The Influence of Manganese Deficiency on Serum IGF-1 and IGF Binding Proteins in the Male Rat (44314)

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Abstract. **Young male rats subjected to a dietary manganese (Mn) deficiency respond to the deficiency by reducing their growth rate. The growth hormone (GH)/insulin-like growth factor (IGF) axis is critical for linear growth; this system is exquisitely sensitive to the nutritional state of the animal. In this study, we examined circulating GH, IGF-1, and insulin levels in Mn-deficient (–Mn; fed a 0.5 μg Mn/g diet) and sufficient (+Mn; fed a 45 μg Mn/g diet) male Sprague-Dawley rats. Additionally, we examined the distribution of circulating IGF binding proteins (IGFBPs) in animals of both dietary groups as these proteins modulate IGF-1 action *in vivo* and *in vitro,* and have been demonstrated to be altered in a number of nutritional and physiological states.**

Body weight was significantly reduced in –Mn relative to +Mn rats. Consistent with other studies, daily food intake was not altered. However, cumulative food intake (over 3 months) was marginally lower in –Mn versus +Mn animals. –Mn animals displayed lower circulating concentrations of IGF-1 (66% of control levels) and insulin (60% of control levels) despite having significant elevations in circulating GH levels relative to +Mn animals (140% of control levels). The IGFBP profile of –Mn animals reflected their elevated GH status, as we observed increased binding of tracer (^{125}I-IGF-1) to the circulating IGFBP-3 complex (120% of control binding) using native chromatography techniques. Interestingly, the lower circulating insulin concentrations of –Mn animals did not result in dramatic elevations in lower-molecular-weight binding proteins.

In summary, we demonstrate that in young male rats, Mn deficiency is associated with alterations in IGF metabolism. These alterations may contribute to the growth and bone abnormalities observed in –Mn animals. [P.S.E.B.M. 1998, Vol 219]

It has been long recognized that manganese (Mn) is essential for normal growth and propagation of animals (1). Evidence points to Mn as an important intracellular messenger (2) and an essential component of carbohydrate metabolism (3–8). Additionally, it has been demonstrated that Mn-deficient rats have elevated pancreatic amylase mRNA levels, particularly when fed high carbohydrate diets (9, 10).

A dramatic manifestation of Mn deficiency in experimental animals is the condition of congenital ataxia, a phenotype characterized by a delayed righting reflex, a lack of equilibrium, and a retraction of the head (11). These collective disorders presumably arise from a common defect associated with abnormally low calcification of the otolith, an inner ear structure. In addition to the calcification defects seen in otiliths, abnormal skeletal development is a common occurrence in Mn-deficient animals (12–18). Taken together, these studies indicate an important role for Mn in normal bone metabolism.

We have observed that body weights of Mn-deficient male rats are reduced relative to Mn-replete litter mates. Interestingly, Mn-deficient animals, unlike zinc (Zn)-

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deficient animals, do not appear to reduce their food intake [7, 9], nor do they develop a cyclic food intake pattern. The observed reduction in body weight suggested the possibility that Mn deficiency might alter growth factor metabolism. This hypothesis is consistent with studies that have demonstrated the sensitivity of the GH/IGF-1 axis to nutritional deficiencies (19–21). Furthermore, given the important roles that the above hormones and insulin play in both soft tissue and bone metabolism, we hypothesized that the reduced body weights and bone abnormalities seen previously were a partial consequence of decreased circulating insulin, GH, and/or its agent of action, IGF-1. The mitogenic potential of circulating IGF-1 is regulated to a varying extent by a family of circulating insulin-like growth factor binding proteins. IGFBPs, similar to IGF-1, are altered by a number of nutrient deficiencies and physiological states (19).

The purpose of the current undertaking was to test the above concepts by examining the effects of a dietary induced Mn deficiency on circulating insulin, GH, and IGF-1 concentrations in a first-generation male Mn-deficient rat model. Furthermore, we examined, under native and denaturing conditions, the distribution of serum IGFBPs in both Mn-deficient and sufficient animals.

Materials and Methods

Animals and Diet. Neonatal Mn deficiency was induced in male Sprague-Dawley pups (Charles Rivers Laboratories, Wilmington, MA) by feeding their dams a Mn-deficient diet (0.5 μg Mn/g diet) after birth and allowing the pups to suckle freely. At the time of weaning (Day 17), the animals were separated into one of two groups and fed either an *ad libitum* purified diet containing 45 μg Mn/g diet (control; +Mn) or 0.5 μg Mn/g diet (deficient; –Mn). Traditionally, our laboratory chooses an earlier day of weaning (i.e., Day 17 vs. Day 21) when conducting metal deficiency studies as these conditions prevent the buildup of appreciable metal stores resulting from the pups consuming maternal chow diet (in this study dams were consuming purified diets at time of weaning). Furthermore, animals consuming deficient diets are placed above those consuming control diet to prevent potential contamination of the former. Detailed components of the diet have been described previously by Baly *et al.* (5). Rats were housed in suspended stainless steel cages in a temperature (22°C)- and light-controlled (12 hr light/dark) room. Animals (107 days old) were anesthetized with pentobarbital and sacrificed by exsanguination at 0900–1000 hr. Serum was quickly separated from RBCs by centrifugation at 4°C and subsequently frozen and stored at −70°C. Liver was frozen for subsequent trace element analysis. The experimental protocol was approved by the University of California at Davis Animal Use and Care Administrative Committee, and the animals were cared for in accordance with the National Research Council's *Guide for the Care of Laboratory Animals.*

Native Column Chromatography. ^{125}I-IGF-1-labeled serum samples were size-fractionated by FPLC (Pharmacia, Piscataway, NJ) under native conditions as described (19). Fractions (250 μl) were collected and assayed for ^{125}I-IGF-1 tracer by gamma counting (Packard Instruments, Meriden, CT). Chromatographic data were integrated with commercially available software.

Radioimmunoassay of Serum IGF-1. To dissociate IGFs from IGFBPs, serum samples (500 μl) were chromatographed in 0.2 *M* formic acid on a 0.9 × 100-cm column containing Sephadex G-50 Fine (Pharmacia) (19). Serum was applied to the column, and fractions containing the IGF peptide (46–71 ml) were collected in 50-ml tubes containing 250 μl radioimmunoassay buffer (0.03 *M* sodium phosphate, 0.25% bovine serum albumin, and 0.02% sodium azide. pH 7.5); samples were then frozen and lyophilized. IGF peptide fractions were solubilized in radioimmunoassay buffer without added bovine serum albumin. IGF-1 content was measured using ^{125}I-IGF-1 as radioligand and a polyclonal antisomatomedin C/IGF-1 generated by Drs. Underwood and Van Wyk, University of North Carolina at Chapel Hill, and distributed through the National Hormone and Pituitary Program. All samples were analyzed in a single assay, which had an intra-assay coefficient of variation (CV) equaling 6%.

Radioimmunoassay of Serum Insulin. The insulin radioimmunoassay was a modification of the method described by Yalow and Berson (22) using 0.05 *M* phosphate buffer containing 0.4% human serum albumin (Cutter Biological, Berkeley, CA) and a precipitation method (23) to separate free from bound insulin. The rat insulin standard (23.1 U/mg) was obtained from Novo Biolabs (Wilton, CT). Porcine anti-rat insulin antisera came from ICN (Costa Mesa, Ca.), ^{125}I-insulin was purchased from Amersham (74 TBq/mmol; Arlington Heights, IL). Polyethlene glycol (PEG; MW = 8 kDa) was obtained from Sigma Chemical Co. (St. Louis, MO).

Radioimmunoassay of Serum GH. Rat GH was iodinated using a modification of the chloramine-T technique (24). Briefly, 5 μg of GH (NIDDK-rGH-1-6) in 0.13 *M* potassium phosphate buffer pH 7.4 (0.16 wt/vol) was incubated with 1.85×10^7 Bq Na^{125}I (Amersham) and 0.08 m*M* chloramine-T in 0.013 *M* phosphate buffer at room temperature for 5 min. The reaction was terminated by the addition of 26 m*M* sodium metabisulfite (in 0.05 *M* phosphate buffer). Tracer purification was performed by gel filtration on a 0.7 × 30 cm Econo-column (Bio-Rad, Richmond, CA) containing Sephadex G-100 Fine (Pharmacia). The column was equilibrated with 0.05 *M* phosphate buffer, 0.1% BSA, pH 7.4 prior to iodination. Serum GH was measured by RIA, using polyclonal anti-rat GH antisera (αGH) provided by the National Hormone and Pituitary Program (NIDDK-anti-rGH-S-5). Serum samples (100 μl) were incubated overnight at room temperature with GH antibody (final dilution of 1:500,000), iodinated GH (approximately 18.5 kBq/100 μl) and buffer (0.15 *M* sodium chloride, 0.01% sodium azide, 0.01 *M* EDTA, pH 7.4) to a final assay volume of 500 μl. Bound radioactivity was precipitated by

the addition of 1% gamma-globulin and 20% PEG followed by centrifugation at 12,000*g* for 25 min. The supernates were removed and pellets then counted by gamma counting (COBRA Auto-Gamma 5000; Packard Instruments). All samples were analyzed within a single assay which had an intra-assay CV of 6%.

Western Ligand Blot (WLB). Molecular forms of serum IGFBPs were further characterized by WLB [20, 25]. Relative intensities of IGFBP bands on autoradiographs were determined by using the FotoAnalyst II Imager System and College Software (Fotodyne, New Berlin, WI).

Trace Element Analysis. Portions of liver and diet (0.5 g) were placed in Erlenmeyer flasks and wet ashed in 5 ml of concentrated nitric acid (12 *M*) for 4 hr at approximately 70°C. Subsequently, the digested samples were quantitatively transferred to volumetric flasks, and trace elements were analyzed on a TJA Video 12 atomic absorption spectrophotometer (Thermo Jarrell Ash Corp., Franklin, MA) (26).

Statistical Analysis. Data were analyzed by an independent *t*-tests (SPSS for Windows: SPSS, Chicago, IL).

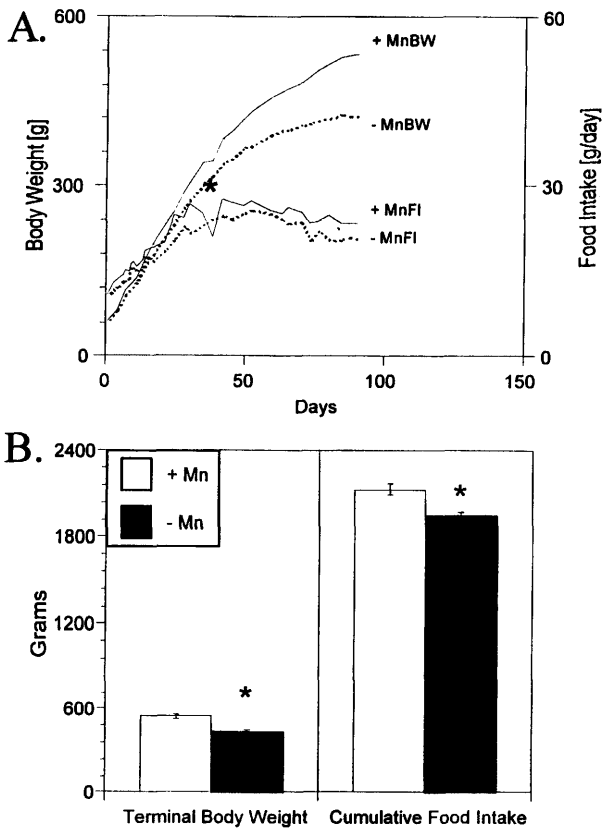

Figure 1. Body weights and food intake of male rats fed either a Mn-deficient (0.5 µg Mn/g diet; –Mn) or Control (45 µg Mn/g diet; +Mn) diet for approximately 90 days. (A) Daily body weights (+MnBW, –MnBW) and food intake (+MnFI, –MnFI) for each group as a function of time. (B) Terminal body weight and cumulative food intake of each dietary group. Data are expressed as mean ± SEM. *indicates significant differences between groups ($P < 0.05$). Note: in IA, mean body weight for the +Mn group was significantly greater than the –Mn group from Day 42. Each group contained at least six animals.

Table I. Serum Hormones and Liver Mn Concentrations in Male Rats Consuming Either a Mn-deficient (-Mn) or Sufficient (+Mn) Diet for 90 Days

Group	GH (µg/l)	IGF-1 (µg/l)	Insulin (µg/l)	Liver Mn (µmoles/g)
+Mn	25 ± 3	213 ± 21	10 ± 2	43 ± 1.0
–Mn	35 ± 3*	141 ± 15*	6 ± 1*	3 ± 0.1*

Note. Data are expressed as mean ± SEM (n = 6/group). *indicates differs significantly from + Mn group ($P < 0.05$).

Results

Body Weights and Food Intake. Body weights in the two groups were similar until Day 42 of the experiment. At Day 42, and for the remainder of the study, the –Mn group displayed significantly lower body weights relative to the +Mn group (Fig. 1A; $P < 0.05$). –Mn animals demonstrated a 20% reduction in terminal body weight relative to +Mn controls (Fig. 1B). Daily food intake was not generally different between the two groups (Fig. 1A). However, there was a small (8.3%), but statistically significant reduction in cumulative food intake (Fig. 1B; $P < 0.05$).

Circulating GH, IGF-1, Insulin, and Liver Mn Concentrations. Single point determination of serum GH concentrations demonstrated higher concentrations in the –Mn group relative to the +Mn group (Table I; $P < 0.05$). The elevated circulating GH concentrations in the deficient group were not associated with a concomitant rise in circulating IGF-1 concentrations. The –Mn group displayed significantly lower serum IGF-1 concentrations than +Mn counterparts (Table I; $P < 0.05$). The –Mn group displayed a 40% reduction in circulating insulin concentrations relative to the +Mn group (Table I; $P < 0.05$). Regression analysis of serum insulin against serum IGF-1 concentrations failed to yield a statistically significant positive correlation although a strong trend was noted (i.e., $P < 0.08$). That the –Mn animals were severely Mn deficient was evident by a 15-fold reduction in liver Mn concentrations (Table I; $P < 0.05$). The Mn deficiency appeared specific as there were no differences between groups with respect to liver calcium, magnesium, zinc, copper, or iron concentrations (data not shown).

Serum Tracer Binding/Native Molecular-Sieve Chromatography and WLB Studies. *In vitro* equilibration of ^{125}I-IGF-1 with sera and its subsequent size fractionation under native conditions led to a distribution of tracer over three major molecular weight areas (Fig. 2A). Area 1 encompassed a molecular weight range of 250-70 kDa and had an elution volume (Ve) of 150 kDa, corresponding to the complex of IGFBP-3, the acid labile subunit (ALS), and IGF-1. Area 2 encompassed the molecular weight range of 70-22 kDa, which is most significantly represented by tracer binding to IGFBP-1/IGFBP-2 (19). This area of tracer binding was rather diffuse with a barely noticeable Ve of 37 kDa; it is possible that minor contribu-

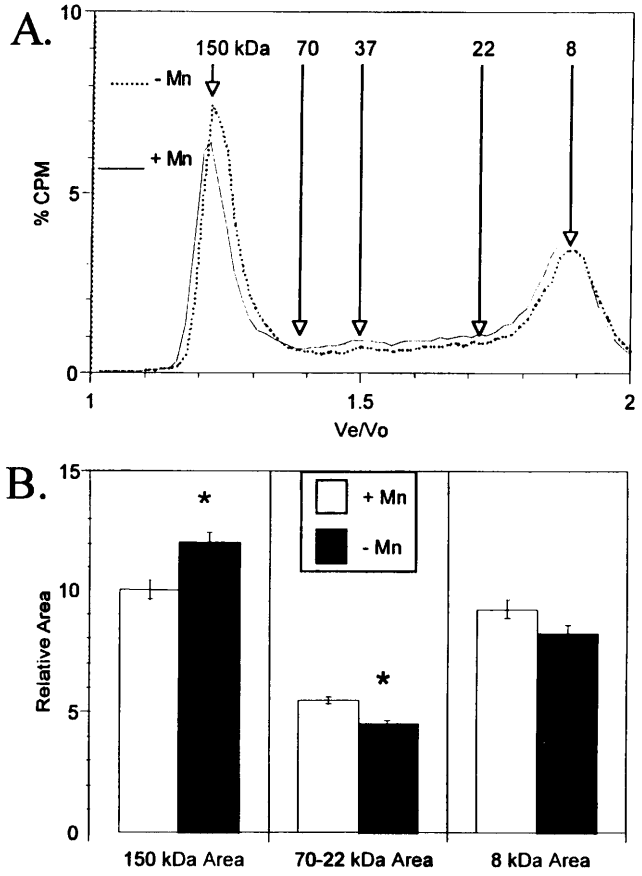

Figure 2. Native gel-permeation chromatography of –Mn and +Mn serum. (A) Serum (125 µl) from –Mn and +Mn animals was incubated with elution buffer (125 µl) and 2×10^5 cpm ^{125}IGF-1 for 4 hr at 4°C. Each chromatogram depicted represents the mean of at least six animals. Numbers appearing above the chromatogram represent elution volumes (Ve) of known molecular weight standards relative to the void volume (V_o). (B) Relative peak areas for the specified molecular weight ranges were obtained by integrating the area under the chromatograms shown in A. Each individual area was normalized to the summed area of the three specified molecular weight range areas. Each relative area represents the mean ± SEM of at least six individual chromatograms for each dietary group. *indicates significant differences between groups ($P < 0.05$).

tions to this peak could be made by IGFBP-3/IGF-1 complex minus the ALS and/or the 29-kDa glycosylated amino terminal fragment of IGFBP-3/IGF-1 minus the ALS. The trailing edge of this area represents tracer binding to the 24-kDa IGFBP-4 species. Finally, Area 3 encompasses a molecular weight range of 22-4.5 kDa with a distinct Ve of 8 kDa representing elution of free tracer.

The magnitude of tracer binding differed among the two groups (Fig. 2A). For example, Area 1 (i.e., the 150-kDa IGFBP-3 complex) represented the largest area of tracer binding for both groups. The –Mn group displayed significantly more binding in this area than the +Mn group (Fig. 2B; $P < 0.05$). In contrast, the –Mn group showed significantly less binding in Area 2 (Fig. 2B; $P < 0.05$) although the magnitude of change was not large. In Area 3, the two groups did not significantly differ, although the trend was for reduced amounts of free tracer in the –Mn group ($P \leq 0.1$).

WLB analysis confirmed the presence of the major IGFBP species in serum of both dietary groups (Fig. 3). The amount of each IGFBP species and the distribution of tracer measured by densitometric analysis failed to demonstrate differences between the two dietary groups (data not shown). The above is consistent with past studies demonstrating that the two analysis systems provide different information (19). For example, the denaturing WLB system, because of its inherent stripping away of endogenous IGF-1/2, gives an estimation of the total binding potential of the various IGFBP species (albeit, to the degree to which the denaturing system and subsequent electrotransfer do not result in loss of post-translational modifications and/or induction of conformational alterations affecting ligand affinity). Conversely, the native chromatography system reflects equilibrium binding of tracer to the various IGFBP species that are saturated to differing extents with endogenous IGF-1/2. This system allows binding to occur under native conditions wherein the IGFBPs are in relative native configuration. However, the resolution of the native system is currently less than that exhibited by the denaturing WLB.

Discussion

In this study, we observed a 20% decrease in body weight in the –Mn group relative to the +Mn group. The decreased body weight gain observed in the –Mn group was associated in part with a reduction in that group's cumulative food intake over the 90-day study period. It remains unclear whether the cumulative decrease in food intake was the driving force behind the lower body weight gains or vice versa. A pair-fed group was not included in this study because it has been the experience of our group [4, 27] and others [7, 9, 12] that Mn deficiency does not result in anorexia, at least in first generation –Mn animals. This observation is supported in our current study, which did not indicate any consistent differences in daily food intake between the two groups. However, we did note a small yet statistically significant decrease in cumulative intake in the

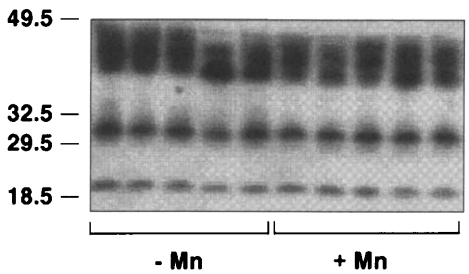

Figure 3. Western Ligand Blot (WLB) of –Mn and +Mn serum. Serum (3 µl) was mixed with sample buffer and fractionated under denaturing and nonreducing conditions on a 12% SDS-PAGE gel. Proteins were transferred to nitrocellulose, probed with 0.5 µCi ^{125}IGF-1, and subjected to radiography. Five representative sera are shown for each dietary group. Molecular weights to the left of the figure represent protein molecular weight standards. There were no significant differences between the groups.

–Mn group. Thus, we were unable to distinguish in the current study between the two following possibilities: 1) a direct effect of the nutrient deficiency on insulin/IGF-1 production/turnover; or 2) an indirect effect of this nutrient on IGF-1 *via* food intake regulation. It is unlikely, in our opinion, that this subtle decrease in cumulative food intake over the course of this 3-month study could account for a 33% decline in circulating IGF-1 concentrations. However, this possibility cannot be entirely discounted given the sensitive nature of the IGF-1 system to caloric and protein deprivation (21). The results of this study are in contrast with the impact of Zn deficiency on circulating IGF-1 levels, where a sharp decline in daily and cumulative food intake were noted and associated with a definitive cyclic intake pattern. The severe Zn deficiency-induced anorexia appeared to drive the precipitous drop in circulating IGF-1 concentrations (>80%) resulting in significantly smaller animals (35% of control body weight) (19).

It has been previously demonstrated that the GH/IGF-1 axis is extremely sensitive to the nutritive state of the animal (21). In many cases, essential nutrient deficiencies result in decreased circulating IGF-1 but not in circulating GH concentrations, which is consistent with the current study. In fact, GH concentrations were higher in the –Mn relative to the +Mn animals ($P < 0.05$). Although this study was not designed to examine the effect of Mn deficiency on the pulsatile release of GH, the data support the concept that GH metabolism was impacted by the deficiency state. A study addressing the effect of Mn deficiency on the 24-hr frequency and amplitude of GH release was outside the scope of the current study, but is needed to unambiguously establish whether Mn deficiency impacts pituitary metabolism of GH. Despite the above limitations, our data are consistent with the concept that the IGF-1 peptide is a feedback inhibitor of GH release and transcription (28). The observation that the increased GH levels seen in the –Mn animals were not associated with increased circulating IGF-1 concentrations suggests an uncoupling of GH with that of IGF-1 metabolism. This uncoupling event, or GH resistance, may be associated with a downregulation of GH receptor (GHr) levels, changes in ligand/receptor affinity, and/or defects associated with the activation and/or propagation of the signaling cascade subsequent to ligand binding. For example, nutritional deprivation can result in decreased GHr transcription (28, 29).

Mn deficiency has resulted in both abnormally low first- and second-phase pancreatic insulin output by perfused pancreata (3, 4, 6). Consistent with these observations, in the current study, circulating insulin concentrations were significantly reduced in –Mn animals. Surprisingly, this outcome had minimal impact on IGFBP expression. For example, both our native chromatography and denaturing WLB systems indicated that Mn deficiency had a minimal impact on the expression of either high (i.e., IGFBP-3 complex) or low (IGFBP-1/2/4) molecular weight species. The increase in *in vitro* tracer binding to the 150-kDa peak suggests that only IGF-1/2 ligand was limiting for IGFBP-3 complex formation. Insulin is thought to be a primary negative modulator of IGFBP-1 gene expression (30, 31). Thus, we had expected to observe markedly elevated IGFBP-1 levels in –Mn animals. Given the limitations of our analytical systems (i.e., column chromatography and WLB), we cannot absolutely say that IGFBP-1 levels were unaffected by Mn deficiency. However, clearly IGFBP-1 is far less impacted in this situation compared with other essential nutrient deficiencies. For example, animals suffering from Zn deficiency and/or starvation show marked increases in the expression of lower molecular weight IGFBPs consistent with decreased circulating insulin concentrations (19). The divergence in IGFBP profile when comparing –Zn and –Mn animals is perhaps a result of the development of a severe catabolic state (and eventual death) with concomitant elevation in circulating glucocorticoids in the former deficiency. A state of low insulin and elevated glucocorticoids would be expected to result in elevated concentrations of IGFBP-1 (30, 31). Mn deficiency, despite significantly reducing the animals' circulating insulin and IGF-1 concentrations, does not lead to severe anorexia or the concomitant energy/macro-nutrient deprivation. Consequently, there appears to be less impact on the animals' production of circulating IGFBPs.

To our knowledge, this is the first report describing alterations in circulating IGF-1 as a consequence of Mn deficiency. This finding is in contrast to Bolze *et al.* (32) who found that Mn-deficient chickens had depressed growth rates, exhibited perosis, and had significantly depressed $^{35}S\text{-}SO_4$ incorporation into cartilage. These findings suggested that IGF metabolism may have been impacted in these animals, although they demonstrated normal circulating glucose, insulin, and IGF-1 concentrations. It has been demonstrated previously that supplementation of GH to hypophysectomized rats leads to a marked increase in chondroitin sulfate synthesis in epiphysial plate cartilage as determined by $^{35}S\text{-}SO_4$ incorporation. GH action on epiphysial plate cartilage chondroitin sulfate synthesis appears to be mediated by IGF-1. Chondroitin sulfate synthesis is particularly reduced in Mn-deficient epiphysial plate cartilage (17). Thus, we suggest that the Mn-deficiency-induced reduction in the circulating IGF-1 concentrations may in part explain the decreased synthesis of chondroitin sulfate reported in earlier studies.

In summary, deficiencies in several essential nutrients leads to a disruption of the GH/IGF-1 axis. Our hypothesis was that nutrient deficiencies, such as Mn deficiency, result in decreased circulating insulin concentrations which subsequently give rise to the disruption of GH, IGF-1, and IGFBP metabolism. Several studies have linked decreases in circulating IGF-1 with those of insulin. For example, the physiological states of starvation, diabetes, and Zn and Mn deficiency result in decreased circulating insulin and IGF-1 concentrations as well as GH resistance. GH resistance has been associated with downregulation of hepatic GH recep-

tors. This hypothesis must be reconciled with the observations of Menon *et al.* (33) who found that exogenous insulin treatment to diabetic rats did not result in upregulation of GH receptors, but did restore circulating IGF-1 concentrations. This observation suggests that insulin may impact IGF-1 metabolism directly, and it is consistent with insulin's emerging role as a co-regulator of hepatic IGF-1 transcription (34). The current study has clearly demonstrated that Mn deficiency results in altered IGF-1 metabolism. This observation may explain the decreases in body weight and bone abnormalities typically associated with –Mn animals. Alterations in circulating IGF-1 levels do not appear to be a consequence of altered IGFBP metabolism. Studies examining the temporal relationship between decreased circulating insulin and IGF-1 concentrations would prove useful. Examination of the impact of Mn deficiency on GH receptor expression and/or signal transduction may yield potential mechanistic explanations for the observed decline in circulating IGF-1 concentrations found in –Mn animals.

Finally, whereas frank Mn deficiency *per se* is not problematic in humans, several population groups may have suboptimal intakes of Mn. However, before Mn supplementation to humans can be recommended, additional studies are required to define more clearly the toxicity threshold for this element. This concept is underscored by the potential risk that Mn supplements could pose to individuals with liver disease (35).

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1. Orent ER, McCollum EV. Effects of the deprivation of manganese in the rat. J Biol Chem **92:**651–678, 1931.
2. Korc M. Manganese as a modulator of signal transduction pathways. Progress in Clinical and Biological Research **380:**235–255, 1993.
3. Baly DL, Curry DL, Keen CL, Hurley LS. Effect of manganese deficiency on insulin secretion and carbohydrate homeostasis in rats. J Nutr **114:**1438–1446, 1984.
4. Baly DL, Curry DL, Keen CL, Hurley LS. Dynamics of insulin and glucagon release in rats: Influence of dietary manganese. Endocrinology **116:**1734–1740, 1985.
5. Baly DL, Keen CL, Hurley LS. Effects of manganese deficiency on pyruvate carboxykinase and phosphoenolpyruvate activity and carbohydrate homeostasis in adult rats. Biol Trace Elem Res **11:**201–212, 1986.
6. Baly DL, Schneiderman JS, Garcia-Welsh AL. Effect of manganese deficiency on insulin binding, glucose transport, and metabolism in rat adipocytes. J Nutr **120:**1075–1079, 1990.
7. Werner L, Korc M, Brannon PM. Effects of manganese deficiency and dietary composition on rat pancreatic enzyme content. J Nutr **117:**2079–2085, 1987.
8. Baly DL, Lee I, Doshi R. Mechanism of decreased insulinogenesis in manganese-deficient rats: Decreased insulin mRNA levels. FEBS Lett **239:**55–58, 1988.
9. Brannon PM, Collins VP, Korc M. Alterations of pancreatic digestive enzyme content in the manganese-deficient rat. J Nutr **117:**305–311, 1987.
10. Chang SC, Brannon PM, Korc M. Effects of dietary manganese deficiency on rat pancreatic mRNA levels. J Nutr **120:**1228–1234, 1990.
11. Hurley LS, Everson GJ, Gieger JF. Manganese deficiency in rats: Congenital nature of ataxia. J Nutr **66:**309–320, 1958.
12. Strause LG, Hegenauer J, Saltman P, Cone R, Resnick D. Effects of long-term dietary manganese and copper deficiency on rat skeleton. J Nutr **116:**135–141, 1986.
13. Hurley LS, Everson LS, Wooten E, Asling CW. Disproportional growth in offspring of manganese-deficient rats. I: The long bones. J Nutr **74:**274–281, 1961.
14. Hurley LS, Wooten E, Everson LS. Disproportional growth in offspring of manganese deficient rats. II: Skull, brain, and cerebrospinal fluid pressure. J Nutr **74:**282–288, 1961.
15. Leach RM Jr., Muenster AM. Studies on the role of manganese in bone formation. I: Effect upon the mucopolysaccharide content of chick bone. J Nutr **78:**51–56, 1962.
16. Leach RM Jr., Muenster AM, Wein EM. Studies on the role of manganese in bone formation. II: Effect upon chondroitin sulfate synthesis in chick epiphyseal cartilage. Arch Biochem Biophys **133:**22–28, 1969.
17. Liu AC-H, Heinrichs BS, Leach M Jr. Influence of manganese deficiency on the characteristics of proteoglycans of avian epiphyseal growth plate cartilage. Poult Sci **73:**663–669, 1994.
18. Leach RL Jr. Mn(II) and glycosyltransferases essential for skeletal development. In: Schramm VL, Wedler FC, Eds. Manganese in Metabolism and Enzyme Function. Orlando: Academic Press, Vol. **I,** pp81–91, 1986.
19. Clegg MS, Keen CL, Donovan SM. Zinc deficiency–induced anorexia influences the distribution of serum insulin-like growth factor–binding proteins. Metabolism **44:**1495–1501, 1995.
20. Donovan SM, Atilano L, Hinz RL, Wilson DM, Rosenfeld RG. Differential regulation of insulin-like growth factors (IGF-1 and -2) and IGF binding proteins during malnutrition in the neonatal rat. Endocrinology **129:**149–157, 1991.
21. Thissen JP, Ketelslegers JM, Underwood LE. Nutritional regulation of the insulin-like growth factors. Endocr Rev **15:**80–101, 1991.
22. Yalow RS, Berson SA. Immunoassay of endogenous plasma insulin in man. J Clin Invest **39:**1157–1161, 1960.
23. Desbuquois B, Aurbach GD. Use of polyethylene glycol to separate free antibody bound peptide hormones in radioimmunoassays. J Clin Endocrinol Metab **33:**732–735, 1971.
24. Ribela MT, Murata Y, Morganti L, Toniolo D, Bartolini P. The use of recombinant human growth hormone for radioiodination and standard preparation in radioimmunoassay. J Immunol Methods **19:**269–274, 1993.
25. Hossenlopp P, Seurin D, Segovia-Quinson B, Hardouin S, Binoux M. Analysis of serum insulin-like growth factor binding proteins using western blotting: Use of the method for titration of the binding proteins and competitive binding studies. Anal Biochem **154:**138–143, 1986.
26. Clegg MS, Keen CL, Lonnerdal B, Hurley LS. Influence of ashing techniques on the analysis of trace elements in animal tissue I: Wet ashing. Biol Trace Elem Res **3:**107–115, 1981.
27. Zidenberg-Cherr S, Keen CL, Hurley LS. The effects of manganese deficiency during prenatal and postnatal development on mitochondrial structure and function in the rat. Biol Trace Elem Res **7:**31–48, 1984.
28. Yamashita S, Melmed S. Insulin-like growth factor I regulation of growth hormone gene transcription in primary rat pituitary cells. J Clin Invest **79:**449–456, 1987.
29. McNall AD, Etherton TD, Fosmire GJ. The impaired growth induced by zinc deficiency in rats is associated with decreased expression of hepatic insulin–like growth factor I and growth hormone receptor genes. J Nutr **125:**874–879, 1995.
30. Suwanichkul A, Morris SL, Powell DR. Identification of an insulin-responsive element in the promoter of the human gene for insulin-like growth factor binding protein-I. J Biol Chem **268:**17063–17068, 1993.

31. Suwanichkul A, Allander SV, Morris SL, Powell DR. Glucocorticoids and insulin regulate expression of the human gene for Insulin-like Growth Factor-Binding Protein 1 through proximal promoter elements. J Biochem **269:**30835–30841, 1994.
32. Bolze MS, Reeves RD, Linbeck FE, Kemp SF, Elders MJ. Influence of manganese on growth, somatomedin, and glycoaminoglycan metabolism. J Nutr **115:**352–358, 1985.
33. Menon RK, Stephan DA, Rao RH, Sheriroff Z, Downs LSJ, LeRoith D, Sterling M. Tissue-specific regulation of growth hormone receptor genes in streptozocin-induced diabetes in the rat. J Endocrinol **142:**453–462, 1994.
34. Krishna A, Pao C, Thule B, Villafuerte B, Phillips L. Transcription initiation of the rat insulin–like growth factor-I gene in hepatocyte primary culture. J Endocrinol **151:**215–223, 1996.
35. Hauser RA, Zesiewicz TA, Martinez C, Rosemurgy AS, Olanow CW. Blood manganese correlates with brain magnetic resonance imaging changes in patients with liver disease. Can J Neurol Sci **23:**95–98, 1996.