

The Reduction in Urinary Glutamate Excretion Is Responsible for Lowering Urinary pH in Pink Urine Syndrome

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We frequently encounter brownish-red, cloudy urine in some obese subjects, which occurs due to pink urine syndrome (PUS). PUS is a phenomenon in which uric acid precipitates into the urine due to reduced urinary pH (UpH). The mechanism underlying urinary acidification has not been elucidated so far. UpH level is adjusted by urinary excretion of ammonia synthesized from glutamate or glutamine, suggesting that renal synthesis of ammonia from glutamate or glutamine is decreased in PUS. However, this hypothesis has not been examined yet. We therefore examined the changes in the urinary excretion of these amino acids in PUS. One-hundred-fifty male students who had undergone a physical examination were enrolled. To determine the presence [PUS (+), n = 72] or absence [PUS (-), n = 78] of PUS, urinary amino acid excretion and UpH were evaluated. Independent risk factors of lower UpH were determined using multiple regression analyses. The PUS (+) subjects, who had lower UpH values than PUS (-) subjects, showed lower urinary excretion of glutamate and some other glucogenic amino acids. Thus, UpH correlated positively with the urinary excretion of glutamate in the PUS (+) subjects. A reduction in urinary glutamate but not in glutamine excretion proved to be an independent risk factor for reduced UpH. In conclusion, PUS appears to occur when a reduction in the synthesis of ammonia from glutamate causes a decrease in UpH. Our results showed that urinary glutamate excretion was reduced in PUS because renal glutamate was consumed by a reaction different from ammonia production.

Keywords: ammonia; glucogenic amino acids; glutamate; pink urine syndrome; urinary pH.

Tohoku J. Exp. Med., 2016 June, 239 (2), 103-110. © 2016 Tohoku University Medical Press

Introduction

While handling urine samples during health checkups, we frequently encounter brownish-red, cloudy urine, which, upon centrifugation, produces pink sediments that are either uric acid crystals or urates. This phenomenon is defined as pink urine syndrome (PUS) (Deitel et al. 1984; Ogawa et al. 2015b). PUS is characterized by cloudy brownish-red urine and the formation of pink sediments in urine upon centrifugation, which occurs due to uric acid (UA) precipitation resulting from urinary acidification. PUS is not a rare phenomenon, with a considerable number of PUS cases (4.4%) found even in healthy young populations (Ogawa et al. 2015b); it is also closely linked to obesity (Deitel et al. 1984; Ogawa et al. 2015b). However, PUS is not a disease; instead, it appears to be a symptom that elicits some sort of physical response. However, the mechanism underlying the phenomenon of PUS is and its significant remains unclear. Not all obese patients manifest symptoms of PUS. In addition,

whether PUS develops in obese patients who fulfill certain criteria or whether the pathology that induces PUS may lead to obesity but does not necessarily cause it is not certain.

PUS results from increased urinary UA (UUA) excretion and a reduction in urinary pH (UpH) level. A reduction in UpH in PUS has been shown to be strongly correlated with oxidative stress (OS) markers in the urine (Ogawa et al. 2015b). UpH is controlled by the secretion of urinary ammonia (NH₃) from the renal proximal tubules (RPTs), where it is converted into ammonium ions (NH₃ + H⁺ → NH₄⁺) (Hems 1975; Zipp and Tannen 1983; Rodriguez-Nichols et al. 1984). The UpH decreases when the supply of H⁺ is so high (acidosis) that it cannot be counteracted by this NH₃, or when the secretion of NH₃ from the RPT decreases. Synthesis of NH₃ in the RPTs involves two reactions: the production of glutamate from glutamine, and the production of α-ketoglutarate from glutamate (Hems 1975; Zipp and Tannen 1983; Rodriguez-Nichols et al. 1984;

Received March 11, 2016; revised and accepted April 28, 2016. Published online May 26, 2016; doi: 10.1620/tjem.239.103.

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Nissim 1999; Tapiero et al. 2002; Karim et al. 2005) (Fig. 1). After filtration in the renal glomeruli, amino acids (AAs) in the blood are reabsorbed and used in the RPT, with the remainder being excreted into the urine (Moret 2007). Therefore, the amount of AAs excreted into the urine might be an important marker that indicates the use of those AAs in the RPTs. Since the UpH is reduced in PUS, it is assumed that, because of some sort of impediment of the RPTs, the use of either glutamine or glutamate and NH_3 synthesis is decreased, while the amount of urinary glutamine or glutamate excretion is likely to have increased. However, there are no reports so far to confirm this hypothesis.

We therefore measured the amount of AAs including glutamine and glutamate and the amount of UA excreted in the PUS (+) subjects and investigated the relationship between UpH and the amount of urinary excretion of these AAs and UA.

Subjects and Methods

Subjects

The subjects enrolled for our previous study (Ogawa et al. 2015b) were used for this study as well. A large proportion of the study population were men, and since numerous cases of UA precipitation have been observed among men (Ogawa et al. 2015b), we decided to conduct our analysis using male subjects only. Of the

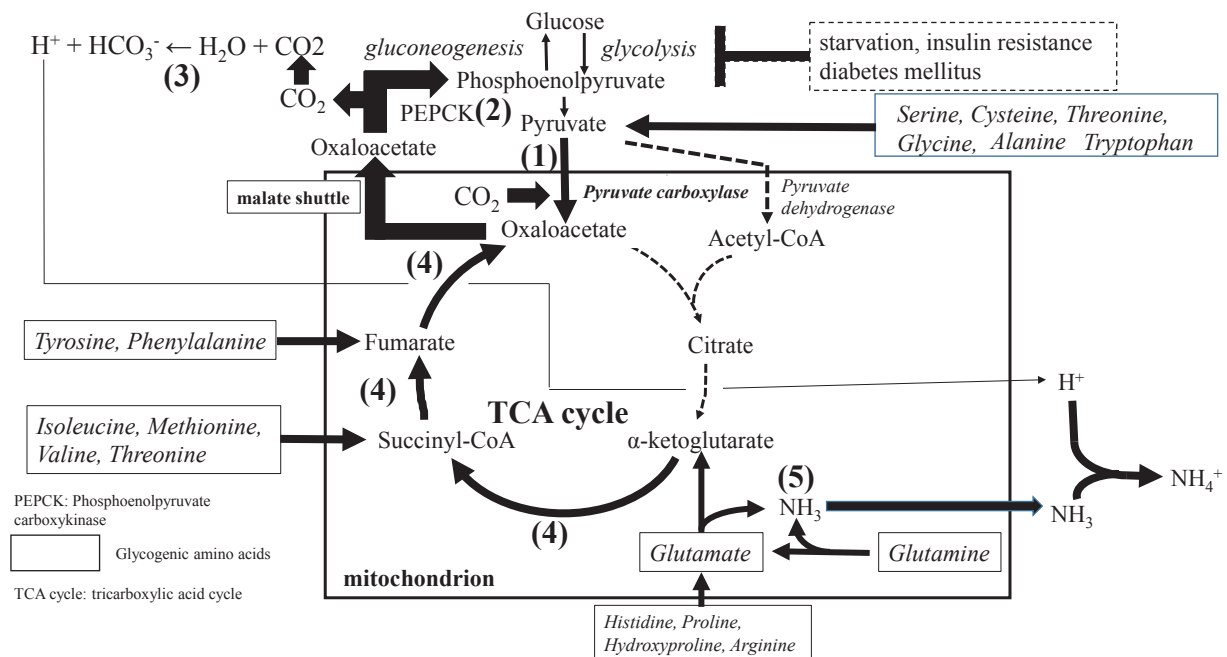


Fig. 1. The relationship, under acidotic conditions, between glucose metabolism and amino acids use in the renal proximal tubules.

The Figure 1 shows the relationship, under acidotic conditions, between glucose metabolism and glucogenic amino acids (GAAs) use in the renal proximal tubules (RPT).

Under conditions such as starvation, dehydration, insulin resistance and diabetes, the glycolysis decreases, so acidosis is liable to be induced. The roles that the kidneys play under acidotic conditions are to increase the urinary excretion of hydrogen (H^+), increase the production of bicarbonate (HCO_3^-). The HCO_3^- is produced by the reaction (3) [water (H_2O) + carbon dioxide (CO_2) \rightarrow H^+ + HCO_3^-]. To move this reaction forward, it is necessary to eliminate H^+ and supply CO_2 . Elimination of H^+ is done by the increased secretion of H^+ into the urine by the Na^+ - H^+ exchanger. As a result, reabsorption of Na^+ is boosted, and body fluid increases. The necessary CO_2 in the RPT cytoplasm is obtained by the reaction (2) (oxaloacetate \rightarrow phosphoenolpyruvate + CO_2). This is a reaction in the cytoplasm. To move this reaction forward, it is necessary to expand the supply of oxaloacetate and reactivate phosphoenolpyruvate carboxykinase (PEPCK). Oxaloacetate is synthesized in the mitochondria through two reactions: a reaction that is caused by the catalysis of pyruvate carboxylase from pyruvate [reaction (1)], and a reaction that is caused by way of the tricarboxylic acid (TCA) cycle [reaction (4)]. The oxaloacetate synthesized in mitochondria is moved to the cytoplasm through the malate shuttle. Under acidotic conditions, pyruvate carboxylase activity is increased, the reaction (1) (pyruvate \rightarrow oxaloacetate) is enhanced. Under reduced glycolysis conditions, therefore, some GAAs that can be used for pyruvate synthesis are used, so the secretion of these GAAs into the urine may be decreased. To rotate the TCA cycle under these circumstances and increase the production of oxaloacetate, it is necessary to enhance the reaction (4) (α -ketoglutarate \rightarrow succinyl-CoA \rightarrow fumarate \rightarrow oxaloacetate). Since some GAAs that can be used for synthesizing each of these substances are used, the urinary excretion of these GAAs is sure to decrease. An important reaction for this α -ketoglutarate synthesis is "glutamine \rightarrow glutamate + ammonia (NH_3), glutamate \rightarrow α -ketoglutarate + NH_3 ." [reaction (5)] In acidosis, both the H^+ that is filtered, and the H^+ that is secreted, have increased. This increased NH_3 production accelerates the urinary excretion of H^+ because of the " H^+ + $\text{NH}_3 \rightarrow \text{NH}_4^+$ " reaction. Urinary pH is adjusted always appropriately by secreting ammonia into the urine.

male students who had undergone freshman health checkups at Tohoku University ($n = 3,651$), individuals whose BMI exceeded 30 kg/m^2 were used as the subjects for our study.

Definition of PUS (+) in the subjects

Of the subjects selected for this study, the ones in whom reddish-brown cloudy urine and pink sediments produced from centrifugation could be confirmed macroscopically and those whose UA crystals or urates could be confirmed microscopically were diagnosed as PUS (+). Subjects who presented no urinary abnormalities either macroscopically or microscopically, and in whom no UA crystals or urates could be confirmed microscopically were diagnosed as PUS (-). The precipitation of UA was confirmed microscopically in many obese subjects with low UpH values, although this could not be confirmed macroscopically. Such cases were excluded from the analysis.

Power analysis

An average difference in UpH of 0.2 is anticipated between uric acid-excreting [PUS (+)] and non-uric acid-excreting [PUS (-)] males (Ogawa et al. 2015b). We therefore conducted a power analysis using a standard deviation of 0.4, a power of 0.8, and a standardized effect volume of 0.5, and obtained the same number of samples for the [PUS (+)] group and [PUS (-)] groups ($n = 64$). Assuming a dropout ratio of 10%, we found that 70 subjects in each group would be required. Of the targeted subjects, we used the body mass index (BMI) as the exclusion factor and obtained 72 subjects in the PUS (+) group and 78 subjects in the PUS (-) group (total number of subjects = 150). We then additionally measured the urinary urea nitrogen (UUN), UUA, and urinary AA excretion (UAA) levels in all the subjects, and compared these values between the PUS (+) and the PUS (-) group.

This cross-sectional study targeted only healthy students who were not undergoing any kind of drug therapy. The evaluation criteria were as follows: age, gender, BMI, systolic blood pressure (SBP), diastolic blood pressure (DBP), UpH, heart rate (HR), UUN, UUA, and UAA. The AAs measured are presented in Table 1.

Measurements

In this study, we conducted investigations after minimizing the influence of age, gender, and BMI. Since UpH is vulnerable to fluctuations through the day, we used the urine taken early in the morning on an empty stomach after fasting for 12 hours, instead of spot urine.

The UpH was measured using an AX-4030 (ARKRAY, Inc., Kyoto, Japan), a fully automated urine analyzer with a preset test-paper. BUN and UUN were measured using the urease-GLDH/ICDH UV method. UUN was measured after eliminating ammonia by the ammonia elimination method. UA and UUA were measured using the uricase peroxidase method (Hitachi LABOSPECT 008).

For measuring the UAA, after the protein had been removed from the urine samples using 6% sulfosalicylic acid, the sample was subjected to derivatization using a MassTrak AAA reagent kit. This kit contains 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate and AQC dissolved in acetonitrile, which is then mixed with the sample with added borate buffer. We then measured the AA concentration using UltraPerformance Liquid Chromatography (MassTrak™ Amino Acid Analysis, column $2.1 \times 150 \text{ mm}$, ACQUITY UPLC® system, and Empower™ 2 software) (UV 260 nm).

This study complied with the Helsinki Declaration and was conducted with the approval of the Medical Ethics Committee of Tohoku University. All the participants provided their full informed

consent.

Statistical analysis

Since all the measurement values showed a normal distribution (Shapiro-Wilk test), they were recorded in the form of mean \pm standard deviation. Numerical figures between the PUS (+) and PUS (-) groups were compared by dispersion analysis (ANOVA on Ranks) using either the Student's t-test (for samples that showed equal variances) or the Kruskal-Wallis method (for samples that did not show equal variances). Single regression tests were conducted using Spearman's rank-order correlation. We also conducted multiple regression analysis using UpH as the dependent variable; factors that showed significant single correlation with UpH were used as the independent variables. Logistic regression analysis was also performed using the presence or absence of PUS as the dependent variable; UpH, age, BMI, SBP, DBP, and HR were used as the independent variables. In all cases, $p < 0.05$ was considered to indicate statistical significance.

Results

Compared with the PUS (-) group, the PUS (+) group showed lower values for UpH, UUN, UUA, and for the urinary excretion of aspartic acid, ornithine, proline, hydroxyproline, isoleucine, hydroxylysine, leucine, and glutamate (Table 1). In a logistic regression analysis conducted using the presence or absence of PUS as the dependent variable and UpH, age, BMI, SBP, DBP, and HR as the independent variables, only UpH was identified as an independent risk factor ($\beta = -0.1060567$, $p = 0.031$). In the logistic regression analysis conducted using PUS as the dependent variable and UpH, UUN, UUA, and the urinary excretion of aspartic acid, ornithine, proline, hydroxyproline, isoleucine, leucine, and glutamate as the independent variables, UUA ($\beta = -0.01$, $p = 0.032$) and the urinary excretion of proline ($\beta = -0.161$, $p = 0.035$), glutamate ($\beta = -0.143$, $p = 0.024$), and ornithine ($\beta = -0.178$, $p = 0.004$) were identified as independent risk factors. However, a decrease in UUA is caused by the precipitation of UUA. Therefore, this decrease in UUA appears to be the result of PUS, rather than its cause. We therefore eliminated UUA and again conducted logistic regression analysis. The following were identified as independent risk factors: UpH ($\beta = -1.428$, $p = 0.013$), and urinary excretion of hydroxyproline ($\beta = -0.145$, $p = 0.043$), proline ($\beta = -0.166$, $p = 0.027$), glutamate ($\beta = -0.0126$, $p = 0.039$), and ornithine ($\beta = -0.201$, $p = 0.001$).

Since it appears likely that PUS is clinically linked to UpH, we examined the correlation between UpH and other factors (Table 2). The results showed that UpH correlated positively with UUA and the excretion of glutamate, threonine, serine, glutamine, isoleucine, glycine, alanine, ornithine, valine, β -alanine, arginine, taurine, carnosine, aspartic acid, leucine, lysine, alpha-amino adipic acid, phenylalanine, citrulline, histidine, 1-methylhistidine, 3-methylhistidine, and alpha-aminobutyric acid, but correlated negatively with HR and SBP. Therefore, a multiple regression analysis using UpH as the dependent variable

Table 1. Comparison between the values of each factor in PUS (+) and PUS (-).

n		PUS (-)				PUS (+)			
		150		78		72		p	
UpH		5.86 ± 0.39		5.94 ± 0.36		5.78 ± 0.40		0.012413	
age	years	18.52 ± 1.40		18.55 ± 1.65		18.48 ± 1.07		0.776618	
BMI	kg/m ²	24.13 ± 4.10		24.07 ± 4.18		24.20 ± 4.04		0.843431	
SBP	mmHg	137.98 ± 17.31		136.05 ± 16.44		139.55 ± 18.09		0.156266	
DBP	mmHg	76.68 ± 12.59		75.15 ± 13.38		78.13 ± 11.54		0.122595	
HR	bpm	90.35 ± 16.13		88.26 ± 16.17		92.17 ± 15.89		0.098713	
UUN	mmol/g Cr	170.57 ± 49.68		180.10 ± 52.01		160.24 ± 45.13		0.013939	
UUA	μmol/g Cr	601.75 ± 428.82		747.55 ± 451.79		443.80 ± 340.43		< 0.00001	
taurine	μmol/g Cr	587.66 ± 398.91		610.78 ± 372.25		568.56 ± 427.14		0.461882	
phosphatidylethanolamines	μmol/g Cr	32.40 ± 12.28		33.20 ± 11.51		31.57 ± 13.08		0.408799	
aspartic acid	μmol/g Cr	11.01 ± 3.90		12.23 ± 4.19		9.72 ± 3.07		0.000041	
hydroxy proline	μmol/g Cr	7.98 ± 3.27		8.79 ± 3.33		7.12 ± 2.98		0.001258	
threonine	μmol/g Cr	92.69 ± 56.81		97.90 ± 58.18		87.62 ± 55.14		0.243621	
serine	μmol/g Cr	252.72 ± 98.00		264.82 ± 96.10		240.46 ± 99.01		0.115823	
asparagine	μmol/g Cr	76.15 ± 37.24		79.25 ± 33.65		73.25 ± 40.75		0.290431	
glutamate	μmol/g Cr	16.21 ± 4.43		16.90 ± 4.61		15.50 ± 4.14		0.047222	
glutamine	μmol/g Cr	286.97 ± 115.01		292.61 ± 113.63		282.01 ± 116.98		0.533569	
sarcosine	μmol/g Cr	ND		ND		ND		-	
alpha-amino adipic acid	μmol/g Cr	24.52 ± 13.79		25.25 ± 12.97		23.92 ± 14.68		0.502364	
proline	μmol/g Cr	14.35 ± 3.83		15.34 ± 4.37		13.31 ± 2.79		0.000847	
glycine	μmol/g Cr	610.86 ± 360.52		635.00 ± 317.17		584.69 ± 402.90		0.395139	
alanine	μmol/g Cr	160.80 ± 82.22		163.67 ± 79.80		159.09 ± 85.22		0.657018	
citrulline	μmol/g Cr	3.13 ± 2.27		3.35 ± 2.24		2.94 ± 2.30		0.265382	
alpha-amino butyric acid	μmol/g Cr	8.21 ± 3.59		7.75 ± 3.51		8.72 ± 3.64		0.107120	
valine	μmol/g Cr	25.18 ± 6.28		26.13 ± 6.24		24.21 ± 6.21		0.054684	
cystine	μmol/g Cr	41.03 ± 10.89		41.14 ± 9.54		41.01 ± 12.26		0.893554	
methionine	μmol/g Cr	6.32 ± 3.73		6.32 ± 3.18		6.34 ± 4.27		0.996254	
cystathionine	μmol/g Cr	12.27 ± 8.05		13.24 ± 8.19		11.23 ± 7.82		0.124624	
isoleucine	μmol/g Cr	9.54 ± 2.56		10.18 ± 2.64		8.88 ± 2.30		0.001337	
leucine	μmol/g Cr	23.75 ± 6.98		24.81 ± 7.32		22.67 ± 6.44		0.042363	
tyrosine	μmol/g Cr	56.52 ± 24.60		57.02 ± 25.36		56.21 ± 23.90		0.798594	
beta alanine	μmol/g Cr	25.11 ± 23.75		24.29 ± 12.56		26.24 ± 31.80		0.658776	
phenylalanine	μmol/g Cr	35.24 ± 10.51		36.55 ± 11.32		33.96 ± 9.43		0.112312	
beta-amino iso butyric acid	μmol/g Cr	346.53 ± 396.03		324.69 ± 392.83		373.49 ± 400.85		0.483926	
homocysteine	μmol/g Cr	ND		ND		ND		-	
gamma amino butyric acid	μmol/g Cr	ND		ND		ND		-	
m-ethanol amine	μmol/g Cr	276.04 ± 63.41		278.47 ± 67.70		272.86 ± 58.76		0.626787	
hydroxylysine	μmol/g Cr	10.93 ± 6.39		12.15 ± 6.61		9.69 ± 5.91		0.015117	
ornithine	μmol/g Cr	15.01 ± 6.91		17.65 ± 6.78		12.19 ± 5.87		< 0.00001	
1 methyl histidine	μmol/g Cr	461.39 ± 503.56		464.82 ± 514.95		462.83 ± 494.51		0.931188	
histidine	μmol/g Cr	648.85 ± 269.71		671.81 ± 286.51		626.11 ± 249.85		0.279325	
lysine	μmol/g Cr	127.09 ± 122.30		131.84 ± 103.71		122.14 ± 140.26		0.622278	
3-methyl histidine	μmol/g Cr	254.96 ± 53.31		260.75 ± 50.74		249.61 ± 55.65		0.167670	
tryptophan	μmol/g Cr	44.87 ± 16.68		45.06 ± 16.90		44.90 ± 16.56		0.888109	
anserine	μmol/g Cr	77.62 ± 49.55		77.15 ± 42.06		78.05 ± 56.16		0.945190	
carnosine	μmol/g Cr	22.17 ± 22.65		21.28 ± 17.80		23.31 ± 27.03		0.616475	
arginine	μmol/g Cr	18.73 ± 5.48		19.30 ± 4.38		18.20 ± 6.43		0.192728	
alloisoleucine	μmol/g Cr	ND		ND		ND		-	

PUS, pink urine syndrome (precipitation of uric acid in the urine.); UpH, urinary pH; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; UUN, urinary urea nitrogen; UUA, urinary uric acid; Cr, urinary creatinine excretion.

and ten factors (excluding UUA) starting from those with the smallest *p*-values as the dependent variables (from the above-mentioned glutamate to β -alanine; Table 3) was conducted, and only urinary glutamate excretion ($\beta = 0.0387$, *p*

< 0.0001) was identified as an independent risk factor.

Discussion

PUS appears to be a phenomenon in which the synthe-

Table 2. Correlations between UpH and various factors.

	UpH	p		UpH	p		UpH	p
age	-0.0242	0.7690	glycine	0.2813	0.0005	1 methyl histidine	0.1794	0.0281
BMI	-0.1574	0.0544	alanine	0.2793	0.0005	histidine	0.1804	0.0272
SBP	-0.1908	0.0194	citruiline	0.1928	0.0240	lysine	0.2058	0.0115
DBP	-0.0992	0.2273	alpha-amino butyric acid	0.1627	0.0466	3-methyl histidine	0.1714	0.0360
HR	-0.1950	0.0160	valine	0.2679	0.0009	tryptophan	0.0650	0.4291
UUN	0.1290	0.1158	cystine	0.1281	0.1182	anserine	0.1633	0.2166
UUA	0.3547	< 0.0001	methionine	0.1379	0.0924	carosine	0.2370	0.0035
taurine	0.2455	0.0026	cystathionine	0.1092	0.1849	arginine	0.2607	0.0013
phosphatidylethanolamines	-0.1555	0.0574	isoleucine	0.2895	0.0003	alloisoleucine	-	-
aspartic acid	0.2344	0.0039	leucine	0.2059	0.0115			
hydroxy proline	0.1029	0.2102	tyrosine	0.0956	0.2447			
threonine	0.3084	0.0001	b alanine	0.2651	0.0010			
serine	0.3226	0.0001	phenylalanine	0.1982	0.0150			
asparagine	0.4064	< 0.0001	beta-amino iso butyric acid	0.0073	0.9292			
glutamate	0.3884	< 0.0001	homocysteine	-	-			
glutamine	0.3028	0.0002	gamma amino butyric acid	-	-			
sarcosine	-	-	m-ethanol amine	0.0389	0.6363			
alpha-amino adipic acid	0.2003	0.0140	hydroxylysine	0.1275	0.1199			
proline	0.1194	0.1457	ornithine	0.2811	0.0005			

UpH, urinary pH; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; UUN, urinary urea nitrogen; UUA, urinary uric acid.

Table 3. Correlation coefficient between UpH and the factors which are related with UpH, starting from those with the smallest *p*-values.

	UpH	<i>p</i>		UpH	<i>p</i>
UUA	0.3547	< 0.0001	taurine	0.2455	0.0026
asparagine	0.4064	< 0.0001	carosine	0.2370	0.0035
glutamic acid	0.3884	< 0.0001	aspartic acid	0.2344	0.0039
threonine	0.3084	0.0001	lysine	0.2058	0.0115
serine	0.3226	0.0001	leucine	0.2059	0.0115
glutamine	0.3028	0.0002	alpha-amino adipic acid	0.2003	0.0140
isoleucine	0.2895	0.0003	phenylalanine	0.1982	0.0150
glycine	0.2813	0.0005	SBP	-0.1908	0.0194
alanine	0.2793	0.0005	citruiline	0.1928	0.0240
ornithine	0.2811	0.0005	histidine	0.1804	0.0272
valine	0.2679	0.0009	1 methyl histidine	0.1794	0.0281
beta alanine	0.2651	0.0010	3-methyl histidine	0.1714	0.0360
arginine	0.2607	0.0013	alpha-amino butyric acid	0.1627	0.0466

UpH, urinary pH; UUA, urinary uric acid; SBP, systolic blood pressure.

sis of NH₃ from glutamate decreases, UpH reduces, and UA precipitates into the urine. We therefore predicted that, in PUS, together with a drop in UpH, the amount of urinary glutamate excreted would increase. We indeed found that the amount of urinary glutamate excreted was decreased to such a level that it reduced UpH. In other words, although glutamate was being actively reabsorbed and used by the RPTs for decreasing the amount excreted into the urine, it was not being used for NH₃ synthesis. What, then, is this reabsorbed glutamate used for?

We observed that a reduction in UpH was strongly related to the increased OS (Ogawa et al. 2015a, b). When OS increases, the body invariably reacts in an attempt to counteract it. One such reaction is the nuclear factor-E2 p45-related factor (Nrf) 2-mediated OS response (Kwak et al. 2004; Sun et al. 2007). When OS increases, intracellular Nrf2 is activated and moves to within the nucleus to exert transcriptional activity. Nrf2 increases the synthesis of UA, lactic acid, and glutathione in an effort to eliminate OS (Mitsuishi et al. 2012). It is believed that, since glutamate

is used for this glutathione synthesis, the supply of glutamate for the NH_3 synthesis reaction is reduced, thereby slowing NH_3 synthesis (Fig. 2).

The next question that we aimed to answer was that if the glutamate in the RPTs is depleted, could the RPTs cope by increasing glutamate reabsorption? The rate of glutamate reabsorption in the RPTs is close to 100% to begin with; therefore, there is very little scope for further increase (around 1% at the most). Thus, there is a limit to the increased reabsorption that results from the increased demand for glutamate. Consequently, if the major share of the reabsorbed glutamate is consumed for glutathione synthesis, the amount of glutamate available for NH_3 synthesis is likely to decrease. Unfortunately, since we did not measure glutathione or urinary NH_4^+ in this study, we were unable to confirm any such changes in glutathione or reductions in NH_3 levels; these topics should be explored in

future studies. Urinary NH_4^+ is reported to decrease in association with diabetes, in which increased OS is observed (Maalouf et al. 2010). In our report, we also noted that UpH correlated negatively with urinary OS markers (Ogawa et al. 2015b).

It also appears unlikely that UA precipitation occurs due to decreased UpH levels alone, leading us to believe that an increase in UA synthesis and UUA excretion are involved in UA precipitation. In PUS, however, since UA is already precipitated into the urine, its UUA concentration is low, making it impossible to evaluate the level of UUA excretion. This, too, appears to be an obstacle that needs to be overcome in further studies. In our previous study targeting diabetes patients, we found that the serum UA concentration correlates negatively with UpH (Ogawa et al. 2015a).

Moreover, although lactic acid synthesis increases with

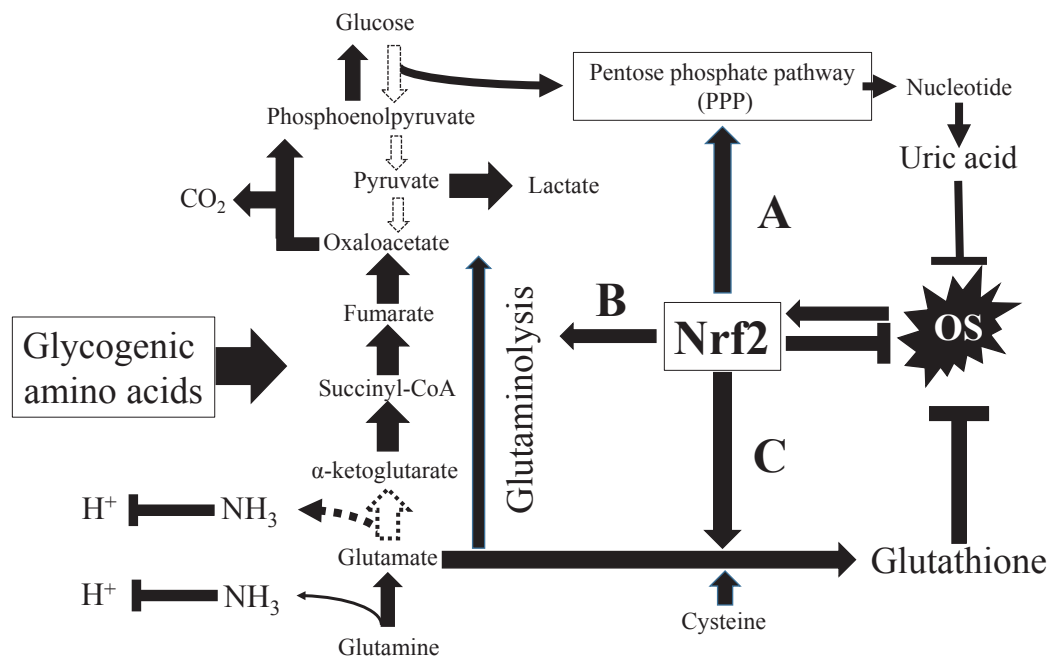


Fig. 2. Relations between synthesis of ammonia and Nrf2 and glucogenic amino acids.

If oxidative stress (OS) increases in the renal proximal tubules (RPT), nuclear factor-E2 p45-related factor (Nrf) 2 becomes reactivated to counteract such OS increases.

Nrf2 increases nucleic acid synthesis via the pentose phosphate pathway, increases the production of UA (A), promotes the synthesis of oxaloacetate from α -ketoglutarate (B), and increases the conversion of glutamate to glutathione (C).

If these actions of Nrf2 are taken into consideration, it becomes easier to explain the following:

A: increased uric acid (UA) production; Nrf2 also activates the pentose phosphate pathway, and increases the production of UA.

B: increased reabsorption of glucogenic amino acids (GAAs); Pink urine syndrome (PUS) (+) subjects showed a greater reduction in the amount of urinary excretion of some GAAs than PUS (-) subjects. This shows that many GAAs are reabsorbed in the RPT and are used actively. The GAAs are used as a substrate of oxaloacetate, fumarate and succinyl CoA syntheses. This reaction is activated by Nrf2 and the lactic acid is synthesized finally.

Nrf2 promotes the glutaminolysis "glutamate \rightarrow α -ketoglutarate \rightarrow succinyl-CoA \rightarrow fumarate \rightarrow oxaloacetate \rightarrow phosphoenolpyruvate \rightarrow pyruvate \rightarrow lactate" reaction, so large amounts of amino acids such as GAAs are consumed. As a result, the amount of GAAs excreted from the kidneys decreases even further.

C: reduced production of ammonia (NH_3) from glutamate and decrease in UpH. NH_3 synthesis is comprised of two reactions: the synthesis of glutamate from glutamine, and the synthesis of α -ketoglutarate from glutamate. As a result of this reactivation of Nrf2, glutamate, not glutamine, is used for glutathione synthesis, so production of NH_3 decreases, which in turn lowers the urinary pH. Under these conditions, the production of NH_3 is dependent on the synthesis of α -ketoglutarate from glutamate, so the relationship between urinary pH and urinary glutamate excretion becomes extremely strong.

the increased transcriptional activity of Nrf2, it is caused the increase in lactic acid synthesis caused by Nrf2. This lactic acid synthesis uses glutamine, glutamate, α -ketoglutarate, succinyl CoA, fumarate, and oxaloacetate as the substrates (Mitsuishi et al. 2012) (Fig. 2). However, in situations where, because of Nrf2 activation, glutamate is consumed for fuel glutathione synthesis and glucose use has also decreased (insulin resistance), these substrates are replaced by glucogenic amino acids (GAAs) (Krebs et al. 1965; Stumpf and Kraus 1977; Jans and Willem 1991; Atlante et al. 1998; Mitsuishi et al. 2012; Kazubek-Zemke et al. 2014) (Fig. 1). Therefore, a reduction in UpH shows a positive correlation with a reduction in the amount of urinary excretion of various kinds of GAAs. Thus, reduced NH_3 synthesis, which is attributable to a shortage of glutamate, is the direct cause of reduced UpH; however, a reduction in the urinary excretion of other GAA types is the result of a different Nrf2 reaction. Thus, in multiple regression analysis, only the amount of glutamate excreted in the urine can be confidently identified as an independent risk factor for UpH.

It is extremely easy to explain PUS as a group of phenomena that include (1) increased UA synthesis due to Nrf2 activation induced by increased OS in the RPTs, (2) increased use of GAAs associated with an increase in lactic acid synthesis, and (3) reduction in NH_3 synthesis caused by the depletion of glutamate that results from increased glutathione synthesis (Mitsuishi et al. 2012) (Figs. 1 and 2). However, in this study, we identified that, in PUS, even a reduction in UpH is strongly related to a reduction in urinary glutamate excretion and that a reduction in UpH is correlated with a reduction in the urinary excretion of some GAAs. In future studies on this subject, Nrf2 activity, glutathione levels, reabsorption rate of various AAs, urinary NH_4^+ , and blood lactic acid levels should also be measured.

In view of the above findings, it appears that PUS is a condition where OS increases in the RPTs due to obesity and other factors (Ogawa et al. 2015b), and where Nrf2 activity is increased to counteract such increased OS. A lower UpH itself increases OS in the RPTs (Souma et al. 2011), indicating a possibility that lowered UpH and increased OS in the RPTs may create a vicious cycle. In diabetes, moreover, a reduction in UpH is related to persistent renovascular disorders (Ogawa et al. 2015a). We believe that even the smallest reduction in UpH in obese and diabetic patients should be paid attention to.

Although PUS is observed in a large number of obese patients and cases with reduced UpH (Ogawa et al. 2015b), obesity and low UpH values are not essential for the development of PUS, neither do all obese cases necessarily show low UpH values or high UUA excretion (Strohmaier et al. 2012; Ogawa et al. 2015b). UA precipitation into the urine appears to occur as a result of a complex interaction between reduced UpH and increased urinary UA excretion. A reduction in UpH can occur either due to an increased supply of H^+ (acidosis) or a reduction in H^+ -eliminating

capabilities (reduced NH_3 synthesis); likewise, an increase in UUA excretion can occur either due to increased UA production or a reduction in UA reabsorption. In obese patients, several of these phenomena are likely to occur simultaneously (Strohmaier et al. 2012). Moreover, increased OS in the RPTs may occur not only in people with obesity but also in those with diabetes and renal diseases (Kazubek-Zemke et al. 2014; Ogawa et al. 2015b). PUS, therefore, is a phenomenon where the precipitation of UA into the urine is induced as a result of a complex combination of the effect of various factors *in vivo*.

In this study, we were unable to measure the urinary levels of NH_3 and NH_4^+ or the local production of CO_2 in the kidneys. We also did not evaluate any changes in arterial plasma levels, AV-differences, or renal metabolism of the likely precursors for renal ammoniogenesis. Furthermore, we did not consider the diet of the subjects. No arterial blood gas analysis was conducted either. This was a study conducted in conjunction with health checkups of university students; therefore, it was not possible to carry out all these investigations. Moreover, since the expression of Nrf2 in the RPT has not been evaluated, our discussions have not been scientifically validated. Further validations with animal experiments are required to confirm our findings.

Acknowledgments

The authors wish to thank Miss Asako Chiba and Mrs. Yasuko Sato for their assistance with gathering the urine samples for the study. We also wish to thank the nurses, Eiko Mitsui, Yoko Hasegawa, Michiko Ota, Megumi Ito, and Hiromi Sato, for their efforts in gathering student data. Our deep appreciation also goes to all the members of the staff who helped us in this study.

This work was supported by Tohoku University's Center for the Advancement of Higher Education President's Research Fund.

Author Contributions

S.O. wrote the manuscript and analyzed the data, J.T., M.S., K.N., and M.O. contributed to collection of the samples, the discussion, and data analysis. S.I. reviewed and edited the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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