Extrarenal Sites of Erythrogenin Production[1] (37309)

STEPHEN M. KAPLAN, SUSAN A. ROTHMANN,[2] ALBERT S. GORDON, IRA A. RAPPAPORT,[2] JAMES F. CAMISCOLI, AND CESARE PESCHLE

Laboratory of Experimental Hematology, Department of Biology, Graduate School of Arts and Science, New York University, New York, New York 10003; and the Institutes of Medical Pathology (I), Universities of Naples and Rome, Italy

Although it is now accepted that the kidney is the chief site of production or activation of erythropoietin (Ep) (1–3), the mechanisms underlying its formation have only recently come under close scrutiny. In 1966 (4, 5), our laboratory reported on the extraction of a principle from the "light-mitochondrial" fraction of hypoxic rat kidneys which, although not erythropoietically active when administered alone, engendered the production of Ep when incubated *in vitro* with normal rat serum (NRS) (6). This principle has been termed the renal erythropoietic factor (REF) or more recently erythrogenin (7).

Attention has been given recently to the phenomenon of extrarenal production of Ep (8). The Ep response to hypoxia in adult nephrectomized rats has been estimated to be approximately 10% that exhibited by unoperated controls (9). A recent finding of significance relates to the fact that the Ep response to hypoxia in nephrectomized rats decreases with increasing times elapsing between renal ablation and exposure to hypoxia (equivalent to 0.45 atm of air) (10, 11). In view of findings that more severe degrees of hypoxia evoke higher levels of plasma Ep in nephrectomized rats than lower intensities (12, 13) and since injections of erythrogenin maintain plasma Ep levels in hypoxic nephrectomized rats (13), the possibility was tested that the liver, implicated as an extrarenal source of Ep (14–16), might respond to severe hypoxia by producing erythrogenin. The possible existence of erythrogenin in the spleens of these animals was also examined. Finally, the levels of erythrogenin were ascertained in the kidneys, livers and spleens of unoperated rats subjected to a similar intensity and duration of hypoxia.

Materials and Methods. Young male rats (Long-Evans strain, 4–5 wk of age weighing 130–160 g) were bilaterally nephrectomized and allowed to recover from surgery for 1 hr. Following the recovery period, the animals were exposed to hypoxia (0.35 atm of air) for 6 hr. Upon termination of the hypoxic period, the animals were immediately decapitated, exsanguinated, and the livers and spleens were removed and immersed in cold 0.25 *M* sucrose. From this point on, all procedures were carried out in the cold (4°). The livers and spleens (and kidneys in controls) were minced in cold 0.25 *M* sucrose which was discarded and replaced by fresh sucrose in a volume of 10 times the original wet weight of each tissue. The tissues were homogenized and centrifuged according to the scheme of Gordon and Zanjani (17) with a modification which eliminated the final centrifugation step after the freeze–thaw procedure. The final pellet was resuspended in distilled water and frozen until assayed. On the day of assay 6 ml of the "light-mitochondrial" extract from each tissue were incubated with 6 ml of saline or NRS. Before use, serum was dialyzed against Na ethylenediaminetetraacetate (EDTA) for 24 hr and then against deionized water for 48 hr. This dialysis procedure prevents the operation of a cation-dependent Ep-inactivating system present in the incubation mixture

[1] Supported by U.S. Public Health Service Research Grant 5-R01-HL03357-16 and a grant from the CNR, Italy.

[2] Predoctoral trainee of the National Institutes of Health (Training Grant 5-T01-HL05645-07).

(18). All dialysis procedures were carried out at 4°. The incubations were conducted for 60 min at 37°. Assay of the incubation mixtures for Ep were carried out in the ex-hypoxic polycythemic mouse (19). Each mouse (5 mice/group) received an ip injection of 2 ml of extract derived from 0.5 to 0.6 g starting wet tissue on the third day posthypoxia; 0.5 μCi of ^{59}Fe (ferrous citrate) was administered intravenously on the fifth day and the percentage RBC-^{59}Fe incorporation values determined on Day 7. The p values were estimated from the distribution of Fisher's t (20).

Results. It will be noted from Table I that considerable erythropoiesis-stimulating activity was generated upon incubation of NRS with the extracts of the light-mitochondrial fractions (erythrogenin) of both livers and spleens of young nephrectomized male rats subjected to severe hypoxia. That erythrogenin was actually being estimated in these organs was indicated by the fact that the incubation mixtures of serum and organ extracts from the hypoxic nephrectomized rats generated significantly higher ($p < 0.05$) erythropoietic activity than the relatively inactive saline–organ extract incubation mixtures. It is of interest that the erythropoiesis-stimulating activity of the liver and spleen extracts from hypoxic nephrectomized rats was not significantly different ($p > 0.05$) from that of kidneys from unoperated hypoxic rats (Table I) when each was incubated with NRS.

Table I also indicates that the liver and spleen extracts of intact hypoxic rats also evoked detectable erythropoiesis-stimulating effects when incubated with NRS. However, this activity was significantly lower ($p < 0.05$) than that of the liver and spleen extract–NRS incubation mixtures from anephric hypoxic rats and the kidney extract–NRS incubation mixtures from intact hypoxic rats.

Discussion. The results of the present study indicate clearly that both the liver and spleen are implicated in extrarenal production of Ep. Thus subjection of young nephrectomized rats to severe hypoxia results in the appearance of highly significant quantities of erythrogenin in the light-mitochondrial ex-

TABLE I. Mean (± SEM) Erythrogenin (Eg) Values in Livers and Spleens of Hypoxic Rats.[a]

Procedure	Liver Eg +:		Spleen Eg +:		Kidney Eg +:		Ep standard (IRP units)		
	Saline	NRS[b]	Saline	NRS	Saline	NRS	0.05	0.20	NRS alone
1. Nephrect. + 0.35[c] atm (6 hr)	1.81 ±0.49	8.30 ±1.61	2.42 ±0.75	10.98 ± 1.31	—	—	3.35 ±0.82	16.81 ± 4.27	1.41 ±0.40
2. Unoper. controls[c] + 0.35 atm (6 hr)	1.29 ±0.49	3.30 ±0.68	1.36 ±0.29	4.11 ± 0.46	1.63 ±0.29	9.11 ±1.63			

[a] Measured as quantity of Ep generated (% ^{59}Fe Inc.) in the exhypoxic polycythemic mouse assay. Values based on 4 separate experiments run at 4 different times.
[b] Normal rat serum.
[c] Fifteen rats per group.

tracts of these organs. The necessity for utilizing severe hypoxia to stimulate sustained production of Ep in anephric rats (12, 13) suggests that the oxygen sensor mechanism for extrarenal erythrogenin and Ep production is less sensitive to oxygen deficiency than is the renal site(s). These heightened levels of erythrogenin most likely account for ability of the renoprival rat to maintain a significant Ep response to intense hypoxia (13).

The young rat was selected for these studies because of previous reports that nephrectomized rats in this age and body weight range more effectively maintain their plasma Ep levels in response to oxygen deficiency than do adult animals (11). This is in accord with findings that young rats possess more potent extrarenal sources of Ep than do adults (21). Further support for this contention is seen from the present results which indicate that hypoxia also induces a slight increase in the erythrogenin content of livers and spleens of young non-nephrectomized rats. However, the erythrogenin elevation in these 2 organs is not as great as that noted in the nephrectomized rat thus suggesting that renal ablation serves as a stimulus for extrarenal production of this factor in response to hypoxia.

The precise tissues within the liver and spleen that serve as extrarenal sites of erythrogenin production remain conjectural. Since both organs represent rich loci of reticuloendothelial cells, it is possible that this system constitutes the extrarenal source. Experiments testing the effects of reticuloendothelial stimulants and depressants on renal and extrarenal production of erythrogenin and Ep are in progress.

Summary. Exposure of young nephrectomized male rats to severe hypoxia results in the appearance of highly significant quantities of erythrogenin in the liver and spleen. The amounts evoked in these 2 organs are greater than in the livers and spleens, and approximately equal to those in the kidneys of young male non-nephrectomized rats exposed to a similar degree of hypoxia. This indicates that nephrectomy serves to potentiate extrarenal erythrogenin production in response to hypoxia. The results also suggest that similar biosynthetic pathways operate in renal and extrarenal production of Ep.

1. Krantz, S. B., and Jacobson, L. O., "Erythropoietin and the Regulation of Erythropoiesis," p. 25. Univ. of Chicago Press, Chicago (1970).

2. Fisher, J. W., Pharmacol. Rev. **24**, 459 (1972).

3. Gordon, A. S., and Zanjani, E. D., *in* "Advances in Internal Medicine" (G. H. Stollerman, ed.), Vol. 18, p. 39. Year Book Med. Pub., Chicago (1972).

4. Contrera, J. F., and Gordon, A. S., Science **152**, 653 (1966).

5. Contrera, J. F., Gordon, A. S., and Weintraub, A. H., Blood **28**, 330 (1966).

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

7. Gordon, A. S., Zanjani, E. D., and McLaurin, W. D., *in* "Renal Pharmacology" (J. W. Fisher, ed.), p. 141. Appleton-Century-Crofts, New York (1971).

8. Mirand, E. A., and Murphy, G. P., *in* "Regulation of Hematopoiesis" (A. S. Gordon, ed.), Vol. 1, p. 495. Appleton-Century-Crofts, New York (1970).

9. Jacobson, L. O., Marks, E. K., Gaston, E. O., and Goldwasser, E., Blood **14**, 635 (1959).

10. Peschle, C., Sasso, G. F., Rossanigo, F., Mastroberardino, G., and Lipparini, F., Riv. Med. Aeronaut. Spaz. **34**, 24 (1971).

11. Schooley, J. C., and Mahlmann, L. J., Blood **39**, 31 (1972).

12. Peschle, C., Sasso, G. F., Rappaport, I. A., and Condorelli, M., J. Lab. Clin. Med. **79**, 950 (1972).

13. Peschle, C., Rappaport, I. A., Sasso, G. F., Chiariello, M., Condorelli, M., and Gordon, A. S., Prog. 15th Annu. Meet. Amer. Soc. Hematol. p. 104 (1972).

14. Reissmann, K. R., and Nomura, T., *in* "Erythropoiesis" (L. O. Jacobson and M. Doyle, eds.), p. 71. Grune and Stratton, New York (1962).

15. Katz, R., Cooper, G. W., Gordon, A. S., and Zanjani, E. D., Ann. N. Y. Acad. Sci. **149**, 120 (1968).

16. Fried, W., Blood **40**, 671 (1972).

17. Gordon, A. S., and Zanjani, E. D., *in* "Hemopoietic Cellular Proliferation" (F. Stohlman, Jr., ed.), p. 97. Grune and Stratton, New York (1970).

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

19. Camiscoli, J. F., and Gordon, A. S., *in* "Regulation of Hematopoiesis" (A. S. Gordon, ed.), Vol. 1, p. 369. Appleton-Century-Crofts, New York (1970).

20. Snedecor, G. W., "Statistical Methods." Iowa State College Press, Ames (1956).
21. Carmena, A. O., Howard, D., and Stohlman, F., Jr., Blood **32**, 376 (1968).

Received Dec. 27, 1972. P.S.E.B.M., 1973, Vol. 143.