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ARTICLE

Effects of hypertension and antihypertensive treatments on sulfatide levels in serum and its metabolism

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Abstract

Serum sulfatides are critical glycosphingolipids present in lipoproteins that work as modulators of thrombosis and hemostasis. Decreased serum sulfatide levels are suggested by our previous work to be related to cardiovascular disease (CVD). Hypertension, known to be an important risk factor for CVD, may affect serum sulfatide levels. However, how hypertension affects serum sulfatides directly and mechanistically is unknown. To elucidate these possible mechanisms, we investigated changes in serum sulfatide levels and their metabolism using an established experimental model of hypertension that uses continuous infusion of angiotensin II (AngII) into mice. Furthermore, we also examined the effects of four different antihypertensive drugs (losartan, irbesartan, nifedipine, and hydralazine) on serum sulfatide metabolism. Serum levels of sulfatides were found to be decreased in groups in which only hypertension was induced (AngII only), whereas they were increased in groups with reduced blood pressure (antihypertensives only) and ameliorated to increasingly normal levels in groups with induced hypertension that were also treated (AngII+antihypertensives). Changes in serum sulfatides were strongly related to hepatic expression levels of cerebroside sulfotransferase (CST), which is a key enzyme involved in sulfatide synthesis. Furthermore, the current study suggests that the primary factors affecting CST expression are oxidative stress, peroxisome proliferator-activated receptor α activity and blood pressure itself. This study demonstrates that hypertension significantly decreases levels of serum sulfatides by reducing hepatic CST expression via various effects mediated by AngII. Antihypertensive treatments can ameliorate abnormalities in serum sulfatide levels and may partially prevent hypertension related CVD by positively affecting sulfatide metabolism.

Keywords blood pressure · serum sulfatides · CST · oxidative stress · PPARα

Introduction

Hypertension is a highly prevalent serious worldwide health problem. Approximately one-third of the global adult population has hypertension, and nearly 13 million of these adults die each year from hypertension and its associated complications [1]. Hypertension is the major risk factor for cardiovascular disease (CVD) due to its contribution to vascular tone, vascular remodeling, and extracellular matrix formations [2]. However, hypertension may affect other underlying mechanisms that may accelerate CVD development and warrant further investigation.

3-O-sulfogalactosylceramides (sulfatides), which are a class of glycosphingolipids composed of ceramide, galactose and sulfate, are known to exert multifunctional biological activities in various organs [3]. Sulfatides exist in serum as major components of glycosphingolipids in serum lipoproteins. Serum sulfatides are produced mainly in the liver and

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are known to work as modulators of thrombosis and hemostasis [4, 5]. Sulfatides are also expressed on cell membranes of leukocytes, platelets, and erythrocytes. These membranous sulfatides on blood cell surfaces accelerate coagulation and thrombosis, possibly through mechanisms that include by activating blood coagulation factor XII, by combining with annexin V, and by helping to stabilize platelet adhesion and aggregation [6, 7]. The previous study suggested that serum sulfatides can intervene in these interactions via membranous sulfatides, disturb the formation of fibrin and directly inhibit thrombin activity [6, 8]. Therefore, abnormalities in serum sulfatide levels may affect the pathogenesis of CVD by exhibiting anticoagulant properties. Indeed, our past clinical study investigating hemodialysis (HD) patients, a very high-risk group for CVD, suggested this relationship. This study demonstrated that CVD incidence is significantly increased in HD patients with lower serum sulfatide levels, compared with those with higher levels. These results suggested that the reduction of serum sulfatide levels might induce development of CVD and that preventing abnormalities in serum sulfatide levels might useful in CVD prevention [9]. Understanding the underlying mechanism is essential for establishing an intervention method. However, the factors influencing serum sulfatide metabolism have not been thoroughly elucidated to date.

Several earlier experimental studies suggest that the reduction of serum sulfatide levels in pathological conditions, such as acute kidney injury [10], was caused mainly by a decreased hepatic expression of cerebroside sulfotransferase (CST), a key enzyme for sulfatide synthesis and that CST expression was decreased by oxidative stress (OS) and increased by activation of peroxisome proliferator-activated receptor α (PPARα) [5, 11], a common transcription factor. We hypothesized that hypertension, which is closely related to CVD pathogenesis, may potentially influence serum sulfatide levels.

To investigate this hypothesis and the associated underlying mechanism, we examined changes of serum sulfatide levels and the associated mechanism using an established experimental model of hypertension where a mouse is given a continuous infusion of angiotensin II (AngII). To eliminate what possible direct effects AngII may have on sulfatide regulation, we used two kinds of angiotensin II receptor blockers (ARBs): a typical ARB (losartan), and a unique ARB (irbesartan) known to activate PPARα [12, 13]. We also wanted to evaluate how serum sulfatide levels changed in conditions where blood pressure was lowered. A representative calcium channel blocker (CCB) (nifedipine) and a direct smooth muscle vasodilator (hydralazine) were chosen, that have minimal effects on sphingolipid metabolism [14]. Four different antihypertensive drugs were administered into sham surgery mice and mice with induced hypertension through AngII infusion, respectively. Changes in serum sulfatide levels and the underlying mechanism for these changes were subsequently investigated.

Methods

Animal experiments

All animal experiments were conducted in accordance with animal study protocols outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and carried out in accordance with the Regulations for Animal Experimentation of Shinshu University. The animal protocol was approved by the Committee for Animal Experiments of Shinshu University (Approval Number 290006). Ten-week-old male C57BL/6J mice weighting 20–25 g were maintained under controlled condition (25 °C; 12-h light/dark cycle) with tap water and randomly assigned into four groups: (1) Control (Con) group ($n = 10$), (2) hypertension (HT) groups that included a low-dose continuous infusion of angiotensin II (AngII; Sigma-Aldrich, MO, USA) into mice (0.2 mg/kg/day; $n = 5$) or a high-dose continuous infusion into mice (0.5 mg/kg/day; $n = 5$), (3) low blood pressure (LBP) groups that included mice that were given losartan (Los; 30 mg/kg/day; LKT Laboratories, MN, USA), mice that were given irbesartan (Irbe; 30 mg/kg/day; Sumitomo Dainippon Pharma, Tokyo, Japan), mice that were given nifedipine (Nif; 24 mg/kg/day; NIHON GENERIC, Tokyo, Japan) or mice that were given hydralazine (Hyd; 5 mg/kg/day; SUN PHARMA, Tokyo, Japan), $n = 5$ for each group, respectively, (4) hypertension treatment (HT-T) groups; high dose AngII infusion was given to mice along with an antihypertensive medication; these groups were AngII+Los, AngII+Irbe, AngII+Nif, or AngII+Hyd, $n = 5$ for each group, respectively. HT and HT-T mice were anesthetized by a mixture of medetomidine hydrochloride, butorphanol tartrate and midazolam, and osmotic minipumps (model 1004 ALZET/DURECT Corporation, Cupertino, CA, USA) were implanted subcutaneously into the interscapular region to allow continuous infusion of an AngII-mixed saline solution [15]. Meanwhile, Con and LBP mice received sham surgery to eliminate impacts of surgery might have had. LBP and HT-T mice were maintained on a standard rodent diet mixed with antihypertensive drugs, whereas Con and HT were only given a standard diet. Each group's food intake was measured every fourth day for the duration of the experiments to verify that the drug dose was constant. The doses of antihypertensive drugs used in present study were adjusted and set by preliminary experiments (Supplementary preliminary experiment methods and supplemental Fig. 1), where various doses of antihypertensive drugs were administered to obtain a very similar reduction of systolic blood pressure

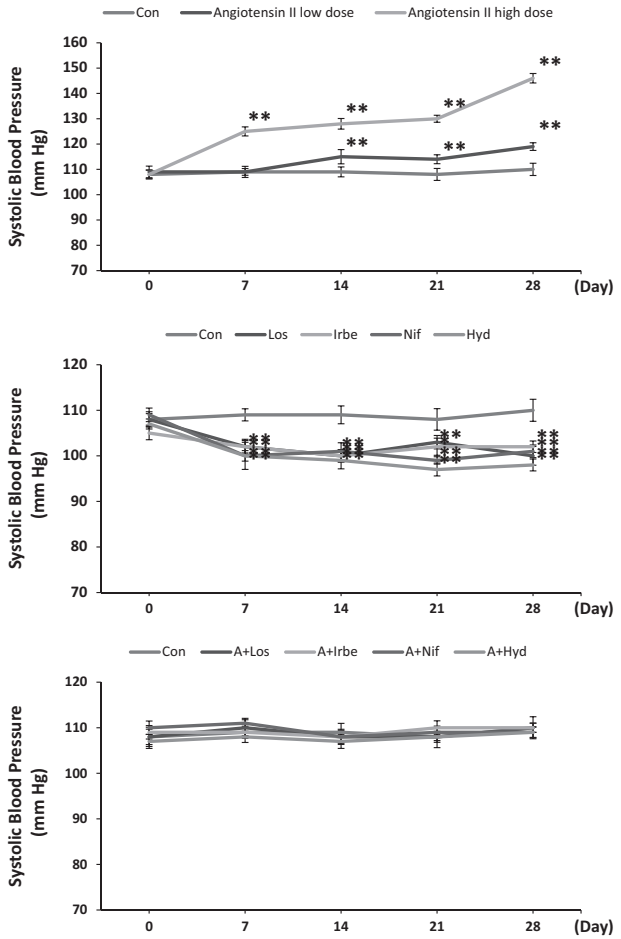

Fig. 1 Systolic blood pressure alterations in mice modeling hypertension (hypertension), mice receiving low blood pressure treatments (low blood pressure) and mice modeling hypertension that also received treatments (hypertension treatment) are expressed in the top, middle, and bottom of the figure, respectively. On day 0, 10-week-old male C57BL/6j mice were treated with a standard rodent diet or a diet with antihypertensive drugs mixed into it. Meanwhile, osmotic minipumps were implanted to infuse AngII. Systolic blood pressure was measured every 7 days and at each time point, with over 20 measurements recorded and averaged. Con control; Los losartan; Irbe irbesartan; Nif nifedipine; Hyd hydralazine; A + Los, AngII+losartan; A + Irbe, AngII+irbesartan; A + Nif, AngII+nifedipine; A + Hyd, AngII+hydralazine. The results are expressed as the mean ± SEM. (Control group $n = 10$, other groups $n = 5$, respectively). $^{*}P < 0.05$, $^{**}P < 0.001$ vs. control

(SBP). All mice were sacrificed under anesthesia at day 28, and serum and livers were collected from each mouse and stored at −80 °C until they were analyzed.

Blood pressure monitoring

SBP was measured in conscious mice using a non-invasive tail-cuff apparatus (BP-98A, Softron Co., Tokyo, Japan) prior to the experiment and every 7 days in experiments with extended durations. At each time point, over 20 measurements were recorded and averaged.

Quantitation and qualitative analyses of sulfatides

Sulfatides were extracted from serum and liver-homogenate of each mouse with n-hexane/isopropanol solution (3:2 v/v), then these extracts were converted to lysosulfatides (LS; sulfatides without fatty acids) by saponification with methanolic sodium hydroxide as described previously [16]. The resulting LS samples were purified through Mono-tip C18 cartridges (GL Sciences, Tokyo, Japan). Following the addition of the internal standard, N-acetylated LS-sphinganine (LS-d 18:0 NAc), the samples were analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) using a TOF/TOF 5800 system (AB Sciex, Framingham, MA, USA) in negative ionization mode. A two-point external calibration was performed with LS-d18:0 NAc ($[M\text{-}H]^- = 584.310$) and LS-(4E)-sphinganine (LS-d18:1) ($[M\text{-}H]^- = 540.284$). The following seven molecular species of LS were detected based on the differences in their sphingoid base structure: LS-sphingadienine (LS-d18:2), LS-d18:1, LS-sphinganine (LS-d18:0), LS-phytosphingosine (LS-t18:0), LS-(4E)-icosasphingenine (LS-d20:1), LS-icosasphingenine (LS-d20:0), and LS-4D-hydroxyicosasphingenine (LS-t20:0). Total levels of sulfatides were calculated as the sum of the levels of these seven LS species [17].

Analysis of mRNA

Liver total RNA was extracted using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). One microgram of total RNA was reverse-transcribed using a PrimeScript RT Reagent kit (Takara Bio, Otsu, Japan). mRNA levels were measured through quantitative real-time polymerase chain reactions (PCR) using a SYBR Premix EX Taq II (Takara Bio) on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster, CA, USA). Gene-specific primers were designed by Primer Express software (Applied Biosystems) as shown in Supplementary Table 1. mRNA encoding for glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as an endogenous control to normalize the samples using the ΔΔCT method and later expressed as fold changes relative to that of mice in the Con group.

Immunoblot analysis

7Liver cytosolic and nuclear fractions were extracted from each mouse using Cytoplasmic Extraction, and NE-PER Nuclear Reagents (Thermo Fisher Scientific, Rockford, IL, USA). Protein concentrations were determined colorimetrically with a BCA Protein Assay kit (Thermo Fisher Scientific). Lysates of nuclear and cytosolic proteins (30 μg) were subjected to 10% sodium dodecyl sulfate-polyacrylamide

gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred onto a nitrocellulose membrane (Amersham Hybond-P; GE Health Care, Little Chalfont, UK). These membranes were blocked for 1 h in 1% non-fat dry milk in Tris-buffered saline and later incubated overnight with the respective primary antibody at 4 °C. The membranes were washed four times and subsequently incubated with peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and treated with ECL Prime Western Blotting Reagents (GE Health). Primary antibodies for long-chain acyl-coenzyme A synthase (LACS), peroxisomal thiolase (PT) and catalase were described previously [18] and others were purchased commercially: antibodies against β-actin, TATA box-binding protein (TBP) and serine palmitoyl-CoA transferase (SPT) were from Abcam (Cambridge, UK), antibody against cerebroside sulfotransferase (CST) was from Abnova (Taipei, Taiwan), anti-arysulfatase A (ARSA) was obtained from Everest Biotech (Oxfordshire, UK), anti-glycolipid transfer protein (GLTP) antibody was obtained from Proteintech (Chicago, IL, USA), and the following primary antibodies were purchased from Santa Cruz Biotechnology (Dallas, CA, USA): ceramide galactosyltransferase (CGT), galactosylceramidase (GALC), neutrophil cytosolic factor 1 (NCF1), NADPH oxidase 2 (NOX2), superoxide dismutase 1 (SOD1), glutathione peroxidase 1 (GPX1) and peroxisome proliferator-activated receptor α (PPARα). The positions of protein bands were determined by co-electrophoresing molecular weight standards (Bio-Rad, Hercules, CA, USA), and the bands of actin and TBP were used as loading controls. Immunoblotting was repeated at least twice for each protein, and the band intensity was measured densitometrically through normalization to the loading control and subsequently expressed as fold changes relative to that of mice in the Con group.

Measurement of lipid peroxides

The concentration of malondialdehyde (MDA) in the liver was measured using a MDA-586 kit (OXIS International, Beverly Hills, CA, USA).

Biochemical analysis

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using enzyme assay kits (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Statistical analysis

Results are expressed as the mean ± SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Fisher's PLSD post hoc test. A probability value less than 0.05 was considered to be statistically significant.

Results

Continuous AngII infusion increased SBP, whereas antihypertensive treatments ameliorated SBP to normal levels

All mice received osmotic minipump implantation surgery or a sham surgery, where no mice died during operation, and systemic conditions indicated all of them had recovered well. For 28-day experiment durations, there were no symptoms of abnormal behavior or eating disorders in these mice.

SBP was measured and analyzed as shown in Fig. 1. A continuous high dose infusion of AngII gradually and significantly increased SBP at the seventh day after initiating infusion compared to before treatment was initiated. Although, the SBP of AngII low dose infusion mice was also raised significantly at the fourteenth day after treatment had begun, the elevation of SBP was mild and did not reach our requirements for a hypertension model. Thus we chose the high dose of AngII infusion as a reliable hypertension model, and we used this experimental condition in our experiments with antihypertensive drugs. Consistent with our preliminary experiment results (Supplementary preliminary experiment methods and supplemental Fig. 1), SBP was similarly and remarkably reduced in LBP groups with suitable doses of the four antihypertensive drugs at the seventh day after infusion had begun until the experiment finished. As expected, each antihypertensive treatment in each HT-T group ameliorated the increased SBP observed in mice, where AngII was infused at a high dose, and SBP was approximately reduced to the SBP level observed at the start of each experiment.

Other basic indices in mice aside from SBP, which included body weight, liver weight, kidney weight, heart weight, food intake, and representative liver injury markers ALT and AST did not differ significantly among groups (Supplementary Table 2).

Serum sulfatide levels were reduced in HT groups, but were increased by antihypertensive treatments in LBP and HT-T groups

To investigate whether hypertension affects serum sulfatide levels, we measured serum sulfatide levels using samples from each experimental mouse. As shown in Fig. 2a, serum sulfatide levels were significantly decreased in HT groups in an AngII dose-dependent manner. In LBP groups, all antihypertensive treatments increased serum sulfatide levels

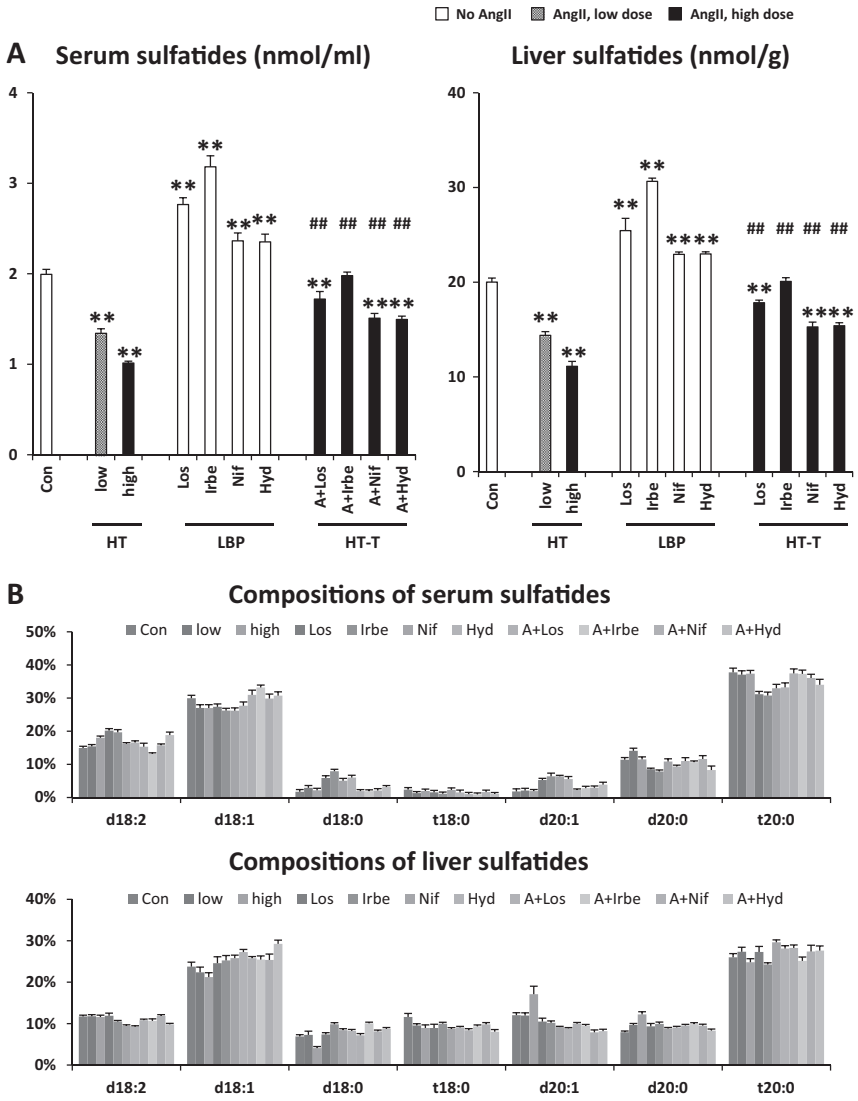

Fig. 2 Serum and liver sulfatide levels and compositions from hypertension, low blood pressure, and hypertension treatment mice. **a** Serum and liver sulfatide levels were measured by MALDI-TOF MS. The levels of sulfatides were calculated as the sum of 7 lysosulfatides species. HT hypertension; LBP low blood pressure; HT-T hypertension treatment. **b** Compositions of 7 lysosulfatides species from serum and liver. d18:2, LS-sphingadienine; d18:1, LS-(4E)-sphinganine; d18:0, LS-sphinganine; t18:0, LS-phytosphingosine; d20:1, LS-(4E)-icosasphingenine; d20:0, LS-icosasphingenine; t20:0, LS-4D-hydroxyicosasphingenine. The results are expressed as the mean ± SEM. (Control group $n = 10$, other groups $n = 5$, respectively). $^{**}P < 0.001$ vs. Control, $\#\#P < 0.001$ vs. AngII high dose. Open bars: no AngII infusion, striped bar: low dose AngII infusion, closed bars: high dose AngII infusion

compared to the control group, and among them, the ARB treatments exhibited the most prominent effects, particularly in Irbe-treated mice. Nif and Hyd had similar effects on elevating serum sulfatide levels, but the effects were milder than those observed with ARBs. These antihypertensive treatments could ameliorate the decline of serum sulfatide levels in mice that were continuously infused with a high dose of AngII. In HT-T groups, serum sulfatide levels in AngII+Irbe mice were almost recovered to control levels. As our previous study reported that serum sulfatides are secreted from the liver, we also measured liver sulfatides [4, 5]. Changes in liver sulfatides mirrored those observed in serum (Fig. 2a). Meanwhile, the sphingoid compositions of serum sulfatides did not differ among groups and were approximately identical to that observed in hepatic sulfatides (Fig. 2b). This finding implies that neither AngII infusion nor antihypertensive treatments affected serum and liver sulfatide compositions. Overall, these results indicate that hypertension decreased serum and liver sulfatide levels, and that antihypertensive treatments can ameliorate those reductions.

Serum sulfatide levels were mainly related to the hepatic expression of CST

To clarify the mechanism causing the serum sulfatide changes mentioned above, hepatic mRNA expression of sulfatide-metabolizing enzymes were measured. mRNA expression of CST was obviously decreased in the HT groups in an AngII dose-dependent manner (Fig. 3a). Inversely, mRNA expression of CST in the LBP groups was significantly increased compared to what was observed in controls, especially in mice treated with ARBs. Irbe-treated mice showed the greatest altered CST mRNA expression of all the LBP groups. CST expressions in Nif and Hyd mice were also identically increased compared to what is

observed in control mice; however, the increase observed was lower than that observed in mice treated with ARBs. In HT-T groups, treatment with ARBs significantly ameliorated the reduced expression of CST in mice continuously infused with a high dose of AngII. The recovered levels of CST in AngII+Los mice were still less than levels that were observed in control mice; however, AngII+Irbe mice nearly reached control levels. CST expressions in AngII+Nif and AngII+Hyd mice tended to show increased levels compared to those observed in mice that were only continuously infused with a high dose of AngII; however, the observed recovery was mild. Immunoblot analysis also confirmed that trends of CST hepatic protein expression in these mice were almost identical to mirrored changes in mRNA expression (Fig. 3b). We also assessed other sulfatide-metabolic enzymes involved CGT, responsible for synthesis of galactosylceramides; SPTLC2, a rate-limiting enzyme in the synthesis of ceramides/sphingolipids that include sulfatides; ARSA, a main enzyme affecting sulfatide degradation; GALC, an enzyme causing degradation of galactosylceramides to ceramides; and GLTP, proteins relating to sphingolipid transfer. mRNA expression and protein levels were not observed to be different in these groups (Fig. 3a and Supplementary Fig. 2). In brief, these results suggested that the hepatic expression of CST is primarily responsible for changes in levels of serum sulfatides.

Hepatic OS level was remarkably increased in HT groups; ARB treatments improved the elevation of hepatic OS but neither nifedipine nor hydralazine caused improvement

As previously reported, increased OS could reduce hepatic CST expression, thereby inducing reduction in serum sulfatide levels [11, 19]. Hence, we measured the level of malondialdehyde (MDA) as a marker for hepatic OS, with the results shown in Fig. 4a. MDA levels were significantly increased in the HT groups when compared to controls. MDA levels were significantly decreased in mice treated with ARBs in the LBP groups compared to controls, but no changes were observed in Nif- and Hyd-treated mice in these LBP groups. In HT-T groups, MDA levels were significantly decreased in AngII+ARBs treated mice compared to mice with a continuous infusion of a high dose of AngII, and reached similar levels to those observed in the control group. However, this reduction in levels of OS was not found in AngII+Nif or AngII+Hyd mice, and MDA levels of these two groups were identical to those in mice receiving a continuous infusion of a high dose AngII.

We assessed hepatic enzymes involved in regulating the generation and elimination of reactive oxygen species (ROS) to further describe the mechanism proposed above. mRNA expression of catalase, which is related to removing ROS, was increased only in Irbe mice in the LBP and HT-T groups. However, mRNA levels of other ROS elimination enzymes, SOD1 and GPX1, did not differ among the groups (Fig. 4b). NOX2 and NCF1, typical enzymes associated with ROS generation, were assessed. Hepatic mRNA expression of NOX2 was increased in the high dose HT group compared to controls and significantly decreased in the AngII+ARBs HT-T group compared to the high dose HT group. The NOX2 suppressive effect was not observed in AngII+Nif or AngII+Hyd HT-T groups. mRNA expression of NCF1 was identical in all groups. Another enzyme associated with ROS generation, ACOX1, was also measured. ACOX1 expression tended to increase in the high dose HT group as compared to controls ($P = 0.054$), but no changes found in any of the LBP groups. Moreover, these four antihypertensive treatments could not suppress the elevation of ACOX1 expressions induced by AngII infusion. Assessing protein levels rendered identical findings (Fig. 4c and Supplementary Fig. 2).

Irbesartan treatment increased the hepatic expression of PPARα and its target enzymes

Another study has demonstrated that PPARα activation could increase hepatic CST expression in mice apart from increased OS affecting hepatic CST expression [5]. Therefore, hepatic PPARα and its representative target enzymes, including LACS and PT, were evaluated. mRNA expression of *Ppara*, *Lacs*, and *Pt* were all only significantly increased in Irbe-treated mice in both the LBP groups and the HT-T groups. All other treatment groups showed no differences compared to controls (Fig. 5a). Irbe-specific PPARα-activation was also observed at the protein level (Fig. 5b). In agreement with the previous study, these results indicate that irbesartan activates hepatic PPARα. This Irbe-specific PPARα-activation might enhance hepatic expression of CST.

Discussion

There has not been research focused on the relationship between hypertension and levels of serum sulfatide. The present study reveals that serum and liver sulfatide levels were remarkably decreased in a hypertension model induced by continuous infusion of AngII in a manner depending on the overall dose of AngII. Administration of different antihypertensive drugs can significantly ameliorate reductions of serum and liver sulfatide levels, with the amelioration effects being dependent on the various mechanisms of each antihypertensive drug. The change in

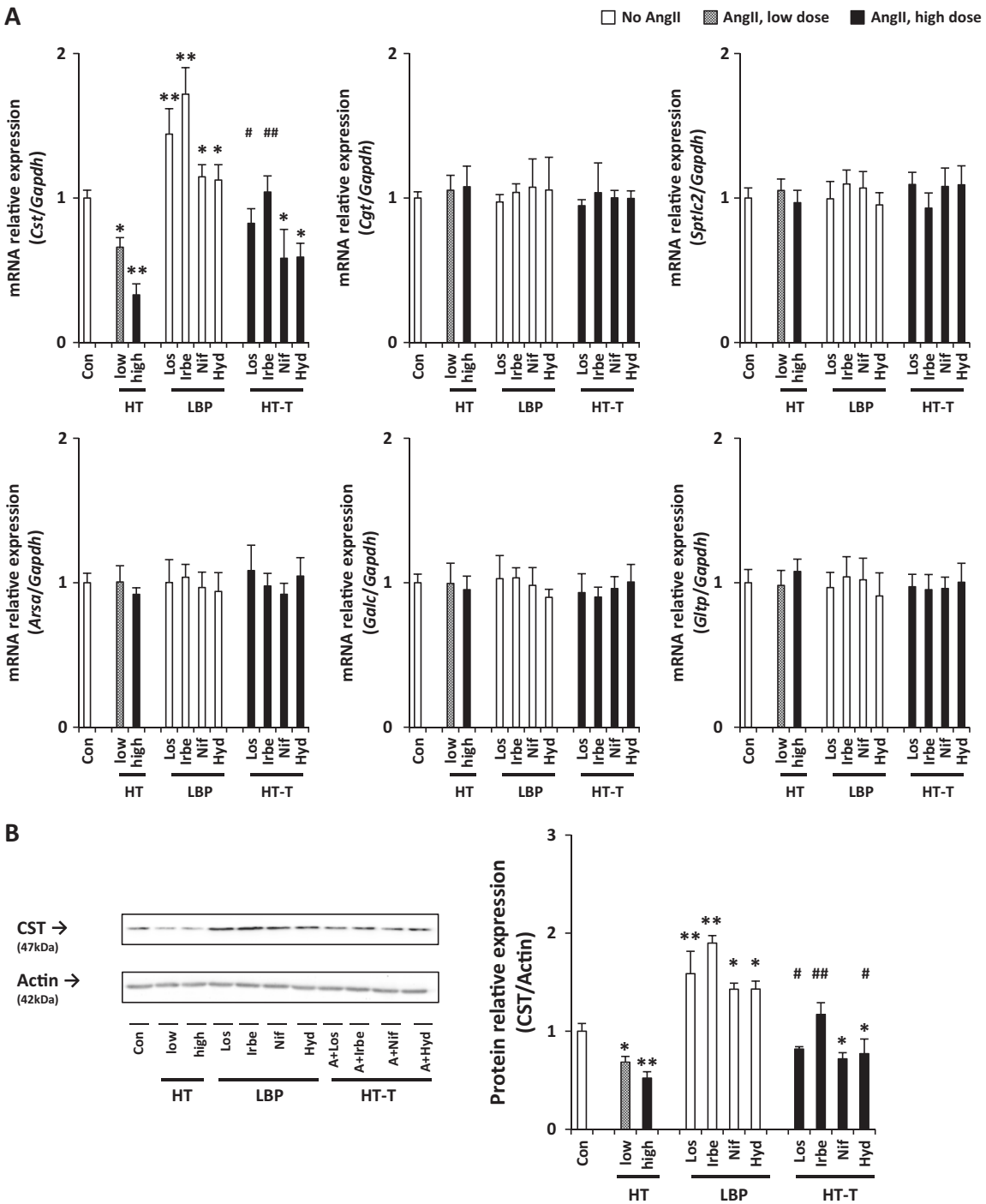

Fig. 3 Expression of enzymes involved in sulfatide biosynthesis, degradation, and transport in livers from hypertension, low blood pressure and hypertension treatment mice. **a** Expression of mRNAs encoding *Cst*, *Cgt*, *Sptlc2*, *Arsa*, *Galc*, and *Gltp*. mRNA levels are normalized to *Gapdh* mRNA levels and are shown as fold changes relative to that observed in mice from the control group. **b** Immunoblot analysis of CST. Thirty micrograms of whole-liver lysate protein prepared from each mouse was loaded into each well for electrophoresis. Actin was used as the loading control. The band intensity was measured densitometrically, normalized to those of actin and shown as values relative to that of mice from control group. The results are expressed as the mean ± SEM. (Control group $n = 10$, other groups $n = 5$, respectively). $^{*}P < 0.05$, $^{**}P < 0.001$ vs. Control, $\#P < 0.05$, $\#\#P < 0.001$ vs. AngII high dose. Open bars: no AngII infusion, striped bar: low dose AngII infusion, closed bars: high dose AngII infusion

sulfatide metabolism was most likely associated with altered hepatic CST expression. Additionally, under the current experimental conditions, we found that hepatic CST expression influenced factors were primarily attributed to

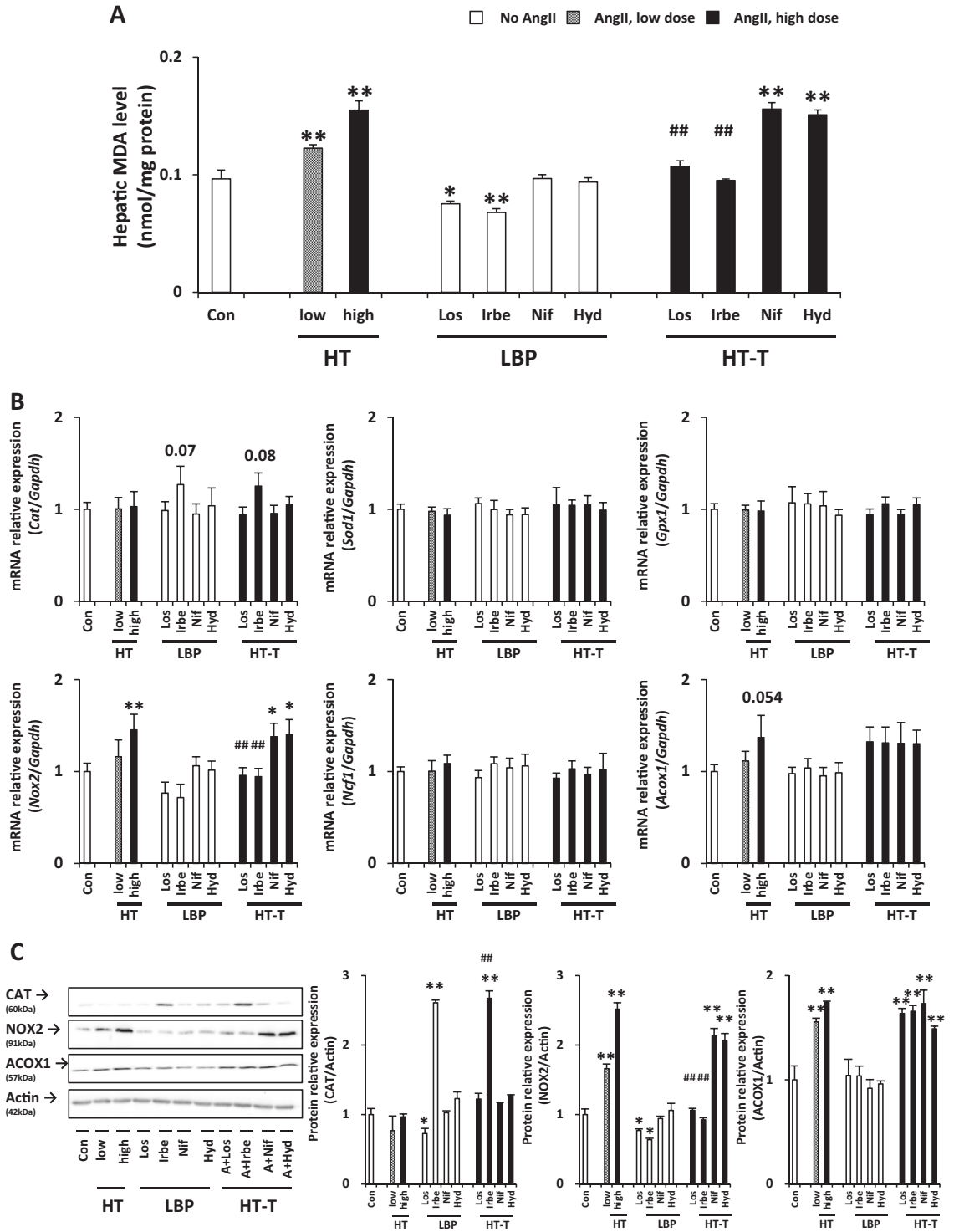

Fig. 4 Expression of enzymes involved in reactive oxygen species metabolism and the tissue oxidation stress marker MDA in livers from hypertension, low blood pressure, and hypertension treatment mice. **a** Hepatic levels of MDA. **b** Expression of mRNAs for *Cat*, *Sod1*, *Gpx1*, *Nox2*, *Ncf1* and *Acox1*. mRNA levels are normalized to *Gapdh* mRNA levels and shown as fold changes relative to that observed in mice from the control group. **c** Immunoblot analysis of CAT, NOX2, and ACOX1. Thirty micrograms of whole-liver lysate protein prepared from each mouse was loaded into each well for electrophoresis. Actin was used as the loading control. The band intensity was measured densitometrically, normalized to those of actin and shown as values relative to that of mice in control group. The results are expressed as the mean ± SEM. (Control group $n = 10$, other groups $n = 5$, respectively). $^{*}P < 0.05$, $^{**}P < 0.001$ vs. Control, $\#\#P < 0.001$ vs. AngII high dose. Open bars: no AngII infusion, striped bar: low dose AngII infusion, closed bars: high dose AngII infusion

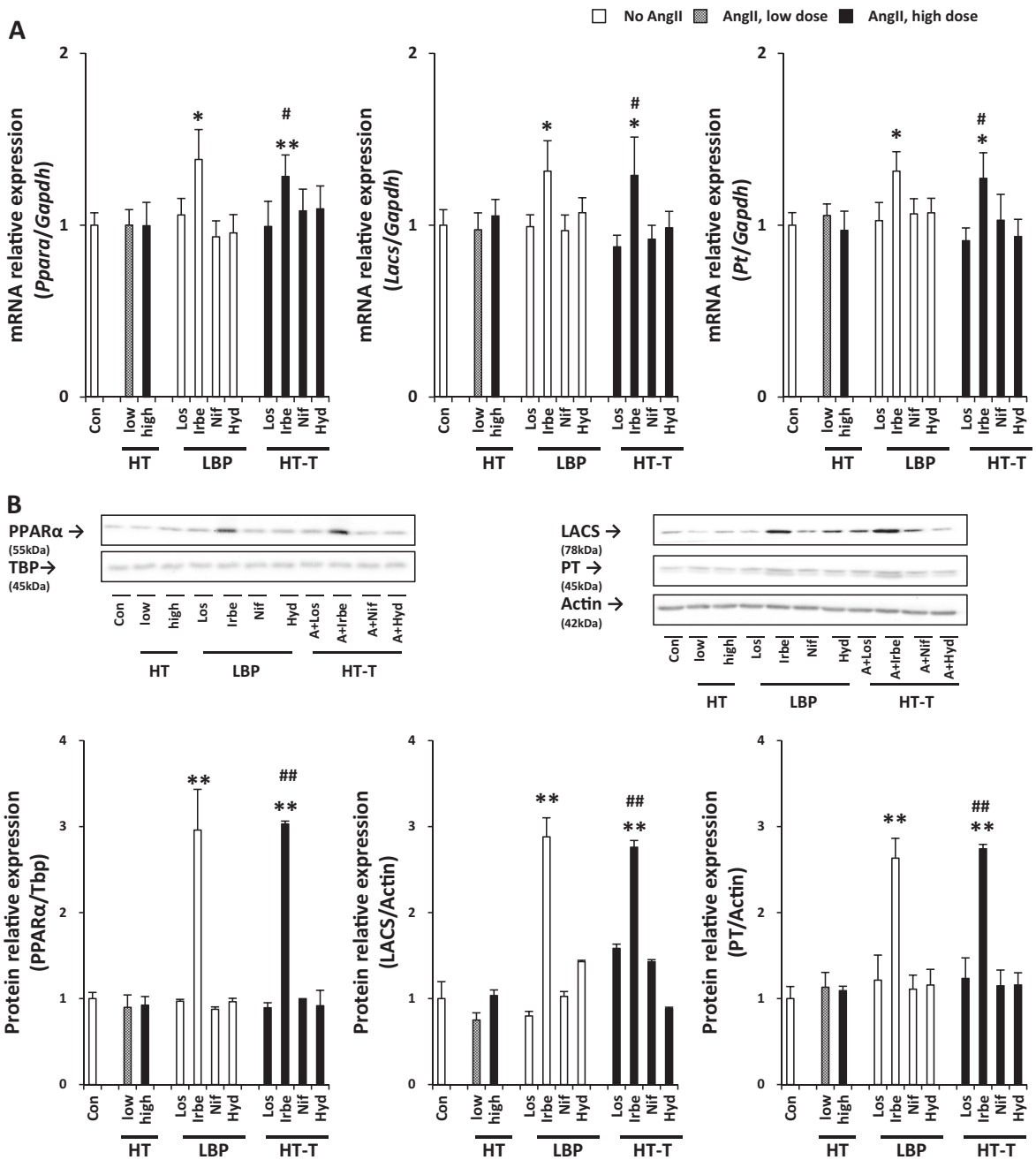

Fig. 5 Expression of PPARα and its target genes LACS and PT in livers from hypertension, low blood pressure, and hypertension treatment mice. **a** Expression of mRNAs encoding *Ppara*, *Lacs*, and *Pt*. mRNA levels are normalized to *Gapdh* mRNA levels and shown as fold changes relative to that observed in mice from the control group. **b** Immunoblot analysis of PPARα, LACS, and PT. Thirty micrograms of the nuclear fraction or whole-liver lysate protein prepared from each mouse was loaded into each well for electrophoresis. TBP or actin was used as the loading control, respectively. The band intensity was measured densitometrically, normalized to those of TBP or actin and shown as values relative to that observed in mice from the control group. The results are expressed as the mean ± SEM. (Control group $n = 10$, other groups $n = 5$, respectively) $^{*}P < 0.05$, $^{**}P < 0.001$ vs. Control, $\#P < 0.05$, $\#\#P < 0.001$ vs. AngII high dose

enhancing OS, activating PPARα and lowering blood pressure itself. Proposed associations of these mechanisms are depicted in Fig. 6.

There is accumulating evidence verifying the interactions between OS and hypertension. Vascular ROS production is previously reported to be increased in a hypertensive situation [20, 21]. Furthermore, the link between vascular OS and hypertension is known to be bidirectional: OS promotes the development of hypertension and contributes to hypertension-induced pathologies [22]. Administrations of antioxidants, such as tempol, or membrane-targeted forms of SOD are reported to possibly lower blood pressure,

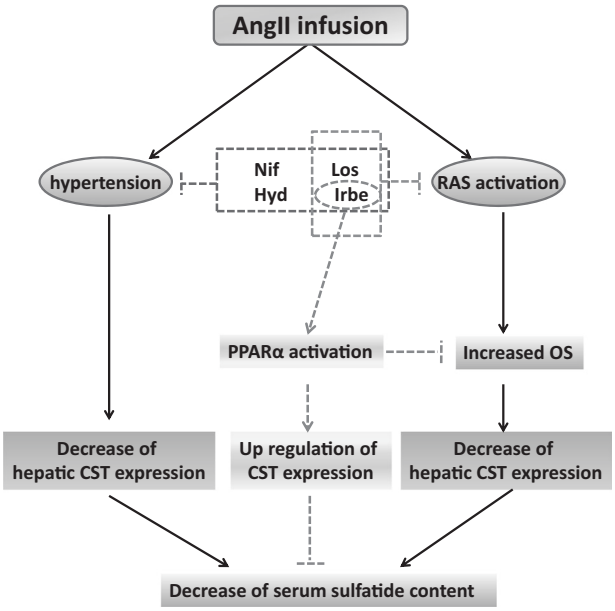

Fig. 6 Scheme relating the complex effects of sulfatide metabolism in livers from hypertension, low blood pressure, and hypertension treatment mice. AngII infusion induced both hypertension and activation of the renin angiotensin system (RAS) in mice. Consequently, these caused reduction of hepatic CST expression, and decreased serum sulfatide levels as shown. All four antihypertensive drugs can alleviate the abnormally reduced serum sulfatide by reducing hypertension. Losartan and irbesartan antagonize RAS activation through an ARB-specific mechanism. This effect of ARBs ameliorated the enhancement of OS, which contributed to improving serum sulfatide levels. The exclusive effect of PPARα activation by irbesartan led to increased CST expression and ameliorated levels of OS. These effects by irbesartan restored the greatest levels of serum sulfatide between the four antihypertensive drugs

similar to an antihypertensive drug [23, 24]. In the current study, nifedipine and hydralazine treatments successfully lowered blood pressure but not the levels of OS, whereas ARBs were shown to improve both hypertension and OS in the same group. Moreover, there are few reports indicating an anti-oxidative ability in non-RAS inhibitor types of antihypertensives. These findings suggest that the related mechanisms between OS and hypertension would be mainly due to RAS activation. It is well accepted that AngII is a potent activator of NADPH oxidase in the cardiovascular system and contributes to ROS production [25]. AngII also enhances the levels of OS in other organs apart from those in the cardiovascular system, such as the kidneys and the liver [26, 27]. A previous study demonstrated that enhancement of OS induced the suppression of hepatic CST expression in a murine model of chronic ethanol consumption [4]. In clinical research, improvement of OS recovers reduced serum sulfatide levels in kidney transplantation patients [11, 28]. Although there is no unambiguous evidence to clarify how improving OS affect hepatic CST expression, OS is considered as a key mechanistic mediator of levels of sulfatides. The current findings demonstrated that AngII-induced HT significantly increased the levels of OS and reduced hepatic CST expression. Furthermore, treatments with ARBs, losartan and irbesartan all completely attenuated increased hepatic levels of OS induced by AngII infusion, and the hepatic expression of CST in these mice was prominently recovered. These findings suggest the close relationship between RAS activation-induced OS and CST expression. However, although non-ARB antihypertensives Nif and Hyd scarcely exert anti-oxidative effects, the CST and serum sulfatide levels of Nif- or Hyd-treated mice in LBP groups were mildly increased. Moreover, reduced CST and serum sulfatide levels in Nif- or Hyd-treated mice in HT-T groups recovered partially. These findings suggest that factors other than OS may influence sulfatide metabolism.

In recent years, we have studied interactions between PPARα and sulfatides, as well as PPARα and ARBs, and made several important discoveries. Our past studies indicated that PPARα activation increased CST gene expression and enhanced sulfatide synthesis in several murine organs, which included the liver, suggesting that CST is a gene targeted through PPARα activation [29]. Recently, we reported that irbesartan, a unique angiotensin II receptor blocker, could activate renal PPARα in mice experiencing protein-overload nephropathy [12]. Other studies from other research groups also demonstrated that irbesartan can upregulate PPARα-activated genes and genes related to fatty acid oxidation in the livers of obese Koletsky rats [13]. These findings suggested that irbesartan is a reliable PPARα agonist. In the present study, hepatic expression of PPARα and its representative target enzymes LACS and PT were increased through an irbesartan-specific effect, which is consistent with past results. In the current study, the degree of increased CST expression in mice treated with irbesartan was much higher than what was observed in mice treated with other antihypertensives in the LBP groups and HT-T groups. This obvious effect of irbesartan causing increased CST might de due to its ability to activate PPARα. Furthermore, PPARα activation is known to exert antioxidant effects by inducting ROS eliminating enzymes, such as catalase [18, 30]. These PPARα-mediated anti-oxidative effects would reduce OS and may contribute to the increase in sulfatide levels. The double-effects of PPARα activation by direct induction of CST and induction of CST mediated by anti-oxidation effects increased sulfatide levels intensively in Irbe-treated mice, adding to the common classic ARB effects of RAS suppression.

Interestingly, although no OS amelioration or PPARα activation was observed in either Nif- or Hyd-treated mice, hepatic CST expression and sulfatide levels in these mice were significantly and similarly improved. Mice blood pressures were downregulated by four different

antihypertensives similarly in LBP groups (SBP 100 ± 2.7 vs. 110 ± 3.7 control) and effectively improved to control levels in HT-T groups (SBP 110 ± 4.8 vs. 110 ± 3.7 control). As these four drugs decreased blood pressure to the same extent via different mechanisms, it is possible that CST expression and sulfatide levels are influenced by blood pressure alterations directly, which may become an important breakthrough point. Previous studies indicated that losartan and hydralazine decreased vascular ceramide levels and plasma ceramide levels in spontaneously hypertensive rats (SHR) possibly due to lowered blood pressure, although the exact mechanism is currently unknown [31]. As sulfatides are derivative of ceramides, the reduction of ceramide levels might reflect the increase of sulfatide synthesis in conditions where blood pressure is decreased. Contrastingly, lowered blood pressure by antihypertensive treatments, including ARB, CCB, and hydralazine, is reported to possibly improve insulin resistance (IR) and suppress inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukins (IL)-6 [32–35]. An earlier study reported that pharmacological intervention in glycosphingolipid biosynthesis may modulate IR in animal models [36]. Furthermore, serum sulfatide levels in type 2 diabetes mellitus patients (where almost all these patients are in an IR state), were significantly decreased [37]. In addition, TNF-α and IL-6 are reported to be critical factors for inducing IR, and expressions of these inflammatory cytokines are reduced by sulfatides [38, 39]. These findings suggest that affected sulfatide metabolism and HT-induced IR, and inflammatory cytokines may be closely linked. Therefore, improving hypertension may change sulfatide metabolism through reducing IR and inflammatory cytokines; however, this hypothesis is not confirmed in the current study, and future studies will be necessary.

Although the present study provides the first suggestion that lowering blood pressure could increase serum sulfatide levels, there are several limitations that must be noted. First, we used a tail cuff method to measure blood pressure. This method is generally associated with wider blood pressure variation than continuous blood pressure monitoring. Therefore, it is possible that we could not detect significant differences in blood pressure among the groups because of variability in measurements. Second, the current study suggests that reversing the reduction of serum sulfatide levels induced by hypertension contributed to normalization of serum sulfatide function. However, it is unclear from the present study whether very high serum sulfatide levels exert beneficial effects. Sulfatides are reported to have both anticoagulant and procoagulant activities. It was reported that when injected into mice with heavily damaged blood vessel walls or when continuously infused into mice through plastic cannulae, sulfatides enhanced thrombosis and blood coagulation [8]. Under in vitro and ex vivo experimental conditions using initially activated platelets, sulfatide-P-selectin interactions are reported to increase platelets activation and aggregation [40]. Additionally, Li et al. recently reported that serum sulfatide levels are abnormally high in ST-segment elevation myocardial infarction (STEMI) patients and that this is positively correlated to STEMI complications [41]. We believe that maintaining serum sulfatides at a normal level is clinically important, but further study is necessary to determine what that level is. Third, we estimated serum sulfatide levels in a single condition of lowering blood pressure; therefore, the accurate correlation curve between continuous blood pressure alterations and serum sulfatide levels could not be determined. Finally, we cannot distinguish whether blood pressure elevation or increased OS levels decreased sulfatide levels in the current study, and more thorough experiments will be needed in the future.

In summary, this study revealed that serum sulfatides decreased in an established experimental model of hypertension by using a continuous infusion of AngII in mice, and can be ameliorated using antihypertensive treatments. The mechanism of serum sulfatide alteration is primarily related to changes in hepatic CST expression. In the present study, CST expression was influenced by multiple factors, which included oxidation stress, peroxisome proliferator-activated receptor α activation and blood pressure itself. Importantly, this relationship between hypertension and serum sulfatides may provide a potential therapeutic direction for the future treatment of hypertension related to CVD.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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