The Action and Neutralization of a Renal Lipid Inhibitor of Erythropoietin[1] (36043)

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Numerous studies have shown that patients with chronic renal disease are incapable of producing enough erythropoietin to adjust their rate of red cell production to the need for oxygen in the tissues. Administration of erythropoietin to such patients would undoubtedly alleviate this maladjustment; but, so far, it has been almost impossible to procure enough erythropoietin for therapeutic trials, much less for maintenance therapy. Far larger quantities are needed than can be obtained from serum and urine, our usual sources for erythropoietin, and it is necessary that erythropoietin be isolated from its source of production. Unfortunately, attempts to isolate erythropoietin from kidney homogenate, the logical source of large scale isolation, have not been very successful. This failure has been explained by suggesting that the kidney produces an erythropoietin-generating enzyme (REF) rather than erythropoietin itself and that erythropoietin is produced in blood by the action of this enzyme on an inactive circulating pro-erythropoietin (1). Although this hypothesis is supported by a considerable amount of experimental data, the interpretation of these data is difficult and there may be room for alternate hypotheses (2). One of these suggests that erythropoietin is present in renal homogenate but in an inactive form because of binding to an inhibitor (3).

There is substantial evidence now for the existence of inhibitors to erythropoietin. Inhibition has been clearly demonstrated with tissue homogenates (4–6). In these investigations much emphasis has been placed on the kidney in view of its important role in the production or release of erythropoietin; however, liver and spleen homogenates also inhibit erythropoietin but bone marrow apparently does not (6).

At the present time, several kinds of inhibitors to erythropoietin or to REF have been described; among these are a basic urinary protein (7), liver histones but not kidney and spleen histones (8), uremic plasma (9), a methanol–acetone extract of uremic serum (10), and a calcium-dependent renal factor that interferes with the erythropoietin-generating system (11).

The existence of a lipid renal inhibitor which acts directly on erythropoietin has been described from our laboratory (3, 12) and the present report deals with its kinetics as an erythropoietin inhibitor and its interaction with normal serum.

Material and Methods. Erythropoietin. Human urinary erythropoietin with a specific activity of 80 units/mg of protein was provided by the National Institutes of Health through the courtesy of the Committee on Erythropoietin.

Renal inhibitor. Washed, decapsulated normal rabbit kidneys (Pel-Freeze) were homogenized in a Waring Blender with 0.5 vol of distilled water per unit weight of tissue. The homogenate was sonicated (Heat Systems Co. Sonifier), strained and lyophilized. The lyophilized powder was then extracted with 2.5 vol (per original weight) distilled ether for 30 min. The extraction was repeated four times, the supernates were filtered through a Whatman No. 2 paper, pooled, and ether was removed by rotary vacuum evaporation. One gram of fresh kidney yielded about 27 mg of lipid material. Further extraction was carried out with 0.5 vol (per original weight) of dis-

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TABLE I. Renal Inhibitor of Erythropoietin (E); Ether and Acetone Solubility.

	^{59}Fe 66 hr utilization (%)
Saline control	0.13 (23)[a]
E, 1 unit	13.4 (28)
E (1 unit) + crude renal homogenate (residue), 0.5 g	0.13 (43)
+ ether-soluble fraction, 14 mg	0.27 (35)
+ ether-insoluble fraction	11.4 (8)
+ acetone extract of ether-soluble fraction, 10 mg	0.19 (52)

[a] Numbers in parentheses designate assays performed.

tilled acetone for 30 min. The extraction was repeated four times, the supernate was decanted, pooled, filtered, and acetone was removed by rotary vacuum distillation. The residue was dissolved in ether, washed three times with 0.2 vol of 0.45% NaCl and once with distilled H_2O, and a final acetone-soluble fraction with a yield of 20 mg/original 1 g of fresh kidney was obtained. All solvents were redistilled; butylated hydroxy toluene was added to the solvents to prevent oxidation (13).

Thin-layer chromatography was performed on silica gel: for neutral lipids with the Applied Science procedure; and for phospholipids, according to the technique described by Marinetti (14). Amino acid analyses were performed on a Technicon Amino Acid Analyzer (15). TAMe hydrolysis was measured with a Radiometer recording pH-Stat.

Incubation procedure. The lipid inhibitor, suspended in 0.5 ml of saline, was mixed with 0.5 ml of saline containing either erythropoietin or normal fresh rabbit serum. The mixture was emulsified by sonication using a continuous 30 sec burst of 20 W energy provided by a microhorn attachment to a Sonifier cell disrupter (Heat Systems Co.) set at Output Control No. 1 with a meter reading of 50–60 divisions. Following sonication, the mixture was incubated for 30 min at 37° in a water bath. Subsequent exposure to erythropoietin or fresh normal rabbit serum was accomplished by adding 1 ml of saline suspension of these materials, repeat sonication for 30 sec and repeat incubation for 30 min at 37° in a water bath. The final 2 ml emulsion containing 1 unit of added erythropoietin was assayed in hypertransfused mice.

Assay. Randomly bred Swiss Webster female mice received 0.9 ml of packed mouse red cells ip on days 0 and 1. On days 6 and 7 they received 1 ml of the incubation mixture; on day 8, 0.5 μCi of ^{59}Fe and on day 11, they were sacrificed and the 66 hr utilization was determined, using 7% of body weight as their blood volume. Four mice were used for each assay, and all results from mice with final hematocrit of less than 55% were discarded. In order to express the degree of inhibition, inhibitor units were defined as the difference between 1 unit and the measured erythropoietic activity in units of the assayed material.

Results. a. Lipid renal inhibitor: Table I shows the inhibitory effect of crude renal homogenate and the fractions obtained by lipid extraction. The ether- and acetone-

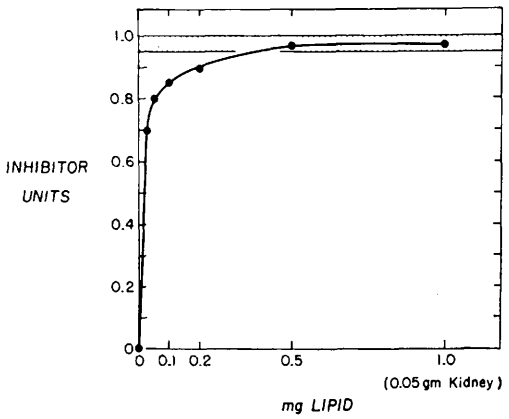

FIG. 1. Inhibition of erythropietin by renal lipid inhibitor (30 min incubation): A dose–response curve of the renal lipid inhibitor. The inhibitor was mixed with 1 unit of erythropoietin, sonicated for 30 sec, and incubated at 37° for 30 min prior assay. The crosshatched area designates that the remaining erythropoietic activity in this range cannot be demonstrated accurately by the assay method.

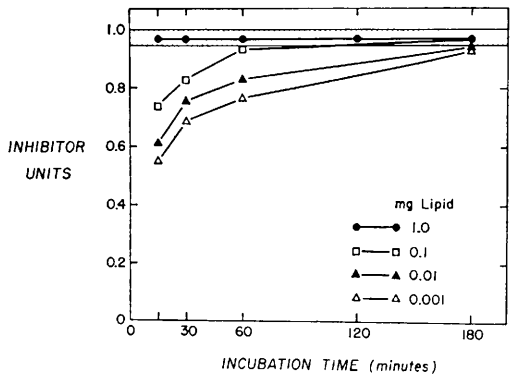

FIG. 2. Inhibition of erythropoietin by renal lipid inhibitor: Inhibitory activity of lipid on 1 unit of erythropoietin at various times of incubation.

soluble material was found (thin-layer chromatography) to contain a variety of neutral lipids but only minimal amounts of phospholipid. Lecithin was also present in the active material. Approximately 0.2 μM of amino acid was obtained from a 6 N HCl hydrolysate of 100 mg of lipid inhibitor. TAMe (tosylarginine methyl ester) was not hydrolyzed by the lipid inhibitor at pH 8.

b. Kinetics of inhibitor activity. 1. When progressively smaller amounts of the lipid inhibitor were incubated for 30 min with erythropoietin, it was found that complete inhibition of 1 unit of erythropoietin could be accomplished by about 0.5 mg of inhibitor (Fig. 1). This corresponds to about 25 mg of kidney tissue.

2. Figure 2 shows the effect of incubation on degree of inhibition. Prolonged incubation with much smaller amounts of inhibitor (0.01 and 0.001 mg) resulted in almost complete inhibition of 1 unit of erythropoietin.

3. Incubation at room temperature or at 4° was found to be about as effective as incubation at 37° (Table II).

c. Inactivation of inhibition by serum. Preincubation of the inhibitor with fresh normal rabbit serum in volumes as small as 0.01 ml was found to cause loss of the inhibitor activity of the lipid material (Table III).

d. Release of erythropoietin from the erythropoietin–lipid complex by serum. 1. Normal fresh rabbit serum released some erythropoietin from the erythropoietin–lipid complex. When this complex was formed by 30 min incubation, the maximal release of erythropoietin after 30 min incubation with serum was about 0.2 units. Incubation with 1 ml or with 0.1 ml of serum released about the same amount of erythropoietin and longer incubation (up to 180 min) did not result in further release. Smaller amounts of serum, however, released progressively less erythropoietic activity (Table IV).

2. When the erythropoietin–lipid complex was formed by 180 min incubation, 0.1 ml of serum incubated with the complex for either 30 min or 180 min failed to release any erythropoietin activity (Table IV).

Discussion. Studies reported previously have shown the existence of an erythropoietic inhibitor in homogenate prepared from normal rabbit kidneys (4). The present report confirms these studies and shows the inhibitor to be ether- and acetone-soluble and most probably of lipid-nature. The possibility that it is a proteolytic enzyme attached to a lipid moiety has not been entirely ruled out, although esterolytic activity against TAMe is absent and the process of inactivation was not temperature dependent. Amino acid analysis disclosed the presence of trace amounts of amino acids. The significance of this observation is not clear but the possibility remains that polypeptide material might be present.

The capacity of this inhibitor to inactivate erythropoietin is considerable, with about 0.01 mg of lipid inactivating the erythropoietic activity of 1 unit of erythropoietin after 3 hr of incubation. Since 0.01 mg of lipid is iso-

TABLE II. Renal Inhibitor of Erythropoietin (E); Effect of Temperature (30 min incubation).

	^{59}Fe 66 hr utilization (%)		
(°):	37	25	4
E (1 unit) + lipid inhibitor, 0.1 mg	3.75 (7)	4.32 (9)	3.25 (9)[a]

[a] Numbers in parentheses designate assays performed.

TABLE III. Neutralization of Inhibitor by Serum.

	^{59}Fe 66 hr utilization (%)
Erythropoietin, 1 unit	
+ lipid inhibitor (1 mg) + saline	0.15 (8)[a]
+ serum, 0.1 ml	13.66 (7)
0.01 ml	4.39 (8)
0.001 ml	1.76 (8)

[a] Numbers in parentheses designate assays performed.

lated from 0.5 mg of renal tissue, a normal rabbit kidney weighing 10 g could theoretically contain 20,000 units of bound and erythropoietically inactive erythropoietin.

The possibility that large amounts of erythropoietin may be present in kidney tissue in an inactive form but potentially recoverable provides a considerable challenge since our needs for erythropoietin far outstrip the present supply from serum or urine. In order to test this possibility, it was first necessary to develop a method by which the bond between erythropoietin and the lipid inhibitors could be broken. Simple lipid extraction of the erythropoietin–lipid complex did not regenerate active erythropoietin. Neither did changes in pH or exposure to pancreatic lipase or to 6 *M* urea. However, incubation with fresh rabbit serum was shown to result in the recovery of about 20% of added erythropoietin. Further studies revealed that this recovery seemed related to the ability of serum to inactivate some inhibitor activity of the lipid. Furthermore, re-extraction of lipid by ether after an initial incubation with serum disclosed that the lipid had lost most of its inhibitory activity.

If the lipid and erythropoietin were permitted to interact for more than the usual 0.5 hr at 37°, the releasing effect of serum was completely abolished suggesting that either erythropoietin is being irreversibly destroyed or inactivated by prolonged exposure to the lipid inhibitor, or that the bond produced is too firm or too concealed to be broken by serum. Despite these difficult problems, the finding of an interaction between erythropoietin and a renal lipid may be of importance for our understanding of the physiologic regulation of red cell production and for development of large-scale isolation of erythropoietin.

Summary. A potent inhibitor of erythropoietin was isolated from rabbit renal homogenates and found to be soluble in ether and acetone. It contained primarily neutral lipids and only trace amounts of phospholipids and amino acids. Its inhibitory activity was not temperature dependent and it was almost completely neutralized by the addition of

TABLE IV. Release of Erythropoietin by Serum.

	^{59}Fe 66 hr utilization (%)
Lipid inhibitor (1 mg) + erythropoietin, 1 unit	
Incubation, 30 min	
+ incubation (30 min) with saline	0.15 (8)[a]
serum, 1 ml	2.28 (8)
0.1 ml	2.80 (7)
0.01 ml	0.84 (8)
0.001 ml	0.27 (7)
+ incubation (180 min) with serum, 0.1 ml	2.36 (4)
Lipid inhibitor (1 mg) + erythropoietin, 1 unit	
Incubation, 180 min	
+ incubation (30 min) with serum, 0.1 ml	0.25 (8)
+ incubation (180 min) with serum, 0.1 ml	0.16 (8)

[a] Numbers in parentheses designate assays performed.

small amounts of normal rabbit serum. When serum was added to the inactive erythropoietin–lipid mixture, some, but not all, erythropoietin could be recovered. These results suggest that erythropoietin may be inactivated by a renal lipid during extraction procedures, thus accounting for the fact that renal extracts are erythropoietically inactive.

1. Gordon, A. S., Cooper, G. W., and Zanjani, E. D., Seminars Hematol. **4**, 337 (1967).

2. Erslev, A. J., and Kazal, L. A., Blood **34**, 222 (1969).

3. Erslev, A. J., Kazal, L. A., and Miller, O. P., Proc. Symp. Erythropoieticum, Prague, 1970, in press.

4. Kuratowska, Z., Ann. N. Y. Acad. Sci. **149**, 128 (1968).

5. Fisher, J. W., Porteus, D. D., Hirashima, K., and Tso, S. C., Proc. Soc. Exp. Biol. Med. **122**, 1015 (1966).

6. Erslev, A. J., and Kazal, L. A., Proc. Exp. Biol. Med. **129**, 845 (1968).

7. Lewis, J. P., Neal, W. A., Moores, R. R., Gardner, E., Jr., Alford, D. A., Smith, L. L., Wright, C-S., and Welch, E. T., J. Lab. Clin. Med. **74**, 608 (1969).

8. Kuratowska, Z., and Kopec, M., Brit. J. Haematol. **16**, 465 (1969).

9. Fisher, J. W., Hatch, F. E., Roh, B. L., Allen, R. C. and Kelley, B. J., Blood **31**, 440 (1968).

10. Kuroyanagi, T., and Saito, M., Tohoku J. Exp. Med. **88**, 117 (1966).

11. Zanjani, E. D., Contrera, J. F., Cooper, G. W., Gordan, A. S. and Wong, K. K., Science **156**, 1367 (1967).

12. Kazal, L. A., Erslev, A. J., and Miller, O. P., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **29**, 628 (1970).

13. Neudoerffer, T. S., and Lea, C. H., J. Chromatogr. **21**, 138 (1966).

14. Marinetti, G. V., J. Lipid Res. **3**, 1 (1962).

15. Hamilton, P. B., Anal. Chem. **35**, 2055 (1963).

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