Impact of Chronic Kidney Dysfunction on Serum Sulfatides and Its Metabolic Pathway in Mice

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Abstract

Serum sulfatides are critical glycosphingolipids that are present in lipoproteins and exert anticoagulant effects. A previous study reported decreased levels of serum sulfatides in hemodialysis patients and suggested an association with cardiovascular disease. However, the mechanism of changes in serum sulfatides in chronic kidney dysfunction has not been well investigated.

The current study examined whether a chronic kidney disease (CKD) state could decrease serum sulfatide levels using 5/6 nephrectomy (5/6NCKD) mice, an established CKD murine model, and studied the mechanisms contributing to diminished sulfatides. 5/6NCKD mice and sham operation control mice were sacrificed at the 4^{th} or 12^{th} postoperative week (POW) for measurement of serum sulfatide levels. Hepatic sulfatide content, which is the origin of serum sulfatides, and the expression of sulfatide metabolic enzymes in liver tissue were assessed as well.

The 5/6NCKD mice developed CKD and showed increased serum creatinine and indoxyl sulfate. The serum levels and hepatic amounts of sulfatides were significantly decreased in 5/6NCKD mice at both 4 and 12 POW, while the degradative enzymes of sulfatides arylsulfatase A and galactosylceramidase were significantly increased. In a Hepa1-6 murine liver cell line, indoxyl sulfate addition caused intracellular levels of sulfatides to decrease and degradative enzymes of sulfatides to increase in a manner comparable to the changes in 5/6NCKD mice liver tissue.

In conclusion, chronic kidney dysfunction causes degradation of sulfatides in the liver to decrease serum sulfatide levels. One explanation of these results is that indoxyl sulfate, a uremic toxin, accelerates the degradation of sulfatides in liver tissue.

Key words

Sulfatides, Chronic kidney disease, 5/6 Nephrectomy model, Indoxyl sulfate

Abbreviations

ACOX; acyl-CoA oxidase

AKI; acute kidney injury

ARSA; arylsulfatase A

CAT; catalase

CGT; ceramide galactosyltransferase

CKD; chronic kidney disease

COX2; cyclooxygenase-2

CST; cerebroside sulfotransferase

CVD; cardiovascular disease

ESRD; end-stage renal disease

GALC; galactosylceramidase

GAPDH; glyceraldehyde-3-phosphate dehydrogenase

HD; hemodialysis

HNE; 4-hydroxynonenal

IS; indoxyl sulfate

LS; lysosulfatides

MALDI-TOF MS; matrix-assisted laser desorption ionization-time of flight mass spectrometry

MDA; malondialdehyde

mRNA; messenger ribonucleic acid

NOX2; nonphagocytic oxidase-2

PBS; phosphate buffered saline

qPCR; quantitative real-time polymerase chain reaction

POW; postoperative week

SPT; serine palmitoyl-CoA transferase

TNFα; tumor necrosis factor-α

5/6NCKD; 5/6 nephrectomy chronic kidney disease

Introduction

In recent years, chronic kidney disease (CKD) patients have been rising in number worldwide due to the aging population and increases in lifestyle-related diseases. [1] Many epidemiological studies have revealed CKD as a risk factor for cardiovascular disease (CVD) such as stroke and myocardial infarction. [2-5] Accordingly, new strategies to prevent CVD are important for the long-term survival and quality of life of CKD patients, [6, 7] but the exact mechanism by which CVD develops in CKD patients remains unclear.

Sulfatides are a type of glycosphingolipid that are present in the serum. Produced in the liver, [8-12] serum sulfatides exert an anti-coagulation effect by inhibiting platelet adhesion to von Willebrand factor in the blood. As serum sulfatides directly affect the function of fibrinogen and thrombin, [13] a decrease in sulfatide levels can contribute to hypercoagulability. Moreover, since sulfatides were accumulated in arteriosclerotic lesions of a familial hypercholesterolemia animal model [14], they might contribute to the progression of arteriosclerosis. An epidemiological study also showed that low serum sulfatide concentration was associated with a history of CVD in hemodialysis (HD) patients to suggest that sulfatide abnormalities enhanced CVD development. [15]

Regarding an association between chronic kidney dysfunction and serum sulfatide perturbations, serum sulfatide levels in end-stage renal disease (ESRD) patients undergoing HD were significantly lower than those in healthy people. [15] Serum sulfatides in HD patients gradually decreased with treatment period duration. [16] This phenomenon was normalized by kidney function improvement following kidney transplantation. [17, 18] The above findings suggest a close relationship between chronic kidney dysfunction and serum sulfatide aberrations. However, it has been difficult to demonstrate a definitive association from epidemiological studies since HD patients possess multiple confounding comorbidities. Animal models of chronic kidney dysfunction are useful for clarifying such

relationships. Our previous reports investigating the influence of kidney dysfunction on serum sulfatide status employed an acute kidney injury (AKI) model of protein-overload nephropathy. [19, 20] With no experimental study clearly assessing whether serum sulfatide levels were decreased in chronic kidney dysfunction and the mechanism of sulfatide change, the present investigation adopted 5/6 nephrectomy chronic kidney disease (5/6NCKD) model mice as a representative disease model. We also examined the mechanism of serum sulfatide reductions by evaluating the changes in sulfatide metabolism in liver tissues of 5/6NCKD mice and in a Hepa1-6 murine liver cell line.

Materials and methods

Mouse experiments

All animal experiments were conducted in accordance with animal study protocols approved by the Shinshu University School of Medicine (Registration number: 290005). Wild-type C57BL/6J male mice were purchased from Japan SLC (Hamamatsu, Japan). To create the 5/6NCKD mice, a 5/6 nephrectomy was performed in 11-week-old mice in 2 stages under 3 types of mixed anesthetic agents (Medetomidine, Butorphanol, and Midazolam). In the first stage, the right kidney was decapsulated via a right flank incision to avoid ureter and adrenal damage, and the upper and lower poles were resected. Bleeding was controlled with thrombin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). After 1 week, the entire left kidney was decapsulated and removed by a left flank incision in the second stage. [21] Eleven 5/6NCKD mice were created. Thirteen control mice underwent sham operations (simple right flank incision in 11-week-old mice and left flank incision in 12-week-old mice without nephrectomy). All mice were maintained in a pathogen-free environment under controlled conditions (25°C; 12-hour light/dark cycle) with tap water and a standard rodent diet provided *ad libitum*. The time points for obtaining sulfatide data were at the 4^{th} and 12^{th} week after the second operation (herewith, "week after the second operation" is indicated as "postoperative week [POW]"). Five of the 11 5/6NCKD mice and 5 of the 13 control mice were sacrificed at 4 POW to observe the time course of sulfatide change. The remaining 6 5/6NCKD mice and 8 control mice were sacrificed at 12 POW. Five 10-week-old mice that had not undergone any operation were sacrificed to obtain preoperative data. Serum, liver, and kidney samples were harvested from each animal. Urine samples and body weight, dietary intake, and blood pressure data were collected 1 week before the first stage operation and at 4, 8, and 12 POW using mice that lived until 12 POW in each group. Urine samples as the total volume per day of

each group were collected using metabolic cages (TECNIPLAST Co., Tokyo, Japan). All samples were stored at −80°C until biochemical analysis. Urinary volume per day per body (mL/day/body) and dietary intake per day per body (g/day/body) were calculated by dividing the total amount of each group by the number of mice. Blood pressure was measured using a non-invasive tail-cuff apparatus (BP-98A, Softron Co., Tokyo, Japan).

Cell line experiments

The Hepa1-6 mouse hepatoma cell line (code no. CRL-1830, ATCC, Manassas, VA, USA) was cultured in DMEM (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% (v/v) fetal bovine serum (MP Biomedicals, Santa Ana, CA, USA), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (FUJIFILM Wako Pure Chemical Corporation) in cell culture dishes at 37°C and a humidified atmosphere of 5% CO_2. The cells were fed every 2-3 days. Cells (2×10^6/dish) were seeded into 10 dishes (55 cm^2) and cultivated in DMEM for 3 days. Then, the cells of 5 dishes were cultivated in DMEM either with 0.2 mM indoxyl sulfate (IS) (Nacalai Tesque, Kyoto, Japan) (IS group) or without (control group) for 2 days. The concentration of IS in the medium (0.2 mM) was determined in accordance with a previous report [22] and the serum IS level in 5/6NCKD mice. Regarding the exposure time of Hepa1-6 cells to IS, since the cells reached confluency at 4-5 days after seeding, there was a risk of characteristic changes if left over 2 days. A previous report [22] also set the maximum exposure time at 2 days. Cells were washed with phosphate buffered saline (PBS) just before collection using PBS and a scraper to prevent mixture with the medium. The samples were centrifuged and the supernatant was removed. The precipitated cells were stored at −80°C until biochemical analysis.

Quantitative analyses of sulfatides

After weighing, liver tissues were homogenized in 4 volumes of cold water. When the Hepa1-6 cells were treated, 6×10^6 cells were homogenized in 500 μL of cold water. A total of 50 μL of liver homogenate, 50 μL of murine serum, and 100 μL of Hepa1-6 cellular homogenate were treated with 18 volumes of n-hexane/isopropanol solution (3:2, v/v) for preparation of lipid extracts. [23] Aliquots of 50 μL of standard human serum samples, whose sulfatide concentration has been established [24], were used as the standard for quantification of sulfatide concentrations in unknown samples. The extracts were then treated with methanolic sodium hydroxide to convert sulfatides to their corresponding lysosulfatides (LS; sulfatides without fatty acids), as described previously. [24] The resulting LS samples were purified through Mono-tip C18 cartridges (GL Sciences, Tokyo, Japan) and analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) together with the calibrator of N-acetylated LS-sphinganine (d18:0). [24] MALDI-TOF MS analysis was performed on a TOF/TOF 5800 system (AB Sciex, Framingham, MA, USA) in negative ion reflector mode with 2-point external calibration using N-acetylated LS-d18:0 ([M–H]−584.310) and LS-(4E)-sphingenine (d18:1) ([M–H]−540.284) peaks. We employed 9-aminoacrydine as the matrix for MALDI-TOF MS, which was an improvement from the method reported previously. [25] Since the main sulfatides existing in the murine serum/liver are galactosylsulfatides (SM4 series) and because sulfatides earlier reported as related to CVD were of the SM4 series [15], the following SM4 series molecular species of LS based on the differences in sphingoid base structure were detected: LS-sphingadienine (d18:2), LS-d18:1, LS-d18:0, LS-phytosphingosine (t18:0), LS-(4E)-icosasphingenine (d20:1), LS-icosasphinganine (d20:0), and LS-4D-hydroxyicosasphinganine (t20:0). The sulfatide concentration of each sample was calculated by comparing the amounts of lyso-sulfatide in murine/Hepa1-6 samples with those of lyso-sulfatide in standard human

serum after correction with the calibrator (N-acetylated LS-sphinganine [d18:0]). For sulfatide levels in liver tissue, the total sulfatide level in a sample (pmol/mg wet liver weight) was calculated as the sum of the above LS species. For sulfatide levels in Hepa1-6 cells, the total protein concentration in homogenates was measured as an internal standard and total sulfatide concentration was divided by total protein concentration. The amount of total sulfatides per 1 g protein in a sample (pmol/g-protein) was calculated.

Immunoblot analyses

Whole-liver lysates and whole Hepa1-6 cell lysates were prepared as described previously [26-29], and their protein concentrations were determined with a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes. [30] After blocking, the membranes were incubated with commercially available primary antibodies followed by horse radish peroxidase secondary antibodies and then treated with ECL Prime Western Blotting Detection Reagent (GE Healthcare, Chicago, IL, USA). Primary antibodies against arylsulfatase A (ARSA), ceramide galactosyltransferase (CGT), cerebroside sulfotransferase (CST), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and serine palmitoyl-CoA transferase (SPT) were purchased from Abcam (Cambridge, UK). Antibodies against galactosylceramidase (GALC) were obtained from Proteintech Group (Chicago, IL, USA), while those against 4-hydroxynonenal (HNE) were procured from ALEXIS Biochemicals (Farmingdale, NY, USA). The positions of the protein bands were determined by Precision Plus Protein Standards (Bio-Rad Laboratories, Hercules, CA, USA). Band intensities were measured densitometrically using an ECL Imager (Thermo Fisher Scientific), normalized to those of GAPDH, and subsequently expressed as fold changes relative to those of the

control mice.

mRNA analyses

Total RNA in liver tissue or Hepa1-6 cell samples was extracted using an RNeasy Mini kit (QIAGEN, Hilden, Germany), and 1 μg of total RNA was reverse-transcribed using a SuperScript III First-Strand synthesis system (Thermo Fisher Scientific). cDNA was subjected to quantitative real-time polymerase chain reaction (qPCR) using a SYBR Premix Ex Taq II (Takara Bio, Shiga, Japan) on a Step One Plus (Thermo Fisher Scientific). Gene-specific primers were designed using Primer Express software (Applied Biosystems, Waltham, MA, USA) as shown in Table 1. Expression data were normalized to those of the GAPDH gene, and the mRNA levels of the target molecules were expressed as fold changes relative to those of control mice. [31]

Other methods

Serum creatinine, aspartate aminotransferase, and alanine aminotransferase concentrations were measured with a JCA-BM6070 clinical analyzer (JEOL, Tokyo, Japan) and urinary protein concentrations were measured with a JCA-BM6050 clinical analyzer (JEOL). Serum IS was quantified by high-performance liquid chromatography (JaICA, Shizuoka, Japan). Histopathological liver and kidney tissue examination was performed as described earlier. [32, 33] The tissue content of malondialdehyde (MDA) was measured using an LPO-586 kit (OXIS International, Beverly Hills, CA, USA).

Statistical analyses

Results are expressed as the mean ± standard error. Statistical analysis was performed using the Student's *t*-test in SPSS software v11.5 J (IBM, New York, NY, USA). A P-value of <0.05 was considered statistically significant.

Results

All C57B6J mice exhibited CKD by 5/6 nephrectomy

A total of 24 mice received 5/6 nephrectomy or a sham surgery. None died during the operation, and systemic findings that indicated all had recovered sufficiently. There were no symptoms of abnormal behavior or eating disorders in the animals during the study period. Body weight and dietary intake were comparable between the test groups (Online Resource, Supplementary Figure S1). Serum levels of creatinine and IS in the 5/6NCKD mice became significantly higher than those in control mice at both 4 POW and 12 POW (Fig. 1A). Serum creatinine and IS were not significantly different between 5/6NCKD mice at 4 POW and at 12 POW. Total urine volume increased in the 5/6NCKD group after surgery (Fig. 1B). Pathological findings of kidney specimens indicated interstitial area fibrosis in all of the 5/6NCKD mice, while none was noted in controls (Fig. 1C). These findings confirmed that CKD had indeed developed in the 5/6 nephrectomy mice. However, blood pressure did not differ significantly between the groups and the amount of urinary protein did not increase after the operation (Online Resource, Supplementary Figure S1).

Serum sulfatide levels decreased while sulfatide degradative enzymes increased in 5/6NCKD mice

Serum sulfatide levels in 5/6NCKD mice were already increased at 4 POW. At both 4 POW and 12 POW, serum sulfatide levels were significantly lower in 5/6NCKD mice than in control mice. (Fig. 2A). The composition of serum sulfatides was similar between the groups (Fig. 2B). Since serum sulfatides are produced in the liver, the amount of liver sulfatides was also measured and found to decrease at both 4 POW and 12 POW (Fig. 2A). The composition of sulfatides in the liver was similar to that in the serum (Fig. 2B). Serum and liver sulfatide levels

were not remarkably different between 5/6NCKD mice at 4 POW and at 12 POW. Using liver samples of mice sacrificed at 12 POW, we next measured the mRNA expression of enzymes associated with sulfatide metabolism in liver tissue using qPCR. The mRNA expressions of sulfatide degradative enzymes such as ARSA, a major enzyme in degradation to galactosylceramides, and GALC, an enzyme causing degradation of galactosylceramides to ceramides, in 5/6NCKD mice were significantly higher than in control mice (Fig. 3A). Other mRNA expressions, including CST, a key enzyme involved in sulfatide synthesis from galactosylceramides, CGT that is responsible for the synthesis of galactosylceramides from ceramides, and SPT, a rate-limiting enzyme in the synthesis of ceramides/sphingolipids that includes sulfatides, were similar between the groups. With liver samples of mice sacrificed at 12 POW, the liver protein amounts of the above enzymes were measured by western blotting. The amounts of ARSA and GALC increased significantly and mirrored the changes in mRNA expression, while the amounts of other associated enzymes did not differ remarkably between the groups (Fig. 3B). Pathological examination of 5/6NCKD mouse livers revealed no obvious abnormalities, and serum aspartate aminotransferase and alanine aminotransferase levels in 5/6NCKD mice were comparable to those in controls (Online Resource, Supplementary Figure S2).

Sulfatide metabolism is known to be affected by oxidative stress and inflammatory responses. Accordingly, the amounts of the representative oxidative stress markers MDA and HNE in liver tissue were measured along with the mRNA amounts of pro-inflammatory response mediators (tumor necrosis factor-α [TNFα] and cyclooxygenase-2 [COX2]) and oxidative stress mediators (nonphagocytic oxidase-2 [NOX2], acyl-CoA oxidase [ACOX], and catalase [CAT]). However, there were no significant differences in the amount of any parameter between the groups (Online Resource, Supplementary Figure S3).

Exposure to IS decreased sulfatide levels and increased sulfatide degradative enzymes in a murine liver cell line

Since the 5/6NCKD mice exhibited a significant increase in serum IS levels, we hypothesized that the decreases in sulfatide levels in the serum and liver could have been caused by uremic toxins accompanying kidney dysfunction. Thus, the representative uremic toxin IS [34-38] was added to Hepa1-6 cells from murine hepatocytes for measurement of sulfatide changes. Intracellular sulfatide levels decreased similarly to as in the liver tissue of 5/6NCKD mice (Fig. 4A). Sulfatides in the Hepa1-6 cells consisted of d18:2, d18:1, d18:0, and d20:1 at a composition that was comparable between the IS and control groups.

The mRNA expression and protein amounts of the sulfatide degradative enzymes ARSA and GALC were increased in the IS group as compared with control mice (Fig. 4B, C), while those of the other sulfatide metabolic enzymes did not differ between the groups. The findings in these in vitro assays strongly indicated a close relationship of changes in sulfatide metabolism with uremic toxins caused by kidney dysfunction.

Discussion

Previous reports investigating the changes in sulfatide metabolism under kidney dysfunction have used AKI protein-overload nephropathy models [19, 20], with none adopting the present CKD model. Serum creatinine and IS levels were elevated and urinary volume was increased, which confirmed that the mice receiving 5/6 nephrectomy had CKD. We could reveal for the first time that chronic kidney dysfunction via 5/6 nephrectomy decreased serum/liver sulfatides as a result of increases in the hepatic sulfatide degradative enzymes GALC and ARSA. Additional in vitro experiments treating Hepa1-6 murine liver cells with IS, a representative uremic toxin, caused the amounts of sulfatides in liver cells to decrease, with ARSA and GALC increasing as in the liver tissue of 5/6NCKD mice. Our findings suggested that the accumulation of uremic toxins in CKD impacted sulfatide metabolism in the liver and accelerated the production of sulfatide-degrading enzymes to decrease serum sulfatide levels (Fig. 5).

Earlier studies employing the AKI model reported that expression of the sulfatide-producing enzyme CST was dramatically decreased by increases in oxidative stress and acute inflammation and that a decreased ability of sulfatide production primarily contributed to reductions in serum sulfatide levels. [19, 20] The accumulation of uremic toxins and mild interstitial fibrosis were observed in both AKI and 5/6NCKD mice. However, urinary protein, hypertension, deterioration of general condition, increases in aminotransferase and oxidative stress, pro-inflammatory response, and pathological liver injury were all absent in the latter. Such 5/6NCKD mice characteristics have been reported elsewhere; Kren et al. found that kidney injury in 5/6NCKD mice did not progress after nephrectomy and that urinary protein did not appear in 5/6NCKD mice, in contrast to 5/6NCKD rats. [39] Moreover, systemic inflammatory markers and oxidative stress markers were scarcely elevated in 5/6NCKD mice. [40, 41] In the AKI model, damage by systemic inflammation and oxidative stress were caused by a rapid decrease

in renal function and/or agents administered to cause kidney injury. Thus, the influence of uremic toxin accumulation due to chronic kidney dysfunction on sulfatide metabolism was difficult to evaluate directly. Here, the influences of inflammation and oxidative stress were considered weak and uremic toxin accumulation from nephron reduction was clearly present in 5/6NCKD mice. A strength of this study is that it considers sulfatide metabolism change in CKD from a different point of view than earlier ones using an AKI model.

We observed that uremic toxins such as IS affected liver cells, which are the origin of serum sulfatides, and accelerated the production of sulfatide-degrading enzymes to decrease sulfatide concentration in the serum and liver tissue. IS modulates the expression of several liver proteins, including cytochrome P450 and uridine diphosphate-glucuronyl transferase. [22, 42] Regarding its molecular biological mechanisms, Jenifer et al. suggested IS to be an agonist for transcription factors in the cytoplasm that regulated the transcription of multiple genes in liver cells. [43] There is a possibility that IS also influences the production of sulfatide-degrading enzymes and decreases serum and liver sulfatides through a mechanism unrelated to inflammation and oxidative stress. More detailed investigation of the mechanisms activating sulfatide degradative enzyme production by IS is needed.

In CKD patients, a decrease in serum sulfatides is suspected to be a cause of CVD. [13-15] As our CKD mouse model indicated that chronic kidney dysfunction could directly decrease serum sulfatides, such diminished sulfatide concentration might evoke hypercoagulability and the progression of arteriosclerosis in CKD patients towards CVD. [13, 14] We are next planning to examine if sulfatide injection into blood vessels can prevent CVD using a CVD-prone mouse model.

This study has several limitations. First, it could not explain all of the changes in sulfatide metabolism seen in the internal environment of CKD patients. Many CKD patients have multiple comorbidities, some of which

(hypertension, diabetes mellitus, hyperlipidemia, obesity, etc.) generate oxidative stress and inflammation. [44-48] Therefore, serum sulfatides in CKD patients may be decreased by both mechanisms: enhancement of sulfatide degradation caused by uremic toxin accumulation and downregulation of sulfatide production caused by some comorbidities. [15-18] Further verification is needed using liver tissue from CKD patients. Second, although Hepa1-6 cells were employed to investigate the influence of IS on liver cells, the composition of sulfatides in Hepa1-6 cells was different from that in mouse liver tissue. Thus, the results from liver tissues may not have been compatible with those from cultured cells, necessitating the future analysis of cell lines and tissue with similar sulfatide composition. However, the CKD environment did not remarkably affect the composition of sulfatides in either in vivo or in vitro experiments; the changes in sulfatide metabolism in CKD are more likely quantitative than qualitative. Third, since the objective of this study was to investigate serum sulfatide metabolic change in the CKD condition, we specifically measured sulfatides using MALDI-TOF MS, which precluded us from investigating other glycosphingolipids or ceramide, a sulfatide precursor. Further study of the change and influence of these molecules are needed using other detection methods. Particularly regarding ceramides, their levels may be altered in CKD mice owing to increases in sulfatide degradative enzymes, as indicated in Figure 5. Indeed, Mitsnefes et al. reported that ceramides were increased in CKD patients. [49] The enhancement of sulfatide decomposition that was seen in this study might also be a cause of ceramide increase. Fourth, we studied the influence of IS, a representative uremic toxin, on sulfatide metabolism in liver tissue using Hepa1-6 cells. However, there are many other kinds of uremic toxins, including p-cresyl sulphate, 3-carboxy-4-methyl-5-propyl-2-furan propionate, and such electrolytes as potassium. [50,51] Sulfatide metabolism in 5/6NCKD mice and CKD patients may accordingly be influenced by many uremic toxins other than IS. Although IS is suggested to be a cause of sulfatide decomposition enhancement in the murine liver, it

is difficult to completely account for decomposition by IS only. Further testing is needed. Lastly, because this study employed mice, its applicability in humans is unclear. Sulfatide composition differs between humans and mice [15-18], but the phenomenon of decreasing sulfatides in the internal environment of CKD appears to be a common finding, indicating that the pathogenetic mechanism of human CKD may be extrapolated somewhat from the results of this investigation.

Conclusion

Serum levels of sulfatides are decreased in 5/6NCKD mice. The accumulation of uremic toxins such as IS caused by chronic kidney dysfunction appears to accelerate the degradation of sulfatides in the liver, which is the origin of serum sulfatides.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Figure Legends

Figure 1. C57BL/6J mice undergoing 5/6 nephrectomy exhibited CKD. A) Serum creatinine and IS concentrations before the first operation (before operation) and at 4 and 12 POW. Mice before operation, N=5; Control mice at 4 POW, N = 5; 5/6NCKD mice at 4 POW, N = 5; Control mice at 12 POW, N = 8; 5/6NCKD mice at 12 POW, N = 6. B) Urinary volume at 1 week before operation and at 4, 8, and 12 POW. Control mice, N = 8; 5/6NCKD mice, N = 6. C) Renal pathological specimens of 5/6NCKD mice at 12 POW (Masson Trichrome staining). Dotted line, control mice; solid line, 5/6NCKD mice. Error bars represent standard error. P-values comparing control mice and 5/6NCKD mice were calculated by the Student's *t*-test. **, P<0.01.

Figure 2. Serum level and hepatic amount of sulfatides in 5/6NCKD mice A) Serum levels and liver amounts of sulfatides before operation and at 4 and 12 POW. B) Composition of serum and liver sulfatides at 12 POW. Mice before operation, N=5; Control mice at 4 POW, N = 5; 5/6NCKD mice at 4 POW, N = 5; Control mice at 12 POW, N = 8; 5/6NCKD mice at 12 POW, N = 6. Dotted line, control mice; solid line, 5/6NCKD mice. Error bars represent standard error. P-values comparing control mice and 5/6NCKD mice were calculated by the Student's *t*-test. **, P<0.01

Figure 3. mRNA expression level and protein amount of sulfatide metabolic enzymes in liver tissue of 5/6NCKD mice. A) Expression of mRNA encoding sulfatide degradative enzymes (ARSA and GALC) and sulfatide-producing enzymes (CST, CGT, and SPT) at 12 POW. mRNA levels were normalized to those of GAPDH, and then normalized to hepatic levels of control mice. B) Protein levels of sulfatide degradative enzymes (ARSA and GALC) and

sulfatide-producing enzymes (CST, CGT, and SPT) at 12 POW. Protein levels were normalized to those of GAPDH, and then normalized to hepatic levels of control mice. Control mice; N = 8, 5/6NCKD mice; N = 6. Open bars, control mice; closed bars, 5/6NCKD mice. Error bars represent standard error. P-values comparing control mice and 5/6NCKD mice were calculated by the Student's *t*-test. *, P<0.05; **, P<0.01

Figure 4. Intracellular sulfatide level, mRNA expression level, and protein amount of sulfatide degradative enzymes in Hepa1-6 cells treated with IS. A) Sulfatide amount in murine Hepa1-6 cells. The IS group was treated with 0.2 mM IS. B) Expression of mRNA encoding sulfatide degradative enzymes (ARSA and GALC) and sulfatide-producing enzymes (CST, CGT, and SPT). mRNA levels were normalized to those of GAPDH, and then normalized to hepatic levels of control mice. C) Protein levels of sulfatide degradative enzymes (ARSA and GALC) and sulfatide-producing enzymes (CST, CGT, and SPT). Protein levels were normalized to those of GAPDH, and then normalized to levels of the control group. Control group, N = 5; IS group, N = 5. Open bars, control group; closed bars, IS group. Error bars represent standard error. P-values comparing the control group and IS group were calculated by the Student's *t*-test. *, P<0.05; **, P<0.01

Figure 5. Conceptual diagram showing sulfatide metabolism changes in CKD. In CKD conditions, the hepatic sulfatide degradative enzymes ARSA and GALC are increased and sulfatide degradation in the liver is accelerated, followed next by a reduction in serum sulfatide levels. Uremic toxins such as IS are thought to contribute to the increases in ARSA and GALC in the liver.

Figure 1

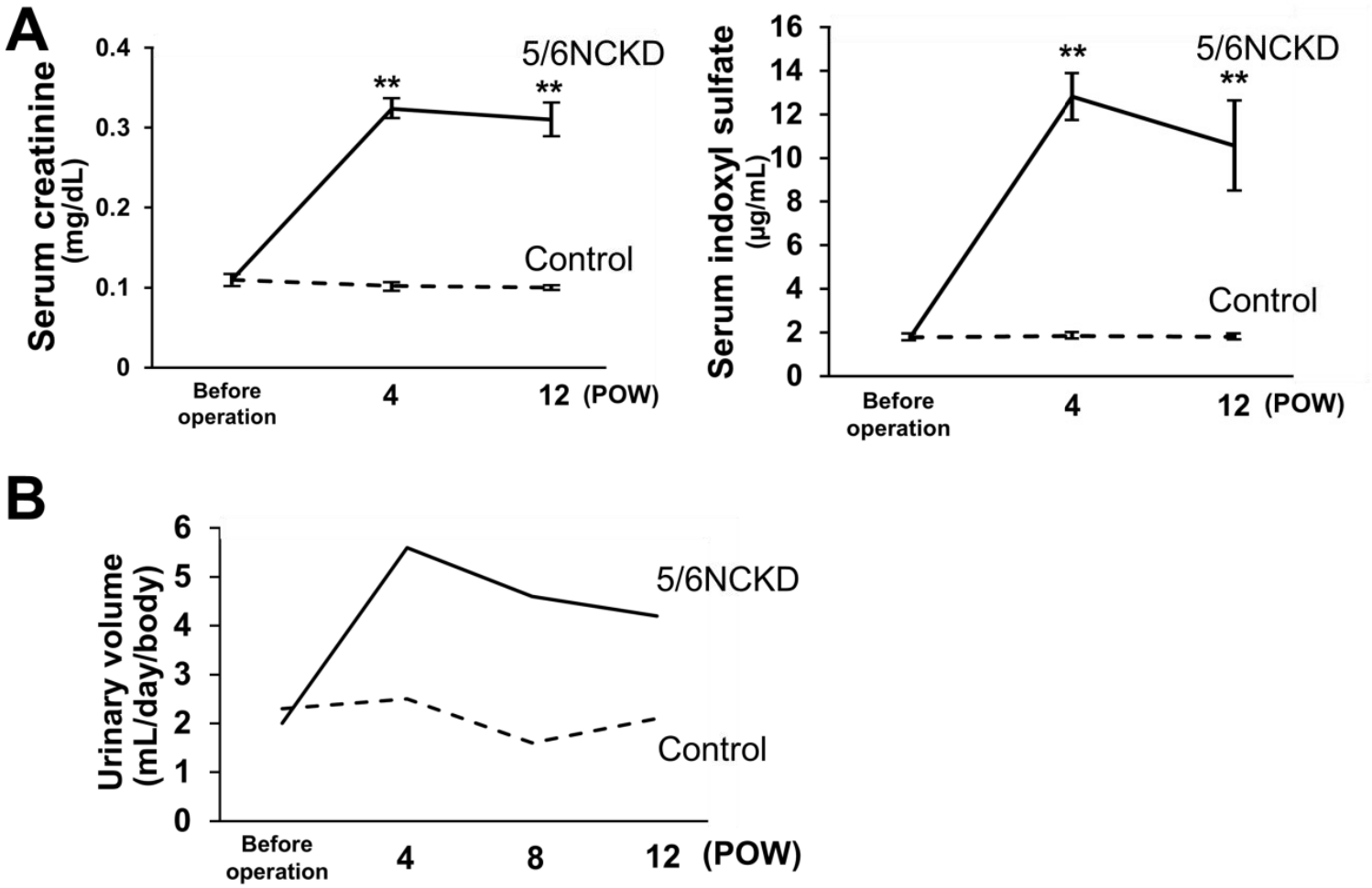

A
Serum creatinine (mg/dL)
0.4
0.3
0.2
0.1
0
5/6NCKD
Control
**
**
Before operation
4
12 (POW)
Serum indoxyl sulfate (μg/mL)
16
14
12
10
8
6
4
2
0
5/6NCKD
Control
**
**
Before operation
4
12 (POW)
B
Urinary volume (mL/day/body)
6
5
4
3
2
1
0
5/6NCKD
Control
Before operation
4
8
12 (POW)
C
Control (12 POW)
300 μm
5/6NCKD (12 POW)
300 μm

Figure 2

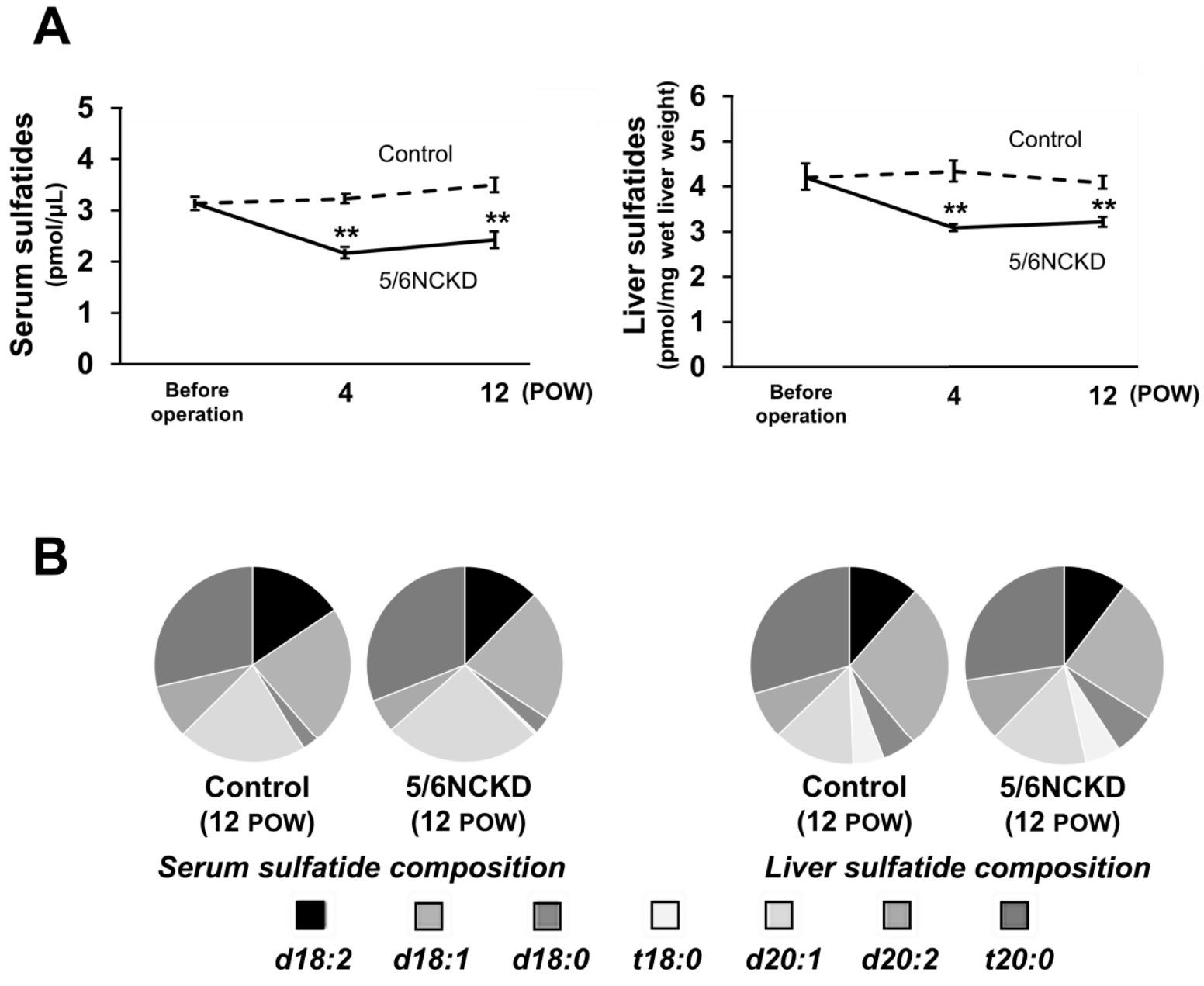

A
Serum sulfatides
(pmol/μL)
5
4
3
2
1
0
Control
5/6NCKD
**
**
Before operation
4
12 (POW)
Liver sulfatides
(pmol/mg wet liver weight)
6
5
4
3
2
1
0
Control
5/6NCKD
**
**
Before operation
4
12 (POW)
B
Control (12 POW)
5/6NCKD (12 POW)
Serum sulfatide composition
Control (12 POW)
5/6NCKD (12 POW)
Liver sulfatide composition
d18:2
d18:1
d18:0
t18:0
d20:1
d20:2
t20:0

Figure 3

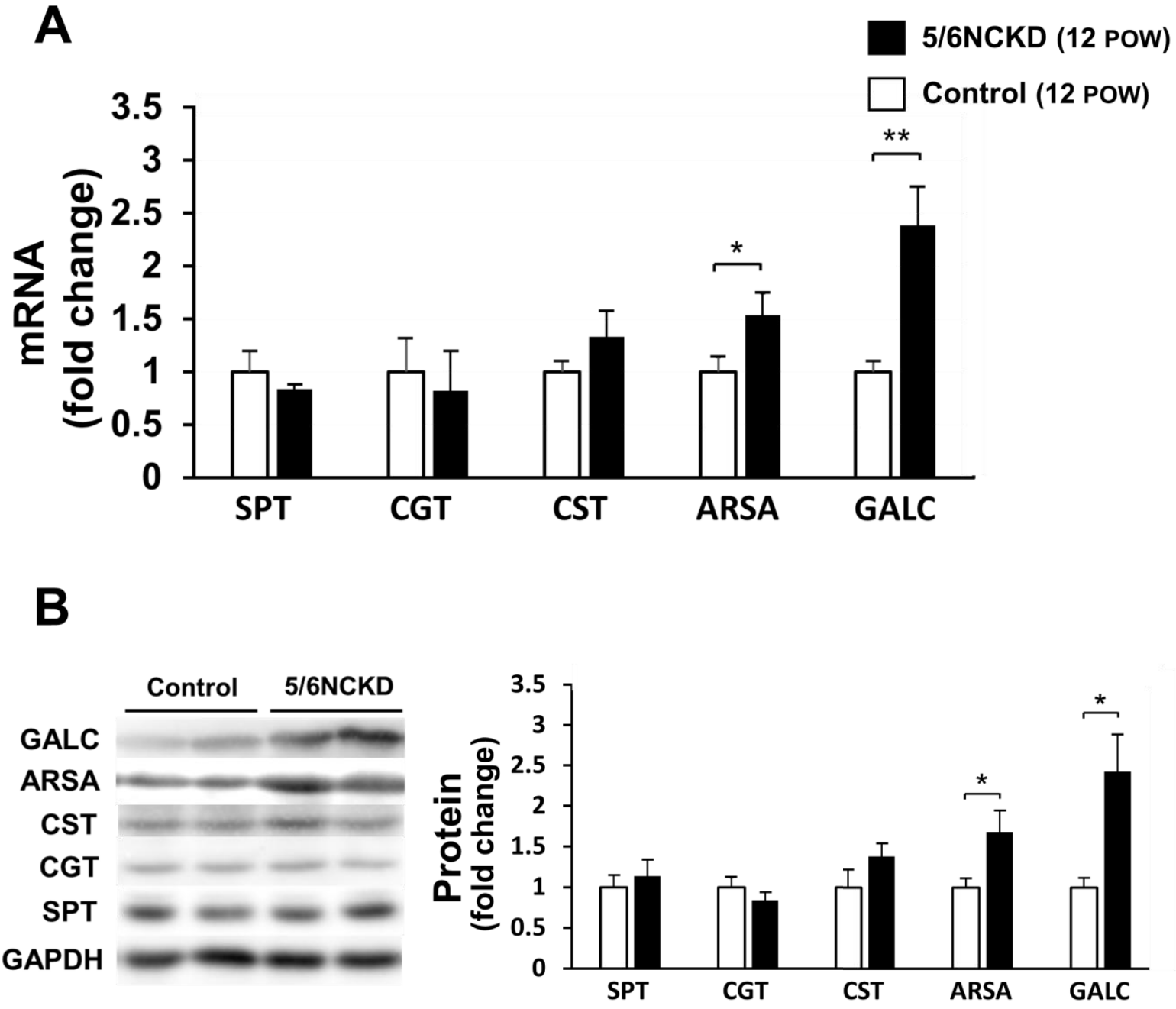

A
5/6NCKD (12 POW)
Control (12 POW)
mRNA (fold change)
3.5
3
2.5
2
1.5
1
0.5
0
**
*
SPT
CGT
CST
ARSA
GALC
B
Control
5/6NCKD
GALC
ARSA
CST
CGT
SPT
GAPDH
Protein (fold change)
3.5
3
2.5
2
1.5
1
0.5
0
*
*
SPT
CGT
CST
ARSA
GALC

Figure 4

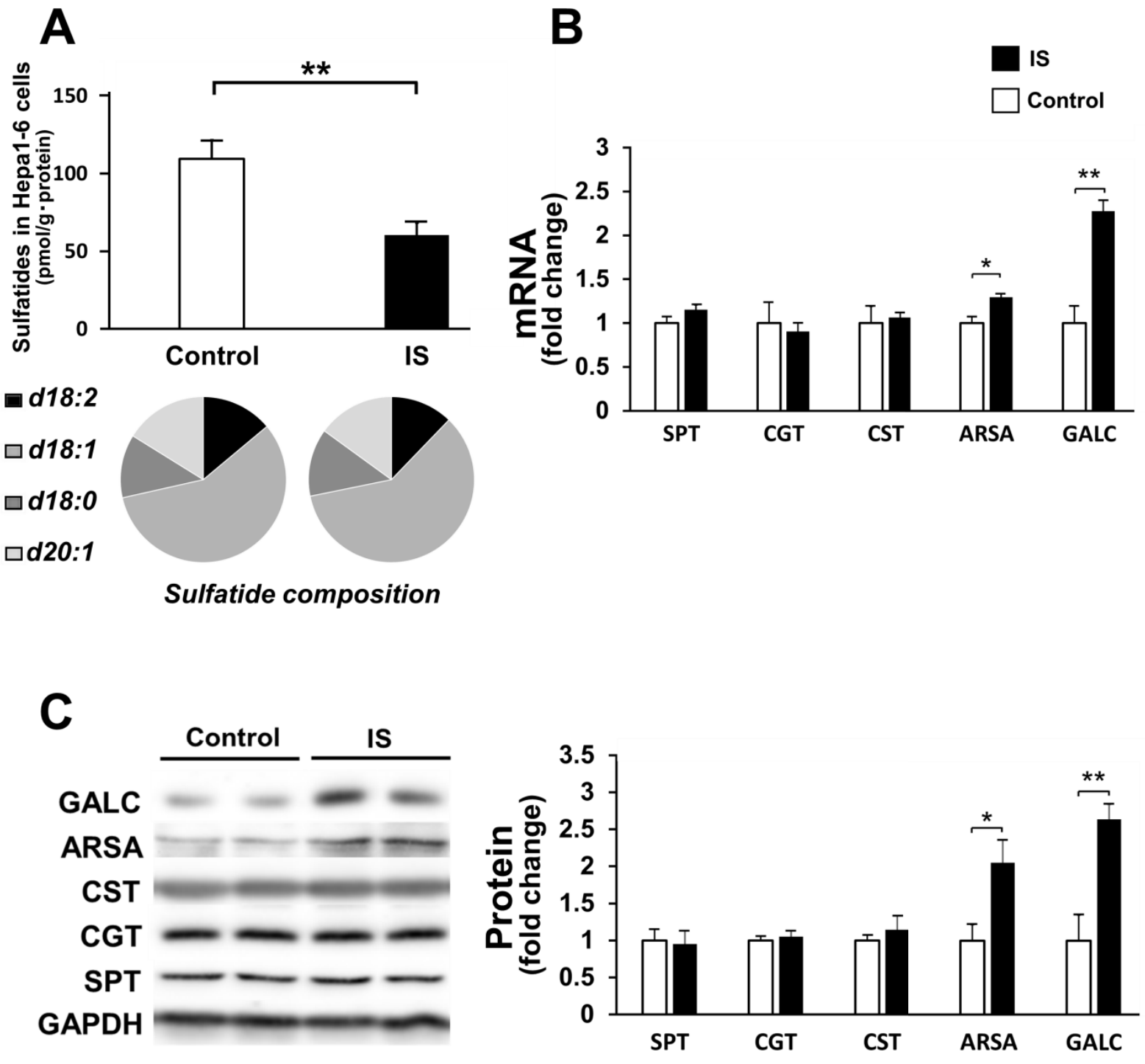
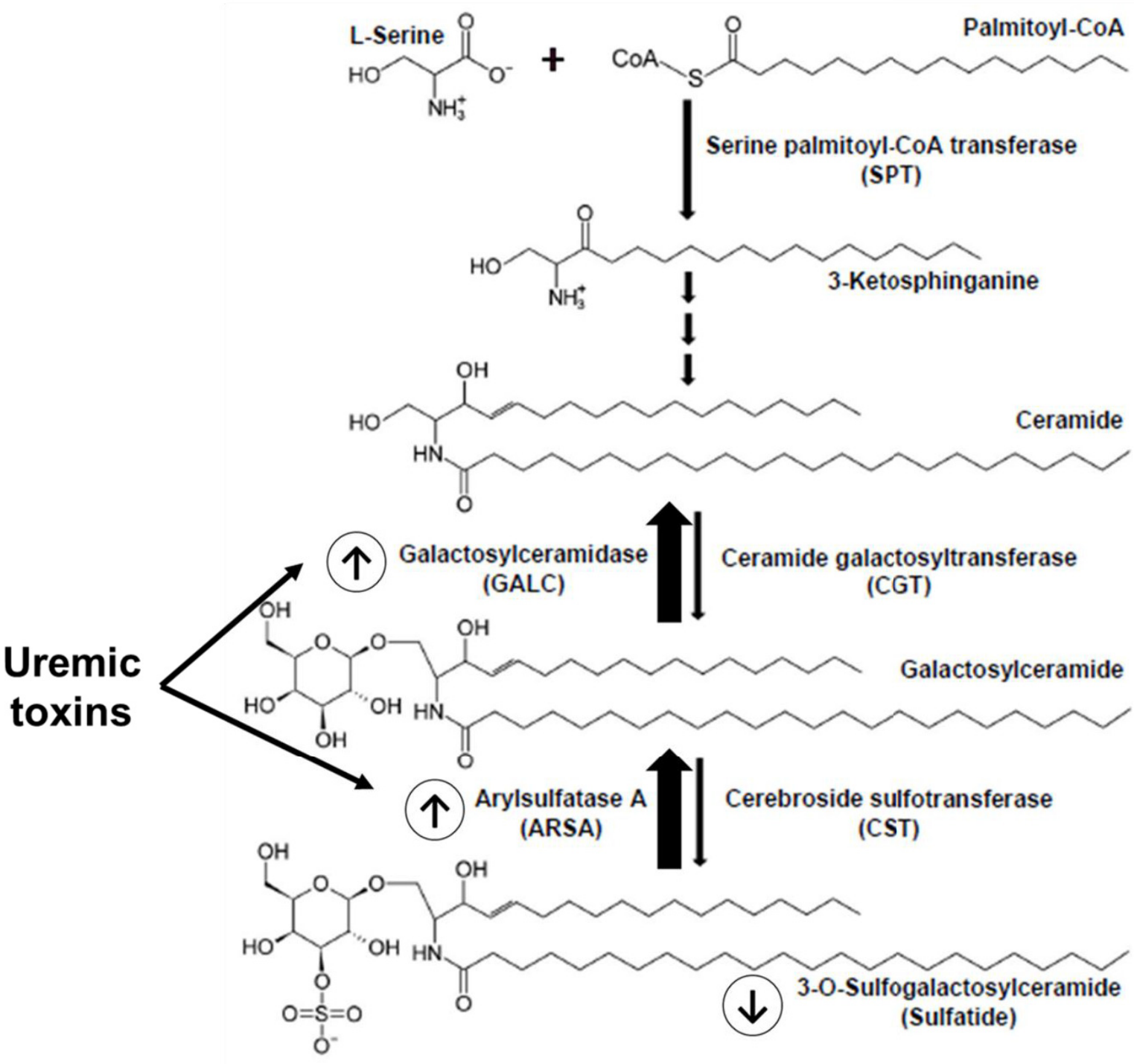

A
**
Sulfatides in Hepa1-6 cells
(pmol/g·protein)
150
100
50
0
Control
IS
d18:2
d18:1
d18:0
d20:1
Sulfatide composition
B
IS
Control
mRNA
(fold change)
3
2.5
2
1.5
1
0.5
0
*
**
SPT
CGT
CST
ARSA
GALC
C
Control
IS
GALC
ARSA
CST
CGT
SPT
GAPDH
Protein
(fold change)
3.5
3
2.5
2
1.5
1
0.5
0
*
**
SPT
CGT
CST
ARSA
GALC

Figure 5


L-Serine
Palmitoyl-CoA
Serine palmitoyl-CoA transferase
(SPT)
3-Ketosphinganine
Ceramide
Galactosylceramidase
(GALC)
Ceramide galactosyltransferase
(CGT)
Uremic
toxins
Galactosylceramide
Arylsulfatase A
(ARSA)
Cerebroside sulfotransferase
(CST)
3-O-Sulfogalactosylceramide
(Sulfatide)

Article title: Impact of Chronic Kidney Dysfunction onSerum Sulfatidesand Its Metabolic Pathwayin Mice

Journal name: Glycoconjugate Journal

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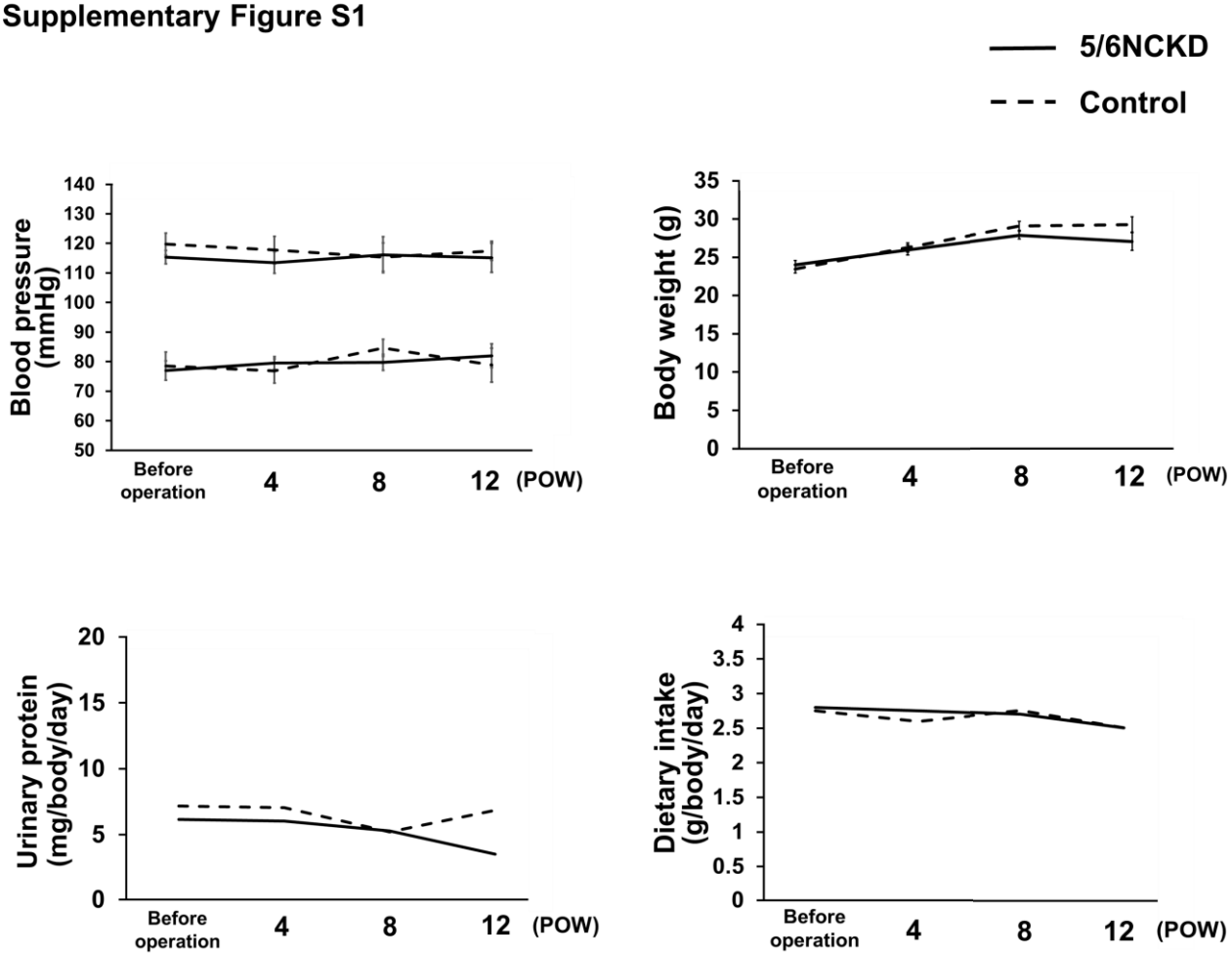
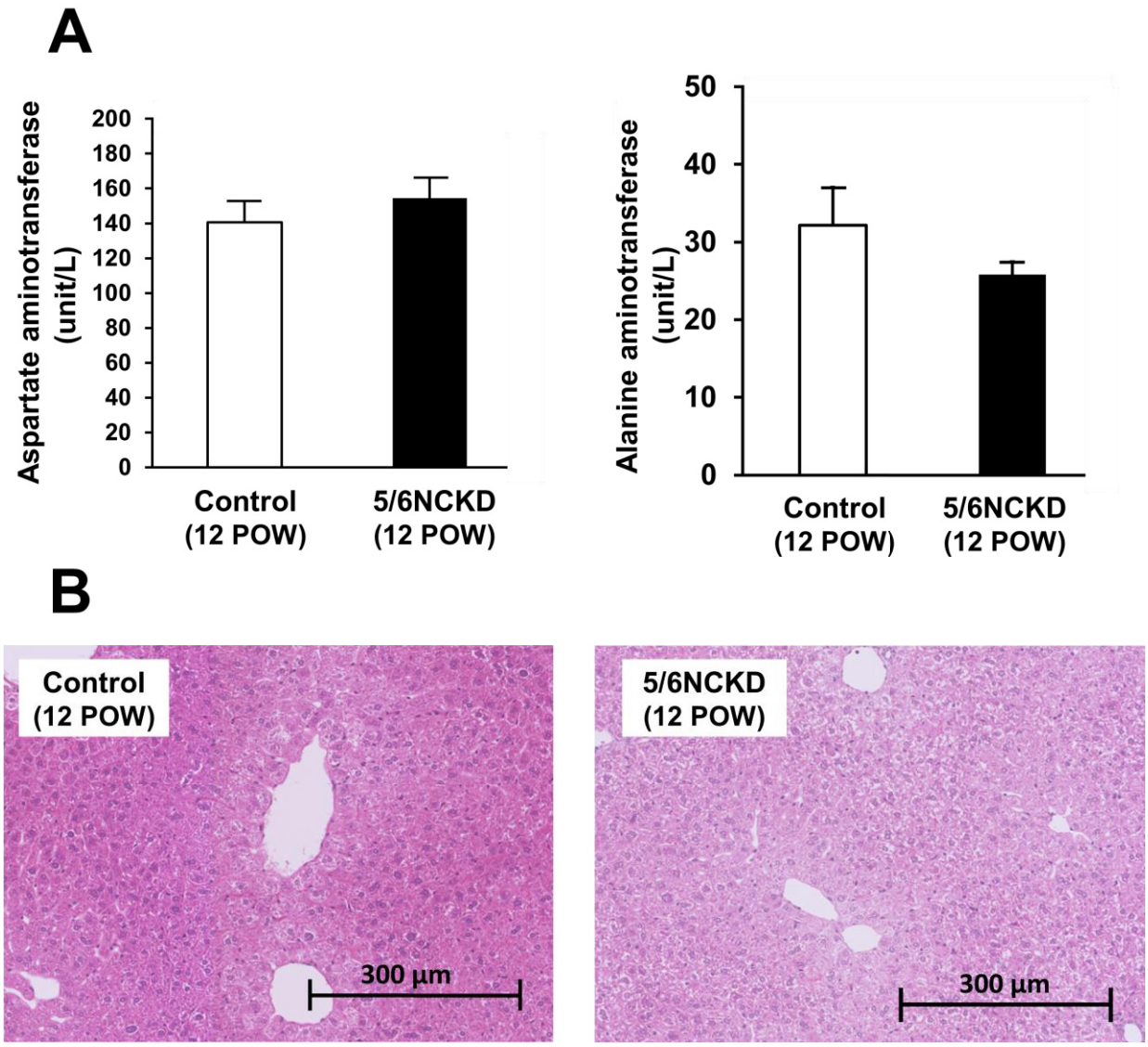
Supplementary Figure S1



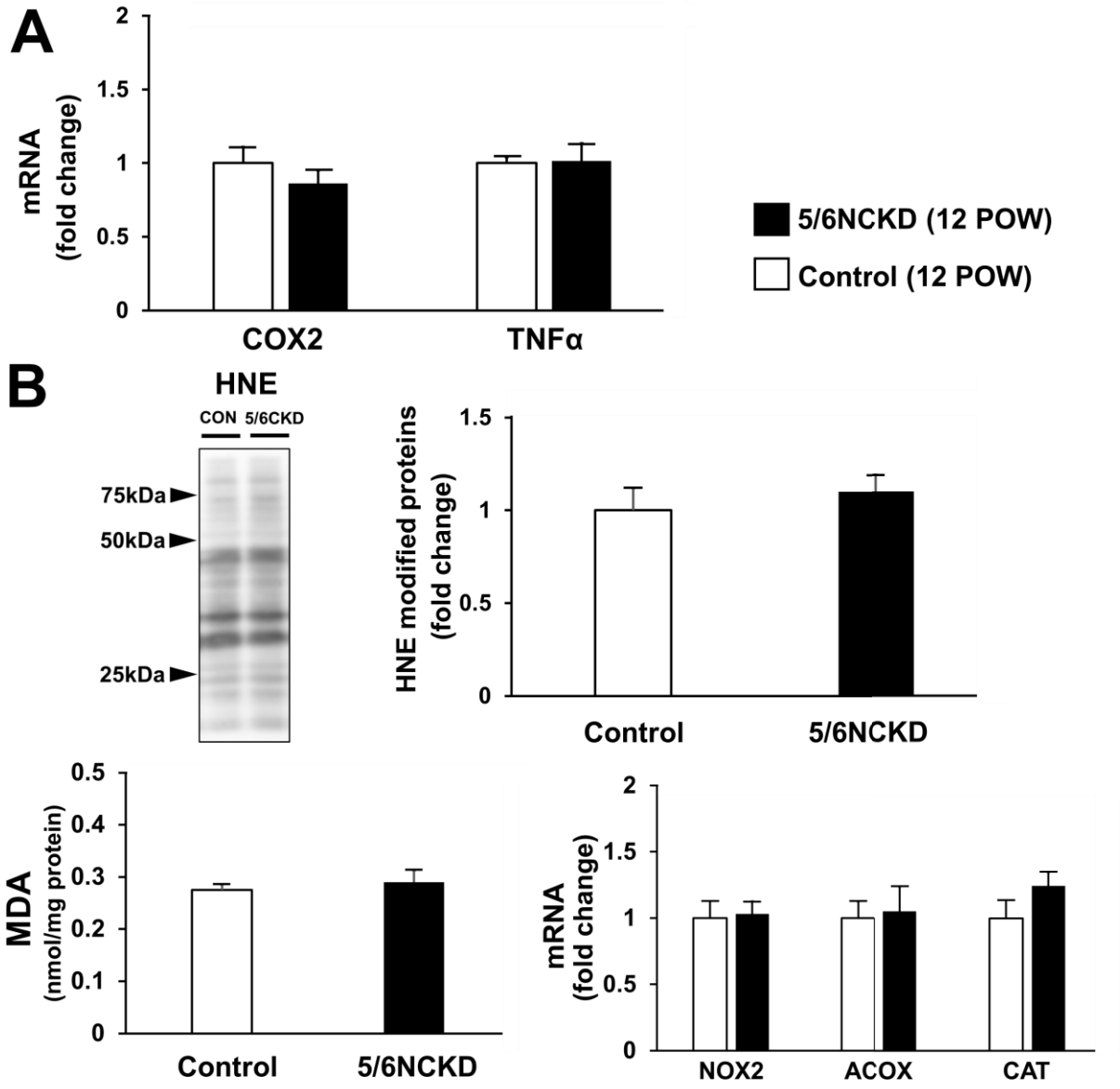
Supplementary Figure S1. *Changes in blood pressure, body weight, urinary protein, and dietary intake in 5/6NCKD mice and controls.* Blood pressure, body weight, urinary protein, and dietary intake from 1 week before the first operation (before operation) to 4, 8, and 12 POW. Blood pressure and body weight did not differ significantly between the groups. Urinary protein did not increase in either group and dietary intake did not decrease. Control mice, N = 8; 5/6NCKD mice, N = 6. Dotted lines, control mice; solid lines, 5/6NCKD mice. Error bars represent standard error. P-values comparing control mice and 5/6NCKD mice were calculated by the Student's *t*-test.

Supplementary Figure S2



Supplementary Figure S2. *Condition of liver tissue in 5/6NCKD mice and controls.* A) Serum levels of aspartate aminotransferase and alanine aminotransferase at 12 POW were comparable between the groups. B) There was no evidence of liver injury in either group in pathological specimens at 12 POW (hematoxylin and eosin staining). Control mice, N = 8; 5/6NCKD mice, N = 6. Open bars, control mice; closed bars, 5/6NCKD mice. Error bars represent standard error. P-values comparing control mice and 5/6NCKD mice were calculated by the Student's *t*-test.

Supplementary Figure S3



Supplementary Figure S3. *Changes in mRNA expression of pro-inflammatory mediators and oxidative stress markers in liver tissue of 5/6NCKD mice and controls.* A) In mRNA analysis of typical pro-inflammatory mediators (COX2 and TNFα) at 12 POW, there were no significant differences between the groups. B) Analysis of oxidative stress markers at 12 POW. Upper: immunoblot of HNE-modified proteins in liver tissues. Results were similar between the groups. Lower left: measurement of MDA in liver tissues. There was no significant difference between the groups. Lower right: mRNA expressions of NOX2, ACOX, and CAT were comparable between the groups. mRNA levels were normalized to those of GAPDH, and then normalized to hepatic levels of control mice. Control

mice, N = 8; 5/6NCKD mice, N = 6. Open bars, control mice; closed bars, 5/6NCKD mice. Error bars represent standard error. P-values comparing control mice and 5/6NCKD mice were calculated by the Student's *t*-test.

Table 1. Primer pairs used for qPCR

Gene	Gene bank accession number	Primer sequence (5' to 3')
Acox	NM_015729	F GGTATGGTGTCGTACTTGAATGAC
		R AATTTCTACCAATCTGGCTGCAC
Arsa	NM_009713	F ACCACCCCTAACCTGGATCAGT
		R ATGGCGTGCACAGAGACACA
Cat	NM_009804	F CGACCAGGGCATCAAAAACTT
		R AACGTCCAGGACGGGTAATTG
Cgt	NM_011674	F TGGGTCCAGCCTATGGATGT
		R GCAGCGTTGGTCTTGGAAAC
Cox2	NM_011198	F TGACCCCCAAGGCTCAAATATG
		R ACCCAGGTCCTCGCTTATGAT
Cst	NM_016922	F ATGGCCTTCACGACCTCAGA
		R CGGTCTTGTGCGTCTTCATG
Galc	NM_008079	F GAGTGAGAATCATAGCGAGCGATA
		R AGTTCCTGGTCCAGCAGCAA
Gapdh	M32599	F TGCACCACCAACTGCTTAG
		R GGATGCAGGGATGATGTTCTG
Nox2	NM_007807	F GAAAACTCCTTGGGTCAGCACT
		R ATTTCGACACACTGGCAGCA
Sptlc2	NM_011479	F TTTCCTGCTACCCCGATCA
		R AGCAGATCCCCAACTTCATCT
Tnfα	NM_013693	F CAGCCGATGGGTTGTACCTT
		R GTGGGTGAGGAGCACGTAGTC

F: forward sequence, R: reverse sequence