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Iron-Induced Accumulation of Hepatic Metallothionein: The Lack of Glucocorticoid Involvement[1,2] (42564)

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Abstract. The process(es) by which parenteral iron effects the accumulation of hepatic metallothionein (MT) is not known. The present study examined glucocorticoids as potential mediators of this process. Chicks were given either one injection (ip) of iron (+1FE) at 10 mg Fe/kg, two injections of iron (+2FE) given 24 hr apart, or a single injection of saline. Plasma corticosterone was evaluated at various times following the last injection. Plasma corticosterone increased approximately 50% following +1FE but more than 200% at 2 and 4 hr following a second injection of iron (+2FE). Plasma zinc showed a transient increase followed by a considerable depression. Coincidentally, the accumulation (determined at 24 hr) of zinc MT in liver of +2FE chicks was three times higher than that of +1FE chicks. In another experiment, markedly greater changes, at similar time intervals, in plasma corticosterone were effected by multiple subcutaneous injections of adrenocorticotropic hormone (ACTH) (either 5 IU ACTH or 20 IU ACTH/kg). Subsequent analysis of hepatic zinc MT showed only minor changes as a result of ACTH injections. These results indicate that a change in the plasma glucocorticoid corticosterone is not a primary component in the process(es) by which parenteral iron effects an increase in hepatic zinc MT. © 1987 Society for Experimental Biology and Medicine.

Metallothioneins (MT) are a group of small molecular weight, metal binding proteins which contain approximately 30% cysteine and are found in a wide variety of organisms (1–3). The synthesis of MT is influenced by a variety of factors, the most widely recognized being metals, zinc, and cadmium. These metals directly stimulate the synthesis of the protein by a mechanism involving the *de novo* synthesis of its messenger RNA (4, 5). The processes by which other factors induce the synthesis of MT are less clear. The metabolic condition of fasting is widely cited to induce the synthesis of hepatic MT (6, 7). Since glucocorticoids are well-established as hormonal inducers of MT synthesis (8–10), it is generally believed that these factors may be responsible for the elevated synthesis of MT during fasting as well as under conditions of stress in general. In yet a different process, the synthesis of MT appears to be influenced by infection and tissue injury, processes which are presumed to be independent of glucocorticoid mediation. This has, in fact, been demonstrated in studies which show that turpentine and inflammation increase hepatic MT synthesis in the absence of changes in circulating glucocorticoids, i.e., adrenalectomized animals (11, 12).

The induction of MT appears then to involve essentially three types of inducers: metals, glucocorticoids, and as yet unidentified factors involved in the induction of MT by infection or bacterial endotoxins (13). A previous report from our laboratory demonstrated that the parenteral administration of iron causes a rapid accumulation of hepatic zinc metallothionein (zinc MT) in chicks (14). Feeding high dietary iron which effected a similar increase in liver iron, however, resulted in no elevation of hepatic zinc MT. These results suggested that iron, a metal which does not bind to MT, when administered intraperitoneally, represents a significant stress and as such promotes the synthesis of hepatic MT. This same suggestion has been presented else-

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where to explain the synthesis of MT subsequent to injections of various elements (15). The present study investigated a glucocorticoid-mediated mechanism by which parenteral iron stimulates the accumulation of hepatic zinc MT. We present evidence that corticosterone, the major circulating glucocorticoid in the chick, is not the primary component involved in the iron-induced accumulation of hepatic zinc MT. The mechanism therefore appears to be glucocorticoid-independent and may involve factors similar to those associated with endotoxin-induced MT synthesis (perhaps interleukins).

Methods. Five-week-old male chicks (250–300 g), Leghorn strain, were used in all studies. Chicks were reared from hatching in batteries with raised wire floors and fed practical corn–soy-based diets containing vitamin and mineral supplements.

The first study was designed to characterize potential changes in circulating corticosterone as a result of the parenteral administration of iron. Chicks were given (ip) either a single injection of iron (+1Fe) as ferric chloride, two injections of iron (+2Fe) at 24-hr intervals, or an equivalent volume of 0.9% NaCl (saline). Each injection of iron was given at a dosage of 10 mg Fe/kg body wt from a solution containing 20 mg Fe/ml. At various intervals in the first 8 hr immmediately following the last injection, blood samples (1 cc) were obtained via the wing vein of chicks within each treatment. In an attempt to provide minimum stress, each treatment comprised three groups of eight chicks, within which, each chick was sampled only twice in the course of the experiment (8 hr). These groups were randomly selected from the entire group of 5-week-old chicks at the initiation of the experiment (prior to injections). In addition, the injection schedule was staggered to provide sufficient time to obtain accurate temporal sampling. Samples were collected in heparinized tubes and held on ice until centrifugation. Plasma was frozen (−20°C) until analysis.

Similar-age chicks and conditions were used in a study of a comparison of the effects of iron injection and the administration of adrenocorticotropic hormone (ACTH). In an attempt to simulate the conditions of iron injection, chicks received either a single series of ACTH injections (comparable to a single injection of iron) or two series of ACTH injections (comparable to two injections of iron given 24 hr apart). The single series consisted of two injections of ACTH (sc, 5 IU/kg) at zero hr and again at 2.5 hr. Blood samples were obtained as previously described at various times following the initial injection. A similar injection protocol was used for chicks receiving two series of ACTH injections except that a second series of injections (sc, 20 IU ACTH/kg) was administered 24 hr following the first injections. Blood samples from this group were obtained only during the second series of injections. The groups which received one and two series of ACTH injections were then designated as +5 ACTH and +20 ACTH, respectively. Plasma samples were obtained and frozen (−20°C) until zinc and corticosterone analyses.

Corticosterone analysis. Plasma corticosterone was determined with some modification by the radioimmunoassay described by Etches (16). One hundred microliters of plasma was combined with an equal volume of distilled/deionized water and extracted by vortex in 4 ml of iso-octane and centrifuged 500*g* for 5 min. After freezing the aqueous fraction, the organic phase was discarded. Four milliliters of methylene chloride was then added and mixed by shaking for 50 min. The methylene chloride was then decanted and dried under reduced pressure at 50°C. Four hundred microliters of a mixture containing antisera (obtained from R. Etches) diluted 1:10,000 with gelatin–phosphate buffer (PBS-Gel, 0.01 *M* KH_2PO_4, pH 7.0, with 1% gelatin and 1% Thimersol), and approximately 10,000 cpm of [^{3}H]corticosterone (1,2,6,7–3H, 98 Ci/mmol, Amersham Corp., Amherst, MA) were added, shaken, and incubated overnight at 4°C. Standards (0 to 1600 pg corticosterone), excess (16 ng), and blanks were included in triplicate for every assay. Following incubation, unbound corticosterone was precipitated by charcoal in gelatin buffer containing dextran (0.01 *M* PBS-Gel, 0.25% charcoal, Norit A, Fisher Scientific Co., Pittsburgh, PA and 0.025% dextran, Sigma Chemical Co., St. Louis, MO.). The charcoal mixture was added (with stirring) and held at 4°C for exactly 15 min followed by centrifugation at 4°C for 15

min at 1475*g*. The supernatant was then decanted, mixed with 5 ml of scintillation fluid (Scintiverse II, Fisher Scientific Co.), and counted by a Beckman liquid scintillation spectrophotometer (Model LS 100) at approximately 30% efficiency. All samples were determined in triplicate and in every assay estimates of extraction recovery were determined by the recovery of [^{3}H]corticosterone added to a control plasma sample. Estimates of plasma corticosterone were then corrected for recovery as well as for assay nonspecific binding which averaged 8%. The percentage of bound [^{3}H]corticosterone in the absence of exogenous corticosterone and corrected for nonspecific binding was from 45 to 55%.

Metallothionein and zinc analyses. Hepatic zinc MT was estimated, with slight modification, by gel filtration chromatography as previously described (14). The exception was the application of 1 ml of cytosol to smaller columns (1.6 × 30 cm) and the collection of 1-ml fractions. Each hepatic cytosol was composed of equal weights of tissue pooled from three chicks. Zinc from the chromatographs was determined by atomic absorption spectrophotometry (AA 575, Varian Associates, Inc., Palo Alto, CA.). Plasma zinc was also determined by AAS after a dilution (1:5) with distilled/deionized water. To verify an increase in the concentration of MT protein (previous measures were indirect via zinc bound), the peak within the VeMT from G75 chromatography of the various treatments was subjected to electrophoresis. An equal aliquot of cytosol from each sample was pooled within each group and chromatographed on columns (2.6 × 50 cm) containing G75 superfine (Pharmacia Fine Chemicals, Piscataway, N.J.). Three milliliters of cytosol was chromatographed at 30 ml/hr and 2-ml fractions were collected after 78 ml of the Vo was discarded. An equal portion of each fraction in the VeMT (fractions 61–72) was combined and concentrated by ultrafiltration (YM2, Amicon Corp., Lexington, MA) to a constant volume (1.8 ml). A portion of the concentrate was subjected to nondenaturing disc PAGE and the gels were stained by Coomassie and silver as described previously (17).

Data were analyzed by one-way analysis of variance and mean differences determined by Dunnett's procedure (18) for determining experimental effects relative to control.

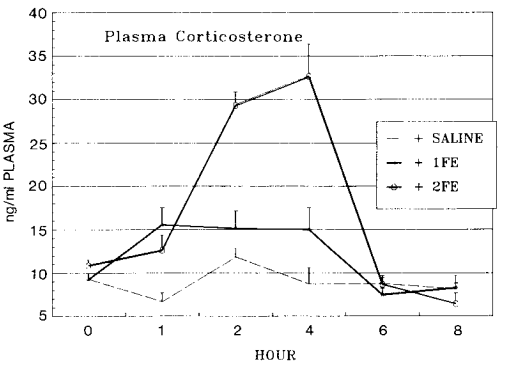

FIG. 1. Effect of parenteral iron administration on plasma corticosterone of chicks. Chicks received ip either one injection of iron (+1FE), two injections (+2FE) of iron given 24 hr apart, or a single injection of 0.9% NaCl (saline). At various times following the final injection, blood samples were obtained for corticosterone and zinc analyses. See Materials and Methods for details of the bleeding schedule. Each point represents a mean ± SEM of seven observations. Values at 1, 2 (only for +2Fe), and 4 hr were significantly ($P < 0.05$) different from saline values.

Results. The parenteral administration of iron caused a significant increase in the concentration of plasma corticosterone (Fig. 1). The nature of this response was influenced by the number of iron injections. In chicks given a single injection of iron (+1FE), plasma corticosterone was elevated 1 to 4 hr after the administration of iron. The levels of plasma corticosterone at similar times following a second injection of iron (24 hr later) were approximately twice as high as those observed in +1FE chicks. Peak concentrations were threefold higher than values observed for saline controls. At 6 hr after either +1Fe or +2Fe, plasma corticosterone values were not different from control. Plasma zinc concentrations were initially elevated (when compared to zero time point) at 2-hr post-iron injection but thereafter decreased, especially in the +2FE group (Fig. 2). Plasma zinc levels for this group at 8 hr post-iron injection were approximately 50% of control values. It is also noteworthy that, at zero time, plasma zinc of this group (+2FE) was markedly lower than control, 0.93 ppm vs 1.6 ppm, respectively.

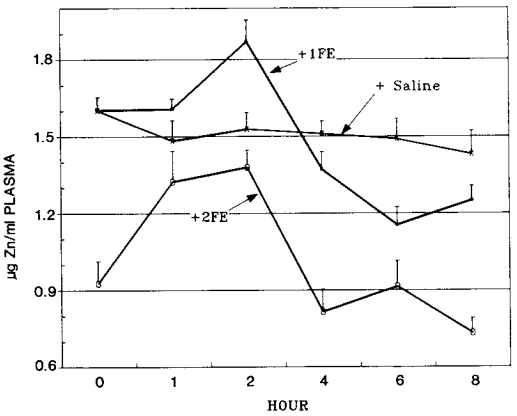

FIG. 2. Effect of parenteral iron administration on the concentration of plasma zinc. Description of experimental treatments is given in the legend to Fig. 1. Each point represents a mean ± SEM of seven to eight observations. Data were not analyzed statistically.

As anticipated, the administration of ACTH resulted in a marked increase of plasma corticosterone (Fig. 3). Two injections of 5 IU

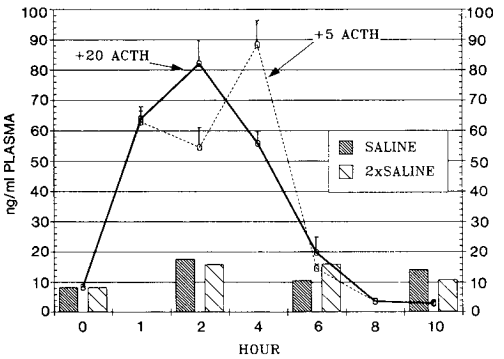

FIG. 3. Plasma corticosterone and the effect of single or multiple injections of ACTH. On the day in which blood samples were obtained, chicks received two injections subcutaneously of either 20 IU ACTH/kg (+20 ACTH) or 5 IU ACTH/kg body weight (+5 ACTH). The first injection was given at 0 hr and the second at 2.5 hr. Control chicks (saline) were treated similarly but received saline. Twenty-four hr prior to this period, the group of chicks designated at +20 ACTH were given injections of 5 IU ACTH/kg at 0 and 2.5 hr. A similar group received saline (2× saline). Details of the sampling schedule are given under Material and Methods. Each point represents a mean ± SEM of seven to eight observations. Values at 1, 2, and 4 hr for both +5 and +20 ACTH groups were significantly ($P < 0.05$) higher than corresponding saline values.

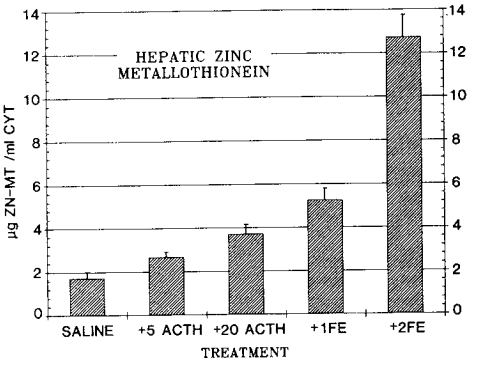

FIG. 4. Hepatic zinc metallothionein of chicks given single or multiple injections either ACTH or iron. The description of ACTH and iron treatments is given in legends to Figs. 3 and 1, respectively. Twenty-four hours after the administration of either the ACTH or iron, chicks were killed and liver obtained. An equal portion of liver from two chicks was combined for cytosol preparation. Zinc metallothionein was determined by gel filtration and expressed as µg zinc/ml cytosol. Each experimental point represents a mean ± of four to five observations (each from pooled tissue). All values except those from +5 ACTH chicks were significantly ($P < 0.05$) higher than saline.

ACTH given at 0 and 2.5 hr caused a six- to eight-fold increase in plasma corticosterone during a 6 hr period. Interestingly, a similar administration of 20 IU ACTH given 24 hr later did not cause a proportionately greater response but resulted in a comparable increase in plasma corticosterone at similar times after the injections. Both treatments resulted in rapid responses and, by 6 hr after the injection, plasma corticosterone was similar to control values. Plasma zinc concentrations were not affected by either ACTH treatment (data not shown).

The results of hepatic zinc MT analysis are shown in Fig. 4. As expected, the parenteral administration of iron especially +2FE caused a marked increase in hepatic zinc MT. A single injection of iron (+1FE) caused an increase in hepatic zinc MT of approximately threefold when compared to values observed in saline controls. Two injections given 24 hr apart (+2FE) caused a sixfold increase in hepatic zinc MT (1.9 vs 12.6 µg zinc MT). These results were consistent with our previous report (14). The administration of ACTH, however,

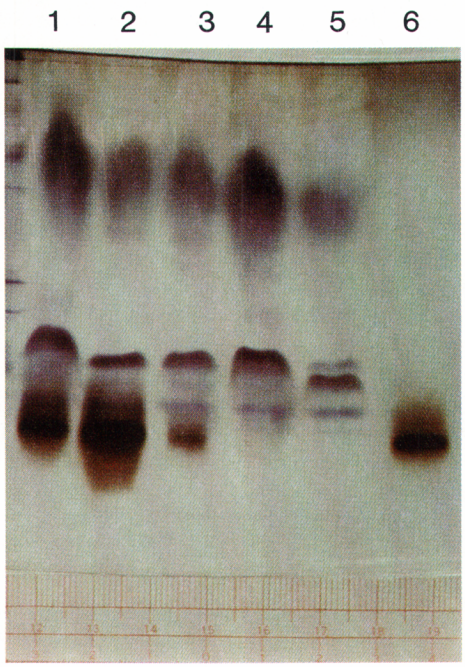

FIG. 5. Gel electrophoresis of G75 "MT peaks" from cytosol of chicks given either ACTH or iron. Sample preparation and chromatographic procedures are given in the legend to Fig. 5. An equal portion from each fraction within the Ve for MT (61 to 72) was pooled and concentrated by ultrafiltration to a equal volume. One hundred microliters was then subjected to gradient discontinuous gel electrophoresis (7.5 to 30%) at constant current for 8 hr. Gels were stained initially with Coomassie and subsequently with silver (MT is clearly distinguished with silver stain). Lanes: (1) +1FE, (2) +2FE, (3) +20 ACTH, (4) +5 ACTH, (5) saline, and (6) 7.7 μg purified chick metallothionein.

resulted in relatively little change in the concentration of hepatic zinc MT. Chicks given a single series of ACTH injections at 5 IU/kg showed hepatic zinc MT levels approximately 2.8 μg zinc MT or an increase of about 30% over saline controls. Those chicks given two series of ACTH injections (the final series consisting of 20 IU ACTH/kg) exhibited slightly higher hepatic zinc MT (3.6 μg zinc MT) representing an increase of 100% over saline control (cf. 600% for +2FE chicks).

Since there were relatively minor changes in hepatic zinc MT of chicks in the two ACTH treatments, MT protein analysis was attempted by gradient PAGE. The electrophoresis of concentrates of the "MT" peaks from G75 (superfine) chromatography is shown in Fig. 5. The analysis revealed that the changes in zinc of the VeMT were accompanied by changes in the MT protein, most notably in the +20 ACTH sample. Again, the relative difference between hepatic levels of MT in chicks given ACTH (+20 ACTH) and those given iron (+2FE) was readily apparent.

Discussion. The results of the present study suggest that parenterally administered iron effects changes in hepatic zinc MT by processes which are not primarily related to changes in plasma corticosterone. The major evidence for this suggestion comes from our results regarding the lack of correlation between responses in hepatic zinc MT and circulating corticosterone. Chicks which received two injections of iron (+2FE) exhibited a marked increase in circulating corticosterone at 2 and 4 hr after the final iron injection (Fig. 1). This response was considerably greater than that of chicks which received only a single iron injection (+1FE). Concomitantly, the accumulation of hepatic zinc MT in the former group was more than twice that observed in the group given +1FE. These particular results would suggest

that a relationship exists between the two responses. However, our data from the second phase of the study regarding the effect of ACTH on the accumulation of hepatic MT do not support this suggestion, i.e., elevated plasma corticosterone (itself) is not the primal cause of elevated hepatic zinc MT.

The experimental approach in this second phase of the study was designed to test the relationship between iron-induced changes in plasma corticosterone and hepatic zinc MT accumulation. To accomplish this, we employed ACTH to effect elevated plasma corticosterone in the absence of other changes. ACTH was given subcutaneously in amounts which we believed would simulate changes observed during iron treatment. To our knowledge, this approach is unique in that it is the first time that MT induction has been examined using the tropic hormone, ACTH. The responses we observed in this study were then responses *in vivo* to naturally secreted glucocorticoids. The results of this phase of our study demonstrated a dramatic effect of exogenous ACTH on plasma corticosterone (Fig. 3). However, they also showed that these treatments have little effect on the concentration of hepatic zinc MT (Fig. 4). Comparing the accumulation zinc MT in chicks given +2FE and those given two series of ACTH injections illustrated that little of the iron-induced accumulation of hepatic zinc MT could be directly attributed to changes in plasma corticosterone. Plasma corticosterone values were nearly twice as high in the ACTH-treated chicks yet hepatic zinc MT levels were only approximately one-third those observed in +2FE chicks. These results suggest that changes in plasma corticosterone as effected by parenteral iron are not related to iron-induced changes in hepatic zinc MT.

In our previous study of the chick, it was noted that hepatic zinc MT could also be elevated in the rat by parenteral iron. To further study the involvement of glucocorticoids in the iron-induced response, we examined the effect of parenteral iron on hepatic zinc MT in the adrenalectomized rat. We found that hepatic zinc MT was elevated approximately fourfold (2.75 vs 0.6 μg Zn MT/ml cytosol) in both intact and adrenalectomized rats (relative to controls) following two intraperitoneal injections of FE at 10 mg Fe/kg (unpublished observations). These results suggest again that glucocorticoids are not primal mediators of the iron-induced response. This is consistent with the reported lack of glucocorticoid involvement in both cadmium- and inflammation-induced (turpentine) hepatic metallothionein synthesis in the rat (11). Interestingly, a third injection of iron resulted in uniform mortality in adrenalectomized rats (but no mortality in intact rats). As noted in our previous work, the response of the rat to parenteral iron is markedly less than that of the chick for reasons presently unknown.

The minimal accumulation of hepatic zinc MT in response to ACTH was not unexpected. Others have indicated that exogenous natural or synthetic adrenocortical steroids when given in relatively large doses result in minor changes in hepatic MT (19). Upon first inspection of the small changes in zinc in the VeMT, we considered that these could have been simply due to changes in zinc bound to proteins in this region of the chromatograph. If true, then the results of metal determination may not reflect actual changes in the MT concentration. However, electrophoretic analysis of equal portions of each G75 peak verified changes in MT in the various samples (Fig. 5). We are therefore confident that the subtle changes observed in zinc MT corresponded to changes in MT protein.

The mechanism by which parenteral iron effects changes in hepatic MT was originally suggested to involve glucocorticoids in a process similar to that of stress (14). It is clear from the results of the present study that changes in circulating corticosterone are not a primal component of this process. It is significant that plasma zinc increased early (within 2 hr) after chicks received iron injections (Fig. 2). This observation is important in that it may suggest that parenteral iron causes elevated plasma zinc which then stimulates the synthesis of hepatic MT. We have previously examined the responses of other tissues to parenteral iron and have shown that neither the pancreas nor the kidney show changes in zinc MT. This observation (especially for pancreas) would suggest that the slight changes in plasma zinc after iron injections, such as that observed in the present

study, are not sufficient to account for the marked changes which occur in hepatic zinc MT. It is noteworthy that in both groups of chicks receiving parenteral iron, plasma zinc was depressed, especially by 24 hr after the first injection of iron (0 hr for +2FE, Fig. 2). Interestingly, depressed levels of both plasma iron and zinc are associated with the response of an animal to infection, inflammatory agents, and interleukin 1 (20, 21). We are currently investigating the potential involvement/role of macrophages as mediating factors in the process(es) by which parenteral iron induces hepatic MT.

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