Taurocholate-Stimulated Leukotriene C$_4$ Biosynthesis and Leukotriene C$_4$–Stimulated Choleresis in Isolated Rat Liver

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Background/Aims: Cysteinyl-containing leukotrienes seem to exert a cholestatic effect. However, leukotriene inhibitors were found to reduce bile salt efflux in isolated rat hepatocytes, suggesting a role for leukotrienes in bile flow formation. *Methods:* In the isolated rat liver, the effects of two different concentrations of leukotriene C$_4$ on bile flow and bile salt excretion are analyzed, as well as the possible effect of taurocholate on the hepatic production of cysteinyl-containing leukotrienes. *Results:* Leukotriene C$_4$ (0.25 fmol) increased bile salt excretion (+22.2%; $P < 0.05$), whereas a much higher dose (0.25 × 10^6 fmol) showed the known cholestatic effect, reducing bile salt excretion (−25.9%; $P < 0.01$). These dose-dependent biphasic effects were specific because they could be prevented by the simultaneous administration of cysteinyl-containing leukotriene antagonists. On the other hand, taurocholate administration induced a dose-dependent increase in biliary excretion of cysteinyl-containing leukotrienes. Furthermore, taurocholate increased messenger RNA levels of 5-lipoxygenase, a key enzyme in leukotriene biosynthesis. Taurocholate increase of hepatocyte intracellular calcium was not significant, suggesting that taurocholate effects are not mediated by stimulation of calcium metabolism. *Conclusions:* These results constitute evidence for the existence of a positive feedback mechanism by which bile salts stimulate the synthesis of leukotrienes that, in turn, stimulate bile salt excretion.

B ile salts are the main promoters of bile flow, and considerable efforts have been made to understand the mechanisms of their uptake, hepatocellular transport, and biliary excretion.[1-3] However, very little is known about the possible role of arachidonic acid derivatives and, in particular, of leukotrienes (LTs) in the secretion of bile salts by the liver.

5-Lipoxygenase is a key enzyme in the cascade leading to LTs. It is responsible for the formation of 5-hydroperoxyeicosatetraenoic acid (5-HPETE) from arachidonic acid, and its further dehydration into LTA$_4$, a pivotal intermediate in leukotriene biosynthesis.[4,5] Hydrolysis of

LTA$_4$ may lead to either LTB$_4$ if catalyzed by LTA$_4$ hydrolase or 5,6-DHETE if catalyzed by cytosolic epoxide hydrolase. The enzymatic addition of glutathione to LTA$_4$ leads to LTC$_4$, which may be subsequently transformed into LTD$_4$ and LTE$_4$. LTC$_4$, LTD$_4$, and LTE$_4$ are referred to as cysteinyl-containing LTs and constitute the slow-reacting substance of anaphylaxis.

Cysteinyl-containing LTs have been found to be the main metabolites of LTA$_4$ in rat liver.[6] Although they have been implicated in the stimulation of secretory processes in several tissues and cells,[7,8] all of the actions ascribed to these LTs in the liver have been related to the production of tissue injury[9] and cholestasis.[10] On the other hand, previous studies in our laboratory have shown that several inhibitors of 5-lipoxygenase impaired bile salt efflux in isolated rat hepatocytes.[11] Also, the cysteinyl-containing LT antagonist FPL 55712 has been reported to reduce the bile flow in anesthetized rats.[12] All of these data taken together strongly suggest that LTs may have a role in the excretion of bile salts and therefore in the genesis of bile flow. In this study, we have analyzed the effect of two different doses of LTC$_4$ on biliary bile salt excretion in the isolated and perfused rat liver. We have also studied the possible effect of taurocholate, a main physiological bile salt in the rat, on LTC$_4$ biosynthesis in the liver.

Materials and Methods

Taurocholic acid (sodium salt), bovine serum albumin, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (HTMP), and L-phenylephrine were obtained from Sigma Chemical Co. (St. Louis, MO). FPL 55712 was a gift from Fisons Pharmaceutical Ltd. (Loughborough, England), and REV 5901 was purchased from Cascade Biochem (Reading, England); Leibovitz L-15

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medium from Whittaker (Walkersville, MD); laminin from ICN Biomedicals (Costa Mesa, CA); Fura-2/acetoxymethyl ester (AM) from Molecular Probes (Eugene, OR); [^{3}H]LTC$_4$, and [^{32}P]deoxycytidine triphosphate were from Amersham (Little Chalfont, England). Synthetic oligonucleotides were purchased from Scandinavian Gene Synthesis AB (Köping, Sweden); reverse transcriptase from GIBCO BRL (Gaithersburg, MD); and *Taq* DNA polymerase from Promega (Madison, WI). All other chemicals were of the highest purity commercially available.

Isolated Rat Liver Perfusion

Isolated livers from male Wistar rats (230–290 g body wt) were used in a recirculating retrograde perfusion system. Liver isolation was as described by Meijer et al.[13] with minor modifications. Briefly, the bile duct was first cannulated with a polyethylene tubing PE-50, followed by cannulation of the vena porta with a catheter Abbocath 16-G. After the catheter was tied in place, the liver was immediately perfused in situ ($\approx$20 mL/min of oxygenated and heparinized Krebs–Ringer bicarbonate buffer at 37°C), and the inferior vena cava was cut simultaneously to avoid an increase in the perfusion pressure. The diaphragm was then opened, the thoracic vena cava was cannulated with a polyethylene cannula (1.5-mm inner diameter), and a ligature previously set around the vena cava, proximal to the insertion of the right renal vein, was closed. Once the adhesions of the liver and the vena cava were carefully cut, the liver was transferred to an acrylic platform and placed into a chamber for perfusion in a recirculating system with approximately 30 mL/min of Krebs–Ringer bicarbonate buffer supplemented with 5.5 mmol/L glucose and 3% bovine serum albumin at 37°C. Oxygenation was ensured by passing the medium through a glass gas exchanger continuously flushed with 95% O$_2$/5% CO$_2$.

The protocol of treatment of the livers was as follows. After a 20-minute stabilization period and a further 20-minute basal period, a bolus of 500 μL of saline buffer containing the substances to be tested was injected, and the experiment was continued for another 20 minutes (stimulation period). We studied the effect of the following substances: 3 or 12 μmol of taurocholate, 0.25 fmol or a 10^6-fold higher amount (i.e., 0.25 $\times$ 10^6 fmol) of LTC$_4$, and the same doses of LTC$_4$ together with cysteinyl-containing LT antagonists (either FPL 55712 or REV 5901, 6.25 $\times$ 10^6 fmol for both). Bile effluents were collected at the end of each 20-minute period.

Some livers were preloaded with tritiated LTC$_4$, and the effect of taurocholate administration on the biliary excretion of LTC$_4$ was tested in these livers. Approximately 100,000 dpm of [^{3}H]LTC$_4$ (dissolved in 500 μL of saline buffer as vehicle) was injected after a basal period of 20 minutes. Thirty minutes later, a bolus with saline vehicle containing 3 μmol of taurocholate was administered, and the biliary excretion of cysteinyl-containing LTs was monitored during a further 45-minute period. In these experiments, bile effluents were collected every 15 minutes.

In the experiments in which the effect of LTC$_4$ on bile flow

and bile salt excretion was tested, a continuous infusion of taurocholate (15 μmol/h) was maintained to counteract the loss of bile salts due to the interruption of the enterohepatic circulation.

In addition to the experiments with bolus injections, a study with continuous infusion of taurocholate (120 or 15 μmol/h) was performed, initiated after the stabilization period and continued for 90 minutes. Bile effluents were collected every 15 minutes. Bile flow was calculated by weighing the collected aliquots.

The viability of the liver during the experiments was assessed by measuring the levels of aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and potassium in the hepatic effluent and by verifying the bile production and the macroscopic aspect of the liver.[13] Liver metabolism was assessed by measuring the hepatic release of glucose, lactate, and pyruvate. No significant differences in the values of AST, LDH, potassium, and glucose were found between groups except after the administration of the highest doses of LTC$_4$ (0.25 $\times$ 10^6 fmol), which altered significantly some of these parameters (see Results).

Bile Salt and LT Analysis

Total bile acids were determined in bile using a commercially available kit from Merck (Darmstadt, Germany). To determine the excretion of LTC$_4$ and LTD$_4$ (the main cysteinyl-containing LTs in bile from isolated rat liver[14]), 25 μL of bile was collected in 200 μL of methanol/water (90:10 by volume), in the presence of 1 mmol/L of the radical scavenger HTMP, followed by solid-phase extraction on C18 (Chromabond column from Macherey Nagel, Düren, Germany) and further reverse phase high-performance liquid chromatography on a 5-μm Novapak C$_{18}$ column (Millipore, Milford, MA) as described.[15] Briefly, the column (0.4 $\times$ 15 cm) was isocratically eluted at 1 mL/min with 33.6:5.4:60:1 acetonitrile/methanol/water/acetic acid (vol/vol), adjusted to pH 5.6 with triethylamine, and the absorbance at 280 nm was continuously recorded. For analysis of LTs, 3-mL fractions eluting at the retention times of standard LTC$_4$ and LTD$_4$ were collected, evaporated under nitrogen, further resuspended in 50 mmol/L Tris buffer (pH 7.5), mixed, and quantitated by a commercial radioimmunoassay kit (Amersham, Little Chalfont, England). Cross-reactivities of this kit for LTC$_4$ and LTD$_4$ were identical.

Analysis of 5-Lipoxygenase Messenger RNA

At the end of each stimulation experiment, a piece of liver was immediately frozen in liquid nitrogen and stored at −80°C until use for the isolation of total RNA by a guanidinium-thiocyanate/phenol/chloroform method.[16] The analysis of 5-lipoxygenase messenger RNA (mRNA) was conducted through a procedure based on the reverse-transcription polymerase chain reaction (RT-PCR), essentially as described.[17] Total RNA (1 μg) was reverse transcribed in a volume of 20 μL. One half of the complementary DNA (cDNA) pool was

used for PCR amplification of a 5-lipoxygenase cDNA fragment (595 base pairs long, located between nucleotides 1500 and 2094 in the reported rat 5-lipoxygenase sequence[18]) using the primers 5' → 3':d(ATCGTGATGATGGACTGCTCG) and d(TGGAATCTGTCTGGTGACAGG). A 314–base pairs long fragment of rat β-actin cDNA was amplified as an internal control for each sample, using 6 μL of the cDNA pool and the primers d(TCTACAATGAGCTGCGTGTG) and d(GGT-GAGGATCTTCATGAGGT), with the underlined nucleotide being different in the rat sequence. This β-actin cDNA fragment corresponds to nucleotides encoding aminoacids between Phe-90 and Thr-194 in the reported rat β-actin sequence.[19] To avoid interference of the plateau effect, 5-lipoxygenase cDNA fragment was amplified by 37 cycles with the step program 94°C, 60°C, and 72°C (1 minute each except 5 minutes for a final extension at 72°C), and β-actin was amplified by 22 cycles with the step program 94°C, 55°C, and 72°C (1 minute each except 5 minutes for the final extension). Blank reactions with no RNA were conducted in all experiments as well. For comparative quantitation, labeled PCR products were obtained by amplification of corresponding DNA fragments in the presence of [^{32}P]deoxycytidine triphosphate. This step and the further processing was as described elsewhere.[17] Values (in cpm) for 5-lipoxygenase mRNA were normalized to those for β-actin mRNA, being the results given as ratios between them (5-lipoxygenase cpm/β-actin cpm). The use of β-actin mRNA as an internal standard has been controverted because it may vary in some circumstances.[20] However, this was not the case in our study. Thus, no differences between groups in β-actin mRNA cpm values were observed in determinations performed simultaneously and with the same batch of labeled deoxycytidine triphosphate.

Validation experiments of PCR assays using known amounts of total RNA (0.25, 0.5, 1, and 2 μg) were performed. Resultant cpm values corresponding to either 5-lipoxygenase or β-actin mRNA had linearity with respect to input of total RNA within the used range. The identity of the PCR products from 5-lipoxygenase cDNA amplification was ascertained both by size and by the restriction patterns after treatment with endonucleases. PstI and PvuII yielded the predicted restriction fragments, whereas EcoRI did not digest the amplified PCR product (no restriction site for EcoRI was expected to be present in the PCR fragment).

Measurement of [Ca²⁺]ᵢ in Hepatocytes

Intracellular concentration of free calcium was measured in isolated rat hepatocytes by fluorescent labeling and image analysis.[21] Hepatocytes were isolated from livers of fed male Wistar rats weighing 240–290 g as previously described.[22] Mean viability by trypan blue exclusion was ≈88%. Cells were resuspended in Leibovitz L-15 medium containing 50 U/mL penicillin, 50 μg/mL streptomycin, and 10% fetal calf serum, plated onto laminin-treated glass coverslips (22 mm diameter), and incubated at 37°C for 2 hours.

Hepatocytes attached to coverslips were then loaded with the dye by incubating (30 minutes) with Fura-2/AM (final concentration, 4–5 μmol/L) in L-15 medium enriched with fetal calf serum. After incubation, coverslips were transferred to a perfusion chamber placed into a Nikon Diaphot inverted microscope (Tokyo, Japan) and continuously superfused with Krebs–Ringer bicarbonate buffer (37°C and gassed with 95% O_2/5% CO_2), at a flow rate of 3 mL/min.[23]

Excitation of the cells was performed with a xenon lamp light filtered alternatively at 340 and 380 nm by means of a computer-controlled rotating filter wheel.[24] Neutral density filters reduced the excitation intensity to 0.1%.[25] Fluorescence was measured in fields of 15–20 cells selected to exclude damaged cells showing blebs or cytosolic granulations. Emission signals were collected with a video camera (Photonic Sciences, Robertsbridge, England), and the 340/380 nm fluorescence ratio was converted to calcium concentrations using a calibration curve prepared with the calcium ionophore ionomycin. The collection of the images and all of the calculations were performed with an Applied Imaging MagiCal image system (Sunderland, England).

Statistical Analyses

Mann–Whitney, Wilcoxon, and Kruskal–Wallis nonparametric statistical tests were used to make comparisons between experimental groups.[26] Values are expressed as the mean ± SEM.

Results

Effects of LTC₄ on Bile Flow, Bile Salt Excretion, and Liver Metabolism

LTC₄, administered as a bolus of 0.25 fmol, was found to increase the excretion of bile salts (+22.2% with respect to the basal values; $P < 0.05$) and the bile flow (+11.1% with respect to the basal values; $P < 0.05$) (Figure 1). These increases were also significant when compared with a control group with only saline vehicle stimulation (both $P < 0.05$). In contrast, a higher dose of LTC₄ (0.25 × 10⁶ fmol) had a cholestatic effect, reducing both biliary bile salt excretion (−25.9% with respect to basal values; $P < 0.01$), and bile flow (−35.9% with respect to basal values; $P < 0.01$) (Figure 1). This high dose of LTC₄ also induced an increase in the rate of glucose release to the effluent (data not shown) and in the lactate/pyruvate ratio as compared with a control with injection of vehicle alone ($P < 0.05$ for both) (Figure 1). The high dose of LTC₄ did not modify either perfusion flow (4.84 ± 0.04 mL·min⁻¹·g liver⁻¹ vs. 4.79 ± 0.07 mL·min⁻¹·g liver⁻¹ in the basal period; NS) or portal pressure (7.75 ± 0.26 mm Hg vs. 7.63 ± 0.27 mm Hg in the basal period; NS).

The specificity of the observed effects of LTC₄ could be assessed by injecting LTC₄ together with LT antagonists (either FPL 55712 or REV 5901; 6.25 × 10⁶ fmol each).
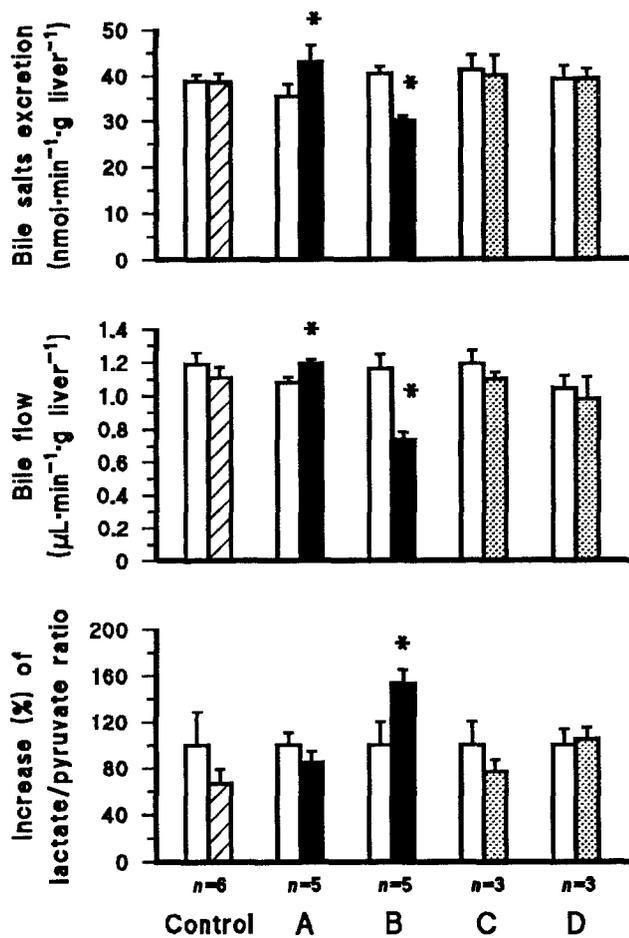
Figure 1. Effect of bolus administrations of two different doses of LTC₄ on bile salt excretion, bile flow, and lactate/pyruvate ratio in comparison with the control group and its antagonism by FPL 55712. Every pair of bars represents the mean value of 3–6 independent experiments of isolated and perfused rat liver either in the respective basal period (□) or after the administration of saline vehicle (▨) (control group), 0.25 fmol of LTC₄ (■) (group A), 0.25 × 10⁶ fmol of LTC₄ (■) (group B), 0.25 fmol of LTC₄ plus 6.25 × 10⁶ fmol of FPL 55712 (▩) (group C), or 0.25 × 10⁶ fmol of LTC₄ plus 6.25 × 10⁶ fmol of FPL 55712 (▩) (group D). The data represent the mean ± SEM. *P < 0.05 vs. the values in the respective basal period.

The simultaneous administration of FPL 55712 and 0.25 × 10⁶ fmol of LTC₄ prevented the reduction of both bile salt excretion and bile flow and the increase in the lactate/pyruvate ratio observed with 0.25 × 10⁶ fmol of LTC₄ alone (Figure 1). Also, the increase in the biliary excretion of bile salts and bile flow observed after injecting 0.25 fmol of LTC₄ alone was prevented by the simultaneous administration of FPL 55712 (Figure 1). FPL 55712 alone induced a slight reduction in both bile flow (−10.5%; n = 3; NS) and bile salt excretion (−6.1%; NS). A similar result was obtained with the other LT antagonist (REV 5901) alone: −13.6% for bile flow and −4.7% for bile salt excretion (n = 3; both NS). REV 5901 also blocked the LT effects on these

parameters in the same way as FPL 55712. Thus, a low dosis of LTC₄ associated with REV 5901 resulted only in minor and nonsignificant decreases of both bile flow (−11.8%; n = 3), and bile salt excretion (−3.4%), similar to those with REV 5901 alone. Also, no significant changes were observed when a high dosis of LTC₄ was administered together with REV 5901 (−14.6% for bile flow and −11.9% for bile salt excretion; n = 3).

Effects of Taurocholate on Bile Secretion and Cysteinyl-Containing LT Biosynthesis

Figure 2 shows the effect of bolus injections with taurocholate (3 and 12 μmol) on both bile flow and biliary excretion of bile salts. As expected, taurocholate caused a significant increase of these parameters in a dose-dependent manner. When the biliary excretion of cysteinyl-containing LTs was measured before and after the administration of taurocholate, it was found that this bile salt also caused a dose-dependent increase in the amount of cysteinyl-containing LTs in the bile (Figure 2). To assess whether this effect was because of hepatic biosynthesis of LTs or merely because of increased excretion of LTs previously produced and maintained in the liver, we performed a series of experiments using livers loaded with [³H]LTC₄ before the stimulation with taurocholate. The recovery of radioactivity in bile and the biliary excretion of cysteinyl-containing LTs were measured simultaneously. Bolus injection of 3 μmol of taurocholate in [³H]LTC₄ preloaded livers induced the expected increase in the biliary excretion of cysteinyl-containing LTs (from 3.17 ± 0.41 to 9.32 ± 1.43 pg·min⁻¹·g liver⁻¹; $P < 0.05$) (Figure 3). This increase was similar to that observed after injecting taurocholate to non−preloaded livers (from 2.26 ± 0.40 to 9.08 ± 2.12 pg·min⁻¹·g liver⁻¹; $P < 0.05$) (Figure 2). However, taurocholate injection did not modify the rate of the biliary excretion of labeled LTC₄ from the preloaded livers (Figure 3), indicating that the increased biliary excretion of LTs on taurocholate administration was mainly due to a de novo production of these compounds in the liver.

The effect of taurocholate was also studied in a continuous infusion model because it may represent a more physiological situation than the bolus injection. Bile flow and biliary excretion of bile salts were higher in livers perfused with taurocholate at a rate of 120 μmol/h than in control livers in which taurocholate was infused at a rate of 15 μmol/h (2.08 ± 0.10 vs. 1.46 ± 0.05 μL·min⁻¹·g liver⁻¹ for the bile flow, respectively, and 123.3 ± 8.5 vs. 24.7 ± 2.4 nmol·min⁻¹·g liver⁻¹ for the excretion of bile salts, respectively; for both $P <$

0.001) (Figure 4). Similarly, the biliary excretion of cysteinyl-containing LTs was significantly higher in livers perfused with 120 μmol/h of taurocholate than in control livers (18.8 $\pm$ 3.6 vs. 3.70 $\pm$ 1.52 pg·min^{-1}·g liver^{-1}, respectively; $P < 0.05$) (Figure 4).

Effect of Taurocholate on the Expression of 5-Lipoxygenase Gene

To further explore the stimulating effect of taurocholate on LT biosynthesis, we studied the expression of 5-lipoxygenase gene in livers challenged by either bolus

injection or continuous infusion. As shown in Figure 5, administration of a bolus of taurocholate enhanced significantly, and in a dose-dependent manner, the levels of 5-lipoxygenase mRNA (represented as 5-lipoxygenase cpm/β-actin cpm ratio). Also, the infusion of taurocholate at a high rate (120 μmol/h) caused an increase in the levels of 5-lipoxygenase mRNA as compared with controls (where taurocholate was infused at a rate of 15 μmol/h) (Figure 5).

Effect of Taurocholate on $[Ca^{2+}]_i$ in Isolated Rat Hepatocytes

To test the possibility that the calcium metabolism might be involved in the stimulation of LT biosynthesis by taurocholate, isolated rat hepatocytes were analyzed for variations of $[Ca^{2+}]_i$. Basal levels of $[Ca^{2+}]_i$ were 174.6 $\pm$ 4.5 nmol/L (n = 60). The α-adrenergic agonist L-phenylephrine (1 μmol/L) was shown to induce, as expected, a significant increase in these levels (+456.1 $\pm$ 24.7 nmol/L; n = 16; $P < 0.001$). However, when taurocholate was continuously superfused (at concentrations of 25 and 50 μmol/L), only a minimal increase in $[Ca^{2+}]_i$
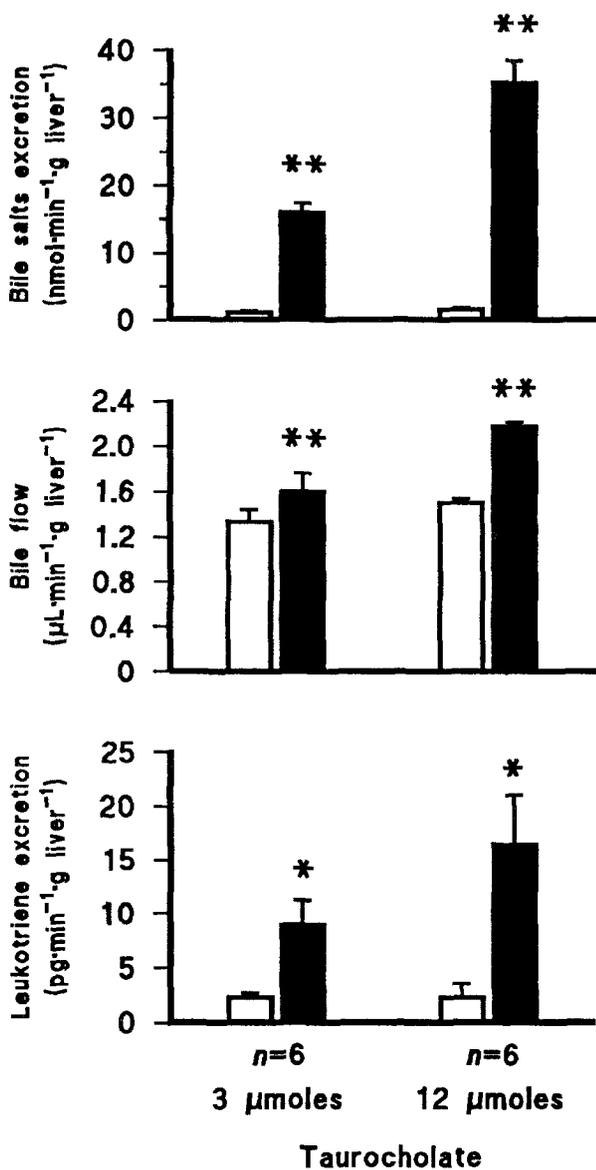
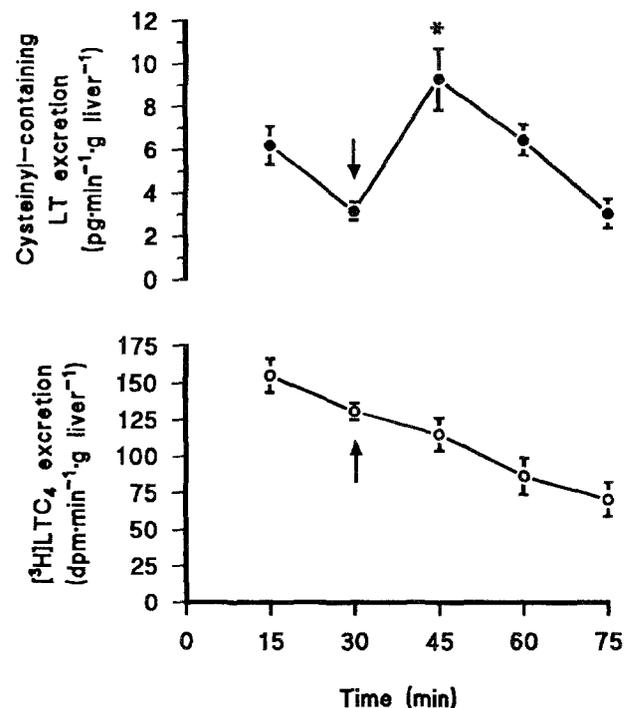Figure 2. Effect of bolus administration of taurocholate on bile salt excretion, bile flow, and cysteinyl-containing LT excretion. □, values in the basal period; ■, values after administration of either 3 or 12 μmol of taurocholate. The data represent the mean $\pm$ SEM (n = 6 each). *$P < 0.05$ and **$P < 0.001$ vs. the values of their respective basal period.

Figure 3. Analysis of the biliary excretion of cysteinyl-containing LTs from livers preloaded with [^{3}H]LTC$_4$ on bolus injections with 3 μmol of taurocholate. ●, values of biliary cysteinyl-containing LTs measured by high-performance liquid chromatography radioimmunoassay; ○, dpm values corresponding to the biliary excretion of the [^{3}H]LTC$_4$ previously added (0 minutes) to the liver. Arrows indicate the injections of taurocholate. The data represent the mean $\pm$ SEM (n = 4). *$P < 0.05$ vs. the basal value.

was observed ($+34.8 \pm 11.1$ and $+26.9 \pm 3.6$ nmol/ L, respectively; NS), which suggests that intracellular changes of calcium are not basically responsible for the stimulation of LT biosynthesis in the liver induced by taurocholate.

Discussion

Using the isolated and perfused rat liver model, we have studied the effects of LTC_4 on bile flow and bile salt excretion as well as the possible effect of taurocholate on the hepatic production of cysteinyl-containing LTs. A bolus with 0.25 fmol of LTC_4 (which presumably reached a concentration of 1 fmol/L in the perfusion fluid) induced a significant increase in both bile flow and biliary excretion of bile salts, although, at a much higher dose
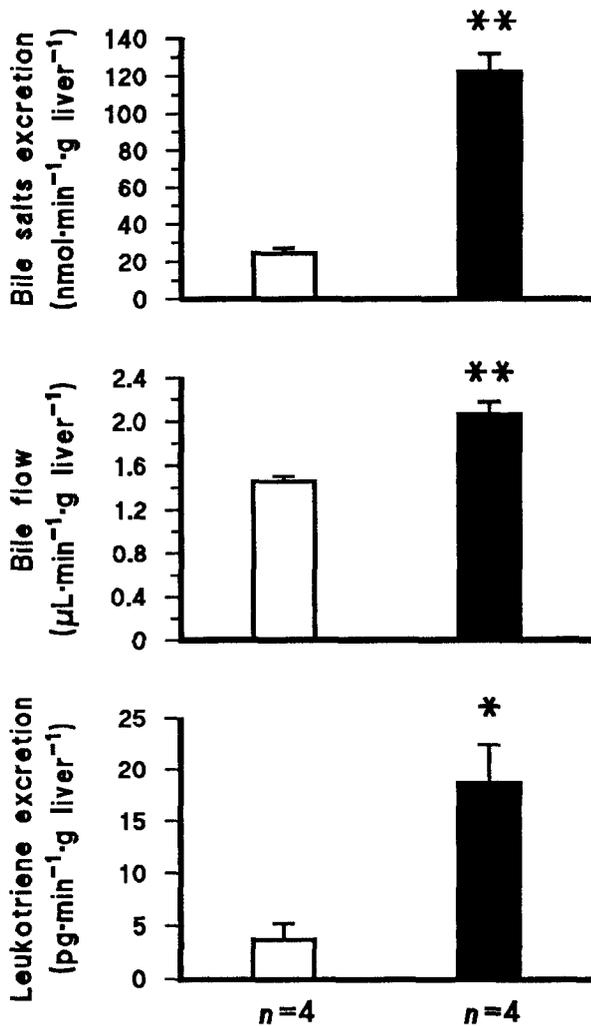
Figure 4. Effect of continuous infusion of taurocholate on bile salt excretion, bile flow, and cysteinyl-containing LT excretion. ■, values in livers infused with taurocholate at a rate of 120 μmol/h; □, values in livers with taurocholate at a rate of 15 μmol/h. The data represent the mean ± SEM of four separated stimulation experiments in each group. *$P < 0.05$ and **$P < 0.001$ between groups.

(0.25 × 10^6 fmol), LTC_4 was found to reduce these parameters (Figure 1). The apparent choleretic effect obtained with the low dose of LTC_4 would be in line with our previous data showing that 5-lipoxygenase inhibitors reduce the efflux of bile salts from isolated rat hepatocytes.[11] Other investigators have found that concentrations of LTC_4 within our range may exert some biological effects in several cell systems, such as stimulation of luteinizing hormone releasing hormone secretion from hypothalamic tissue,[27] increase of the contractile activity of the heart,[28] and stimulation of mitogenesis in human airway epithelial cells.[29] These effects occurred in a narrow range of LTC_4 concentrations ($0.1-10$ fmol/L), disappearing at higher concentrations.[27-29] Our present data in the perfused liver agree with this phenomenon. Thus, increasing the concentration of the injected LTC_4 turned a seemingly physiological choleretic effect into a cholestatic one, the latter also being in accordance with previous observations by Krell and Dietze[10] that LTC_4 may induce cholestasis. The pathogenetic role of high concentrations of cysteinyl-containing LTs in the liver has also been shown in a variety of experimental models, including fulminant hepatitis induced by D-galactosamine/endotoxin,[9,30,31] carbon tetrachloride-induced liver injury,[32] and frog virus 3–induced liver injury.[33] In our study,
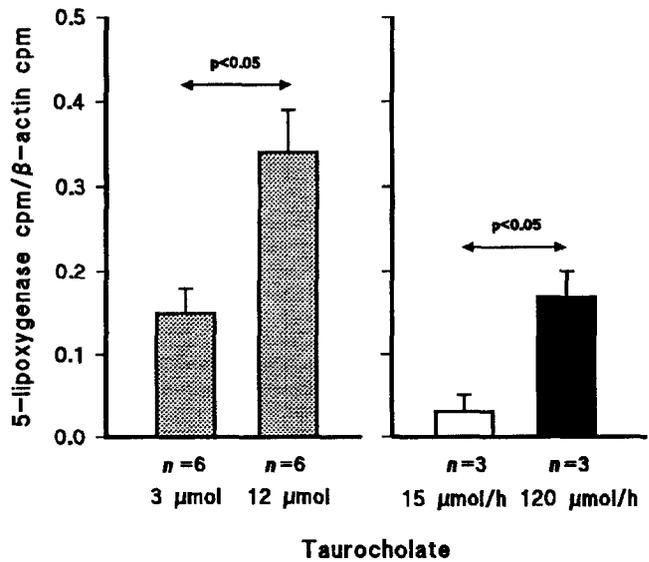
Figure 5. Effect of taurocholate on the levels of 5-lipoxygenase mRNA in liver tissue. Taurocholate was administered to isolated livers either as a bolus or in continuous infusion. Total RNA was extracted from liver samples, and 5-lipoxygenase mRNA was analyzed by RT-PCR (see Materials and Methods). Values of cpm corresponding to 5-lipoxygenase mRNA were normalized to those obtained for β-actin mRNA. The data are given as mean ± SEM. □, livers in which taurocholate was infused at a rate of 15 μmol/h; ▦, livers stimulated with bolus injection of taurocholate; ■, livers stimulated with a continuous infusion of taurocholate at a high rate (120 μmol/h).

all of the parameters of liver metabolism investigated were in the normal range throughout the experiments using the choleretic dose of LTC_4, whereas a higher dose of LTC_4 (0.25×10^6 fmol) was found to enhance both the lactate/pyruvate ratio (Figure 1) and the hepatic release of glucose. This increase in glucose release in response to LTC_4 has been already reported by others.[34,35]

The effects of LTC_4 on bile salt excretion seem specific because they were reversed by LT antagonists. FPL 55712 prevented both the choleretic and the cholestatic effect shown by low and high LTC_4 doses, respectively. Also, FPL 55712 was able to block the increased release of glucose to the effluent and the abnormally high lactate/pyruvate ratio induced by high doses of LTC_4 (Figure 1). Similar effects on bile flow and bile salt excretion were obtained when LT administrations were associated with REV 5901 (see Results).

Our data do not permit any conclusion about the mechanisms by which LTC_4, at a relatively high concentration, decreases the biliary excretion of bile salts. According to Iwai and Jungerman,[34] it is unlikely that the hemodynamic actions of LTC_4 are responsible for this effect in the isolated and perfused rat liver because there was no correlation with changes in either the portal pressure or the perfusion flow (see Results). It also seems unlikely that high concentrations of LTC_4 could interfere with the sinusoidal uptake of bile salts because in other reports it has been shown that concentrations of LTC_4 higher than 1 nmol/L scarcely could influence the uptake of taurocholate from perfusate in the isolated rat liver.[35] A competition between bile salts and LTC_4 for their excretion to the canaliculi is unlikely as well because the excretion of taurocholate is mediated by a 110-kilodalton glycoprotein,[36–38] whereas cysteinyl-containing LTs seem to use a different export carrier.[37,38] It is possible that the cholestatic effect of LTC_4 be mediated by interference with the hepatocytic transport of bile salts. In this context, it may be noted that some cytosolic glutathione transferases, which possess high-binding affinity for LTC_4, are involved in the intrahepatocytic transport of bile salts.[39] Thus, high amounts of exogenous LTC_4 reaching the liver could interfere with the binding of bile salts to GSH transferases, impairing their canalicular excretion. In addition, as suggested by the elevation in the lactate/pyruvate ratio, high concentrations of LTC_4 might reduce the availability of energy, which is essential for bile salt secretion.

In this study, we also found that taurocholate may influence the production of cysteinyl-containing LTs in the liver. Bolus injections of taurocholate were followed by a dose-dependent increase in the biliary excretion of both bile salts and cysteinyl-containing LTs (Figure 2). Continuous infusion of taurocholate had a similar effect on these parameters (Figure 4). Experiments in which livers were preloaded with labeled LTC_4 show that taurocholate stimulates the excretion of LTs by enhancing their hepatic biosynthesis rather than through a washout effect of preformed cysteinyl-containing LTs maintained in the liver (Figure 3). Our finding of a taurocholate-stimulated 5-lipoxygenase mRNA expression in the liver (Figure 5) suggests that bile salts may modulate the liver biosynthesis of LTs by influencing, either directly or indirectly, the 5-lipoxygenase gene expression in hepatocytes or, most probably, in nonhepatocyte cells (e.g., mast cells and Kupffer cells, see below).

It is known that the liver is a major organ for the disposal of cysteinyl-containing LTs of systemic origin,[40] but, according to our data, the liver also seems to be a source of cysteinyl-containing LT production. This production has been shown to take place in the liver only after immunopathologic stimuli.[41–43] Interestingly, our results indicate that also physiological stimuli, such as bile salts, may enhance the liver production of cysteinyl-containing LTs.

The mechanism by which taurocholate stimulates the production of LTs by the liver was not fully analyzed here. It has been shown that the synthesis of cysteinyl-containing LTs requires the membrane translocation of 5-lipoxygenase, and its specific binding to a membrane protein termed 5-lipoxygenase-activating protein, both activation steps being dependent on $[Ca^{2+}]_i$.[44,45] On the other hand, it has been reported that bile salts, mainly in the form of taurine conjugates, may act as calcium ionophores.[46] However, when we studied the effect of taurocholate on $[Ca^{2+}]_i$ in isolated rat hepatocytes, only a very small increase in its levels was observed, as previously shown by others.[23] Apparently, this increase is not enough to account for the membrane translocation of 5-lipoxygenase and synthesis of LTs.[47] Therefore, it is unlikely that intrahepatocytic calcium movements be involved in the coupling between taurocholate transport and cysteinyl-containing LT biosynthesis.

Regarding the cellular origin of LT formation, it has been reported that during anaphylaxis, the resident mast cells are the main intrahepatic cells initiating LT biosynthesis.[43] Similarly, the Kupffer cells are known to possess the complete enzymatic set for the synthesis of cysteinyl-containing LTs, being able to produce these compounds when properly stimulated.[48] Also, hepatocytes have been described to synthesize cysteinyl-containing LTs from LTA_4 produced by Kupffer cells,[49] as well as from 5-HPETE in the presence of glutathione.[50] Therefore, there

is room for speculations about intercellular cooperation between diverse cell types such as Kupffer cells and hepatocytes, leading to transcellular metabolism of LT precursors, as reported in several cell systems in vitro.[51–56]

In conclusion, our results show that LTC_4 at low dose (0.25 fmol) exerts a choleretic effect, increasing the biliary secretion of bile salts. LTC_4, however, induces cholestasis when administered at a relatively high dose $(0.25 \times 10^6 \text{ fmol})$ (it should be emphasized that the two LTC_4 concentrations differ in six orders of magnitude). This suggests a biphasic effect of the cysteinyl-containing LTs on the liver function, as already pointed out by Iwai and Jungermann in relation to the carbohydrate metabolism.[57] On the other hand, this study also shows that taurocholate stimulates the hepatic synthesis of cysteinyl-containing LTs. Accordingly, it is suggested the existence of a positive feedback mechanism by which bile salts would stimulate the synthesis of cysteinyl-containing LTs, and these compounds, in turn, would promote bile salt secretion. These observed interrelationships between bile salts and cysteinyl-containing LTs in the liver might be of physiological significance for the formation of bile.

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