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IMMUNOREACTIVE ERYTHROPOIETIN CONCENTRATIONS IN NEONATAL RATS
AND THE EFFECTS OF HYPOXIA

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ABSTRACT

Immunoreactive erythropoietin (Ep) was measured in intact neonatal normoxic and hypoxic (0.5 atm for 18 hrs) rats daily from birth to weaning and compared to the adult rat. Plasma and tissue Ep levels varied greatly during the first three weeks regardless of whether the animals were hypoxic or not. With the exception of the first and ninth day of life, circulating Ep levels were higher than adult levels in normal newborn rats. Neonatal rats responded to hypoxia with increasing Ep levels, and the response improved with age such that during the third week of life plasma Ep levels were significantly higher than measured in adult hypoxic rats. No sex difference in male and female response to hypoxia could be documented until sexual maturity (day 42). In the normoxic neonatal rat more Ep originated from the liver than the kidneys until day 10, while under hypoxic conditions the switch occurred as early as 2 days after birth. These present studies suggest that renal erythropoietin production, both in normoxic and hypoxic newborn intact rats, occurs at an earlier age than had been previously shown.

INTRODUCTION

Several studies have shown that the neonatal rat is able to increase circulating Ep levels in response to hypoxic hypoxia (1-4). On the other hand, erythropoiesis cannot be further stimulated in the newborn rat by exposure to hypoxia or exogenous Ep administration (5-8), nor can erythropoiesis be suppressed in these young animals by starvation, bilateral nephrectomy or hypertransfusion (1,2,9-11). Since the kidney is considered to be the primary organ responsible for Ep production in adult mammals (12), several authors concluded that erythropoiesis in the neonatal rat may be independent of renal Ep during the first three weeks of life. These above studies were carried out
in several different laboratories, using animals at different ages and atmospheric pressures for varying lengths of time, and the samples were analyzed primarily in the in vivo polycythemic mouse assay.

The high sensitivity of the radioimmunoassay and the relatively small sample size required for analysis made this study possible. We attempted to find answers to the following questions: What are the circulating, hepatic and renal Ep levels in normal neonates measured daily through the first three weeks of life and when they attain sexual maturity (day 42)? How are these Ep levels altered by exposure to hypoxic hypoxia? Can immunoreactive Ep be measured in milk obtained from hypoxic lactating rats, and can it be transferred to the neonatal animals? Since we established earlier (13) that in the fetal rat the liver is the main Ep producing tissue, we attempted to determine the age at which the switch from the liver to the kidney occurred in both the normal and hypoxic neonatal rat.

MATERIALS AND METHODS

Animals: Pregnant Sprague-Dawley rats were obtained from Simonson Laboratory, Gilroy, CA. The animals arrived 7 days before the expected delivery and were housed with free access to food and water. Two days before the presumed delivery date the cages were checked every two hours from 7 am to 8 pm, and the time and litter sizes were recorded. Approximately 75% of the deliveries occurred between 7 am and 2 pm. The litter sizes ranged from 4 to 15 pups and were adjusted to 8-10 per mother in order to assure equal nutritional status for the neonates during the experiment. At the age of 10 days, mothers were selected for hypoxia each day such that the same number of male and female pups could be exposed.

Experimental Design: Mothers and their pups were exposed to hypoxia of 0.5 atm
for 18 hrs (3 pm - 9 am) while for each day an age-matched group served as controls. The animals were anaesthetized with ether and weighed. Throughout the experiment the hypoxic pups were killed first. Blood and tissues were obtained immediately after hypoxia, and the total collection time was less than one hour (9-10 am). During this time the tissues were stored on ice in small covered dishes in order to reduce possible degradation and evaporation and homogenized as fast as possible, although this step was slower than obtaining the tissues. While these tissues were processed, the control animals were killed during the next hour (10-11 am) and the tissues handled in an identical manner. From 1 to 10 days of age, blood was collected in heparinized hematocrit tubes after decapitation. Older animals were bled by heart puncture. Samples had to be pooled to make analysis by RIA possible. From day 1 to 21 six pools were generated daily for the control and six for the hypoxic groups: in the latter group six pools were obtained for males and females separately from day 10 to 21. On day 42 analysis was possible on individual animals and 8 animals were used in each group; i.e. 8 control, 8 hypoxic male and 8 hypoxic female rats. During the first four days 4 animals were pooled for analysis, a total of 24 rats were used for the control and hypoxic groups, respectively. From days 5-7 we pooled three animals; i.e. 18 rats for each group per day, and thereafter, two animals were sufficient. From 10-21 days of age we used a total of 12 control, 12 hypoxic male and 12 hypoxic female rat in order to generate 6 pools for each group. The kidneys and whole liver tissues were excised and pooled corresponding to the pups whose plasmas had been pooled. Salivary glands were collected and combined for all animals in each group, i.e., controls, hypoxic (days 1-9), and hypoxic males and females (days 10-21 and 42). All tissues were weighed and homogenized in 0.05 M phosphate buffer (pH 7.5) with 5% fetal bovine serum (w/v), centrifuged at 10,000 x g for
15 minutes in an Eppendorf Micro Centrifuge (Sybron-Brinkman Instruments). Plasma and tissue homogenates were frozen and stored at -20°C until RIA analysis.

Since the neonatal animals were exposed to hypoxia with their mothers, it had to be ascertained whether the levels measured in the pups could have been derived from the mother via the milk. In order to do this, two groups of experiments were carried out. In the first experiment three lactating rats were removed from their litters and exposed to hypoxia overnight for 18 hours. After returning to ambient pressure, they and three control rats were anaesthetized with pentobarbital (60 mg/kg i.p.) and lactation was induced by injection of pitocin (4-5 U/animal), obtained from Sigma Chemical Co., St. Louis, MO. Within 5-10 minutes milk could be obtained slowly by mild suction through a glass pipette connected to a vacuum source.

In a second experiment groups of pups at the age of seven days were separated from their mothers. Three adults were exposed to hypoxia overnight while three others were kept in the colony at ambient pressure. At 8 am the pups were returned for nursing for three hours to their respective groups of mothers. The animals were killed three hours later and plasma, kidneys and liver tissues from the young animals were collected and analyzed for Ep content.

Immunoreactive erythropoietin concentrations were measured by RIA (14). The reference preparation was a previously bioassayed rat serum pool obtained from animals exposed to hypoxia. The lower sensitivity of this assay is approximately 4 mU/ml, interassay variation is < 12% and intraassay variation < 9%. All samples were initially analyzed in single 100 µl aliquots and repeated in two separate RIAs in duplicate at the appropriate dilutions which could be compared to the standard curves. The data presented here are the means of the
four determinations from the latter two assays. In addition, some plasmas and kidney tissue homogenates were serially diluted as a check for parallelism. Results were analyzed with the sigmoid computer program developed by Rodbard and Hutt (15) on the LBL CDC 7600 Computer.

RESULTS

The results of this study are shown both in graphic and tabulated form. Plasma Ep levels in normoxic neonatal rats vary profoundly during the first three weeks of life (Table 1 and Fig. 1). With the exception of day 1 and 9, the circulating levels were significantly higher than those measured in 42 day old and adult rats. Peak values of 190 mU/ml were measured on day 10 and 20. Liver tissue Ep concentrations rose five-fold on the second day of life and are most likely responsible for the increase of Ep observed in plasma. Thereafter, every plasma peak also coincided with elevated renal Ep concentrations, the most pronounced one occurring on day ten. After the second day of life, liver Ep levels decline to less than 20 mU/g of tissue with the exception of days 10 and 11 when a small increase could be observed.

Hypoxic exposure showed that neonatal rats increase Ep production as early as the first day of life, and the increase in circulating Ep levels closely resembled the increase in renal Ep concentrations (Figure 2). While on the whole Ep production increased with age, it is of interest that on day 10 and 13 we observed sharp declines in renal and plasma Ep, at a time when unstimulated control animals showed unexpected peaks. Nevertheless, the Ep levels in the hypoxic groups were still significantly higher than those measured in the controls. Liver Ep concentrations fluctuated relatively little. No difference in the response of males and females to hypoxia could be documented from day 10 through weaning, but at day 42, the levels in male rats were
significantly higher than in female rats and were similar to those measured in adults rats of both sexes. The sharp decline on day 10 and 11 also cannot be attributed to a female response because, as can be seen in Table 1, even though the levels were all reduced, plasma, kidney and liver Ep content of the female group was actually higher than that of the male rats. Analysis of salivary gland tissue homogenates for the presence of immunoreactive Ep were negative throughout the study from day 1-42 of age. However, some male rats left over from this study were used in a different experiment at the age of 65 days and salivary gland tissue content of Ep was similar to that measured in adult rats, approximately 1000 mU/g (16).

We have established earlier that the fetal rat kidney is able to synthesize Ep and respond to maternal hypoxia two days before birth, but that during the last week of fetal life the liver is the main Ep producing tissue in the rat fetus. Taking into account tissue weights and their Ep concentrations, it is possible to calculate how much of the hormone is available from renal or hepatic sources. Figure 3 shows that in the normoxic neonatal rat more Ep originates from the liver than the kidneys until day 10. The presence of the profound increase on day 10 seems to be connected with the liver to kidney switch of Ep production in the normal rat, because thereafter the availability of Ep from the kidney is always higher than that from the liver. During hypoxic conditions, however, the switch occurred as early as 2 days after birth, and in spite of increased liver weight from day 2 on, the kidney provided more Ep.

No erythropoietin could be measured in milk from normal or hypoxic lactating rats (< 4 mU/ml). Similarly, no increases were measured in the plasma of pups which had been removed from their mothers overnight and been returned for nursing the morning after hypoxic exposure. Plasma values in
neonates nursed by hypoxic mothers measured 60.7 ± 3.2 (SEM, n=7 pools of three pups each), and in those nursed by normoxic mothers the values were 58.6 ± 4.5 (n=6 pools of three pups each). Similarly, no differences were measured in liver and kidney tissue homogenates of these young rats. Because of the intraassay variation of approximately 9%, there may still be some transfer from the mother to the pup via the milk, but the RIA is unable to detect this.

DISCUSSION

Immunoreactive erythropoietin was measured daily in intact neonatal normoxic and hypoxic rats during the first three weeks of life and was compared to adult levels. Plasma and tissue Ep levels were determined by radioimmunoassay, a method which provides the high sensitivity necessary to determine normal or below normal values in unmanipulated small animals. In addition, the determinations require a small sample size and are not as costly as the in vivo bioassay. However, a few neonatal hypoxic samples of this study were bioassayed, and the higher than adult levels were confirmed (data not shown).

Previous studies which attempted to measure Ep in the normal neonatal rat have been unsuccessful because of the insensitivities of the assay systems used (2,4). This is not surprising because in our study more than half of the plasma and all of the tissue values, with the exception of four determinations, were either at or below the detectable bioassay range. However, using a cell culture assay system, Meberg and Haga (17) found measurable plasma Ep levels in non-hypoxic mice 9 and 20 days old.

With the exception of the first and ninth day of life circulating Ep levels were higher than adult levels in the normal newborn rat. The initial rise was attributable to hepatic Ep synthesis. From 10 days of age, renal Ep
content was reflected in plasma levels. The liver continued to be a contributor until days 10 and 11. The elevation in hepatic Ep content during this time cannot be simply attributed to residual blood content because a similar increase could not be measured on day 19 when plasma levels were equal to those observed on day 10.

The data presented here show that rat plasma and tissue Ep levels vary greatly during the first three weeks of life regardless of whether the animals were kept at ambient pressure or were exposed to 18 hours of hypoxia. A periodicity in reticulocytes has been shown by Scribner (18), in the neonatal mouse, and by Morley and Stohlman (19), during the recovery of erythropoiesis following irradiation. A similarly high variation of the plating efficiency of neonatal mouse CFU-E bone marrow cells has been reported by Hall et al. (20).

In addition, the plating efficiencies of neonatal marrow cells during the first three weeks of life were always higher than those obtained from adult bone marrows, possibly reflecting higher than adult Ep receptor occupancy as a result of elevated endogenous Ep levels. However, the temporal pattern in the mouse may not be identical to that observed here in the neonatal rat because these authors found a low plating efficiency between days 9-11, a time when we observed the first major peak in renal and circulating Ep levels.

Exposure of neonates to hypoxia confirms results reported earlier that neonatal rats respond to hypoxia with increasing Ep levels and that the response improves steadily with age (1-3). In fact, during the third week of life, the levels measured in this study significantly exceeded those found in adult male rats exposed to the same hypoxic stimulus. Of the above cited studies, only Gruber et al. (1) found the levels in intact neonatal hypoxic rats to be higher than in adults when intact rats were tested at the ages of 2 and 3 weeks. It appears that at these ages an exposure of 6 hours at 0.35 atm...
may be comparable to the 18 hours at 0.5 atm used in this study. Other authors found the response in the neonatal rat to be below that measured in adult animals, but this may be due to either a shorter time of exposure and/or less hypoxic stress (2,3). From days 10 to 21 of age, female and male rats responded equally to hypoxic exposure, in terms of renal Ep production and resulting circulating Ep levels. At the onset of maturity (day 42), the response was equal to that found in adult rats.

It is of interest that at 10 and 13 days of age when Ep in the unstimulated group reached the first significant peaks in kidney tissues and plasma levels, there were sharp, and highly significant, reductions in Ep production in hypoxic rats. Even though it is tempting to assume that Ep production may be reduced in these animals because of rising endogenous levels in the unstimulated animals, Fried and Barone-Varelas (21) have shown in adult rats that plasma Ep levels do not affect the rate of Ep production or secretion. We have no evidence at the present time whether this feedback mechanism might be different in the young rat.

This present study could neither confirm the presence of Ep in milk obtained from normoxic and hypoxic mothers, nor establish if the Ep levels were elevated in the newborns nursed by hypoxic mothers. Lactating rats exposed to the same hypoxia usually have plasma levels of approximately 300-400 mU/ml, and we were not able to show any transfer from the mother to the young. The age of 7 days was chosen for this particular experiment, because at this time endogenous renal and hepatic Ep levels were low and the plasma levels were declining and still separated from the 10-day peak by three days (Table 1 and Figure 1). It also appears unlikely that Ep was inactivated by acid hydrolysis in the stomach because the pH in the neonatal rat stomach is reported not to fall below six before day 10 (22), and we have found that Ep becomes...
immunologically unreactive below pH 4.5. Our results appear to be in contrast to Carmichael et al. (23), who observed increased erythropoiesis in neonates suckled by anemic mothers, or after oral Ep administration at 10 days of age. We did not analyze peripheral blood and bone marrow values. Increased erythropoiesis is not necessarily excluded here, even though Lucarelli et al. (9) also could document no change in erythropoiesis in young rats nursed by normal or hypertransfused mothers. Some of the effects observed in milk, including early measurements obtained with the in vivo polycythemice mouse assay, do not exclude the presence of growth factors possibly having similar effects, both on erythropoiesis and $^{59}$Fe uptake in the polycytemie mouse assay. This hypothesis does not seem unlikely because milk has been reported to contain several different growth factors (24). In addition, Kurtz et al. (25) found insulin-like growth factor I (IGF I) to be a potent growth and colony formation factor in vitro within the physiological concentration range of the hormone.

Whether the neonatal rat derives most of its erythropoietin from the liver or the kidney has been the subject of investigation for years (1,2,6,9,26). These studies showed that Ep production in nephrectomized newborn rats after hypoxia is only slightly reduced or not affected at all. Our data in intact rats showed that the liver to kidney switch of Ep production in the normoxic neonatal rat occurred at day 10, and in the hypoxic newborn as early as day 2 of life. The conclusion reached by Gruber et al. (1) and Lucarelli et al. (9), that Ep production in significant measure is independent of renally produced Ep during the neonatal period in the rat, appears not to be correct when applied to the intact rat, and is only true in the nephrectomized neonatal rat. The effects observed with nephrectomized rats were rather due to the fact that during the first three weeks of life, the liver retains the ability to
synthesize Ep and fully compensates in the absence of the kidneys. This ability is mostly lost in the adult, although the liver has been found to be the principal site of extrarenal Ep production (27) and is able to maintain baseline quantities of Ep in the absence of the kidneys. Recent evidence has shown that in the fully compensated hemolytic state induced in the rat by long-term phenylhydrazine administration the liver can become the primary source of Ep (28), indicating that it is even possible to reverse the switch from renal to extrarenal in the adult rat.

While it is important to be able to measure circulating plasma and tissue concentrations and determine the time of liver to kidney switch in the developing rat, no explanation can be offered at the present time to the following questions: why would an animal whose erythropoiesis cannot be stimulated further by exogenous Ep administration or hypoxia maintain higher than adult levels during the first three weeks of life, and during hypoxic conditions, elaborate even more than is necessary for the adult to reach erythropoietic homeostasis? This is especially puzzling because Miller et al. (29) found that very small increases in the levels of Ep (approximately 5 mU/ml) are capable of significant increases in red blood cell production in the adult rat. The possibility has to be considered that the function(s) of Ep in neonatal rats may be multifold and not just limited to those necessary for erythropoiesis. Rather, Ep may work in conjunction with other growth factors until adult homeostasis is achieved, a fact that would also support the suggestion by Lucarelli et al. (6,9) that erythropoiesis in the neonatal rat is governed differently from that in the adult.

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FIGURE LEGENDS

Figure 1. The upper shows the daily variation in plasma erythropoietin in the normoxic neonatal rat. The lower panel shows the liver and kidney erythropoietin concentrations during the same period. Values are means ± SEM.

Figure 2. Erythropoietin concentrations in plasma (upper panel) and kidney and liver (lower panel) in the neonatal rat exposed to hypoxia (0.5 atm x 18 hrs) as a function of age. Values are means ± SEM.

Figure 3. Total liver (0) and kidney (0) tissue erythropoietin content (mU/g X tissue weight) in the neonatal rat as a function of age under hypoxic (upper panel) and normoxic (lower panel) conditions. M = Male; F = Female.
Fig. 1
Fig. 2
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