Lysosomal Enzymes in Aortas of Species Susceptible and Resistant to Atherosclerosis[1] (36363)

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There have been numerous suggestions that a relationship may exist between lysosomal activity in the aorta and the development of atherosclerosis. The lysosome, thought of as a grouping of acid hydrolytic enzymes having a specific subcellular localization, is part of the body's cellular scavenging system. As such, lysosomes respond to excess protein, lipid, and mucopolysaccharide by hydrolyzing the material to more easily disposable units. Miller and Kothari (1) reported a definite increase of percent lysosome-like activity of β-glucuronidase, cathepsin, acid phosphatase, and arylsulfatase B in human atherosclerotic aortas. Curreri *et al.* (2) studied lysosomal enzymes in aortas of rabbits with cholesterol-induced atherosclerosis. There was a marked increase in activity of the lysosomal enzymes in the atherosclerotic aortas. Bonner, Kothari, and Miller (3), in a preliminary report observed higher lysosomal enzyme levels in aortas of some species resistant to atherosclerosis. The present paper gives a more detailed comparison of aorta lysosomal enzyme activity in several animal species. The enzymes studied were β-glucuronidase, acid phosphatase, arylsulfatase B, cathepsin D, cholesteryl ester hydrolase, and malate dehydrogenase. All are believed to be lysosomal with the exception of malate dehydrogenase which is a marker for mitochondria. Using density gradient techniques, Kothari, Bonner, and Miller (4) found human aortic cholesteryl ester hydrolase to satisfy the usual biophysical criteria for the lysosomal nature of an enzyme.

Materials and Methods. Chemicals were obtained from Sigma Chemical Co., St. Louis, MO or General Biochemicals, Chagrin Falls, OH. Aortas were obtained from Rockland, Gilbertsville, PA. The aortas were all from males. One-gram samples were employed in the study. For rat and guinea pig 15 aortas were required, for the rabbit two aortas, and for the dog and swine sections along the aorta were taken.

Preparation of Fractions. After thawing, aortas were rinsed in preparation media (5) to remove adhering blood. Intima and media of the aorta were homogenized in 0.25 *M* sucrose solution (1:10 w/v) in an ice-cooled, glass–glass, hand driven homogenizer. The whole homogenate was centrifuged at 2000*g* for 10 min using the Sorvall RC2-B centrifuge. The residue containing unbroken cells, nuclei, and cellular debris was discarded. The supernatant was centrifuged at 17000*g* for 10 min. The two 17000*g* fractions, the supernatant and the lysosome-like residue, were used for the study. The latter fraction was taken up in 0.1% Triton X-100 (1:5 w/v). Immediately prior to enzyme assay, both fractions were quick frozen and thawed 10 times in an acetone–dry ice bath.

Preparation of Substrates and Assay. All assays were performed at 37°, with exception of malate dehydrogenase which was assayed at room temperature, on 0.2 ml of enzyme preparation (0.5 ml for cholesteryl ester hydrolase).

β-glucuronidase activity was determined

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TABLE I. Total Lysosomal Enzyme Activity as Units[a]/g Tissue (± SD).[b]

Species	Beta-glucuronidase	Acid phosphatase	Arylsulfatase	Cathepsin	Cholesteryl ester hydrolase
Rat (8)[c]	209 ± 154	89 ± 19	1056 ± 352	7.6 ± 2.1	679 ± 38
Guinea pig (7)	134 ± 42	114 ± 21	465 ± 41	6.9 ± 1.4	471 ± 44
Dog (7)	59 ± 27	80 ± 29	2526 ± 228	4 ± 1.5	741 ± 20
Rabbit (6)	74 ± 19	46 ± 18	745 ± 156	5.1 ± 1.1	263 ± 19
Swine (5)	13 ± 4	61 ± 19	202 ± 58	4.3 ± 0.8	192 ± 40

[a] See text for definition of units.

[b] Intima-media preparations were homogenized in 0.25 *M* sucrose solution (1:10 w/v). Homogenates were fractionated at 17000*g*. Total activity is the sum of the supernatant and the lysosome-like residue activities. Incubation of 0.2–0.5 ml of enzyme preparation at 37° was in total volume of 1–2 ml for 2 to 6 hr.

[c] Number of samples.

by the method of Fishman *et al.* (6) as modified by Plaice (7). Incubation was 5 hr in 0.2 *M* acetate buffer pH 4.5 containing phenolphthalein glucuronide in a final concentration of 0.006 *M*.

The modified method of Valentine and Beck (8) was used in determining acid phosphatase activity. Incubation was 4 hr in 1.8 ml of 0.052 *M* Na-β-glycerophosphate and 0.05 *M* acetate buffer pH 5.0.

Arylsulfatase B activity was determined by the method of Roy (9) using 0.06 *M* nitrocatechol sulfate as substrate in 0.05 *M* acetate buffer pH 5.0 containing 1 *M* KCl with 5 hr incubation. The end product was determined by alkaline quinol reagent. The addition of phosphotungstic acid was omitted.

Cathepsin D activity was determined using 0.26 m*M* denatured hemoglobin in 0.1 *M* acetate buffer pH 3.6 with a 2-hr incubation based on the method of Anson (10). The product of reaction was measured on the filtrate by the method of Lowry *et al.* (11).

Cholesteryl ester hydrolase was determined according to the method of Vahouny, Kothari and Treadwell (12) using micellar solubilized cholesterol oleate. An emulsion in 0.154 *M* phosphate buffer pH 6.6 was prepared containing 2.56 μmol cholesterol oleate, 5 μmol sodium taurocholate, 7.7 μmol oleic acid, and 3 mg lecithin/ml of the mixture. The organic solvent was evaporated at 37° and the mixture homogenized. The emulsified substrate was then sonicated for 30 min or longer to near clarity. For each assay 0.25 ml of the substrate preparation was used in the final volume of 1.0 ml. Incubation was for 6 hr. The reaction was stopped and the lipids were extracted by addition of 5 ml acetone–ethanol (1:1 v/v). Cholesteryl ester and free cholesterol were determined by the method of Sperry and Webb (13).

Malate dehydrogenase was determined by the method of Mehler *et al.* (14) in which the rate of oxidation of NADH by an excess of oxaloacetic acid in the presence of enzyme preparation was measured by following the resulting decrease in absorbance at 340 nm.

Protein was determined by the method of Lowry *et al.* (11).

Definition of Units. The units for β-glucuronidase and arylsulfatase are the amount of enzyme hydrolyzing 1 μg of the substrate/hr under the conditions of assay. The units for acid phosphatase and cathepsin are the amount of enzyme hydrolyzing 1 nmol of the substrate/hr. For cholesteryl ester hydrolase, 1 unit represents the amount of enzyme catalyzing the production of 1 nmol of free cholesterol/hr. The unit for malate dehydrogenase is the amount of enzyme which causes the oxidation of 1 nmol of NADH/min under the specific conditions.

Results and Discussion. Total Lysosomal Enzyme Activity. Table I presents results obtained for the total lysosomal enzyme activity on a wet weight basis in the aortas of the species studied. The total activity is the sum of the 17000*g* supernatant activity and that released from the lysosome-like residue.

TABLE II. Total Lysosomal Enzyme Activity in Aortas as Units[a]/mg Protein (± SD).

Species	Beta-glucuronidase	Acid phosphatase	Arylsulfatase	Cathepsin	Cholesteryl ester hydrolase
Rat (8)[b]	9.9 ± 2.5	4.5 ± 1	60 ± 22	0.4 ± 0.1	40.4 ± 2
Guinea pig (7)	4 ± 1	3 ± 0.7	14.5 ± 2.7	0.25 ± 0.06	14 ± 3.2
Dog (7)	1.3 ± 0.4	1.7 ± 0.5	31.1 ± 8.2	0.1 ± 0.04	11.9 ± 3.2
Rabbit (6)	2 ± 0.51	1.3 ± 0.2	19 ± 7	0.15 ± 0.02	9 ± 2
Swine (5)	0.45 ± 0.6	1.9 ± 0.4	6.5 ± 2.3	0.13 ± 0.02	6.5 ± 2

[a] See text for definition of units.
[b] Number of samples.

Eight groups of rat aortas, 7 groups of guinea pig, 7 dog aortas, 6 groups of rabbit, and 5 swine aortas were studied.

As can be seen from Table I the total activity of lysosomal enzymes tends to vary by enzyme and species. Thus the cathepsin activity is low for all five species studied; arylsulfatase activity was very high in dog and rat and low in swine; acid phosphatase activity was of the same order of activity in all the aortas examined; and β-glucuronidase levels were high in the rat and guinea pig and quite low in swine aorta. Cholesteryl ester hydrolase activity was highest in the aortas of the two species most resistant to experimental atherosclerosis, the dog and rat, and lowest in the susceptible rabbit and swine. Table II presents lysosomal aortic enzyme activity on a per mg protein basis. The rankings of activities are generally similar to those observed in Table I. On the base of hydrolysis/mg protein all the rat aorta lysosomal enzymes were most active.

Table III summarizes the percentage of total lysosomal enzyme activity present in the 17000*g* pellet (lysosome-like fraction). The fraction of activity present in the pellet is similar for all species with only few exceptions. These are the low β-glucuronidase activity in the rat and guinea pig preparations and the low cathepsin and high arylsulfatase in the dog. The differences in total enzyme activities seen in Table I may represent leakage of enzyme during preparation or may be related to ease of release of the particular enzyme.

In Table IV the activity of the mitochondrial enzyme, malate dehydrogenase, in 750*g* supernatant fractions was measured. All five species, whether susceptible or resistant, had similar activity levels.

It has been shown with a given species that when the atherosclerotic process develops there is an increase in several of the lysosomal enzymes. This is true for human and rabbit as shown in our laboratory (1, 2). It seems reasonable to expect that a scavenging system such as the lysosomes would be ac-

TABLE III. Lysosome-Like Fraction—Percent Activity.[a]

Species	Beta-glucuronidase	Acid phosphatase	Arylsulfatase	Cathepsin	Cholesteryl ester hydrolase
Rat (8)[b]	9	11	12	13	14
Guinea pig (7)	9	10	11	14	12
Dog (7)	20	16	30	4	11
Rabbit (6)	18	9	10	19	14
Swine (5)	15	11	13	16	20

[a] Activities released from the 17000*g* lysosome-like fraction were measured. The residue was taken up in Triton X-100 (1:5 w/v), and quick frozen and thawed 10 times in an acetone-dry ice bath. Activity is expressed as percent activity in the 17000*g* lysosome-like fraction based on U/g tissue value.
[b] Number of samples.

TABLE IV. Malate Dehydrogenase Activity in 750*g* Supernatant.[a]

Species	Units/g tissue	Units/mg protein
Rat	84	2.21
Guinea pig	74	2.27
Dog	115	2.53
Rabbit	93	3.66
Swine	70	2.57

[a] Malate dehydrogenase activity was measured using 750*g* supernatant fractions of the species studied. The rate of oxidation of NADH by an excess of oxaloacetic acid in the presence of enzyme preparation was measured by following the resulting decrease in absorbance at 340 nm. One unit of activity is the amount of enzyme which causes the oxidation of 1 nmole of NADH/min under the specified conditions.

tivated to resist the development of a slowly progressive, chronic pathologic process such as atherosclerosis. Even more interesting is our finding that some aortic lysosomal enzymes appear to be set at a higher level in species resistant to spontaneous or induced atherosclerosis. Thus, the resistant rat showed a considerably higher activity of all lysosomal hydrolases than did either the susceptible rabbit or swine. The data suggest that the aortas of resistant species are better adapted to resist the chemical changes which represent the early atherosclerotic lesion.

Summary. Lysosomal enzyme activity in grossly normal aortas of species susceptible and resistant to atherosclerosis has been studied. β-Glucuronidase, cathepsin, acid phosphatase, arylsulfatase B, and cholesteryl ester hydrolase were the lysosomal-like enzymes measured. Resistant species, the rat, guinea pig and dog, showed higher total lysosomal enzyme activity than did the susceptible species, rabbit and swine. Using malate dehydrogenase as an indicator, no difference in mitochondrial enzyme activity was observed in the various species.

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