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Hyaline membrane disease lung plasminogen prematurity respiratory distress syndrome

Pulmonary Plasminogen-Activator Activity in Hyaline Membrane Disease

A Reevaluation on Human, Bovine, and Rabbit Fibrin Substrates

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Extract

Plasminogen-activator activity was measured in lungs from 7 newborn premature infants examined at autopsy (control lungs) and 11 infants dying of hyaline membrane disease (hyaline membrane lungs) and was reevaluated by using human and rabbit fibrin substrates in addition to the traditional bovine fibrin substrate. The whole homogenate and the supernatant and sediment fractions from the lungs of the normal infants were active when tested on unheated human, bovine, and rabbit fibrin plates. In comparison, 10 of the 11 specimens of hyaline membrane lungs failed to lyse either bovine or human fibrin when the whole homogenate or the supernatant or sediment fractions were tested. Rabbit fibrin substrate was digested by all the hyaline membrane lung homogenates, but only after a prolonged incubation period. Mixtures of the supernatant fraction from any of the 10 hyaline membrane specimens, plus equal volumes of lung homogenate from a normal infant, resulted in total inhibition of normal plasminogen-activator activity.

Mixture of 0.05 ml 2M KSCN with 0.1 ml normal lung homogenate enhanced plasminogen-activator activity by approximately 80% using human, bovine, or rabbit fibrin substrates. The addition of thiocyanate also induced plasminogen-activator activity of homogenates from 11 hyaline-membrane lungs. Three of these 11 specimens caused lysis of heated human fibrin plates in the presence of thiocyanate, indicating direct protease activity.

Extraction of the hyaline-membrane lungs with thiocyanate by the method of ASTRUP and ALBRECHTSEN[5] revealed plasminogen-activator activity in all specimens. None of the thiocyanate extracts caused lysis of heated fibrin plates. Thus, the direct proteolytic activity, induced in some lung homogenates by the addition of thiocyanate, did not survive the acid precipitation phase of the thiocyanate extraction procedure.

Saline extracts (supernatants) of 11 hyaline membrane and 7 control lungs were tested on unheated and heated human fibrin plates with the addition of either 2M KSCN or water to determine whether thiocyanate enhanced activator activity by increasing the solubility of the enzyme or by direct enhancement of enzyme activity. Activator activity of the supernatant fractions from 7 control lungs was enhanced by the addition of 2M KSCN; three of the 7 specimens showed a low degree of protease activity on heated plates. The enhancement of soluble enzyme activity in the controls indicated a direct effect of the electrolyte on enzyme activity. Hyaline membrane lungs, however, failed to develop plasminogen-activator activity in 8 of 11 supernatant fractions, and 2 of the 3 specimens that did develop lytic activity also lysed heated fibrin. It appeared, therefore, that the majority of hyaline-membrane lungs lacked saline-soluble plasminogen activator in contrast to control lungs. Acid precipitates prepared from the inactive saline extracts of hyaline membrane lungs and dissolved

in 2M KSCN also lacked activator activity, in contrast to the presence of such activity in normal lungs. This finding suggested that the activator was absent from saline extracts of hyaline membrane lung rather than being merely inhibited.

It is concluded that the plasminogen activator from the lungs of human infants has no preference for human fibrin substrate over bovine or rabbit fibrin. The tissue activator of plasminogen could not be readily extracted from lung, although normal infant lung does contain a saline-soluble fraction. The activator could be solubilized more readily in 2M KSCN, but activity was also greatly enhanced by the presence of thiocyanate or other electrolytes in high concentration. The high ionic strengths may enhance enzyme activity directly, or may reduce the effect of an inhibitor normally present in tissues. In contrast to normal lung homogenates, hyaline membrane lungs are mostly inactive against either human or bovine fibrin substrates. Plasminogen-activator activity can be manifest in hyaline membrane lungs by adding 2M thiocyanate to the lung homogenates or by extracting the lung tissue with thiocyanate. The effect of a strong inhibitor in hyaline membrane lung is apparently overcome by an increase in ionic strength or by the thiocyanate extraction procedure. By adding electrolyte to induce plasminogen-activator activity and by testing acid precipitates of saline extracts, the absence of saline-soluble plasminogen activator is shown to be the most consistent abnormality of fibrinolysis in lungs with hyaline-membranes.

Speculation

The deficiency of intrapulmonary fibrinolysis associated with pulmonary hyaline membrane formation may reflect immediate response by the organism to inflammation or stress. Inhibition of plasminogen activation within the alveolar spaces of the lung, however, may be disastrous since any retained precipitate is compressed into compact, obstructive hyaline membranes.

Introduction

A deficiency of fibrinolytic activity has been detected in the lungs of newborn infants in association with hyaline membrane formation (hyaline membrane lung) [12–14]. This deficiency was caused by an inhibitor in these lungs of the tissue activator of plasminogen that could be detected in the supernatant and sediment fractions of lung homogenates. Normal placental tissue also contains an inhibitor of plasminogen activation; this inhibitor resembles that detected in hyaline membrane lung in that it has the ability to attach to the sediment fraction of a normal lung homogenate. It has been postulated that damage to the placenta from anoxia or acidosis caused release of the inhibitor from the placenta into the blood stream and eventual adsorption by lung tissue in the fetus [14].

The assays of plasminogen activator on which these conclusions and postulates have been based were performed with a bovine fibrin substrate. Subsequently, AMBRUS *et al.* [2] using a human fibrin substrate, were unable to detect a deficiency of plasminogen activator in hyaline membrane lung. The enzyme characteristics described by these investigators, however, suggested that they may not have been measuring the tissue activator of plasminogen [3, 16]. Alternatively, it was suggested that the contradictory results obtained by them could have been the result of their use of human fibrin substrate. A comparison of the two substrates for measuring pulmonary plasminogen activator and a reevaluation of such activity in hyaline membrane lung was indicated. The stability of the tissue activator of plasminogen in frozen specimens allowed this reappraisal of enzyme activity in lungs stored over a number of years.

Materials and Methods

Materials

Lungs from 7 normal premature infants (normal lungs) and 11 infants with pulmonary hyaline membrane disease (hyaline membrane lungs) were obtained at autopsy and stored at −20° for approximately five to seven years.

Bovine fibrinogen [23], 'dried human fibrinogen' [24], rabbit fibrinogen [25], and bovine thrombin [26] were used in the studies.

Preparation of Fibrin Plates

Fibrin plates were prepared from human, bovine, or rabbit fibrinogen in 9.0 cm diameter flat-bottomed petri dishes by adding 0.3 ml of 0.2 % bovine thrombin (dissolved in a 50:50 mixture of 0.1 M borate buffer at pH 8.0 and glycerin containing 5 mg calcium chloride per ml) to 10.0 ml 0.4 % fibrinogen in 0.1 M borate buffer at pH 8.0. The plates were swirled immediately to mix and kept at room temperature for two to four hours before use, or stored at 4° for a period as long as one week. Some fibrin plates were heated at 85° for 30 minutes to destroy intrinsic plasminogen.

Fibrin plates prepared from rabbit fibrinogen were not as firm as the clots resulting from human or bovine fibrinogen and could not be stored longer than 24 to 48 hours without disintegration during the assay. When heated at 85° for 30 minutes, the rabbit clots exuded much of the water content. Rabbit fibrinogen, therefore, was not easily utilized for fibrin-plate assay techniques.

Preparation of Lung Homogenates

A ten percent suspension of lung tissue (wet weight/volume) was prepared by grinding the lung in ice-cold physiological saline with a Potter Elvehjem type ground glass homogenizer. A portion of the homogenate was centrifuged at 30,000 × g for 20 minutes at 4°. The supernatant fraction was removed and the sediment washed by repeating the three previous steps. The washed sediment was finally resuspended in saline to the original volume. Thus, the whole homogenate, supernatant fraction, washed sediment, and the final wash water were available for enzymatic assay.

Thiocyanate Extraction of Plasminogen Activator

Potassium thiocyanate extracts of lung tissue were prepared by the method of ASTRUP and ALBRECHTSEN [5]. The final acid precipitates were dissolved in 2M KSCN and brought to neutral pH with crystals of sodium bicarbonate.

Assay of Plasminogen Activator

A sample of lung homogenate, 0.1 ml, was pipetted onto human or bovine fibrin plates. These were incubated at 37° for 22 hours. Using rabbit fibrin substrate, only 0.05 ml of the enzyme preparation was pipetted onto three individual fibrin plates for each assay to allow more room for lysis. Fibrinolysis was more rapid with this substrate. Activity was quantitated in square millimeters by multiplying cross diameters of the area of lysis. Direct fibrinolytic activity was detected on heated fibrin plates incubated at 37° for periods as long as seven days.

The effects of various electrolytes upon plasminogen activator were studied by adding 0.05 ml (0.025 ml for rabbit fibrin plates) of electrolyte solutions to the drop of lung homogenate on the fibrin plate. Distilled water served as a control. Activity was measured as described above.

The effect of calcium concentration on plasminogen-activator activity was investigated by varying the concentration of calcium chloride in the thrombin solution used for preparing the fibrin plates.

The inhibitor to plasminogen activator in hyaline membrane lungs was detected by mixing equal amounts of supernatant fraction from hyaline-membrane lung, or saline as control, with whole homogenate of normal infant lung and assaying 0.1 ml aliquots of the mixtures on unheated fibrin plates.

Results

Plasminogen-Activator Activity of Lung Fractions on Various Fibrin Substrates

Data concerning the presence or absence of plasminogen-activator activity in homogenates of normal and hyaline membrane lung are summarized in table I. The whole homogenate and the supernatant and sediment fractions from 7 normal lungs were active on

Table I. Effect of plasminogen activator of normal and hyaline membrane lung on bovine, human, and rabbit fibrin substrates

Specimen	Plasminogen-activator activity		
	Bovine fibrin	Human fibrin	Rabbit fibrin
Normal infant lung (7 specimens)			
Whole homogenate	+	+	+[1]
Supernatant fraction	+	+	+
Sediment fraction	+	+	+
Hyaline-membrane lung (10 specimens)			
Whole homogenate	0	0	+[2]
Supernatant fraction	0	0	+
Sediment fraction	0	0	+
Hyaline-membrane supernatant (10 specimens) plus normal infant lung homogenate	0	0	

[1] Marked lysis by all normal lung fractions on rabbit fibrin within 5 hours incubation.

[2] Definite lysis by hyaline membrane lung fractions on rabbit fibrin only after an incubation period of 48 hours.

Table II. Effect of 2M KSCN upon fibrinolytic activity of lung homogenates

	Specimen	Plasminogen-activator activity			Fibrinolytic activity (heated fibrin plates)		
		Whole homogenate	Whole homogenate +2M KSCN	KSCN extract	Whole homogenate	Whole homogenate +2M KSCN	KSCN extract
		mm²			mm²		
Normal infant lung	A	448	803	850	0	0	0
Hyaline membrane lung	1	0	221 (at 96 h)	486	0	0	0
	2	0	375 (at 48 h)	275	0	0	0
	3	0	381	499	0	0	0
	4	0	558	489	0	440	0
	5	0	394	592	0	0	0
	6	0	314 (at 48 h)	511	0	0	0
	7	0	335	594	0	0	0
	8	0	164 (at 48 h)	473	0	0	0
	9	0	435	700	0	221	0
	10	0	178	594	0	0	0
	11	221	600	621	110	400	0

unheated human, bovine, and rabbit fibrin plates. None of these specimens caused lysis of heated fibrin plates. In comparison, 10 of 11 specimens of hyaline membrane lung failed to lyse either bovine or human fibrin when the whole homogenate or the supernatant or sediment fractions were tested. Rabbit fibrin substrate, however, was digested by all homogenates of hyaline membrane lung, but only after an incubation period of 48 hours. Normal lungs caused marked lysis of rabbit fibrin within 5 hours. Mixtures of the supernatant fraction from any of the 10 hyaline membrane lungs, plus equal volumes of homogenate from normal lung, resulted in total inhibition of normal plasminogen-activator activity.

The eleventh specimen of hyaline membrane lung caused lysis of both bovine and human fibrin plates, but this specimen also caused lysis of heated fibrin, indicating the presence of a protease.

Effect of Thiocyanate on Pulmonary Plasminogen-Activator Activity

Mixture of 0.1 ml normal lung homogenate with 0.05 ml 2M KSCN enhanced plasminogen-activator activity by approximately 80 % on human fibrin substrates (table II). Activity was enhanced in a similar fashion on bovine and rabbit fibrin by thiocyanate or other electrolytes (NaCl, NaBr, NaI). These electrolytes alone had no effect on unheated fibrin containing calcium [18].

Eleven homogenates of hyaline membrane lung were mixed with 2M KSCN and tested on unheated and heated human fibrin plates (table II). Seven of the 11 specimens induced lysis of the unheated fibrin in the presence of thiocyanate during an incubation period of 24 hours; three others after 48 hours, and a single specimen after 96 hours. Three of the 11 specimens of hyaline membrane lung also caused lysis of heated human fibrin plates in the presence of thiocyanate, indicating direct protease activity. These three specimens were otherwise unable to lyse heated fibrin in the absence of added electrolyte.

Thiocyanate Extraction of Plasminogen-Activator

Extraction of hyaline membrane lungs with thiocyanate by the method of ASTRUP and ALBRECHTSEN [5] revealed plasminogen-activator activity in all specimens (table II). The specimens that developed slow activity (48–96 hours) when 2M KSCN was merely mixed with the lung homogenate now caused fibrinolysis by 24 hours. None of the thiocyanate extracts, however, caused lysis of heated fibrin plates, whereas three of these lungs lysed heated fibrin when thiocyanate was merely mixed with the homogenate. Thus, the direct proteolytic activity, induced in some lung homogenates

Table III. Effect of 2M KSCN upon fibrinolytic activity in homogenates of the soluble fraction of normal and hyaline membrane lungs

	Lung specimen	Plasminogen-activator activity		Fibrinolytic activity (heated fibrin plates)	
		Lung super. $+H_2O$	Lung super. +KSCN	Lung super. $+H_2O$	Lung super. +KSCN
		mm^2		mm^2	
Normal infant lung	A	187	380	0	0
	B	Trace	276	0	0
	C	100	393	0	0
	D	144	509	0	0
	E	161	387	0	100 (at 168 h)
	F	169	611	0	183 (at 168 h)
	G	209	539	0	100 (at 168 h)
Hyaline membrane lung	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
	4	Trace	235	0	400 (at 72 h)
	5	0	163	0	0
	6	0	0	0	0
	7	0	0	0	0
	8	0	0	0	0
	9	0	0	0	0
	10	0	0	0	0
	11	273	429	80	100 (at 168 h)

by thiocyanate, did not survive the acid precipitation phase of the thiocyanate extraction procedure.

Effect of Thiocyanate upon Saline-Soluble Plasminogen-Activator

In order to determine whether thiocyanate increased activator activity by increasing the solubility of the enzyme or by direct enhancement of enzyme activity, the clear supernatant (saline extract) fractions of 11 hyaline membrane and 7 control lungs were tested on unheated and heated human fibrin plates with the addition of either 2M KSCN or water to the drop of extract on the fibrin plates. The activator activity of the supernatant fractions from the 7 control lungs was enhanced by the addition of 2M KSCN (table III). Three of the 7 specimens, however, showed a low degree of protease activity on heated plates with the addition of KSCN. The hyaline membrane lungs, however, failed to develop plasminogen-activator activity in 8 of 11 supernatant fractions; two of the three hyaline membrane specimens that did develop lytic activity also lysed heated fibrin. It appeared, therefore, that the majority of hyaline membrane lungs lacked the saline-soluble plasminogen activator present in control lungs. Activity of the soluble enzyme in the controls was enhanced by thiocyanate, indicating a direct effect of the electrolyte on enzyme activity.

Acid precipitates were prepared from the inactive saline extracts of hyaline membrane lungs and dissolved in 2M KSCN. These preparations lacked activator activity in contrast to the presence of such activity in acid precipitates from normal lungs. This finding suggested that the activator was missing from the saline extracts, not merely inhibited.

Effect of Calcium on Plasminogen-Activator Activity

The lytic activity of normal lung homogenate was markedly accelerated when assayed on unheated human and bovine fibrin plates in the absence of added calcium. Whole homogenates of hyaline membrane lung, however, still lacked activity on unheated fibrin plates in the absence of added calcium.

Discussion

The plasminogen activator from the lungs of human infants has no preference for human fibrin substrate over bovine or rabbit fibrin. The differences in activity of these substrates probably result from variations in plasminogen content of the fibrinogen preparation. Similarly, sensitivity of the assay may also vary with different batches of fibrinogen from a single species. The rabbit fibrin used in this study was extremely sensitive for the detection of activator activity and was found to have a high plasminogen content [18], but this substrate was unstable and not easily utilized for this assay.

The tissue activator of plasminogen cannot be extracted readily from lung [6], although normal infant lung does contain a saline-soluble fraction. The activator can be solubilized more readily in 2M KSCN [5], and activity is greatly enhanced by thiocyanate or high concentrations of other electrolytes. The increase in activity resulting from the addition of electrolyte, however, is not merely the result of increased solubility of the enzyme, since activity of the saline-soluble enzyme fraction is also enhanced by the electrolytes. The higher ionic strength may enhance enzyme activity directly or may reduce the effect of an inhibitor normally present in the tissue. Addition of calcium ion, in contrast to other electrolytes, reduces plasminogen-activator activity of human lung. Calcium however, is needed to stabilize the fibrin clot and to prevent nonenzymatic lysis of the clot [18].

In contrast to normal lung homogenates, most hyaline membrane lungs were found to be inactive when either human or bovine fibrin substrates were used, and we were able thus to reproduce our previous results with these lungs and to extend our observations to include data with human fibrin substrate. Rabbit fibrin substrate was dissolved by hyaline membrane lungs, but at an extremely slow rate (48 hours), in contrast to the rapid action (less than 5 hours) of normal infant lung.

Plasminogen-activator activity could be manifest in hyaline membrane lungs by adding 2M thiocyanate to the lung homogenates, or by extracting the lung tissue with thiocyanate. This finding confirms that the activator is present in these lungs, but is bound to an inhibitor of plasminogen activation. The effect of the inhibitor is apparently overcome by increases in ionic strength or by extraction with thiocyanate.

Another aspect of deficient fibrinolysis in hyaline membrane lungs involves the absence of saline-soluble plasminogen-activator activity. In an earlier study [14], 13 of 16 lungs with hyaline membranes were reported to lack uninhibited activator activity, but 2 of the remaining 3 specimens lacked only saline-soluble enzyme activity. In this study, the addition of electrolyte to induce plasminogen-activator activity and the testing of acid precipitates of saline extracts have shown that the absence of saline-soluble plasminogen activator is the most consistent abnormality of fibrinolysis in hyaline membrane lungs. ALBRECHTSEN [1] has reported that the activity obtained in saline extracts reflects the immediate availability of plasminogen activator for the organism.

In three instances, the mere addition of 2M KSCN to a lung homogenate induced true proteolytic (fibrinolytic) activity, whereas acid-precipitated thiocyanate extracts of these lungs did not have such activity. This fibrinolytic activity is probably of leukocytic origin; leukocytes contain a fibrinolytic enzyme that is activated by high electrolyte concentration [17]. ASTRUP *et al.* [7] have found that a leukocytic fibrinolysin is destroyed during the acid precipitation phase of thiocyanate extraction. These studies suggest that the presence or absence of plasminogen activator can be evaluated more readily by increasing the ionic strength of the tissue homogenate to approximately 0.67 at the time of assay. If these tissue preparations are also active on heated fibrin plates, assay of the acid precipitate from a thiocyanate extract will differentiate activator activity from that of a leukocytic fibrinolysin. Whole tissue homogenates, however, must be tested without added electrolyte to reveal the presence of an inhibitor to the plasminogen activator.

Studies by other investigators [4, 13, 20] have failed to confirm our earlier reports of a defect in plasminogen-activator activity in the majority of lungs with hyaline-membrane formation. These authors did not attempt to repeat our experiments precisely. SANDBERG *et al.* [20] reported that three specimens of hyaline membrane lung, *extracted with thiocyanate*, showed normal pulmonary plasminogen-activator activity on bovine fibrin plates, leading them to deny an enzymatic deficiency. These investigators, in addition, tried to demonstrate the presence of an inhibitor in the thiocyanate extracts by mixing concentrates of these extracts with thiocyanate extracts of various tissues. They did not realize, however, that the inhibitor was inactivated by the thiocyanate extraction procedure [14] and could not be detected by use of this methodology.

KLOSS and LIBAL [11] were also 'unable to reproduce our findings', but they used an assay technique in which Streptokinase (Varidase) was added to the assay mixture. This technique would not necessarily measure plasminogen-activator activity, since Streptokinase will activate any residual proactivator in tissue preparation and will result in lysis of the fibrin clot. The authors state, however, that they only *occasionally* observed fibrinolysis without addition of Streptokinase,

implying that they may indeed have observed the same deficiency reported by us.

In still another conflicting report, AMBRUS *et al.* [2] were unable to substantiate our findings of a deficiency of plasminogen activator in the lungs of infants with hyaline membrane disease by using human fibrin substrate rather than bovine fibrin. They did report that the activity assayed was rapidly dissipated during the interval between death and autopsy and during storage at 4°. The tissue-activator of plasminogen, however, is stable indefinitely (to at least 7 years in the freezer). The activity measured by these investigators was probably a labile 'tissue lysokinase' that would require the presence of a human proactivator for its fibrinolytic effect and, therefore, would be unmeasurable using bovine fibrin. A lysokinase will not activate plasminogen directly; it must activate a proactivator present in human, but not in bovine, blood [21]. Thus, the presence of a labile 'lysokinase' was not detected in our studies of fresh tissue by use of bovine fibrin substrate. The current study, in which we utilized tissue stored in the freezer for a number of years, reveals the presence of the same inhibition detected initially and confirms our first observations. The tissue 'lysokinase' that AMBRUS *et al.* must have detected is thought by ALBRECHTSEN [1] to originate solely from the blood present in the organs, so that an assay of the 'true tissue activator' would be more indicative of the fibrinolytic potential in lung than would a measure of tissue (blood) 'lysokinase' activity.

A number of other reports by investigators who used a bovine fibrin plate assay similar to that employed by us confirms our observations that a fibrinolytic defect exists in association with pulmonary hyaline membrane formation [8–10, 15]. The deficiency of plasminogen-activator activity in the lungs of infants with hyaline-membrane disease is obviously not the primary cause of this condition [15], but this deficiency could play a role in the retention of intrapulmonary fibrin deposits and the formation of hyaline membranes. Knowledge of the fibrinolytic defect in hyaline membrane disease provides better rational for the therapeutic approach to the disease employing fibrinolytic enzymes [4].

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