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Mechanism of decreased insulinogenesis in manganese-deficient rats

Decreased insulin mRNA levels

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Manganese-deficient rats exhibited seven-fold lower preproinsulin mRNA levels compared to control, as detected by dot blot hybridization of both total and poly(A)$^+$ RNA using a preproinsulin cDNA probe. No differences in the size of the insulin mRNA were observed. Thus, decreased mRNA levels may be a major contributing factor to the decreased insulinogenesis observed in manganese-deficient rats.

Insulinogenesis; Manganese deficiency; mRNA level

1. INTRODUCTION

A fundamental problem in diabetes is the inability to utilize blood glucose efficiently. In type 1 diabetics, the apparent defect is inadequate pancreatic insulin secretion, resulting in low circulating plasma insulin levels that are inadequate for maintenance of glucose homeostasis. A role for manganese (Mn) in pancreatic insulin synthesis and secretion has been proposed [1,2]. Consistent with this idea, we have previously shown that manganese deficiency leads to impaired pancreatic insulin secretion [3]. Our studies suggested that the decreased insulin output from Mn-deficient pancreata was due to decreased insulin biosynthesis as well as increased degradation [4].

Pancreatic insulin synthesis and secretion is regulated by a number of factors [5]. However, glucose appears to be a primary signal for these processes. Several studies suggest that glucose stimulates insulin synthesis at both the transcriptional and translational level [6–10]. While glucose has been shown to induce the synthesis of preproinsulin mRNA, this process appears to be more important in the long-term regulation of insulinogenesis [6–8,10]. Several recent studies suggest that insulin biosynthesis is acutely controlled at the translational level by glucose [9,11]. Glucose appears to stimulate elongation rates, stimulate protein synthesis, and increase the transfer of initiated insulin mRNA molecules from the cytoplasm to microsomal membranes [9]. In addition, glucose has also been shown to protect insulin mRNAs from degradation [12].

The purpose of the present study was to examine the cellular mechanisms by which Mn deficiency affects pancreatic insulinogenesis. With this in mind, we measured preproinsulin mRNA levels in Mn-deficient rats. We found that Mn-deficient rats had significantly lower preproinsulin mRNA levels compared to controls. Characterization of the message by Northern analysis indicated no differences in the size of the insulin mRNAs from control or Mn-deficient rats. These studies provide insight into how Mn affects cellular processes that may ultimately be important in understanding the pathogenesis and potential treatments of diabetes.

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2. MATERIALS AND METHODS

2.1. *Animals and diets*

Weanling female Sprague-Dawley rats (Charles River, Wilmington, DE) were fed a purified diet containing either 45 μg Mn/g (Control) or 1 μg Mn/g (Mn-). At maturity (approx. 2 months of age), rats were mated with stock-fed (Purina Rat Chow, Ralston Purina Co., St. Louis, MO) males. Animals were fed their respective diets throughout pregnancy and allowed to deliver their young. Offspring remained with their dams until weaning (30 days postpartum) and had free access to the maternal diet at all times during lactation [2]. Male offspring (3 months of age) of both control (n = 6) and Mn- (n = 7) rats were used in these studies. Tissue manganese was analyzed by atomic absorption spectrophotometry to confirm the deficiency [3].

2.2. *Pancreatic RNA extraction*

Fresh pancreatic tissue was rapidly homogenized in 5 vols of 4 M guanidinium isothiocyanate per gram of tissue. Total RNA was isolated using the hot phenol/chloroform extraction method of Maniatis et al. [12]. The final RNA pellet was rinsed with 70% ethanol and dissolved in sterile water for further analysis. The purity of the RNA was assessed by absorbance at 260/280 nm. The integrity of the RNA isolated was assessed by an in vitro wheat germ translation system. The RNA was translated in the presence of [^{35}S]methionine, followed by SDS-PAGE autoradiography. The presence of high molecular mass ^{35}S-labeled proteins confirmed that the mRNAs isolated had not been degraded.

2.3. *Poly(A)$^+$ RNA isolation*

Total RNA samples were heated to 65°C for 5 min in 20 mM Tris-HCl, 0.5 M NaCl, 1 mM EDTA, 0.1% SDS sample buffer (pH 7.4). Poly(A)$^+$ RNA was isolated from these samples using oligo(dT)-cellulose chromatography, as described by Maniatis et al. [12].

2.4. *Preproinsulin mRNA determination*

2.4.1. Dot blot hybridization

RNA, extracted as above, was denatured by heating to 100°C for 5 min and the RNA bound to nitrocellulose paper by vacuum filtration. The nitrocellulose paper, baked for 2 h at 80°C, was prehybridized and hybridized as described by Thomas [14]. Rat preproinsulin gene I in PBR322 was nick-translated (Bethesda Research Labs) using [α-^{32}P]dCTP and subsequently purified by Sephadex G-50 chromatography. The specific activity of the probe averaged 3.4×10^7 cpm/mg. Yeast tRNA was also blotted to monitor nonspecific binding of the probe. No binding of the labeled cDNA probe to yeast tRNA was observed. Increasing amounts of both total (20–100 μg) and poly(A)$^+$ (1–6 μg) RNA were hybridized to the labeled preproinsulin cDNA probe to determine the linear range of RNA hybridization to the probe. For visualization of the dots, autoradiography was performed at −70°C for 18–40 h. In addition, hybridization assays were quantitated by cutting out the ^{32}P-labeled dot blot and counting in a scintillation counter.

Duplicate hybridization experiments were carried out on both total and poly(A)$^+$ RNA using a B-actin cDNA probe. Hybridization of the labeled actin cDNA probe revealed no differences in mRNA content between Mn-deficient and control animals, ruling out a nonspecific effect of Mn deficiency on mRNA content or variable recovery of poly(A)$^+$ RNA during oligo(dt) chromatography.

2.4.2. Northern analysis

The poly(A)$^+$ RNA precipitates were denatured with 2.2 M formaldehyde and 50% (v/v) formamide in 40 mM Mops, 10 mM sodium acetate, 1 mM EDTA at 55°C for 15 min. The samples were electrophoresed on 1.5% agarose gels before transfer to nitrocellulose [13]. Hybridization was carried out as described previously [14]. Visualization was carried out by autoradiography as described previously. Northern blot analyses of islet RNA prepared by the above method revealed no degradation of insulin mRNA.

2.4.3. Statistical analysis

Data were analyzed using a one-way analysis of variance (ANOVA). Group comparisons were made using the least significant difference method when a significant F was reached.

3. RESULTS

Pancreatic manganese content was significantly ($p < 0.05$) lower in manganese-deficient animals (0.67 ± 0.10 μg/g pancreas) compared to controls (2.40 ± 0.16 μg/g pancreas). No significant differences in body weight or pancreatic weight were observed. The 260/280 absorbance ratio of the pancreatic RNA isolated averaged 1.94. In vitro translation of the RNA yielded both high and low molecular mass bands on SDS-PAGE (not shown), indicating the mRNAs isolated had not been significantly degraded.

3.1. *Preproinsulin mRNA levels*

Hybridization of ^{32}P-labeled probe to increasing amounts of total RNA (20–100 μg) and poly(A)$^+$ RNA (1–6 μg) showed a linear increase in the amount of labeled probe hybridized (not shown). Hybridization of labeled probe to total RNA (100 μg) demonstrates lower preproinsulin mRNA levels in Mn-deficient rats (n = 7) compared to controls (n = 6). A typical experiment is shown in fig.1. Similar results were observed with poly(A)$^+$ RNA extracts (fig.1B). Fig.2 summarizes the results of 12 hybridizations of poly(A)$^+$ RNA from control and Mn-deficient rats. Preproinsulin mRNA levels were 9-fold lower in Mn-deficient animals compared to those of controls.

3.2. *Northern analysis*

Northern blot hybridization (fig.3) of poly(A)$^+$

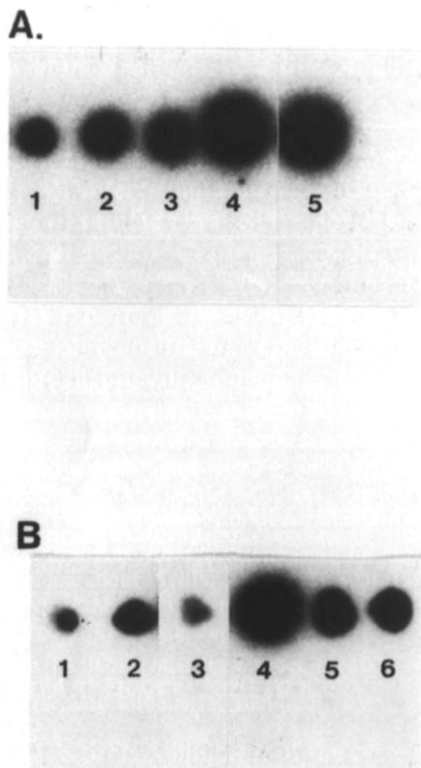

Fig.1. Effect of manganese deficiency on pancreatic insulin mRNA levels. Pancreatic RNA from three Mn-deficient and two control rats was phenol extracted and ethanol precipitated. The content of insulin mRNA was quantitated by dot hybridization as described in section 2. (A) Dot hybridization of insulin mRNA from total RNA extracts of Mn-deficient (1–3) and control (4 and 5) rats. (B) Dot hybridization of insulin RNA from poly(A)$^+$ RNA extracts of Mn-deficient (1–3) and control (4–6) rats.

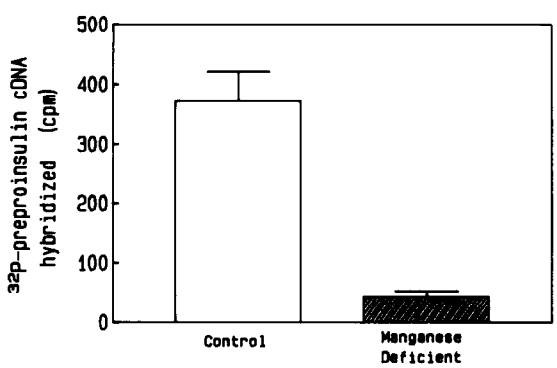

Fig.2. Insulin mRNA levels in poly(A)$^+$ RNA extracts from control and Mn-deficient rats. Poly(A)$^+$ RNA extracts were prepared by oligo(dt)-cellulose chromatography as described in section 2. The vertical bar represents mean ± SE of 6 control rats and 7 Mn-deficient rats.

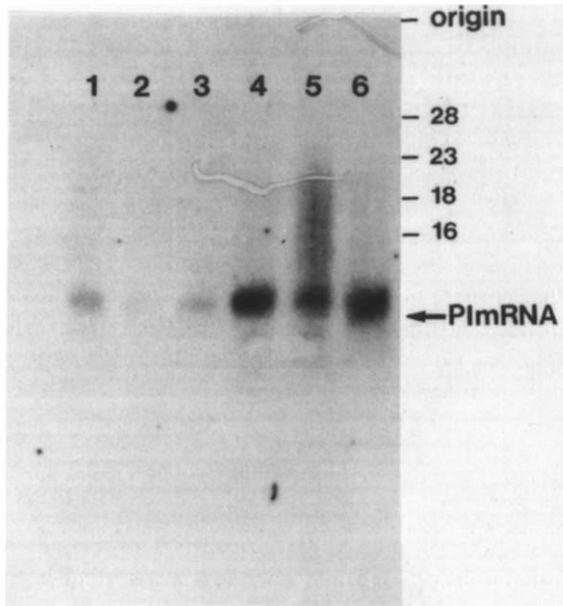

Fig.3. Northern analysis of insulin mRNA from control and Mn-deficient rats. Poly(A)$^+$ RNA extracts were denatured with 2.2 M formaldehyde and 50% (v/v) formamide, 10 mM sodium acetate, 1 mM EDTA at 55°C for 15 min. The samples were subjected to electrophoresis on 1.5% agarose gels and then transferred to nitrocellulose. Hybridization was carried out as described in section 2. Lanes 1–3 are from Mn-deficient rats and lanes 4–6 are from control rats. The relative mobilities of 16–28 S standards are shown on the right. PImRNA represents the preproinsulin mRNA bands.

RNA demonstrates that both control and Mn-deficient rats had preproinsulin mRNAs of the same size. In addition, the same pattern of decreased amounts of preproinsulin mRNA in Mn-deficient rats was observed.

4. DISCUSSION

We have previously demonstrated that insulin biosynthesis in response to a maximally stimulating glucose concentration (300 mg/dl) is decreased 9-fold in Mn-deficient rats compared to controls [1]. This decrease in insulinogenesis could be due to several factors, including decreased insulin gene transcription, altered insulin mRNA processing, or impaired conversion of proinsulin to insulin. The first two possibilities could lead to decreased mRNA levels as well as alterations in the size of the insulin mRNA synthesized in Mn-deficient rats. In the present study, we have begun

to explore at what level low intracellular concentrations of Mn affect insulinogenesis. The data demonstrate that insulin mRNA levels are decreased 9-fold in Mn-deficient rats compared to controls. The fold reduction in insulin mRNA levels in this study is strikingly similar to the decrease in insulinogenesis previously observed in Mn-deficient rats. Thus, the data strongly suggest that the decreased amount of insulin mRNA is a major factor contributing to the decreased glucose-induced insulin synthesis seen in islets from Mn-deficient rats. This decreased insulin mRNA probably represents a long-term adaptation of these animals to the deficiency. The specific mechanisms by which low intracellular Mn may affect insulin mRNA content remains to be elucidated. Several likely possibilities include impaired insulin gene transcription, altered insulin mRNA processing or translatability, and altered cytosolic stability of existing mRNAs.

We have begun to address these possibilities by characterizing the insulin message using Northern analysis. In these studies mRNAs from control and Mn-deficient animals were similar with respect to size. This would suggest that mRNA processing was not affected by Mn deficiency. However, effects of Mn deficiency on mRNA processing cannot be ruled out since most defective transcripts never leave the nucleus. Thus, examining transcription rates and message turnover in nuclei will be important. We have also examined the ability of glucose to induce new insulin mRNA synthesis by injecting fasted rats with glucose over a 12 h period. Preliminary data from these studies suggest that glucose was equally able to induce new mRNA synthesis as evidenced by similar mRNA content in glucose-injected deficient and control animals. Thus, insulin gene transcription may not be affected by Mn deficiency. However, effects of manganese on gene transcription cannot as yet be ruled out as these studies were done in whole animals, where blood glucose concentrations are tightly regulated (5–9 mM following glucose injections). Maximal effects of glucose on insulin mRNA synthesis require 16–20 mM glucose [9–11]. In addition, fasting followed by glucose injection will affect many physiological events in the whole animals, which may impinge on pancreatic insulin homeostasis, masking specific effects of glucose on insulin mRNA levels. Thus definitive studies will require the use of isolated islets. Studies designed to address the biochemical mechanisms underlying manganese's effect on insulin mRNA content are currently being done in our laboratory.

In conclusion, these studies provide insight into how Mn affects cellular processes that may ultimately be important in understanding the pathogenesis and potential treatments of diabetes.

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