Immunoreactive Erythropoietin Concentrations in Fetal and Neonatal Rats and the Effects of Hypoxia

By Gisela K. Clemons, Sherry L. Fitzsimmons, and Darlene DeManincor

Immunoreactive erythropoietin (Ep) was measured in normoxic and hypoxic (0.5 atm; 18 hours) fetal rats from day 14 to day 21 of gestation and in neonatal rats from birth to weaning, and was compared to the adult rat. Amniotic fluid (AF) Ep was approximately 100 mU/mL on day 14 and 15, and decreased to 20 mU/mL on day 20, with no difference between the hypoxic and normoxic mothers. Only on day 21 did the Ep in the AF increase slightly in the hypoxic group, while the Ep in the control group continued to fall to 15 mU/mL on day 21, the last day of pregnancy. Before day 17 of gestation the rat fetus appears to have hypoxia-independent, extrahematopoietic Ep available which is followed by hepatic and renal Ep production, both of which become sensitive to maternal hypoxia during the last days of pregnancy. In the neonatal rat plasma and tissue, Ep levels varied greatly during the first three weeks of life regardless of whether the animals were hypoxic or not. With the exception of the first and ninth days of life, circulating Ep levels were higher than adult levels in neonatal rats. Neonatal rats responded to hypoxia with increasing Ep levels, and the response increased with age such that during the third week of life the plasma Ep levels were significantly higher than 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 two days after birth.

PREVIOUS STUDIES have established that erythropoiesis in the fetal and adult mammal is regulated by erythropoietin (Ep). It has also been shown that fetal Ep production responds and increases during periods of fetal hypoxemia. Zanjani et al. have provided evidence that the liver is the primary site of Ep production in the fetus, at least in the sheep during the last trimester, and the switch from hepatic to renal Ep synthesis occurs around the time of birth, whereas in the rat previous studies showed that Ep is predominantly derived from the liver until after birth. While it has been shown that the neonatal rat is able to increase Ep levels in response to hypoxic hypoxia, erythropoiesis cannot be further stimulated in the newborn rat by exposure to hypoxia or exogenous Ep administration. Several investigations have suggested that erythropoiesis in the neonatal rat may be independent of renal Ep during the first three weeks of life. These 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 mostly in the in vivo polycythemic mouse assay.

The high sensitivity of the radioimmunoassay and the relatively small sample size required for analysis made these studies possible. We attempted to identify the tissue(s) responsible for Ep production in the normal rat fetus and to determine the effects of maternal hypoxia on Ep production during the last week of fetal life. In addition we tried to answer the following questions: what are the circulating, hepatic, and renal Ep levels in normal newborn rats, and can it be transferred to the neonatal animals? We also attempted to determine the age at which the switch from the liver to the kidney occurred both in the normal and hypoxic neonatal rat.

MATERIALS AND METHODS

Animals

Pregnant Sprague-Dawley rats with timed pregnancies, obtained from Simonsen Laboratory, Gilroy, Calif., were housed with free access to food and water for at least five days before the start of the experiments (day 8 to day 9 after mating). For the neonatal study the cages were checked two days before the presumed delivery date every two hours from 7 am to 8 pm, and the time and litter size was recorded. Approximately 75% of the deliveries occurred between 7 am and 2 pm. The litter size ranged from four to 15 pups and was adjusted to eight to ten per mother in order to assure equal nutritional status for the neonates during the experiment. When pups reached 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. All animals in the hypoxic groups were exposed to a high altitude equivalent to 18,000 feet (0.5 atm) for 18 hours (3 pm to 9 am). Every day hypoxic mothers, fetal rats, and neonatal rats were killed within one hour after hypoxia (9 to 10 am) and immediately followed by control animals for each group (10 to 11 am).

Experimental Design

Fetal study. From day 13 of gestation, each day four pregnant rats were exposed to hypoxia. The mothers were bled by cardiac puncture under ether anesthesia and amniotic fluid was aspirated before the fetus was removed from the uterus. Placental tissue was

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not collected because we had established earlier that placental Ep content did not exceed 20 mU/g at any time after 14 days of gestation. The litter size varied from eight to 13 animals. All fetuses were blotted dry and weighed before tissues and blood were obtained. Fetal blood, liver tissue, and kidney tissue were collected as early as possible and pooled for each litter. Fetal liver tissue was collected from day 15 on, blood from day 17 and kidney tissue from day 19 of gestation. On day 14 tissue could not be obtained for analysis; therefore Ep was measured in the whole fetus and on days 15 to 18 in fetuses without liver and kidney anlage from which most of the blood had been removed. Plasma from each litter was pooled after centrifugation with the exception of day 17 from the hypoxic group. Plasma pools from all fetuses of four mothers had to be pooled in order to make repeated radioimmunoassay (RIA) analysis possible.

**Neonatal study.** Mothers and their pups were exposed to hypoxia; each day an age-matched group served as controls. From one to 10 days of age blood was collected in heparinized hemocrit tubes after decapitation. Older animals were bled by heart puncture. From day 1 to day 21 six pools were generated for the control and hypoxic group, except that in the latter group six pools were obtained for males and females separately from day 10 to day 21. Samples had to be pooled to make RIA analysis possible. During the first four days four animals were pooled, three from days 5 to 7, and thereafter until day 21 two animals were sufficient. On day 42 analysis was possible on individual animals and eight animals were used in each group: 8 control, 8 hypoxic male, and 8 hypoxic female rats. The kidney and whole liver tissues were excised and pooled identically to the pups whose plasmas had been pooled. Salivary glands were collected and combined for all animals in each group: controls, hypoxic (days 1 through 9), and hypoxic males and females (days 10 through 21 and day 42).

During the collection 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. All tissues were weighed and homogenized in 0.05 mol/L phosphate buffer (pH 7.5) with 5% fetal bovine serum (wt/vol), and centrifuged at 10,000 g for 15 minutes in an Eppendorf Micro Centrifuge (Sybron-Brinkman Instruments, Palo Alto, Calif.). Plasma, amniotic fluid, and tissue homogenates were stored frozen 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 anaesthetized with pentobarbitral (60 mg/kg IP) and lactation was induced by injection of pitocin (4 to 5 U/animal), obtained from Sigma Chemical, St Louis. Within five to ten 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 both groups of mothers. The animals were killed three hours later and plasma, kidneys, and liver tissues were collected and analyzed for Ep content.

### Radioimmunoassay of Rat Erythropoietin

Immunoreactive erythropoietin concentrations were measured by RIA. Even though the tracer is highly purified Ep of human origin and the antiserum was generated against human urinary Ep extract, a bioassayed standard preparation of high-altitude rat serum yields a dose-response curve in the RIA similar, although not identical, to that obtained with the Second International Reference preparation of human Ep. The technical details of the RIA and the validation of the rat Ep RIA have been published. The sensitivity limit of this assay is approximately 4 mU/mL, interassay variation is <12%, and intrassay variation <9%. All samples were initially analyzed in single 100-μL aliquots and repeated in two separate RIAs in duplicates, sample size permitting, at appropriate dilutions that could be compared to the standard curves. The results presented in Table 1 represent the means of each of the four pools generated each day per group and three to five determinations per pool. The neonatal data in Table 2 are the means of four determinations from the latter two assays. Because the tissues were homogenized in an equal volume of buffer to tissue weight, this volume in the RIA represented a 1:2 dilution and was accounted for in the computer program as such. In addition, some plasma 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 on the LBL CDC 7600 Computer.

### RESULTS

**Erythropoietin Concentrations in Fetal Plasma, Tissues, and Amniotic Fluid**

There appears to be no immunological difference between fetal and adult Ep because all sample dilutions parallel the rat Ep standard. The Ep concentrations measured in the whole fetus on day 14 were relatively high and appear to be in equilibrium with the amniotic fluid (AF) (Table 1). Thereafter a steady decline in Ep levels was measured in both fetuses and AF with advancing pregnancy. This is particularly interesting in light of the fact that the volume of the AF diminishes during the last week of gestation and becomes increasingly more viscous. Amniotic fluid of control animals reached the lowest value on day 21, a time at which there was the first significant increase of Ep measured in the hypoxic group due to their increased urinary contribution (p < 0.05). There was no difference between the Ep concentrations in the AF as a result of maternal hypoxia with the exception of day 21. Similarly, no significant difference could be measured in whole pups on day 14 of gestation or later when most of the plasma, all of the liver, and later all of the kidney had been removed.

On day 15 maternal exposure to hypoxia did not affect Ep production in fetal liver tissue and the increase measured on day 16 is still statistically insignificant. Fetal hepatic Ep production in response to maternal hypoxia, however, could first be demonstrated in tissue content on day 17 (p < 0.01). Throughout the remainder of gestation liver Ep concentrations were significantly higher in the hypoxic fetuses than in those from control mothers. Ep content in control liver tissue remained fairly stable between days 15 and 19. Beginning with day 19 there was a significant decline in hepatic tissue Ep concentration, both in the control and hypoxic groups, and this decline was reflected in the lowest plasma values on day 20.

The earliest obtainable renal tissue on day 19 was also not affected by maternal hypoxia but increased its competence of
<table>
<thead>
<tr>
<th>Fetal Age (days)</th>
<th>Pups/Pool*</th>
<th>Plasma (mU/mL)</th>
<th>Liver (mU/g)</th>
<th>Kidney (mU/g)</th>
<th>Pups (mU/g)</th>
<th>Amniotic Fluid (mU/mL)</th>
<th>Maternal Plasma (mU/mL)</th>
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<tr>
<td>15</td>
<td>9–12</td>
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<td>14.4 ± 1.5</td>
<td>13.5 ± 0.8</td>
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<td>16</td>
<td>8–12</td>
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<td>—</td>
<td>13.4 ± 0.9</td>
<td>20.3 ± 3.7</td>
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<td>17</td>
<td>8–13</td>
<td>32.6 ± 4.0†</td>
<td>42.5 (1)</td>
<td>16.5 ± 1.7</td>
<td>30.6 ± 3.3**</td>
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<td>18</td>
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<td>28.0 ± 2.1</td>
<td>56.1 ± 4.5††</td>
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<td>37.8 ± 8.3††</td>
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<td>19</td>
<td>8–12</td>
<td>6.6 ± 1.6</td>
<td>26.6 ± 2.2††</td>
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*Each determination consisted of four pools with the exception of fetal HA plasma on day 17.
†Mean ± SEM.
‡Whole pup.
§Pup minus liver tissue.
∥Pup minus liver and kidney tissue.
††All HA values are significantly higher (p < 0.001).

The index shown for significance is always between the HA group and control and not within each group: #p < 0.05; **p < 0.01; ††p < 0.001.
control kidneys.

After the concentrations rose fivefold on the second day of life and are the first three weeks of life (Table 2), we observed sharp declines in plasma Ep levels. Thereafter the high plasma peaks also coincided with elevated renal Ep concentrations, the most pronounced 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.

### Erythropoietin Concentrations in Normoxic Neonatal Rats

The results of this study are shown both graphically and in tabulated form. Plasma Ep levels in normoxic neonatal rats vary profoundly during the first three weeks of life (Table 2 and Fig 1). With the exception of days 1 and 9 the circulating levels in the neonates were significantly higher than those measured in 42-day-old and adult rats. Peak values of 190 mU/mL were measured on days 10 and 20. Liver tissue Ep concentrations rose fivefold on the second day of life and are most likely responsible for the increase observed in plasma Ep levels. Thereafter the high plasma peaks also coincided with elevated renal Ep concentrations, the most pronounced 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.

### Erythropoietin Concentrations in Hypoxic Neonatal Rats

Hypoxic exposure showed that neonatal rats increase Ep production as early as the first day of life, and circulating Ep levels closely resembled renal Ep concentrations (Fig 2). While on the whole Ep production increased with age it is of interest that on days 10 and 13 we observed sharp declines in renal and plasma Ep in hypoxic animals, 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 sex 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 adult rats of both sexes. The sharp decline on days 10 and 11 also cannot be attributed to a female response because, as can be seen in Table 2, even though they were all reduced, plasma, kidney, and liver 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 one to 42 days of age.

Total Renal and Hepatic Erythropoietin Content and Time of Switching

Taking into account tissue weights and their Ep concentrations, it is possible to calculate how much of the hormone is present in renal and hepatic tissues. Figure 3 shows that in the normoxic neonatal rat the Ep content in the liver is higher than in the kidney until day 10. The profound increase on day 10 seems to be connected with the liver-to-kidney switch of Ep production in the rat, because thereafter renal Ep content is always higher than liver content. During hypoxic conditions, however, the switch occurred as early as two days after birth, and in spite of increased liver weight from day 2 on, kidney content was higher.

Lack of Measurable Erythropoietin in Rat Milk and Transfer to Neonates

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 that had been removed from their mothers overnight and returned in the morning after hypoxic exposure. Plasma values in neonates nursed by hypoxic mothers measured $60.7 \pm 3.2$ mU/mL (SEM, $n = 7$ pools of three pups each), and in those nursed by normoxic mothers were $58.6 \pm 4.5$ mU/mL (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

The data presented here show that erythropoietin can be measured in the rat during the last week of fetal life and that
the fetal response to maternal hypoxia is a function of age or, more specifically, a function of the developmental maturity of the Ep-producing tissues involved. The observation of relatively high levels of Ep in the AF and in the pups on day 14, and the fact that Ep levels in pups were still high on day 15 of gestation even though the liver with lower Ep levels had been removed, seems to suggest that in the fetal rat, and possibly also in other species, fetal hepatic Ep production is preceded by extrahepatic Ep synthesis that does not respond to maternal hypoxia. Early renal origin of Ep can be ruled out because from the first day the liver was removed (day 15), the early kidney tissue (either kidney anlage or pronephron) was also removed even though it could not be analyzed. It has been shown by Wells that the fetal rat kidney is only able to secrete urine during the last 2 days of gestation and the elevated levels in AF on day 21 are most likely the result of increased renal Ep synthesis in response to maternal hypoxia. The importance of these relatively high levels of Ep at this stage of development is not understood. Cole and Paul have reported that yolk sac erythropoiesis cannot be enhanced by exogenous Ep. The elevated levels in fetal rats could either be necessary for initiation of hepatic erythropoiesis until hepatic Ep synthesis can occur, or, more likely, Ep might have additional functions. It is possible that Ep acts as a growth factor alone or in conjunction with other growth factors in order to facilitate growth.

It is of interest that Ep levels and concentrations decline with advancing gestation except in renal tissue. This phenomenon occurred in the AF and pups and, apparently independently, in the fetal liver tissues and plasma during the last three days of gestation (irrespective of whether the mothers were exposed to hypoxia or not). There appear to be two possible explanations for this observation. The first possibility is that less Ep is needed because of increased sensitivity of the target tissue(s). This has been shown for somatomedins in fetal sheep where low levels were measured at a time of greatest need. The other possibility might be that target tissue sensitivity has not changed, but rather that the erythropoietic system is already "turned on" and operating at the highest rate. Both situations would explain the fact that at birth the newborn rat has a reticulocyte count between 90% and 95% and therefore the decline in Ep at this time is unlikely to be the result of suppressed erythropoiesis.

In the newborn rat immunoreactive 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 exposed to 18 hours of hypoxia. With the exception of the first and ninth days of life the circulating Ep levels were higher than adult levels in the normal newborn rat. The initial rise was probably attributable to hepatic Ep synthesis. From ten days of age renal Ep content was reflected in plasma levels. The liver continued to be a contributor up to days 10 and 11. The elevation in hepatic Ep content during this time cannot be due simply 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.

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. 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 al17 found the levels in intact neonatal hypoxic rats to be higher than in adults when intact rats were tested at the age of 2 and 3 weeks. It appears that at this age an exposure of six hours at 0.35 atm may be comparable to 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. 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.

This present study neither could confirm the presence of Ep in milk obtained from normoxic and hypoxic mothers nor establish whether 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 to 400 mU/mL, and we were not able to show any transfer from the mother to the young. The age of seven 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 ten-day peak by three days (Figure 1 and Table 2). It also appears unlikely that Ep was inactivated by acid hydrolysis

**Fig 3.** Total liver (O) and kidney (b) tissue erythropoietin content (mU/g x tissue weight) in the neonatal rat as a function of age under hypoxic (A) and normoxic (B) conditions. M. male; F. female.
in the stomach because the pH in the neonatal rat stomach is reported not to fall below six before day 10,24 and we have found that Ep becomes immunologically unreactive below pH 4.5. Our results appear to be in contrast to Carmichael et al.29 who observed increased erythropoiesis in neonates suckled by anemic mothers, and after oral Ep administration at ten days of age. We did not analyze peripheral blood and bone marrow values and increased erythropoiesis is not necessarily excluded here, even though Lucarelli et al.31 also could document no change in erythropoiesis in young rats nursed by normal or hypertransfused mothers.

Whether the neonatal rat derives most of its erythropoietin from the liver or the kidney has been the subject of investigation for years.9,12,14,30 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 suggest that the liver-to-kidney switch of Ep production in the normoxic neonatal rat occurred at day 10 and in the hypoxic newborn on day 2 of life. The conclusions reached by Gruber et al.9 and Lucarelli et al.31 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 only true in the nephrectomized neonatal rat. The effects observed with nephrectomized rats were rather due to the fact that during the first 3 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 production31 and is able to maintain baseline quantities of Ep in the absence of the kidneys. Recent evidence has shown that in fully compensated hemolytic state induced in the rat by long-term phenylhydrazine administration the liver can become the primary source of Ep,32 indicating that it is even possible to reverse the switch in the adult with time.

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 explanations can be offered at the present time to the following question: Why would an animal whose erythropoiesis can neither be stimulated further by exogenous Ep administration or hypoxia nor be totally suppressed by hypertransfusion29 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 be limited to 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.31 that erythropoietin in the neonatal rat is governed differently from that in the adult.

ACKNOWLEDGMENT

It is with great pleasure that we thank Drs George Brecher and Shirley Ebbe for critically reviewing the manuscript, and for their valuable suggestions and interest in the work.

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