Developmental Regulation of Hepatic Ceruloplasmin mRNA and Serum Activity by Exogenous Thyroxine and Dexamethasone (44380)

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Abstract. **Ceruloplasmin (Cp) is a copper-dependent oxidase with roles that include the regulation of iron metabolism, participation in the acute-phase response to inflammation, and antioxidant systems. Although developmental increases in hepatic Cp gene expression and serum activity have been described, the molecular mechanisms that are responsible for this regulation are not fully understood. The studies described here explored the possible role of glucocorticoids and thyroxine (T_4) in the early neonatal development of Cp by the administration of these hormones on postnatal Day 1 (24 hr after birth), and the measurement of both hepatic Cp mRNA and serum activity through postnatal Day 10. Administration of the synthetic glucocorticoid hormone, dexamethasone (2 μg/g body wt), resulted in an increase in Cp mRNA on Days 3–7 that was accompanied by an increase in serum Cp activity that reached statistical significance at Day 10. Exogenous T_4 (2 μg/g body wt) significantly increased Cp mRNA 24 hr after administration. Serum Cp activity was also significantly elevated by the early neonatal administration of T_4. Furthermore, gestational hypothyroidism resulted in a significant decrease in Cp activity after postnatal Day 3. These data suggest a role for thyroid hormone and possibly glucocorticoids in the normal developmental regulation of Cp.** [P.S.E.B.M. 1999, Vol 221]

Ceruloplasmin (Cp), a developmentally regulated α_2-glycoprotein, is a blue copper oxidase that contains six atoms of copper per molecule. At least 60% of serum copper is associated with Cp in both humans and rodents (1). The amino acid (2), cDNA (3–5), and genomic promoter (6) sequences of Cp have all been determined. Although Cp is synthesized by a variety of organs such as the choroid plexus, testes, placenta (4), uterus, lung (7), and lymphocytes (8), the primary site of adult synthesis and secretion is the hepatocyte (4).

Cp is a multifunctional protein. It has ferrioxidase activity and thus plays a major role in iron metabolism (9). It has also been shown to act as a powerful serum antioxidant (10). Furthermore, as an acute phase protein of the inflammatory response, its synthesis and activity increase in response to inflammation (4). The acute phase response of Cp has been shown to be the result of transcriptional regulation (5). Other work has suggested that Cp may be a source of copper for nonhepatic organs (11–13).

Immediately after birth and during the early neonatal period, serum Cp concentration and activity rise (14, 15). This corresponds to a reduction in hepatic copper content and a rise in serum copper concentration (15, 16). The rise in serum Cp and its associated activity is the result of increased hepatic Cp gene expression and Cp synthesis during development (14). Cp mRNA can first be detected in rat liver around gestational Day 15 (7). In the first several days after birth, hepatic Cp mRNA abundance increases rapidly, reaches a plateau around the first week of life, and then

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continues to rise gradually to adult levels through the first 3 postnatal weeks (7, 17).

The mechanisms responsible for the postnatal regulation of hepatic Cp are not completely understood. Recent work has shown that retinoic acid may play a role in the post-transcriptional regulation of serum Cp activity during development (17). While the transcriptional regulation of Cp during development has not been fully explored, hepatic gene expression during the developmental period is known to be regulated by a variety of transcriptional events (18). Fleming and Gitlin have identified a transcription factor that binds to the 5′-flanking region of the Cp gene and appears to play a role in the regulation of Cp gene expression during development (6). Additionally, hormones such as glucocorticoids and thyroxine play a role in hepatic maturation, as exogenous administration of these hormones has been shown to result in precocious development (19). These hormones have also been implicated in the mechanisms responsible for the developmental pattern of expression seen for a variety of genes such as hepatic glucokinase, which is regulated by thyroxine (20), and hepatic tryptophan oxygenase, which is regulated by glucocorticoids (21).

Thus, the current studies used the exogenous administration of either synthetic glucocorticoid hormones or thyroid hormone during the early neonatal period as well as gestational hypothyroidism to determine whether these hormones play a role in the postnatal increase in hepatic Cp mRNA abundance and the resulting developmental increase in serum Cp activity.

Materials and Methods

Animals and Hormone Treatments. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Florida State University. Pregnant Sprague-Dawley dams were housed in polyethylene cages with bedding in a temperature-controlled room with a 12:12-hr light:dark cycle. Dams were fed a standard commercial diet (Rodent Laboratory Chow No. 5001, Ralston-Purina, St. Louis, MO) and water, *ad libitum*. The day of parturition was considered Day 0.

In the first study, 24 hr after birth (postnatal Day 1), pups were weighed and injected subcutaneously (sc) with the synthetic glucocorticoid, dexamethasone (Dex), in 0.9% NaCl (2 μg/g body weight, dexamethasone 21-phosphate, disodium salt, Sigma Chemicals) (22). Control pups were injected with the same volume of the saline vehicle. Pups were chosen randomly for hormone treatment and developmental day. On postnatal Days 1 (prior to injection), 2, 3, 5, 7, and 10, Dex-treated and vehicle-treated animals were sacrificed for collection of trunk blood for serum preparation and measurement of ceruloplasmin activity (n = 5–8). Liver was also collected for measurement of hepatic ceruloplasmin mRNA abundance by Northern analysis (n = 4). Body weights were measured throughout the developmental period. In a second study, pups (n = 4–6) were injected subcutaneously with 2 μg thyroxine/g body weight (23, 24) on postnatal Day 1. The thyroxine (T_4) was first dissolved in 3 m*M* NaOH/0.9% NaCl. As above, control animals were injected with equal volumes of vehicle on postnatal Day 1. Liver and serum were collected on postnatal Days 1, 2, 3, 5, and 7 for measurement of Cp mRNA abundance and Cp activity, respectively. Additionally, pregnant dams were given 6-*N*-propyl-2-thiouracil (PTU) in their drinking water(0.05%, w/v) beginning on gestational Day 7 to induce hypothyroidism (25).

Serum Ceruloplasmin Activity. Trunk blood (≈0.5 ml) was collected from each animal on the appropriate developmental day. Blood was centrifuged at 1000*g* for 10 min at 4°C, and serum was stored frozen at −80°C until analysis. Cp activity was measured using 50 μl serum by the p-phenylenediamine (ppd) oxidase method (26). Oxidation of the ppd substrate was measured by spectrophotometry at A_{540}. Care was taken to prevent hemolysis as this has been shown to interfere with the assay for activity (14). Samples with evidence of hemolysis were excluded from the analysis. Repeated measures of the same sample showed an intersample variation of <5% (range 2.6%–3.8%).

Northern Analysis of Cp mRNA. Total cellular RNA was first isolated from fresh liver tissue using a modification of the guanidium-isothiocyanate-phenol-chloroform method (27). In addition to the standard protocol, an extra phenol-chloroform extraction was employed to ensure the elimination of RNases from the samples. RNA samples (20 μg) from hormone-treated and vehicle-treated animals were subjected to agarose gel electrophoresis (1% w/v, 0.66 *M* formaldehyde, MOPS). Prior to loading, RNA was stained with ethidium bromide to allow visualization under UV light and confirmation of the integrity of the RNA. RNA was then transferred to a nylon membrane (GeneScreen Plus, NEN DuPont, Boston, MA) and hybridized to random primed ^{32}P-labeled rat Cp cDNA and a ^{32}P-labelled 28S rRNA probe, which served as a control for equal loading of lanes. Blots were exposed to x-ray film (Fujifilm RX) at −80°C. Densitometry was used to quantitate the relative amounts of bound cDNA probes. In all cases Cp mRNA abundance was normalized to 28S rRNA abundance and reported as mean ± SD. Differences were considered significant at the $P \leq 0.05$ level as determined by ANOVA and posthoc t tests.

Results

The effect of exogenous hormones on body weight during the neonatal period is seen in Table I. Treatment with 2 μg/g dexamethasone (Dex) resulted in a decrease in average body weight compared to age-matched, vehicle-treated controls. This reduction in body weight began as early as 24 hr after the administration of exogenous Dex and persisted through Day 7. Body weights of animals treated with thyroxine (T_4) on postnatal Day 1 were not significantly different at any age (Table I).

Table I. Body Weights of Hormone- and Vehicle-Treated Rat Pups[a]

	Postnatal day					
	1	2	3	5	7	10
Study 1						
Vehicle	7.71 ± .99	8.51 ± .44	9.85 ± .49	13.8 ± .58	19.8 ± 1.0	24.4 ± 1.5
Dex	7.71 ± .99	7.50 ± .88[b]	8.41 ± 1.1[b]	12.5 ± 1.5[c]	16.7 ± 2.3[c]	18.9 ± 5.6
Study 2						
Vehicle	7.12 ± .82	7.90 ± .95	9.23 ± 1.2	12.7 ± 1.8	17.3 ± 2.8	ND
T_4	7.12 ± .82	8.11 ± 1.1	8.56 ± 1.3	11.9 ± 1.8	16.7 ± 2.6	ND

[a] Body weight in g (mean ± SD).
[b] Statistically different from age-matched, vehicle-treated controls at $P \leq 0.001$.
[c] Statistically different from age-matched, vehicle-treated controls at $P \leq 0.05$.

In the first study, the effect of Dex on hepatic Cp mRNA abundance during the neonatal period was examined by Northern analysis. As expected, developmental increases in hepatic Cp mRNA were observed, such that hepatic Cp mRNA abundance increased 4-fold between postnatal Day 1 and Day 10 ($P \leq 0.01$, Fig. 1). The exogenous administration of Dex on postnatal Day 1 did not result in an increase in hepatic Cp mRNA above control levels until postnatal Day 3 (48 hr after Dex administration). This elevation persisted through postnatal Day 7 ($P \leq 0.002$). By postnatal Day 10, there was no difference in Cp mRNA between vehicle-treated and Dex-treated pups (Fig. 1). Serum Cp activity increased in control pups during the developmental period ($P \leq 0.01$). Mean Cp activity in Dex-treated pups was elevated compared to vehicle-treated controls (Fig. 2) but did not reach statistical significance until postnatal Day 10 ($P \leq 0.05$).

In the second study, which tested the possible role of T_4 in the developmental regulation of Cp, significant developmental increases in hepatic Cp mRNA levels were seen in vehicle-treated control animals, $P \leq 0.001$ (Fig. 3). Exogenous administration of T_4 on postnatal Day 1 produced a 3-fold elevation in hepatic Cp mRNA abundance 24 hr after administration of T_4 (Day 2) compared to controls ($P \leq 0.01$). The effect of T_4 on Cp mRNA was transient as there were no significant differences in Cp mRNA abundance in the control and T_4-treated groups by postnatal Day 5 (Fig. 3).

Corresponding serum Cp activity was also measured in Study 2. In vehicle-treated control animals, Cp activity increased during the developmental period such that Days 5, 7, and 10 were all significantly different from Day 1 ($P \leq 0.05$) (Fig. 4). T_4 treatment induced serum activity on postnatal Day 3 (mean ± SD, 24.1 ± 6.7 vs. 29.2 ± 2.7 I.U.) and postnatal Day 5 (28.1 ± 0.65 vs. 42.31 ± 7.5 I.U., $P \leq 0.05$). The increase in Cp activity in T_4-treated rats was consistent with the amount of increase seen in hepatic Cp mRNA at Day 3 in T_4-treated animals (Fig. 3). Like the mRNA, the T_4-induced Cp activity was transient. By postnatal Day 7, Cp activity had returned to control values (Fig. 4). Treatment with PTU during gestation to induce hypothyroidism resulted in a significant decrease in pup Cp activity by post-

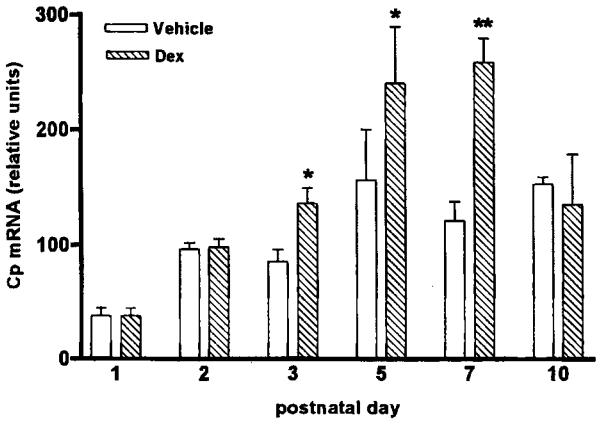

Figure 1. The effect of exogenous dexamethasone (Dex) on hepatic ceruloplasmin mRNA. Pups were injected with a vehicle or Dex (2 μg/g body weight) on postnatal Day 1. Cp mRNA was measured by Northern analysis during the first 10 days after birth. Cp mRNA data were normalized to the abundance of 28S rRNA. Bars represent mean ± SD ($n = 3$). Statistically different from age-matched controls at *$P \leq 0.05$, ** $P \leq 0.01$. Control values increased significantly during the developmental period ($P \leq 0.01$).

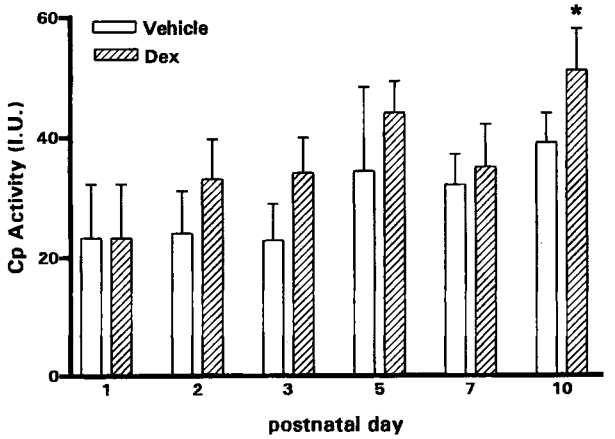

Figure 2. The effect of exogenous dexamethasone (Dex) on serum ceruloplasmin activity. Pups were injected with a vehicle or Dex (2 μg/g body weight) on postnatal Day 1. Cp activity was measured during the first 10 days after birth. Bars represent mean ± SD (n = 5–8). *Significantly different from age-matched controls at $P \leq 0.05$. Control values increased significantly during the developmental period, $P \leq 0.01$.

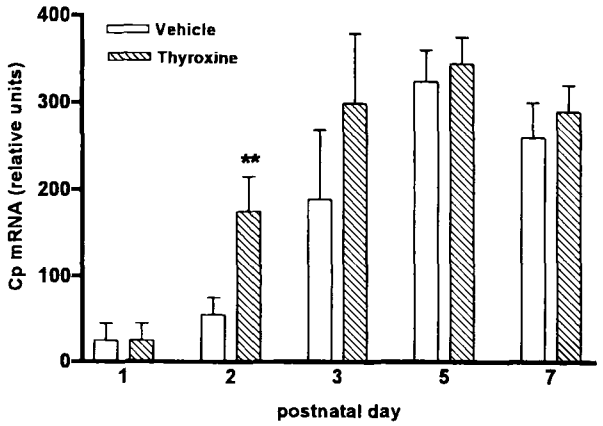

Figure 3. The effect of exogenous thyroxine (T_4) on hepatic ceruloplasmin mRNA. Pups were injected with a vehicle or T_4 (2 μg/g body weight) on postnatal Day 1. Cp mRNA was measured by northern analysis during the first 7 days after birth. Cp mRNA data were normalized to the abundance of 28S rRNA. Bars represent mean ± SD ($n = 4$). **Significantly different from age-matched controls at $P \leq 0.01$. Control values increased significantly during the developmental period, $P \leq 0.001$.

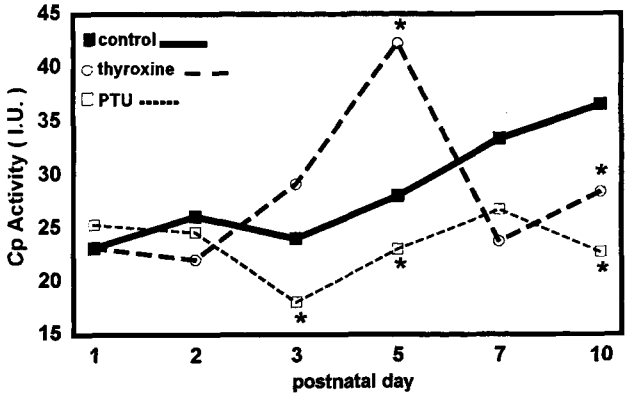

Figure 4. The effect of alterations in thyroid hormone on serum ceruloplasmin activity. Thyroxine- treated pups were injected with a vehicle or T_4 (2 μg/g body weight) on postnatal Day 1. Hypothyroid pups (PTU) were born to dams treated wtih PTU (0.05% in drinking water) from gestational Day 7. Cp activity was measured during the first 10 days after birth. Bars represent mean ± SD ($n = 4$–6). *Significantly different from age-matched controls at $P \leq 0.05$. Control values increased significantly during the developmental period, $P \leq 0.001$.

natal day 3 ($P \leq 0.05$). This decrease persisted throughout the study and remained significantly lower at Day 10 (Fig. 4).

Discussion

Both hepatic Cp mRNA and serum Cp activity increased during normal postnatal development. The relative increases in mRNA between postnatal Days 1 and 10 were greater than the overall increases in serum activity during the same period. This may be due to postnatal extra-hepatic synthesis of Cp (4, 7, 8). Beginning at the end of gestation, Cp is synthesized in fetal lung and exported into the plasma (7). Lung Cp expression decreases during the first 3–4 postnatal weeks (7) as hepatic synthesis and export increase. Thus the presence of Cp from extra-hepatic sources such as neonatal lung would result in high serum Cp activity relative to hepatic Cp mRNA during the early postnatal period as seen in the current report.

The exogenous administration of Dex in Study 1 resulted in a reduction in total body weight that was consistent with earlier studies showing that treatment with cortisol on postnatal Day 1 resulted in a significant reduction in body weight that persisted throughout adulthood (24). Despite this characteristic weight loss, administration of glucocorticoids during the early neonatal period results in precocious development (19, 24). The precocious synthesis of a variety of hepatic enzymes, such as tryptophan oxygenase (21) and ornithine aminotransferase (28), as well as hepatic α-fetoprotein gene expression (29) has been reported. Given these data on hepatic gene expression, and other data showing that Dex increases the synthesis and secretion of Cp in primary cultures of adult rat liver parenchymal cells (30), we hypothesized that glucocorticoids may play a role in the regulation of hepatic Cp mRNA during the developmental period. Indeed, administering Dex to 1-day-old pups transiently induced Cp mRNA, but this induction did not occur until 48 hr after the initial exogenous dose. The observed lag in mRNA induction may be due to the fact that glucocorticoids are not acting here *via* a classic glucocorticoid response element-mediated (GRE) mechanism (31); rather, glucocorticoids may be regulating other transacting factors that in turn increase Cp mRNA abundance. Alternative mechanisms include glucocorticoid-mediated alterations in message stability. For example, glucocorticoids have been shown to increase fatty-acid synthase mRNA stability in the fetal rat (32).

Thyroid hormone has repeatedly been shown to play a critical role in postnatal development (19). Hypothyroidism results in generalized developmental delays including late ear and eye opening, delayed development of the righting reflex, and lags in the development of other behaviors (19). The fact that no changes in body weight were observed with T_4 administration in Study 2 confirms earlier observations (24), and is likely due to the fact that early neonatal administration of thyroxine does not alter metabolic rate as it does in adults (20). Whereas very low levels of thyroid hormone have been shown in the fetus, circulating thyroxine levels increase beginning on postnatal Day 4 and peak around Day 14 (33), about the same time that Cp mRNA levels peak. The induction of Cp activity and hepatic mRNA following administration of T_4, and the reduced Cp activity in pups born to hypothyroid dams (PTU-treated) suggest a role for T_4 in the developmental regulation of Cp.

The exact mechanism whereby T_4 regulates Cp mRNA abundance and activity during development is not known. Thyroid hormone is known to regulate the expression of many genes *via* thyroid hormone response elements (TRE) in the 5′-flanking region of these genes (31). However, sequence analysis of the genomic promoter did not reveal classical TRE consensus sequences in the Cp gene (6). Alternatively, administration of T_4 has been shown to induce

glucocorticoids (34). Thus, one might hypothesize that the exogenous thyroxine induced glucocorticoid release leading to an increase in Cp mRNA. This explanation cannot fully account for the current data, however, because T_4 induced Cp mRNA by postnatal Day 2, whereas Dex treatment did not induce Cp mRNA until Day 3. Thus, post-transcriptional mechanisms may be at work to regulate Cp mRNA abundance, or T_4 may regulate uncharacterized transcription factors. Fleming and Gitlin (6) have identified a novel transcription factor that plays a role in the developmental pattern of Cp gene expression. The possible role of T_4 in the regulation of this transcription factor has not been tested.

Although the exact molecular mechanisms are still uncertain, the data presented in this report suggest roles for glucocorticoids and thyroid hormone in the developmental regulation of hepatic Cp gene expression and serum Cp activity.

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