Identification of bile acid-CoA:amino acid *N*-acyltransferase in rat kidney

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A novel location of the bile-acid-conjugating enzyme bile acid-CoA:amino acid *N*-acyltransferase (BAT) has been discovered in the cytosolic fraction of rat kidney. Both taurine and glycine were utilized as substrates. Formation of bile acid *N*-acyl amidates was verified by h.p.l.c. by comparison with authentic standards and by specific hydrolysis using cholyglycine hydrolase. Immunoblot analysis using a human liver anti-BAT polyclonal antibody indicated that rat kidney BAT has the same molecular mass as rat liver BAT. These findings suggest that the kidney has a role in bile acid metabolism and physiology.

INTRODUCTION

Bile acids (BA) are formed in the liver from the metabolism of cholesterol and are conjugated with either taurine or glycine before secretion into bile (Hofmann, 1989). Conjugation of BA to form BA *N*-acyl amidates facilitates their secretion into bile; it is also an important determinant of bile flow (Schertén *et al.*, 1971), and it enables BA to act as detergents in the intestine.

Formation of BA *N*-acyl amidates occurs in two steps. Initially, BA form thioesters with CoA in a reaction catalysed by choly-CoA synthetase (cholate-CoA ligase; EC 6.2.1.7). The second step involves conjugation of bile acid-CoA thioesters with either taurine or glycine, and is catalysed by bile acid-CoA:amino acid *N*-acyltransferase (BAT; EC 2.3.1.65) (Schertén, 1967).

A specific polyclonal antibody against purified human liver BAT has been raised in rabbits (Johnson *et al.*, 1991). Immunoblot analysis using this specific human anti-BAT antibody revealed that a single protein band (molecular mass 40 kDa) was detected in rat, but not dog or pig, liver cytosol (Kwakye *et al.*, 1991). This protein has subsequently been confirmed as BAT by both immunohistochemical and immunoabsorption studies (Kwakye *et al.*, 1991; Romeo *et al.*, 1991). In preliminary immunohistochemical experiments using the human liver anti-BAT antibody, BAT has been tentatively identified in rat kidney.

The purpose of the present study was to confirm that BAT enzyme activity was present in rat kidney and to characterize partially its properties.

MATERIALS AND METHODS

Materials

[2-³H]Taurine (20.9 Ci/mmol), and [2-³H]glycine (20.0 Ci/mmol) were purchased from New England Nuclear Corp., Boston, MA, U.S.A. Radiolabelled taurine gave a single radioactive peak on reversed-phase h.p.l.c. Radiolabelled glycine was purified by reversed-phase h.p.l.c. (Sweeny *et al.*, 1987) before use. Cholic acid, unlabelled amino acids and bile acids were purchased from Calbiochem, San Diego, CA, U.S.A. SDS, CoA and cholyglycine hydrolase (EC 3.5.1.24) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Acrylamide and nitrocellulose were purchased from Bio-Rad Laboratories, Rich-

mond, CA, U.S.A. Biotin-labelled goat anti-rabbit antibody, avidin and biotinylated alkaline phosphatase were obtained from Pierce, Rockford, IL, U.S.A. Choly-CoA was synthesized by the method of Shah & Staple (1968) and purified as previously described (Johnson *et al.*, 1989). All other solvents and reagents were purchased in the highest grade possible.

Preparation of cytosolic fractions

Male Sprague–Dawley rats were killed by cervical dislocation and the kidneys were removed, washed in cold physiological saline and homogenized in 3 vol. of 100 mM-potassium phosphate buffer, pH 7.2. The homogenate was centrifuged at 100 000 *g* in a Beckman Type 50.2 Ti rotor (*r*_{av}, 8.12 cm) for 60 min. The supernatant was filtered through gauze and used for all subsequent experiments. These procedures were performed at 4 °C.

Assay of BAT activity

BAT activity was determined by a radioassay that specifically measures the formation of BA *N*-acyl amidates (Johnson *et al.*, 1989). The reaction mixtures contained 0.05 µCi of [³H]taurine or [³H]glycine, 100 mM-potassium phosphate, 1.5 mM-choly-CoA and 100 µg of kidney cytosol in a volume of 75 µl at a final pH of 8.2, and were incubated at 37 °C. The reaction was terminated at 30 min and the radioactive product was extracted into *n*-butanol. Assay blanks were performed in the presence of heat-denatured kidney cytosol protein.

Identification of bile acid conjugates

Confirmation of the radioactivity in the *n*-butanol phase as BA *N*-acyl amidates utilized reversed-phase h.p.l.c. The *n*-butanol extract was dried under nitrogen and reconstituted in 100 µl of mobile phase [64 % (v/v) methanol/2 mM-potassium phosphate, pH 4.0]. Authentic unlabelled cholytaurine or cholyglycine was added to the sample, which was then injected on a Beckman 110B h.p.l.c. apparatus fitted with a 250 mm × 4.6 mm reversed-phase C₁₈ column (Jones Chromatography, Littleton, CO, U.S.A.) and eluted at flow rate of 1 ml/min. Elution of the authentic unlabelled cholytaurine or cholyglycine was monitored at 210 nm. Fractions (1 ml) were collected, and radioactivity in each fraction was measured in a Beckman LS 5801 scintillation counter after the addition of 5.4 ml of scintillation

Abbreviations used: BA, bile acid(s); BAT, bile acid-CoA:amino acid *N*-acyltransferase.

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cocktail (Research Products International Corp., Mount Prospect, IL, U.S.A.). The radioactive peak which was co-eluted with the authentic unlabelled BA *N*-acyl amidate was pooled and dried under N_2 . The sample was reconstituted in 100 μ l of 100 mM-sodium acetate buffer, pH 5.6, and incubated for 30 min at 37 °C in the presence or absence of cholyglycine hydrolase. The reaction mixture was then analysed by reversed-phase h.p.l.c. under the same conditions as those described above.

Kinetic and competitive-inhibition studies

Initial rates of reaction were determined for various concentrations of taurine (0.5, 1, 2, 4, 8 and 16 mM) at fixed concentrations of glycine (0, 40 and 80 mM), using saturating concentrations of choly-CoA (1.5 mM). The incubation time and protein concentration used were such that no more than 10% of the limiting substrate was consumed in the reaction. The kinetic parameters were calculated from these data using non-linear regression analysis, as described previously (Johnson *et al.*, 1990).

Immunoblot studies

Western-blot and immunoabsorption methods were carried out as previously described (Kwakye *et al.*, 1991). The intensity of staining was enhanced severalfold by using the avidin-biotin-alkaline phosphatase-complex staining procedure (Leary *et al.*, 1983). Protein concentration was determined by the Lowry method (Lowry *et al.*, 1951), with BSA as standard.

RESULTS AND DISCUSSION

The presence of BAT was demonstrated for the first time in the kidney by using methods which demonstrated both its enzyme activity and its immunological response to a specific BAT polyclonal antibody raised in rabbits against purified human liver BAT.

BAT enzyme activity in rat kidney cytosol was verified using a specific radioassay that measures the production of BA *N*-acyl amidates (Johnson *et al.*, 1989). Confirmation that the radioactivity in the butanol phase was BA *N*-acyl amidate was carried out by reversed-phase h.p.l.c. In each case, the major peak of radioactivity was co-eluted with authentic unlabelled cholytaurine (Fig. 1a) or cholyglycine (Fig. 2a). The h.p.l.c.-purified products were subjected to hydrolysis with cholyglycine hydrolase, an enzyme that specifically hydrolyses BA *N*-acyl amidates (Nair *et al.*, 1967). H.p.l.c. analysis of the reaction mixtures showed complete hydrolysis of the putative BA *N*-acyl amidates (Figs. 1b and 2b). There was no hydrolysis when incubation was done in the absence of cholyglycine hydrolase.

The rate of formation of cholytaurine was linearly related to the added kidney cytosol protein concentration over the range 50–250 μ g (Fig. 3a). The reaction rate was also linearly related to incubation time for the first 150 min (Fig. 3b, ●); reactions with heat-denatured kidney cytosol protein did not result in product formation (Fig. 3b, ○).

The optimum pH for rat kidney BAT activity was between 8.2 and 8.4 (Fig. 4a), compared with 7.8–8.0 for rat liver BAT (Killenberg & Jordan, 1978). Kidney BAT activity was largely independent of incubation temperature in the range 37–50 °C; however, temperatures above 50 °C rapidly caused the activity to decline (Fig. 4b). The pattern of BAT activity from rat liver with temperature is similar, with a decline of activity above 48 °C (Killenberg & Jordan, 1978). When assayed under optimized reaction conditions, BAT activity in the kidney was 4.2 nmol/min per mg, which is 8% of the BAT activity in rat liver.

Increasing the concentration of taurine in the presence of saturating concentrations of choly-CoA led to increased BAT

activity, which exhibited saturation above 16 mM (Fig. 5a). The initial reaction rates of rat kidney BAT were fitted to the Michaelis-Menten equation by non-linear regression analysis, yielding an apparent K_m of 5.2 ± 0.9 mM, which is similar to our previously reported value of 2.7 mM for partially purified BAT from rat liver (Kwakye *et al.*, 1991).

Like the enzyme from rat liver, BAT from rat kidney has a

Fig. 1. H.p.l.c. profile of the rat kidney cytosol-catalysed radioactive product and an authentic cholytaurine standard before (a) and after (b) incubation of the radioactive product with cholyglycine hydrolase

The reaction was carried out as described in the Materials and methods section.

Fig. 2. H.p.l.c. profile of the rat kidney cytosol-catalysed radioactive product and an authentic cholyglycine standard before (a) and after (b) incubation of the radioactive product with cholyglycine hydrolase

The reaction was carried out as described in the Materials and methods section.

Fig. 3. Linearity of the rate of radioactive product formation with protein concentration (a) and incubation time (b)

A 100 μg portion of rat kidney cytosol (●) or heat-denatured protein (○) was incubated with 0.05 μCi of [³H]taurine and 1.3 mM-cholyl-CoA in 100 mM-potassium phosphate for 30 min at 37 °C.

Fig. 4. Dependence of radioactive product formation on pH (a) and temperature (b)

The reaction mixture contained 100 mM-potassium phosphate, 0.05 μCi of [³H]taurine, 100 μg of rat kidney cytosol and 1.3 mM-cholyl-CoA.

very low affinity for glycine; kinetic experiments with glycine were particularly difficult to perform, owing to low levels of product formed. However, by adding increasing fixed amounts of unlabelled glycine (0, 40 and 80 mM), it was possible to demonstrate that glycine decreased the formation of [³H]cholyl-*N*-taurine at all concentrations of taurine studied (0.5, 1, 2, 4, 8 and 16 mM). Double-reciprocal Lineweaver-Burk plots of the rates of reaction and taurine concentration intersect on the abscissa (Fig. 5b), indicating that glycine competitively inhibited BAT activity with [³H]taurine as substrate. A replot of apparent K_m versus inhibitor concentration (Segel, 1975) gave a K_i value of 155 ± 20 mM for glycine.

Immunoblot analysis of 100 000 *g* rat kidney and liver cytosol proteins utilizing a human liver anti-BAT polyclonal antibody

Fig. 5. Effect of increasing concentrations of taurine (a) and unlabelled glycine (b) on the rate of radioactive product formation

The taurine concentration was varied between 0.5 and 16 mM in both experiments. Inhibition experiments were done with fixed concentrations of glycine [0 (●), 40 (○) and 80 mM (▲)].

Fig. 6. Western-blot analysis of 100 000 *g* rat liver and kidney cytosol using affinity-purified human liver anti-BAT polyclonal antibody

Lanes 1 and 2 contained 700 μg of rat kidney cytosol respectively before (1) and after (2) passage through a human liver anti-BAT antibody-Sepharose affinity column. Lanes 3 and 4 contain 400 μg of rat liver cytosol each, before (3) and after (4) passage through a human liver anti-BAT antibody-Sepharose affinity column.

identified a 40 kDa protein (Fig. 6, lanes 1 and 3). Evidence that this protein is BAT was shown by the complete disappearance of this band (lanes 2 and 4), as well as the removal of all of BAT enzyme activity, from the eluant after passage of 100 000 *g* rat kidney and liver cytosol over a human liver anti-BAT polyclonal antibody affinity column.

The presence of an enzyme catalysing the formation of BA *N*-acyl amides in the kidney suggests that our knowledge of the metabolism and physiology of BA is incomplete. Most BA filtered by the glomerulus are efficiently reabsorbed in the proximal tubules (Weiner *et al.*, 1964; Barnes *et al.*, 1977). BAT may conjugate BA absorbed into the renal tubular cells to prevent their diffusion back into the tubular lumen. In hepatobiliary disease, BAT may have a role in protecting the kidney from the toxic effects of elevated serum levels of unconjugated BA. It is also possible that, at an earlier stage in evolution, the kidney had a role in the metabolism of BA that entered the systemic circulation, a role that became less important as a more efficient enterophepatic circulation of BA evolved.

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