Post-hatching ontogeny of intestinal proton-coupled folate transporter and reduced folate carrier in broiler chickens

M. Jing\(^1,2\), G. B. Tactacan\(^1\) and J. D. House\(^1,2^+\)

\(^1\)Department of Animal Science, University of Manitoba, Winnipeg, Canada R3T 2N2; \(^2\)Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, Canada R3T 2N2

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Folate transporters, including the reduced folate carrier and the proton-coupled folate transporter, encoded by \textit{Slc19a1} and \textit{Slc46a1} genes respectively, play important roles in the transport of folate across biological membranes given the hydrophilic nature of folates. Although a number of studies have demonstrated that these two transporters are regulated ontogenetically in mammals, little data are available on their developmental patterns of expression in poultry. The objective of this study was to investigate the expression patterns of \textit{Slc19a1} and \textit{Slc46a1} in jejunal and cecal tissue of broiler chickens during post-hatching development. Post-hatch male chicks (Ross \textit{\times} Ross) had free access to water and a soybean/wheat-based diet. Jejunal, cecal and blood samples were collected on day-of-hatch but before feeding (D0), and on D2, D7, D14, D21 and D35 post-hatch (\(n = 8\) at each time point), respectively. Plasma folate concentrations were low on the day of hatch and increased with maturation; by contrast, plasma homocysteine, a marker of folate status, was highest (\(P < 0.05\)) in the day-of-hatch birds and decreased thereafter. Increasing age reduced mRNA abundance of \textit{Slc19a1} (\(P < 0.05\)) in the jejunum and cecum. Abundance of \textit{Slc46a1} mRNA (\(P < 0.05\)) gradually decreased in the cecum with increasing age and that of \textit{Slc46a1} in the jejunum initially decreased and then increased to level similar to that of day-of-hatch. The study provides some initial data on ontogenetic regulation of \textit{Slc19a1} and \textit{Slc46a1} in the jejunum and cecum of the chicken and lays the ground work for future nutritional studies. Moreover, the expression of \textit{Slc19a1} and \textit{Slc46a1} transcripts in the cecum provides evidence of the potential for cecally derived folate to contribute to the folate status of the host.

Keywords: reduced folate carrier, proton-coupled folate transporter, mRNA abundance, ontogeny, broiler

Implications

There is a scarcity of knowledge regarding the ontogenetic patterns of expression for folate transporters in poultry species. Results generated in the study showed that the mRNA expression pattern of \textit{Slc19a1} and \textit{Slc46a1} genes, which encode the reduced folate carrier and the proton-coupled folate transporter proteins, respectively, appears to be differentially regulated with age in the broiler chicken. In addition, such results indicate the possible importance of cecal folate absorption to folate homeostasis in chickens. Future studies will investigate the contribution of bacterially synthesized folate to folate requirements and the regulation of folate transporters in the hind gut in chickens.

Introduction

Folate is an essential micronutrient that acts as a key one-carbon donor required for \textit{de novo} nucleotide and methionine biosynthesis (Lucok, 2000). Humans and other animals do not have the capability to synthesize folate, and thus must obtain the vitamin from exogenous sources via intestinal absorption. The intestine is exposed to two sources of folate: a dietary source, where the vitamin is mainly absorbed in the jejunum (Said \textit{et al.}, 1987; Said, 2004) and a bacterial source, where the vitamin is synthesized by the normal microflora in the large intestine and distal part of the small intestine (Crittenden \textit{et al.}, 2003; Kim \textit{et al.}, 2004; Asrar and O’Connor, 2005). Owing to the hydrophilic nature of folate, its passive diffusion across biological membranes is negligible. Therefore, the mechanisms for folate transport in the intestine are subject to considerable interest. Two facilitative transporters, the reduced folate carrier (RFC; also called solute carrier family 19, member 1) and a newly identified proton-coupled folate transporter (PCFT; also called solute carrier family 46, member 1), encoded by \textit{Slc19a1} and \textit{Slc46a1} genes, respectively, have been reported to be expressed on the apical brush border membrane of intestinal epithelial cells and involved in intestinal folate absorption.
transport in mammals (Matherly and Goldman, 2003; Qiu et al., 2006). Before the discovery of PCFT, RFC had long been regarded as the primary carrier-mediated intestinal folate transport protein (Chiao et al., 1997). However, RFC-mediated transport has been characterized to be optimal at near-neutral pH, whereas intestinal folate transport activity functions optimally at acidic pH (Wang et al., 2005; Said and Seetharam, 2006). PCFT has high affinity for folates and operates optimally at low pH, and has recently been identified as the major molecular entity of the intestinal folate transport system (Qi et al., 2006 and 2007; Inoue et al., 2008). Members of our group recently found that the transport of folic acid in the intestine of laying hens demonstrated maximal activity at an acidic pH of 6.0, but was significantly reduced when H\textsuperscript+ gradients were eliminated, indicating PCFT may be the primary functional protein in the transport process of folate in chickens (Tactacan et al., 2011).

Research in mammals has shown that the overall intestinal transport process of folate is regulated ontogenetically, which is associated with changes at the transcriptional level of RFC and/or PCFT. Previous studies in rats have demonstrated that the transport system for folate was fully developed at weaning, but decreased at weaning. The affinity of the transport carriers increased with age. Therefore, the objective of the present study was to investigate the post-hatching ontogeny of jejunal and cecal folate transporters in chickens. We hypothesize that the expression of genes encoding folate transporter proteins in the jejunum and cecum of broiler chickens decreases with age. Therefore, the objective of the present study was to investigate the post-hatching ontogeny of jejunal and cecal mRNA abundance of genes Scl19a1 and Scl46a1 in male broiler chickens.

**Material and methods**

**Animals**

Male day-of-hatch chicks (Ross × Ross; Granny’s Poultry Co-operative) were randomly assigned to brooder batteries equipped with heated floor pens with wood shavings. All pens had 24-h lighting and chicks were maintained in brooder batteries until 2 weeks of age and then transferred to grower batteries without heating but maintained at room temperature. Birds had free access to water and a soybean/wheat-based diet that was formulated to contain 3040 kcal/kg metabolizable energy, 20.0% CP, 1.15% total Lys, 0.47% total Met, 0.87% total Met and Cys, 0.77% total Thr, 1.22% total Arg, 0.28% total Tryp, 0.98% calcium, 0.49% available phosphorus and 0.21% sodium. The diet was additionally supplemented with 4.0 mg/kg synthetic folic acid (96%). The birds were managed in accordance with recommendations established by the Canadian Council on Animal Care (1993) following an animal care protocol approved from the University of Manitoba’s Animal Care Protocol Management and Review Committee.

**Tissue sampling**

Birds were sacrificed by cervical dislocation at the following time points post-hatch: D0 (within 9 h after hatch but before feeding), D2, D7, D14, D21 and D35 after feeding. For all time points, the proximal part of jejunum and middle part of cecum were collected from eight birds that had similar BW and rinsed with ice-cold PBS solution (pH 7.4), and then stored in RNAlater\textsuperscript® Tissue Collection solution (Applied Biosystems Inc., Foster, CA, USA). Blood samples were collected into heparinized tubes, and the resultant plasma was used for subsequent folate and homocysteine (Hcy) analysis.

**Analysis of plasma folate and Hcy**

Plasma folate concentrations were determined using a competitive binding assay, SimulTRAC-S Radioassay Kit Vitamin B\textsubscript{12} [\textsuperscript{57}Co]/Folate [\textsuperscript{125}I] (MP Biomedicals, Orangeburg, NY, USA) according to the manufacturer’s protocol. Plasma Hcy was measured by reverse-phase HPLC with fluorescence detection, using the method of Araki and Sako (1987), as modified by Gilfix et al. (1997).

**RNA isolation and quantitative real-time PCR (qRT-PCR)**

Approximately 15 mg of intestinal tissue was used for total RNA isolation using RNeasy Mini kit (QIAGEN Canada Inc., Mississauga, ON, Canada) following the manufacturer’s instructions, and RNA quality and quantity were assessed on a DU800 Spectrophotometer (Beckman Coulter Canada Inc., Mississauga, ON, Canada). The absorbance ratio at wavelength 260/280 nm was within 1.8 to 2.1. The prepared RNA was treated, in order to eliminate the possibility of genomic DNA contamination, through the use of a TURBO DNA-free\textsuperscript® kit (Applied Biosystems Inc.). The cDNA for respective tissues was synthesized via reverse transcription (RT) using 1 \( \mu \)g of total RNA and the SuperScript\textsuperscript® VILO\textsuperscript® cDNA Synthesis Kit (Invitrogen Canada Inc., Burlington, ON, Canada). The RT conditions for each cDNA synthesis were 25°C for 10 min, 42°C for 90 min and 85°C for 5 min. Gene expression was quantified by SYBR green real time PCR as described previously (Jing et al., 2009). The primer sequences for Scl19a1, Scl46a1 and Actb encoding beta-actin and their specificity have been reported previously (Jing et al., 2009 and 2010).
Conditions for the qRT-PCR were 95°C for 20 s and 40 cycles of denaturation at 95°C for 3 s, and combined annealing and extension at respective annealing temperature (60°C for both Slc19a1 and Slc46a1; 62°C for Actb) for 30 s, then followed by one three-segment cycle of product melting (95°C/15 s, 60°C/1 min, 95°C/15 s). Dissociation curves confirmed the specific amplification of the Slc19a1, Slc46a1 and Actb cDNA and the absence of non-specific products. Real-time PCR was performed in duplicate in 20-μl reactions using a StepOneTM Real-Time PCR System (Applied Biosystems Inc.). Corresponding mean values were used for further analysis. The relative level of target gene expression was analyzed using the ΔΔCt method (User Bulletin #2: Relative Quantitation of Gene Expression; Applied Biosystems). Changes in the mRNA abundance were calculated after normalization to Actb. The stability of Actb as an internal control was validated by the data that showed that the absolute values of ΔCt among intestinal samples at different developmental ages were less than 1 (Figure 1). The formulas used in the ΔΔCt calculation are provided below:

\[ \Delta C_t = C_{target} - C_{Actb} \]

\[ \Delta \Delta C_t = C_{treatment} - C_{control} \]

**Gene expression level** = \(2^{-\Delta \Delta C_t}\)

For both genes, the control values used in the calculation of relative mRNA abundance were the respective values derived from day 0 for both the cecum and the jejunum.

**Figure 1** mRNA abundance of Actb gene in both the jejunum and cecum of male broiler chickens at different ages, with day 0 representing day of hatch (DOH). Data are presented as means plus standard deviation (n = 4).

### Results

**BW of broiler chickens**

The BWs of birds were recorded before sacrificing at each time point. The values (mean ± s.e.m.) were 40 ± 1, 61 ± 2, 164 ± 8, 443 ± 27, 1005 ± 34 and 2230 ± 35 g on D0 (day of hatch (DOH) but before feeding), D2, D7, D14, D21 and D35 post-hatch, respectively, which were in agreement with growth curves reported in the commercial management guide for these broiler chickens (Aviagen, 2007).

**Plasma folate and Hcy concentrations**

Plasma folate and Hcy concentrations for broiler chickens at different ages are given in Table 1. In general, plasma folate concentrations were lowest on the DOH and then increased with age up to day 7, with no significant differences observed between time points post-day 7. The plasma concentrations for Hcy were highest on day-of-hatch and decreased thereafter (P < 0.05).

**Jejunal and cecal RFC and PCFT mRNA abundance**

The ΔΔCt method was used to determine the mRNA abundance of genes examined in this study. The fold changes in the expression of Slc19a1 and Slc46a1 during development are presented in Figure 2 for the jejunum, and in Figure 3 for the cecum, respectively. Overall, Slc19a1 mRNA in the jejunum and cecum, while not directly compared, revealed similar expression patterns with age, namely, its mRNA abundance was highest on DOH and then declined (P < 0.05). The expression of Slc46a1 in the jejunum decreased (P < 0.05) in the first week and then gradually increased back to the level of DOH at D35 post-hatch. However, Slc46a1 expression in the cecum progressively decreased (P < 0.05) with advancing age.

### Table 1 Plasma folate and homocysteine concentrations in male broiler chickens at different ages

<table>
<thead>
<tr>
<th></th>
<th>D0</th>
<th>D2</th>
<th>D7</th>
<th>D14</th>
<th>D21</th>
<th>D35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folate (ng/ml)</td>
<td>(49.1\ ± \ 5.2^c)</td>
<td>(100.4\ ± \ 23.0^{bc})</td>
<td>(199.6\ ± \ 25.0^a)</td>
<td>(191.2\ ± \ 19.3^b)</td>
<td>(153.0\ ± \ 9.2^{ab})</td>
<td>(167.5\ ± \ 17.0^a) **</td>
</tr>
<tr>
<td>Homocysteine (μmol/l)</td>
<td>(37.98\ ± \ 3.74^a)</td>
<td>(24.43\ ± \ 1.38^c)</td>
<td>(21.52\ ± \ 1.47^{rd})</td>
<td>(29.37\ ± \ 1.07^b)</td>
<td>(20.26\ ± \ 1.27^{cd})</td>
<td>(17.56\ ± \ 1.39^d) **</td>
</tr>
</tbody>
</table>

^a,b,c,d^ Values (mean ± s.e.m.) not sharing a common superscript within a row differ significantly (P < 0.05) between age groups (n = 4 to 8).

\(^{1}\)D0, D2, D7, D14, D21 and D35: day of hatch and days 2, 7, 14, 21 and 35 after hatch of male broilers.

**P < 0.01.**

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Figure 2 Relative mRNA abundance of Slc19a1 (n = 4; s.e.m. = 0.20) and Slc46a1 (n = 4; s.e.m. = 0.37) in the jejunum of male broiler chickens at different ages, with day 0 representing day of hatch (DOH). For each tissue, means not sharing a common letter differ between age groups (P < 0.05). For both genes, the ΔCt value at day 0 was used as the control in calculating relative mRNA abundance.

Figure 3 Relative mRNA abundance of Slc19a1 (n = 4; s.e.m. = 0.17) and Slc46a1 (n = 4; s.e.m. = 0.24) in the cecum of male broiler chickens at different ages, with day 0 representing day of hatch (DOH). Means not sharing a common letter differ between age groups (P < 0.05). For both genes, the ΔCt value at day 0 was used as the control in calculating relative mRNA abundance.

Discussion

Intestinal development is likely to be associated with changes in the levels of expression of various genes related to the transport of nutrients. Existing research in mammals has shown that the process of folate transport undergoes physiological development-dependent regulation accompanied with the changes in the expression of folate carriers RFC and PCFT in the intestine. However, relevant studies in poultry have not yet been reported. Thus, the present study was designed to investigate whether the expression of folate transporters in the intestine is regulated by ontogeny in chickens, which will offset the relative scarcity of knowledge on the folate transport system in avian species, and also contribute to how these might differ from mammalian expression patterns.

The ontogeny of folate transporters has been determined in mammals, in which there is a downregulation in their expression with development. Higher expression of Slc19a1 transcripts was detected in suckling rats compared with weanling or adult animals (Balamurugan and Said, 2003). Shafizadeh and Halsted (2009) recently showed that Slc19a1 mRNA transcripts were present in the small intestine at birth and declined significantly throughout development to 6 months in pig. Balamurugan et al. (2007) also found that there was a gradual decrease with development in the abundance of mRNA and protein of a specific folate transporter (encoded by folt-1 gene) in the intestine of nematodes. There is little data available on the ontogeny of PCFT expression because of its new identification as a major transport system for folate.

Studies in chickens have provided evidence that nutrient transporters were highly expressed in the immediate post-hatch period in response to increasing needs for the assimilation of numerous nutrients in support of growth, metabolism and development (Gilbert et al., 2007). In the current study, we found that the expression of Slc19a1 and Slc46a1 in the intestine was affected during post-hatching development. The mRNA abundance of Slc19a1 in jejunum and cecum, while not directly compared, exhibited similar expression patterns, and the overall trend was a decrease in expression with maturation. Although Slc46a1 mRNA in the cecum decreased gradually with age, its abundance in the jejunum was reduced in the first week and then progressively restored to post-hatching levels. In accordance with previous research in mammals, our current findings support the possibility that the ontogeny-dependent regulation of intestinal folate transporters was mediated, at least in part, via a transcriptional mechanism(s) in chickens. However, additional studies are needed to identify the exact mechanisms that regulate the expression of these gene products during development, and whether alterations in mRNA stability, post-transcription or translation are also involved in this ontogenic regulation of folate transport in the intestine.

The ontogenic downregulation of Slc19a1 expression was in agreement with the findings of Li et al. (2008) who reported that intestinal Slc19a1 mRNA abundance at D14 post-hatch was lower than that at DOH in broiler chickens analyzed by RNA microarrays, however data regarding PCFT was not presented. Taken together, the available evidence from both avian and mammalian species support the existence of mechanisms to prepare the developing animal for folate uptake post-hatch or post-natally. The higher expression of RFC and/or PCFT during the early post-natal or post-hatching period is consistent with the fact that folate is very important for early growth and development because of its critical role in DNA, RNA and protein synthesis. Determining the precise expression patterns of folate transporters may lead to the development of better folate supplementation in the formulations that more closely match folate availability with its uptake capacity. Moreover, information gained from this study will be useful in improving
our understanding of the processes associated with folate assimilation in the chicken intestine during post-hatching development.

In order to provide additional insights into the potential biological relevance of the observed ontogenic changes in the expression patterns of the folate transporters, we measured plasma folate and Hcy as markers of folate availability. Plasma folate was low at initial hatch and then increased to a plateau by day 7 post-hatch. In general, plasma Hcy concentrations mirrored observed changes in folate status, being highest at DOH and decreasing thereafter, with the exception of an elevation at day 14. Folate plays an important role in lowering Hcy (van der Put et al., 2001; House et al., 2003), and is a major determinant of plasma Hcy concentrations. Other nutrients including vitamins B6 and B12 and non-nutritional factors may influence Hcy metabolism (House et al., 1999). With respect to plasma folate, the dramatic increase observed immediately post-hatch likely reflects the transition from maternal (i.e. yolk sac) to dietary sources of folate, as the diets contain sufficient amounts of both natural and synthetic folate. Changes in plasma folate did not parallel the ontogenic patterns of mRNA abundance of the folate transporters. Mammalian studies have demonstrated that the expression of intestinal folate transporters is downregulated by folate oversupplementation (Ashokkumar et al., 2007; Dev et al., 2011) and upregulated under conditions of folate deficiency (Said et al., 2000; Liu et al., 2005). The authors suggest the existence of homeostatic and adaptable responses to intestinal folate uptake processes via the transport system in response to levels of dietary folate intake. When faced with folate over-supplementation, the animal tries to minimize net uptake of dietary folates from the intestinal lumen and into the bloodstream via decreasing folate transporter(s) expression; in contrast, when faced with the challenge of folate deficiency, the increase in the expression of the transport protein(s) should serve to maximize its uptake of dietary folates. On the basis of these suggestions, it is proposed in the present study that the reduced expression of key folate transporters immediately after hatch may respond to abundant folate, in other words, high folate may downregulate the expression of the genes. Such a change probably works to ensure that folate absorption does not occur at a rate that exceeds the capacity of the chick to metabolize excess folate at a critical transition period between limited maternal folate supply and the new and abundant sources of folate available from diet.

Our previous studies have shown that both Slc19a1 and Slc46a1 transcripts were expressed in the ceca of laying hens (Jing et al., 2009 and 2010). As expected, their expression was also found in the ceca of broiler chickens, and the mRNA abundance of both Slc19a1 and Slc46a1 decreased with maturation. These data may serve to highlight the potential importance of the ceca in folate metabolism and homeostasis, given the fact that folates derived from commensal microorganisms may serve as potential complementary sources of bioavailable folate (Kim et al., 2004; Asrar and O’Connor, 2005).

In conclusion, the results obtained in this study demonstrate that the expression of jejunal and cecal folate transporters decreased as the chicks aged. The exact mechanisms regulating the reduction in expression are not known, but may relate to factors in play during the critical transition period from maternal to dietary sources of folate. Adequate folate absorption is critical to ensure its sufficient supply for critical biological functions, including DNA and RNA synthesis, and the regulation of one carbon metabolism. In addition, the current data regarding the expression of folate transporters in the ceca serve to provide evidence that the endogenous production of folates from commensal microorganisms may contribute to the folate status of the developing chick.

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