Hepatic caeruloplasmin-gene expression during development in the guinea-pig

Correlation with changes in hepatic copper metabolism

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Decreased amounts of the serum copper-binding protein caeruloplasmin (hypocaeruloplasminaemia) are one of the characteristic abnormalities of copper metabolism that are found in all neonatal mammals. In the present study we have investigated the mechanism responsible for hypocaeruloplasminaemia found in neonatal guinea pigs. Northern-blot analysis of guinea-pig liver RNA revealed the presence of two caeruloplasmin mRNA species. A marked developmental change in expression was observed, with no detectable mRNA before birth, when hepatic copper is elevated. Expression was initiated soon after birth, when copper levels in the liver are falling, and was closely linked to the rise in enzymic activity and protein levels in serum. There was no differential regulation of the two mRNA species; however, a marked inter-animal variation in mRNA levels was observed. Using gel-filtration chromatography we were able to show that, before birth, serum copper was associated with a low-molecular-mass species and that, with the advent of expression of the caeruloplasmin gene after birth, copper was increasingly associated with caeruloplasmin.

INTRODUCTION

Caeruloplasmin (EC 1.16.3.1) a Blue α_2 -globulin, containing six atoms of copper per molecule, is the major serum copper-binding protein (Gutteridge & Stocks, 1981). Synthesized primarily in the liver (Ettinger, 1984), it circulates in the serum as a single polypeptide chain with an apparent molecular mass of 132 kDa (Takahashi *et al.*, 1984). The complete amino acid sequence of human and rat caeruloplasmin has been determined by protein sequence analysis (Takahashi *et al.*, 1984) and by cDNA cloning (Koschinsky *et al.*, 1986; Yang *et al.*, 1986; Aldred *et al.*, 1987; Gitlin, 1988; Fleming & Gitlin, 1990). There is a high degree of sequence identity between rat and human proteins: 93 % at the amino acid level and 84 % at the nucleotide level (Fleming & Gitlin, 1990).

Caeruloplasmin appears to be multi-functional, and its exact role remains unresolved (Freiden, 1986). It is involved in copper transport (Dameron & Harris, 1987), iron metabolism (Freiden, 1986), antioxidant defence (Goldstein *et al.*, 1979; Samokyszyn *et al.*, 1989) and possibly tissue angiogenesis (Raju *et al.*, 1982). As befits a protein which exhibits these host defence functions, caeruloplasmin is an acute-phase protein, increasing 2–3-fold after trauma or infection. Caeruloplasmin was originally considered to be synthesized exclusively by the liver (Gutteridge & Stocks, 1981); however, recently both protein synthesis and mRNA expression have been detected in non-hepatic tissue (Skinner & Griswold, 1983; Yang *et al.*, 1986; Aldred *et al.*, 1987; Fleming & Gitlin, 1990).

During late gestation and soon after birth, neonatal mammals exhibit marked changes in copper metabolism (Ettinger, 1984). Copper, which accumulates in the liver during late gestation, decreases rapidly towards adult levels after birth with the advent of biliary copper excretion (Srai *et al.*, 1986). Serum copper, which is low at birth, increases rapidly towards adult levels after parturition (Gutteridge & Stocks, 1981). Decreased amounts of

caeruloplasmin, a condition known as hypocaeruloplasmin-aemia, is another characteristic of all neonatal animals, including man (Gutteridge & Stocks, 1981). Recent studies have suggested that, in neonatal rats, caeruloplasmin mRNA is present in the liver from day 15 of gestation (Fleming & Gitlin, 1990), whereas the holoprotein is not present in the circulation until later, suggesting that some post-translational control mechanism is involved. In Wilson's disease, an inborn error of copper metabolism that is characterized by variable extents of hypocaeruloplasmin-aemia, it has been postulated that both transcriptional and translational impairment of normal caeruloplasmin metabolism are involved (Czaja *et al.*, 1987). In the present study we have investigated the developmental changes in hepatic caeruloplasmin-gene expression in the guinea pig, an animal in which developmental changes in copper metabolism are similar to those found in humans (Srai *et al.*, 1986). The results suggest that caeruloplasmin is regulated at the pre-translational stage during development. Additionally the results also show that 'non-caeruloplasmin' copper plays an important role in copper transport in the developing foetus.

EXPERIMENTAL

Sample collection

Time-mated Dunkin–Hartley guinea pigs, fed on a standard diet (SDS Ltd., Witham, Essex, U.K.), were used throughout. Gestation is between 67–69 days and day 0 is the first 24 h after birth. Prenatal littermates were delivered by Caesarian section using halothane anaesthesia. Blood samples were collected into copper-free plastic tubes and allowed to clot at 4 °C; serum was removed after centrifugation and divided into portions, which were used immediately or stored at –20 °C. Tissue samples were removed with ethanol-washed instruments, snap-frozen in liquid N₂, and stored at –70 °C.

Abbreviations used: PBS, phosphate-buffered saline; poly(A)⁺, polyadenylated; 1 × SSPE, 180 mM-NaCl/10 mM-NaH₂PO₄/1 mM-EDTA, pH 7.4; 1 × Denhardt's, 0.02 % Ficoll/0.02 % polyvinylpyrrolidone/0.02 % albumin; SOD, superoxide dismutase.

Analytical procedures

Serum caeruloplasmin oxidase activity was determined using the rate of oxidation of *p*-phenylenediamine (Henry *et al.*, 1960), using bovine caeruloplasmin (Sigma) as standard. Copper in serum and column fractions was determined by electrothermal atomic-absorption spectroscopy using a Perkin-Elmer 3030 instrument equipped with a HGA-400 graphite furnace and autosampler. Protein was determined using the Bio-Rad protein assay reagent (Bradford, 1976), using bovine γ -globulin as standard.

SDS/PAGE and Western blotting

Serum samples were subjected to electrophoresis in 7.5% gels under denaturing conditions using a Bio-Rad Mini-Protein II gel system, at 40 mA/gel for 1 h. Coloured molecular-mass markers (Amersham) were run on all gels. At the end of the electrophoresis proteins were transferred electrophoretically on to nitrocellulose (Bio-Rad) using a constant current of 60 mA for 1 h. One portion of the resultant blot was stained with Amido Black and the other was stained for caeruloplasmin using a sheep anti-human antibody (Serotec) as described below. The blot was blocked for 2 h in phosphate-buffered saline (0.01 M-disodium phosphate/0.15 M-NaCl, pH 7.2) (PBS) containing 3% (w/v) de-fatted Marvel (non-fat dried milk) and 1% (v/v) Tween 20. The blot was incubated overnight with the primary antibody (1:500) in PBS, washed four times, incubated with a second antibody conjugated to horseradish peroxidase (1:500) (Sigma) for 1 h and then developed with H_2O_2 and horseradish peroxidase developer (Bio-Rad).

Gel-filtration studies

Samples (0.5 ml) of guinea-pig serum were subjected to gel filtration on Sephacryl S-200 (Pharmacia) (70 cm $\times$ 1 cm) at 4 °C, using 10 mM-potassium phosphate, pH 7.4, as the eluant. The column was calibrated with the appropriate molecular-mass standards, including BSA and caeruloplasmin (Sigma).

RNA extraction, Northern and slot-blotting

Total RNA was extracted from tissue by homogenization in guanidinium isothiocyanate followed by density-gradient centrifugation through CsCl (Chirgwin *et al.*, 1979). The RNA concentration was estimated by A_{260} . Polyadenylated [poly(A)⁺] RNA was purified using oligo(dT)-cellulose (Sambrook *et al.*, 1989). RNA samples were denatured in formaldehyde-containing buffer and electrophoresed in 1%-agarose/2.2 M-formaldehyde gels. To confirm equal gel loading, gels were stained with ethidium bromide. RNA was transferred to nylon filters (Hybond-N; Amersham, Bucks., U.K.) overnight and fixed by u.v.-light-induced cross-linking. Slot-blot of total RNA were prepared on nitrocellulose (Schleicher and Schuell) or nylon using samples from different animals. RNA was immobilized on to the filters by baking for 2 h in a vacuum oven at 80 °C or by u.v. cross-linking. Caeruloplasmin mRNA was determined by using a ³²P-labelled cRNA probe corresponding to amino acids 560–786 of the human protein sequence (Gitlin, 1988). Hybridization was performed at 58.5 °C in 50% formamide/5 $\times$ SSPE/5 $\times$ Denhardtts/50 mM-sodium phosphate (pH 6.5)/salmon sperm DNA (200 μ g/ml)/1 mM-EDTA/0.1% SDS. Filters were washed for 20 min in 2 $\times$ SSPE, 0.2 $\times$ SSPE and twice in 0.2 $\times$ SSPE/0.1% SDS at 65 °C. Blots were reprobed with either a cRNA probe to the full sequence of human superoxide dismutase (CuZn-SOD) (Sherman *et al.*, 1983), a cRNA probe to rat actin (Fleming & Gitlin, 1990) or an excess of an oligonucleotide complementary to rat 28 S rRNA. Blots were dried and exposed to film at -70 °C with an intensifying

screen. Slot-blot were quantified by laser densitometry (LKB; Ultra gel XL).

RESULTS

Serum copper and caeruloplasmin oxidase activity

Measurement of serum copper confirmed that levels in foetal serum were low (25–40% of adult) and that these levels increased markedly after birth (Table 1). Caeruloplasmin oxidase activity was absent from serum before birth, but increased rapidly towards adult levels immediately after parturition (Table 1).

Western blotting of caeruloplasmin

It was initially confirmed that the anti-(human caeruloplasmin) antibody recognized guinea-pig protein. The guinea-pig protein appeared to be smaller (by 2–3 kDa) than the human protein (results not shown). Fig. 1 shows that no immunoreactive caeruloplasmin was present in serum from prenatal and day-0 animals. Abundant immunoreactive caeruloplasmin was, however, detected in serum from day-4, day-12 and adult animals.

Gel-filtration studies

Once it became clear that neonatal guinea-pig serum contained a large quantity of 'non-caeruloplasmin'-associated copper, gel-filtration chromatography was used to help identify the nature of the copper-binding components. The chromatographic profile of serum separated on Sephacryl S-200, shown in Fig. 2, clearly shows that adult serum separated into three peaks. The major

Table 1. Developmental changes in serum copper and caeruloplasmin activity in guinea pig

Results are expressed as means $\pm$ S.E.M. At least seven observations were made at each time point.

Age (days)	Copper (μ mol/l)	Caeruloplasmin (μ g/l)
60*	2.96 $\pm$ 0.5	0
67	3.59 $\pm$ 1.1	0
0†	3.91 $\pm$ 1.1	3.1 $\pm$ 2.8
1	5.47 $\pm$ 1.1	10.5 $\pm$ 4.4
4	6.41 $\pm$ 2.2	19.9 $\pm$ 8.2
12	6.09 $\pm$ 1.4	17.9 $\pm$ 4.2
28	8.13 $\pm$ 1.6	32.1 $\pm$ 11.8

* Gestation.

† After birth.

Fig. 1. Developmental changes in caeruloplasmin protein in guinea-pig serum

A 100 μ g sample of guinea-pig serum was electrophoresed and Western-blotted as described in the text. Serum samples were from day 60 of gestation (1), day 0 (2), day 4 (3), day 12 (4) and adult (5). Lanes 6–10 contain duplicates of lanes 1–5 stained for total protein. The positions of the molecular-mass markers are as indicated.

Fig. 2. Developmental shift in copper-binding profiles in guinea-pig serum

Serum samples were subjected to gel filtration as described in the text, and fractions (2 ml) were collected for protein and copper determination. Profiles from six ages are illustrated: days 60 (□) and 67 of gestation (▲), days 1 (●), 4 (○) and 10 (△) after birth, and adult (■).

Fig. 3. Cross-reactivity of human caeruloplasmin cRNA probe with guinea-pig RNA

Samples (1 µg) of poly(A)⁺ RNA were electrophoresed, blotted and processed as described in the text. Human liver is in lane H, guinea pig in G and rat in lane R. The blot was exposed for 1.5 h.

peak (120–150 kDa) was eluted in fractions 28–30 and is assumed to represent caeruloplasmin, because of the presence of oxidase activity and immunoreactive caeruloplasmin in these fractions. The second major peak was eluted in fractions 33–35 (60–70 kDa) and is assumed to represent albumin, as these fractions contained immunoreactive albumin. Some samples also contained a peak in the void volume of the column; however, this peak was not found consistently. Profiles from late-gestation animals or from animals on the day of birth were markedly different (Fig. 2). In prenatal animals, copper appeared only in a peak towards the total volume of the column and is assumed to be of low molecular mass (< 5 kDa). On the day of birth, copper was present in this peak, although a small amount was also present in the caeruloplasmin and albumin peaks. Increasing amounts of copper were found in the caeruloplasmin peak with development (results not shown).

Developmental changes in hepatic caeruloplasmin mRNA

By hybridizing Northern blots of poly(A)⁺ RNA from adult-human, rat and guinea-pig liver we were able to confirm that the human cRNA probe specifically cross-reacted with guinea-pig RNA (Fig. 3). The guinea-pig liver contains two mRNA species, as does the human, whereas rat liver contains only one. A similarly sized mRNA species (3.7 kb) is present in all livers. The additional band in guinea-pig liver is approx. 5.0 kb, and in the

human is approx. 4.2 kb (Fig. 3). Fig. 4 shows a Northern-blot developmental profile of liver caeruloplasmin mRNA. Before birth (days 56 and 63 of gestation; lanes A and B), no caeruloplasmin mRNA is present and on the day of birth (lane C) the level is low. By day 2 expression is markedly increased and remains high into adulthood. There is no clear differential expression of the two mRNA species. Equal RNA loading was confirmed by re-hybridizing the same blot with a rRNA probe. We attempted to see if the data from the Northern blot were representative by the use of slot blots (Fig. 5) and these confirm that, before birth, mRNA is undetectable, and after birth levels increase dramatically. It is also clear that, at all ages, there is a marked inter-animal variation. This blot was subsequently re-hybridized with a probe to CuZn-SOD. We expected this probe to act as a control, as it has been shown by a number of workers that steady-state levels of this mRNA remain unchanged under a variety of conditions, including development (Visner *et al.*, 1990, Fleming & Gitlin, 1990). It is clear that, in guinea-pig liver, steady-state levels of SOD mRNA increase gradually with development. However, we believe that levels of expression are constant at any given age and are, therefore, a reflection of loading of RNA on to the filter. To study further the variability of levels of expression in the adult animals, we prepared a series of slot-blots using RNA extracted from a further nine day-0 and nine adult animals. All of the adult animals were ex-breeding females of similar age. The slot-blots were hybridized with the caeruloplasmin probe, and mRNA was quantified by densitometry. The blot was also hybridized with a probe to actin, and the results are expressed as absorbance units relative to actin. By using an unpaired *t* test, the level of caeruloplasmin mRNA in adult animals (1.71 ± 0.39 units; mean $\pm$ S.E.M.) was found to be significantly higher ($P = 0.0018$) than in day-0 animals (0.26 ± 0.06 unit). The marked variation seen in Fig. 5 was still present both in adult and day-0 animals. A similar significant difference was found when the blot was probed with SOD and the caeruloplasmin mRNA levels expressed relative to SOD mRNA. In this case adult caeruloplasmin levels were 1.29 ± 0.22 units, whereas the day-0 value was 0.36 ± 0.07 unit ($P = 0.0028$). In the adult animals caeruloplasmin oxidase act-

Fig. 4. Northern-blot profile of caeruloplasmin mRNA in developing guinea-pig liver

Samples (15 μ g) of total hepatic RNA were electrophoresed, blotted and processed as described in the text. A, gestation day 56; B, gestation day 63; C, day 0; D, day 2 after birth; E, day 4; F, day 6; G, day 11; H, day 21; I, adult. The positions of the rRNA bands are indicated on the right. The blot was exposed for 2.5 h.

Fig. 5. Developmental changes in caeruloplasmin (CP) expression

Slot-blots were prepared and processed as described in the text, using 10 μ g of total hepatic RNA. RNA was prepared from three livers at all ages, except day 2, when only two livers were used. The blot was stripped and rehybridized with a probe to CuZn-SOD to confirm equal loading of RNA. Blots were exposed for 2 h. 63, gestation day 63; B, birth; 2, 4, 6, 11 and 21, days after birth; A, adult.

ivity was found to correlate with mRNA levels ($r = 0.71$, $P = 0.034$). SOD mRNA levels expressed relative to actin were significantly greater in the adult animals ($P = 0.027$).

DISCUSSION

The exact role that caeruloplasmin plays in copper homeostasis remains to be elucidated. In neonatal mammals levels of caeruloplasmin are markedly reduced or may be completely absent (Gutteridge & Stocks, 1981), an observation that we have previously made in the developing guinea pig (Bingle *et al.*, 1990). The present study confirms and extends our previous work and suggests that the hypocaeruloplasminaemia seen in the developing guinea pig is the result of a marked decrease in the

steady-state amount of caeruloplasmin mRNA. These results suggest that, in the guinea pig, copper availability in itself does not regulate caeruloplasmin expression, as before birth liver copper levels are greatest and decrease on the day of birth when caeruloplasmin expression is initiated (Bingle *et al.*, 1990). Recently it has been reported that the hypocaeruloplasminaemia seen in developing rats is not the result of transcriptional down-regulation of the caeruloplasmin gene (Fleming & Gitlin, 1990). Unlike the situation in guinea pigs, caeruloplasmin is present in rat serum during development, albeit in diminished amounts; however, steady-state caeruloplasmin mRNA levels are greater than might be expected, given the holoprotein levels, suggesting some form of post-translational regulation (Fleming & Gitlin, 1990). It is possible that such regulation is at the level of copper incorporation into the apoprotein. Up to 10% of caeruloplasmin in the circulation is found in the apo form in adults (Holtzman & Gaumnitz, 1970), a value that is elevated in neonatal serum (Shokeir, 1971). It is unclear whether the reduced caeruloplasmin levels found in the developing human (Shokeir, 1971) are the result of transcriptional or post-transcriptional regulation. Studies on tissue from early human embryos has shown that caeruloplasmin is expressed in human liver up to 18 weeks of gestation (Ley *et al.*, 1989).

In both guinea pigs and humans caeruloplasmin is encoded by at least two distinct mRNA species, whereas in rats only a single transcript is present (Aldred *et al.*, 1987; Fleming & Gitlin, 1990). All species express a mRNA of 3.7 kb, which contains all the information required to encode the full-length protein. The additional mRNA in guinea-pig liver is slightly larger (5.0 kb) than that found in human liver (4.2 kb). The larger transcript in human liver also encodes the mature protein and arises as a result of differential polyadenylation (J. D. Gitlin, unpublished work). Human monocytes and macrophages express a further mRNA species of 3.9 kDa in addition to those expressed in the liver. The reason for the larger-sized transcript in the guinea pig awaits sequencing of the cDNA. A probe to the untranslated region of the human sequence did not hybridize specifically to the larger guinea-pig RNA (C. D. Bingle & J. D. Gitlin, unpublished work). In both guinea pig and human, no clear differential expression of the mRNA species has been described.

Caeruloplasmin levels in patients with the inborn error of copper metabolism, Wilson's disease, may vary from undetectable to within the normal range (Scheinberg & Sternlieb, 1984). In addition, levels may be elevated by external stimuli, such as hormones or pregnancy, even in patients with initially undetectable or very low levels of circulating protein (Gutteridge & Stocks, 1981). It has been suggested that hypocaeruloplasminaemia in Wilson's disease is the result of a combination of both reduced caeruloplasmin-gene transcription and some form of translational impairment, as caeruloplasmin mRNA has been shown to be present in a patient with undetectable levels of the protein in the circulation (Czaja *et al.*, 1987). Studies with human liver-derived cell lines suggest that impairment of incorporation of copper into the apoprotein during biosynthesis does not result in failure of normal secretion or increased turnover of the apoprotein within the cells (Sato & Gitlin, 1991). It is possible that impairment of copper incorporation into the apoprotein may be the underlying cause of hypocaeruloplasminaemia of Wilson's disease. It is clear that the reduced levels of caeruloplasmin in Wilson's disease are not due to a structural defect of the caeruloplasmin gene, as Wilson's disease has been linked to chromosome 13 (Frydman *et al.*, 1985) whereas the caeruloplasmin gene is on chromosome 3 (Yang *et al.*, 1986).

The complete lack of caeruloplasmin from guinea pig serum before birth also casts further light on the role of the protein in copper metabolism. Transport of copper to sites of utilization

has long been suggested to be one of the major roles of caeruloplasmin (Freiden, 1986). However, it is apparent that caeruloplasmin does not play a role in transport of copper in the neonatal serum. The observation that caeruloplasmin is also expressed and synthesized by a number of tissues (Yang *et al.*, 1986; Aldred *et al.*, 1987; Fleming & Gitlin, 1990) suggests that it may play a more localized role in host defence. The occurrence of copper with low-molecular-mass ligands in the neonatal serum may also help to explain the marked hepatic accumulation of copper that occurs during development (Lui, 1987; Bingle *et al.*, 1990). Low-molecular-mass copper binders, possibly representing peptides, have been suggested to present the form of copper that is most readily absorbed by the liver and other tissues (Ettinger, 1984). It is interesting that these low-molecular-mass serum copper complexes are present in the period when hepatic copper accumulation is greatest (Srai *et al.*, 1986; Bingle *et al.*, 1990). The developmental shift of serum copper from low-molecular-mass copper binders to caeruloplasmin via albumin suggests that the specific copper-binding site on albumin plays a role when these other binders are absent. Copper binding to the albumin peak is unlikely to reflect changes in the levels of serum albumin during development, as it has been shown, in other species, that albumin increases steadily during development, to reach adult levels after birth.

In view of the similarities between the mRNA species in guinea-pig and human liver and the observation that the reduced levels of caeruloplasmin in the guinea pig and, to a lesser extent, in Wilson's disease are regulated at the pre-translational level, suggests that a study of the factors responsible for such regulation in the guinea pig may give us insights into the cause of hypocaeruloplasminaemia occurring in Wilson's disease.

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