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Mechanisms of caeruloplasmin biosynthesis in normal and copper-deficient rats

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To examine the mechanisms of holo-caeruloplasmin biosynthesis, we measured the serum caeruloplasmin concentration and oxidase activity, hepatic caeruloplasmin mRNA content and hepatocyte caeruloplasmin biosynthesis and secretion in normal and copper-deficient rats. Copper deficiency resulted in a near-complete loss of serum caeruloplasmin oxidase activity, yet only a 60% reduction in serum caeruloplasmin concentration and no change in the abundance of hepatic caeruloplasmin mRNA or the rate of caeruloplasmin biosynthesis. Both interleukin-1α and lipopolysaccharide increased hepatic caeruloplasmin mRNA content and caeruloplasmin biosynthesis in normal and copper-deficient animals, but neither mediator increased caeruloplasmin oxidase activity in the copper-deficient group. Pulse–chase studies in primary hepatocytes from normal and copper-deficient rats revealed that the secretory rates for newly synthesized caeruloplasmin were identical, despite little or no holo-caeruloplasmin synthesis in hepatocytes of copper-deficient rats. We conclude that hepatocyte copper content has no effect on hepatic caeruloplasmin-gene expression or caeruloplasmin biosynthesis and that the incorporation of copper into newly synthesized caeruloplasmin is not a rate-limiting step in the biosynthesis or secretion of the apoprotein from rat hepatocytes.

INTRODUCTION

Caeruloplasmin is a blue-copper oxidase found in the sera of all vertebrate species, accounting for 90–95% of the plasma copper. The protein is synthesized in hepatocytes as a single polypeptide chain and is secreted into the plasma with six atoms of copper per molecule [1,2]. The functions of the holoprotein include roles in copper and iron transport and metabolism, tissue angiogenesis, antioxidant defence and coagulation [3,4]. Consistent with these roles, caeruloplasmin is an acute-phase protein, and the serum concentration increases during infection, inflammation and tissue injury [5]. Marked decreases in serum caeruloplasmin concentration are observed in inherited disorders of copper metabolism in man and mice [6].

Although the biosynthesis and secretion of caeruloplasmin have been studied, the mechanisms of copper incorporation during biosynthesis have not been elucidated. Copper is incorporated into caeruloplasmin within hepatocytes before secretion, and turnover data indicate that very little copper exchanges from the protein in the circulation [7,8]. About 10% of circulating caeruloplasmin occurs as the apoprotein, but it is unclear if this is secreted from the liver without copper or results from a low rate of copper exchange in the plasma and extracellular fluids [9]. The incorporation of copper into caeruloplasmin appears to require specific intracellular steps, because this process is deficient in patients with Wilson's Disease, who have a marked limitation of copper excretion into bile and no incorporation of copper into newly synthesized caeruloplasmin [10].

Copper deficiency resulting from dietary copper restriction provides a unique model to examine the mechanisms of holo-caeruloplasmin synthesis because the caeruloplasmin present in the sera of copper-deficient animals is greater than 90% apoprotein [11]. Kinetic studies suggest a lag between caeruloplasmin synthesis and holoprotein secretion in copper-deficient animals re-fed copper [12]. Although these data were not obtained under steady-state conditions, such kinetic differences in the synthesis and/or secretion rates for apo- and holo-caeruloplasmin imply that the availability of intracellular copper may be rate-limiting for caeruloplasmin secretion. Recent metabolic studies utilizing ^{67}Cu indicate that the kinetics of apo- and holo-caeruloplasmin synthesis and secretion are identical in a human liver cell line [13]. To elucidate the mechanisms of holo-caeruloplasmin synthesis, we have studied the effect of dietary copper content on caeruloplasmin-gene expression and caeruloplasmin biosynthesis and secretion in hepatocytes from normal and copper-deficient rats.

EXPERIMENTAL

Materials

Chemicals and reagents were obtained from the following sources: Dulbecco's modified minimal essential media (MEM), with and without methionine, from Gibco (Grand Island, NY, U.S.A.); bovine fetal-calf serum, L-glutamine and penicillin/streptomycin from Flow (McLean, VA, U.S.A.); formalin-fixed *Staphylococcus aureus* (IgG sorb) from the Enzyme Center (Malden, MA, U.S.A.); nylon membranes (Hybond-N) from Amersham Corp. (Arlington Heights, IL, U.S.A.); electrophoresis reagents, including SDS and acrylamide, from Bio-Rad (Richmond, CA, U.S.A.); 2,5-diphenyloxazole (En^{3}Hance) from du Pont–New England Nuclear (Boston, MA, U.S.A.); scintillation fluid (Instagel) from Packard (Downer's Grove, IL, U.S.A.); [^{14}C]methylated protein standards from Amersham (Arlington Heights, IL, U.S.A.); *Escherichia coli* 127:B8 lipopolysaccharide (LPS) from Sigma Chemical (St. Louis, MO, U.S.A.); [^{35}S]methionine from ICN Radiochemicals (Wil-

Abbreviations used: IL-1α, interleukin-1α; LPS, lipopolysaccharide; i.p., intraperitoneal; PBS, phosphate-buffered saline (0.01 M-potassium phosphate/0.25 M-NaCl, pH 7.4).

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mington, DE, U.S.A.); recombinant human interleukin-1α (IL-1α) was supplied by Hoffman–LaRoche (Nutley, NJ, U.S.A.).

Animals

Adult male and female CD strain (Charles River Breeding Laboratories, Wilmington, MA, U.S.A.) and Sprague–Dawley (Harlan/Sprague–Dawley, Indianapolis, IN, U.S.A.) rats were housed in the animal care facility and maintained on rodent laboratory chow with free access to food and water. A 12 h light/dark cycle was used and, in some experiments, the copper intake was controlled by 4-week feeding of a purified diet based on formulation AIN-76A to contain either 0.6 or 6.0 mg of Cu/kg [12]. After anaesthetization with intraperitoneal (i.p.) pentobarbital sodium (100 mg/kg), animals were killed by exsanguanition. All animals were handled according to the regulations of the American Association of Accreditation of Laboratory Animal Care and the methods of killing, the animals were consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

Experimental inflammation

Experimental inflammation was induced by the single (i.p.) injection of either 100 μg of LPS or 5 μg of IL-1α. Control animals received a single 1 ml i.p. injection of phosphate-buffered saline (PBS). In some experiments, copper-deficient rats received i.p. injections of copper (1.25 mg/kg) given as copper acetate. After injection, rats were killed at 24 h, and blood was collected, allowed to clot overnight at 4 °C and centrifuged at 4500 ***g*** for 15 min at 4 °C. The supernatant was re-centrifuged at 2000 ***g*** for 5 min, and the final serum samples were stored at −70 °C. Serum caeruloplasmin oxidase levels were determined by using a *p*-phenylenediamine oxidase assay [14]. Serum caeruloplasmin concentration was determined by an e.l.i.s.a. assay using purified rabbit anti-(rat caeruloplasmin) antiserum [15]. Serum copper and zinc concentrations were determined by flame atomic-absorption spectrophotometry using a 1:5 dilution with 1 % HCl [16].

RNA isolation and blot analysis

For analysis of caeruloplasmin-gene expression, animals were killed, the skin was sterilized with 70 % (v/v) ethanol and the organs were removed and processed as previously described [17]. RNA was isolated by dissolution of tissues in guanidinium isothiocyanate, followed by CsCl-gradient centrifugation [18]. RNA samples were analysed after agarose/formaldehyde-gel electrophoresis and transferred to nylon membranes by hybridization with ^{32}P-labelled complementary RNA antisense transcripts [19]. RNA dot-blots were prepared from total RNA samples in serial dilution (0.5–2 μg of RNA/sample), and in all cases the relative hybridization varied directly with the amount of input RNA [19]. After autoradiography, individual dots were excised and quantified by liquid-scintillation counting.

Isolation of hepatocytes and biosynthetic studies

Hepatocytes were isolated from normal and copper-deficient rats by collagenase perfusion, and 2.5×10^6 cells were added per 60 mm-diameter collagen-coated culture disc. After a 3 h attachment period the cells were cultured overnight at confluence in DMEM without serum [20]. The copper concentration was measured in sample hepatocytes both before and after overnight culture to be certain that a copper-deficient state was maintained under these conditions. For biosynthetic studies, monolayers were rinsed three times with PBS and incubated at 37 °C in methionine-free DMEM containing [^{35}S]methionine (50 μCi/dish). To determine the rate of caeruloplasmin synthesis, cells were pulse-labelled for 45 min. To determine the rate of caeruloplasmin secretion, cells were pulsed for 10 min, rinsed with PBS and incubated in serum-free media with 1000-fold excess methionine for determined chase periods. After labelling, cell monolayers were rinsed with PBS, lysed by freeze–thawing and the lysate was solubilized as described [21]. Lysate and media were clarified by centrifugation at 4 °C for 10 min at 10000 ***g*** and stored until use at −80 °C. Total protein synthesis was determined in all samples by trichloroacetate precipitation of an aliquot of cell lysate or media [22]. Clarified lysates and media from [^{35}S]methionine-labelled cells were incubated overnight in PBS containing 1 % SDS, 1 % Triton × 100, 0.5 % deoxycholic acid and 2 mM-phenylmethanesulphonyl fluoride with excess antiserum. Immune complexes were precipitated with formalin-fixed *Staphylococcus aureus*, released by boiling in sample buffer and applied to SDS/7.5 %-PAGE under reducing conditions [23]. [^{14}C]Methylated size markers were induced on all gels and, after electrophoresis, gels were impregnated with 2,5-diphenyloxazole and dried for fluorography on XAR-5 film. [^{35}S]-Methionine-labelled caeruloplasmin was quantified in individual gel slices after digestion with 15 % (v/v) H_2O_2 for 24 h and addition of Instagel scintillation fluid [13].

RESULTS

Serum copper, zinc and caeruloplasmin concentrations were measured in normal and copper-deficient rats (Table 1). Serum copper concentration was markedly reduced in animals maintained on a copper-deficient diet for 4 weeks. In addition, a single dose of copper acetate (i.p.) was sufficient to normalize the serum copper levels within 24 h. Serum caeruloplasmin oxidase activity was also markedly diminished in copper-deficient rats, but was restored to normals levels within 24 h after copper acetate injection. Serum caeruloplasmin concentration was 35–40 % of that seen in normal animals and was not completely restored to normal 24 h after injection of copper. Treatment with either IL-1 or endotoxin increased the concentration of caeruloplasmin in the serum of both normal and copper-deficient animals, but did not increase the serum caeruloplasmin oxidase activity in those animals which were maintained on a copper-deficient diet. The serum zinc concentrations were unchanged throughout these manipulations.

To determine the mechanisms for the decrease in serum caeruloplasmin concentration in copper-deficient animals and the basis for the changes in serum caeruloplasmin concentrations in copper-deficient and -sufficient animals after copper repletion and injection with LPS or IL-1, we first examined the abundance of hepatic caeruloplasmin mRNA in these animals. As Fig. 1 shows, a single 3.7 kb caeruloplasmin transcript was detected by RNA blot analysis in normal (lane 1), copper-deficient (lanes 2, 4 and 6) and copper-repleted animals (lanes 3, 5 and 7) after injection of saline (0.9 % NaCl) (lanes 2 and 3), LPS (lanes 4 and 5) or IL-1 (lanes 6 and 7). Because these studies failed to reveal any qualitative changes in caeruloplasmin mRNA associated with the copper state of the animal or acute-phase stimulation, the abundance of hepatic caeruloplasmin mRNA was quantified using dot-blot analysis (Table 2). There were no differences in the abundance of hepatic caeruloplasmin mRNA between normal and copper-deficient animals (control). A 2- or 3-fold increase in hepatic caeruloplasmin mRNA was observed after injection of IL-1 or LPS respectively, and these changes were similar regardless of the copper state of the animal (normal versus deficient). In addition, extra-hepatic pulmonary expression of caeruloplasmin was also demonstrated by RNA blot analysis after injection of LPS. The abundance of pulmonary caerulo-

Table 1. Serum caeruloplasmin, copper and zinc concentrations in normal and copper-deficient rats

Results are means ± S.D. for the number of measurements (n) shown.

Group	Serum caeruloplasmin		Serum copper (g/ml)	Serum zinc (g/ml)	n
	Oxidase activity (mol/min per litre)	E.l.i.s.a. (mg/dl)			
Control (copper-sufficient)*	24.5 ± 4.5	27.6 ± 1.6	0.81 ± 0.17	1.33 ± 0.03	4
Copper-deficient	1.6 ± 0.5‡	11.0 ± 1.4	0.014 ± 0.012	1.44 ± 0.16	4
Copper-deficient + copper†	25.1 ± 2.4	20.0 ± 3.1	0.73 ± 0.06	1.46 ± 0.17	3
Copper-deficient + IL-1	2.3 ± 1.0	17.5 ± 5.6	0.05 ± 0.03	1.51 ± 0.12	4
Copper-deficient + copper + IL-1	31.1 ± 5.1	26.6 ± 16.8	0.74 ± 0.10	1.29 ± 0.21	4
Copper-deficient + LPS	0	28.0 ± 3.4	0.04 ± 0.04	1.49 ± 0.15	4
Copper-deficient + copper + LPS	24.9 ± 4.0	32.6 ± 10.2	0.79 ± 0.12	0.99 ± 0.42	3

* The mineral mix of the diet for animals in the study was controlled such that the final diet contained either 6.0 mg of Cu/kg (sufficient) or 0.6 mg of Cu/kg (deficient).

† Animals received 1.25 mg of copper acetate i.p./kg and were killed 24 h later. In some experiments IL-1α or LPS was given with or without copper as noted in the Experimental section. All measurements were made 24 h after treatment.

‡ $n = 2$ for this measurement.

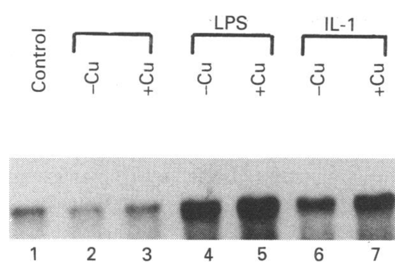

Fig. 1. Hepatic caeruloplasmin mRNA content in copper-sufficient and copper-deficient animals

A 10 μg portion of total cellular RNA was isolated from normal (lane 1), copper-deficient (lanes 2, 4 and 6) and copper-replete (lanes 3, 5 and 7) rats. Animals were treated with a single i.p. injection of saline (lanes 2 and 3), LPS (lanes 4 and 5) or IL-1 (lanes 6 and 7) as described in the Experimental section. RNA was electrophoresed, transfered to nylon and hybridized with a ^{32}P-labelled cRNA antisense probe to rat caeruloplasmin as described in the text. The blot was exposed to X-ray film at −70 °C for 4 h and subsequently rehybridized with a ^{32}P-labelled cRNA probe to β-actin.

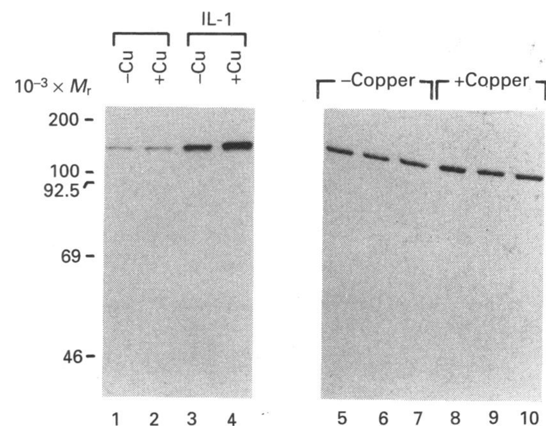

Fig. 2. Caeruloplasmin biosynthesis in primary rat hepatocytes

Primary rat hepatocytes were isolated from copper-deficient (−Cu) or copper-sufficient (+Cu) rats by collagenase perfusion and exposed for 20 h to saline (lanes 1 and 2) or IL-1α (lanes 3 and 4) as described in the text. After treatment, hepatocytes were labelled with [^{35}S]-methionine, biosynthetically labelled caeruloplasmin was immunoprecipitated from a portion of cell lysate with rabbit anti-(rat caeruloplasmin)antiserum, immune complexes were isolated with *Staphylococcus aureus* protein and subjected to SDS/PAGE. [^{14}C]-Methylated molecular-mass markers are indicated on the left. Additional immunoprecipitations from primary rat hepatocytes of copper-deficient (lanes 5–7) and copper-sufficient (lanes 8–10) animals are also shown. The dried gel was exposed to film at −70 °C for 5 h.

plasmin mRNA was equivalent in copper-sufficient and copper-deficient animals (results not shown).

We next examined the rate of caeruloplasmin biosynthesis in primary hepatocytes derived from copper-sufficient and copper-deficient rats. Hepatocytes were isolated by collagenase perfusion, labelled with [^{35}S]methionine, and newly synthesized caeruloplasmin was immunoprecipitated and analysed by SDS/PAGE (Fig. 2). Caeruloplasmin biosynthesis was identical in copper-deficient (Fig. 2, lane 1) and copper-sufficient (Fig. 2, lane 2) rats. Furthermore, the increase in the rate of caeruloplasmin biosynthesis after treatment of hepatocytes with IL-1 was equivalent in both groups of animals (Fig. 2, lanes 3 and 4). These data were identical when the experiment was repeated using hepatocytes derived from copper-deficient (Fig. 2, lane 5–7) and copper-sufficient (Fig. 2, lanes 8–10) rats. Quantification of the rate of biosynthesis of caeruloplasmin by analysis of the total radioactivity in specific eluted caeruloplasmin bands revealed no differences in the rates of biosynthesis in copper-deficient (412 ± 87 c.p.m.) and copper-sufficient (373 ± 22 c.p.m.) rats.

Because the analysis of caeruloplasmin mRNA abundance and caeruloplasmin biosynthesis failed to reveal differences to account for the observed changes in serum caeruloplasmin concentrations in copper-deficient rats, we next examined the secretory rate of caeruloplasmin from primary rat hepatocytes. As Fig. 3 shows, the rate of secretion of newly synthesized caeruloplasmin from primary rat hepatocytes, as determined by pulse–chase labelling with [^{35}S]methionine, was identical in hepatocytes derived from copper-sufficient (Fig. 3*a*) and copper-deficient (Fig. 3*b*) animals. Caeruloplasmin was secreted from primary hepatocytes with a half-life of approx. 75–80 min, as determined by quantification of eluted bands from the gels (results not shown), and this rate was the same in multiple experiments (six for each group) using hepatocytes derived from both copper-sufficient and copper-deficient animals. The rate of

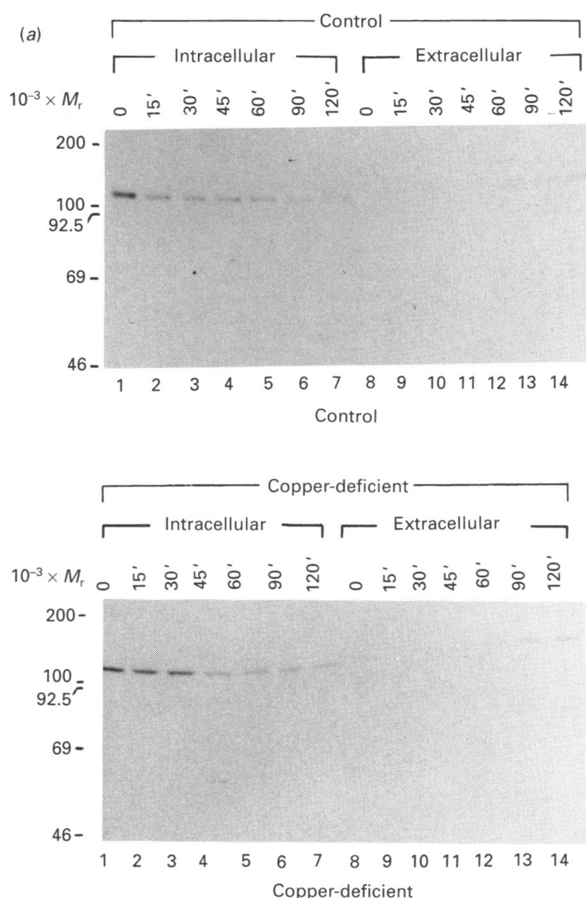

Fig. 3. Secretory rate of pulse-labelled caeruloplasmin in primary rat hepatocytes

Primary rat hepatocytes were pulse-labelled with 50 μCi of [^{35}S]-methionine for 10 min and then chased for 0 (lanes 1, 8), 15 (lanes 2 and 9), 30 (lanes 3 and 10), 45 (lanes 4 and 11), 60 (lanes 5 and 12), 90 (lanes 6 and 13) or 120 (lanes 7 and 14) min(′) with media containing a 1000-fold excess of methionine. Immunoprecipitates from the cell lysates (intracellular, lanes 1–7) or culture media (extracellular, lanes 8–14) were analysed by SDS/PAGE and fluorography as described in the text. Primary rat hepatocytes were isolated from cooper-sufficient (*a*) and copper-deficient animals (*b*).

secretion of caeruloplasmin was unchanged in both copper-sufficient and copper-deficient animals after treatment with IL-1 (results not shown).

DISCUSSION

The results of the present study reveal that despite the lack of serum caeruloplasmin oxidase activity in copper-deficient animals serum caeruloplasmin is detectable using immunochemical methods. These findings are consistent with previous data suggesting that copper-deficient rats have circulating apo-caeruloplasmin [9]. Despite previously obtained results suggesting a direct effect of copper or other transition metals on caeruloplasmin biosynthesis, there have been no studies which have directly addressed this question [24–27]. The data presented here indicate that the copper state of the animal is without effect on the basal level of hepatic caeruloplasmin mRNA. This observation is consistent with data from murine hepatocytes indicating that the intracellular copper content does not affect the caeruloplasmin mRNA content of these cells [28]. In addition, the data also indicate that the increase in hepatic caeruloplasmin mRNA after IL-2 or LPS administration is independent of copper status of the animal. Because this increase has previously been shown to occur at the transcriptional level [17], the results imply that hepatic caeruloplasmin-gene transcription is not dependent on the hepatocyte copper content. Furthermore, because the induction of pulmonary caeruloplasmin mRNA in copper-deficient animals was identical with that previously described in normal animals [29], we conclude that extrahepatic caeruloplasmin-gene expression, at least in this tissue, is similarly independent of copper status.

The biosynthetic studies reported here indicate that changes in the rate of caeruloplasmin synthesis or secretion do not account for the differences in serum caeruloplasmin concentrations observed in the animals in the present study. The data are consistent with a previous observation that, in whole animals, the rate of release of apo-caeruloplasmin into the circulation proceeds at the same rate in copper-deficient and copper-sufficient rats, despite the fact that the concentration of caeruloplasmin in copper-deficient rats is approx. 35–40 % of that seen in normal animals [11]. The finding that the biosynthesis and secretion of the protein from hepatocytes is independent of copper availability is consistent with recent studies using a hepatoma cell line where the biosynthesis and secretion of apo- and holo-protein from HepG2 cells were shown to occur at identical rates [13]. It is unlikely that these results were complicated by rapid repletion of copper within the hepatocytes after culture *in vitro* since previous studies have indicated that copper-deficient cells maintain a copper-deficient status *in vitro* for a prolonged period of time in culture [30]. The conclusion that caeruloplasmin biosynthesis is independent of the copper status of the animal is also consistent with the discordance observed between hepatic copper concentrations and caeruloplasmin production in the neonatal animal [31] and indicates that copper availability for inclusion into apo-caeruloplasmin is rate-limiting only in terms of expression of the holoprotein from hepatocytes.

These data suggest a model for copper incorporation into caeruloplasmin where availability of copper determines only the ratio of holo- to apo-protein secreted from the cell. Ultimately, then, the serum caeruloplasmin will be the result of the known differences in plasma levels based on known differences in the serum half-lifes, the apoprotein turnover being much greater in rodents and humans [4,32]. Such a model is consistent with data obtained in newborn human infants and pregnant women, where, despite a 10-fold difference in caeruloplasmin biosynthesis, the normal ratio of apo- to holo-caeruloplasmin in the plasma is maintained [33]. Other studies with rat hepatocytes in culture also support this hypothesis [34]. Thus, under normal conditions, the pool of available hepatocyte copper would not appear to be

Table 2. Hepatic caeruloplasmin mRNA content in normal and copper-deficient rats

At 24 h after the injection of saline (control), IL-1α or LPS in normal or copper-deficient rats, the animals were killed and RNA was isolated. Total RNA was analysed by dot-blot analysis as described in the text. Values are presented as percentages of the control value, where the radioactivity for normal rats in the control (saline) group was assigned a value of 100 %. Results are means $\pm$ S.D. for three separate experiments.

Group	Normal	Copper-deficient
Control	100 $\pm$ 18	107 $\pm$ 28
IL-1α	257 $\pm$ 37	236 $\pm$ 56
Endotoxin	387 $\pm$ 29	366 $\pm$ 71

rate-limiting, as further evidenced by the net increase in holo-caeruloplasmin biosynthesis as measured by ^{67}Cu incorporation in patients taking oestrogen [7]. The data observed here also suggest a potential mechanism for reduced serum caeruloplasmin in patients with Wilson's disease who have elevated hepatic copper but functional copper-deficiency in terms of copper incorporation into caeruloplasmin or biliary excretion. Although a previous study suggested abnormalities in caeruloplasmin-gene expression in these patients [35], the present results suggest an alternative explanation, namely that turnover differences account for reduced serum caeruloplasmin in such patients, since copper appears to be without effect on caeruloplasmin-gene expression or biosynthesis and secretion. Future studies should be designed to assess this question directly in these patients.

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