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Dietary supplementation with zinc oxide stimulates ghrelin secretion from the stomach of young pigs☆

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Abstract

Dietary supplementation with zinc is known to enhance food intake and growth in young children. However, the underlying mechanisms remain largely unknown. Ghrelin, a peptide derived mainly from stomach, plays an important role in food-intake regulation. The present study was conducted with the piglet model to test the hypothesis that zinc may increase gastric ghrelin secretion. In Experiment 1 (Exp. 1) , thirty-six 28-day-old weaned pigs were assigned to two groups (18 pigs/group), receiving four-week supplementation of 0 or 2000 mg/kg Zn (as ZnO) to the basal diet containing 100 mg/kg Zn. In Experiment (Exp. 2), sixteen 28-day-old piglets were assigned to the same treatments (n=8/group) as in Exp. 1, except that they were pair-fed an equal amount of diet. At the end of the experiments, blood, stomach and duodenum samples were obtained for biochemical analysis, including assays of ghrelin protein and insulin-like growth factor-I (IGF-I) in plasma, as well as quantification of ghrelin and IGF-I mRNA levels in the duodenum and gastric mucosa. Further, gastric mucosal cells from unsupplemented piglets were cultured with 0–0.5 mM ZnO for 2–24 h for assays of ghrelin production and gene expression. Dietary Zn supplementation increased plasma concentrations of ghrelin, IGF-I and cholecystokinin; IGF-I gene expression in the duodenum as well as food intake and piglet growth (Exp. 1). The effects of ZnO on plasma levels of ghrelin, intestinal IGF-I expression and piglet growth were independent of food intake. Addition of ZnO to culture medium enhanced ghrelin production from gastric mucosal cells without affecting ghrelin mRNA levels. Collectively, our results indicate that ZnO stimulates ghrelin secretion from the stomach at the post-transcriptional level. This novel finding aids in elucidating the cellular and molecular mechanism for a role of zinc in promoting food intake and growth of young children.

Keywords: Zinc oxide; Ghrelin; Growth; Food intake; Piglets

1. Introduction

The whole body content of zinc in humans ranks second among trace elements only to that of iron [1]. There are at least 300 zinc-dependent enzymes (including carbonic anhydrase, alkaline phosphatase and cytosolic superoxide dismutase) and zinc-binding proteins (including metallothionein, insulin and numerous transcription factors) in mammals [2,3]. Thus, zinc plays an important role in regulating gene expression, nutrient metabolism, immune function and health [4–7]. Notably, over 40 years ago, dietary supplementation with zinc was reported to enhance food intake and growth of young children with zinc deficiency [8]. However, the underlying mechanisms remain largely unknown. Given the current widespread deficiency of zinc in humans, which affects more than 2 billion people worldwide [9], new knowledge about the cellular and molecular mechanisms for the action of supplemental zinc will guide development of new means for the effective prevention and treatment of zinc malnutrition.

Recent work with the young pig, an excellent animal model for studying human nutrition and metabolism [10,11], has shown that dietary ZnO supplementation at levels between 1500 and 3000 mg Zn/kg markedly improves

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gastrointestinal function and daily weight gain [12–14]. Because ZnO possesses antimicrobial properties [15], it was previously assumed that high levels of dietary ZnO enhanced the growth of weaned pigs by controlling pathogenic bacterial scours. However, ZnO promotes growth in early- and conventionally weaned pigs, regardless of diarrhea prevalence or intestinal microbial numbers [16–18]. Additionally, high levels of ZnO had no effect on the number of excreted *Escherichia coli* and enterococci per gram feces or intestinal morphology in young pigs under practical conditions [17]. Therefore, it is unlikely that ZnO enhances piglet growth primarily through an inhibition of intestinal microbial flora but possibly via a systemic effect.

The gastrointestinal (GI) tract is the largest hormone-producing organ in the body, and its hormones play versatile roles in signal communication between the digestive tract and the central neural system [19]. Ghrelin is a GI hormone secreted from the stomach and takes part in the regulation of appetite and growth. Other GI hormones, including cholecystokinin (CCK), may also be involved in this regulation [20]. Based on these findings, we hypothesize that ZnO may increase the plasma levels of ghrelin and other GI hormones, thereby interacting with the hypothalamus-pituitary growth axis to regulate piglet growth. This hypothesis was tested in the present study using both in vivo and in vitro studies.

2. Materials and methods

2.1. Experimental animals

All animals used in these experiments were maintained according to the principles of the China Agricultural University Animal Care and Use Committee. The experimental pigs were purchased from the Sino-Dutch Animal Husbandry Training and Demonstration Centre (Beijing, China) and were subjected to typical piglet management procedures prior to purchase such as iron injection, teeth clipping, tail docking and creep feeding.

2.1.1. Experiment 1

Thirty-six 28-day-old Large White×Pietran×Landrace crossbred barrows, with an initial body weight of 7.5±0.5 kg, were housed in 180×170-cm^2 raised weaner-decks equipped with a fully slatted mesh floor. Each pen was equipped with a self-feeder and a nipple waterer, allowing pigs free access to feed and water. The temperature in the nursery facility was controlled at 27–28°C. Pigs were allotted to one of two dietary treatments by balancing initial body weight and litter. One group was fed the basal diet containing 100 mg/kg Zn as $ZnSO_4$, and the other group was fed the basal diet supplemented with ZnO to provide 2000 mg/kg Zn by replacement of an equal amount of rice bran (Table 1). Each diet was fed to six pens of pigs with three piglets per pen. The experimental diets were based on corn and soybean meal with additional dietary protein supplied by whey powder, spray-dried porcine plasma and fishmeal.

Table 1
Ingredients and chemical composition of the basal diet

Ingredient	0–14 days	15–28 days
	(%)	
Corn	56.09	57.81
Soybean meal	21	24
Whey powder	10	10
Spray-dried plasma protein	3.5	–
Fish meal	4	2.5
Soybean oil	2	2
Rice bran	0.67	0.78
Dicalcium phosphate	1	1.4
Salt	0.3	0.3
Limestone	0.75	0.5
L-Lysine·HCl	0.25	0.35
DL-Met	0.11	0.14
Chloride choline	0.11	0.11
Premix[a]	0.22	0.22
Nutritional level		
DE (MJ/kg)	3400	3400
Crude protein[b]	20.39	18.28
Total lysine	1.40	1.25
Calcium[b]	0.81	0.79
Total phosphate[b]	0.60	0.59

[a] Premix contained the following (mg/kg diet): retinyl palmitate, 4.94; cholecalciferol, 0.063; all-rac-α-tocopheryl acetate, 20; menadione sodium bisulfite 3.0; thiamin, 1.5; riboflavin, 4.0; pyridoxine, 3.0; vitamin B-12, 0.012; niacin, 30; pantothenic acid, 15; folic acid, 0.75; biotin, 0.05; choline, 550; Mn, 40 ($MnSO_4·H_2O$); Fe, 90 ($FeSO_4·H_2O$); Zn, 100 ($ZnSO_4·H_2O$); Cu, 8.8 ($CuSO_4·5H_2O$); I, 0.35 (KI) and Se, 0.30 (Na_2SeO_3).

[b] Value determined in the present study.

The experimental diets were formulated to meet or exceed National Research Council (NCR) recommendations for all nutrients [21] (Table 1).

Pig weight and feed intake were determined at a 2-week interval to calculate average daily gain (ADG), average daily feed intake (ADFI) and feed conversion during the period of 4-week study. On day 28, pigs were deprived of feed for 12 h before blood sampling to avoid the effect of feed intake on GI hormone secretion. Blood samples were collected from 18 pigs per treatment via the anterior vena cava into 9-ml vacutainer tubes (Greiner Bio-One, Kremsmunster, Austria) and were transferred into centrifuge tubes containing 500 U of aprotinin and 1.25 mg of EDTA-2Na per milliliter of whole blood. Plasma was obtained by centrifugation (Heraeus Biofuge 22R Centrifuge, Osterode, Germany) at 3000×*g* for 30 min at 4°C, and stored at −20°C until analyses for insulin, insulin growth factor (IGF)-1, gastrin, cholecystokinin (CCK), somatostatin, and ghrelin. One pig selected randomly from each pen was euthanized, and the fundic mucosal layer of stomach, duodenum and liver were sampled and the samples stored at −80°C until analyses for mRNA levels of IGF-1, ghrelin and growth hormone receptor (n=6).

2.1.2. Experiment 2

Sixteen 28-day-old crossbred (Large White×Pietran×Landrace) barrows, with initial body weight of 8.1±0.2 kg, were

individually housed in stainless steel metabolism pens (0.8×0.4 m) with a total slatted floor. The pigs were randomly assigned to one of the two dietary treatments used in Experiment 1 (Exp. 1) (n=8). However, in this experiment, pigs in the control group were allowed free access to feed, whereas the ZnO-supplemented pigs were individually pair-fed the amount of feed consumed by the control group of pigs on the previous day as described by Swamy et al. [22]. The pigs were fed twice daily and feed intake was recorded daily. Piglet weight gain and feed conversion (an indicator of food efficiency) were determined at a 2-week interval during the 28-day period of experiment. Blood and tissue samples from the stomach, liver, and duodenum (n=8) were obtained as described in Exp. 1.

2.2. Determination of zinc content in the plasma and gastric mucosal tissue

Zinc concentrations in plasma and gastric mucosal tissue were determined by atomic absorption spectrophotometry, as described by Burch et al. [23].

2.3. Measurement of plasma hormones

In addition to ghrelin, other GI hormones [insulin, IGF-I, gastrin, CCK, and somatostatin (SS)] in plasma that participate in the regulation of animal growth were analyzed to determine the systemic effect of Zn supplementation on the endocrine status. Insulin was measured using a commercially available swine ELISA kit (BioSource International, Camarillo, CA, USA). Ghrelin was determined by a radioimmunoassay [24] using an acyl-ghrelin (n-octanoylated at Ser-3) — (Active) RIA kit (Linco Research, St. Charles, MO, USA). IGF-I was analyzed using a commercially available human IRMA kit (DSL-5600 ACTIVE Insulin-like growth factor-1 coated-tube kit, Diagnostic System Laboratories, Webster, TX, USA), which was validated to measure porcine plasma IGF-1, as described in the manufacturer's protocol and previous reports [25,26]. Gastrin and CCK were assayed using human RIA kits as described [27]. Gastrin was measured using a commercially available gastrin RIA kit (Beijing Northern Biotechnology Institute, Beijing, China). The sensitivity of the gastrin assay was 10 pg/ml and intra- and inter-assay CVs were <10% and 15%, respectively. Cholecyctokinin was assayed on ethanol-extracted plasma using the ^{125}I Cholecystokinin-33 kit (Department of Endocrinology, Royal Postgraduate Medical School, UK). Somatostatin was assayed using a commercially available RIA kit (human, rat, mouse and porcine) (Phoenix Pharmaceuticals, Belmont, CA, USA). Assays were performed in duplicate and analyzed according to the instructions of the manufacturers.

2.4. Cell culture

To further test the effects of ZnO on the secretion of ghrelin, porcine gastric mucosal cells were cultured. Gastric mucosal cells were obtained from additional eight 28-day-old weaned pigs with a body weight of 7.9±0.1 kg that had been fed the basal diet containing 100 mg Zn/kg from $ZnSO_4$ but no ZnO. The gastric mucosal cells were obtained as previous reported [28] with some modifications. Briefly, stomach was surgically removed from pigs and washed three times with D-Hank's Balanced Salt solution (Sigma, St. Louis, MO, USA). The fundic mucosal layer was dissected from the submucosa, minced into <1 mm^3 portion and washed with D-Hank's solution supplemented with 0.1% bovine serum albumin. The tissue was incubated with 0.1% collagenase I at 37°C for 1–3 h. This digestion mixture was centrifuged at 1000×g for 5 min and filtered through a 100-μm nylon cell strainer (BD Filcon, San Jose, CA, USA). The cells which passed through the strainer were pelleted by centrifugation at 1000×g, resuspended, pooled and washed in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) and filtered again through a 40-μm nylon cell strainer (BD Filcon) into 50-ml tubes (BD Filcon) and then centrifuged for another 10 min at 1000×g. The isolated cells were then incubated in 24-well plastic cell-culture plates (1×10^6 cells/well, Costar) in DMEM/F12 (GIBCO, Invitrogen) supplemented with 20% fetal bovine serum (GIBCO), 200 U/L penicillin and 200 μg/L streptomycin at a density of 1×10^6 cells/ml. Cells were attached to the culture plate within 24 h and became confluent in 3 days. Under phase contrast microscopy, over 90% of cells possessed epithelial characteristics.

The concentration of ZnO in culture medium and the ZnO dilutions used in DMEM/F12 or DMEM were prepared according to a previous study [29]. Six replicates of primary cultures of gastric mucosal cells were treated with various concentrations of ZnO (0, 0.05, 0.10 and 0.50 mmol/L), respectively. All cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 (Heraeus, Osterode, Germany). After 24 h of incubation, the medium was removed and the plates were washed with DMEM/F12 to remove any unattached cells and cell debris. At confluence, cells were treated with ZnO for 2, 4, 8, 16 or 24 h. At the end of the culture periods, media from three wells were combined as one replicate and stored at −20°C until analyses. Concentrations of ghrelin from the stomach mucosal cells were assayed using a commercially available kit, as described above. Experiments were repeated four times.

2.5. Extraction of RNA and cDNA synthesis

Total RNA was extracted using Trizol reagent (Invitrogen Life Technologies) from the liver, duodenum and stomach according to the manufacturer's instructions. The total RNA from cultured gastric mucosal cells were isolated and purified using the tissue/cell RNA mini kit (Watson Biotechnologies, Shanghai, China) according to the manufacturer's instructions. RNA integrity was verified by electrophoresis. RNA in samples was quantified using UV-clear Microplates

(Corning) in TECAN Genios (Tecan, Austria) at OD_{260} and OD_{280} (OD_{260}/OD_{280} ratio of all samples must be >1.80). Total RNA was reverse-transcribed as follows. Briefly, 2 μg of each RNA isolated from each pig was added to a 40-μl reaction system containing 1.0 μl of Oligo-dT_{18} (Promega, Madison, WI, USA), 1.0 μl of 25 mmol/L dNTP (Sigma), 10 U of RNasin inhibitor (Promega), 10 U of M-MLV transcriptase (Promega), 8.0 μl of M-MLV reaction buffer (Promega) and 25 μl of RNase-free water. Cycle parameters for the reverse transcription (RT) procedure were one cycle at 75°C for 5 min, one cycle at 37°C for 2 h and one cycle at 4°C for 5 min. The RT products (cDNA) were stored at −20°C for quantification by fluorescence real-time polymerase chain reaction (PCR).

2.6. Real-time fluorescence PCR for quantification of mRNA levels

Quantitative PCR analysis was carried out in a DNA Engine Opticon 2 fluorescence detection system (MJ Research, San Francisco, CA, USA) according to our optimized PCR protocols [30]. The PCR reaction system (10 μl) contained 5 μl of DyNAmo SYBR Green qPCR mix (Finnzymes, Espoo, Finland), 0.5 μl of primer (25 μmol/L forward and 25 μmol/L reverse), 1 μl of cDNA template (<10 μg/μl) and 3.5 μl of double-distilled water. The PCR cycles were set at 95°C for 5 min for first cycle and then 35 cycles at 94°C for 30 s, variable annealing temperature for 30 s and 72°C for 30 s. The melting curve from 65 to 95°C at 0.1°C/s was monitored continuously with fluorescence measurement; the last cycle was set at 72°C for 10 min. The annealing temperature for IGF-1, ghrelin, GHR and β-actin was 64.0, 58.0, 54.5 and 64.0°C, respectively. All samples were measured in triplicate.

To amplify IGF-1, ghrelin, GHR and β-actin cDNAs, the following sequences of PCR primer pairs were used: forward 5′-CTGTAACCATGAGGCTGAGA-3′, reverse 5′-CTCCA-TACTTCCTGTACTCC -3′ for IGF-1 (254 bp); forward 5′-TGTGTGCTGATGGCGGT CCT-3′, reverse 5′-CGGGGTCTTCTAGGAGGTAT-3′ for CCK (281 bp); forward 5′-GGAGTCCAAGAAGCCAGCAG-3′, reverse 5′-ACAGAGGTGGCTGGTCTCAG-3′ for ghrelin (269 bp); forward 5′-CTCGATAT TGATGACCCTGA-3′, reverse 5′-GATGAGTTGAGTCAGTTCCA-3′ for GHR (360 bp) and forward 5′-TGCGGGACATCAAGGAGAAG-3′, reverse 5′-AGTTGAAGG TGGTCTCGTGG-3′ for β-actin (216 bp).

The standard curve method was used to quantify gene expression [31]. Briefly, copy numbers were determined from three independent cDNA preparations of all samples. Copy numbers were calculated relative to dilution series of the respective reference plasmids, comprising 10^3–10^7 copies. The housekeeping gene, β-actin, was used as an internal standard for the PCR reaction. The Ct value (number of cycles halfway through the exponential phase) was determined. mRNA levels for study genes were normalized to the house-keeping gene of β-actin.

2.7. Statistical analysis

Data are expressed as mean±S.E.M. Data on performance were analyzed by the randomized complete block design, using analysis of variance (ANOVA). Data on plasma IGF-1, gut-brain hormones, mRNA levels as well as zinc concentrations in plasma and gastric mucosal tissue were analyzed by unpaired *t* test for the animal feeding studies. For the in vitro study, data on ghrelin protein and its mRNA levels were analyzed by one-way time-repeated ANOVA using the general linear model (GLM) procedure of SAS [32]. In Exp. 1, the pen was used as the experimental unit for growth performance data, plasma parameters, and gene expression. In Experiment 2 (Exp. 2), pigs were individually housed, and therefore, the pig was the experimental unit. $P<05$ was considered significant difference.

3. Results

3.1. Pig growth performance

In Exp. 1, body weight gain and feed intake were enhanced ($P<05$) during the first 2-wk and over the entire 4-wk period when 2000 mg/kg of ZnO was fed, in comparison with the control pigs which were fed 100 mg/kg as $ZnSO_4$ (Table 2). Feed conversion was improved by supplemental ZnO during the first 2-wk ($P<05$), while there was no difference over the whole 4-wk period.

Because the results of Exp. 1 indicated that dietary supplementation with 2000 mg/kg of ZnO increased feed

Table 2
Effect of dietary supplementation with ZnO on growth performance in weaned pigs (Exp. 1 and 2)

Item	Control	ZnO supplementation	S.E.M.	*P*
Exp. 1 (Ad lib)				
0–14 (d)				
ADG[a] (g)	203	278	10.12	.003
ADFI[b] (g)	372	455	14.25	.030
Feed/gain	1.85	1.66	0.09	.042
0–28 (d)				
ADG[a] (g)	388	435	12.81	.048
ADFI[b] (g)	679	723	9.49	.021
Feed:gain	1.79	1.67	0.06	.130
Exp. 2 (Pair-fed)				
0–14 (d)				
ADG[a] (g)	276	305	8.06	.015
ADFI[b] (g)	440	440	12.37	.351
Feed/gain	1.58	1.45	0.04	.026
0–28 (d)				
ADG[a] (g)	414	449	13.73	.037
ADFI[b] (g)	628	628	15.61	.139
Feed:gain	1.52	1.40	0.05	.038

Values are means and pooled S.E.M. (n=6 in Exp.1 and n=8 in Exp. 2).
[a] Average daily gain.
[b] Average daily feed intake.

intake in weaned piglets, we conducted a pair-feeding experiment (Exp. 2) to ensure similar intakes of feed between the control and Zn-supplemented group. In Exp. 2, supplementation of ZnO to the diet increased piglet body weight gain ($P<.05$) and feed conversion ($P<.05$) during the first 2 weeks and the entire 4-week period.

3.2. Zinc contents in the plasma and gastric mucosal tissue

We determined zinc concentrations in plasma and gastric mucosal tissue of piglets fed the basal (control) diet (containing 100 mg Zn/kg from $ZnSO_4$) and piglets that were pair-fed the same amount of the basal diet supplemented with 2000 mg ZnO/kg (Exp. 2; Table 2). Zinc concentrations in plasma and gastric mucosal tissue of ZnO-supplemented piglets were 168% ($P<.01$) and 26% greater ($P<.05$), respectively, than those in pigs fed the control diet (Table 3).

3.3. Plasma concentrations of IGF-I and GI hormones

In Exp. 1, dietary supplementation of ZnO increased plasma concentrations of ghrelin ($P<.05$), IGF-I ($P<.03$) and CCK ($P<.01$) but had no effects on insulin, gastrin or SS on day 28 (Table 4). However, when ZnO-supplemented pigs were pair-fed the same amounts of diet as the control pigs (Exp. 2), only ghrelin was increased ($P<.001$) in plasma, while the other gut-brain peptides tested did not differ, in comparison with the control group (Table 4).

3.4. Secretion of ghrelin by cultured gastric mucosal cells

The effects of ZnO treatment on the secretion of ghrelin from cultured gastric mucosal cells are illustrated in Fig. 1. Ghrelin secretion was increased by adding 0.05, 0.10 and 0.50 mmol/L of ZnO in culture medium for 4 and 8 h ($P<.05$ and $P<.01$, respectively). There was no difference in ghrelin levels among the various doses of ZnO treatments after 2, 16 or 24 h of incubation.

3.5. mRNA levels for ghrelin, GHR and IGF-I

The mRNA levels are expressed as ratios of study genes to the beta-actin gene. In Exp. 1, dietary supplementation with ZnO increased ($P<.05$) mRNA levels for IGF-1 and growth hormone receptor in the liver (Fig. 2A). The ZnO treatment also enhanced ($P<.05$) mRNA levels for IGF-1 and cholecyctokinin in the duodenum and the stomach, respectively (Fig. 2A). In contrast, in Exp. 2, the mRNA levels for IGF-1, and growth hormone receptor in the liver and cholecyctokinin mRNA in the stomach did not differ between control and ZnO-supplemented pigs when they were pair-fed the same amount of diet (Fig. 2B). Thus, the increase in mRNA levels for gastric cholecyctokinin, hepatic IGF-1 and GHR resulted from enhanced feed intake rather than dietary ZnO supplementation.

In both Experiments 1 and 2, ghrelin mRNA levels were not altered in the stomach of weaned pigs in response to dietary supplementation with ZnO on d 28 (Fig. 2A and 2B). In the in vitro study, it was further demonstrated that ghrelin

Table 3
Effect of dietary supplementation with ZnO on zinc concentrations in plasma and gastric mucosal tissue (Exp. 2)

Item	Control	ZnO supplementation	S.E.M.	P
Plasma (μg/ml)	1.20	3.22	0.13	<.001
Gastric mucosal tissue (μg/g)	87.4	110.2	6.46	.041

Values are means and pooled S.E.M. (n=8).

Table 4
Effect of dietary supplementation with ZnO on the plasma levels of gastrin, insulin, somatostatin and IGF-1 (Exp. 1 and 2)

Item	Control	ZnO Supplementation	S.E.M.	P
Exp. 1 (Ad lib)				
Gastrin (pg/ml)	18.5	16.4	1.3	.340
Insulin (μIU/ml)	4.19	3.73	0.29	.124
IGF-1 (ng/ml)	227	386	24	.012
SS (pg/ml)	28.6	29.7	1.6	.536
Ghrelin (pmol/L)	186	247	8	.021
CCK (pmol/L)	4.37	6.91	0.43	.004
Exp. 2 (Pair-fed)				
Gastrin (pg/ml)	37.7	44.5	3.5	.217
Insulin (μIU/ml)	9.98	12.6	1.2	.153
IGF-1 (ng/ml)	174	177	7.1	.784
SS (pg/ml)	33.4	22.8	4.3	.121
Ghrelin (pmol/L)	182	821	25	<.001
CCK (pmol/L)	2.63	2.57	0.26	.900

Values are means and pooled S.E.M. (n=6 in Exp.1 and n=8 in Exp. 2).

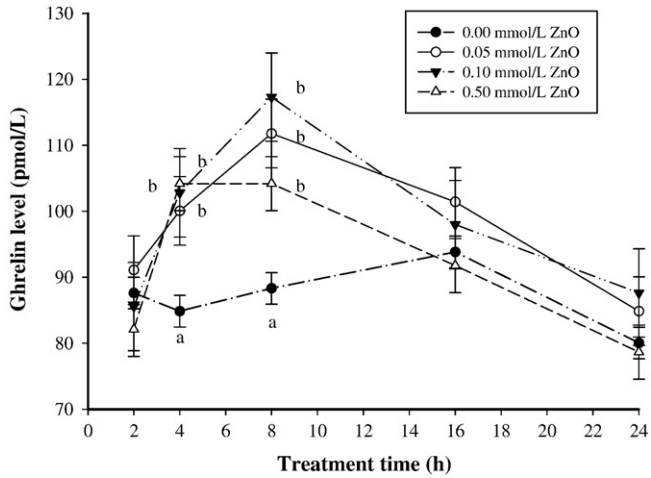

Fig. 1. Effects of various concentrations of ZnO on the release of ghrelin from primary gastric mucosal cells in vitro. At confluence, cultured gastric mucosal cells were treated with 0.0, 0.05, 0.10 and 0.50 mmol/L ZnO for 2, 4, 8, 16 and 24 h. Culture media from three wells were combined as one replicate for ghrelin measurement. Values are means±S.E.M. of four independent experiments carried out in triplicate. Means at the same treatment time sharing different superscript letters are different ($P<.05$).

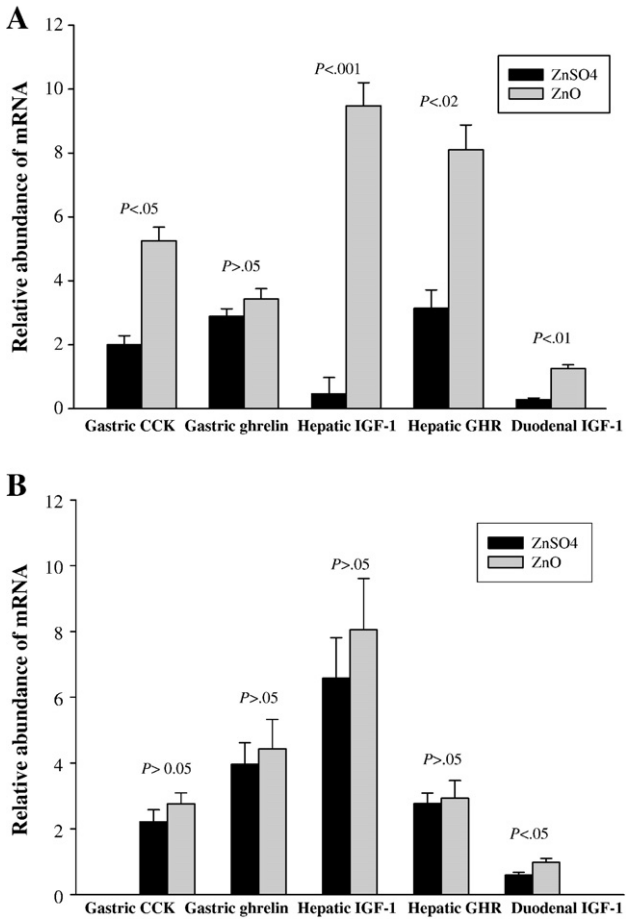

Fig. 2. Effects of dietary ZnO supplementation on relative abundance of mRNA levels for gastric ghrelin, hepatic IGF-1 and GHR and duodenal IGF-1. Data are means±SEM, n=6 in Exp. 1 and n=8 in Exp. 2. The duodenum, stomach and liver of treated pigs were sampled at the end of 28-day period of Exp. 1 (A) and of Exp. 2 (B). The mRNA levels for gastric ghrelin, hepatic IGF-1 and GHR, and duodenal IGF-1 were determined using real-time fluorescence reverse transcriptase-PCR. The relative abundance of mRNA of a study gene was expressed on the basis of β-actin mRNA levels.

mRNA levels in cultured gastric mucosal cells were not altered after exposure to ZnO for 2, 8 and 24 h (Fig. 3).

4. Discussion

Despite a large body of knowledge about the requirement of zinc for the functions of many enzymes and proteins [2,3], zinc deficiency remains a significant problem in human nutrition worldwide largely because of inadequate intake [9]. It is truly remarkable that zinc provision rapidly improved the growth of zinc-deficient young children [8]. Importantly, dietary supplementation with zinc also increased heights and body weight gains in adolescent individuals who had a marginal zinc status [33]. Similarly, adding zinc to the basal diet that was thought to provide a sufficient level of the mineral could enhance both food intake and whole-body growth in young pigs [12–14]. However, the underlying mechanisms are largely unknown. Given the availability of zinc supplementation for both preventative and therapeutic purposes in human and animal nutrition [9], it is important that the mode of the action of zinc be fully understood. Because of the similarity in digestion, absorption, metabolism and physiology between pigs and humans [10,11], the young pig provides a useful model to understand how dietary zinc supplementation stimulates food intake and growth in children. This study was conducted to test the hypothesis that supplementing ZnO to the basal diet meeting NRC-recommended requirement of zinc by piglets may stimulate ghrelin secretion by stomach, therefore enhancing feed consumption and growth performance. Our work did not aim at determining a role of zinc for piglets under zinc deficiency.

Some studies suggested that the growth-promoting effect of dietary ZnO in pigs resulted primarily from the increased voluntary feed intake [13,34]. However, extensive work demonstrated that ZnO supplementation was effective in increasing piglet growth when feed intake was not altered [12,35,36]. Consistent with this observation, we found that ZnO-supplemented pigs had a higher body weight gain than the control pigs even when the two groups of pigs were pair-fed the same amounts of diets (Exp. 2). This result indicates that the increased growth performance of weaned pigs in response to dietary ZnO supplementation can be achieved independent of an increase in feed intake, even though these two parameters are usually positively correlated. Therefore, it is likely that ZnO exerts its effect through a more complex mechanism than simply by enhancing feed intake. Additionally, the piglet provides a useful model to study the molecular mechanism whereby ZnO regulates animal growth.

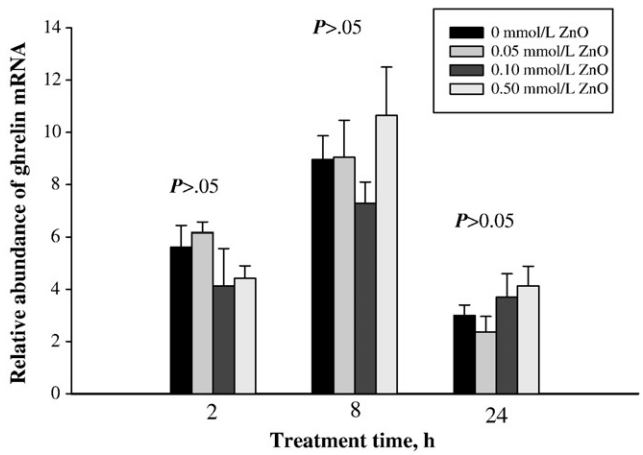

Fig. 3. Effects of various concentrations of ZnO on ghrelin mRNA levels in incubated primary gastric epithelial cells in vitro. At the end of 2-, 8- or 24-h culture in the presence of various concentrations of ZnO, total mRNA of cultured gastric mucosal cells from three wells of the same treatment was extracted, combined and purified as one sample and, then, was reverse-transcribed to cDNA. The levels of the ghrelin gene were determined using fluorescence real-time reverse transcriptase-PCR with a reference gene of β-actin fragment. Values are means±S.E.M. of four independent experiments carried out in triplicate.

In mammals, ghrelin is secreted primarily from the stomach and acts on the small intestine and brain to stimulate food intake via yet unknown mechanisms [37]. Ghrelin interacts with the growth-hormone releasing hormone and somatostatin axis, resulting in augmented production of IGF-I and CCK by the liver and GI tract, respectively [38–40]. IGF-I promotes muscle protein synthesis [26], whereas CCK stimulates feed intake [37]. A novel and important finding from the current study is that dietary ZnO supplementation increased plasma levels of ghrelin in weaned pigs (Table 4). Further, we found that ZnO stimulated the release of ghrelin from stomach mucosal cells (Fig. 1). To our knowledge, such effects of ZnO have not previously been reported for any other animal species. Consistent with the action of ghrelin on IGF-I and CCK secretion [38–40], the ZnO treatment increased mRNA levels for IGF-I in both the liver and small intestine (Fig. 2), mRNA levels for CCK in the gut (Fig. 3), plasma levels of both IGF-I and CCK (Table 4) as well as feed intake and body weight gain in weaned pigs (Table 2).

Intriguingly, when feed intake was unaltered in pair-fed pigs, dietary supplementation with ZnO did not affect plasma levels of IGF-I or CCK (Table 4) but remained effective in enhancing circulating levels of ghrelin and animal growth (Table 2). These results indicate that IGF-I or CCK did not mediate the beneficial effect of ZnO on growth performance of weaned pigs. Likewise, gastrin , insulin and SS are unlikely to be responsible for the action of ZnO because plasma levels of these hormones did not differ between control and ZnO-supplemented pigs (Table 4). Based on the previous report that intravenous administration of exogenous ghrelin increased piglet growth without altering feed intake [39], we suggest that this hormone may play an important role in mediating the effect of ZnO supplementation on enhancing protein synthesis in weaned piglets.

The intestinal microflora is highly complex [41], and quantification of its dynamic changes (both numbers and species) in ZnO-supplemented piglets is beyond the scope of the present study. Nonetheless, the published work shows that ZnO promotes growth in early- or conventionally weaned pigs, independent of intestinal microbial numbers [16–18]. Additionally, high levels of ZnO do not affect either the number of excreted *Escherichia coli* and enterococci per gram feces or intestinal morphology in young pigs [17]. Moreover, changes in the numbers and species of intestinal bacteria are not necessarily associated with changes in feed intake by piglets [42]. Therefore, the emerging consensus is that ZnO enhances feed intake and piglet growth primarily through mechanisms that do not merely involve alterations in the intestinal microflora [12,14]. In support of this view, results from the in vitro culture of gastric mucosal cells in the presence of 0.0, 0.05, 0.10 and 0.50 mM ZnO, but the absence of gastrointestinal microorganisms, clearly indicated that ZnO directly stimulated ghrelin secretion (Fig. 1). Taken together, these findings suggest that the stimulatory effect of ZnO on ghrelin secretion by stomach does not depend on microbial changes.

As an initial step to understand the molecular mechanisms responsible for the effect of ZnO on ghrelin secretion by stomach mucosal cells, we determine ghrelin production and its mRNA levels in the cells. Addition of ZnO to culture medium consistently increased ghrelin production by stomach mucosal cells in a time- and dose-dependent manner (Fig. 1). However, no change in ghrelin mRNA levels was observed in these cells in response to elevated levels of extracellular ZnO (Figs. 2 and 3). These results indicates that ZnO had a direct effect on stimulating ghrelin production from these cells via mechanisms involving steps beyond gene transcription. It is possible that ZnO promotes the translation of ghrelin mRNA for protein synthesis and/or inhibits ghrelin degradation in ghrelin cells, which are known to be closed-type endocrine cells [40]. Further studies are warranted to test this novel hypothesis.

Available evidence from studies with piglets shows that dietary ZnO is not effectively absorbed into the blood circulation, compared with $ZnSO_4$ and Zn-methionine [13,17]. Interestingly, among various resources of zinc, only supplemental ZnO in diet could promote growth in piglets, although high levels of all resources of zinc from ZnO, $ZnSO_4$ and Zn-Methionine could increase zinc concentrations in plasma and liver [13–18]. Supplementing a pharmacological level of ZnO to the diet is clearly relevant to the management of neonatal pigs under production conditions [13–18]. We are not aware of its use for children. However, a pharmacological level of $ZnSO_4$ has recently been used to prevent diarrhea and enhance growth in 1- to 6-month-old human infants [43]. Results from the current study with piglets may help understand the mechanisms responsible for the beneficial effect of ZnO on neonates and also provide a framework to guide future biochemical studies of the action of $ZnSO_4$.

In conclusion, dietary supplementation with ZnO increased ghrelin secretion from the stomach, plasma concentrations of ghrelin, and intestinal IGF-I expression in young pigs independent of feed intake. ZnO had a direct effect on stimulating ghrelin production from gastric mucosal cells without affecting ghrelin mRNA levels. These novel findings help to define cellular and molecular mechanisms responsible for the beneficial effect of dietary zinc supplementation on enhancing food intake and growth of children.

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