Embryonic Cadaverine Signaling: Implications for Plasma Free Amino Acid and Skeletal Muscle Energy Metabolism in Newly Hatched Chicks

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Cadaverine is a bioactive substance derived from lysine degradation by lysine decarboxylase and has gained attention for its physiological effects. Studies in rodents have revealed its role as a cell growth regulator, particularly intestinal bacterial-produced cadaverine. However, the nutritional and physiological roles of cadaverine during the embryonic period remain unclear, especially considering the immature state of the gut microbiota and digestive functions during this stage. This study explored the potential functions of cadaverine as a nutritional and metabolic signal during chicken embryonic development. Experiments were conducted using an *in ovo* administration method to evaluate the effects of nutritional bioactive substances on developing chicken embryos. Although there were no observable changes in body or organ weights of newly hatched chicks following *in ovo* cadaverine administration to day 18 chick embryos, plasma tryptophan, N^τ-methylhistidine, and N^π-methylhistidine concentrations decreased and the gene expression of insulin/insulin-like growth factor 1 signaling in skeletal muscle was upregulated. These findings imply that cadaverine influences tryptophan metabolism and skeletal muscle catabolism during the embryonic period, suggesting its role as a bioactive factor contributing to energy metabolism signaling in skeletal muscle.

Key words: cadaverine, embryo, newly hatched chicks, polyamine, skeletal muscle catabolism

Introduction

Cadaverine is a naturally occurring nitrogen-containing organic compound (alkaloid) generated through decarboxylation of the essential amino acid lysine. It falls under the category of polyamines because of its structure, which comprises a carbon chain with two amino groups[1]. Polyamines are generic terms for cationic organic compounds containing multiple amino groups. The chemical characteristics of polyamines allow them to function in cellular homeostasis, including in cell proliferation and differentiation[2], nucleic acid protection[3], apoptosis[4], autophagy[5], and antioxidant activity[6]. Other common polyamines found in vertebrates include putrescine, spermidine, and spermine, which are synthesized by the decarboxylation of ornithine[7]. Polyamines in the body originate from both *in vivo* production pathways (decarboxylation in tissues) and *ex vivo* pathways (derived from food intake and decarboxylation by intestinal bacteria)[8,9]. However, tissue production of cadaverine suggests the significance of its action, especially considering the relatively low activity of decarboxylase[2,5,8,9].

There are many reports of the function of cadaverine in birds, including its association with negative indicators of productivity, mainly as an indicator of spoilage (bacterial fermentation) of livestock products (muscle) and feed[10,11] and induction of glandular stomach damage in chickens[11]. In contrast, a study using metabolome analysis, a technology that comprehensively analyzes nutrients and their metabolites in the body, including amino acids, has shown that when broiler and layer chicks that have been improved through breeding and selection are compared, plasma cadaverine concentrations in broiler chicks are higher than those in layer chicks[12]. It has also been suggested that the function of metabolites produced by the gut microbiota is important and that the gut microbiota develops in the digestive tract of the chick embryo via inoculation from the mother hen's oviduct[13,14]. Since insulin and insulin-like growth factors in the blood of chick embryos regulate subsequent energy metabolism, it is important to evaluate the relationship between the actions of these hormones and nutrient metabolites produced

Table 1.   Gene-specific polymerase chain reaction primers used for chick gene expression analysis.

Gene	Accession No.		Primer sequence (5'→3')	Annealing Temperature (°C)	Product size (bp)
RPS17	NM_204217.1	F:	AAGCTGCAGGAGGGAGGAGAGG	68.2	136 bp
		R:	GGTTGGACAGGCTGCCGAAGT		
INSR	AF111857.1	F:	GTCTGCTTTTCTCCCCTCCACA	68.6	146 bp
		R:	GACAACCAGTCAACTTGGCAAA		
IGF1R	NM_205032.3	F:	GGCCATACGGATTGAGAAGAAC	65.5	110 bp
		R:	TCGGAGGCTTATTTCCAACAAT		
IRS-1	NM_001031570.1	F:	TCGCCTTCTCTATGCTGCAA	65.9	122 bp
		R:	GAACCTGATGGTGGGGATGT		
IRS-2	XM_425588.4	F:	ACTCGGACAGCTTCTTCTTCAT	63.1	152 bp
		R:	GAACTCGGACAGCTCCTTTAGA		

F, forward primer; INSR, insulin receptor; IGF1R, insulin-like growth factor 1 receptor; IRS-1, insulin receptor substrate-1; IRS-2, insulin receptor substrate-2; RPS17, ribosomal protein S17; R, reverse primer.

by gut microbiota[15]. However, the relationship between early growth and nutritional and physiological functions of cadaverine in chicks and chickens is not well understood. In this study, the potential of cadaverine signaling as a nutrient signal during early growth was investigated by examining the variation in plasma free amino acid concentrations in newly hatched chicks after *in ovo* cadaverine stimulation and analyzing its effects on amino acids and related energy metabolism.

Materials and Methods

In ovo cadaverine administration and organ weights in newly-hatched chicks

Fertilized broiler eggs (Ross 308) were purchased from a local hatchery (ISHII, Iwate, Japan). The eggs were incubated at 37.8 °C and 60% relative humidity, and on the 14th day of embryonic development, eggs that had started to develop normally were selected and classified so that the average egg weights were equal. On the 18th day of incubation, a perforation was made in the eggshell using a needle and 500 µL of cadaverine or deionized water was administered into the egg using the method of Ohta *et al.* [16]. After administration, the needle holes were closed with cellophane, incubation was resumed, and eggs were allowed to hatch. The cadaverine (Fujifilm Wako Chemical, Osaka Japan) used in the experiments was a five-step dilution of a cadaverine solution based on a report by Barnes *et al.* [11]. Hatched chicks were weighed and euthanized after blood collection. Blood samples were collected after anticoagulation with heparin. Euthanized chicks were opened and sexed by observing the shape of their gonads. The weight of each organ (the whole brain, heart, pectoral muscle, liver, sartorius muscle, pancreas, and residual yolk sac) was measured. Pectoral muscle samples were flash-frozen in liquid nitrogen, blood samples were centrifuged at 4 °C, 4,000 × *g*, for 10 min, and plasma samples were collected. Each sample was stored at -80 °C until analysis. This study was approved by the Animal Experimentation Committee of Nippon Veterinary and Life Science University (Approval Nos. 2019 K-34, 2020 K-37, and 2021 K-62).

Determination of plasma free amino acid concentrations in newly hatched chicks

Following the method described by Sakano *et al.*[17], plasma free amino acid (arginine, lysine, methionine, isoleucine, leucine, valine, phenylalanine, threonine, tryptophan, histidine, glycine, glutamic acid, glutamine, aspartic acid, serine, alanine, cysteine, tyrosine, proline, taurine, N^{π}-methylhistidine, and N^{τ}-methylhistidine) concentrations were determined using a fully automated amino acid analyzer (JLC-500/V2, Japan Electron Optics Laboratory, Tokyo, Japan) after plasma samples were deproteinized with 3% sulfosalicylic acid solution.

Gene expression analysis of insulin/insulin-like growth factor 1 signaling in skeletal muscle of newly hatched chicks

Total RNA was extracted and purified from the collected pectoral muscle samples using IsoPlus RNA (Takara Biosciences, Shiga, Japan). A cDNA library derived from each organ was constructed using a PrimeScript cDNA Synthesis Kit (Takara Biosciences, Shiga, Japan). The expression levels of insulin receptor (*INSR*), insulin-like growth factor 1 receptor (*IGF1R*), and insulin receptor substrate 1/2 (*IRS-1/2*) genes in the pectoral muscles were amplified using real-time polymerase chain reaction (PCR) (Applied Biosystems 7500 Fast Real-Time PCR System, CA, USA). Gene expression levels were compared by relative quantification using the comparative Ct method, with ribosomal protein S17 (*RPS17*) serving as an internal standard. The PCR primer sequences are listed in Table 1.

Statistics

Differences in organ weight and plasma free amino acid content were compared using the Mann-Whitney *U*-test. Hatchability and skeletal muscle gene expression levels were analyzed using the Kruskal-Wallis test. All analyses were performed using the commercially available software JMP version 11 (SAS Institute, Cary, NC, USA). Results are presented as the means ± standard error of the mean (SEM) and are considered statistically different at *P* < 0.05.

Table 2. **Influence of cadaverine administration on chick hatchability.**

	Cadaverine conc. (mM)					
	0	0.25	0.5	2	10	50
Number of embryos	31	19	19	12	12	12
Hatchability (%)	87.1 [a]	89.47 [a]	84.21 [a]	50 [b]	25 [b]	0.08 [b]

Dissimilar letters are significantly different at *P* < 0.05.

Table 3. **Hatching weight and tissue weight of *in ovo* cadaverine-administrated chicks.**

	Control			Cadaverine			*P* value
BW P0 (g)	44.35	±	0.82	46.3	±	1.51	0.61
Brain/BW	2.24	±	0.06	2.13	±	0.07	0.63
Heart/BW	0.76	±	0.02	0.68	±	0.03	0.2
Pectoral muscle/BW	0.59	±	0.03	0.63	±	0.04	0.31
Liver/BW	2.41	±	0.03	2.22	±	0.05	0.18
Sartorius muscle/BW	0.42	±	0.01	0.47	±	0.03	0.07
Pancreas/BW	0.17	±	0.01	0.15	±	0.02	0.64
Yolk/BW	10.05	±	0.45	12.04	±	0.81	0.06

Values are the mean ± the standard error of the mean. Number of chicks in the control group: n = 8, cadaverine-treated group: n = 5. BW, body weight; P0, post-hatch day 0

Results

In ovo cadaverine administration and organ weights in newly hatched chicks

When administered to embryos at various concentrations (0–50 mM) on the 18th day of incubation, cadaverine caused a dose-dependent decrease in chicken hatchability. Notably, no significant differences were observed between the concentrations of 0.25 and 0.5 mM; however, hatchability decreased significantly at concentrations of 2 mM and above (Table 2). Table 3 presents the body and organ weights of newly hatched chicks from embryos treated with 0.25 mM cadaverine on the 18th day after the start of incubation. No differences were observed in the body weight at hatching or in the weights of the whole brain, heart, pectoral muscle, liver, thigh muscle, pancreas, or remaining yolk sac weight per body weight between the cadaverine-treated and control groups.

Plasma free amino acid concentrations in newly hatched chicks

The plasma free amino acid concentrations of newly hatched chicks administered 0.25 mM cadaverine to embryos 18 d after the start of incubation are shown in Table 4. Among the free amino acids measured in this study, tryptophan, N^{π}-methylhistidine, and N^{τ}-methylhistidine levels were significantly lower after cadaverine administration. However, the levels of other amino acids were not affected.

Gene expression analysis of INSR/IGF1R signaling in the skeletal muscle of newly hatched chicks

INSR (Fig. 1 A) and *IGF1R* (Fig. 1 B) levels were significantly upregulated by 0.25 mM cadaverine administration and the gene expression of the intracellular signal proteins *IRS1* and *IRS2* was also significantly upregulated by 0.25 mM cadaverine exposure (Fig. 1 C-D).

Discussion

Some alkaloids, including polyamines, exert important biological effects; some alkaloids are toxic, whereas others have useful pharmacological effects depending on their actions[1,18]. In the present study, the subsequent growth of chick embryos after treatment with cadaverine was investigated and the hatchability rate was 50% or less at doses of 2 mM or higher, whereas the target zone and hatchability rate were not affected at concentrations between 0.25 mM and 0.5 mM. These data indicated the toxic effects of high concentrations of cadaverine on individuals, as high concentrations resulted in developmentally arrested eggs. Although there were no significant changes in hatchling weight, brain, heart, liver, skeletal muscle, pancreas, or remaining yolk sac weights of chicks in the 0.25–0.5 mM treatments, regulation of nutrient metabolism involving these metabolic organs is important during the embryonic period[19–22]. In the regulation of nutrient metabolism during the embryonic period, free amino acids in the blood are a source of raw materials for protein synthesis, proteolytic products, and glucose synthesis, and are used as important indicators of energy metabolism and nutritional status assessment[23,24]. The variations in plasma free amino acid concentrations in chicks immediately after hatching were investigated. Plasma tryptophan, N^{τ}-methylhistidine and N^{π}-methylhistidine concentrations were significantly decreased.

The low plasma free tryptophan concentration in chicks hatched after cadaverine administration was attributed to tryptophan metabolism. Tryptophan is an essential amino acid that

Table 4. Effect of *in ovo* cadaverine administration on plasma free amino acid concentrations in newly hatched chicks.

	Control			Cadaverine			P value
Arginine	100	±	7.95	101.24	±	12.49	0.93
Lysine	100	±	6.25	97.85	±	14.04	0.87
Methionine	100	±	5.94	83.21	±	7.22	0.08
Isoleucine	100	±	3.04	97.19	±	5.94	0.64
Leucine	100	±	3.72	97.4	±	7.69	0.73
Valine	100	±	2.73	93.89	±	4.97	0.24
Phenylalanine	100	±	3.18	97.23	±	6.86	0.67
Threonine	100	±	9.97	81.01	±	6.64	0.16
Tryptophan	100	±	2.86 [a]	80.22	±	8.57 [b]	0.02
Histidine	100	±	7.99	86.69	±	9.23	0.28
Glycine	100	±	3.58	97.7	±	5.10	0.69
Glutamic acid	100	±	6.44	89.73	±	5.43	0.25
Glutamine	100	±	5.76	87.57	±	3.39	0.11
Aspartic acid	100	±	6.03	99.71	±	7.27	0.97
Serine	100	±	2.96	95.21	±	4.68	0.35
Alanine	100	±	5.75	81.45	±	8.60	0.07
Cysteine	100	±	4.67	91.85	±	5.69	0.27
Tyrosine	100	±	6.35	86.33	±	10.81	0.24
Proline	100	±	8.92	103.62	±	5.61	0.75
Taurine	100	±	9.34	69.26	±	18.56	0.11
N^{π}-methylhistidine	100	±	5.01 [a]	75.22	±	7.96 [b]	0.01
N^{t}-methylhistidine	100	±	6.97 [a]	61.33	±	15.73 [b]	0.02

Data are expressed as percent change (%) with the mean value of the control area set at 100, and then expressed as the mean ± the standard error of the mean. Dissimilar letters are significantly different at $P < 0.05$.

is metabolized as a substrate in the kynurenine, serotonin, and indole pathways[25,26]. These metabolic pathways function as biomarkers for immune and stress responses in chickens, as well as in intestinal microflora[27–29]. In other words, these data suggest that low tryptophan levels in the blood due to cadaverine signaling during egg incubation may be responsible for the functionality of these tryptophan metabolites.

Furthermore, plasma N^{t}-methylhistidine and N^{π}-methylhistidine levels decreased after cadaverine administration. Since these amino acids are metabolites of histidine, an essential amino acid, and are often used as indicators of muscle proteolysis in various animals[30–33], these data suggest that skeletal muscle proteolysis in hatched chicks is inhibited by cadaverine administration during the embryonic period. In fact, the gene expression levels of receptors for *INSR* and *IGF1R*, which regulate the suppression of muscle proteolysis, and intracellular signaling regulatory proteins for these receptors, were upregulated by cadaverine administration. Since cadaverine stimulation promotes insulin secretion in cultured mouse pancreatic beta cell lines[34], the phenomenon observed in this study suggests that cadaverine signaling during the embryonic period may act on pro-insulin/insulin-like growth factor synthesis or secretion. These findings suggested that cadaverine signaling during the embryonic period promoted the synthesis or secretion of insulin and insulin-like growth factors. Although no obvious differences in metabolic organ weights were observed in these experiments, cadaverine signaling might have contributed to the action of insulin/insulin-like growth factors during chick embryogenesis. Further analysis of the molecular mechanisms, such as transcriptional regulation and post-translational modulation, is anticipated with regard to organ development.

During the late embryonic period (E14-) of chick embryogenesis, energy metabolism, including blood glucose regulation, is activated in preparation for hatching[15]. Blood glucose is an essential nutrient utilized as an energy source in the body before and after hatching[35]. During late embryogenesis, glycogenesis facilitates a rapid increase in glucose levels[36]. In birds, including chickens, the embryonic stage is a closed trophic environment in the eggshell, independent of the mother; the substrates for glycogenesis are amino acids and glycogen in the egg, as well as amino acids derived from the degradation of muscle proteins[35,36]. Considering these data, inhibition of skeletal muscle proteolysis by cadaverine signaling during the embryonic period suggests that cadaverine itself may act as a nutrient signal and control energy consumption by the entire embryo in a labor-saving manner. Indeed, in studies using organ culture techniques, fluctuations in TCA cycle metabolite levels were observed after cadaverine stimulation (unpublished data). These data suggest
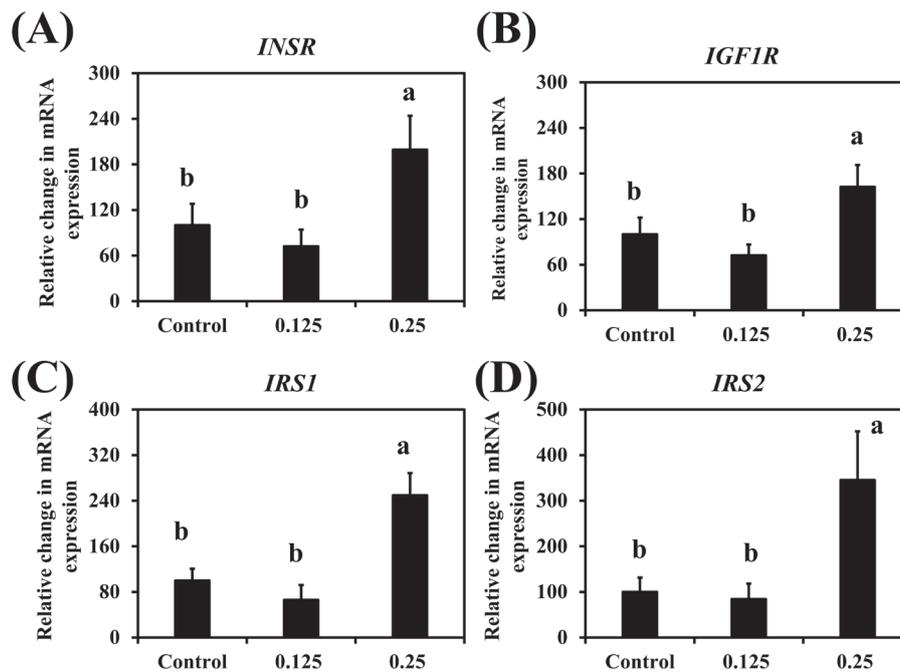
Fig. 1.   **Gene expression of insulin/insulin-like growth factor signaling in pectoral muscle of newly-hatched chicks after cadaverine administration to 18-day-old embryos.** (A) Insulin receptor (*INSR*), (B) Insulin-like growth factor I receptor (*IGF1R*), (C) Insulin receptor substrate 1 (*IRS1*), and (D) Insulin receptor substrate 2 (*IRS2*) are expressed relative values (%) to the mean of the control. The values are the mean ± the standard error of the mean (SEM). Number of chicks used: Control, 8; 0.125 mM cadaverine, 7; 0.25 mM cadaverine, 7. Dissimilar letters are significantly different at $P < 0.05$.

that cadaverine may act as a substrate for energy metabolism; therefore, in the future, the relationship between each organic acid as a TCA cycle metabolite, its effect on mitochondrial function in energy delivery, and its direct bioactive action in metabolic organs should be analyzed. Findings from these studies may lead to better nutritional control of chicks before and after hatching, and improve animal management methods to mitigate stress responses, including excessive muscle protein catabolism during hatching. There were no detected sex-related differences attributable to the administration of cadaverine. Because sex differences in plasma beta-alanine, hypotaurine, and thyroid hormones have been observed in newly hatched broiler chicks[12,15], investigating these amino acid metabolic pathways and their relationship with steroid hormones may reveal new nutritional functions in cadaverine signaling.

In summary, this study revealed that cadaverine, a lysine metabolite, affected tryptophan and histidine metabolism in embryonic chicks and acted as an anabolic signal that inhibited catabolism, promoting insulin/insulin-like growth factor signaling, especially in skeletal muscle tissue.

Author Contributions

Jun-ichi Shiraishi conceptualized and designed the experiments. Jun-Ichi Shiraishi, Naoko Shimakura, Kazuki Kimura, Ai-Saiga Egusa, and Yoshiyuki Ohta performed the experiments and provided reagents, materials, and analytical tools. Jun-Ichi Shiraishi wrote the manuscript. Naoko Shimakura, Kazuki Kimura, Ai-Saiga Egusa and Yoshiyuki Ohta reviewed and revised the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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