

Age-related Regulation of Active Amino Acid Transport in the Ileum of Broiler Chickens

Mikako Shibata¹, Tatsuyuki Takahashi¹, Kanari Endo¹, Takaharu Kozakai³,
Yoshiyuki Azuma² and Yohei Kurose¹

¹Laboratory of Animal Metabolism and Function, School of Veterinary Medicine,
Kitasato University, 35-1 Higashi 23, Towada, Aomori, Japan

²Laboratory of Animal and Human Nutritional Physiology, School of Veterinary Medicine,
Kitasato University, 35-1 Higashi 23, Towada, Aomori, Japan

³Faculty of Education, Art and Science, Yamagata University, 1-4-12 Koshirakawamachi, Yamagata, Japan

Broiler chickens grow rapidly within a short period; in this regard, our group had previously reported a decrease in the active transport of glucose in the intestines of broiler chickens with their growth. Therefore, in this study, we compared the active transport process of amino acids in the intestines between 1- and 5-week-old broilers using everted sac, Ussing chamber techniques, and real-time quantitative polymerase chain reaction (RT-PCR). The everted sac experiment showed that amino acids were absorbed from all segments of the small intestine in both age groups. There were no significant differences in the serosal to mucosal ratio between 1- and 5-week-old broilers. The Ussing chamber experiment showed that amino acid-induced short-circuit current (ΔI_{sc}) in the ileal epithelium was significantly greater in the 5-week-old chickens than in the 1-week-old chicks ($P=0.035$). Membrane conductance, an indicator of ion permeability, showed no significant difference between the two groups. Moreover, the mRNA expression levels of amino acid transporters (ASCT1, EAAT3, B⁰AT1, and y⁺LAT1) were significantly elevated in the distal ileum of the 5-week-old broilers compared to those in the 1-week-old broilers ($P<0.05$), while no significant differences were observed in the mRNA levels of ATB0⁺, B^{0/+}AT, rBAT, CAT1, and CAT2 in both groups. Our study provides clear evidence that age-dependent increase in the active transport of amino acid across the ileal epithelium is caused by the high expression of Na⁺-dependent amino acid transporters in broiler chickens.

Key words: age, amino acid, active transport, broiler, ileum

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Introduction

Genetic selection and high-energy feeds have led to a rapid growth rate and efficient meat production in broiler chickens (Zuidhof *et al.*, 2014). However, metabolic diseases, excessive abdominal fat deposit, ascites, sudden death syndrome, and leg problems, often occur in broiler chickens (Julian, 2005). Understanding various dynamics in intestinal nutrient absorption upon growth may, therefore, contribute to the development of efficient strategies toward addressing these

problems.

In our previous study, we showed that the active transport of glucose across the jejunal epithelium decreased with growth in broiler chickens, but it is not accompanied by any variations in maltase activity or the expression of glucose-absorption-related genes (Shibata *et al.*, 2019). Our result suggests that the intestinal absorption of nutrients other than glucose may increase with growth, e.g., the intestinal absorption of amino acids and fructose was shown to change upon growth in broilers (Obst and Diamond, 1992).

Amino acids are crucial for fetal and postnatal growth and development (Wu, 2009). Deficiency in dietary lysine (Lys) can delay chick development (Sterling *et al.*, 2003). Moreover, dietary supplementation of methionine increases body weight but decreases abdominal fat in 5-week-old broilers (Mohamed and Talha, 2011). In incubated chick skeletal muscles, the addition of glutamine induces an anabolic effect by increasing protein synthesis and decreasing protein degradation (Wu and Thompson, 1990).

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Correspondence: Yohei Kurose, School of Veterinary Medicine, Kitasato University, 35-1 Higashi 23, Towada, Aomori, 034-8628, Japan.

(E-mail: kurose@vmas.kitasato-u.ac.jp)

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In the intestine, the amino acid is transported via Na^+ -dependent and/or Na^+ -independent transporters expressed in the cell membrane as well as glucose (Hyde *et al.*, 2003). The ileum and jejunum are the active sites for amino acid absorption (Tauqir, 2016). Dietary amino acids are transported across the brush border membrane of the intestine via mainly Na^+ -dependent and Na^+ -independent transporters in response to acidic, neutral, and/or basic amino acids (Bröer, 2008). An age-dependent increase in mRNA expression levels of Na^+ -dependent neutral amino acid transporter ($\text{B}^0\text{AT1}$), anionic amino acid (aspartate and glutamate) transporter (EAAT3) and Na^+ -independent cationic and neutral amino acid exchanger ($\text{B}^{0/+}\text{AT}$) was observed in the ileum, but not in the duodenum and jejunum, of broilers (Gilbert *et al.*, 2007).

In this study, we compared the transport of active amino acids in the intestine between 1- and 5-week-old broilers to evaluate the hypothesis that 5-week-old broilers substitute amino acids for glucose to meet their rapid growth. The everted sac technique and short-circuit current method (I_{sc}) were used to accurately assess the active transport of amino acids across the intestinal epithelium. Furthermore, mRNA expression levels of amino acid transport-related genes (ASCT1, $\text{ATB}^{0/+}$, EAAT3, $\text{B}^0\text{AT1}$, $\gamma\text{-LAT1}$, $\text{B}^{0/+}\text{AT}$, rBAT, CAT1, and CAT2) were quantified in the ileum. In all experiments, 1-week-old chicks showing the highest growth rate and 5-week-old chickens reaching slaughter weight were compared.

Materials and Methods

Animals and Diets

All experiments performed in this study were approved by the Institutional Animal Care and Use Committee of Kitasato University (Approval #16-064). Male broiler chickens (White Cornish \times White Plymouth Rock) aged between 1 and 5 weeks were used for the study. Day-of-hatch broiler chicks were obtained from Prifoods Co., Ltd. (Aomori, Japan). The chicks were housed into brooders at $28 \pm 2^\circ\text{C}$ until they reached 1 or 3 weeks of age, and the 3-week-old chicks were individually housed at $26 \pm 2^\circ\text{C}$ until they reached 5 weeks of age. The brooders and room had 24-hr lighting. All chickens consumed the same standard diet (Chubushiryō Co., Ltd., Chita, Japan) to minimize dietary effects through the experiments and had access to feed and water *ad libitum*. To empty different amounts of intestinal digesta prior to tissue preparation, chickens were fasted for 12 h at 1 week of age and 24 h at 5 weeks of age.

Tissue Preparation

Broilers were euthanized using pentobarbital (NACALAI TESQUE, INC., Kyoto, Japan) overdose (50 mg/kg BW, i.p.) and exsanguination at 1 and 5 weeks of age. The entire small intestine was collected and divided into five regions: the duodenum, proximal jejunum, distal jejunum, proximal ileum, and distal ileum. All regions were used in everted sacs experiments, and the distal ileum was used in I_{sc} experiments. Some pieces of fresh mucosal epithelial tissues were placed in RNAlater[®] stabilization solution (Thermo

Fisher Scientific K.K., Tokyo, Japan) for quantitative real-time PCR (RT-PCR) analysis. Tissue samples were all stored at -80°C until use.

Measurement of Intestinal Amino Acid Absorption using the Everted Sac Technique

Intestinal amino acid absorption was evaluated in 1-week-old ($n=6$: 208 ± 5.2 g BW) and 5-week-old broilers ($n=6$: 2650 ± 149.4 g BW) using the everted sac technique based on our previous report (Shibata *et al.*, 2019). Briefly, each intestinal tissue was cut into approximate segments of 5 cm and was rinsed with phosphate buffer saline (PBS) on ice. These tissues were everted and filled with Krebs Ringer Buffer (KRB: 140 mM NaCl, 5.0 mM KCl, 1.0 mM MgCl_2 , 2.0 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 10 mM HEPES, pH 7.4) supplemented with equimolar mixtures of 10 amino acids (each 1 mM: L-alanine (Ala), L-asparagine (Asn), L-glutamate (Glu), L-lysine (Lys), L-histidine (His), L-glutamine (Gln), L-serine (Ser), L-methionine (Met), L-tryptophan (Trp), glycine (Gly)) gassed with 100% O_2 at 40°C for at least 30 min. The everted tissues were then incubated with 50 mL of KRB supplemented with the same amino acids in a 40°C water bath for 60 min. The content of the sacs represents the amount absorbed in the serosal compartments, whereas the content of the flask represents the mucosal compartments. The contents of the sac sample were stored at -30°C until use. The serosal to the mucosal ratio (S/M) of amino nitrogen concentrations was calculated as an index of amino acid transport.

Measurement of I_{sc} and Tissue Conductance in Intestinal Amino Acid Absorption

The I_{sc} experiment was conducted following the method previously described (Shibata *et al.*, 2019). Briefly, the fresh mucosal tissue of the distal ileum was mounted in Ussing chambers (exposed area: 0.50 cm^2) in either 1-week-old ($n=8$: 157.5 ± 13.4 g BW) or 5-week-old broilers ($n=8$: $2,291.3 \pm 171.3$ g BW). Tissue was equilibrated with modified KRB buffer (127 mM NaCl, 4.0 mM KCl, 2.0 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 10 mM D-glucose, 10 mM HEPES, pH 7.4) at 40°C for 15 min while gassing with 100% O_2 . Both buffers were then replaced with KRB without D-glucose. Equimolar mixtures of 10 amino acids (each 1 mM: Ala, Asn, Glu, Lys, His, Gln, Ser, Met, Tyr, Gly) or water were added to the mucosal chambers, and the tissue was incubated for 10 min.

As electrogenic ion transport, I_{sc} was continuously recorded using a voltage-clamp amplifier (CEZ-9100, NIHON KOHDEN Co., Ltd., Tokyo, Japan) and a MacLab 8 (AD Instruments, Pty Ltd., Australia) at baseline and after the stimulation with amino acids to the mucosal chambers. To estimate tissue conductance (G_t , mS/cm^2), voltage command pulses (10 mV, 1 sec duration) were also applied at 1 min intervals through the experiment. G_t was calculated based on Ohm's law. The difference in I_{sc} (ΔI_{sc} , $\mu\text{A/cm}^2$) was calculated by subtracting the basal I_{sc} from the peak I_{sc} after amino acids challenge. The difference in G_t (ΔG_t) was calculated by subtracting the basal average G_t ($-5-0$ min) before challenge from average G_t (5-10 min) after amino acid challenge.

Amino Nitrogen Concentrations

Amino nitrogen concentrations in everted sac samples

were measured using a ninhydrin reaction method based on previous reports with slight modification (Moore *et al.*, 1954; Matthews *et al.*, 1964). First, 100 μ l of sample solution was added to 300 μ l of 5% trichloroacetic acid and subsequently centrifuged at 10,000 \times g for 10 min at 4°C. Then 40 μ l of supernatant was mixed with 2 mL of color reagent (254 mM citric acid monohydrate, 14 mM ninhydrin, 0.6 mM ascorbic acid, 20% 2-methoxyethanol) in a glass tube. These samples were boiled at 100°C for 20 min and kept in ice at least for 15 min prior to analysis. Amino nitrogen concentrations were measured using a multi microplate reader (MTP-800Lab, CORONA ELECTRIC Co., Ltd., Ibaraki, Japan).

RNA Isolation and Quantitative RT-PCR.

Total RNA was extracted from the RNAlater[®]-immersed distal ileum tissues in both chicken groups using RNAiso Plus (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. cDNA was synthesized from 500 ng total RNA using the ReverTra Ace qPCR RT Master Mix kit (TOYOBO CO., LTD., Osaka, Japan). Specific primers were designed using Primer 3Plus software and selected according to the previous report (Table 1) (Gilbert *et al.*, 2007). qRT-PCR was performed using THUNDERBIRD SYBR qPCR Mix (TOYOBO CO., LTD., Osaka, Japan) on a StepOnePlus[™] Real-Time PCR System (Thermo Fisher Scientific K.K.). Melting curves in all primers were confirmed in the specificity of the single-target amplification. The housekeeping gene β -actin was used for normalization. All tests were done in duplicate, and the relative mRNA expression levels were calculated from the Ct value of samples compared to a standard curve. Each target band was confirmed by sequence analysis after direct sequencing analysis.

Statistical Analysis

All data were presented as mean \pm standard error (SE). In all data, differences between the two age groups were determined as statistically significant at $P < 0.05$. In the everted sac experiment, the two-way ANOVA was used to compare the S/M ratios based on two independent factors (region and age). In other experiments, statistical significance between the two age groups was analyzed by Student's *t*-test for equal variances or by Welch *t*-test for unequal variances.

Results

Analysis of S/M Ratio on Intestinal Amino Acid Absorption using the Everted Sac Technique

S/M ratio was more than 1 in all segments (Fig. 1), but there were no significant differences in the S/M ratio between 1- and 5-week-old broilers.

Changes in I_{sc} and Tissue Conductance in the Distal Ileum After Amino Acid Stimulation

After stimulation with amino acids, a gradual elevation of the amino acid-induced I_{sc} response was observed in the 1-week-old chicks while an immediate elevation was observed in the 5-week-old chickens (Fig. 2A). The ΔI_{sc} in the 5-week-old chickens was significantly higher than that in the 1-week-old chicks ($P = 0.035$) (Fig. 2B). However, there was no significant difference in tissue conductance between the two age groups ($P = 0.262$) (Fig. 2C).

mRNA Expression of Amino Acid Transport-related Genes in the Distal Ileum

ASCT1, EAAT3, B⁰AT1, and y⁺LAT1 mRNA levels were significantly up-regulated in the 5-week-old chickens compared to those in the 1-week-old chicks ($P < 0.05$) (Fig. 3), while no significant differences were observed in ATB^{0/+}, B^{0/+}AT, rBAT, CAT1, and CAT2 mRNA levels between the

Table 1. Primers used for real-time PCR

Primer	Sequence	Accession No.	Product size (bp)	
Slc1a4	Forward	TTG GAG GCC ATT GGA CTT CC	XM_001232899.5	102
	Reverse	GGC ATC CCC TTC CAC ATT CA		
Slc6a14	Forward	CTG CAG TAA AAC ACG CCT CG	XM_426267.1	97
	Reverse	CGT GAG GTT GTT GCT TGC AA		
Slc1a1	Forward	CGG ACA GGA AGG GAT GTA GC	XM_424930.6	91
	Reverse	ACT CCA ATA CCC AGC AGC AC		
Slc6a19	Forward	TTG AGC AAA TGC AGC AGC TG	XM_419056.6	112
	Reverse	CCG GTT CCT TCA ACT CCC TC		
Slc7a7	Forward	GCT CCT GCT AAA TGC CCT TTG	XM_418326.6	110
	Reverse	AGA TCA GCA CGA CGT ACA GC		
Slc7a9	Forward	CCA GGT TGT GAT CCA CCT CC	NM_001199133.1	99
	Reverse	GCT TCC CAG CTT CAC ACT CA		
Slc3a1	Forward	CCA TAC ATC GGG GCT GGA TG	XM_004935370.3	86
	Reverse	GAC GCT GTC TAA CCC ATC CA		
Slc7a1	Forward	CAA GAG GAA AAC TCC AGT AAT TGC A	NC_006088.4	75
	Reverse	AAG TCG AAG AGG AAG GCC ATA A		
Slc7a2	Forward	TGC TCG CGT TCC CAA GA	NC_006091.4	67
	Reverse	GGC CCA CAG TTC ACC AAC AG		
Actb	Forward	TGC GTG ACA TCA AGG AGA AG	NM_205518.1	110
	Reverse	GAC CAT CAG GGA GTT CAT AGC		

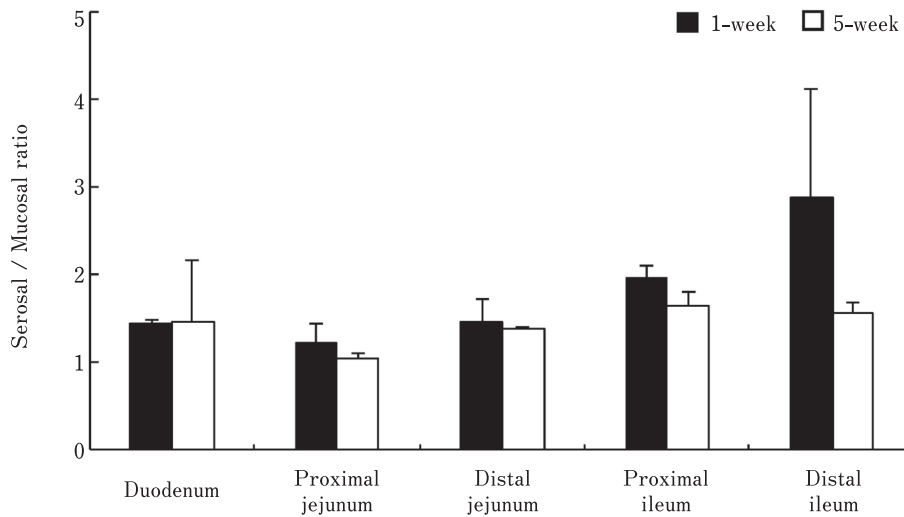


Fig. 1. **Amino acid transport in everted sacs of 1- and 5-week-old chickens in the presence of amino acids.** The serosal (S): mucosal (M) amino nitrogen concentrations ratio (S/M) was calculated after incubation with Krebs ringer buffer (KRB) containing 1 mM of each of the 10 amino acids (Ala, Asn, Glu, Lys, His, Gln, Ser, Met, Tyr, Gly) and analysis in 1- (■: $n=4-6$) and 5-week-old (□: $n=4-6$) chickens. Data are represented as mean \pm SE. Two-way ANOVA was used to compare the S/M ratios based on two independent factors (region and age) to assess treatment effects. Statistical significance was defined as $*P < 0.05$.

1- and 5-week-old broilers.

Discussion

We have previously demonstrated that the active transport of glucose in the intestine of 5-week-old broilers is weaker than that in 1-week-old broilers (Shibata *et al.*, 2019). In this study, we compared the ability of active amino acid transport in the intestine between 1- and 5-week-old chickens to evaluate the hypothesis that 5-week-old broiler chickens substitute amino acids for glucose to meet their rapid growth.

The result of the everted sac experiment suggests that amino acids are transported from the mucosal side to the serosal side in all segments of the small intestine in both age groups. Previous studies indicate that the distal ileum is a more active site of amino acid absorption compared to the duodenum, proximal jejunum, distal jejunum, and proximal ileum in chickens (Yokota, 1969; Webb, 1990). In this study, however, the everted sac experiment did not show any regional and age-dependent differences in amino acid absorption, probably due to the difficulty in normalizing surface area and filling quantity of the everted sac.

To better understand age-dependent amino acid absorption in the distal ileum of broiler chickens, we adopted the I_{sc} method, which can standardize the exposed area and media volume. The I_{sc} response to amino acid load was rapid and large in the 5-week-old broilers compared to that in 1-week-old broilers, supporting a previous study that showed an increase in intestinal uptake of proline with age, that peaked at 6 weeks in domestic chickens (Obst and Diamond, 1992).

In contrast, the intestinal uptake of tyrosine is lower in aged rats than in young rats (Huang, 1985). Therefore, age-dependent changes in intestinal absorption of amino acids may depend on the types of amino acids and animal species. Further studies will be needed to clarify the absorption of each amino acid in chickens. Furthermore, we did not observe differences in tissue conductance; this implies tight junction permeability between 1- and 5-week-old broilers and suggests that the different I_{sc} responses to amino acids should be associated with electrogenic amino acid transport, but not with paracellular transport across epithelial cells.

In our study, mRNA expression levels of Na^+ -dependent amino acid transporters (ASCT1, EAAT3, $\text{B}^0\text{AT1}$, and $\text{y}^+\text{LAT1}$) in the distal ileum were up-regulated in the 5-week-old broilers compared to those in the 1-week-old broilers, while Na^+ -independent amino acid transporter ($\text{B}^{0/+}\text{AT}$, rBAT, CAT1, and CAT2) did not show any significant differences. In broiler chicks, glutamate transporter EAAT3 and neutral amino acid transporter $\text{B}^0\text{AT1}$ are expressed at the highest level in the ileum, and their gene expression levels increase linearly with age (Gilbert *et al.*, 2010). In rats, EAAT3 mRNA expression levels also exhibit a gradual increase from postnatal day 4 to 21 (Rome, 2002). Glu and Gln, precursors of other amino acids (Windmueller and Spaeth, 1975; Blachier *et al.*, 1999), are utilized as an oxidative fuel source in enterocytes (Blachier, 2009). Therefore, an age-dependent rise in mRNA expression levels for their transporters may represent the maturation of enterocytes in broilers.

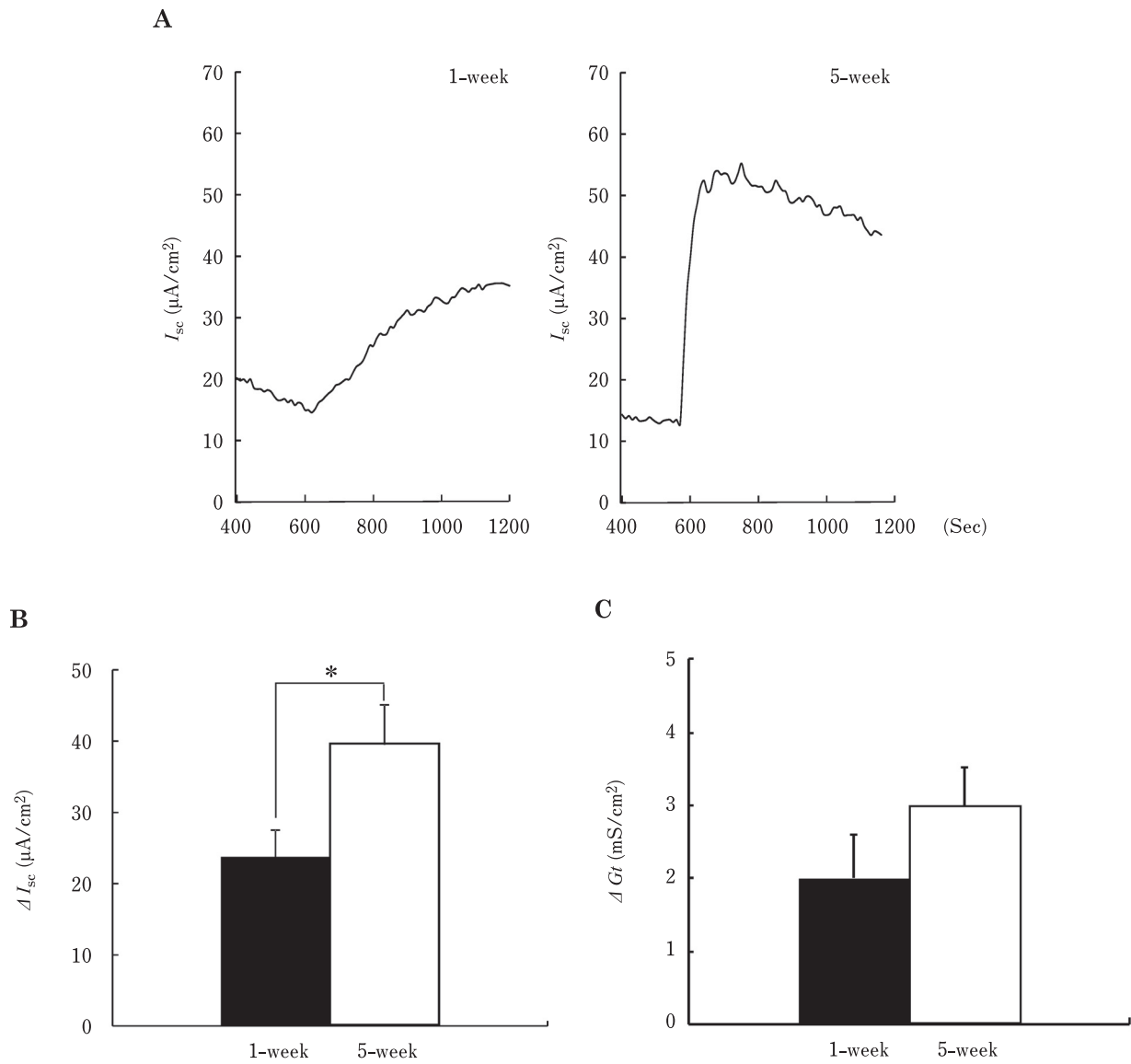


Fig. 2. Amino acid-induced I_{sc} in Ussing chamber of 1- and 5-week-old chickens after stimulation with amino acids. (A) Representative trace of I_{sc} increases in response to a luminal challenge with an amino acid in 1-week-old and 5-week-old chickens. Panel (B) shows the amino acid-stimulated I_{sc} in the distal ileum of 1- and 5-week-old chickens. I_{sc} was measured after addition of amino acid mixture solution (each 1 mM: Ala, Asn, Glu, Lys, His, Gln, Ser, Met, Tyr, Gly) into the mucosal compartment of 1- (■: $n=8$) and 5-week-old (□: $n=8$) chickens. Values (ΔI_{sc} : $\mu A/cm^2$) were calculated by subtracting basal I_{sc} values from peak I_{sc} values after challenge with amino acid mixture solution. (C) The difference in tissue conductance (ΔG_t , mS/cm^2) was calculated by subtracting the basal average conductance (−5–0 min) from the average conductance (5–10 min) after amino acids challenge. Data are represented as mean \pm SE. Student's or Welch t -test was used to assess treatment effects. Statistical significance was defined as * $P<0.05$.

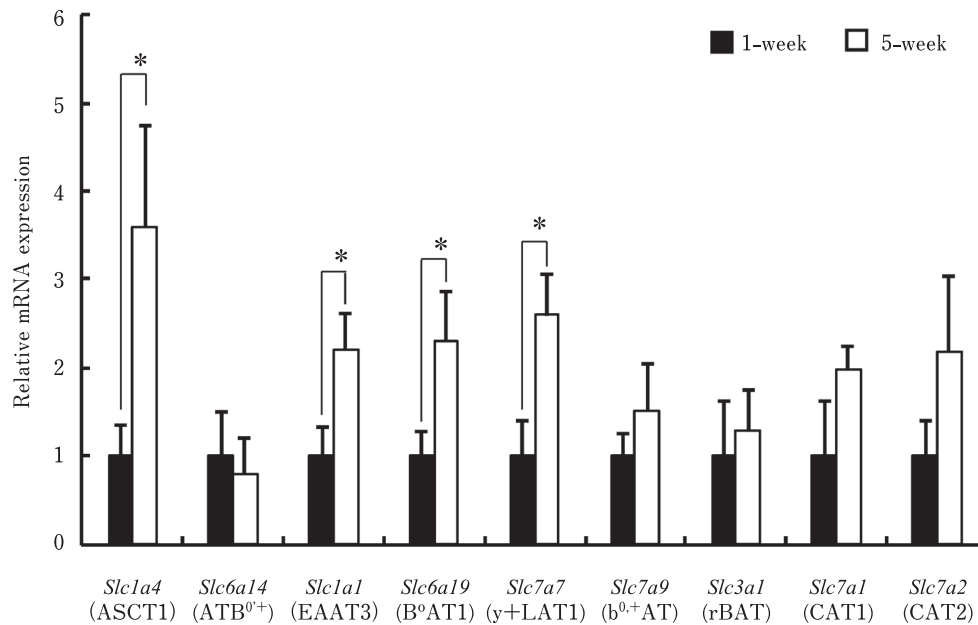


Fig. 3. mRNA expression levels of amino acid transport-related genes in the distal ileum of 1- and 5-week-old chickens. *Slc1a4* (ASCT1), *Slc6a14* (ATB⁰⁺), *Slc1a1* (EAAT3), *Slc6a19* (B⁰AT1), *Slc7a7* (y+LAT1), *Slc7a9* (B⁰⁺AT), *Slc3a1* (rBAT), *Slc7a1* (CAT1), and *Slc7a2* (CAT2) mRNA expression levels were determined using real-time PCR analysis in 1- (■: *n*=8) and 5-week-old (□: *n*=8) chickens. The PCR data were normalized using β -actin. Data are represented as mean \pm SE. Student's *t*-test was used to assess treatment effects. Statistical significance was defined as **P*<0.05.

In conclusion, we provide clear evidence that age-dependent increase in the active transport of amino acids across the ileal epithelium is caused by increased expression of Na⁺-dependent amino acid transporters in broiler chickens. Dietary supplementation of amino acids, e.g., glutamate and neutral amino acids, and avoiding excessive dietary carbohydrate supply upon growth will result in efficient meat production in broiler chickens.

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Conflicts of Interest

The authors declare no conflict of interest.

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