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Comparative Biochemistry and Physiology, Part A 145 (2006) 328–333
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Zinc attenuates forskolin-stimulated electrolyte secretion without involvement of the enteric nervous system in small intestinal epithelium from weaned piglets

Zike Feng [a,b], Dorthe Carlson [b,*], Hanne Damgaard Poulsen [b]

[a] *Ministry of Agriculture Feed Industry Centre, No.2 Yuanmingyuan West Road, Beijing, Post Code 100094, PR China*
[b] *Department of Animal Health, Welfare and Nutrition, Danish Institute of Agricultural Sciences, Research Centre Foulum, 8830 Tjele, Denmark*

Received 1 May 2006; received in revised form 26 June 2006; accepted 6 July 2006
Available online 14 July 2006

Abstract

In a previous study, we found that secretagogue-stimulated electrolyte secretion was attenuated by dietary and serosal zinc in piglet small intestinal epithelium in Ussing chambers. Several studies show that the enteric nervous system (ENS) is involved in regulation of electrolyte and/or fluid transport in intestinal epithelium from many species. The aim of the present study is to examine the mechanisms behind the attenuating effect of zinc on electrolyte secretion and to study whether the ENS is involved in this effect of zinc *in vitro*. Twenty-four piglets (six litters of four piglets) were allocated randomly to one of two dietary treatments consisting of a basic diet supplemented with 100 mg zinc/kg (Zn_{100}) or 2500 mg zinc/kg (Zn_{2500}), as ZnO. All the piglets were killed at 5–6 days after weaning and *in vitro* experiments with small intestinal epithelium in Ussing chambers were carried out. Furthermore, zinc, copper, alkaline phosphatase (AP) and metallothionein (MT) in mucosa, liver, and plasma were measured. These measurements showed that zinc status was increased in the Zn_{2500} compared to the Zn_{100} fed piglets. The *in vitro* studies did not confirm previous findings of attenuating effects of dietary zinc and zinc *in vitro* on the 5-HT induced secretion. But it showed that the addition of zinc at the serosal side attenuated the forskolin (FSK) (cAMP-dependent) induced ion secretion in epithelium from piglets fed with Zn_{100} diet. Blocking the ENS with lidocaine or hexamethonium apparently slightly reduced this effect of zinc *in vitro*, but did not remove the effect of zinc. Consequently, it is suggested that zinc attenuates the cAMP dependent ion secretion mainly due to an effect on epithelial cells rather than affecting the mucosal neuronal pathway.

Keywords: Ussing chamber; Electrolyte transport; 5-HT; Forskolin; Lidocaine; Hexamethonium; Zinc status; Diarrhoea; Metallothionein; Alkaline phosphatase

1. Introduction

For economic reasons, farm-raised piglets are weaned at a younger age than in the wild (Boudry et al., 2002). However, weaning is a very critical period in a pig's life due to separation from the sow and abrupt change from milk to cereal-based diets (Melin et al., 2004), resulting in transient alterations in the morphology and function of the gastrointestinal tract (Miller and Skadhauge, 1997; McCracken et al., 1999). This might, in the presence of pathogenic microorganisms, lead to outbreak of diarrhoea after weaning.

Over the past two decades, it has been documented that supplementation of 2500–3000 mg/kg of dietary zinc, as ZnO, for weaned piglets in a period of 2 weeks after weaning results in reduced occurrence of diarrhoea and improves feed intake and daily weight gain (Poulsen, 1989, 1995; Hahn and Baker, 1993; Smith et al., 1997). Recently, few studies have explored the mechanisms by which zinc reduces diarrhoea with *in vitro* or in vivo investigations. The *in vitro* studies demonstrated that the serotonin (5-HT) and theophylline induced chloride (Cl^-) secretion in the ileum was significantly reduced in weaned piglets fed with 2500 mg zinc/kg feed compared with 100 mg zinc/kg feed (Carlson et al., 2004). Furthermore, new *in vitro* studies showed that zinc added at the serosal side of intestinal epithelium reduced secretagogue stimulated secretion in a similar manner (Carlson et al., 2005). Hoque et al. (2005) concluded that zinc inhibits cAMP-stimulated Cl^- secretion by selectively inhibiting cAMP-activated K^+ channels on the basolateral membrane of ileal epithelial cells. Furthermore, Klein et al. (2002) found that

* Corresponding author. Tel.: +45 89 99 13 88; fax: +45 89 99 13 78.
E-mail address: dorthe.carlson@agrsci.dk (D. Carlson).

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doi:10.1016/j.cbpa.2006.07.006

zinc inhibited adenylate cyclase activity in the membrane of N18TG2 neuroblastoma cells, which raises the possibility that zinc may function as either a neuromodulator or neurotoxic agent via altering intracellular cAMP levels. The enteric nervous system (ENS), located in the intestinal wall is composed of two major nerve plexuses, the myenteric and the submucous neurons. The submucous motor neurons predominantly innervate the mucosa and regulate secretion, absorption and blood flow, while myenteric motor neurons are primarily involved in the control of motility (Brookes, 2001). Quantitatively, the ENS is responsible for at least 60% of the evoked secretory response (Lundgren, 2002). On this background, it was hypothesized that zinc attenuates secretagogue evoked Cl^- secretion and that the ENS is involved in this action of zinc in post weaning piglets. Consequently, it was aimed to study the effect of zinc (dietary zinc and zinc *in vitro*) in combination with or without nerve blockers (lidocaine and hexamethonium) on the short circuit current responses to different secretagogues (5-HT and forskolin (FSK)) in piglet small intestinal epithelium in Ussing chambers.

2. Materials and methods

The protocol used in the present experiment complied with the Danish Ministry of Justice concerning animal experimentation and care of experimental animal.

2.1. Animals and diets

The present study comprised 24 piglets (six litters of four piglets) with an initial average weight at weaning of 8.1 (±1.0) kg. At weaning the four littermates were allocated randomly to one of two dietary treatments consisting of a basic diet (Table 1) supplemented with 100 mg zinc/kg (Zn_{100}) or 2500 mg zinc/kg (Zn_{2500}). The zinc source used was ZnO. All piglets were Landrace/Yorkshire/Duroc cross-breeds and they were allowed *ad libitum* access to creep feed (the Zn_{100} diet) from 14 days of age and they were weaned at 28 days of age. After weaning, the piglets were individually housed in pens with *ad libitum* access to feed and water. The feed intake and weight gain of the piglets were recorded during the 5–6 days long experimental period. The consistency of faeces was recorded and classified as 0 (normal), 1 (slightly liquid) and 2 (very liquid, indicating diarrhoea). However, none of the piglets were treated against diarrhoea as diarrhoea only appeared on the day that they, due to the experimental protocol, were to be killed. At 5 or 6 days after weaning, the piglets were killed with dietary groups evenly distributed at 08:00 or 13:00 h. The piglets were stunned with a bolt gun followed by exsanguinations.

Table 1
Dietary ingredients (g/kg) of the basic diet and analysed dry matter and zinc content of the two experimental diets

Barley	313.0
Wheat	313.2
Soybean meal, toasted	216.6
Fish meal	79.8
Animal fat	50.0
Monocalcium phosphate	9.1
Calcium carbonate	9.2
Lysine, 40%	3.9
DL-methionine	0.4
Sodium chloride	2.9
Vitamin and mineral mixture [a]	2.0

	Zn_{100} [b]	Zn_{2500}
Dry matter (%) [c]	90.7	90.6
Zinc (mg/kg DM) [c]	142	2122

[a] Provided (mg/kg): iron 50, manganese 27.7, copper 20, iodine 0.2, selenium 0.3, vitamin E 60, vitamin K_3 2.2, vitamin B_1 2.2, vitamin B_2 4, vitamin B_6 3.3, D-pantothenic acid 11, niacin 22, biotin 0.06, vitamin A 4400 IU/kg, vitamin D 1000 IU/kg.

[b] Zn subscript designates supplied amount of zinc (mg/kg) from zinc oxide to the basic diet.

[c] Analysed content.

2.2. Sample collection and measurements

The piglets were bled from the anterior vena cava with heparinized vacutainer tubes at 08:00 h at the day of slaughter. Immediately after the piglets were killed, a midline abdominal incision was made and 40 cm of the small intestine (located 5 m proximal to the ileocecal junction) was removed and placed in an oxygenated and phosphate-buffered bathing media at room temperature. The bathing media (Ringer's solution) contained (in mmol/L): 25$NaHCO_3$, 120NaCl, 1.0$MgSO_4$, 6.3KCl, 2.0$CaCl_2$, 0.32 phosphate buffer (pH 7.4) and 16 glucose. For metallothionein (MT), zinc and copper measurements, 100 cm of the mid-ileum (located proximal to the first site) was cut open, washed thoroughly with ice-cold 0.9% NaCl and cut longitudinally. Subsequently, the intestinal mucosa was scraped of. Liver samples were also excised for mineral (zinc and copper) and MT analysis. Half of the mucosa and liver samples were stored at −80 °C for MT protein analysis and the other half was stored at −20 °C until zinc and copper measurements. Full blood was centrifuged at 12 620 ×*g* for 3 min (sigma 201 M) and haematrocrit was measured.

2.3. Measurement of electrophysiological parameters

The electrophysiological parameters of the intestinal epithelium were detected by a modification of the procedure from Carlson et al. (2004). Briefly, the intestinal epithelium were stripped of the muscles layers and mounted in Ussing chambers (WPI, Sarasota, FL, USA) in 8 replicates within 15 min after slaughter. The bathing media were replaced 10 min later and the tissues equilibrated for 20 min, before basal Isc and PD were recorded. Thereafter, 23 μM zinc (from a 1.15 mM $ZnSO_4$ stock solution) was added at the serosal side to four of the chambers. After 20 min (new steady state) 4 mM lidocaine (local anaesthetic) or 20 μM hexamethonium (nicotinic receptor blocker) was added to the serosal side of four chambers each to block the enteric nervous system (ENS). Subsequently, with 20 min intervals, 0.1 mM serotonin (5-HT) and 10 μM FSK (an adenylate cyclase activator), were added at the serosal side of all chambers.

2.4. Mineral, metallothionein and alkaline phosphatase analysis

Diets, mucosa, liver, and plasma were analysed for zinc and copper by atomic absorption spectrophotometry (Unicam SP9,