] glucose release from hepatocytes. Cells were perifused with 50 nM vasopressin (●), 25 μM tBuBHQ (▲) or Me_2SO (○) for 5 min. One experiment representative of three is shown. (Modified from Ref.[9]).

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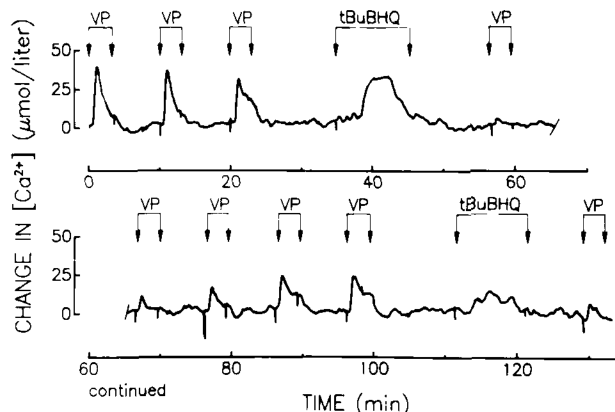

FIGURE 6 Effects of tBuBHQ on Ca^{2+} fluxes in the isolated perfused rat liver. Changes in perfusate Ca^{2+} concentrations were measured as described in *Materials and Methods*. The infusion times for vasopressin (10 nM) and tBuBHQ (25 μM) are indicated by arrows.

release from suspensions of hepatocytes (Figure 5). The rate and magnitude of glucose release by vasopressin and tBuBHQ were similar. This result is significant because to our knowledge it is the first time that a low-molecular-weight hydroquinone has been demonstrated to induce a hormone-like response.

Mobilization of Endoplasmic Reticular Ca^{2+} by tBuBHQ in the Isolated Perfused Liver

The Ca^{2+}-mobilizing effects of tBuBHQ and vasopressin were also examined in the isolated perfused rat liver system. As shown in Figure 6, repeated infusion of vasopressin into the perfused liver resulted in repeated transients of Ca^{2+} efflux. In contrast, infusion of tBuBHQ resulted in a single, slow and prolonged release of Ca^{2+} from the liver. This release only occurred after a considerable lag as compared with vasopressin (Figure 6) or phenylephrine (not shown). Addition of vasopressin after tBuBHQ infusion had ceased produced no Ca^{2+} efflux into the perfusate; however, the responsiveness to vasopressin then gradually returned over time. We have previously shown that inhibition of liver microsomal Ca^{2+} sequestration is associated with an inhibition of (Ca^{2+}–Mg^{2+})-ATPase activity.[8] The extent of inhibition of both ATP-dependent Ca^{2+} sequestration and (Ca^{2+}-Mg^{2+})-ATPase activity in microsomes prepared from livers at different times after tBuBHQ infusion (Figure 7) correlated with inhibition of vasopressin-stimulated Ca^{2+} efflux.

CONCLUSIONS

The experiments described in this paper show that tBuBHQ increases [Ca^{2+}]$_i$ in isolated hepatocytes by mobilizing the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} pool. It is clear that IP_3 mobilization does not mediate the effects of tBuBHQ, because the hydroquinone does not stimulate inositol phosphate accumulation (Table 2). The possibility that tBuBHQ non-specifically increases the endoplasmic reticular or plas-

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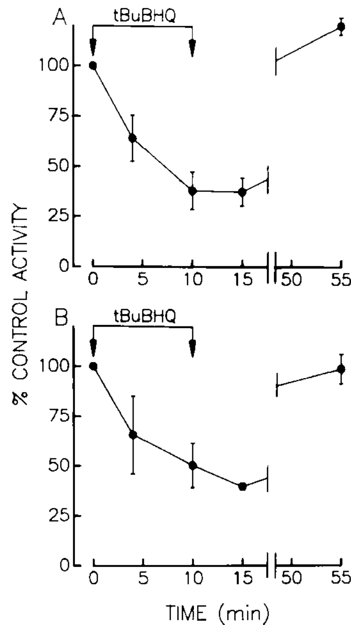

FIGURE 7 tBuBHQ-induced inhibition of microsomal Ca^{2+} sequestration and (Ca^{2+}-Mg^{2+})-ATPase activity in the isolated perfused rat liver. Perfused livers were infused with tBuBHQ (25 μM) for 10 min (arrows). Ca^{2+} sequestration (A) and (Ca^{2+}-Mg^{2+})-ATPase activity (B) were measured in microsomes prepared from lobes removed from the isolated perfused liver at the indicated time points. Each point represents the mean $\pm$ S.E. of 3–4 separate determinations.

ma membrane permeability, or both, seems unlikely because tBuBHQ neither stimulated Ca^{2+} efflux from Ca^{2+}-loaded liver microsomes nor did tBuBHQ alter $^{45}Ca^{2+}$ and Mn^{2+} movement across the plasma membrane.[9] Our results are consistent with the proposal that tBuBHQ mobilizes the endoplasmic reticular Ca^{2+} pool by inhibiting the (Ca^{2+}-Mg^{2+})-ATPase activity. Hence, tBuBHQ may be used as a tool for determining the role of the endoplasmic reticular Ca^{2+} pool in a variety of physiological and toxicological processes.

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