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≪Research Note≫

Glycated Tryptophan PHP-THβC Incorporated into Various Chicken Embryonic Cells Constitutes Cellular Proteins

Ryosuke Makino[1,2*], Kayoko Abe[1*,†] and Kazumi Kita[1]

[1] Faculty of Agriculture, Iwate University, Morioka, Iwate 020−8550, Japan
[2] Faculty of Agriculture, Ehime University, Matsuyama, Ehime 790−8566, Japan
[†] Present address: Graduate School of Systems Life Sciences, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819−0395, Japan

Glycation is a non-enzymatic reaction, and amino acids are glycated by glucose in vivo. Tryptophan is glycated with glucose to form two types of glycated compounds, tryptophan-Amadori product and (1R, 3S)-1-(D-gluco-1, 2, 3, 4, 5-pentahydroxypentyl)-1, 2, 3, 4-tetrahydro-β-carboline-3-carboxylic acid (PHP-THβC). Although PHP-THβC can be incorporated into various chicken embryonic cells, the mechanism of its incorporation into intracellular fluids has not been clarified. In this study, we examined whether PHP-THβC once incorporated into various chicken embryonic cells can combine with proteins. Embryonic cells from the breast muscle, liver, spleen, kidney, proventriculus, gizzard, and skin were prepared and ^{3}H-PHP-THβC was added to the culture medium at final concentrations of 0, 200, 400, 600, and 800 μM to examine the incorporation of PHP-THβC. After 18 h of incubation, radioactivity was measured in the whole-cell and protein fractions of the chicken embryonic cells. As PHP-THβC concentration increased from 0 to 600 μM, its accumulation in the whole-cell fractions of all types of chicken embryonic cells linearly increased and reached the maximum level. The saturated PHP-THβC accumulation in the whole-cell fractions suggests that PHP-THβC could be incorporated into intracellular fluids across cellular membranes by some transporter proteins. As PHP-THβC concentration increased from 0 to 800 μM, its accumulation in the protein fractions of all types of chicken embryonic cells increased in a linear manner and reached a maximum level in the 800 μM PHP-THβC treatment group. This is the first study to indicate that a part of PHP-THβC incorporated into the whole-cell fraction was detected in the protein fraction of various chicken embryonic cells.

Key words: chicken embryo, glycation, PHP-THβC, protein synthesis, tryptophan

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Introduction

Glycation, the so-called Maillard reaction or amino-carbonyl reaction, is characterized with the dehydration condensation reaction between the carbonyl group of reducing sugars and the amino group of proteins and amino acids (Maillard, 1912). This reaction is a non-enzymatic reaction, wherein glycated amino compounds form Schiff bases and undergo rearrangement to produce stable Amadori products. Amadori products undergo further complex reactions and form advanced glycation end-products (AGEs). Acceleration of glycation during hyperglycemia, as observed in diabetes mellitus, increases the production and accumulation of AGEs, thereby contributing to the development of diabetic complications (Brownlee, 2001; Singh *et al*., 2001).

Hyperglycemia is commonly observed in avian species. The blood glucose level of birds is two to three times higher than that of humans (Farhat and Chavez, 2000; Quist *et al*., 2000; Katavolos *et al*., 2008; Atakisia *et al*., 2009; Prinzinger and Misovic, 2010). Hence, glycation proceeds quickly and produces glycated compounds in the plasma and tissue fluids of chickens. In our previous study, radiolabeled AGEs prepared by a reaction between ^{14}C-glucose and amino acid mixture were intravenously administered to chickens and their tissue distribution was examined. The study revealed that AGEs could be predominantly incorporated into the spleen, kidney, and liver of chickens (Kita, 2014). We also reported that glycated-tryptophan, (1R, 3S)-1-(D-gluco-1, 2,

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Correspondence: Dr. Kazumi Kita, Laboratory of Animal Nutrition, Faculty of Agriculture, Iwate University, Morioka, Iwate 020−8550, Japan. (E-mail: kitak@iwate-u.ac.jp)
* Equal contribution

3, 4, 5-pentahydroxypentyl)-1, 2, 3, 4-tetrahydro-β-carboline-3-carboxylic acid (PHP-THβC), and valine-Amadori product were incorporated into various cells from the skeletal muscle, spleen, kidney, and liver (Makino *et al*., 2016). Although PHP-THβC was found to be incorporated into various chicken embryonic cells, the mechanism for its incorporation into intracellular fluids has not been clarified. Moreover, the manner in which PHP-THβC is incorporated into cells is yet unknown.

In the present study, we prepared radiolabeled PHP-THβC and examined whether it combined with proteins upon incorporation into various chicken embryonic cells.

Materials and Methods

Preparation of Radiolabeled PHP-THβC

Radiolabeled PHP-THβC was prepared as previously reported (Nishimagi and Kita, 2012; Makino *et al*., 2015). In brief, 5 mM non-radioactive L-tryptophan and a 50 μM radioactive tryptophan solution containing L-[5-^{3}H]-tryptophan (37 MBq/mL, 50 nmol/mL; American Radiolabeled Chemicals Inc., MO, USA) were added to a 2 M D-glucose solution. The mixture was incubated at 37℃ for 3 days, and a cation-exchange resin (Dowex 50W-X8) was added to remove unreacted glucose. The solution eluted from the resin using 3 M ammonia was evaporated, and the condensed solution was incubated at 4℃ for several days to precipitate PHP-THβC. The crude product of PHP-THβC was rinsed with ultrapure water until white crystals of PHP-THβC were obtained. PHP-THβC was dried using a centrifugal evaporator, and its specific radioactivity (1.11 MBq/mmol) was measured.

Preparation of Cells from Various Tissues of Chicken Embryos

Thirty fertilized eggs of Single Comb White Leghorn chickens were purchased from a local hatchery (Koiwai Farm Co., Ltd, Shizukuishi, Iwate, Japan). Embryonic cells from various tissues were prepared as previously described (Kita and Makino, 2014). In brief, fertilized eggs were incubated for 17 days, and embryos were obtained from eggs. After decapitation, the breast muscle, liver, spleen kidney, proventriculus, gizzard, and skin were excised and finely minced with scissors. Minced tissues were gently digested using 0.25% (w/v) trypsin, pipetted several times, and passed through a gauze to remove any undigested tissue pieces. Cells from each tissue were divided into two groups to measure the radioactivity of whole-cell and protein fractions. Cells were seeded on Type-1 collagen-coated 48-well plates (Corning Inc., NY, USA) with Medium 199 supplemented with 2.5 μg/mL amphotericin, 100 units of 100 μg/mL penicillin-streptomycin, 50 μg/mL gentamycin, and 10% fetal calf serum and incubated at 37℃ in 5% CO_2/95% air (v/v). All reagents except penicillin-streptomycin for the preparation of various tissue cells were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Penicillin-streptomycin was obtained from Biological Industries Ltd. (Kibbutz Beit Haemek, Israel). Animal care was in compliance with the applicable guidelines from the Iwate University Animal Care and Use Committee (No. A201549).

Measurement of PHP-THβC Radioactivity

After overnight incubation of cells, the culture medium was drawn and the cells were incubated in fresh Medium 199 supplemented with PHP-THβC. The final concentrations of PHP-THβC were 0, 200, 400, 600, and 800 μM. To examine the incorporation of PHP-THβC, ^{3}H-PHP-THβC was also added to the medium. After 18 h of incubation, the medium was removed and the cells were rinsed thrice with ice-cold Medium 199. The rinsed medium was confirmed to exhibit no radioactivity. To prepare whole-cell fractions, cells removed from Medium 199 were lysed with 0.5% (w/v) sodium hydroxide (NaOH)/0.1% (v/v) Triton X-100. To prepare protein fractions, ice-cold Medium 199 was removed and replaced with ice-cold 5% (w/v) trichloroacetic acid (TCA) to extract intracellular free amino acids. TCA was rinsed off, and the cells were rinsed with ice-cold Dulbecco's phosphate-buffered saline (DPBS). Precipitated cells were lysed with 0.5% (w/v) NaOH/0.1% (v/v) Triton X-100, and their radioactivity in NaOH/Triton X-100 was measured using a liquid scintillation counter as an index of the amount of PHP-THβC. The protein contents in the whole-cell and protein fractions were measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Radioactivity was corrected for each protein content.

Statistical Analysis

Statistical analysis of data was performed by one-way analysis of variance (ANOVA) and Tukey's HSD test for multiple comparisons ($P<0.05$) using the general linear model (GLM) procedures of SAS (SAS/STAT version 9.4). The main effect was the PHP-THβC concentration. Linear regression equations were also calculated using GLM.

Results

The accumulation of PHP-THβC in the whole-cell fraction of various chicken embryonic cells is shown in Fig. 1, and the regression equations between the medium PHP-THβC concentration and PHP-THβC accumulation in the whole-cell fraction are shown in Table 1. A significant interaction was observed between the medium PHP-THβC concentration and different types of tissues. As PHP-THβC concentration increased from 0 to 600 μM, its accumulation in all types of chicken embryonic cells linearly increased and reached the maximum level. The accumulation of PHP-THβC remained stable or declined in the 800 μM PHP-THβC treatment group. At 600 μM, the accumulation of PHP-THβC was the highest in the proventriculus, followed by the skin, liver, and gizzard. PHP-THβC accumulation in the whole-cell fraction of the kidney, spleen, and muscle was significantly lower than that in other tissues.

The accumulation of PHP-THβC in the protein fraction of various chicken embryonic cells is shown in Fig. 2, and the regression equations between the medium PHP-THβC concentration and PHP-THβC accumulation in the protein fraction are shown in Table 1. A significant interaction was reported between the medium PHP-THβC concentration and

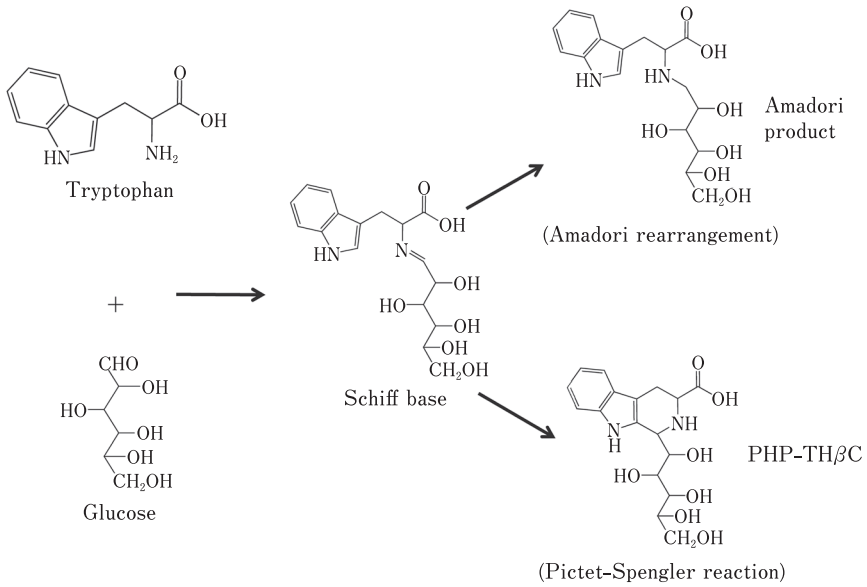

Fig. 1. **The non-enzymatic glycation of tryptophan to form tryptophan-Amadori product and PHP-THβC.**

Table 1. **Regression equations between medium PHP-THβC concentration and PHP-THβC accumulation in whole-cell and protein fractions**

Whole-cell fraction (Medium PHP-THβC concentration 0–600 μM)								
Tissue	Gizzard	Heart	Kidney	Liver	Proventriculus	Muscle	Skin	Spleen
Intercept	−2.2502	−0.7929	0.5357	−0.3953	−0.6523	0.1160	−1.4395	0.9026
Slope	0.0319	0.0265	0.0155	0.0292	0.0348	0.0194	0.0313	0.0170
Correlation coefficient	0.8893	0.9598	0.9303	0.9094	0.9317	0.9158	0.8993	0.8741
Probability	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Protein fraction (Medium PHP-THβC concentration 0–800 μM)								
Tissue	Gizzard	Heart	Kidney	Liver	Proventriculus	Muscle	Skin	Spleen
Intercept	−0.9753	−1.0600	−0.3747	−0.3887	−0.4060	−0.3620	−1.1133	−0.5527
Slope	0.0095	0.0143	0.0095	0.0097	0.0101	0.0102	0.0101	0.0106
Correlation coefficient	0.8081	0.9448	0.9500	0.8968	0.8884	0.9287	0.9128	0.8617
Probability	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

different types of tissues. As PHP-THβC concentration increased from 0 to 800 μM, PHP-THβC accumulation in the protein fractions of all types of chicken embryonic cells increased in a linear manner and reached a maximum level in the 800-μM treatment group. PHP-THβC accumulation in the heart was higher than that in other tissues.

Discussion

Glycation, like oxidation, is a non-enzymatic chemical reaction that may be caused in vivo in the presence of reduced sugars such as glucose and fructose. In our previous studies, we used chicken as a hyperglycemic animal model and demonstrated glycated amino acids such as PHP-THβC and valine-Amadori products in its blood (Makino *et al*., 2015; Honma *et al*., 2017). These amino acid-Amadori products could be incorporated into various cells derived from chicken embryos (Makino *et al*., 2016). As shown in Fig. 1, the accumulation of glycated tryptophan, PHP-THβC, in various chicken embryonic cells was consistent with the observations reported in our previous study. In the present study, we used different concentrations of PHP-THβC in the culture medium to examine the mechanism of PHP-THβC incorporation into intracellular fluids. There are two types of mechanisms to carry solutes across the cellular membrane,

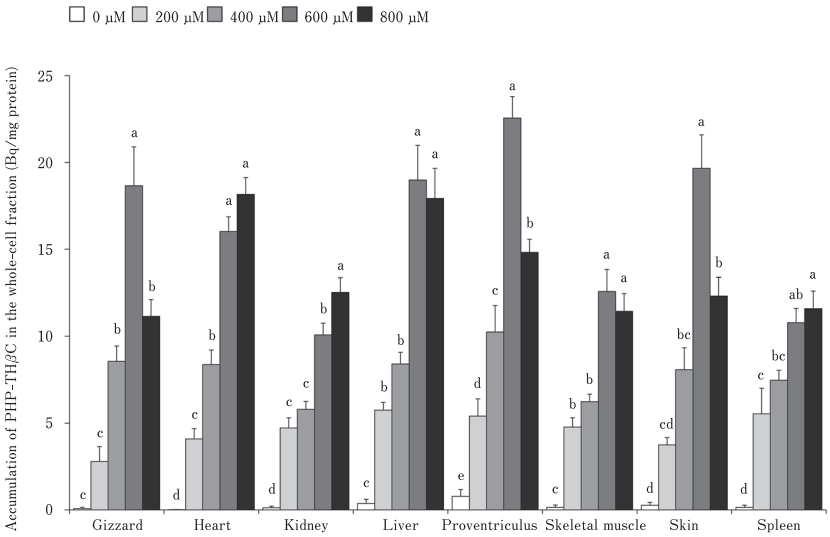

Fig. 2. **Influence of different concentrations of PHP-THβC from 0 to 800 μM in the culture medium on its accumulation in the whole-cell fraction of various chicken embryonic cells.** Bars represent means± SE. $^{a-e}$. The number of wells was six. Means with different superscripts in each tissue are significantly different at $P<0.05$.

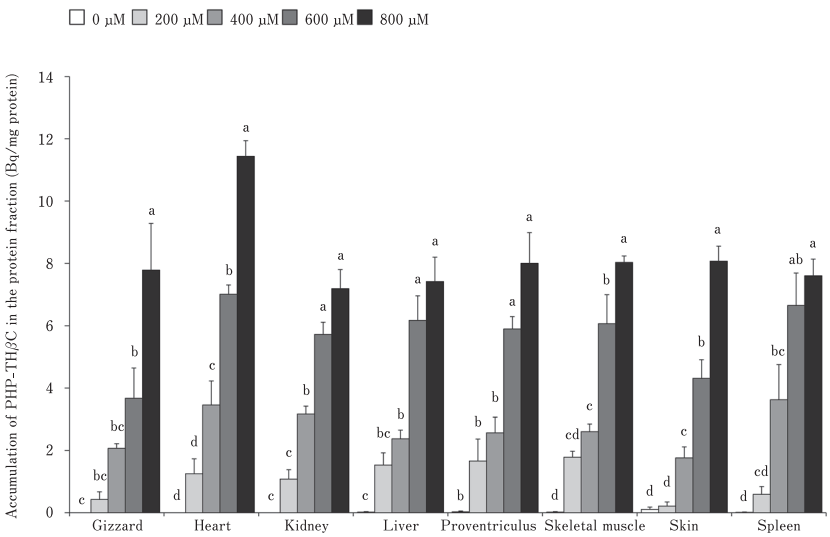

Fig. 3. **Influence of different PHP-THβC concentrations from 0 to 800 μM in the culture medium on its accumulation in the protein fraction of various chicken embryonic cells.** Bars represent means± SE. $^{a-d}$. The number of wells was six. Means with different superscripts in each tissue are significantly different at $P<0.05$.

passive transport and active transport. Passive transport includes simple diffusion, which involves transport of molecular substances from high to low concentrations across the cellular membrane without any carrier protein and energy supply. Facilitated diffusion, another mode of passive transport, is characterized with the transport of molecular substances with a carrier protein but without energy supply. Active transport is the movement of molecular substances driven by the consumption of ATP with a transporter protein (Fielding and Fielding, 2001). As the number of transporter proteins can be limited, both facilitated diffusion and active transport are saturated in the presence of a very high concentration of a molecule. As shown in Fig. 1 and Table 1, as the medium PHP-THβC concentration increased from 0 to 600

μM, its accumulation in the whole-cell fractions of all types of chicken embryonic cells linearly increased and reached the maximum level. The levels remained stable or declined thereafter as the medium PHP-THβC concentration reached 800 μM. The saturated PHP-THβC accumulation in the whole-cell fraction suggests that PHP-THβC could be incorporated into intracellular fluids across the cellular membrane by some transporter proteins. As PHP-THβC has the same chemical structure as tryptophan and glucose, it may be possibly incorporated by tryptophan transporters such as LAT1, LAT2, TAT1, B^0AT1/HND, and $ATB^{0,+}$ (Christensen, 1990; Bröer, 2008); further studies are warranted to test this hypothesis.

We have previously reported that PHP-THβC could not serve as a precursor for protein synthesis in chicken embryonic myoblasts cultured in a tryptophan-free culture medium (Makino *et al*., 2015); this observation seems to be inconsistent with that reported in the present study. In our previous study, the concentration of PHP-THβC added to the culture medium ranged from 0 to 49 μM, while that tested in the present study varied from 0 to 800 μM. The minimum concentration tested was 200 μM, suggesting that PHP-THβC at concentrations higher than 200 μM could be incorporated from the culture medium into the intracellular fluids.

In conclusion, glycated tryptophan, PHP-THβC, can be incorporated into various chicken embryonic cells and serve as a precursor for protein synthesis when the concentration of the glycated tryptophan is higher than 200 μM.

Acknowledgments

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Conflicts of Interest

The authors declare no conflict of interest.

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