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Nutrition

Impact of high dietary zinc on zinc accumulation, enzyme activity and proteomic profiles in the pancreas of piglets

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ABSTRACT

The exocrine pancreas plays an important role in zinc homeostasis. Feeding very high (2000–3000 mg zinc/kg diet) levels of zinc oxide to piglets for short periods is a common practice in the swine industry to improve performance and prevent diseases. The impact on pancreatic function and possible side effects during long-term feeding of high dietary zinc levels are still poorly understood. A total of 54 weaned piglets were either fed with low (57 mg/kg, LZn), normal (164 mg/kg, NZn) or high (2425 mg/kg, HZn) zinc concentration in the diets. After 4 weeks of feeding, ten piglets per treatment were euthanized and pancreas samples were taken. Tissue zinc concentration and metallothionein abundance was greater with HZn compared with NZn and LZn ($P < 0.05$). Similarly, activity of α-amylase, lipase, trypsin and chymotrypsin was higher with HZn as compared with NZn and LZn diets ($P < 0.05$), whereas elastase activity was unchanged. Total trolox equivalent antioxidative capacity of pancreas tissue was higher with HZn diets compared with the other treatments ($P < 0.05$). Pancreatic protein profiles of NZn and HZn fed piglets were obtained by 2D-DIGE technique and revealed 15 differentially expressed proteins out of 2100 detected spots ($P < 0.05$). The differentially expressed proteins aldose reductase, eukaryotic elongation factor II and peroxiredoxin III were confirmed by immunoblotting. Identified proteins include zinc finger-containing transcription factors and proteins mainly associated with oxidative stress response and signal transduction in HZn compared with NZn pigs. Histologic examination however showed no morphologic changes. The results suggest that long-term supply of very high dietary zinc increases zinc and metallothionein concentration, and digestive enzyme activity, but also triggers oxidative stress reactions in the pancreas of young pigs. The data provide new insights into pancreatic function under outbalanced zinc homeostasis.

Introduction

Zinc is an essential trace element that is involved in more than 300 processes in the body and zinc deficiency can lead to severe disorders in both man and animals [1,2]. In pigs, dietary recommendations are in the range of approximately 80–100 mg zinc/kg, whereas the maximum authorized dietary level in the EU is 150 mg zinc/kg. On the other hand, very high dietary amounts of zinc oxide (e.g. 2000–4000 mg/kg diet) are commonly used as an alternative for antibiotics to prevent or treat diarrhoea [3,4], such high amounts of dietary zinc oxide have been reported to improve feed intake and growth rates [5]. We have recently shown that improved performance can only be observed during the early supplementation period of 2 weeks, whereas longer

Abbreviations: ALB, albumin; ALR, aldose reductase; ATP5B, mitochondrial ATP synthase transporting F1 complex beta subunit; BW, body weight; CPB1, carboxypeptidas B1; DHDH, trans-1,2-dihydrobenzene-1,2-diol dehydrogenase; DM, dry matter; eEF II, eukaryotic elongation factor II; ENO1, α-enolase; ER, endoplasmatic reticulum; GLRX3, glutaredoxin-3-like; GNB2L1, guanine nucleotide-binding protein subunit beta-2-like 1 (RACK1); HSP90B1, heat shock protein 90B1 (endoplasmin, GRP94); HZn, high dietary zinc group; IRE1α, inositol-requiring enzyme 1α; LMNB1, lamin-B1-like; LZn, low dietary zinc group; MT, metallothionein; NZn, normal dietary zinc group; PRDX III, peroxiredoxin III; RPL19, 60S ribosomal protein L19; SDHA, succinate dehydrogenase subunit A; TEAC, trolox equivalent antioxidative capacity; TXNL2, thioredoxin-like protein 2; UPR, unfolded protein response.

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supplementation can even turn into the opposite [6]. In addition, feeding high levels of dietary zinc oxide to piglets over 4 weeks down-regulated intestinal transporters for zinc uptake and increased expression of those transporters for export from epithelial cells, but did not prevent zinc accumulation in various tissues including the pancreas [6–8]. When zinc is supplied at recommended dietary levels, homeostasis is maintained through the interplay of various zinc transporters [9] and specific intracellular heavy-metal binding proteins such as metallothionein [10]. Besides bile and through transporter-mediated secretion from epithelial cells, excretion of zinc into the intestinal tract occurs through secretion from the exocrine pancreas to maintain body zinc homeostasis [11,12]. The pancreas plays also a key role in nutrient digestion and energy balance, and there are indications linking zinc supply and tissue zinc concentration with organ functionality. For example, high dietary zinc oxide increased pancreatic enzyme activities in weaning piglets [13], whereas zinc toxicity and pancreatic necrosis have been reported in parenteral nourished piglets with over-supplementation of zinc at 50 mg zinc/L total parenteral nutrition solution [14]. Since high increases in pancreatic zinc concentrations in piglets fed high amounts of dietary zinc oxide over 4 weeks were determined previously [6], the question arose, whether the associated pancreatic zinc accumulation could have adverse effects in piglets. The current study, which was an additional part of previous studies [6,8], thus aimed at determining the influence of high dietary zinc levels on exocrine pancreatic function and global protein expression in weaning piglets.

Materials and methods

Animals, diets and sampling

All procedures involving pig handling and treatments were approved by the local state office of occupational health and technical safety 'Landesamt für Gesundheit und Soziales Berlin' (LaGeSo Reg. Nr. 0347/09).

The study was part of a comprehensive study aimed at determining the impact of dietary zinc on intestinal physiology and parts including detailed experimental conditions were published previously [6,8]. Briefly, a total of 54 weaning piglets (26 ± 1 days of age) were randomly allocated into three treatment groups (balanced for gender and body weight), and received one of three experimental diets (Supplemental Table 1), based on wheat, barley, and soybean meal, as described previously [8]. Dietary zinc level was adjusted by partially replacing cornstarch with analytical grade zinc oxide (Sigma, Taufkirchen, Germany). The analyzed zinc concentration of the three diets was 57 (low dietary zinc, LZn), 164 (normal dietary zinc, NZn), and 2425 (high dietary zinc, HZn) mg/kg DM, respectively. Water and feed were provided ad libitum. After 4 weeks, ten pigs per group (5 male, 5 female) were euthanized, the pancreas dissected from the intestines and immediately stored at −80 °C until further analyses. For histological examinations, one piece of pancreas tissue from the right pancreatic lobe was fixed in formaldehyde (4%).

Tissue zinc concentration

Pancreas tissue was freeze-dried and incinerated at 600 °C for 8 h prior to hydrolysis in hydrochloric acid. Zinc concentration was determined by atomic absorption spectrometry in an AAS vario 6 spectrometer (Analytik Jena, Jena, Germany). Accuracy and precision of the analysis were checked routinely using replicate samples and certified reference zinc solution (Titrisol, Merck, Schwalbach, Germany, #9953). An external standard (PerkinElmer Pure XVI, #N9300281; PerkinElmer, Shelton USA) was used for calibration. The within measurement error was below 5%. Detection limit of the measurement was approximately 0.02 mg/L and limit of quantification was 0.06 mg/L.

Digestive enzyme activity and antioxidative capacity

Tissue was ground over liquid nitrogen and total protein extracted in duplicates. For normalization of enzyme activity data, total protein concentration was determined using the Bradford assay in microtitration plates as described previously [15]. Lipase activity was determined by measurement of colour intensity caused by liberation of methylresorufin from 1,2-O-dilauryl-glycero-3-glutaric acid-(6-methylresorufin) ester (Analyticon Biotechnologies, Lichtenfels, Germany) after activation of the enzyme with co-lipase. Pancreatic α-amylase activity was also determined colorimetrically after cleavage of 4,6-ethylidene-(G_7) p-nitrophenyl-(G_1)-α,D-maltoheptaoside (Analyticon Biotechnologies, Lichtenfels, Germany) by the enzyme and subsequent hydrolysis of the respective fragments by α-glucosidase to glucose and p-nitrophenol. For the determination of trypsin, chymotrypsin, and elastase activities, trypsin was activated from trypsinogen with enterokinase (Sigma, Taufkirchen, Germany), which in turn activated chymotrypsin and elastase. Enzyme activity was determined by measuring the colour intensity of p-nitroanilide cleaved from specific substrates Nα-Benzoyl-L-arginine 4-nitroanilide, SucAAA-pNA (BaChem, Weil am Rhein, Germany) and SucAAPF-pNA (CalBiochem, Pfullingen, Germany), for trypsin, elastase and chymotrypsin, respectively.

To test antioxidative capacity, the TEAC assay was performed using the protocol established by Fischer et al. [16]. The within measurement error for all enzyme activity measurements was below 5%.

Global protein expression in pancreatic tissue

Proteins were extracted by addition of 1 mL lysis buffer (9 M urea, CHAPS 2%, biolyte pH 3–10 supplemented with 60 mM DTT, 5 μM PMSF) and protease inhibitor mixture (CalBiochem, Pfullingen, Germany) and treatment in a FastPrep FP120 homogenizer (MP Biomedicals, Eschwege, Germany). Protein lysates were further purified by a modified TCA-acetone precipitation method (2-D-Clean Up kit, GE Healthcare, München, Germany) and mixed with DIGE labelling buffer (8 M urea, 4% (w/v) CHAPS, 30 mM Tris, pH 8.5). Concentration was determined by using a 2-D Quant Kit (GE Healthcare, München, Germany).

The 2D-DIGE analysis was performed as described previously [17]. Briefly, a pool consisting of equal amounts of all samples from animals was prepared as the internal standard and labelled with Cy2. Each gel contained the internal standard, one sample obtained from NZn and one sample of HZn fed animals, respectively. Samples were rehydrated, isoelectrically focused and equilibrated as described previously. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) for the second dimension was carried out in an ETTAN DALT *six* electrophoresis unit (GE Healthcare, München, Germany), first at 0.2 W per gel for 1 h and thereafter at 2 W per gel for further 18 h.

Protein spots were visualized by using the Typhoon 9400 laser imager (GE Healthcare, München, Germany) choosing the appropriate wavelength for each CyDye (Cy2 = 520 nm; Cy3 = 580 nm; Cy5 = 670 nm) at a resolution of 100 μm, were cropped and imported into DeCyder V.7.0 software (GE Healthcare, München, Germany). During spot detection by a co-detection algorithm in the software, the estimated number of spots were set at 2500, and the exclude filter was set a slope >1.7 and area <200. The DeCyder differential in the gel analysis (DIA) module was used to process the images from a single gel and enables the pair-wise comparison