This is to certify that the
dissertation entitled

EFFECTS OF PHARMACOLOGICAL ZINC AND PHYTASE SUPPLEMENTATION ON METALLOTHIONEIN, HEPATIC DIFFERENTIAL GENE EXPRESSION, ZINC TRANSPORTER-1, MINERAL EXCRETION, AND APPARENT RETENTION IN NEWLY WEANED PIGS

presented by

Michelle Marie Martínez

has been accepted towards fulfillment
of the requirements for the

Doctor of Philosophy degree in Animal Science

Major Professor's Signature

September 24, 2004

Date

EFFECTS OF PHARMACOLOGICAL ZINC AND PHYTASE SUPPLEMENTATION ON METALLOTHIONEIN, HEPATIC DIFFERENTIAL GENE EXPRESSION, ZINC TRANSPORTER-1, MINERAL EXCRETION, AND APPARENT RETENTION IN NEWLY WEANED PIGS

By

Michelle Marie Martínez

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Animal Science

2004

ABSTRACT

EFFECTS OF PHARMACOLOGICAL ZINC AND PHYTASE SUPPLEMENTATION ON METALLOTHIONEIN, HEPATIC DIFFERENTIAL GENE EXPRESSION, ZINC TRANSPORTER-1, MINERAL EXCRETION, AND APPARENT RETENTION IN NEWLY WEANED PIGS

By

Michelle Marie Martínez

The swine industry feeds pharmacological zinc (Zn) as Zn oxide to newly weaned pigs to improve growth, while exact mechanisms are unknown, metallothionein (MT) may be involved. Environmental concerns arise due to high Zn excretion. Exogenous phytase improves mineral availability from swine feedstuffs. We hypothesized that adding phytase would reduce the amount of Zn needed to elicit a beneficial response. Experiment 1: pigs (n=96, 5.5kg, 18d) were fed 150,1000 or 2000 mgZn/kg with or without phytase (+P or -P, Natuphos®, 500 FTU/kg) for 14d post-weaning. Liver and kidney Zn, and relative MT mRNA abundance and protein were greater ($P<0.05$) in pigs fed 1000+P, and 2000-P or 2000+P vs. the remaining treatments. Duodenal MT mRNA abundance and protein were greatest ($P<0.05$) in pigs fed 2000+P. Zinc Transporter-1 (ZnT-1) protein (~50 kDa) was identified. Pigs fed 150 and 1000 had similar ZnT-1 protein expression in the liver, while pigs fed 2000 had reduced expression ($P<0.02$). Fifty-two amplicons putatively differentially expressed were identified by differential display RT-PCR in the liver. Five

transcripts with increased expression in pigs fed pharmacological Zn with sequence similarities to glyoxalase 1 (*GLO1*), peroxiredoxin 4 (*PRDX4*), aminoacylase I (*ACY1*), orosomucoid 1 (*ORM1*), and carboxypeptidase U (*CPB2*) were confirmed. Relative *GLO1* ($P<0.01$), *PRDX4* ($P<0.01$) and *ACY1* ($P<0.01$) mRNA abundances were greater in pigs fed 1000 and 2000 than in pigs fed 150, while relative *ORM1* and *CPB2* abundances were not affected. Experiment 2: pigs (n=24, 7.2kg, 20d) were individually fed 100, 1000 or 4000 mgZn/kg with or without phytase (+P or -P, Natuphos®, 500 FTU/kg) for 14d post-weaning, followed by a 100 mgZn/kg diet for 7d. Feeding 4000 resulted in greater ($P<0.01$) plasma, hepatic, and renal Zn, and hepatic, renal, and jejunal MT protein than pigs fed 100 or 1000. Duodenal MT was greater ($P<0.01$) in pigs fed 1000 and 4000 vs. pigs fed 100. Phytase did not reduce Zn excretion. Daily fecal excretion indicated that feeding pharmacological Zn for 11d vs. 14d reduced fecal Zn by 50%. Phytase and pharmacological Zn beneficial effects are independent and not additive, our hypothesis is rejected.

I dedicate this to my dear partner in love and life Carlos M. Laó Dávila and our growing family. Your constant support, undoubted love, fantastic sense of humor, steady perspective, persistence at large, fabulous evaluation of life, questioning everything, uncommonness, passion outdoors, routine nonsense, outliving star kept me going all these years like essential fluids required for life. *Junto a tí he descubierto muchas cosas. Entre ellas que los límites no existen, aprendí que la ley es ficción, a pararme, y que en el mundo de los locos el cuerdo es el loco. Como dice Silvio: "Eres como esos días en que eres la vida y todo lo que tocas se hace primavera". Iluminas mis días y mi vida con tu presencia. Te amo.* May our future together be full of joy, health and wonderful moments, m

ACKNOWLEGDEMENTS

My most sincere thank you to Carlos and my family, I appreciate the support and love throughout the years invested in MSU.

Dr. Catherine W. Ernst for your constant support and for teaching me many things of science and life, many of which I will further practice in my future and evolving career. Dr. Gretchen M. Hill, my major professor and Drs. Dale Rozeboom, Kevin Roberson, Wanda Chenoweth, members of my guidance committee, for their helpful suggestions and ideas, accessibility and time.

I thank the Michigan Agricultural Experiment Station, the USDA, and EPA for financial support for my research projects, The Graduate School for the Competitive Doctoral Enrichment Fellowship, the Department of Animal Science and the College of Agriculture and Natural Resources for your support throughout my years at MSU.

The molecular genetics laboratory, I thank for all those special moments of troubleshooting PCR's, or oligonucleotide random primer labeling. Nancy Raney, Valencia Rillington, Megan Closs, Dr. Kwan Suk Kim, Leslie Bach, Katie Rodriguez, thanks for advise, constructive criticism and help in the lab. Jane Link, Dana Dvoracek-Driska, Jessica Green, and Allison Meyer in the non-ruminant nutrition laboratory for your technical assistance, help in the lab and at the farm. Also, to the MSU Swine Research and Teaching Facility staff for all the help while I was conducting my field research studies.

Thanks to the Center for Animal Functional Genomics for their real time RT-PCR technical assistance, especially Dr. Steve Suchyta, Brian Tooker, Chris Colvin and Dr. Paul Coussens. To the muscle biology laboratory, for your assistance with the protein assays especially Dr. Matt Doumit, Chuck Allison, and Emily Helman. Dr. Robert Tempelman, for all our great statistic discussions, especially those with the dot blot spatial variation. Dr. Jeanne Burton, Dr. Patty Weber and the Immunogenetics Lab for your support and troubleshooting expertise, and to Dr. Steve Bursian, the department of Animal Science Graduate Coordinator.

Juliana Perez Laspiur, for all the emotional, physical and personal assistance, you are without a doubt one of the brightest people I know. Thank you for every single explanation, chickens in good time, advice and friendship. Guillermo Ortiz Colón for your exceptional friendship and true values, your honesty and help are truly appreciated. Thanks for the political discussions, western blot lectures and RNA troubleshooting days, I wish you the absolute best in life. Raquel Bastos Tabet for genuinely being you. Thanks for being there and providing your support.

To my cat, my cute Chiqui-Chiqui, for the at home support and snootiness.

"¡A TOD@S MUCHAS GRACIAS!"

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER TWO

CHAPTER FOUR

CHAPTER FIVE

LIST OF FIGURES

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

CHAPTER FIVE

CHAPTER ONE

Literature Review

INTRODUCTION

The capacity of pigs to adapt to new diets and properly digest dry feed during the immediate post-weaning period influences their subsequent performance (1). At weaning, pigs are transferred to a nursery facility, commingled, vaccinated and exposed to a new social and physical environment. Collectively, these changes challenge the health status of the animals. Post-weaning stress symptoms include low feed intake, diarrhea, atrophy of small intestinal villi and ultimately reduced growth rate, which can translate into significant financial losses for the swine industry (2).

Nutritional stress arises when piglets feeding patterns are changed, from consuming sow's milk (water based and highly digestible) to a dry diet of different temperature, smell, taste, texture, composition and digestibility (3). Voluntary feed intake during the first days after weaning is low, thus insufficient to cover the maintenance energy requirement (4). Low feed intake correlates with alterations in the gastrointestinal tract, and the combination of these factors may cause lower nutrient digestive and absorptive capacity (5). Poor performance often observed in the newly weaned pig might result from an immunological response to soy protein antigens found in starter diets and may lead to a compromised intestinal morphology and subsequent diarrhea (6,7).

Digestive capacity and diet composition

The digestive system of the piglet at weaning is adapted to secrete enzymes for milk digestion. Lactase activity is high, while the activity of lipases and proteases is sufficient to digest milk fat and protein (8). As early as one wk post-weaning (~ 5 wk of age), pigs display lower exocrine pancreatic enzyme activity compared to pre-weaned or 6 wk old pigs (9). Similar observations were obtained by Jensen et al. (1997), who speculated that decreases in enzyme activity might be due to depleted enzymatic stores caused by higher rates of secretion than synthesis, suggesting that pigs need to adapt by increasing enzyme secretion immediately post-weaning when they are exposed to the new dietary regimen (10). Thus the selection of dietary ingredients for post-weaning diets is crucial for proper digestion and growth.

Nursery diets can be formulated based on a feed budget and/or in several phases that differ in ingredient composition. Generally, the feedstuffs found in the initial post-weaning diet are highly digestible quality ingredients, such as lactose, plasma protein and spray-dried whey. The length of time this first diet is fed depends on the age at weaning. Many byproducts such as spray-dried red blood cells, egg protein and fishmeal are also used in early diets. Also, refined soy products, such as soy protein concentrate, with reduced allergenicity are utilized.

Corn is high in digestible starch, low in fiber and protein, and deficient in lysine and tryptophan. These deficiencies are corrected by adding other feedstuffs to the diet, such as soybean meal. Soybean meal is a good protein

source, due to its amino acid composition and balance. Since, it is high in lysine, tryptophan and threonine, it supplies the amino acids that are limiting in corn. However, young pigs may display hypersensitivity to some indigestible oligosaccharides found in soybean meal. In addition, soybean meal contains immunologically active proteins that can affect gut morphology, by increasing crypt depth and reducing villous height of the piglets (6).

Minerals are required nutrients supplied in weanling pig diets, and can be fed in organic or inorganic forms to meet the pigs' requirements. Copper (Cu) as copper sulfate ($CuSO_4$) and zinc (Zn) as zinc oxide (ZnO) have been used in pharmacological concentrations as growth promoting agents. Interestingly, an additive growth response is not obtained when these minerals are fed together (11,12). However, when an antibacterial agent (i.e. Carbadox) is fed together with pharmacological Cu (13) or Zn (14), the growth response obtained is additive.

The biological responses seen in pigs fed these minerals are influenced by chemical form and feeding level (15). However, their mechanisms of action are not clear. Antibacterial properties are attributed to Cu as $CuSO_4$ when it is fed at concentrations of 200 to 250 mg Cu/kg of diet, and its use is effective in the presence or absence of antibiotics (16). However, higher levels of Cu (> 500 mg/kg) should not be fed to pigs because they are toxic (17,18).

Numerous studies demonstrate that feeding 2000 to 3000 mg Zn/kg diet as ZnO to traditionally (18 – 24 d) or early-weaned (10 – 14 d) pigs, results in enhanced growth performance (11,14,19). Studies feeding pharmacological Zn

as acetate, carbonate, sulfate (20), complexed to individual amino acids such as methionine or lysine (21), amino acid peptides or polysaccharide complex (22) are not as effective in enhancing growth as ZnO. However, comparing the effects of feeding Zn at the required concentration (100 mg Zn/kg) vs. feeding pharmacological concentrations of up to 4000 mg Zn/kg, as ZnO to newly weaned pigs on tissue accretion and daily fecal Zn excretion has not been investigated.

Environmental concerns

Currently, ZnO is included in most nursery diets at a dietary concentration of 2000 to 3000 mg Zn/kg diet as a growth promotant and it may aid in preventing post-weaning diarrhea (14). However, supplementing pharmacological Zn to pigs creates environmental concerns due to increased Zn excretion. One study estimated that pigs fed 2000 or 3000 mg Zn/kg for 21 d excrete 14.1 or 21.5 times more fecal Zn, respectively, than pigs fed 150 mg/kg of Zn (23), which is close to the NRC (24) Zn requirement (100 mg Zn/kg). Furthermore, in a nursery balance study during which pigs were fed pharmacological Zn for 10 d prior to fecal collection, pigs were in negative balance, excreting more Zn than they consumed (22). This is likely because the balance study was conducted following 10 days of feeding pharmacological Zn.

High Zn accumulation in the soil has been implicated in reduced plant growth (25). Feces and urine composition are related to the diet fed to the animal; phosphorus (P), nitrogen (N), Cu and Zn are of greatest environmental concern. Phosphorus concentration in the waste material is often used to

regulate the amount of swine and poultry manure that may be applied as fertilizer (26).

Phytic acid

Phytic acid is an organic form of P, chemically known as myo-inositol hexakis dihydrogen phosphate (IP6). Common animal feedstuffs contain 60-80% of the total P in the phytic acid molecule (27). This molecule is the primary source of inositol and a storage form of P in plant seeds (28). It contains a six-carbon ring with six phosphate groups (OPO_3H_2), making phytic acid a strong chelating agent that binds cations with different affinities. Phosphate group number 5 has been shown to preferentially bind Zn *in vitro* (29).

Phytase

In the past two decades microbial and plant acid phosphatases have been isolated, purified, characterized and commercialized as feed additives to increase nutrient availability. The International Union of Pure and Applied Chemistry and the International Union of Biochemistry recognize two types of phytase (myo-inositol hexakisphosphate phosphohydrolase) phosphatases (30). The 3-phytase (E.C. 3.1.3.8) attacks phytic acid at the 3-position, and the 6-phytase (E.C. 3.1.3.26) removes the phosphate group at the 6-position of the phytate molecule. The removal of these groups represents P release, thus any cation attached to this group is released as well. In theory, once the first phosphate is released, the resulting IP5 molecule can be re-attacked by the enzyme, releasing another phosphate group, yielding an IP4 molecule. Hydrolysis of the additional phosphate groups may continue until an IP molecule is obtained (31).

Commercially available phytases such as Natuphos® (BASF, Mt. Olive, NJ) and Ronozyme® P (Roche Vitamins, Parsippany, NJ) are examples of the 3 and 6 phytases, respectively. Additional available phytases are Phyzyme™ XP (Diversa Corp., San Diego, CA), Zymetrics Quantum™ (Diversa Corp., San Diego, CA), Eco-Phos™ (Phytax, Portland, ME), Allzyme Phytase™ (Alltech, Nicholasville, KY), Biofeed® (Novo Nordisk, Princeton, NJ), and Finase™ (Alko Ltd. Biotechnology, Rajamäki, Finland).

Possible phytase sources for pigs

There are four possible sources of phytases for pigs: 1) endogenous intestinal phytase produced in digestive secretions, 2) endogenous phytase in feedstuffs, 3) phytase from resident bacteria, or 4) supplemented phytase produced by exogenous microorganisms (26).

Endogenous intestinal phytase. The activity of the intestinal phytase in pigs receiving a corn-soybean meal diet is negligible. As much as 60-74% of the phytic acid hydrolysis occurs in the stomach and duodenum of pigs fed supplemental fungal phytase compared to a 10% hydrolysis in pigs fed unsupplemented diets (32). *In vitro* studies using rat, human, chicken and calf intestinal mucosa to determine endogenous phytase activity, conclude that pH plays a key role in phytase activity. In the rat, optimal activity is achieved at a pH range from 7.0 to 7.5. In human homogenates, maximal activity is obtained at pH 7.4, with no detectable activity at pH 9. In chicken and calf intestinal mucosa, optimal pH is at 8.3 and 8.6, respectively (33).

Endogenous phytase in feedstuffs. Phytase is naturally found in several dietary feedstuffs such as wheat, rice and barley, all of which could be used for swine diets. However, it has been reported that the phytic acid P released from animal diets formulated with wheat is below the amounts released with phytase supplemented diets (28). Therefore, in order to use wheat as a phytase source, diets must be formulated with at least 40% wheat to hydrolyze all the phytic acid P, which is not economically feasible (26,28). In 1992, Cromwell estimated that 29 to 49% of the P in wheat by-products is bioavailable, while the P bioavailability from fish meal, blood meal, whey and alfalfa meal is estimated to be 100% (34). Phytase can also be obtained from soybeans (35). However, because phytase is an enzyme subject to degradation at high temperatures, its activity is not sustained during the high temperatures used in the production of soybean meal (36).

Supplemental and resident bacterial phytase. High temperature resistance is desirable for phytase use in animal feed, because of feed pelleting and processing (31). In 1996, Yi and Kornegay suggested that in 17 kg pigs the main site of dietary fungal phytase activity (51% of maximum activity) was in the stomach at pH 4, with reduced activity (31% of maximum activity) in the upper part (2 m section past the pylorus) of the small intestine at pH 6.8 (37). However, the exact location within the gastrointestinal tract and the pH at which the P is released from phytic acid in pigs fed corn-soybean meal diets are unknown. Considering temperature and pH as required characteristics, several microbial phytases have been developed from the bacteria *Bacillus sp.*, (38), and

Escherichia coli (*E. coli*) (39), the fungus *Aspergillus niger* (40), plants, or by genetic modifications (36).

Bacterial phytase yields are low, their optimum pH for maximum activity is neutral to alkaline, and since most bacteria produce intracellular phytase that is unable to hydrolyze phytic acid efficiently, their use as an additive is not practical (28,30). The fungal phytase produces extracellular enzyme and is widely used commercially (Natuphos®, Allzyme™, Ronozyme® P) in swine diets as a feed additive to improve phytate-P bioavailability and to reduce fecal P excretion of pigs (41,42). Fungal phytase has maximal activity at both pH 2.5 and pH 5.5, which resembles the pH in the stomach of pigs (3.4 - 4.8), suggesting that the pH of stomach digesta is favorable for high phytase activity, whereas the pH in the small intestine (6.4 –7.2) is favorable for pancreatic enzymes which hydrolyze proteins from the chyme, potentially destroying any leftover phytase (37).

Using genetic engineering techniques, the *E. coli* phytase gene (appA) was isolated and incorporated into a yeast expression system (*Pichia pastoris*), producing a thermostable phytase that works at a pH range of 2.5 - 3.5, which also resembles the physiological pH of the proventriculus of chickens and stomach of pigs. Another experimental phytase known as a "consensus" phytase, is heat-stable, and derived from the sequences of 13 known fungal phytases. This enzyme's optimal activity occurs at 71°C, and its catalytic activity and maximal pH are comparable to those of *Aspergillus fumigates* at 37°C (43). Growth performance, bone strength and plasma inorganic P were improved by supplementation (1250 U/kg) to weanling pigs, however more research is needed

to compare its effects on bone mineralization and mineral tissue concentrations with other available phytases (44).

Phytase supplementation

Phytic acid is a strong chelating agent, attracting multivalent cations to form phytic acid-cation complexes. It is considered an important anti-nutritional factor for the availability of minerals (45). At neutral and alkaline pH, phytic acid forms insoluble salts with cations like calcium (Ca), Cu, iron (Fe) or Zn (46). O'Dell and Savage (47) were the first to suggest that phytic acid reduces the availability of dietary Zn in the chick. When rats are fed diets containing 3000 mg Zn/kg and phytic acid, less Zn is absorbed and fecal Zn is increased (48). A plausible mechanism to explain how phytic acid affects Zn absorption was proposed by Oberleas and co-workers (49). Zinc, phytic acid and Ca in a molar ratio of 1:1:2 respectively, form a precipitate in a pH range of 5 to 9, which contains 97% of the originally added Zn (49), suggesting that Zn combines with the phosphate groups of the phytic acid and Ca to form a Zn-Ca-phytic acid insoluble complex. Both the formation of mineral-phytic acid complexes and the negligible amount of endogenous phytase production in pigs increases the tendency to excrete phytic acid bound minerals in the feces, resulting in decreased mineral absorption and increased environmental concerns.

Commercial phytase supplementation to pigs fed corn-soybean meal diets is a good alternative for enhancing mineral absorption. Phytase addition to animal feeds is currently widely implemented in the Netherlands and the United States (50). Phytase hydrolyses the ester bond phosphate groups, and this

hydrolysis is needed for releasing inorganic P and the trace minerals that may be attached to the phytic acid molecule (51). Phytase supplementation studies in pigs demonstrate improved performance, increased plasma Zn concentration (52,53), greater Zn retention (54), and improved bone mineralization (55), while adding phytase to human diets also results in increased Fe absorption (56). Thus, phytase supplementation is a promising solution to improve Zn availability and possibly limit environmental pollution (57).

Zinc essentiality and biochemical properties

In 1869, Raulin first showed that Zn was required for the growth of *Aspergillus niger*; and it was later reported that Zn was essential for the growth of rats (58). In animals, signs of Zn deficiency include growth failure, hair loss, hyperkeratinization of the skin, and testicular atrophy (59). The discovery that Zn prevents and cures parakeratosis in pigs (60) and the demonstration of Zn deficiency and stunted growth in chicks (47), initiated an interest in studying the nutritional physiology of Zn in livestock. In humans, it was discovered that Zn deficiency caused growth retardation in male Middle Eastern adolescents consuming primarily unleveaned bread. Subsequent growth was obtained upon Zn supplementation (58).

Zinc chemical characteristics include no variable valance (low risk of free radical production), rapid exchange of ligands, and binding mostly to sulfur or nitrogen in biological systems (61). These properties enable Zn to play three major biological roles: catalytic, structural and regulatory (62). Zinc is required by more than 300 metalloenzymes, and can be directly involved in the catalysis of

an enzyme. The removal of the catalytic Zn results in an apoenzyme that retains its tertiary structure but has decreased activity (61). Zinc is usually bound by three protein ligands and a water molecule, that work in coordination during catalysis (63). The function of Zn is to polarize the substrate and activate the water molecule that later acts as a nucleophile. The Zn acts as a template that brings together the substrate and the nucleophile (64). This lowers the transition energy thereby accelerating the conversion of substrates to products. Structurally, Zn is part of the structure of insulin, DNA and RNA polymerases, proteins involved in DNA replication, transcription factors and Zn finger proteins (65). Finally, in its regulatory role, Zn serves as cofactor for enzymes, enhances protein stability and regulates transcription of several genes. Zinc is essential for proper immune function, including natural killer cell activity (66), and can potentially modulate secondary messenger metabolism, thus affecting cell signaling and transduction (67).

Benefits of zinc supplementation

Children that consume diets that are primarily plant based, with low or no animal protein, benefit from Zn supplements (68). Zinc supplementation reduces persistent diarrhea (69) and is used along with oral rehydration therapy to treat acute watery diarrhea in developing countries (70-72). Supplementing Zn is an economically feasible alternative for growth enhancement and diarrhea treatment.

European researchers hypothesize that the mode of action of ZnO in weanling pigs is by controlling diarrhea caused by *E. coli* (73). However, studies

demonstrate no change in number of *E. coli* or enterococci per gram of feces of pigs fed pharmacological Zn diets (74,75). Furthermore, studies in Caco-2 cells exposed to enterotoxigenic *E. coli* demonstrate that ZnO protects these cells by inhibiting bacterial adhesion and internalization into cells, thus modulating subsequent cytokine expression that might occur in response to pathogenic exposure, and not by a direct antibacterial effect (76).

Numerous studies of pigs fed pharmacological Zn have been reported in an effort to explain the mechanism of action of dietary Zn. In 1995, McCully et al. reported that feeding 3000 mg Zn/kg diet to nursery pigs for 21 d improved fecal consistency versus animals fed 150 mg Zn/kg (20). These results are in accordance with an NCR-42 study in which pigs fed 3000 mg Zn/kg and/or 250 mg Cu/kg had firmer stools compared with animals fed 100-150 mg Zn/kg diet or 10-15 mg Cu/kg diet (12). The reason for fecal consistency improvement is unknown. However, immunohistochemical analysis of intestinal tissue of nursery pigs fed 3000 mg Zn/kg diet for 28 d post-weaning demonstrated deeper duodenal crypts and greater total thickness from the villus tip to serosa layer, thus improving gut morphology and possibly increasing the absorption capacity of the duodenum (77).

Microscopic examinations of the duodenal and jejunal intestinal segments from nursery pigs challenged with transmissible gastroenteritis virus (TGE) fed 3000 mg Zn/kg diet for 14 d, suggested an improvement in gut morphology when compared to TGE-challenged pigs fed 250 mg Zn/kg diet (78). Perhaps the beneficial effects of ZnO supplementation are the result of a combination of

mechanisms including gut morphology improvement, and possible cytokine and gene expression changes that act in concert to improve growth and fecal consistency.

Metallothionein

Expression of proteins has been studied to understand Zn metabolism. Among these is metallothionein (MT), a low molecular weight (< 7 kDa) protein with a Cys-X-Cys amino acid sequence (X = any non-cyclic, non-Cys or heterocyclic amino acid), a primary protein structure of 61 to 68 amino acids (79) and a high metal content of 7 g atoms of metal / mol of protein (80). This protein was first isolated from the equine renal cortex in the late 50's by Margoshes and Vallee (81), and is found throughout the animal kingdom (82). Binding affinity for metals is as follows: mercury (Hg) ≈ lead (Pb) > Cu^{+1} ≈ silver (Ag) > cadmium (Cd) > Zn (83).

There are two major MT genes: MT-I and MT-II. They differ slightly in their sequence, and their respective proteins are separable by high performance liquid chromatography (84,85). Two additional genes exist, MT-III and MT-IV, with heterogeneity being a post-translational acetylation and/or variations due to metal composition (86). In mammals, MT-I and MT-II are found in all organs, while MT-III is mainly expressed in the brain and MT-IV is most abundant in squamous epithelial tissue (87). In the pig, a total of ten MT isoforms have been identified, seven of them for MT-I, two for MT-II, and one for MT-III (88).

Despite many decades of research, the precise physiological function of MT has not been identified. Proposed functions are metal transport and storage,

protection against reactive oxygen species (89), detoxification of heavy metals (90,91), and metal transfer to ligands or proteins with higher affinity for the particular metal (92). Although this protein is primarily found in the cytoplasm, nuclear expression has also been observed. Nuclear localization could be related to DNA protection against oxidative damage, or regulation of Zn supply to enzymes and transcription factors (93). Therefore, MT does not have a single biological function but its role is multifaceted (94).

Liver, kidneys and intestinal mucosa play a key role in Zn homeostasis (95), often with MT, a protein often considered as an intracellular marker of excess Zn (96). Our laboratory fed 3000 mg Zn/kg for 7, 14, or 28 d post-weaning and found elevated renal and hepatic Zn, and increased duodenal, renal and hepatic MT protein concentrations (19). Tran et al. (96) fed 10, 100, 400 or 1000 mg Zn/kg to rats for 7 d and reported that Zn concentrations increased from the stomach to the colon, with significantly higher concentrations in rats fed 400 and 1000 mg Zn/kg than in rats fed 10 or 100 mg Zn/kg.· In addition, MT concentrations were higher in the duodenum of rats fed 400 and 1000 mg Zn/kg than MT concentrations in the jejunum, ileum, caecum, and colon. Metallothionein was higher in all tissues of these rats than rats fed 10 or 100 mg Zn/kg. These studies suggest that an adaptive mechanism, which includes MT, exists to protect from excess Zn accumulation.

Regulation of gene expression by dietary zinc

Nutrition influences gene expression. In the last ten to fifteen years, nutrients have been recognized as direct and independent regulators of gene

expression, having the ability to influence transcription, mRNA processing, mRNA stability, translation and post-translational modifications (97). Thus, understanding the interaction between Zn and gene expression may yield important information (98).

Transcriptional regulation of MT. In 1981, Durnam and Palmiter identified the MT-I gene in the mouse (99). By combining DNA sequence with results of deletion mapping studies, short sequences were found to mediate metal response of the MT gene. These sequences are known as metal response elements (MREs) and are present in multiple copies in the 5' regulatory region upstream of the transcription initiation start site. Once Zn is inside the cell, it binds to metal transcription factor-1 (MTF-1), which acts as a Zn sensor (100,101). The MREs serve as binding sites for transcription factors such as MTF-1, increasing transcription rate of MT upon metal activation (102).

Mechanisms for MT gene regulation by Zn have been studied. Different concentrations (5 – 180 mg Zn/kg) of dietary Zn regulate MT in mouse kidney, liver, and intestine (103). The regulation by Zn is due to increased transcription, with maximal levels of MT mRNA abundance observed shortly before changes in protein synthesis are seen (104,105). Richards and Cousins (106) were able to block MT mRNA induced by high dietary Zn using the transcription inhibitor actinomycin D, confirming transcriptional regulation. Additional studies in rats fed a Zn depleted diet for 4 d, then gavaged with either no Zn or 125 mg Zn/kg BW after an overnight fast showed that MT mRNA abundance was threefold higher in

rats fed Zn, and this increase preceded the accumulation of Zn in the liver by a few hours, indicating a rapid transcriptional induction by Zn (104).

Metallothionein synthesis in mucosal cells is triggered by both fasting and high luminal Zn concentrations (95). Feeding a Zn-deficient diet to rats for 1 d decreased MT concentrations from normal to nearly undetectable concentrations in both liver and intestine. However, repletion with a diet containing 150 mg Zn for one additional day caused a 10-fold increase in MT synthesis (104). When rats were fed 1000 to 2000 mg Zn/kg diet for up to 8 wks, increases in hepatic MT concentration were reported, but these dropped to trace amounts after feeding the same rats a Zn deficient diet for 3 d (107). Similarly, traditionally and early weaned pigs fed 3000 mg Zn/kg diet for 14 d showed increased MT protein concentrations in the liver compared to pigs fed 150 mg Zn/kg (19). These studies suggest that Zn induces MT synthesis *de novo,* and that MT is involved in trafficking or processing newly acquired cellular Zn.

Hepatic MT can be induced by an inflammatory response. Glucocorticoids produced as a consequence of an inflammatory response induced MT-I and MT-II through sequences known as glucocorticoid response elements (86). Metallothionein synthesis during inflammation may also be regulated by acute phase reactant proteins, catecholamines, glucagon, interleukin-6, interleukin-1, tumor necrosis factor α, and γ-interferon (108,109). Additional nucleotide sequences in the MT promoter known as antioxidant response elements induce MT synthesis upon activation due to oxidative stress (110). None of these biochemical mechanisms have been studied in the pig.

Transcriptional regulation of additional genes. Gene expression studies in the intestine by Blanchard and Cousins (111) in male Sprague-Dawley rats fed Zn deficient diets show upregulation of pre-prouroguanylin (pre-PUG) and cholecystokinin (CCK). Pre-PUG is the precursor of uroguanylin, an intestinal hormone that controls electrolyte balance in the small intestine, and CCK has a neuroendocrine function of satiety control and endocrine roles of gall bladder contraction and gastric emptying (112). The regulation of pre-PUG mRNA and uroguanilyn by dietary Zn might have a role in diarrheal disease, while CCK's role in satiety control may explain the well-documented anorexia seen in a Zn deficient state (113). Interestingly, uroguanylin and CCK proteins show overexpression during Zn deficiency, strengthening the hypothesis that the involvement of these genes is part of the mechanism that causes diarrhea and anorexia in Zn deficient animals (114).

Most Zn studies to assess gene expression have focused on deficiency. To observe effects of Zn deficiency on intestinal mucosa gene expression, rats were individually fed a Zn deficient or adequate diet for 14 d. Results showed that expression of genes associated with redox state of the cell, and carbohydrate and lipid metabolism were affected (115). Also, DDRT-PCR studies show that the differential expression of thymic genes of rats fed Zn deficient diets precedes phenotypic effects on thymic function (116).

Zinc homeostasis

Zinc absorption responds to alterations in dietary Zn. Cousins (95) suggested that high Zn intakes resulted in a decrease in absorption efficiency,

however the mechanism involved in Zn transport in tissues within the body has not been elucidated. To achieve Zn homeostasis, regulation occurs through intestinal uptake, fecal excretion, renal resorption and excretion, and intracellular storage (117). On a cellular level, under variable Zn concentrations, an adjustment in Zn uptake and secretion, intracellular protein binding, re-distribution and sequestration within vesicles or organelles is required for homeostatic control (118).

When Zn is internalized, it is thought to be available in four intracellular pools. First, it can bind tightly to metalloproteins, serving a structural role (119). Second, MT binds Zn with low affinity and may become part of a labile Zn pool considered a reservoir for cytosolic Zn (86). Third, it may be compartmentalized in intracellular organelles to supply Zn to dependent proteins, or be stored. Compartmentalization may occur in organelles such as Golgi apparatus, synaptic vesicles and secretory granules (120). Fourth, Zn may be free, which is considered to be in the nanomolar range (121).

Zinc is a charged, hydrophilic ion that cannot move across membranes by passive diffusion, requiring the presence of transmembrane proteins to aid the passage of "free" Zn across cell membranes (100). Transporter-mediated movement, which is a time, concentration, pH, and temperature-dependent process, is likely responsible for "free" Zn movement across cellular membranes (118).

Zinc transporters. In eukaryotes, there are two known families of Zn transporters. The SLC39 transporters belong to the ZIP family and are involved

in Zn uptake from outside the cell into the cytoplasm and from intracellular compartments into the cytoplasm. The second family, SLC30 transporters, belong to the cation diffusion facilitator (CDF) family and promote Zn efflux by pumping Zn from the cytoplasm out of the cell and from the cytoplasm into intracellular organelles and vesicles (122,123).

The CDF family can be divided into three different sub-groups based on their sequence similarities. The CDF-I subgroup is found only in prokaryotes, whereas CDF-II and III are found in both eukaryotes and prokaryotes (122). In mammals, a total of 9 mammalian CDF members have been characterized including two recently identified members (124). These carriers are known as Zn transporters (ZnT). All contain 6 transmembrane domains and have both N- and long C- termini on the cytoplasmic side with the exception of ZnT-5, which has a large N- terminal extension and an estimated six to nine additional transmembrane domains (122,124). The ZnT's contain a cytoplasmic histidine-rich loop between transmembrane domains IV and V, where it is thought that Zn may be bound, and transported across the membranes. The greatest degree of conservation between these transporters is found within transmembrane domains I, II and V (122,125).

In the rat, the mRNA abundance of ZnT-1 and ZnT-4 is distributed ubiquitously, with varied mRNA abundance among tissues, while ZnT-2 mRNA abundance is greatest in the small intestine and kidney (126). Duodenum and jejunum ZnT-1 mRNA expression is greater in the villus cells than in crypt cells and the protein is localized in the basolateral surface in the upper portion of the

villus cells (100). The mRNA of ZnT-3 has only been observed in brain and testis (127), while ZnT-5 is ubiquitously expressed with highest expression in the pancreas and is associated with Zn enriched vesicles involved in insulin secretion (128). The ZnT-6 facilitates translocation of cytoplasmic Zn into the trans Golgi network and vesicular compartment (129). The ZnT-7 is expressed primarily in the Golgi apparatus membrane of the small intestine, liver and lungs, and functions in transporting Zn from the cytoplasm into the Golgi apparatus. Because of ZnT-7's high abundance in the small intestine, it may play a role in Zn absorption (125). According to Palmiter and Huang (124), ZnT-8 and ZnT-9 were identified as transporters via database analysis, and no experimental data are available.

A mutation in the ZnT-4 gene causes insufficient Zn transport into milk from the lactating mammary glands of lethal milk mice (130). As a consequence, the pups of any genotype that are raised by lethal milk mothers die of Zn deficiency before weaning (131). However, maternal Zn supplementation improves pup survival, indicating that there are additional transport mechanisms within the gland to facilitate Zn export into milk (132).

Palmiter and Findley (133) first discovered ZnT-1 in mutagenized baby hamster kidney cells (BHK) transfected with a Zn-responsive reporter gene. The BHK cell line was grown in high Zn culture media and was extensively mutagenized to screen for genes affected by Zn concentrations. One mutant variant of these cells showed elevated basal expression of the reporter gene, and resistance to Zn toxicity. A Zn export function was suggested for this gene.

Expression of ZnT-1 as a fluorescent fusion protein showed localization on the plasma membrane of the BHK cells, supporting the theory of the transporter having an exporter function. In addition, a study using transient forebrain ischemic cells in gerbils demonstrated ZnT-1 up-regulation during ischemia, a condition that causes Zn influx into neurons (134). Thus, ZnT-1 transcriptional regulation by Zn may contribute to cellular Zn balance by promoting Zn efflux.

Transcription of the ZnT-1 gene is under the control of the MRE-binding transcription factor MTF-1. The ZnT-1 promoter contains two MRE consensus sequences and MTF-1 binds to both sites, resulting in transcriptional activation (135). Expression of MT occurs in a similar fashion; therefore Zn homeostasis may be achieved through the induction of both these genes.

In the rat, two ZnT-1 transcripts (~2.4 and 7.0 kb) have been identified (133). Liver and intestinal membrane protein preparations indicate the presence of ~38 and ~42 kDa proteins, respectively (100). However, the estimated size of ZnT-1 based on its amino acid sequence is ~55 kDa. Protein size differences with various tissues suggest that ZnT-1 may be post-translationally modified (100,132,136). Potential modifications include phosphorylation or glycosylation, which may modulate the stability and/or transport activity of ZnT-1 (125). Another possibility for the multiple ZnT-1 sizes may be due to its suggested multimeric structure (133).

The ZnT-1 transporter is oriented toward the basolateral surface of enterocytes, consistent with the function of Zn efflux towards the portal circulation (100). Dietary Zn intakes of 180 mg Zn/kg show a significant increase in rat ZnT-

1 mRNA in the intestine but not in kidney or liver. Furthermore, 2 hr after an oral gavage dose of Zn (32.5 mg/kg BW) to rats, MT and ZnT-1 mRNA increase in liver and intestine; however, no increases in ZnT-1 protein are obtained. At 6 hr post-dose, both MT and ZnT-1 mRNA and ZnT-1 protein increase significantly (100). Therefore, following a high oral Zn dose, up-regulation of ZnT-1 and MT mRNAs occurs rapidly, although protein induction of ZnT-1 is slightly slower than MT, suggesting that ZnT-1 and MT translational mechanisms differ.

Palmiter (137) suggests that these proteins work together to prevent Zn toxicity in cells, however their inductive mechanisms are indeed different. High intracellular Zn induces apoMT, which sequesters Zn and makes cells more tolerable to increased Zn concentrations. In contrast, ZnT-1 facilitates Zn efflux, thus lowering total cellular Zn content (133). However, both mechanisms work together to handle Zn disposal when excess Zn enters the cell.

LITERATURE CITED

1. Marion, J., Rome, V., Savary, G., Thomas, F., Le Dividich, J. & Le Huerou Luron, I. (2003) Weaning and feed intake alter pancreatic enzyme activities and corresponding mRNA levels in 7-d-old piglets. J. Nutr. 133: 362-368.

2. Pollmann, D. S. (1993) Effects of nursery feeding programs on subsequent grower-finisher pig performance. In: Proceedings of the Fourteenth Western Nutrition Conference (J. Martin), pp. 243-254. Edmonton.

3. Zijlstra, R. T., Whang, K. Y., Easter, R. A. & Odle, J. (1996) Effect of feeding a milk replacer to early-weaned pigs on growth, body composition, and small intestinal morphology, compared with suckled littermates. J. Anim. Sci. 74: 2948-2959.

4. Bark, L. J., Crenshaw, T. D. & Leibbrandt, V. D. (1986) The effect of meal intervals and weaning on feed intake of early weaned pigs. J. Anim. Sci. 62: 1233-1239.

5. Pluske, J. R., Williams, I. H. & Aherne, F. X. (1996) Maintenance of villous height and crypt depth in piglets by providing continuous nutrition after weaning. Anim. Sci. 62: 131-144.

6. Li, D. F., Nelssen, J. L., Reddy, P. G., Blecha, F., Klemm, R. & Goodband, R. D. (1991) Interrelationship between hypersensitivity to soybean proteins and growth performance in early-weaned pigs. J. Anim. Sci. 69: 4062-4069.

7. Bailey, M., Miller, B. G., Telemo, E., Stokes, C. R. & Bourne, F. J. (1993) Specific immunological unresponsiveness following active primary responses to proteins in the weaning diet of piglets. Int. Arch. Allergy Immunol. 101: 266-271.

8. Maxwell, C. V. & Carter, S. D. (2001). Feeding the weaned pig. In: Swine Nutrition (A.J. Lewis, & L.L. Southern) 2nd Ed, pp. 691-706. CRC Press, Boca Raton, FL.

9. Lindemann, M. D., Cornelius, S. G., el Kandelgy, S. M., Moser, R. L. & Pettigrew, J. E. (1986) Effect of age, weaning and diet on digestive enzyme levels in the piglet. J. Anim. Sci. 62: 1298-1307.

10. Jensen, M. S., Jensen, S. K. & Jakobsen, K. (1997) Development of digestive enzymes in pigs with emphasis on lipolytic activity in the stomach and pancreas. J. Anim. Sci. 75: 437-445.

11. Smith, J. W., Tokach, M. D., Goodband, R. D., Nelssen, J. L. & Richert, B. T. (1997) Effects of the interrelationship between zinc oxide and copper sulfate on growth performance of early-weaned pigs. J. Anim. Sci. 75: 1861-1866.

12. Hill, G. M., Cromwell, G. L., Crenshaw, T. D., Dove, C. R., Ewan, R. C., Knabe, D. A., Lewis, A. J., Libal, G. W., Mahan, D. C., Shurson, G. C., Southern, L. L. & Venum, T. L. (2000) Growth promotion effects and plasma changes from feeding high dietary concentrations of zinc and copper to weanling pigs (regional study). J. Anim. Sci. 78: 1010-1016.

13. Roof, M. D. & Mahan, D. C. (1982) Effect of carbadox and various dietary copper levels for weanling swine. J. Anim. Sci. 55: 1109-1117.

14. Hill, G. M., Mahan, D. C., Carter, S. D., Cromwell, G. L., Ewan, R. C., Harrold, R. L., Lewis, A. J., Miller, P. S., Shurson, G. C. & Veum, T. L. (2001) Effects of pharmacological concentrations of zinc oxide with or without the inclusion of an antibacterial agent on nursery pig performance. J. Anim. Sci. 79: 934-941.

15. Cromwell, G. L., Stahly, T. S. & Monegue, H. J. (1989) Effects of source and level of copper on performance and liver copper stores in weanling pigs. J. Anim. Sci. 67: 2996-3002.

16. Stahly, T. S., Cromwell, G. L. & Monegue, H. J. (1980) Effects of the dietary inclusion of copper and (or) antibiotics on the performance of weanling pigs. J. Anim. Sci. 51: 1347-1351.

17. Suttle, N. F. & Mills, C. F. (1966) Studies of the toxicity of copper to pigs. 1. Effects of oral supplements of zinc and iron salts on the development of copper toxicosis. Br. J. Nutr. 20: 135-148.

18. Suttle, N. F. & Mills, C. F. (1966) Studies of the toxicity of copper to pigs. 2. Effect of protein source and other dietary components on the response to high and moderate intakes of copper. Br. J. Nutr. 20: 149-161.

19. Carlson, M. S., Hill, G. M. & Link, J. E. (1999) Early and traditionally weaned nursery pigs benefit form phase-feeding pharmacological concentrations of zinc oxide: effect on metallothionein and mineral concentrations. J. Anim. Sci. 77: 1199-1207.

20. McCully, G. A., Hill, G. M., Link, J. E., Weavers, R. L., Carlson, M. S. & Rozeboom, D. W. (1995) Evaluation of zinc sources for the newly weaned pig. J. Anim. Sci. 73: 72 (abs).

21. Schell, T. C. & Kornegay, E. T. (1996) Zinc concentration in tissues and performance of weanling pigs fed pharmacological levels of zinc from ZnO, Zn-methionine, Zn-lysine, or $ZnSO_4$. J. Anim. Sci. 74: 1584-1593.

22. Case, C. L. & Carlson, M. S. (2002) Effect of feeding organic and inorganic sources of additional zinc on growth performance and zinc balance in nursery pigs. J. Anim. Sci. 80: 1917-1924.

23. Meyer, T. A., Lindemann, M. D., Cromwell, G. L., Monegue, H. J. & Inocencio, N. (2002) Effects of pharmacological levels of zinc as zinc oxide on fecal zinc and mineral excretion in weanling pigs. Prof. Anim. Sci. 18: 162-168.

24. NRC (1998) Nutrient Requirements of Swine, 10^{th} Ed. Washington, DC.

25. Chaney, R. L. (1993). Zinc phytotoxicity. In: Zinc in Soils and Plants (A.D. Robson), pp. 135-150. Kluer Academic Publishers, Dordrecht, Netherlands.

26. Kornegay, E. T. (1996). Nutritional, environmental and economic considerations for using phytase in pig and poultry diets. In: Nutrient Management of Food Animals to Enhance and Protect the Environment (E.T. Kornegay), pp. 277-302. CRC Press, Inc., Boca Raton, FL.

27. Harland, B. F. & Morris, E. R. (1995) Phytate: A good or a bad food component. Nutr. Res. 15: 733-754.

28. Wodzinki, R. J. & Ullah, A. H. (1996) Phytase. Adv. Appl. Microbiol. 42: 263-302.

29. Champagne, E. T. & Fisher, M. S. (1990) Binding differences of Zn (II) and Cu (II) ions with phytate. J. Inorg. Biochem. 38: 217-223.

30. Dvoráková, J. (1998) Phytase: sources, preparation and exploitation. Folia Microbiol. 43: 323-338.

31. Vohra, A. & Satyanarayana, T. (2003) Phytases, microbial sources, production, purification, and potential biotechnological applications. Crit. Rev. Biotechnol. 23: 29-60.

32. Jongbloed, A. W., Mroz, Z. & Kemme, P. A. (1992) The effect of supplementary *Aspergillus niger* phytase in diets for pigs on concentration and apparent digestibility of dry matter, total phosphorus, and phytic acid in different sections of the alimentary tract. J. Anim. Sci. 70: 1159-1168.

33. Bitar, K. & Reinhold, J. G. (1971) Phytase and alkaline phosphatase activities in intestinal mucosae of rat, chicken, calf and man. Biochim. Biophys. Acta 268: 442-452.

34. Cromwell, G. L. (1992) The biological activity of phosphorus in feedstuffs for pigs. Pig News Info. 13: 75N-78N.

35. Li, J., Hegeman, C. E., Hanlon, R. W., Lacy, G. H., Denbow, M. D. & Grabau, E. A. (1997) Secretion of active recombinant phytase from soybean cell-suspension cultures. Plant Physiol. 114: 1103-1111.

36. Lei, X. & Stahl, C. H. (2001) Biotechnological development of effective phytases for mineral nutrition and environmental protection. Appl. Microbiol. Biotechnol. 57: 474-481.

37. Yi, Z. & Kornegay, E. T. (1996) Sites of phytase activity in the gastrointestinal tract of young pigs. Anim. Feed Sci. Technol. 61: 361-368.

38. Kim, Y., Kim, H. K., Bae, K. S., Yu, J. H. & Oh, T. K. (1998) Purification and properties of a thermostable phytase from Bacillus sp. DS11. Enzyme Microb. Technol. 22: 2-7.

39. Greiner, R., Kontietzny, U. & Jany, K. D. (1993) Purification and characterization of two phytases from *Escherichia coli*. Arch. Biochem. Biophys. 303: 107-113.

40. van Hartingsveldt, W., van Zeijl, C. M., Harteveld, G. M., Gouka, R. J., Suykerbuyk, M. E., Luiten, R. G., van Paridon, P. A., Selten, G. C., Veenstra, A. E. & van Gorcom, R. F. (1993) Cloning, characterization and overexpression of the phytase-encoding gene (phyA) of *Aspergillus niger*. Gene 127: 87-94.

41. Matsui, T., Nakagawa, Y., Tamura, A., Watanabe, C., Fujita, K., Nakajima, T. & Yano, H. (2000) Efficacy of yeast phytase in improving phosphorus bioavailability in corn-soybean meal-based diet for growing pigs. J. Anim. Sci. 78: 94-99.

42. Mullaney, E. J., Daly, C. B. & Ullah, A. H. (2000) Advances in phytase research. Adv. Appl. Microbiol. 47: 157-199.

43. Lehmann, M., Kostrewa, D., Wyss, M., Brugger, R., D'Arcy, A., Pasamontes, L. & van Loon, A. P. (2000) From DNA sequence to improved functionality: using protein sequence comparisons to rapidly design a thermostable consensus phytase. Protein Eng. 13: 49-57.

44. Gentile, J. M., Roneker, K. R., Crowe, S. E., Pond, W. G. & Lei, X. G. (2003) Effectiveness of an experimental consensus phytase in improving dietary phytate-phosphorus utilization by weanling pigs. J. Anim. Sci. 81: 2751-2757.

45. Ravindran, V., Bryden, W. L. & Kornegay, E. T. (1995) Phytates: occurrence, bioavailability and implications in poultry nutrition. Poult. Avian Biol. Rev. 6: 125-143.

46. Vohra, P., Gray, G. A. & Kratzer, F. H. (1965) Phytic acid-metal complexes. Proc. Soc. Exp. Biol. Med. 120: 447-449.

47. O'Dell, B. L. & Savage, J. E. (1957) Symptoms of zinc deficiency in the chick. Fed. Proc. 16: 394.

48. Davies, N. T. & Nightingale, R. (1975) The effects of phytate on intestinal absorption and secretion of zinc, and whole-body retention of zinc, copper, iron and manganese in rats. Br. J. Nutr. 34: 243-258.

49. Oberleas, D., Muhrer, M. E. & O'Dell, B. L. (1966) Dietary metal-complexing agents and zinc availability in rats. J. Nutr. 90: 56-62.

50. Holm, P. B., Kristiansen, K. N. & Pedersen, H. B. (2002) Transgenic Approaches in Commonly Consumed Cereals to Improve Iron and Zinc Content and Bioavailability. J. Nutr. 132: 514S-516.

51. Pallauf, J. & Rimbach, G. (1997) Nutritional significance of phytic acid and phytase. Arch. Anim. Nutr. 50: 301-319.

52. Lei, X., Ku, P. K., Miller, E. W., Ullrey, D. E. & Yokoyama, M. (1993) Supplemental microbial phytase improves bioavailability of dietary zinc to weanling pigs. J. Nutr. 123: 1117-1123.

53. Martínez, M. M., Hill, G. M., Link, J. E., Raney, N. E., Tempelman, R. J. & Ernst, C. W. (2004) Pharmacological zinc and phytase supplementation enhance metallothionein mRNA abundance and protein concentration in newly weaned pigs. J. Nutr. 134: 538-544.

54. Adeola, O., Lawrence, B., Sutton, A. & Cline, T. (1995) Phytase-induced changes in mineral utilization in zinc-supplemented diets for pigs. J. Anim. Sci. 73: 3384-3391.

55. Cromwell, G. L., Coffey, M. T., Monegue, H. J. & Randolph, J. H. (1995) Efficacy of low-activity, microbial phytase in improving the bioavailability of phosphorus in corn-soybean meal diets for pigs. J. Anim. Sci. 73: 449-456.

56. Sandberg, A. S., Hulthen, L. R. & Turk, M. (1996) Dietary *Aspergillus niger* phytase increases iron absorption in humans. J. Nutr. 126: 476-480.

57. Jondreville, C., Revy, P. S. & Dourmad, J. Y. (2003) Dietary means to better control the environmental impact of copper and zinc by pigs from weaning to slaughter. Livest. Prod. Sci. 84: 147-156.

58. Prassad, A. (1993). Historical aspects of zinc. Biochemistry of Zinc (E. Frieden), pp. 1-15. Plemum Press, New York.

59. Prassad, A. (2003) Zinc and Immunity: Molecular Mechanisms of Zinc Action on T Helper Cells. J. Trace. Elem. Exp. Med. 16: 139-163.

60. Tucker, H. F. & Salmon, W. D. (1955) Parakeratosis or zinc deficiency disease in the pig. Proc. Soc. Exp. Biol. Med. 88: 613-616.

61. Mocchegiani, E., Muzzioli, M. & Giacconi, R. (2000) Zinc, metallothioneins, immune responses, survival and ageing. Biogerontology 1: 133-143.

62. Vallee, B. L. & Falchuk, K. N. (1993) The biochemical basis of zinc physiology. Phys. Res. 73: 79-118.

63. Vallee, B. L. & Auld, D. S. (1990) Active-site zinc ligands and activated H_2O of zinc enzymes. Proc. Natl. Acad. Sci. U S A 87: 220-224.

64. Mathews, B. W. (1988) Structural basis of the action of thermolysis and related zinc peptidases. Acc. Chem. Res. 21: 333-340.

65. Coleman, J. E. (1992) Zinc proteins, enzymes, storage proteins, transcription factors and replication proteins. Ann. Rev. Biochem. 61: 897-946.

66. Wellinghausen, N., Kirchner, H. & Rink, L. (1997) The immunobiology of zinc. Immunol. Today 18: 519-521.

67. Beyersmann, D. & Haase, H. (2001) Functions of zinc in signaling, proliferation and differentiation of mammalian cells. Biometals 14: 331-341.

68. Ferguson, E. L., Gibson, R. S., Opare Obisaw, C., Ounpuu, S., Thompson, L. U. & Lehrfeld, J. (1993) The zinc nutriture of preschool children living in two African countries. J. Nutr. 123: 1487-1496.

69. Sazawal, S., Black, R. E., Bhan, M. K., Jalla, S., Sinha, A. & Bhandari, N. (1997) Efficacy of zinc supplementation in reducing the incidence and prevalence of acute diarrhea-a community-based, double-blind, controlled trial. Am. J. Clin. Nutr. 66: 413-418.

70. Roy, S. K., Tomkins, A. M., Akramuzzaman, S. M., Behrens, R. H., Haider, R., Mahalanabis, D. & Fuchs, G. (1997) Randomized controlled trial of zinc supplementation in malnourished Bangladeshi children with acute diarrhoea. Arch. Dis. Child. 77: 196-200.

71. Dutta, P., Mitra, U., Datta, A., Niyogi, S. K., Dutta, S., Manna, B., Basak, M., Mahapatra, T. S. & Bhattacharya, S. K. (2000) Impact of zinc supplementation in malnourished children with acute watery diarrhoea. J. Trop. Pediatr. 46: 259-263.

72. Al-Sonboli, N., Gurgel, R. Q., Shenkin, A., Hart, C. A. & Cuevas, L. E. (2003) Zinc supplementation in Brazilian children with acute diarrhoea. Ann. Trop. Paediatr. 23: 3-8.

73. Holm, A. (1988) *Escherichia coli* associated diarrhea in weaner pigs: zinc oxide added to the feed as a preventative measure. Dan. Veterinaertidsskr 72: 1118-1126.

74. Jensen Waern, M., Melin, L., Lindberg, R., Johannisson, A., Petersson, L. & Wallgren, P. (1998) Dietary zinc oxide in weaned pigs-effects on performance, tissue concentrations, morphology, neutrophil functions and faecal microflora. Res. Vet. Sci. 64: 225-231.

75. Katouli, M., Melin, L., Jensen-Waern, M., Wallgren, P. & Mollby, R. (1999) The effect of zinc oxide supplementation on the stability of the intestinal flora with special reference to composition of coliforms in weaned pigs. J. Appl. Microbiol. 87: 564-573.

76. Roselli, M., Finamore, A., Garaguso, I., Britti, M. S. & Mengheri, E. (2003) Zinc oxide protects cultured enterocytes from the damage induced by *Escherichia coli*. J. Nutr. 133: 4077-4082.

77. Carlson, M. S., Hoover, S. L., Hill, G. M., Link, J. E. & Turk, J. R. (1998) Effect of pharmacological zinc on intestinal metallothionein concentration and morphology in the nursery pig. J. Anim. Sci. 76: 57 (abs).

78. Stanger, B. R., Hill, G. M., Link, J. E., Turk, J. R., Carlson, M. S. & Rozeboom, D. W. (1998) Effects of high Zn on TGE-challenged, early weaned pigs. J. Anim. Sci. 76: 57 (abs).

79. Kägi, J. H. R. & Schaffer, A. (1988) Biochemistry of metallothionein. Biochemistry 27: 8509-8515.

80. Kojima, Y., Berger, C., Vallee, B. L. & Kagi, J. H. (1976) Amino-acid sequence of equine renal metallothionein-1B. Proc. Natl. Acad. Sci. U S A 73: 3413-3417.

81. Margoshes, M. & Vallee, B. L. (1957) A cadmium protein from equine kidney cortex. J. Am. Chem. Soc. 79: 4813-4814.

82. Hamer, D. H. (1986) Metallothionein. Annu. Rev. Biochem. 55: 913-951.

83. Nielson, K. B., Atkin, C. L. & Winge, D. R. (1985) Distinct metal-binding configurations in metallothionein. J. Biol. Chem. 260: 5342-5350.

84. Suzuki, K. T. & Maitani, T. (1981) Metal dependent properties of metallothionein. Biochem. J. 199: 289-295.

85. Miles, A. T., Hawksworth, G. M., Beattie, J. H. & Rodilla, V. (2000) Induction, regulation, degradation and biological significance of mammalian metallothioneins. Crit. Rev. Biochem. Mol. Biol. 35: 35-70.

86. Coyle, P., Philcox, J. C., Carey, L. C. & Rofe, A. M. (2002) Metallothionein: the multipurpose protein. Cell. Mol. Life Sci. 59: 627-647.

87. Romero Isart, N. & Vasak, M. (2002) Advances in the structure and chemistry of metallothioneins. J. Inorg. Biochem. 88: 388-396.

88. Huang, M. C., Pan, P. K., Zheng, Z. F., Chen, N. C., Peng, J. Y. & Huang, P. C. (1998) Multiple isoforms of metallothionein are expressed in the porcine liver. Gene 211: 49-55.

89. Sato, M. & Bremner, I. (1993) Oxygen free radicals and metallothionein. Free Radic. Biol. Med. 14: 325-337.

90. Kelly, E. J., Quaife, C. J., Froelick, G. J. & Palmiter, R. D. (1996) Metallothionein I and II protect against zinc deficiency and zinc toxicity in mice. J. Nutr. 126: 1782-1790.

91. Klaassen, C. D. & Liu, J. (1998) Metallothionein transgenic and knock-out mouse models in the study of cadmium toxicity. J. Toxicol. Sci. 23: 97-102.

92. Schmidt, C. & Beyersmann, D. (1999) Transient peaks in zinc and metallothionein levels during differentiation of 3T3L1 cells. Arch. Biochem. Biophys. 364: 91-98.

93. Cherian, M. G. & Apostolova, M. D. (2000) Nuclear localization of metallothionein during cell proliferation and differentiation. Cell. Mol. Biol. 46: 347-356.

94. Vallee, B. L. (1991). Introduction to Metallothionein. In: Methods in Enzymology. Metallobiochemistry. Part B. Metallothionein and Related Molecules (J.F. Riordan, & B.L. Vallee), pp. 3-8. Academic Press, Inc., Boston, MA.

95. Cousins, R. J. (1985) Absorption, transport and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin. Physiol. Rev. 65: 238-309.

96. Tran, C. D., Butler, R. N., Howarth, G. S., Philcox, J. C., Rofe, A. M. & Coyle, P. (1999) Regional distribution and localization of zinc and metallothionein in the intestine of rats fed diets differing in zinc content. Scand. J. Gastroenterol. 34: 689-695.

97. Clarke, S. D. (1999) Nutrient regulation of gene and protein expression. Curr. Opin. Clin. Nutr. Metab. Care 2: 287-289.

98. Hesketh, J. E., Vasconcelos, M. H. & Bermano, G. (1998) Regulatory signals in messenger RNA: determinants of nutrient-gene interaction and metabolic compartmentation. Br. J. Nutr. 80: 307-321.

99. Durnam, D. M. & Palmiter, R. D. (1981) Transcriptional regulation of the mouse metallothionein-I gene by heavy metals. J. Biol. Chem. 256: 5712-5716.

100. Mc Mahon, R. J. & Cousins, R. J. (1998) Regulation of the zinc transporter ZnT-1 by dietary zinc. Proc. Natl. Acad. Sci. USA 95: 4841-4846.

101. Andrews, G. K. (2000) Regulation of metallothionein gene expression by oxidative stress and metal ions. Biochem. Pharmacol. 59: 95-104.

102. Stuart, G. W., Searle, P. F., Cen, H. Y., Brinster, R. L. & Palmiter, R. D. (1984) A 12-base-pair DNA motif that is repeated several times in metallothionein gene promoters confers metal regulation to a heterologous gene. Proc. Natl. Acad. Sci. USA 81: 7318.

103. Dunn, M. A., Blalock, T. L. & Cousins, R. J. (1987) Metallothionein. Proc. Soc. Exp. Biol. Med. 185: 107-119.

104. Mc Cormick, C. C., Menard, P. M. & Cousins, R. J. (1981) Induction of hepatic metallothionein by feeding zinc to rats of depleted zinc status. Am. J. Physiol. 240: E414-E421.

105. Blalock, T. L., Dunn, M. A. & Cousins, R. J. (1988) Metallothionein gene expression in rats: tissue-specific regulation by dietary copper and zinc. J. Nutr. 118: 222-228.

106. Richards, M. P. & Cousins, R. J. (1975) Mammalian zinc homeostasis: requirement for RNA and metallothionein synthesis. Biochem. Biophys. Res. Commun. 64: 1215-1223.

107. Chen, R. W., Vasey, E. J. & Whanger, P. D. (1977) Accumulation and depletion of zinc in rat liver and kidney metallothionein. J. Nutr. 107: 805-813.

108. Schroeder, J. J. & Cousins, R. J. (1990) Interleukin 6 regulates metallothionein gene expression and zinc metabolism in hepatocyte monolayer cultures. Proc. Natl. Acad. Sci. USA 87: 3137-3141.

109. Coyle, P., Philcox, J. C. & Rofe, A. M. (1995) Metallothionein induction in cultured rat hepatocytes by arthritic rat serum, activated macrophages, interleukin-6, interleukin-11 and leukaemia inhibitory factor. Inflamm. Res. 44: 475-481.

110. Lee, D. K., Carrasco, J., Hidalgo, J. & Andrews, G. K. (1999) Identification of a signal transducer and activator of transcription (STAT) binding site in the mouse metallothionein-I promoter involved in interleukin-6-induced gene expression. Biochem. J. 337: 59-65.

111. Blanchard, R. K. & Cousins, R. J. (1996) Differential display of intestinal mRNAs regulated by dietary zinc. Proc. Natl. Acad. Sci. USA 93: 6863-6868.

112. Greenberg, R. N., Hill, M., Crytzer, J., Krause, W. J., Eber, S. L., Hamra, F. K. & Forte, L. R. (1997) Comparison of effects of uroguanylin, guanylin, and *Escherichia coli* heat-stable enterotoxin STa in mouse intestine and kidney: evidence that uroguanylin is an intestinal natriuretic hormone. J. Investig. Med. 45: 276-282.

113. Chesters, J. K. & Quarterman, J. (1970) Effects of zinc deficiency on food intake and feeding patterns of rats. Br. J. Nutr. 24: 1061-1069.

114. Cui, L., Blanchard, R. K., Coy, L. M. & Cousins, R. J. (2000) Prouroguanylin overproduction and localization in the intestine of zinc-deficient rats. J. Nutr. 130: 2726-2732.

115. Blanchard, R. K., Moore, J. B., Green, C. L. & Cousins, R. J. (2001) Modulation of intestinal gene expression by dietary zinc status: effectiveness of cDNA arrays for expression profiling of a single nutrient deficiency. Proc. Natl. Acad. Sci. U S A 98: 13507-13513.

116. Moore, J. B., Blanchard, R. K. & Cousins, R. J. (2003) Dietary zinc modulates gene expression in murine thymus: results from a comprehensive differential display screening. Proc. Natl. Acad. Sci. U S A 100: 3883-3888.

117. Krebs, N. F. (2000) Overview of zinc absorption and excretion in the human gastrointestinal tract. J. Nutr. 130: 1374S-1377S.

118. Reyes, J. G. (1996) Zinc transport in mammalian cells. Am. J. Physiol. 270: C401-C410.

119. Vallee, B. L. & Auld, D. S. (1990) Zinc coordination, function and structure of zinc enzymes and other proteins. Biochemistry 29: 5647-5659.

120. Zalewski, P. D., Millard, S. H., Forbes, I. J., Kapaniris, O., Slavotinek, A., Betts, W. H., Ward, A. D., Lincoln, S. F. & Mahadevan, I. (1994) Video image analysis of labile zinc in viable pancreatic islet cells using a specific fluorescent probe for zinc. J. Histochem. Cytochem. 42: 877-884.

121. Williams, R. J. P. (1989). An introduction to the biochemistry of zinc. In: Zinc in Human Biology (C.F. Mills), pp. 15-31. Springer-Verlag, New York.

122. Gaither, L. A. & Eide, D. J. (2001) Eukaryotic zinc transporters and their regulation. Biometals 14: 251-270.

123. Kambe, T., Yamaguchi-Iwai, Y., Sasaki, R. & Nagao, M. (2004) Overview of mammalian zinc transporters. Cell Mol. Life. Sci. 61: 49-68.

124. Palmiter, R. D. & Huang, L. (2004) Efflux and compartmentalization of zinc by members of the SLC30 family of solute carriers. Pflugers Arch. 447: 744-751.

125. Kirschke, C. P. & Huang, L. (2003) ZnT7, a novel mammalian zinc transporter, accumulates zinc in the Golgi apparatus. J. Biol. Chem. 278: 4096-4102.

126. Liuzzi, J. P., Blanchard, R. K. & Cousins, R. J. (2001) Differential regulation of zinc transporter 1, 2 and 4 mRNA expression by dietary Zn in rats. J. Nutr. 131: 46-52.

127. Palmiter, R. D., Cole, T. B., Quaife, C. J. & Findley, S. D. (1996) ZnT-3 a putative transporter of zinc into synaptic vesicles. Proc. Natl. Acad. Sci. USA 93: 14934-14939.

128. Kambe, T., Narita, H., Yamaguchi-Iwa, i. Y., Hirose, J., Amano, T., Sugiura, N., Sasaki, R., Mori, K., Iwanaga, T. & Nagao, M. (2002) Cloning and characterization of a novel mammalian zinc transporter, zinc transporter 5, abundantly expressed in pancreatic β cells. J. Biol. Chem. 277: 19049-19055.

129. Huang, L., Kirschke, C. P. & Gitschier, R. J. (2002) Functional characterization of a novel mammalian zinc transporter, ZnT-6. J. Biol. Chem. 277: 26389-26395.

130. Huang, L. & Gitschier, R. J. (1997) A novel gene involved in zinc transport is deficient in the lethal milk mouse. Nat. Genet 17: 292-297.

131. Piletz, J. E. & Ganschow, R. E. (1978) Zinc deficiency in murine milk underlies expression of the lethal milk (*lm*) mutation. Science 199: 181-183.

132. Kelleher, S. L. & Lönnerdal, B. (2003) Zn transporter levels and localization change throughout lactation in rat mammary gland and are regulated by zinc in mammary cells. J. Nutr. 133: 3378-3385.

133. Palmiter, R. D. & Findley, S. D. (1995) Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc. The EMBO J. 14: 639-649.

134. Tsuda, M., Imaizumi, K., Katayama, T., Kitagawa, K., Wanaka, A., Tohyama, M. & Takagi, T. (1997) Expression of zinc transporter gene, ZnT-1, is induced after transient forebrain ischemia in the gerbil. J. Neurosci. 17: 6678-6684.

135. Langmade, S. J., Ravindra, R., Daniels, P. J. & Andrews, G. K. (2000) The transcription factor MTF-1 mediates metal regulation of the mouse ZnT1 gene. J. Biol. Chem. 275: 34803-34809.

136. Kelleher, S. L. & Lönnerdal, B. (2002) Zinc transporters in the rat mammary gland respond to marginal zinc and vitamin A intakes during lactation. J. Nutr. 132: 3280-3285.

137. Palmiter, R. D. (2004) Protection against zinc toxicity by metallothionein and zinc transporter 1. Proc. Natl. Acad. Sci. U S A 101: 4918-4923.

CHAPTER TWO

Martínez, Michelle M., Hill, Gretchen M., Link, Jane E., Raney, Nancy E., Tempelman, Robert J., Ernst, Catherine W. (2004) Pharmacological Zinc and Phytase Supplementation Enhance Metallothionein mRNA Abundance and Protein Concentration in Newly Weaned Pigs. Journal of Nutrition, 134:538-544.

Pharmacological Zinc and Phytase Supplementation Enhance Metallothionein mRNA Abundance and Protein Concentration in Newly Weaned Pigs[1,2]

Michelle M. Martínez, Gretchen M. Hill,[3] Jane E. Link, Nancy E. Raney, Robert J. Tempelman and Catherine W. Ernst

Department of Animal Science, Michigan State University, East Lansing, MI

Running title: ZINC & PHYTASE IMPACT ON METALLOTHIONEIN IN PIGS

[1] Presented in part at the 11th meeting of Trace Elements in Man and Animals (TEMA), in Berkeley, California, June 2-6, 2002 [Martínez, M.M., Hill, G.M., Link, J.E., Ernst, C.W., and Raney, N.E. (2002) Impact of Pharmacological Zinc and Phytase on Liver Metallothionein Concentration and mRNA Abundance in the Young Pig. (Abstract 59)], and at the American Society of Animal Science Midwestern section meeting, March 17-19, 2003 [Martínez, M.M., Hill, G.M., Link, J.E., Raney, N.E., and Ernst, C.W. (2003) Pharmacological Zn and phytase enhance renal and intestinal mucosa cell metallothionein protein and relative mRNA abundance in the nursery pig. J. Anim. Sci. 81:56 (Abstract 226).]

[2] Supported in part by an Animal Health Formula Fund Grant from the Michigan Agricultural Experiment Station, East Lansing, Michigan.

[3] To whom correspondence should be addressed. E-mail: hillgre@msu.edu. Michigan State University, Department of Animal Science, 2209 Anthony Hall, East Lansing, MI 48824. Fax (517) 432-0190. Tel. (517) 355-9676.

[4] Abbreviations used: APC, American Protein Corporation; CI, confidence interval; MT, metallothionein; Zn, zinc; ZnO, zinc oxide; Z_{150}, 150 mg Zn/kg; $Zn_{150}P$, 150 mg Zn/kg with phytase; $Zn_{1,000}$, 1,000 mg Zn/kg; $Zn_{1,000}P$, 1,000 mg Zn/kg with phytase; $Zn_{2,000}$, 2,000 mg Zn/kg; $Zn_{2,000}P$, 2,000 mg Zn/kg with phytase.

ABSTRACT

The swine industry feeds pharmacological zinc (Zn) to newly weaned pigs to improve health. Since most swine diets are plant-based with high phytic acid contents, we hypothesized that adding phytase to diets could reduce the amount of Zn needed to obtain beneficial responses. Metallothionein's (MT) role in Zn homeostasis could be important in this positive response. Thus, the goal of this study was to investigate the effect of dietary Zn and phytase on relative MT mRNA abundance and protein concentration in newly weaned pigs. Diets containing adequate (150 mg Zn/kg) or pharmacological concentrations of Zn (1,000 or 2,000 mg Zn/kg), as zinc oxide, with or without phytase (0, 500 FTU/kg, Natuphos® BASF) were fed in a 3 x 2 factorial design. Plasma and tissue minerals were measured in pigs killed after 14 d of dietary intervention. Hepatic and renal relative MT mRNA abundance and protein were greater ($P < 0.05$) in pigs fed 1,000 mg Zn/kg with phytase, or 2,000 mg Zn/kg with or without phytase versus the remaining treatments. Intestinal mucosa MT mRNA abundance and protein were greatest ($P < 0.05$) in pigs fed 2,000 mg Zn/kg with phytase. Pigs fed 1,000 mg Zn/kg plus phytase or 2,000 mg Zn/kg with or without phytase had higher plasma, hepatic and renal Zn than those fed the adequate Zn diets or 1,000 mg Zn/kg. We conclude that feeding 1,000 mg Zn/kg with phytase enhances MT mRNA abundance and protein and Zn absorption just as 2,000 mg Zn/kg with and without phytase.

KEY WORDS: • *metallothionein* • *pharmacological zinc* • *phytase* • *pig*

INTRODUCTION

In recent years, Zn supplementation has been used in developing countries to treat diarrheal disease in young children (1,2). In addition, the U.S. swine industry adds 2,000 – 3,000 mg Zn/kg as zinc oxide (ZnO) to the diets of newly weaned pigs for the first 14 d post-weaning to promote growth (3,4) and fecal consistency (5,6), both of which can be impaired by weaning stress. Feeding pharmacological Zn (3,000 mg/kg) for 14 d to newly weaned pigs improves gut morphology by increasing villous height and reducing crypt depth in the duodenum and jejunum, thus potentially increasing the absorptive capacity of the small intestine and consequently improving growth (7).

Since the 1960's there has been increasing evidence that phytic acid, an organic molecule found in cereal grains, possesses anti-nutritional properties (8,9). Phytic acid forms insoluble salts with bivalent cations such as calcium (Ca) and Zn, thus affecting their bioavailability at neutral and alkaline pH, leading to increased mineral excretion and reduced mineral absorption (10). Since pigs consume grain diets and lack the ability to produce sufficient phytase endogenously, supplementation of this enzyme in feed has been shown to improve mineral bioavailability and decrease nutrient excretion (11). However, the effect of phytase when pharmacological Zn is fed has not been evaluated.

Metallothionein is a low molecular weight (~7 kDa), cysteine-rich protein present in many living organisms (12). This protein has been considered an intracellular marker of excess Zn inside cells, based on the increased induction of MT when dietary Zn intakes are well above normal (13). Despite many decades

of research, MT's precise physiological function has not been elucidated. However, some suggested functions attributed to this protein are detoxification of nonessential heavy metals (cadmium and mercury), homeostasis of Zn and copper (Cu), metal-transfer, free-radical scavenger, and metal-storage (14,15).

Transcriptional regulation of MT by dietary Zn has been demonstrated in rats and cultured cells treated with Actinomycin D (16). Moreover, evidence suggests that transcriptional regulation of the MT gene occurs by activation of metal response elements (MREs) located in the promoter region of the MT gene, in response to the binding of intracellular Zn to metal transcription factor-1 (MTF-1) (17). The MTF-1 acts as an intracellular Zn sensor able to coordinate several genes involved in Zn homeostasis (18).

The purpose of this experiment was to determine the effects of supplementing pharmacological Zn with or without phytase on liver, kidney and intestinal mucosa MT mRNA abundance and protein concentration. In previous research when swine diets contained pharmacological Zn, an interaction between Zn, Cu and iron (Fe) was observed (5,19). Thus, the dietary effect of pharmacological Zn and phytase on hepatic, renal and plasma mineral (Cu, Fe, Zn and P) concentration of the pigs was also investigated.

MATERIALS AND METHODS

Animals and diets. Ninety-six crossbred [(Landrace x Yorkshire) x Duroc] gilts and barrows (5.5 kg, 18 d of age) were housed in pens (2.23 m^2), in a biosecure, environmentally controlled room (25-30°C) at the Swine Teaching and Research Center at Michigan State University (East Lansing, MI). At weaning, pigs (4 / pen) were randomly allotted to one of six different treatments based on weight and litter for a 14-d study. Diets met or exceeded the recommendations of the National Research Council (20). The Zn source used in this experiment was ZnO which contained 72% Zn (Prince Agri Products, Quincy, IL). The phytase was added at 500 FTU/kg (Natuphos®, BASF Corp., Mount Olive, NJ). One phytase unit (FTU) is the amount of enzyme that releases 1μmol inorganic phosphorus from sodium phytate per minute at pH 5.5 and 37°C. Treatments in a 3 x 2 factorial arrangement were fed for 14 d (**Table 1**). The dietary treatments were: 1) adequate Zn diet containing 150 mg Zn/kg (Zn_{150}), 2) Zn_{150} plus 500 FTU/kg ($Zn_{150}P$), 3) pharmacological Zn diet containing 1,000 mg Zn/kg ($Zn_{1,000}$), 4) $Zn_{1,000}$ plus 500 FTU/kg ($Zn_{1,000}P$), 5) pharmacological Zn diet containing 2,000 mg Zn/kg ($Zn_{2,000}$), or 6) $Zn_{2,000}$ plus 500 FTU/kg ($Zn_{2,000}P$). Pigs were provided feed and water *ad libitum*. This project was approved by the Michigan State University All University Committee on Animal Use and Care (12/99-159-00).

Sample collection and measurements. The pigs and feed were weighed weekly and daily gain, daily feed intake and feed efficiency were calculated. On d-14, one pig from each pen (4 pigs / treatment) was randomly

selected and bled from the anterior vena cava with heparinized vacutainer tubes (BD Vacutainer™, Becton Dickson, Franklin Lakes, NJ) for determination of plasma minerals (Cu, Fe, Zn and P). These pigs were then killed by a lethal injection (0.05 mL/kg BW) of sodium pentobarbital (392 g/L). Kidney and liver samples were excised for mineral (Cu, Fe, Zn and P) and MT protein analysis, while separate tissue samples were collected and flash frozen in liquid nitrogen for RNA isolation. The small intestine was ligated at the ligament of Treitz to remove the first 20 cm segment of intestine. The intestinal sample was cut longitudinally starting at the pylorus to expose mucosa cells and washed three times in 20 mL of ice-cold phosphate buffered saline (PBS). The intestinal mucosa was scraped and cells were prepared following the procedure of Carlson et al. (4). Half of the cells were weighed and collected in 15 mL polypropylene tubes (Corning Inc., Corning, NY) followed by the addition of 250 mmol/L sucrose buffer (a volume of 4 times the weight) for the detection of MT. The remaining cells were flash frozen in liquid nitrogen for RNA isolation.

Mineral analysis. All glassware used during the analysis was soaked for 12 h in 500 mmol/L nitric acid solution, followed by two rinses with deionized-distilled water. To determine mineral concentrations, the tissue and feed were microwave digested (21). The concentrations of Cu, Fe and Zn were determined by flame atomic absorption spectometry (Unicam 989 Atomic Absorption Spectometer, SOLAAR Series, Cambridge, UK) while tissue P concentration was detected by a colorimetric method (22) utilizing a Beckman DU7400 spectrophotometer (Beckman Coulter Inc., Fullerton, CA). Bovine liver was used

as standard reference (15771, U.S. Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD) and was analyzed with the tissue samples for instrument standardization and quality control.

Plasma samples were deproteinized with 800 mmol/L trichloroacetic acid (TCA) solution prior to the Cu, P, and Zn analyses. Briefly, plasma samples were added 1:4 (v/v), vortexed and centrifuged at 2,000 x g for 15 min and analyzed for Cu and Zn by flame atomic absorption spectometry, and P by the colorimetric method (22). The number of plasma samples available for the P analysis varied among the different dietary treatments (Zn_{150}, n=3; $Zn_{150}P$, n=2; $Zn_{1,000}$, n=4; $Zn_{1,000}P$, n=2; $Zn_{2,000}$, n=2; $Zn_{2,000}P$, n=4) due to inadequate sample volume for each animal. Plasma Fe was determined by flame atomic absorption spectrometry (4).

Metallothionein assay. Metallothionein protein concentration was determined by a modification of the silver saturation assay (23). A red blood cell hemolysate was prepared from fresh porcine blood (24). Reagents were prepared daily. Tissue (0.2 - 0.5g) was homogenized (Janke & Kunkel Ultramax T25, Tekmar Company, Cincinnati, OH) in 250 mmol/L sucrose buffer (1:4) and centrifuged at 18,000 x g for 20 min. For renal and intestinal samples from pigs fed Zn_{150} and $Zn_{150}P$, two additional repetitions of the hemolysate step were performed.

RNA isolation. Total RNA was isolated from kidney, liver and intestinal mucosa cells using TRIzol® Reagent (GIBCO BRL, Life Technologies, Gaithersburg, MD) according to the manufacturers protocol. RNA concentrations

were determined by absorption at 260 nm (A_{260}) using a Beckman DU650 spectophotometer (Beckman Coulter, Inc, Fullerton, CA). RNA quality and integrity were determined by calculating the $A_{260/280}$ ratio and by agarose gel electrophoresis, respectively.

Dot blot and northern blot analysis. Duplicate dot blots were prepared as previously described (25) by spotting 1, 3 and 5 μg of denatured kidney, liver or intestinal mucosa RNA (n=6) onto nylon membranes (Hybond-XL, Amersham Biosciences Corp., Piscataway, NJ). Total RNA samples were also subjected to northern blot analysis as previously described (25) to determine the number and size of MT transcripts. The dot and northern blots were hybridized with a mouse MT-1 cDNA probe (provided by J. Carrasco and J. Hidalgo of the Universidad Autónoma de Barcelona, Barcelona, Spain) using modifications of standard procedures (26,27). Blots were also probed with a porcine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe for normalization (28). Probes were synthesized using ^{32}P-dCTP (Perkin Elmer, Life Sciences Inc., Boston MA) and the Multiprime DNA Labeling System (RPN 1601Z, Amersham Biosciences Corp., Piscataway, NJ). Hybridizations were done at 65°C for 16-22 h. Membranes were rinsed as previously described (25) and exposed to Kodak BioMax film (Eastman Kodak Co, Rochester, NY) in the presence of intensifying screens at -80°C for 1-4 d. Quantification of MT and GAPDH relative mRNA abundance was achieved by densitometry scan of autoradiographs using a Fluor-S MultiImager (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis. The data was analyzed using regular analysis of variance procedures based on the MIXED procedure of SAS (29). Factors of interest always included zinc, phytase and their interaction. The experimental unit in the growth performance analysis was the pen, while for plasma and tissue minerals, and MT protein concentration, the individual pigs served as experimental units. For MT mRNA abundance, blot by treatment (zinc-phytase) interaction was additionally modeled along with pig as random effects to define the experimental units for this analysis, with Satterthwaite's approximation used to determine the error degrees of freedom for test. Furthermore, GAPDH mRNA abundance was modeled as a covariate for a regression-based normalization of MT mRNA abundance in accordance with recent recommendations by Poehlman (30).

With the exception of Fe values, a logarithmic transformation was required to make the data more normally distributed. Thus, back-transformed means and their respective 95% confidence intervals (lower and upper limits) are provided as point and interval estimates, respectively. Scheffé's test was used to determine the presence of linearity of response to treatments.

The Fe concentrations and remaining log-transformed values were used for a residual correlation analysis between plasma and tissue mineral concentrations, MT relative mRNA abundance and protein concentrations. Each pair of traits is jointly modeled using a series of bivariate mixed effects models that each include the specified between-trait residual correlation to be estimated. SAS PROC MIXED is used to do this in a very similar way to what was also

recently considered by Thiebaut et al. (31). The residual correlation is an adjusted or partial correlation. That is, after the mixed model effects (e.g. zinc, phytase, zinc x phytase, blot, blot x zinc x phytase, pig) are accounted or adjusted for, the correlation for whatever is left over (hence the term residual correlation) between responses of interest is estimated. Residual correlations were considered to be different from 0 when $P < 0.05$.

RESULTS

Plasma and tissue mineral concentrations. Feeding pharmacological Zn (1,000 or 2,000 mg Zn/kg) with or without phytase to the nursery pigs did not affect plasma Cu (**Table 2**). However, a significant interaction of phytase and dietary Zn on renal Cu was obtained ($P < 0.05$). Different dietary treatments did not significantly affect hepatic Cu or Fe, or plasma or renal Fe concentrations (data not shown). Plasma P concentration was higher ($P < 0.05$) in the pigs fed the phytase-supplemented diets, except in the pigs fed $Zn_{2,000}P$. In this study, the pigs' renal P concentration was not affected by the dietary treatments. However, both dietary Zn ($P < 0.05$) and supplemental phytase ($P < 0.05$) increased hepatic P (Table 2).

A statistically significant effect of zinc ($P < 0.05$) and phytase ($P < 0.05$) was observed for plasma Zn concentrations (**Figure 1**A). In the liver (**Figure 1**B), there was a statistically significant interaction of zinc and phytase supplementation ($P < 0.05$). However, renal Zn responded similarly to plasma Zn (**Figure 1**C) where a significant effect of dietary Zn ($P < 0.05$), as well as by phytase supplementation ($P < 0.05$) was obtained. The Scheffe's test confirmed that regardless of phytase supplementation, increasing the concentration of dietary Zn caused a linear increase in plasma, kidney and liver Zn ($P < 0.0001$).

Metallothionein protein and mRNA abundance. Northern blot analysis was performed to determine the number and size of MT transcripts in kidney, liver and intestinal mucosa. The analysis revealed the presence of a single MT transcript (~ 0.5 kb) in all three tissues, although no transcript was

detected in the intestinal mucosa of pigs fed Zn_{150} (data not shown). The blots were stripped and re-probed with GAPDH and a single transcript (~ 1.2 kb) was detected in all of the samples (data not shown).

Dot blot analysis was performed to determine relative MT mRNA abundance in liver (**Figure 2**), kidney and intestinal mucosa of individual pigs. The pigs fed $Zn_{1,000}P$, $Zn_{2,000}$ or $Zn_{2,000}P$ had greater MT mRNA abundance when compared to pigs fed adequate Zn (Zn_{150}, $Zn_{150}P$) or 1,000 mg Zn/kg ($Zn_{1,000}$). To correct for loading differences, all dot blots were striped and re-probed with porcine GAPDH (data not shown).

Relative metallothionein mRNA abundance (**Figure 3**) in the liver tissue of pigs fed $Zn_{1,000}P$ was not statistically different from that of pigs fed $Zn_{2,000}$ or $Zn_{2,000}P$. A significant interaction ($P < 0.01$) of phytase and zinc supplementation on liver relative MT mRNA abundance was obtained with values being four to five-fold greater in pigs fed $Zn_{1,000}P$, $Zn_{2,000}$ or $Zn_{2,000}P$ than for those fed Zn_{150}, $Zn_{150}P$ or $Zn_{1,000}$. No significant interaction was observed for hepatic MT protein concentration (Figure 3). However, MT protein concentration increased with dietary Zn ($P < 0.05$) and with supplemental phytase ($P < 0.05$).

In the kidney, a significant zinc and phytase interaction on MT mRNA abundance (**Figure 4**) was observed ($P < 0.04$). In addition, there was a significant effect of Zn on MT protein (Figure 4) concentration in the kidney ($P < 0.0003$), but no phytase effect was obtained.

The response of MT in the intestinal mucosa (**Figure 5**) was different than that observed in the other tissues. The relative MT mRNA abundance and the

protein concentration obtained from intestinal mucosa cells of pigs fed Zn_{150}, $Zn_{150}P$ or $Zn_{1,000}$ was below the detectable limits of the assays (0.29 arbitrary units and 186 µmol Ag/g, respectively). However, there was a significant Zn and phytase interaction on relative MT mRNA abundance and MT protein concentration (Figure 5). In both cases, the greatest response was in pigs that received $Zn_{2,000}P$ when compared with the pigs that were fed $Zn_{1,000}P$ or $Zn_{2,000}$ ($P < 0.05$).

Residual correlations. The results of the mineral residual correlation (**Table 3**) demonstrated that in the liver and kidney, Cu and Fe were significantly correlated. Moreover, in the kidney, Zn was correlated with kidney Cu and Fe ($P < 0.01$ and $P < 0.02$, respectively). However, residual correlations between plasma mineral concentrations and tissue minerals, relative MT mRNA abundance and protein were not significant (data not shown).

The MT residual correlation (Table 3) confirmed that liver MT mRNA abundance and liver MT protein were significantly correlated. Furthermore, liver Zn was correlated with liver relative MT mRNA abundance and protein concentration ($P < 0.03$ and $P < 0.01$, respectively). A similar relationship was obtained for the correlation analysis performed in the renal tissue. Kidney MT mRNA and protein were correlated ($P < 0.01$), while kidney Zn was correlated with both relative MT mRNA abundance and protein ($P < 0.01$ and $P < 0.01$, respectively). The intestinal mucosa MT mRNA and MT protein displayed a trend to significantly correlate ($r = 0.36$, $P = 0.08$, data not shown).

Growth performance. Significant differences in daily gain were observed on the first week of the study. Pigs fed supplemental phytase gained faster (203.49 ± 33.20 vs. 238.76 ± 34.55, $P < 0.03$). For the remainder of the experiment, there were no differences in gains, feed intake or feed efficiency (data not shown).

DISCUSSION

Weaning is a major stress that involves a change of diet and environment for pigs resulting in decreased performance and increased incidence of diarrhea. In addition, the corn and soybean meal included in swine diets are high in phytic acid, which causes adverse effects on mineral bioavailability and may contribute to depressed growth (8). Nutritional approaches have been investigated to promote nutrient availability and growth in newly weaned pigs. Supplementing phytase to weaned pig diets results in improved Zn utilization (32), while pharmacological Zn enhances growth performance and reduces the incidence of diarrhea in nursery pigs through an undetermined mechanism (33). However, feeding a combination of supplemental phytase and pharmacological Zn to pigs has not been reported.

Proposed mechanisms for enhanced growth with pharmacological Zn include 1) metallothionein, a protein that plays a key role in Zn homeostasis by sequestering Zn in the intestine, and 2) improved gut morphology (7). In agreement with studies of rats and mice that demonstrate dietary Zn regulation of the MT gene (16,34), in this study we confirm that pharmacological Zn and phytase supplementation increase the abundance of MT mRNA in the liver, kidney and intestinal mucosa cells of nursery pigs. By performing northern blot analysis of liver, kidney and intestinal mucosa RNA, we revealed the presence of an approximately 0.5 kb MT transcript, which is in accordance with a rat transcript size previously published for MT (35).

The liver, where the highest concentrations of Zn and MT were found, was the most responsive tissue, similar to the work of Carlson and collaborators (4). Pigs fed 1,000 mg Zn/kg with no phytase had liver MT mRNA abundance and protein concentration similar to pigs fed adequate Zn, regardless of phytase inclusion. However, when phytase is added to 1,000 mg Zn/kg, MT mRNA and protein concentration increase and are similar to pigs fed 2,000 mg Zn/kg with or without phytase. Interestingly, this dietary treatment effect was also obtained with Zn concentrations in the liver. This suggests that supplementing zinc and phytase increases the amount of Zn absorbed, characterized by an overall greater MT mRNA abundance, MT protein and Zn concentration.

In renal tissue, a different response pattern for MT mRNA abundance and protein was seen than that obtained in the liver. A significant interaction of zinc and phytase on MT mRNA abundance was obtained. Metallothionein protein concentration increased with dietary Zn, whereas phytase did not have a synergistic effect. Nevertheless, MT mRNA abundance, protein and Zn concentrations were positively correlated ($P < 0.01$), similar to the work of Blalock et al. (36) where rats fed 180 mg Zn/kg had kidney MT mRNA abundance linearly correlated with total MT protein. Blalock and collaborators also observed a tissue difference on the induction of both metallothionein mRNA and protein.

The amount of intestinal mucosa MT differed from that observed in the liver and kidney, emphasizing tissue specificity. The amount induced in pigs fed Zn_{150}, $Zn_{150}P$, or $Zn_{1,000}$ was below the detection limits of the MT mRNA abundance and protein assays, hence no data is reported. Metallothionein

mRNA abundance and protein concentrations from pigs fed $Zn_{2,000}P$ were higher than in pigs fed $Zn_{1,000}P$ or $Zn_{2,000}$. This suggests that in the upper intestine, MT is induced to a greater extent when exposed to high dietary Zn, 10 to 20 times the requirement, and this effect is more marked by supplementing phytase along with pharmacological Zn (2,000 mg Zn/kg), an effect not seen with other tissues. In addition, the early work of Richards and Cousins (16) showed that dietary Zn regulates MT, which plays a key role in Zn absorption by controlling the amount of Zn entering the body and transferred into portal circulation.

Using the Zn chelator Zinquin and immunohistochemical analysis, Tran et al. (13) reported that rats fed increasing concentrations of Zn (0, 400 mg Zn/kg) displayed an increased proportion of the Zn attached to the luminal surface of the gut, but when Zn was fed at a pharmacological concentration (1,000 mg Zn/kg) more Zn was internalized and sequestered by MT synthesized *de novo*. Perhaps the unique MT increase observed in the intestinal mucosa in pigs fed $Zn_{2,000}P$ prevents additional Zn from crossing into circulation, thus preventing higher Zn concentrations in the liver. Moreover, it is possible that the hepatic Zn pool of pigs fed Zn_{150}, $Zn_{150}P$ or $Zn_{1,000}$, can be processed efficiently by several mechanisms, one of which might be MT. However, when pigs are fed $Zn_{1,000}P$, $Zn_{2,000}$ or $Zn_{2,000}P$ the MT concentrations plateau, indicating that an alternate pathway might be needed to metabolize this Zn overload.

In the plasma, Zn concentrations increased linearly ($P < 0.05$) with dietary Zn. However, no statistically significant residual correlations between plasma and tissue mineral concentrations, MT protein or MT mRNA abundance were

obtained. Similar results were observed in young broilers by Sandoval et al. (37). Thus, the use of plasma Zn as an absorption or mineral status indicator in pigs is of little value.

Feeding the combination of Zn and phytase to pigs increases renal Cu concentration ($P < 0.05$) similar to observations by Carlson et al. (4) when nursery pigs were fed 3,000 mg Zn/kg (ZnO) for up to 28 d. This documented effect of Zn on Cu metabolism was previously observed in nursery pigs whose mothers were fed pharmacological Zn (5,000 mg Zn/kg) for two parities (19). Hepatic Cu concentrations were depressed in the young pig, which has also been observed in humans and rats. The lack of response in hepatic Cu in this study may be due to the limited time of the dietary intervention.

Even though it has been suggested that the Ca to P ratio can be adjusted to obtain a significant phytase effect (38), our experimental diets were formulated to meet the P requirement as established by the NRC for swine (20). Pigs fed the highest Zn diets as well as those fed phytase had increased ($P < 0.05$) P concentrations in the liver, in accordance to similar results obtained by our lab, where a numerical increase in hepatic P concentration was obtained in pigs fed 2,000 mg Zn/kg for 14 d (39). This unique pharmacological Zn effect on P metabolism deserves further investigation. In addition, plasma P was also higher ($P < 0.05$) when pigs were fed supplemental phytase. These results demonstrate an apparent increase in P availability primarily caused by phytase supplementation, which is in accordance with previous research demonstrating that phytase supplementation increases overall P retention in pigs (11).

In conclusion, these findings support the hypothesis that pigs fed pharmacological Zn have increased organ Zn characterized by increased MT mRNA abundance and MT protein concentrations. Moreover, supplementing phytase further enhanced these effects when a minimum of 1,000 mg Zn/kg was fed. Due to MT's role in sequestering Zn when excess Zn is fed, one might hypothesize that adequate dietary Zn concentrations (150 mg Zn/kg) did not provide sufficient Zn to significantly induce MT, even when phytase was supplemented. However, adding phytase to 1,000 mg Zn/kg diet did provide the additional Zn necessary for higher MT mRNA abundance and protein compared to 1,000 mg Zn/kg alone. These data suggest that current pharmacological doses of Zn fed to pigs (2,000 mg Zn/kg) could be reduced to 1,000 mg Zn/kg by adding phytase. This dietary modification could result in the enhanced growth benefits of pharmacological Zn, as well as reduced nutrient excretion, especially Zn, by newly weaned pigs.

ACKNOWLEDGEMENT

The authors wish to express appreciation to J. Carrasco and J. Hidalgo of the Universidad Autónoma de Barcelona, Barcelona, Spain for providing the mouse MT-1 cDNA probe, and BASF Corporation, Prince Agri Products and APC Inc for supplying ingredients used in this study. The technical assistance of Dana Dvoracek-Driksna and Kim Hargrave is gratefully acknowledged.

LITERATURE CITED

1. Sazawal, S., Black, R. E., Bhan, M. K., Jalla, S., Sinha, A., & Bhandari, N. (1997) Efficacy of zinc supplementation in reducing the incidence and prevalence of acute diarrhea - a community-based, double-blind, controlled trial. Am. J. Clin. Nutr. 66: 413-418.

2. Dutta, P., Mitra, U., Datta, A., Niyogi, S. K., Dutta, S., Manna, B., Basak, M., Mahapatra, T. S., & Bhattacharya, S. K. (2000) Impact of zinc supplementation in malnourished children with acute watery diarrhoea. J. Trop. Pediatr. 46: 259-263.

3. Stanger, B. R., Hill, G. M., Link, J. E., Turk, J. R., Carlson, M. S., & Rozeboom, D. W. (1998) Effects of high Zn on TGE-challenged, early weaned pigs. J. Anim. Sci. 76: 57.

4. Carlson, M. S., Hill, G. M., & Link, J. E. (1999) Early and traditionally weaned nursery pigs benefit form phase-feeding pharmacological concentrations of zinc oxide: effect on metallothionein and mineral concentrations. J. Anim. Sci. 77: 1199-1207.

5. Hill, G. M., Cromwell, G. L., Crenshaw, T. D., Dove, C. R., Ewan, R. C., Knabe, D. A., Lewis, A. J., Libal, G. W., Mahan, D. C., Shurson, G. C., Southern, L. L., & Venum, T. L. (2000) Growth promotion effects and plasma changes from feeding high dietary concentrations of zinc and copper to weanling pigs (regional study). J. Anim. Sci. 78: 1010-1016.

6. McCully, G. A., Hill, G. M., Link, J. E., Weavers, R. L., Carlson, M. S., & Rozeboom, D. W. (1995) Evaluation of zinc sources for the newly weaned pig. J. Anim. Sci. 73: 72.

7. Carlson, M. S., Hoover, S. L., Hill, G. M., Link, J. E., & Turk, J. R. (1998) Effect of pharmacological zinc on intestinal metallothionein concentration and morphology in the nursery pig. J. Anim. Sci. 76: 57.

8. O'Dell, B. L., & Savage, J. E. (1960) Effects of phytic acid on zinc availability. Proc. Soc. Exp. Biol. Med. 103: 304-305.

9. Ravindran, V., Bryden, W. L., & Kornegay, E. T. (1995) Phytates: occurrence, bioavailability and implications in poultry nutrition. Poult. Avian Biol. Rev. 6: 125-143.

10. Oberleas, D., Muhrer, M. E., & O'Dell, B. L. (1966) Dietary metal-complexing agents and zinc availability in rats. J. Nutr. 90: 56-62.

11. Adeola, O., Lawrence, B. V., Sutton, A. L., & Cline, T. R. (1995) Phytase-induced changes in mineral utilization in zinc-supplemented diets for pigs. J. Anim. Sci. 73: 3384-3391.

12. Dunn, M. A., Blalock, T. L., & Cousins, R. J. (1987) Metallothionein. Proc. Soc. Exp. Biol. Med. 185: 107-119.

13. Tran, C. D., Butler, R. N., Howarth, G. S., Philcox, J. C., Rofe, A. M., & Coyle, P. (1999) Regional distribution and localization of zinc and metallothionein in the intestine of rats fed diets differing in zinc content. Scand. J. Gastroenterol. 34: 689-695.

14. Bremner, I., & Beattie, J. H. (1990) Metallothionein and the trace minerals. Annu. Rev. Nutr. 10: 63-83.

15. Davis, S. R., & Cousins, R. J. (2000) Metallothionein expression in animals: a physiological perspective on function. J. Nutr. 130: 1085-1088.

16. Richards, M. P., & Cousins, R. J. (1975) Mammalian zinc homeostasis requirement for RNA and metallothionein synthesis. Biochem. Biophys. Res. Comm. 64: 1215-1223.

17. Radtke, F., Heuchel, R., Georgiev, O., Hergersberg, M., Gariglio, M., Dembic, Z., & Schaffner, W. (1993) Cloned transcription factor MTF-1 activates the mouse metallothionein I promoter. EMBO J. 12: 1355-1362.

18. Andrews, G. K. (2001) Cellular zinc sensors: MTF-1 regulation of gene expression. BioMetals 14: 223-237.

19. Hill, G. M., Ku, P. K., Miller, E. R., Ullrey, D. E., Losty, T. A., & O'Dell, B. L. (1983) A copper deficiency in neonatal pigs induced by a high zinc maternal diet. J. Nutr. 113: 867-872.

20. NRC (1998) Nutrient Requirements of Swine, 10th Ed. National Academy Press, Washington, DC.

21. Shaw, D. T., Rozeboom, D. W., Hill, G. M., Booren, A. M., & Link, J. E. (2002) Impact of vitamin and mineral supplement withdrawal and wheat middling inclusion on finishing pig growth performance, fecal mineral concentration, carcass characteristics, and the nutrient content and oxidative stability of pork. J. Anim. Sci. 80: 2920-2930.

22. Gomori, G. (1942) A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. J. Clin. Lab. Med. 27: 955-960.

23. Scheuhammer, A. M., & Cherian, M. G. (1986) Quantification of metallothionein by silver saturation. In J.F. Riordan, & B.L. Vallee, Eds. Methods in Enzymology. Metallobiochemistry. Part B. Metallothionein and related molecules. Academic Press, Inc, San Diego, Ca.

24. Onosaka, S., & Cherian, M. G. (1982) Comparison of metallothionein determination by polarographic and cadmium-saturation methods. Toxicol. Appl. Pharmacol. 63: 270-274.

25. Ernst, C. W., McFarland, D. C., & White, M. E. (1996) Expression of insulin-like growth factor II (IGF-II), IGF binding protein-2 and myogenin during differentiation of myogenic satellite cells derived from the turkey. Differentiation 61: 25-33.

26. Brown, T., & Mackey, K. (1997) Analysis of RNA by Northern and Slot Blot Hybridization. In F.M. Ausubel, ed. Current Protocols in Molecular Biology. John Wiley & Sons, New York.

27. Brown, T. (2000) Dot and Slot Blotting of DNA. In F.M. Ausubel, ed. Current Protocols in Molecular Biology. John Wiley & Sons, New York.

28. Yao, J., Coussens, P. M., Saama, P., Suchyta, S., & Ernst, C. W. (2002) Generation of expressed sequence tags from a normalized porcine skeletal muscle cDNA library. Animal Biotechnol. 13: 211-222.

29. SAS Systems (1999-2000) The SAS System for Windows 98, 8.1 ed. SAS Institute, Inc., Cary, N.C.

30. Poehlman, E. T. (2003) Reduced metabolic rate after caloric restriction-can we agree on how to normalize the data? J. Clin. Endocrinol. Metab. 88: 14-15.

31. Thiebaut, R., Jacqmin-Gadda, H., Chene, G., Leport, C., & Commenges, D. (2002) Bivariate linear mixed models using SAS proc MIXED. Comput. Methods Programs Biomed. 69: 249-256.

32. Lei, X., Ku, P. K., Miller, E. W., Ullrey, D. E., & Yokoyama, M. (1993) Supplemental microbial phytase improves bioavailability of dietary zinc to weanling pigs. J. Nutr. 123: 1117-1123.

33. Poulsen, D. (1989) Zinc oxide for weaned pigs. 40th Annu. Mtg. Eur. Assoc. Anim. Prod., Dublin, Ireland.

34. Durnam, D. M., & Palmiter, R. D. (1981) Transcriptional regulation of the mouse metallothionein-I gene by heavy metals. J. Biol. Chem. 256: 5712-5716.

35. Huber, K. L., & Cousins, R. J. (1988) Maternal zinc deprivation and interleukin-1 influence metallothionein gene expression and zinc metabolism of rats. J. Nutr. 118: 1570-1576.

36. Blalock, T. L., Dunn, M. A., & Cousins, R. J. (1988) Metallothionein gene expression in rats: tissue-specific regulation by dietary copper and zinc. J. Nutr. 118: 222-228.

37. Sandoval, M., Henry, P. R., Luo, X. G., Littell, R. C., Miles, R. D., & Ammerman, C. B. (1998) Performance and tissue zinc and metallothionein accumulation in chicks fed a high dietary level of zinc. Poult. Sci. 77: 1354-1363.

38. Wodzinki, R. J., & Ullah, A. H. (1996) Phytase. Adv. Appl. Microbiol. 42: 263-302.

39. Martínez, M. M., Hill, G. M., Link, J. E., Green, J. G., & Driksna, D. D. (2002) Effects of the addition of phytase and pharmacological concentrations of zinc oxide on phosphorus excretion in the nursery pig. J. Anim. Sci. 80: 73.

TABLE 1

Composition of experimental diets for nursery pigs[1,2]

Ingredients	0-7 d g/kg diet (as fed)	7-14 d g/kg diet (as fed)
Corn	390.90	531.40
Soybean meal (44% CP)	202.00	201.80
Spray-dried whey	200.00	200.00
Plasma protein[3]	60.00	———
Lactose	100.00	———
Blood cells[3]	———	20.00
DL-Methionine	1.10	0.60
L-Lysine • HCL	1.50	1.50
Soybean oil	10.00	10.00
Monocalcium phosphate[4]	14.10	15.20
Ground limestone	5.50	5.20
Salt	3.50	3.30
Trace mineral pre-mix[5]	5.00	5.00
Vitamin pre-mix[6]	6.00	6.00

[1]The basal diet was formulated to meet or exceed recommended concentrations (20). Analysis of the diets indicated that dietary Zn was within approximately 10% of the targeted dietary concentration.

[2] ZnO (1.25, 2.50 g / kg of final diet) and Natuphos® phytase (0.84 g / kg of final diet) were substituted for corn to provide 1,000 and 2,000 mg of Zn and the necessary phytase were appropriate.

[3]APC, Inc (AP 920 - sprayed dried plasma, AP 301G - granulated sprayed dried animal blood cells).

[4]Monocalcium phosphate = 21% elemental P.

[5]Trace Mineral pre-mix, (mineral / kg of final diet): $CuSO_4 \cdot 5H_2O$, 10 mg; $FeSO_4 \cdot H_2O$, 100 mg; $C_2H_4(NH_2)_2 \cdot 2HI$, 0.15 mg; $MnSO_4 \cdot H_2O$, 10 mg; Na_2SeO_3, 0.3 mg; ZnO, 100 mg.

[6]Vitamin pre-mix, (vitamin / kg of final diet): retinyl acetate, 1.65 mg; cholecalciferol, 0.165 mg; DL-α-tocopherol, 0.0198 mg; menadione sodium bisulfite, 4.41 mg; niacin, 26.0 mg; riboflavin, 4.41 mg; Ca-D-panthotenate, 17.62 mg; thiamine mononitrate, 1.10 mg; pyridoxine hydrochloride, 0.991 mg; cyanocobalamin, 33 mg.

TABLE 2

Concentration of copper, and phosphorus in plasma, kidney, and liver of pigs fed adequate or pharmacological levels of zinc with or without supplemental phytase[1]

Phytase[2], FTU/kg	0			500			P value		
Zinc, mg/kg	150	1,000	2,000	150	1,000	2,000	Zn	Phy	Zn x Phy
Copper									
Plasma (μmol/L)	24.32 (21.29 - 27.79)	21.98 (19.25 - 25.12)	21.57 (18.89 - 24.63)	23.55 (20.41 - 26.69)	24.42 (21.38 - 27.90)	21.98 (18.84 - 23.55)	0.21	0.73	0.51
Kidney (mmol/g)	0.091 (0.058 - 0.140)^{a}	0.132 (0.085 - 0.206)^{ab}	0.466 (0.279 - 0.776)^{c}	0.064 (0.041 - 0.100)^{a}	0.283 (0.182 - 0.438)^{bc}	0.634 (0.408 - 0.986)^{c}	0.01	0.19	0.05
Liver (mmol/g)	0.793 (0.515 - 1.239)	0.743 (0.479 - 1.154)	0.524 (0.338 - 0.815)	0.804 (0.518 - 1.248)	0.804 (0.518 - 1.248)	0.700 (0.452 - 1.088)	0.37	0.47	0.78
Phosphorus									
Plasma (μmol/L)	0.129 (0.032 - 0.420)	0.420 (0.097 - 2.067)	0.226 (0.065 - 0.646)	1.163 (0.226 - 5.588)	1.357 (0.291 - 6.589)	0.226 (0.065 - 0.711)	0.19	0.05	0.21
Kidney (mmol/g)	0.087 (0.084 - 0.094)	0.087 (0.081 - 0.090)	0.087 (0.081 - 0.094)	0.084 (0.081 - 0.087)	0.090 (0.087 - 0.097)	0.087 (0.084 - 0.094)	0.63	0.81	0.18
Liver (mmol/g)	0.100 (0.097 - 0.103)	0.097 (0.094 - 0.100)	0.107 (0.103 - 0.110)	0.103 (0.100 - 0.107)	0.103 (0.097 - 0.110)	0.107 (0.103 - 0.113)	0.01	0.02	0.21

[1] The values presented are backtransformed means (lower and upper limits of the 95% confidence intervals), n=4. Means in a row without a common superscript differ, $P < 0.05$.

[2] One phytase unit (FTU) is the amount of enzyme that releases 1μmol of inorganic phosphorus from sodium phytate per minute at pH 5.5 and 37°C, (Natuphos BASF Corp, Mount Olive, NJ).

TABLE 3

Residual correlation of the copper, iron, zinc, metallothionein mRNA abundance and metallothionein protein concentrations in liver and kidney of pigs fed pharmacological levels of zinc with or without supplemental phytase

Response variable	Estimate	P value
Liver		
Cu vs. Fe	0.41	0.04
MT mRNA vs. MT protein	0.40	0.04
MT mRNA vs. Zn	0.41	0.03
MT protein vs. Zn	0.63	0.01
Kidney		
Cu vs. Fe	0.59	0.01
Zn vs. Cu	0.86	0.01
Zn vs. Fe	0.45	0.02
MT mRNA vs. MT protein	0.66	0.01
MT mRNA vs. Zn	0.48	0.01
MT protein vs. Zn	0.77	0.01

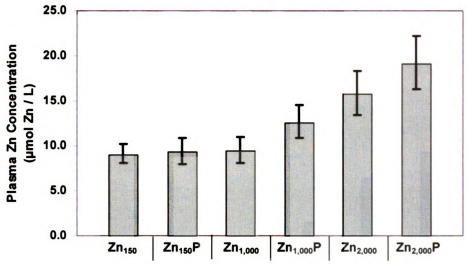

Figure 1A. Concentration of zinc in plasma of pigs fed zinc (150, 1,000 and 2,000 mg Zn/kg) with or without phytase for 14-d post-weaning. Values are backtransformed means (bar height) with 95% CI (error bars), n=4. Main effects of zinc and phytase were statistically significant, $P < 0.05$.

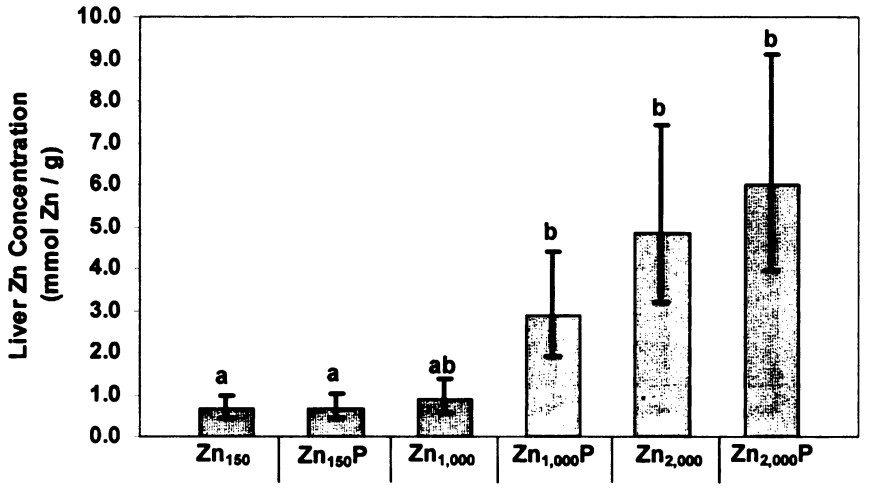

Figure 1B. Concentration of zinc in liver of pigs fed zinc (150, 1,000 and 2,000 mg Zn/kg) with or without phytase for 14-d post-weaning. Values are backtransformed means (bar height) with 95% CI (error bars), n=4. The zinc x phytase interaction was significant; means without a common letter differ, $P < 0.05$.

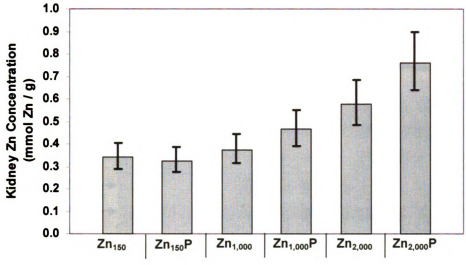

Figure 1C. Concentration of zinc in kidney of pigs fed zinc (150, 1,000 and 2,000 mg Zn/kg) with or without phytase for 14-d post-weaning. Values are backtransformed means (bar height) with 95% CI (error bars), n=4. Main effects of zinc and phytase were statistically significant, $P < 0.05$.

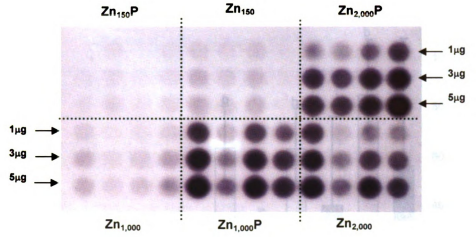

Figure 2. Dot blot analysis of liver MT mRNA of pigs fed zinc (150, 1,000 and 2,000 mg Zn/kg) with or without phytase for 14-d post-weaning. Each quadrant contains total RNA from 4 pigs fed the designated diet. RNA samples from each pig were spotted at 1, 3, and 5 μg.

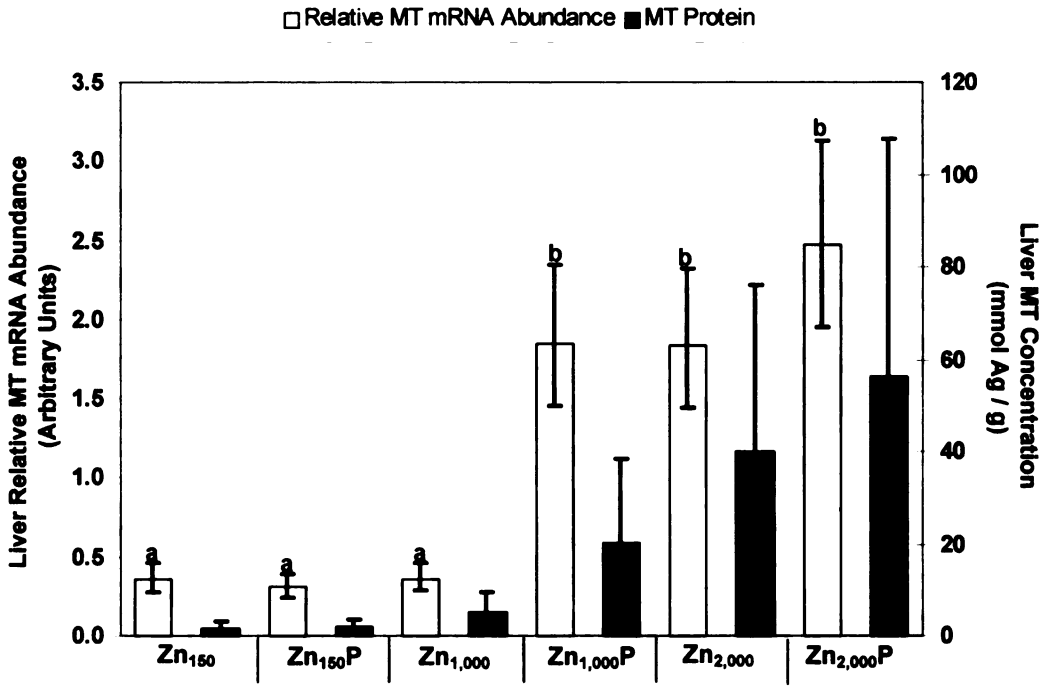

Figure 3. Relative MT mRNA abundance and protein concentration in the liver of pigs fed zinc (150, 1,000 and 2,000 mg Zn/kg) with or without phytase for 14-d post-weaning. Values are backtransformed means (bar height) with 95% CI (error bars), n=4. A significant zinc x phytase interaction was detected for relative hepatic MT mRNA abundance; means without a common letter differ, $P < 0.01$. Main effects of zinc and phytase on MT protein concentration were statistically significant, $P < 0.05$.

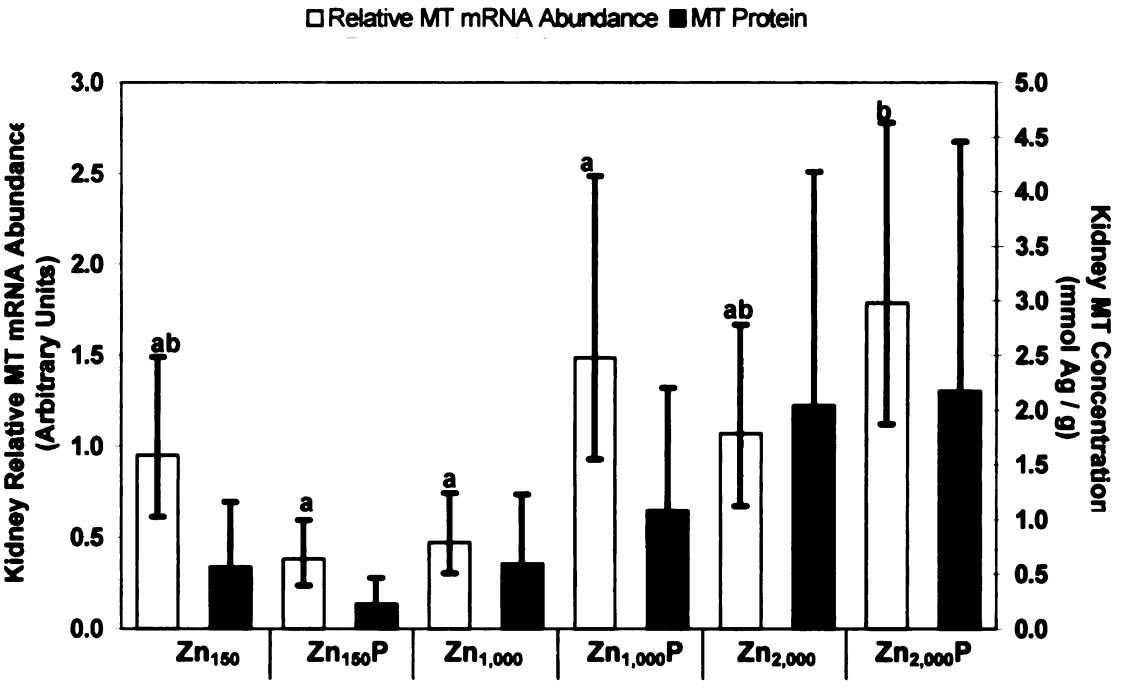

Figure 4. Relative MT mRNA abundance and protein concentration in the kidney of pigs fed zinc (150, 1,000 and 2,000 mg Zn/kg) with or without phytase for 14-d post-weaning. Values are backtransformed means (bar height) with 95% CI (error bars), n=4. A significant zinc x phytase interaction was detected for relative renal MT mRNA abundance; means without a common letter differ, $P < 0.04$. The effect of zinc on MT protein concentration was statistically significant, $P < 0.0003$.

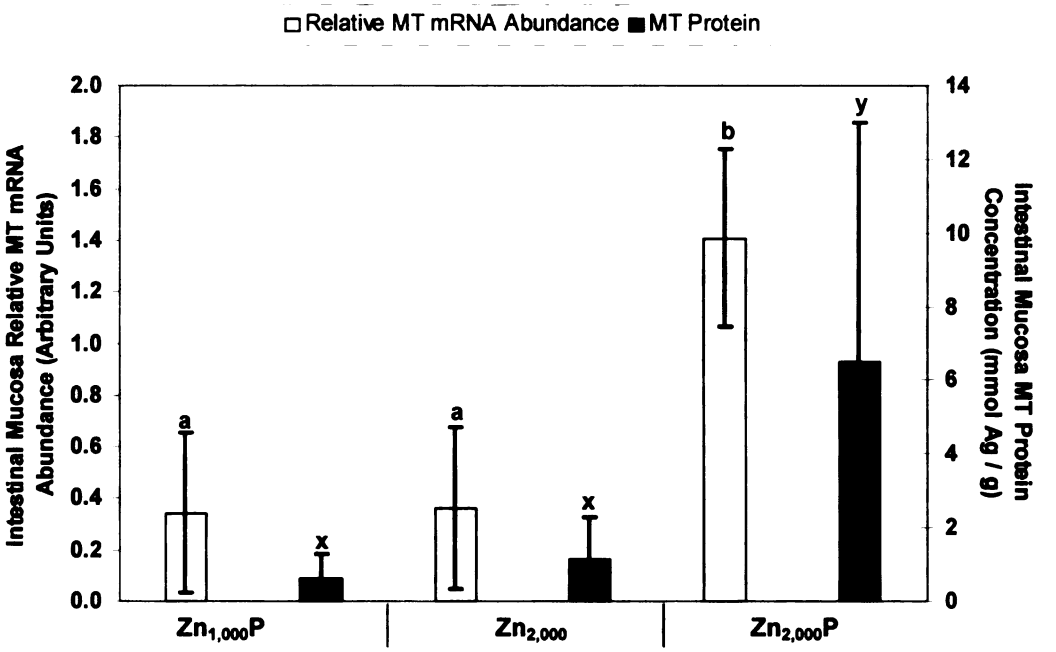

Figure 5. Relative MT mRNA abundance and protein concentration in the intestinal mucosa of pigs fed zinc (1,000 mg Zn/kg with phytase and 2,000 mg Zn/kg with or without phytase) for 14-d post-weaning. Data for pigs fed 150 mg Zn/kg with or without phytase and 1,000 mg Zn/kg without phytase were below the detectable limits of the assays. Values are backtransformed means (bar height) with 95% CI (error bars), n=4. A zinc x phytase interaction for relative intestinal mucosa MT mRNA abundance and MT protein concentration was detected; means without a common letter differ, $P < 0.05$.

CHAPTER THREE

Identification of ZnT-1 in Porcine Liver of Newly Weaned Pigs Fed Pharmacological Zinc and Phytase Supplemented Diets[1]

Michelle M. Martínez[*], Gretchen M. Hill[*], Jane E. Link[*], Catherine W. Ernst[*], Bo L. Lönnerdal[†], Shannon L. Kelleher[†], Emily E. Helman[*] and Matthew E. Doumit[*‡]

[*]Department of Animal Science, Michigan State University, East Lansing, MI; [†]Department of Nutrition, University of California, Davis, CA; [‡]Department of Food Science and Human Nutrition, Michigan State University, East Lansing, MI.

Running Title: ZnT-1 IN YOUNG PIGS FED HIGH ZINC

[1] Supported in part by Six State Consortium on Animal Waste Management of Environmental Protection Agency, an Animal Health Formula Funds Grant from the Michigan Agricultural Experiment Station, and The Graduate School - Competitive Doctoral Enrichment Fellowship, Michigan State University, East Lansing, MI.

[2] Abbreviations used: BCA, bicinchonic acid; EST, expressed sequence tag; FTU, phytase unit; HES, hepes / sucrose / EDTA; IgG, immunoglobulin; MT, metallothionein; MRE, metal response element; MTF, metal transcription factor; TBS-T, tris buffered saline with tween-20; Zn, zinc; ZnO, zinc oxide; ZnT, zinc transporter; Z_{150}, 150 mg Zn/kg; $Zn_{150}P$, 150 mg Zn/kg with phytase; $Zn_{1,000}$, 1,000 mg Zn/kg; $Zn_{1,000}P$, 1,000 mg Zn/kg with phytase; $Zn_{2,000}$, 2,000 mg Zn/kg; $Zn_{2,000}P$, 2,000 mg Zn/kg with phytase.

ABSTRACT

Pharmacological zinc (Zn) is routinely fed to newly weaned pigs to improve growth. We recently demonstrated that pharmacological Zn and phytase supplementation increases metallothionein (MT) mRNA abundance and protein in the liver, kidney and intestinal mucosa of newly weaned pigs. Since Zn transporter-1 (ZnT-1) appears to act in concert with MT to effectively manage Zn, we hypothesized that ZnT-1 protein is involved in hepatic Zn homeostasis of pigs fed pharmacological Zn diets. Adequate (150 mg Zn/kg) or pharmacological (1000 or 2000 mg Zn/kg) Zn diets with or without phytase (0 or 500 phytase units /kg) were fed to pigs for 14 d post-weaning. The ZnT-1 was identified as a ~50 kDa protein found exclusively in pig liver crude membrane fractions. Pigs fed 150 and 1000 mg Zn/kg had comparable ZnT-1 protein expression; pigs fed 2000 mg Zn/kg showed reduced expression of this protein. Phytase did not affect ZnT-1 protein. Our results suggests that hepatic cells of pigs fed pharmacological Zn (2000 mg Zn/kg) for 14 d, preferentially increase intracellular MT rather than ZnT-1 to manage the excess Zn.

KEY WORDS: • *liver* • *pharmacological zinc* • *phytase* • *pig* • *zinc transporter-1*

INTRODUCTION

The mechanism involved in Zn transport within and between tissues has not been elucidated (1). To achieve Zn homeostasis, regulation occurs through intestinal uptake, endogenous secretions, fecal excretion, renal resorption and excretion, and intracellular storage (2). Under variable Zn concentrations, cells adjust: i) Zn uptake and secretion, ii) intracellular protein binding, iii) re-distribution and/or iv) sequestration within vesicles or organelles for homeostatic control (3).

Numerous studies demonstrate that feeding 2000 to 3000 mg Zn/kg as zinc oxide (ZnO) to traditionally (18 – 21 d) or early-weaned (10 – 14 d) pigs, results in enhanced growth performance (4,5), improved fecal consistency (6,7) and gut morphology (8). However, the mechanism of action has not been elucidated. Recently, studies from our laboratory reported that feeding 1000 mg Zn/kg plus 500 phytase units (FTU)/kg of phytase or 2000 mg Zn/kg with or without phytase for 14 d post-weaning increased relative metallothionein (MT) mRNA abundance and protein concentrations in liver, kidney and intestinal mucosa. Furthermore, a positive correlation between Zn concentration and MT in liver and kidney suggests that MT plays a homeostatic role in pigs fed high Zn diets (9).

Exposing cells to high concentrations of Zn activates many protective mechanisms (10), such as downregulation of Zn uptake transporters (11), sequestration of Zn into intracellular compartments (12), induction of binding proteins such as MT (13) and increased Zn efflux (14). Palmiter (10) suggested

that as excess Zn enters the cell, both MT and the Zn efflux transporter, ZnT-1 compete with each other for handling Zn disposal.

Transcription of the MT and ZnT-1 genes is under the control of the metal transcription factor –1 (MTF-1). Once Zn is inside the cell, it binds to MTF-1, which acts as a Zn sensor (15). Short sequences known as metal response elements (MREs) found in the promoter region of genes serve as binding sites for transcription factors such as MTF-1 increasing transcription rate of MT (16) and ZnT-1 (17) upon metal activation. The MT gene contains six MREs in the 5' regulatory region upstream of the transcription initiation site (18), while the ZnT-1 promoter contains two MRE consensus sequences and MTF-1 binds to both sites, resulting in transcriptional activation (17). These reports led us to hypothesize that ZnT-1 plays a key role with MT in hepatic Zn homeostasis of pigs fed pharmacological Zn diets. Thus, the objectives of this experiment were to identify ZnT-1 in the liver, and to determine the effects of pharmacological Zn and phytase supplementation on the expression of ZnT-1.

MATERIALS AND METHODS

Animals and diets. Animal description, allotment and diet composition have been previously published (9). The Zn source, ZnO, contained 72% Zn (Prince Agri Products), and phytase was added at 500 phytase units (FTU)/kg diet (Natuphos®, BASF Corp., Mount Olive, NJ). One FTU is the amount of enzyme that releases 1 μmol inorganic phosphorous from sodium phytate per minute at pH 5.5 and 37°C. The basal diet was formulated to meet or exceed NRC recommendations (19). The dietary treatments were: 1) a basal diet containing 150 mg Zn/ kg (Zn_{150}), 2) Zn_{150} plus 500 FTU/kg ($Zn_{150}P$), 3) basal plus pharmacological Zn diet containing 1000 mg Zn/kg (Zn_{1000}), 4) Zn_{1000} plus 500 FTU/kg ($Zn_{1000}P$), 5) basal plus pharmacological Zn diet containing 2000 mg Zn/kg (Zn_{2000}) or 6) Zn_{2000} plus 500 FTU/kg ($Zn_{2000}P$). Pigs consumed feed and water *ad libitum*. This project was approved by the Michigan State University All University Committee on Animal Use and Care (12/99-159-00).

Sample collection. On d-14, one pig from each pen (n = 24) was randomly selected and killed by an injection (0.22 mL/kg body weight) of sodium pentobarbital (392 g/L). Liver tissue samples were excised and flash frozen in liquid nitrogen for protein isolation. Additionally, liver tissue was obtained from a 21 d lactating Sprague-Dawley rat that was fed a standard rat chow for use in optimizing the western blot analysis, and to compare band patterns.

Total membrane protein preparation. Approximately 500 mg of liver tissue was homogenized in 10 mL of ice cold HES buffer [20 mM HEPES, pH 7 / 250 mM sucrose / 1 mM EDTA / protease inhibitor mixture containing 4-(2-

aminoethyl) benzenesulfonyl fluoride, trans-epoxysuccinyl-L-leucyl-amido(4-guanidino) butane, bestatin, leupeptin, aprotonin and sodium EDTA (Sigma-Aldrich, St. Louis, MO)] using a tissue homogenizer (Janke & Kunkel Ultramax T25, Tekmar Company, Staufeni, Berlin, Germany) for 20 s. The homogenate was centrifuged at 21000 x g for 30 min at 4°C. The supernatant was removed and the pellet was resuspended in 0.5 mL HES buffer. An aliquot (0.5 mL) of both fractions was mixed in electrophoresis treatment buffer (125 mM Tris, 4% SDS, 20% glycerol) and heated at 50°C for 20 min. Protein concentrations were immediately determined in both soluble and insoluble protein fractions using the bicinchonic acid (BCA) protein assay kit (Pierce, Rockford, IL). Beta-mercaptoethanol was added at 5% (v/v) and bromophenol blue was added at 4% (v/v) to the samples prior to storage at -20°C. Also, 50 and 100 μg of protein from all samples were loaded on 10% SDS/PAGE gels stained with Coomassie™ brilliant blue R250 (Amersham Biosciences, Buckinghamshire, UK).

Production of ZnT-1 antibody. A polyclonal rabbit anti-rat ZnT-1 antibody (GTRPQVHSGKE, Life Technologies, Carlsbad, CA) was used for this study (20). The only porcine sequence corresponding to ZnT-1 is a 424 bp expressed sequence tag (EST) consensus sequence (TC155407) in The Institute for Genomic Research (TIGR) database. In the identified epitope region within the translated porcine sequence, 3 out of the 11 amino acids are different in the pig sequence (GTRPQAQSGKD).

Western blot analysis. Equal amounts of membrane and soluble protein (50 μg) from one randomly selected pig from each treatment, plus the rat sample

were resolved in a 10% SDS/PAGE gel and transferred onto an Immobilon-P^{SQ} (Millipore, Billerica, MA) membrane for 2 hr at 100 V. Blots (n = 8) were blocked for 1 hr at room temperature with 100 g/L nonfat milk in Tris-buffered saline (20mM Tris, 137mM NaCl, 2mM KCl) / 0.1% Tween-20 (TBS-T). Membrane strips (0.5 cm) were stained with amido black and destained to visualize protein presence. Membranes were incubated with the rabbit anti-rat ZnT-1 polyclonal antibody (1:1000) for 8 hr at 4°C, and then washed three times at room temperature with blocking solution.

Membranes were incubated at room temperature with goat-anti-rabbit immunoglobulin (IgG) conjugated to alkaline phosphatase (1:500, Sigma-Aldrich, St. Louis, MO) for 1 hr, and washed three times with blocking solution, three times with TBS-T and one additional time with TBS. Rabbit pre-immune serum (1:1000) and goat anti-rabbit IgG (1:500) served as negative controls. Immunoreactive bands were visualized by treatment with an alkaline phosphatase conjugate substrate (Bio-Rad, Hercules, CA) for 2 min.

Blots were re-blocked with 50 g/L nonfat milk in TBS-T for 1 hr, then incubated with a mouse β-actin (1:10000) monoclonal antibody (Abcam Ltd, Cambridge, UK) for 45 min at room temperature. Membranes were washed three times with blocking solution, and subsequently incubated with goat-anti-mouse IgG conjugated to alkaline phosphatase (1:20000, Sigma-Aldrich, St. Louis, MO) for 45 min, washed three times with blocking solution, three times with TBS-T and one additional time with TBS. Immunoreactive bands were

visualized by treatment with an alkaline phosphatase conjugate substrate for 8 min.

Statistical analysis. The data were analyzed using analysis of variance procedures based on the MIXED procedure of SAS (21). The model included the main effects (zinc and phytase) and their interaction. Blot by treatment (zinc x phytase) interaction was additionally modeled with pig as random effect to define the experimental units for this analysis, with Satterthwaite's approximation used to determine the error degrees of freedom for test (22). Furthermore, β-actin values were modeled as a covariate for a regression-based normalization of ZnT-1 protein expression in accordance with recent recommendations by Poehlman (23). Due to lack of normal distribution, data were log transformed. Thus, back-transformed means and their respective 95% confidence intervals (lower and upper limits) are provided as point and interval estimates, respectively. Scheffé's test was used to determine the presence of linearity of response to treatments (24).

RESULTS

Identification of ZnT-1 in porcine liver. Utilizing western blot analysis, ZnT-1 was identified in the pellet fraction and was absent in the supernatant of liver homogenates (**Figure 1**). Specificity was confirmed with rabbit preimmune serum, a total IgG fraction, or the ZnT-1 antibody (**Figure 2**).

Effect of diet on zinc transporter protein concentrations. A ~ 50 kDa immunoreactive band was identified as ZnT-1 protein in the rat and pig samples (**Figure 3A**). An additional band (~ 38 kDa) was identified in all samples, but was more intense in rat liver (Figure 3A) in agreement with results reported by McMahon and Cousins (14). Both sizes are in agreement with the ZnT-1 predicted sizes and the sizes previously observed in rat liver using this antibody (S. Kelleher, unpublished data). Beta-actin blots exhibited a ~ 42 kDa band, which was present in all liver samples (**Figure 3B**), and is in agreement with its published size (25).

A significant Zn effect ($P < 0.02$) was detected on the expression of ZnT-1 protein (**Figure 3C**), which was higher in pigs fed Zn_{1000} compared with pigs fed Zn_{2000}. Liver of pigs fed Zn_{150} had comparable ZnT-1 expression to that of pigs fed Zn_{1000}. Neither a Zn x phytase interaction nor a main phytase effect were observed for relative ZnT-1 protein abundance.

DISCUSSION

In this study, we present evidence of the presence and regulation of ZnT-1 in the liver of pigs fed adequate or pharmacological Zn concentrations without an effect by supplemental phytase. Using a highly specific affinity-purified rat antibody (20), we identified for the first time the presence of ZnT-1 in liver samples of newly weaned pigs, suggesting the existence of common epitopes for this protein. Despite the fact that the epitope sequences are only 72% homologous, ZnT-1 was successfully identified in all hepatic membrane fraction samples. Zinc transporter - 1 protein had been previously identified in intestinal and hepatic membrane fractions of rats fed 30 and 180 mg Zn/kg (14), as well as in the serosal membrane of cells in the mammary gland of lactating rats fed 10 and 25 mg Zn/kg (20).

Young pigs adapt and benefit from pharmacological dietary Zn, which suggests that regulation of Zn in tissues, such as the liver, is effective in managing excess Zn. Previously, we documented that hepatic Zn concentration of pigs fed phytase and pharmacological Zn diets increased linearly, and these values were correlated to the increase in relative MT mRNA abundance and protein (9). The role of ZnT-1, a member of the ZnT transporters, has not been investigated in the pig when Zn is fed at 10 to 20 times its requirement. The function of the transporters in the ZnT family is to reduce cytoplasmic Zn through efflux or secretion (26). Therefore, we propose that ZnT-1 is part of the mechanism involved in regulating Zn homeostasis in newly weaned pigs fed pharmacological Zn diets.

Feeding a marginal Zn diet (10 mg Zn/kg) to lactating rats decreased mammary gland ZnT-1 and MT proteins while plasma, milk or mammary gland Zn concentrations remained unchanged (20) suggesting regulation of Zn upon exposure to a low Zn diet. An oral Zn dose (35 mg Zn/kg BW) given to rats has been shown to increase liver ZnT-1 protein and serum Zn 6 h after dosing compared with rats receiving saline (14). These results suggest that rats receiving 2.5 to 3 times adequate dietary Zn (0.17 – 0.22 mmol/kg BW) (27), have increased hepatic Zn efflux via ZnT-1 transporter. In our study, ZnT-1 protein expression was greater in pigs fed Zn_{1000} than in those fed Zn_{2000}, but hepatic MT protein and Zn were greater in pigs fed Zn_{2000} than those fed Zn_{1000}. These data suggest that Zn efflux through this transporter was reduced. The decrease in ZnT-1 combined with the increase in hepatic Zn and MT concentrations suggests an altered hepatic Zn efflux mechanism that has not been previously reported.

Feeding 500 or 1000 mg Zn/kg diet is not as effective as 2000 or 3000 mg Zn/kg for promoting growth (5,28) in the newly weaned pig. Perhaps reduced ZnT-1 expression in pigs fed Zn_{2000} occurs as a response to an overwhelmed homeostatic system or to the adequate sequestering methods of MT. This intracellular Zn increase may affect other signaling pathways (29), which may also affect the expression of the transporter. Recently, Palmiter (10) suggested that when cells are suddenly exposed to increased Zn concentrations, both MT and ZnT-1 work in concert, and as intracellular Zn increases, Zn efflux by pre-existing ZnT-1 predominates. Perhaps when pigs are exposed to high Zn

concentrations for 14 d, cells preferentially increase intracellular MT rather than ZnT-1 to manage the excess Zn.

Preferential MT gene amplification compared to ZnT-1 was observed in baby hamster kidney cells (BHK) exposed to high concentrations of Zn (~600 μM). Palmiter proposed that this could be due to: 1) the close linkage of the MT-1 and MT-2 genes (7 kb apart) in the hamster cells and consequent amplification of both genes upon high Zn exposure, and/or 2) an energetic advantage to sequester Zn rather than effluxing it (10). Also, there are more MRE sequences present in the promoter region of MT (16), compared to ZnT-1 (17), which may affect the differential regulation of these proteins at the gene level, upon exposure to Zn. Although we demonstrated that ZnT-1 plays a role in regulating Zn in the liver of pigs fed pharmacological Zn, there are still unanswered questions.

ACKNOWLEDGEMENTS

The authors thank Dr. Jeanne Burton at Michigan State University, for providing the mouse β-actin antibody; BASF Corporation, Prince Agri Products, International Ingredients Corporation, and American Protein Corporation for supplying ingredients used in this study; and Guillermo Ortiz-Colón, and Chuck Allison for technical assistance.

LITERATURE CITED

1. Cousins, R. J. (1985) Absorption, transport and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin. Physiol. Rev. 65: 238-309.

2. Krebs, N. F. (2000) Overview of zinc absorption and excretion in the human gastrointestinal tract. J. Nutr. 130: 1374S-1377S.

3. Reyes, J. G. (1996) Zinc transport in mammalian cells. Am J Physiol 270: C401-410.

4. Smith, J. W., Tokach, M. D., Goodband, R. D., Nelssen, J. L. & Richert, B. T. (1997) Effects of the interrelationship between zinc oxide and copper sulfate on growth performance of early-weaned pigs. J. Anim. Sci. 75: 1861-1866.

5. Hill, G. M., Mahan, D. C., Carter, S. D., Cromwell, G. L., Ewan, R. C., Harrold, R. L., Lewis, A. J., Miller, P. S., Shurson, G. C. & Veum, T. L. (2001) Effects of pharmacological concentrations of zinc oxide with or without the inclusion of an antibacterial agent on nursery pig performance. J. Anim. Sci. 79: 934-941.

6. Stanger, B. R., Hill, G. M., Link, J. E., Turk, J. R., Carlson, M. S. & Rozeboom, D. W. (1998) Effects of high Zn on TGE-challenged, early weaned pigs. J. Anim. Sci. 76: 57 (abs).

7. Hill, G. M., Cromwell, G. L., Crenshaw, T. D., Dove, C. R., Ewan, R. C., Knabe, D. A., Lewis, A. J., Libal, G. W., Mahan, D. C., Shurson, G. C., Southern, L. L. & Venum, T. L. (2000) Growth promotion effects and plasma changes from feeding high dietary concentrations of zinc and copper to weanling pigs (regional study). J. Anim. Sci. 78: 1010-1016.

8. Carlson, M. S., Hoover, S. L., Hill, G. M., Link, J. E. & Turk, J. R. (1998) Effect of pharmacological zinc on intestinal metallothionein concentration and morphology in the nursery pig. J. Anim. Sci. 76: 57 (abs).

9. Martínez, M. M., Hill, G. M., Link, J. E., Raney, N. E., Tempelman, R. J. & Ernst, C. W. (2004) Pharmacological zinc and phytase supplementation enhance metallothionein mRNA abundance and protein concentration in newly weaned pigs. J. Nutr. 134: 538-544.

10. Palmiter, R. D. (2004) Protection against zinc toxicity by metallothionein and zinc transporter 1. Proc. Natl. Acad. Sci. U S A 101: 4918-4923.

11. Gaither, L. A. & Eide, D. J. (2001) Eukaryotic zinc transporters and their regulation. Biometals 14: 251-270.

12. Cousins, R. J. & McMahon, R. J. (2000) Integrative aspects of zinc transporters. J. Nutr. 130: 1384S-1387S.

13. Hamer, D. H. (1986) Metallothionein. Annu. Rev. Biochem. 55: 913-951.

14. McMahon, R. J. & Cousins, R. J. (1998) Regulation of the zinc transporter ZnT-1 by dietary zinc. Proc. Natl. Acad. Sci. USA 95: 4841-4846.

15. Andrews, G. K. (2000) Regulation of metallothionein gene expression by oxidative stress and metal ions. Biochem. Pharmacol. 59: 95-104.

16. Stuart, G. W., Searle, P. F., Cen, H. Y., Brinster, R. L. & Palmiter, R. D. (1984) A 12-base-pair DNA motif that is repeated several times in metallothionein gene promoters confers metal regulation to a heterologous gene. Proc. Natl. Acad. Sci. USA 81: 7318.

17. Langmade, S. J., Ravindra, R., Daniels, P. J. & Andrews, G. K. (2000) The transcription factor MTF-1 mediates metal regulation of the mouse ZnT1 gene. J. Biol. Chem. 275: 34803-34809.

18. Thiele, D. J. (1992) Metal-regulated transcription in eukaryotes. Nucleic Acids Res. 20: 1183-1191.

19. NRC (1998) Nutrient Requirements of Swine, 10th Ed. Washington, DC.

20. Kelleher, S. L. & Lönnerdal, B. (2002) Zinc transporters in the rat mammary gland respond to marginal zinc and vitamin A intakes during lactation. J. Nutr. 132: 3280-3285.

21. SAS Systems (1999-2000) The SAS System for Windows 98, 8.1 Ed. SAS Institute, Inc., Cary, N.C.

22. Satterthwaite, F. E. (1946) An approximate distribution of estimates of variance components. Biom. Bull. 2: 110-114.

23. Poehlman, E. T. (2003) Reduced metabolic rate after caloric restriction-can we agree on how to normalize the data? J. Clin. Endocrinol. Metab. 88: 14-15.

24. Scheffé, H. (1956) A method for judging all contrasts in an analysis of variance. Biometrika 40: 87-104.

25. Huang, P., Steplock, D., Weinman, E. J., Hall, R. A., Ding, Z., Li, J., Wang, Y. & Liu-Chen, L.-Y. (2004) {kappa} Opioid receptor interacts with Na+/H+-exchanger regulatory factor-1/Ezrin-Radixin-Moesin-binding phosphoprotein-50 (NHERF-1/EBP50) to stimulate Na+/H+ exchange independent of Gi/Go proteins. J. Biol. Chem. 279: 25002-25009.

26. Palmiter, R. D. & Huang, L. (2004) Efflux and compartmentalization of zinc by members of the SLC30 family of solute carriers. Pflugers Arch. 447: 744-751.

27. Davis, S. R., McMahon, R. J. & Cousins, R. J. (1998) Metallothionein knockout and transgenic mice exhibit altered intestinal processing of zinc with uniform zinc-dependent zinc transporter-1 expression. J. Nutr. 128: 825-831.

28. Case, C. L. & Carlson, M. S. (2002) Effect of feeding organic and inorganic sources of additional zinc on growth performance and zinc balance in nursery pigs. J. Anim. Sci. 80: 1917-1924.

29. Watjen, W., Benters, J., Haase, H., Schwede, F., Jastorff, B. & Beyersmann, D. (2001) Zn2+ and Cd2+ increase the cyclic GMP level in PC12 cells by inhibition of the cyclic nucleotide phosphodiesterase. Toxicology 157: 167-175.

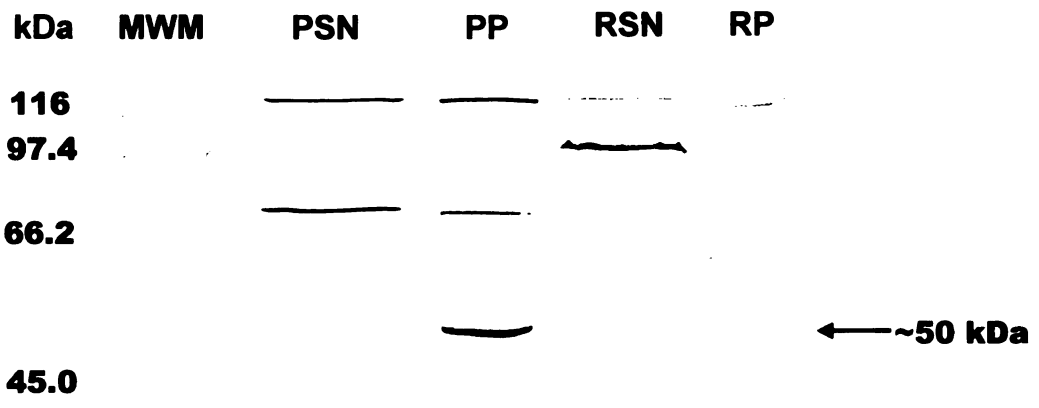

Figure 1. Detection of ZnT-1 in two protein fractions. The transporter was absent in the soluble protein fraction (supernatant) and identified in the crude membrane fraction (pellet) in liver homogenates of pigs and rat. Soluble or membrane protein fractions (50 μg) from pig and rat livers were resolved in 10% SDS/PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes. PSN, pig supernatant; PP, pig pellet; RSN, rat supernatant; RP, rat pellet. Molecular weight markers (MWM) are shown on the left. Location of ZnT-1 is shown by an arrow.

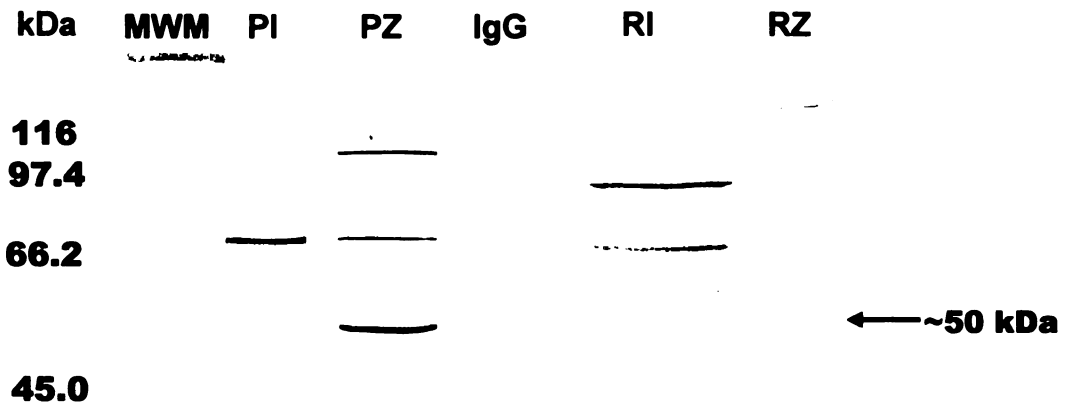

Figure 2. Specificity of rabbit anti-rat ZnT-1 antibody. Membrane protein fractions (50 μg) from pig and rat livers were resolved in 10% SDS/PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes. PI, pig sample incubated with rabbit preimmune serum, 1:1000 dilution; PZ, pig sample incubated with ZnT-1, 1:1000 dilution; IgG, total IgG fraction (no 1° antibody incubation), 1:500 dilution; RI, rat sample incubated with rabbit preimmune serum, 1:1000 dilution; RZ, rat sample incubated with ZnT-1 antibody, 1:1000 dilution. Blots were incubated with a 2° antibody-alkaline phosphatase conjugate, and then visualized with an alkaline phosphatase substrate. Molecular weight markers (MWM) are shown on the left. Location of ZnT-1 is shown by an arrow.

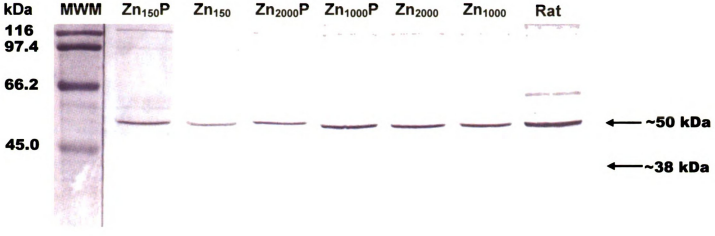

Figure 3A. Expression of ZnT-1 protein in liver homogenates of pigs fed dietary Zn (150, 1000 or 2000 mg Zn/kg) with or without supplemental phytase (P) and in liver of an adult rat. Equal amounts of membrane protein (50 μg) from each animal were used for western blot analysis. A representative blot incubated with rabbit anti-rat ZnT-1 is shown. Molecular weight markers (MWM) are shown on the left. Location of ZnT-1 (~38 and 50 kDa), is shown by arrows.

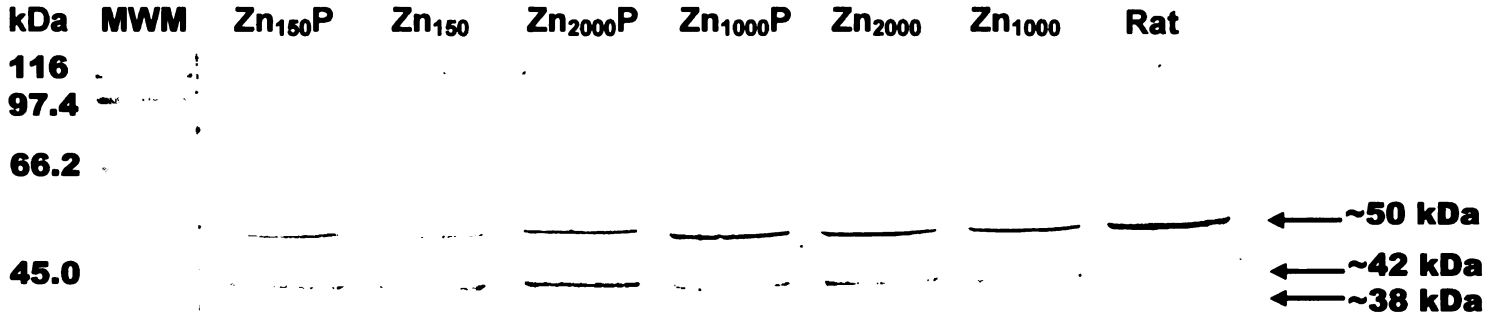

Figure 3B. Expression of ZnT-1 and β-actin proteins in liver homogenates of pigs fed dietary Zn (150, 1000 or 2000 mg Zn/kg) with or without supplemental phytase (P) and in liver of an adult rat. Equal amounts of membrane protein (50 μg) from each animal were used for western blot analysis. A representative blot incubated with rabbit anti-rat ZnT-1 and rabbit anti-mouse β-actin as described in Materials and Methods is shown. Molecular weight markers (MWM) are shown on the left. Locations of ZnT-1 (~38 and 50 kDa), and β-actin (~42 kDa) are shown by arrows.

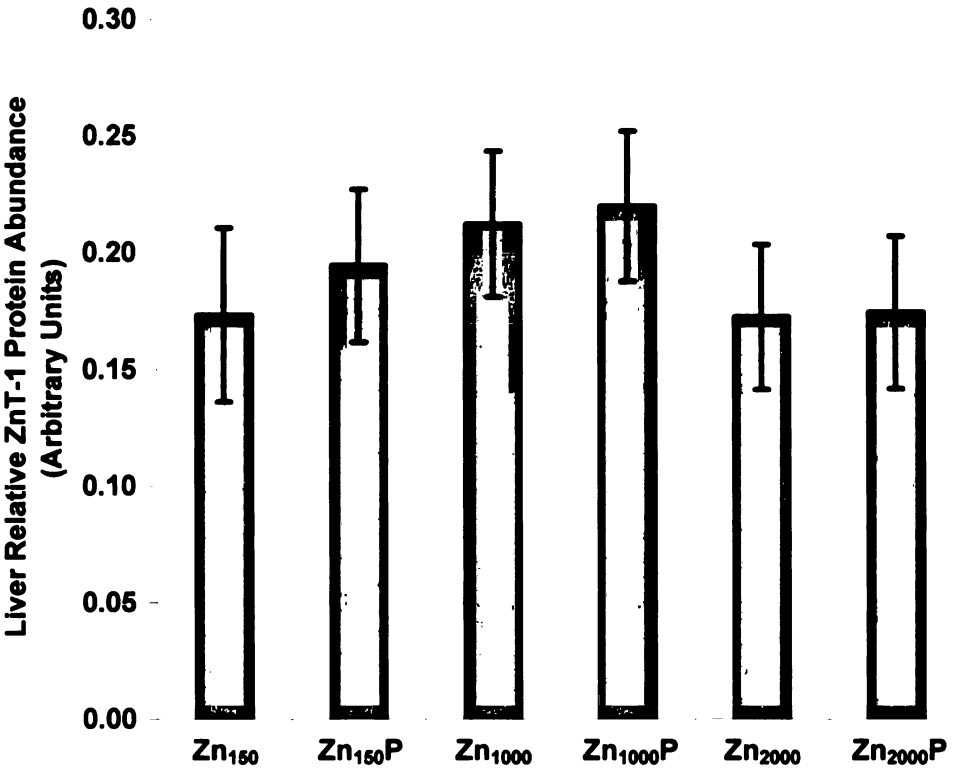

Figure 3C. Relative ZnT-1 protein abundance in liver homogenates of pigs fed dietary Zn (150, 1000 or 2000 mg Zn/kg) with or without supplemental phytase. Equal amounts of membrane protein (50 μg) from each animal were used for western blot analysis. Densitometric analysis of relative hepatic ZnT-1 protein abundance was performed. Values are backtransformed means with 95% CI, n = 4. A significant zinc effect was detected, $P < 0.02$.

CHAPTER FOUR

Dietary Pharmacological or Excess Zinc and Phytase Effects on Tissue Mineral Concentrations, Metallothionein, and Apparent Mineral Retention in the Newly Weaned Pig

M. M. Martínez, J.E. Link and G.M. Hill

Department of Animal Science, Michigan State University,

2209 Anthony Hall, East Lansing, MI 48824

Running title: High Zinc & Phytase on Tissue Minerals & MT in Pigs

ABSTRACT

Feeding pharmacological zinc (Zn) to weaned pigs improves growth, and dietary phytase improves phosphorus and Zn availability. Metallothionein (MT) increases in the duodenum, kidney and liver of pigs fed 1,000 mg Zn/kg with phytase or 2,000 mg Zn/kg with or without phytase when fed for 14 d post-weaning. The goal of this study was to determine the effects of feeding pharmacological Zn and phytase on tissue minerals, MT, mineral excretion and apparent retention. Twenty-four newly-weaned pigs (20 d, 7.2 kg) were individually fed twice daily, a basal diet supplemented with 0, 1,000 or 4,000 mg Zn/kg as Zn oxide, without or with phytase (500 FTU/kg) for 14 d, followed by a basal diet (100 mg Zn/kg) without phytase for 7 d. Pigs fed 4,000 mg Zn/kg without phytase had higher (p = 0.01) plasma, hepatic, renal Zn, renal Cu, and hepatic, renal, and jejunal MT than pigs fed the basal diet or 1,000 mg Zn/kg. Duodenal MT was higher (p = 0.0001) in pigs fed 1,000 and 4,000 mg Zn/kg than in pigs fed the unsupplemented diets. In pigs fed 1,000 and 4,000 mg Zn/kg, Zn loading occurred during the first 11 d of supplementation; by d 14 excess Zn was being excreted in the feces.

Index Entries: apparent retention, metallothionein, mineral excretion, pharmacological zinc, phytase, nursery pig

INTRODUCTION

The U.S. swine industry adds pharmacological (1,500 to 3,000 mg Zn/kg) zinc (Zn) as zinc oxide (ZnO) to post-weaning diets to enhance growth (1, 2) and improve fecal consistency (3). The National Research Council estimates that a 5 to 10 kg pig requires 100 mg Zn/kg (4). Supplementing Zn at 30 times (3,000 mg Zn/kg) its requirement has been shown by our laboratory to increase villous height and reduce crypt depth, potentially increasing intestinal absorptive capacity (5). The effects of even higher dietary Zn concentrations in the newly weaned pig on tissue accretion and Zn excretion have not been investigated.

Phytate phosphorus (P) found in cereal grains is known to complex with cations reducing their bioavailability (6-8). Because non-ruminants produce negligible amounts of phytase (9), exogenous supplementation of phytase is an alternative for enhancing mineral availability (10), and retention (11, 12).

Metallothionein (MT) is a cysteine rich protein that is involved in Zn and copper (Cu) homeostasis, transfer and storage (13, 14), and is considered an intracellular Zn marker when Zn is fed in excess (15). Our laboratory has shown that feeding pharmacological Zn (1,000 and 2,000 mg Zn/kg) with 500 phytase units (FTU)/kg of phytase (Natuphos®, BASF Corp., Mount Olive, NJ) or 2,000 mg Zn/kg without phytase for 14 d post-weaning increases kidney Cu, liver Zn, and MT mRNA and protein concentrations in the duodenum, kidney and liver (16). Liver, kidneys and intestinal mucosa play a key role in Zn homeostasis (17). No studies have focused on the effects of adequate and pharmacological Zn on MT concentration in jejunum in the young pig. Our laboratory fed 3,000

mg Zn/kg for 7, 14, or 28 d post-weaning and found elevated renal and hepatic Zn and MT protein concentrations (2). Tran et al. (15) fed 10, 100, 400 or 1,000 mg Zn/kg to rats for 7 d and demonstrated that Zn concentrations increased constantly from the stomach to the colon, with higher concentrations in rats fed 400 and 1,000 mg Zn/kg than in rats fed 10 or 100 mg Zn/kg. In addition, MT concentrations were higher in the duodenum of rats fed 400 and 1,000 mg Zn/kg compared with MT concentrations in the jejunum, ileum, caecum, and colon, and compared with rats fed the remaining treatments. However, Reeves et al. (18) reported that feeding 350 mg Zn/kg to rats initially increased intestinal mucosa, liver and kidney Zn and MT, until d 14 of supplementation and declined to concentrations similar to those of rats fed 50 mg Zn/kg by the end of the 42 d experiment. These results suggest that an adaptive mechanism exists which protects tissues when pharmacological Zn concentrations are fed. This adaptive mechanism may include MT and increased mineral excretion.

The objectives of this study were to determine the effects of feeding adequate (100 mg Zn/kg), pharmacological (1,000 mg Zn/kg) or excess (4,000 mg Zn/kg) Zn without or with phytase (500 FTU/kg) on tissue minerals [calcium (Ca), P, Cu, iron (Fe) and Zn], MT concentrations, mineral excretion and apparent retention.

MATERIALS AND METHODS

Animals, Diets and Measures

At weaning, 24 [(Landrace x Yorkshire) x Duroc] gilts and barrows (20 d of age, 7.2 kg) were blocked by weight, sex and litter and individually penned in stainless steel pens (1.40 m x 0.56 m) equipped with a stainless steel self-feeder and nipple waterer in an environmentally controlled room (25-30°C) for a 21 d study. This study was approved by the Michigan State University All University Committee on Animal Use and Care (AUCAUC, 12/99-159-00).

The six dietary treatments were fed in two phases (d 1 to 7 and 8 to 14) in a complete randomized block design with a 3 x 2 factorial arrangement of treatments, followed by a common diet from d 15 to 21(Table 1). The basal diet, fed in a meal form, was formulated to meet or exceed the recommendations of the National Research Council (4) and contained 100 mg Zn/kg provided as ZnO. The dietary treatments were: 1) adequate Zn diet with 100 mg Zn/kg (Zn_{100}), 2) Zn_{100} plus 500 FTU/kg ($Zn_{100}P$), 3) pharmacological Zn diet with 1,000 mg/kg added Zn ($Zn_{1,000}$), 4) $Zn_{1,000}$ plus 500 FTU/kg ($Zn_{1,000}P$), 5) excess Zn diet with 4,000 mg/kg added Zn ($Zn_{4,000}$), or 6) $Zn_{4,000}$ plus 500 FTU/kg ($Zn_{4,000}P$). The dietary phytase used in this experiment was Natuphos® (BASF Corp., Mount Olive, NJ). One phytase unit (FTU) is defined as the amount of enzyme that releases 1μmol inorganic phosphorus from sodium phytate per minute at pH 5.5 and 37°C. The Zn source was ZnO which contained 72% Zn (Prince Agri

Products, Quincy, IL). Pigs were fed to appetite twice daily with water available *ad libitum*.

During the study, feed waste, total feces, and urine were collected twice daily. Fecal samples were stored (-20°C) in whirl pack bags (NASCO, Modesto, CA). Urine was filtered through glass wool and volume was recorded. An aliquot was frozen (-20°C) in 50 mL screw cap polypropylene tubes (Corning Inc., Corning, NY). Body weight of pigs was measured weekly and ADG, ADFI and G:F were calculated.

Tissue Mineral and Metallothionein Analysis

On d-21, blood samples were collected in heparinized evacuated tubes (BD Vacutainer, Becton Dickson, Franklin Lakes, NJ) from the anterior vena cava. Pigs were then killed by intra-cardiac injection (0.22 mL/kg body weight) of sodium pentobarbital (392 g/L). Plasma was collected by centrifugation (2,000 x g, 10 min, 4°C, GS-6KR, Beckman Coulter, Palo Alto, CA) and frozen (-80°C) for later determination of plasma minerals (Ca, Cu, Fe, and Zn) by atomic absorption spectroscopy (Unicam 989, Thermo Electron Corp., Franklin, MA) as previously described (16). Plasma P was determined spectrophotometrically (DU 7400, Beckman Coulter, Palo Alto, CA) by the method of Gomori (19). Kidney and liver samples were excised and frozen (-80°C) for later analysis. Intestinal mucosa cells were collected and processed from the duodenum and jejunum according to the method described by Carlson et al. (2). Tissues were microwave-digested (MARS 5, CEM Corp., Matthews, NC) for mineral analysis as described by Shaw et al. (20). The MT protein concentration was determined by modification of the

silver saturation assay of Scheuhammer and Cherian (21) as described by Martínez et al. (16).

Fecal and Urine Analysis

Feces were oven (Isotemp 650G, Fisher Scientific, St. Louis, MO) dried at 80°C for 24 h, then ground (Thomas-Wiley Mill model ED-5, Thomas Co., Philadelphia, PA). They were further processed through a 1 mm screen in a Cyclotec 1093 Mill (Foss Tecator, Höganäs, Sweden). Fecal mineral concentrations were determined after microwave digestion as described above for tissues. Urine samples were thawed and centrifuged at 1,000 x g for 15 min prior to mineral analysis. They were diluted as needed for determination of the individual mineral elements.

All glassware used for mineral analyses was washed in 30% nitric acid and rinsed with double-deionized water. Instrument accuracy for all mineral analyses was established using bovine liver standard (1577b; NIST, Gaithersburg, MD).

Statistical analysis

Feed intake and performance of one pig fed the $Zn_{100}P$ diet was extremely low throughout the 21 d study. Residual and scatter plots (22) characterized it as an outlier, and its data was excluded from the experiment. Weight gain, feed intake, feed efficiency and apparent mineral retention were analyzed using the MIXED procedure of SAS (23) with repeated measures. The mixed model included the pig as random effect, while the fixed effects included zinc, phytase, their interaction, day as the repeated measures variable, and the subject of the

repeated measures specified as the pig within the dietary treatments. The following covariance structures for the repeated measurements were compared: compound symmetry, autoregressive order one, and heterogeneous autoregressive order one (24). The appropriate covariance structure was determined utilizing Akaike's information criterion (25). Apparent retention was calculated as mineral intake minus mineral in feces and urine. For fecal, urinary and retained Ca, P, Cu, and Fe values, their respective mineral intake was modeled as a covariate for a regression based-normalization (26). Apparent Ca, P, Cu, and Fe retention are expressed as least square means ± standard error.

For plasma and tissue mineral concentrations, the experimental unit was the individual pig nested within dietary treatments. Least square mean estimates were used as treatment means, and Bonferroni adjustments were performed as multiple comparison test. Residual diagnostic plots revealed that Zn concentration in the feces, urine, and tissues, as well as, Zn retention and tissue MT concentration required logarithmic transformation to improve normal distribution. Back-transformed means and their respective 95% CI (lower and upper limits) are given in tables and figures. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Growth Performance

Pharmacological Zn and supplemental phytase did not affect (p = 0.66) ADG or ADFI during the 21 d study (data not shown). There was a significant Zn and phytase interaction (p = 0.04) on feed efficiency during d 15-21 (data not shown). The pigs may not have responded to pharmacological Zn concentrations because of their high health status, the small number of pigs per treatment (n = 4) or because newly weaned pigs were fed and kept in individual stainless steel pens. Our results are in agreement with Schell and Kornegay (27), Smith et al., (1), and Hill and Miller (28) where 1,000, 3,000 or 5,000 mg Zn/kg respectively, did not improve growth parameters and with studies reported by Augspurger et al. (29) and Stahl et al. (30), where phytase supplementation did not improve growth.

Plasma, Kidney and Liver Mineral Analysis

Feeding newly weaned pigs $Zn_{4,000}$ resulted in higher plasma Zn (p = 0.01), and kidney Zn (p = 0.01) concentrations than when pigs were fed Zn_{100} or $Zn_{1,000}$; no phytase effect was detected (Table 2). Lei et al. (10) reported that feeding 1,350 FTU/kg to pigs increased plasma Zn. Others have observed that feeding 3,000 mg Zn/kg resulted in higher plasma and kidney Zn (2, 31, 32). There was a significant Zn and phytase interaction (p = 0.01) on hepatic Zn concentrations (Figure 1), which is in agreement with previous reports from our laboratory where Zn and phytase interacted when pigs were fed 150, 1,000 or 2,000 mg Zn/kg with or without phytase for 14 d post-weaning (16). This

interaction resulted in pigs fed $Zn_{1,000}P$ having a greater hepatic Zn concentrations than pigs fed Zn_{100}, $Zn_{100}P$ or $Zn_{1,000}$. Pigs fed $Zn_{4,000}$ and $Zn_{4,000}P$ had similar hepatic Zn concentrations that were greater than liver Zn of pigs fed the other diets. As with our previous work (16) feeding phytase caused an increase in hepatic Zn concentrations with 1,000 mg Zn/kg, but not with 2,000 mg Zn/kg (16) or 4,000 mg Zn/kg (present study). This finding may be due to the 4,000 mg Zn/kg diet masking the effect of phytase or reducing its usefulness. Also, when Zn is fed in excess of requirement with or without phytase, its concentration remains elevated in plasma and storage organs even when dietary Zn concentration was decreased 7 d before the pigs were killed indicating that Zn from the diet (100 mg Zn/kg) is adequate to meet the pigs needs and that the tissue concentrations are not toxic to these young animals.

Supplementing Zn or phytase did not affect Ca, P, Cu or Fe in plasma, or Ca, P, or Fe concentrations in tissues (Table 3). Similarly, Adeola et al. (11) found no change in plasma Ca or Cu concentrations after feeding 1,500 FTU/kg of phytase to pigs for 21 d post-weaning. Murry et al. (33) found that the addition of phytase (700 or 1,000 FTU/kg) to adequate P diets for 35 d post-weaning did not affect serum Ca, P or Cu concentrations. In contrast to our results, Meyer et al. (34) found that feeding 2,000 or 3,000 mg Zn/kg to 22 old pigs for 21 d post-weaning decreased liver Cu. In the present study, pigs fed $Zn_{4,000}$ had greater ($p = 0.01$) kidney Cu concentration than pigs fed Zn_{100} or $Zn_{1,000}$, independent of phytase supplementation. Similar results were observed by Carlson et al. (2), after 28 d of supplementing 3,000 mg Zn/kg, and Hill et al. (28) when feeding

5,000 mg Zn/kg to gilts and sows for two parities. The increase in renal Cu may be due to MT sequestering Cu resulting in increased urinary Cu excretion, which we observed (data not shown).

Tissue Metallothionein Protein Analysis

The MT response in duodenum and jejunum differed. Duodenal MT was greater (p = 0.0001) in pigs fed $Zn_{1,000}$ and $Zn_{4,000}$ compared with pigs fed Zn_{100} (Figure 2A). However, in the jejunum pigs fed $Zn_{4,000}$ had greater (p = 0.009) MT concentrations than pigs fed the remaining treatments (Figure 2B). Furthermore, the jejunal MT concentrations were less than those observed in the duodenum, regardless of phytase addition. Carlson et al. (2) reported that feeding 3,000 mg Zn/kg for 14 d post-weaning resulted in increased duodenum MT compared to pigs fed 100 mg Zn/kg. In mice, MT concentrations were greater in the duodenum when 400 mg Zn/kg was fed compared with MT in remaining segments of the intestine (35), and with mice fed 10 and 150 mg Zn/kg. In addition, feeding 400 mg Zn/kg or 1,000 mg Zn/kg to rats for 7 d increased duodenal MT compared to jejunal, ileal, caecal and colon MT concentrations (15). Furthermore, MT concentrations were greater in rats fed 400 and 1,000 mg Zn/kg than those fed 10 and 100 mg Zn/kg. These data suggest that pigs fed 1,000 and 4,000 mg Zn/kg have an increased need to synthesize duodenal MT to sequester and/or transfer Zn into portal circulation. However, after Zn uptake by the duodenum, the cells of the jejunum are not stimulated to synthesize as much MT when fed 100 or 1,000 mg Zn/kg as when 4,000 mg Zn/kg is fed indicating that the Zn concentration in the jejunum of pigs fed 4,000 mg Zn/kg was greater

and stimulated the production of MT. This indicates that the duodenum and jejunum respond independently to Zn concentrations. The increased MT sequesters excess Zn, which can be excreted in sloughed mucosal cells (17).

Similar to kidney Zn, MT was greater (p = 0.01) in pigs fed $Zn_{4,000}$ than in pigs fed the other treatments (Figure 2C). However in the liver (Figure 2D), pigs fed $Zn_{1,000}$ had greater (p = 0.01) MT protein concentration than pigs fed Zn_{100}, while those fed $Zn_{4,000}$ had greater liver MT than pigs fed Zn_{100} and $Zn_{1,000}$. Metallothionein concentration was not affected by phytase in any other tissue. Our renal and hepatic MT results are in agreement with other studies where MT responds to the amount of Zn fed in the diet (2, 36). We have also shown that pharmacological Zn (2,000 mg Zn/kg) increased kidney and liver MT mRNA as well as protein concentration in pigs 14 d post-weaning, and these values were positively correlated with their respective tissue Zn concentrations (16). The present study suggests that after removing pharmacological or excess dietary Zn, endogenous Zn continues to stimulate MT production in the duodenum and/or jejunum, and that liver MT continues to sequester and manage excess Zn.

Fecal, Urinary and Apparent Zn Retained

Intake, fecal, urinary and retained Zn during phase I (d 1-7), II (d 8-14), III (d 15-21) and overall (21 d) are shown in Table 4. During phase I and II, Zn in feces, urine and that retained increased as the concentration of Zn in the diet increased, with no effect of supplemental phytase. In this study, pigs fed 1,000 and 4,000 mg Zn/kg for 14 d, excreted 12.2 (292 mg/d) and 43.5 (1,046 mg/d) times more fecal Zn respectively, than pigs fed 100 mg Zn/kg (24 mg/d). The

greater Zn retention of pigs fed diets that exceed NRC recommendations ($Zn_{1,000}$ and $Zn_{4,000}$) is due to Zn loading of tissues prior to increased fecal and urinary Zn excretion. This is consistent with results of previous studies (32, 34, 37).

During phase III after pharmacological and excess Zn feeding was discontinued and all pigs were fed 100 mg Zn/kg, pigs previously fed 1,000 and 4,000 mg Zn/kg diets continued to excrete more urinary and fecal Zn than pigs fed 100 mg Zn/kg; pigs fed 4,000 mg Zn/kg were in negative balance. Pigs fed the NRC requirement (Zn_{100}) excreted a consistent amount of fecal and urinary Zn throughout the 21 d of the study, maintaining their balance thus indicating that Zn was not in excess but adequate. Case and Carlson (32) fed pharmacological Zn (3,000 mg Zn/kg) to 17 d old pigs for 10 d prior to fecal collections. Their pigs were in negative balance, most likely due to Zn loading occurring for 10 d prior to collection which was similar to what we observed after 11 d of dietary intervention (data not shown).

Overall, fecal Zn excretion was greater ($p = 0.01$) for pigs fed $Zn_{4,000}$, than pigs fed $Zn_{1,000}$ and Zn_{100} (Table 4). However, there was a significant Zn by phytase interaction ($p = 0.03$) on urinary Zn excretion, where pigs fed $Zn_{4,000}$ and $Zn_{4,000}P$ had greater urinary Zn excretion compared with pigs fed $Zn_{1,000}P$, but not $Zn_{1,000}$. Zinc intake and retention was greater ($p = 0.01$) for pigs fed $Zn_{4,000}$ and $Zn_{4,000}P$ compared with pigs fed the remaining diets.

Daily Zn Fecal Excretion and Retention Patterns

We are the first to report day by day fecal Zn excretion (Figure 3) starting on d 1 after weaning. Fecal Zn excretion for all treatments was similar for d 1 to

5. On d 6, pigs fed $Zn_{4,000}$ excreted 3 times more Zn than pigs fed $Zn_{1,000}$ and about 10 times more than pigs fed Zn_{100}. From d 8-14, the elevated Zn excretion pattern plateau. In phase III, when dietary Zn was fed at 100 mg Zn/kg, within 3 d all pigs had comparable fecal Zn excretion values averaging 55 mg/d, (Figure 3). These data confirm that when Zn is fed at a greater concentration than its requirement, Zn loads into tissues during the first 11 d of supplementation before increased Zn excretion occurs. This pattern is absent in pigs fed Zn at their requirement (100 mg Zn/kg) for 21 d post-weaning. According to our data, if $Zn_{1,000}$ or $Zn_{4,000}$ were fed for only 11 d instead of 14 d, fecal Zn excretion would be reduced by 50% (168 mg/d and 578 mg/d, respectively).

Mineral (Ca, P, Cu, Fe) Apparent Retention

Apparent Ca, P, Cu, and Fe retention are presented for phase I (d 1-7), II (d 8-14), III (d 15-21) and overall (d 1-21) in Table 5. Supplementing phytase increased ($p = 0.05$) Ca retention throughout the entire study, and enhanced P ($p = 0.02$) apparent retention, during phase II, III and overall. These results are consistent with those of Murry et al. (33) who fed weanling pigs phytase (0, 700 or 1,000 FTU/kg) and adequate P diets and reported increased P retention and a tendency for increased Ca retention. Lei et al. (38) showed that Ca retained as a percent of intake was increased with supplemental phytase. While retention was improved, Ca and P concentrations were not increased in kidney, liver and plasma. Perhaps the retained Ca and P was present in bone tissue, which was not measured in this study. The retention of Cu and Fe was not affected by feeding phytase.

Feeding $Zn_{4,000}$ increased urinary and decreased fecal Cu (data not shown) resulting in increased Cu retention ($p = 0.05$). Similarly, Carlson et al. (37) showed that pigs fed 3,000 mg Zn/kg as ZnO retained more Cu. However, Meyer et al. (34) showed decreased Cu and Fe retention due to greater fecal Cu and Fe excretion in pigs fed 3,000 mg Zn/kg for 21 d. Our results of increased urinary Cu could be associated with the increased renal Cu concentrations (Table 3) observed in pigs fed $Zn_{4,000}$, confirming an increased urinary Cu output as a result of feeding high Zn.

Phytase supplementation and pharmacological Zn improved Ca, P, Cu and Zn retention with no interaction. It is unclear why phytase did not respond with Zn in an additive manner. *In vitro* studies by Maenz et al. (39) showed that at pH 7 and a Zn to phytic acid molar ratio of 0.35:1, phytate-P hydrolysis by phytase was reduced by 50%. It was proposed by these authors that two phytic acid molecules binding Zn, may bridge together, causing a conformational change in the phytic acid molecule that is inaccessible for phytase hydrolysis. However, in this study Ca and P retention were improved by phytase supplementation, an indication that some hydrolysis indeed occurred.

Our study results suggest that supplementing phytase (500 FTU/kg) plus 1,000 or 4,000 mg Zn/kg increases liver Zn but does not affect Ca, P, Cu or Fe in tissues, MT or reduce Zn excretion. Feeding 4,000 mg Zn/kg as ZnO to newly weaned pigs for 14 d, does not produce toxic effects. Supplementing dietary Zn at 10 or 40 times the Zn requirement resulted in an adaptive response, characterized by increased MT concentrations in the intestinal mucosa, liver and

kidney, which persist 7 d after pharmacological or excess Zn are withdrawn. Daily fecal Zn excretion plot confirms that Zn loading occurs by d 11 of feeding pharmacological or excess Zn, suggesting a shorter supplementation period (11 d) may be effective in reducing Zn excretion by 50% thus reducing environmental concerns about feeding pharmacological Zn as a growth stimulant in the swine industry.

ACKNOWLEDGEMENTS

This work was supported in part by a Cooperative State Research Education and Extension Service – Initiative for Future Agriculture and Food Systems grant from the United States Department of Agriculture, and by The Graduate School – Competitive Doctoral Enrichment Fellowship, Michigan State University, East Lansing, MI. The authors express their appreciation to BASF Corporation, Prince Agri Products, American Protein Corporation, and International Ingredient Corporation for supplying dietary ingredients used in this study.

LITERATURE CITED

1. Smith, J. W., Tokach, M. D., Goodband, R. D., Nelssen, J. L. and Richert, B. T., Effects of the interrelationship between zinc oxide and copper sulfate on growth performance of early-weaned pigs. *J. Anim. Sci.* 75, 1861-1866 (1997).

2. Carlson, M. S., Hill, G. M. and Link, J. E., Early and traditionally weaned nursery pigs benefit form phase-feeding pharmacological concentrations of zinc oxide: effect on metallothionein and mineral concentrations. *J. Anim. Sci.* 77, 1199-1207 (1999).

3. McCully, G. A., Hill, G. M., Link, J. E., Weavers, R. L., Carlson, M. S. and Rozeboom, D. W., Evaluation of zinc sources for the newly weaned pig. *J. Anim. Sci.* 73 (Supplement 2), 72 (1995).

4. NRC, *Nutrient Requirements of Swine*, 10th, Washington, DC (1998).

5. Carlson, M. S., Hoover, S. L., Hill, G. M., Link, J. E. and Turk, J. R., Effect of pharmacological zinc on intestinal metallothionein concentration and morphology in the nursery pig. *J. Anim. Sci.* 76, 57 (1998).

6. O'Dell, B. L. and Savage, J. E., Effect of phytic acid on zinc availability. *Proc. Soc. Exp. Biol. Med.* 103, 304-305 (1960).

7. Oberleas, D., Muhrer, M. E. and O'Dell, B. L., Dietary metal-complexing agents and zinc availability in rats. *J. Nutr.* 90, 56-62 (1966).

8. Davies, N. T. and Nightingale, R., The effects of phytate on intestinal absorption and secretion of zinc, and whole-body retention of zinc, copper, iron and manganese in rats. *Br. J. Nutr.* 34, 243-258 (1975).

9. Jongbloed, A. W., Mroz, Z. and Kemme, P. A., The effect of supplementary *Aspergillus niger* phytase in diets for pigs on concentration and apparent digestibility of dry matter, total phosphorus, and phytic acid in different sections of the alimentary tract. *J. Anim. Sci.* 70, 1159-1168 (1992).

10. Lei, X., Ku, P. K., Miller, E. W., Ullrey, D. E. and Yokoyama, M., Supplemental microbial phytase improves bioavailability of dietary zinc to weanling pigs. *J. Nutr.* 123, 1117-1123 (1993).

11. Adeola, O., Lawrence, B., Sutton, A. and Cline, T., Phytase-induced changes in mineral utilization in zinc-supplemented diets for pigs. *J. Anim. Sci.* 73, 3384-3391 (1995).

12. Yi, Z., Kornegay, E. T., Ravindran, V., Lindemann, M. D. and Wilson, J. H., Effectiveness of Natuphos phytase in improving the bioavailabilities of phosphorous and other nutrients in soybean meal-based semipurified diets for young pigs. *J. Anim Sci.* 74, 1601-1611 (1996).

13. Bremner, I., Metallothionein III. Biological Roles and Medical Implications, in K. T. Suzuki, N. Imura and M. Kimura, Birkhauser, Basel, Switzerland, pp. 111-124 (1993).

14. Davis, S. R., McMahon, R. J. and Cousins, R. J., Metallothionein knockout and transgenic mice exhibit altered intestinal processing of zinc with uniform zinc-dependent zinc transporter-1 expression. *J. Nutr.* 128, 825-831 (1998).

15. Tran, C. D., Butler, R. N., Howarth, G. S., Philcox, J. C., Rofe, A. M. and Coyle, P., Regional distribution and localization of zinc and metallothionein in the intestine of rats fed diets differing in zinc content. *Scand. J. Gastroenterol.* 34, 689-695 (1999).

16. Martínez, M. M., Hill, G. M., Link, J. E., Raney, N. E., Tempelman, R. J. and Ernst, C. W., Pharmacological zinc and phytase supplementation enhance metallothionein mRNA abundance and protein concentration in newly weaned pigs. *J. Nutr.* 134, 538-544 (2004).

17. Cousins, R. J., Absorption, transport and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin. *Physiol. Rev.* 65, 238-309 (1985).

18. Reeves, P. G., Adaptation responses in rats to long-term feeding of high-zinc diets: emphasis on intestinal metallothionein. *J. Nutr. Biochem.* 6, 48-54 (1995).

19. Gomori, G., A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. *J. Clin. Lab. Med.* 27, 955-960 (1942).

20. Shaw, D. T., Rozeboom, D. W., Hill, G. M., Booren, A. M. and Link, J. E., Impact of vitamin and mineral supplement withdrawal and wheat middling inclusion on finishing pig growth performance, fecal mineral concentration, carcass characteristics, and the nutrient content and oxidative stability of pork. *J. Anim. Sci.* 80, 2920-2930 (2002).

21. Scheuhammer, A. M. and Cherian, M. G., Quantification of metallothionein by silver saturation, in *Methods in Enzymology. Metallobiochemistry. Part B. Metallothionein and related molecules*, 205, J. F. Riordan and B. L. Vallee, Academic Press, Inc, San Diego, Ca, pp. 78-83 (1986).

22. Ott, R. L., *An Introduction to Statistical Methods and Data Analysis*, 4th ed., Duxbury Press, Belmont, California (1993).

23 SAS Systems (1999-2000) The SAS System for Windows 98, 8.1 ed. SAS Institute, Inc., Cary, N.C.

24. Littell, R. C., Henry, P. R. and Ammerman, C. B., Statistical analysis of repeated measures data using SAS procedures. *J. Anim Sci.* 76, 1216-1231 (1998).

25. Akaike, H., Fitting autoregressive models for prediction. *Ann. Inst. Stat. Math.* 21, 243-247 (1969).

26. Poehlman, E. T., Reduced metabolic rate after caloric restriction-can we agree on how to normalize the data? *J. Clin. Endocrinol. Metab.* 88, 14-15 (2003).

27. Schell, T. C. and Kornegay, E. T., Zinc concentration in tissues and performance of weanling pigs fed pharmacological levels of zinc from ZnO, Zn-methionine, Zn-lysine, or $ZnSO_4$. *J. Anim. Sci.* 74, 1584-1593 (1996).

28. Hill, G. M. and Miller, E. R., Effect of dietary zinc levels on the growth and development of the gilt. *J. Anim Sci.* 57, 106-113 (1983).

29. Augspurger, N. R., Spencer, J. D., Webel, D. M. and Baker, D. H., Pharmacological zinc levels reduce the phosphorus-releasing efficacy of phytase in young pigs and chickens. *J. Anim Sci.* 82, 1732-1739 (2004).

30. Stahl, C. H., Roneker, K. R., Pond, W. G. and Lei, X. G., Effects of combining three fungal phytases with a bacterial phytase on plasma phosphorus status of weanling pigs fed a corn-soy diet. *J. Anim Sci.* 82, 1725-1731 (2004).

31. Hill, G. M., Miller, E. R. and Stowe, H. D., Effect of dietary zinc level on health and productivity of gilts and sows through two parities. *J. Anim Sci.* 57, 114-122 (1983).

32. Case, C. L. and Carlson, M. S., Effect of feeding organic and inorganic sources of additional zinc on growth performance and zinc balance in nursery pigs. *J. Anim. Sci.* 80, 1917-1924 (2002).

33. Murry, A. C., Lewis, R. D. and Amos, H. E., The effect of microbial phytase in a pearl millet-soybean meal diet on apparent digestibility and retention of nutrients, serum mineral concentration, and bone mineral density of nursery pigs. *J. Anim Sci.* 75, 1284-1291 (1997).

34. Meyer, T. A., Lindemann, M. D., Cromwell, G. L., Monegue, H. J. and Inocencio, N., Effects of pharmacological levels of zinc as zinc oxide on fecal zinc and mineral excretion in weanling pigs. *Prof. Anim. Sci.* 18, 162-168 (2002).

35. Tran, C. D., Butler, R. N., Philcox, J. C., Rofe, A. M., Howarth, G. S. and Coyle, P., Regional distribution of metallothionein and zinc in the mouse gut. Comparison with metallothionein-null mice. *Biol. Trace. Elem. Res.* 1998, 239-251 (1998).

36. Blalock, T. L., Dunn, M. A. and Cousins, R. J., Metallothionein gene expression in rats: tissue-specific regulation by dietary copper and zinc. *J. Nutr.* 118, 222-228 (1988).

37. Carlson, M. S., Boren, C. A., Wu, C., Huntington, C. E., Bollinger, D. W. and Veum, T. L., Evaluation of various inclusion rates of organic zinc either as polysaccharide or proteinate complex on the growth performance, plasma, and excretion of nursery pigs. *J. Anim Sci.* 82, 1359-1366 (2004).

38. Lei, X. G., Ku, P. K., Miller, E. R. and Yokoyama, M. T., Supplementing corn-soybean meal diets with microbial phytase linearly improves phytate phosphorus utilization by weanling pigs. *J. Anim Sci.* 71, 3359-3367 (1993).

39. Maenz, D. D., Engele-Schaan, C. M., Newkirk, R. W. and Classen, H. L., The effect of minerals and mineral chelators on the formation of phytase-resistant and phytase-susceptible forms of phytic acid in solution and in a slurry of canola meal. *Anim. Feed. Sci. Tech.* 81, 177-192 (1999).

Table 1

Composition of experimental diets for nursery pigs (g / 100 g diet)[1]

Ingredients	Phase I	Phase II	Phase III
Corn	39.09	53.14	54.75
Soybean meal (44% CP)	20.20	20.18	23.75
Spray-dried whey	20.00	20.00	10.00
Plasma protein[2]	6.00	———	———
Lactose[3]	10.00	———	———
Blood cells[2]	———	2.00	———
Menhaden fish meal	———	———	5.00
DL-Methionine	0.11	0.06	———
L-LysineHCL	0.15	0.15	0.15
Choice White Grease[4]	———	———	3.00
Soybean oil	1.00	1.00	———
Monocalcium Phosphate[5]	1.45	1.52	1.35
Ground limestone	0.55	0.52	0.60
Salt	0.35	0.33	0.30
Trace mineral pre-mix[6]	0.50	0.50	0.50
Vitamin pre-mix[7]	0.60	0.60	0.60

Dietary treatments	Analyzed total zinc concentration (as fed), mg/kg diet		
Zn_{100} = (100 mg/kg Zn, ZnO)	130.14	129.40	117.73
$Zn_{1,000}$ = (Zn_{100} + 1,000 mg Zn/kg)	1,281.46	1,114.30	
$Zn_{4,000}$ = (Zn_{100} + 4,000 mg Zn/kg)	4,654.90	3,913.15	
$Zn_{100}P$ = (Zn_{100} + 500 FTU/kg)	130.30	131.84	
$Zn_{1,000}P$= (Zn_{100} + 500 FTU/kg + 1,000 mg Zn/kg)	1,226.48	1,110.05	
$Zn_{4,000}P$ = (Zn_{100} + 500 FTU/kg + 4,000 mg Zn/kg)	4,841.74	4,044.98	

[1]The basal diet was formulated to meet or exceed nutrient needs (4). Phase I (d 0-7), Phase II (d 8-14), Phase III (d 15-21). Zinc as zinc oxide was added at the expense of corn.

[2]American Protein Corp., Inc (AP 920 - sprayed dried plasma, AP 301G - granulated sprayed dried animal blood cells)

[3]International Ingredients Corp.

[4]Titre = 36°C min., 4% max. free fatty acids, 1% MIU max.

[5]Monocalcium phosphate = 21% elemental P

[6]Trace Mineral pre-mix (g mineral/kg premix): $CuSO_4 \cdot 5H_20$, 2; $FeSO_4 \cdot H_20$, 20; $C_2H_4(NH_2)_2 . 2HI$, 0.03; $MnSO_4 \cdot H_20$, 2; Na_2SeO_3, 0.06; ZnO, 20.

[7]Vitamin pre-mix (IU/kg premix): vitamin A, 91,8562; vitamin D, 91,865; vitamin E, 11,022; (g vitamin /kg premix): vitamin K, 2.20; niacin, 4.40; riboflavin, 0.73; panthotenic acid, 2.94; thiamine, 0.18; vitamin B_6, 0.17; vitamin B_{12}, 5.50.

Table 2

Effects of adequate, pharmacological or excess zinc with or without supplemental phytase on plasma and kidney Zn concentrations[1,2,3].

Phytase, FTU/kg	0			500			*p* value		
Zinc, mg/kg	100	1,000	4,000	100	1,000	4,000	Zn	Phy	Zn*Phy
Zinc									
Plasma (mg/L)	0.91 (0.78 - 1.06)	0.91 (0.78 - 1.06)	1.34 (1.15 - 1.55)	0.96 (0.81 - 1.14)	1.13 (0.98 - 1.32)	1.45 (1.25 - 1.69)	0.01	0.06	0.49
Kidney (ug/g)	24.0 (19.3 - 29.9)	25.8 (20.7 - 32.1)	52.4 (42.1 - 65.4)	23.4 (18.2 - 30.3)	31.4 (25.3 - 39.3)	64.8 (51.9 - 80.6)	0.01	0.16	0.49

[1]Twenty three pigs with an average initial weight of 5.5 kg were housed in individual stainless steel pens and were used for a 21 d study.

[2] All pigs were fed dietary treatments for d 1-14, and a common diet for d 15-21.

[3]The values presented are back-transformed means (lower and upper limits of the 95% CI), n=4 per treatment (except $Zn_{100}P$, n=3).

Table 3

Effects of adequate, pharmacological or excess zinc with or without supplemental phytase on plasma, kidney and liver Ca, P, Cu and Fe concentrations.[1,2,3]

Phytase, FTU/kg	0			500				*p* value		
Zinc, mg/kg	100	1,000	4,000	100	1,000	4,000	SEM[4]	Zn	Phy	Zn*Phy
Calcium										
Plasma (g/L)	0.112	0.118	0.116	0.119	0.108	0.115	0.004	0.79	0.70	0.13
Kidney (mg/g)	0.69	0.69	0.68	0.73	0.68	0.69	0.02	0.39	0.45	0.60
Liver (mg/g)	0.44	0.45	0.46	0.48	0.48	0.45	0.02	0.88	0.43	0.60
Phosphorus,										
Plasma (g/L)	0.071	0.069	0.068	0.068	0.067	0.069	0.002	0.91	0.50	0.57
Kidney (mg/g)	0.26	0.27	0.28	0.26	0.28	0.28	0.012	0.17	0.93	0.97
Liver (mg/g)	0.33	0.34	0.34	0.36	0.36	0.33	0.008	0.47	0.28	0.16
Copper										
Plasma (mg/L)	1.2	1.3	1.5	1.4	1.1	1.3	0.09	0.06	0.62	0.12
Kidney (ug/g)	7.9	10.8	46.8	6.9	16.8	45.8	5.02	0.01	0.76	0.72
Liver (ug/g)	32.6	31.6	27.6	36.2	43.6	32.7	5.58	0.41	0.15	0.73
Iron,										
Plasma (mg/L)	1.2	1.5	1.6	1.3	1.1	1.0	0.33	0.92	0.24	0.66
Kidney (ug/g)	33.9	39.1	41.9	40.4	38.0	39.7	4.73	0.74	0.78	0.63
Liver (ug/g)	155.0	159.4	179.0	161.4	181.9	158.1	15.18	0.70	0.83	0.36

[1]Twenty three pigs with an average initial weight of 5.5 kg were housed in individual stainless steel pens.

[2] All pigs were fed dietary treatments for d 1-14, and a common diet for d 15-21.

[3]The values presented are means, n=4 (except $Zn_{100}P$, n=3)

[4] Average SEM due to an unbalanced design. The largest SEM is within 10% of the reported value.

Table 4

Effects of feeding adequate, pharmacological or excess zinc with or without supplemental phytase on Zn balance[1,2,3].

Phytase, FTU/kg	0			500			*p* value		
Zinc, mg Zn/kg	100	1,000	4,000	100	1,000	4,000	Zn	Phy	Zn*Phy
d1-7 (mg/d)									
Intake	74 (60 - 93)	952 (770 - 1,190)	2,738 (2,196 - 3,415)	95 (74 - 121)	706 (566 - 881)	2,853 (2,288 - 3,559)	0.01	0.09	0.06
Fecal	28 (18 - 43)	130 (86 - 196)	547 (355 - 844)	27 (17 - 42)	72 (42 - 121)	451 (273 - 746)	0.01	0.13	0.45
Urine	0.32 (0.16 - 0.61)	1.96 (1.02 - 3.77)	4.33 (2.25 - 8.32)	0.56 (0.27 - 1.15)	0.94 (0.49 - 1.81)	3.09 (1.61 - 5.94)	0.01	0.53	0.14
Retained	45 (-86 - 111)	820 (292 - 1,233)	2,186 (1,460 - 2,425)	67 (-91 - 132)	633 (156 - 1,025)	2,398 (1,419 - 3,076)	0.01	0.59	0.57
d8-14 (mg/d)									
Intake	106 (87 - 130)	1,153 (943 - 1,471)	3,367 (2,752 - 4,119)	103 (84 - 126)	954 (779 - 1,167)	3,822 (2,124 - 4,676)	0.01	0.70	0.28
Fecal	60 (46 - 78)	762 (587 - 989)	2,777 (2,138 - 3,605)	70 (52 - 95)	756 (582 - 981)	2,761 (2,126 - 3,584)	0.01	0.65	0.77
Urine	0.31 (0.14 - 0.69)	2.87 (1.30 - 6.36)	5.40 (2.44 - 11.96)	0.53 (0.21 - 3.58)	0.84 (.38 - 1.85)	3.84 (1.73 - 8.50)	0.01	0.29	0.11
Retained	46 (-11 - 146)	388 (-92 - 414)	585 (56 - 779)	32 (-11 - 143)	197 (-51 - 373)	1,057 (258 - 2,815)	0.01	0.24	0.07
d15-21 (mg/d)									
Intake	58 (47 - 69)	67 (55 - 78)	50 (38 - 61)	53 (42 - 65)	54 (43 - 65)	57 (46 - 69)	0.43	0.47	0.19
Fecal	34 (24 - 48)	57 (41 - 80)	75 (54 - 105)	27 (19 - 40)	48 (34 - 67)	76 (54 - 105)	0.01	0.36	0.78
Urine	0.37 (0.13 - 1.04)	1.36 (0.48 - 3.83)	1.83 (0.65 - 5.14)	0.78 (0.24 - 2.57)	0.56 (0.20 - 1.56)	2.22 (0.79 - 6.24)	0.05	0.97	0.28
Retained	24 (-11 - 41)	9 (-63 - 22)	-27 (-96 - -148)	25 (-15 - 46)	5 (-64 - 24)	-21 (-86 - -140)	0.01	0.77	0.88
d1-21 (mg/d)									
Intake	107 (94 - 121)	583 (512 - 663)	1,070 (940 - 1,217)	109 (96 - 125)	459 (403 - 522)	1,177 (1,035 - 1,339)	0.01	0.43	0.32
Fecal	21 (18 - 25)	103 (88 - 121)	278 (236 - 328)	21 (18 - 26)	80 (67 - 95)	265 (223 - 316)	0.01	0.28	0.50
Urine	0.24 (0.14 - 0.41)^{x}	1.41 (0.82 - 2.42)^{yz}	2.50 (1.46 - 4.29)^{z}	0.45 (0.25 - 0.83)^{x}	0.54 (0.32 - 0.93)^{xy}	2.13 (1.24 - 3.65)^{z}	0.01	0.47	0.03
Retained	86 (-32 - 102)^{x}	487 (292 - 633)^{y}	790 (546 - 943)^{y}	130 (-38 - 223)^{x}	378 (135 - 502)^{xy}	910 (742 - 1,176)^{y}	0.01	0.20	0.05

[1]Twenty three pigs with an average initial weight of 5.5 kg were housed in individual stainless steel pens and were used for a 21 d study.

[2] All pigs were fed dietary treatments for d 1-14, and a common diet for d 15-21.

[3]The values presented are back-transformed means (lower and upper limits of the 95% confidence interval), n=4 (except $Zn_{150}P$, n=3). Means in a row without a common superscript differ, $p < 0.05$.

Table 5

Effects of feeding adequate, pharmacological or excess zinc with or without supplemental phytase on apparent Ca, P, Cu and Fe retention[1,2,3].

Phytase, FTU/kg	0			500				*p* value		
Zinc, mg Zn/kg	100	1,000	4,000	100	1,000	4,000	SEM[4]	Zn	Phy	Zn*Phy
d1-7										
Calcium (g/d)	2.10	1.95	1.96	2.25	2.20	2.05	0.10	0.23	0.05	0.69
Phosphorus (g/d)	1.37	1.31	1.37	1.44	1.43	1.40	0.05	0.87	0.13	0.71
Copper (mg/d)	2.14	1.89	2.10	2.67	2.50	2.27	0.37	0.81	0.17	0.82
Iron (mg/d)	59.93	56.76	53.66	66.07	66.98	56.75	6.50	0.47	0.24	0.86
d8-14										
Calcium (g/d)	2.53	2.37	2.27	2.54	2.78	2.71	0.14	0.84	0.03	0.29
Phosphorus (g/d)	1.56	1.51	1.55	1.56	1.77	1.68	0.61	0.50	0.02	0.16
Copper (mg/d)	2.58	2.37	3.22	1.80	2.37	2.61	0.35	0.21	0.14	0.52
Iron (mg/d)	57.24	59.39	45.60	56.91	57.73	61.49	9.30	0.80	0.43	0.42
d15-21										
Calcium (g/d)	4.57	4.21	4.66	4.76	4.77	4.92	0.17	0.23	0.03	0.53
Phosphorus (g/d)	2.45	2.28	2.48	2.49	2.55	2.57	0.06	0.24	0.02	0.22
Copper (mg/d)	1.50	1.04	1.99	2.01	1.65	1.98	0.28	0.10	0.13	0.53
Iron (mg/d)	41.28	35.65	43.74	47.86	42.04	42.71	6.20	0.65	0.45	0.80
d1-21										
Calcium (g/d)	3.04	2.81	2.96	3.18	3.23	3.22	0.08	0.56	0.01	0.27
Phosphorus (g/d)	1.78	1.70	1.79	1.84	1.91	1.88	0.03	0.58	0.01	0.07
Copper (mg/d)	2.02	1.72	2.49	2.10	2.11	2.30	0.18	0.05	0.55	0.30
Iron (mg/d)	51.66	50.81	49.19	57.22	54.59	54.47	3.59	0.77	0.11	0.96

[1]Twenty three pigs with an average initial weight of 5.5 kg were housed in individual stainless steel pens.

[2] All pigs were fed dietary treatments for d 1-14, and a common diet for d 15-21.

[3]The values presented are means, n=4 (except $Zn_{100}P$, n=3).

[4] Average SEM due to an unbalanced design. The largest SEM is within 10% of the reported value.

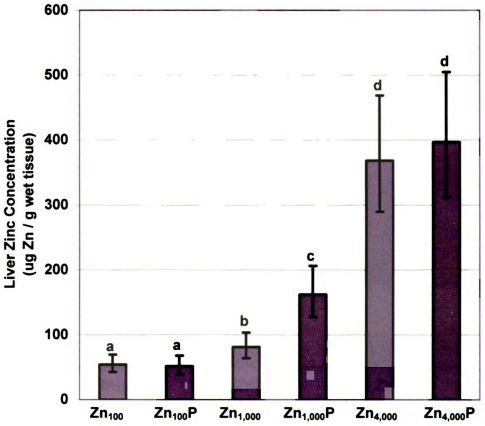

Figure 1. Concentration of zinc in liver of pigs fed 100, 1,000 or 4,000 mg Zn/kg as zinc oxide, with or without 500 FTU/kg of phytase for 14 d post-weaning and a common diet (100 mg Zn/kg) for the subsequent 7 d. Values are backtransformed means with 95% CI, n = 4 (except $Zn_{100}P$, n=3). The zinc x phytase interaction was significant. Means without a common superscript differ (p = 0.01).

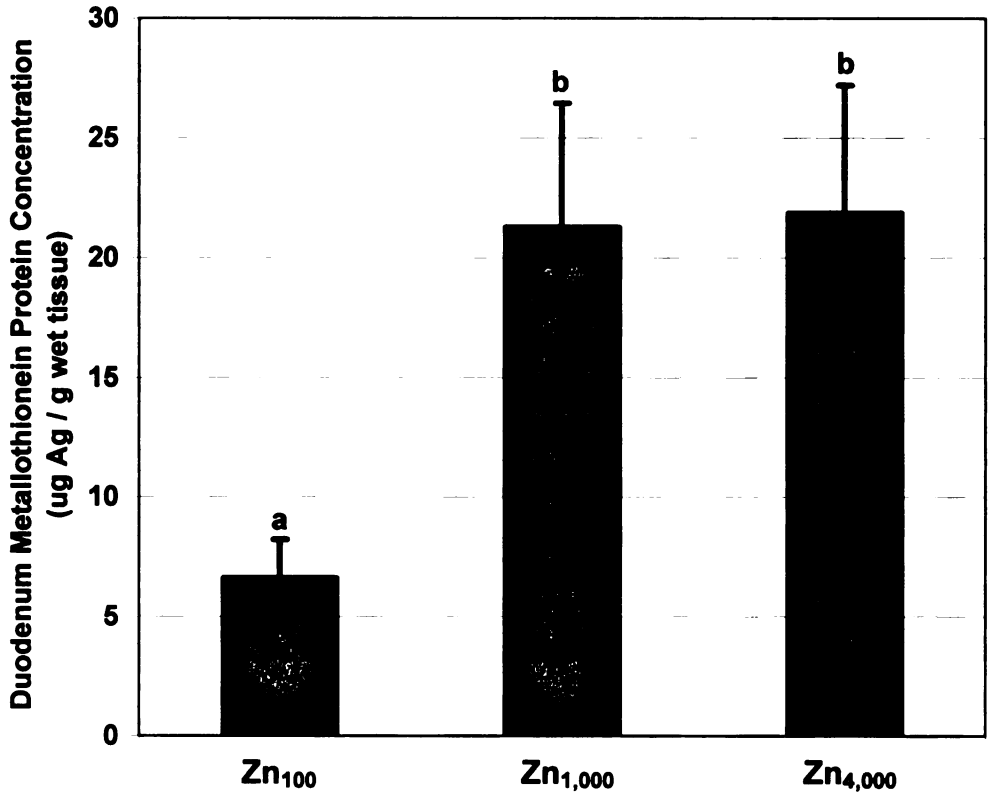

Figure 2A. Concentration of metallothionein in duodenum of pigs fed 100, 1,000 or 4,000 mg Zn/kg as zinc oxide, with or without 500 FTU/kg of phytase for 14 d post-weaning and a common diet (100 mg Zn/kg) for the subsequent 7 d. Values are backtransformed means with 95% confidence interval, n = 8, (except Zn_{100}, n=7). Main effect of zinc was significant (p = 0.0001).

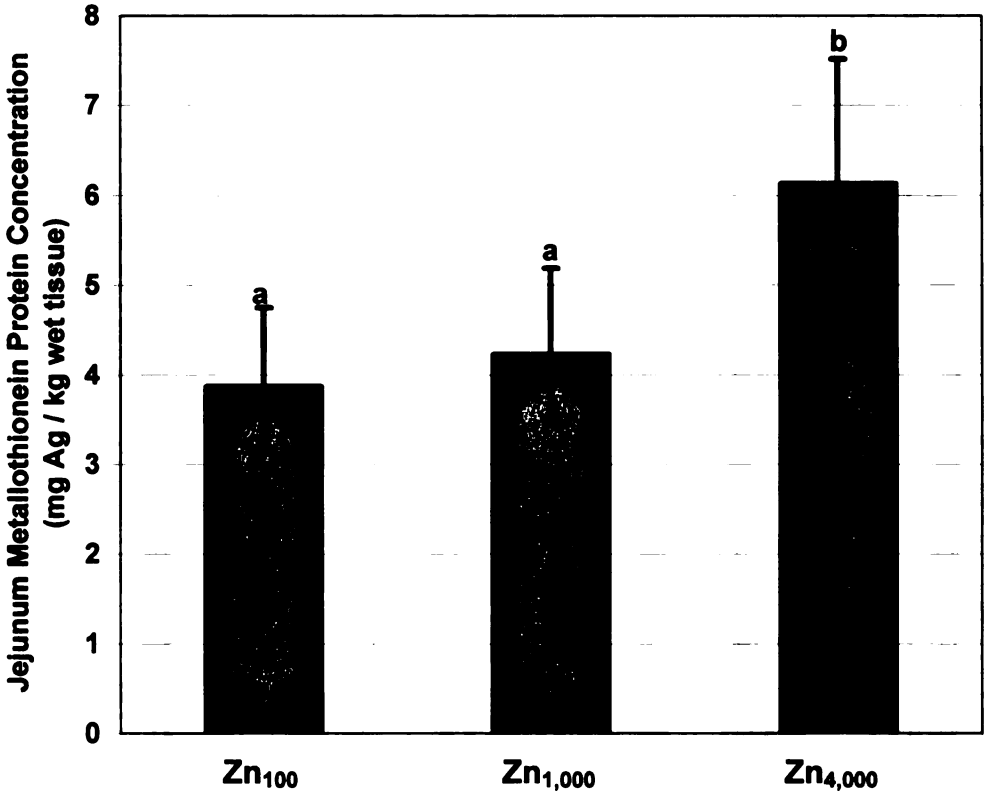

Figure 2B. Concentration of metallothionein in jejunum of pigs fed 100, 1,000 or 4,000 mg Zn/kg as zinc oxide, with or without 500 FTU/kg of phytase for 14 d post-weaning and a common diet (100 mg Zn/kg) for the subsequent 7 d. Values are backtransformed means with 95% confidence interval, n = 8, (except Zn_{100}, n = 7). Main effect of zinc was significant (p = 0.01).

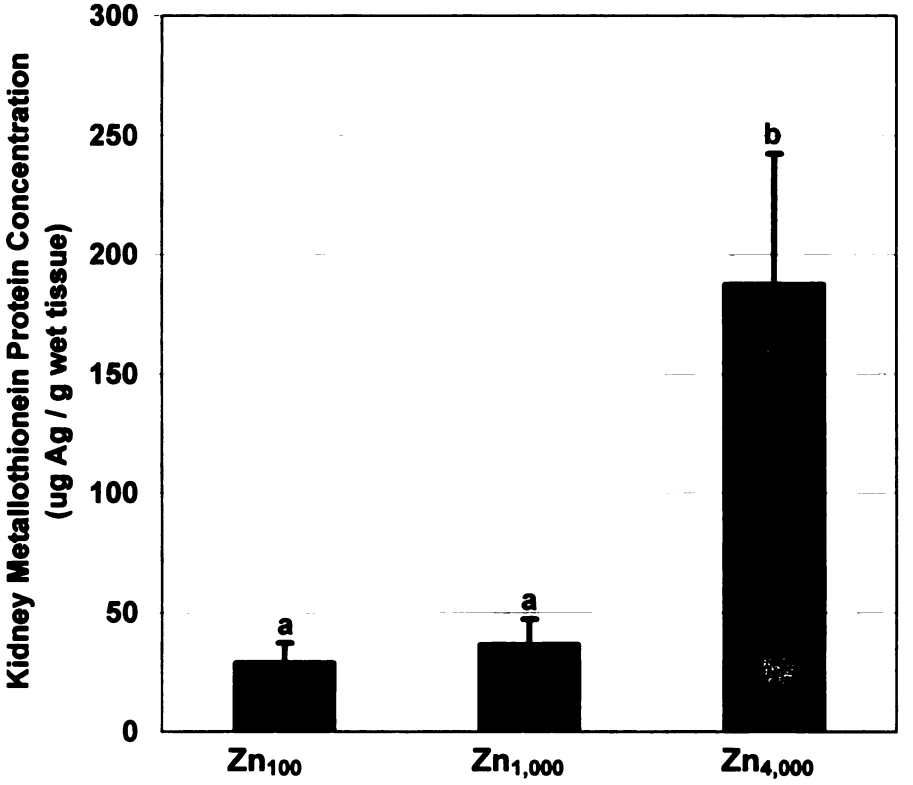

Figure 2C. Concentration of metallothionein in kidney of pigs fed 100, 1,000 or 4,000 mg Zn/kg as zinc oxide, with or without 500 FTU/kg of phytase for 14 d post-weaning, and a common diet (100 mg Zn/kg) for the subsequent 7 d. Values are backtransformed means with 95% confidence interval, n = 8, (except Zn_{100}, n=7). Main effect of zinc was significant (p = 0.01).

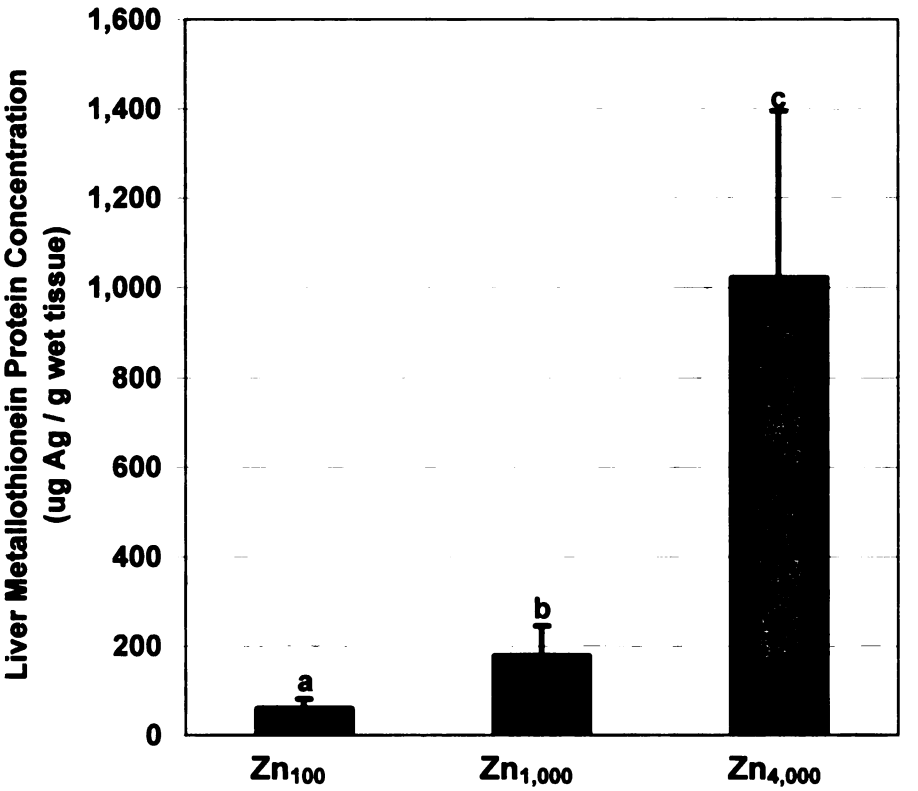

Figure 2D. Concentration of metallothionein in liver of pigs fed 100, 1,000 or 4,000 mg Zn/kg as zinc oxide, with or without 500 FTU/kg of phytase for 14 d post-weaning and a common diet (100 mg Zn/kg) for the subsequent 7 d. Values are backtransformed means with 95% confidence interval, n = 8, (except Zn_{100}, n=7). Main effect of zinc was significant (p = 0.01).

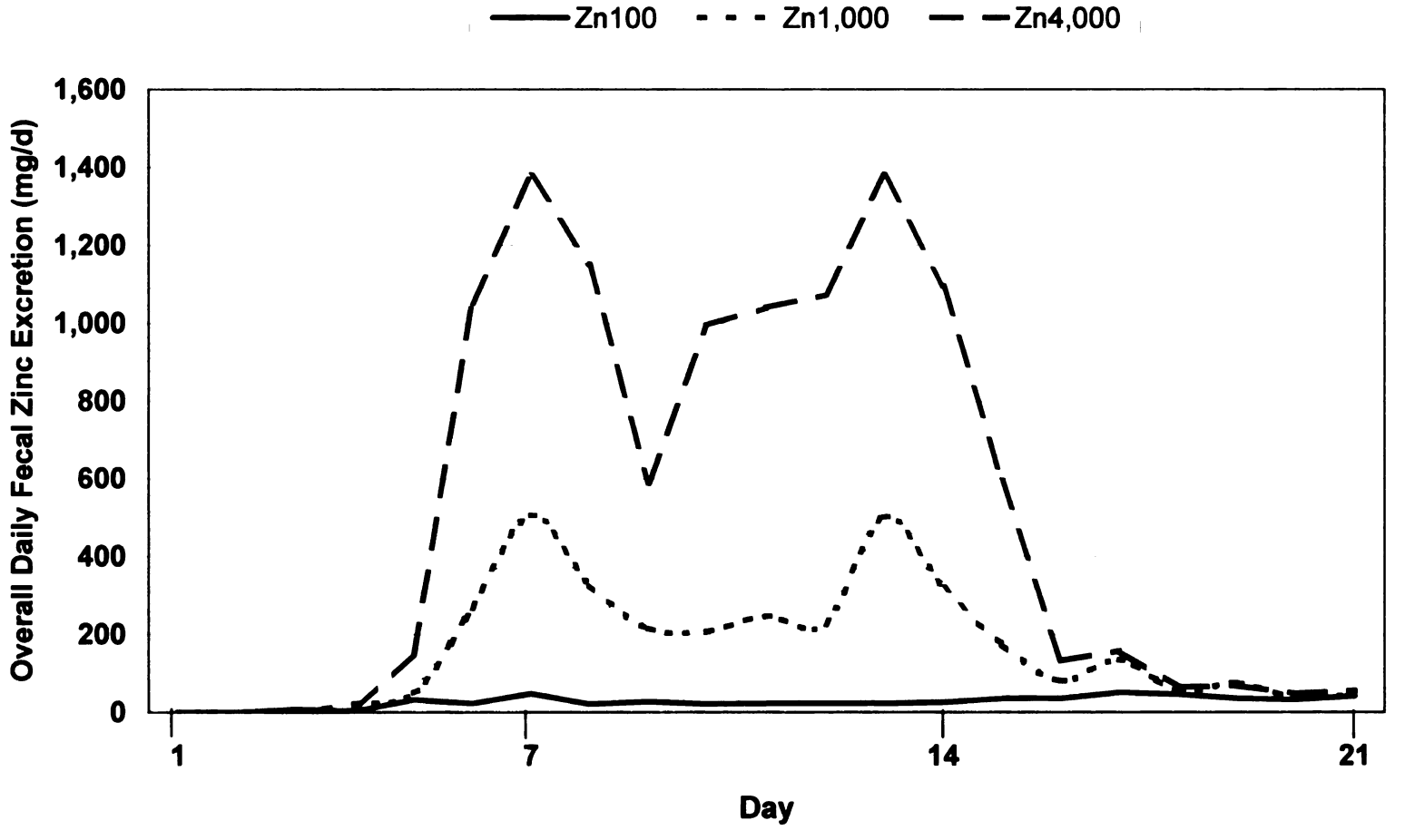

Figure 3. Daily fecal zinc excretion patterns of pigs fed 100, 1,000 or 4,000 mg/kg as zinc oxide, with or without 500 FTU/kg of phytase for 14 d post-weaning and a common diet (100 mg Zn/kg) for the subsequent 7 d.

CHAPTER FIVE

Identification of Differentially Expressed Genes in Hepatic Tissue of Newly Weaned Pigs Fed Pharmacological Zinc and Phytase Supplemented Diets[1,2]

Michelle M. Martínez, Gretchen M. Hill, Jane E. Link, Nancy E. Raney and Catherine W. Ernst

Department of Animal Science, Michigan State University, East Lansing, MI

Running title: ZINC & PHYTASE AFFECTS GENE EXPRESSION IN PIGS

[1] Presented in part at the American Society of Animal Science Midwestern section meeting, March 17-19, 2004 [Martínez, M.M., G.M. Hill, J.E. Link, N.E. Raney and C.W. Ernst. (2004) Pharmacological zinc supplementation in nursery pigs regulates glyoxalase 1 and peroxiredoxin 4 gene expression. J. Anim. Sci. 82: 19 (Supplement 2)].

[2] Supported in part by an Animal Health Formula Funds Grant from the Michigan Agricultural Experiment Station, by a Six State Consortium on Animal Waste Management of Environmental Protection Agency, and by The Graduate School – Competitive Doctoral Enrichment Fellowship, Michigan State University, East Lansing, MI.

[3] Abbreviations used: *ACY1*, aminoacylase 1; *CPB2*, carboxypeptidase U; DDRT-PCR, differential display RT-PCR; EST, expressed sequence tag; FTU, phytase unit; *GLO1*, glyoxalase I; MT, metallothionein; MRE, metal response element; MTF, metal transcription factor; *ORM1*, orosomucoid 1; *PRDX4*, peroxiredoxin 4; Zn, zinc; ZnO, zinc oxide; Z_{150}, 150 mg Zn/kg; $Zn_{150}P$, 150 mg

Zn/kg with phytase; $Zn_{1,000}$, 1,000 mg Zn/kg; $Zn_{1,000}P$, 1,000 mg Zn/kg with phytase; $Zn_{2,000}$, 2,000 mg Zn/kg; $Zn_{2,000}P$, 2,000 mg Zn/kg with phytase.

ABSTRACT

Porcine post-weaning stress symptoms include low feed intake, diarrhea, small intestinal villi atrophy and reduced growth. Pharmacological zinc (Zn) improves fecal consistency, intestinal morphology, and growth. Furthermore, adding phytase to high Zn diets increases hepatic Zn concentration and metallothionein (MT) mRNA abundance and protein. We hypothesized that pharmacological Zn and phytase supplementation to newly weaned pigs would result in changes in gene expression. The goal of this study was to investigate the effects of feeding newly weaned pigs dietary Zn (150, 1000, or 2000 mg Zn/kg) as Zn oxide (ZnO) with or without phytase (0 or 500 phytase units (FTU)/kg) for 14 d on hepatic differential gene expression using differential display reverse transcriptase polymerase chain reaction (DDRT-PCR). Liver DNAase treated RNA was reverse transcribed using a 3' poly-T primer ending in the anchor bases AC, labeled with ^{33}P-dATP and amplified with six 5'arbitrary primers. A total of 52 amplicons were putatively identified as differentially expressed, cloned and sequenced. Five transcripts exhibiting increased expression in pigs fed pharmacological Zn that had sequence similarities to genes encoding glyoxalase 1 (*GLO1*), peroxiredoxin 4 (*PRDX4*), aminoacylase I (*ACY1*), orosomucoid 1 (*ORM1*) and carboxypeptidase U (*CPB2*) were selected for confirmation. Relative hepatic *GLO1* ($P < 0.0007$), *PRDX4* ($P < 0.009$) and *ACY1* ($P < 0.01$) mRNA abundances were greater in pigs fed 1000 and 2000 mg Zn/kg than in pigs fed 150 mg Zn/kg. Relative *ORM1* and *CPB2* mRNA abundances were not affected by dietary treatments. Results under these

experimental conditions suggest that feeding pharmacological Zn (1000 or 2000 mg Zn/kg) affects genes involved in reducing oxidative stress and amino acid metabolism, which are essential for cell detoxification and proper cell function.
KEY WORDS: • *differential display RT-PCR* • *liver* • *nursery pig* • *pharmacological zinc* • *phytase*

INTRODUCTION

The ability of pigs to adapt to and digest dry feed during the post-weaning period influences their subsequent performance (1). Changes in the pig's social and physical environment, as well as in texture and nutritional composition of the diet, preset a stressful setting. These stressors alter many systems including immunity and are involved in the etiology of common diseases (2). Post-weaning stress symptoms include low feed intake, diarrhea, atrophy of small intestinal villi and ultimately reduced growth rate, which can translate into significant financial losses for the swine industry (3).

Pharmacological Zn has been successful in increasing growth and improving fecal consistency (4,5). Furthermore, feeding pharmacological Zn (3000 mg Zn/kg) to newly weaned pigs for 14 d improves gut morphology by increasing villous height and reducing crypt depths in the duodenum and jejunum (6).

Differential gene expression is responsible for morphological and phenotypical differences, since the transcriptome is dynamic (7). Studies on how micronutrients affect gene expression will help to clarify the role of trace elements in health and metabolism and their connection to biochemical events (8).

Mechanistically, Zn is involved in gene expression in numerous ways including DNA replication, RNA transcription, through the activity of transcription factors, DNA and RNA polymerases and playing a role in programmed cell death (9). Metallothionein (MT), a Zn binding protein, exerts a protective effect against

stress by acting as an antioxidant, a Zn storage protein and a metal transfer protein (10). Transcriptional regulation of the MT gene by dietary Zn has been demonstrated in rats (11).

Recently, we reported that feeding newly weaned pigs phytase (500 FTU/kg) and pharmacological Zn (1000 - 2000 mg/kg) increased MT mRNA abundance and protein concentrations in the liver, kidney and intestinal mucosa (12). Other genes may be affected by this dietary intervention. Therefore, the objective of this experiment was to determine the identity of genes that are differentially expressed in the liver of pigs fed a pharmacological Zn diet with or without phytase supplementation for 14 d post-weaning.

MATERIALS AND METHODS

Animals and diets. Animal description, allotment and diet composition have been previously described (12). The dietary treatments fed to pigs for 14 d after weaning were: 1) adequate Zn diet containing 150 mg Zn/kg (Zn_{150}), 2) Zn_{150} plus 500 FTU/kg ($Zn_{150}P$), 3) pharmacological Zn diet containing 1000 mg Zn/kg (Zn_{1000}), 4) Zn_{1000} plus 500 FTU/kg ($Zn_{1000}P$), 5) pharmacological Zn diet containing 2000 mg Zn/kg (Zn_{2000}), or 6) Zn_{2000} plus 500 FTU/kg ($Zn_{2000}P$). Pigs were provided feed and water *ad libitum*. This project was approved by the Michigan State University All University Committee on Animal Use and Care (12/99-159-00).

Sample collection and total RNA isolation. Details about euthanasia, liver sample collection methods and total RNA isolation were previously published (12). RNA concentrations were determined with the RNA 6000 Pico LabChip® kit using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). Additionally, RNA quality and integrity were determined by calculating the $A_{260/280}$ ratio and by agarose gel electrophoresis, respectively.

Differential display reverse transcription polymerase chain reaction. Using modifications (13) of Liang and Pardee (14), genomic DNA contamination was minimized by treating 1 μg of total RNA with 1 U of amplification grade DNAse I, (Invitrogen, Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Individual DNAase treated RNA samples (200 ng) from animals fed Zn_{150}, Zn_{1000}, $Zn_{1000}P$ and Zn_{2000} (n = 4 per treatment), were reverse transcribed by using a common anchor primer (5'-$d(T)_{12}$AC-3') and RT-mix [1X

Buffer, 25 μM dNTP's, 10 mM DTT and 40 U of Superscript II (Invitrogen, Life Technologies, Carlsbad, CA)]. Samples were incubated at 40°C for 5 min, 50°C for 50 min, and 70°C for 15 min in a Peltier Thermal Cycler (PTC-200, MJ Research, Waltham, MA). A final temperature drop to 4°C stopped the reaction.

Oligonucleotide primers for DDRT-PCR were obtained from the U.S. Pig Genome Coordination Program (http://www.genome.iastate.edu/ resources/ ddprimer.html), and were randomly selected (**Table 1**). Using one anchor primer paired with six arbitrary primers on 16 cDNA samples, a total of 96 PCR reactions were generated and ~ 2% of all mRNA species present were screened. PCR reactions were performed using cDNA (400 ng) in a solution containing 0.2 μM 3'- anchor primer, 20 μM of each dNTP, 1.5 mM $MgCl_2$, 1X $MgCl_2$ - free PCR buffer, 0.2 μM 5' - arbitrary primer, 2.5 μCi [α-^{33}P] dATP (Perkin Elmer, Life Sciences Inc., Boston, MA) and 0.5 U Taq DNA Polymerase (Promega, Madison, WI). PCR cycling parameters were: 95°C for 2 min, 4 cycles at 92°C for 15 s, 50°C for 30 s, and 72°C for 2 min, followed by an additional 25 cycles with annealing at 60°C for 30 s and extension at 72°C for 2 min.

Denaturing loading dye (formamide, bromophenol blue and xylene cyanol) was mixed with each DDRT-PCR sample (8 μl), and samples were dried on medium heat for 5 min using a speed-vacuum (Savant Instruments Inc., Farmingdale, NY) followed by a 3 min 95°C denaturing step. The samples were electrophoresed on 0.4 mm 5.2% polyacrylamide denaturing gels. Following a 5-6 hr run at 60 W on a vertical gelbox (Model S2, Invitrogen, Life Technologies / GIBCO BRL Sequence Systems, Carlsbad, CA), gels were transferred onto

Whatman chromatography paper (Whatman®, Maidstone, England) and dried at 80°C for 30 min using a slab dryer (SGD 2000, Savant). Gels were exposed to Biomax™ film (Eastman Kodak Co., Rochester, NY) overnight.

Excision and re-amplification of DDRT-PCR products. Radiographs were developed and the selected bands were circumscribed and rehydrated in 100 μl DEPC treated water and heated at 50°C for 30 min. Re-amplification reactions included 2 μl of gel band eluate and the anchor and arbitrary primers used in the DDRT-PCR step under the same reaction conditions and cycling parameters described above, excluding the isotope. To assess quality of the reactions, 1 and 2 μl of re-amplification products were electrophoresed with a λ Hind III marker (Invitrogen, Life Technologies, Carlsbad, CA) in 1% agarose gels stained with ethidium bromide (Sigma-Aldrich, St. Louis, MO).

Cloning and sequencing of DDRT-PCR products. Products of interest obtained from the DDRT-PCR gels were cloned into pGEM-T-Easy Vector-System I (Promega, Madison, WI). Recombinant vectors were transformed into *E. coli* DH5α competent cells (Invitrogen, Life Technologies, Carlsbad, CA). Plasmid DNA was purified with the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA) and digested with *EcoR* I to confirm the presence of an insert. Sequencing was performed using SP6 or M13 forward primers (Michigan State University Genomics Technology Support Facility). Putative identification of the amplicons was determined using the basic local alignment search tool (BLASTn) software, with the non-redundant and EST databases of GenBank and The Institute for Genomic Research (TIGR) database.

Independent confirmation by relative real-time PCR. Transcript levels for 5 genes from 23 liver samples were compared. The hepatic RNA sample for one pig fed $Zn_{150}P$ was of poor quality, and was excluded from the analysis. The genes were aminoacylase-1 (*ACY1*), glyoxalase I (*GLO1*), peroxiredoxin 4 (*PRDX4*), orosomucoid 1 (*ORM1*), carboxipeptidase U (*CPB2*) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the normalizing gene (**Table 2**). Relative real-time PCR primers were designed using Primer Express v. 2.0 (Applied Biosystems, Foster City, CA), and the assays were performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Michigan State University Center for Animal Functional Genomics).

First strand cDNA synthesis was performed with an oligo $(dT)_{14}$ primer using SuperScript II RNase H (Invitrogen, Life Technologies, Carlsbad, CA), following the manufacturers protocol. The cDNA was purified with QuickClean resin (BD Biosciences Clontech, Palo Alto, CA) followed by precipitation with sodium acetate and ethanol. Purified cDNAs were suspended in DNase/RNase-free sterile water, and quantified using a spectrophotometer. The cDNA samples were diluted to a final concentration of 10 ng/µl, and stored at -20°C until use. The real time PCR reactions included 50 ng of cDNA, 300 µM primer, and 1X SYBR Green Master Mix (Applied Biosystems, Foster City, CA).

Relative quantification was determined using duplicate cDNA samples from each animal. PCR amplification efficiency plots were generated using serially diluted cDNA (4 dilutions) to confirm the use of GAPDH as the

normalizing gene. Results were recorded relative to a common liver cDNA sample from a pig fed Zn_{150} after normalizing for GAPDH. Relative gene expression changes were then computed using the $2^{-\Delta\Delta Ct}$ method (15).

Independent confirmation by northern blot hybridization. When PCR amplification plots did not reveal equal amplification efficiency for GAPDH with the target gene, confirmation of DDRT-PCR results was done by northern blot analysis using the cDNA clones obtained by DDRT-PCR, labeled as described previously (12) with 18S rRNA for normalization.

Statistical analysis. The data were analyzed using analysis of variance based on the MIXED procedure of SAS (16). If Zn, phytase (main effects) or their interaction, were not statistically significant, the datasets were merged. For the relative real time PCR analyses, the model included pig nested within the treatment interaction with the plate as random effect. Satterthwaite's approximation was used to determine the error df for test (17). A base 2 logarithmic transformation (Log_2) of the target and normalization gene Cts was made to account for the exponential amplification of cDNA prior to analyzing Ct values with the MIXED procedure of SAS. The transformed target Cts and GAPDH Cts were modeled as covariates and used in a regression-based normalization of Cts in accordance with recommendations by Poehlman (18). These values were compared to the results calculated by using the $2^{-\Delta\Delta Ct}$ method (15), and data are presented as fold changes.

For northern blot analyses, the blot by treatment interaction was used in the model with pig as a random effect. The 18S rRNA values were modeled as

covariates for a regression-based normalization of the genes' relative mRNA abundance. Differences were considered to be significant when $P < 0.05$.

RESULTS

Identification of expressed sequences displayed by DDRT-PCR. A total of 56 putatively differentially expressed amplicons were cloned and sequenced. High quality nucleotide sequence data was obtained for 52 of these ESTs and submitted to GenBank (accession numbers CF106636 – CF106687). A FASTA search of the GenBank and TIGR databases revealed that 24 of the ESTs (46%) had significant similarities to genes with known identities, 7 were similar to mitochondrial DNA (13%), and the remainder had unknown identities (40%). Identified genes were similar to genes involved in protein and amino acid metabolism, oxidative stress response, regulation of transcription, and membrane transport (**Table 3**). From the dietary treatment groups on the DDRT-PCR gels (Table 3), 5 genes were selected for subsequent mRNA abundance analyses.

Confirmation of differential expression as affected by dietary treatment. From the display gels, the five selected genes exhibited increased relative mRNA abundance in the liver of pigs fed pharmacological Zn diets (Zn_{1000}, Zn_{2000}) without phytase. To confirm differential expression, a combination of relative real time PCR and northern blot analyses was utilized.

Two oxidative stress response genes involved in the reduction of peroxides [peroxiredoxin 4 (*PRDX4*)], and in detoxification of glycating agents [glyoxalase I (*GLO1*)] showed differential expression. Northern blot analysis of *PRDX4* revealed a single transcript of ~0.95 kb (**Figure 1**). Relative abundance of *PRDX4* mRNA (**Figure 2**) in liver of pigs fed pharmacological Zn diets was 2

fold higher in pigs fed Zn_{1000}, and 4 fold higher in pigs fed Zn_{2000} when compared with pigs fed Zn_{150} ($P < 0.009$). PCR amplification efficiency plots revealed that GAPDH was not a suitable normalizing gene for *GLO1*, thus northern blot hybridization was performed. A single transcript (~ 1.9 kb) was obtained for *GLO1* (Figure 1). Relative *GLO1* mRNA abundance was greater ($P < 0.0007$) in pigs fed pharmacological Zn diets (Zn_{1000} and Zn_{2000}) when compared with pigs fed Zn_{150} (**Figure 3**). Supplemental phytase did not affect the mRNA abundance of *PRDX4* or *GLO1*.

N-aminoacylase I (*ACY1*), which is a gene involved in amino acid metabolism, also displayed differential expression. Northern blot analysis revealed the presence of a single transcript (~ 1.3 kb, Figure 1), and that relative *ACY1* mRNA abundance increased ($P < 0.01$) in pigs fed pharmacological Zn (Zn_{1000} and Zn_{2000}). No phytase effect was observed (**Figure 4**).

ORM1 and *CPB2* were not confirmed to have differential expression in pigs fed any of the dietary treatments using real time PCR (data not shown).

DISCUSSION

Differential display reverse transcription PCR is a technique that allows the simultaneous comparison of multiple treatments, the identification of differentially regulated mRNAs, and generation of ESTs which can be compared with DNA sequences in the databases (14,19).

The PRDX (EC 1.11.1.-) family is the most recently identified oxidative stress enzyme to be characterized. It encodes for a ubiquitous cytosolic enzyme, which reduces hydrogen peroxide via redox-active cysteine residues. In addition to hydrogen peroxides, PRDXs can also regulate peroxide-mediated signaling cascades, and their overexpression reduces hydrogen peroxides in response to tumor necrosis factor α (TNF-α). In this study, a single transcript for *PRDX4* was revealed, which agrees with the size reported for human *PRDX4* (20).

Peroxiredoxin 4 overexpression in mice suppresses Nf-κB activation through regulation of I-κB phosphorylation (20). Additionally, it has been reported that Zn supplementation (1000 mg Zn/kg) to streptozotozin treated mice inhibits Nf-κB activation, thus providing protection against oxidative stress and inflammatory responses (21). Similarly, MT has also been implicated in inhibiting TNF-induced activation of Nf-κB DNA binding, by inhibition of IκB degradation and subsequent NF-κB suppression (22). Kim et al. (23) suggest that MT modulates intracellular signaling molecules such as transcription factors (Nf-κB) by sequestering intracellular Zn, which is required for its DNA binding activity. Peroxiredoxins are considered redox regulators of signal transduction

(20), acting like switches to control intracellular pathways. Perhaps the increase in intracellular Zn causes an alteration in cell signaling (24), and *PRDX4* upregulation occurs as a result. The suppressive effect by *PRDX4* on Nf-κB may be a mechanism involved in pharmacological Zn's enhancement of health in the newly weaned pig.

Glyoxalase 1 (EC 4.4.1.5) is a cytosolic ubiquitous Zn metalloenzyme that catalyzes the glutathione dependent conversion of glycating agents such as methylglyoxal to S-D-lactoylglutathione via a 1,2-hydrogen transfer (25). This enzyme works in conjunction with glyoxalase II to further convert S-D-lactoylglutathione to D-lactate (26). Methylglyoxal can react with arginine and lysine residues in proteins, and at high concentrations it inhibits glycolytic enzymes (27), enhances lipid peroxidation, and binds DNA and RNA causing macromolecular damage (28). Genomic analysis of the *GLO1* gene revealed the presence of functional metal response elements (MREs) located 647 bp downstream of the transcription initiation site (27). Another gene containing MREs in its promoter region is the MT gene (29). The MREs control transcriptional activation of MT upon exposure to Zn, through the action of metal transcription factor-1 (30). Transcriptional activity of *GLO1* in transfected HepG2 cells was elevated twofold upon exposure to 25 and 75 μM $ZnCl_2$ for 48 hr (27). We have previously shown that MT mRNA abundance and protein concentration are increased in liver, kidney and intestinal mucosa of pigs fed pharmacological Zn (12), similar to the liver expression patterns observed for *PRDX4* and *GLO1* in

this study. The present study showed a single *GLO1* transcript, which was in accordance with the size of human *GLO1* (27).

Zinc involvement in oxidative stress is well known. Its deficiency causes oxidative DNA damage by impairment of antioxidant defense, and compromises DNA repair mechanisms (31). Pharmacological Zn supplementation (3000 mg Zn/kg) to early weaned pigs for 21 d post-weaning has been shown to reduce red blood cell superoxide dismutase (EC 1.15.1.1) activity compared with pigs fed 150 mg Zn/kg (6). However, the mechanism of how Zn exerts its antioxidant action is not well defined. It has been suggested that MT induction, and the ability of Zn to occupy iron and copper binding sites on proteins and DNA, may contribute toward its antioxidant action (32). The present study shows that pharmacological Zn supplementation is associated with increased mRNA abundance of *PRDX4* and *GLO1*, genes that participate in the oxidative stress response through differing mechanisms. Our results for hepatic *GLO1* expression are in agreement with observations in *GLO1* transfected HepG2 cells (27). Perhaps Zn transcriptionally regulates *GLO1* through MRE activation, in a similar fashion to the MT gene, therefore offering protection against glycating agents. To our knowledge, we provide the first evidence that pharmacological Zn in the diet increases *PRDX4* mRNA abundance.

Of the three additional genes selected for confirmation: 1) *ACY1*, encodes for a Zn metalloenzyme involved in peptide metabolism, 2) *ORM1*, encodes for an acute phase reactant protein, and 3) *CPB2*, encodes a plasma carboxypeptidase. N-acyl-L-amino-acid aminohydrolase (EC 3.5.1.14) is a

cytosolic enzyme with greatest abundance in pig kidney versus the liver (33,34). The biological role of *ACY1* is to hydrolyze neutral and hydrophobic α-*N*-acyl-L-amino acids generated during protein degradation (35). This Zn dependent enzyme has been identified in mammals, primarily in kidney and liver (34), and contains a single Zn atom per subunit; it is proposed to have catalytic roles (36). However, Heese et al. (37) suggested that Zn plays a structural role, because the Zn binding site was too far from the catalytic site of the enzyme. In the present study, the presence of a single *ACY1* transcript was revealed, which is in accordance with the size reported for pig *ACY1* by Mitta et al. (33). Furthermore, we confirmed that pharmacological Zn supplementation increased the relative abundance of *ACY1* mRNA. No differences were observed on the abundance of this transcript between animals fed Zn_{1000} and Zn_{2000}, suggesting that at dietary concentrations greater than 1000 mg Zn/kg, relative *ACY1* abundance reaches a plateau. To our knowledge, this study provides the first evidence of Zn effects on the relative *ACY1* mRNA abundance.

Orosomucoid 1 and carboxypeptidase U are genes that encode an acute phase reactant protein known as α-1-acid glycoprotein, and plasma pro-carboxypeptidase (EC 3.4.17.20), respectively. These plasma proteins are secreted into the blood after being synthesized in the liver (38,39). Serum concentrations of *ORM1* are high in fetal (40) and neonatal pigs, decreasing markedly to a constant level by 2 wk of age (41) and declining further by 112 d. In a similar pattern, liver *ORM1* mRNA is relatively abundant in late fetal and neonatal pigs then declines rapidly after birth (42). Alpha-1-acid glycoprotein

plasma concentration rises during inflammation, thus its function is related to modulation of an inflammatory response primarily caused by interleukin 1, interleukin 6 and glucocorticoids (43). Sequence analysis of the murine *ORM1* gene revealed the presence of four MRE sequences in the 5' flanking region, and one in intron 5 (44). Furthermore, relative liver *ORM1* mRNA abundance in mice injected with 0.5 mg Hg/kg (i.p.) was increased in a time dependent manner. Zinc was also used to investigate *ORM1* mRNA response upon heavy metal exposure, but its effect on *ORM1* mRNA was not as marked as Hg (45). The authors concluded that Hg regulates *ORM1* at the transcriptional level. In our study, we did not observe a significant effect of dietary Zn on liver *ORM1* mRNA abundance. Additional studies on *ORM1* protein indicated that 2000 mg Zn/kg diet or the antibiotic, Tylan® (88 g/kg), fed to newly weaned pigs for 14 d did not affect *ORM1* plasma protein concentrations (46).

Plasma *CPB2* was reported in 1988 as a carboxypeptidase involved in blood clotting (47). Several names have been used for this enzyme according to its general catalytic mechanism, including carboxypeptidase U (U = unstable), carboxypeptidase R (R = cleaves Arg and Lys residues in C-termini), plasma carboxypeptidase B (B = basic) and most recently Thrombin-Activatable Fibrinolysis Inhibitor (TAFI) (48). The activity of *CPB2* has been shown to be affected by the availability of Zn, as demonstrated by the loss of activity with a Zn chelating agent, 1,10-phenantroline (49). The presence of *CPB2* has been demonstrated in the pig, rabbit and mouse, with activity ranging from 20% (mouse) to 500% (pig) compared to human serum *CPB2* (50).

Our display gels indicate that pharmacological Zn increases the mRNA abundance of *ORM1* and *CPB2* in pig liver. However, relative real time PCR results did not confirm their differential expression. This may be due to the low number of pigs in our treatments (n = 4) or the increased sensitivity of the real time PCR assays. Future research should be conducted to evaluate additional genes affected by the dietary treatments and to confirm the roles of these genes at the protein level.

ACKNOWLEDGEMENTS

The authors express their appreciation to Dr. Max Rothschild, U.S. Swine Genome Coordinator, for distribution of the DDRT-PCR oligonucleotide primers, Dr. Robert J. Tempelman for assisting with the statistical analysis, and the staff of the Michigan State University Center for Animal Functional Genomics for technical assistance.

LITERATURE CITED

1. Marion, J., Rome, V., Savary, G., Thomas, F., Le Dividich, J. & Le Huerou Luron, I. (2003) Weaning and feed intake alter pancreatic enzyme activities and corresponding mRNA levels in 7-d-old piglets. J. Nutr. 133: 362-368.

2. Kelley, K. W. (1980) Stress and immune function: a bibliographic review. Ann. Rech. Vet. 11: 445-478.

3. Pollmann, D. S. (1993) Effects of nursery feeding programs on subsequent grower-finisher pig performance. In: Proceedings of the Fourteenth Western Nutrition Conference (J. Martin), pp. 243-254. Edmonton, Canada.

4. McCully, G. A., Hill, G. M., Link, J. E., Weavers, R. L., Carlson, M. S. & Rozeboom, D. W. (1995) Evaluation of zinc sources for the newly weaned pig. J. Anim. Sci. 73: 72 (abs).

5. Hill, G. M., Cromwell, G. L., Crenshaw, T. D., Dove, C. R., Ewan, R. C., Knabe, D. A., Lewis, A. J., Libal, G. W., Mahan, D. C., Shurson, G. C., Southern, L. L. & Veum, T. L. (2000) Growth promotion effects and plasma changes from feeding high dietary concentrations of zinc and copper to weanling pigs (regional study). J. Anim. Sci. 78: 1010-1016.

6. Carlson, M. S., Hill, G. M. & Link, J. E. (1999) Early- and traditionally weaned nursery pigs benefit from phase-feeding pharmacological concentrations of zinc oxide: effect on metallothionein and mineral concentrations. J. Anim. Sci. 77: 1199-1207.

7. Junghans, P., Kaehne, T., Beyer, M., Metges, C. C. & Schwerin, M. (2004) Dietary protein-related changes in hepatic transcription correspond to modifications in hepatic protein expression in growing pigs. J. Nutr. 134: 43-47.

8. Hesketh, J. E., Vasconcelos, M. H. & Bermano, G. (1998) Regulatory signals in messenger RNA: determinants of nutrient-gene interaction and metabolic compartmentation. Br. J. Nutr. 80: 307-321.

9. Falchuk, K. H. (1998) The molecular basis for the role of zinc in developmental biology. Mol. Cell. Biochem. 188: 41-48.

10. Kagi, R. H. J. (1993) Evolution, structure and chemical activity of class I metallothioneins: overview. In: Metallothioneins III (K.T. Suzuki, N. Imura, & M. Kimura), pp. 29-56. Birkhauser Verlag, Basel, Switzerland.

11. Richards, M. P. & Cousins, R. J. (1975) Mammalian zinc homeostasis requirement for RNA and metallothionein synthesis. Biochem. Biophys. Res. Comm. 64: 1215-1223.

12. Martínez, M. M., Hill, G. M., Link, J. E., Raney, N. E., Tempelman, R. J. & Ernst, C. W. (2004) Pharmacological zinc and phytase supplementation enhance metallothionein mRNA abundance and protein concentration in newly weaned pigs. J. Nutr. 134: 538-544.

13. Wesolowski, S. R., Raney, N. E. & Ernst, C. W. (2004) Developmental changes in the fetal pig transcriptome. Physiol. Genomics 16: 268-274.

14. Liang, P. & Pardee, A. B. (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257: 967-971.

15. Livak, K. J. & Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-[Delta][Delta]CT method. Methods 25: 402-408.

16. SAS Systems (1999-2000) The SAS System for Windows 98, 8.1 ed. SAS Institute, Inc., Cary, N.C.

17. Satterthwaite, F. E. (1946) An approximate distribution of estimates of variance components. Biom. Bull. 2: 110-114.

18. Poehlman, E. T. (2003) Reduced metabolic rate after caloric restriction-can we agree on how to normalize the data? J. Clin. Endocrinol. Metab. 88: 14-15.

19. Cousins, R. J., Blanchard, R. K., Moore, J. B., Cui, L., Green, C. L., Liuzzi, J. P., Cao, J. & Bobo, J. A. (2003) Regulation of zinc metabolism and genomic outcomes. J. Nutr. 133: 1521S-1526.

20. Jin, D. Y., Chae, H. Z., Rhee, S. G. & Jeang, K. T. (1997) Regulatory role for a novel human thioredoxin peroxidase in Nf-κB activation. J. Biol. Chem. 272: 30952-30961.

21. Ho, E., Quan, N., Tsai, Y. H., Lai, W. & Bray, T. M. (2000) Dietary zinc supplementation inhibits NF-κB activation and protects against chemically induced diabetes in CD1 mice. Exp. Biol. Med. 226: 103-111.

22. Sakurai, A., Hara, S., Okano, N., Kondo, Y., Inoue, J.-i. & Imura, N. (1999) Regulatory role of metallothionein in NF-[kappa]B activation. FEBS Letters 455: 55-58.

23. Kim, C. H., Kim, J. H., Lee, J. & Ahn, Y. S. (2003) Zinc-induced NF-κB inhibition can be modulated by changes in the intracellular metallothionein level. Toxicol. Appl. Pharmacol. 190: 189-196.

24. Watjen, W., Benters, J., Haase, H., Schwede, F., Jastorff, B. & Beyersmann, D. (2001) Zn2+ and Cd2+ increase the cyclic GMP level in PC12 cells by inhibition of the cyclic nucleotide phosphodiesterase. Toxicology 157: 167-175.

25. Creighton, D. J. & Hamilton, D. S. (2001) Brief history of glyoxalase I and what we have learned about metal ion-dependent, enzyme catalyzed isomerizations. Arch. Biochem. Biophys. 387: 1-10.

26. Creighton, D. J., Migliorini, M., Pourmotabbed, T. & Guha, M. K. (1988) Optimization of efficiency in the glyoxalase pathway. Biochemistry 20: 7376-7384.

27. Ranganathan, S., Ciaccio, P. J., Walsh, E. S. & Tew, K. D. (1999) Genomic sequence of human glyoxalase-I: analysis of promoter activity and its regulation. Gene 240: 149-155.

28. Brambilla, G., Sciaba, L., Faggin, P., Finollo, R., Bassi, A. M., Ferro, M. & Marinari, U. M. (1985) Methylglyoxal-induced DNA-protein cross-links and cytotoxicity in Chinese hamster ovary cells. Carcinogenesis 6: 683-686.

29. Stuart, G. W., Searle, P. F., Cen, H. Y., Brinster, R. L. & Palmiter, R. D. (1984) A 12-base-pair DNA motif that is repeated several times in metallothionein gene promoters confers metal regulation to a heterologous gene. Proc. Natl. Acad. Sci. USA 81: 7318.

30. Radtke, F., Heuchel, R., Georgiev, O., Hergersberg, M., Gariglio, M., Dembic, Z. & Schaffner, W. (1993) Cloned transcription factor MTF-1 activates the mouse metallothionein I promoter. EMBO J. 12: 1355-1362.

31. Ho, E. & Ames, B. N. (2002) Low intracellular zinc induces oxidative DNA damage, disrupts p53, NFkappaB and AP1 binding and affects DNA repair in a rat glioma cell line. Proc. Natl. Acad. Sci. U S A 99: 16770-16775.

32. Har-el, R. & Chevion, M. (1990) Zinc (II) protects against meta-mediated free radical induced damage: studies on single and double-strand DNA breakage. Free. Radic. Res. Commun. 12-13: 509-515.

33. Mitta, M., Ohnogi, H., Yamamoto, A., Kato, I., Sakiyama, F. & Tsunasawa, S. (1992) The primary structure of porcine aminoacylase 1 deduced from cDNA sequence. J. Biochem. 112: 737-742.

34. Lindner, H., Hopfner, S., Tafler-Naumann, M., Miko, M., Konrad, L. & Rohm, K. H. (2000) The distribution of aminoacylase I among mammalian species and localization of the enzyme in porcine kidney. Biochimie 82: 129-137.

35. Giardina, T., Biagini, A., Dalle Ore, F., Ferre, E., Reynier, M. & Puigserver, A. (1997) The hog intestinal mucosa acylase I: Subcellular localization, isolation, kinetic studies and biological function. Biochimie 79: 265-273.

36. Loffler, H. G., Rohm, K. H. & Schneider, F. (1986) Studies on metal ion dependence, kinetics and SH (S-S) groups of acylaminoacid aminohydrolase.

In: Zinc Enzymes (In I. Bertini, C. Luchinat, W. Maret, & M. Zeppezauer), pp. 281-288. Birkhauser, Boston, MA.

37. Heese, D., Berger, S. & Rohm, K. H. (1990) Nuclear magnetic relaxation studies of the role of the metal ion in Mn^{+2}-substituted aminoacylase I. Eur. J. Biochem. 188: 175-180.

38. Dente, L. (1989) Human alpha-1-acid glycoprotein genes. Prog. Clin. Biol. Res. 300: 85-98.

39. Tan, A. K. & Eaton, D. L. (1995) Activation and characterization of procarboxypeptidase B from human plasma. Biochemistry 34: 5811-5816.

40. Lampreave, F. & Pineiro, A. (1984) The major serum protein of fetal and newborn pigs: biochemical properties and identification as a fetal form of alpha 1-acid glycoprotein. Int. J. Biochem. 16: 47-53.

41. Son, D. S., Shimoda, M. & Kokve, E. (1996) Implication of altered levels of plasma alpha (1)-acid glycoprotein and its derived sialic acid on plasma protein binding of trimethoprim in pigs in physiological and pathological states. Vet. Q. 18: 10-13.

42. Stone, R. T. & Maurer, R. A. (1987) Cloning and developmental regulation of alpha 1 acid glycoprotein in swine. Dev. Genet. 8: 295-304.

43. Mackiewicz, A., Dewey, M. J., Berger, F. G. & Baumann, H. (1991) Acute phase mediated change in glycosylation of rat α_1-acid glycoprotein in transgenic mice. Glycobiology 1: 265-269.

44. Cooper, R., Eckley, D. M. & Papaconstantinou, J. (1987) Nucleotide sequence of the mouse alpha 1-acid glycoprotein gene 1. Biochemistry 26: 5244-5250.

45. Yiangou, M., Ge, X., Carter, K. & Papaconstantinou, J. (1991) Induction of several actute-phase protein genes by heavy metals: a new class of metal-responsive genes. Biochemistry 30: 3798-3806.

46. Green, J. G., Hill, G. M., Link, J. E., Martínez, M. M., Dvoracek-Driksna, D. M., Raney, N. E. & Ernst, C. W. (2003) Comparison of tylosin and pharmacological zinc on acute phase reactant proteins and minerals. J. Anim. Sci. 81: 112 (abs.).

47. Hendriks, D., Scharpé, S., van Sande, M. & Lommaert, M. P. (1989) Characterization of a carboxypeptidase in human serum distinct from carboxypeptidase N. J. Clin. Chem. Clin. Biochem. 27: 277-285.

48. Bouma, B. N., Marx, P. F., Mosnier, L. O. & Meijers, J. C. M. (2001) Thrombin-Activatable Fibrinolysis Inhibitor (TAFI, Plasma Procarboxypeptidase B, Procarboxypeptidase R, Procarboxypeptidase U). Thromb. Res. 101: 329-354.

49. Eaton, D. L., Malloy, B. E., Tsai, S. P., Henzel, W. & Drayna, D. (1991) Isolation, molecular cloning, and partial characterization of a novel carboxypeptidase B from human plasma. J. Biol. Chem. 266: 21833-21838.

50. Schatteman, K. A., Goossens, F. J., Scharpe, S. S. & Hendriks, D. (1999) Activation of plasma carboxypeptidase U in different mammalian species points to a conserved pathway of inhibition of fibrinolysis. Thromb. Haemost. 82: 1718-1721.

TABLE 1

Anchor and arbitrary primers used in this experiment[1]

No.	Primer sequence
Anchor # 9 $d(T)_{12}$ AC	5'-TTTTTTTTTTTTAC-3'
Arbitrary # 4	5'-GCTAGCAGAC-3'
Arbitrary # 7	5'-TGGATTGGTC-3'
Arbitrary # 9	5'-TAAGCCTAGC-3'
Arbitrary # 13	5'-GTTGCACCAT-3'
Arbitrary # 16	5'-TCGGTCATAG-3'
Arbitrary # 20	5'-TCGATACAGG-3'

[1] Source: http://www.genome.iastate.edu/resources/ddprimer.html

TABLE 2

List of real time PCR primers used for DDRT-PCR confirmation[1]

Gene	Direction	Primer sequence
CPB2	Forward	5'-TGG CAT GTC ATC AGA AAT GGT T-3'
CPB2	Reverse	5'-CTT GCT GGA ATC AGT AAA TTT CAC TCT-3'
ORM1	Forward	5'-TTG AGT GCA CGG GAA TCC A-3'
ORM1	Reverse	5'-CCA GCG GCC CAC ACA-3'
PRDX4	Forward	5'-ATG ACC TCC CTG TGG GTA GAT CT-3'
PRDX4	Reverse	5'-ACA GAC TTC TCC ATG TTT GTC AGT GT-3'
GAPDH	Forward	5'-TGG AAA GGC CAT CAC CAT CT-3'
GAPDH	Reverse	5'-CCA GCA TCG CCC CAT TT-3'

[1]Primers were designed using Primer Express v. 2.0 (1995-2000)

TABLE 3

List of differentially expressed products and sequence identity information[1,2]

EST ID[3] (Length - bp)	EST Acc. No.	Sequence identity	Species / Accession No.[4]	Pig TC number	Pattern of transcript abundance[5]
13E1 (152)	CF106654	Activator heat shock 90kDa protein ATPase homologue 1 (*AHSA1*)	H. sapiens / BC007398.2	TC128680	3=4>2>1
13N2 (151)	CF106663	Activator heat shock 90kDa protein ATPase homologue 1 (*AHSA1*)	H. sapiens / BC007398.2	BF077793	3=4>2>1
13C1 (492)	CF106652	Basic Transcription Factor 3 (*BTF3*)	H. sapiens / BC008062.1	TC129241	4=2>3>1
20K1 (278)	CF106687	Carboxypeptidase U (*CPB2*)	H. sapiens / NM_001872.2	TC152650	3=4>2=1
04D3 (374)	CF106639	Coagulation factor IX (*F9*)	H. sapiens / HUMFIXG6	TC151707	4>2>3=1
20E2 (396)	CF106681	DNA Polymerase II Beta (*POLB*)	H. sapiens / HUMLNCAP	TC130004	4=1>3>2
07C1 (364)	CF106641	Flavin containing monooxygenase 3 (*FMO3*)	B. taurus / AF488422.1	TC143905	1>2=3=4
13Q3 (479)	CF106665	Glyoxalase I (*GLO1*)	H. sapiens / BC001741.1	TC133110	4>3>2>1
20C1 (354)	CF106679	GTP binding protein (*RAB2A*)	C. familiaris / DOGRAB2A	TC129194	4=3>2>1
13N1 (269)	CF106662	Histidine Rich Glycoprotein (*HRG*)	B. taurus / NM_173919.2	TC128876	3>4>2=1
13G1 (384)	CF106657	Integrin alpha-6 (*ITGA6*)	H. sapiens / BC050585.1	TC133250	1>2=3=4
13D2 (407)	CF106653	Laminin Receptor 1 (*LAMR1*)	H. sapiens / BC071971.1	TC128421	3>4=2>1
13T2 (441)	CF106669	Melanoma differentiation associated protein - 5 (MDA5)	H. sapiens / NM_022168.2	TC138162	3>4>2>1
13A1 (196)	CF106650	N-Aminoacylase I (*ACY-1*)	S. scrofa / AB017196.1	TC146027	4=3=2>1
04C1 (484)	CF106636	Orosomucoid 1 (*ORM1*)	S. scrofa / PIGA1AG	TC129371	4>3>2=1
20J1 (546)	CF106686	Orosomucoid 1 (*ORM1*)	S. scrofa / PIGA1AG	TC129371	3=4>2=1
13I1 (349)	CF106659	Peroxiredoxin (*PRDX4*)	H. sapiens / NM_006406.1	TC146545	4=3>2>1
20H1 (295)	CF106684	Polyubiquitin (*UBB*)	B. taurus / BTPOLYUB	TC145474	3=4>2=1
07A1 (240)	CF106640	Progesterone receptor membrane protein 1 (*PGRMC1*)	S. scrofa / NM_213911.1	TC129474	4=3>2>1
20D2 (272)	CF106680	Putative membrane steroid receptor	S. scrofa / NM_213739.1	TC133666	3>4>2=1
13R5 (620)	CF106667	Ribosomal protein L17 (*RPL17*)	B. taurus / AB099057.1	TC145700	4>3>2>1
20A1 (464)	CF106677	Synaptic Vesicle Protein 2B (*SV2B*)	R. norvegicus / NM_057207.2	TC146651	1>2=3=4
09E1 (228)	CF106648	Tyr 3-monooxygenase / Trp 5-monooxygenase activation protein (*YWHAB*	H. sapiens / NM_003404.3	TC128901	3=4=2>1
13B2 (310)	CF106651	Wilms' tumour associating protein (*WTAP*)	H. sapiens / AK131393.1	TC129293	4=3>2>1
07E1 (300)	CF106643	Mitochondrial DNA	S. scrofa / AF304200	TC137448	1>3>2=4
07H2 (177)	CF106645	Mitochondrial DNA	S. scrofa / AF304200	TC137448	1>2>3=4
16B2 (297)	CF106671	Mitochondrial DNA	S. scrofa / AF304200	TC146285	4=3>2>1
09B3 (156)	CF106646	Mitochondrial DNA	S. scrofa / AF304202	TC132929	3>4>2=1
13P1 (336)	CF106664	Mitochondrial DNA	S. scrofa / AF304202	TC145580	1>2>3=4
16J4 (334)	CF106675	Mitochondrial DNA	S. scrofa / AF304202	TC145580	3>4=2=1
09D1 (207)	CF106647	Mitochondrial DNA	S. scrofa / AF304203	TC145618	3>4=2=1

[1] Amplicons were compared to GenBank database using the BLAST software v. 2.9.9 May 12, 2004. NGH = matched to genes with unknown identity.

[2] Amplicons were compared to TIGR Sus scrofa gene index v.9.0 updated May 14, 2004 using the BLAST software v. 2.0

[3] First two digits of EST number designate arbitrary primer used for DDRT-PCR.

[4] Species, GenBank accession number for most significant match.

[5] Pattern of transcript abundance observed on DDRT-PCR gels. 1=Zn150; 2=Zn1000; 3=Zn1000P; 4=Zn2000. Pattern observed in the display gel from highest (>) to lowest comparable (=) abundance.

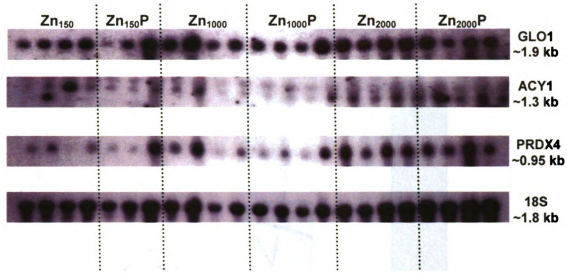

Figure 1. Northern blot analysis of liver *GLO1*, *ACY1* and *PRDX4* mRNA of pigs fed 150, 1000 or 2000 mg Zn/kg (with or without phytase) for 14 d post-weaning. Liver total RNA (12 μg) isolated from each pig was analyzed using the cDNA clones derived from differential display gels as probes. Also shown is 18S rRNA hybridization to assess equal loading of mRNA.

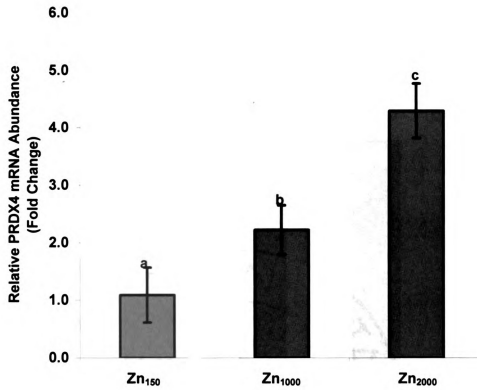

Figure 2. Relative *PRDX4* mRNA abundance in the liver of pigs fed 150, 1000 or 2000 mg Zn/kg (with or without phytase) for 14 d post-weaning. Relative real time PCR was performed and fold changes relative to GAPDH and a common Zn_{150} reference sample are presented. Values are means ± SEM, n=8, (except Zn_{150}, n=7). A significant zinc effect was detected for relative *PRDX4* mRNA abundance, $P < 0.009$. No Zn by phytase interaction, or phytase effect were detected.

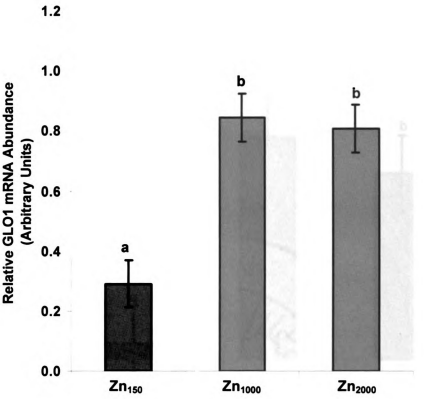

Figure 3. Relative *GLO1* mRNA abundance in the liver of pigs fed 150, 1000 or 2000 mg Zn/kg (with or without phytase) for 14 d post-weaning. Northern blot analysis was performed and values are mean optical density readings ± SEM, n=8 (except Zn_{150}, n=7). A significant zinc effect was detected for relative hepatic *GLO1* mRNA abundance, $P < 0.0007$. No Zn by phytase interaction, or phytase effect were detected.

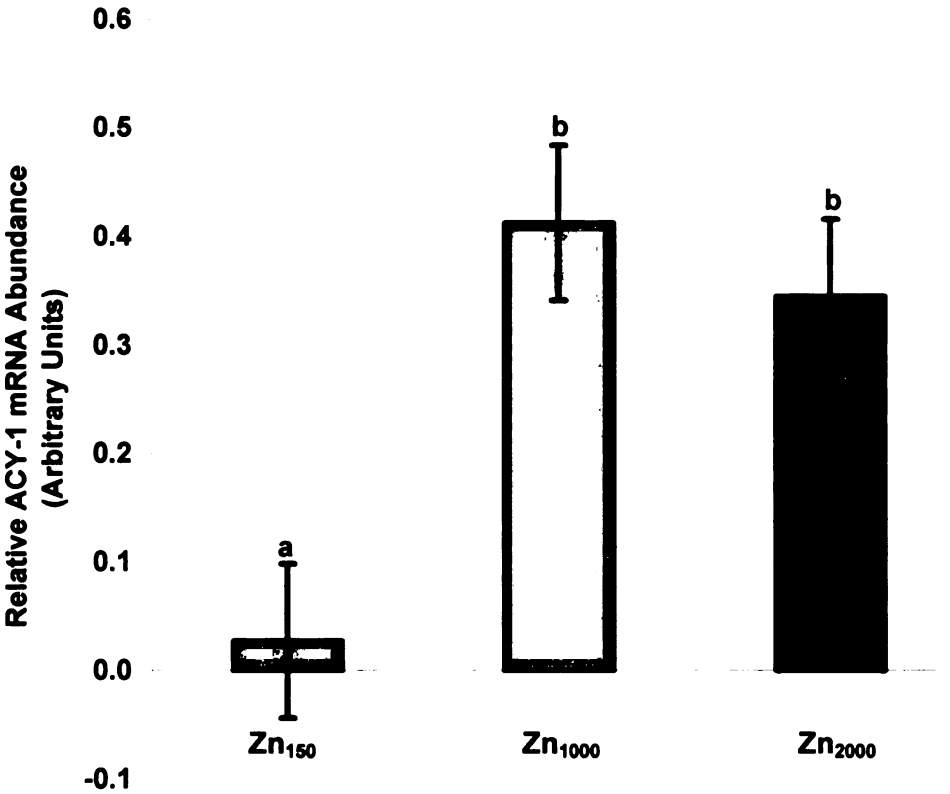

Figure 4. Relative *ACY1* mRNA abundance in the liver of pigs fed 150, 1000 or 2000 mg Zn/kg (with or without phytase) for 14 d post-weaning. Northern blot analysis was performed and values are mean optical density readings ± SEM, n=8 (except Zn_{150}, n=7). A significant zinc effect was detected for relative hepatic *ACY1* mRNA abundance, $P < 0.01$. No Zn by phytase interaction, or phytase effect were detected.