Research Note: Jejunum phosphatases and systemic *myo*-inositol in broiler chickens fed without or with supplemented phytase

Fernando Gonzalez-Uarquin,* Edwin Molano,† Franziska Heinrich,* Vera Sommerfeld,* Markus Rodehutscord ,* and Korinna Huber*[,1]

Institute of Animal Science, University of Hohenheim, 70599 Stuttgart, Germany; and †Laboratory of Veterinary Physiology and Pharmacology, Universidad Nacional de Colombia, Bogota, Colombia

ABSTRACT As a constituent of animal cells, *myo*-inositol (**MI**) has been hypothesized to be crucial in several metabolic and regulatory pathways. Recently, it was shown that dietary phytase contributes to release of MI from phytate in the poultry digestive tract, increasing its systemic concentrations. This study investigated the activities of phosphatases in the jejunum and systemic plasma MI concentration in broilers not supplemented or supplemented with phytase through analyses based on modifications from commercial enzyme activity kits. Three hundred sixty male Ross 308 broilers were randomly allocated to 24 pens (15 birds per pen) in 4 dietary groups. The positive control group was fed with an adequate basal diet. The negative control group (**NC**) was fed with a reduced level of P and Ca. Groups Phy1500 and Phy3000 were fed with the NC diet plus 1,500 or 3,000 FTU of phytase per kilogram of feed, respectively. One bird per pen was selected for the measurement of jejunal phosphatase activity; MI concentration in plasma, the liver, and the kidney; and key MI enzyme concentrations (liver inositol monophosphatase 1 [**IMPase 1**] and kidney *myo*-inositol oxygenase [**MIOX**]). Endogenous phytase and alkaline phosphatase activity as well as IMPase 1 and MIOX expression were not statistically different among the dietary groups. The supplementation of 1500 FTU of phytase per kilogram of feed resulted in increase of plasma ($P < 0.001$) and kidney ($P < 0.05$) but not liver MI concentrations. The results indicated that systemic MI might reflect MI released from dietary sources; however, it did not appear to change expression of enzymes related to endogenous MI synthesis in the liver and catabolism in the kidney. New and larger studies are necessary to reach stronger evidence on the effects of dietary phytase on intestinal and systemic MI concentrations in broilers.

Key words: broiler, kidney, liver, *myo*-inositol, phytase activity

2020 Poultry Science 99:5972–5976
https://doi.org/10.1016/j.psj.2020.08.045

INTRODUCTION

Myo-inositol hexakisphosphate (**$InsP_6$**) is an antinutritional factor contained in the feed. In the gastrointestinal tract of broiler chickens, $InsP_6$ may be hydrolyzed to inorganic phosphate, intermediate inositol phosphates with different degrees of dephosphorylation (**InsPs**), and *myo*-inositol (**MI**) by the action of enzymes such as *myo*-inositol hexakisphosphate phosphohydrolase (phytase) and alkaline phosphatase (**ALP**).

Endogenous mucosal phosphatases (enzymes produced in the intestinal tract by enterocytes)—besides microbial phosphatases—have been shown to exist in the jejunum of broiler chickens (Huber et al., 2015). They appeared to have an important role in reducing the content of $InsP_6$ in the small intestine, indicating that broilers have a high endogenous capacity to degrade $InsP_6$ when the phosphorus (**P**) and calcium (**Ca**) supply overall is low (Sommerfeld et al., 2019). Supplementation with exogenous phytase results in higher $InsP_6$ degradation and increased P digestibility in broiler chickens, and at high doses, exogenous phytase (>500 FTU per kilogram of feed) triggered higher dephosphorylation activity of $InsP_6$ and increased MI concentration in the small intestine, which in turn was associated with increased MI concentrations in plasma of broilers (Sommerfeld et al., 2018). *Myo*-inositol has recently been suggested to be a relevant molecule for several

© 2020 Published by Elsevier Inc. on behalf of Poultry Science Association Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Received May 3, 2020.
Accepted August 24, 2020.

[1]Corresponding author: korinna.huber@uni-hohenheim.de

processes in chicken metabolism (Gonzalez-Uarquin et al., 2019).

The concentrations of MI in chicken organs and tissues are rarely reported (EFSA FEEDAP Panel, 2016). It is known that MI is endogenously synthesized de novo from D-glucose or generated by dephosphorylation of intracellular InsPs. A key enzyme for MI synthesis is inositol monophosphatase 1 (**IMPase 1**), which is responsible for the dephosphorylation of $InsP_1$ to MI. In the kidney, MI is reabsorbed via the proximal tubule and degraded by *myo*-inositol oxygenase (**MIOX**) (Gonzalez-Uarquin et al., 2020). Therefore, expression and activity of IMPase 1 and MIOX might indicate endogenous capacity for MI release from $InsP_1$ and MI catabolism, respectively. However, it is not yet possible to distinguish between MI in chicken metabolism originating from InsPs dephosphorylation and other metabolic precursors of MI.

Methods for quantification of phosphatase activity and MI metabolism are not readily available for use on chickens, so new and accessible strategies must be developed. Hence, the objective of this study was to determine the concentrations of jejunum phosphatases and systemic MI in broilers based on modifications from commercial enzymatic activity kits. Hypothetically, mucosal phosphatase activity and systemic MI concentrations would increase on phytase supplementation of the feed.

MATERIALS AND METHODS

Animals and Diets

Animals, diet composition, and experimental design of this trial were described previously in the study by Ajuwon et al. (2020). In brief, 360 male Ross 308 hatchlings were allocated to 24 floor pens. From the 8th to 22nd D of life, broilers were fed with the experimental diets, which were composed as follows: 1) nutrient-adequate basal diet based on soybean meal and corn (**PC**), 2) basal diet with a reduced P and Ca level (**NC**), 3) NC plus 1,500 FTU of phytase per kilogram of feed (**Phy1500**), and 4) NC plus 3,000 FTU of phytase per kilogram of feed (**Phy3000**). The concentrations of P and Ca in NC, Phy1500, and Phy3000 diets were reduced to balance for the expected effect of phytase-based P release and reduced Ca binding (1.5 g of P, 1.6 g of Ca). These levels were as per the recommendations of the phytase manufacturer for an inclusion level of 500 FTU per kilogram of feed (Quantum Blue; AB Vista, Marlborough, UK). Broilers were stunned with a gas mixture of 35% CO_2, 35% N_2, and 30% O_2 and euthanized by CO_2 asphyxiation. All procedures and protocols were approved by the Regierungspräsidium Tübingen, Germany (project no. HOH 51/17 TE), in accordance with the German Animal Welfare Legislation.

Tissue Sampling

After CO_2 asphyxiation, the broilers were weighed and immediately eviscerated. Six birds per diet (i.e., 1 bird per pen with body weight close to the average) were selected for this study, whereas the other birds were used for measurements made in the accompanying study (Ajuwon et al., 2020).

Liver and kidney samples were collected. About 15 cm of the proximal jejunum was removed and freed of any fat, and the jejunal mucosa was stripped off carefully using microscopic slides. All the tissue samples were washed in 1× PBS (0.02 mol/L, pH 7.0–7.2), cut in small pieces, shock frozen into liquid nitrogen, and collected in prechilled cryotubes. Immediately after finishing the collection, the organ samples were transported on dry ice and subsequently stored at −80°C for further analysis.

Tissue Grinding and Homogenization

The liver and kidney samples were ground under liquid nitrogen by using mortar and pestle. For homogenization, 420 mg of the sample was mixed with 500 µl of 1× PBS plus protease inhibitor (Complete mini; Hoffmann-La Roche, Mannheim, Germany) in lysing matrix tubes (MP Biomedicals, France) containing silica beads. Fast-prep homogenization (FastPrep-24 5G; MP Biomedicals, Shanghai, China) was performed 3 times at a rate of 6 m/s for 30 s (i.e., 90 s in total with 30 s of cooling intervals on wet ice to avoid heating of samples). After homogenization, the samples were centrifuged for 15 min at 1,500× g (Centrifuge 5424R; Eppendorf, Hamburg, Germany). Supernatants were collected, aliquoted, and stored at −80°C for further analysis.

Brush Border Membrane Enrichment

Brush border membrane (**BBM**) enrichment of enterocytes was performed as per the method described by Huber et al. (2015). In short, mucosa samples of the jejunum were ground under liquid nitrogen, and BBM were enriched by $MgCl_2$ precipitation and high-speed centrifugation. Resuspensions were made by gently passing the final BBM preparation through a syringe cannula with a 22-G needle (10×). Fifty microliters of BBM aliquots was shock frozen in liquid nitrogen and stored at −80°C until analysis.

Protein Quantification

One aliquot of each homogenate was diluted to 1:400 in distilled water for both organs: livers and kidney. Protein concentrations of the homogenates were determined using a protein assay according to the method of Bradford (Bradford Reagent, 5×; SERVA, Heidelberg, Germany). Analyses were performed in triplicate according to the manufacturer's protocol.

Endogenous Phosphatase Activity

Activity of phosphatases associated with the BBM was measured by using the phytic acid (phytate)/total phosphorus kit (K-PHYT 05/17 assay; Megazyme,

Bray, Ireland) with modifications. This kit is commonly used to determine phytate content of feedstuffs by complete enzymatic degradation of phytate to MI and P. For that, the kit contains a phytase and an ALP, which is added consecutively to the feedstuff sample. Finally, the released P is quantified using a spectrometric measure also included in the kit. To adapt the kit for measurement of endogenous mucosal phosphatases, the kit enzymes were replaced by BBM preparations (160 μg of protein); 5 μg of phytate was added as the substrate for the BBM-associated phosphatases. Phytate degradation was performed at a pH of 5.5 (optimal pH for phytase activity) for 10 min at 40°C according to the manufacturer's protocol. After finishing the reaction by adding trichloric acid, released phosphate (P_i) was quantified. The amount of P_i per milligram of protein per time released at pH 5.5 indicated the activity of endogenous phytase. In a second preparation from the same animal, activity of BBM-associated phosphatases was determined at a pH of 10.4 (optimal for ALP) and after finishing the reaction with addition of trichloric acid, released P_i was quantified spectrophotometrically. The amount of P_i per milligram of protein per time released at pH 10.4 indicated the activity of endogenous ALP. Total phosphatase activity was measured by processing the assay at both pH values with a further aliquot of BBM. The total amount of P_i per milligram of protein per time released indicated the activity of total endogenous phosphatases.

Myo-inositol Determination in Plasma, the Liver, and the Kidney

Plasma MI concentration was measured using gas chromatography-mass spectrometry after derivatization (Ajuwon et al., 2020). In the liver and kidney, diluted homogenates from each sample were used. The MI concentrations were measured using a commercially available enzymatic kit (K-INOSL 02/14; Megazyme International, Ireland) as per previous studies (Gonzalez-Uarquin et al., 2019). The K-INOSL assay was downscaled to 96-well microtiter plates (655101; Greiner Bio-One GmbH, Frickenhausen, Germany), and eight samples were run per assay. All samples were assessed in duplicate on the same plate, and concentrations were calculated as per the standards provided by the kit. Values were given as mg/g of DM.

Inositol Monophosphatase 1 and MIOX Expression

Inositol monophosphatase 1 expression and MIOX expression were determined using commercial enzyme-linked immunoassay kits (Chicken MIOX ELISA Kit, MBS7215577; Chicken IMPA1 ELISA Kit, MBS7235623; MyBioSource, San Diego, CA) in liver and kidney homogenates, respectively. Measurements were performed according to the manufacturer's protocol. Reported intra-assay and interassay CV% were 5.5 and 7.3%, respectively. In brief, 100 μl (containing 56.9 ± 0.8 mg/ml of protein, mean ± SEM) of each liver and kidney homogenates was buffered with the balance solution (provided by the kit) and incubated for 1 h at 37°C together with IMPase and MIOX horseradish peroxidase, respectively. After the incubation period, the plates were washed manually 5 times. Thereafter, the homogenates were incubated with a substrate for horseradish peroxidase. Once the product of the enzyme–substrate reaction formed a blue-colored complex, the stop solution was added to end the reaction, generating a yellow color. The intensity of yellow color was then measured spectrophotometrically at 450 nm using a microplate reader (Infinite M Nano, TECAN, Salzburg, Austria). Because both kits were competitive ELISA, the intensity of the color was inversely proportional to the IMPase 1 and MIOX concentration in the samples. Concentration of the standards was plotted as per the intensity of color (absorbance); then, sample enzyme concentrations were extrapolated from the standard values by a 5-parameter logistic curve fit (Magellan software, Tecan GmbH 2016, Salzburg, Austria). All values were normalized against tissue homogenate protein concentration. Values were given as pg of enzyme/mg of total protein.

Statistical Analyses

Dietary treatments were compared by one-way ANOVA followed by Tukey's Honest Significant Difference test using GraphPad Prism, version 6.07, for Windows (GraphPad Software, La Jolla, CA). F-values indicated the between-group-to-within-group variance ratio. Degrees of freedom are shown in the tables and figures as subscripted F-values. Laboratory analyses were performed in triplicate. The level of significance for all tests was set at $P < 0.05$.

RESULTS AND DISCUSSION

To the best of our knowledge, this study presents the first results showing MI and MI key enzyme concentrations in broilers using accessible experimental methods based on modifications from commercial enzymatic activity kits.

Activity of Endogenous Phosphatases

Total endogenous phosphatase, phytase, and ALP activities in the BBM were not different among the treatments (Table 1). The interindividual variation of measures was high for all treatments. These analyses were performed with 6 replicates per treatment; however, owing to technical reasons, phytase and ALP activity could not be analyzed in all samples.

It is difficult to distinguish between the extent of $InsP_6$ hydrolysis exerted by endogenous mucosal or microbial phosphatases. However, $InsP_6$ hydrolysis up to

Table 1. Effect of P, Ca, and phytase on endogenous phosphatase activity in the jejunum mucosa of broiler chickens fed with the experimental diets from day 8 to day 22.[1,2]

	PC	NC	Phy1500	Phy3000	*P-value*	F_{df}[3]
	μg of Pi[4]/mg of BBM[5] protein/min					
Total phosphatase activity	(n = 6) 0.95 ± 0.09	(n = 6) 0.99 ± 0.11	(n = 5) 1.38 ± 0.23	(n = 6) 1.28 ± 0.16	0.15	$1.93_{3,19}$
Phytase activity	(n = 5) 2.48 ± 0.25	(n = 5) 2.49 ± 0.35	(n = 4) 2.95 ± 0.56	(n = 4) 3.36 ± 0.45	0.39	$1.06_{3,14}$
ALP[6] activity	(n = 5) 1.07 ± 0.03	(n = 4) 0.98 ± 0.11	(n = 4) 0.98 ± 0.06	(n = 3) 1.08 ± 0.05	0.66	$0.54_{3,12}$

The n value varied among the treatments owing to technical reasons.
[1]The positive control group (PC) was fed with an adequate basal diet. The negative control group (NC) was fed with a reduced level of P and Ca. Groups Phy1500 and Phy3000 were fed with the NC diet plus 1,500 or 3,000 FTU of phytase per kilogram of feed, respectively.
[2]Data are given as treatment means ± SEM, and n values per treatment are given in brackets. One-way ANOVA was used as the statistical analysis of data.
[3]F-values indicated the between-group-to-within-group variance ratio. Degrees of freedom (df) are shown in the table as subscripts of F-values.
[4]Inorganic phosphate.
[5]BBM: brush border membrane.
[6]ALP: alkaline phosphatase.

the terminal ileum was found to be 42% in gnotobiotic broilers fed without a phytase supplement (Sommerfeld et al., 2019), which indicates a remarkable contribution of mucosal phytase to phytate hydrolysis. Total mucosal phytase activity appeared to be increased in growing broilers more because of dietary P rather than exogenous phytase (Abudabos, 2012). The underlying functions of the adaptive capacity are not fully understood, and endogenous enzyme activity may belong to the functions changed.

Myo-*inositol Concentrations in Plasma, the Liver, and the Kidney*

Plasma MI concentrations of the NC and PC groups were significantly lower than those of the Phy1500 and Phy3000 groups (Phy1500 vs. PC, $P < 0.001$; Phy1500 vs. NC, $P < 0.001$; Phy3000 vs. PC, $P < 0.002$; and Phy3000 vs. NC, $P < 0.04$) (Table 2). Liver MI concentrations did not differ among the treatments. In kidneys, the Tukey post hoc test indicated a higher MI concentration in the Phy1500 group than in the NC group ($P = 0.04$).

MI concentrations of about 11.0 mg/g of DM in the broiler liver has been reported by the EFSA FEEDAP Panel (2016); nonetheless, although this reference reported concentrations similar to the present study, it did not mention detailed information about the source of chickens and further experimental details. In the present study, broilers supplemented with 1500 FTU per kilogram of feed showed increase in their MI concentrations in kidneys compared with broilers fed with NC diet. This increase appeared to follow the increases seen in plasma, and because the kidney is the only organ where MI is catabolized, MI concentrations in kidneys may reflect MI surplus from plasma MI levels.

Effects of phytase supplementation on organ MI concentrations were not yet reported; however, potential

Table 2. Concentrations of MI and key MI enzymes in plasma, the liver, and the kidney of day 22 broilers (6 broilers per treatment).[1,2]

	PC	NC	Phy1500	Phy3000	*P-value*	F_{df}[4]
Concentration in mmol/L						
Plasma MI[3]	0.26 ± 0.01[b]	0.29 ± 0.01[b]	0.41 ± 0.02[a]	0.36 ± 0.01[a]	<0.001	$15.78_{3,20}$
Concentration in mg/g of DM						
Liver MI	7.93 ± 0.68	8.1 ± 1.4	10.3 ± 0.7	8.3 ± 0.07	0.38	$1.07_{3,20}$
Kidney MI	9.38 ± 1.3[a,b]	8.6 ± 0.6[b]	12.3 ± 0.9[a]	9.4 ± 0.6[a,b]	<0.05	$3.09_{3,20}$
Concentration in pg/mg of total protein						
Liver IMPase 1[5]	1.24 ± 0.11	1.36 ± 0.09	1.12 ± 0.08	1.20 ± 0.07	0.34	$1.16_{3,20}$
Kidney MIOX[6]	1.38 ± 0.22	1.39 ± 0.30	1.17 ± 0.20	1.36 ± 0.22	0.90	$0.18_{3,20}$

Abbreviation: MI, *myo*-inositol.
[1]The positive control group (PC) was fed with an adequate basal diet. The negative control group (NC) was fed with a reduced level of P and Ca. Groups Phy1500 and Phy3000 were fed with the NC diet plus 1,500 or 3,000 FTU of phytase per kilogram of feed, respectively.
[2]Data are given as group means ± SEM, and n values per group are given in brackets. One-way ANOVA was used as the statistical analysis of data.
[3]Plasma concentration was reported previously by Ajuwon et al. (2020).
[4]F-values indicated the between-group-to-within-group variance ratio. Degrees of freedom (df) are shown in the table as subscripts of the F-value. Different letters indicate differences among the groups.
[5]IMPase 1: inositol monophosphatase 1.
[6]MIOX: myo-inositol oxygenase.

linkages between supplemented MI in the liver and kidney MI concentrations have been studied in rodents. *Myo*-inositol–supplemented rats presented increases in kidney MI concentrations in comparison with MI-deprived rats from birth until adulthood (Burton and Wells, 1976). No differences were seen in liver MI concentration between the birth and 14 D of age; however, differences existed from day 18 of age until adulthood. Apparently, young rats suckled with MI-deficient milk showed rise in liver endogenous MI synthesis to the level of MI-supplemented rats; however, young and adult rats appeared to need dietary MI sources to maintain their optimal MI levels. Interestingly, female but not male MI-deprived rats presented high concentrations of MI in the liver and kidneys at day 21 of age (day of weaning), reaching the levels of MI-supplemented rats (Burton and Wells, 1976), indicating females may have a MI metabolism different from males. Although results from rat studies should not be extrapolated to chickens, they are indicative of metabolic differences between sex that are worth being investigated in poultry.

Liver IMPase 1 and Kidney MIOX Expression

Phytase supplementation did not affect IMPase 1 and MIOX protein expression in the liver and kidney, respectively (Table 2). Nonetheless, expression of both enzymes was numerically lower in the Phy1500 group than in other treatment groups. Walk et al. (2018) did not find differences in the mRNA expression of hepatic and renal MI transporters (SMIT1/2 and HMIT) of broilers not supplemented and supplemented with phytase. These results, together with the already discussed unaffected liver MI concentrations, indicate that the liver of broilers could rely more on intracellular MI synthesis than on blood levels of MI. However, measuring mRNA expression of MI transporters may not reflect the amount of functional protein in the cell, and measurement of the activity of MI key enzymes and transporters may reflect biological function better than mere quantitation of their expression.

Supplementation of 1500 FTU of phytase per kilogram of feed increased plasma and kidney MI concentrations; however, more research is necessary to understand and elucidate the underlying causes and mechanisms. It is expected that results of the present study may prompt further research on the mechanistic level such as the rate of MI synthesis in kidneys, the rate of MI excretion, IMPase 1/MIOX activity, and endogenous MI metabolism in organs.

ACKNOWLEDGMENTS

The authors are grateful to Kolapo Ajuwon (Department of Animal Sciences, Purdue University, West Lafayette, USA) for excellent collaboration in the animal trial and publishing of the accompanying article in which the experimental design of this article relied.

Conflict of Interest Statement: The authors did not provide a conflict of interest statement.

REFERENCES

Abudabos, A. M. 2012. Intestinal phytase activity in chickens (Gallus Domesticus). Afr. J. Microbiol. Res. 6:4932–4938.

Ajuwon, K. M., V. Sommerfeld, V. Paul, M. Däuber, M. Schollenberger, I. Kühn, O. Adeola, and M. Rodehutscord. 2020. Phytase dosing affects phytate degradation and Muc2 transporter gene expression in broiler starters. Poult. Sci. 99:981–991.

Burton, L. E., and W. W. Wells. 1976. Myo-Inositol metabolism during lactation and development in the rat. The prevention of lactation-induced fatty liver by dietary myo-inositol. J. Nutr. 106:1617–1628.

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). 2016. Scientific opinion on the safety and efficacy of inositol as nutritional additive for dogs and cats. EFSA J. 14:e04511.

Gonzalez-Uarquin, F., A. Kenéz, M. Rodehutscord, and K. Huber. 2019. Dietary phytase and myo-inositol supplementation are associated with distinct plasma metabolome profile in broiler chickens. Animal 14:549–559.

Gonzalez-Uarquin, F., M. Rodehutscord, and K. Huber. 2020. Myo-inositol: its metabolism and potential implications for poultry nutrition—a review. Poult. Sci. 99:893–905.

Huber, K., E. Zeller, and M. Rodehutscord. 2015. Modulation of small intestinal phosphate transporter by dietary supplements of mineral phosphorus and phytase in broilers. Poult. Sci. 94:1009–1017.

Sommerfeld, V., S. Künzel, M. Schollenberger, I. Kühn, and M. Rodehutscord. 2018. Influence of phytase or myo-inositol supplements on performance and phytate degradation products in the crop, ileum, and blood of broiler chickens. Poult. Sci. 97:920–929.

Sommerfeld, V., A. G. van Kessel, H. L. Classen, M. Schollenberger, I. Kühn, and M. Rodehutscord. 2019. Phytate degradation in gnotobiotic broiler chickens and effects of dietary supplements of phosphorus, calcium, and phytase. Poult. Sci. 98:5562–5570.

Walk, C. L., M. R. Bedford, and O. A. Olukosi. 2018. Effect of phytase on growth performance, phytate degradation and gene expression of myo-inositol transporters in the small intestine, liver and kidney of 21 day old broilers. Poult. Sci. 97:1155–1162.