Glucocorticoids and dietary iron regulate postnatal intestinal heavy and light ferritin expression in rats

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Yeh, Kwo-Yih, Mary Yeh, and Jonathan Glass. Glucocorticoids and dietary iron regulate postnatal intestinal heavy and light ferritin expression in rats. Am. J. Physiol. Gastrointest. Liver Physiol. 278: G217–G226, 2000.—To cope with increasing dietary iron exposure, the intestinal epithelium of weaning rats must control intracellular labile iron pools. Intestinal expression of heavy (H) and light (L) ferritin subunits during early weaning and after cortisone administration and/or iron feeding was investigated. Changes in H and L ferritin gene expression were determined by nuclear runoff transcriptional assay, Northern blot analysis, and metabolic labeling of protein synthesis. H ferritin mRNA levels did not change between days 12 and 15, doubled on day 18, and tripled on day 24. L ferritin mRNA was reduced by 50% on days 18 and 24. The protein level of the H and L subunits paralleled the change in mRNAs. Cortisone treatment on day 12 induced a precocious increase of H and decrease of L mRNA expression on day 15. Nuclear runoff assays showed that cortisone did not change H and reduced L ferritin gene transcription. The increased level of H mRNA by cortisone was not translated, unless the rats were fed an iron-fortified diet, which reduced iron regulatory protein activity and stimulated a three- to sixfold increase of ferritin synthesis. This changes in intestinal H and L ferritin expression in weaning rats are modulated by glucocorticoids and iron; the former stabilizes H mRNA and suppresses L ferritin gene transcription, and the latter derepresses translation of ferritin mRNA.

FERRITIN SERVES as an intracellular iron reservoir and plays a cardinal role in iron homeostasis. Ferritin proteins assemble into a shell consisting of 24 mixed heavy (H) and light (L) subunits. Iron enters into the shell via channels and is stored as ferricydride crystal with inorganic phosphate (17). The H and L subunits perform distinct functions in the process of iron storage in ferritin. In vertebrates, the H subunit exerts the critical oxidative step of iron uptake and the L subunit confers nucleation center for iron core formation (17, 29, 37). Ferroxidase activity gives the H subunit an important property for sequestering and limiting intra-cellular labile iron from catalyzing formation of highly toxic hydroxyl radicals. The formation of iron cores on L subunits greatly enhances iron deposition. The preferential expression of H-rich ferritin in the heart and brain and L-rich ferritin in the liver and spleen (7, 17, 40) has functional significance relating to iron storage, mobilization, and protection from toxic free radicals. The mechanisms underlying organ-specific ferritin subunit expression remain, however, mostly unknown.

Translational regulation is the best known mechanism for ferritin expression. This regulatory step is mediated by an iron regulatory element (IRE) in the 5' untranslated region (UTR) of H and L mRNAs and cytosolic iron regulatory proteins 1 and 2 (IRP1 and IRP2) (2, 15, 16, 17, 21, 24). Because IRPs exert their translational repression by interacting with the IRE (2, 15, 16, 18, 20), and because both IRPs have the same binding affinity to the IRE of H and L ferritin mRNAs (2, 7), the lack of differential binding to IREs indicates that IRPs do not differentially regulate H and L mRNA translation. In rat heart and liver, H and L ferritin mRNA differ in neither recruitment of polyribosomes nor translational efficiency (7). Thus tissue-specific ferritin isoform expression appears to be regulated by gene transcription and/or mRNA stabilization and by posttranslational stabilization of protein subunits.

Previously, we reported (41) a shift of rat ferritin isoform expression from L-rich to H-rich ferritin in the intestine at postnatal day 18. The developmental increase of intestinal H subunit expression is tissue specific, because in the liver the expression of H subunit does not increase. The intestine-specific change in H and L subunit expression during development provides an unique animal model for the investigation of tissue-specific regulatory mechanisms. Because iron feeding induces intestinal expression of ferritin with unaltered low H-to-L ratios (41), the increase of iron intake during weaning is unlikely to contribute to developmental increase in H subunit expression. In many cultured cell types, increased H ferritin expression is associated with cell differentiation (11, 31, 33). The postnatal increase of intestinal H ferritin expression might be related to intestinal maturation.

It is well documented that glucocorticoids modulate rat intestinal maturation in rats at postnatal days 18–20 (20, 42, 44–46). In the present study, we investigated whether glucocorticoids could account for the
change in H and L subunit expression seen with development. We hypothesized that if hormonal regulation takes place, the developmental increase of H and decrease of L ferritin expression would be precociously induced by exogenous glucocorticoids. To test this hypothesis, we have carried out studies on the effect of cortisone on ferritin gene expression. In this report, we demonstrate that exogenous glucocorticoid stimulates specific increases of H ferritin mRNA expression in the intestine but not in the liver. The mechanism underlying this stimulation is mediated by specific stabilization of the ferritin H mRNA and suppression of L gene transcription. Elevation of ferritin H mRNA by cortisone is not accompanied by the increase of ferritin H subunit protein synthesis, unless iron is provided for translational derepression. The elevation of H and reduction of L ferritin mRNA after cortisone and the subsequent translation after iron exposure result in a greater rate of H subunit synthesis than L subunit synthesis.

MATERIALS AND METHODS

Materials. Tris, hydrochloric acid, phenylmethylsulfonyl fluoride (PMSF), HEPES, dithiothreitol (DTT), sucrose, gelatin, NP-40, leupeptin, pepstatin, aprotinin, β-mercaptoethanol, TCA, DEAE-cellulose, Triton X-100, ferrous sulfate, sodium citrate, and formaldehyde were purchased from Sigma Chemical (St. Louis, MO). SDS, acrylamide, bisacrylamide, Chelex, and agarose were obtained from Bio-Rad Laboratories (Richmond, CA). Guanidinium thiocyanate and 2-mercaptoethanol were from Eastman Kodak (Rochester, NY). [35S]methionine, [32P]UTP, and [32P]dCTP were from NEN (Boston, MA). Hybond-N nylon membrane was from Amersham (Arlington Heights, IL). QuikHyb rapid hybridization solution, Prime-It II random primer labeling kit, and RNA transcription kit were from Stratagene (La Jolla, CA).

Animals and experimental treatments. Sprague-Dawley rats from Charles River Breeding Laboratories (Wilmington, MA) were mated and bred in animal rooms with a 12-h light-dark cycle. Rats were fed Purina Laboratory Chow and water ad libitum. Litters were reduced to 10 animals at 24 days postnatal age the animals had developed physiological anemia (39) and were lightly anesthetized by pentobarbital sodium, intragastric tubing was implanted, and 1 µg/ml pepstatin. Incubated at 80°C for 10 min, cooled on ice for 15 min, and centrifuged at 16,000 g for 30 min. The supernatants containing 5 mg of protein were incubated with 2× excess of anti-rat ferritin antisera overnight, and the immunoprecipitate was washed four times with PBS containing 0.1% Tween 20. To determine radioactivity in ferritin subunits, immunoprecipitates were subjected to SDS-PAGE to separate H and L subunits. As the positive control of mRNA translation, intestinal sucrase activity was measured according to the method described previously (43). A unit (U) of sucrase activity is...
expressed as micromoles of sucrose hydrolyzed per milligram of protein per minute.

For determinations of [32P]methionine incorporation in ferritin subunits, the immunoprecipitate was subjected to electrophoresis in a 5–22% polyacrylamide gradient gel under reducing conditions as described previously to separate H and L subunits (41). Purified rat liver ferritin was run in parallel for the identification of ferritin subunit bands. Radioactivity in H and L subunits was determined with a PhosphorImager. Protein bands in the resolving gel were stained with Coomassie blue. H and L subunit proteins visualized by Coomassie blue staining were quantitated by transmittance densitometry using volume integration with ImageQuant application software (Molecular Dynamics).

Isolation of nuclei from intestinal mucosa and nuclear runoff transcription assays. The nuclei were isolated as described previously (43). Briefly, duodenal mucosal scrapes from three rats were pooled, washed with 20 ml of ice-cold PBS, suspended in 10 ml of cell lysis buffer containing 10 mM HEPES (pH 7.5), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 200 mM sucrose, 0.5% NP-40, 0.5 mM PMSF, 1 µg/ml leupeptin, and 1 µg/ml aprotinin, homogenized by 60–80 strokes in a Dounce homogenizer with pestle B to obtain >80% lysed cells (monitored by phase-contrast microscopy), filtered through four layers of cheesecloth, and centrifuged at 800 g for 5 min to obtain the crude nuclear pellet. The pellet was resuspended in 25 ml of cell lysis buffer without NP-40 but containing 1.65 M sucrose. The crude nuclear suspension was layered on a sucrose cushion solution (2 M sucrose, 2 mM MgCl2, and 10 mM Tris·HCl, pH 7.5) and centrifuged at 25,000 g for 1 h. The nuclear pellet was then resuspended in 200 µl of storage buffer (50 mM Tris·HCl, pH 8.3, 40% sucrose, 0.5 M sucrose, pH 8.0, 3 mM MgCl2, 0.3 mM KCl, 0.1 DTT, and 0.5 each of ATP, CTP, and GTP with 0.1 mCi [α-32P]UTP for 30 min at 30°C (13)). After incubation, the reaction mixture was digested with RNase-free DNase I and subsequently by protease K. The RNA was then extracted from the mixture, hydrolyzed in 250 mM NaOH on ice for 10 min, neutralized by adding a half-volume of 1 M HEPES, and precipitated by ethanol.

The newly synthesized RNA was used to hybridize ferritin H, ferritin L, sucrase-isomaltase, and GAPDH cDNAs immobilized on nitrocellulose membrane strips for 12 h at 65°C. For negative controls, pBluescript II SK (+) DNA was also blotted on the membrane. High-stringency washing and radioactive signal detection were performed as described in Northern and dot blot analyses of mucosal H and L ferritin mRNA levels. Determination of IRP activity by electrophoretic mobility shift assay. Unlabeled and [32P]-labeled RNA containing the IRE was prepared by in vitro transcription of the linearized (by Sma I) pGL-66 as described previously (28). For labeled RNA, the transcription reaction was carried out in a reaction mixture containing 1.5 mM each of ATP, GTP, and CTP and 50 µCi of [α-32P]UTP (800 Ci/mmol, NEN) with SP6 RNA polymerase. [32P]-labeled RNA was purified after urea gel electrophoresis. Unlabeled RNA was similarly prepared except that the labeled UTP was replaced by 1.5 mM UTP. To prepare the cytoplasmic extract the mucosal scrape was gently homogenized in 10 vols of ice-cold lysis buffer (10 mM HEPES, pH 7.5, 3 mM MgCl2, 40 mM KCl, 5% glycerol, 0.3% Nonidet P-40, and 0.5 mM PMSF) with a Dounce homogenizer. The cytoplasmic extract in the supernatant was collected after centrifugation at 13,000 g for 10 min at 4°C and was stored at −70°C. Interaction of RNA and protein was analyzed by electrophoretic mobility shift assay (EMSA). Briefly, aliquots of cytoplasmic extracts containing 20 µg of protein were added into a reaction mixture containing 10 mM HEPES, pH 7.5, 3 mM MgCl2, 40 mM KCl, 5% glycerol, 2 mM DTT, and 1 × 105 cpm of [32P]-labeled RNA probe. After 10 min of incubation at room temperature, RNAase T1 (1 unit per reaction) and heparin (5 mg/ml) were added into the reaction mixture and incubated for 15 min to degrade unbound probe and displace nonspecific protein-RNA interaction. Protein-RNA complexes were resolved on a 5% nondenaturing PAGE. Radioactive signals were detected by PhosphorImager analysis.

Statistical analysis. ANOVA was computed from the experimental data among animal groups. When the F-value obtained from ANOVA was significant, Bonferroni’s test was applied to test for differences among groups. Unpaired Student’s t-test was used to calculate the data between H and L ferritin expression within an animal group.

RESULTS

Effect of cortisone on H and L ferritin mRNA expression. Intestinal mucosal H and L ferritin mRNA levels did not change between postnatal days 12 and 15. On postnatal days 18 and 24, mucosal H mRNA levels had increased approximately two- to fourfold, whereas L mRNA levels were reduced by 50% (Fig. 1). The developmental change did not occur in the liver, because H and L mRNA levels remained the same at 12, 15, 18, and 24 days of age (Fig. 1). Administration of cortisone at day 12 induced a two- to threefold increase of the intestinal H and a 60% reduction in L mRNA levels at day 15 (Fig. 2). The cortisone-induced increase of H and decrease of L mRNA levels in the intestine was consistent with the result of separate dot blot analysis (not shown). In contrast to the intestine, in the liver cortisone changed neither H nor L mRNA levels (Fig. 2). The effect of cortisone on intestinal differentiation was confirmed by precocious expression of sucrase-isomaltase mRNA (Fig. 2).

Effect of cortisone on ferritin H and L transcription. Increases in intestinal ferritin H mRNA levels could be the result of either increased gene transcription or mRNA stabilization. Nuclear runoff transcription assay showed that cortisone had no influence on ferritin H gene transcription (Fig. 3). In contrast, cortisone reduced L gene transcription by 50% (Fig. 3). As an additional positive control of the transcriptional regulation, transcription of intestinal sucrase-isomaltase gene was analyzed and found to be precociously induced by cortisone (Fig. 3).

Effect of cortisone on ferritin subunit protein expression. Postnatal increases of intestinal H mRNA expression were associated with an increase of H protein subunit at days 18 and 24 (Fig. 4), resulting in a greater amount of H than L protein subunits. In the liver, there was no differential change in the expression of H and L protein subunits during development, although increases in both L and H subunits also occurred at days 18 and 24 (Fig. 4). In cortisone-treated rats, however, the increase of intestinal H mRNA was not accompanied by an increase of H subunit protein (Fig. 5). In contrast, in parallel with the decrease of L ferritin...
mRNA, there was a decrease of L subunit protein (Fig. 5B). Cortisone had no effect on hepatic H and L ferritin expression (Fig. 5A and data not shown). The marked increase of sucrase-isomaltase mRNA induced by cortisone paralleled with the increased protein synthesis, resulting in sucrase activity increasing from undetectable to 62 ± 8 mU (n = 3). The lack of H subunit protein production suggests that the translation of ferritin mRNA is independent of glucocorticoids.

Effect of dietary iron on ferritin H and L mRNA translation. It is well documented that translation of ferritin mRNAs is regulated by iron (2, 16, 18). It is likely that the failure of the elevated H ferritin mRNA to be translated was the result of physiological iron deficiency in the neonatal pups. To address the issue of whether iron was needed for the translation of H ferritin mRNA in cortisone-treated rat pups, we investi-
gated the influence of dietary iron on ferritin protein synthesis by metabolic labeling with \([^{35}\text{S}]\)methionine. In this study, cortisone increased duodenal H ferritin and decreased L ferritin proteins (Fig. 6A). The relative changes were very similar to the data for H and L mRNA levels shown in Fig. 2. The iron-fortified diet did not alter intestinal H ferritin mRNA levels and induced a modest increase of L mRNA in control rats (Fig. 6A). In control rats fed a low-iron diet, the incorporation of \([^{35}\text{S}]\)methionine into ferritin was relatively low and was 70% higher in H than L ferritin (Fig. 6A). However, after adjustment for five methionines in the H and L subunits, the synthetic rates of H and L subunits were similar. In rats fed a low-iron diet, cortisone did not increase ferritin subunit synthesis despite a marked increase of H ferritin mRNA (Fig. 6, B and D). The low ferritin synthetic rates were associated with a relatively low steady-state level of ferritin in both control and cortisone-treated rats fed a low-iron diet (Fig. 6, C and E). The steady-state level of H-to-L subunit ratios, however, was lower in normal than in cortisone-treated rats (0.89 \pm 0.07 vs. 1.26 \pm 0.06, \(P<0.05\)). Dietary iron markedly increased ferritin mRNA translation in control and cortisone-treated rats, because \([^{35}\text{S}]\)methionine incorporation into ferritin increased dramatically (Fig. 6, B and D). The increased H ferritin mRNA in the cortisone-treated rats was now clearly translated, because the \([^{35}\text{S}]\)methionine incorporation into the H subunit increased significantly (Fig. 6, B and D). The greater increase of H than L ferritin subunit synthesis resulted in an increase of ferritin H-to-L ratio in the steady state to 2.55 \pm 0.22 in
Maternal milk contains mainly carbohydrates that differ between an adult diet and maternal milk. An adult diet is rich in disaccharides (lactose). For the digestion of α-linked carbohydrates into monosaccharides, the enzymes needed to hydrolyze these sugars must be expressed in the intestinal epithelium. In rats, postnatal expression of the intestinal sucrase-isomaltase gene exemplifies this functional demand placed on the developing intestine (20, 45).

Iron is another important nutrient that differs in quantity in the neonatal versus adult diet. Iron is in low concentration in the maternal milk and is present in iron-binding proteins such as lactoferrin or transferrin and lipid. In the adult diet, iron is more abundant and is present as heme and various iron compounds that are potentially toxic. To cope with the increased iron in adult diets, the intestine of weaning rats must develop a mechanism to protect itself from the influx of excessive iron. H ferritin is one of the major proteins involved in the regulation of intracellular labile iron pools (17, 34). The H ferritin subunit provides a strategy for cell survival in an iron-rich environment, under oxidative stress and UV irradiation (3, 6, 12). Thus an increase of H ferritin would protect the intestinal epithelium from the potential toxicity of iron.

The present observation that the increase of ferritin H expression occurred in the intestine but not in the liver during the early weaning period confirms our previous report (41). The present study further demonstrates that cortisone induced a precocious increase of intestinal H ferritin mRNA and decrease of L mRNA levels, indicating that glucocorticoids modulate developmental changes in both H and L genes albeit in opposite directions. The increase of H and decrease of L ferritin mRNAs was regulated at different steps of gene expression. Nuclear runoff transcription data showed that cortisone did not increase H ferritin gene transcription. This data implies that H ferritin mRNA is regulated by transcriptional suppression. The mechanisms underlying the specific stabilization of H ferritin mRNA in the intestine are unknown. Tissue-specific ferritin mRNA stabilization has not been previously described in vivo tissue, although it has been suggested that H ferritin mRNA is more stable than L ferritin mRNA in the rat liver (36). In cultured cells, such as human monocytes (33) and erythroleukemia K562 cells (31), an increase of H mRNA during differentiation is regulated by mRNA stabilization. Changes in intestinal ferritin H and L mRNA levels induced by cortisone, however, were not reflected in the H and L ferritin proteins unless the rats were fed the iron-fortified diet. Thus the present data reveal that the developmental increase of intestinal expression of H ferritin and decrease of L ferritin expression is regulated by two different categories of regulators at distinct levels of gene expression.

With weaning, the young of a mammal substitutes an adult diet for maternal milk to survive independently. Because the composition of the diets differ greatly, digestion and absorption of the adult diet depend on the adaptability and maturation of the digestive system. Carbohydrates are the best-known dietary components that differ between an adult diet and maternal milk. An adult diet is rich in α-linked di- or polysaccharides. Maternal milk contains mainly β-disaccharide (lactose). For the digestion of α-linked carbohydrates into monosaccharides, the enzymes needed to hydrolyze these sugars must be expressed in the intestinal epithelium. In rats, postnatal expression of the intestinal sucrase-isomaltase gene exemplifies this functional demand placed on the developing intestine (20, 45).

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In contrast to H ferritin expression, the present data show that intestinal L ferritin mRNA expression was reduced during development and by cortisone. The opposite directions of regulation allow tissue-specific expression of ferritin shells with defined H and L ferritin subunits. The mechanism underlying cortisone suppression of L ferritin gene expression in the intes-
tine remains undefined. High levels of iron could positively regulate L ferritin gene expression in the liver (40) and in the intestine (Ref. 23; unpublished observation).

The presence of two separate regulatory steps for the expression of both the H and L ferritin genes represents a stringent control of ferritin protein production. This regulation might be closely related to the metabolic need of intracellular labile iron, which would be quickly sequestered if H ferritin protein was produced under low iron status. In K562 cells, overexpression of the H ferritin subunit impairs $\beta$-globin mRNA accumulation and hemoglobin synthesis (35). The translational control of ferritin expression is different from other intestinal transcripts, such as sucrase-isomaltase and lactase, to which glucocorticoids induce a coordinate increase of mRNA and protein expression in the absence of dietary substrate (42).

Developmental expression of intestinal sucrase-isomaltase is modulated by glucocorticoids (20, 32, 44–46). We previously reported that the increase of intestinal sucrase-isomaltase mRNA paralleled sucrase activity during postnatal development (26) and after cortisone administration (46). The mechanisms by which glucocorticoids induce sucrase-isomaltase gene expression remain undefined. Although the intention of the present study was to use sucrase-isomaltase as a positive marker for cortisone-induced intestinal maturation, the nuclear runoff data demonstrate for the first time that the rate of sucrase-isomaltase gene transcription was increased in cortisone-treated rats. These data suggest that glucocorticoids induce postnatal intestinal sucrase-isomaltase expression by an increase of gene transcription. The present data raise an important question about the mode of glucocorticoid action. Consensus sequences of glucocorticoid receptor binding sites in the promoter and enhancer regions of sucrase-isomaltase gene have not been described (30). Meso-dermal products appear to be required, because the glucocorticoid induction of sucrase-isomaltase gene expression depends on the presence of mesodermal-derived cells or their products (25, 44). Because the induction of sucrase-isomaltase expression by glucocorticoids is confined to crypt cells (44, 45), the hormonal
rats fed a low-iron diet and was significantly reduced in activity was high in both control and cortisone-treated rats. The crypt cells might also be the target of glucocorticoid-induced suppression of L ferritin gene expression.

The present observation showed that intestinal IRP activity was high in both control and cortisone-treated rats fed a low-iron diet and was significantly reduced in rats fed an iron-fortified diet. These changes in IRP activities had the expected relationship to ferritin protein expression, because the intestinal ferritin content was low in the rats fed a low-iron diet and was increased in the rats fed an iron-fortified diet. These data are consistent with the role of IRP repressing ferritin mRNA translation (17, 21, 24) and indicate that the translational repression also occurs in the neonatal intestine. In addition, our study of ferritin biosynthesis demonstrated that the synthetic rates of both the H and L ferritin subunits were significantly higher in rats fed an iron-fortified than in those fed a low-iron diet. These data further support the interpretation that the iron-fortified diet increases ferritin mRNA translation through the decrease of IRP activities. The observation that the changes in the H and L ferritin protein synthesis occurred in parallel with mRNA levels is consistent with other reports indicating a coordinated control of H and L mRNA at the translational level (2, 7, 40).

Both IRP1 and IRP2 activities in the intestine of rat pups were decreased by the iron-fortified diet. Iron modulates IRP1 activity by inactivation and IRP2 activity by degradation through the proteasome pathway (16–19, 22, 24). A growing body of evidence indicates that oxidative processes mediate iron-induced reduction of both the IRP1 and IRP2 activities (17, 22). We do not know whether oxidative processes mediate dietary iron-induced decrease of IRP1 and IRP2 activities in the intestine of rat pups.

The mechanism(s) by which cortisone increases IRP activity is also unknown. Nevertheless, the increase of IRP activity in cortisone-treated rats may have physiological importance. Because glucocorticoids are strong mitogens for the intestinal epithelial cell in weaning rats (42, 45), and because iron is required for cell proliferation (1, 9), the increase of IRP activity would result in the increase of enterocyte iron uptake mediated by transferrin receptor (TfR) and perhaps the recently identified divalent cation transporter (DCT1) (14, 24). Both TfR and DCT1 contain IREs in 3'-UTR. Interactions of the IRP and IRE would stabilize TfR and DCT1 mRNAs, with a subsequent increase of TfR and DCT1 expression (14, 24). TfR is expressed in the basolateral membrane of enterocytes and is responsible for iron uptake by the transferrin-TfR-mediated endocytic pathway. DCT1 is expressed in the brush-border membrane of enterocytes (47) and is important for active transport of iron or other divalent metals (14).

An increase of intestinal expression of TfR has been reported to occur at the time of weaning and to correlate with the increase of crypt cell proliferation in rat pups (1). Steroid hormones have also been shown to increase TfR expression in the testes of hypophysectomized rats (36). It remains to be determined whether glucocorticoids modulate TfR and/or DCT1 expression in the small intestine of weaning rats, placing the developmental control of the entire scope of iron uptake under hormonal regulation.

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REFERENCES


