Title

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IMMUNOREACTIVE ERYTHROPOIETIN STUDIES IN HYPOXIC RATS AND THE ROLE OF THE SALIVARY GLANDS

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ABSTRACT

Rat erythropoietin (Ep) crossreacts in the radioimmunoassay (RIA) developed in this laboratory. Immunoreactive Ep was measured in serum and tissues of male rats in response to short-term hypoxia (0.43 atm x 24 hrs). In the unstimulated rat all tissues examined had low levels of Ep with the exception of the submaxillary or salivary gland (SG). Exposure to hypoxia for 24 hrs resulted in significant increases in kidney and serum levels of Ep with no apparent change in SG content. Sialectomy immediately prior to exposure reduced renal Ep production and serum levels significantly after 4 hrs of exposure. Nephrectomy (N) confirmed previous results by others that Ep production after exposure to hypoxia is reduced but not abolished. The effect of N plus sialectomy was identical to N alone, thus excluding the SG as a source of extrarenal Ep in nephrectomized rats. The long-term effect of SG ablation to the same constant hypoxic stimulus showed a steady decline of the Ep response during the first week after surgery, both in renal production and serum levels. Thereafter, from one to six weeks the serum levels remained constant, being higher than in the unstimulated rat but significantly lower than in intact hypoxic animals. No crossreactivity in the RIA was found with renin, renin substrate, nerve and epidermal growth factor or somatomedins. If this Ep-like substance in the SG were the source of extrarenal Ep it should have been possible to document an increase in serum concentration before an increase can be measured in renal content. It appears, however, that the presence of the SG is necessary for renal tissue to be able to synthesize Ep during hypoxia.

INTRODUCTION

Exposure to reduced oxygen tension increases circulating erythropoietin

levels. In adult animals the kidney is the major organ to produce the hormone but extrarenal production sites have also been shown to exist. The present evidence available for renal and extrarenal Ep production site(s) has been recently reviewed by Anagnostou (1), and the salivary gland has been suggested by Zangheri et al. (2,3) as a possible source of extrarenal Ep. These authors demonstrated Ep in salivary glands of rats and mice by immunochemical methods, and also found a significant reduction of circulating Ep levels in young nephrectomized rats after removal of the SG and short hypoxic exposure. A reduction could not be demonstrated in adult rats unless anemia was superimposed to nephrectomy, ablation of the salivary gland and hypoxia. Even then the magnitude of reduction was significantly less than that observed in young animals.

The purpose of the present study was to determine the kinetics between kidney and salivary gland Ep content with respect to circulating hormone levels, the effect of salivary gland ablation on intact kidney tissue Ep production during hypoxia, and whether the SG can be considered to be one of the proposed extrarenal sources of Ep.

MATERIALS AND METHODS

Adult male Sprague-Dawley rats (300-350 g), obtaind from Simonson Laboratory, Gilroy, CA, were housed with free access to food and water in our colony at least one week before the experiments. Surgery was performed under metafane anesthesia and the animals were allowed to recover for at least one hour before they were placed in the hypoxic chamber. The salivary glands were removed for varying lengths of time before exposure while studies with nephrectomized animals were completed within 24 hrs after surgery. At the end of the experiments, the animals were anaesthetized, blood was drawn from the

vena cava and allowed to clot at room temperature before centrifugation. Tissues were collected and homogenized in an equal volume of phosphate buffer (pH 7.5) containing 5% bovine serum albumin (BSA). The samples were stored at -20°C until RIA analysis.

EXPERIMENTAL DESIGN

Experiment 1:

Normal, intact rats were exposed to hypoxia of 0.43 atm for 24 hrs. Under metafane anaesthesia blood, urine and kidney, liver, spleen, brain, lung and salivary gland tissues were collected from hypoxic and normoxic animals. Saliva and lymph were obtained from rats which had been anaesthetized with pentobarbital (60 mg/kg). Salivation was induced by intraperitoneal injection of epinephrine (2 mg/kg). Pooled salivary secretions were collected in capillary tubes placed between the tongue and the floor of the mouth over a 30-45 minute period after the injection of epinephrine. Lymph was collected from the thoracic duct over a time span of 1-2 hrs. A separate group of rats were fasted for four days with free access to water and exposed to the same hypoxia between the third and fourth day.

Experiment 2:

Normal, intact rats were exposed to 0.4 atm from 5 to 120 minutes. Serum, kidney and salivary gland tissues were collected for analysis.

Experiment 3:

Four groups of rats were exposed to 0.5 atm for varying length of time. This higher oxygen tension was chosen to insure the survival of the nephrectomized rats. From the first group, the intact animals, the serum, kidneys and salivary glands were collected. The second group underwent salivary gland ablation immediately prior to hypoxia and serum and kidney

tissues were collected. The third group was nephrectomized prior to hypoxia and serum, liver and salivary glands were collected, while the fourth group was both sialectomized and nephrectomized and serum and liver tissues were analyzed for Ep content. Intact and sialectomized rats were exposed from 1-48 hours. Experiments with nephrectomized rats were terminated within 24 hours.

Experiment 4:

Salivary glands were removed in 12 groups of rats (8 rats per group) on day zero. Thereafter, a group of 8 rats was challenged with hypoxia (0.5 atm x 24 hrs) at weekly intervals for up to six weeks after surgery. At each time interval another group of sialectomized rats served as controls. The animals were weighed weekly as well as before and after hypoxia. Serum and kidney tissues were collected for Ep analysis.

Erythropoietin concentrations were measured by radioimmunoassay as described by Garcia et al. (4) and Clemons (5). 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; the interassay variation is < 12% and intraassay variation < 9%. Most of the samples were analyzed in at least three separate RIA's, either in serial dilutions, as a check for parallelism, or repeated as duplicates at appropriate dilutions which could be compared to the standard curve. BSA was added to the urine samples in the RIA in order to approximate the protein content of serum (100 ul 5% BSA in phosphate buffer, previously lyophilized). Results were analyzed with the sigmoid computer program developed by Rodbard and Hutt (6) on the LBL CDC 7600 Computer.

RESULTS

Experiment 1:

The changes of immunoreactive Ep concentrations in fluids and tissues of normal and serum of fasted rats in response to a simulated high altitude of 0.43 atm for 24 hrs are shown in Table 1. In normal rats Ep levels are low in all fluids and tissues tested with the exception of salivary gland tissue. Serum Ep levels in rats which had been fasted for four days were significantly lower than control values ($p < 0.001$). As expected, the effects of hypoxia were reflected in highly elevated Ep concentrations in the serum and kidney, while the Ep levels in the SG tissue were essentially unaltered. Increased Ep concentrations were measured in urine, saliva and lymph, although the levels varied with respect to origin of the fluids (see Table 1).

Experiment 2:

This short-term experiment was undertaken in order to determine the time of hypoxic exposure at which the earliest changes in kidney Ep content could be measured and how these changes could be correlated with serum and SG Ep levels. As can be seen in Table 2, the earliest increase of Ep was measured in the kidney after 60 minutes of hypoxia. This increase was measured at a time when no changes in serum levels were detected. During the next hour kidney concentrations of Ep rose 10-fold and serum levels 3-fold. However, during this 2 hr time period there was no change in SG Ep levels.

Experiment 3:

The results of this experiment are shown in Figure 1. Serum Ep levels (upper panel) in intact rats rise progressively with the length of hypoxic exposure and reach peak values between 12 and 18 hours. This is in accordance with previously published observations (7,8). A similar temporal pattern was established for kidney Ep content in intact rats (lower panel). As in

experiment 2, the increase in kidney Ep was faster and higher than that observed in the serum. Removing the salivary glands immediately prior to hypoxia had no effect on either kidney or serum Ep levels during the first 4 hrs of exposure when compared to intact animals. However, during prolonged exposure, renal tissue did not increase its Ep content further and actually declined after 12 hours of exposure. A similar decline was also reflected in serum values, and, in fact, after 18 hours serum and kidney values were 1/10 of those in intact animals but still significantly higher than in unstimulated control rats.

A small but significant increase in serum Ep could be measured in nephrectomized rats and in nephrectomized rats which also had the salivary glands removed. There was no difference between these two groups. Between 2-4 hrs the levels increased 4-fold when compared to normoxic control which had undergone the same surgical procedures. Where the temporal pattern of serum Ep in the nephrectomized group with intact salivary glands and the sialectomized group with intact kidneys is compared, it seems to indicate that the Ep measured in the salivary gland is not a contributor to either elevated kidney or serum levels of Ep during hypoxia.

Salivary gland Ep content was measured in intact and nephrectomized rats. At no time was there a significant decline but the levels were slightly but not significantly increased after 6 hours in the intact group (Figure 3) and remained constant in the nephrectomized group (data not shown).

Experiment 4:

Figure 2 shows the effects of sialectomy on kidney and serum Ep levels in response to hypoxia as a function of time after surgery. Experiment 3 showed that during the first 4 hours after surgery the response in the experimental and intact groups was identical, while significant differences occurred after

the initial 4 hours. Delaying the exposure from 2-7 days after surgery showed a steady decline of both renal Ep production and circulating Ep levels. This effect was maintained throughout the remainder of the experiment from 2 to 6 weeks after surgery. The Ep levels were significantly reduced, but still were approximately 5 times those in the control groups. This reduction cannot be explained by starvation. Body weight increased from 260 ± 10 g at time of surgery to 420 ± 8 g six weeks after surgery when unoperated controls weighed 450 ± 16 g. In addition, there were no differences in hematocrits between the experimental and control groups at the end of the experiments, confirming the results reported by Menendez-Patterson et al. (9).

DISCUSSION

Although it has been firmly established that the kidney is the primary tissue for erythropoietin production in adult mammals, extrarenal sites of Ep synthesis have been shown to exist in the absence of renal tissue (7,10), presumably mostly of hepatic origin (11). Evidence was also presented that renal and extrarenal Ep can be neutralized by antisera to Ep, thus suggesting that both molecules are immunologically similar (10,12).

The presence of Ep or an erythropoietin-like substance in rat and mouse salivary glands has been reported, and it was considered by Zangheri et al. as a source of extrarenal Ep in rats and mice (2,3,13). Our data confirms the presence of immunoreactive Ep in salivary glands, but these hormone levels were high in the unstimulated rat and apparently not altered by exposure to hypoxia, thus making the SG as an extrarenal source of immunoreactive Ep unlikely.

Attempts to confirm these high levels of Ep in the in vivo polycythemic mouse assay were unsuccessful because the homogenates were exceedingly toxic to the mice. The samples were still toxic after either fractionation on

Sephadex-G 150 and/or dialysis and heat inactivation followed by fractionation before bioassay.

The circumstantial evidence for this substance being erythropoietin are the following: SG gland homogenates from mice and rats parallel their respective reference standards in the RIA. After heat inactivation (56 degrees for 30 minutes) parallelism was retained but there was a loss of approximately 40% of activity. We have observed similar decreases in rat serum, plasma and kidney homogenates after heat inactivation, observations which have also been reported by Jelkmann (14). The high levels of immunoreactive Ep measured are not due to the presence of proteolytic enzymes possibly interfering in the RIA. Addition of known amounts of either rat or human Ep to the homogenates prior to the assay were fully recovered (greater than 95%). The salivary gland is known to contain a variety of hormones and growth factors which might show immunological crossreactivity in the assay, especially since renin and angiotensin have been implicated in extrarenal Ep production (15,16) and renin substrate has been suggested to be a possible precursor for Ep (17). We have tested a variety of hormones, growth factors and substrates and have found no crossreactivity with any purified compound, thus the RIA neither measures renin nor renin substrate (5). The close correlation reported by Gould et al. (18) between increased Ep levels and renin, renin substrate and angiotensin levels in response to hypoxia can be assumed to be either independent from each other, or the increased levels of Ep in the kidney are the result of a direct action of the renin-angiotensin system on renal tissue during hypoxia.

Even though the data presented here confirm Zangheri's results in showing the presence of high levels of Ep in the salivary glands they do not support the notion that this Ep contributes to extrarenal Ep in times of need because there was no difference between the nephrectomized and nephrectomized-

sialectomized hypoxic groups with respect to serum levels. Liver and spleen tissues were also analyzed in these two groups and the levels in both groups were slightly elevated but this increase could be accounted for by residual blood content in the tissues (data not shown).

As can be seen in Figure 3, the temporal relationship between kidney, serum and salivary gland Ep was such that kidney Ep concentrations rose and preceded serum levels through the first 12 hours of hypoxia. If the salivary gland were a contributor of extrarenal Ep it should have been possible to document a decline of SG Ep but instead a relatively small increase was observed after 6 hours of exposure. Because there was no decline in SG Ep it also seems unlikely that this material might be a precursor for Ep which gets activated in the kidney. In the latter case the serum levels should have increased before any increases in the kidney could be measured. It is difficult to imagine why this material should be immunoreactive in the SG and not immunoreactive while in transit to the kidney where it could be measured again. The presence and/or function of Ep in SG is still difficult to explain. It is interesting that Ep can be found in intact adult male and female rats but not in neonatal animals. SG tissue content of Ep in neonatal rats (day 1-21) and in 42 day old male and female rats was undetectable, but it was present in high amounts in males at 65 days of age.

While the high levels and functions of immunoreactive Ep in the SG in the rat cannot be explained at the present time, it is of interest that in the absence of the SG the kidney tissue apparently looses most of its ability to increase Ep production in response to hypoxia. Even though the serum levels measured 1 to 6 weeks after SG ablation resemble those of the 4 day fasted rat exposed to hypoxia (Table 1), it has to be recognized that the animals in this experiment only experienced a 10% reduction in weight gain during the 6 weeks

while the fasted animals actually lost 30% of their body weight in four days.

The data presented here seems to exclude the SG as a source of extrarenal Ep in the intact and nephrectomized hypoxic rat. There appears to be, however, a dependence of renal tissue on the presence of the salivary gland in order to be able to synthesize Ep during hypoxic exposure of more than four hours. Experiments are under way to establish whether the reduced Ep synthesis during hypoxia in rats without SG can be corrected by replacement therapy of either renin, renin substrate, epidermal or nerve growth factors.

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Table 1

Immunoreactive erythropoietin concentrations in fluids and tissue of rats exposed to hypoxia (0.43 atm x 24 hrs)[a]

	Control	n	Hypoxia	n
FLUIDS (mU/ml)				
Serum	23.4 ± 0.9	39	1490 ± 228	8
Serum 4d fasted	12.0 ± 1.0	8	60.3 ± 6.7	8
Saliva	21.7	*	134	*
Urine	ND[b]	8	176 ± 40	8
Lymph	20.4	*	560	*
TISSUES (mU/g)				
Kidney	21.4 ± 1.4	23	1430 ± 236	8
Liver	13.5 ± 1.8	8	22.7 ± 4.1	8
Spleen	14.7 ± 1.2	8	26.5 ± 6.3	8
Brain	6.8 ± 2.5	5	8.4 ± 1.2	5
Lung	9.8 ± 0.9	5	44.0 ± 4.2	5
Salivary Gland	1310 ± 107	23	1050 ± 119	8

(a) Mean ± SEM

(b) Not detectable

(*) Pools of two animals each

Table 2

Erythropoietin levels in male rats exposed to hypoxia (0.4 atm)

(n=8)	Serum (mU/ml)	Kidney (mU/g)	Salivary Gland (mU/g)
Control	21.3 ± 1.2	20.4 ± 1.5	959 ± 229
5 min 8% O_2	22.9 ± 1.1	20.4 ± 1.5	988 ± 188
10 min 8% O_2	22.5 ± 1.3	18.3 ± 1.4	871 ± 107
15 min 8% O_2	21.4 ± 1.6	23.4 ± 0.4	907 ± 54
30 min 8% O_2	21.1 ± .0	23.9 ± 2.9	895 ± 120
45 min 8% O_2	19.1 ± 0.4	25.5 ± 1.9	855 ± 77
60 min 8% O_2	21.1 ± .0	45.9 ± 6.1	976 ± 83
120 min 8% O_2	64.8 ± .5	199.4 ± 34	872 ± 187

a) Mean ± SEM Kidney

Legends

Figure 1: Erythropoietin levels in serum and kidney tissues during hypoxia in intact, Salivariectomized (S), nephrectomized (N), and nephrectomized rats after SG removal.

Figure 2: Serum and kidney erythropoietin levels in normal and hypoxic rats as a function of time after SG abaltion (n=8)

Figure 3: Ratio of erythropoietin concentrations in intact rat serum and tissues relative to control values during hypoxia.

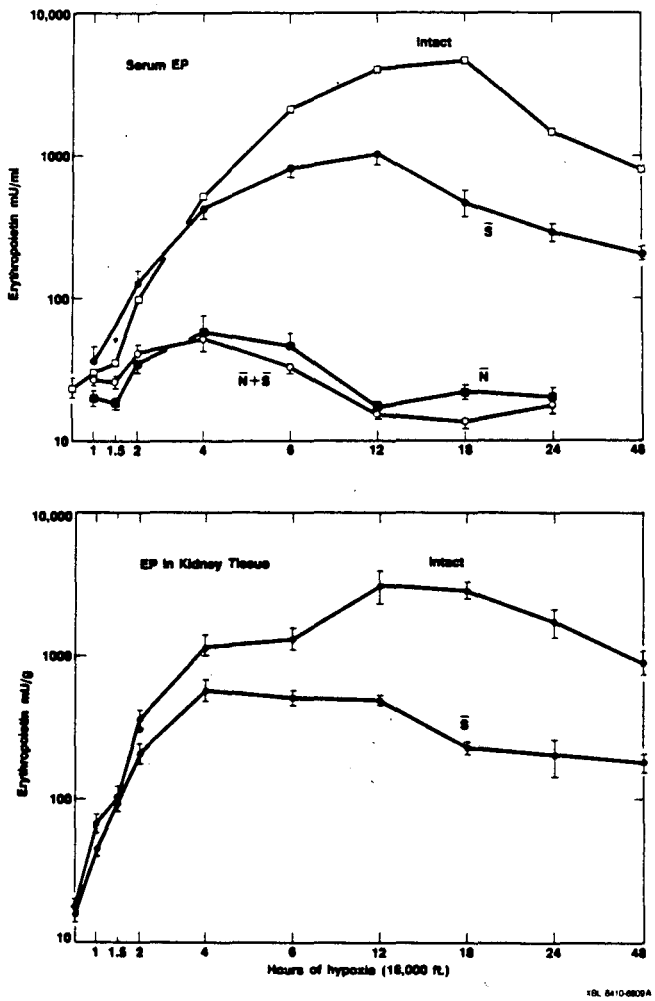
Serum EP
Intact
S̄
N̄+S̄
N̄
Erythropoietin mU/ml
10,000
1000
100
10
1 1.5 2 4 6 12 18 24 48
EP in Kidney Tissue
Intact
S̄
Erythropoietin mU/g
Hours of hypoxia (18,000 ft.)

Fig. 1

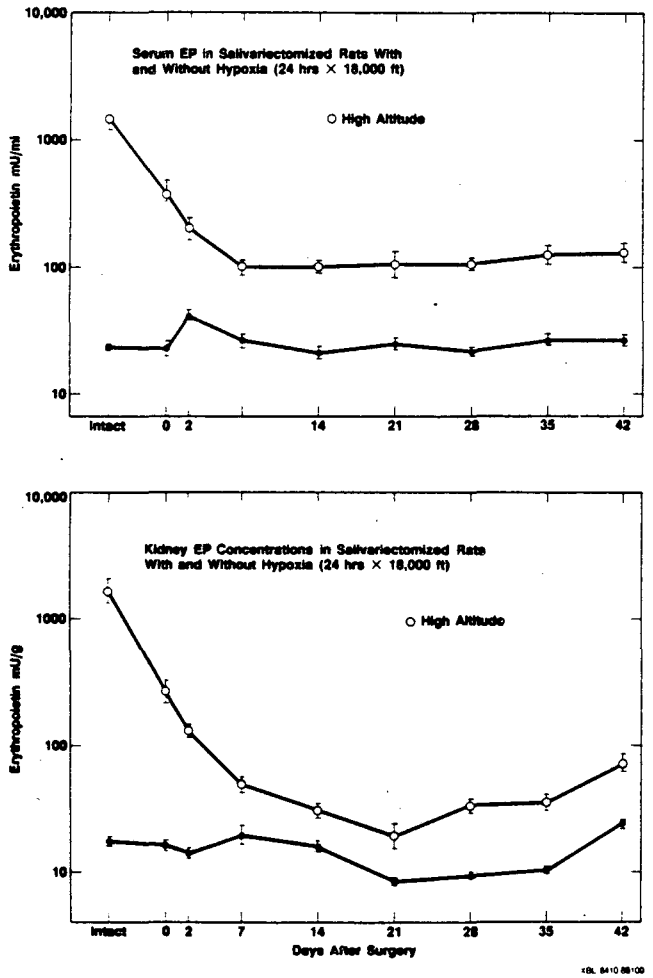
Serum EP in Salivariectomized Rats With and Without Hypoxia (24 hrs × 18,000 ft)
○ High Altitude
Erythropoietin mU/ml
10,000
1000
100
10
Intact
0
2
14
21
28
35
42
Kidney EP Concentrations in Salivariectomized Rats With and Without Hypoxia (24 hrs × 18,000 ft)
○ High Altitude
Erythropoietin mU/g
10,000
1000
100
10
Intact
0
2
7
14
21
28
35
42
Days After Surgery

Fig. 2

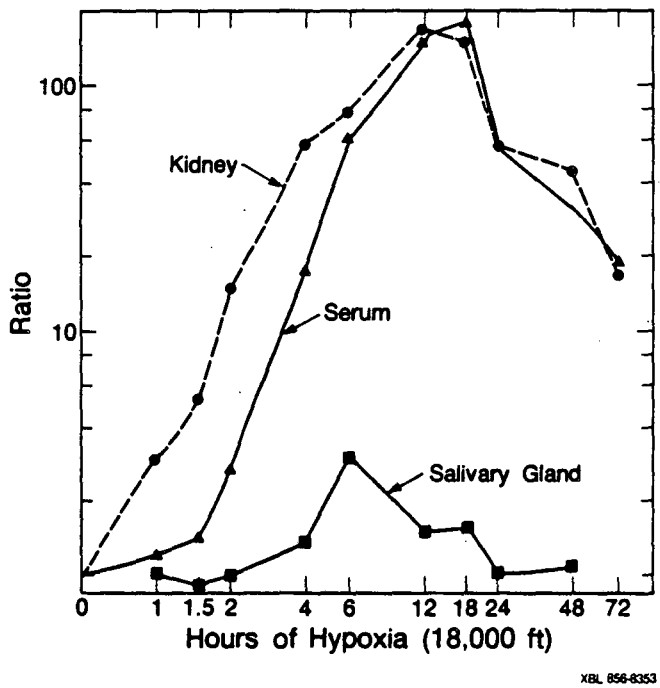
100
10
Ratio
Kidney
Serum
Salivary Gland
0
1
1.5
2
4
6
12
18
24
48
72
Hours of Hypoxia (18,000 ft)
XBL 856-8353

Fig. 3

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