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EICOSANOIDS RELEASED FOLLOWING INHIBITION OF THE ENDOPLASMIC RETICULUM Ca^{2+} PUMP STIMULATE Ca^{2+} EFFLUX IN THE PERFUSED RAT LIVER

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Abstract—In the isolated perfused rat liver 2,5-di(*tert*-butyl)hydroquinone (tBuHQ), a selective inhibitor of the endoplasmic reticulum Ca^{2+} pump, induces a prolonged glucose output and stimulates Ca^{2+} efflux. The present study shows that tBuHQ depleted the hormone-sensitive Ca^{2+} pool in the perfused liver, abolishing the vasopressin- or phenylephrine-induced Ca^{2+} efflux. The effects of tBuHQ were reversible, since the response to these agonists gradually returned within 1 hr of perfusion, and protein synthesis was not required for this recovery. Since tBuHQ does not cause Ca^{2+} efflux from isolated hepatocytes, we examined the mechanism responsible for the tBuHQ-induced Ca^{2+} efflux observed in the intact liver. The cyclooxygenase inhibitor indomethacin prevented the Ca^{2+} extrusion stimulated by tBuHQ, but not that induced by vasopressin. During infusion of tBuHQ there was a 9-fold increase in the concentration of thromboxane B_2 in the perfusate. The Ca^{2+} efflux response to tBuHQ was inhibited by the thromboxane/prostaglandin endoperoxide receptor antagonist, L-655,240 {3-[1-(4-chlorobenzyl)-5-fluoro-3-methyl-indol-2-yl]2,2-dimethylpropanoic acid} in the absence of any effect on thromboxane B_2 release. Thus, the inhibition of the endoplasmic reticulum Ca^{2+} pump by tBuHQ results in a rise in the cytosolic Ca^{2+} concentration in non-parenchymal cells, leading to the formation of cyclooxygenase products. The released eicosanoids, in turn, stimulate Ca^{2+} efflux from hepatocytes.

Many hormones and growth factors alter the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$‡) as part of their intracellular signal transduction system [1]. In hepatocytes, these hormones include vasopressin and α_1-adrenergic agonists [2, 3]. The initial response to stimulation of their plasma membrane receptors is the hydrolysis of phosphatidylinositol 4,5-bisphosphate and the release of two second messengers, diacylglycerol and inositol(1,4,5)-trisphosphate [Ins(1,4,5)P_3] [4]. The latter binds to specific receptors, which are Ca^{2+}-permeable channels, in the endoplasmic reticulum or some fraction thereof [4, 5], giving rise to a rapid increase in $[Ca^{2+}]_i$. Concomitantly, or shortly thereafter, a Ca^{2+} entry response is observed. The Ca^{2+} transient is terminated by both Ca^{2+} reuptake into intracellular stores and efflux from the liver cells [6–10]. Thus, in the perfused liver system, vasopressin and α_1-adrenergic agonists stimulate extrusion of Ca^{2+} into the perfusate [6, 8]. Glucose output is also enhanced, as the rise in $[Ca^{2+}]_i$ activates phosphorylase *a*.

Previous studies from this laboratory have demonstrated that 2,5-di(*tert*-butyl)hydroquinone (tBuHQ§) is a potent inhibitor of the endoplasmic reticulum Ca^{2+} pump [11], but does not affect Ca^{2+} uptake or release in isolated liver mitochondria [11] or nuclei [12]. Furthermore, tBuHQ does not inhibit the Ca^{2+}-stimulated ATPase activity of a liver plasma membrane fraction [11]. In isolated hepatocytes, tBuHQ releases Ca^{2+} from the Ins(1,4,5)P_3-sensitive Ca^{2+} store, without itself causing accumulation of inositol phosphates [13], and indirectly stimulates Ca^{2+} entry from the extracellular medium [14]. This results in a prolonged increase in $[Ca^{2+}]_i$ with activation of glycogenolysis.

In the perfused liver, tBuHQ also mobilizes the hormone-sensitive Ca^{2+} pool and causes increases in $[Ca^{2+}]_i$ and Ca^{2+} efflux into the perfusate. Thus, when vasopressin or phenylephrine are infused after tBuHQ, the hormone-stimulated Ca^{2+} efflux is abolished [15]. The rise in $[Ca^{2+}]_i$ is demonstrated by glucose release into the perfusion medium [16]. The observation that tBuHQ also induces a Ca^{2+} efflux response in the perfused liver is in clear contrast with the findings with isolated hepatocytes, where Ca^{2+} extrusion seems to depend on the presence of the hormone. Thus, whereas vasopressin induced a 9-fold stimulation of Ca^{2+} extrusion with decrease of $[Ca^{2+}]_i$ to basal levels in isolated hepatocytes, tBuHQ produced a weak (2-fold) stimulation of Ca^{2+} efflux that failed to restitute basal $[Ca^{2+}]_i$ [10].

Therefore, the aim of the present work was to further investigate the effects of tBuHQ on hepatic Ca^{2+} fluxes, in particular the mechanism by which tBuHQ stimulates Ca^{2+} efflux to the perfusate. We can now report that; (i) the inhibition of the endoplasmic reticulum Ca^{2+} pump by tBuHQ is reversible and protein synthesis is not required for

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‡ Abbreviations: L-655,240, 3-[1-(4-chlorobenzyl)-5-fluoro-3-methyl-indol-2-yl]2,2-dimethylpropanoic acid; tBuHQ, 2,5-di(*tert*-butyl)hydroquinone; $[Ca^{2+}]_i$, cytosolic free calcium concentration; TX, thromboxane; Ins(1,4,5)P_3, inositol(1,4,5)trisphosphate.

§ This compound has been previously referred to as 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone [11, 13].

recovery of hormone response after tBuHQ, and (ii) the elevation of $[Ca^{2+}]_i$ by tBuHQ elicits the release of eicosanoids, most likely from non-parenchymal cells, that stimulate Ca^{2+} efflux from the liver.

MATERIALS AND METHODS

Materials. 2,5-Di(*tert*-butyl)hydroquinone was purchased from EGA-Chemie (Steinheim, Germany). Arsenazo III (98% pure), [Arg^8]-vasopressin, L-phenylephrine, cycloheximide, and indomethacin were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). NADH and lactate dehydrogenase (L-lactate: NAD oxidoreductase, EC 1.1.1.27) were from Boehringer Mannheim (Germany). The thromboxane (TX)/prostaglandin endoperoxide receptor antagonist L-655,240 (3-[1-(4-chlorobenzyl)-5-fluoro-3-methylindol-2-yl]2,2-dimethylpropanoic acid) [17] was a gift from Dr A. W. Ford-Hutchinson, Merck-Frosst (Canada). All other chemicals and solvents were of the highest purity commercially available.

Isolated liver perfusion. Male Wistar rats (ALAB AB, Sollentuna, Sweden) weighing 240–340 g and fed *ad lib.* were used. Anesthesia was induced by sodium pentobarbital (60 mg/kg body weight, i.p.). Livers were prepared as in Farrell *et al.* [18] and perfused in a constant-pressure system and single-pass mode with modified Krebs–Henseleit buffer containing 12.5 mM Hepes (pH 7.4) and 1.3 mM $CaCl_2$ (or 0.1 mM $CaCl_2$, where indicated), as previously described [16, 19]. Liver viability was assessed by appropriate perfusion flow rates (>3.8 mL/g/min) and lack of leakage of lactate dehydrogenase into the perfusate, as described by Farrell *et al.* [16].

Measurement of changes in perfusate $[Ca^{2+}]$ *and* TXB_2. The $[Ca^{2+}]$ of the effluent perfusate was recorded continuously using a Ca^{2+}-selective electrode (F2112, Radiometer, Copenhagen) coupled to a combination reference electrode (Radiometer K-401) via a salt bridge (2% agarose in 3 M KCl), as previously described [16]. Chemical determination of perfusate $[Ca^{2+}]$ (using arsenazo III as the Ca^{2+}-indicator) confirmed the Ca^{2+} electrode measurements, thus excluding interference of the test chemicals or transient changes in flow rate or temperature with the Ca^{2+} electrode. Infusion of a 50 μM Ca^{2+} standard was used to calibrate changes in perfusate $[Ca^{2+}]$. TXB_2 was determined by radioimmunoassay using a kit from New England Nuclear (Dupont, Boston, MA, U.S.A.) and was used as an index of the production of TXA_2.

Experimental design. A period of 20–30 min was allowed for equilibration of the liver. Test substances were then infused into the hepatic inflow tract 10 cm from the liver by means of a precision, syringe-driven infusion pump (Carnegie Medicin AB, Stockholm, Sweden) calibrated to deliver a volume corresponding to 0.05% of perfusate flow (typically, 20–23 μL/min). L-Phenylephrine and tBuHQ were administered in ethanol; infusion of ethanol alone had no effect on the parameters of interest. Other test substances were diluted in perfusion buffer. Samples of effluent perfusate were obtained from the outflow tract at appropriate time intervals for the determination of lactate dehydrogenase and TXB_2. The maximum duration of each experiment was 120 min.

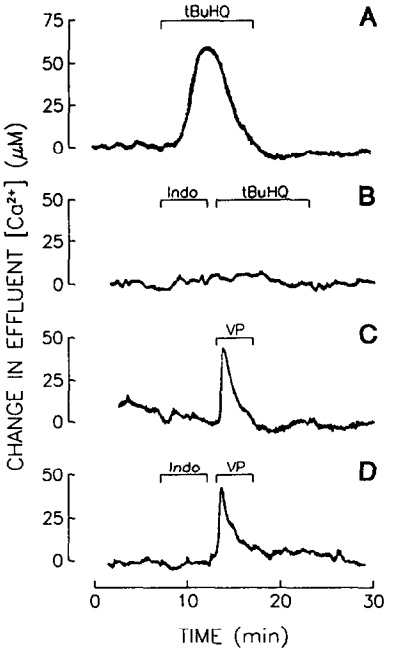

Fig. 1. Effect of indomethacin on tBuHQ- and vasopressin-induced Ca^{2+} efflux from the perfused rat liver. tBuHQ (25 μM), vasopressin (VP, 10 nM) and the cyclooxygenase inhibitor indomethacin (indo, 10 μM) were infused into the liver as indicated by the bars. The effluent perfusate $[Ca^{2+}]$ was continuously measured using the in-line Ca^{2+}-selective electrode. The $[Ca^{2+}]$ in the perfusion medium was 1.3 mM.

RESULTS

Infusion of the endoplasmic reticulum Ca^{2+} pump inhibitor, tBuHQ, stimulated Ca^{2+} efflux from the perfused liver (Fig. 1A), as previously reported [16]. In addition, prior infusion of tBuHQ (25 μM, 10 min) reduced Ca^{2+} efflux in response to vasopressin (10 nM, 3 min) or phenylephrine (2 μM, 3 min) to less than 10% of control values (Table 1). Prolongation of tBuHQ infusion to 20 min completely abolished hepatic Ca^{2+} efflux in response to these agonists, but the hormone responsiveness gradually returned within 1 hr of perfusion. Pretreatment of rats with cycloheximide (at a dose that has previously been shown to inhibit hepatic microsomal protein synthesis by 85% [20]) did not markedly alter this recovery (Table 1).

As mentioned above, the Ca^{2+} efflux response evoked by tBuHQ in the perfused liver is in contrast with the lack of such an effect in isolated hepatocytes. One possible explanation for this discrepancy is that non-parenchymal cells present in the intact organ affect hepatic Ca^{2+} efflux. Non-parenchymal cells are able to release arachidonic acid metabolites (eicosanoids) and other mediators upon stimulation [21, 22], and it has been reported that some

Table 1. Recovery of hormone-stimulated Ca^{2+} efflux from the perfused rat liver following tBuHQ infusion

Treatment	(N)	Amplitude, % of control hormonal response					
		Time* (min)					
		10	20	35	50	60–65	75–80
Control							
Vasopressin	(4)	98 ± 4	86 ± 7	86 ± 18	88 ± 8	97 ± 7	—
Phenylephrine	(3)	80 ± 2	75 ± 3	81 ± 4	83 ± 5	68 ± 3	78 ± 6
tBuHQ 10 min							
Vasopressin	(4)	—	10 ± 2	23 ± 6	51 ± 16	55 ± 10	77 ± 10
Phenylephrine	(3)	—	2 ± 2	13 ± 5	45 ± 10	58 ± 10	76 ± 6
tBuHQ 20 min							
Vasopressin	(3)	—	2 ± 2	1 ± 1	14 ± 3	34 ± 6	52 ± 5
Phenylephrine	(3)	—	3 ± 2	3 ± 3	9 ± 6	28 ± 14	51 ± 9
Cycloheximide, tBuHQ 10 min							
Vasopressin	(3)	—	6 ± 2	17 ± 5	41 ± 11	47 ± 7	58 ± 4
Phenylephrine	(3)	—	5 ± 5	11 ± 5	23 ± 11	36 ± 18	56 ± 7

* Time after initial hormone infusion.

In each experiment, the amplitude of the transient change in perfusate $[Ca^{2+}]$ produced by an initial (control) hormone infusion was taken as a 100% response (40 ± 10 μM for vasopressin, N = 7; 40 ± 10 μM for phenylephrine, N = 5). This was followed 10 min later by infusion of tBuHQ (25 μM for 10 or 20 min) or, for the 'Control' group, a second infusion of hormone. The values listed in the table indicate the relative amplitude of subsequent hormone-induced Ca^{2+} transients, expressed as a percentage of the initial control response. For each experiment, the same hormone was used throughout. Vasopressin (10 nM) or phenylephrine (2 μM) were infused for 3 min. Finally, the effect of treatment *in vivo* with cycloheximide on the recovery of hormone-induced Ca^{2+} efflux from the perfused rat liver following tBuHQ is shown. Cycloheximide (3 mg/kg body wt) in 0.15 M NaCl was injected i.p. 60 min prior to preparation of the livers for perfusion.

The results are expressed as the mean ± SD of three to four (N) experiments for each protocol. The $[Ca^{2+}]$ in the perfusate was reduced to 0.1 mM in order to increase the signal to noise ratio for the Ca^{2+}-selective electrode measurements. No significant difference in hormone-stimulated Ca^{2+} efflux was observed between 1.3 and 0.1 mM $[Ca^{2+}]$ in the perfusate.

eicosanoids elicit metabolic and vasomotor effects, and alter whole-organ Ca^{2+} fluxes in the perfused liver [23–25]. We therefore investigated the possible contribution of eicosanoids to the effects of tBuHQ in the perfused liver. Infusion of the cyclooxygenase inhibitor, indomethacin (10 μM for 5 min) abolished, or markedly inhibited, the Ca^{2+} efflux response to subsequently added tBuHQ (Fig. 1B and Table 2). This was not due to a nonspecific effect of indomethacin on plasma membrane Ca^{2+} fluxes, since vasopressin-induced Ca^{2+} efflux was not affected (Fig. 1C and D, and Table 2). Moreover, indomethacin did not alter tBuHQ-induced rise in $[Ca^{2+}]_i$ in isolated hepatocytes, nor did it block the Ca^{2+}-dependent stimulation of glucose output from the perfused liver by tBuHQ (data not shown). These results suggest the involvement of cyclooxygenase products in tBuHQ-induced Ca^{2+} efflux from the liver.

Since the limiting factor in eicosanoid biosynthesis in most tissues is the availability of free arachidonic acid, measurement of one or several metabolites can be used as an index of arachidonate release. Figure 2A shows that the concentration of TXB_2, the stable rearrangement product of TXA_2, increased in the effluent perfusate during infusion of tBuHQ (25 μM, 10 min). The levels of TXB_2 started to rise 3 min after infusion of tBuHQ, reached maximal values within 10 min of initiating tBuHQ infusion, and

Table 2. Effects of indomethacin and the TX/prostaglandin endoperoxide receptor antagonist L-655,240 on tBuHQ- and vasopressin-induced Ca^{2+} efflux from the perfused liver

Treatment	Ca^{2+} efflux (nmol/min/g liver)	(N)
Vasopressin	87.4 ± 16.0	5
tBuHQ	89.9 ± 31.1	6
Indomethacin + vasopressin	88.3 ± 20.8	3
Indomethacin + tBuHQ	21.6 ± 14.9*	5
L-655,240 + tBuHQ	32.6 ± 21.9*	4

The livers were perfused with buffer containing 1.3 mM $CaCl_2$. Vasopressin (10 nM for 3 min), tBuHQ (25 μM for 10 min, or for 7 min in the experiments with L-655,240), indomethacin (10 μM for 5 min, commencing 6 min before tBuHQ or vasopressin, as shown in Fig. 1), or L-655,240 (1 μM, for 8 min, commencing 1 min before tBuHQ) were infused into the hepatic inflow tract. The $[Ca^{2+}]$ in the effluent perfusate was measured with a Ca^{2+}-selective electrode, and Ca^{2+} efflux was calculated from the area under the curves during addition of the compounds by comparison with Ca^{2+} standards spiked in each experiment.

The results are expressed as the mean ± SD of the number of experiments indicated under (N). Duncan's multiple range test was performed, and the significance of differences among group means compared to the controls is shown (* $P < 0.05$).

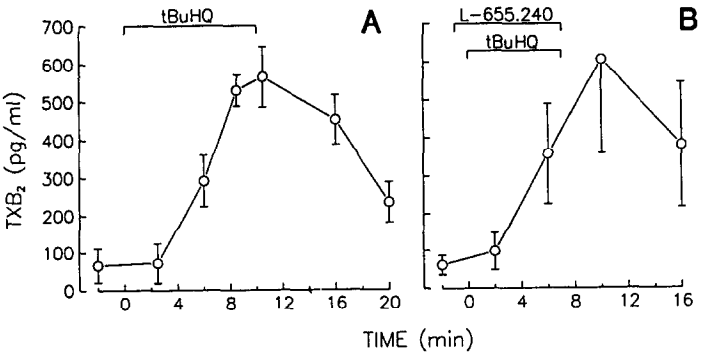

Fig. 2. Stimulation of TXB_2 release from the perfused liver by tBuHQ. Effect of the TX/prostaglandin endoperoxide receptor antagonist L-655,240. tBuHQ (25 μM) was infused for 10 min (panel A), and L-655,240 (1 μM) was administered prior and during tBuHQ (25 μM, 7 min) infusion (panel B). TXB_2 appearing in the effluent perfusate (single-pass mode) was determined as described in Materials and Methods. The results are shown as the mean ± SD of four to six independent experiments.

progressively decreased thereafter when tBuHQ was withdrawn. Since hepatocytes metabolize TXB_2 [26, 27], the figures shown in Fig. 2 represent the net balance between formation and hepatic clearance of TXB_2.

In a series of experiments we examined the effect of the TX/prostaglandin endoperoxide receptor antagonist, L-655,240 [17] on the tBuHQ-mediated Ca^{2+} efflux response. In four separate experiments, L-655,240 (1 μM, 8 min infusion, starting 1 min before tBuHQ) markedly inhibited the Ca^{2+}-efflux response to tBuHQ (Table 2), but did not affect the release of TXB_2 evoked by tBuHQ (Fig. 2B). This suggests that, at variance with the findings in platelets [28], there is no self-amplifying TX release in liver cells.

DISCUSSION

We report here that the effect of tBuHQ on Ca^{2+} efflux in the perfused liver was reversible, and that synthesis of new protein was not required for this reversibility. The time-course for recovery of hormone responsiveness after tBuHQ is in agreement with that of ATP-dependent Ca^{2+} sequestration and (Ca^{2+}-Mg^{2+})-ATPase activity in microsomes prepared from livers at various times after infusion of tBuHQ [15]. Thus, the present findings are consistent with the proposal that inhibition of the Ca^{2+} pump by tBuHQ is via a non-covalent (reversible) interaction. This is in apparent contrast with the inhibition of hormone-induced Ca^{2+} responses after treatment of rats with the hepatotoxicant, bromotrichloromethane, the effects of which were irreversible [29].

Whereas tBuHQ caused a prolonged release of Ca^{2+} from the perfused liver, it only weakly stimulated Ca^{2+} efflux in suspensions of isolated hepatocytes [10], as discussed above. Our present findings suggest a mechanism that may account for this discrepancy and which is consistent with the assertion that the rise in $[Ca^{2+}]_i$ caused by mobilization of the hormone-sensitive Ca^{2+} pool by tBuHQ activates the formation of eicosanoids. This effect is most likely via phospholipase A_2 (EC 3.1.1.4), the activity of which is known to be Ca^{2+}-dependent [30–32]. Although the origin of the TX released was not investigated here, previous studies have shown that the major producers of eicosanoids in the liver are the Kupffer cells, while sinusoidal endothelial cells contribute less [21, 26]. Furthermore, in primary cultures of Kupffer cells elevation of $[Ca^{2+}]_i$ promotes eicosanoid synthesis [22], and the translocation of phospholipase A_2 from the cytosol to a membrane fraction by an increase in $[Ca^{2+}]$ in the physiological range has been recently reported [33]. Rat hepatocytes in primary culture do not seem to produce physiologically significant amounts of prostanoids and leukotriene B_4 [21, 26]. Thus, our findings are compatible with the assumption that tBuHQ-induced mobilization of intracellular Ca^{2+} stores also occurs in Kupffer cells and that the concomitant rise in $[Ca^{2+}]_i$ stimulates eicosanoid release. In keeping with this view, tBuHQ induced an increase in $[Ca^{2+}]_i$ in the macrophage cell line $P388D_1$ (Gerard Bannenberg and Juan Llopis, unpublished observations).

The present results demonstrating tBuHQ-induced TX release in liver are in contrast with the reported inhibition of cyclooxygenase activity by tBuHQ in platelets [28]. However, in agreement with our findings, tBuHQ has been found to stimulate release of TX in a perfused lung preparation [34].

The observation that TX, and/or possibly other cyclooxygenase products, can activate plasma membrane Ca^{2+} efflux in the perfused rat liver, is in agreement with previous reports [23–25]. Vasopressin was shown to stimulate a Ca^{2+} efflux pathway in hepatocytes [10]; the activation of Ca^{2+} extrusion by cyclooxygenase products could involve a similar, receptor-mediated mechanism. There is now considerable evidence that the effects of several compounds (e.g. phorbol ester, endotoxin, platelet activating factor, and extracellular nucleotides) in hepatocytes differ from those found in the perfused liver and that intercellular communication, by release

of eicosanoids or other mediators, determines the metabolic and vascular effects in the perfused organ [35–39]. Likewise, prostaglandins and TX have been found to mediate some effects of sympathetic nerve stimulation in the perfused liver [40].

In conclusion, it appears that an increase in $[Ca^{2+}]_i$ by release of an intracellular Ca^{2+} store, independently of receptor stimulation, is sufficient to trigger arachidonate release and metabolism in non-parenchymal cells and that TX and/or other cyclooxygenase products stimulate a Ca^{2+} efflux mechanism in the hepatocytes. Finally, the different effects of tBuHQ on Ca^{2+} extrusion in isolated hepatocytes and the perfused liver illustrate the importance of studying phenomena at different levels of biological organization in order to clarify their physiological relevance.

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