Inhibitory Effects of Quaternary Ammonium Compounds on Lysosomal Degradation of Endogenous Proteins

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The effects of quaternary ammonium compounds on the degradation of endogenous proteins in isolated lysosomes were studied. Proteolysis was assayed by measuring the trichloroacetic acid-soluble radioactivity in the lysosomes which had been labeled *in vivo* with ^{14}C-leucine. *p*-Biphenylmethyl-(*dl*-tropyl-α-tropinium)bromide (BTTB), at concentrations higher than 0.85 mM, was found to inhibit the lysosomal proteolysis stoichiometrically. Other quaternary ammonium compounds, such as *N*-methylatropinium bromide (NMA), cetyltrimethylammonium bromide (CTAB), tubocurarine and gallamine, were also examined in the presence or absence of Triton X-100 which is able to destroy the lysosomal membranes. Of these four compounds, NMA was the most effective proteolysis inhibitor, showing 25% inhibition for intact lysosomes at a concentration of 1 mM of the compound. When these compounds were assayed after Triton X-100 treatment of the lysosomes, the effects of all the drugs were augmented, suggesting that they, after accumulation in the lysosomes, act through direct interactions with lysosomal proteases. This was supported by a kinetic analysis of the action of NMA on cathepsin B, a typical lysosomal protease.

Keywords lysosome; proteolysis; quaternary ammonium compound; *p*-biphenylmethyl-(*dl*-tropyl-α-troponium)bromide (BTTB); *N*-methylatropinium bromide (NMA); cetyltrimethylammonium bromide (CTAB); tubocurarine; tropane alkaloid

The process of intracellular protein degradation is not understood as fully as the mechanism of protein synthesis. The former process has been reported to depend primarily on the action of lysosomes. Endogenous protein digestion by lysosomes is known to be inhibited by lysosome inhibitors such as chloroquine and methylamine.[1–4] Most previous attempts to inhibit the lysosomal degradation of proteins have been conducted on isolated cells[5–7] or perfused organs.[8,9] However, no systematic examination has been made of the effects of quaternary ammonium compounds on protein degradation by using isolated lysosomes.

p-Biphenylmethyl-(*dl*-tropyl-α-troponium)bromide (BTTB), a quaternary ammonium derivative of tropane alkaloid, is an antispasmodic drug, and we have shown that this drug can be taken up specifically by rat liver lysosomes *in vivo*.[10] Also, *N*-methylatropinium bromide (NMA), a similar compound to BTTB, competitively reduced the affinity of BTTB for lysosomal membrane.[11] Lysosomal proteolysis might thus be influenced by these compounds.

In the present study, we examined the effects of various quaternary ammonium compounds on proteolysis in isolated mouse liver lysosomes and also the effects of these compounds on the activity of cathepsin B, a typical lysosomal protease.

Materials and Methods

Animals and Chemicals Male dd/y mice (20—25 g) were used. L-[1-^{14}C]Leucine was obtained from Amersham International plc (57 mCi/mmol). BTTB was a kind gift from Funai Pharmaceutical Co., Ltd. (Japan). Chloroquine and *d*-tubocurarine were purchased from Sigma Chemical Co. (U.S.A). NMA was obtained from Merk (Germany). Cetyltrimethylammonium bromide (CTAB) was obtained from Eastman Kodak (U.S.A.).

Preparation of Lysosomes At 16 h after intraperitoneal administration of ^{14}C-leucine, mice were sacrificed, and the livers were removed, immersed in 0.25 M sucrose–0.04 M Tris-acetate buffer (pH 7.4) and 10% (w/v) homogenates were prepared. The light mitochondrial (LM) fraction, which was a lysosome-rich fraction, was prepared according to the method of de Duve *et al.*[12] Part of the aliquots was suspended in the above buffered sucrose solution containing 5 mM ethylenediaminetetraacetic acid (EDTA), and the residual part was suspended in 0.25 M sucrose (pH 7.4)–1% Triton X-100 to destroy the lysosomal membranes. The LM fraction from control mouse liver was subjected to freeze-thawing (7 times). The resultant suspensions were centrifuged at 15000 × *g* for 20 min and the supernatants were used as the enzyme preparation for the experiment on the effects of NMA on cathepsin B activity.

Measurement of Endogenous Protein Degradation Aliquots (2 ml) of each LM fraction containing various basic compounds (0.5 ml) were allowed to stand for 60 min at 4 °C and the pH was adjusted to 5.5 with 1 N HCl. The suspensions were then incubated for 30 min at 37 °C. The reactions were terminated by adding an equal volume of 10% trichloroacetic acid (TCA). The radioactivity in the supernatants obtained after centrifugation at 15000 × *g* for 10 min was measured. The net TCA-soluble radioactivity was determined by subtracting the control value. (The control value here means the TCA-soluble radioactivity of the LM sample treated in the same manner except for the initial addition of the drugs).

Biochemical Assays Cathepsin B activity was measured by the method of Barret.[13] One unit of activity was defined as the amount of the enzyme liberating 1 μmol 2-naphthylamine/min from α-*N*-benzoyl-D,L-arginine-2-naphthylamide (BANA) used as substrate.

Results and Discussion

The effect of BTTB, one of the tropane alkaloids, which is lysosome tropic, on the lysosomal degradation of endogenous protein was examined.

As shown in Table I, BTTB at 0.85 mM inhibited the proteolysis and did so progressively up to a concentration

TABLE I. Effect of *p*-Biphenylmethyl-(*dl*-tropyl-α-tropinium)bromide (BTTB) on the Degradation of Endogenous Proteins in Lysosomes

Compound	Concentration (mM)	Degradation (%)	Inhibition (%)
None	—	18.0 ± 1.1	0
BTTB	0.85	15.5 ± 1.2	13.9
	1.70	11.3 ± 1.2	37.2
	3.30	5.2 ± 1.2	71.1
Chloroquine	0.05	18.6 ± 1.2	0
	0.10	14.6 ± 1.1	18.9
	0.20	12.2 ± 1.1	32.2

See Materials and Methods for experimental details. Degradation (%) denotes the trichloroacetic acid-soluble activity divided by the total radioactivity in the fraction incubated. Blanks were run at 4 °C. Values represent the means ± S.D. (n = 6).

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Table II. Effects of Quaternary Ammonium Compounds on the Degradation of Endogenous Proteins in Lysosomes

Compound (1 mM)	− Triton X-100		+ Triton X-100	
	Degradation (%)	Inhibition (%)	Degradation (%)	Inhibition (%)
None	15.5±0.7	0	7.8±0.5	0
NMA	11.6±1.6[b]	25.2	4.9±1.2[b]	37.2
CTAB	14.0±0.7	9.7	6.3±1.6	19.2
Tubocurarine	14.0±0.5	9.7	6.5±0.2	16.7
Chloroquine	8.7±0.2[a]	43.9	1.4±0.2[a]	82.1

See Materials and Methods for experimental details. − Triton X-100: lysosomes were suspended in 0.25 M sucrose–0.04 M Tris-acetate buffer (pH 7.4)–5 mM EDTA solution. + Triton X-100: lysosomes were suspended in 0.25 M sucrose–1% Triton X-100. *a*) $p<0.01$. *b*) $p<0.05$, *vs.* none.

of 3.3 mM. In the case of chloroquine as a positive reference, a strong inhibitory effect was observed at a concentration lower than that of BTTB. The effects of several quaternary ammonium compounds including NMA on the degradation in the presence or absence of Triton X-100 were also investigated. The results are summarized in Table II. NMA strongly inhibited the protein degradation. Although CTAB is a long chain aliphatic quaternary ammonium compound with a detergent action, its potency of inhibition of proteolysis was lower than that of NMA. Tubocurarine, a bis-quaternary ammonium compound, inhibited the degradation to the same degree as CTAB (about 10% at 1 mM). Recently, Meijer *et al.*[14] reported confirmation in their experiments on subcellular fractionation of the liver at 15 min after intravenous administration of *d*-tubocurarine, that this drug was localized in the lysosomes. In a previous paper,[11] we showed that the binding of BTTB to lysosomal membranes did not decrease following addition of tubocurarine at a final concentration of below 1 mM. These findings indicate that the action of tubocurarine on lysosomes is weaker than that of BTTB.

When the lysosomal membranes were destroyed by the addition of 1% Triton X-100, the degree of inhibition of proteolysis by quaternary ammonium compounds exceeded that in the absence of the detergent. BTTB has been reported to be taken up by lysosomes.[10] This suggests that the mechanism of inhibition of protein degradation by these quaternary ammonium compounds may involve their direct interaction with lysosomal enzymes. The potencies of these compounds as an inhibitor of protein degradation might thus be related to the membrane permeability.

Figure 1 shows the results of a kinetic analysis of the effect of NMA on the reaction of cathepsin B. NMA inhibited the cathepsin B activity. The K_m and V_{max} values of cathepsin B were 2 mM and 8.00 U/g liver, respectively, and the apparent K_m value when NMA was present was almost unchanged. However, the V_{max} of the reaction in the presence of NMA decreased depending on the concentration of the drug. NMA up to 100 mM acted as a non-competitive inhibitor for cathepsin B. Cowey *et al.*[15] reported that chloroquine at 10 mM inhibited the bacterial collagenase, clostridiopeptidase A, and the inhibition was reversible and non-competitive. Wibo *et al.*[16] also reported that chloroquine at a high concentration of 100 mM inhibited cathepsin B. The detailed inhibition mechanism,

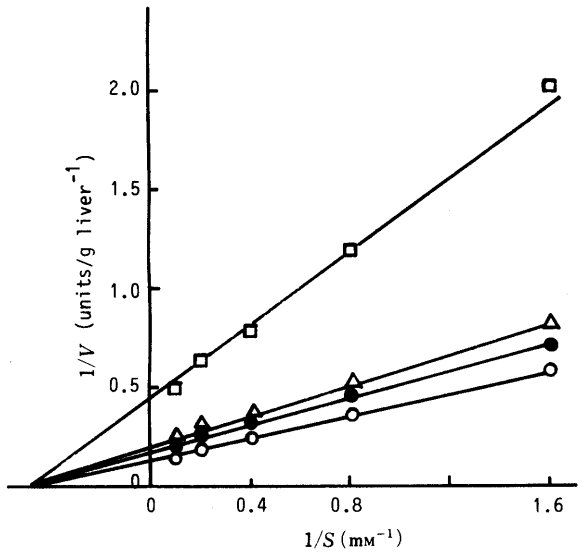

Fig. 1. Kinetic Analysis of the Reaction of Cathepsin B with *N*-Methylatropinium Bromide (NMA) at Various Concentrations

The enzyme preparations were preincubated for 5 min with each compound at concentration of 1, 10 and 100 mM. The reactions were initiated by adding BANA at 37.5 °C. The figure shows Lineweaver–Burk plots of BANA concentration against cathepsin B activity in the presence or absence of NMA. ○—○, none; ●—●, 1 mM; △—△, 10 mM; □—□, 100 mM.

however, has not yet been clarified.

When lysosomes were not treated with Triton X-100, the degree of proteolysis inhibition was relatively low. Thus, the lysosomal membranes may act to some degree as a barrier to the permeation of these drugs. The inhibition of lysosomal proteolysis as measured by the isotopic method exceeded that as measured from the cathepsin B inhibition alone, indicating that the tropane alkaloids also inhibit lysosomal proteases other than cathepsin B. Allison *et al.*[17] have proposed two mechanisms for the incorporation of substances into lysosomes, that is, endocytosis and direct incorporation involving a transmembrane mechanism. The binding affinity of the tropane alkaloids employed in this experiment to serum proteins was very weak, so that these compounds must have been incorporated into lysosomes by the latter mechanism. The incorporated compounds inhibit lysosomal protease including cathepsin B, resulting in suppression of the lysosomal protein degradation. Furthermore, when these tropane alkaloids are condensed in lysosomes, the intralysosomal pH should be shifted to the alkaline side. The suppression of lysosomal protein degradation induced by these tropane alkaloids might thus be due to direct inhibition of the lysosomal protease and also a shift of the intralysosomal pH from the optimum for these enzyme reactions to the alkaline side.

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