Plasminogen Activator Activity of Rat Lysosomes* (33429)

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Mammalian tissues have long been known to contain activators of blood fibrinolytic activity (1–7). Lack and Ali (8) found the plasminogen activator to be associated with lysosomes isolated from rabbit and mouse tissues. Increased permeability of the lysosomes following treatment with Triton X-100 heightened their plasminogen activator activity (9). Sugiyama extracted plasminogen activator in salt solutions from mitochondria, microsomes, and lysosomes isolated from rat brain, rat and rabbit kidney, and canine parotid glands. He measured the greatest plasminogen activator levels in the lysosomal and microsomal fractions of these tissues but did not isolate plasminogen activator from rat liver organelles.

Plasminogen is an abundant protein in mammalian blood. Its activation resembles that of trypsinogen, being enzymatically controlled and requiring partial hydrolytic digestion of the precursor molecule (11, 12). In searching for plasminogen activator in tissues one would logically seek a hydrolytic enzyme there. Lysosomes are intracellular organelles which contain a wealth of hydrolytic enzymes. Twelve such enzymes have been identified within lysosomes (13–16). Lipoprotein membranes form the boundary of the lysosomes, enclose the enzymes, regulate their release and presumably their activation. As long as the enzymes are retained by the lysosomal membrane they appear to be inactive. Interference with the stability of the lysosomal membranes will allow an increase of outflow of active lysosomal enzymes into the cytoplasm and body fluids.

In the present experiments we isolated cellular organelles from rat tissues and analyzed them for proteolytic and plasminogen activator activity. In the first study, rat liver mitochondria, lysosomes, microsomes, and a final supernate were assayed for proteolytic activity and plasminogen activator activity using a caseinolytic method. Also, an aliquot of the lysosomal fraction was labilized by sonification prior to undergoing these assays. Next the permeability of rat liver lysosomes was altered by subjection to alternate freezing and thawing. The plasminogen activator activity of the lysosomes was assessed before and after this treatment by a fibrinolytic assay.

Finally the plasminogen activator activity of lysosomes extracted from several rat tissues was measured using a fibrin-^{131}I clot assay. The acid phosphatase content of the lysosomal fractions obtained in this last experiment was determined. The percentage of acid phosphatase of each lysosomal fraction as compared to the total acid phosphatase activity, i.e., sedimentable and nonsedimentable activity, was used to evaluate the adequacy of lysosomal isolation in the various tissues.

Methods and Materials. Albino, male rats (Sprague-Dawley Strain) were maintained on Purina lab chow and water *ad libitum.* They varied in weight from 375–410 g. The rats were anesthetized by intraperitoneal injection of 0.5 ml of 6% Nembutal solution prior to tissue removal. One-g samples of tissue were homogenized in 3 ml of 0.25 *M* sucrose using a Potter-Elvehjem apparatus. The homogenate was subjected to the differential centrifugation method of De Duve *et al.* (13) to obtain mitochondrial, lysosomal, microsomal, and final supernate fractions from each tissue. Centrifugations were conducted at 4° in an RC-2 automatic, refrigerated Sorvall centrifuge. Total protein for 0.1 ml of the cell fractions was determined by the Hycel biuret procedure (Hycel Co., Houston, Texas). Six ml of the Hycel biuret reagent

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TABLE I. Plasminogen Activator Activity of Rat Liver Cell Fraction.

		% Casein digestion[a] resulting from		
Cell fraction	Total protein (g/100 ml)	Proteolytic activity[b]	Plasminogen activator activity[c]	Activation of rat serum plasminogen[d]
Mitochondria	0.51	2.4 (±0.05)	28.1 (±2.5)	22.5 (±1.5)
Microsomes	0.93	4.4 (±0.6)	29.4 (±2.6)	23.3 (±1.6)
Lysosomes: Plain	0.87	7.7 (±0.6)	35.7 (±2.2) p 0.001	30.0 (±1.6) p 0.001
Sonified	0.93	8.5 (±0.8)	40.8 (±2.8) p 0.001	36.1 (±1.9) p 0.001
Final supernate	0.55	3.4 (±0.7)	29.5 (±2.4)	23.0 (±1.6)

[a] Mean values for 12 rats per datum. Values for spontaneous caseinolysis were subtracted from the lysis values obtained with tissue fractions. Spontaneous caseinolysis averaged 8.2% (±1.3). The addition of plasminogen to casein-^{131}I did not increase this value (7.9% ± 1.4), nor did the addition of rat sera to combined plasminogen and casein-^{131}I.

[b] Cell fraction incubated with casein-^{131}I—to measure proteolytic activity of the fraction.

[c] Cell fraction incubated with bovine plasminogen in addition to casein-^{131}I. Activation of plasminogen by the cell fraction increases the caseinolysis of Column I.

[d] Cell fractions incubated with rat serum and casein-^{131}I. Activation of serum plasminogen by the cell fractions would also increase caseinolysis of Column I.

was added to 0.1 ml of tissue fraction. The mixture was incubated at 37° in a shaking water bath for 30 min and the optical density was read at 540 mμ.

In the first experiment, 0.1 ml of mitochondrial, lysosomal, microsomal, and final supernate fractions isolated from rat liver were tested for plasminogen activator activity or proteolytic activity. In addition half of the lysosome fraction was subjected to minimal sonification using a Branson Sonifier S-75 set at 7 A and applied to the suspension for approximately 5 sec. This procedure was included to increase the lability of the lysosomal membranes (17). Proteolytic activity was determined on 0.1 ml of the tissue fractions by the procedure previously described (18), i.e., lysis of casein-^{131}I was the basis for determining tissue fraction proteolytic activity. Plasminogen activator activity of tissue fractions was determined by the same method except that 0.1 ml of bovine plasminogen obtained from the Nutritional Biochemical Corporation (1 mg of plasminogen/ml of physiological saline) was added to casein-^{131}I prior to the addition of the tissue fraction. Spontaneous activation of plasminogen within rat serum was assessed by deleting the bovine plasminogen and adding 0.1 ml rat serum to casein-^{131}I in its place.

Fibrinolytic assays were also conducted (Table II and III) using the fibrin clot method of Alkjaersig and Fletcher (19). These clots were made with 0.4 ml of aged human blood plasma, 0.1 ml of bovine thrombin (50 NIH units/ml, Parke Davis Co.), 0.1 ml of fibrinogen-^{131}I and were enriched with 0.1 ml of bovine plasminogen (Nutritional Biochemical Corporation). The resulting clots were stable at room temperature and at 37° for more than 18 hr. Contamination with protease or plasminogen activator was not apparent. One-tenth ml of the tissue lysosome fractions was incubated with these clots in a shaking water bath for 30 min at 37°. The radioactivity of an aliquot of the lysed fluids was then determined in a deep-well scintillation detector. One set of fibrin clots was heated to 85° for 30 min to inactivate the plasminogen. Digestion of these clots would be a measure of fibrinolytic activity.

Alternate freezing and thawing of liver lysosomal fractions was conducted to increase their permeability (17) in the second and third experiments (Table II and III). Each fraction was subjected to 8 (Table II) and later 6 (Table III) of these sequences prior to measuring its plasminogen activator activity.

Maximum plasminogen activator activity

TABLE II. Fibrinolysis of Plasminogen Enriched Clots by Rat Liver Lysosomes.

	Total protein (g/100 ml)	% Fibrin-^{131}I lysed[a]	
		Plasminogen activator activity	% Maximum plasminogen activator activity[b]
Lysosomes: Plain	1.05	20.1 (±2.1)	30.8
Freeze–thawed	1.03	28.3 (±2.2)	43.3
Streptokinase[c]		65.3	100

[a] Mean values for 5 rats per datum.

[b] Fibrin lysed by the lysosomal fractions was determined as the percentage of the lysis induced by 600 units of streptokinase, a potent plasminogen activator.

[c] 600 units of streptokinase.

in Table II is arbitrarily taken as the degree of lysis of plasminogen enriched fibrin-^{131}I clots within the incubation period by 600 units of streptokinase (Nutritional Biochemical Corporation), a potent plasminogen activator. The plasminogen activation produced by 100 units of streptokinase was later found to yield approximately the same activator activity. This concentration was used in the experiments of Table III in determining maximum plasminogen activator activity.

Acid phosphatase activity was determined on the tissue homogenate and on the lysosomal fraction following its isolation from the homogenate using the method of Bessey *et al.* (20). The percentage of the acid phosphatase activity of the lysosomal fraction was determined in comparison with that of each component of the homogenate.

Controls for spontaneous caseinolysis, fibrinolysis, and bovine plasminogen activation were included. These consisted of the casein assay system or fibrin-^{131}I clot with 0.1 ml of physiological saline substituted for cell fractions and were otherwise treated as were the test assays. The percentage of spontaneous digestion in these controls was subtracted from that produced by the tissue fraction. Controls for fibrinolytic activity of tissue lysosomes consisted of plasminogen enriched fibrin,^{131}I clots incubated at 85° for 30 min in order to inactivate all plasminogen in the system. Lysosomes of the various organs used in these experiments (Table II and II) were incubated with these clots as well as the unheated ones. Lysis of the heated clots by the tissue fractions would be due to inherent fibrinolytic activity rather than plasminogen activator activity.

Result and Discussion. The mitochondria, lysosomes, microsomes, and final supernate separated from rat tissue homogenates were tested for proteolytic activity and plasminogen activator activity. As seen in Table I, minimal proteolytic activity was obtained for mitochondria, microsomes, and final supernate. Proteolytic activity of the rat liver lysosomes exceeded that of the other fractions. Sonification increased protein content and proteolytic activity of the lysosomes by less than 1%. When the cell fractions were incubated with plasminogen and casein-^{131}I, proteolysis was markedly increased following the 16-hr incubation period by 5- to 10-fold. Increased caseinolysis apparently reflected the degree of conversion of plasminogen to the proteolytic enzyme plasmin. Maximal activation of bovine plasminogen in the middle column of Table I and of plasminogen in rat serum (third column) was achieved by the lysosomal fractions. Mild sonification increased these values by about 5%. Bovine plasminogen activation by mitochondria, microsomes, and the final supernate was essentially the same in the bovine plasminogen assay indicating that soluble plasminogen activator had become equilibrated among the fractions separated from the original cell homogenates other than the lysosomes. Similarly, the activation of plasminogen inherent in rat serum was essentially the same for the cell fractions other than the lysosomes.

TABLE III. Plasminogen Activator Activity of Lysosomes Isolated from Rat Tissue.[a]

	Total protein (mg/100 ml)	% of total acid phosphatase activity[b]	% Lysis of plasminogen enriched clot[c]	% Maximum plasminogen activator activity[d]
Brain	1.12	63.1 (±3.5)	27.3 (±1.4)	49.8
Lung	0.32	21.8 (±3.0)	41.9 (±2.0)	76.5
Liver	0.82	57.3 (±2.0)	24.8 (±1.9)	45.3
Lymph node[e]	0.51	54.4 (±3.3)	28.0 (±1.8)	51.1
Spleen[f]	0.36	26.5 (±5.0)	25.4 (±1.5)	46.4
Kidney	0.47	26.0 (±5.5)	25.2 (±1.6)	46.0

[a] Mean values for 7 rats per datum. Lysosome extracted from 1 g of tissue and subjected to freeze–thaw 6 times.

[b] Acid phosphatase activity of lysosomal fraction determined as a percentage of the total activity for all the fractions of the tissue homogenate.

[c] Clots heated at 85° for 30 min prior to lysosome addition were not lysed by the lysosomal fraction tests. Lysis of unheated clots is reported here.

[d] Clot digestion by lysosomes determined as percentage of that induced by 100 units of streptokinase.

[e] Lysosomes extracted from 0.23 g of tissue.

[f] Lysosomes extracted from 0.85 g of tissue.

Again, plasminogen activator activity appeared to be equilibrated in all fractions but the lysosomes. In each case, the *p* value (student *t* test) for the difference in plasminogen activation by the lysosomes as compared to the other fractions was <0.001.

Digestion of fibrin-^{131}I clots containing human serum and enriched with added bovine plasminogen was readily achieved by incubation with lysosomes from rat liver (Table II). Eight sequences of freezing and thawing increased the availability of plasminogen activator activity of the rat liver lysosomes. Fibrin-^{131}I digestion was increased by 8.2% following this treatment. Lysis of plasminogen enriched clots by these lysosomal fractions was compared to that induced by 600 units of streptokinase in the right hand column. The latter was arbitrarily considered to cause maximum activation of the available plasminogen in the fibrin clots.

Finally lysosomes were separated from a variety of rat tissues by the same procedure used for liver lysosome separation (Table III). An indication of the adequacy of lysosome separation of the various tissue homogenate is given by the comparison of acid phosphatase measured in each fraction of the homogenate. De Duve (21) has reported that rat liver gives about the same ratio of sedimentable acid hydrolase activity as unsedimentable activity if the tissue homogenization has not been severe enough to seriously damage the lysosomes. The recovery of lysosomes in brain, liver, and lymph node tissues from rats appeared to be quite good since the acid phosphatase of these lysosomes was greater than 50% of the total activity.

The lower acid phosphatase activity of lung, spleen, and kidney may mean that the lysosomes of these tissues were not as well isolated by the method developed for liver lysosome separation. It is also quite possible that the fragility of the lysosome of these tissues exceeds that of the first group causing the release of their acid phosphatase from the sedimentable fraction into the nonsedimentable component of the homogenate prior to analysis. Indeed De Duve (21) had found splenic lysosomes to be highly sensitive to mechanical breakage induced by a Potter-Elvehjem homogenizer. In addition to the trauma of separation, these lysosomes also were repeatedly frozen and thawed 6 times to increase their permeability. Plasminogen enriched clots were readily digested by lysosomes from all the tissues studies whereas none digested the heated clots. Therefore none of the lysosomal fractions registered fibrinolytic activity using the heated clot assay. The plasminogen activator activity was especially marked in the lung lysosomes. Lung has previously been reported to show strong fibrinolytic activator activity (4). Since lysosomes were extracted from only 0.23 g of lymph node, their plasminogen activation content was remarkably high.

It seems probable then that lysosomes of mammalian tissues are an important source of plasminogen activator. Its release is controlled by the stability of the binding membrane. The outflow of this activator into the cytoplasm and ultimately its body fluids may be the prime regulator of fibrinolysis in blood.

Summary. Rat tissues have been fractionated in order to assay their plasminogen activator activity. Mitochondria, microsomes, and the final supernate separated from rat liver were essentially equal in this activity suggesting that the soluble plasminogen activator in the cell homogenate had equilibrated among them. Lysosomes showed significantly greater plasminogen activator activity than the other fractions. The plasminogen activator release from lysosomes could be increased by sonification and freezing and thawing. When the lysosomes of a series of six tissues were compared, lung lysosomes were richest in plasminogen activator. The activator activity was approximately the same for lysosomes of rat spleen, kidney, liver, brain, and lymph node. Proteolytic activity of rat liver cell fractions was less than ¼ that measured in the presence of plasminogen. Fibrinolytic activity of cell fractions measured with heated fibrin clots was negligible.

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