Oral Glucose Tolerance and Tryptophan Metabolism in Non-Obese and Non-Insulin-Dependent Diabetic Goto–Kakizaki Rats Fed High-Tryptophan Diets

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Summary We investigated oral glucose tolerance and tryptophan (Trp) metabolism in non-obese and non-insulin-dependent diabetic Goto–Kakizaki (GK) rats fed high-Trp diets. Five-week-old male Wistar and GK rats were fed a 20% casein diet (control diet) or the same diet supplemented with 1%, 2%, 3%, or 5% Trp for 58 d. Oral glucose tolerance tests were performed on Days 14 and 28 of the experimental period. Urine as well as livers and blood were collected on the last day of the experiment. The glucose concentration and the amount of Trp metabolites were measured. On Day 14 of the experiment, the incremental blood glucose concentrations integrated over a period of 2 h (ΔAUC_{0-2h}) of blood glucose in rats fed the 3% and 5% Trp diets had decreased by 13% and 18%, respectively, compared with that of the control-GK rats. However, no significant differences were found in the rats fed +1% or +2% Trp diets compared with control-GK rats. On Day 28, there were no significant differences found in the ΔAUC_{0-2h} of blood glucose levels in any group including the control-GK group. On the last day, the concentrations of plasma glucose, total cholesterol, and triglyceride did not show differences in any group. There were no specific phenomena observed in the metabolism of Trp in GK rats even when fed an excess of Trp, compared with that of Wistar rats. Oral Trp administration and its continuous use may not improve blood glucose levels in type 2 diabetic rats.

Key Words GK rat, tryptophan metabolism, glucose tolerance, diabetes, excessive tryptophan

Nicotinamide (Nam), serotonin, and melatonin are important bioactive compounds derived from the essential amino acid, tryptophan (Trp) (1–3). Several studies have demonstrated that Trp can regulate physiological processes and may be the basis for novel therapeutics to prevent or treat chronic diseases (4).

Previously, it was shown that plasma Trp levels in diabetic rats and children with insulin-dependent diabetes were significantly lower than normal (5–7). Recently, metabolome analysis demonstrated that plasma concentrations of Trp and its metabolite kynurenine were lower in type 2 diabetic Torii rats than in normal rats (7). It is known that Trp deficiency modulates glucose tolerance (8). Lower concentrations of Trp in blood lead to lower concentrations of serotonin, which could contribute to depression in diabetes (6).

Data on Trp intake and blood glucose concentrations in diabetic conditions are limited. In the two reports published, oral administration of Trp did not modify glycemia in type 2 diabetic rats (9) and oral administration of Trp suppressed the elevation of blood glucose concentration in type 2 diabetic rats when the

rats were glucose-loaded (10). Based on these results, we hypothesized that a single intravenous injection of Trp suppresses blood glucose concentration, whereas oral intake of Trp over several days will not influence glucose concentration in diabetic rats. Previously, we demonstrated that the Trp–Nam conversion ratio was significantly lower in streptozotocin-induced type 1 diabetic rats than in healthy control rats (11); however, the activity of Trp 2,3-dioxygenase was accelerated by diabetic conditions (12). In addition, the catabolism of Nam in these groups of rats was not similar (11). These results suggest that the Trp requirement is higher in diabetic conditions than in normal conditions.

To the best of our knowledge, no previous studies have investigated the effect of continuous intake of Trp on blood glucose concentrations in type 2 diabetic rats. Therefore, we investigated oral glucose tolerance and Trp metabolism in non-obese and non-insulin-dependent diabetic Goto–Kakizaki (GK) (an animal model of type 2 diabetes) rats fed high-Trp diets.

MATERIALS AND METHODS

Chemicals. Vitamin-free milk casein, sucrose, L-methionine, gelatinized cornstarch, Trp, Nam, anthranilic acid (AnA) and quinolinic acid (QA) were

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purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Kynurenic acid (KA), xanthurenic acid (XA), 3-hydroxyanthranic acid (3-HA), and *N*¹-methyl-nicotinamide (MNA) chloride were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). *N*¹-methyl-2-pyridone-5-carboxamide (2-Py) and *N*¹-methyl-4-pyridone-3-carboxamide (4-Py) were synthesized using the methods of Pullman and Colowick (13) and Shibata et al. (14) respectively. Corn oil was purchased from Ajinomoto Co., Inc. (Tokyo, Japan). The mineral mixture (AIN-93G-MX) (15) and nicotinic-acid-free vitamin mixture (AIN-93-VX) (15) were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). All of the other chemicals used in this study were of the highest purity available from commercial sources.

Experimental animals and diets. Animals were maintained in a temperature-controlled room (22°C) with 60% relative humidity and a 12-h light/dark cycle (lights on: 06:00–18:00). Five-week-old male Wistar and GK rats weighing around 40 g were obtained from CLEA Japan, Inc. (Tokyo, Japan). The GK rat is a genetic animal model of type 2 diabetes that was created by selective breeding of an outbred colony of Wistar rats, with selection for high glucose levels in an oral glucose tolerance test (OGTT) (16). General characteristics of the GK rat include fasting hyperglycemia, impaired secretion of insulin in response to glucose both in vivo and in isolated pancreata, raised glycosylated hemoglobin, hepatic and peripheral insulin resistance and a variety of late complications, including nephropathy and neuropathy (16–18). The rats were divided into six groups of five rats each, and each group was placed in an individual metabolic cage (CT-10; CLEA Japan, Inc.). Rats in the control-Wistar and control-GK group were fed a 20% casein diet (containing 0.26% Trp) until the end of the experiment. The other groups were fed a 20% casein diet supplemented with 1%, 2%, 3%, or 5% Trp. The rats were fed the diet and water *ad libitum* for 58 d. The first day of the experiment was designated Day 1. Body weights and food consumption were recorded daily around 09:00 am. On the last day (Day 58, 09:00 am–Day 59, 09:00 am), 24-h urine samples were collected in amber bottles containing 1 mL of 1 mol/L HCl and were stored at –20°C until needed. After urine collection had been finished (Day 59, 09:00 am), the rats were sacrificed by decapitation, blood was collected in heparinized tubes, and livers were removed. The care and treatment of the experimental animals conformed to the University of Shiga Prefecture guidelines for the ethical treatment of laboratory animals (approval number, No. 21-4).

Measurements of Trp and its metabolites. Nam (19) and its catabolites, MNA (20), 2-Py, and 4-Py (19), in urine were measured directly by high-performance liquid chromatography (HPLC). To measure the total Nam (pyridine nucleotides+free type of Nam) content in the blood and liver, whole blood and liver homogenate were autoclaved at 121°C for 20 min to convert the pyridine nucleotides to Nam. The resulting Nam measurement was then determined by HPLC (14, 19). The urinary

concentration of 3-HA and XA was measured using the methods of Shibata and Onodera (21), the urinary concentration of KA was measured using the method of Shibata (22), and the urinary concentration of QA was measured using the method of Mawatari et al. (23).

Oral glucose tolerance test (OGTT). OGTT was performed on Days 14 and 28 during the experiment. Five rats were used in each group and fasted for 17–20 h before the experiments. For OGTT, 0.4 mL of 50% glucose solution per 100 g body weight (2 g glucose/kg body weight) was orally administered. Blood was collected through the tail artery immediately before (0 min) and 30, 60, 90, and 120 min after glucose administration. Plasma glucose concentrations were measured using a glucose oxidase method with the help of a blood glucose meter (Glutest Every; Sanwa-Kagaku, Nagoya, Japan). Glucose concentrations were calculated as the incremental blood glucose concentrations integrated over a period of 2 h ($\Delta\text{AUC}_{0-2\text{h}}$) after glucose loading.

General biomarkers in blood and urine. Glucose, total cholesterol, triglycerides, albumin, and urea nitrogen in plasma samples were measured using a FUJI DRI-CHEM 3500V (Fujifilm Medical Co., Ltd., Tokyo Japan). Plasma and urine creatinine were measured using a colorimetric method (24).

Statistical analysis. All experimental data are expressed as means with the standard error of the mean for each group. The statistical significance was determined by one-way analysis of variance (ANOVA), followed by Tukey's test or a two-way ANOVA to evaluate the effect of Trp diet group and OGTT performance day (Day 14 and Day 28), followed by Bonferroni's post hoc test. Body weights, food intake, clinical parameters, organ weights, and Nam and its metabolites were compared between control-Wistar, control-GK rats and rats with different concentrations of Trp intake. The area under the curve (AUC) was calculated using the trapezoidal rule. Differences at $p < 0.05$ were considered statistically significant. Prism 4.0 (Graph Pad Software, San Diego, CA) was used for all analyses.

RESULTS

Effects of Trp intake on body weight, food intake, and organ weights of GK rats

Table 1 shows the body weight, food intake, and organ weights of Wistar and GK rats. The total food intakes during the experiment in the control-Wistar group were higher than in the GK groups, and the intakes of the control-GK, +1% Trp, and +2% Trp diet groups were the same. Meanwhile, they were lower in the +3% Trp and +5% Trp diet groups compared with those of the control-GK, +1% Trp and +2% Trp diet groups.

The body weight gains during the experiment in the control-Wistar group were higher than in the GK groups, and these were the same among the control-GK and +1% Trp diet groups. They were lower in the groups fed the +2% Trp, +3% Trp, and +5% Trp diets compared with those of the control-GK and +1% Trp diet groups.

Liver weights were lower in the +5% Trp diet group

Table 1. Effects of Trp intake on body weight, food intake, urine volume, organ weights and clinical parameters in GK rats.

	Control-Wistar	Control-GK (+0% Trp)	+1% Trp	+2% Trp	+3% Trp	+5% Trp
Initial body weight (g)	112.7±0.7	92.2±4.1	92.4±4.0	92.9±4.2	92.7±4.3	92.9±4.9
Final body weight (g)	419.6±11.1 ^a	330.1±4.1 ^b	308.4±7.0 ^c	299.4±2.1 ^c	297.7±3.6 ^c	274.8±5.7 ^d
Body weight gain (g)	306.8±11.4 ^a	237.8±3.0 ^b	215.9±10.1 ^b	206.5±4.6 ^c	205.0±3.6 ^{cd}	181.9±4.5 ^d
Total food intake (g/58 d)	1,059.1±15.3 ^a	880.5±12.9 ^b	849.3±9.3 ^b	841.5±25.2 ^b	797.5±19.8 ^c	743.1±10.7 ^c
Trp intake ¹ (μmol/d)	253±4 ^a	209±3 ^a	1,127±13 ^b	1,876±53 ^c	2,513±70 ^d	3,710±68 ^e
Organ weights						
Liver (g)	14.5±0.7 ^a	12.3±0.4 ^b	12.1±0.3 ^b	11.8±0.4 ^b	10.9±0.3 ^b	9.9±0.5 ^c
Kidney (g)	2.55±0.12	2.65±0.12	2.65±0.03	2.59±0.07	2.57±0.09	2.57±0.07
Clinical parameters						
Plasma glucose (mg/dL)	89±7 ^a	205±8 ^b	167±23 ^b	174±33 ^b	207±15 ^b	212±17 ^b
Plasma total cholesterol (mmol/L)	2.83±0.08 ^b	4.01±0.04 ^a	3.51±0.35 ^{ab}	3.67±0.11 ^a	3.35±0.04 ^{ab}	3.19±0.07 ^b
Plasma triglyceride (mmol/L)	2.42±0.31 ^c	1.26±0.14 ^b	1.26±0.12 ^{bc}	1.27±0.11 ^{ab}	1.12±0.12 ^{bc}	1.15±0.09 ^{ac}
Plasma creatinine (mmol/L)	0.02±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.02±0.00
Plasma albumin (mmol/L)	0.66±0.31	0.71±0.01	0.69±0.01	0.69±0.01	0.67±0.01	0.68±0.01
Plasma urea nitrogen (mmol/L)	20.9±0.6	17.8±1.0	20.4±1.0	22.1±1.8	18.6±1.2	18.1±1.3
Urine creatinine (mg/d)	4.3±0.1 ^a	15.0±1.3 ^{bc}	15.7±2.1 ^{bc}	18.0±2.4 ^b	14.7±0.9 ^{bc}	10.9±1.0 ^c

Trp, tryptophan.

Data are presented as mean±SE for five rats.

Mean values within a row with unlike superscript letters are significantly different as determined by one-way analysis of variance with Tukey's multiple comparison test ($p<0.05$).

¹ Trp intake: food intake (g/d)×0.2×0.875×0.013×1/204×10⁶.

Fig. 1. Oral glucose tolerance test (OGTT) in Wistar and GK rats. OGTT was performed on Days 14 (A) and 28 (B) of the experiment. GK rats were fasted for 17–20 h before the OGTT. Mean values with unlike superscript letters were significantly different among GK-groups as determined by one-way analysis of variance with Tukey's multiple comparison test ($p<0.05$).

than in the other diet groups. No differences in kidney weights were observed among the groups.

Effects of Trp intake on the clinical parameters of GK rats

Table 1 shows the clinical parameters in Wistar and GK rats. The plasma glucose concentrations on Day 58 (the last day of the experiment) in the control-Wistar group were higher than in the GK groups (Table 1). Meanwhile, no differences in the plasma glucose concentrations on Day 58 were observed among any diet groups in GK-rats.

The plasma total cholesterol levels in the +5% Trp diet group was lower than in the control-GK group, although no differences in these values for the other Trp diet groups were observed compared with those of the

control-GK group. The plasma triglyceride concentration in the control-Wistar group was higher than in the control-GK group and +2% Trp diet group. The plasma triglyceride concentration in the +5% Trp diet group was lower than in the control-GK group, although no differences in this value for the other Trp diet groups were observed compared with that of the control-GK group. No differences in concentrations of plasma albumin and plasma urea nitrogen were observed among any diet groups. The urine creatinine concentration was lower in the +5% Trp diet group than in the +2% Trp diet groups.

Effects of Trp intake on blood glucose tolerance of GK rats

To estimate the effect of Trp on blood glucose con-

Table 2. Effects of Trp intake on the fasting, 120-min blood glucose levels and $\Delta\text{AUC}_{0-2\text{h}}$ concentrations in GK rats on Day 14 and Day 28 of the experiment.

Group	Fasting blood glucose (mg/dL)		120-min blood glucose (mg/dL)		$\Delta\text{AUC}_{0-2\text{h}}^1$ (mg/dL per min)		
	Time	Day 14	Day 28	Day 14	Day 28	Day 14	Day 28
Control-Wistar		78±2	63±3	84±5	87±6	106±1	103±2
Control-GK (+0% Trp)		173±11 ^a	172±11	332±8 ^a	351±14 ^a	330±8 ^a	336±17
+1.0% Trp		147±10 ^a	178±12	323±28 ^a	334±21 ^{ab}	318±15 ^a	327±22
+2.0% Trp		157±3 ^{ac}	167±9	318±19 ^a	267±9 ^b	308±9 ^a	297±17
+3.0% Trp		138±7 ^{ab}	158±6	283±19 ^{ab}	299±17 ^{ab}	288±10 ^b	321±18
+5.0% Trp		121±6 ^b	169±6	223±7 ^b	280±16 ^b	270±6 ^b	329±13*
One-way ANOVA ²							
Group effect		<i>p</i> <0.05	NS	<i>p</i> <0.05	<i>p</i> <0.05	<i>p</i> <0.05	NS
Two-way ANOVA ³							
Group effect		<i>p</i> <0.05		<i>p</i> <0.05		NS	
Time effect		<i>p</i> <0.05		NS		<i>p</i> <0.05	
Interaction effect (Group×Time)		NS		<i>p</i> <0.05		NS	

Trp, tryptophan; AUC, area under the curve, NS, not significant.

Data are presented as mean $\pm$ SE for five rats.

¹ $\Delta\text{AUC}_{0-2\text{h}}$ increment blood glucose concentration integrated over a period of 2 h.

² Significance was determined by one-way ANOVA among GK-rat groups, and mean values within a row with unlike superscript letters are significantly different by Tukey's multiple comparison test ($p<0.05$).

³ Significance was determined by two-way ANOVA ($p<0.05$) among GK-rat groups.

Table 3. Effects of Trp intake on the Nam concentration in blood and liver in GK rats.

	Control-Wistar	Control-GK (+0% Trp)	+1% Trp	+2% Trp	+3% Trp	+5% Trp
Blood total Nam ($\mu\text{mol/mL}$)	135 $\pm$ 4 ^a	101 $\pm$ 3 ^b	115 $\pm$ 3 ^d	132 $\pm$ 3 ^{acd}	135 $\pm$ 5 ^{acd}	152 $\pm$ 8 ^{ac}
Liver Nam ($\mu\text{mol/g}$ of liver)	1.96 $\pm$ 0.06 ^a	2.00 $\pm$ 0.05 ^a	3.24 $\pm$ 0.13 ^b	3.38 $\pm$ 0.16 ^b	3.24 $\pm$ 0.23 ^b	3.00 $\pm$ 0.09 ^b

Trp, tryptophan; Nam, nicotinamide.

Data are presented as mean $\pm$ SE for five rats.

Mean values within a row with unlike superscript letters are significantly different from those of control-Wistar rats as determined by one-way ANOVA with Tukey's multiple comparison test ($p<0.05$).

centrations, these were measured after glucose loading. Blood glucose reached the highest concentration (369 mg/dL) 1 h after glucose loading in the control-GK group (Fig. 1). On Day 14, the fasting and 120-min (after glucose loading) blood glucose concentrations were not significantly different between the control-GK group and the +1% Trp, +2% Trp or +3% Trp diet groups, whereas in the +5% Trp diet groups, they were significantly decreased compared with the control-GK and +1% Trp diet group ($p<0.05$). On Day 14, the blood glucose concentration increased and finally reverted over a period of 2 h ($\Delta\text{AUC}_{0-2\text{h}}$ values) after the administration of glucose in the control-GK diet group, which was 330 mg/dL/min (Table 2). The +1% Trp and +2% Trp diet did not affect the $\Delta\text{AUC}_{0-2\text{h}}$ of blood glucose on Day 14, whereas in the +3% Trp and +5% Trp diet group, the $\Delta\text{AUC}_{0-2\text{h}}$ fell by 13% and 18%, respectively, compared with the control-GK diet group ($p<0.05$). On Day 28, no significant differences were observed among

any of the groups with respect to increases in blood glucose concentrations. There was a significant time-effect change in fasting blood glucose and $\Delta\text{AUC}_{0-2\text{h}}$, but significant interaction was not observed (two-way ANOVA: time effect, $p<0.05$; interaction effect, NS). In a 120-min blood glucose reading, the time-effect change had no significant difference, but significant interaction was observed (two-way ANOVA: time effect, NS; interaction effect, $p<0.05$). Multiple comparisons were made on a 120-min blood glucose result with interaction observed: the 120-min blood glucose level on Day 14 was significantly lower in the +3% Trp diet than in the control-GK group, whereas the 120-min blood glucose readings on Day 28 in the +2% and +5% Trp diet were lower than in the control-GK group.

Effects of Trp intake on total Nam concentrations of blood and liver in GK rats

Table 3 shows the total Nam concentrations in the blood and liver. The total Nam concentrations in the

blood were higher in the control-Wistar than in the control-GK group, and these concentrations in the +2% Trp, +3% Trp, and +5% Trp diet groups were higher than in the control-GK group; however, there were no differences among the +2% Trp, +3% Trp and +5% Trp diet groups.

The Nam concentrations per gram of liver were also higher in the Trp diet groups than in the control-Wistar and control-GK group; however, there were no differences among the Trp diet groups.

Effects of Trp intake on the Trp–Nam pathway of GK rats

Table 4 shows the effects of Trp on the Trp–Nam metabolism. The relative Trp contents based on the diet composition in the control-GK, +1% Trp, +2% Trp, +3% Trp, and +5% Trp diet groups were 1.0, 4.8, 8.7, 12.5, and 20.2 times, respectively. In comparison with the control-Wistar group, the control-GK group of excretion ratio of (2-Py+4-Py)/MNA was lower and the conversion ratio of Trp–Nam was higher. On the other hand, the other metabolites were essentially the same among the control-Wistar and control-GK groups. A particular phenomenon observed was that the urinary excretion amounts of Trp metabolites increased with Trp intake. Specifically, the urinary excretion of AnA dramatically increased in the +5% Trp diet group. The urinary excretion amounts of QA were increased in rats fed the +1% Trp diet group; however, there were no differences among the Trp diet groups.

Although the excretion amounts of SUM (Nam and its catabolites such as MNA, 2-Py, and 4-Py) were also increased in a dose-dependent manner, its content was at the same level in the +2% and +3% Trp diet groups and its content was decreased in the +5% Trp diet group. The urinary excretion ratio of (2-Py+4-Py)/MNA decreased with increasing dietary Trp levels. And the conversion percentage of Trp–Nam decreased with increasing dietary Trp levels.

DISCUSSION

In this study, we first clarified that a high dose of Trp prior to the development of type 2 diabetes did not suppress blood glucose concentrations. Some other reports (10, 25) have not been consistent with our results. Hundley et al. (25) reported that administration of Trp via stomach tube increased the blood glucose concentration in fasting alloxan-induced type 1 diabetic rats. Meanwhile, Inubushi et al. (10) demonstrated that a Trp-rich diet (0.32% Trp diet) suppressed the elevation of blood glucose in type 2 diabetic Torii rats. The difference between these two experiments could be due to differences in study design. In the former study, Trp was administered after the development of diabetes (25), while in the latter study, the Trp-rich diet was provided prior to the development of diabetes (10). Furthermore, an oral dose of Trp was more effective than intraperitoneal injection with regard to the control of blood glucose (10).

In the present study, the oral administration of Trp did not suppress the elevation of blood glucose concentrations, despite administering Trp prior to the development

Table 4. Effects of Trp intake on the urinary excretion of Nam and its metabolites and Trp–Nam pathway of the GK rats.

	Control-Wistar	Control-GK (+0% Trp) (1.0)	+1% Trp (4.8)	+2% Trp (8.7)	+3% Trp (12.5)	+5% Trp (20.2)
AnA (nmol/d)	404±112 ^a	109±12 ^a (1.0)	660±92 ^a (6.1)	2,141±480 ^a (19.6)	7,838±1,674 ^a (72.0)	32,892±4,686 ^b (302)
KA (nmol/d)	2,811±519 ^{ac}	1,024±73 ^c (1.0)	6,671±1,124 ^a (6.5)	8,555±1,915 ^b (8.4)	8,822±376 ^b (8.6)	14,579±1,149 ^d (14.2)
XA (nmol/d)	1,279±223 ^a	555±21 ^a (1.0)	1,869±157 ^a (3.4)	7,012±1,168 ^b (12.6)	8,520±506 ^b (15.4)	12,022±722 ^c (21.7)
3-HA (nmol/d)	106±3 ^a	90±3 ^a (1.0)	128±58 ^b (1.4)	323±56 ^c (3.6)	480±32 ^c (5.3)	756±54 ^d (8.4)
QA (μmol/d)	1.18±0.09 ^a	1.22±0.09 ^a (1.0)	6.08±0.33 ^b (5.0)	7.25±1.58 ^b (5.9)	5.08±0.81 ^b (4.2)	6.59±0.33 ^b (5.4)
(2-Py+4-Py)/MNA	13.24±1.04 ^a	10.62±0.82 ^b (1.0)	4.36±0.43 ^c (0.41)	1.60±0.18 ^d (0.15)	1.25±0.05 ^d (0.12)	1.14±0.04 ^d (0.11)
SUM (μmol/d)	6.54±0.32 ^a	8.61±0.57 ^a (1.0)	39.4±1.24 ^b (4.6)	48.3±3.5 ^c (5.6)	45.4±0.73 ^{bc} (5.3)	35.7±2.45 ^d (4.2)
Conversion ratio of Trp–Nam ¹ (%)	2.59±0.11 ^a	3.83±0.16 ^b (1.0)	3.19±0.11 ^c (0.83)	2.41±0.14 ^{ad} (0.63)	1.76±0.06 ^e (0.46)	0.94±0.07 ^f (0.25)

Trp, tryptophan; Nam, nicotinamide; AnA, anthranilic acid; KA, kynurenic acid; XA, xanthurenic acid; 3-HA, 3-hydroxyanthranilic acid; QA, quinolinic acid; MNA, N¹-methylnicotinamide; 2-Py, N¹-methyl-2-pyridone-5-carboxamide; 4-Py, N¹-methyl-4-pyridone-3-carboxamide; SUM, Nam + MNA + 2-Py + 4-Py.

¹ Conversion ratio of Trp–Nam: SUM (μmol/d)/Trp intake (μmol/d)×100.

Data are presented as mean±SE for five rats.

Mean values within a row with unlike superscript letters are significantly different from those of control-Wistar rats as determined by one-way ANOVA with Tukey's multiple comparison test ($p<0.05$).

Numbers in parentheses are the relative values versus the control.

Fig. 2. Trp metabolism. ACMS, aminocarboxymuconate-semialdehyde; AMS, aminomuconate-semialdehyde; AnA, anthranilic acid; KA, kynurenic acid; NMN, nicotinamide mononucleotide; MNA, N¹-methylnicotinamide; 2-Py, N¹-methyl-2-pyridone-5-carboxamide; 4-Py, N¹-methyl-4-pyridone-3-carboxamide; XA, xanthurenic acid.

of type 2 diabetes. However, in comparisons between the OGTT administration days and between Trp addition amounts, the interaction effects were essentially not observed. The reduced pancreatic β -cell mass (26) and elevated basal plasma glucose (18) were detected in GK rats as young as 1 mo post-weaning. These symptoms are seen earlier than in Torii rats (27). It might be that Trp-rich chow consumed from a young age preserved the secretion of insulin and delayed the progression of type 2 diabetes. Together, the above results and our findings suggest that high-dose oral Trp is not useful in delaying the progression of mild hyperglycemia.

The reduction in food intake and the delay in body weight gain of the GK rats fed +3% Trp and +5% Trp diets were small compared with those of the GK rats fed the control diet. Therefore, the adverse effects of excessive intake of Trp were very weak in GK rats.

In addition, we revealed the Trp metabolism in GK rats. Figure 2 shows the Trp metabolism. We found that Nam is synthesized from Trp even in GK rats. Generally speaking, the synthesized amount of Nam is dependent on intake of Trp when it is within physiological amounts (28). In the present experiment, the conversion of Trp to Nam was maximized with the +1% Trp diet. The same phenomenon was observed in the urinary excretion

of QA. This finding observed in GK rats was a similar phenomenon to that seen in normal healthy animals (27). An abnormal increase in the urinary excretion of AnA in GK rats fed the +5% Trp diet was also reported in normal healthy animals (27). This phenomenon is attributed to the properties of kynureninase (a pyridoxal phosphate-dependent enzyme) (29). The K_m value of kynurenine with kynureninase is much higher than that of 3-hydroxykynurenine (29), so the reaction of kynurenine $\rightarrow$ AnA occurs very little under normal Trp intake conditions. Meanwhile, with high Trp intake, the accumulation of kynurenine occurs, so the reaction of kynurenine $\rightarrow$ AnA occurs. Under physiological conditions, kynurenine is metabolized to 3-hydroxykynurenine, which is then hydrolyzed to 3-HA by kynureninase. 3-HA is metabolized to aminocarboxymuconate-semialdehyde, which then spontaneously cyclizes to form QA. The formation of QA increased 5-fold with the +1% Trp diet compared with the control diet (0.26% Trp diet), which was almost the same as the increase in Trp intake, and it did not increase with the higher Trp diets. These results indicate that the enzyme 3-HA 3,4-dioxygenase (a heme-containing enzyme) (30) that catalyzes the reaction 3-HA $\rightarrow$ aminocarboxymuconate-semialdehyde is optimized owing to the +1% Trp diet.

3-HA 3,4-dioxygenase requires Fe^{2+} ; QA binds Fe^{2+} (31). Therefore, the increased QA from the Trp-rich diet might inhibit the activity of 3-HA 3,4-dioxygenase. We previously revealed that the formation of Nam from Trp is subject to the QA formation, and the enzyme that governs it is 3-HA 3,4-dioxygenase (32). Therefore, these findings indicate that the same mechanism exists in GK rats as in normal healthy animals.

The Trp catabolites KA and XA are side intermediates. KA spontaneously forms from 4-(2-aminophenyl)-2,4-dioxobutanoic acid and glutamic acid from kynurenine and 2-oxoglutaric acid by kynurenine aminotransferase (a pyridoxal phosphate-dependent enzyme). XA spontaneously forms from 4-(2-amino-3-hydroxyphenyl)-2,4-dioxobutanoic acid and glutamic acid from 3-hydroxykynurenine and 2-oxoglutaric acid by the same enzyme, kynurenine aminotransferase. The urinary excretion amounts of these two side metabolites KA and XA were mildly increased with the increase in Trp intake. Thus, the activity of kynurenine aminotransferase could be strong enough to metabolize a large amount of Trp.

In the present experiment using GK rats, the urinary excretion ratio (2-Py+4-Py)/MNA decreased with Trp-rich diets as well as in normal healthy animals (27). This phenomenon is due to the inactivation of 4-Py-forming MNA oxidase being suppressed by the substrate MNA (33). A Trp-rich diet causes MNA formation to increase, and the accumulated MNA inactivates 4-Py-forming MNA oxidase (33).

We found in the present study that the high doses of Trp prior to the development of type 2 diabetes did not suppress blood glucose levels. The metabolic response was found to be nearly the same in normal healthy animals and GK rats. We previously reported on the metabolism of Trp in healthy humans (34, 35). The pathophysiological relevance of Trp metabolism in type 2 diabetes should be examined in humans.

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Author contributions

E.I. and K.S. designed the study. E.I. conducted the experiments. E.I. drafted the manuscript, and K.S. revised and reviewed the manuscript. All authors read and approved the final manuscript.

REFERENCES

- 1) Wurtman RJ. 1982. Nutrients that modify brain function. *Sci Am* **246**: 50–59.
- 2) Cardinali DP. 1981. Melatonin. A mammalian pineal hormone. *Endocr Rev* **2**: 327–346.
- 3) Fernstrom JD. 2000. Can nutrient supplements modify brain function? *Am J Clin Nutr* **71**: 1669S–1675S.
- 4) Chen T, Zheng X, Ma X, Bao Y, Ni Y, Hu C, Rajani C, Huang F, Zhao A, Jia W, Jia W. 2016. Tryptophan predicts the risk for future type 2 diabetes. *PLoS One* **11**: e0162192.
- 5) MacKenzie RG, Trulsson ME. 1978. Does insulin act directly on the brain to increase tryptophan levels? *J Neurochem* **30**: 1205–1208.
- 6) Herrera R, Manjarrez G, Nishimura E, Hernandez J. 2003. Serotonin-related tryptophan in children with insulin-dependent diabetes. *Pediatr Neurol* **28**: 20–23.
- 7) Yokoi N, Beppu M, Yoshida E, Hoshikawa R, Hidaka S, Matsubara T, Shinohara M, Irino Y, Hatano N, Seino S. 2015. Identification of putative biomarkers for prediabetes by metabolome analysis of rat models of type 2 diabetes. *Metabolomics* **11**: 1277–1286.
- 8) Wittman JS 3rd. 1976. Alteration of glucose tolerance by dietary L-tryptophan in rats. *J Nutr* **106**: 631–635.
- 9) Tormo MA, Romero de Tejada A, Morales I, Paredes S, Sanchez S, Barriga C, Hernandez R. 2004. Orally administered tryptophan and experimental type 2 diabetes. *Mol Cell Biochem* **261**: 57–61.
- 10) Inubushi T, Kamemura N, Oda M, Sakurai J, Nakaya Y, Harada N, Suenaga M, Matsunaga Y, Ishidoh K, Katunuma N. 2012. L-tryptophan suppresses rise in blood glucose and preserves insulin secretion in type-2 diabetes mellitus rats. *J Nutr Sci Vitaminol* **58**: 415–422.
- 11) Imai E, Sano M, Fukuwatari T, Shibata K. 2011. Tryptophan-nicotinamide metabolism in rats with streptozotocin-induced diabetes: association of dietary vitamin content. *Nippon Eiyo Shokuryo Gakkaishi (J Jpn Soc Nutr Food Sci)* **64**: 313–321.
- 12) Shibata K. 1987. Tryptophan-niacin metabolism in alloxan diabetic rats and partial prevention of alloxan diabetes by nicotinamide. *Agric Biol Chem* **51**: 811–816.
- 13) Pullman ME, Colowick SP. 1954. Preparation of 2- and 6-pyridones of N1-methylnicotinamide. *J Biol Chem* **206**: 121–127.
- 14) Shibata K, Kawada T, Iwai K. 1987. High-performance liquid chromatographic determination of nicotinamide in rat tissue samples and blood after extraction with diethyl ether. *J Chromatogr* **422**: 257–262.
- 15) Reeves PG. 1997. Components of the AIN-93 diets as improvements in the AIN-76A diet. *J Nutr* **127**: 838S–841S.
- 16) Goto Y, Kakizaki M, Masaki N. 1975. Spontaneous diabetes produced by selective breeding of normal Wistar rats. *Proc Jpn Acad* **51**: 80–85.
- 17) Ostenson CG, Khan A, Abdel-Halim SM, Guenifi A, Suzuki K, Goto Y, Efendic S. 1993. Abnormal insulin secretion and glucose metabolism in pancreatic islets from the spontaneously diabetic GK rat. *Diabetologia* **36**: 3–8.
- 18) Portha B, Serradas P, Bailbe D, Suzuki K, Goto Y, Giroix MH. 1991. Beta-cell insensitivity to glucose in the GK rat, a spontaneous nonobese model for type II diabetes. *Diabetes* **40**: 486–491.
- 19) Shibata K, Kawada T, Iwai K. 1988. Simultaneous micro-determination of nicotinamide and its major metabolites, N¹-methyl-2-pyridone-5-carboxamide and N¹-methyl-4-pyridone-3-carboxamide, by high-performance liquid chromatography. *J Chromatogr* **424**: 23–28.
- 20) Shibata K. 1987. Ultramicro-determination of N¹-methylnicotinamide in urine by high-performance liquid chromatography. *Vitamins* **61**: 599–604.

- 21) Shibata K, Onodera M. 1992. Simultaneous high-performance liquid chromatographic measurement of xanthurenic acid and 3-hydroxyanthranilic acid in urine. *Biosci Biotechnol Biochem* **56**: 974.
- 22) Shibata K. 1988. Fluorimetric micro-determination of kynurenic acid, an endogenous blocker of neurotoxicity, by high-performance liquid chromatography. *J Chromatogr* **430**: 376–380.
- 23) Mawatari K, Oshida K, Iinuma F, Watanabe M. 1996. Determination of quinolinic acid by liquid chromatography with fluorimetric detection. *Adv Exp Med Biol* **398**: 697–701.
- 24) Slot C. 1965. Plasma creatinine determination. A new and specific Jaffe reaction method. *Scand J Clin Lab Invest* **17**: 381–387.
- 25) Hundley JM, McDaniel EG, Sebrell WH. 1956. Tryptophan-niacin metabolism in alloxan diabetic rats. *J Nutr* **59**: 407–423.
- 26) Movassat J, Saulnier C, Portha B. 1995. Beta-cell mass depletion precedes the onset of hyperglycaemia in the GK rat, a genetic model of non-insulin-dependent diabetes mellitus. *Diabete Metab* **21**: 365–370.
- 27) Shinohara M, Masuyama T, Shoda T, Takahashi T, Katsuda Y, Komeda K, Kuroki M, Kakehashi A, Kanazawa Y. 2000. A new spontaneously diabetic non-obese Torii rat strain with severe ocular complications. *Int J Exp Diabetes Res* **1**: 89–100.
- 28) Shibata K, Matsuo H. 1990. Effect of dietary tryptophan levels on the urinary excretion of nicotinamide and its metabolites in rats fed a niacin-free diet or a constant total protein level. *J Nutr* **120**: 1191–1197.
- 29) De Antoni A, Costa C, Allegri G. 1975. Studies on 3-hydroxykynureninase from rat liver. *Acta Vitaminol Enzymol* **29**: 339–343.
- 30) Okuno E, Kohler C, Schwarcz R. 1987. Rat 3-hydroxyanthranilic acid oxygenase: purification from the liver and immunocytochemical localization in the brain. *J Neurochem* **49**: 771–780.
- 31) Muller AC, Dairam A, Limson JL, Daya S. 2007. Mechanisms by which acyclovir reduces the oxidative neurotoxicity and biosynthesis of quinolinic acid. *Life Sci* **80**: 918–925.
- 32) Shibata K, Morita N, Shibata Y, Fukuwatari T. 2013. Enzymes that control the conversion of L-tryptophan-nicotinamide and the urinary excretion ratio (N^1 -methyl-2-pyridone-5-carboxamide + N^1 -methyl-4-pyridone-3-carboxamide)/ N^1 -methylnicotinamide in mice. *Biosci Biotechnol Biochem* **77**: 2105–2111.
- 33) Shibata K, Matsuo H. 1989. Inhibition of N^1 -methylnicotinamide oxidase activity by a large amount of N^1 -methylnicotinamide injected into rats. *Agric Biol Chem* **53**: 2393–2397.
- 34) Hiratsuka C, Fukuwatari T, Sano M, Saito K, Sasaki S, Shibata K. 2013. Supplementing healthy women with up to 5.0 g/d of L-tryptophan has no adverse effects. *J Nutr* **143**: 859–866.
- 35) Hiratsuka C, Fukuwatari T, Shibata K. 2012. Fate of dietary tryptophan in young Japanese women. *Int J Tryptophan Res* **5**: 33–47.