Zinc ions in the endocrine and exocrine pancreas of zinc deficient rats

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Summary. Objective: Zinc deficiency is a problem world-wide. Zinc and insulin are intimately related, and a reduced zinc intake may affect glucose metabolism. The present study investigates how subclinical zinc deficiency in rats affects glucose metabolism and zinc distribution in the pancreas. Methods: Glucose metabolism was evaluated by blood-glucose, serum insulin, homeostasis model assessment (HOMA), and intraperitoneal glucose tolerance tests. Immersion zinc-sulphide autometallography (iZnSAMG) was used to describe zinc ion distribution. Results: After 4 weeks on a zinc deficient diet (<10 ppm), the zinc deficient rats had a slightly impaired glucose metabolism characterized by significantly increased blood-glucose levels. No differences in serum insulin, insulin resistance, beta-cell function were observed. The zinc deficient rats had significantly decreased serum zinc without any clinical signs of zinc deficiency. Zinc ion staining intensity of the islets of Langerhans was unaffected by the zinc deficiency. In contrast, the acinar cells in the exocrine pancreas appeared depleted of iZnSAMG grains in the zinc deficient rats when compared with their controls. Though statistically non-significant, a reduction in total zinc of the pancreas was found. Conclusions: The present findings suggest that the endocrine pancreas is able to compensate for the subclinical zinc deficiency as it maintains an adequate zinc ion level in the secretory vesicles for insulin storage. The exocrine pancreas lacks this ability; it exhibits decreased levels of zinc ion staining as a consequence of 4 weeks of reduced zinc intake.

Key words: Autometallography, Beta-cells, Diabetes, Insulin, Secretory vesicles

Introduction

Zinc is an essential trace element, involved in virtually all aspects of metabolism. In the pancreas, the beta-cells contain large amounts of zinc; one of the major roles of zinc is the binding of insulin in hexamers (Derewenda et al., 1989; Dodson and Steiner, 1998). Zinc ions and insulin create a hexameric, crystalline structure, comprising 2 zinc ions and 6 insulin molecules, which is stored in the secretory vesicles until secreted in response to metabolic demands (Emdin et al., 1980; Hutton et al., 1983). Furthermore, zinc ions have been found in the vesicles of alfa-cells, as well as in the zymogen vesicles of acinar cells (Kristiansen et al., 2001). Due to the close relation between zinc and insulin, studies addressing zinc deficiency, glucose metabolism and diabetes have been carried out. Many diabetic patients are zinc deficient, probably caused by a combination of increased zinc loss with urine and intestinal zinc malabsorption, which has been observed in both type 1 and type 2 diabetic individuals (Salgueiro et al., 2001). Due to the close relation between zinc and insulin, studies addressing zinc deficiency, glucose metabolism and diabetes have been carried out. Many diabetic patients are zinc deficient, probably caused by a combination of increased zinc loss with urine and intestinal zinc malabsorption, which has been observed in both type 1 and type 2 diabetic individuals (Salgueiro et al., 2001). In diabetic rats, the total pancreatic zinc content has been found to be lower as compared to controls (Levine et al., 1983), whereas total pancreatic zinc in zinc deficient rats was found to be unaltered (Boquist and Lernmark, 1969; Huber and Gershoff, 1973). Although there are contradictory results of oral glucose tolerance tests performed in zinc deficient animals, it is possible that insulin resistance is related to zinc deficiency (Kinlaw et al., 1983; Levine et al., 1983; Faure et al., 1992). Mechanisms proposed for insulin resistance during zinc deficiency are many: 1) interference with the insulin receptor binding, 2) decreased insulin receptor synthesis, and 3) abnormal glucose carrier structure and/or translocation inside the cell as a consequence of increased lipid peroxidation (Faure et al., 1992; Kennedy et al., 1998). There are also contradictory results as to the serum insulin concentration in zinc deficiency. In some studies unchanged insulin levels were found (Kennedy et al.,...
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In 1998, whereas other studies showed decreased serum insulin levels in zinc deficient animals when compared to pair fed controls (Levine et al., 1983). In one study a decreased beta-cell granulation in zinc deficient Chinese hamsters using the sulphide silver method was found, indicating that less zinc was present in the vesicles (Boquist and Lernmark, 1969). Beta-cell secretory vesicles contain large amounts of zinc, probably more than needed for insulin binding (Foster et al., 1993), indicating that the vesicles may have a reservoir function.

Zinc ions can be visualised with autometallographic techniques, like the Neotimm (Danscher, 1981), the selenium (Danscher, 1982) or the in-vivo sulphide methods (Danscher, 1996; Kristiansen et al., 2001). Immersion zinc sulphide autometallography (iZnS<sub>AMG</sub>) is the newest member of the zinc ion specific autometallographic techniques. iZnS<sub>AMG</sub> can, in contrast to the previous methods, be used for zinc ion tracing in fresh tissue (Danscher et al., 2004). The sensitivity of the methodology is at the level of a few zinc atoms if two or more are located close (within nanometres) to one another (Danscher, 1996). Using ZnS<sub>AMG</sub> we have previously demonstrated zinc ions in the zymogen vesicles of acinar cells (Kristiansen et al., 2001), although the role of these zinc ions has not been established. Zinc deficiency leads to a reduction in size and volume fraction of the zymogen vesicles (Perez-Jimenez et al., 1996). The exocrine pancreas seems to be more sensitive to changes in zinc than the endocrine pancreas since a low dosage of a single sub-cutaneous injection of zinc induces injury in pancreatic exocrine cells, but not in endocrine cells (Minami et al., 2001). Some studies show that the acinar cells concentrate zinc and that intestinal zinc absorption could be modulated by an exocrine ligand (Montgomery et al., 1943; Van Wouwe and Uijlenbroek, 1994).

A previous study on the insulinoma-derived INS-1E cells showed that iZnS<sub>AMG</sub> detectable zinc ions were able to respond to both acute and chronic changes in the glucose concentration (Søndergaard et al., 2005), probably mediated by membrane zinc transporters. The aim of the present study is to examine the impact of subclinical zinc deficiency on iZnS<sub>AMG</sub> detectable zinc ions in the pancreas, as well as possible changes in glucose metabolism.

Materials and methods

Animals and treatment

All experiments were performed using a total of 30 female Wistar rats, 8 weeks old at the start of the experiment. The animals were housed individually in plastic cages at constant temperature (23°C) and humidity (53%), and a fixed 12:12 hrs night and day cycle. All animals were obtained from Taconic M&B Breeding Laboratory, (Ry, Denmark). Institutional, national and international guidelines for animal welfare were obeyed.

Zinc deficiency was induced by maintaining rats on distilled water and semi-synthetic zinc deficient fodder, i.e. <10 parts per million (ppm) (C1040, Altromin, Lage, Germany). The control animals were maintained on the same diet supplemented with zinc (70 ppm).

The animals were randomly divided into 3 groups containing 10 rats in each group. Group 1 received the zinc deficient fodder, group 2 was pair-fed controls, maintained on the same amount of fodder on a body-weight basis as the rats on the zinc deficient fodder. A third group were ad libitum fed controls, given free access to fodder. Before the start of the dietary regime, the rats was acclimatized to the semi-synthetic fodder by being fed the zinc supplemented fodder for 7 days. Thereafter the animals lived for 4 weeks on their respective dietary regime before they were sacrificed. The rats were anesthetized with isoflurane, blood samples were collected from the heart, and they were sacrificed by decapitation. Immediately after sacrifice, the abdomen was opened and the pancreas removed.

Pancreata from 8 rats in each of the three groups were immersed in 3% glutaraldehyde (GA) stored at 4°C for 2 hrs. After post-fixation in 4% GA for 2 hrs, the pancreas was cut into 2 mm slabs using the HistOtech slicing machine (HistOtech ApS, Aarhus, Denmark).

Half of the pancreata from 4 of the ad libitum fed control rats and from 4 of the zinc deficient rats were fast frozen in liquid nitrogen and analysed for zinc using atomic absorption spectrometry (AAS).

Methodological controls: 1) One 2 mm slab from each of the 8 rats in each group served as blank controls. They were treated as the corresponding slabs, apart from being exposed to sulphide ions. 2) Two animals from each group were treated with the chelator diethylthiocarbamate (DEDTC) in order to block all zinc ions as zinc-DEDTC molecules, i.e. during pentobarbiturate anaesthesia the animals were injected intraperitoneally with 1000 mg/kg body weight DEDTC, and allowed to survive for one hour before sacrifice (Danscher et al., 1973). One 2 mm slab from each of the 8 rats in each group were DEDTC-immersion controls, they were immersed in 5 mM DEDTC for 1 h and afterwards treated as the corresponding slabs (Danscher et al., 2004).

Blood samples

Blood samples for measuring serum insulin, serum glucose and serum zinc after 8 hours of fasting were drawn from the heart during isoflurane anaesthesia. The samples were centrifuged at 3000 rpm for 3-5 min and serum was frozen and kept at -80°C until further analysis. The samples were analysed using an ultra-sensitive rat insulin ELISA Kit (DRG Diagnostics, Marburg, Germany), with an intra- and interassay CV<5% and 10%, respectively. Serum zinc was measured by means of inductively coupled plasma mass spectrometry. Before sacrifice, an intraperitoneal glucose
tolerance test (IPGTT) was performed after an 8 hrs fast. Blood glucose was measured on samples obtained by tail bleeding before the intraperitoneal injection of 2 g glucose/kg body weight as well as 30, 60, 90, and 120 minutes after the glucose injections. Blood-glucose was measured using a Precision Xtra (MediSense®, Gentofte, Denmark).

In order to estimate insulin resistance in the rats, the homeostasis model assessment (HOMA) index was calculated by the formula: (fasting serum insulin (µU/ml) x fasting serum glucose (mmol/l))/22.5 (Minami et al., 2001). The β-cell function was estimated by the formula: (20 x fasting serum insulin (µU/ml))/ (fasting serum glucose (mmol/l – 3.5) (Matthews et al., 1985).

Immersion ZnS\textsuperscript{AMG} staining

The 2 mm slabs were immersed in the NeoTimm solution (0.1% sodium sulphide dissolved in 3% GA) at 4ºC for 24 hrs (Danscher, 1981; Danscher et al., 2004). Afterwards the tissue slabs were rinsed in phosphate buffer (PB) and cryoprotected in 30% sucrose for 24 hrs. They were frozen with CO\textsubscript{2} gas. Cryostat sections (20 µm) were cut and placed on Farmer rinsed slides (1 ml potassium ferricyanide 10%, 1 ml sodium thiosulphate 10%, 90 ml water). The glass slides were then immersed in the AMG developer, i.e. 60 ml gum arabic (33% aqueous solution), 10 ml citrate buffer at pH 3.8 (25.5 g of citric acid · H\textsubscript{2}O + 23.5 g sodium citrate · 2 H\textsubscript{2}O to 100 ml distilled water), 15 ml hydroquinone (aqueous solution containing 0.85 g), and 15 ml silver lactate (aqueous solution containing 0.12 g). The AMG development took place in a 26ºC warm water bath covered with a light tight box for 40 minutes. The AMG process was stopped by replacing the developer with 5% sodium thiosulphate. After ten minutes the slides were rinsed in 40ºC warm tap water, rinsed in distilled water, and finally counterstained with 1% toluidine blue (Søndergaard et al., 2005).

Light microscopy was performed. The amount of intracellular zinc in the exocrine and endocrine pancreas was graded semi-quantitatively on a scale from 0 to 5: 0) no visible grains; 1) a few just visible grains; 2) few small, but distinct grains; 3) many medium to large-sized grains; 4) numerous grains; 5) extensive silver deposition leading to diffuse staining of the cells (Ellermann-Eriksen et al., 1987).

Statistical analysis

All data are presented as means ± SEM. The significance of difference was assessed by the unpaired student’s t test. Any P value above 0.05 was considered not significant.

Results

Zinc deficiency and glucose metabolism

The zinc deficient rats were given food with up to 10 ppm zinc for 4 weeks. They had significantly lower serum levels of zinc with a 53% reduction as compared to the ad libitum fed rats, and a 44% reduction in serum zinc when compared to the pair fed controls (Fig. 2). However, they did not develop any of the classical signs of zinc deficiency such as weight loss, anorexia, alopecia, or skin lesions and were therefore classified as

<table>
<thead>
<tr>
<th>GROUP TREATMENT</th>
<th>1 Ad libitum fed controls (n=8)</th>
<th>2 Pair fed controls (n=8)</th>
<th>3 Zn-deficient (n=8)</th>
<th>Statistical significance of difference 1 vs 3: P</th>
<th>Statistical significance of difference 2 vs 3: P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>213.8 ± 3.0</td>
<td>210.0 ± 5.9</td>
<td>219.1 ± 1.6</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Food consumption (g/g body weight/24 hrs)</td>
<td>0.059 ± 0.003</td>
<td>0.055 ± 0.003</td>
<td>0.055 ± 0.003</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Total pancreas zinc (mg/ kg wet weight)</td>
<td>30.20 ± 2.34</td>
<td>NM</td>
<td>NM</td>
<td>23.73 ± 0.57</td>
<td>0.06 (NS)</td>
</tr>
<tr>
<td>S-insulin (µg/l) fasting</td>
<td>1.54 ± 0.13</td>
<td>1.78 ± 0.39</td>
<td>1.87 ± 0.24</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>HOMA index</td>
<td>15.9 ± 1.5</td>
<td>18.0 ± 4.0</td>
<td>18.6 ± 2.6</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>B-cell function (%)</td>
<td>137.7 ± 17.6</td>
<td>167.4 ± 34.8</td>
<td>161.4 ± 16.9</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>B-glucose (mmol/l)</td>
<td>Fasting: 5.28 ± 0.08</td>
<td>5.08 ± 0.09</td>
<td>5.67 ± 0.14</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
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<tr>
<td></td>
<td>Non-fasting: 5.61 ± 0.34</td>
<td>5.82 ± 0.14</td>
<td>6.45 ± 0.20</td>
<td>NS</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Mean values with standard errors; number of animals in parentheses; NS, not significant, NM, not measured.
having subclinical zinc deficiency. Analysing the whole pancreas for zinc using AAS we found that the zinc deficient rats had a reduction in total pancreas zinc content of 21% (P=0.06) as compared to the ad libitum fed rats, although this was not significant (Table 1).

The zinc deficient rats had an impaired glucose metabolism. Both their fasting and non-fasting blood-glucose levels were significantly higher than the controls (Table 1), and during the IPGTT their blood-glucose levels were higher compared to the 2 control groups at 0 and 120 min (Fig. 1). Even though the zinc deficient rats had increased glucose levels it was not reflected in the serum insulin values, no differences in HOMA index or in the beta-cell capacity were found as compared to the ad libitum fed and pair-fed controls (Table 1).

Zinc deficiency and iZnSAMG detectable grains

iZnSAMG staining was used to determine how zinc deficiency affected the distribution of zinc ions in the rat pancreas. We found an intense ZnSAMG staining in the islets of Langerhans. No difference in staining intensity was found between the islets from the zinc deficient rats and the two control groups. In contrast, when we examined the exocrine pancreas, it was obvious that the acinar cells in the zinc deficient rats were almost completely void of iZnSAMG grains, whereas the ad libitum fed and the pair fed controls were loaded with AMG grains. This was supported by grading the silver accumulation in the cells semi-quantitatively on a scale from 0-5. The ad libitum fed control rats were graded as 2, the pair fed controls as 2 and the zinc deficient rats as 0. The islets in all three groups were graded as 5 (Fig. 3). Both the DEDTC-rats, the immersion DEDTC-rats, and the blank controls had absolutely no iZnSAMG staining, thereby proving the zinc specificity of the applied autometallographic technique.

Discussion

Zinc in the beta-cell secretory vesicles is involved in the storage and stabilization of the insulin hexamere (Emdin et al., 1980; Dodson and Steiner, 1998) and our findings that iZnSAMG detectable zinc is unchanged when comparing the zinc deficient rats with the controls suggest that the beta-cell secretory vesicles are able to compensate for the low level of zinc in the circulation, ensuring that enough zinc is present in the vesicles for insulin hexamerisation and storage. We hypothesize that the pancreatic beta-cells act as a zinc reservoir. One single study has previously described a reduced sulphide-silver staining in the islets together with a decreased beta-cell granulation after zinc deficiency (Boquist and Lernmark, 1969). These animals received a diet with a lower zinc content and were more severely zinc deficient than the rats in the present study (Fig. 2) and were not able to compensate for the reduced zinc intake. Such a notion is further supported by the present findings of reduced levels of total zinc. The reduction in total pancreas zinc that has been reported in previous studies (Canton and Cremin, 1990; van Wouve and Uijlenbroek, 1994). As the islets constitute 1-2% of the whole organ this reduction most likely reflects a decrease in zinc in the exocrine part of the pancreas. Our semi-quantitative grading of zinc ions in the exocrine and endocrine pancreas further supports this notion. We found that the exocrine part of the pancreas in the zinc deficient rats was almost depleted of zinc ions whereas the islets were loaded with zinc ions and appeared no different from the controls (Fig. 3). The presence of zinc ions in the acinar cells has previously been described using sulphide silver staining techniques (Kristensen et al., 2001; Voigt, 1959), and it is known that zinc is important for the function of carboxypeptidase, a zinc metalloenzyme present in the pancreatic juice (Vallee and Neurath, 1955), and that the acinar zinc has a faster turn-over than the islet zinc (McIsaac, 1955). But using

Fig. 1. Effect of subclinical zinc deficiency on the intraperitoneal glucose test. rhomb, ad libitum fed controls; square, pair fed controls; triangle, zinc deficient rats. P<0.05 zinc deficient vs ad libitum fed and pair fed controls.

Fig. 2. Effect of eating a hypo-zinc compared to a normo-zinc diet on serum zinc. Data are given as ± SEM. Mean values for serum zinc are significantly lower for the zinc deficient rats as compared to the ad libitum fed and the pair fed controls. ** P<0.01, *** P<0.001.
Fig. 3. Effect of subclinical zinc deficiency on the iZnsAMG stainability in the exocrine and endocrine pancreas. A, B. Micrographs of the exocrine and endocrine pancreas of a pair fec control, arrowheads show the islets of Langerhans. Note the intensely stained islets, and the delicate iZnsAMG grains in the exocrine pancreas. C, D. Micrograph of a zinc deficient rat. the islets are again intensely stained, but the acinar cells in the exocrine pancreas are totally depleted of iZnsAMG grains (arrows). Scale bar: 100 micron.
the iZnS\textsuperscript{AMG} method we only visualise the small amount of free zinc ions not firmly bound to enzymes, transcription factors or proteins (Vallee and Falchuk, 1993), and the exact role of these free zinc ions in the vesicles of the acinar cells of the exocrine pancreas is unknown.

Even though the rats fed the zinc deficient diet developed reduced serum zinc levels and total pancreas zinc they did not develop the classical signs of zinc deficiency such as anorexia, weight loss or alopecia. This is probably due to the fact that the rats received just enough zinc to maintain their body weight. This is supported by a study in which the limits for developing clinical signs of zinc deficiency disappeared gradually with increasing zinc in the fodder. When the zinc supplementation reached 12 ppm zinc no difference in weight gain or food consumption was found when comparing the zinc deficient rats with the zinc supplemented rats (Williams and Mills, 1970). Even though the subclinical zinc deficient rats did not develop any of the classical signs of zinc deficiency they did develop an impaired glucose metabolism (Fig. 1 and Table 1), which is consistent with previous observations made on severely zinc deficient animals (Quarterman et al., 1966; Hendricks and Mahoney, 1972; Quarterman and Florence, 1972; Huber and Gershoff, 1973; Faure et al., 1991). Studies on blood insulin levels are contradictory (Boquist and Lernmark, 1969; Huber and Gershoff, 1973; Levine et al., 1983; Kennedy et al., 1998). Pancreas insulin seems unchanged by zinc supplementation (Huber and Gershoff, 1973; Brown et al., 1975), indicating that insulin synthesis is not impaired. Furthermore, the zinc deficient rats were not insulin resistant estimated by the HOMA index and their beta-cell function was intact.

The reason for choosing the iZnS\textsuperscript{AMG} method for this experimental set-up instead of the previously used ZnS\textsuperscript{AMG} method is that the iZnS\textsuperscript{AMG} method gives us the opportunity to select a part of the pancreas for zinc AAS analysis since the tissue is fresh and has not been fixed in vivo. Furthermore, this new method is excellent for semi-quantitative purposes since it is possible to optimize and standardize all the steps, making sure that all the tissues we compared received exactly the same amount of sodium-sulphide, got the same exposure time and Table 1, which is consistent with previous observations made on severely zinc deficient animals (Quarterman et al., 1966; Hendricks and Mahoney, 1972; Quarterman and Florence, 1972; Huber and Gershoff, 1973; Faure et al., 1991). Studies on blood insulin levels are contradictory (Boquist and Lernmark, 1969; Huber and Gershoff, 1973; Levine et al., 1983; Kennedy et al., 1998). Pancreas insulin seems unchanged by zinc supplementation (Huber and Gershoff, 1973; Brown et al., 1975), indicating that insulin synthesis is not impaired. Furthermore, the zinc deficient rats were not insulin resistant estimated by the HOMA index and their beta-cell function was intact.

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In conclusion, we found that rats with subclinical zinc deficiency developed a slightly impaired glucose metabolism without any changes in serum insulin. Furthermore, the islets of Langerhans were found to be loaded with iZnS\textsuperscript{AMG} detectable zinc ions, with no difference when comparing the zinc deficient rats with controls. However, the acinar cells of the exocrine pancreas were almost completely void of zinc ions in contrast to the controls. Our findings suggest that during subclinical zinc deficiency, the pancreatic beta-cells are able to compensate for the reduced amounts of dietary zinc, whereas the exocrine acinar cells suffer from significantly reduced amounts of zinc ions.

Acknowledgements. We wish to thank D. Jensen, A. Meier, H. Mikkelsen, J. Lund, Thorkild Nielsen, K. Nyborg, and K. Wiedemann, for their excellent technical assistance. This study was supported by The Danish Diabetes Association.

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Accepted January 20, 2006