early phase of virus infection, e.g., at 1 dpi, but return to normal levels within a few days in a cell culture system. On the other hand, the virus-infection-induced expression of other genes, such as the extracellular signal-regulated kinase (ERK) gene, remains for a prolonged period of time (data not shown). Also, some of the gene products induced in the acute phase may suppress the expression of other genes. Under these balanced conditions, it is quite possible that certain genes are induced only at a later time, e.g., 3 to 5 dpi, but not immediately after virus infection.

It was reported previously that HCV core protein-expressing transgenic mice exhibit marked insulin resistance by inhibiting IRS-1 tyrosine phosphorylation and Akt phosphorylation (45, 58). However, our present results showed that HCV NS5A, but not the core protein, was associated with increased gluconeogenesis. Moreover, it was recently reported that HCV infection significantly inhibited cellular glucose levels at 10 dpi (69), which is quite the opposite of what we observed in the present study. These results collectively suggest the possibility that multiple pathways are involved in glucose metabolism in HCV-infected cells. Also, the possible effect(s) of the dysregulation of hepatic gluconeogenesis on the HCV life cycle needs to be clarified.

In conclusion, our present results collectively suggest that HCV promotes hepatic gluconeogenesis, resulting in increased glucose production in hepatocytes via an NS5A-mediated, FoxO1-dependent pathway.

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Use of serum and urine metabolome analysis for the detection of metabolic changes in patients with stage 1-2 chronic kidney disease

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ABSTRACT

Background: Chronic kidney disease (CKD) is a major health problem throughout the world, and understanding the pathological condition of CKD has become increasingly important. The recent development of advanced metabolomic assay techniques now allows the human metabolic condition to be evaluated sensitively and comprehensively.
Objectives: The aim of this study was to use metabolomic analysis to perform a preliminary survey of metabolic changes occurring in patients with stage 1-2 CKD.
Patients and Methods: Serum and urine metabolomic profiles of 15 patients with stage 1-2 CKD were analyzed using our previously reported capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) systems, and compared to 7 healthy volunteers.
Results: The CE-TOFMS systems in three different modes for cation, anion, and nucleotide analyses detected multiple metabolites in serum and urine samples. In cation analysis mode, several increases in nonessential amino acids were identified in patients with stage 1-2 CKD, similar to those reported for end-stage renal disease (ESRD). Free-radical scavengers carnosine and hypotaurine were decreased in the urine, whereas serum hypotaurine and taurine were increased, consistent with changes in renal and/or systemic oxidative stress. Moreover, the cardiotoxin hypoxanthine was markedly increased in the serum, whereas serum and urine adenosine and urine guanine were decreased, suggesting changes in purine nucleotide metabolism which could affect cardiovascular prognosis. Changes in other unidentified metabolites were also detected.
Conclusions: These results suggest that multiple changes in the metabolism are already detectable in stage 1-2 CKD using metabolome analysis. Further studies on these metabolic changes may result in new strategies to prevent cardiovascular events and progression to ESRD in patients with CKD.

▸ *Implication for health policy/practice/research/medical education:*
The results of this study suggest that multiple changes in the metabolism are already detectable in stage 1-2 CKD, and these changes may be detected using metabolome analysis.

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1. Background

It has been reported that the prevalence of chronic kidney disease (CKD) has increased to over 10% (1), and is now

a major health problem throughout the world. A number of co-morbidities including cardiovascular diseases are associated with CKD and prognosis is poor, with many patients experiencing disease progression (2). CKD is not only a strong risk factor for cardiovascular diseases (3, 4), but also the precursor of end stage renal failure (ESRD, also known as CKD stage 5), which needs renal replacement therapy such as dialysis. Previous studies using ion-exchange chromatography or HPLC have identified several changes in amino acid metabolism in patients with ESRD (5-8), but a survey of metabolomic changes has not been reported. Metabolomics is a discipline dedicated to the global study of metabolites, their dynamics, composition, interactions, and responses to interventions or to changes in their environment (9). The recent developments of advanced metabolomic assay techniques now allow the human metabolic condition to be evaluated sensitively and comprehensively. Metabolomics has already been reported to be effective for the discovery of biomarkers for disease diagnosis, such as cancer (10, 11) and cardiovascular diseases (12), but its effectiveness for the assessment of renal physiology and kidney disease is still uncertain. Metabolomics may be a useful tool for analyzing the condition of CKD, because CKD is recognized to be a disease affecting multiple biochemical pathways, and thus may cause multiple changes in systemic metabolism. Moreover, CKD is a strong risk factor for cardiovascular disease, which is highly correlated with metabolic changes. Finally, blood and urine examinations are non-invasive procedures, and due to the fact that they identify changes in both systemic and renal metabolism, may be particularly useful for the non-invasive assessment of patients with CKD.

2. Objectives

Because of the potential importance of understanding metabolic changes in patients with CKD, the aim of this study was to examine the serum and urine metabolites of patients with stage 1-2 CKD, using our recently developed CE-TOFMS system (13), and to compare the results to healthy volunteers, in order to see if metabolic changes can be detected at an early stage in CKD.

3. Materials and Methods

3.1. Patient recruitment and sample collection

This study followed the ethical standards of the Helsinki Declaration and was approved by the Ethics Committee of Keio University. Informed consent was obtained from each participant. A total of fifteen patients with stage 1-2 CKD who were admitted to Keio University Hospital, Tokyo, Japan from January 2008 to March 2009 for kidney biopsy were enrolled in this study. Seven healthy volunteers (5 male and 2 female, with no medical problems including urine abnormalities) participated in this study as controls. CKD was defined according to the criteria of the KDIGO group based on the K/DOQI clinical practice guidelines for CKD (3). In accordance with these criteria, stage 1 CKD was defined as CKD with normal or increased GFR (≥ 90 mL/min/1.73 m 2), and stage 2 CKD was defined as CKD with a mild decrease in GFR (60-89 mL/min/1.73 m

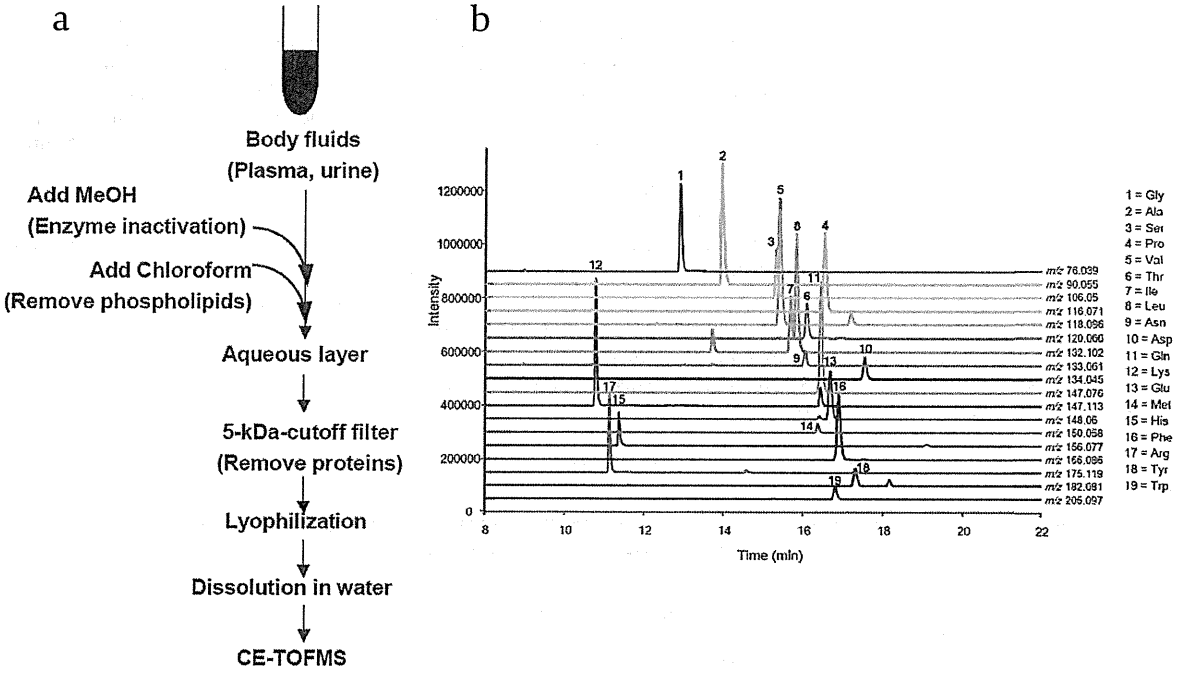

Figure 1. (a) Schematic representation of metabolite extraction method prior to CE-TOFMS analysis. (b) Representative electropherogram of CE-TOFMS.

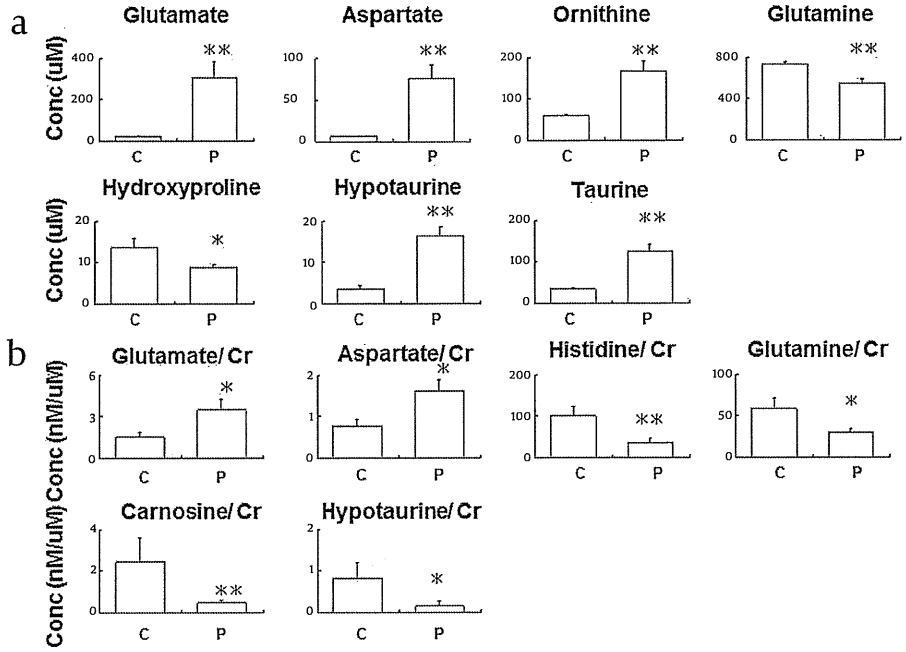

Figure 2. Changes in amino acid metabolites in (a) serum and (b) urine of patients with stage 1-2 CKD. C: Control, P: CKD patients. *, **: $p < 0.05$, $p < 0.01$ vs. controls.

2) (14). Because these patients were candidates for renal biopsy, the presence of proteinuria (≥ 0.5 g/g creatinine) had been checked on at least 3 separate occasions during a period of over 3 months. Moreover, the kidney biopsies were checked in all the patients to confirm that there were no false positive or false negatives in the diagnosis of CKD. Blood and urine samples for metabolomic studies were obtained after an overnight fast. The protocol for urine collection was to void the bladder at bedtime, then to obtain a mid-stream sample from the first morning sample. Samples were then centrifuged without delay at 3000 rpm for 10 min at 4 °C, and the supernatant was stored at - 80 oC until extraction and assay. Values of serum chemistries were obtained using standard hospital laboratory techniques, and GFR was calculated from the age and serum creatinine concentrations.

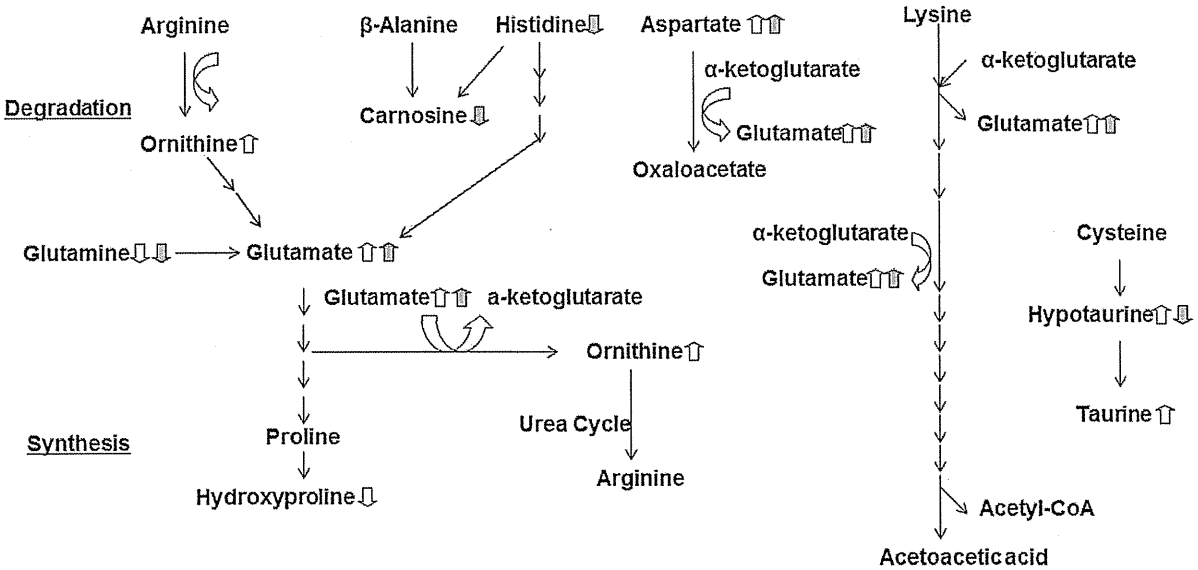

Figure 3. Observed metabolite changes in the serum and urine of patients with stage 1-2 CKD mapped onto the pathways involved in amino acid degradation and synthesis. Open arrows represent significant increases or decreases in serum samples, and closed arrows represent significant changes in urine samples.

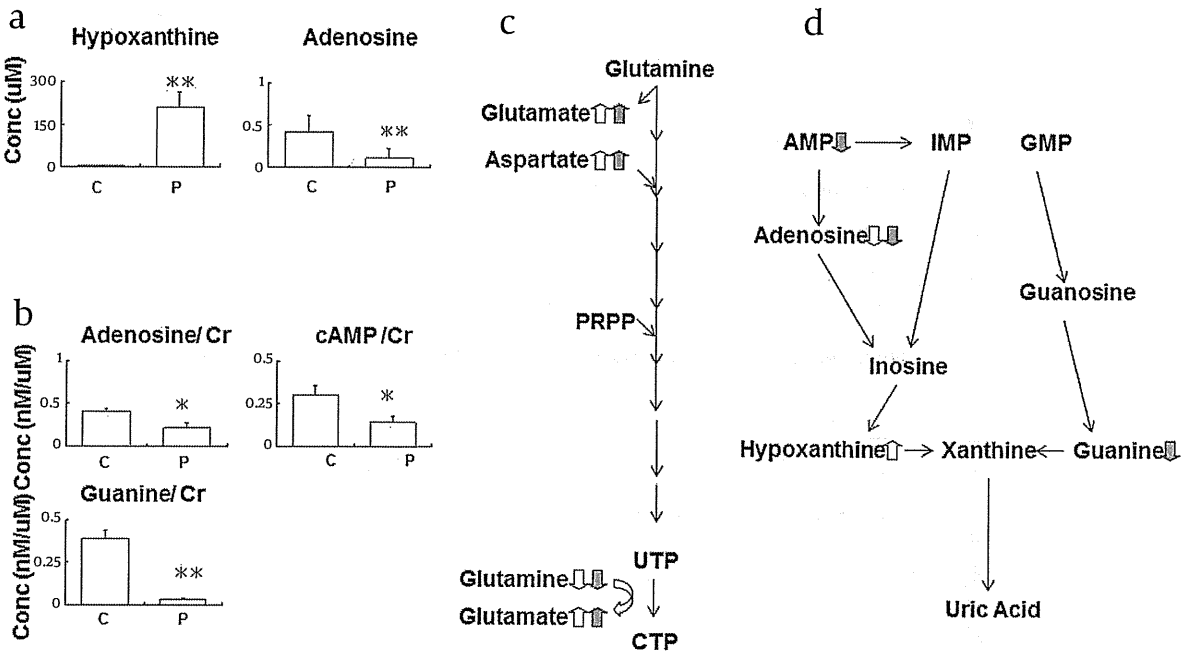

Figure 4. Changes in nucleic acid metabolites in (a) serum and (b) urine of patients with stage 1-2 CKD. Observed metabolite changes mapped onto the pathways involved in (c) pyrimidine nucleotide synthesis and (d) purine nucleotide degradation. Open arrows represent significant increases or decreases in serum samples, and closed arrows represent significant changes in urine samples. *, **: $p < 0.05$, $p < 0.01$ vs. controls.

3.2. Metabolites Extraction

Serum or urine samples (100 ul) were added to methanol (900 ul) containing internal standards (20 uM each of methionine sulfone, MES, and D-Camphol-10-sulfonic acid) and mixed to inactive enzymes. After adding deionized water (400 ul) and chloroform (1 ml), the solution was centrifuged at 4600 g for 5 min at 4 °C and the 300 ul upper aqueous layer was filtered through a Millipore 5-kDa cutoff centrifuge filter to remove proteins. The filtrate was lyophilized and dissolved in 50 ul of Milli-Q water containing reference compounds (200 µl each of 3-aminopyrorridine and trimesate) prior to capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) analysis. Samples from patients and controls were prepared and quantified simultaneously to avoid inter-assay variations *(Figure 1)*.

3.3. CE-TOFMS Conditions for Cationic Metabolite Analysis

The instrumentation and measurement conditions of CE-TOFMS are described elsewhere (13, 15, 16). Separations were carried out in a fused silica capillary (50 µm inner diameter x 100 cm total length) filled with 1M formate as the electrolyte. Approximately 3 nl of sample solution were injected at 50 mbar for 3 sec, and 30 kV of Voltage was applied. The capillary was maintained at 20 °C, and the sample tray was cooled below 5 oC. Methanol water (50 % v/v) containing 0.1 µM Hexakis (2,2-difluorothoxy) phosphazene was delivered as the sheath liquid at 10 µl/min.

3.4. CE-TOFMS Conditions for Anionic Metabolite Analysis

A cationic polymer-coated COSMO (+) capillary was used as the separation capillary. A 50mM ammonium acetate solution (pH 8.5) was used as electrolyte solution for CE separation. Sample solution (30 nl) was injected at 50 mbar for 30 s and -30 kV of voltage was applied. Ammonium acetate (5 mM) in 50 % methanol-water (v/v) containing 0.1 µM Hexakis (2, 2-difluorothoxy) phosphazene was delivered as the sheath liquid at 10 µl/min.

3.5. CE-TOFMS Conditions for Nucleotide-related Metabolite Analysis

Separations were carried out in a fused silica capillary filled with 50 mM ammonium acetate (pH 7.5). ESI-TOFMS was operated in the negative ion mode, and the capillary voltage was set at 3500 V. A flow rate of heated dry nitrogen gas (heater temperature 300 °C) was maintained at 7 l/min. Other conditions were identical to those used in anionic metabolite analysis.

3.6. Data processing and statistical analysis

Raw data were analyzed with our proprietary software named MasterHands-1.0.6.16 and JDAMP-128, as described previously (13, 15). For each urine sample, the measured metabolite concentrations were normalized using concentration of creatinine to obtain the amount of metabolite contained (nmol) per creatinine level (µmol) of each sample. Statistical comparisons were made by

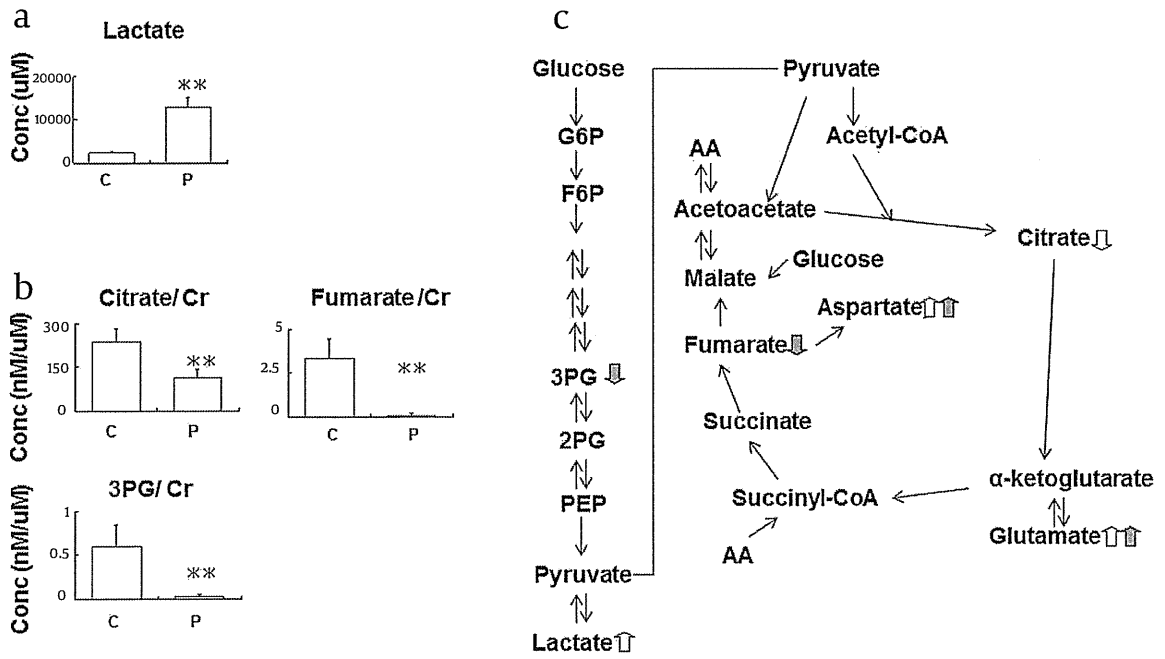

Figure 5. Changes in carbohydrate metabolites in (a) serum and (b) urine of patients with stage 1-2 CKD. (c) Observed metabolite changes mapped onto carbohydrate metabolic pathways. Open arrows represent significant increases or decreases in serum samples, and closed arrows represent significant changes in urine samples. C: Control, P: CKD patients. **: $p < 0.01$ vs. controls.

Mann-Whitney's U-test. P values < 0.05 were considered to be statistically significant.

4. Results

4.1. Baseline characteristics

Serum and urine metabolite profiles were compared between 15 patients with stage 1-2 CKD and 7 healthy volunteers. The baseline characteristics of the patients and controls are shown in *Table 1*. The two groups were similar in age, gender, BMI, and blood pressure. Estimated GFR (eGFR) in patients with stage 1-2 CKD showed a significant decrease (74.84 ± 4.30 ml/min/1.73m2, $p < 0.01$), which was compatible with the definition of stage 1-2 CKD patients, where stage 1 is defined as renal function of 90 ml/min/1.73 m2 or greater, and stage 2 as 60-89 ml/min/1.73 m2 respectively. Blood urea nitrogen and creatinine levels were not significantly different between the two groups. 24-h urinary protein excretion was increased in the patient group, whereas the value in controls was below the detectable threshold.

4.2. Changes in amino acid metabolites in the serum and urine of patients with stage 1-2 CKD

The CE-TOFMS systems in three different modes for cation, anion, and nucleotide analyses detected multiple metabolites, in serum and urine samples. In cation analysis mode, various changes in amino acid metabolites were found in the serum and urine of patients with early stage CKD compared to healthy volunteers *(Figure 2, 3)*. Several nonessential amino acids, in particular glutamate, asparatate, and ornithine were significantly increased in the serum (glutamate: from 22.7 ± 2.7 to 294.7 ± 78.8; aspartate: from 5.7 ± 0.5 to 76.2 ± 16.2; ornithine: from 59.4 ± 3.1 to 167.1 ± 24.4 μM, $p < 0.01$). Similar increases were found in the urine for glutamate and aspartate. In contrast, the essential amino acid histidine was decreased in the urine (from 100.6 ± 24.2 to 36.6 ± 9.1 nM/uM, $p < 0.01$). Both serum and urine glutamine were significantly decreased, suggesting a possible change in the equilibrium for glutamine-glutamate conversion by glutamine synthetase. Hydroxyproline was significantly

Table 1. Clinical characteristics of patients included in the study

Variable	Control (No.=7)	CKD patients (No.=15)
Age	30.9 ± 5.0	43.7 ± 4.2
Sex (male/female)	5/2	10/5
Body Mass Index (kg/m2)	20.4 ± 0.6	21.7 ± 0.9
Systolic BP (mmHg)	112 ± 4	121 ± 3
Diastolic BP (mmHg)	73 ± 4	75 ± 3
Blood urea nitrogen (mg/dl)	12.4 ± 1.0	14.3 ± 1.0
Serum creatinine (mg/dl)	0.74 ± 0.05	0.83 ± 0.03
eGFR (ml/min/1.73m2)	101.71 ± 4.88	74.84 ± 4.30 [a]
Serum uric acid (mg/dl)	5.4 ± 0.4	6.1 ± 0.4
Urine protein (g/day)	below threshold	1.35 ± 0.32

[a] $p < 0.01$ vs. controls

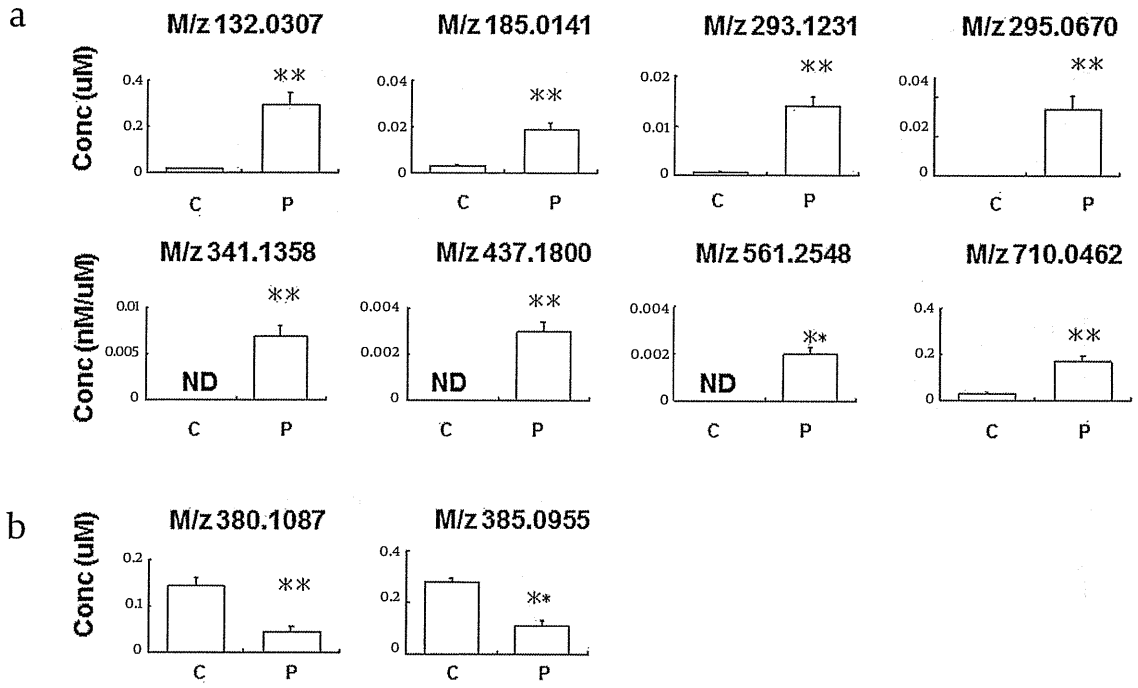

Figure 6. Changes in unidentified metabolites which were (a) increased or (b) decreased in the serum of patients with stage 1-2 CKD. C: Control, P: CKD patients. M/z: mass-to-charge ratio of the metabolite. ND: not detectable. **: $p < 0.01$ vs. controls.

decreased in the serum (from 13.8 ± 2.0 to 8.7 ± 0.8 μM, $p < 0.05$). Several changes were also found in oxidative stress-related amino acids. In particular, carnosine and hypotaurine were significantly lower in the urine of CKD patients, whereas hypotaurine and taurine were elevated in the serum.

4.3. Changes in nucleic acid metabolites in the serum and urine of patients with stage 1-2 CKD

In cation and anion analysis mode, multiple changes in nucleic acid metabolites were observed in the serum and urine of patients with early stage CKD *(Figure 4)*. In particular, hypoxanthine in the serum was markedly elevated (from 4.0 ± 0.7 to 209.8 ± 53.3 μM, $p < 0.01$), whereas adenosine was decreased in both the serum and urine of patients.

4.4. Changes in carbohydrate metabolites in the serum and urine of patients with stage 1-2 CKD

Several changes in carbohydrate metabolites were also observed in the serum and urine of patients with CKD *(Figure 5)*. Serum lactate increased from 2331 ± 422 to 12903 ± 2273 μM ($p < 0.01$), whereas urine citrate, fumarate and 3-phosphoglycerate were decreased compared to controls.

4.5. Changes in unidentified metabolites in the serum of patients with stage 1-2 CKD

The metabolome analysis revealed that serum levels of several novel unidentified metabolites were also markedly increased *(Figure 6a)* or decreased *(Figure 6b)* in the patients with CKD compared to controls.

5. Discussion

There have been several reports about serum amino acid patterns in advanced (stage 5) CKD, also known as end-stage renal disease (ESRD) (5-8). In general, the essential amino acid levels are decreased, while the nonessential amino acids are either within the normal range or increased, so the ratio of essential to nonessential amino acids is decreased in ESRD. It has been assumed that these changes are due to low protein intake, deficiency of excretory and metabolic functions of the diseased kidneys, toxic effect of uremia and, in dialysis patients, loss of protein and amino acids by the dialytic procedure (6, 7). The results of this study were compatible with the previous reports on patients with ESRD, and suggest that the changes in amino acid metabolism were already detectable at an early stage of CKD. It is interesting that these changes were seen even without marked renal insufficiency, suggesting that changes in amino acid metabolism are an early event in the course of CKD, and do not require the presence of uremia. Interestingly, not all nonessential amino acids were increased. In particular, glutamine was decreased in both the serum and urine, whereas glutamate was increased, suggesting possible changes in the conversion equilibrium of these two amino acids in these patients. We also found evidence for changes in oxidative stress in early stage CKD. In particular, the free-radical scavengers carnosine

and hypotaurine were decreased in the urine of patients, but conversely hypotaurine and taurine were increased in the serum. We speculate that these free-radical scavengers may have been decreased in the urine because of increased oxidative stress in the kidney, and this was counteracted by increases in the serum.

An important advantage of this method is that multiple metabolic pathways could be analyzed simultaneously using the three modes of electropherogram analysis. Regarding nucleic acid metabolites, serum and urine adenosine and urine guanine were decreased and serum hypoxanthine increased in the patient group, suggesting the possibility that degradation of purine nucleotide was elevated in these patients with stage 1-2 CKD. Interestingly, hypoxanthine was markedly increased in the serum of patients, to about 50 times the level of controls. Previous report in patients on dialysis showed that plasma concentrations of hypoxanthine and uric acid were increased in patients with ESRD (17). In this study hypoxanthine was already increased in patients with stage 1-2 CKD, even though serum uric acid was unchanged. These results may be important because hypoxanthine may act as a cardiotoxin (18), possibly by causing mitochondrial damage through increased oxidative stress (19). A recent report also suggested that hypoxanthine accumulation in xanthine oxidoreductase depletion mice caused progression of renal interstitial fibrosis, also by an oxidative stress-related mechanism (20). These results suggest the hypothesis that increased hypoxanthine may be one reason for the increased incidence of cardiovascular disease in patients with CKD (3, 4), as well as a potential risk factor for progression of renal disease.

Concerning carbohydrate metabolism, we found that serum lactate was increased, but other TCA cycle metabolites, such as citrate and fumarate, were decreased, suggesting that changes in glucose metabolism may also be evident from an early stage in CKD. An important advantage of metabolome analysis is the potential to identify new and unidentified metabolites which could have important pathophysiological functions. In our studies, we found that several novel unidentified metabolites were significantly increased in the serum of patients with CKD, whereas others were decreased. At present, the molecular structures of these metabolites are unknown. It is possible that these unidentified products may have novel pathophysiological functions, or may be new disease markers for renal injury. We are therefore planning further extended studies to examine these possibilities. One caveat of this study is that the patients with stage 1-2 CKD in our study were all candidates for renal biopsy, and may not be representative of the general population of stage 1-2 CKD. Thus, the possibility that these changes specifically appeared in proteinuric kidney diseases, but may not seen in early stage CKD without proteinuria, cannot be completely ruled out. Moreover, CKD of various etiologies were considered together in the patient group, because we were unable to discover a clear correlation between specific etiologies and their metabolomic profiles. Based on our current findings, further studies are warranted for comparisons between different renal diseases. In summary, the results of this study suggest that metabolic analysis may be used for detecting changes in amino acid, nucleic acid, and carbohydrate metabolites in the serum and urine of patients with early stage CKD, as well as for detecting unidentified metabolites which may have novel functions. Understanding these changes may be important for developing new strategies to prevent cardiovascular events and progression to ESRD in patients with CKD.

Financial support

None declared.

Conflict of interest

The authors declare that they have no conflicts of interests related to this study.

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Analysis of liver metabolism in a rat model of heart failure☆

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ABSTRACT

Background: Cachexia, namely body wasting, is a common complication in cases of congestive heart failure (CHF). Although, neurohumoral and immune abnormalities are associated with the condition, precisely how the imbalance of catabolism and anabolism is responsible for the wasting process is not known.
Methods: We analyzed markers of cachexia in Dahl salt-sensitive rats which show marked hypertension with preserved systolic function at 11 weeks and CHF at 17–19 weeks of age. We also analyzed the change in hepatic metabolism associated with CHF since liver plays a central role in the systemic regulation of catabolism and anabolism.
Results: In CHF rats, a failure to grow was observed and blood hepatic protein levels were decreased associated with increased blood proinflammatory cytokine levels, indicating that Dahl rats serve as a model of cardiac cachexia. Food intake was reduced, and blood sugar and insulin levels were decreased. Despite the apparent fasting condition, blood fatty acid levels were decreased and triglycerides levels were increased. In CHF rats, liver incorporated more glucose, the gene expression related to gluconeogenesis was decreased, the gene expression related to lipogenesis was increased, and the triglyceride content of the liver was increased. The paradoxical production of triglycerides synthesis in fasting rats was associated with a proinflammatory response in liver.
Conclusions: The Dahl salt-sensitive rat can be used as a model of cardiac cachexia. The cachexia was associated with abnormal hepatic metabolism that might work as a maladaptive response during the progression of CHF.

1. Introduction

Congestive heart failure (CHF) is becoming a serious health care problem. CHF is associated with a significant change in energy metabolism of the heart, and the altered energetics is hypothesized to play an important role in the progression of CHF [1]. Using a Dahl rat model which shows a distinct transition from compensated left ventricular hypertrophy to CHF, we recently found that left ventricular hypertrophy or CHF was associated with a distinct change in the metabolic profile of the heart and that the metabolic remodeling of heart might be a therapeutic target [2].

CHF is also associated with abnormal energy metabolism in extracardiac tissues. Cachexia, namely body wasting, is a common complication among CHF patients [3,4]. Cardiac cachexia is associated with a poor prognosis and disability. Several lines of evidence suggest that neurohumoral and immune abnormalities play a critical role, and a complex imbalance of catabolism and anabolism is likely to be responsible for the development of the wasting process [4].

Although cardiac cachexia is an important complication and a potential target of therapeutic intervention in cases of CHF, its pathophysiology is poorly understood. One reason for this is the limited number of animal models of cardiac cachexia available. There is a report that the skeletal muscle atrophy caused by reduced activity is significantly different from the muscle atrophy observed in CHF rats [5]. However, the mechanism by which the imbalance between catabolism and anabolism is induced is not clear. In this study, we have shown that the Dahl salt-sensitive rat fed a high-salt diet is an animal model of cardiac cachexia. To gain insight into the mechanism of cardiac cachexia, we have analyzed the change of hepatic

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Table 1
Primer sequences used in real time quantitative RT-PCR.

Gene	Forward	Reverse	Ref.	GenBank entry
18SrRNA	AGTCCCTGCCCTTTGTACACA	CGATCCGAGGGCCTCACTA	[26]	M11188
Pyruvate carboxylase	CCGTTCTAAGGTGCTAAAGGA	GACGAGTATTCAGGCTATCCA		BC085680.1
Phosphoenolpyruvate carboxykinase (PEPCK)	ATGTCAGAAGAGGACTTCGAGA	CTCAATACCAATCTTGGCCAGA		BC085680.1
ATP Citrate lyase	GGCAAGATCCTCATCATTGGA	CAACTTCTCCCATCACTCGTA		BC100618.1
Acetyl-CoA carboxylase (ACC) α	ATGATTGCTGGGGAATCCTCA	GAGGTGTATACTTCCCGACCA		NM_022193.1
Fatty acid synthase	CAAGTTATTCGACCACCCAGA	TCACCCAGTTGTCTTTCCAGA		NM_017332.1
Sterol regulatory element-binding protein (SREBP) 1c	TCACTGAAAGACCTGGTGTCA	GCTTTCACCTGGTTATCCTCA		AF286470.2
Sterol regulatory element-binding protein (SREBP) 2	CAAGTACCTGCAGCAGGTCA	AGTCAATGGAATAGGGGGAGA		NM_001033694
Tumor necrosis factor (TNF)α	ATGGTCCTCTTTCAGTCG GAG	TGTC TACTGAACTTCGGGGTG	[27]	NM_012675.3
Interleukin (IL)1β	CTTCCCCAGGACATGCTAGG	CAAAGGCTTCCCCTGGAGAC	[28]	NM_031512.2
C-reactive protein (CRP)	ACATTGTGGGGACAAATGCA	ACATTGGGGCTGAATACCCTA		NM_017096.3
Transthyretin	GGCTCACCACAGATGAGAAGTTC	ACAAATGGGAGCTACTGCTTTGGC	[29]	NM_012681.1
Retinol-binding protein (RBP) 4	AGAAGGGTCATATGAGCGCTA	GTATCGATGATCCAGTGGTCA		NM_013162.1
Hepatocyte nuclear factor (HNF) 4	AAATGTGCAGGTGTTGACCA	CACGCTCCTCCTGAAGAATC	[29]	EF193392

metabolism since the liver plays a central role in the systemic regulation of catabolism and anabolism.

2. Materials and methods

2.1. Animals

Inbred male Dahl salt-sensitive (DS) rats (Japan SLC, Hamamatsu, Shizuoka, Japan) were fed a 0.3% NaCl (low salt; LS) diet until the age of 6 weeks, then an 8% NaCl (high salt; HS) diet [2]. DS rats fed only the low-salt diet were used as controls. Animal care and the experiments were approved by the Institutional Animal Care and Use Committee of Kyoto University and conducted by the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

2.2. Protocols

Serial measurements of body weight, food intake, and cardiac function by echocardiography were performed from 11 weeks of age. Blood samples were obtained at age 18 weeks without fasting. The measurement of mRNAs, proteins, and metabolites was performed at the age of 11 and 18 weeks without fasting. The hepatic uptake of ^{18}F-deoxyglucose (FDG) and ^{125}I-15-(*p*-iodophenyl)-9-*R,S*-methylpentadecanoic acid (9MPA) was measured at age 11 and 18 weeks after an overnight fast.

2.3. Cardiac echocardiography

Echocardiography was performed based on a previously reported protocol [2]. Briefly, rats were anesthetized with inhaled diethyl ether (Wako Pure Chemical Industries, Osaka, Japan), and transthoracic echocardiography was performed using a Sonos-5500 echocardiograph (Agilent Technologies, Santa Clara, CA) with a 15-MHz linear transducer. M-mode echocardiograms were obtained at the papillary muscle level. At least two independent M-mode measurements for each animal were carried out.

2.4. Blood analysis

Blood samples were collected without fasting via the right ventricle, and the samples were centrifuged at 3000 rpm for 15 min and analyzed as described [2]. Plasma concentration of insulin was analyzed using a commercial kit (Shibayagi Co., Shibukawa, Gunma, Japan).

2.5. Sampling of hepatic tissue

To obtain liver tissues for biochemical analyses, 11-week-old LS (n=6), 11-week-old HS (n=8), and 17-week-old HS (n=6) rats were sacrificed by decapitation without fasting. A piece of the liver was obtained from the right lobe, rapidly divided, snap frozen in liquid nitrogen, and stored at −80 °C. The liver tissues were also used for the measurement of mRNA, glycogen, and triglyceride (TG).

2.6. Metabolome analysis (CE-TOFMS)

A targeted metabolomic approach was performed using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) [6]. Hepatic tissue from 11-week-old LS (n=6), 11-week-old HS (n=6), and 17-week-old HS (n=6) rats were analyzed. The conditions used were described in Supplementary materials.

2.7. Levels of glycogen and triglycerides

Glycogen and TGs were extracted from liver tissue of 11-week-old LS, 11-week-old HS, and 17-week-old HS rats (n=6–8 in each group) using previously described methods [7,8], and analyzed with commercial kits (BioAssay Systems, Hayward, CA, and Cayman Chemical, Ann Arbor, MI, respectively).

2.8. Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the liver tissue (n=6–8 in each group) by the acid guanidinium thiocyanate–phenol–chloroform method. Quantitative RT-PCR was carried out as described previously [2]. The sequences of primers used are listed in Table 1. The mRNA level of each gene was standardized with the corresponding 18S ribosomal RNA as an internal control. The Genbank accession numbers are also included in Table 1.

2.9. Western blotting

Liver lysate was obtained by homogenization in ice-cold buffer [10% glycerol, 137 mM NaCl, 20 mM Tris–HCl pH 7.4, 4 g/ml aprotinin, 4 g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 4 g/ml pepstatin, 20 mM NaF, 1 mM sodium pyrophosphate, and 1 mM orthovanadate] [9]. The lysate was kept on ice for 15 min and cleared by centrifugation at 15,000 g for 20 min at 4 °C. Protein concentrations were determined by the Bradford method (BioRad, Hercules, CA). 200 μg of liver tissue lysate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were transferred onto polyvinylidene difluoride membranes. The primary antibodies used for western blotting were for sterol regulatory element-binding protein (SREBP) 1 (2A4, 1:500, Santa Cruz, Santa Cruz, CA), SREBP2

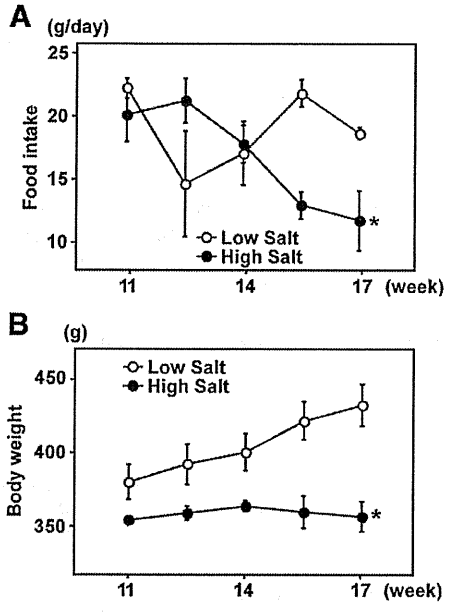

Fig. 1. Food intake and body weight of Dahl rats fed a high-salt diet. (A) Serial measurements of food intake. Rats with congestive heart failure (CHF) ate less than control rats. (B) Serial measurements of body weight. CHF rats showed a failure to grow. n=4 for each group. *p<0.05 versus control rats, namely Dahl rats fed a low-salt diet.

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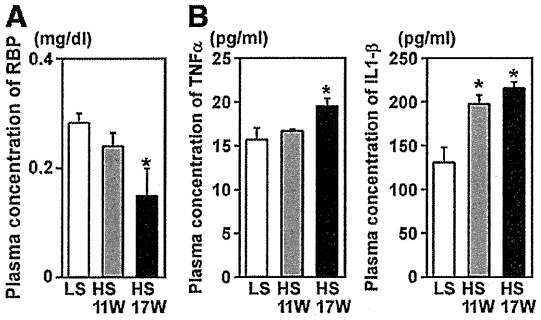

Fig. 2. Markers of cachexia in Dahl rats. (A) The concentration of retinol-binding protein (RBP) in plasma was decreased in CHF rats (HS 17W). (B) The plasma concentration of tumor necrosis factor (TNF)-α and interleukin (IL)1-β was increased in CHF rats. n = 6–8 in each group. LS; low salt, HS; high salt. *p<0.05 versus control LS rats.

(1:500, (Cayman Chemical), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1000, Chemicon, Temecula, CA).

2.10. Hepatic uptake of ^{18}FDG and ^{125}I-9MPA

The hepatic uptake of glucose and fatty acids was estimated by measuring the incorporation of an analog of glucose (deoxyglucose) and a fatty acid (9MPA) as described [2]. The 11-week-old LS (n = 7), 11-week-old HS (n = 10), and 17-week-old HS rats (n = 12) were injected with 1 mCi of ^{18}FDG and 20 μCi of ^{125}I-9MPA. The animals were fasted overnight before the injection since variation in the uptake of the isotope-labeled molecule was found when fed animals were used. The rats were sacrificed by decapitation 45 min after the injection, and the livers were removed and washed in cold saline. Specimens of the left lobe were collected and frozen in liquid nitrogen and radioisotopic activity was measured using a scintillation counter (Packard Cobra2 ™ Auto-gamma, GMI, Ramsey, Minnesota) [2]. To measure ^{18}FDG uptake, radioisotopic activity was measured just after sacrifice because the half decay time of ^{18}FDG is 110 min. To measure ^{125}I-9MPA uptake, another radioisotopic measurement was made 48 h after the first. The amount of radioisotope incorporated was presented as a percentage of the administered dose corrected by liver weight in grams. Using this method, cross-talk between the two tracers was negligible [2].

2.11. Plasma and tissue concentrations of inflammatory cytokines

The levels of tumor necrosis factor (TNF)-α and interleukin (IL)1-β in tissue homogenate and plasma were measured using sandwich enzyme-linked immunosorbent assay (ELISA) kits (Shibayagi Co. and R&D Systems; Minneapolis, MN), according to the manufacturers' instructions. The tissue homogenate for the protein analysis was used in this assay.

2.12. Statistical analysis

Values are expressed as means ± SEMs. Differences among experimental groups were tested by ANOVA with post hoc comparisons using the Bonferroni test. In all tests, a value of $p<0.05$ was considered significant.

3. Results

3.1. Dahl rats fed a high-salt diet develop hypertension, heart failure, and cachexia

As we reported previously, DS rats fed a high-salt (HS) diet developed hypertension (HT) at 11 weeks of age (systolic blood pressure; 211 ± 12 mm Hg, diastolic blood pressure; 160 ± 6 mm Hg) [2]. On echocardiographic examination, fractional shortening (FS) was

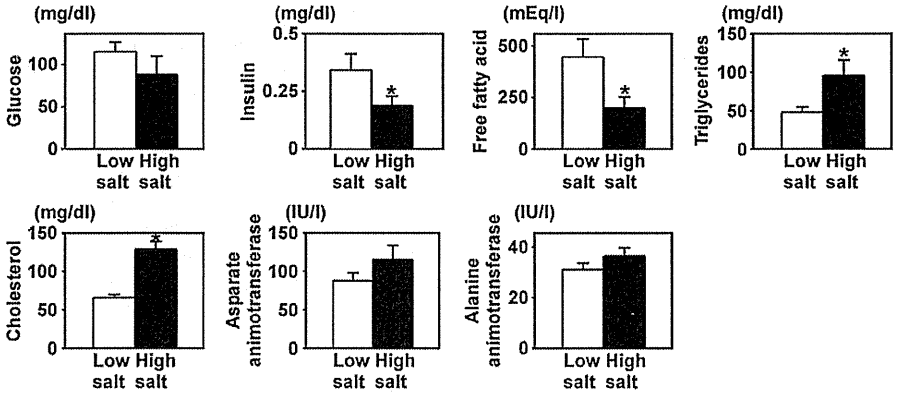

Fig. 3. Blood analysis of Dahl rats. Blood sugar and insulin levels were decreased, and triglyceride and cholesterol levels were increased in CHF rats. n = 5–6 in each group. *p<0.05 versus control rats.

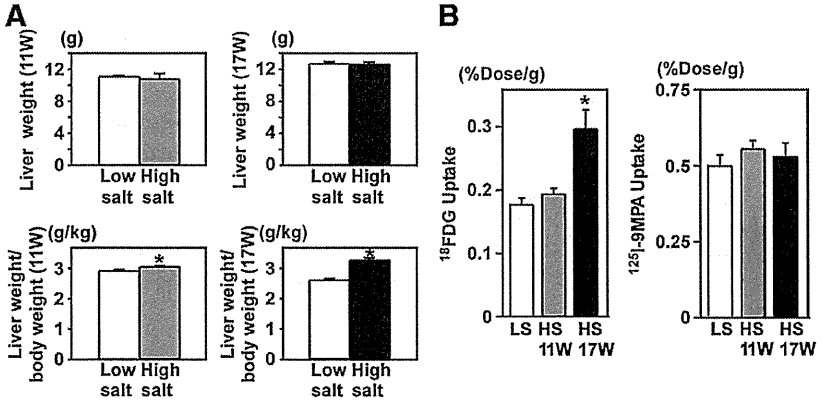

Fig. 4. Liver weight and the uptake of glucose and fatty acids. (A) Liver weight and liver weight corrected by body weight at 11 and 17 weeks of age. Liver weight corrected by body weight was increased in CHF rats (HS 17W) compared with control rats. n = 6–12 in each group. LS; low salt, HS; high salt. *p<0.05 versus control rats. (B) The uptake of ^{18}FDG, a glucose analog, was increased in CHF rats (HS 17W) compared to control rats. The uptake of ^{125}I-9MPA, a fatty acid analog, in the liver was not changed. n = 7–12 in each group. *p<0.05 versus control rats.

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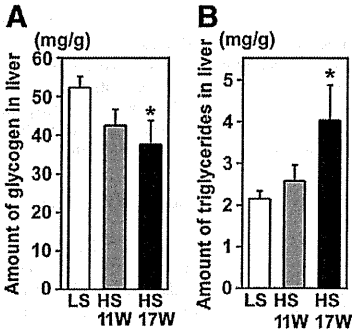

Fig. 5. Glycogen and triglyceride content in liver. (A) The amount of glycogen in liver was decreased in CHF rats. (B) The amount of triglycerides in liver was increased in CHF rats. n = 6–8 in each group. LS; low salt, HS; high salt. *p<0.05 versus control rats.

found to be preserved (62.4 ± 0.9%). At around 17 weeks of age, the rats showed signs of CHF, such as tachypnea and immobilization, and decreased FS (39.7 ± 1.5%). DS rats fed a low-salt (LS) diet did not develop hypertension or CHF, and were used as controls.

Serial measurements of food intake and body weight showed that CHF rats ate less than controls (Fig. 1A) and had a lower body weight (Fig. 1B). The failure to grow in the CHF rats led us hypothesize that the animals may serve as a model of cardiac cachexia. Blood levels of hepatic proteins, such as albumin, transthyretin, and transferrin, can serve as markers of a nutritional index to screen for malnutrition and monitor the metabolic response to dietary intervention [10,11]. Blood hepatic proteins are synthesized mainly in liver and have short half lives in blood. We measured the concentration of retinol-binding protein (RBP), a blood hepatic protein, in plasma and found that it was decreased in CHF rats (Fig. 2A). Cachexia is also known to be associated with neurohumoral and immune abnormalities [4]. Indeed, plasma concentrations of two representative proinflammatory cytokines, TNF-α and IL-1β, were elevated in CHF rats (Fig. 2B).

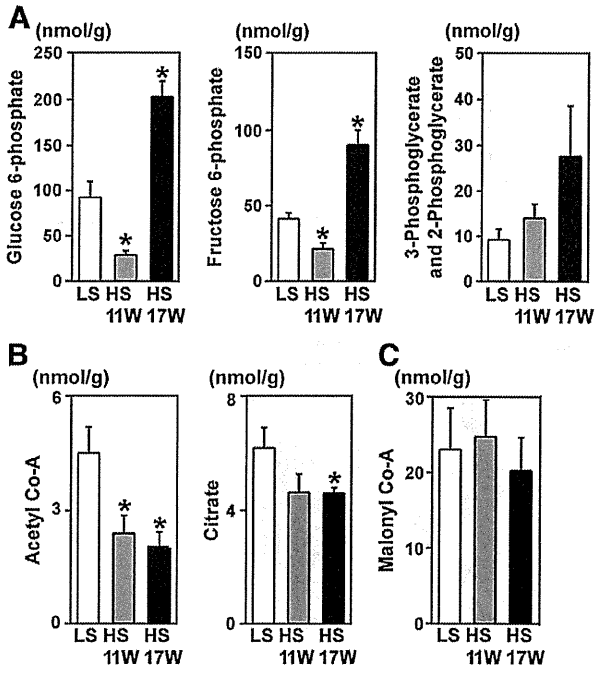

Fig. 6. The amounts of metabolites in liver determined by metabolome analysis. (A) Concentrations of metabolites of glycolysis. (B) Concentrations of metabolites in the Krebs cycle. (C) Malonyl CoA concentration. n = 6 in each group. LS; low salt, HS; high salt. *p<0.05 versus control rats.

3.2. Blood analysis of CHF rats

To investigate the systemic metabolic profile of Dahl rats, we examined blood chemistry. Both glucose and insulin levels were lower in CHF rats than control rats in the fed condition (Fig. 3), as reported [2]. Plasma levels of cholesterol and TG were increased, and free fatty acids (FFAs) were decreased in DS rats with CHF, as reported [2]. Concentrations of representative liver enzymes, such as aspartate amino transferase and alanine transaminase, did not differ among the groups. Lower food intake, associated with lower glucose and insulin levels, suggested that the animals were starved. However, the plasma level of FFAs was decreased and that of TG increased, which is not consistent with a starved condition. Based on these observations, we sought to examine the hepatic metabolism in this model since the liver plays a key role in the homeostasis of systemic catabolism and anabolism.

3.3. Analysis of energy metabolic pathways in the liver

Liver weight corrected by body weight was increased in CHF rats compared with control rats (Fig. 4A). The increase in liver weight is likely to be due to congestion since venous dilation in liver tissue is reported in this model [12]. It is of interest that increased right atrial pressure is reported to indicate malnutrition in CHF patients [13]. Despite lower blood sugar levels, the uptake of ^{18}FDG increased in CHF rats (Fig. 4B). The uptake of ^{125}I-9MPA, a fatty acid analog, was not changed (Fig. 4B). The amount of glycogen in liver was decreased, and that of TG was increased in the CHF rats (Fig. 5A, B). The metabolome analysis (Supplementary table) revealed that levels of some metabolites of glycolysis increased (Fig. 6A) and some metabolites in the Krebs cycle, such as acetyl-CoA and citrate, were decreased (Fig. 6B). Overall, these results may suggest hepatic lipogenesis to be increased and acetyl-CoA to be used for the synthesis of TG and cholesterol.

Next, we examined the gene expression related to lipogenesis and gluconeogenesis. The gene expression of enzymes related to lipogenesis, such as ATP citrate lyase, acetyl-CoA carboxylase, and fatty acid synthase, was up-regulated in CHF rats (Fig. 7A). In contrast, the expression of rate-limiting enzymes in gluconeogenesis, such as pyruvate carboxylase and phosphoenolpyruvate carboxykinase (PEPCK), was down-regulated. Next, we examine the expression of sterol regulatory element-binding protein (SREBP1)1-c and SREBP2, which increase lipogenesis and suppress gluconeogenesis [14,15]. The gene expression of SREBP1-c and SREBP2 was increased in CHF rats. The amount of SERBP proteins was also increased (Fig. 7B and C).

3.4. Expression of rapid turnover protein in liver

Since RBP, a blood hepatic protein, was decreased in the blood of CHF rats, we examined the gene expression of serum hepatic proteins. mRNA levels of transthyretin and retinol-binding protein (RBP) 4 were decreased in CHF rats (Fig. 8A). To examine the mechanism responsible for the decrease of rapid turnover proteins, we measured the expression of hepatocyte nuclear factor (HNF) 4. HNF4 is known to regulate transthyretin gene expression [16,17]. HNF4 mRNA levels started to decrease in rats with HT, and decreased significantly in rats with CHF (Fig. 8B).

3.5. Expression of proinflammatory genes

Inflammatory responses are reported to enhance lipogenesis [18] and induce cachexia [19]. It is well established that CHF is associated with the inflammation of cardiac and extra-cardiac tissue [20]. Thus, local inflammatory responses might be a mechanism causing

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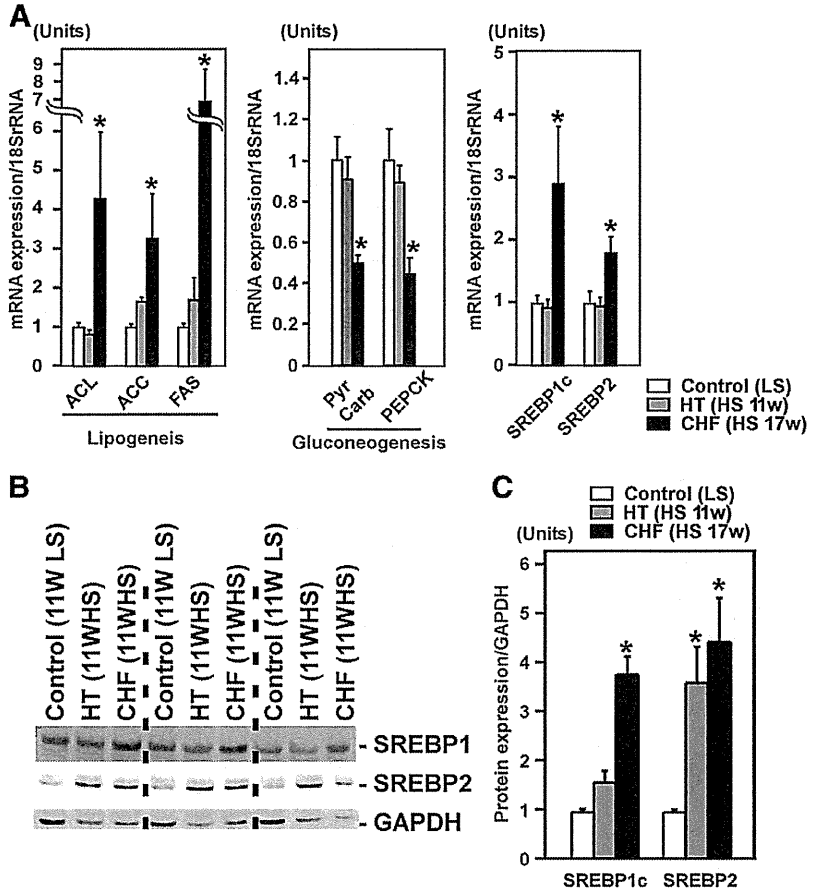

Fig. 7. Expression of mRNA and protein levels related to lipogenesis and gluconeogenesis. (A) The gene expression related to lipogenesis was increased in liver of CHF rats (HS 17W). The gene expression related to gluconeogenesis was decreased. The gene expression of sterol regulatory element-binding protein (SREBP)1c and SREBP2 was increased. n = 6–8 in each group. *p<0.05 versus control rats. (B) Representative images of the Western blotting of SREBP1 and SREBP2. (C) The protein levels of SREBP1 and SREBP2 in liver tissue were increased. n = 3 in each group. LS; low salt, HS; high salt. *p<0.05 versus control rats.

increased lipogenesis and the decreased synthesis of blood hepatic proteins. Therefore, we examined the expression of genes related to proinflammatory responses. The gene expression of TNF-α, IL1-β, and C-reactive protein, started to increase in liver of rats with HT but not with CHF, and significantly increased in CHF (Fig. 9A). The protein levels of TNF-α and interleukin1-β in liver also started to increase in HT rats, and remained increased in CHF rats (Fig. 9B).

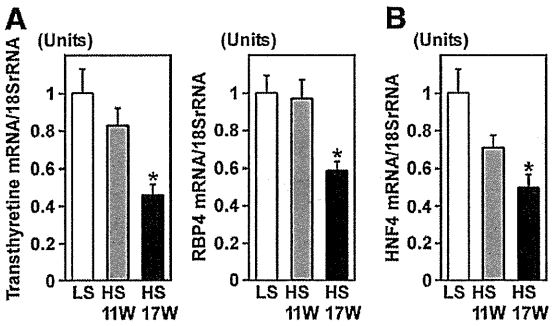

Fig. 8. Analysis of gene expression of hepatic proteins. (A) The gene expression of hepatic proteins, transthyretin and RBP4, was decreased in CHF rats (HS 17W). (B) Hepatocyte nuclear factor (HNF) 4 mRNA was decreased in CHF rats. n = 6–8 in each group. LS; low salt, HS; high salt. *p<0.05 versus control rats.

4. Discussion

In this study, we have shown that Dahl rat fed a high-salt diet developed CHF associated with a failure to grow, decreased blood hepatic protein, and increased blood proinflammatory cytokines. These results indicate the Dahl rat with CHF to be a model of cardiac cachexia.

4.1. Abnormal lipid metabolism in liver of CHF rats

During fasting, liver metabolism changes from anabolism to catabolism. Glucose is produced from glycogen breakdown and gluconeogenesis, and lipid accumulated as TG is used to produce FFAs. The glucose and FFAs are released into the circulation and delivered to the brain, skeletal muscle, the heart etc. However, despite decreased food intake and lower blood sugar and insulin levels, lipogenesis was increased in the liver of CHF rats (Fig. 10). The mechanism by which lipogenesis is increased on fasting in these rats is unknown. However, Fon Tacer et al. reported that giving TNF-α to fasted mice changed the metabolic profile of the liver from lipid usage to synthesis [18]. In that report, SREBP levels were are decreased in fasted mice, but increased on addition of TNF-α, associated with increased lipogenesis. Another report also showed abnormal lipid metabolism to be associated with inflammatory responses in liver [21]. Indeed, SREBPs increased in association with the amount of TNF-α mRNA and protein in this study. The TNF-α concentration in

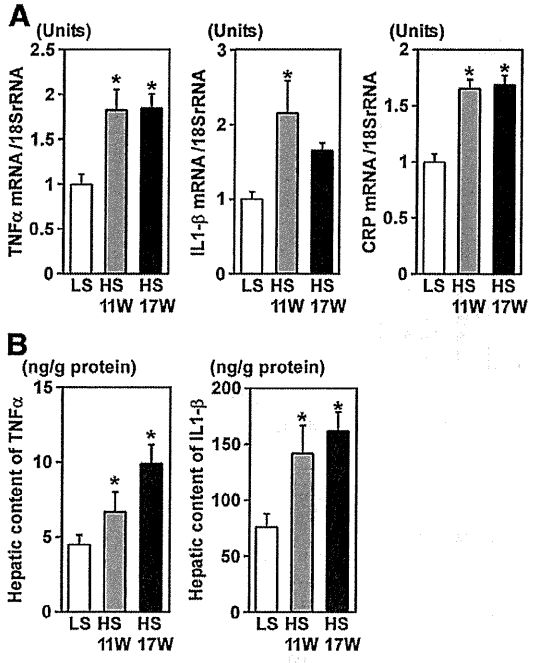

Fig. 9. Expression of proinflammatory cytokines in liver. (A) The gene expression of proinflammatory cytokines, TNF-α, IL1-β, and C-reactive protein (CRP), was increased in HT (HS 11W) and CHF rats (HS 17W). n=6–8 in each group. (B) The amounts of TNF-α and IL1-β started to increase in the liver in HT rats, and remained increased in the liver in CHF rats assessed by ELISA. n=6 in each group. LS; low salt, HS; high salt. *p<0.05 versus control rats.

liver was reported to be increased in an animal model of pacing-induced heart failure [22]. Thus, CHF-associated proinflammatory responses may be a mechanism of abnormal lipid metabolism in CHF rats.

4.2. Abnormal blood hepatic protein synthesis in CHF

Blood levels of hepatic proteins are used to evaluate nutritional status, but they are also influenced by many factors other factors. Particularly, the presence of inflammation is known to inversely correlate with circulating levels of hepatic proteins. Indeed, the gene expression of hepatic proteins was decreased associated with local increases in proinflammatory molecules of the liver in this study. However, the mechanism by which inflammation affects hepatic protein is not known [10]. HNF4 regulates the gene expression of transthyretin [16,17]. Although the mechanism by which proinflammatory molecules act on HNF4 expression is unclear, increased levels of SREBPs are known to repress HNF4 gene expression [23].

4.3. Measurement of metabolites using metabolome analysis

The measurement of metabolites in glycolysis and the Krebs cycle provides a snap shot of metabolism. However, based on the findings that ^{18}FDG uptake was increased, the amount of metabolites in glycolysis was increased, the amount of metabolites in the Krebs cycle was decreased, and the TG content was increased, we speculate that acetyl-CoA might be used for *de novo* lipogenesis. Further analysis, in which isotope-labeled glucose or fatty acid is injected and isotope-labeled metabolites are measured, is needed to know how glucose or fatty acids are used to test if the speculation is correct [24,25].

4.4. Potential role of abnormal liver metabolism in cardiac cachexia

Food intake was reduced and blood sugar levels were decreased in this model. During fasting, the liver is expected to deliver energy substrates, such as glucose and FFAs, to peripheral tissues. However, paradoxically, liver incorporated more glucose, the expression of genes related to gluconeogenesis was decreased, the expression of genes related to lipogenesis was increased, and TG content was increased. The response appears to be maladaptive, when the body is

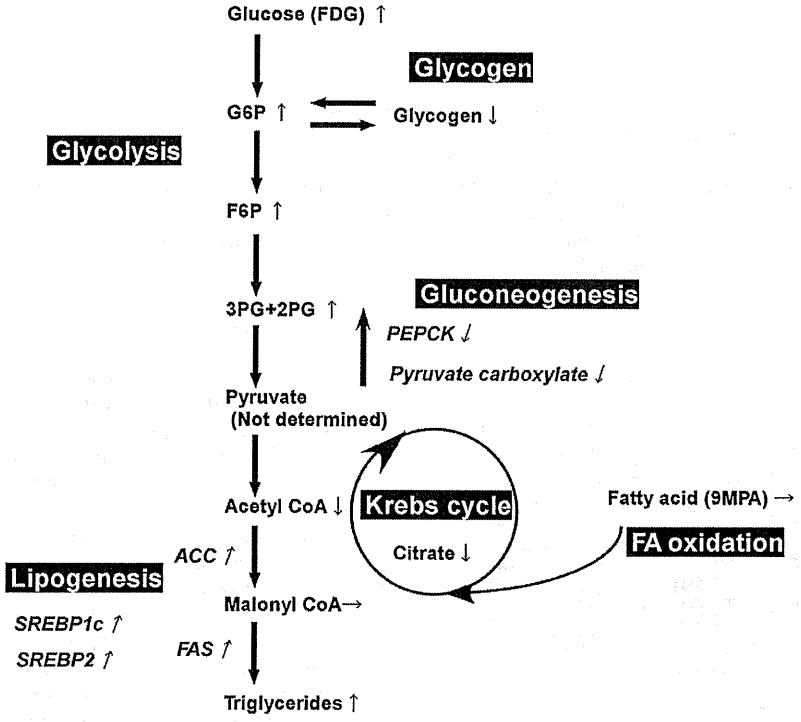

Fig. 10. Summary of the metabolic prolife in liver of CHF rats. Liver incorporated more glucose, gene expression related to gluconeogenesis was decreased, gene expression related to lipogenesis was increased, and TG content was increased in CHF rat liver.

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losing weight and peripheral tissues need more substrates to maintain tissue homeostasis. Although this study is an observational one, the findings indicate the abnormal liver metabolism to be a maladaptive process and worsen the CHF. Further study of whether the modulation of liver metabolism ameliorates CHF is needed.

Acknowledgements

The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology [30].

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ijcard.2011.07.056.

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Transcriptional Regulation of Organic Anion Transporting Polypeptide SLCO4C1 as a New Therapeutic Modality to Prevent Chronic Kidney Disease

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ABSTRACT: Uremic toxins accumulate in patients with chronic kidney diseases (CKDs) and cause further progression of renal damage and cardiovascular diseases. Recently, it was reported that some of the organic anion transporting polypeptides (OATPs) and the organic anion transporters (OATs) are involved in the renal elimination of uremic toxins. SLCO4C1 is the only OATP expressed at the basolateral side of proximal tubular cells in human kidney, and it mediates the excretion of uremic toxins. The overexpression of human SLCO4C1 in rat kidney promotes the renal excretion of uremic toxins and reduces hypertension, cardiomegaly, and renal inflammation in renal failure. Statins induce SLCO4C1 expression thorough transcriptional factor Aryl hydrocarbon receptor through binding of the xenobiotic responsive element at its promoter region. The administration of statin in a rat renal failure model facilitated the elimination of uremic toxins and mitigated organ damage. In addition, metabolomic analysis of rat renal failure models and patients with CKD by capillary electrophoresis–mass spectrometry is a useful method for identifying new uremic solutes and explores surrogate biomarkers for detecting the progression of early stage CKD. © 2011 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 100:3696–3707, 2011

Keywords: Organic anion-transporting polypeptide transporters; Organic anion transporter; ABC transporters; Membrane transport; Uremic toxin; Statin; Chronic kidney disease; Metabolome analysis; Capillary electrophoresis; Mass spectrometry

INTRODUCTION

The kidney is involved in the elimination of various intrinsic and extrinsic compounds from the body, and in the regulation of homeostasis and pharmacokinetics. Various water-soluble substances are excreted from the blood into the urine by the kidney. These renal excretory systems comprise three major components, that is, glomerular filtration, tubular secretion, and tubular reabsorption. Glomerular filtration can be partially compensated for by hemodialysis (HD), but the accumulation of uremic toxins that cannot be effectively eliminated by dialysis leads to the progression of renal damage, hypertension, and cardiovascular diseases (CVDs). The selective and specific excretion and reabsorption of various metabolic compounds and urinary-eliminated drugs and metabolites is not possible by artificial means. Many of the urinary-eliminated drugs and metabolites are water soluble and cannot easily penetrate the lipid bilayer

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of cell membranes. Cell membrane transporter proteins or ATP-dependent pump proteins are needed for selective and efficient transport in renal tubular cells.

The organic anion transporting polypeptide (OATP/SLCO/SLC22) family and the organic anion transporter (OAT/SLC21) family include cell membrane transporting proteins that carry various organic anions and neutral compounds. Organic anion transporters are expressed on both the apical and basolateral (vascular) sides of renal tubular cells and transport various compounds across the cell membrane.[1]

Recently, OATs and OATPs were reported to transport not only urinary-excreted substances such as drugs and metabolites but also uremic toxins that accumulate in patients with chronic kidney diseases (CKDs).[2–8]

Chronic kidney disease is defined as an estimated glomerular filtration rate (eGFR) less than 60 mL/min per 1.73 m^2 of body surface area.[9] The prevalence of CKD is now estimated at approximately 10% of the population, so CKD is becoming an increasingly more important public health problem. CKD is strongly associated with cardiovascular events and the prognosis.[9] With the progression of CKD, various uremic toxins accumulate, causing renal damage and hypertension worsening the prognosis.[10,11] More than 110 organic compounds have now been identified as uremic toxins.[12] Among these toxins, guanidino compounds, such as guanidino succinate (GSA) and asymmetric dimethylarginine (ADMA), increase in patients with CKD and correlate with the prognosis.[11,13] Particularly, ADMA is an inhibitor of nitric oxide synthase and is associated with hypertension, renal damage, cardiac hypertrophy, and cardiovascular events.[14,15]

The mortality from CVD in patients undergoing dialysis is markedly higher than that in the general population, with about one half of the deaths in dialysis patients are attributed to CVDs.[16] The cardinal features of uremic cardiac disease are left ventricular hypertrophy (LVH), reduced capillary density, fibrosis, and ventricular remodeling. Those cardiovascular outcomes, such as heart failure, ischemic heart disease, stroke, and peripheral vascular disease, aggravate the mortality.[16] Cardiac hypertrophy is a powerful independent predictor of the patient's survival in CKD, and the regression of LVH is associated with reduced risk in CVDs and improves the survival rate.[17]

A reduction of the accumulated uremic toxins protects against the development of hypertension, renal damage, and CVD in patients with CKD, but there is no established treatment. In addition, early detection of an impairment of renal function could enable treatment to prevent further deterioration and complications.

Recently, we isolated a human kidney-specific OATP, termed SLCO4C1, and functionally characterized it as a digoxin transporter.[2] The OATP family is involved in the membrane transport of bile acids, conjugated steroids, thyroid hormone, eicosanoids, peptides, cardiac glycosides (digoxin, digitoxin, and ouabain), and numerous drugs (antibiotics, hydroxymethylglutaryl-CoA reductase inhibitors/stains, and anticancer drugs).[18,19] In the kidney, SLCO4C1 might be the first step in the transport of digoxin and various compounds into the urine. We revealed that many compounds accumulate during renal failure, and that the kidney-specific OATP SLCO4C1 excretes uremic toxins resulting in reductions of the blood pressure (BP) and renal inflammation.[7] To generalize these results for clinical use, it is necessary to examine the accumulation of uremic solutes precisely. We have revealed that (1) human kidney-specific OATP SLCO4C1 is the responsible molecule for excreting uremic toxins,[2] (2) the overexpression of human SLCO4C1 in rat kidney promotes the renal excretion of uremic toxins and reduces hypertension, cardiomegaly, and inflammation in renal failure,[7] and (3) metabolomic analysis of blood and urine samples from patients with CKD can be used to evaluate each substance to detect early stage CKD.[8]

Compounds highly correlated with eGFR and whose plasma concentrations change in a manner approximated by the first-degree equation are excellent candidates for detecting CKD and identifying uremic toxins that might aggravate the kidney function in the early stage of CKD. The above-mentioned results identified a number of uremic compounds, many of which are novel, that predict worsening renal function. These compounds provide diagnostic information and may be targets for therapies designed to treat the complications of CKD.[8]

Renal transporter proteins are potential therapeutic targets for CKD to improve the prognosis of patients with damaged kidneys and related CVDs.

Molecular Entity of Renal Tubular Transporting System

Organic anion transporters are membrane proteins composed of 12 transmembrane domains, and both the *n*-terminal and *c*-terminal are located in the cytosol. Organic anion transporters are subdivided into two major gene families, that is, OAT (OAT, SLC21)[20,21] and OATP (OATP, SLC22/SLCO)[18,19] based on the amino acid sequence homology.

OATs transport relatively small organic anions (generally <400 Da) such as *p*-aminohippurate (PAH), probenecid, and fluorescein. OATPs can transport comparably bulky compounds (generally >500 Da) such as steroid hormones, thyroid hormone, and bile acids.

Both OAT and OATP families are expressed in renal tubular proximal cells that conduct the renal tubular excretion and reabsorption of various anionic compounds.[22,23] ATP-binding cassette (ABC) transporters regulate the ATP-dependent active efflux transport of many kinds of organic anions and cations. Members of the ABC transporter family, that is, multidrug resistance gene1 product (MDR1, pgp1),[24] multidrug resistance protein (MRP)2,[25] and MRP4[26] are distributed in the apical membrane of renal tubular cells and regulate tubular excretion into urine.

OATs are localized in both the apical (OAT4, URAT1) and basolateral sides (OAT1,OAT2, OAT3) of renal proximal tubular cells.[22,23] The apical side OATs are considered to control tubular reabsorption from urine into cells but also some excretion of anionic compounds into the urine. OATs localized in the basolateral membrane are thought to mainly regulate the uptake of organic anions from blood into cells as the first step of renal tubular excretion. Although several ABC transporters and OATs are expressed in human kidney proximal tubular cells,[22,23,27–30] only SLCO4C1 is exclusively expressed and localized in the basolateral side of human renal proximal tubular cells[2] (Fig. 1a).

OAT Family

In human, at least, five OATs are expressed in proximal tubules.[23,30] OAT1,[31] OAT2,[32,33] and OAT3[31,34] are expressed in the basolateral side and OAT4[35] and URAT1[36] are localized at the apical lumen. Basolateral OATs mainly uptake various organic anions from blood into tubular cells. However, the transport by OAT at the apical membrane is still controversial.[30,37] In addition, URAT1 predominantly transports uric acid from urine into tubular cells and plays a role in renal tubular uric acid reabsorption.[36,38,39]

Chronic Kidney Disease and Organic Anion Transporter

In patients with renal failure, various organic anion uremic toxins such as indoxyl sulfate (IS), 3-carboxyl-4-methyl-5-propyl-2-furanopropionic acid (CMPF), indoleacetate (IAA), and hippuric acid (HA) accumulate.[12,40]

Among them, IS is an uremic toxin derived from dietary tryptophan. Tryptophan is converted to indole by tryptophanase in intestinal bacteria such as *Escherichia coli*. Indole is absorbed into the blood stream from the intestine and subsequently undergoes oxidization and sulfate conjugation in the liver to form IS. IS is water soluble and is normally excreted by kidney into urine. In serum, around 90% of IS is bound to albumin and excreted mainly via active secretion by renal proximal tubular cells.[41]

In CKD patients, as the kidney function declines, reduced renal excretion results in elevated serum concentrations of IS. The oral adsorbent AST-120 prevents the intestinal absorption of indole, reduces the accumulation of IS, and might ameliorate nephrotoxicity.[42]

Niwa et al.[43,44] reported that administration of IS in rat renal failure models promotes the renal damage progression, and oral uremic toxin absorbents reduced the level of IS in both serum and urine. Enomoto et al.[3] also showed that IS is accumulated in proximal tubules of IS-overloaded rats with renal failure. Rat Oat1/Slc22a6 and Oat3/Slc22a8 are also expressed in the proximal tubular cells.[3]

Because the cytotoxicity of IS was enhanced by the overexpression of rat Oat1/Slc22a6 or Oat3/Slc22a8 in a cell culture system, this transporter was thought to be one of the responsible molecules for uptaking IS.[3] Deguchi et al.[6] reported that not only IS but also CMPF, IAA, and HA were taken up by both human OAT1/SLC22A6 and OAT3/SLC22A8 from blood into tubular cells. Because OATs are also expressed in bone osteoblasts, muscle cells, and the blood–brain barrier, it is possible that OATs are involved in the transport of uremic toxins and the pathogenesis of uremia in various organs.[42,45]

The expression profiles of OATs were studied in animal experimental models. The downregulation of rat Oat1/Slc22a6 and Oat3/Slc22a8 expression was reported in 5/6-nephrectomized rats as a chronic renal failure (CRF) model,[46] and in a rat ischemic–reperfusion model[47,48] and cisplatin-induced nephropathy model[49,50] as acute renal failure models. These reports suggest that the reduced expression of OATs in renal failure is one of the causes of the decreased renal excretory function.[51,52]

In human, quantitative real-time polymerase chain reaction (PCR) analyses of renal biopsy specimens from patients with kidney diseases have been reported. Indeed, OAT1/SLC22A6 and OAT3/SLC22A8 expressions were decreased in patients with CKD.[53,54] Furthermore, downregulation of OAT3/SLC22A8 was significantly related to the renal excretion of the antibiotic cefazolin, a known substrate of OAT.[54] These results suggest the importance of OATs in CKD.

In mice, Oat1/Slc22a6 and Oat3/Slc22a8 are also localized on the basolateral membrane, so they are considered to be responsible for tubular uptake in renal proximal tubules. Knockout mice of these OATs have been established. Despite the lack of morphological changes in Oat1-null and Oat3-null mice, there are considerable alterations in renal uptake and/or secretion of organic anions in these two knockout mice. In Oat1-null mice, the decreased renal secretion of PAH and reduced renal uptake of the diuretic furosemide with impaired diuretic responsiveness to this drug were reported.[55] In Oat3-knockout mice, renal uptake of taurocholate, estrone-3-sulfate, and PAH were greatly decreased in an *in vitro* study

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