Mechanism of Erythropoietin Production by Cobaltous Chloride

By Marilyn E. Miller, Donald Howard, Frederick Stohlman, Jr., and Patricia Flanagan

Normal and nephrectomized Sprague-Dawley rats were treated with CoCl₂ at three dose levels, 10, 20, and 25 μm/100 g body weight. The effects of this drug on acid-base balance were related to the production of erythropoietin. Within 6 hr after the administration of CoCl₂ to normal rats, a dose-related respiratory alkalosis occurred associated with an increase in the affinity of hemoglobin for oxygen. This was followed by an increase in the production of erythropoietin. Nephrectomy altered the acid-base balance of the animal such that a profound acidosis occurred after the administration of CoCl₂ with an associated decrease in the affinity of hemoglobin for oxygen. Erythropoietin could not be detected in these nephrectomized rats given CoCl₂. These findings demonstrate that the production of erythropoietin after the administration of CoCl₂ is related in significant measure to changes in acid-base balance with its subsequent effect on the affinity of hemoglobin for oxygen.

Cobaltous chloride (CoCl₂) is known to stimulate erythropoiesis and produce polycythemia in both man and animals. This effect was first shown to be related to an increase in the production of erythropoietin (Ep) by Goldwasser and his associates. Several mechanisms of action have been proposed to explain this effect of cobalt on erythropoiesis, the most generally accepted of which has been that cobalt affects Ep production by producing renal "histotoxic hypoxia." Recent observations of man during exposure to low ambient P₀₂ have related the erythropoietin response seen under these conditions to an increase in the affinity of hemoglobin for oxygen secondary to the respiratory alkalosis which develops during the initial 6 hr of exposure to low ambient P₀₂. Rather than evoking a separate mechanism for the Ep response seen after the administration of CoCl₂, it seemed appropriate to investigate the effect of CoCl₂ on the affinity of hemoglobin for oxygen and to relate the production of Ep to this more general mechanism. For this reason both normal and nephrectomized rats were treated with CoCl₂ and its effect on pH, P₀₂, PO₂, and in vivo P₀₂ were related to the production of Ep.
Fig. 1. Mean ± SEM of venous $P_{CO_2}$ after the administration of CoCl$_2$ at two dose levels, 10 and 25 $\mu$moles/100 g body weight. $N = 10$.

Fig. 2. Mean ± SEM of venous pH after the administration of CoCl$_2$ at two dose levels, 10 and 25 $\mu$moles/100 g body weight. $N = 10$.

Fig. 3. Mean ± SEM of $P_{SO_2}$ values after the administration of CoCl$_2$, $N = 10$. 

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 350-400 g were given a single subcutaneous injection of CoCl$_2$·6H$_2$O at three dose levels, 10, 20, and 25 µmoles/100 g body weight. A 0.025 M stock solution of CoCl$_2$·6H$_2$O was made, and aliquots were subsequently diluted to ensure that the volume injected was the same regardless of the concentration used. The animals were sacrificed at 3, 6, 12, 18, 24, and 48 hr after the administration of CoCl$_2$; immediately prior to sacrifice they were anesthetized with chloral hydrate, 36 mg/100 g body weight intraperitoneally. Blood was sampled anaerobically, from the inferior vena cava without stasis, into heparinized glass syringes for the measurement of pH, P$_{CO_2}$, P$_{O_2}$, oxygen saturation, and carboxyhemoglobin. A second sample was obtained by cardiac puncture. The plasma from the cardiac samples was pooled for each time point and dose level and assayed for erythropoietin.

A second group of rats were bilaterally nephrectomized under ether anesthesia. After recovery from the anesthesia (approximately 1 hr) they were given a single subcutaneous injection of CoCl$_2$ (20 µm/100 g body weight). Blood was sampled in the same manner as in the preceding group. Venous whole blood pH, P$_{CO_2}$, and P$_{O_2}$ were measured at 37°C with the appropriate electrodes (Model 213 pH Blood Gas Analyzer; Instrumentation Laboratory). Oxygen saturation and carboxyhemoglobin were measured spectrophotometrically (Model 182 Cooximeter, Instrumentation Laboratory). The actual oxygen affinity, in vivo P$_{50}$, (partial pressure of O$_2$ at which 50% of hemoglobin is saturated with O$_2$) was calculated with the use of the Hill Equation from the measured P$_{O_2}$ and oxygen saturation of the venous blood, assuming $n = 2.7$.

Plasma levels of erythropoietin were measured in hypertransfused CF$_1$ virgin female mice 12-16 wk old, as previously described. $0.5$-ml aliquots of pooled plasma from each time point and dose level were injected subcutaneously on 2 consecutive days. Erythropoietin is expressed as the 24-hr uptake of radioactive iron ($^{59}$Fe citrate).

Normal values were obtained from male Sprague-Dawley rats who were bled under the same conditions as the experimental group.

RESULTS

During the first 3 hr after the injection of cobalt chloride (CoCl$_2$) the animals developed acidosis, the extent of which was dose related. During the ensuing 3 hr respiratory alkalosis developed, as evidenced by a marked decrease in P$_{CO_2}$ and an increase in pH (Figs. 1 and 2). The degree of alkalosis in rats receiving 25 µm was greater and more sustained than in those receiving 10 µm. The only significant difference between rats receiving 20–25 µm and CoCl$_2$ was in the pH at 12 and 18 hr, being greater in rats given 25 µm. The changes in pH were accompanied by changes in P$_{50}$ values as shown in Fig. 3. In rats receiving 20–25 µm of CoCl$_2$ there was an initial decrease in the affinity of hemoglobin for oxygen which was secondary to the acidosis; this was not observed in animals given 10 µm. As the animals developed alkalosis the P$_{50}$ fell in all groups. In those rats receiving 25 µm of CoCl$_2$ the increased oxygen affinity was more pronounced between 12 and 18 hr than in the group receiving 10 µm; the changes in P$_{50}$ of rats receiving 20 or 25 µm were similar (Fig. 3 and Table 1).

The production of erythropoietin was estimated from serum concentrations, and these data are shown in Fig. 4. A dose-response effect was evident, those animals receiving 25 µm of cobalt having a substantially greater production of erythropoietin than did those receiving 10 µm. Differences between 20 and 25 µm were not appreciated until 24 hr after the injection, at which time the serum concentrations of Ep were lower in the animals receiving 20 µm (Table 1).

The difference in response between anephric and normal animals to CoCl$_2$
Table 1. Comparison of the Difference in pH, $P_{CO_2}$, $P_{O_2}$, and Ep Values Between the 20 and 25 $\mu$m Dose Level (Mean Value ± SEM)

<table>
<thead>
<tr>
<th>Hours*</th>
<th>pH</th>
<th>$P_{CO_2}$ mm Hg</th>
<th>$P_{O_2}$ mm Hg</th>
<th>Per cent $^{39}$Fe Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20</td>
<td>25</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>7.25 ± 0.02</td>
<td>7.21 ± 0.03</td>
<td>48.2 ± 0.7</td>
<td>47.3 ± 1.0</td>
</tr>
<tr>
<td>6</td>
<td>7.34 ± 0.01</td>
<td>7.35 ± 0.01</td>
<td>33.7 ± 1.1</td>
<td>34.2 ± 0.7</td>
</tr>
<tr>
<td>12</td>
<td>7.37 ± 0.01</td>
<td>7.41 ± 0.01</td>
<td>29.2 ± 0.9</td>
<td>29.1 ± 0.6</td>
</tr>
<tr>
<td>18</td>
<td>7.42 ± 0.01</td>
<td>7.45 ± 0.01</td>
<td>24.4 ± 1.0</td>
<td>25.1 ± 1.0</td>
</tr>
<tr>
<td>24</td>
<td>7.35 ± 0.0</td>
<td>7.35 ± 0.01</td>
<td>28.3 ± 1.0</td>
<td>26.1 ± 0.5</td>
</tr>
<tr>
<td>48</td>
<td>7.28 ± 0.01</td>
<td>7.32 ± 0.01</td>
<td>38.6 ± 1.3</td>
<td>35.9 ± 0.7</td>
</tr>
<tr>
<td>Control</td>
<td>7.32 ± 0</td>
<td>46.5 ± 0.8</td>
<td>35.1 ± 0.3</td>
<td>0.02 ± 0</td>
</tr>
</tbody>
</table>

*Hours after the administration of CoCl$_2$·6H$_2$O.
†$\mu$m/100 g body weight.
N = 10.
Fig. 4. Mean ± SEM of plasma erythropoietin after the administration of CoCl₂, pooled for each time point and dose level.

Fig. 5. Mean ± SEM of venous $P_{CO_2}$ in both nephrectomized and normal rats given the same dose of CoCl₂. $N = 8-10$ rats per time point.

Fig. 6. Mean ± SEM of venous pH in both nephrectomized and normal rats given the same dose of CoCl₂. $N = 8-10$ rats per time point.
can be seen in Figs. 5 and 6. Initially, the anephric rats became more acidotic in response to cobalt than did controls. Subsequently, the $P_{CO_2}$ decreased but to a lesser extent than the controls, so that the renoprival animals did not develop alkalosis; the pH, although rising between 6 and 24 hr after injection, remained in the acidotic range. The persistent acidosis of the nephrectomized animals was associated with a continued decrease in oxygen affinity as shown in Fig. 7. Erythropoietin production could not be demonstrated in the nephrectomized animal but was strikingly elevated in the control animals (Fig. 7).

**DISCUSSION**

It is well established that acute administration of high doses of CoCl$_2$ will result in an increase in the serum concentrations of erythropoietin. It has been generally accepted that the increased production of erythropoietin under these circumstances is due to histotoxic hypoxia. The present studies do not exclude the possibility that cobalt directly affects the renal tissue responsible for eryth-
Erythropoietin production, but they do indicate that this is not the sole mechanism responsible for the changes in Ep production associated with the administration of CoCl₂. Changes in the oxygen dissociation curve and hence delivery of oxygen to the tissues appears to be a critical determinant.

In the present study there was an initial acidosis which we think was due to the infusion of large amounts of chloride ion. Subsequently, we observed a decrease in P₉₀ and an increase in pH, indicating that respiratory alkalosis had developed. One can only speculate on the mechanism of respiratory alkalosis, but a central nervous system effect of the cobalt ion seems likely. The pH is known to affect the oxygen dissociation curve by virtue of the Bohr effect on hemoglobin. In the alkalotic subject this results in an increased affinity of hemoglobin for oxygen and in consequence a decrease in the delivery of oxygen to the tissues; in acidosis the converse obtains. This was manifest in the present studies by changes in the P₉₀. Initially, after higher doses of cobalt there was a decrease in O₂ affinity as evidenced by an increased P₉₀. After 6 hr there was a striking increase in O₂ affinity. The increase in O₂ affinity preceded the rise in the erythropoietin concentrations observed in the serum. Further, the degree of change in P₉₀ seemed to correlate with the extent of erythropoietin production in that the changes in O₂ affinity were greatest in those animals receiving 20 or 25 μm of cobalt as was erythropoietin production. Thus, the erythropoietic response to cobalt appeared to be due in significant measure to shifts in the oxygen dissociation curve, the increased O₂ affinity leading to decreased tissue oxygenation and hence erythropoietin production.

As has been reported previously, anephric rats were unable to produce erythropoietin in response to the cobalt challenge. Extrarenal erythropoietin production was not seen in anephric rats treated with cobalt, as has been observed shortly after exposing nephrectomized rats to hypoxia. Removal of the prime source of erythropoietin production, the kidneys, does not account for the failure of extrarenal erythropoietin production. The kidney, however, is one of the principal regulators of acid-base balance in mammals, and renoprival animals develop severe acidosis. This was observed in the present series of experiments. The initial infusion of the chloride ion further increased the degree of acidosis, and, although the animals hyperventilated after the administration of cobalt and there was a rise in pH, the animals remained acidotic. As in control animals, the oxygen affinity decreased during the initial 3 hr, but in the absence of the kidneys and in the presence of continued acidosis the shift in the P₉₀ to the higher oxygen affinity seen in control animals did not occur in anephric rats. Thus, the oxygen affinity remained decreased, and oxygen delivery to the tissues increased. This seems the most likely explanation for the failure of extrarenal production of erythropoietin under these experimental circumstances.

We previously reported that changes in the oxygen dissociation curve in normal human beings exposed to hypoxia have a profound effect on the extent of erythropoietin production. Thus, when we acidified the subjects by the administration of acetazolamide prior to exposure to hypoxia, erythropoietin production was substantially reduced. This points to the major importance of pH, which through the Bohr effect on hemoglobin, affects oxygen delivery and
as a result erythropoietin production. The present studies on the erythropoietic response to cobalt further support this concept.

REFERENCES

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