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Metabolomics approach to serum biomarker for loperamide-induced constipation in SD rats

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Loperamide has long been known as an opioid-receptor agonist useful as a drug for treatment of diarrhea resulting from gastroenteritis or inflammatory bowel disease as well as to induce constipation. To determine and characterize putative biomarkers that can predict constipation induced by loperamide treatment, alteration of endogenous metabolites was measured in the serum of Sprague Dawley (SD) rats treated with loperamide for 3 days using ^{1}H nuclear magnetic resonance (^{1}H NMR) spectral data. The amounts and weights of stool and urine excretion were significantly lower in the loperamide-treated group than the No-treated group, while the thickness of the villus, crypt layer, and muscle layer was decreased in the transverse colon of the same group. The concentrations of aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and creatinine (Cr) were also slightly changed in the loperamide-treated group, although most of the serum components were maintained at a constant level. Furthermore, pattern recognition of endogenous metabolites showed completely separate clustering of the serum analysis parameters between the No-treated group and loperamide-treated group. Among 35 endogenous metabolites, four amino acids (alanine, glutamate, glutamine and glycine) and six endogenous metabolites (acetate, glucose, glycerol, lactate, succinate and taurine) were dramatically decreased in loperamide-treated SD rats. These results provide the first data pertaining to metabolic changes in SD rats with loperamide-induced constipation. Additionally, these findings correlate the changes in 10 metabolites with constipation.

Key words: loperamide, constipation, metabolomics, serum, transverse colon

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Loperamide is a synthetic piperidine derivative that is effective for the treatment of a variety of diarrhea syndromes, including acute, nonspecific (infectious) diarrhea, traveler's diarrhea, and chemotherapy-related and protease inhibitor associated diarrhea [1,2]. Loperamide is effective for the gut-directed symptoms of diarrhea in patients with painless diarrhea or diarrhea-predominant irritable bowel syndrome. Loperamide can also be used to induce an increase in anal sphincter tone, which may improve fecal continence in patients with and without diarrhea [2]. Loperamide is an opioid-receptor agonist that peripherally acts on the μ-opioid receptors located in the myenteric plexus of the large intestine, although it does not affect the central nervous system [3]. When loperamide binds opioid receptor, this complex transfers the signal to decrease the activity of the myenteric plexus, which subsequently decreases the tone of the longitudinal and circular smooth muscles of the intestinal wall. The low activity of the myenteric plexus enhances total stay time of substances in the intestine, allowing more water to be absorbed out of the fecal matter. Furthermore, this low activity leads to decreased

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movements of colonic mass and suppresses the gastrocolic reflex to improve diarrhea syndromes [4].

Although loperamide causes side effects related to its impact on bowel motility including abdominal pain, distention, bloating, nausea, vomiting, and constipation, it is generally well tolerated at recommended nonprescription doses [2]. Owing to these side effects, loperamide has been used to induce constipation in a variety of studies to determine the cause of constipation and identify novel compounds with therapeutic effects [5-7]. When used to treat animals, it can stimulate the extension of stool evacuation time and delay intestinal luminal transit through inhibition of water secretion [8] and smooth movement in the intestinal wall [9,10]. Although the dose and time for loperamide treatment have been found to vary among studies, constipation was successfully induced by treatment with 1.5-3 mg/kg body weight of loperamide for 3-7 days [5-7,11,12]. However, screening of metabolomics biomarkers that can anticipate the constipation induced by loperamide treatment has not been conducted to date. Generally, metabolomics analyze metabolomes changed in response to stressors or xenobiotics and then identify biomarkers [13,14]. This methodology has also been widely applied in preclinical studies investigating the toxicity, safety and efficacy of chemical compounds [15-17].

As part of the search for sensitive and reliable biomarkers of loperamide-induced constipation, this study was designed to comparatively compare serum biomarkers obtained from loperamide-treated SD rats with No-treated rats using the metabolomics-based proton (NMR) platform. The results indicated that the metabolomics profile of serum collected from loperamide-treated SD rats may provide useful information to develop novel sensitive and reliable biomarkers.

Materials and Methods

Care and use of animals

The animal protocol used in this study was reviewed and approved by the Pusan National University-Institutional Animal Care and Use Committee (PNU-IACUC; Approval Number PNU-2012-0010). Adult SD rats were purchased from SamTacho (Osan, Korea) and handled at the Pusan National University-Laboratory Animal Resources Center accredited by Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International (Accredited Unit Number-001525) in accordance with the United States of America (USA) National Institutes of Health (NIH) guidelines and the Korea Food and Drug Administration (Accredited Unit Number-00231) in accordance with the Laboratory Animals Act. All rats were provided with standard irradiated chow diet (Purina Mills, Seoungnam, Korea) *ad libitum* and maintained in a specific pathogen-free state under a strict light cycle (lights on at 06:00 h and off at 18:00 h) at a temperature of 22±2°C and a relative humidity of 50±10%.

Induction of constipation

Constipation was induced in SD rats by subcutaneous injection of loperamide (1.5 mg/kg weight) in 0.9% sodium chloride twice a day (9:00 AM, 6:00 PM) for 3 days, whereas the non-constipation group was injected with 0.9% sodium chloride alone [5]. For the animal experiment, 8-week-old SD rats (n=6-10) were assigned to either a non-constipation group (loperamide-treated group, n=3-5) or a constipation group (No-treated group, n=3-5). At 72 h after loperamide treatment, all animals were sacrificed using CO_2 gas until assay.

Measurement of stool parameters and urinary volume

All SD rats were bred in metabolic cages during the experimental period to avoid contamination of samples. The stools and urine excreted from each SD rat were collected at 10:00 am. Stool weight was weighed three times per sample using an electric balance, whereas the water content was determined as the difference between the wet and dry weights of the stool as previously described [5,6]. Changes in the urine volume were measured three times per sample using a cylinder.

Histological analysis

Transverse colons collected from SD rats were fixed with 10% formalin for 48 h, embedded in paraffin wax, and then sectioned into 4 μm thick slices that were stained with hematoxylin and eosin (H&E, Sigma-Aldrich, MO, USA). Morphological features of these sections were observed under light microscopy, after which the length of the villus layer, thickness of crypt and muscle layer, diameter of crypt, and number of crypts were measured using Leica Application Suite (Leica Microsystems, Switzerland).

Serum biochemical analysis

For serum biochemical analysis, blood was collected

from abdominal veins of rats that were fasted for 8 hr and incubated for 30 min at room temperature. Serum was then obtained by centrifugation of blood. The serum alkaline phosphatase (ALP), alanine aminotransferase (ALT), AST, LDH, blood urea nitrogen (BUN), and Cr levels were subsequently assayed using an automatic serum analyzer (HITACHI 747, Tokyo, Japan). All assays were measured using fresh serum and conducted in duplicate.

Metabolomics analysis

Appropriate serum samples (300 μL) obtained from animals were placed in micro centrifuge tubes containing 300 μL D_2O solutions with 4 mM TSP as a qualitative standard for the chemical shift scale. After vortexing, serum samples were analyzed by NMR spectrometry within 48 h. All spectra were determined using a Varian Unity Inova 600 MHz spectrometer operating at a temperature of 26°C supported by Pusan National University (Busan, Korea). The one-dimensional NMR spectra were acquired with the following acquisition parameters: spectral width 24,038.5 Hz, 7.55 min acquisition time, and 128 nt. Additional conditions of a relaxation delay time of 1 s and saturation power of 4 were set to suppress a massive water peak. NMR spectra were reduced to data using the program Chenomx NMR Suite (ver 4.6, Chenomx Inc., Edmonton, Alberta, Canada). The spectral region of δ 0.0-10.0, excluding the water peak (δ 4.5-5.0), was segmented into regions of 0.04 ppm, which provided 250 integrated regions in each NMR spectrum. This binning process endowed each segment with integral values, giving an intensity distribution of the whole spectrum with 250 variables prior to pattern recognition analysis.

All data were converted from the NMR suite Professional software format into Microsoft Excel format (*.xls). One-dimensional NMR spectra data were imported into the SIMCA-P (version 12.0, Umetrics Inc., Kinnelon, NJ, USA) for multivariate statistical analysis to examine the intrinsic variation in the data set. These data were scaled using centered scaling prior to principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA). With the scaling process, the average value of each variable is calculated and then subtracted from the data. Score plots of PCA and PLS-DA were used to interpret the intrinsic variation of the data. Variable importance plots (VIP) were also utilized to select putative metabolites related to loperamide.

Table 1. Alteration of stool and urine secretion in loperamide-induced constipated SD rats

Category	No-treated group	Loperamide-treated group
Stool Number	35.8±4.2	18.2±3.1*
Stool Weight (g)	4.1±0.5	2.5±0.4*
Water content (%)	53.0±5.9	28.9±8.6*
Urine Volume (ml/day)	13.7±0.8	10.0±0.7*

Data represent the mean±SD from three replicates. *P<0.05 compared to the No-treated group.

Statistical analysis

One-way ANOVA (SPSS for Windows, Release 10.10, Standard Version; SPSS, Chicago, IL, USA) was used to identify significant differences between No- and loperamide-treated SD rats. All values are reported as the mean±SD. A P value of <0.05 was considered significant.

Results

Induction of constipation

Constipation is generally determined based on altered excretion from laboratory animals. To investigate whether loperamide treatment could induce constipation, alterations in excretion parameters including stool weight and urine volume were measured in No- and loperamide-treated SD rats. Excretion volumes of stool and urine were significantly reduced after administration of loperamide compared to the No-treated group. Indeed, the weight of stool in the loperamide-treated group was approximately twice that of the No-treated group. Furthermore, the water content of stool in the loperamide-treated group was roughly 45-55% lower than that in the No-treated group (Table 1). Therefore, these results suggest that loperamide treatment could successfully induce constipation in SD rats through inhibition of stool and urine excretion.

Histological alteration of the transverse colon

To investigate the effects of loperamide treatment on the histological structure of the transverse colon, the villus length, crypt layer thickness, and muscle thickness were measured in transverse colons of SD rats following H&E staining. The average length of the villus layer was significantly shorter in the loperamide-treated group than the No-treated group. The loperamide-treated group also showed greatly reduced crypt layer thickness and muscle thickness relative to those in the No-treated group. Furthermore, the number of crypts per restrict area as

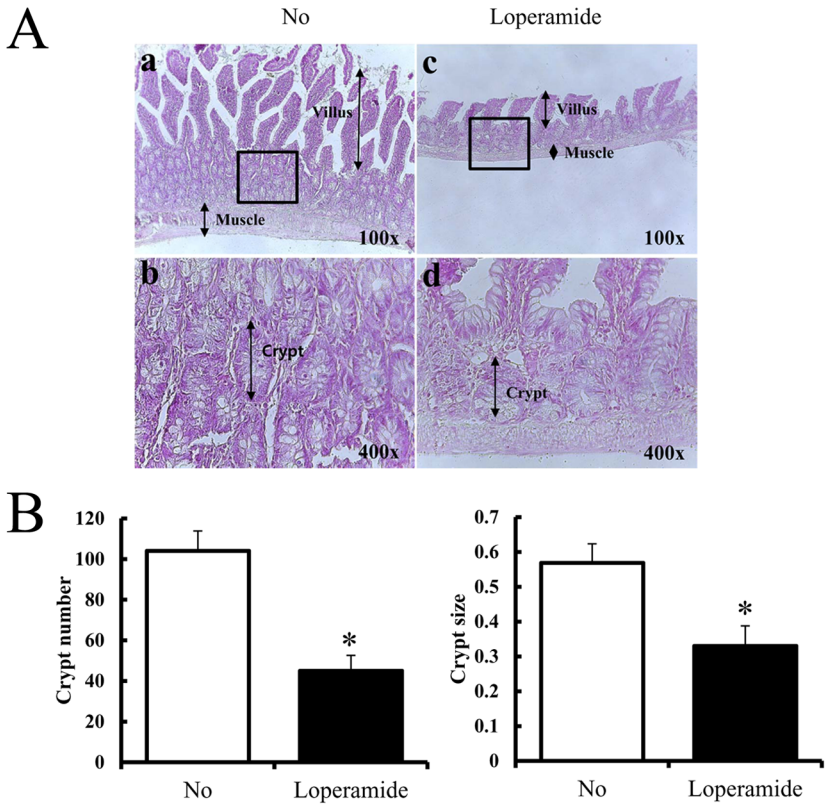

Figure 1. Effects of loperamide treatment on histological parameters in the transverse colon of SD rats. (A) H&E-stained sections of transverse colons collected from No-treated rats (a and b) andloperamide-treated rats (c and d) were observed at two different magnifications using a light microscope. (B) The crypt number per specific area and diameter per crypt was measured with Leica Application Suite. Five to six rats per group were assayed in triplicate by H&E. Data represent the mean±SD from three replicates. **P*<0.05 compared to the No-treated group.

well as diameter of the crypt was decreased after loperamide treatment (Figure 1). These results indicated that loperamide treatment may induce constipation-like phenotypes including short villus length, decreased crypt layer and muscle layer, and a small number of crypts in the transverse colon of SD rats.

Effects of loperamide on serum biochemical components

To investigate whether loperamide treatment could affect the serum biochemical components, alteration of several components related to liver and kidney metabolism was investigated in blood serum using serum biochemical analysis. Liver toxicity analysis revealed no differences in the concentrations of two liver toxicity indicators, ALT and LDH, between the No-treated group and loperamide-treated group. However, the concentration of ALP and AST was slightly increased in the loperamide-treated group than that of the No-treated group (Table 2). In the case of kidney toxicity analysis, similar patterns to those observed upon liver toxicity analysis were found for the BUN and Cr concentrations. The concentration of BUN in serum did not differ significantly among rats, but that of Cr was lower in the loperamide-treated group than the No-treated group (Table 2). Therefore, these results suggest that loperamide treatment may induce some toxicity in the liver and kidneys of SD rats.

Table 2. Serum biochemical analysis of loperamide-induced constipated SD rats

	No-treated group (mg/dL)	Loperamide-treated group (mg/dL)
BUN	23.6±1.81	24.2±2.21
Cr	0.7±0.07	0.4±0.12*
ALP	346.8±41.93	775.2±186.94*
ALT	25.4±2.60	29.8±4.86
AST	54.6±5.68	67.8±4.65*
LDH	150.6±12.12	154.6±14.38

Data represent the mean±SD of three replicates. **P*<0.05 compared to the No-treated group.

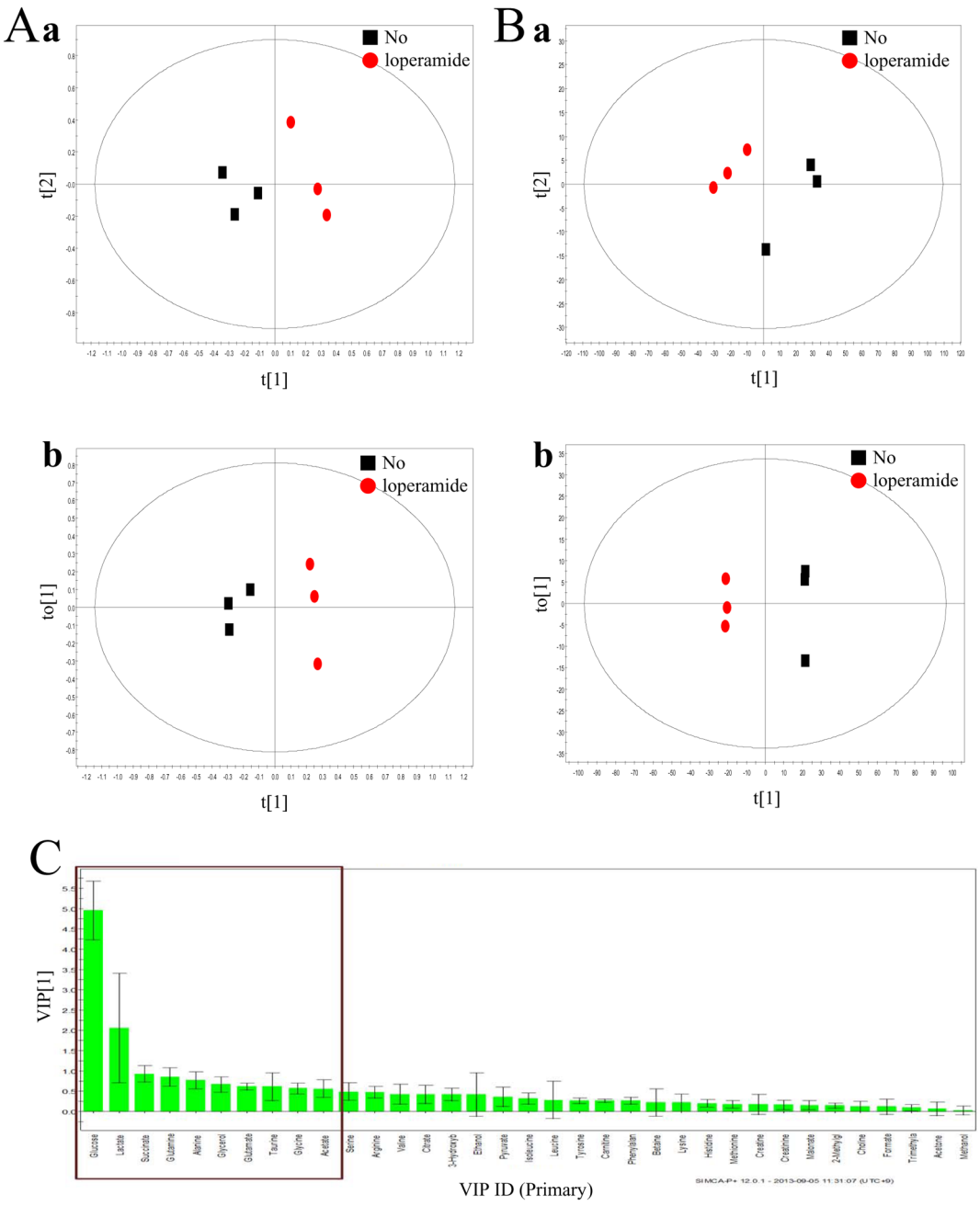

Figure 2. Metabolomics pattern recognition using PCA (a) and OPLS-DA (b). (A) Global profiling of loperamide treatment in the serum samples. (B) Targeted profiling of loperamide treatment in the serum samples. (C) The VIP showed the major metabolites contributing to cluster separation.

Effects of loperamide treatment on endogenous metabolites in serum

Upon NMR analysis of serum, pattern recognition using PCA and OPLS-DA of the NMR spectra in global profiling revealed clustering between the No-treated and loperamide treated groups (Figure 2A). Chenomx NMR Suite (ver. 4.6, Chenomx Inc., Edmonton, Alberta, Canada) was used for targeted NMR spectral analysis. A total of 35 metabolites were determined, and PCA and OPLS-DA score plots differed between the No-treatment and loperamide treatment groups (Figure 2B). Based on these results, endogenous metabolites were selected to investigate the effects of loperamide treatment using VIP. The threshold for meaningful contributions to identify significantly influential metabolites was established as VIP >0.5 (Figure 2C). Among 35 endogenous

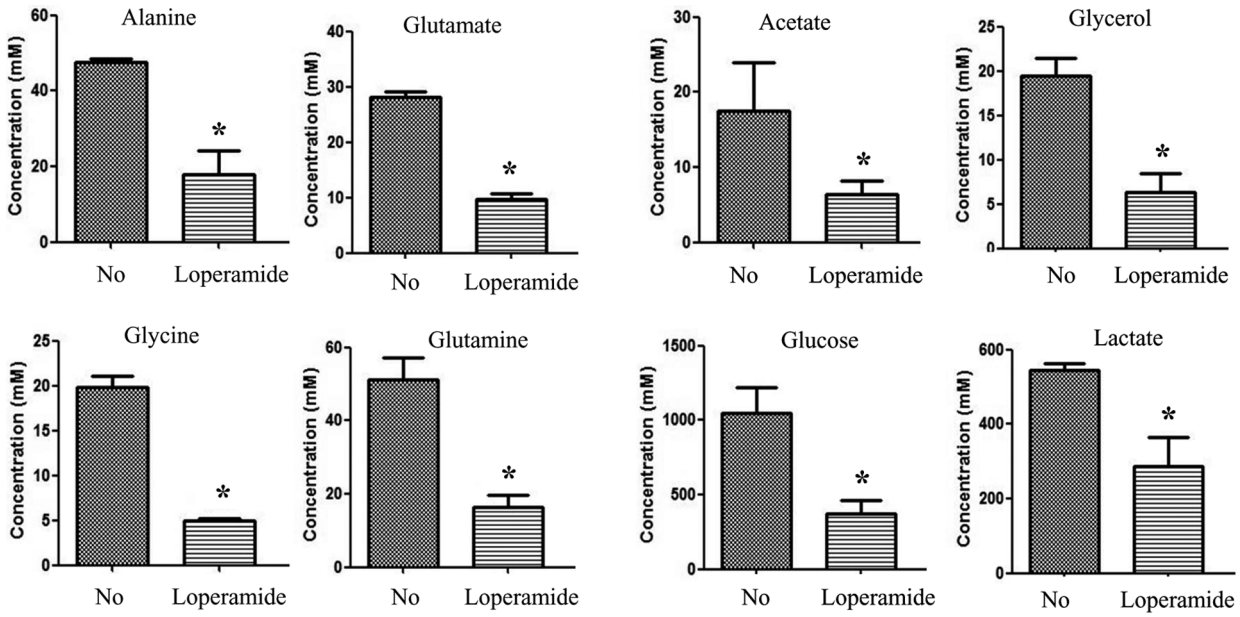

Figure 3. Concentration of four amino acids after loperamide administration in SD rats. Data represent the mean±SD from three replicates. *P<0.05 compared to the No-treated group.

Figure 4. Concentration of six endogenous metabolites after loperamide administration in SD rats. Data represent the mean±SD from three replicates. *P<0.05 compared to the No-treated group.

metabolites, ten metabolites were selected as putative candidates: acetate, alanine, glucose, glutamate, glutamine, glycerol, glycine, lactate, succinate, taurine (Figure 3 and 4). These metabolites were categorized into two major groups, an amino acid group containing alanine, glutamine, glutamate and glycine, and a metabolite group containing acetate, glucose, glycerol, lactate, succinate and taurine. In the serum sample of loperamide-treated rats, the concentrations of the ten metabolites were significantly decreased relative to those of No-treated rats. The highest decrease of the concentration was observed in glycine, followed succinate, taurine, glycerol and glutamine. Most metabolites in the loperamide-treated rats were roughly 2-3 times lower than those in the No-treated group (Figure 3 and 4). Therefore, these results suggested that loperamide treatment could cause a decrease of endogenous metabolites relative to the controls.

Discussion

Constipation is a chronic gastrointestinal disorder characterized by symptoms such as infrequent bowel movements, difficulty during defecation, and sensation of incomplete bowel evacuation that affects almost 25% of the western population [18-21]. Generally, chronic constipation is categorized into three groups by assessment of colonic transit and anorectal function, normal transit or irritable bowel syndrome, pelvic floor dysfunction (functional defecatory disorders) and slow transit constipation [22]. Among patients with chronic constipation, the most prevalent form is normal transit (59%), followed by functional defecatory disorders (25%), slow transit (13%) and a combination of defecatory disorders and slow transit (3%) [23]. This disorder is often caused by insufficient dietary fiber intake, inadequate fluid intake, decreased physical activity, side effects of medication, hypothyroidism, and obstruction by colorectal cancer [24]. Constipation can also be induced as side effects of the administration of drugs applied to treat clinical diseases [6,7]. In this study, we selected loperamide to induce constipation and observed the human-like symptoms of constipation in SD rats injected with 4 mg/kg of loperamide without

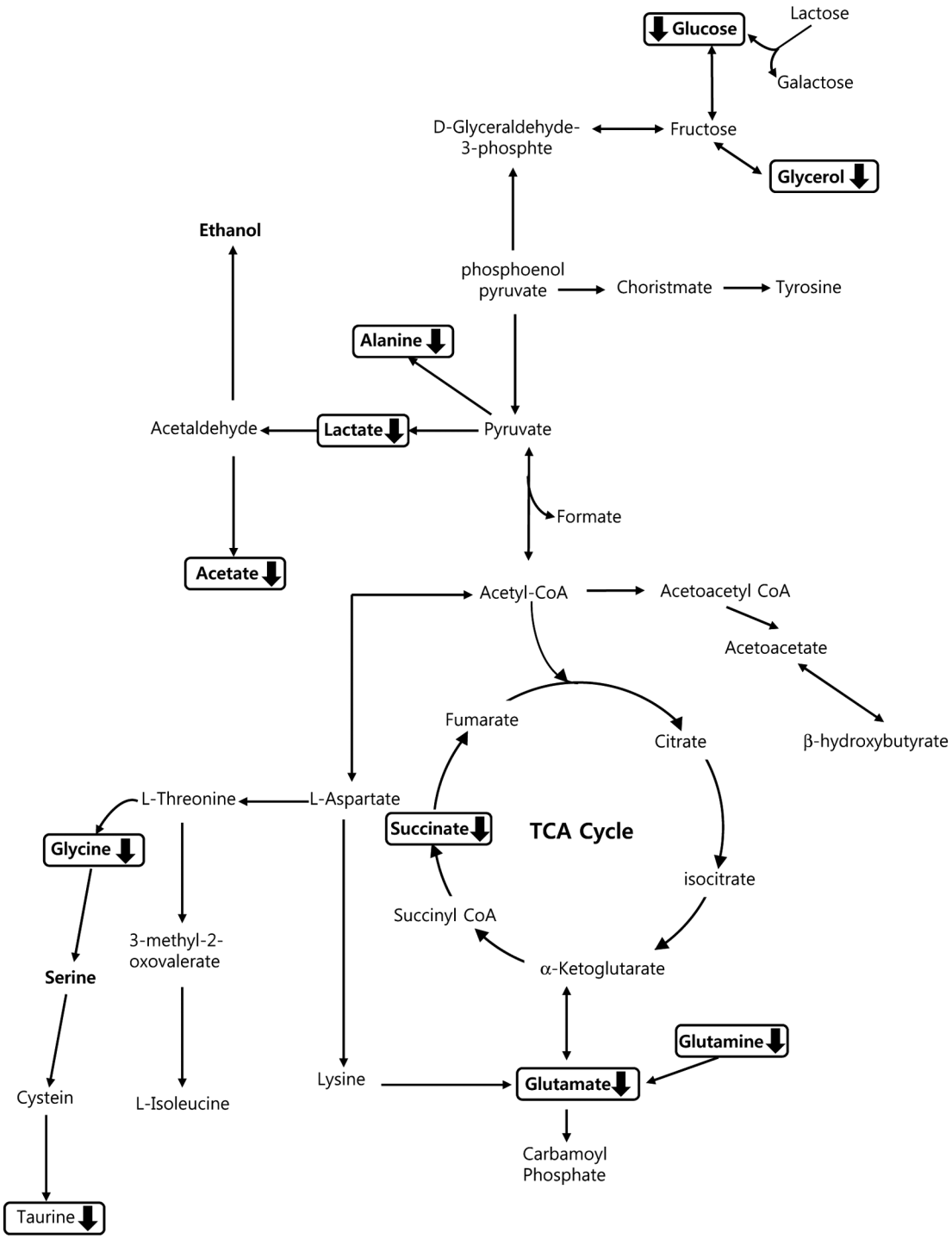

Figure 5. Pathway related to endogenous metabolites that were changed after loperamide treatment. Bold arrows indicate the alteration of metabolites, while the box shows the altered metabolites.

any specific problems. However, the rat model of pharmacological constipation induced by loperamide treatment can only reflect slow transit constipation, which is characterized by less daily fecal excretion, lower water content, lower numbers of fecal pellets and thinner fecal mucus [11,25,26]. Therefore, metabolomics profiles obtained from the loperamide-induced constipation cannot be applied to all forms of chronic constipation detected in human patients.

In loperamide-induced rats, a significant reduction of

fecal excretion and histological structure is considered one of the key markers of constipation in most studies. Administration of loperamide dramatically induced a decrease of stool-related factors such as pellet number, weight, and water content [5,6,11,27]. Furthermore, the average thickness of the distal colon layer and mucus layer is thinner in loperamide-treated rats than in control rats [6,25]. In the present study, a similar effect on stool-related factors and histological structure was observed in loperamide-treated rats (Figure 1 and 2).

Only a few studies have investigated the metabolomics profiles associated with constipation. Rodriguez et al. [28] examined the metabolic profile before and after a meal challenge in a cohort of children with constipation and determined its relationship with postprandial colon motility patterns to identify metabolic targets for treatment of constipation. Of 187 metabolites, 16 amino acid and 22 lipid metabolites had changed significantly in the postprandial group. Some of the metabolites including methylhistamine, histamine and Gamma-Amino Butyric Acid (GABA) were decreased in the same group at 60 min after the meal. A significant correlation between normal and abnormal postprandial motility pattern was observed in specific metabolites including glycerol, carnosine, alanine, asparagine, cytosine, choline, phosphocholine, thyroxine and triiodothyronine [28]. In this study, most metabolites were significantly decreased in SD rats treated with loperamide relative to the No-treated group. These findings are very different from those of previous results in which the level of most metabolites was increased in the postprandial group with constipation. However, glycerol showed a similar pattern in both studies. The level of glycerol decreased in a time-dependent manner from 14 to 56% in a study of children with constipation, while their level decreased by 3 times in loperamide-induced constipation rats. Therefore, this concomitant feature demonstrates the possibility that glycerol is tightly correlated with the phenotypes of constipation in mammals (Figure 5). And also TCA cycle is expected to be down-regulated when succinate concentration is decreased in constipation-induced rats (Figure 5).

Overall, this is the first study to examine the metabolic changes in SD rats treated with loperamide and to correlate changes in specific metabolites with loperamide-induced constipation. In addition, the results presented herein provide evidence that glycerol may be considered a biomarker for prediction of constipation induced by loperamide treatment.

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