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# Mobilization of the hormone-sensitive calcium pool increases hepatocyte tight junctional permeability in the perfused rat liver

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Hepatocyte tight junctional permeability has been shown to be regulated by hormones that exert their effects via phospholipase C activation. However, the precise transduction pathway involved in this effect is not known. The present study has employed the selective inhibitor of microsomal $Ca^{2+}$ sequestration, 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (tBuBHQ), to examine the effect of the mobilization of the endoplasmic reticular $Ca^{2+}$ pool on tight junctional permeability in the perfused rat liver. Infusion of tBuBHQ followed by a bolus infusion of horseradish peroxidase (HRP) resulted in a significant increase in the first peak of biliary HRP, a measure of junctional permeability, whereas transcellular (vesicular) transport of HRP was not affected. Therefore, we conclude that the effect of hormones on tight junctional permeability is mediated, at least in part, by the mobilization of intracellular $Ca^{2+}$.

Tight junction; Calcium; Horseradish peroxidase; Rat liver

## 1. INTRODUCTION

Within the liver, hepatocytes maintain a permeability barrier between blood and bile by means of intercellular tight junctions. This barrier prevents the free diffusion of plasma constituents into bile, as well as the reflux of biliary components into the vascular compartment. Hepatocytes regulate the transport of electrolytes and other molecules into bile by several mechanisms. In addition to active hepatocellular secretion of electrolytes and organic anions into the bile canaliculus, two routes for passage of plasma molecules into bile have been described [1]: a rapid paracellular route, which involves sieving through the tight junctions, and a slow transcellular pathway, inhibitable by colchicine, which involves vesicular transport. The latter mechanism is exemplified by the receptor-mediated transport of IgA [2]. Several permeability probes have been used to study the passage of molecules from blood to bile in the perfused rat liver [1,3]. The continuous infusion of low $M_r$ molecules, such as inulin or [$^{14}$C]sucrose, can be used to monitor the paracellular route. In contrast, horseradish peroxidase (HRP) infused as a bolus, has been used to follow both the paracellular and transcellular pathways. Lowe et al. [3] have demonstrated with histochemical techniques that the early entry of HRP into bile truly reflects passage through the hepatocyte tight junctions.

Tight junctions were once considered as static pores that allowed the selective diffusion of molecules depending on their size and charge. However, recent evidence indicates that the hepatocyte tight junctional permeability, in common with that of vascular endothelium and many epithelia, is subject to hormonal regulation. Thus, Lowe et al. [3] have shown that vasopressin, epinephrine and angiotensin II induce a dose-dependent increase in junctional permeability to HRP and [$^{14}$C]sucrose in the perfused rat liver, although the exact transduction pathway involved is not known.

We have previously shown that 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (tBuBHQ) is a selective inhibitor of the liver microsomal $Ca^{2+}$ translocase, but does not affect uptake or release of $Ca^{2+}$ by isolated liver mitochondria [4] or nuclei [5]. Furthermore, tBuBHQ does not inhibit the plasma membrane $Ca^{2+}/Mg^{2+}$-ATPase activity and does not affect $Ca^{2+}$ fluxes across the hepatocyte plasma membrane [4,6]. In isolated hepatocytes, tBuBHQ releases $Ca^{2+}$ from the inositol 1,4,5-trisphosphate-sensitive $Ca^{2+}$ pool without itself causing accumulation of inositol phosphates [6]. This results in an increase in $[Ca^{2+}]_i$. Similarly, in the perfused liver tBuBHQ induces mobilization of intracellular hormone-sensitive $Ca^{2+}$ stores and net glucose output from the liver [7] (Llopis, J., Farrell, G.C., Duddy, S.K., Kass, G.E.N., Gahm, A., Moore, G.A. and Orrenius, S., submitted). We have therefore used tBuBHQ to examine whether an in-

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*Abbreviations:* tBuBHQ, 2,5-di(*tert*-butyl)-1,4-benzohydroquinone; HRP, horseradish peroxidase; $[Ca^{2+}]_i$, cytosolic free $Ca^{2+}$ concentration



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crease in $[Ca^{2+}]_i$ is the mechanism by which hormones enhance hepatic tight junctional permeability.

## 2. MATERIALS AND METHODS

### 2.1. *Chemicals*

3,3',5,5'-tetramethylbenzidine, HRP (Donor: hydrogen-peroxide-oxidoreductase, EC 1.11.1.7) type VI-A and $Arg^8$-vasopressin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). $H_2O_2$ was from Fluka Chemie (Buchs, Switzerland). NADH and pyruvate were from Boehringer Mannheim GmbH (Mannheim, Germany). taurodehydrocholic acid was from Calbiochem (La Jolla, CA, USA) and tBuBHQ was from EGA-Chemie (Steinheim, Germany). All other chemicals were of at least analytical reagent grade.

### 2.2. *Liver perfusion*

Male Wistar rats (ALAB AB, Sollentuna, Sweden) weighing 210–270 g and fed ad libitum, were used as liver donors. Anesthesia was induced using sodium pentobarbital (60 mg/kg body weight i.p.). Livers were prepared as described [7,8]. Liver perfusion was carried out in single-pass mode via the portal vein with modified Krebs-Henseleit buffer, containing 12.5 mM Hepes, 1.3 mM $CaCl_2$, and 22 μM taurodehydrocholic acid (pH 7.4), continuously gassed with $O_2/CO_2$ (19:1) and maintained at 37°C, using a constant pressure system as described elsewhere [9]. Perfusate flow rate was approximately 4 ml · $min^{-1}$ · g $liver^{-1}$. Taurodehydrocholic acid was included in the perfusion medium in order to avoid interference of low bile flow with the measurement of junctional permeability. Liver viability was assessed by an adequate perfusate flow rate, bile flow rate > 2.3 μl · $min^{-1}$ · g $liver^{-1}$, and no detectable leakage of lactate dehydrogenase (L-lactate:$NAD^+$ oxidoreductase, EC 1.1.1.27) into the perfusate.

### 2.3. *Experimental design*

Livers were allowed to equilibrate for a period of 15 min. tBuBHQ (25 μM final concentration) or vehicle (ethanol, 0.05% v/v final concentration) was then infused into the hepatic inflow tract for 10 min. During this initial 25-min period bile was continuously collected in tared tubes to assess bile flow. One minute after termination of compound administration, HRP (0.5 mg in 0.5 ml of perfusate) was infused as a bolus over a 30-s period into the hepatic inflow tract 10 cm from the liver. Thereafter, bile was continuously sampled every minute during the subsequent 45 min, and collected into tubes containing 0.5 ml of citrate buffer (0.2 M, pH 3.95). The assay for peroxidase activity was performed immediately after termination of the experiment. Effluent perfusate was collected regularly for determination of lactate dehydrogenase activity.

### 2.4. *Measurement of enzymatic activities*

HRP activity in bile was assayed spectrophotometrically at 450 nm as described by Gallati and Pracht [10], using $H_2O_2$ and 3,3',5,5'-tetramethylbenzidine as substrates. Lactate dehydrogenase activity in the perfusate was assayed according to Vassault [11].

## 3. RESULTS AND DISCUSSION

The effect of infusion of the vehicle (ethanol) followed by a pulse of 0.5 mg HRP on the subsequent biliary output of HRP is shown in Fig. 1A. Two peaks of biliary HRP can be observed, a small peak reaching a maximum 3 min after infusion of the probe and a larger peak at 16 min.

Fig. 1B depicts the effect of tBuBHQ infusion on biliary HRP activity following a one-pass bolus infusion of HRP into the liver. As was observed in control

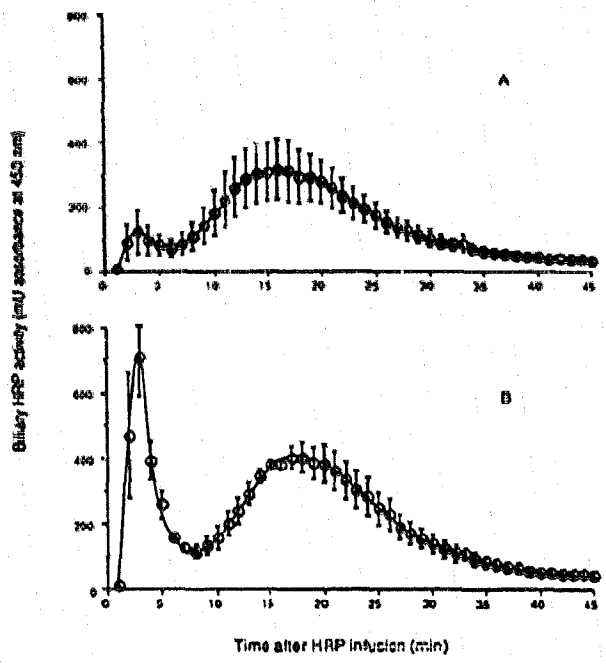


Fig. 1. Appearance of HRP activity in bile after a bolus infusion of 0.5 mg into the perfused rat liver. Vehicle (ethanol, panel A) or tBuBHQ (25 μM, panel B) were infused for 10 min starting 11 min before HRP injection (time = 0). Values are means ± SE (shown by bars) of 5 experiments for controls (A) and 4 experiments for tBuBHQ (B).

perfusions, the first HRP peak occurred at 3 min. However, the height of this peak was significantly greater than that obtained with infusion of the vehicle (about 5.7-fold, $P < 0.005$ by Student's $t$-test). Furthermore, the kinetics of the increase were similar to those reported for vasopressin, epinephrine and angiotensin II [3]. The time at which the first peak was observed is consistent with an instantaneous passage of the enzyme into the biliary canaliculi, as the calculated time for passage of the probe through the biliary dead space was 2.96 min, after taking into account catheter volume (13 μl), estimated biliary tree volume (35 μl) [12] and bile flow rate (16 μl · $min^{-1}$ for the tBuBHQ experiments). The second peak maximum of HRP appearance was at 18 min for tBuBHQ infusions. However, neither the magnitude of the second peak nor its time of appearance were significantly different from those of the control experiments. Therefore, it appears that tBuBHQ, like the above mentioned hormones [3], did not affect transcellular transport of HRP.

Experiments were also performed in the absence of the choleretic taurodehydrocholic acid (data not shown) and, although basal bile flow rates were lower, tBuBHQ similarly increased the height of the first peak of biliary HRP. Again, the kinetics and magnitude of the tBuBHQ response were virtually identical to those produced by vasopressin (10 nM, infused for 3 min, not shown).

Despite the perturbation of $Ca^{2+}$ homeostasis produced by tBuBHQ, there was no evidence of hepatocellular injury as indicated by the absence of lactate dehydrogenase leakage into the perfusate [7]. In the presence of taurodehydrocholic acid, bile flow transiently decreased during infusion of tBuBHQ (1.88 ± 0.26 $\mu l \cdot min^{-1} \cdot g\ liver^{-1}$, mean ± SD, $n = 4$) as compared to infusion of the vehicle (2.28 ± 0.43 $\mu l \cdot min^{-1} \cdot g\ liver^{-1}$, mean ± SD, $n = 5$). In the absence of taurodehydrocholic acid, tBuBHQ infusion also induced a reversible decrease in bile flow, which was similar in magnitude to that caused by the vehicle [7]. Lowe et al. [3] have shown that vasopressin also produces a small and transient decrease in bile flow. Therefore, the tBuBHQ-induced increase in biliary HRP activity was more likely due to increased junctional permeability than to an enhancement of diffusion rate through tight junctions as a result of lower bile flow rates.

Kan and Coleman [13] have reported that the calcium ionophore A23187 increases the tight junctional permeability in rat liver. However, in addition to the increase in the first biliary HRP peak, the second peak, which represents pinocytosis and transcellular transport of the probe, was also enhanced. A23187 promotes $Ca^{2+}$ influx as well as permeabilization of intracellular $Ca^{2+}$ stores, raising the question of whether extracellular $Ca^{2+}$ influx is necessary to elicit the reported enhancement of tight junctional permeability. As recent studies in our laboratory have demonstrated, tBuBHQ does not stimulate $Ca^{2+}$ influx across the hepatocyte plasma membrane [6,14]. Therefore, it can be concluded from the present work that the tBuBHQ-induced mobilization of the hormone-responsive $Ca^{2+}$ pool with subsequent elevation of $[Ca^{2+}]_i$ [6,7], is sufficient to elicit an increase in hepatocyte tight junctional permeability. $Ca^{2+}$ could act through $Ca^{2+}$-dependent kinases and/or the cytoskeleton. Evidence of intimate cytoskeletal-tight junction association has been presented in intestinal mucosal cells [15,16], providing a morphological basis for a possible cytoskeletal control of tight junctional permeability.

The observation that $Ca^{2+}$-mobilizing hormones and release of the endoplasmic reticular $Ca^{2+}$ pool by chemicals enhance hepatocyte tight junctional permeability may have physiological and toxicological implications, i.e. in the modulation of bile composition, since small changes in permeability may considerably alter the appearance in bile of proteins abundant in blood or stimulate reflux of biliary components into the sinusoidal compartment. The present results also suggest the possibility that toxicants which impair liver $Ca^{2+}$ homeostasis may very likely alter tight junctional permeability in vivo, supporting the recent observations that menadione increases junctional permeability to HRP in the perfused rat liver [17] and that thiol-oxidants increase bile/perfusate ratio of [$^{14}$C]sucrose [18]. Thus, alteration of tight junctional permeability is an additional mechanism whereby toxicant-induced changes in intracellular $Ca^{2+}$ homeostasis may produce impairment of functional integrity in the intact liver.

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