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Nature of Tissue-bound Lithocholic Acid and Its Implications in the Role of Bile Acids in Carcinogenesis[1]

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Abstract

Lithocholic acid, a monohydroxy secondary bile acid, is present in tissues in two forms. One form is extractable with 95% ethanol-0.1% ammonia (soluble lithocholate), and the other form is firmly bound to tissue residues and can be released only by the bile salt-deconjugating enzyme, clostridial cholanoylamino acid hydrolase (tissue-bound lithocholate). Studies on bile salt-protein interactions revealed that lithocholic acid had amino group-modifying activity specifically directed against the basic side group of lysine residues. Degradative procedures yielded *N*-ε-lithocholyllysine, confirmed by comparison with the authentic compound synthesized in our laboratories. Studies on the distribution of tissue-bound lithocholate in tissues have revealed high concentrations of this form of lithocholate in livers of rats treated with the carcinogen, methylazoxymethanol. In light of these observations, the role of bile acids, and specifically lithocholic acid, as promoters of tumorigenesis must be further investigated.

Introduction

In 1933, Wieland and Dane (21) and Cook and Haslewood (1) reported that methylcholanthrene, a highly potent carcinogen, could be obtained chemically from either cholic acid or dehydronorcholene. This was confirmed by Fieser and Newman (4) in 1935, when they described the chemical synthesis of 20-methylcholanthrene using cholic acid as the intermediate. These early observations aroused the suspicion that bile acids, as possible precursors of carcinogenic aromatic hydrocarbons, could be involved in the etiology of carcinogenesis.

The tumorigenic potential of a bile acid was first demonstrated with deoxycholic acid by Ghiron (5) and confirmed by Cook *et al.* (2). Since then, apocholic acid and 3β-acetoxybisnor-Δ^5-cholenic acid have been shown to have similar biological properties (8, 9).

Although these early studies did not lead to the identification of any of the naturally occurring bile acids as potent carcinogens, there has been a strong resurgence in interest in this field. Recent epidemiological studies suggest that bile acids may act as colon-tumor promoters in high-risk populations (14, 16). It has been suggested that secondary bile acids and their metabolic products formed by the action of microbial flora in the colonic environment might act in concert with carcinogens in promoting neoplastic transformation. In conventional and germ-free rats, both lithocholic and deoxycholic acids promote the formation of colonic adenocarcinoma (14, 16), and the administration of lithocholic acid has been shown to enhance the ethionine-induced development of hyperplastic nodules and hepatomas in rat liver (6). More recently, *in vitro* studies have shown that lithocholic acid and its conjugates exhibit comutagenic activity in the *Salmonella*-mammalian microsome mutagenicity test (18) as well as promote transformation of hamster embryo cells in culture (7).

Normal livers have yielded significant amounts of lithocholic acid extractable with 95% ethanol containing ammonium hydroxide (15). However, while studying tissue bile acids in hepatic injury following the small bowel bypass procedure (17) and in various pathological specimens of human livers, we found unusually low levels of this bile acid.

Subsequent studies revealed the existence of lithocholic acid in tissue-bound form TBL[2] extractable only after subjecting the tissue residue to enzymatic hydrolysis with the bile salt-deconjugating enzyme, clostridial cholanoylamino acid hydrolase (10, 11). In this paper, we describe the nature of TBL and its possible significance in carcinogenesis.

Isolation and Quantitation of TBL

The procedure for the isolation of TBL from tissues and its separation from soluble lithocholate is outlined in Chart 1 (12). Since in TBL lithocholic acid appears to be conjugated to protein via ε-amino groups of lysine, it is probable that not all of the conjugated lithocholic acid would be sterically accessible to the action of the hydrolase. Pretreatment of the protein residue with a variety of proteolytic enzymes before the hydrolase step significantly improved the yield of lithocholate (19) (Table 1). In the modified procedure, the tissue precipitate is predigested with trypsin to enhance the action of clostridial hydrolase in the release of lithocholate from the bound form.

Identification of ε-Lithocholyllysine in Tissues

Since lithocholate is released from tissue-bound form only by the mediation of cholanoylamino acid hydrolase (peptide bond hydrolase), it was postulated that the bile acid is probably covalently bound to protein through a peptide bond. In model experiments using labeled and unlabeled lithocholic acid coupled to either bovine serum albumin or polylysine, the hydrolase was active in releasing the bile acid from the bound form. Further insight into the nature of the lithocholylamino acid residue was gained by the observation that lithocholic acid exhibited amino group-modifying activity specifically on the ε-amino groups of lysine in model compounds such as α-*t*-butoxycarbonyl-L-lysyl-β-naphthylamide. In contrast, lithocholic acid exhibited no such activity towards arginine in α-benzyloxycarbonyldiarginyl-β-naphthylamide (13). Since the peptide bond

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[2] The abbreviation used is: TBL, tissue-bound lithocholate.

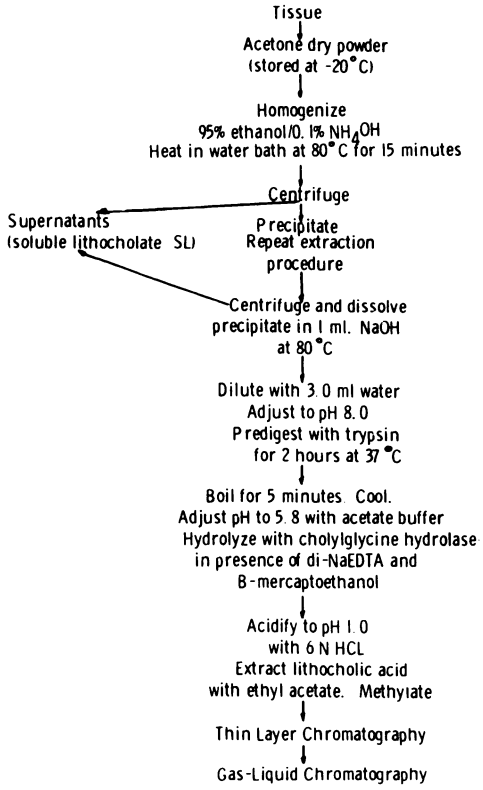

Chart 1. Analysis of TBL (19).

Table 1

Effect of proteolytic enzymes on release of lithocholate by clostridial hydrolase

Incubation mixture	Lithocholic acid released (μg/g dry wt)
Control (no proteolytic enzyme), pH 6.0	1.8
Trypsin (2 mg/ml), pH 6.0[a]	71
Protease (2 mg/ml), pH 6.0	36
Pepsin (2 mg/ml), pH 6.0	46
Peptidase (2 mg/ml), pH 6.0	24
Pronase (2 mg/ml), pH 6.0	27

[a] Adjustment of pH to 8.0 during tryptic digestion, followed by acidification to pH 5.8 treatment with hydrolase, further improved the yield by 2-fold.

in bile acid conjugates is resistant to acid hydrolysis, tissues containing TBL were subjected to 6 N HCl hydrolysis from which a lithocholylamino acid fragment was isolated by thin-layer chromatography. This ninhydrin-positive product was compared with *N*-ε-lithocholyl-L-lysine (I), *N*-α-lithocholyl-L-lysine (II), and *N*-α-ε-bislithocholyl-L-lysine synthesized in our laboratories (12) (Chart 2). Among the 3 synthetic compounds, only Compound I was ninhydrin positive. In addition, the mass spectrometric pattern of Compound I was conspicuous by the absence of the fragment (Chart 3) at *m/e* 170 (given by Compound II by McLafferty rearrangement) derived from the steroid lactam at *m/e* 486, a characteristic of *N*-α-amino-substituted bile salt conjugates. The native product was identical to Compound I and showed similar characteristics by infrared spectroscopy and thin-layer chromatography (12). Both the native product and *N*-ε-lithocholyllysine migrated together on thin-layer chromatography and yielded free lithocholic acid when treated with the clostridial hydrolase. The combined results of these studies showed *N*-ε-lithocholyllysine to be the predominant residue in TBL.

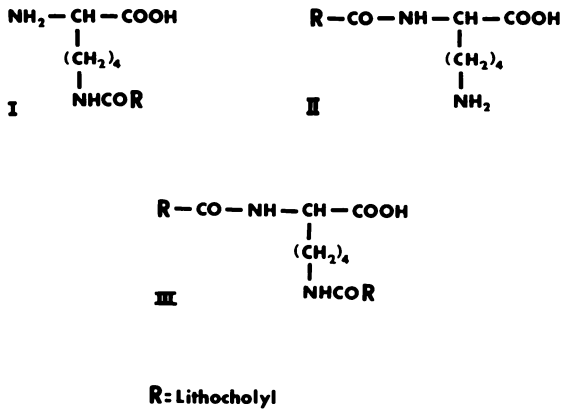

Chart 2. Structures of *N*-ε-lithocholyl-L-lysine (*I*), *N*-α-lithocholyl-L-lysine (*II*), and *N*-α-ε-bis-lithocholyl-L-lysine(*III*).

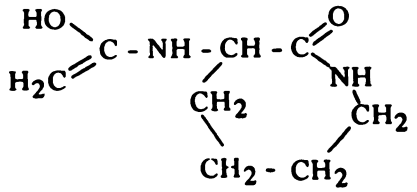

Chart 3. Mass spectrometric fragment of *N*-ε-lithocholyl-L-lysine.

Table 2

TBL in livers of methylazoxymethanol-treated rats

Methylazoxymethanol (35 mg/kg) was administered i.v. to Charles River rats (5 to 6 weeks old). The corresponding controls received an equal volume of 0.9% NaCl solution vehicle. The animals were sacrificed 11 months later.

Treatment	Lithocholic acid (μg/g dry wt)
0.9% NaCl solution	6.0 ± 4.7[a]
Methylazoxymethanol	199 ± 44

[a] Mean ± S.D.

TBL in Tumor Tissues

We have been studying TBL in a variety of normal and tumor tissues. Livers from male rats treated with methylazoxymethanol (35 mg/kg i.v. were administered to 5 to 6-week-old male Charles River rats sarcrificed 11 months later) had significantly higher concentrations of TBL as compared to the corresponding 0.9% NaCl solution-treated controls (20) (Table 2). Similarly, in a random sampling of 12 colonic polyps from subjects with familial polyposis coli, we observed a very high concentration (180 μg/g net weight) of TBL in one case, the only one which showed carcinoma *in situ* histologically.[3]

Possible Significance of TBL in Carcinogenesis

At this time, there is no direct evidence to postulate a role for TBL in the neoplastic process. However, it is interesting to note that, in TBL, lithocholic acid appears to have a specificity for lysine residues and that this is the only bile acid we have thus far detected in the tissue-bound form. Most carcinogens, some of which are metabolized to electrophilic intermediates, covalently bind to cellular macromolecules (3). Although lithocholic acid and other monohydroxylated bile acids as a class show unique cellular toxicity not seen with other dihydroxy or

[3] A. Krush, G. Pavlides, and P. P. Nair, unpublished observations.

trihydroxy bile acids, mechanistically we are unable to propose a mode of formation of TBL.

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