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Research Article

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Release of Ca^{2+} from the Endoplasmic Reticulum Is Not the Mechanism for Bile Acid–induced Cholestasis and Hepatotoxicity in the Intact Rat Liver

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Abstract

The hypothesis that monohydroxy bile acids exert their cholestatic and hepatotoxic effects via a sustained elevation of cytosolic [Ca^{2+}] was tested in the isolated perfused rat liver. Infusion of the specific inhibitor of microsomal Ca^{2+} sequestration, 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (tBuBHQ) (25 μM for 10 min) produced efflux of Ca^{2+} from the liver and a sustained (20 min) increase in cytosolic [Ca^{2+}] as indicated by the threefold increase in hepatic glucose output. Release of the endoplasmic reticular Ca^{2+} pool was demonstrated by the complete abolition of vasopressin- and phenylephrine-induced Ca^{2+} exchange between the liver and perfusate. Despite the profound perturbation of intracellular Ca^{2+} homeostasis produced by tBuBHQ, there was no decrease in bile flow and no evidence of hepatocellular injury (for 60 min), as indicated by lactate dehydrogenase release. In contrast, lithocholic acid (25 μM for 10 or 30 min) or taurolithocholic acid (5 μM for 10 or 30 min) produced an 80–90% inhibition of bile flow and a progressive increase in perfusate lactate dehydrogenase activity. During and after bile acid infusion, there was no change in Ca^{2+} fluxes between liver and perfusate, no stimulation of glucose output from the liver, and hormone-stimulated Ca^{2+} responses were preserved. It is concluded that the mechanisms for bile acid–induced cholestasis and hepatotoxicity in the intact liver are not attributable to changes in intracellular Ca^{2+} homeostasis, and especially not to prolonged release or depletion of Ca^{2+} sequestered in the endoplasmic reticulum. (*J. Clin. Invest.* 1990. 85:1255–1259.) cholestasis • monohydroxy bile acids • hepatotoxicity • endoplasmic reticular Ca^{2+} pool • hepatic calcium fluxes

Introduction

Calcium plays a critical role in metabolic regulation, motility, secretion, cell division, cytoskeletal structural integrity, and other cellular activities (1–3). To modulate such diverse biological functions, cell Ca^{2+} homeostasis is closely regulated by the dual processes of active extrusion of Ca^{2+} and sequestration into subcellular compartments (4, 5). Together, these processes maintain the basal cytosolic [Ca^{2+}] in the 100–200 nM range (4). The endoplasmic reticulum is a key site for Ca^{2+} sequestration because of its affinity for Ca^{2+} and capacity for Ca^{2+} storage (5–7). In addition, the endoplasmic reticular Ca^{2+} store, or some portion of it, has been demonstrated to be rapidly released by inositol 1,4,5-trisphosphate during the process of signal transduction after the binding of many hormones and growth factors to their cell surface receptors (8). Since Ca^{2+} is also a cofactor for catabolic enzymes such as proteases, phospholipases, and endonucleases, it is not surprising that sustained increases in cytosolic [Ca^{2+}] have been implicated mechanistically in some types of cell death (9, 10).

Little is known about the mechanism of monohydroxy bile acid toxicity. However, it seems likely that the cholestatic and hepatotoxic effects of this class of compounds, which include lithocholic acid (LCA)[1] and taurolithocholic acid (TLC), probably result from their ability to alter the permeability characteristics of biological membranes (11–14). Recent attention has focused on bile acid–induced alterations in intracellular [Ca^{2+}]; however, there is considerable variation in the literature regarding mechanistic aspects of bile acid–induced perturbation of Ca^{2+} homeostasis. For example, LCA has been demonstrated to enhance the Ca^{2+} permeability of erythrocytes and model membranes (15). More recently, LCA and TLC have been shown to increase the cytosolic [Ca^{2+}] of isolated hepatocytes (16, 17). Whereas in one study (17) the bile acid–induced changes in intracellular [Ca^{2+}] could be modulated by removing Ca^{2+} from the incubation medium, suggesting that influx of extracellular Ca^{2+} into hepatocytes contributed to the loss of viability observed, it has also been demonstrated that bile acids selectively permeabilize the endoplasmic reticulum to release the inositol 1,4,5-trisphosphate–mobilizable Ca^{2+} pool (16, 18). Mechanistic differences notwithstanding, it has been postulated that depletion of an intracellular Ca^{2+} store that is essential for bile acid secretion and/or prolonged elevation of cytosolic [Ca^{2+}] is responsible for bile acid–induced cholestasis and hepatotoxicity (15–18).

The isolated perfused rat liver system has been used to examine both physiological and pathological aspects of Ca^{2+} homeostasis, such as the rapid efflux of Ca^{2+} from the liver which occurs within seconds of hormone binding to cell surface receptors (19, 20) or Ca^{2+} entry into the liver as an early event in hepatotoxicity produced by menadione (21) and cystamine (22). In these studies we used the isolated perfused liver to examine the potential role of Ca^{2+} fluxes in bile acid–induced liver injury. The hypothesis that a sustained release of endoplasmic reticular Ca^{2+} would cause impairment of bile flow and hepatocellular necrosis was tested using the potent and specific inhibitor of ATP-dependent microsomal Ca^{2+} sequestration, 2,5-di(*tert*-butyl)-1,4-benzohydroquinone (tBuBHQ). In recent studies from this laboratory using subcellular fractions (23) and isolated hepatocytes (24), it has been

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1. *Abbreviations used in this paper:* LCA, lithocholic acid; LDH, lactate dehydrogenase; tBuBHQ, 2,5-di(*tert*-butyl)-1,4-benzohydroquinone; TLC, taurolithocholic acid.

demonstrated that tBuBHQ specifically depletes the inositol 1,4,5-trisphosphate–mobilizable intracellular Ca^{2+} pool and causes a prolonged increase in cytosolic [Ca^{2+}].

Methods

Materials and animals. [Arg^8]Vasopressin, phenylephrine, LCA, TLC, and sodium pyruvate were purchased from Sigma Chemical Co. (St. Louis, MO). NADH and lactate dehydrogenase (LDH; E.C. 1.1.1.27) were from Boehringer Mannheim GmbH (Mannheim, FRG), while tBuBHQ was from EGA-Chemie (Steinheim, FRG). All other chemicals were of at least analytical reagent grade. Male Wistar rats (ALAB AB, Sollentuna, Sweden) weighing 260–360 g and fed ad lib were used as liver donors in all experiments.

Isolated liver perfusion. Anesthesia was induced by sodium pentobarbital (60 mg/kg body weight i.p.). Livers were prepared for perfusion as described in reference 25; bile ducts were cannulated with polyethylene tubing (0.28 mm i.d., 0.61 mm o.d.; Clay Adams, Parsippany, NJ). Bile was collected in preweighed tubes and flow was calculated by weight, assuming that the specific gravity of bile was 1 g/ml.

Perfusion was carried out in single-pass mode with modified Krebs-Henseleit buffer (pH 7.4, with 1.3 mM [Ca^{2+}]). Liver temperature was kept at 37°C. The constant pressure system used in these studies has been diagrammatically described elsewhere (21). Oxygenation was ensured by passing the perfusate through a glass gas exchanger continually flushed with 95% O_2, 5% CO_2. The perfusate reservoir level was maintained at 21 cm above the tip of the inflow cannula placed in the portal vein, and this provided perfusate flow rates of at least 3.5 ml/g liver weight per min in all experiments. An oxygen electrode (model 0260; Beckman Instruments, Inc., Fullerton, CA) inserted in a flow cell placed in the outflow tract from the liver was used to continuously record the perfusate oxygen concentration.

Viability criteria were as follows: (*a*) adequate perfusate flow rates; (*b*) bile flow rates of > 0.90 μl/g liver weight per min during the first 60 min of perfusion, with no more than a 30% decrease in bile flow over the total perfusion time; (*c*) oxygen consumption > 3 μmol/g liver per min; and (*d*) no detectable leakage of hepatic intracellular enzymes for 90 min. For this purpose, LDH was selected as a marker since it proved to be a more sensitive indicator of enzyme leakage than aminotransferases (21). None of the compounds added to the perfusion system interfered with the LDH assay.

Determination of changes in perfusate [Ca^{2+}]. The [Ca^{2+}] of the hepatic effluent perfusate was recorded continuously using a Ca^{2+}-selective electrode (F2112; Radiometer, Copenhagen, Denmark) coupled to a combination reference electrode (K-401; Radiometer) via an agarose salt bridge (2% in 3 M KCl), as previously described (21). The signals from the electrodes were amplified using a pH meter (PHM 84; Radiometer), and the recorder input voltage was regulated by a bucking device. The perfusate was earthed to decrease electrical interference. At a perfusate [Ca^{2+}] of 1.3 mM, a signal to noise ratio of > 4 was given by a change in perfusate [Ca^{2+}] of 5 μM.

Experimental design. After establishment of liver perfusion, a period of 15 min was allowed for equilibration of the system. Thereafter, effluent perfusate samples and bile were collected during a control period of 30 min. Experimental periods were then begun by infusion of test substances as described in figure and table legends. Test chemicals were 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 (i.e., typically 20–23 μl/min). Compounds were administered in ethanol, and control experiments were carried out with infusion of ethanol for 10 or 30 min, or without any additions. To control for any confounding effect of ethanol itself, some experiments were performed with TLC or LCA delivered in dimethyl sulfoxide. For experiments designed to test hepatic responsiveness to Ca^{2+}-mobilizing hormones, vasopressin and the α_1-adrenergic agonist, phenylephrine, were dissolved in perfusate buffer to give concentrations in inflow perfusate of 10 nM and 2 μM, respectively. The maximum duration of experiments was 150 min.

Measurement of hepatic glucose release. Concentrations of glucose in the effluent perfusate were determined by the glucose oxidase method using a commercially available kit (Glucose-Trinder; Sigma Chemical Co.).

Statistical methods. Data are expressed as mean±SE. For individual experimental groups, differences in mean values for bile flow at different stages were compared using the paired *t* test.

Results

Effect of TLC, LCA, and tBuBHQ on bile flow and LDH leakage. Infusion of 25 μM LCA produced progressive cholestasis, the onset of which was evident by 15–20 min and which eventually resulted in virtual cessation of bile flow (Fig. 1 *A* and Table I). Bile flow was also reduced 80–95% by TLC (5 μM) (Fig. 1 *B* and Table I). The extent of reduction in bile flow depended on the duration of bile acid infusion. In contrast to the bile acids, tBuBHQ only slightly altered bile flow during infusion (Fig. 1 *C*), an effect that was reversible and similar in magnitude to that of the vehicle (ethanol; Table I).

The onset of cholestasis during bile acid infusion was accompanied by an increasing leakage of LDH from the liver into the perfusate (Fig. 1, *B* and *C*). Perfusate levels of LDH activity continued to rise after cessation of bile acid infusion. No significant LDH activity was detected in perfusate samples for 50 min after initiation of tBuBHQ infusion (Fig. 1 *C*) or for the corresponding time in control experiments (data not shown).

Ca^{2+} fluxes between liver and perfusate during infusion of bile acids and tBuBHQ: comparison with Ca^{2+}-mobilizing hormones. Infusion of either 10 nM vasopressin or 2 μM phenylephrine for 3 min provoked Ca^{2+} release from the liver, which is in agreement with previous reports (19, 20). Hormone-induced Ca^{2+} releases were only slightly attenuated (< 15%) upon repeated hormone exposures administered at intervals of 10 min or longer (not shown). Release of free Ca^{2+} into the hepatic outflow tract was undetectable during bile acid infusion (Fig. 2, *B* and *C*). A slight negative deflection in the Ca^{2+} signal was consistently noted at the onset of bile acid infusion; however, this probably represented a direct effect on

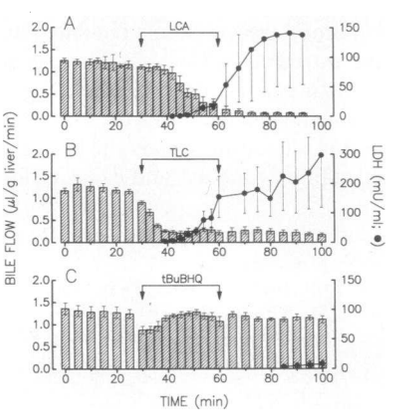

Figure 1. Effects of TLC, LCA, and tBuBHQ on bile flow (*bars*) and LDH leakage (*solid circles*) from isolated perfused rat livers. Test chemicals dissolved in ethanol were infused for 30 min (*arrows*). Bile was collected and quantitated as described in Methods. LDH was determined in samples of perfusate collected from the outflow tract of the liver (single-pass perfusion). *A*, LCA (25 μM); *B*, TLC (5 μM); *C*, tBuBHQ (25 μM). Values represent mean±SE of three to five separate perfusions for each treatment.

Table I. Effect of tBuBHQ, LCA, TLC, and Vehicle on Bile Flow in the Isolated Perfused Rat Liver

Addition (*n*)	Bile flow		
	Before addition	During addition	After addition
	μl/g liver per min		
Ethanol, 10 min (3)	1.19±0.10	0.91±0.22	0.98±0.07
Ethanol, 30 min (6)	1.35±0.11	1.17±0.10	1.16±0.08
25 μM tBuBHQ, 10 min (4)	1.15±0.21	0.84±0.17	1.12±0.19
25 μM tBuBHQ, 30 min (4)	1.29±0.12	1.12±0.04	1.14±0.03
25 μM LCA, 10 min (5)	1.19±0.09	1.21±0.14	0.45±0.15
25 μM LCA, 30 min (4)	1.19±0.14	0.63±0.14	0.09±0.05
5 μM TLC, 10 min (3)	1.28±0.15	0.59±0.04	0.40±0.02
5 μM TLC, 30 min (6)	1.24±0.06	0.36±0.05	0.20±0.04

Average bile flow rates were determined for the 30-min period before infusion of the test substances or vehicle, the period of infusion of the test substances (10 or 30 min), and the postinfusion period (40 min after the 30-min infusions, and 60 min after the 10-min infusions). Thus, the total perfusion time was 100 min for all treatments. Data represent mean±SE of the average bile flow rates determined for n = 3–6 separate livers. For individual livers, average bile flow rates for each period were calculated from 3–10 fractions of bile, which was continuously collected throughout the experiments.

the responsiveness of the calcium electrode, since similar effects were observed when the test substances were infused into the circuit without the liver being present (not shown). In contrast to the bile acids, infusion of tBuBHQ caused a substantial efflux of Ca^{2+} from liver into the vascular space (Fig. 2 *A*). Release of Ca^{2+} into the perfusate began 2–3 min after starting tBuBHQ infusion, rose to a peak at 5–7 min, and then declined to basal values by 9–12 min. When vasopressin or phenylephrine were infused immediately after the infusion of tBuBHQ, there was complete abolition of hormone-stimulated Ca^{2+} efflux into the perfusate (Fig. 2 *A*). During the ensuing 70 min, the ability to mobilize hepatic Ca^{2+} in response to hormone infusions gradually returned (shown in part, Fig. 2 *A*). These observations in the intact liver indicate that tBuBHQ initially produced complete release of the hormone-responsive endoplasmic reticular Ca^{2+} pool, and that inhibition of Ca^{2+} reuptake into this compartment was sustained for at least 30 min.

In contrast with the results obtained with tBuBHQ, vasopressin and phenylephrine stimulated apparently normal efflux of hepatic Ca^{2+} both during and after infusion of TLC and LCA (Fig. 2, *B* and *C*). The onset of cholestasis and release of LDH produced by these agents were not associated with diminished hormone-induced Ca^{2+} signals. Moreover, repeated infusions of hormones continued to elicite the physiological Ca^{2+} response after the development of bile acid–induced cholestasis and enzyme leakage (Fig. 2, *B* and *C*). These observations indicate that the inositol 1,4,5-trisphosphate–mobilizable Ca^{2+} pool was not released by the bile acids, and that Ca^{2+} reuptake after prior inositol 1,4,5-trisphosphate–mediated release was preserved.

Hepatic glucose release during infusion of bile acids and tBuBHQ. Since cytosolic [Ca^{2+}] is a major regulator of phosphorylase *a* activity, hepatic glucose release was used as an indicator of cytosolic free [Ca^{2+}] (19, 26, 27). Infusion of tBuBHQ resulted in enhanced glucose output from the perfused liver (Fig. 3), which is consistent with the Ca^{2+}-mobilizing activity of tBuBHQ (24). The change in effluent perfusate glucose concentration after infusion of tBuBHQ was greater than that produced by vasopressin (Fig. 3), and the temporal profile differed in that enhanced glucose release, and thus by implication increased cytosolic [Ca^{2+}], continued for at least 20 min after cessation of tBuBHQ infusion (Fig. 3). Unlike the effects of hormones or tBuBHQ, hepatic glucose release was not significantly altered during infusion of either LCA or TLC (Fig. 3).

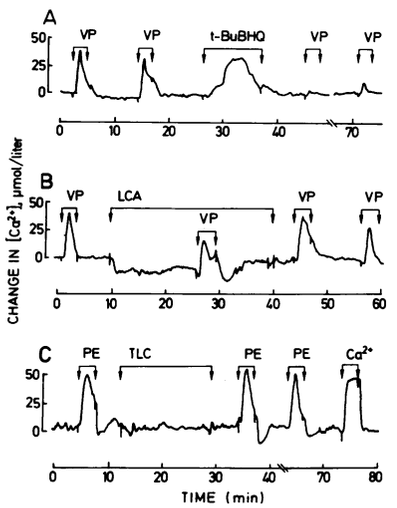

Figure 2. Changes in perfusate (Ca^{2+}) during infusion of hormones, tBuBHQ, LCA, and TLC. Traces are hand-drawn copies of recordings from a Ca^{2+}-sensitive electrode placed in-line in the outflow tract of the isolated liver perfusion circuit as described in Methods. Additions to the perfusion system, abbreviations, and concentrations were as follows: vasopressin for 3 min (*VP*, 10 nM); phenylephrine for 3 min (*PE*, 2 μM); tBuBHQ for 10 min (25 μM); LCA for 30 min (25 μM); and TLC for 15 min (5 μM). Each tracing is representative of at least two experiments. The calibration of the calcium electrode (indicated on the vertical axis to the left of each recording) was determined by infusion of standard calcium solutions, such as that at far right of *C*, recorded during infusion of 50 μM Ca^{2+}.

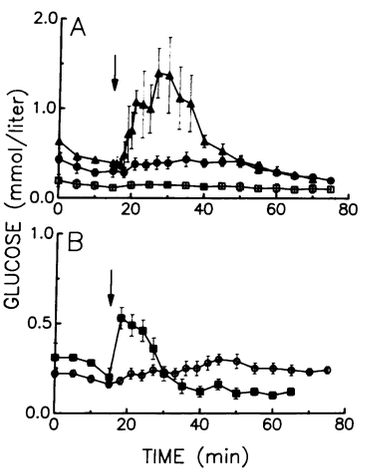

Figure 3. Effects of tBuBHQ, LCA, TLC, ethanol, and vasopressin on the concentration of glucose in hepatic effluent perfusate. No glucose was present in the perfusion medium entering the liver, so that the concentrations indicated are a direct reflection of hepatic glucose output. Time 0 indicates the start of the experimental period, which followed a 15–30-min equilibration period (see Methods). Infusion of the test substances was initiated at time = 15 min (*arrow*). *A*, tBuBHQ (25 μM infused for 10 min; ▲); ethanol (vehicle control; 0.05% infused for 30 min; □); and LCA (25 μM infused for 30 min; ●). *B*, TLC (5 μM infused for 30 min; ○) and vasopressin (10 nM infused for 10 min; ■). Data represent the mean±SE of three to five separate perfusions for each treatment.

Discussion

A potential link between disruption of hepatic Ca^{2+} homeostasis and bile acid–induced liver injury has been suggested (16) in light of the observation that Ca^{2+} appears to play an important role in canalicular bile flow, perhaps as a requirement for nonmuscular canalicular contractile properties (28–31), and that cholestatic bile acids significantly disrupt Ca^{2+} handling by membranes and isolated cells in vitro (15–18).

In the present studies, we have used two approaches to investigate a possible mechanistic role for altered cellular Ca^{2+} homeostasis in bile acid–induced cholestasis and hepatotoxicity. In one set of experiments, tBuBHQ was used to selectively perturb hepatic Ca^{2+} homeostasis in the same way as has been proposed to result from exposure of isolated hepatocytes to monohydroxy bile acids, i.e., selective depletion of the endoplasmic reticular Ca^{2+} store (16, 18). We have previously demonstrated that tBuBHQ is a potent and specific inhibitor of ATP-dependent microsomal Ca^{2+} sequestration (23), and in isolated hepatocytes tBuBHQ (EC_{50} = 1–2 μM) releases the inositol 1,4,5-trisphosphate–mobilizable Ca^{2+} pool (24). One consequence of Ca^{2+} release from endoplasmic reticulum by tBuBHQ is a sustained increase in cytosolic [Ca^{2+}], leading to enhanced glycogenolysis and glucose output from the hepatocytes by activating phosphorylase *a* (27). In the present studies, tBuBHQ produced identical effects in the isolated perfused rat liver. However, despite the clear-cut and prolonged nature of these tBuBHQ effects, there was no reduction of bile flow that could be attributable to the disruption of Ca^{2+} homeostasis. There was also no leakage of hepatic intracellular enzymes after the infusion of tBuBHQ. These observations allow two conclusions to be drawn. Not only is tBuBHQ devoid of hepatotoxicity in the short term, but release of endoplasmic reticular Ca^{2+} and the associated prolonged elevation of the cytosolic [Ca^{2+}] to physiologically relevant levels (24) produces neither cholestasis nor hepatocellular necrosis.

To complement the tBuBHQ experiments, we examined the effects of cholestatic bile acids on Ca^{2+} fluxes under conditions leading to hepatotoxicity. Infusion of LCA or TLC rapidly initiated cholestasis, which progressed to almost complete cessation of bile flow and was associated thereafter with leakage of LDH from the liver. Despite arrested bile flow and hepatic injury, there was no significant change in perfusate [Ca^{2+}] or glucose to indicate either efflux of mobilized intracellular Ca^{2+} or entry of extracellular Ca^{2+} into the liver. In addition, the liver continued to respond to hormone receptor activation with normal mobilization and extrusion of Ca^{2+} and glucose at all stages of the development of bile acid–induced injury. Unless a bile acid–sensitive, tBuBHQ-insensitive subcellular pool of Ca^{2+} that was not identified in these or previous (16) studies is responsible for the maintenance of bile flow, it seems that endoplasmic reticular Ca^{2+} depletion does not mediate bile acid–induced cholestasis. It is improbable for such a hypothetical bile acid–sensitive Ca^{2+} pool to exist, because no stimulation of glucose output or alteration in Ca^{2+} fluxes across the plasma membrane were observed during and after bile acid infusion. It can also be concluded from the present observations that the toxic effects produced by monohydroxy bile acids in isolated perfused liver do not occur through a sustained increase in cytosolic [Ca^{2+}].

Exposure of isolated hepatocytes to LCA and TLC results in a rapid increase in cytosolic [Ca^{2+}] (16–18), which is followed by LDH leakage from the cells (17). An EC_{50} of ~ 25 μM has been reported for Ca^{2+} mobilization by cholestatic bile acids in intact hepatocytes (16); however, studies from this laboratory (Kass, G. E. N., S. K. Duddy, and S. Orrenius, unpublished data) and others (17) have found that significantly higher concentrations of bile acids (50–100 μM) are required to produce an increase in cytosolic [Ca^{2+}]. From available data, it seems improbable that such high concentrations of monohydroxy bile acids are ever attained in vivo (11, 32). Nevertheless, our present data show that the abnormalities of cell Ca^{2+} homeostasis observed in isolated hepatocytes exposed to toxic bile acids are not reproduced in the isolated perfused liver, despite clear manifestation of cholestasis and cytotoxicity. It is likely that the difference in response of isolated hepatocytes vs. isolated perfused liver is a function of differences in the dose of administered bile acids that actually reach target sites within the cell.

In summary, studies with the model inhibitor of microsomal Ca^{2+} sequestration, tBuBHQ, showed that prolonged elevation of cytosolic [Ca^{2+}] together with depletion of the endoplasmic reticular Ca^{2+} pool caused neither cholestasis nor hepatotoxicity in the isolated perfused rat liver. Monohydroxylated bile acids, at concentrations which arrested bile flow and caused leakage of LDH from the liver, failed to mobilize endoplasmic reticular Ca^{2+} or alter Ca^{2+} exchange at the sinusoidal pole of the plasma membrane. It is concluded that alterations of cellular Ca^{2+} homeostasis are not the mechanism by which cholestatic bile acids initiate hepatotoxicity.

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