Effect of ceruloplasmin on plasma iron in copper-deficient swine

Department of Medicine, University of Utah Medical School, Salt Lake City, Utah 84112

COPPER-DEFICIENT SWINE develop severe anemia, the morphological characteristics of which are indistinguishable from those seen in iron deficiency (9). The anemia does not result from reduced activity of hem biosynthetic enzymes (11). On the other hand, several abnormalities in iron metabolism are observed. These include: 1) defective iron absorption, 2) an inability to transfer iron from damaged erythrocytes to plasma at a normal rate, and 3) increased deposition of iron in reticuloendothelial and hepatic parenchymal cells. These abnormalities have been shown to be due to an inability to transfer iron from cells to plasma (10).

The anemia results from reduced activity of ceruloplasmin (10). On the other hand, several abnormalities in iron metabolism are observed. These include: 1) defective iron absorption, 2) an inability to transfer iron from damaged erythrocytes to plasma at a normal rate, and 3) increased deposition of iron in reticuloendothelial and hepatic parenchymal cells. These abnormalities have been shown to be due to an inability to transfer iron from cells to plasma (10). The copper-containing serum globulin, ceruloplasmin, has been reported by Curzon and O’Reilly (6) to catalyze the oxidation of ferrous iron in vitro. Osaki and co-workers (16) confirmed and extended these observations and suggested that, by this mechanism, ceruloplasmin may play a biological role in the transfer of iron from cells to plasma.

The following assumptions are implicit in their hypothesis: 1) that iron is presented to plasma in the form of ferrous iron, 2) that oxidation to the ferric form is necessary for optimal binding by plasma transferrin, and 3) that the rate of iron oxidation in the absence of ceruloplasmin is not adequate to meet the demands of the erythroid bone marrow for iron. If the hypothesis is correct, a defect in this reaction for iron. If the hypothesis is not supported, a defect in this reaction for iron. If the hypothesis is not supported, an in vivo system.

In previously reported studies, the intravenous administration of 0.1 mg of inorganic copper per kilogram of body weight to copper-deficient swine was followed by an increase in the plasma iron concentration (10). This phenomenon might have been due either to an effect of the administered copper or to an effect of the ceruloplasmin synthesized and released from the copper-deficient pigs. The administration of 0.1 mg of copper per kilogram of body weight to copper-deficient pigs resulted in a rapid, marked, and sustained increase in plasma iron concentration. An amount of inorganic copper equivalent to that contained in the ceruloplasmin produced only a minimal, transient increase in plasma iron.

Copper-deficient swine develop severe anemia, the morphological characteristics of which are indistinguishable from those seen in iron deficiency (9). The anemia does not result from reduced activity of hem biosynthetic enzymes (11). On the other hand, several abnormalities in iron metabolism are observed. These include: 1) defective iron absorption, 2) an inability to transfer iron from damaged erythrocytes to plasma at a normal rate, and 3) increased deposition of iron in reticuloendothelial and hepatic parenchymal cells. These abnormalities have been shown to be due to an inability to transfer iron from cells to plasma (10). The copper-containing serum globulin, ceruloplasmin, has been reported by Curzon and O’Reilly (6) to catalyze the oxidation of ferrous iron in vitro. Osaki and co-workers (16) confirmed and extended these observations and suggested that, by this mechanism, ceruloplasmin may play a biological role in the transfer of iron from cells to plasma transferrin. The following assumptions are implicit in their hypothesis: 1) that iron is presented to plasma in the form of ferrous iron, 2) that oxidation to the ferric form is necessary for optimal binding by plasma transferrin, and 3) that the rate of iron oxidation in the absence of ceruloplasmin is not adequate to meet the demands of the erythroid bone marrow for iron. If the hypothesis is correct, a defect in this reaction for iron. If the hypothesis is not supported, a defect in this reaction for iron. If the hypothesis is not supported, an in vivo system.

In previously reported studies, the intravenous administration of 0.1 mg of inorganic copper per kilogram of body weight to copper-deficient swine was followed by an increase in the plasma iron concentration (10). This phenomenon might have been due either to a direct effect of the administered copper or to an effect of the ceruloplasmin synthesized therefrom. A preliminary study of the time relations between the change in plasma iron and plasma ceruloplasmin following the above dose of copper failed to distinguish between these two possibilities. The studies to be reported employed a smaller dose of inorganic copper, one that induced no measurable change in plasma ceruloplasmin. The effects of such an injection were compared with the effect of the same amount of copper in the form of ceruloplasmin. A preliminary report of this work has been published elsewhere (19).

METHODS

Fifteen 4-day-old pigs were obtained from a single supplier. All were maintained at least 70 days on a copper- and iron-deficient diet consisting of canned, condensed milk (9). In addition, each pig was given a total of 2.0 g of iron as iron dextrin (Pigdex) in multiple intramuscular injections between 5 and 30 days of age. No other source of iron was available to them throughout the study period.

The pigs were divided into three groups. Those in group I (seven animals) initially were given ceruloplasmin. After 8–10 days, when changes due to the initial injection were no longer apparent, four of these pigs were given copper sulfate. Pigs in group II (six animals) were given copper sulfate initially and four of these were given ceruloplasmin 8–10 days later. Thus there were 11 studies in which ceruloplasmin was given and 10 studies with inorganic copper. The effect of the administered agent was the same whether given as the initial or subsequent injection. Group III consisted of two pigs which were given plasma from a copper-deficient pig as a control of nonspecific effects of homologous protein administration.

The administered dose of ceruloplasmin copper or of copper sulfate was related to estimated plasma volume (3), rather than body weight. Each pig was given 15 μg of copper per 100 ml plasma volume through a catheter inserted into the anterior vena cava. On a weight basis, the dose averaged 9.1 μg of copper per kilogram of body weight in both groups I and II and ranged from 7.1 to 13.0 μg/kg in individual pigs because of variations in plasma volume. This dose was selected on the basis of preliminary experiments which indicated that such an amount of inorganic copper would induce no appreciable change in plasma ceruloplasmin levels.

Ceruloplasmin used in these studies was prepared by chromatography on diethylaminoethyl Sephadex columns.
CERULOPLASMIN EFFECTS ON PLASMA IRON IN SWINE

RESULTS

Injections of ceruloplasmin, copper sulfate, or copper-deficient plasma were made after about 70 days on the copper-deficient diet. At this time, all pigs in the experimental groups were profoundly copper deficient, as indicated by the presence of anemia, hypocupremia, and hypoceruloplasminemia (Table 1). Wide variations in serum iron concentration were found in all groups. No significant differences among the three experimental groups with respect to these measures were found prior to injection. The manifestations of copper deficiency in these pigs did not differ from those reported in more detail elsewhere (10).

With the dose levels used, no increase in plasma ceruloplasmin was detected after the administration of copper sulfate (Fig. 1). There was a prompt increase in plasma ceruloplasmin after the administration of ceruloplasmin, and essentially all of the injected ceruloplasmin was found in the circulation at 30 min. Thereafter, plasma ceruloplasmin levels decreased with a $T_{1/2}$ of approximately 2 days. A significant difference ($P < .01$) between the plasma ceruloplasmin levels of pigs receiving inorganic copper and those of pigs receiving ceruloplasmin was maintained for 6 days.

Following the administration of ceruloplasmin, the plasma iron concentration increased promptly, reaching a peak about 4 hr after the injection. The increased values were sustained for 4 days and then gradually returned toward control levels (Fig. 1). In the pigs given inorganic copper, only a slight increase in plasma iron concentration was observed over the next few hours. At 24 and 48 hr after the injections, the plasma iron concentration was less than that observed prior to the infusion. The two groups remained significantly different ($P < .001$) with respect to the change in plasma iron concentrations for 5 days. No change in plasma iron concentration was observed over a 6-hr period in either of the pigs given homologous, copper-deficient plasma (Fig. 1). The rapidity with which ceruloplasmin injection affects plasma iron is shown in Table 2. The increase in plasma iron in the ceruloplasmin-treated group was apparent in 5 min and plasma iron concentration rose rapidly thereafter.

In order to determine whether the increase in plasma iron concentration following ceruloplasmin administration was due to an increased flow of iron into plasma or to decreased iron outflow, ferrokinetic studies were performed in eight copper-deficient swine. Autologous plasma tagged with $^{59}$Fe was injected intravenously, followed in 25 min by ceruloplasmin injection. Frequent blood samples were obtained

### TABLE 1. Measures of copper deficiency

<table>
<thead>
<tr>
<th>No. of Studies</th>
<th>VPRC, ml/100 ml</th>
<th>Hemoglobin, g/100 ml</th>
<th>Plasma Copper, µg/100 ml</th>
<th>Plasma pPD Oxidase Activity, A$_{440}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal range (95% limits)</td>
<td>10 33±47</td>
<td>10.3±14</td>
<td>13±25</td>
<td>0.47±0.71</td>
</tr>
<tr>
<td>Prior to ceruloplasmin injection</td>
<td>11 24±3</td>
<td>6.9±0.8</td>
<td>19±1</td>
<td>0.006±0.0005</td>
</tr>
<tr>
<td>Prior to copper sulfate injection</td>
<td>10 24±2</td>
<td>6.6±0.6</td>
<td>17±3</td>
<td>0.008±0.0008</td>
</tr>
<tr>
<td>Prior to plasma injection</td>
<td>2 31, 13</td>
<td>9, 4</td>
<td>12, 9</td>
<td>0.014, 0.010</td>
</tr>
</tbody>
</table>

Values in experimental groups are means ± SE. *VPRC = volume of packed red cells.

The routine hematologic methods and those for determining plasma iron concentration and plasma iron binding capacity have been published (5). Plasma copper concentrations were determined with oxalylhydrazide (1), and plasma ceruloplasmin by the paraphenylenediamine (pPD) oxidase method of Ravin (17). All glassware used throughout the study was iron and copper free.

Eight additional copper-deficient pigs were used in ferrokinetic studies that were performed by previously reported techniques (7). Kinetics during the nonsteady state were analyzed by means of a digital computer simulation technique as reported by Bishop et al. (2).

### TABLE 2. Increase in plasma iron during first hour after injection of ceruloplasmin or copper sulfate

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Increase in Plasma Iron, µg/100 ml</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceruloplasmin-injected pigs</td>
<td>Copper sulfate-injected pigs</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td>15</td>
<td>26</td>
<td>3</td>
</tr>
<tr>
<td>30</td>
<td>64</td>
<td>2</td>
</tr>
<tr>
<td>60</td>
<td>136</td>
<td>14</td>
</tr>
</tbody>
</table>

*Four pigs in each group. †Paired t test.
over a 2-hr period to monitor changes in plasma iron concentration and specific activity. These data were then simulated with a computer programmed to calculate time-dependent changes in specific activity and concentration in a pool as a function of altered inflow and outflow rates (2).

The results of a representative study in a single copper-deficient pig given ceruloplasmin are depicted in Fig. 2. The best match between observed and computer-simulated data was achieved by holding plasma iron outflow constant and increasing inflow (Fig. 2A). By contrast, a poor match was obtained when inflow was held constant and outflow was decreased (Fig. 2B). A number of attempts were made to match the data by varying inflow and outflow simultaneously. When a significant change in outflow was combined with an increased inflow, it was possible to simulate the curve for either plasma iron or specific activity, but not both. Therefore, it appears that the primary effect of ceruloplasmin is to increase plasma iron inflow.

**DISCUSSION**

Defective movement of iron from reticuloendothelial (R-E) cells, hepatic cells, and gastrointestinal mucosa to plasma is a characteristic abnormality in copper-deficient swine (10). The present studies demonstrate that this defect can be reversed promptly by the intravenous administration of ceruloplasmin. The effect of ceruloplasmin could not have been due simply to partial correction of the copper-deficient state because inorganic copper, in an amount identical to that contained in the injected ceruloplasmin, induced little or no increase in plasma iron levels.

It should be noted that the most probable source of iron which entered the plasma after ceruloplasmin administration was the R-E and hepatic cells. The gut could not have been a source, since these animals were maintained on an iron-deficient diet.

These observations suggest, therefore, that the R-E iron "block" previously demonstrated in copper-deficient swine (10) is a consequence, at least in part, of hypoceruloplasminemia. They further suggest that ceruloplasmin plays a physiologic role in mediating R-E iron release.

The mechanism of action of ceruloplasmin was not determined in these studies. However, the observations support the concept of ceruloplasmin function proposed by Osaki and associates (16). According to their concept, iron is presented to the cell membrane in the form of ferrous ion. Subsequent binding of iron by plasma transferrin would depend on the rate of oxidation to ferric ion, a rate which is markedly accelerated by ceruloplasmin. In the absence of ceruloplasmin, the rate of cell-to-plasma iron transfer could be no faster than the autooxidation reaction. The rate of the latter reaction, under physiologic conditions, appears to be inadequate to account for the amount of iron normally passing into plasma (16). In the absence of ceruloplasmin, however, iron oxidation is so rapid that it could not be a rate-limiting step in cellular iron release.

Another possible mechanism of action of ceruloplasmin is that it delivers copper to a critical intracellular site more efficiently than does albumin-bound copper and by this means regenerates an intracellular copper enzyme essential for iron release. A defect in such a system could account for the development of the R-E iron "block" in copper-deficient swine and for the corrective effect of ceruloplasmin. This mechanism, however, seems unlikely because ceruloplasmin was effective within 5 min after administration (Table 2) and at a time when all of the ceruloplasmin activity and its copper appeared to be in the plasma.

The above concepts of ceruloplasmin function have not been supported by observations in human subjects with Wilson's disease. Decreased ceruloplasmin levels are regularly observed in this disorder, but abnormalities in iron metabolism apparently are rare. The reasons for the differences between copper-deficient swine and patients with Wilson's disease are not immediately apparent, but several possibilities exist: 1) The copper-deficient pigs are rapidly growing animals, and their red cell mass must expand to keep pace with growth. In such a setting, a restriction in the availability of iron is more likely to be apparent than in hematologically stable subjects with Wilson's disease. 2) In general, the hypoceruloplasminemia of copper-deficient pigs is more severe than the hypoceruloplasminemia of patients with Wilson's disease. This quantitative difference may be of particular significance since the present studies suggest that the amount of ceruloplasmin required for normal iron metabolism is very small. A marked effect on plasma iron inflow was observed when plasma ceruloplasmin was increased by only 10% of the normal level. A minimum effective dose was not determined. Ceruloplasmin levels of less than 10% of normal were found in only 3 of 28 patients with untreated Wilson's disease studied in this laboratory (4). Unexplained abnormalities in iron metabolism have been reported in an occasional patient with Wilson's disease (15), but the relation to the degree of ceruloplasmin deficiency has not been studied. 3) Other mechanisms for oxidizing iron may partially compensate for ceruloplasmin lack in Wilson's disease. Acceleration of iron oxidation by citrate in serum is one such mechanism (13). Another might be inferred from the data of Johnson and co-workers (8), who found that ferroxidase activity in dialyzed serum from 8 of 11 patients with Wilson's disease exceeded that which could be accounted for by the ceruloplasmin content. 4) It is possible that some iron leaves the cell by way of pathways which are...
CERULOPLASMIN EFFECTS ON PLASMA IRON IN SWINE

not ceruloplasmin dependent. For example, a soluble ferric chelate, as hypothesized by Saltman et al. (18), might cross cell membranes and attach directly to transferrin without requiring oxidation of iron. Such a pathway might increase in Wilson’s disease and compensate for the lack of cerulo-

Finally, it should be noted that although ceruloplasmin has been administered to patients with Wilson’s disease (19), serial determinations of serum iron were not made.

REFERENCES


The technical assistance of Mr. George Trappett, Mr. Dale Chlarson, Miss Jacqueline Thomas, and Mrs. Alice Tustison is gratefully acknowledged. We thank Dr. Wayne H. Linkenheimer of the American Cyanamid Company for supplying the intramuscular iron preparation and Dr. Fenimore T. Johnson of the Upjohn Company for supplying heparin for use as an anticoagulant.

This study was supported by a Research Grant (AM-04189), a Training Grant (AM-5098), and a Special Fellowship (No. 1-F3-GM 35, 479-01) from the National Institutes of Health.

G. R. Lee is a Markle Scholar in Academic Medicine.

Received for publication 21 March 1969.

17. Ravin, H. A. An improved colorimetric enzymatic assay of cerulo-