Effect of Manganese Deficiency on Insulin Secretion and Carbohydrate Homeostasis in Rats[1,2]

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ABSTRACT The effects of manganese (Mn) deficiency on carbohydrate metabolism in the Sprague-Dawley rat were investigated. Oral glucose tolerance tests were performed on offspring from Mn-sufficient female rats fed 45 μg Mn per gram diet (C-C), offspring from Mn-deficient female rats fed 1 μg Mn per gram diet (D1-D1) and rats not exposed to Mn deficiency in utero but fed D1 diet from weaning to maturity (D1). Mn-deficient rats, D1 and D1-D1, had significantly lower concentrations of Mn in liver, kidney, heart and pancreas than controls. D1-D1 rats responded with a diabetic type of glucose tolerance curve when given oral glucose. Insulin levels of D1-D1 rats were not commensurate with their high glucose levels. Measurements made by using an isolated perfused-pancreas preparation indicate that insulin output due to release of stored hormone was 76% of control levels in D1-D1 rats. This suggests either lower insulin stores or impaired release of insulin in D1-D1 rats. Second phase insulin release was also significantly lower in D1-D1 rats than in controls. Pancreatic insulin content was 63% of control levels in D1-D1 rats, further supporting the idea of lower insulin stores in Mn-deficient rats. No differences in plasma glucose levels were observed between D1 rats and controls after an oral-glucose load. These findings indicate that dietary Mn deficiency can result in impaired insulin secretion producing impaired carbohydrate metabolism; however, the timing of the deficiency may be a critical factor in the expression of this abnormality. J. Nutr. *114*: 1438–1446, 1984.

INDEXING KEY WORDS manganese • glucose • glucose tolerance • insulin secretion • carbohydrate metabolism

The essential nature of manganese has been recognized since 1931 when Kemmerer and co-workers (1) and Orent and McCollum (2) demonstrated poor growth in mice and abnormal reproduction in rats fed diets deficient in the element. It is now well recognized that dietary manganese deficiency can result in a wide variety of structural and metabolic defects (3). Despite recent interest in intracellular free Mn^{2+} as an important mechanism of cellular metabolic control (4) and evidence for the essential nature of this element for several biological functions, precise biochemical roles for manganese are poorly understood.

A relationship between manganese and abnormal carbohydrate metabolism was first suggested by Rubenstein and co-workers in 1962 (5). They reported the case of a diabetic patient, resistant to insulin therapy, who responded to oral doses of manganese chloride with a consistent drop in blood

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glucose levels. Manganese supplementation was tried because of the ineffectiveness of high doses of insulin in maintaining normal blood glucose and the patient's statement that his diabetic condition could be controlled to some extent by an extract of lucerne (alfalfa, *Medicago sativa*). Analysis of the alfalfa extract revealed a high concentration of manganese. In contrast to the effectiveness of manganese, oral supplements of zinc, magnesium, cobalt or iron had no effect on the patient's blood sugar levels, indicating a specific role for manganese in insulin release or action.

A similar observation was reported by Shani et al. (6). They found that the sand rat, whose natural diet is high in manganese, developed an insulin-resistant diabetes when fed a commercial rat feed containing relatively low levels of manganese. The diabetic condition was reversed after reintroduction of the manganese-rich natural diet.

Everson and Shrader (7) reported that guinea pigs, born to manganese-deficient dams and fed manganese-deficient diets from birth to 60 days of age, had abnormal glucose tolerance curves. Histological examination of the pancreas from these animals indicated that the deficient animals had hypertrophied pancreatic islet tissue with degranulated β-cells and an increased proportion of α-cells (8). All of these signs of manganese deficiency were reversed following dietary manganese supplementation for 2 months.

Based on the latter observations, we formulated the hypothesis that manganese deficiency might result in altered insulin secretion. Such an abnormality could explain, at least partially, the altered glucose metabolism observed in manganese-deficient animals. The specific effect of manganese on insulin metabolism might occur at several levels, including reduced synthesis and/or secretion of insulin (in addition to reduced peripheral insulin sensitivity). In this study, we have examined the relationship of manganese deficiency and pancreatic function in rats.

MATERIALS AND METHODS

Animals and diets. Sprague-Dawley rats (Simonsen Laboratory, Gilroy, CA) were used in this study. Beginning at 21 days of age, weanling rats of both sexes were assigned to one of two dietary groups and fed a purified diet containing either 45 μg Mn/g (control) or 1 μg Mn/g (D1). The diet contained 30% casein, 54.5% glucose (Cerelose, CPC International Inc., Englewood Cliffs, NJ), 8% corn oil, 6% salt mix and 1.5% vitamin mix. The detailed composition of the diet has been described previously (9). Diet and distilled water were provided ad libitum. The rats were individually housed in suspended stainless-steel cages in a temperature- and light-controlled room (22-23°C, 12-hour light-dark cycle). At maturity (approximately 3 months of age), the male rats were used in carbohydrate studies, and the female rats were mated with stock-fed (Purina Rat Chow, Ralston Purina Co., St. Louis, MO) males.

Pregnancy was confirmed by the presence of a copulatory plug, which was considered day 0 of pregnancy. During pregnancy and lactation the dams previously fed the diet containing 1 μg Mn/g were now fed a diet of the same composition but with 3 μg Mn/g. It was necessary to increase manganese to 3 μg/g during pregnancy because 90% of the offspring from dams fed 1 μg Mn/g diet died during the first 7 days postpartum (10). The rats in the control group continued to receive the diet containing 45 μg Mn/g. Offspring remained with their dams until weaning (30 days postpartum) and had free access to the maternal diet at all times during lactation. Beginning at day 30, the deficient male offspring were fed the diet containing 1 μg Mn/g (D1-D1) until maturity.

Tissue analysis. At maturity, both control and manganese-deficient rats were killed by ether overdose. Deficient and control rats were age matched in this study. Deficient rats weighed slightly less than the controls. The reason for this difference is not known as there were no differences in food intake. Blood samples were collected by cardiac puncture with heparinized syringes, transferred into centrifuge tubes and centrifuged at 4°C for 20 minutes at 300 *g* (Beckman, Model TJ-6 centrifuge, Beckman Instruments, Palo Alto, CA). Plasma was removed and frozen at −5°C. Liver, kidney, heart and pancreas were removed, rinsed free of blood, blotted dry, weighed and stored frozen

in plastic vials for later determination of tissue manganese, copper, zinc and iron levels. These elements were measured because deRosa and associates (11) reported that minor fluctuations in these elements can occur in manganese-deficient animals. Tissue samples for trace element analysis were wet ashed with 16 *N* nitric acid (2 ml of Ultrex grade, J. T. Baker Co., San Francisco, CA), concentrated by evaporation and diluted with distilled deionized water (12). Trace element concentrations were determined by flame atomic absorption spectrophotometry (Instrumentation Laboratories, Model 551, Wilmington, MA).

Glucose tolerance studies. Rats were fasted overnight and weighed. An initial blood sample was taken from the tail vein in a heparinized capillary tube for measurement of plasma glucose and insulin. The animals were then given an oral glucose load of 180 mg glucose/100 g body weight. Blood samples were taken 20, 40, 60, 120, 180 and 240 minutes after the glucose loading. Blood samples were immediately centrifuged, and plasma was removed and frozen at −5°C. Glucose was measured by the glucose oxidase method with a Beckman Glucose Analyzer 2. Plasma insulin was determined by radioimmunoassay as previously described by Stern and co-workers (13). Rat insulin (20.7 mU/ng, NOVO Industri, Copenhagen, Denmark) was used as the reference standard and porcine ^{125}I-insulin (Cambridge Nuclear, Billerica, MA) was used as the tracer. Standard additions were performed during each assay to ensure interassay reproducibility.

Pancreas perfusion studies. The pancreas with the adjacent proximal portion of the duodenum, the spleen and the stomach were removed from anesthetized nonfasted rats and perfused in vitro as previously described by Curry and co-workers (14). The artificial perfusate was a modified Krebs-Ringer bicarbonate solution with 4% dextran as the colloid (15). Immediately at the start of the perfusion, a 10-minute equilibration period was begun in which tissue temperature was stabilized at 37.5°C by use of an externally controlled heat source as well as the warmed (37.5°C) perfusate. The perfusate was continually oxygenated in an atmosphere of 95% O_2 and 5% CO_2. Flow rate was constant at 9.4 ml/minute. The pressure developed by the constant flow pump (Cole-Palmer Instrument Co., Masterflex, Chicago, IL) was continually monitored to detect any vascular resistance changes within the pancreatic preparation. No recycling of perfusate occurred. Following the equilibration period, a constant glucose infusion (to produce a stimulus of 300 mg/dl) was begun at time zero of the perfusion and continued through 80 minutes. Venous outflow was collected continuously and analyzed for insulin content as previously described. Total insulin output was calculated from the volume of perfusate collected per unit time and its insulin concentration.

Pancreatic insulin extraction. The pancreata removed for determination of insulin content were prepared by a combination of methods (16–18). The pancreas was removed from the rat, washed in ice-cold saline and cleaned of any bits of fat or intestine. It was then blotted dry, weighed, flash frozen in liquid N_2 and stored at −70°C until extraction. The pancreas was minced and sonicated in ice-cold acid ethanol by using an Insonator Model 500 (Savant Instruments, Inc., Hicksville, NY). Trasylol (aprotinin, FBA Pharmaceuticals, New York, NY) was added to the extraction solution to prevent proteolysis. After an overnight extraction, samples were centrifuged for 20 minutes at 3000 × *g* at 4°C. Supernatants were removed and the pellet resuspended in the acid ethanol/trasylol buffer. After a 4-hour extraction period, samples were centrifuged as before, and the supernatants were combined with those from the previous centrifugation. Insulin was precipitated by adding four volumes of an alcohol/diethyl ether solution to the supernatant. After an overnight incubation, samples were centrifuged at 4°C for 20 minutes at 3000 × *g*. Supernatants were discarded, and the pellets were resuspended in 0.01 *N* HCl. The pH was adjusted to 2.5, and the samples were centrifuged as above. The resulting supernatant was frozen at −70°C for later assay.

Statistics. Data were analyzed by analysis of variance (19) followed by group comparisons (Student's *t*) when a significant *F*-value was obtained. Polynomial regression was used to analyze the relationship between

plasma glucose and insulin in control and deficient rats.

RESULTS

The offspring from the manganese-deficient rats (D1-D1) exhibited characteristics typical of their deficiency, including retraction of the head and the inability to right themselves from a supine position. This congenital ataxia is thought to be due to an abnormality in mucopolysaccharide synthesis in the inner ear (20). Galactosyl transferase, a manganese-activated enzyme required for this process, is depressed in manganese-deficient animals (21).

Tissue trace element levels. The concentration of manganese, copper, zinc and iron in various tissues of control and Mn-deficient rats is shown in table 1. No differences in trace element levels were observed between first and second generation control rats. Therefore, data from these animals were combined. Liver manganese concentration was lower ($P < 0.001$) in both groups of manganese-deficient rats than in the controls. Liver manganese concentrations averaged 15% of the control levels in rats not exposed to manganese deficiency in utero (D1 rats) and 25% of the control levels in offspring of manganese-deficient dams (D1-D1 rats). No significant differences were observed in the concentrations of liver iron, copper and zinc among the groups. Thus, tissue manganese levels were lower in rats fed the deficient diets than in controls, while no consistent differences were found in tissue levels of copper, zinc and iron.

Glucose tolerance. The response of control and manganese-deficient rats to an oral glucose load is shown in figure 1. No differences in plasma glucose levels in response to oral glucose were observed between first and second generation control rats. Thus, values for these animals were combined into a single group. No differences in fasting plasma glucose levels were observed between controls and D1 rats. In addition, plasma glucose levels of D1 rats were similar to controls after oral glucose; blood glucose increased in both groups from a fasting level of 100 ± 5 mg/dl to 160 ± 7 mg/dl after 60

TABLE 1

Trace element levels in tissues of manganese-deficient and control rats[1,2]

Tissue and group	n	Mn	Cu	Zn	Fe
			μg/g tissue		
Liver					
Control	8	2.28 ± 0.20	4.64 ± 0.25	30.7 ± 2.1	139 ± 16
D1	8	0.32 ± 0.08[a]	4.44 ± 0.49	36.9 ± 4.7	150 ± 15
D1-D1	12	0.58 ± 0.11[a]	4.77 ± 0.24	29.8 ± 1.9	139 ± 12
Kidney					
Control	8	0.76 ± 0.05	6.79 ± 0.45	22.5 ± 1.6	72 ± 5
D1	8	0.27 ± 0.03[ab]	7.20 ± 0.69	24.4 ± 0.5	100 ± 6[ab]
D1-D1	12	0.40 ± 0.03[a]	7.80 ± 1.00	23.0 ± 1.6	85 ± 5
Heart					
Control	8	0.38 ± 0.02	5.4 ± 0.34	16.4 ± 0.7	82 ± 4
D1	8	0.15 ± 0.03[a]	5.0 ± 0.10	17.7 ± 1.8	99 ± 3[a]
D1-D1	12	0.15 ± 0.04[a]	5.4 ± 0.10	14.4 ± 0.01	98 ± 5[a]
Pancreas					
Control	8	1.60 ± 0.10	1.41 ± 0.17	23.1 ± 1.6	37 ± 3
D1	8	0.48 ± 0.02[a]	1.20 ± 0.05	23.0 ± 1.4	44 ± 3
D1-D1	12	0.53 ± 0.16[a]	1.23 ± 0.10	22.0 ± 1.5	36 ± 5

[1]Values are means ± SEM for tissue wet weight and for number of samples in *n* column. Controls were fed 45 μg Mn/g diet; D1 and D1-D1 were fed 1 μg Mn/g diet. See text for experimental details. [2]Significant differences: [a]from control level ($P < 0.001$); [b]from D1-D1 level ($P < 0.002$).

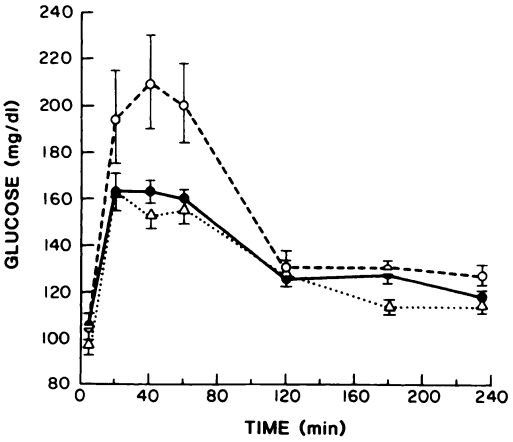

Fig. 1 Oral glucose tolerance curves for control (●), Dl (△), and Dl-Dl (○) rats. Animals were given an oral glucose load of 180 mg/100 g body weight. Values shown are means ± SEM.

minutes and had nearly returned to baseline levels by 2 hours. In contrast, plasma glucose levels in Dl-Dl rats were greater ($P < 0.05$) than control levels at 20, 40 and 60 minutes after the glucose load. One hour after oral glucose, plasma glucose averaged 210 ± 11 mg/dl.

Plasma immunoreactive insulin levels after an oral glucose load are shown in figure 2. Despite similar levels of fasting blood glucose, Dl and Dl-Dl rats had lower fasting blood insulin levels than controls ($P < 0.05$). Insulin levels of the Dl-Dl rats tended to remain lower than control levels over the time course studied, even though their plasma glucose levels were significantly greater. This inadequacy of plasma insulin levels in response to changing blood glucose in Dl-Dl rats, compared to controls, is shown in figure 3. Polynomial regression analysis indicates that a first-degree polynomial (or a linear relationship) best describes the relationship between plasma glucose and insulin levels in control rats ($F = 2.34$, $P > 0.1654$). However, a second-degree polynomial regression equation, indicating a curvilinear relationship, best describes the relationship between these two parameters in deficient rats ($F = 7.39$, $P < 0.009$). No differences in plasma insulin levels following glucose loading were observed between Dl and control rats.

Pancreatic perfusion. Pancreatic insulin output in response to a glucose infusion is shown in figure 4. First phase insulin output (0–10 minutes) by Dl-Dl rats, reflecting insulin output due to release of stored hormone (14), was only 76% of control values. The second phase of insulin release (fig. 4), representing release of newly synthesized as well as preformed hormone, was also lower ($P < 0.02$) in the Dl-Dl rats fed the 1 μg Mn/g of diet. When second phase insulin output of Dl-Dl rats is expressed as a percentage of control levels (or percent inhibition), it is apparent that the degree of inhibition of insulin release by Dl-Dl rats became progressively more pronounced with continued glucose stimulation (fig. 5).

Pancreatic insulin content. Insulin content of the pancreata of control and Dl-Dl rats is shown in table 2. Insulin content was lower in deficient rats both fed ($P < 0.05$) and fasted ($P < 0.001$) than in control rats; the average was 63% of control levels in fed rats and 84% of control levels in fasted rats.

DISCUSSION

Our findings of abnormal glucose tolerance in manganese-deficient rats support and extend the work of Everson and Shrader (7). In our study, offspring of manganese-deficient dams fed 1 μg Mn/g of diet from

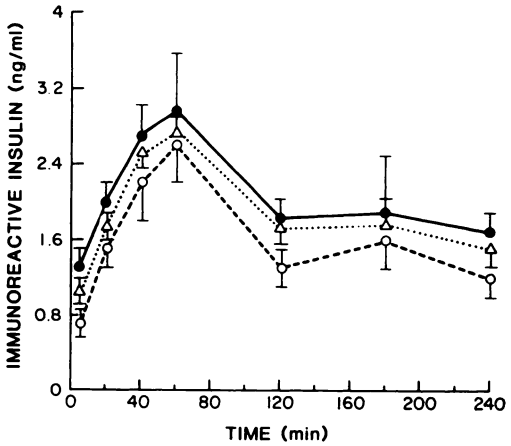

Fig. 2 Plasma insulin levels following oral glucose in control (●), Dl (△) and Dl-Dl (○) rats. Values shown are means ± SEM.

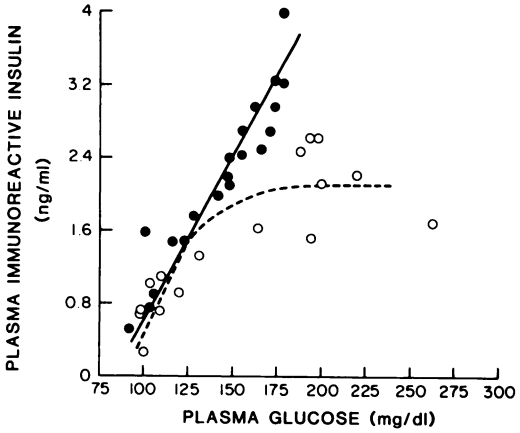

Fig. 3 Relationship of plasma insulin to increasing plasma glucose levels after a glucose load. Points represent values from control (●) and Dl-Dl (○) rats.

weaning to maturity (Dl-Dl) showed a diabetic type of glucose tolerance curve. In contrast to the Everson and Shrader study, we did not observe prolonged hyperglycemia lasting 4 hours after glucose loading. This difference between the two studies is probably due to species differences in sensitivity to manganese deficiency as well as to the high susceptibility of the guinea pig to disorders of carbohydrate metabolism, including spontaneous diabetes (22). The rapid return (by 2 hours) of plasma glucose to near baseline levels after oral glucose in manganese-deficient rats indicates that peripheral insulin sensitivity is not altered significantly in these animals.

The normal glucose tolerance observed in Dl rats indicates that a complex relationship exists between manganese and glucose homeostasis. The lack of an effect of manganese deficiency on glucose tolerance in these rats, despite very low tissue levels of manganese, argues for a developmental component in the abnormal glucose tolerance observed in Dl-Dl rats. In other words, the manganese deficiency must be present while the animal is in utero, or during its suckling period, or both. This is not surprising because many metabolic changes are occurring during these two periods of time. The development of the rat pancreas has begun by the twelfth day of embryological development (23). Both glucagon (24, 25) and insulin (26–28) appear by midgestation and increase as gestation progresses. Appropriate stimuli may cause a rapid hormone release during embryogenesis (29–31), although the mechanism for insulin release does not become fully mature until the end of gestation or after birth (32–34). Nutritional factors are important in the regulation of insulin secretion; exogenous secretagogues such as the high glucose levels of a diabetic mother can stimulate the fetal pancreas and may affect later control of insulin secretion (35). Prenatal abnormalities in nutrient supply, such as may occur in prematurity, in postmaturity, in infants of diabetic mothers, in maternal starvation, or as a result of other manipulations of early feeding, can cause profound and possibly permanent endocrine, enzymatic and metabolic changes (23).

Another aspect of manganese deficiency that might affect insulin production is the destruction of pancreatic β-cells. Diabetogenic agents such as alloxan have been postulated to function via the production of superoxide anion radicals (36). The activity of manganese superoxide dismutase (MnSOD) in pancreatic islet cells is low relative to that in other tissues (37); thus, a reduction in MnSOD activity with manganese deficiency

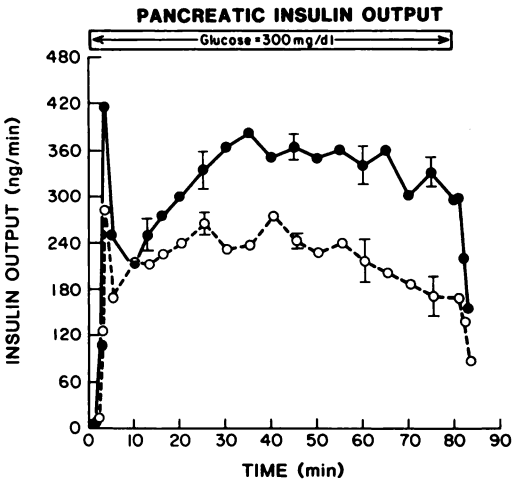

Fig. 4 Time course of insulin secretion in response to a 300 mg/dl glucose stimulus (minute 0–80) in control (●) and Dl-Dl (○) rats. Values shown are means ± SEM.

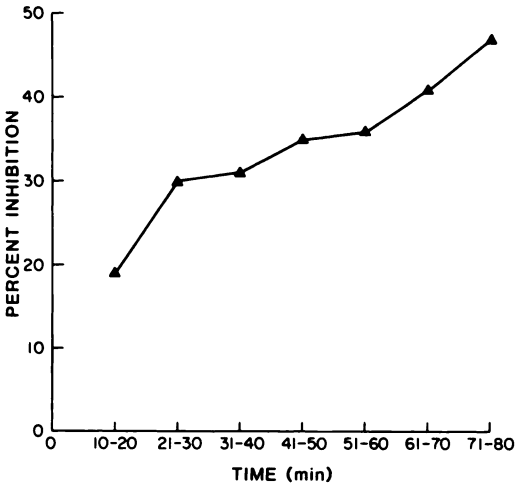

Fig. 5 Inhibition of insulin release over time in Dl-Dl rats. Each point represents percent inhibition = [C − Dl-Dl)/C × 100], where C = mean insulin release of control rats; Dl-Dl = mean insulin release of second generation manganese-deficient rats.

may make the pancreas particularly susceptible to free radical damage. Consistent with this possibility is the finding of low levels of MnSOD activity and increased lipid peroxidation in the livers of offspring from manganese-deficient rats compared to controls (38).

Tissue manganese concentration does appear to be important. Offspring of manganese-deficient rats fed 3 μg Mn/g of diet (rather than 1 μg/g) from weaning to maturity had twofold higher liver and pancreatic manganese concentrations as well as normal glucose tolerance (Baly, D. L., Keen, C. L. and Hurley, L. S., unpublished data). Total tissue manganese concentration, however, may be of secondary importance compared to the distribution of free and bound manganese within the islet cell (4). In agreement with this hypothesis, we have observed dramatic changes in carbohydrate homeostasis after i.p. injections of high doses of $MnCl_2$ (39). At 2 hours postinjection, plasma-glucose levels had increased 4-fold over initial values, while plasma-insulin levels had decreased 6.4-fold.

The observation of low fasting plasma insulin levels in Dl-Dl rats compared to controls, despite both groups having similar plasma glucose levels, is difficult to interpret and may reflect a complex glucoregulatory adaptation to manganese deficiency. This observation allows us to suggest that the hypoinsulinemic manganese-deficient rat is capable of compensatory mechanisms to maintain normal glucose homeostasis during moderate fasting. In contrast, however, the plasma-insulin levels of these deficient animals during glucose tolerance studies were not commensurate with their high circulating-glucose levels. Thus, the manganese-deficient rat is much more susceptible to hyperglycemic episodes after feeding or any other hyperglycemic situation.

TABLE 2

Pancreatic insulin content[1,2]

Group	*n*	Pancreatic wt[3]	Insulin	
		g	*μg*	*μg/g*
Fed				
Control	8	1.08 ± 0.040	59.4 ± 4.2	52.0 ± 6.1
Dl-Dl	8	0.987 ± 0.128	37.5 ± 7.0[a]	39.4 ± 5.0[a]
% of control			63%	76%
Fasted				
Control	8	1.04 ± 0.042	51.8 ± 1.6	59.2 ± 5
Dl-Dl	12	0.954 ± 0.044	43.7 ± 3.2[a]	43.9 ± 4[b]
% of control			84%	74%

[1]Values are means ± SEM for the number of samples in *n* column. [2]Significant differences: [a]from control level ($P < 0.05$); [b]from control level ($P < 0.001$). [3]Wet weight.

First phase insulin release (comprised of preformed stored hormone) was only 76% of the control values in these D1-D1 rats. This is in agreement with our finding that deficient rats had lower insulin stores, which were approximately 65% of those in fed control rats. Insulin output from the pancreas of manganese-deficient animals during the second phase of insulin secretion (a combination of newly synthesized as well as preformed hormone) (14, 40), was also significantly lower than the control levels. It is of particular interest that the degree of inhibition of insulin secretion by manganese-deficient pancreata became progressively more pronounced as the period of glucose stimulation was extended. Since the influence of insulinogenesis is considered to become progressively more pronounced as a function of continual glucose stimulation (14, 40), the data presented in this paper strongly imply that manganese deficiency results in an impaired insulin biosynthetic mechanism. Additional experiments must be performed to address this issue directly.

It is important to stress that the effects reported here may be strain dependent. Rat-strain differences in neonatal mortality (10) and lipid metabolism (41) have been reported in response to dietary manganese deficiency. In any event, however, the data presented in this paper clearly indicate that the low circulating plasma insulin levels seen in manganese-deficient Sprague-Dawley rats are due, at least in part, to impaired pancreatic insulin secretion. More importantly, we suggest that appropriate manganese levels are required in utero, as well as during suckling, for the development of the normal insulin synthesis/secretion process. Failure to ensure proper manganese levels may result in abnormal glucose homeostasis as a consequence of depressed pancreatic function.

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