

Acute kidney injury induced by protein-overload nephropathy down-regulates gene expression of hepatic cerebroside sulfotransferase in mice, resulting in reduction of liver and serum sulfatides

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Abbreviations: ARSA, arylsulfatase A; CGT, ceramide galactosyltransferase; COX2, cyclooxygenase-2; CST, cerebroside sulfotransferase; GALC, galactosylceramidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HNE, 4-hydroxynonenal; LS, lysosulfatides; d18:2, sphingadienine; d18:1, (4*E*)-sphingenine; d18:0, sphinganine; t18:0, phytosphingosine; d20:1, (4*E*)-icosasphingenine; d20:0, icosasphinganine; t20:0, 4*D*-hydroxyicosasphinganine; MALDI-TOF MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; MDA, malondialdehyde; NF- κ B, nuclear factor- κ B; NOX, nonphagocytic oxidase; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; SD, standard deviation; SEM, standard error of the mean; Sp1, specificity protein 1; TNF α , tumor necrosis factor- α .

Abstract

Sulfatides, possible antithrombotic factors belonging to sphingoglycolipids, are widely distributed in mammalian tissues and serum. We recently found that the level of serum sulfatides was significantly lower in hemodialysis patients than that in normal subjects, and that the serum level closely correlated to the incidence of cardiovascular disease. These findings suggest a relationship between the level of serum sulfatides and kidney function; however, the molecular mechanism underlying this relationship remains unclear. In the present study, the influence of kidney dysfunction on the metabolism of sulfatides was examined using an established murine model of acute kidney injury, protein-overload nephropathy in mice. Protein-overload treatment caused severe proximal tubular injuries within 4 days, and this treatment obviously decreased both serum and hepatic sulfatide levels. The sphingoid composition of serum sulfatides was very similar to that of hepatic ones at each time point, suggesting that the serum sulfatide level is dependent on the hepatic secretory ability of sulfatides. The treatment also decreased hepatic expression of cerebroside sulfotransferase (CST), a key enzyme in sulfatide metabolism, while it scarcely influenced the expression of the other sulfatide-metabolizing enzymes, including arylsulfatase A, ceramide galactosyltransferase, and galactosylceramidase. Pro-inflammatory responses were not detected in the liver of these mice; however, potential oxidative stress was increased. These results suggest that down-regulation of hepatic CST expression, probably affected by oxidative stress from kidney injury, causes reduction in liver and serum sulfatide levels. This novel mechanism, indicating the crosstalk between kidney injury and specific liver function, may prove useful for helping to understand the situation where human hemodialysis patients have low levels of serum sulfatides.

Keywords: sulfatides; sphingoglycolipids; acute kidney injury; protein-overload nephropathy; peroxisome proliferator-activated receptor α (*Ppara*)-null mice; cerebroside sulfotransferase (CST).

Introduction

Sulfatides are sphingoglycolipids consisting of sphingoid, fatty acid, galactose and sulfate [1]. They are widely distributed in mammalian tissues such as brain, kidney, liver and the digestive tract [1-3]. Sulfatides are also contained in serum as components of lipoprotein [1,4]. However, their physiological functions in blood are not fully understood. We have reported the antithrombotic potential of sulfatides in some experimental studies [4-8] and recently showed that the level of serum sulfatides might be a novel biomarker for cardiovascular diseases in human hemodialysis patients [9]. These findings suggest that the investigation of the *in vivo* kinetics of sulfatides affected by kidney dysfunction would be important.

Our clinical research demonstrated that the serum sulfatide level was significantly lower in human hemodialysis patients than that in normal subjects, suggesting a relationship between the serum level and kidney function [9]. To clarify the relationship, we investigated the influence of kidney dysfunction on sulfatide metabolism using an established murine model of acute kidney injury, protein-overload nephropathy in peroxisome proliferator-activated receptor α (*Ppara*)-null mice [10]. Our earlier study demonstrated that *Ppara*-null mice exhibit higher sensitivity to tubular toxic factors accompanied with proteinuria, and that protein-overload treatment induces severe proximal tubular injuries within 4 days in these mice [10].

Materials and methods

Mice and protein-overload treatment

All animal experiments were conducted in accordance with animal study protocols approved by the Shinshu University School of Medicine. *Ppara*-null mice were on a Sv/129 genetic background as described elsewhere [11]. The mice were raised in a specific pathogen-free environment under controlled conditions (25°C; 12 h light/dark cycle) and maintained with tap water and a standard rodent diet *ad libitum*. Eighteen-week-old male *Ppara*-null mice weighing 30-35 g were used in this study. The mice were given consecutive daily intraperitoneal bolus injections of 0.4 g of bovine serum albumin (Sigma-Aldrich Corporation, St. Louis, MO, USA) for 4 days [10]. At days 1 and 4, the mice were sacrificed under anesthesia for collection of blood and tissues. Isolated liver was cannulated via the portal vein and perfused with 20 ml of ice-cold buffer (0.15 M KCl and 1 mM EDTA, pH 7.25) to wash out blood. Kidneys were not perfused because of the difficulties involved in applying this method. The numbers of mice subjected to analysis were as follows: control group (untreated), $n = 4$; day 1 group, $n = 6$; and day 4 group, $n = 8$. To neglect the blood contamination in all tissues, we also tried a systemic perfusion method in the sample preparation process, using the other *Ppara*-null mice of protein-overload nephropathy. After the protein-overload treatment, 30 ml of the ice-cold KCl/EDTA buffer was injected in to each mouse through the left heart ventricle ($n = 3$ in each group). Both samples prepared by each method were used for measurement of the tissue content of sulfatides.

Quantitation and identification of sulfatides

Sulfatides were extracted from 50 μ l of serum or tissue homogenate obtained from each mouse according to the hexane/isopropanol method [12] and were converted to lysosulfatides (LS; sulfatides without fatty acids) by saponification with sodium hydroxide [13]. After purification, the LS samples were desalted by Mono-tip C18

cartridges (GL Sciences, Tokyo, Japan) followed by addition of *N*-acetyl LS possessing sphinganine (LS-d18:0 NAc) as the internal standard. They were then analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) using a Voyager Elite XL Biospectrometry Workstation (PerSeptive Biosystems, Framingham, MA, USA) in negative ionization mode [13]. A two-point external calibration was performed with LS-d18:0 NAc ($[M-H]^-$, 584.31) and LS-(4*E*)-sphinganine (LS-d18:1) ($[M-H]^-$, 540.28). Seven molecular species of LS were detected, based on the differences in sphingoid base structure: LS-sphingadienine (LS-d18:2), LS-d18:1, LS-d18:0, LS-phytosphingosine (LS-t18:0), LS-(4*E*)-icosasphinganine (LS-d20:1), LS-icosasphinganine (LS-d20:0), and LS-4*D*-hydroxyicosasphinganine (LS-t20:0) [1,13]. The level of sulfatides was calculated as the sum of the levels of these seven LS species.

Immunoblot analysis

Whole-tissue lysates (liver and kidney) were prepared from each mouse as described previously [14-17]. Protein concentrations were measured using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) [18]. The lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking, the membranes were incubated with primary antibodies followed by alkaline phosphatase-conjugated secondary antibodies [19,20]. Mouse polyclonal primary antibodies against cerebroside sulfotransferase (CST) and ceramide galactosyltransferase (CGT), as well as mouse monoclonal primary antibodies against galactosylceramidase (GALC), were purchased from Abnova Corporation (Jhousih St., Taiwan). Goat polyclonal primary antibodies against arylsulfatase A (ARSA), rabbit polyclonal primary antibodies against actin and 4-hydroxynonenal (HNE) were obtained from Everest Biotech (Upper Heyford, Oxfordshire, UK), Santa Cruz Biotechnology (Santa Cruz, CA, USA), and ALEXIS Biochemicals (Farmingdale, NY, USA), respectively. Band intensities were measured densitometrically, normalized to those of actin and then normalized to hepatic levels of control *Ppara*-null mice.

Analysis of mRNA expression

Total tissue RNA (liver and kidney) was extracted using an RNeasy Mini Kit (QIAGEN, Hilden, Germany), and 2 µg of total RNA were reverse-transcribed using oligo-dT primers and SuperScript II reverse transcriptase (Invitrogen Corporation, Carlsbad, CA, USA). Levels of mRNA were quantified by real-time polymerase chain reaction (PCR) using a SYBR Premix Ex Taq™ II (Takara Bio, Otsu, Japan) on a Thermal Cycler Dice TP800 system (Takara Bio) [19,20]. The specific primers were designed by Primer Express software (Applied Biosystems, Foster City, CA, USA) as follows: 5'-ATGGCCTTCACGACCTCAGA-3' and 5'-CGGTCTTGTGCGTCTTCATG-3' for CST (GenBank accession number, NM_016922); 5'-ACCACCCCTAACCTGGATCAGT-3' and 5'-ATGGCGTGCACAGAGACACA-3' for ARSA (NM_009713); 5'-TGGGTCCAGCCTATGGATGT-3' and 5'-GCAGCGTTGGTCTTGGAAC-3' for CGT (NM_011674); 5'-GAGTGAGAATCATAGCGAGCGATA-3' and 5'-AGTTCCTGGTCCAGCAGCAA-3' for GALC (NM_008079); 5'-CAGCCGATGGGTTGTACCTT-3' and 5'-GTGGGTGAGGAGCACGTAGTC-3'

for tumor necrosis factor- α (TNF α) (NM_013693); 5'-TGACCCCAAGGCTCAAATATG-3' and 5'-ACCCAGGTCCTCGCTTATGAT-3' for cyclooxygenase-2 (COX2) (NM_011198); 5'-GAAAACTCCTTGGGTCAGCACT-3' and 5'-ATTCGACACACTGGCAGCA-3' for nonphagocytic oxidase (NOX) 2 (NM_007807); 5'-AGCATCTGCATCTGTCCTGAAC-3' and 5'-GACTGTCCGGCACATAGGTA AAA-3' for NOX4 (NM_015760); 5'-TTCCCCTTTGTCTCCAAGACTCT-3' and 5'-TCCCCGATCATCACCTTTGT-3' for carbamoyl-phosphate synthase I (NM_001080809); 5'-AAATTCGGGATGCACCTTCA-3' and 5'-GCATACTGCTCTGCTAGCTTGACT-3' for ornithine carbamoyltransferase (NM_008769); and 5'-TGCACCACCAACTGCTTAG-3' and 5'-GGATGCAGGGATGATGTTCTG-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (M32599). The GAPDH mRNA was used as an internal control. The mRNA levels are shown as the ratio of target gene/GAPDH.

Other methods

Serum urea nitrogen and creatinine concentrations were measured with a clinical analyzer (JCA-BM2250; JEOL, Tokyo, Japan). Serum transaminase levels were determined by a Transaminase CII-test kit (Wako Pure Chemical Industries, Osaka, Japan). Histopathological examination of liver and kidney tissues was performed as described earlier [10,21]. The tissue content of malondialdehyde (MDA) was measured using a LPO-586 kit (OXIS International, Beverly Hills, CA, USA).

Statistical analysis

Data from immunoblot and real-time PCR are expressed as mean \pm standard error of the mean (SEM). Other data are shown as mean \pm standard deviation (SD). Statistical analysis was performed using one-way analysis of variance followed by Dunnett's test (SPSS Statistics 17.0; SPSS inc., Chicago, IL, USA). A probability value of less than 0.05 was considered to be statistically significant.

Results

Sulfatide levels in serum, liver and kidney

As in the previous study [10], protein-overload treatment caused acute kidney injuries in *Ppara*-null mice (serum urea nitrogen, 21.4 ± 3.3 , 31.2 ± 3.9 , and 77.4 ± 10.8 mg/dl for control, day 1, and day 4 groups, respectively; serum creatinine, 0.31 ± 0.05 , 0.78 ± 0.06 , and 1.01 ± 0.14 mg/dl for control, day 1, and day 4 groups, respectively), due to obvious proximal tubular damages. On the other hand, this treatment barely induced apparent liver injuries (serum transaminases were within the normal limit; pathological findings of hematoxylin & eosin stain had minimal changes). These results were consistent with the previous findings showing the acute kidney injuries without apparent hepatic damages in the *Ppara*-null mice of protein-overload nephropathy. The level of serum sulfatides was markedly decreased by protein-overload treatment in a time-dependent manner (Fig. 1A, bar graph). Hepatic content of sulfatides was also decreased in parallel to the serum reduction (Fig. 1B, bar graph). In contrast, renal content of sulfatides was increased (Fig. 1B, bar graph). The content of hepatic sulfatides was lower than that of kidney; however, the sphingoid composition of hepatic

sulfatides was very similar to that of the serum ones at each time point (Fig. 1, pie charts). To neglect the possibility of blood contamination in each tissue, we measured the tissue content of sulfatides again using organs after systemic perfusion. The hepatic content of sulfatides in the control, day 1, and day 4 groups was 34.3 ± 3.3 , 22.3 ± 3.4 , and 10.8 ± 2.4 pmol/mg, respectively, and that of kidney was 1523.3 ± 46.3 , 1725.6 ± 51.2 , and 1911.6 ± 54.9 pmol/mg, respectively. The contents and sphingoid composition of these organs were consistent with those in Fig. 1B. Therefore, blood contamination would not have influenced the sulfatides data. These findings suggest that the levels of serum sulfatides would be dependent on the hepatic secretory ability of the sulfatides.

Altered expression of sulfatide-metabolic enzymes via protein-overload treatment

To investigate the mechanism underlying the alteration of sulfatide content in each tissue, the expression of sulfatide-metabolic enzymes was examined. Protein and gene expression of CST, a key enzyme of sulfatide synthesis from galactosylceramides [22], was significantly decreased in the liver in a time-dependent manner (Fig. 2). In contrast, renal expression of CST was markedly increased (Fig. 2). The expressions of the other sulfatide-metabolic enzymes including ARSA (an enzyme degrading sulfatides to galactosylceramides), CGT and GALC (enzymes functioning in the synthesis and degradation of galactosylceramides, the precursor of sulfatides, respectively) were not changed by protein-overload treatment in each tissue (Fig. 2 and 3). These results suggest that the alteration of hepatic and renal sulfatide contents is due to gene expression of CST in each tissue. The down-regulation of hepatic CST gene expression induced by protein-overload treatment might cause reduction of liver and serum sulfatide levels.

Pro-inflammatory responses and oxidative stress induced by protein-overload

To elucidate the mechanism underlying the alteration of sulfatide metabolism in liver and kidney, we investigated the influences of protein-overload treatment on these organs. Since the earlier study demonstrated that protein-overload treatment induced apparent inflammation and oxidative stress in kidney [10], we evaluated these pathogenic factors. As shown in our previous study, protein-overload treatment markedly increased the renal expression of mRNAs encoding pro-inflammatory mediators, including TNF α and COX2, known targets of the nuclear factor- κ B (NF- κ B) signaling (Fig. 4A). Furthermore, this treatment greatly elevated the oxidative stress markers such as HNE and MDA in the kidney, and increased the renal expression of mRNA encoding NADPH oxidase, a representative enzyme of superoxide generation (Fig. 4B). In the liver, this treatment scarcely affected the expressions of the pro-inflammatory cytokines, while it mildly increased the oxidative stress and the mRNA expression of NADPH oxidase (Fig. 4). We could not detect the obvious changes in hepatic mRNA expression of other oxidative enzymes, such as acyl-CoA oxidase and cytochrome P450 4A, as well as those of antioxidative enzymes including catalase, glutathione peroxidase, and superoxide dismutase (data not shown). These results suggest that protein-overload treatment greatly induced both pro-inflammatory responses and oxidative stress in the kidney, while the treatment induced potential oxidative stress without apparent inflammation in the liver. The differences of pathogenic situations in each organ might result in different regulations in sulfatide metabolism.

Discussion

The present study demonstrated that both serum and hepatic sulfatide levels were decreased in the established murine model of acute kidney injuries, protein overload nephropathy in *Ppara*-null mice. The sphingoid composition of serum sulfatides was very similar to that of hepatic ones, suggesting that the serum level would be dependent on the hepatic secretory ability of the sulfatides. Hepatic gene expression of CST alone was considerably decreased, which might be responsible for the reduction in liver and serum sulfatide levels. In contrast to the obvious kidney injuries, apparent liver injuries accompanied with inflammation were not detected, however, potential oxidative stress was increased in these mice. These findings indicate the presence of crosstalk between kidney injury and specific liver function through pathogenic effects of oxidative stress.

The potential of oxidative stress might affect other liver functions such as amino acid metabolism. To evaluate this hypothesis, we measured the hepatic expression of mRNAs encoding key enzymes in the urea cycle, carbamoyl-phosphate synthase I and ornithine carbamoyltransferase, in *Ppara*-null mice of protein-overload nephropathy. These levels were elevated by approximately 4.3- and 6.2-fold at day 1 and 3.5- and 3.7-fold at day 4, respectively. These findings suggest that protein-overload treatment could affect diverse liver functions without apparent hepatocyte damages. These functional alterations in the liver might be derived from the enhancement of oxidative stress. Many past studies demonstrated that patients with kidney disease are under a considerable level of oxidative stress [23,24]. Therefore, the systemic oxidative stress of these patients might interfere with specific hepatic functions, such as metabolism of sulfatides.

Today there is no information concerning transcription factors affecting gene expression of CST. An earlier study has identified multiple sites of exon 1 on the murine gene of CST [25]. Based on this information, we surveyed the transcription factor-binding sites on the CST gene promoter region, using two different programs (MATCH, <http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi>; and TFSEARCH, <http://www.cbrc.jp/research/db/TFSEARCH.html>). This investigation detected the consensus sequences for specificity protein 1 (Sp1)-binding sites and NF- κ B-binding sites: Sp1-binding sites, -98/-89 of exon 1a (GGGGCGGGGG), -273/-259 of exon 1c (CCCCGCCCCGCCCC), and -25/-16 of exon 1g (GCCCCGCCCT); NF- κ B-binding sites, -2,105/-2,096 of exon 1a (GGGACTTCTC), -2,080/-2,071 of exon 1a (GGAAATGCCT), -570/-561 of exon 1c (GGAATCTCCC), and -704/-695 of exon 1e (GGAGATTCCC). Many previous studies have demonstrated that oxidative stress influences the DNA-binding activity of these transcription factors [26,27]. A study using a rat diabetic nephropathy model reported that the elevated oxidative stress decreased Sp1 transcriptional activity in both the liver and kidney via modulation of intracellular redox homeostasis, and it increased NF- κ B transcriptional activity only in the kidney [28]. The present study also demonstrated that protein-overload treatment increased oxidative stress in both the liver and kidney while it induced NF- κ B activation only in the kidney. Therefore, the reduction of hepatic CST gene expression in these mice might be derived from a decrease in Sp1 activity via oxidative stress. On the other hand, the elevation of renal CST gene expression might be strongly affected by excess activation of the NF- κ B pathway rather than the effect of Sp1 modulation. Further investigation about the precise molecular mechanism of CST

gene-regulation is necessary.

We previously found that the level of serum sulfatides was significantly decreased in human hemodialysis patients via an unknown mechanism [9]. The results obtained by the present study indicate that the reduction in serum sulfatide levels in these patients would be caused by down-regulation of hepatic CST gene expression. However, we have to consider differences between the human hemodialysis patients and this murine acute kidney injury model. First, this animal model developed acute kidney injury via intensive tubular damages, while most human patients developed chronic kidney diseases over an extended period of time. Therefore, there might be differences in the severity of oxidative stress, cytokine production, and inflammation. Hemodialysis patients are known to be exposed to a considerable amount of oxidative stress and hypercytokinemia, related to the accumulation of various uric toxins, insulin resistance, hormonal changes, promotion of the renin-angiotensin system, hemodialysis procedure, and so on [29]. The virulence of these factors might be weak compared with protein-overload treatment; however, these toxic effects are on going and might affect hepatic CST gene expression. Second, we used genetically modified animals, *Ppara*-null mice, in the present study. Earlier studies have established that human organs including liver and kidney contain considerably low levels of PPAR α , like *Ppara*-null mice [17,30]. Since PPAR α exerts anti-inflammatory, anti-oxidative, and anti-apoptotic effects, the gap in PPAR α content between rodent and human is expected to cause species differences in the phenotype. Therefore, the alteration of hepatic function responding to kidney injuries in *Ppara*-null mice might be better applied to human cases as compared with the situation using wild-type mice.

In conclusion, the results indicate that kidney injuries down-regulate the hepatic gene expression of CST, resulting in reduction of liver and serum sulfatide levels. This novel mechanism, indicating the crosstalk between kidney injuries and specific liver function, should be useful for improving the understanding of cases of human hemodialysis patients with low serum sulfatide levels. Additionally, the proposed mechanism helps to explain why human kidney dysfunction frequently accompanies cardiovascular disease [31,32].

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References

- [1] I. Ishizuka, Chemistry and functional distribution of sulfoglycolipids, *Prog. Lipid Res.* 36 (1997) 245-319.
- [2] K. Honke, Y. Zhang, X. Cheng, et al., Biological roles of sulfoglycolipids and pathophysiology of their deficiency, *Glycoconj. J.* 21 (2004) 59-62.
- [3] K. Nagai, K. Tadano-Aritomi, Y. Niimura, et al., Higher expression of renal sulfoglycolipids in marine mammals, *Glycoconj. J.* 25 (2008) 723-726.
- [4] A. Hara, T. Taketomi, Occurrence of sulfatide as a major glycosphingolipid in WHHL rabbit serum lipoproteins, *J. Biochem.* 102 (1987) 83-92.
- [5] A. Hara, T. Taketomi, Characterization and changes of glycosphingolipids in the aorta of the Watanabe hereditary hyperlipidemic rabbit, *J. Biochem.* 109 (1991) 904-908.
- [6] M. Kyogashima, The role of sulfatide in thrombogenesis and haemostasis, *Arch. Biochem. Biophys.* 426 (2004) 157-162.
- [7] A. Hara, K. Uemura, T. Taketomi, Sulfatide prolongs blood-coagulation time and bleeding time by forming a complex with fibrinogen, *Glycoconj. J.* 13 (1996) 187-194.
- [8] A. Hara, Y. Kutsukake, K. Uemura, et al., Anticoagulant activity of sulfatide and its anti-thrombotic effect in rabbit, *J. Biochem.* 113 (1993) 781-785.
- [9] R. Hu, G. Li, Y. Kamijo, et al., Serum sulfatides as a novel biomarker for cardiovascular disease in patients with end-stage renal failure, *Glycoconj. J.* 24 (2007) 565-571.
- [10] Y. Kamijo, K. Hora, K. Kono, et al., PPAR α protects proximal tubular cells from acute fatty acid toxicity, *J. Am. Soc. Nephrol.* 18 (2007) 3089-3100.
- [11] S.S. Lee, T. Pineau, J. Drago, et al., Targeted disruption of the α isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators, *Mol. Cell. Biol.* 15 (1995) 3012-3022.
- [12] A. Hara, N.S. Radin, Lipid extraction of tissues with a low-toxicity solvent, *Anal. Biochem.* 90 (1978) 420-426.
- [13] G. Li, R. Hu, Y. Kamijo, et al., Establishment of a quantitative, qualitative, and high-throughput analysis of sulfatides from small amounts of sera by matrix-assisted laser desorption ionization-time of flight mass spectrometry, *Anal. Biochem.* 362 (2007) 1-7.
- [14] T. Aoyama, Y. Uchida, R.I. Kelley, et al., A novel disease with deficiency of mitochondrial very-long-chain acyl-CoA dehydrogenase, *Biochem. Biophys. Res. Commun.* 191 (1993) 1369-1372.
- [15] T. Aoyama, M. Souri, S. Ushikubo, et al., Purification of human very-long-chain acyl-coenzyme A dehydrogenase and characterization of its deficiency in seven patients, *J. Clin. Invest.* 95 (1995) 2465-2473.
- [16] T. Aoyama, J.M. Peters, N. Iritani, et al., Altered constitutive expression of fatty acid-metabolizing enzymes in mice lacking the peroxisome proliferator-activated receptor α (PPAR α), *J. Biol. Chem.* 273 (1998) 5678-5684.
- [17] T. Nakajima, Y. Kamijo, N. Tanaka, et al., Peroxisome proliferator-activated receptor α protects against alcohol-induced liver damage, *Hepatology* 40 (2004) 972-980.
- [18] T. Aoyama, S. Yamano, D.J. Waxman, et al., Cytochrome P-450 hPCN3, a novel

- cytochrome P-450 IIIA gene product that is differentially expressed in adult human liver, *J. Biol. Chem.* 264 (1989) 10388-10395.
- [19] Y. Kamijo, K. Hora, N. Tanaka, et al., Identification of functions of peroxisome proliferator-activated receptor α in proximal tubules, *J. Am. Soc. Nephrol.* 13 (2002) 1691-1702.
- [20] N. Tanaka, K. Moriya, K. Kiyosawa, et al., PPAR α activation is essential for HCV core protein-induced hepatic steatosis and hepatocellular carcinoma in mice, *J. Clin. Invest.* 118 (2008) 683-694.
- [21] Y. Kamijo, K. Hora, T. Nakajima, et al., Peroxisome proliferator-activated receptor α protects against glomerulonephritis induced by long-term exposure to the plasticizer di-(2-ethylhexyl)phthalate, *J. Am. Soc. Nephrol.* 18 (2007) 176-188.
- [22] K. Honke, Y. Hirahara, J. Dupree, et al., Paranodal junction formation and spermatogenesis require sulfoglycolipids, *Proc. Natl. Acad. Sci. U.S.A.* 99 (2002) 4227-4232.
- [23] S. Granata, G. Zaza, S. Simone, et al., Mitochondrial dysregulation and oxidative stress in patients with chronic kidney disease, *BMC Genomics* 10 (2009) 388.
- [24] J. Kendrick, M.B. Chonchol, Nontraditional risk factors for cardiovascular disease in patients with chronic kidney disease, *Nat. Clin. Pract. Nephrol.* 4 (2008) 672-681.
- [25] Y. Hirahara, M. Tsuda, Y. Wada, et al., cDNA cloning, genomic cloning, and tissue-specific regulation of mouse cerebroside sulfotransferase, *Eur. J. Biochem.* 267 (2000) 1909-1917.
- [26] R. Schreck, K. Albermann, P.A. Baeuerle, Nuclear factor kappa B: an oxidative stress-responsive transcription factor of eukaryotic cells (a review), *Free Radic. Res. Commun.* 17 (1992) 221-237.
- [27] R. Ammendola, M. Mesuraca, T. Russo, et al., The DNA-binding efficiency of Sp1 is affected by redox changes, *Eur. J. Biochem.* 225 (1994) 483-489.
- [28] O. Ramon, H.K. Wong, M. Joyeux, et al., 2'-deoxyguanosine oxidation is associated with decrease in the DNA-binding activity of the transcription factor Sp1 in liver and kidney from diabetic and insulin-resistant rats, *Free Radic. Biol. Med.* 30 (2001) 107-118.
- [29] M. Zanetti, R. Barazzoni, G. Guarnieri, Inflammation and insulin resistance in uremia, *J. Ren. Nutr.* 18 (2008) 70-75.
- [30] F.J. Gonzalez, J.M. Peters, R.C. Cattley, Mechanism of action of the nongenotoxic peroxisome proliferators: role of the peroxisome proliferator-activator receptor α , *J. Natl. Cancer Inst.* 90 (1998) 1702-1709.
- [31] A.S. Levey, J.A. Beto, B.E. Coronado, et al., Controlling the epidemic of cardiovascular disease in chronic renal disease: what do we know? What do we need to learn? Where do we go from here? National Kidney Foundation Task Force on Cardiovascular Disease, *Am. J. Kidney Dis.* 32 (1998) 853-906.
- [32] M.J. Sarnak, A.S. Levey, A.C. Schoolwerth, et al., Kidney disease as a risk factor for development of cardiovascular disease: a statement from the American Heart Association Councils on Kidney in Cardiovascular Disease, High Blood Pressure Research, Clinical Cardiology, and Epidemiology and Prevention, *Circulation* 108 (2003) 2154-2169.

Figure legends

Fig. 1

Sulfatide content and composition in serum, liver, and kidney from *Ppara*-null mice of protein-overload nephropathy.

Serum levels (**A**) and tissue contents (**B**) of sulfatides (bar graphs), as well as their compositions (pie charts), were measured by MALDI-TOF MS. The levels of sulfatides were calculated as the sum of those of seven LS species classified by type of sphingoid base structure. Con, control group ($n = 4$); Day 1, BSA treatment group at day 1, ($n = 6$); and Day 4, BSA treatment group at day 4, ($n = 8$). Results of the level and composition are expressed as mean \pm SD and mean percentage, respectively. $*P < 0.05$, $**P < 0.01$.

Fig. 2

Expression of enzymes involved in sulfatide biosynthesis and degradation in liver and kidney from *Ppara*-null mice of protein-overload nephropathy.

(**A**) Immunoblot analysis of CST and ARSA. One hundred micrograms of whole-liver or whole-kidney lysate protein prepared from each mouse were loaded into each well and subjected to electrophoresis. Actin was used as the loading control. Band intensities were measured densitometrically, normalized to those of actin, and then normalized to hepatic levels of control *Ppara*-null mice. (**B**) Analysis of mRNA encoding CST and ARSA. Hepatic and renal mRNA levels were measured using quantitative real-time PCR and normalized to those of GAPDH, respectively. Data are expressed as mean \pm SEM. The groups and numbers of mice were the same as **Fig. 1**. $*P < 0.05$.

Fig. 3

Expression of enzymes involved in biosynthesis and degradation of galactosylceramides, the precursor of sulfatides, in liver and kidney from *Ppara*-null mice of protein-overload nephropathy.

(**A**) Immunoblot analysis of CGT and GALC. The same samples in **Fig. 2A** (100 μ g of protein) were used. Band intensities were measured densitometrically, normalized to those of actin, and then normalized to hepatic levels of control *Ppara*-null mice. (**B**) Analysis of mRNA encoding CGT and GALC. Hepatic and renal mRNA levels were measured using quantitative real-time PCR and normalized to those of GAPDH, respectively. Data are expressed as mean \pm SEM. The groups and numbers of mice were the same as **Fig. 1**.

Fig. 4

Pro-inflammatory responses and oxidative stress induced by protein-overload treatment.

(**A**) Analysis of mRNA for typical pro-inflammatory mediators. Hepatic and renal mRNA levels were measured using quantitative real-time PCR and normalized to those of GAPDH, respectively. (**B**) Analysis of oxidative stress markers. *Upper*: Immunoblot of HNE. The same samples in **Fig. 2A** (100 μ g of protein) were used. Band intensities were measured densitometrically, normalized to those of actin, and then normalized to hepatic levels of control *Ppara*-null mice. *Lower left*: Measurement of MDA. *Lower right*: Real-time PCR of tissue-specific NADPH oxidase. The hepatic and renal mRNA levels were normalized to those of GAPDH. All data are expressed as mean \pm SEM. The

groups and numbers of mice were the same as **Fig. 1**. * $P < 0.05$, ** $P < 0.01$.

Figure 1

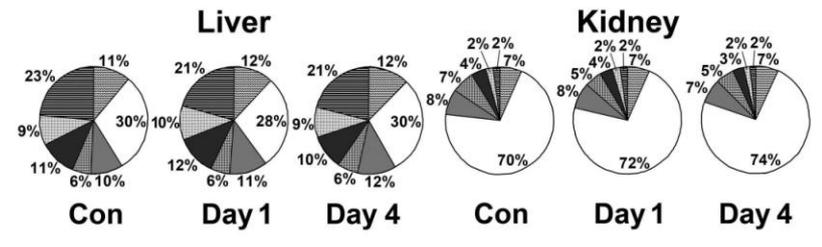
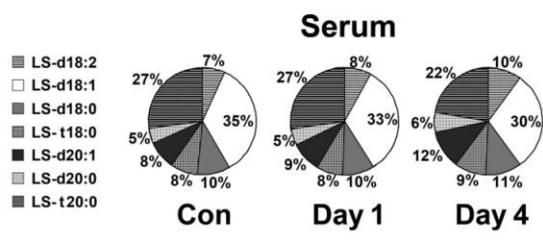
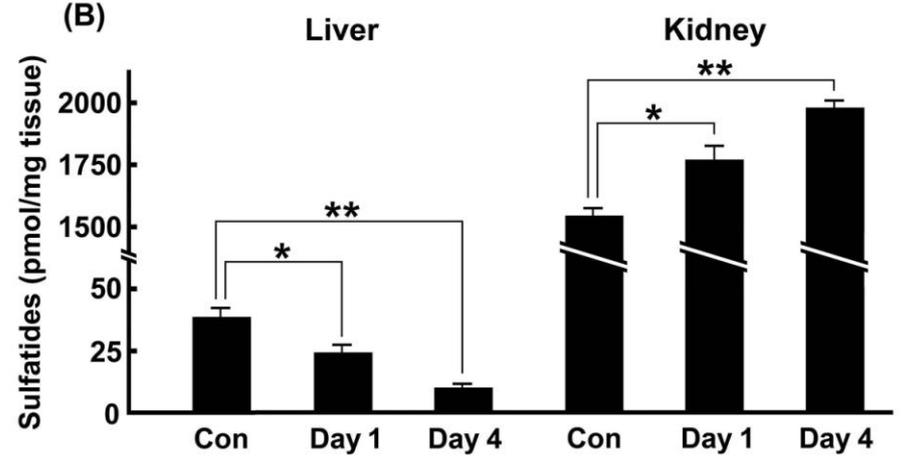
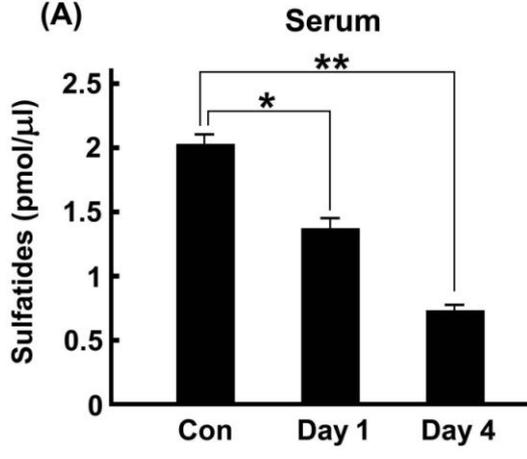


Figure 2

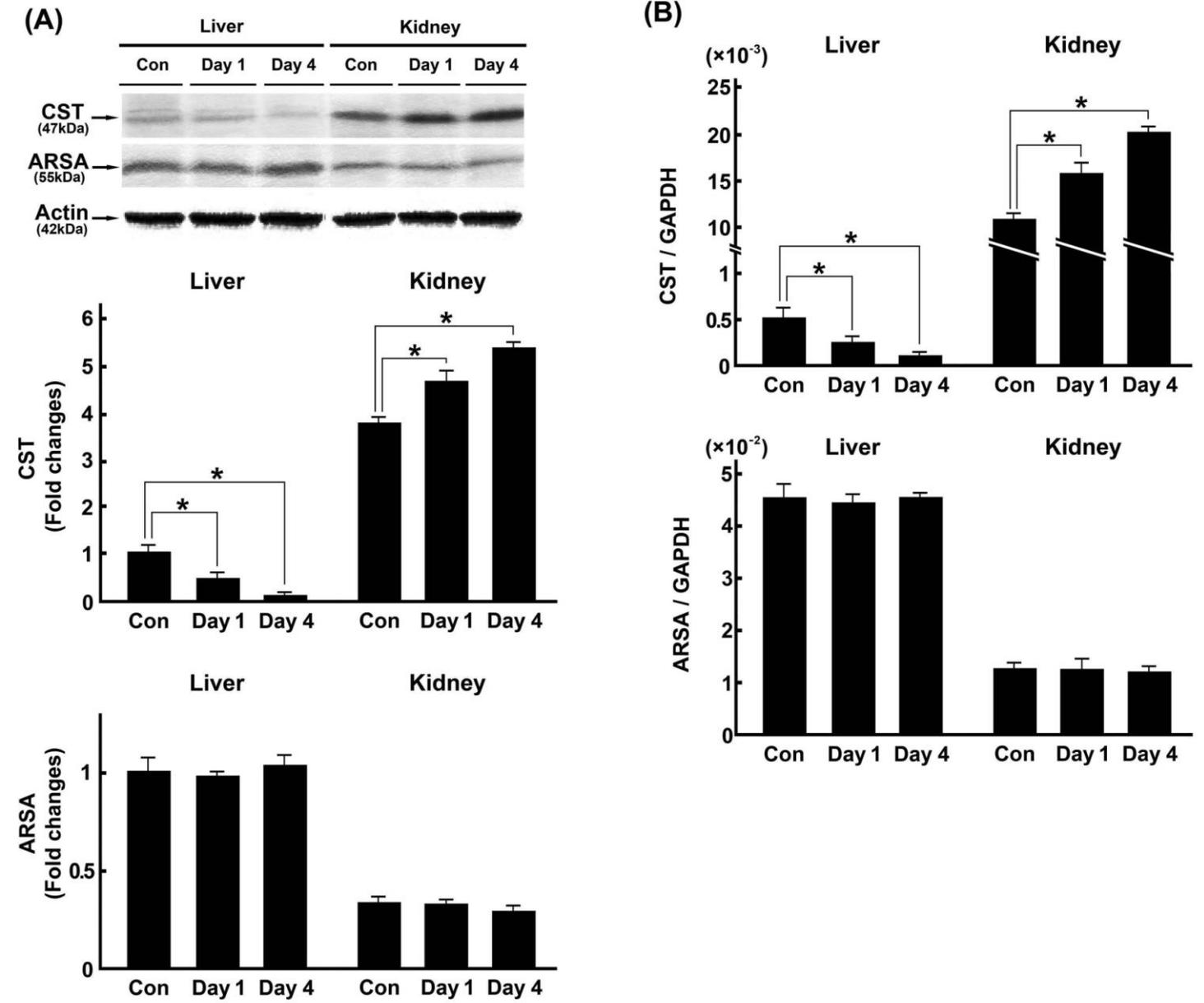


Figure 3

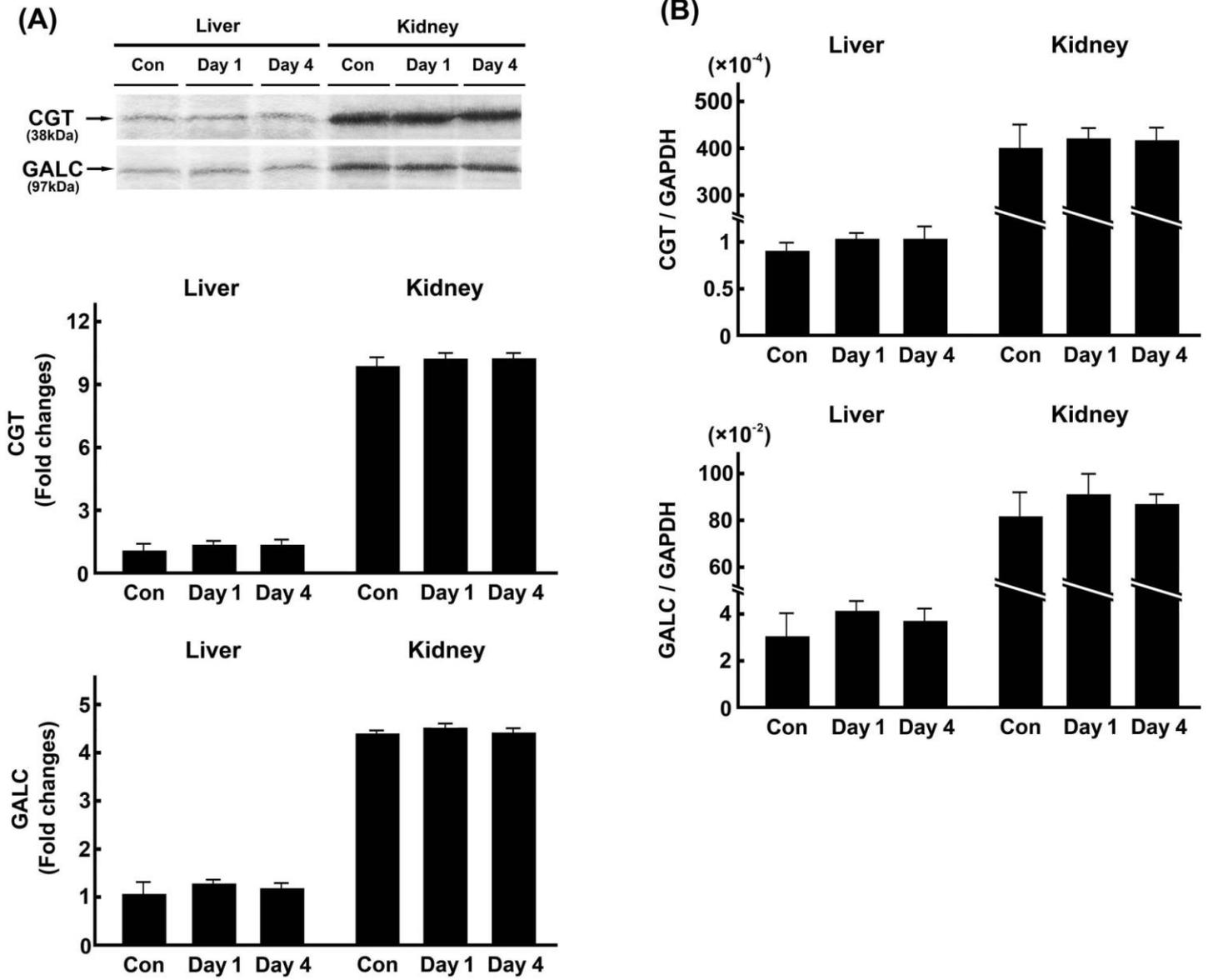


Figure 4

