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Effects of bile salts on brush-border and cytosolic proteolytic activities of intestinal enterocytes

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Abstract

The effects of bile salts, i.e., sodium deoxycholate, sodium glycocholate, sodium glycodeoxycholate, sodium taurocholate, and sodium taurodeoxycholate, on the activities of individual rat intestinal brush-border membrane peptidases and cytosolic insulin-degrading activities were examined. At 2 mM, bile salts had no effects on aminopeptidases P and W, but did inhibit other brush-border peptidases though no single bile salt was able to inhibit every peptidase. Even at concentrations higher than the CMC, inhibition of brush-border insulin degradation is incomplete while that of cytosolic insulin degradation is complete at low concentrations. The results suggest that bile salts, inhibiting brush-border membrane and cytosolic proteolytic hydrolysis, may be useful for reducing intestinal degradation of peptide drugs; and that bile salts have stronger inhibitory effects on soluble than on membrane-bound insulin-degrading activity.

Key words: Bile salt; Brush-border membrane; Insulin degradation; Proteolysis

1. Introduction

Bile salts not only enhance absorption of hydrophilic small molecules and macromolecules across various epithelial tissues, but also inhibit proteolytic enzymes (Chowdhary et al., 1985; Kidron et al., 1987; Hoogdalem et al., 1989; Yamamoto et al., 1990). Deoxycholate (2.4 mM) and glycocholate (20 mM) were shown to inhibit insulin degradation by hepatocyte particulates and intestinal homogenates, respectively (Chowdhary et al., 1985; Yamamoto et al., 1990). Deoxycholate had stronger inhibitory effects than other enzyme inhibitors, such as aprotinin and *p*-chloromercuriphenylsulfonic acid (Yamamoto et al., 1990). Above the CMC, lipophilic enzyme substrates can be trapped inside the micelles, resulting in reduced enzymatic hydrolysis. However, the effects of bile salts on proteolytic activities at concentrations lower than the CMC are not well understood. It is unknown whether their effects on membrane-bound and soluble peptidases are similar. Studies of the effects of bile salts at low concentrations on enterocyte proteolytic activities will provide additional information for their use in promoting absorption of peptide and protein drugs. The present study examines the effects of sodium deoxycholate, sodium glycocholate, sodium glycodeoxycholate, sodium taurocholate, and sodium taurodeoxycholate on the activities of

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individual brush-border membrane peptidases, and on brush-border membrane and cytosolic insulin-degrading activities.

2. Material and methods

Benzyloxycarbonyl-Pro-Ala, benzyloxycarbonyl-Pro, Ala, Gly-Pro-*p*-nitroanilide, Glu-Trp, insulin B-chain, [D-Ala^2, Leu^5]-enkephalinamide, *p*-nitrophenyl phosphate, *p*-nitroaniline, 1,10-phenanthroline, phosphoramidon, $MnCl_2$, Tris, Tyr-D-Ala-Gly, and pentobarbital, sodium glycocholate, sodium taurodeoxycholate, sodium taurocholate, sodium deoxycholate and sodium glycodeoxycholate were obtained from Sigma Chemical Co. (St. Louis, MO). Arg-Pro-Pro was from Bachem Bioscience Inc. (Philadelphia). Cilastatin was a gift from Dr Helmut Krop (Merck Sharp & Dohme Research Laboratories). Bovine γ-globulin and dye reagent for the protein assay were obtained from Bio-Rad Lab. (Richmond, CA). All other chemical reagents and buffer components were of analytic grade. Acetonitrile was of HPLC grade. All the chemicals were used as obtained.

2.1. Preparation of brush-border membranes and cytosol

Young male Sprague-Dawley rats, 300 g, were used as the animal model. The first 8 cm of rat small intestine was treated as the duodenum, the next 35 cm as the jejunum, the last 25 cm proximal to the ileocecal junction as the ileum, and the segment between the jejunum and the ileum as the jejunoileal junction. Brush-border membranes were prepared from the jejunoileal junction and ileum as published previously (Kessler et al., 1978; Bai, 1993). Briefly, the intestinal mucosa of each segment was scraped off, suspended in a hypotonic solution (50 mM mannitol in the 2 mM pH 7.5 Hepes/Tris buffer), and homogenized using a blender and a glass/Teflon potter homogenizer. $CaCl_2$ (1 M) was added to the homogenates to achieve a final concentration of 10 mM and the mixture was centrifuged at $3000 \times g$ for 15 min. The supernatant was collected and centrifuged at $27\,000 \times g$ for 30 min to obtain pellets. Pellets were resuspended in a 150 mM NaCl, 10 mM Tris/HCl (pH 6.8) buffer and homogenized with a glass/Teflon potter homogenizer. Then centrifugations at $3000 \times g$ and $27\,000 \times g$ were repeated to obtain pure brush-border membranes. Protein concentrations were determined using the Bradford method and γ-globulin as the protein standard (Bai, 1993). Activity of alkaline phosphatase was determined to assess the purity of brush border membranes. On average, brush-border membranes were 20 (± 0.7) times enriched. In a separate preparation, intestinal homogenate was subjected to centrifugation at $100\,000 \times g$ at 4°C for 1 h to obtain cytosol (Bai et al., 1992).

2.2. Effects of bile salts

In studies of the effects of bile salts, the incubation mixture (300 μl) consisted of 10 mM pH 6.8 Tris/HCl buffer, NaCl (150 mM), a substrate, a bile salt (2 mM) and brush-border proteins (0.03–0.006 mg) or homogenate proteins (0.1–2 mg). Bile salts were sodium deoxycholate, sodium glycocholate, sodium glycodeoxycholate, sodium taurocholate, and sodium taurodeoxycholate. Activities of dipeptidyl peptidase IV (DPP IV) and endopeptidase-24.11 were studied using Gly-Pro-*p*-nitroanilide and [D-Ala^2,Leu^5]-enkephalinamide, respectively. Benzyloxycarbonyl-Pro-Ala was used as the substrate for carboxypeptidase P, Glu-Trp for aminopeptidase W, Arg-Pro-Pro for aminopeptidase P, and insulin B-chain for endopeptidase-2 (Auricchio et al., 1978; Jackson et al., 1988; Lasch et al., 1988; Barnes et al., 1989). The incubation mixture for the aminopeptidase W assay also contained 0.05 mM cilastatin and 5 mM 1,10-phenanthroline, which were added to eliminate any contribution to the aminopeptidase W activity from aminopeptidase N, aminopeptidase A, and microsomal dipeptidase. For assaying endopeptidase-2 activity, phosphoramidon was added to the reaction mixture in order to eliminate endopeptidase-24.11 activity (Barnes et al., 1989). $MnCl_2$ (3 mM) was included in the reaction mixture for aminopeptidase P assay in order to maximize its activity (Lasch et al., 1988). The

final substrate concentrations in the incubation mixture were 0.5 mM for Glu-Trp, 0.45 mM for Gly-Pro-*p*-nitroanilide, 0.018 mM for [D-Ala^2, Leu^5]enkephalinamide, 0.2 mM for Arg-Pro-Pro, 0.02 mM for the insulin B-chain, and 0.027 mM for benzyloxycarbonyl-Pro-Ala. Activities of all the enzymes were studied at 37°C except those of DPP IV and endopeptidase-24.11 were determined at room temperature because substrates of the latter two enzymes were hydrolyzed too rapidly at 37°C. Aliquots were taken periodically and 0.1 N HCl was used to stop enzymatic degradation.

Insulin degradation was studied using the trichloroacetic acid method (Duckworth et al., 1972). The incubation mixture consisted of 50 mM Tris/HCl buffer (pH 7.5 at 37°C), 1% BSA (w/v), 125 mM NaCl, 30 pM ^{125}I-(A14)-insulin, a bile salt (2 mM) and cytosolic proteins (100–600 μg/ml). Periodically, 200 μl aliquots were taken, and 200 μl of 10% trichloroacetic acid was added to stop the reaction. The mixture was then subjected to $3000 \times g$ centrifugation. The supernatant containing insulin fragments was then counted using a gamma counter.

2.3. Analysis

HPLC systems consisted of an SIL autoinjector, an LC-600 pump, an SPD-6A UV spectrophotometric detector, and a CR 601 recorder (Shimadzu Corporation, Kyoto, Japan), a C_8 (Altex Ultrasphere-ODS, 5 μm, 4.6 mm × 15 cm) (Beckman Instrument, Berkley, CA) or a C_{18} (Vydac 5 μm, 4.6 mm × 25 cm) (Vydac, Hesperia, CA) reversed-phase column, or a strong cation-exchange column (Partisil 10 SCX, 25 cm, Whatman, Maidstone, U.K.). HPLC methods for Gly-Pro-*p*-nitroanilide and [D-Ala^2]Leu-enkephalinamide were as published previously (Bai, 1993). Using the cation-exchange column, Arg-Pro-Pro eluted at 8 min using a mobile phase of pH 2.5 0.05 M $(NH_4)_2H_2PO_4$: acetonitrile 90 : 10 and Glu-Trp at 10 min using a mobile phase of pH 2.5 0.005 M $(NH_4)_2H_2PO_4$: acetonitrile 90 : 10. Insulin B-chain eluted at 6 min from the C_{18} column using 0.05% TFA : acetonitrile 65 : 35 while *N*-CBZ-Pro-Ala eluted at 8 min from the C_8 column using 0.05% TFA : acetonitrile 75 : 25. ^{125}I-(A14)-human recombinant insulin was assayed using a gamma counter.

2.4. Data analysis

Initial hydrolysis rates of each substrate were obtained from the first 10–20% proteolysis. Student's *t*-test was used for statistical analysis.

3. Results and discussion

Sodium deoxycholate and sodium glycodeoxycholate at 2 mM significantly inhibited endopeptidase-24.11 while the others did not show any effect (Table 1). The former achieved 17% inhibition and the latter 29%. Activity of endopeptidase-2 was significantly inhibited by sodium glycocholate (20%), sodium taurocholate (47%), and sodium taurodeoxycholate (41%). None of the bile salts inhibited the activities of aminopeptidase W and aminopeptidase P (Table 1). DPP IV was significantly inhibited by sodium taurocholate (32%) and sodium taurodeoxycholate (19%). Carboxypeptidase P was significantly inhibited by sodium deoxycholate (50%) and sodium taurodeoxycholate (57%). Although endopeptidase-24.11 and endopeptidase-2 have similar substrate specificities, those bile salts to which endopeptidase-24.11 was sensitive did not inhibit endopeptidase-2, or vice versa. This seems to suggest that inhibition of these two enzymes by bile salts is not through competition for the substrate binding sites but takes place via other mechanism(s). Sodium glycocholate and sodium glycodeoxycholate had no effect on any exopeptidases. In summary, effects of bile salts on brush-border peptidases showed the order of taurodeoxycholate > taurocholate and deoxycholate > glycocholate and glycodeoxycholate.

The CMCs of deoxycholic, glycocholic, glycodeoxycholic, and taurocholic acids in water are 10, 12, 6, and 10 mM, respectively; those in 150 mM NaCl are 3, 10, 2, and 6 mM, respectively (Roda et al., 1990). Although the CMC of taurodeoxycholic acid is unknown, it is likely that its CMC is slightly lower than that of taurocholic

Table 1
Effects of bile salts on intestinal brush-border peptidase activities

	Control	NadC	NaGC	NaGdC	NaTC	NaTdC
Aminopeptidase W	37.75 (2.33)	47.10 (3.82)	35.65 (0.75)	36.29 (1.59)	37.05 (0.81)	48.20 (1.40)
Dipeptidylpeptidase IV	107.86 (9.66)	92.09 (12.51)	95.71 (2.06)	96.07 (2.04)	73.74 (9.67) ($p < 0.025$)	87.86 (6.86) ($p < 0.05$)
Carboxypeptidas P	0.14 (0.03)	0.07 (0.01) ($p < 0.05$)	0.10 (0.01)	0.21 (0.02)	0.14 (0.02)	0.06 (0.01) ($p < 0.025$)
Aminopeptidase P	72.98 (15.19)	88.05 (7.21)	85.10 (11.44)	97.72 (6.31)	97.10 (10.59)	76.40 (4.69)
Endopeptidase-24.11	0.59 (0.02)	0.49 (0.05) ($p < 0.05$)	0.98 (0.08)	0.42 (0.02) ($p < 0.005$)	0.56 (0.07)	0.57 (0.04)
Endopeptidase-2	6.16 (0.21)	5.91 (0.32)	4.91 (0.27) ($p < 0.025$)	5.90 (0.27)	3.26 (0.33) ($p < 0.005$)	3.61 (0.14) ($p < 0.005$)

[a] NadC, NaGC, NaGdC, NaTC, and NaTdC: sodium deoxycholate, sodium glycocholate, sodium glycodeoxycholate, sodium taurocholate, and sodium taurodeoxycholate, respectively.
[b] The concentration of each bile salt is 2 mM.
[c] The data represent the average of hydrolysis rate (μmol/min per g protein) of three experiments and are expressed as mean ± S.E.

acid, since both deoxycholic and glycodeoxycholic acids have slightly lower CMCs than cholic acid and glycocholic acid, respectively (Rado et al., 1990). The CMCs of cholic acid in water and in 150 mM NaCl are 13 and 11 mM, respectively. It is expected that the CMCs of sodium salts of individual bile acids in the buffer containing 10 mM pH 6.8 Tris/HCl and NaCl (150 mM) used for this study would be different from those of bile acids in 150 mM NaCl. The influence of the Tris molecules, having positive charge at pH 6.8, on the CMCs of bile salts may be through two possible mechanisms: (1) interacting with individual anionic bile salt molecules via electrostatic attraction to form ion pairs; and (2) interacting with the micelle via electrostatic attraction to reduce charge repulsion within the micelle at higher ionic strength. The effect of 10 mM Tris buffer on reducing the chage repulsion within the micelle is likely significantly much lower than that of 150 mM Na^+, since the concentration of Tris buffer was much lower than that of Na^+ and the Tris molecule may not fit as well as Na^+ in the electrical double layer due to its larger size. It is likely that the interaction between Tris and bile salt molecules dominated under the experimental conditions used, and that, at 2 mM, the bile salts tested will not form micelles. The mechanism of the inhibitory effects of bile salts on proteolytic activities at concentrations lower than the CMCs is unknown. Presumably, bile salts, being anions at pH 7.5, can interact with proteins through electrostatic and van der Waals' interactions. Consequently, the three-dimensional structures of membrane-bound proteins may be influenced, and thus their proteolytic activities may be affected. Moreover, bile salts can also perturb membrane lipids and impose some effects on the

Table 2
Effects of bile salts on insulin-degrading activity in supernatant of intestinal mucosal homogenates

	Control	NadC	NaGC	NaGdC	NaTC	NaTdC
1 mM bile salt	2.27 (0.06)	1.27 (0.24) ($p < 0.02$)	1.72 (0.05) ($p < 0.002$)	1.24 (0.11) ($p < 0.002$)	1.55 (0.10) ($p < 0.002$)	1.21 (0.14) ($p < 0.002$)
10 mM bile salt	2.27 (0.06)	0	0	0	0	0

[a] NadC, NaGC, NaGdC, NaTC, and NaTdC: sodium deoxycholate, sodium glucocholate, sodium glycodeoxycholate, sodium taurocholate, and sodium taurodeoxycholate, respectively.
[b] The data represent the average of hydrolysis rates (μmol/min per g protein) of thee experiments and are expressed as mean ± S.E.

Table 3
Effects of bile salts on insulin degradation by ileal brush-border membranes

	Control	1 mM NaGdC	10 mM NaGdC	44 mM NaGdC
Insulin-degrading activity	1.18 (0.12)	0.94 (0.20)	0.38 (0.01) ($p < 0.005$)	0.53 (0.16) ($p < 0.025$)

[a] NaGdC: sodium glycodeoxycholate.
[b] The data represent the average hydrolysis rates (pmol/min per g protein) of three experiments.

stability of membrane proteins (Vallet-Strouve et al., 1985).

Insulin aggregates at concentrations higher than 0.1 μM, and at 30 pM it exists as monomers in solution (Derewenda et al., 1990). Therefore, the observed results are due to the intrinsic inhibitory effects of bile salts. At 1 mM, all the bile salts significantly inhibited cytosolic insulin-degrading activity (Table 2). Inhibition was 44% by sodium deoxycholate, 24% by sodium glycocholate, 45% by sodium glycodeoxycholate, 32% by sodium taurocholate, and 47% by sodium taurodeoxycholate. At 1 mM, the sodium salts of glycodeoxycholate, deoxycholate, and taurodeoxycholate had similar inhibition (45%) on cytosolic insulin degradation; the other two bile salts had weaker effects. It is unknown why the deoxylated bile salts had similar inhibition. At 10 mM, all the bile salts completely abolished cytosolic insulin degradation. The effects of sodium glycodeoxycholate on insulin degradation increased with its concentration (Tables 3 and 4). At 10 mM and above, it also significantly inhibited insulin degradation by ileal brush-border membranes. This inhibition was 68% at 10 mM and 55% at 44 mM (Table 3). At 0.01 and 0.1 mM, sodium glycodeoxycholate had no effects on cytosolic insulin degradation; however, it achieved 100% inhibition above 3 mM (Table 4). At 44 mM, it only achieved 50% inhibition on brush-border insulin degradation, indicating that insulin-degrading activity is more resistant to the inhibitory effects of this bile salt. The mechanisms of the effects of bile salts on cytosolic proteolytic activities are likely similar to those on membrane peptidases. Stronger inhibition of cytosolic insulin-degrading activities at low bile salt concentrations may be explained by higher availability of surface area of peptidases for interaction with bile salts. Cytosolic enzymes have the whole surface available for interaction with bile salts while membrane proteins only have a small portion exposed at the membrane surface to be influenced by bile salts. The higher the concentrations of bile salts, the stronger will be the interaction and inhibition.

Table 4
Inhibition of NaGdC on cytosolic insulin-degrading activity of intestinal enterocytes

Control	0.01 mM	0.1 mM	1 mM	3 mM	5 mM
	0%	0%	45%	100%	100%

Data represent the average of three experiments. NaGdC: sodium glycodeoxycholate.

The results suggest that bile salts can inhibit brush-border membrane and cytosolic peptidases at low concentrations and that their inhibitory effects are stronger on cytosolic peptidases.

References

Auricchio, S., Greco, L., Vizia, B.D.G. and Buonocore, V., Dipeptidyl aminopeptidase and carboxypeptidase activities of the brush border of rabbit small intestine. *Gastroenterology*, 75 (1978) 1073–1079.

Bai, J.P.F., Distribution of brush-border membrane peptidases along the rabbit intestine: Implication for oral delivery of peptide drugs. *Life Sci.*, 52 (1993) 941–947.

Bai, J.P.F., Hu, M., Subramanian, P., Mosberg, H.I. and Amidon, G.L., Utilization of peptide carrier system to improve the intestinal absorption: Targeting prolidase as a prodrug converting enzyme. *J. Pharm. Sci.*, 81 (1992) 113–116.

Barnes, K., Ingram, J. and Kenny, A.J., Proteins of the kidney microvillar membrane. *Biochem. J.*, 264 (1989) 335–346.

Chowdhary, B.K., Smith, G.D. and Peters, T.J., Subcellular localization and partial characterization of insulin proteolytic activity in rat liver. *Biochim. Biophys. Acta*, 840(1985) 180–186.

Derewenda, U., Derewenda, Z.S., Dodson, G.G. and Hubbard, R.E., Insulin structure. In Cuatrecasas, P. and Jacobs, S. (Eds), *Insulin*, Springer, Berlin, 1990, pp. 23–39.

Duckworth, W.C., Heinemann, M.A. and Kitabchi, A., Purification of insulin-specific protease by affinity chromatography. *Proc. Natl. Acad. Sci. USA,* 69 (1972) 3698–3702.

Hoogdalem, E.J., De Boer, A.G. and Breimer, D.D., Intestinal drug absorption enhancement: an overview. *Pharmacol. Ther.,* 44 (1989) 407–443.

Jackson, M.C., Choudry, Y., Bourne, A., Woodley, J.F. and Kenny, A.J., A fluorimetric assay for aminopeptidase W. *Biochem. J.,* 253 (1988) 299–302.

Kessler, M., Acuto, O., Storelli, C., Murer, H., Muller, M. and Semenza, G., A modified procedure for the rapid preparation of efficiently transporting vesicles form small intestinal brush border membranes. *Biochim. Biophys. Acta,* 506 (1978) 136–154.

Kidron, M., Bar-On, H., Berry, E.M. and Ziv, E., The absorption of insulin from various regions of the rat intestine. *Life Sci.,* 31 (1982) 2837–2841.

Lasch, J., Koelsch, R., Steinmetzer, T., Meumann, U. and Demuth, H.U., Enzymic properties of intestinal aminopeptidase P: a new continuous assay. *FEBS Lett.,* (1988) 171–174.

Roda, A, Minutelo, A., Angellotti, M.A. and Fini, A., Bile acid structure-activity relationship: Evaulation of bile acid lipophilicity using 1-octanol/water partition coefficient and reverse phase HPLC. *J. Lipid Res.,* 31 (1990) 1433–1443.

Vallet-Strouve, C., Tellier, C., Poignant, S. and Boucrot, P., Effect of micellar lipids on rabbit intestinal brush-border membrane phospholipid bilayer integrity studied by ^{31}P-NMR. *J. Membrane Biol.,* 84 (1985) 73–79.

Yamamoto, A., Hayakawa, E. and Lee, V.H.L., Insulin and proinsulin proteolysis in mucosal homogenates of the rabbit: implications in peptide delivery from nonoral route. *Life Sci.,* 47(1990) 2465–2474.