Sequestration of Cytoplasmic Enzymes in an Autophagic Vacuole-Lysosomal System Induced by Injection of Leupeptin*

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Administration of leupeptin to rats induces the accumulation of numerous autophagic vacuoles in the liver. Furuno et al. (Furuno, K., Ishikawa, T., and Kato, K. (1982) J. Biochem. (Tokyo) 91, 1485-1494) have recently devised a method for Percoll density gradient equilibrium fractionation of crude lysosomal fractions to isolate a highly enriched preparation of autophagic vacuoles. This system was used to determine whether cytoplasmic enzymes are normally sequestered into autophagic vacuoles in fed animals. Within 30 min following the administration of leupeptin to fed rats, several cytoplasmic enzymes could be demonstrated in vacuolar fractions heavier than mitochondrial and normal lysosomes. The activities of tyrosine aminotransferase and lactic dehydrogenase as well as antigens of fructose-bisphosphate aldolase were detectable in fractions with densities of 1.115 to 1.15 g/ml containing cathepsins and acid phosphatase. The cytoplasmic enzymes in these fractions exhibited latency and were sequestered within membranous organelles. Six hours after the administration of leupeptin, the autophagic vacuoles gradually disappeared from these fractions concurrently with the loss of both cytoplasmic and lysosomal marker enzymes. For 6 h after injection of leupeptin the activities of cathepsin D and acid phosphatase increased in autophagic vacuoles and decreased in the postvacuolar lysosomal fraction. Administration of dexamethasone, which induces the synthesis of tyrosine aminotransferase and cytosolic aspartate aminotransferase, selectively increased the sequestration of these enzymes to proportional degrees. Cycloheximide administered simultaneously with leupeptin rapidly inhibited formation of autophagic vacuoles and the sequestration of both cytoplasmic and lysosomal enzymes. However, when cycloheximide was administered 1 h after leupeptin, the formation of autophagosomes and the sequestration of cytoplasmic enzymes were inhibited but the vacuolar uptake of acid phosphatase and cathepsin D continued to increase for several hours. When cycloheximide was injected 1 h after leupeptin, losses of lactic dehydrogenase and aldolase proteins were observed in autophagic vacuoles isolated 1 and 2 h later.

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Autophagy and proteolysis in the liver are enhanced by deprivation of amino acids and insulin (1-3) and by the administration of glucagon (4-6), and these enhancements are reversed by administration of amino acids and insulin (2, 7, 8). The appearance and subsequent rapid disappearance of autophagic vacuoles have been suggested to reflect alterations in the degradation of intracellular proteins (5, 6). However, there have been few biochemical studies on autophagy, especially in undeprived, basal conditions.

Recently, Furuno et al. (9) showed that administration of leupeptin, a nonreversible inhibitor of lysosomal thiol proteinases, caused the appearance and persistence of numerous autophagic vacuoles in the liver. They suggested that because of the retarded digestion of sequestered materials, the autophagic vacuoles constitute most of the lysosomal population.

Using leupeptin-treated rats, we examined whether cytoplasmic enzymes are sequestered in the lysosomal-vacuolar system in fed rats. Results showed that tyrosine aminotransferase, an enzyme with a short half-life, and lactic dehydrogenase and aldolase, enzymes with long half-lives, were detectable in autophagic vacuoles isolated from the livers of leupeptin-treated rats.

This paper describes changes in activities and properties of cytoplasmic enzymes sequestered into autophagic vacuoles accumulated in the liver following the administration of leupeptin.

EXPERIMENTAL PROCEDURES

Materials
Leupeptin was purchased from the Protein Research Foundation (Osaka, Japan). Percoll was obtained from Pharmacia Fine Chemicals. [1-14C]Valine (40-60 mCi/mmol) was obtained from New England Nuclear. Nitrocellulose membranes were from Bio-Rad. Horseradish peroxidase-conjugated second antibodies (rabbit anti-goat IgG and goat anti-rabbit IgG) were obtained from Cappel Laboratories, Inc.

Methods
Treatment of Animals—Male Wistar rats weighing 170-200 g were given standard chow and water ad libitum. Dexamethasone (100 μg/100 g of body weight) was injected intraperitoneally as a solution in saline twice a day for 3 days and 9 h after the last injection leupeptin (2 mg/100 g of body weight) was injected intraperitoneally as a solution in saline. Cycloheximide (0.1 mg/100 g of body weight) was injected intraperitoneally as a solution in saline. Liver proteins were prelabeled by intraperitoneal injections of 30 μCi of [1-14C]valine (NEN-171) at 24 and 4 h before sacrifice.

Percoll Density Gradient Centrifugation—Livers were perfused with cold saline and then 4 g of liver were homogenized as described by Furuno et al. (9). The resultant 20% homogenate was centrifuged at
650 × g for 5 min and the supernatant was centrifuged at 1,700 × g for 10 min. The resulting precipitate was suspended in 30 ml of iso-osmotic Percoll at a density of 1.10 mg/ml, and centrifuged at 63,600 × g for 60 min. Fractions of 1 ml were collected by pumping 80% sucrose into the bottom of tubes and the marker enzyme and protein concentrations of these fractions were measured.

The distribution of lysosomal enzyme activities in autophagic vacuoles and the postvacuolar lysosomal fraction, a 20% homogenate of liver from leupeptin-treated rats was centrifuged at 650 × g for 5 min and the supernatant was centrifuged at 11,000 × g for 20 min. The resultant pellet was suspended in 30 ml of Percoll at a density of 1.10 g/ml and centrifuged at 63,600 × g for 60 min. Fractions of 1 ml were collected as described above and their lysosomal enzyme activities were measured. Activities of lysosomal enzymes in autophagic vacuoles and the postvacuolar lysosomal fraction were calculated from the areas of activities distributed in each fraction.

Isolation of Autophagic Vacuoles—Autophagic vacuoles obtained from the livers of leupeptin-treated rats by differential centrifugation and Percoll density gradient centrifugation were purified further as described by Furuno et al. (10). The layer with a density of 1.13–1.15 g/ml was carefully collected from the Percoll gradient, diluted with iso-osmotic Percoll with a density of 1.10 g/ml, and recentrifuged under the same conditions as before. Material with a density of 1.13–1.15 g/ml was again collected, and centrifuged at 105,000 × g for 120 min to obtain a pellet covered by a fluffy layer. The pellet and fluffy layer were carefully collected, suspended in 0.25 M sucrose, pH 7.0, and centrifuged at 10,000 × g for 30 min. The pellet was carefully resuspended in 0.25 M sucrose, pH 7.0, and recentrifuged at 10,000 × g for 30 min. The resulting pellet was used as isolated autophagic vacuoles. The autophagic vacuole fraction obtained from 4 g of liver was suspended in 2 ml of 0.025 M sucrose, pH 7.0, containing 0.2% Triton X-100, and aliquots were used for assay of enzyme activities or antigenicities.

Immunogens and Immunization Procedures—Aldolase was purified from rat liver by the method of Rutter et al. (11), except that material was fractionated with 45–70%, not 45–60%, (NH₄)₂SO₄. Anti-aldolase serum was obtained as described before (12). Lactic dehydrogenase was purified from rat liver by the method of Scopes (13). Aspartate aminotransferase was purified from the cytosol fraction of rat liver by a modification of the method of Jenkins et al. (15). Anti-lactic dehydrogenase and anti-aspartate aminotransferase were raised in rabbits by injection of 200 μg of protein emulsified with complete Freund's adjuvant intracutaneously followed by two booster injections of the same dose at 2-week intervals. The rabbits were bled 7 days after the second booster injection.

Electrophoretic Blotting Procedures—Autophagic vacuoles isolated from the livers of leupeptin-treated rats were suspended in 0.025 M sucrose, pH 7.0. After two cycles of freezing-thawing, the suspension was placed on the top of a discontinuous sucrose gradient formed, from bottom to top, with 1.5 ml of 45% sucrose (w/v), 1.7 ml of 57%, and 0.8 ml of 80% sucrose. The gradient was centrifuged for 2.5 h at 40,000 × g in a Hitachi RPS 50 rotor. The precipitate was dissolved in 0.01 M Tris-HCl, pH 8.0, containing 1 mM EDTA, 1% sodium dodecyl sulfate, and 5% β-mercaptoethanol, and vascular proteins were separated by electrophoresis on a 10% polyacrylamide gel containing sodium dodecyl sulfate. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets was performed by the method of Towbin et al. (16). Transfer was usually carried out at 20 V for 3 h. The electrophoretic blots were soaked in 3% bovine serum albumin in saline (0.9% NaCl, 10 mM Tris-HCl, pH 7.4) for 1 h at 37 °C. Then they were rinsed in saline and incubated with antigen diluted fivefold in 3% bovine serum albumin in saline also containing 10% rabbit or goat serum. The sheets were then washed with saline and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or horseradish peroxidase-conjugated rabbit anti-goat IgG. These second antibody preparations were used at 1:1000 dilution in saline containing 3% bovine serum albumin and 10% carrier serum. The blots were incubated for 2 h at room temperature and washed with saline. The color reaction was carried out with o-dianisidine as described by Towbin et al. (16).

Analytical Procedure—The densities of Percoll fractions were determined with density gradient markers from Pharmacia Fine Chemicals. Acid phosphatase was assayed as described by Igarashi and Hollander (17). Cathepsin D activity was determined by a modification (12) of the method of Matsuda and Misaka (18). Bz'-Arg-2-naphthylamide hydrolyzing activity was determined by the method of Barrett (19). Cytochrome oxidase was measured as described by Wharton and Tragoloff (20). Tyrosine aminotransferase activity was assayed by the method of Granner and Tomkins (21). Lactic dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, phosphoglucomutase, and phosphoenolpyruvate carboxykinase were assayed by the methods of Stolar et al. (22), Furuno et al. (23), Tanaka et al. (24), Noltmann (25), and Lane et al. (26). In these methods activity was determined by measuring the decrease in absorbance at 340 nm at 25 °C in reaction mixture in a final volume of 1 ml. Fructose-bisphosphate aldolase was measured at 25 °C by the method of Rutter et al. (11). Aldolase antigens were precipitated quantitatively with monospecific antibodies raised to purified rat liver aldolase. Then the antibody-antigen complexes were washed 3 times with 0.17 M NaCl and solubilized in 0.3 ml of NCS (Amersham Corp.), and their radioactivity was counted in Aquasol in a Beckman LS-150 scintillation counter. Aspartate aminotransferase was determined by the method of Schwartz (27). Anti-cyttoplasmic aspartate aminotransferase was used to determine the amount of soluble aspartate aminotransferase in autophagic vacuoles. For this aspartate aminotransferase preparations were incubated with and without anti-cyttoplasmic aspartate aminotransferase at 37 °C for 60 min, the insoluble complex formed was precipitated by centrifugation, and aminotransferase activity of the supernatant was measured. The amount of soluble aspartate aminotransferase activity was then calculated as the difference between the activities with and without the antibody. Protein was measured by the method of Lowry et al. (28) with crystalline bovine serum albumin as a standard.

RESULTS

Detection of Cyttoplasmic Enzymes in Autophagic Vacuoles from the Livers of Leupeptin-treated Rats—Leupeptin was injected intraperitoneally at a dose of 2 mg/100 g of body weight into test rats, and control rats received 0.9% NaCl only. The animals were sacrificed 1 h later and the heavy mitochondrial-lysosomal fraction was obtained from the livers of controls and leupeptin-treated rats as described under "Experimental Procedures." This fraction was subjected to Percoll density gradient centrifugation. The typical distribution patterns of several marker enzymes in the various fractions obtained by Percoll density gradient centrifugation are shown in Fig. 1. Cytochrome oxidase was recovered in fractions with densities of 1.08–1.115 g/ml and there was no significant difference in its distribution in control (Fig. 1, top) and leupeptin-treated (Fig. 1, bottom) animals. Acid phosphatase, lysosomal marker, was recovered in fractions of lower density (1.07–1.10 g/ml) than the mitochondrial marker in control liver. But in liver from leupeptin-treated rats, this enzyme was mainly recovered in fractions with a density of 1.15–1.15 g/ml, which were clearly separated from the mitochondrial marker. Lysosomal fractions of heavier density from the livers of leupeptin-treated rats contained not only acid phosphatase but also many other lysosomal enzymes, including arylsulphatase and cathepsin D (not shown).

Morphological examination indicated that these lysosomal fractions of heavier density separated by Percoll density gradient centrifugation mainly consisted of autophagic vacuoles (9).

If cyttoplasmic proteins are normally degraded via the lysosomal-vacuolar system, they should be detected in fractions rich in autophagic vacuoles. Therefore, we measured the activities of several cytoplasmic enzymes in all fractions obtained by Percoll density gradient centrifugation after solubilizing these fractions in Triton X-100 to a final concentration of 0.2%. Tyrosine aminotransferase, an enzyme with a short half-life, and lactic dehydrogenase and fructose-bis-phosphate aldolase, two enzymes with long half-lives, were detected in fractions of heavier density than mitochondria (Fig. 1, bottom). The activities of various cytoplasmic enzymes in isolated autophagic vacuoles and in the cytoplasm are shown in Table I.

Next we tested the possibility that these cyttoplasmic en-

1 The abbreviation used is: Bz-, benzoyl-.
FIG. 1. Isopyknic distributions of acid phosphatase, cytochrome oxidase, tyrosine aminotransferase, lactic dehydrogenase, and aldolase antigen in Percoll density gradients after centrifugation of crude heavy mitochondrial lysosomal fractions of livers from control (top) and leupeptin-treated rats (bottom). Gradients were collected in 30 fractions of 1 ml each ranging in density from 1.03 to 1.16 g/ml, and analyzed for acid phosphatase (A), cytochrome oxidase (O), tyrosine aminotransferase (TAT, □), lactic dehydrogenase (LDH, ○), and aldolase antigen (▲) after treatment with 0.2% Triton X-100. — — density. Aldolase antigen was measured with specific antibody against rat liver aldolase as described under "Experimental Procedures."

TABLE I
Activities of cytoplasmic enzymes in autophagic vacuoles
Leupeptin was injected intraperitoneally into rats at a dose of 2 mg/100 g of body weight. Rats were killed 1 h later and the cytosol fraction and autophagic vacuoles were prepared from their livers as described under "Experimental Procedures." Various cytoplasmic enzyme activities were measured and values are shown as deviations for five rats.

| Enzyme                          | Cytosol (A) | Autophagic vacuoles (B) | (B)/(A) |
|---------------------------------|-------------|-------------------------|---------|
| Tyrosine aminotransferase       | 1.01 ± 0.31 | 4.9 ± 0.8               | 0.49    |
| Aspartate aminotransferase      | 34.2 ± 3.8  | 150 ± 27                | 0.44    |
| Lactic dehydrogenase (soluble form) | 240 ± 18     | 810 ± 10                | 0.34    |
| Pyruvate kinase                 | 18 ± 4.2    | 13 ± 1.4                | 0.072   |
| Glyceraldehyde-3-phosphate      | 79 ± 12     | 24 ± 2.8                | 0.090   |
| Aldolase                        | 4.2 ± 0.6   | >0.8                    | >0.02   |
| Phosphoglucoisomerase           | 52 ± 6.8    | 5.1 ± 1.2               | 0.01    |
| Phosphoenolpyruvate carboxykinase | 1.7 ± 0.4  | >0.25                   | >0.015  |

zymes were not present in the vacuoles but adsorbed to the outside of their membranes by the following two experiments. First, autophagic vacuoles from livers of leupeptin-treated rats were purified further as described under "Experimental Procedures," and the latencies of enzymes were tested by measuring their activities with and without Triton X-100. Results showed that the activities of both cytoplasmic and lysosomal enzymes in intact autophagic vacuoles were less than 40% of those in preparation treated with Triton X-100 (not shown). Second, intact and Triton X-100-treated autophagic vacuoles were incubated with anti-lactic dehydrogenase for 30 min at room temperature, and then lactic dehydrogenase was assayed immediately after Triton X-100 was added to the intact vacuoles. As shown in Fig. 2, the lactic dehydrogenase activities were significantly inhibited in Triton X-100-treated autophagic vacuoles. Values are means of three different experiments.
Fig. 3. Time course of changes in acid phosphatase, protein concentration, tyrosine aminotransferase, and lactate dehydrogenase in isolated autophagic vacuoles from livers of leupeptin-treated rats. Leupeptin was injected intraperitoneally into rats at a dose of 2 mg/100 g of body weight. Rats were killed at the times indicated and autophagic vacuole fractions were isolated from the liver as described under "Experimental Procedures" and analyzed for acid phosphatase (△), protein (○), tyrosine aminotransferase (TAT, □) and lactate dehydrogenase (LDH, ◦). Values are means for five rats.

Fig. 4. Distribution patterns of acid phosphatase and cathepsin D activities in Percoll density gradients after centrifugation of total lysosome fractions of livers from control (апример) and leupeptin-treated rats (2). Leupeptin was injected intraperitoneally into rats at a dose of 2 mg/100 g of body weight. Rats were killed 3 h after the injection and mitochondrial lysosomal fractions were prepared from their livers. Gradients after Percoll density centrifugation were collected in 30 fractions of 1 ml, and were analyzed for acid phosphatase and cathepsin D activities.

The rapid disappearance of cytoplasmic enzyme activities may be partly due to reappearance of inhibited lysosomal cathepsins and partly to inactivation and degradation of the enzymes in the autophagic vacuoles. The difference in the rates of decrease of tyrosine aminotransferase and lactate dehydrogenase activities as well as the differences in the percentages of cytoplasmic enzymes sequestered (Table I) may be attributed to differences in the rates of inactivation of the enzymes in the autophagic vacuoles.

Redistribution of Acid Phosphatase and Cathepsin D Activities between Lysosomes and Autophagic Vacuoles after Administration of Leupeptin—Leupeptin (2 mg/100 g of body weight) was injected intraperitoneally and then rats were killed at intervals, and the autophagic vacuole fraction was isolated from their livers. Fig. 3 shows the activities of the lysosomal marker enzyme (acid phosphatase and the cytoplasmic enzymes) tyrosine aminotransferase and lactate dehydrogenase and the protein concentration in isolated autophagic vacuoles.

The activity of acid phosphatase began to increase within 30 min after injection of leupeptin, reached a plateau at 3-6 h, and then gradually decreased. Protein concentration showed a similar change with time. Cytoplasmic enzyme activities in autophagic vacuoles also increased in parallel with lysosomal enzyme activity until 6 h after injection of leupeptin, but then they decreased rapidly, and were scarcely detectable 12 h after the injection. The rapid disappearance of cytoplasmic enzyme activities may be partly due to reappearance of inhibited lysosomal cathepsins and partly to inactivation and degradation of the enzymes in the autophagic vacuoles. The difference in the rates of decrease of tyrosine aminotransferase and lactate dehydrogenase activities as well as the differences in the percentages of cytoplasmic enzymes sequestered (Table I) may be attributed to differences in the rates of inactivation of the enzymes in the autophagic vacuoles.

Acid phosphatase activity in autophagic vacuoles initially treated with Triton X-100 was inhibited 80% by incubation with antibody, whereas that of the intact vacuoles was inhibited only about 10%.

The above results show that the cytoplasmic enzymes detected in autophagic vacuoles were not adsorbed to the outside the membranes of these vacuoles, but were actually present within the vacuoles.

Changes in Lysosomal Marker Enzyme, Cytoplasmic Enzyme Activities, and Protein Concentration in Isolated Autophagic Vacuoles from Liver at Different Times after Administration of Leupeptin—Leupeptin (2 mg/100 g of body weight) was injected intraperitoneally and then rats were killed at intervals, and the autophagic vacuole fraction was isolated from their livers. Fig. 3 shows the activities of the lysosomal marker enzyme (acid phosphatase and the cytoplasmic enzymes) tyrosine aminotransferase and lactate dehydrogenase and the protein concentration in isolated autophagic vacuoles.
that is, the sum of the activities in autophagic vacuoles and postvacular lysosomes, did not change appreciably during the 24-h examination period. Thus, there was a redistribution of acid phosphatase and cathepsin D activities between autophagic vacuoles and the postvacular lysosomal fractions after administration of leptopin, suggesting that lysosomal hydrolases in autophagic vacuoles are mainly derived from primary or pre-existing secondary lysosomes.

**Inhibition of Bz-Arg-2-naphthylamide Hydrolase in Autophagic Vacuoles and Lysosomes**—Bz-Arg-2-naphthylamide is hydrolyzed by cathepsins B and H in the liver and these enzymes are inhibited by leptopin in *vitro* and *in vivo* (29). The changes in Bz-Arg-2-naphthylamide hydrolyzing activities in autophagic vacuoles and postvacular lysosome fractions of the liver with time after leptopin treatment are shown in Fig. 6. Bz-Arg-2-naphthylamide hydrolyase activity in lysosomes decreased rapidly to a very low level 3–6 h after injection of leptopin and no increase in its activity in autophagic vacuoles was observed. The total activity also decreased markedly after injection of leptopin, indicating that Bz-Arg-naphthylamide hydrolyzing activity in both autophagic vacuoles and the postvacular lysosome fraction is inhibited by leptopin.

**Table II**

*Effect of dexamethasone on activities of cytoplasmic enzymes in autophagic vacuoles from the livers of leptopin-treated rats.*

Dexamethasone (100 μg/100 g of body weight) was injected intrapectonitely as a solution in saline twice a day for 3 days. Nine hours after the last injection leptopin (2.0 mg/100 g of body weight) was injected. Rats were killed 1 h later and the cytosol fraction and autophagic vacuoles were prepared from their livers as described under "Experimental Procedures" for measurement of cytoplasmic enzyme activities. Values are means ± standard deviations for five rats. Values in parentheses show activities in units or milliunits/mg of protein.

| Enzymes                        | Control         | Dexamethasone | Control         | Dexamethasone |
|--------------------------------|-----------------|---------------|-----------------|---------------|
|                                | units/g liver   |               | units/g liver   |               |
| Lactic dehydrogenase           | 245 ± 27        | 240 ± 35      | 0.806 ± 0.068   | 0.780 ± 0.098 |
|                                | (4.47 ± 0.52)   | (4.87 ± 0.56) | (1.10 ± 0.12)   | (1.15 ± 0.20) |
| Aspartate aminotransferase (soluble) | 34.2 ± 3.2     | 138 ± 15      | 0.150 ± 0.017   | 0.608 ± 0.085 |
|                                | (0.702 ± 0.070) | (3.00 ± 0.47) | (0.145 ± 0.014) | (0.625 ± 0.108) |
| Tyrosine aminotransferase      | 840 ± 202       | 2870 ± 520    | 4.32 ± 0.60     | 11.0 ± 2.2    |
|                                | (17.0 ± 3.7)    | (46.7 ± 6.8)  | (4.20 ± 0.51)   | (11.9 ± 2.5)  |

**FIG. 5.** Redistribution of acid phosphatase and cathepsin D activities between autophagic vacuoles and lysosomes following administration of leptopin. Leptopin was injected intraperitoneally into rats at a dose of 2 mg/100 g of body weight. Rats were killed at the times indicated and mitochondrial lysosomal fractions were prepared from their livers. Then autophagic vacuoles and postvacular lysosomes were separated by Percoll density gradient centrifugation as described under "Experimental Procedures," and acid phosphatase and cathepsin D activities were measured in both fractions. Values are means for five rats for the activities in autophagic vacuoles (●—●), the postvacular lysosomal fraction (○—○), and the total mitochondrial lysosomal fraction (Δ—Δ).

**FIG. 6.** Inhibition of Bz-Arg-2-naphthylamide hydrolase (*BANA* hydrolase) in autophagic vacuoles and postvacular lysosome fractions from the livers of leptopin-treated rats. Leptopin was injected intraperitoneally into rats at a dose of 2 mg/100 g of body weight. Rats were killed at the times indicated and autophagic vacuoles and postvacular lysosome fractions were prepared from their livers as described under "Experimental Procedures." Values are means for five rats for Bz-Arg-2-naphthylamide hydrolyzing activity in autophagic vacuoles (●—●), the postvacular lysosome fraction (○—○) and the total mitochondrial lysosomal fraction (Δ—Δ).

**TABLE II**

*Effect of dexamethasone on activities of cytoplasmic enzymes in autophagic vacuoles from the livers of leptopin-treated rats.*

Dexamethasone (100 μg/100 g of body weight) was injected intraperitoneally as a solution in saline twice a day for 3 days. Nine hours after the last injection leptopin (2.0 mg/100 g of body weight) was injected. Rats were killed 1 h later and the cytosol fraction and autophagic vacuoles were prepared from their livers as described under "Experimental Procedures" for measurement of cytoplasmic enzyme activities. Values are means ± standard deviations for five rats. Values in parentheses show activities in units or milliunits/mg of protein.

| Enzymes                        | Cytosol | Autophagic vacuoles |
|--------------------------------|---------|---------------------|
|                                | Control | Dexamethasone       | Control | Dexamethasone |
|                                |         | units/g liver       |         | units/g liver |
|                                |         | (milliunits/g liver)|         | (milliunits/g liver)|
| Lactic dehydrogenase           | 245 ± 27| 240 ± 35            | 0.806 ± 0.068| 0.780 ± 0.098 |
|                                | (4.47 ± 0.52)| (4.87 ± 0.56) | (1.10 ± 0.12) | (1.15 ± 0.20) |
| Aspartate aminotransferase (soluble) | 34.2 ± 3.2 | 138 ± 15            | 0.150 ± 0.017| 0.608 ± 0.085 |
|                                | (0.702 ± 0.070)| (3.00 ± 0.47) | (0.145 ± 0.014)| (0.625 ± 0.108) |
| Tyrosine aminotransferase      | 840 ± 202| 2870 ± 520          | 4.32 ± 0.60 | 11.0 ± 2.2 |
|                                | (17.0 ± 3.7) | (46.7 ± 6.8) | (4.20 ± 0.51) | (11.9 ± 2.5) |
Effects of Dexamethasone on Tyrosine Aminotransferase, Soluble Aspartate Aminotransferase, and Lactic Dehydrogenase Activities in Autophagic Vacuoles from the Livers of Leupeptin-treated Rats—Next we examined whether an increase in selected enzymes in the cytosol causes a proportional increase in the sequestration of these enzymes into autophagic vacuoles. Rat liver tyrosine aminotransferase and soluble aspartate aminotransferase are known to be induced by glucocorticoids (30, 31). Therefore, dexamethasone was injected into rats twice a day for 3 days and 9 h after the last injection leupeptin was injected. The rats were sacrificed 1 h after injection of leupeptin and autophagic vacuoles were isolated from their livers and from those of control rats. This treatment resulted in a 3-fold increase in tyrosine aminotransferase and aspartate aminotransferase but no change in lactic dehydrogenase in the cytosol fraction (Table II). Coincident with these increases in the cytosol, the tyrosine aminotransferase and aspartate aminotransferase activities in autophagic vacuoles also increased 3- and 4-fold, respectively, while the activity of lactic dehydrogenase in these organelles did not change. The specific activities of these three cytoplasmic enzymes in isolated autophagic vacuoles were all lower than those in the cytoplasmic fraction.

Effect of Cycloheximide on the Formation of Autophagic Vacuoles—When cycloheximide was injected into rats simultaneously with leupeptin, no accumulation of cytoplasmic and lysosomal enzymes in the autophagic vacuole fraction was observed (Fig. 7). When cycloheximide was injected 1 h after leupeptin, the activities of both tyrosine aminotransferase and lactic dehydrogenase were rapidly inhibited, and there was no further increase in the accumulation of proteins in the vacuolar fraction. Surprisingly, the activity of acid phosphatase continued to increase for 2 h after cycloheximide administration. The activity of cathepsin D also showed a similar change with time to that of acid phosphatase (not shown).

Loss of Lactic Dehydrogenase and Aldolase Proteins in Autophagic Vacuoles after Injection of Leupeptin—Cycloheximide was injected into rats 1 h after leupeptin and autophagic vacuoles were isolated from the liver 0, 1, and 2 h after injection of cycloheximide. The isolated autophagic vacuoles were treated with hypotonic sucrose solution and the proteins in the vacuolar contents were isolated by discontinuous sucrose gradient centrifugation. Then proteins in vacuolar contents were separated on 10% polyacrylamide gel containing sodium dodecyl sulfate. The electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets and detection of lactic dehydrogenase and aldolase proteins on sheets with their antibodies were performed as described under “Experimental Procedures.”
DISCUSSION

Intracellular proteins in hepatocytes and other mammalian cells can be separated into two major groups on the basis of the rate of their catabolism (32). The first group, which constitutes as much as 15–30% of newly synthesized proteins but only about 1% of the total protein content, turns over rapidly with a half-life of less than 1 h (33). Since this group fails to respond to alterations in the nutritional or hormonal status of the cell (34–37) or to lysosomotropic inhibitors (33–38), it has been suggested that its degradation is nonlysosomal in origin (36, 38) and could well represent post-translational processing of intracellular and secretory proteins. The second group consists of long-lived and relatively stable proteins whose degradation is inhabitable by lysosomotropic agents (37, 38) and is responsive to physiological regulation by amino acids and hormones such as insulin and glucagon (1–3, 6–8). This group is likely to be degraded primarily in autophagic vacuoles (13, 6–8), although some investigators still consider that the basal rate of turnover may be carried out by nonlysosomal proteolytic mechanisms (35, 39).

Morphologically one can distinguish two forms of autophagic vacuoles: newly formed autophagosomes containing sequestering cytoplasmic products but lacking lysosomal enzymes, and secondary lysosomes formed within 8 min by fusion of the autophagosomes with primary lysosomes or dense bodies and containing hydrolytic enzymes. The evidence presented in this paper strongly suggests that several cytoplasmic enzymes are continuously being sequestered into autophagosomes. Blocking of intralysosomal thiol cathepsins by the administration of leupeptin leads to the accumulation of secondary lysosomes containing both undegraded sequestered intracellular proteins and lysosomal enzymes.

Inhibition of intralysosomal proteolysis by the administration of leupeptin does not result in reduction of the rate of sequestration of cytoplasmic proteins into autophagic vacuoles. The fact that the intralysosomal half-lives of cytoplasmic enzymes are shorter than those of the enzymes in vivo supports the idea of the absence of such regulation.

The sequestration of cytoplasmic enzymes into autophagosomes does not appear to be selective. When tyrosine aminotransferase and soluble aspartate aminotransferase were induced 3-fold by the administration of dexamethasone, we found that the rates of sequestration of aminotransferases into autophagic vacuoles were selectively increased 3-fold without a concomitant increase in the uptake of lactate dehydrogenase or total protein into the organelles. We also found that the specific activities of all the cytoplasmic enzymes we examined in isolated autophagic vacuoles were lower than those of the enzymes in the cytoplasm (Table II), although this could be due to possible loss of cytoplasmic enzymes from autophagic vacuoles during isolation of the vacuoles. We recently found2 that when the rate of autophagy was increased by starvation, the observed rate of uptake of aldolase antigen and total protein into the autophagic vacuoles was increased 3-fold without a concomitant increase in the amount or activity of the enzyme in the cytoplasm. Thus, if the inhibition of Bz-Arg-2-naphthylamide hydrolase activity for 3–6 h after the administration of leupeptin (Fig. 6) is indicative of a block in intralysosomal proteolysis of the same duration, then the accumulation of cytoplasmic enzymes in the autophagic vacuoles must be a function of both the rate of autophagosome formation and the concentration of the enzymes in the cytoplasm.

It is conceivable that the sequestration of cytoplasmic proteins may be more highly selective than that demonstrated in these experiments. Inhibiting intralysosomal proteolysis by the administration of leupeptin may be depriving liver cells of some amino acids critical for the de novo synthesis of some essential proteins or peptides. It may, therefore, be the absence of an adequate supply of these amino acids that triggers the sequestration of any available protein in proportion to its amount in the cytoplasm.

The observation that cycloheximide administration simultaneously with leupeptin blocks the formation of autophagic vacuoles suggests a specific block in the synthesis of some protein or peptide essential for the autophagic process. Alternatively, this finding could more likely be ascribed to an increase in intracellular amino acids that results from the inhibition of synthesis (40). The cell will only internalize as much protein as it needs, but once sequestered, the protein must be fully degraded to maintain cellular homeostasis. This can explain why the administration of cycloheximide 1 h after leupeptin should block further sequestration of protein, the internalization of more proteolytic enzymes from primary lysosomes into autophagic vacuoles, and the inactivation and degradation of the proteins already sequestered.

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