The Renal Erythropoietic Factor (REF). IX. Its Subcellular Distribution* (33695)

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Hypotonic extracts of light mitochondrial and microsomal fractions of kidneys from normal, phenylhydrazine-treated and hypoxic rats contain a principle (the renal erythropoietic factor, REF) which, although not erythropoietic when administered alone, engenders the production of erythropoietin (ESF) when incubated *in vitro* with normal serum (1–4). Kinetic studies suggest that the REF is an enzyme which acts on a protein substrate in serum to produce the ESF (5).

The presence of ESF-generating activity in both the light mitochondrial and microsomal subcellular fractions of the kidney (4) is interpretable in two ways: (a) the REF is formed in subcellular particles of both fractions, or (b) it is made in one fraction and stored in the other. The present studies were performed in a preliminary attempt to differentiate between these two possibilities.

Materials and Methods. Groups of five male Long-Evans rats (200–250 g) were exposed to 0.45 atm of air for varying times ranging from 0.25 to 22.5 hr. Immediately after interruption of hypoxic stimulation, the rats were bled by cardiac puncture under ether anesthesia, and the kidneys were collected. Heparinized plasma was obtained and frozen until used.

The kidneys were kept cold until homogenized. They were cut into small pieces with scissors and homogenized in 0.25 *M* sucrose (10 ml/g of kidney) in a loose and then in a tight-fitting homogenizer. The resulting homogenate was centrifuged at 6300*g* for 10 min. The supernatant fluid was removed, recentrifuged at 21,000*g* for 20 min and the precipitate (light mitochondrial fraction) was collected. The supernatant fluid was recentrifuged at 45,000*g* for 30 min and the precipitate (premicrosomal fraction) was removed and discarded. The supernatant fluid derived here was recentrifuged at 105,000*g* for 45 min and the precipitate (microsomal fraction) was collected. The light mitochondrial and the microsomal fractions were rehomogenized in distilled water (2 ml/g of kidney) with a drop of detergent (Cutscum) and frozen until assayed.

Before assay the material was thawed and centrifuged at 37,000*g* for 10 min, and the precipitate was discarded. The resulting supernatant fluids were used in the incubations described below.

The incubation procedure consisted of mixing equal volumes of either light mitochondrial extract (LME) or microsomal extract (MCE) with EDTA-dialyzed normal serum (3) for 60 min at 37°. At the end of this period, the incubation mixtures were assayed for erythropoietic activity in the hypoxia-induced polycythemic mouse (6). Plasmas obtained at the different times of hypoxia were also assayed. Four to five mice were used to test each sample. Each mouse received a single 2 ml i.p. injection of the incubation mixture or a single 1 ml i.p. injection of plasma on day 3 posthypoxia. On day 5, they were injected i.v. with 0.5 μCi ^{59}Fe in 0.2 ml of saline. Percentage RBC-radioiron incorporation values were estimated on day 7. These values were converted into ESF units by reference to the dose–response curve for the International Reference Preparation for ESF (6). Using this system, data from separate assays became comparable. Most points were assayed two to three times. Other points were checked by exposing additional donor rats to the same duration of hypoxic stimulation.

Protein determinations on all incubation mixtures were made using the Biuret

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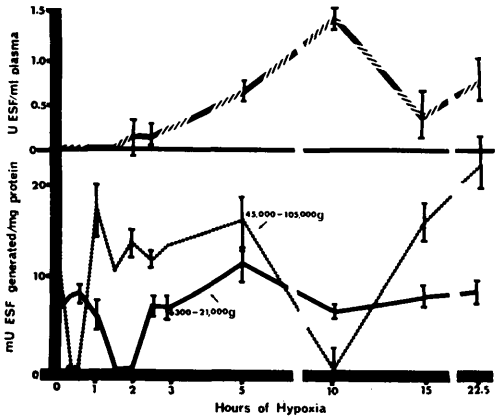

FIG. 1. ESF-generating activity/mg of protein of extracts of renal fractions and ESF present in plasma of rats during a 48-hr exposure to hypoxia. Values are given as milliunits of ESF generated/mg of renal extract (6300–21,000g is the light mitochondrial fraction and 48,000–105,000g is the microsomal fraction). All extracts of renal fractions were incubated with equal amounts of EDTA-dialyzed serum for 60 min. Activity is that present in 2 ml of the incubate. Vertical lines through the mean values represent ± 1 SEM.

procedure (7). The specific activity of the kidney samples was calculated as the milliunits of ESF generated/2 ml of incubate (containing 1 ml of REF) divided by the mg of protein/1 ml of the RED.

Results. The intermediate kidney fraction (premicrosomal) was collected and discarded to preclude contamination between the higher and lower sedimenting fractions. The specific activity of the REF in extracts of the light mitochondrial and microsomal fractions with increasing times of hypoxic exposure, was related to the quantities of ESF present in the plasma (Fig. 1). These REF values were similar to those obtained for total activity (not taking amount of protein into consideration), but it was felt that specific activity calculations were more desirable since the concentration of protein differed among the kidney fractions.

At 15 min of hypoxic stimulation, ESF-generating activity was present only in the LME. By 1 hr, activity was detectable in the MCE and remained present here through the fifth hour of exposure. On the other hand, from 1.5 to 2 hr, the LME was devoid of activity. At 2.5 hr ESF-generating activity was again detectable in the LME, and activity persisted through 22.5 hr. The REF activity underwent a diminution in the MCE from hours 5 to 10 of hypoxia. Thereafter it began to increase, attaining a maximum value at 22.5 hr.

ESF did not appear in the plasma until after 2 hr of hypoxia. Following this, the ESF titers rose steadily until a maximum was attained at 10 hr. The quantities decreased by 15 hr and thereafter began to rise again.

Discussion. The results indicate fluctuations in the quantity of the REF in two different subcellular fractions of rat kidney during 22.5 hr of continuous exposure to hypoxia. It would seem unlikely that the REF is generated and stored in both fractions of the kidney, since if this were the case, a differential appearance in time of the substance during hypoxic stimulation would not be expected to occur.

The observed data are interpretable in terms of two mechanisms: (a) the REF is made in the light mitochondrial fraction and is then transported to the microsomal fraction where it is stored and later released; and (b) the REF is produced in the microsomal fraction and is then conducted to the light mitochondrial fraction where it is stored and subsequently released.

In this regard, our unpublished electron microscope studies reveal the light mitochondrial fraction to contain an occasional mitochondrion, some lysosomes and mostly microbodies or the peroxisomes of de Duve (8). Further treatment of the light mitochondrial fraction with sucrose gradient centrifugation by a modification of the method of Leighton *et al.* (9) showed the REF-active fraction to be composed almost exclusively of peroxisomes. The microsomal fraction contains elements derived from the rough-surfaced portion of the endoplasmic reticulum. Experiments with pancreatic tissue (10–12) have shown that alpha-chymotrypsinogen is synthesized in or on ribonucleoprotein particles attached to the membrane of the endoplasmic reticulum, following which it migrates through the channels of the reticulum to the

Golgi zone where it is enveloped with membrane to become zymogen granules. Other experiments (13) have demonstrated that the enzyme ribonuclease is synthesized first in the microsomal fraction of the mouse pancreas.

Since the REF appears to be an enzyme which generates ESF (5), it seems possible that it is formed, as are pancreatic enzymes (10–13), on the membranes of the endoplasmic reticulum. Thus the following mechanism is suggested for the production and distribution of the REF during hypoxic stimulation: (a) exposure to hypoxia results first in an emergency shunting the REF from the microsomal to the light mitochondrial fraction where it becomes incorporated mostly into the peroxisomes. Thus by 0.25 hr of hypoxic stimulation, little REF is detectable in the microsomal fraction and at this time the light mitochondrial fraction contains the material. By 1.5 hr no REF remains in the light mitochondrial fraction which remains depleted until more enzyme is formed and migrates there from the endoplasmic reticulum of the microsomal fraction; and (b) continued exposure results in a more prolonged stimulation of generation of new REF in the ribosomal complex of the endoplasmic reticulum in the microsomal fraction with continued migration occurring from there to the peroxisomes of the light mitochondrial fraction.

Summary. The appearance of the REF in the light mitochondrial and microsomal fractions of rat kidney was studied during a continuous 22.5 hr exposure to hypoxia. Within 15 min a depletion of the REF occurred in the microsomal fraction (105,000*g*) but ESF-generating activity was detectable at this time in the light mitochondrial fraction (21,000*g*). Beginning with 1 hr of hypoxia, REF activity reappeared in the microsomal but decreased in the light mitochondrial fraction. At 10 hrs, REF activity was again present in the light mitochondrial but not in the microsomal fraction. With further exposure, the REF was again evident in both fractions up to 22.5 hr. Plasma ESF values rose steadily from hours 2 to 10 followed by a decrease at hour 15 and a secondary rise at 22.5 hours. It is suggested that the REF is generated in the microsomal fraction from which the factor is transported to the peroxisomes contained within the light mitochondrial fraction.

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