Peroxisome proliferator-activated receptor α mediates enhancement of gene expression of cerebroside sulfotransferase in several murine organs

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Abstract

Sulfatides, 3-*O*-sulfogalactosylceramides, are known to have multifunctional properties. These molecules are distributed in various tissues of mammals, where they are synthesized from galactosylceramides by sulfation at C3 of the galactosyl residue. Although this reaction is specifically catalyzed by cerebroside sulfotransferase (CST), the mechanisms underlying the transcriptional regulation of this enzyme are not understood. With respect to this issue, we previously found potential sequences of peroxisome proliferator-activated receptor (PPAR) response element on upstream regions of the mouse CST gene and presumed the possible regulation by the nuclear receptor PPARα. To confirm this hypothesis, we treated wild-type and *Ppara*-null mice with the specific PPARα agonist fenofibrate and examined the amounts of sulfatides and CST gene expression in various tissues. Fenofibrate treatment increased sulfatides and CST mRNA levels in the kidney, heart, liver, and small intestine in a PPARα-dependent manner. However, these effects of fenofibrate were absent in the brain or colon. Fenofibrate treatment did not affect the mRNA level of arylsulfatase A, which is the key enzyme for catalyzing desulfation of sulfatides, in any of these six tissues. Analyses of the DNA-binding activity and conventional gene expression targets of PPARα has demonstrated that fenofibrate treatment activated PPARα in the kidney, heart, liver, and

small intestine but did not affect the brain or colon. These findings suggest that PPARα activation induces CST gene expression and enhances sulfatide synthesis in mice, which suggests that PPARα is a possible transcriptional regulator for the mouse CST gene.

(less than 250 words)

Keywords: CST; fenofibrate; PPARα; sphingoglycolipid; sulfoglycolipid.

Introduction

Sulfatides are sulfoglycolipids composed of ceramides, galactoses, and sulfates. Sulfatides are widely expressed in mammalian tissues, such as brain, kidney, liver, and digestive tract, mainly at the outer leaflet of the biological membrane [1]. Several studies have proposed the biological functions of sulfatides in various fields, including the nervous system, immune system, glucose metabolism, osmotic regulation, spermatogenesis, thrombosis/hemostasis, microbial infection, and cancer [1-3]. Therefore, sulfatide metabolism dysfunction might lead to the development of various diseases [2]. The multifunctional property of sulfatides is thought to be associated with the diversity of their chemical structures. Depending on the ceramide moiety, various molecular species of sulfatides exist, whose expression pattern and level are specific in tissues [1, 2]. Therefore, understanding the regulatory mechanism of sulfatide metabolism in various tissues may be important.

Sulfatides are synthesized from galactosylceramides by sulfation at C3 of the galactosyl residue. This reaction is catalyzed only by cerebroside sulfotransferase (CST) [3]. In contrast, sulfatides are degraded in this reverse reaction that is catalyzed by arylsulfatase A (ARSA) [1, 2]. However, there is little information about the transcriptional regulators for these key enzymes in sulfatide metabolism. We previously

reported that CST gene expression was affected by oxidative stress and inflammation in mice [4, 5]. We have proposed several transcription factors involved in these phenomena by searching through the upstream regions of the mouse CST gene [4-7]. During this search, we also found putative sequences of peroxisome proliferator-activated receptor (PPAR) response element (PPRE): -1,434/-1,422 (AGGTCTAAGGGCA), -1,202/-1,190 (TGGACTTTGCCCT), and -896/-884 (AGGACAAAGAGCA) from exon 1a; -1,499/-1,487 (AGGCTACAGTTCA) from exon 1e; and -1,569/-1,557 (AGGTCAGAGCACA) and -302/-290 (AGGACAGAGCCCA) from exon 1f. Therefore, we hypothesized the possible regulation of CST gene expression by the nuclear receptor PPARα. To confirm this hypothesis, we first examined whether PPARα activation influenced murine hepatic CST because the liver is highly sensitive to PPARα-induced effects. We demonstrated that the amounts of both hepatic CST mRNA and protein, as well as the hepatic contents of sulfatides, were increased with PPARα activation [8]. However, it is unknown whether PPARα plays an important role in the transcriptional regulation of CST in other organs, such as kidney, heart, brain, and digestive tract. Specifically, we previously reported that opposing responses concerning CST induction and tissue sulfatide contents between liver and kidney were found in the kidney dysfunction murine model [4, 5],

which suggests organ-specific regulation of sulfatide metabolism. Therefore, extended studies using multiple murine tissues are needed to determine the role of PPARα in murine CST gene regulation. In the present study, we examined the amounts of sulfatides and CST gene expression in various tissues from wild-type and *Ppara*-null mice that were treated with the PPARα agonist fenofibrate for the first time.

Materials and Methods

Mice and treatment

All of the mouse experiments were conducted in accordance with animal study protocols that were approved by the Shinshu University School of Medicine. Wild-type and *Ppara*-null mice were on a SV129 genetic background as previously described [9, 10]. These mice were maintained in a pathogen-free environment under controlled conditions (25 °C; 12-h light/dark cycle) with municipal water and a standard rodent diet *ad libitum*. Twelve-week-old male wild-type and *Ppara*-null mice weighing 25-30 g were used in this study. The mice of each genotype were divided into two groups (n = 6/group). One mouse group was treated with a regular diet that contained 0.1% (w/w) fenofibrate (Wako Pure Chemical Industries, Osaka, Japan) for 7 days, and the other group continued on a regular diet as a control. Our previous study demonstrated that more than 4 days of PPARα agonist treatment were required to sufficiently induce most of the PPARα target genes in mouse liver [11]. Furthermore, our other studies revealed that the induction levels of typical PPARα target genes by fibrate treatments in kidney and liver were almost identical between the 5 to 7-day treatment group and the 14-day treatment group [12-15]. Therefore, the present study used a 7-day fenofibrate treatment. Seven days after commencing the treatment, the mice were sacrificed under anesthesia

for tissue collection. The brain was minced well by scissors prior to freezing on dry ice in order to prepare whole-brain sample. The other tissues were cut into several pieces and then frozen in a similar manner. The tissue samples were stored at -80 °C until analysis.

Extraction and measurement of sulfatides

The sulfatides were extracted according to the hexane/isopropanol method [16] and were converted to lysosulfatides that consisted of sphingoids, galactoses, and sulfates [17]. The lysosulfatide samples were analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) on a Voyager Elite XL Biospectrometry Workstation (PerSeptive Biosystems, Framingham, MA, USA) [17]. The sulfatide amounts were calculated as the total amounts of the seven molecular species of lysosulfatides as shown in Table 2.

Analysis of mRNA

Total RNA 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). The generated

cDNA was analyzed by quantitative real-time polymerase chain reaction (qPCR) using SYBR Premix Ex Taq II (Takara Bio, Otsu, Japan) on a Thermal Cycler Dice TP800 System (Takara Bio). The gene-specific primers were designed with Primer Express software (Applied Biosystems, Foster City, CA, USA) and ordered from Sigma Aldrich Corporation (St. Louis, MO, USA). The primer pairs are as follows: 5'-ATGGCCTTCACGACCTCAGA-3' and 5'-CGGTCTTGTGCGTCTTCATG-3' for CST (NCBI GenBank accession number, NM_016922); 5'-ACCACCCCTAACCTGGATCAGT-3' and 5'-ATGGCGTGCACAGAGACACA-3' for ARSA (NM_009713); 5'-TGGGTCCAGCCTATGGATGT-3' and 5'-GCAGCGTTGGTCTTGGAAAC-3' for ceramide galactosyltransferase (CGT) (NM_011674); 5'-GAGTGAGAATCATAGCGAGCGATA-3' and 5'-AGTTCCTGGTCCAGCAGCAA-3' for galactosylceramidase (GALC) (NM_008079); 5'-TGGTATGGTGTCGTACTTGAATGAC-3' and 5'-AATTTCTACCAATCTGGCTGCAC-3' for peroxisomal acyl-CoA oxidase (AOX) (NM_015729); and 5'-TGCACCACCAACTGCTTAG-3' and 5'-GGATGCAGGGATGATGTTCTG-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (M32599). GAPDH was used as an endogenous control gene. The qPCR analysis was performed by a standard curve method, and the data were

expressed as the ratio of target molecule/GAPDH mRNA levels.

PPARα DNA-binding assay

The DNA-binding activity of PPARα was determined using a PPARα transcription factor assay kit (Cayman Chemical, Ann Arbor, MI, USA). This assay is based on an enzyme-linked immunosorbent assay using PPRE-immobilized microplates and PPARα-specific antibodies, which provides similar results to those from a conventionally used radioactive gel shift assay. The DNA-binding assay was performed according to the manufacturer's instructions using whole-tissue lysates (60 μg protein) prepared from fenofibrate-treated and untreated wild-type mice. The sample preparation was performed as described [18-20]. The protein concentrations were measured by a BCA protein assay kit (ThermoFisher Scientific, Waltham, MA, USA). The results were shown as the fold changes relative to those of the wild-type mice without fenofibrate treatment.

Statistical analysis

All data were expressed as the mean ± standard deviation (SD). The statistical analysis was performed using an unpaired *t*-test (SPSS Statistics 17.0; SPSS Inc.,

Chicago, IL, USA). A probability value of less than 0.05 was considered statistically significant.

Results

The amounts and compositions of sulfatides

The amounts of sulfatides in six mouse tissues are presented in Table 1. The constitutive amount of sulfatides differed among the organs. A greater amount of sulfatides was detected in the brain and kidney compared with the heart, liver, small intestine and colon in the control mice of both genotypes. There was no difference in the constitutive tissue amounts of sulfatides between the genotypes. The amounts were markedly increased by fenofibrate treatment in the kidney, heart, liver, and small intestine of the wild-type mice, whereas this effect was not observed in the brain or colon of the wild-type mice or in any of the six tissues from the *Ppara*-null mice.

The sphingoid base structures of the tissue sulfatides in the fenofibrate-treated and untreated wild-type mice are presented in Table 2. Although tissue specificity in the sphingoid base structures was found in both groups, fenofibrate-induced compositional changes of these molecular species were not detected. In *Ppara*-null mice, a similar tissue distribution of these sphingoids was also observed with no fenofibrate-induced compositional changes (data not shown).

The expression of CST and other enzymes involved in sulfatide metabolism

The mRNA level of CST was increased by fenofibrate treatment in the kidney, heart, liver, and small intestine of the wild-type mice (Figure 1). However, CST induction by fenofibrate was not found in the brain or colon of the wild-type mice or in any of the tissues of the *Ppara*-null mice. The tissue levels of ARSA mRNA were unchanged by fenofibrate in either genotype (Figure 2). In any of the wild-type mouse tissues, there was no significant alteration by fenofibrate in the mRNA expression levels of CGT and GALC (data not shown), which are the enzymes that catalyze the forward and reverse reactions in the synthesis of galactosylceramides from ceramides, respectively. These results suggest that high CST expression was associated with increased sulfatide contents in fenofibrate-treated wild-type mice.

The function of PPARα

As shown in Figure 3, the DNA-binding activity of PPARα was increased by fenofibrate treatment in the kidney, heart, liver, and small intestine of wild-type mice, but these increases were absent in the brain and colon. Consistent with this result, the mRNA expression of AOX, the established PPARα target molecule [21], was also increased by fenofibrate in these four tissues of the wild-type mice only (Figure 4). Therefore, the enhanced function of PPARα resulting from fenofibrate treatment

occurred in the kidney, heart, liver, and small intestine but not in the brain or colon. These results correlated with the data concerning the levels in sulfatides and CST mRNA.

Discussion

This study demonstrated that fenofibrate treatment increased the amounts of sulfatides and the expression level of CST mRNA in the kidney, heart, liver, and small intestine in a PPARα-dependent manner. However, these effects were not found in the brain or colon. Fenofibrate treatment activated PPARα in the former four tissues but did not affect the brain or colon. Through this evidence, we propose that PPARα activation induces CST gene expression and enhances sulfatide synthesis in mice.

In the current study, there was no significant difference in the constitutive amount of the sulfatides in any of the tissues in the *Ppara*-null mice compared with the wild-type mice. This was also the case with the constitutive mRNA level of sulfatide-metabolizing enzymes including CST. Furthermore, the profile of tissue distribution of sulfatides in this study (Table 1; sulfatide content was higher in the brain and kidney compared with the other organs) was different from the known tissue expression profile of PPARα (i.e., PPARα was abundant in the kidney, heart, liver, and small intestine, whereas its expression level was markedly low in the brain and colon) [22, 23]. These findings indicate that PPARα would not be essential in the regulation of basal tissue sulfatide amounts. It has been reported that the constitutive amounts of distributed tissue sulfatides are mainly regulated by tissue-specific CGT gene

expression [24]. In fact, considerable amounts of CGT and its products, galactosylceramides, are known to be markedly detected in sulfatide-rich organs, such as brain and kidney [25]. Therefore, the constitutive tissue sulfatide amounts would be primarily dependent on tissue CGT expression levels and galactosylceramide content. Because other mechanisms that influence basal tissue sulfatide levels, such as the excretion of sulfatides from each tissue and modulating factors of basal sulfatide metabolism, are unknown, further studies would be required.

In the current study, the large increases in CST mRNA expression and tissue sulfatide levels were detected only in PPARα-rich organs with PPARα activation, which suggests that PPARα plays an important role in enhancing sulfatide synthesis. It has been reported that the effective transcriptional response of PPARα by its agonist was scarcely detected in low PPARα-expressing tissues, such as white adipose tissues [26-28], which indicates the significance of tissue levels of PPARα for its activity. In the current study, the different fenofibrate-induced CST expression levels in each organ might be caused by different tissue-specific PPARα expression levels. Moreover, the difference in tissue-specific fenofibrate bioavailability might be important. It is known that fenofibrate hardly enters into the central nervous system because of the blood-brain barrier system [29, 30], which might explain the lack of fenofibrate action in the brain.

In contrast, it has been reported that fenofibrate is distributed to the colon via the blood stream after absorption from the small intestine, and the drug concentration in the colon is almost identical to that in small intestine [31]. Therefore, the poor action of colon PPARα via fenofibrate treatment would be mainly due to low tissue expression levels of PPARα.

Although this study proposed a mechanism whereby the increased tissue sulfatide level was caused by CST gene induction via PPARα activation, there might be an alternative explanation, i.e., fenofibrate treatment increased the synthetic substrate of sulfatides, galactosylceramides, followed by facilitation of sulfatide synthesis. The tissue amounts of galactosylceramides are primarily regulated by the following 4 enzymes: CST, CGT, ARSA, and GALC. In the present study, transcriptional up-regulation by fenofibrate treatment was observed only in CST, which suggests that the unchanged protein amounts of CGT, ARSA, or GALC scarcely influenced tissue amounts of galactosylceramides. However, we cannot deny the possibility that compensatory modulation of enzymatic activity of sulfatide metabolism via fenofibrate treatment, as well as that of galactosylceramide contents, might affect tissue sulfatide levels. Further research to investigate protein amounts and/or enzymatic activity of sulfatide metabolic enzymes and measurement of galactosylceramide content in each

organ are needed.

Several previous studies using the C57BL mouse strain have reported that IV^3-sulfogangliotetraosylceramides were major sulfoglycolipids in the small intestine, whereas I^3-sulfogalactosylceramides, sulfatides, were scarcely detected [1, 32, 33]. In contrast, our recent study using the SV129 mouse strain revealed a large concentration of sulfatides in the small intestine [34]. With respect to this discrepancy, a previous report has noted the possibility of murine strain-specific differences in the composition and quantity of glycosphingolipids [35]. Furthermore, there might be another methodological difference. The earlier studies that investigated sulfoglycolipids generally used the following method. First, extracted lipids from each organ were separated by thin-layer chromatography, and then, the lipid composition was analyzed by mass spectrometry [36]. This method was useful for identifying novel sulfoglycolipid molecules; however, small sulfoglycolipid concentrations might be difficult to detect. In contrast, our assay system was based on the analysis of the seven types of lysosulfatides on a MALDI-TOF MS system that was optimized for detecting sulfatides, and the detection sensitivity was greater than that of previous methods [17]. Therefore, the discrepancy in murine small intestine sulfatides might be related to murine genetic background differences and/or the analytical procedures used to detect

sulfoglycolipids. Today, it is unclear whether IV^3-sulfogangliotetraosylceramides are contained in the small intestine of SV129 mice at a significant level. It is known that this molecule is also generated by CST [37]; further research to investigate whether PPARα regulates the synthesis of other sulfoglycolipids via CST as in the case of sulfatides, will be needed.

PPARα is the central regulator of fatty acid metabolism, particularly for long-chain fatty acids. PPARα maintains the constitutive expression of genes for several enzymes that are involved in the mitochondrial fatty acid β-oxidation system, such as long-chain acyl-CoA synthetase, and PPARα activation induces gene expression of these enzymes and stimulates β-oxidation [11, 38-43]. Because the synthesis of sphingolipids is initiated by the transfer of L-serine onto activated long-chain fatty acids, such as palmitoyl-CoA, which arises from the reaction with long-chain acyl-CoA synthetase [44], PPARα presumably participates in the regulation of overall sphingolipid metabolism. In fact, a previous study has shown ceramide accumulation in human skin cells that are treated with the potent PPARα agonist Wy-14,643, together with increases in gene expression and activity of serine palmitoyltransferase [45], which is the key enzyme for the initial step in sphingolipid synthesis [46]. Therefore, more studies on sphingolipid metabolism would improve our understanding of the biological function of

PPARα.

Last, the present study provides the possibility of the clinical application of PPARα agonists to disorders caused by decreased sulfatide levels. Although it remains unclear whether such disorders occur in the kidney, heart, liver, or small intestine, serum sulfatides have been recently recognized as a possible internal inhibitory factor in thrombogenesis [47-49]. Serum sulfatides are produced and secreted mainly by liver as the major component of glycosphingolipids in lipoproteins [50]. In fact, the sphingoid base composition of serum sulfatides is similar to those of liver [34]. Recent clinical studies have revealed a significant reduction in serum sulfatide levels in the final stage of chronic kidney disease, which is strongly correlated with a high incidence of cardiovascular disease at this stage [51-53]. Other studies using a kidney dysfunction mouse model have demonstrated that kidney injuries cause simultaneous reductions in the levels of sulfatides in serum and liver, which is associated with the down-regulation of hepatic CST expression [4, 5]. Furthermore, our recent study demonstrated that hepatic PPARα activation and resulting hepatic CST induction caused an increase in serum sulfatide concentration [8]. These findings suggest that new PPARα-activating drugs, which efficiently stimulate hepatic PPARα while maintaining renal function, might be effective to treat kidney disease patients. Future studies that focus on serum

sulfatide function are needed.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Figure legends

Figure 1

CST mRNA expression levels in various tissues from fenofibrate (FF)-treated wild-type (+/+) and *Ppara*-null (-/-) mice.

The mRNA levels were analyzed by qPCR and normalized to GAPDH mRNA. The data are expressed as the mean ± SD (n = 3/group). *****$P < 0.05$.

Figure 2

ARSA mRNA expression levels in various tissues from fenofibrate (FF)-treated wild-type (+/+) and *Ppara*-null (-/-) mice.

The mRNA levels were analyzed by qPCR and normalized to GAPDH mRNA. The data are expressed as the mean ± SD (n = 3/group).

Figure 3

The PPRE-binding activity of PPARα in various tissues from fenofibrate (FF)-treated wild-type mice.

Whole-tissue lysate was used for this assay. The data are expressed as the mean ± SD (n = 3/group). The mean value of each control tissue is shown as 1. *****$P < 0.05$.

Figure 4

AOX mRNA expression levels in various tissues from fenofibrate (FF)-treated

wild-type (+/+) and *Ppara*-null (-/-) mice.

The mRNA levels were analyzed by qPCR and normalized to GAPDH mRNA. The data are expressed as the mean ± SD (n = 3/group). *$P < 0.05$.

Table 1

Sulfatide amounts in various tissues from fenofibrate-treated wild-type and *Ppara*-null mice

	Ppara (+/+)		*Ppara* (-/-)	
	Control	Fenofibrate	Control	Fenofibrate
Brain	5,138 ± 308	4,960 ± 275	5,550 ± 366	5,330 ± 234
Kidney	1,315 ± 30	1,597 ± 31 *	1,402 ± 46	1,343 ± 41
Heart	17 ± 1	44 ± 2 *	18 ± 1	17 ± 1
Liver	33 ± 2	72 ± 4 *	31 ± 3	32 ± 2
Small intestine	66 ± 5	78 ± 6 *	60 ± 4	61 ± 5
Colon	27 ± 2	28 ± 2	26 ± 2	28 ± 2

Sulfatides were converted to lysosulfatides and were analyzed by MALDI-TOF MS. The amounts of sulfatides (nmol/g tissue) were calculated as the total amounts of the seven molecular species of lysosulfatides as shown in Table 2. The data are expressed as the mean ± SD (n = 6/group). * $P < 0.05$, vs. the control of the same genotype.

Table 2

Sphingoid composition of sulfatides in various tissues from fenofibrate-treated wild-type mice

	Ppara (+/+) Control/Fenofibrate						
	d18:2	d18:1	d18:0	t18:0	d20:1	d20:0	t20:0
Brain	3/3	71/70	9/10	3/3	9/10	3/2	2/2
Kidney	7/6	69/72	8/7	7/6	4/3	3/3	2/3
Heart	17/15	36/38	1/1	14/13	13/12	10/11	9/10
Liver	12/11	29/30	11/10	6/6	12/11	10/9	20/23
Small intestine	10/9	16/17	11/10	12/13	11/10	6/5	34/36
Colon	15/14	36/34	9/10	21/23	6/5	10/11	3/3

Sulfatides were converted to lysosulfatides and were analyzed by MALDI-TOF MS. The seven molecular species of lysosulfatides are as follows: sphingadienine (d18:2), (4*E*)-sphingenine (d18:1), sphinganine (d18:0), 4D-hydroxysphinganine (t18:0), (4*E*)-icosasphingenine (d20:1), icosasphinganine (d20:0), and 4D-hydroxyicosasphinganine (t20:0). The data are expressed as the mean percentages (n = 6/group).

Figure 1

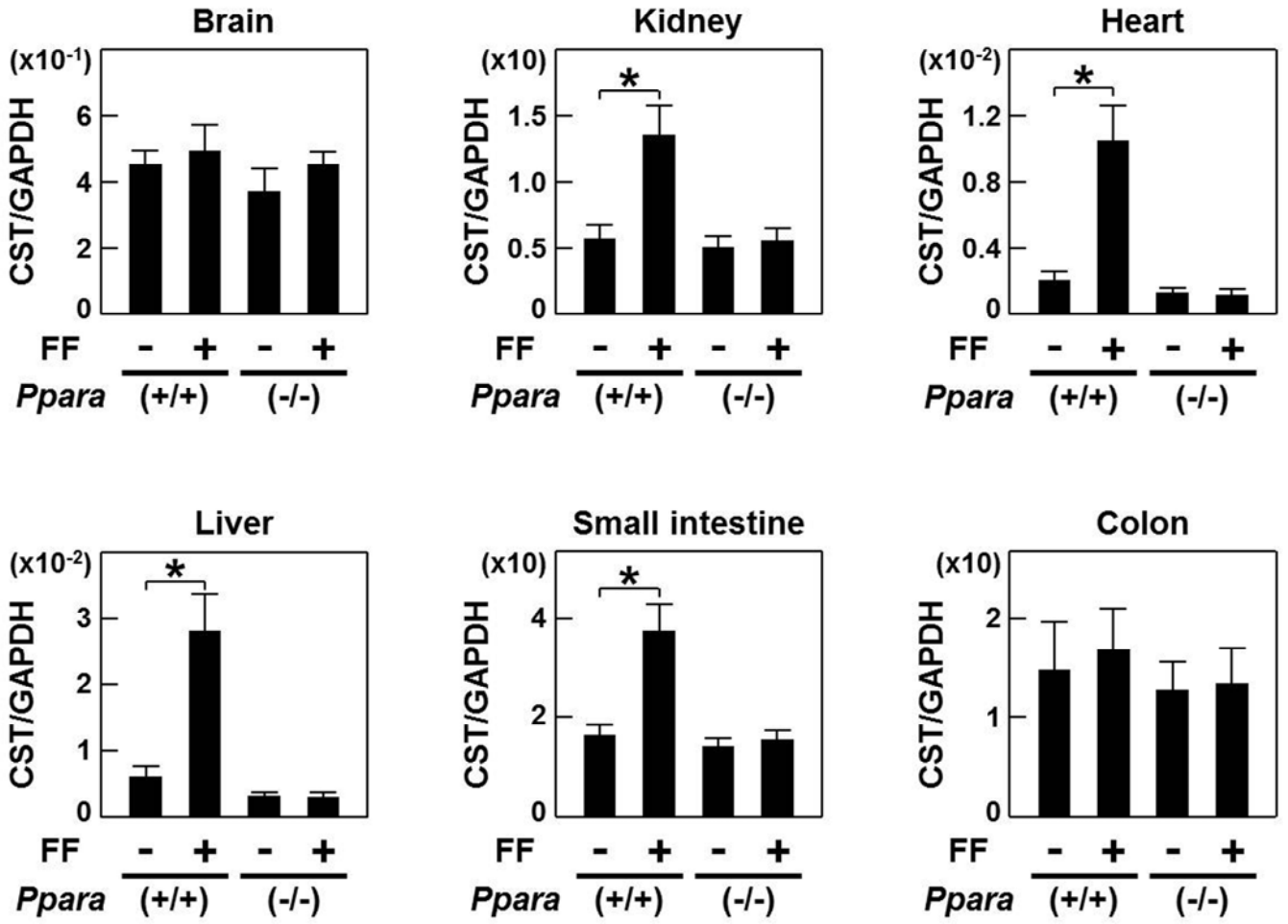

Brain
(x10-1)
CST/GAPDH
6
4
2
0
FF - + - +
Ppara (+/+) (-/-)
Kidney
(x10)
*
1.5
1.0
0.5
0
Heart
(x10-2)
1.2
0.8
0.4
Liver
3
2
1
Small intestine
4
Colon
(x10)
2
1

Figure 2

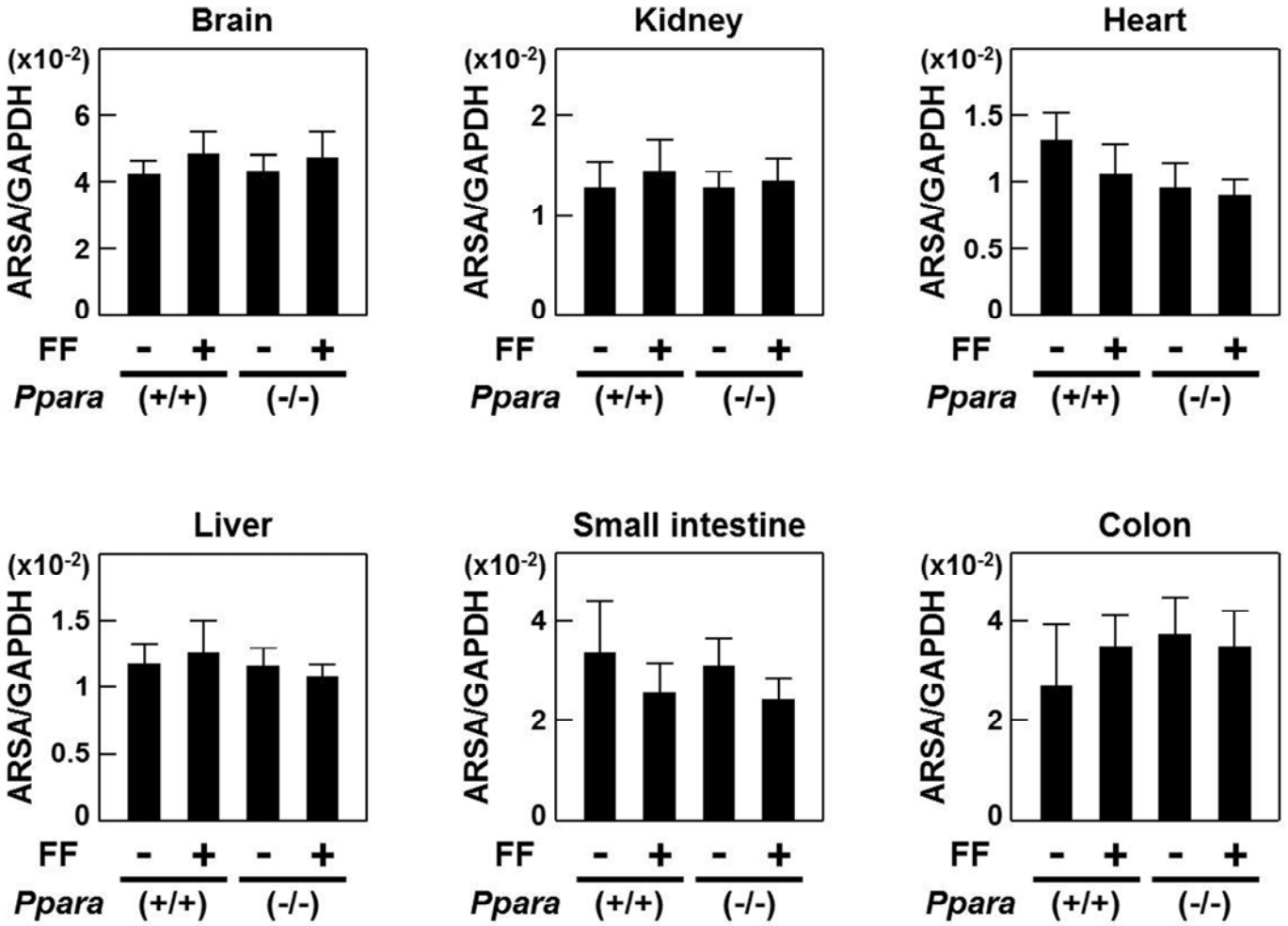

Brain
Kidney
Heart
Liver
Small intestine
Colon
(x10-2)
ARSA/GAPDH
FF
Ppara
(+/+)
(-/-)

Figure 3

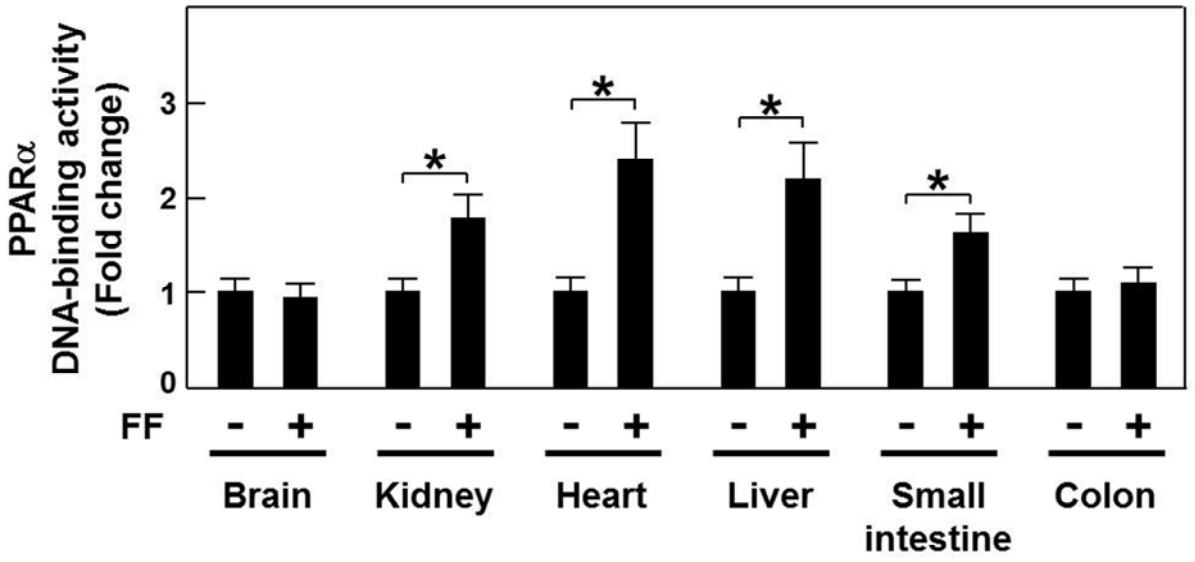
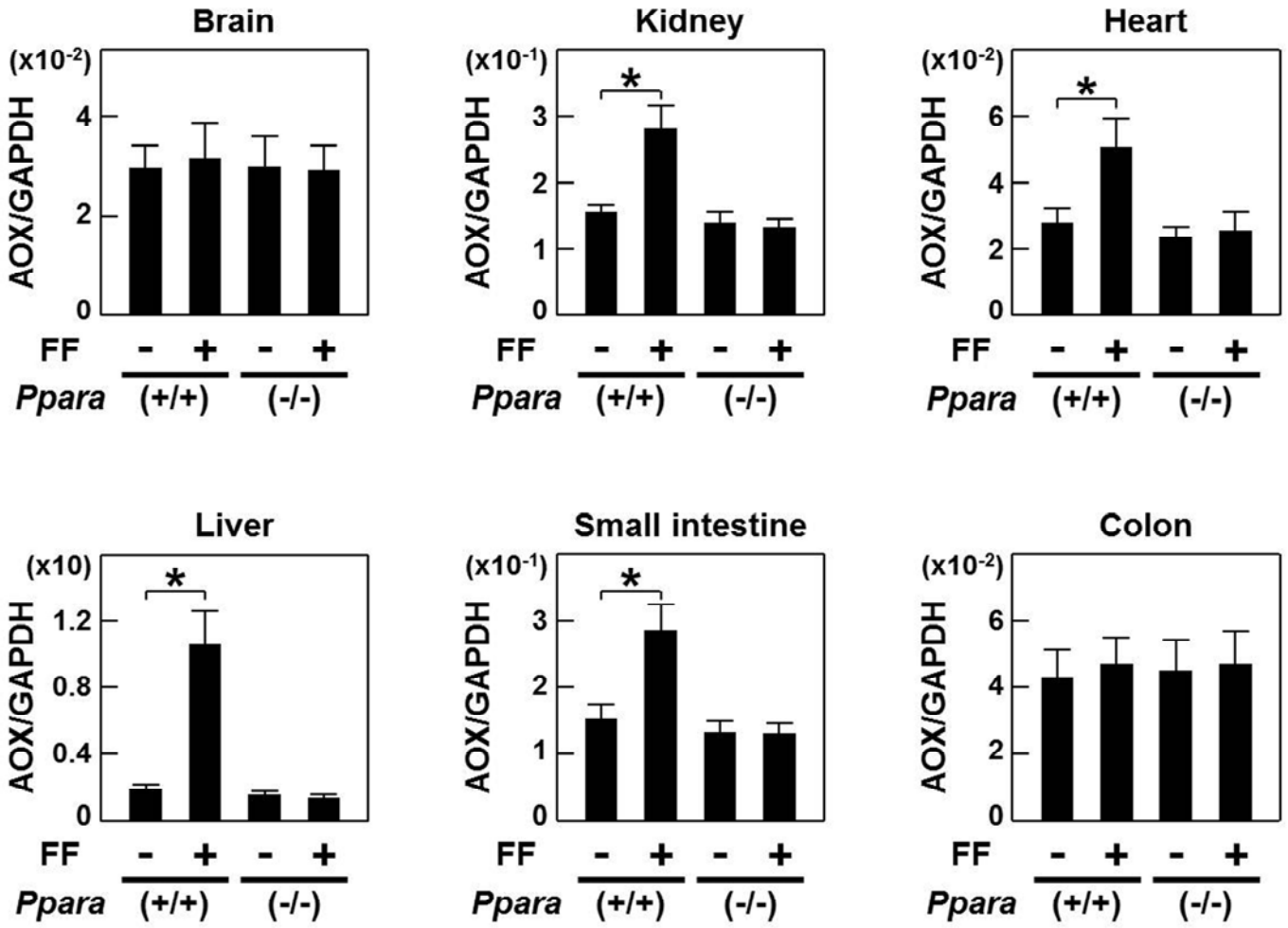

PPARα
DNA-binding activity
(Fold change)
0
1
2
3
*
*
*
*
FF
- +
- +
- +
- +
- +
- +
Brain
Kidney
Heart
Liver
Small intestine
Colon

Figure 4


Brain
(x10-2)
AOX/GAPDH
4
2
0
FF - + - +
Ppara (+/+) (-/-)
Kidney
(x10-1)
*
3
2
1
0
Heart
(x10-2)
*
6
4
2
0
Liver
(x10)
*
1.2
0.8
0.4
Small intestine
(x10-1)
Colon
(x10-2)