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Huyen Tran
University of Nebraska-Lincoln

Phillip Miller
University of Nebraska - Lincoln, pmiller1@unl.edu

Thomas E. Burkey
University of Nebraska - Lincoln, tburkey2@unl.edu

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In Vivo and *In Vitro* Expression of Porcine Zinc Transporter (Znt) 1 mRNA

Zinc transporter (ZnT1) mRNA is differentially expressed *in vivo*, in weaned pig tissues, and *in vitro*, in response to additions of zinc sulphate or antibiotics.

Huyen Tran
Phillip S. Miller
Thomas E. Burkey[1]

Summary

Preliminary experiments were carried out to evaluate in vivo expression of zinc transporter (ZnT) 1 mRNA in a panel of tissues obtained from weaned pigs and to evaluate in vitro *changes in ZnT1 mRNA in a porcine jejunal epithelial cell line (IPEC-J2) treated with low (40 µM) or high (80 µM) concentrations of zinc sulphate ($ZnSO_4$) or, in a separate experiment, with low (50 µg/ml) or high (100 µg/mL) concentrations of antibiotics (as gentamicin; GENT). Lipopolysaccharide (LPS; 10 ng/mL) was included as a negative control in both experiments. Zinc transporter 1 mRNA was detected in all tissues evaluated with the greatest level of expression observed in the tonsil. A treatment × time interaction was not observed for IPEC-J2 cells treated with $ZnSO_4$; however, the addition of high $ZnSO_4$ tended ($P = 0.08$) to increase ZnT1 mRNA expression when means were averaged among all time points. Exposure of IPEC-J2 cells to GENT resulted in a significant treatment × time interaction ($P < 0.005$) with increases in ZnT1 mRNA observed at 3 (low GENT) and 6 (low and high GENT) hours post-treatment compared to CTL- or LPS-treated cells. This research indicates that ZnT1 mRNA is differentially expressed* in vivo*, in a panel of porcine tissues, and* in vitro*, in IPEC-J2 cells exposed to low or high concentrations of zinc or antibiotics.*

Introduction

Zinc (Zn) is a trace mineral which has a functional role in many metalloenzymes and is essential for normal growth because of the association with nucleic acids and protein synthesis. In pigs, a Zn-deficient diet has been reported to result in appetite reduction as a result of the lack of gustin, a protein involved in acuity. Conversely, pigs fed pharmacological levels of Zn experience improved growth performance and decreased incidence of diarrhea. In addition, other effects have been attributed to zinc, including anti-inflammatory and infectious resistance mediation, maintenance of membrane function and stability via stabilization of membrane structure or anti-oxidation, and protection of cells from free-oxygen radicals. Thus, it appears that Zn may have an effect on reducing pathogen colonization of cell surfaces, especially when fed at pharmacological concentrations.

Zinc transporter 1 (ZnT1) is a cellular zinc exporter which is predominantly located in intracellular compartments and plasma membrane to promote zinc efflux from the cells when cellular zinc is abundant. In addition, ZnT1 is distributed in a number of tissues but has been observed to be most abundantly expressed in the proximal small intestine of humans. Although the mechanism for ZnT1 expression in all species and tissues has not been fully elucidated, it has been shown that ZnT1 mRNA is regulated by zinc supplementation or deletion in human cultured cells or in pigs fed high concentrations of zinc. The induction of ZnT1 gene transcription has also been explained by the binding of the metal-specific transcription factor MTF-1 on two metal response elements in the ZnT1 promoter region in mice.

Most of the research evaluating the expression and regulation of ZnT1 has been conducted in humans and mice and little emphasis has been placed on studies evaluating ZnT1 expression and regulation in pigs. Previous work has demonstrated that expression of ZnT1 protein in the liver was greater in pigs fed 1,000 ppm Zn than pigs fed 2,000 ppm Zn (with or without phytase). It has also been observed that ZnT1 is involved in regulating Zn homeostasis in nursery pigs fed pharmacological concentrations of Zn. However, the mechanisms for ZnT1 mRNA expression in different types of tissues in weaned pigs or the *in vitro* response of ZnT1 mRNA in porcine gut epithelial cells to different culture conditions is not clear. Therefore, the objectives of the current experiments are to evaluate the *in vivo* expression of ZnT1 mRNA in a panel of tissues obtained from weaned pigs, and to assess the *in vitro* changes in ZnT1 mRNA in IPEC-J2 cells treated with low and high concentrations of $ZnSO_4$ or GENT.

Materials and Methods

In Vivo *Expression of ZnT1 mRNA in Porcine Tissues*

Four crossbred barrows (typical of commercial pigs), approximately 5 weeks of age, were used and the experimental protocol was approved by the University of Nebraska Institutional Animal Care and Use Committee of the University of Nebraska–Lincoln. Weaned pigs (n = 4) were sacrificed and tissues were collected at the UNL Veterinary Diagnostic Center. Tissues (spleen, jejunum, ileum, liver, tonsil, and thymus) were excised and immediately frozen in liquid nitrogen for subsequent analyses.

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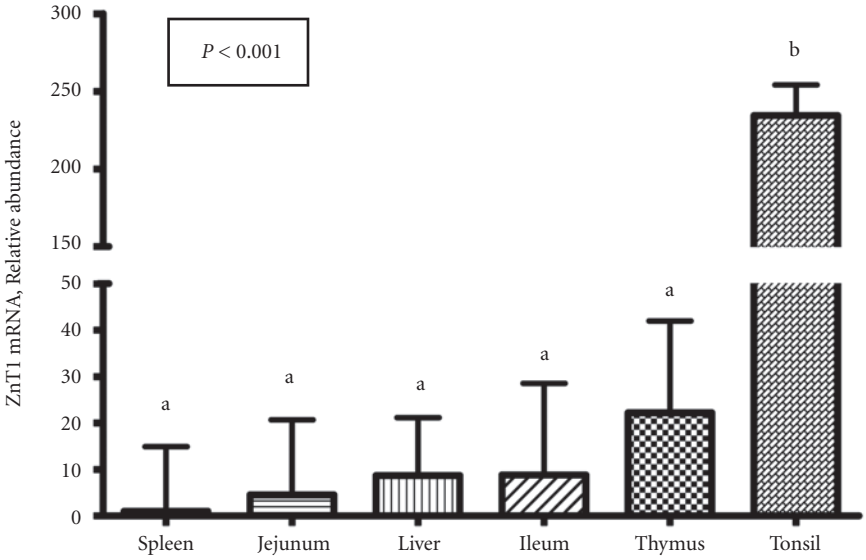

Figure 1. Relative abundance of zinc transporter (ZnT) 1 mRNA in porcine spleen, jejunum, ileum, liver, and thymus. Each bar represents the least squares mean (± SEM) of four observations. Bars without common superscripts differ ($P < 0.001$).

In Vitro *Expression of ZnT1 mRNA in IPEC-J2 Cells*

Two separate experiments were conducted using IPEC-J2 cells in order to characterize ZnT1 mRNA expression. The IPEC-J2 cells have been characterized previously and are nontransformed, jejunal epithelial cells derived from neonatal pigs and are maintained as a continuous culture. Cell cultures were maintained in DMEM-F12 growth medium supplemented with insulin/transferrin/Na selenite media supplement, epidermal growth factor, antibiotic and fetal bovine serum. For experimentation, IPEC-J2 cells were seeded onto six-well cell culture plates and maintained in the above mentioned media. The cells were allowed to adhere for 24 hours before being washed and re-fed every other day for seven days to allow for the formation of a model epithelium. Twenty-four hours before experimentation, cells were washed and replacement media was as above but devoid of antibiotics. Experiment 1 included the following treatments: 1) control (CTL; growth media devoid of antibiotics); 2) CTL + LPS (10 ng/mL); 3) CTL + low $ZnSO_4$ (40 µM); and 4) CTL + high $ZnSO_4$ (80 µM). Experiment 2 included the following treatments: 1) CTL; 2) CTL + LPS (10 ng/mL); 3) CTL + low GENT (50 µg/mL); and 4) CTL + high GENT (100 µg/mL). For both experiments, total RNA was harvested at 1.5, 3 and 6 hours following the addition of the respective treatments.

RNA Isolation and Quantitative Real-Time PCR Analysis

Total RNA was extracted and contaminating DNA was removed from all RNA samples. Samples were reconstituted in nuclease-free water and frozen for further analysis. The quality of RNA was assessed by agarose gel electrophoresis and visualization of the 28S and 18S rRNA bands. The quantity of RNA was determined by spectrophotometry (OD 260 nm). Complementary DNA (cDNA) was synthesized from 1.0 µg of RNA. Reverse transcription was carried out using reverse transcription reagents in a 50 µL final volume that included 25 mM $MgCl_2$, 500 µM dNTP's, 2.5 µM random hexamers, 0.4 U/µL Rnase inhibitor, 50 U/µL MultiScribe reverse transcriptase, and TaqMan RT buffer. The reverse transcription mixture was incubated at 25°C for 10 minutes, heated to 37°C for 60 minutes, and inactivated at 95°C for 5 minutes. The resultant cDNA was used as a template for real-time, quantitative polymerase chain reaction (qPCR) in order to quantify ZnT1 mRNA relative to the quantity of the endogenous control (18S rRNA). The qPCR reactions were carried out in 384-well plates with the ZnT1-specific forward and reverse primers and TaqMan TAMRA probe, PCR Mastermix, and 3.5 µL cDNA template. The porcine specific ZnT1 primers and detection probe were synthesized from published GenBank (Accession No. AY918800) sequences using PrimerExpress software. Commercially available eukaryotic 18S rRNA primers and probe were used as an endogenous control. PCR reactions, run in triplicate wells, were carried out with the Applied Biosystems 7900HT Fast Detection System using 40 cycles of amplification with alternating 15 seconds, 95°C denaturation and 1 minute, 60°C anneal/extension cycles.

Statistical Analyses

Relative abundance of ZnT1 mRNA in IPEC-J2 cells were calculated with the ΔΔCT method using the average ΔCT values of cells from control wells as the reference expression. These ΔΔCT values were analyzed using the MIXED procedure (SAS Inst. Inc., Cary, N.C.) to determine the effect of treatment on ZnT1 relative gene expression. The model included effects of treatment, time, and their interaction. Gene expression data from porcine tissues were handled similarly except that the average spleen ΔCT values were used as the reference gene expression (n = tissue from four pigs).

Results and Discussion

In Vivo *Expression of ZnT1 mRNA in Porcine Tissues*

The relative abundance of ZnT1 mRNA in porcine tissues is presented in Figure 1. Expression of ZnT1 mRNA was expressed in all tissues obtained from weaned pigs. In addition, significant differences in ZnT1 mRNA abundance were observed among tissues ($P < 0.001$). The greatest relative abundance of ZnT1 mRNA was observed in the tonsil and was greater ($P < 0.05$) than all other tissues (in

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order of decreasing ZnT1 mRNA: thymus, ileum, liver, jejunum, and spleen). This result differs from previous work in humans where ZnT1 mRNA was observed to be most abundant in the duodenum and jejunum. More research is warranted to evaluate ZnT1 mRNA expression in a greater number of animals, over a broader panel of tissues, as well as within different cell types.

In Vitro *Expression of ZnT1 mRNA in IPEC-J2 Cells*

The relative abundance of ZnT1 mRNA from the *in vitro* experiments is presented in Figure 2 ($ZnSO_4$ supplementation) and Figure 3 (GENT supplementation). The relative abundance of ZnT1 mRNA for each of the respective treatments are presented in comparison to CTL cells where the relative abundance of ZnT1 mRNA equals one.

$ZnSO_4$ Supplement on Cultured Media

With respect to experiments where IPEC-J2 cells were supplemented with low and high $ZnSO_4$, no time × treatment interaction was observed. However, ZnT1 mRNA expression tended ($P < 0.1$) to increase over time when means were averaged among all treatments, and ZnT1 mRNA tended ($P < 0.07$) to be increased in response to $ZnSO_4$ supplementation when means were averaged across all time points. These results are in agreement with previous work by others where ZnT1 mRNA in Caco-2 cells (a human colonic epithelial cell line) was increased with supplemental zinc within four hours of exposure.

In the second *in vitro* experiment, GENT supplementation resulted in a significant time × treatment interaction ($P < 0.005$). At 1.5 hours following initial exposure of IPEC-J2 cells to their respective treatments, no effects of treatment were observed on the relative abundance of ZnT1 mRNA. At 3 hours post-treatment, cells treated with high GENT had greater ($P < 0.05$) ZnT1 mRNA compared to all other treaments. At six hours post-treatment, the relative abundance of ZnT1 mRNA was greater ($P < 0.05$) in low and high GENT treated cells compared to CTL or LPS treated cells. In addition, the relative abundance of ZnT1 mRNA of high GENT treated cells was greater ($P < 0.05$) compared to all other treatments at all time points.

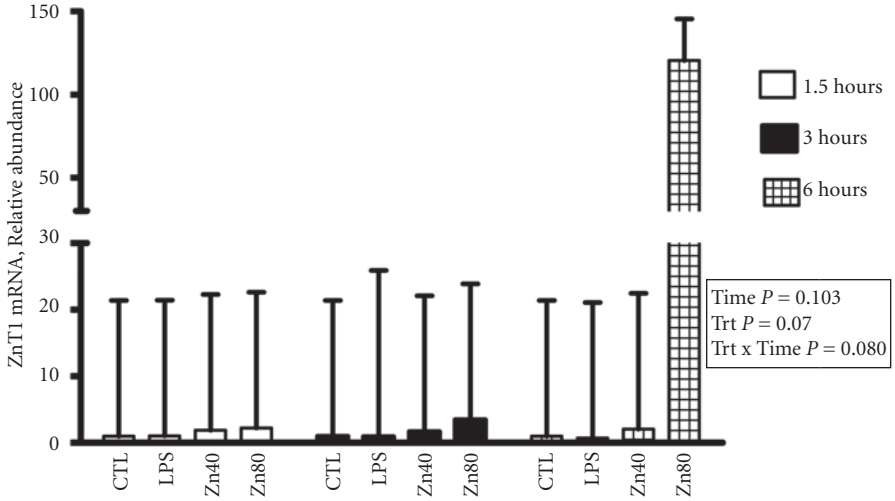

Figure 2. Relative abundance of zinc transporter (ZnT) 1 mRNA from cultured porcine jejunal epithelial cells (IPEC-J2) treated with media alone (CTL), 10 ng/mL lipopolysaccharide (LPS), 40 µM zinc (as zinc sulphate; Zn40), or 80 µM zinc (as zinc sulphate; Zn80). Total RNA extracted at 1.5, 3.0, and 6.0 hours post treatment. Each bar represents the least squares mean (± SEM) of three observations.

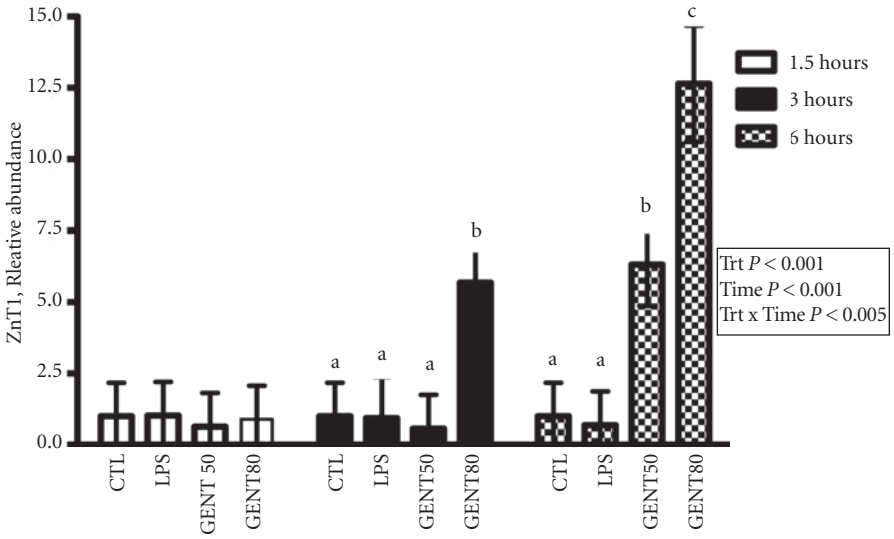

Figure 3. Relative abundance of zinc transporter (ZnT) 1 mRNA from cultured porcine jejunal epithelial cells (IPEC-J2) treated with media alone (CTL), 10 ng/mL lipopolysaccharide (LPS), 50 µM antibiotic (as gentamicin; GENT 50), or 100 µM antibiotic (gentamicin; GENT 100). Total RNA extracted at 1.5, 3, and 6 hours post-treatment. Each bar represents the least squares mean (± SEM) of three observations. Within time periods, bars without common superscripts differ ($P < 0.05$).

Conclusions

This research indicates that ZnT1 mRNA is differentially expressed in tissues obtained from weaned pigs and that ZnT1 mRNA is differentially regulated in IPEC-J2 cells in response to zinc and antibiotic supplementation. Additional studies are underway to evaluate ZnT1 mRNA expression in pigs and to determine the mechanisms by which zinc supplementation affects animal growth and health.

[1]Thomas E. Burkey is an assistant professor, Phillip S. Miller is a professor, and Huyen Tran is a graduate student in the Animal Science Department at the University of Nebraska–Lincoln.

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