Inhibitory Effect of Glucagon on Erythropoiesis

By J. P. Naets and M. Guns

The effect of glucagon on erythropoiesis has been studied in mice and rats. Long-acting glucagon protamine zinc (Novo) was injected subcutaneously twice daily at 9 a.m. and 4 p.m. After administration of 2 × 200 μg/day for 10 days, the erythropoietic response of male rats was markedly depressed. Total normoblast counts per femur, reticulocytes, and 59Fe uptake into red cells fell respectively to 35%, 50%, and 17% of control values. The same results were observed with male and female mice injected twice daily with 50 μg of glucagon. The erythropoietic response of mice to hypoxia was also inhibited. After 12 days of hypoxia (320 mm Hg 16 hr/day), the red cell mass increased in controls from 3.12 to 6.31 and only to 5.19 ml/100 g body weight in the glucagon-treated group (p < 0.02). Response of polycythemic mice to exogenous erythropoietin (ESF) was reduced after glucagon injection, and the inhibition was proportional to the logarithm of the dose. The lowest active dose (3 μg) reduced the 59Fe uptake to 77% of controls, whereas 50 μg administration reduced it to 43%. This inhibitory effect decreased gradually as a function of the time interval between glucagon injections and ESF administration. Production of ESF seemed unaffected by glucagon administration. Since after hypoxia (4 hr, 300 mm Hg), the ESF titer of rats and mice was similar in controls and glucagon-treated groups. As the inhibitory effect of glucagon was only elicited if administrated close to the ESF injection, and since no effect on ESF production could be demonstrated, it was inferred that the hormone acts mainly at the level of erythroid stem cell differentiation. It was suggested that hyperglucagonemia was responsible for the anemia of glucagonomas and might be implicated in the anemia of other clinical conditions.

ANEMIA OF OBSCURE ETIOLOGY is a common feature in glucagon-secreting tumors. Regression of the anemia after surgical removal has been reported.1,2 This association between hyperglucagonemia and anemia might be fortuitous. We have therefore studied the effect of glucagon on erythropoiesis in mice and rats and demonstrated an inhibitory effect of the hormone. The mechanism of this effect has been investigated.

MATERIALS AND METHODS

Erythropoiesis was studied in mice and rats. TO female mice (males in only one experiment), weighing 20–25 g, and male Wistar rats (190–220 g) were used, respectively, in groups of 6 and 4 per experimental point. Variable dosages of glucagon protamine zinc (Novo Novo Industrie, Amsterdam, The Netherlands) in 0.1 ml were injected subcutaneously twice daily (at 9 a.m. and 4 p.m.) in the different experiments. Controls were likewise injected with saline or protamine zinc solution (PZ). Incorporation of radioiron into red cells was measured 24 hr after injection of 0.5 μCi (59Fe) FeCl3/0.2 ml saline, into a tail vein in normal mice or rats, and after 72 hr in polycythemic mice. Counts of normoblasts per femur were determined. Nucleated cells were counted on total femoral marrow. Smears were stained with benzidine and counterstained with May-Grunwald-Giemsa. Differential counts were performed on 1000 cells. Reticulocytes were stained with Brilliant Cresyl Blue. Polychromasia was achieved in mice by exposure to hypoxia (320 mm Hg during 16 hr for 3 wk daily), and experiments were started 5 days after removal of the altitude chamber. Determination of red cell mass was performed as follows: 5 donor mice were injected with 7 μCi 59Fe, and blood was collected 2 days later by cardiac puncture. The blood was pooled, centrifuged 20 min at 4000 rpm, and red cells were washed twice with saline. One-tenth milliliter of a suspension of radioactive washed red cells in saline (hematocrit 50%) was injected into a tail vein. A blood sample was obtained 10 min later by cardiac puncture and radioactivity measured. Blood for ESF assay was collected with heparin by puncture and by cardiac puncture and its radioactivity measured. Blood for ESF assay was collected with heparin by puncture of the heart in mice and of the abdominal aorta in rats. Pooled plasma was stored at −20°C until used. ESF assay was performed on erythropoietic TO mice following a technique previously described.3 Six polycythemic mice per experimental point were injected subcutaneously with 1 ml of the plasma to be assayed 5 and 6 days after removal of hypoxia. 59Fe was injected 53 hr after the first injection of plasma, and 59Fe uptake into red cells measured 72 hr later, assuming the blood volume to be 7% of body weight. For glucagon assay, blood was drawn in chilled tubes containing EDTA and trasyol (Bayer, Leverkusen, Germany), centrifuged immediately, and plasma was stored at −20°C until assay. The hormone was measured by a double-antibody radioimmunoassay procedure following the technique of Leichter et al.4 The 30-K glucagon antibody was purchased from Dr. Unger (University of Texas Medical School).

RESULTS

Effect of Glucagon on Erythropoiesis of Normal Rats and Mice

As shown in Table 1, after glucagon administration of 2 × 100 or 2 × 200 μg/day for 10 days, the erythropoiesis of male rats was strikingly depressed. Total normoblasts counts per femur in 2 experiments fell respectively from 45.3 and 31.9 × 109 after PZ injection to 13.6 and 13.1 × 109 after glucagon (200 μg) administration. In both experiments the same effect was observed on hemoglobin level, reticulocyte counts, 59Fe uptake into red cells, and the percentage of marrow normoblasts. The differences between these parameters measured after PZ or glucagon administration are highly significant. p Values were < 0.005 for the Hb level, < 0.02 for reticulocytes, and < 0.001

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Table 1. Effect of Glucagon on Erythropoiesis of Normal Male Rats

<table>
<thead>
<tr>
<th>Experiment #1</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hb (g/dl)</td>
</tr>
<tr>
<td>Saline</td>
<td>15.5 ± 0.2</td>
</tr>
<tr>
<td>PZ</td>
<td>15.5 ± 0.4</td>
</tr>
<tr>
<td>Glucagon (100 µg)</td>
<td>14.5 ± 0.2</td>
</tr>
<tr>
<td>Glucagon (200 µg)</td>
<td>13.6 ± 0.1</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hb (g/dl)</td>
</tr>
<tr>
<td>Saline</td>
<td>15.5 ± 0.4</td>
</tr>
<tr>
<td>PZ</td>
<td>15.2 ± 0.2</td>
</tr>
<tr>
<td>Glucagon (100 µg)</td>
<td>13.8 ± 0.5</td>
</tr>
<tr>
<td>Glucagon (200 µg)</td>
<td>13.9 ± 0.5</td>
</tr>
</tbody>
</table>

Four rats per group; 2 injections daily for 10 days (mean ± SEM).

for the other parameters. The same inhibitory effect was observed on the erythropoiesis of mice of both sexes. After injection of 2 × 50 µg glucagon for 10 days, the hematocrit and the hemoglobin level in 5 experiments averaged, respectively, 47.6% ± 0.4% (mean ± SEM) and 15.6 ± 0.1 g/dl in PZ-treated mice and 44.4% ± 0.6% and 14.4 ± 0.2 g/dl after glucagon administration. The difference of these values amounting to 9% were highly significant (p < 0.001). As indicated in Fig. 1, the other erythropoietic parameters were also reduced, and the difference between PZ and glucagon groups was also significant. No differences between saline- and PZ-injected groups were observed. After twice daily injections of glucagon for 10 consecutive days, all the animals were in very good condition. In rats, weight averaged 193 and 202 g, respectively, before and 228 and 222 g after injections of PZ or glucagon. In mice, similar weights were observed before and after injections, averaging, respectively, 25, 26, and 25 g before and 27, 26, and 25 g after injection for 10 days of saline, PZ, or glucagon.

Effect of Glucagon on the Erythropoietic Response to Hypoxia

Normal mice were exposed to hypoxia (320 mm Hg, 16 hr/day) for 12 days during a period of 16 days. They were injected twice daily either with glucagon (20 or 50 µg), PZ, or saline. Table 2 shows the mean values of 3 experiments. The increase in red cell mass per 100 g body weight observed in PZ- and saline-injected mice was inhibited by the administration of glucagon, averaging, respectively, 105% and 102% in PZ- and saline-treated mice as compared to only 66% after glucagon injection. Values of the glucagon-treated groups are significantly different from PZ-
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Table 2. Effect of Glucagon Injections on Increased Red Cell Mass Induced by Hypoxia

<table>
<thead>
<tr>
<th>Weight* (g)</th>
<th>Hct (%)</th>
<th>Red Cell Volume/100 g Body Weight (ml)</th>
<th>Hb (g)</th>
<th>Hb/100 g Body Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After hypoxia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>24.8 ± 0.5</td>
<td>68.3 ± 1.7</td>
<td>6.39 ± 0.42</td>
<td>0.43 ± 0.04</td>
</tr>
<tr>
<td>PZ</td>
<td>23.3 ± 0.4</td>
<td>67.3 ± 1.6</td>
<td>6.31 ± 0.35</td>
<td>0.41 ± 0.03</td>
</tr>
<tr>
<td>Glucagon</td>
<td>24.3 ± 0.9</td>
<td>62.5 ± 1.3</td>
<td>5.17 ± 0.30</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td>Glucagon (50 μg)†</td>
<td>23.3 ± 0.5</td>
<td>60.6 ± 1.3</td>
<td>5.19 ± 0.27</td>
<td>0.32 ± 0.02</td>
</tr>
</tbody>
</table>

Each value represents the mean result from 3 experiments ± SEM.
*Weight at the time of red cell mass measurement.
†One experiment only.

Injected mice. Values obtained for p were < 0.005 for PCV, < 0.025 for total red cell mass and total hemoglobin, and < 0.02 for red cell mass and hemoglobin per 100 g body weight.

Effect of Glucagon on the Response to Exogenous ESF

Polycythemic mice were given subcutaneously 1 U of human ESF (extracted from the urine of an anemic patient) on days 1 and 2. Increasing doses of glucagon were injected twice daily on days 1, 2, and 3. Radioiron was injected on day 3. In Fig. 2, results are expressed as percentages of the erythropoietic response of the controls injected with ESF and PZ (considered as 100%). Glucagon significantly reduced the response to exogenous ESF, and the effect was directly proportional to the logarithm of the dose. The lowest dose of glucagon with a detectable response was 3 μg. The mean ⁵⁹Fe uptake after PZ injection was 19.44% ± 0.70%, and after 3 μg glucagon administration, 16.17% ± 1.12% (p < 0.025). Figure 3 shows the inhibitory effect of glucagon on the reticulocyte response of polycythemic mice to a single intravenous injection of 3 U ESF. Administration of glucagon started the day of ESF injection (day 0). A dose of 50 μg × 2 for 5 days nearly suppressed the reticulocyte response.

![Fig. 2. Inhibitory effect of increasing doses of glucagon on the response of polycythemic mice to ESF (2 × 1 U s.c.). Percentage of the response of controls injected with ESF and PZ (considered as 100%). Glucagon significantly reduced the response to exogenous ESF, and the effect was directly proportional to the logarithm of the dose. The lowest dose of glucagon with a detectable response was 3 μg. The mean ⁵⁹Fe uptake after PZ injection was 19.44% ± 0.70%, and after 3 μg glucagon administration, 16.17% ± 1.12% (p < 0.025). Figure 3 shows the inhibitory effect of glucagon on the reticulocyte response of polycythemic mice to a single intravenous injection of 3 U ESF. Administration of glucagon started the day of ESF injection (day 0). A dose of 50 μg × 2 for 5 days nearly suppressed the reticulocyte response.](image_url)

![Fig. 3. Effect of glucagon (20 μg (x), 50 μg (Ⅲ), saline (○), and PZ (□) injected twice daily for 5 days, on the reticulocyte response of polycythemic mice to a single i.v. injection of 3 U ESF (mean ± SEM).](image_url)
Effect of Glucagon on the Erythropoietic Response to ESF in Polycythemic Mice, as a Function of Time Interval Between ESF Injection and Glucagon Administration

Administration of glucagon (50 μg x 2) for 5 consecutive days before a single i.v. injection of ESF (1 IU) did not affect the erythropoietic response of polycythemic mice to exogenous ESF. Similarly, the injection of glucagon (50 μg x 2) on 1 day only, at different intervals after injection of ESF, had no inhibitory effect. Daily administration of glucagon (2 x 30 μg/day), starting simultaneously or close to a single injection of ESF (1.5 U s.c.), as already shown in Fig. 2, exerted an inhibitory effect on the erythropoietic response. This effect decreased gradually as a function of time interval between glucagon injections and ESF administration (Fig. 4).

Effect of Glucagon on ESF Production in Mice and Rats

Experimental groups of 20 mice and 4 rats were exposed to hypoxia (4 hr, 300 mm Hg) after the administration of glucagon during 5 days (50 μg in mice, 200 μg in rats). Blood was sampled immediately after removal of the altitude chamber. The incorporation of radioiron into red cells in assay mice averaged 1.48% ± 0.15% for saline-injected controls and, respectively, 18.49% ± 1.96% and 15.62% ± 0.77% in assay mice given hypoxic plasma of rats treated with PZ or glucagon (p > 0.10, mean of 3 experiments), and likewise, 19.69% ± 1.97% and 19.68% ± 1.79% with the hypoxic plasma of mice (mean of 2 experiments).

Plasma Level of Glucagon

At different time intervals after injection, glucagon was measured in pooled plasma of 5 mice or in 3 individual plasma samples. The value of 11 pg/ml found in normal mice must not be considered as the normal titer of glucagon in mice, but as a baseline, since the antigen used in the RIA was of porcine origin as in our experiments. As shown in Table 3, after administration of 50 μg glucagon, the plasma level fell from 605 pg/ml (1 hr after injection) to 60 pg/ml 6 hr later. Whatever the dose of glucagon, all values were low 7 and 17 hr after administration of the hormone.

DISCUSSION

The present study reports the inhibitory effect of glucagon on erythropoiesis. This effect was demonstrated by the depression of reticulocytosis, 59Fe uptake, normoblast percentage, and absolute normoblast counts in femoral marrow. Normoblasts decreased, respectively, from 5.3 to 1.2 x 10⁶ per femur in male mice and from 3.6 to 1.7 x 10⁶ in females (Fig. 1). The same effect was observed in the rat (Table 1). Furthermore, the erythropoietic response to hypoxic stimulation was also reduced, as the red cell mass measured after 12 days of hypoxic stimulation averaged, respectively, 6.31 and 5.19 ml/100 g body weight in PZ- and glucagon-treated groups (Table 2). The action of glucagon was also demonstrated by the inhibition of the erythropoietic response of polycythemic mice to exogenous ESF (Figs. 2 and 3). The depressor effect was proportional to the log-dose administered and already detectable for a dose as small as 3 μg.

As the disappearance rate of glucagon is very fast, slow-acting glucagon protamine zinc was used. However, as shown in Table 3, glucagonemia was only moderately increased in our experiments. Basal values of glucagonemia in mice averaged 11 pg/ml, whereas
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If glucagon was given for 5 days before and 7 hr after the first injection of 50 μg of the hormone, erythropoiesis seemed to occur if administration of glucagon started 0 or 1 day after injection of the hormone. These values were well below glucagonomas of 1000–4000 pg/ml reported in patients with glucagon-secreting tumors.1,2

If the inhibitory effect of glucagon on erythropoiesis is well established by the present data, its mechanism has still to be clarified. The experiments suggest an action in the early stages of erythropoiesis and exclude that this effect is related to the inhibition of ESF production. The absence of inhibitory effect on erythropoiesis if glucagon was given for 5 days before a single injection of ESF in polycythemic mice seems to indicate that the pool of ESF-sensitive cells was not affected. As shown on Fig. 4, maximal inhibition of erythropoiesis seemed to occur if administration of glucagon started 0 or 1 day after injection of the hormone. Accordingly, it was assumed that glucagon interfered with red cell production at the level of stem cells or ESF-sensitive cell differentiation, and not with heme synthesis. However, the major inhibitory effect of glucagon injections started the day of ESF administration could also be due to the greater number of injections performed. On the other hand, administration of glucagon during 5 days before hypoxic stimulation had no effect on ESF plasma titer.

Inhibition of protein synthesis, an enhancement of protein catabolism, and neoglucogenesis by glucagon could depress erythropoiesis by the shortage of protein precursors required for red cell formation. The mechanism involved could be similar to the one implicated in the anemia of protein deprivation. This hypothesis is unlikely, since the response of protein-deprived rats to exogenous ESF is not decreased, contrary to the depression induced by glucagon. Moreover, it has been shown in rats that the anemia of protein deprivation is mainly related to the decrease in ESF production.10 The action of glucagon also seems different from that of starvation, where production as well as response to exogenous ESF are reduced. Because the only known direct biochemical action of glucagon is the stimulation of the production of cAMP,13 and because this nucleotide depresses the cellular growth of fibroblasts and mesodermal cells in vitro,14 it could be tempting to speculate that the depressing effect of glucagon is mediated through cAMP. An argument against this view is that cAMP enhances erythropoiesis in mice.15

Since extrapolation of observations made in rodents to the human species is conjectural, a depressor effect of glucagon on erythropoiesis in man can only be suggested. However, it must be recalled that estrogens and androgens have been found to exert similar effects on erythropoiesis in rodents and in man. It is therefore tempting to relate the high levels of glucagon and the anemia usually reported in patients with glucagon-secreting tumors. A possible role of the hormone in the depression of erythropoiesis in several diseases with hyperglucagonemia might be evocated. This could be the case of anemia in hepatic cirrhosis,16 chronic inflammatory syndrome,17 and renal failure,18 where the lack of ESF is probably not the only factor involved.19 The hyperglucagonemia of starvation20 could also be implicated in the reduced response to ESF reported in fasted rats.12 It is clear that further investigations are required to assess the mechanism of the action of glucagon on erythropoiesis and to establish its eventual role in certain anemias.

ACKNOWLEDGMENT

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