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Expression Analysis of the Aldo-Keto Reductases Involved in the Novel Biosynthetic Pathway of Tetrahydrobiopterin in Human and Mouse Tissues

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Tetrahydrobiopterin (BH_4) acts as a cofactor of the aromatic amino-acid hydroxylases, and its deficiency may result in hyperphenylalaninemia (HPA) and decreased production of the neurotransmitters. BH_4 is synthesized by sepiapterin reductase (SPR) from 6-pyruvoyl-tetrahydropterin (PPH_4). A patient with SPR deficiency shows no HPA; however, an SPR knockout mouse exhibits HPA. We have reported on the SPR-unrelated novel biosynthetic pathway from PPH_4 to BH_4 (salvage pathway II) in which 3α-hydroxysteroid dehydrogenase type 2 and aldose reductase work in concert. In this study, we performed the expression analysis of both proteins in humans and wild-type mice. The results of expression analysis indicated that salvage pathway II worked in human liver; however, it did not act in human brain or in mouse liver and brain. For this reason, a patient with SPR deficiency may show progressive neurological deterioration without HPA, and SPR knockout mice may exhibit HPA and abnormal locomotion activity.

Key words: AKR1B1, AKR1C3, Aldo-keto reductase, BH_4 deficiency, SPR deficiency.

Tetrahydrobiopterin (BH_4) is a cofactor for aromatic amino-acid hydroxylases (*1*, *2*), which catalyses the initial steps in phenylalanine degradation in the liver and the rate-limiting steps in the biosynthesis of catecholamine and indoleamine neurotransmitters in the brain. BH_4 is also required by nitric oxide synthase, which generates nitric oxide, a messenger molecule involved in various processes in many tissues (*3*, *4*).

The pathway of the *de novo* biosynthesis of BH_4 from GTP involves GTP cyclohydrolase I (GTPCH-I, EC 3.5.4.16), 6-pyruvoyl-tetrahydropterin synthase (PTPS, EC 4.6.1.10) and sepiapterin reductase (SPR, EC 1.1.1.153). SPR catalyses the last step of the biosynthesis, in which the diketo group on the side chain of PPH_4 is converted into the corresponding diol form in BH_4 (*5*–*7*). A deficiency of BH_4 causes hyperphenylalaninemia (HPA), which leads to the abnormal development of mammalian neonates.

In 2001, SPR deficiency was first discovered in a patient with progressive psychomotor retardation and dystonia. However, the patient showed normal urinary pterins without HPA (*8*–*10*). These findings suggest that an enzyme or enzymes other than SPR may be involved in the formation of BH_4 from PPH_4.

Park *et al*. (*11*) previously reported that human monomeric carbonyl reductase (CBR) reduces PPH_4 to both 1′-oxo-2′-hydroxypropyl-tetrahydropterin (1′-$OXPH_4$) and 1′-hydroxy-2′-oxopropyl-tetrahydropterin (2′-$OXPH_4$) and that aldose reductase (AKR1B1, EC 1.1.1.21) catalyses the reduction of 2′-$OXPH_4$ to BH_4. Therefore, if both AKR and CBR proteins exist in the tissue, BH_4 can be synthesized from PPH_4 without SPR. However, the 2′-$OXPH_4$-forming activity of CBR is quite low compared to its 1′-$OXPH_4$-forming activity. Therefore, the BH_4-forming activity, which involves CBR and AKR1B1, functions with difficulty in humans.

Blau *et al*. (*9*) proposed that BH_4 is synthesized through salvage pathway I in the case of SPR deficiency (Fig. 1). In salvage pathway I, sepiapterin, which is generated non-enzymatically from 1′-$OXPH_4$, is converted to dihydrobiopterin (BH_2) by CBR. The final reduction to BH_4 in the liver is catalysed by the enzyme dihydrofolate reductase (DHFR, EC 1.5.1.3). The activity of DHFR is ∼10× lower in the brain than in the liver. Thus, sepiapterin is reduced to dihydrobiopterin by CBR but cannot be further reduced to BH_4 owing to low DHFR activity in the brain. Therefore, they concluded that a patient with SPR deficiency shows progressive psychomotor retardation without HPA.

We previously discovered two carbonyl reductases (CRI and CRII) that are involved in the formation of BH_4 from PPH_4 in the fat body of the *lemon* mutant and the

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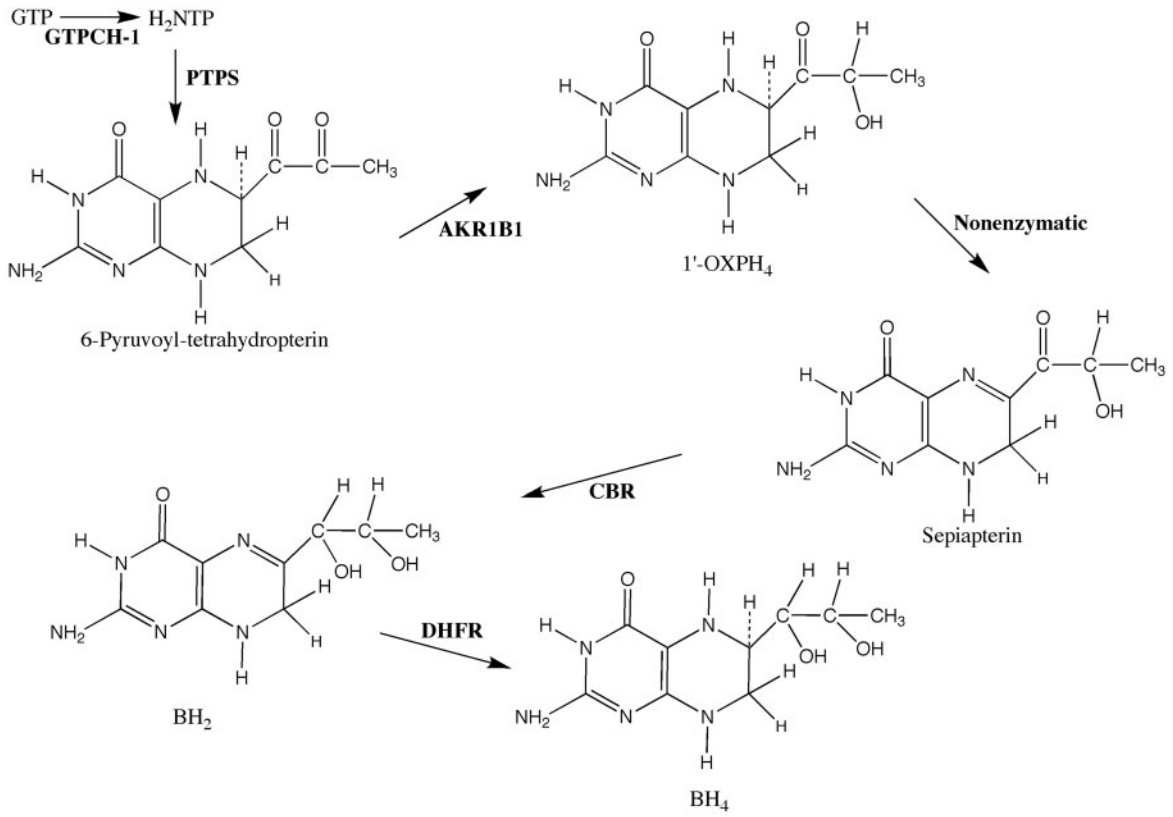


Fig. 1. **The SPR-unrelated BH_4 biosynthetic pathway (salvage pathway I).** In the absence of SPR, 1′-$OXPH_4$ is oxidized non-enzymatically to sepiapterin, which is reduced to BH_4 by CBR and DHFR.

normal strain of the silkworm, *Bombyx mori* (*12–14*). Furthermore, we have reported on a novel alternative biosynthetic pathway (salvage pathway II) from PPH_4 to BH_4, in which 3α-hydroxysteroid dehydrogenase type 2 (AKR1C3, EC 1.1.1.213) and AKR1B1 work in concert (Fig. 2) (*15*). Salvage pathway II shows that AKR1C3 efficiently catalyses the reduction of PPH_4 to the intermediate metabolite, 2′-$OXPH_4$, which is reduced to BH_4 by AKR1B1.

Recently, Yang *et al*. (*16*) indicated that the SPR knockout mouse exhibited HPA, dwarfism and impaired body movement. Furthermore, $Spr^{-/-}$ mice, as reported by Takazawa *et al*. (*17*), also showed HPA. In spite of adequate activity of CBR and DHFR in the mouse liver, SPR knockout mice show HPA. Thus, salvage pathway I, which is proposed by Blau *et al*. (*9*), may not function in mouse liver. We believe that salvage pathway II works in human liver but not in wild-type mouse liver. Consequently, humans who lack *SPR* may show no HPA, and SPR knockout mice show HPA. In order to verify this hypothesis, we examined the expression analysis of AKR1B1 and AKR1C3 proteins in human and wild-type mouse tissues using anti-AKR1B1 or anti-AKR1C3 antibodies. In the case of mice, western blot and immunohistochemical analyses showed that the AKR1C3 protein was expressed in the liver but not in the brain. In contrast, the AKR1B1 protein was detectable in the brain but not in the liver.

In the case of humans, western blot analysis showed that the AKR1B1 and AKR1C3 proteins were both expressed in the liver; however, AKR1B1 was only expressed in the brain, and AKR1C3 could not be detected in the brain.

These results of the expression analysis of AKR1B1 and AKR1C3 by means of immunohistochemistry and western blot analysis could be explained by the relationship between HPA in SPR knockout mouse and the absence of HPA in patients with SPR deficiency. Moreover, they suggest that the SPR-unrelated BH_4 formation pathway from PPH_4, which is involved in the AKR enzymes, functions in the human liver. In this report, for the first time, we explain the reasons that SPR knockout mice show abnormal locomotion activity with HPA and a patient with SPR deficiency exhibits progressive neurological deterioration without HPA.

MATERIALS AND METHODS

Chemicals and Enzymes—BH_4 and sepiapterin were purchased from Dr Schircks (Jona, Switzerland). Dihydroneopterin triphosphate (NH_2TP) was synthesized enzymatically from GTP by the method of Yoshioka *et al*. (*18*) using purified GTP cyclohydrolase I from chicken liver (*19*). 1′-$OXPH_4$ and 2′-$OXPH_4$ standards were prepared as described previously (*12*). Other chemicals were of analytical grade and obtained from

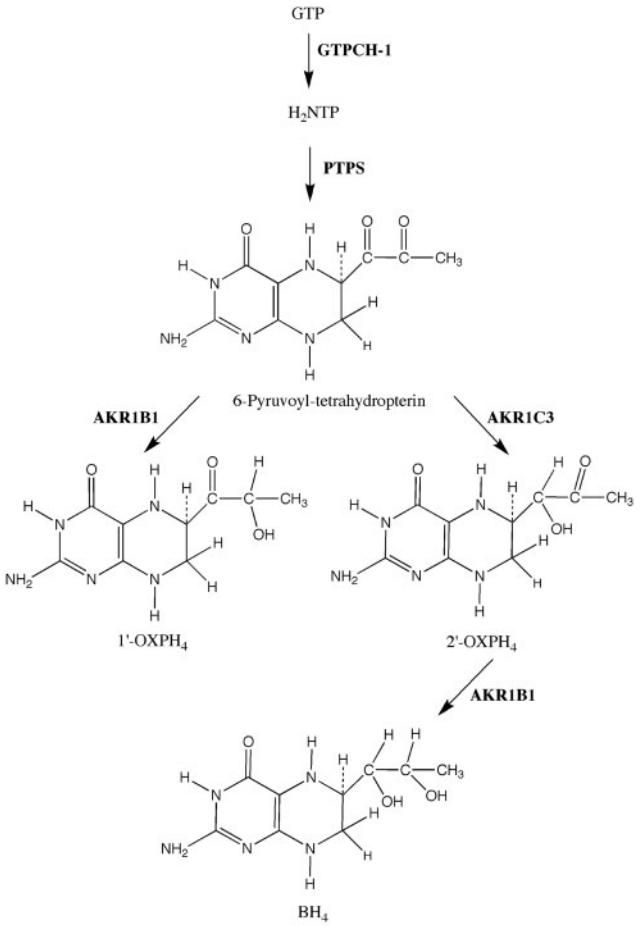


Fig. 2. **The SPR-unrelated BH_4 biosynthetic pathway (salvage pathway II).** BH_4 is synthesized from PPH_4 by AKR1C3 and AKR1B1.

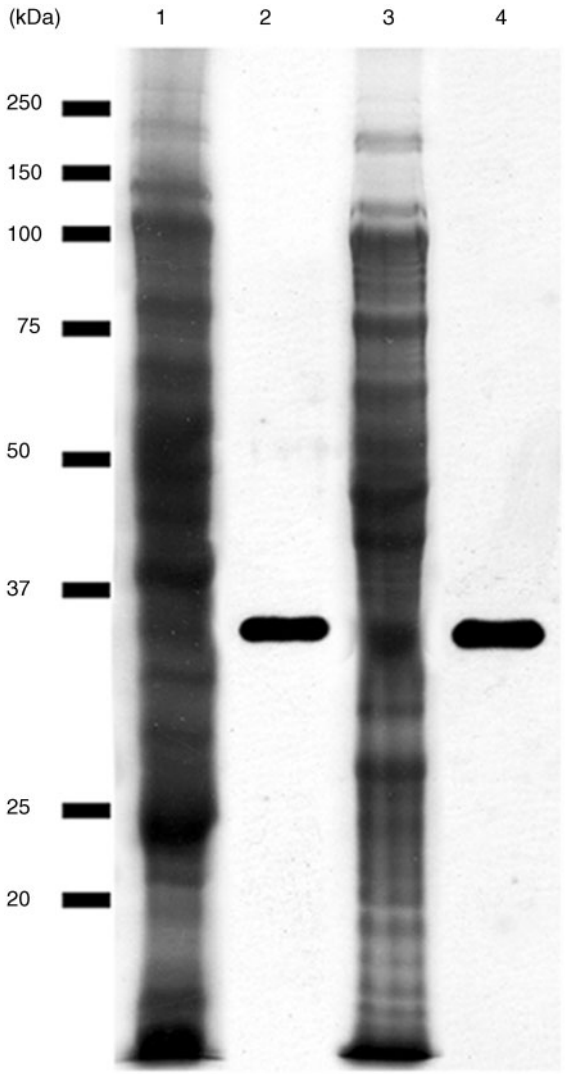


Fig. 3. **Specificity of anti-AKR1B1 and anti-AKR1C3 antibodies.** Lanes 1 and 3 show the SDS/PAGE analysis of the protein from the *E. coli* lysate, which expressed human AKR1B1 and AKR1C3, respectively. The gel was stained with Coomassie Brilliant Blue R-250. Lanes 2 and 4 are immunoblot analyses using the anti-AKR1B1 antibody and the anti-AKR1C3 antibody, respectively. Immunoblot analysis shows that both antibodies can only react with one species of ~36 kDa. Ten micrograms of crude *E. coli* lysate was used.

commercial sources. PPH_4 synthase was purified from chicken liver by the method of Takikawa *et al*. (*20*).

Human Tissues and Animals—Human tissues were obtained in compliance with the Ethical Committee of Wakayama Medical University and Osaka City University Medical School's Ethics Committee. Three human brains were obtained following autopsies: Patient 01 (P01) (female) was 68 years old with Alzheimer's disease; Patient 02 (P02) (female) was 78 years old with oophoroma; and Patient 03 (P03) (female) was 48 years old with bacterial meningitis. One human liver was obtained following an autopsy: Patient 04 (P04) (male) was 1 year old with galactosialidosis. Another was obtained as a result of a biopsy: Patient 05 (P05) (male) was 5 years old with amylopectinosis. The autopsy samples from brain and liver were obtained between 5 and 7 h post mortem. The autopsy samples and biopsy liver sample were stored at –70°C until use. Male mice of BALB/c (3 weeks old), an example of wild-type mouse, were obtained from a breeder.

Production of an Anti-AKR1B1 and an Anti-AKR1C3 Antibody—Rabbit polyclonal antibodies against the purified recombinant human AKR1B1 and AKR1C3 proteins (*21*, *22*) were raised. For immunization, a solution containing the purified proteins (1 mg/ml) was emulsified with Freund's complete adjuvant having twice the volume of the antigen solution. Rabbits received a dose of 0.5 mg of the proteins (AKR1B1, AKR1C3) intradermally at multiple sites on their backs. Doses of 0.5 mg of the proteins in Freund's incomplete adjuvant were then given as booster injections at 2 weeks intervals by subcutaneous injections. More than six booster injections were necessary to obtain a satisfactory antibody titer.

Western Blot Analysis—Western blot analyses of mouse liver, brain, kidney, heart and lung lysates and of human

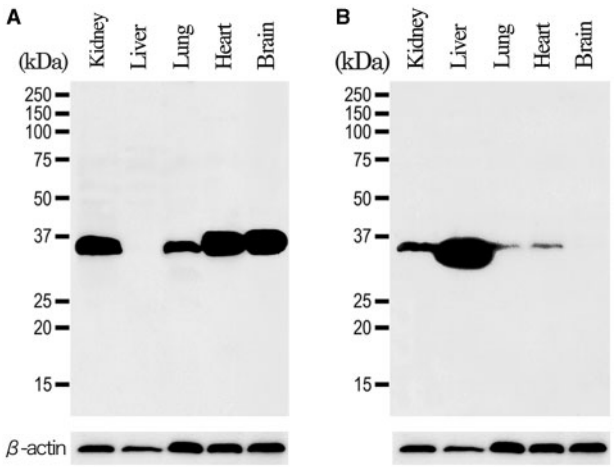


Fig. 4. **Western blot analysis of extracts from mouse tissues.** Western blot was performed using (A), an anti-AKR1B1 antibody; (B) an anti-AKR1C3 antibody. The AKR1B1 protein was strongly expressed in the brain, heart, lung, and kidney; however, the anti-AKR1B1 antibody could not be recognized in liver lysate. The AKR1C3 protein was strongly expressed in the liver and kidney but not in the brain. Lower panels show immunostaining of β-actin using the same membrane. Ten micrograms of lysate protein of each tissue was used.

brain extracts from the cerebellar cortex, spinal cord, substantia nigra, hippocampus, hypothalamus and caudate nucleus and liver lysate were performed. The tissues were homogenized in a 20 mM potassium phosphate buffer (pH 7.0) containing 1 tablet/50 ml protease inhibitor cocktail (Roche) and 0.05% Igepal CA-630 (Sigma). The homogenates were then centrifuged at $15{,}000 \times g$ for 20 min, and solid ammonium sulfate was added to the supernatant to 70% saturation. The precipitate was collected by centrifugation at $15{,}000 \times g$ for 20 min and dissolved in 0.3 vol. of the extracted buffer. The solution was dialysed overnight against the same buffer and centrifuged at $15{,}000 \times g$ for 20 min. The supernatant was stored at –70°C until used. The extract was subjected to 12% SDS–PAGE, and the proteins were transferred to a PVDF membrane (Bio-Rad) at 80 mA for 120 min. After being blocked with 3% skim milk, the membranes were first incubated with an adequate dilution of the antibodies and then with anti-rabbit IgG conjugated to horseradish peroxidase. Following repeated washing of the membrane, the signals were visualized with ECL plus (GE Healthcare).

Immunohistochemistry—Three-week-old male mice of BALB/c were purchased from a breeder (Japan SLC, Inc., Hamamatsu, Japan). They were deeply anesthetized with diethyl ether and subsequently sacrificed by perfusion with physiological saline followed by 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4). The brain and liver were dissected 30 min after perfusion, post-fixed in the same fixative overnight at 4°C, and rinsed three times at 30 min intervals with a 0.1 M phosphate buffer. They were then automatically processed through paraffin embedding with a vacuum infiltration processor (Tissue-Tek VIP5 Jr., Sakura FineTek Japan). Four serial sections of a median plane in the liver were cut to a thickness of 5 μm with a sliding microtome (Microm HM 430, Zeiss, Germany). The first slide was stained with the hematoxylin-eosin (HE) stain. Brain sections in the sagittal, cross (transversal) and horizontal planes were cut into serial sections with a thickness of 5 μm with a sliding microtome. The first slide was stained with the Kluver–Barrera's (KB) stain. The second to fourth slides were used for immunostaining. After treatment with 0.3% H_2O_2 in methanol, the sections were incubated overnight with anti-AKR1B1 antisera (1:200 dilution) for the second slide, anti-AKR1C3 antisera (1:200 dilution) for the third slide, and normal rabbit serum (1:200 dilution) for the fourth slide at 4°C. They were incubated with goat anti-rabbit peroxidase-conjugated immunoglobulin (IgG) [N-Histofine Simple Stain Mouse MaxPO(R), Nichirei Biosciences, Inc., Japan]. The peroxidase activity was visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB) in 0.05 M Tris (pH 7.6) with H_2O_2 as a chromogen solution, and the sections were counterstained with the hematoxylin stain.

Assay of Sepipterin Reductase Activity—The reaction mixture contained the following components: a 50 mM potassium phosphate buffer (pH 7.0), 1 mM NADPH, 15 μM sepiapterin, 0.5 mM N-acetyl serotonin (NAS, a potent inhibitor of SPR) or DW, and an appropriate amount of human liver extract from P04 or mouse liver extract in a final volume of 100 μl. The reaction mixture was incubated at 37°C for 20 min in darkness. The reaction was stopped by the addition of 10 μl of a 20% trichloroacetic acid solution and 20 μl of an iodine solution (1% I_2, 2% KI). After allowing the mixture to stand for 30 min at room temperature in darkness, excess iodine was reduced by the addition of 10 μl of a 2% ascorbic acid solution (*23*), and the mixture was centrifuged at $15{,}000 \times g$ for 5 min. The amount of biopterin (BP) in the resulting supernatant was measured by HPLC with fluorometric detection, as previously described (*12*).

Assay of Tetrahydropterin-Producing Activity—Analysis of tetrahydropterins was performed by HPLC with electrochemical detection, as described previously (*14*, *15*). The reaction mixture contained the following components: a 50 mM potassium phosphate buffer (pH 7.0), 100 μM NADPH, 10 μl of a concentrated solution of PPH_4 synthase, 5 mM dithiothreitol, 8 mM $MgCl_2$, 14 μM NH_2TP, 0.5 mM NAS and an appropriate amount of human liver extract from P04 or mouse liver extract in a final volume of 100 μl. The reaction mixture was flushed with N_2 gas, sealed, and incubated at 37°C for 30 min in darkness. The reaction was stopped by the addition of 10 μl of a 20% trichloroacetic acid solution and the mixture was centrifuged at $15{,}000 \times g$ for 5 min. The amount of tetrahydropterins in the resulting supernatant was measured by HPLC, as previously described (*12*).

RESULTS

Specificity of Anti-AKR1B1 and Anti-AKR1C3 Antibodies—To demonstrate the ability of the antibodies to specifically distinguish AKR1B1 and AKR1C3 from other cellular proteins, western blot analysis was

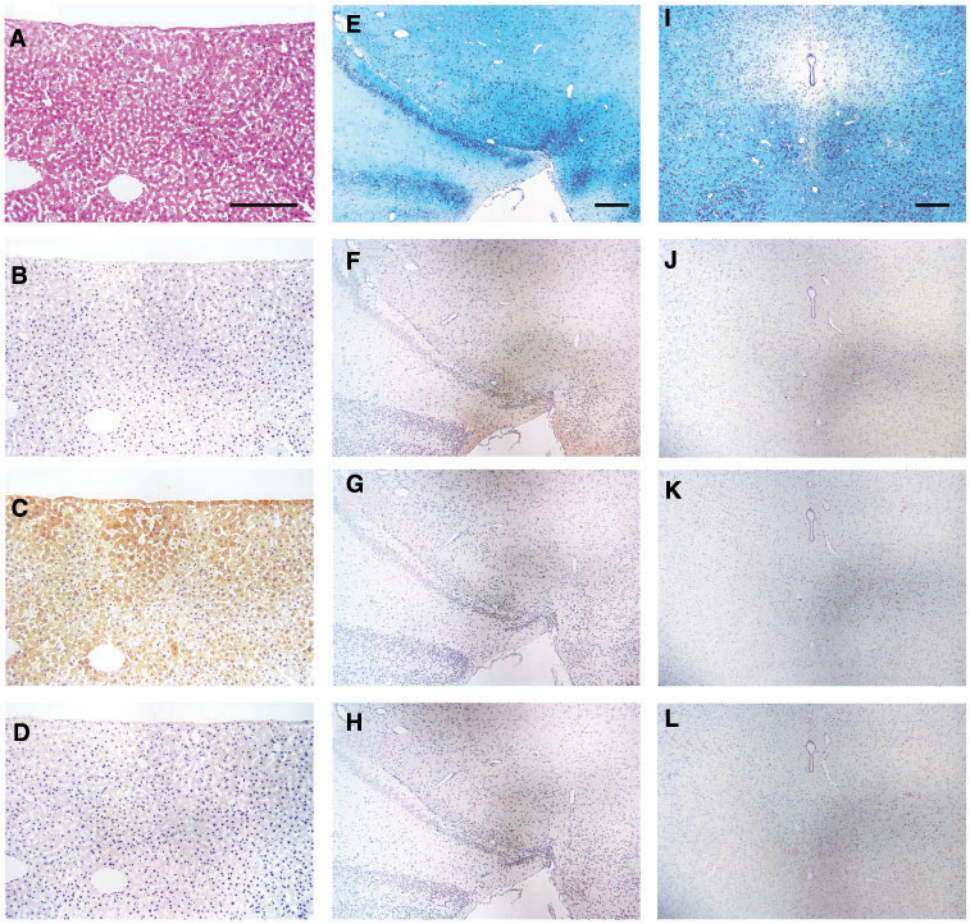
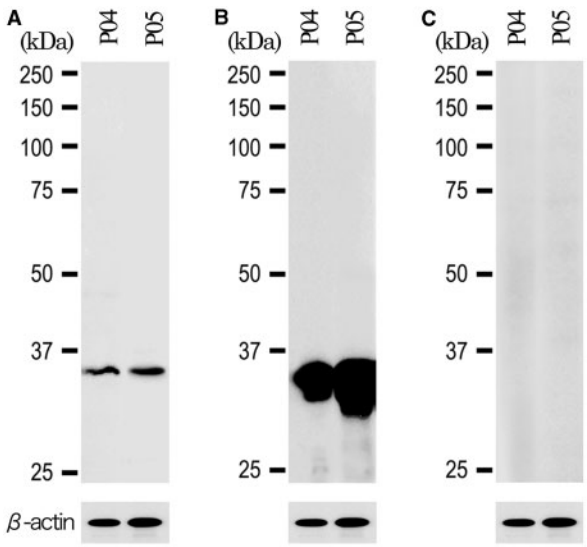


Fig. 5. **Immunohistochemical analysis of AKR1B1 and AKR1C3 in mouse liver and brain.** Paraformaldehyde-fixed paraffin sections of liver were reacted with: (A) hematoxylin-eosin (HE) stain; (B) anti-AKR1B1 serum; (C) anti-AKR1C3 serum; (D) negative control. The substantia nigra (dopaminergic neuron) sections were reacted with (E); KB stain; (F) anti-AKR1B1 serum; (G) anti-AKR1C3 serum; (H) negative control. The dorsal nucleus raphe (serotonergic neuron) sections were reacted with (I); KB stain; (J) anti-AKR1B1 serum; (K) anti-AKR1C3 serum; (L) negative control. The cross-reacting protein was visualized with 3,3′-diaminobenzidine tetrahydrochloride. The immunoreactivity of AKR1C3 is shown in the cell bodies of mouse liver. AKR1B1 immunoreactivity is not shown elsewhere in the liver regions. On the contrary, AKR1B1 immunoreactivity is shown weakly in the cell bodies of monoaminergic neurons in the brain. No staining is shown in the sections of mouse brain incubated with an anti-AKR1C3 antibody.



performed in the total cellular lysate from *E. coli* cells that expressed human AKR1B1 or AKR1C3. A single protein species with a molecular mass of ~36 kDa was detected in this total cellular preparation (Fig. 3, lanes 2 and 4). The recognized protein species had the same size as the AKR1B1 and AKR1C3 proteins (*24*, *25*).

Western Blot Analysis from Mouse Tissues—Western blot analysis showed strong expression of the AKR1B1 protein in the brain, heart, lung and kidney. However, the anti-AKR1B1 antibody could not be recognized against the liver lysate (Fig. 4A). On the other hand,

Fig. 6. **Western blot analysis of human liver lysate.** Western blot was performed using (A), an anti-AKR1B1 antibody; (B) an anti-AKR1C3 antibody; and (C), a normal rabbit serum. Lower panels show immunostaining of β-actin using the same membrane. Both AKR1B1 and AKR1C3 proteins were detected with each antibody from the human liver lysate. Ten micrograms of lysate protein was used.

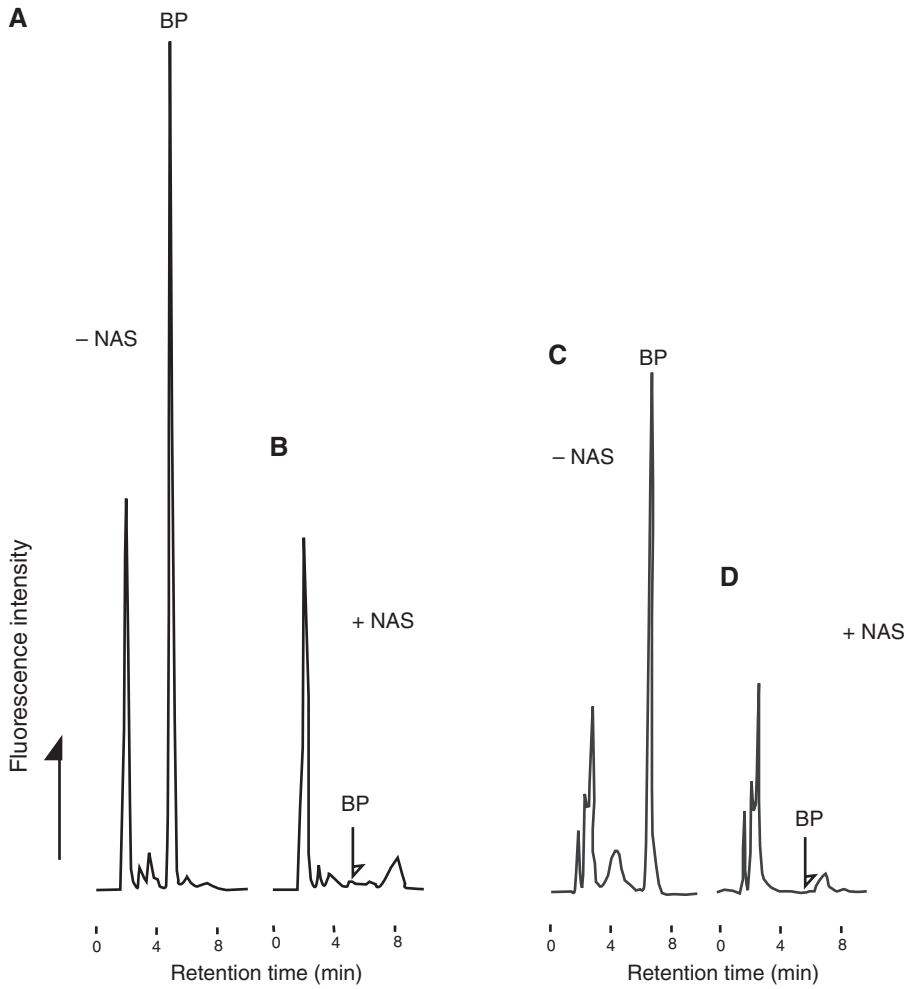


Fig. 7. **Analysis by HPLC of SPR activity.** The reaction mixture contained 15 μM sepiapterin, 1mM NADPH, 0.5 mM NAS or DW, and an appropriate amount of human liver extract from P04 or an appropriate amount of mouse liver extract in a final volume of 100 μl. It was incubated for 20 min at 37°C in darkness. The SPR activity was measured in terms of the amount of biopterin (BP) produced by fluorimetry. (A, B) human liver extract; (C, D) mouse liver extract.

the AKR1C3 protein was strongly expressed in the liver and kidney but not in the brain (Fig. 4B).

Immunohistochemical Analysis of AKR1B1 and AKR1C3 in Mouse Liver and Brain—AKR1B1 immunoreactivity was not shown in the liver, but AKR1C3 immunoreactivity was shown in the cell bodies of the liver (Fig. 5A–D). In some monoaminergic neurons in the brain, AKR1B1 immunoreactivity weakly showed cell bodies of the substantia nigra and dorsal nucleus raphe, but AKR1C3 immunoreactivity could not be detected in some monoaminergic neurons in the brain, as in the negative control (Fig. 5E–L).

Western Blot Analysis from Human Liver—Western blot analysis for AKR1B1 and AKR1C3 was conducted in human brain and liver lysates. Both AKR1B1 and AKR1C3 proteins were detected with each antibody from the human liver lysate but not in the negative control (Fig. 6A–C).

Sepiapterin Reductase Activity in Human Liver and Mouse Liver—The BP-forming activity by SPR was assayed in human liver or mouse liver extracts. Strong SPR activity was shown in human liver and mouse liver; however, the activity was completely inhibited by the addition of 0.5 mM NAS in the reaction mixture (Fig. 7A–D).

Tetrahydropterin-Producing Activity in Human Liver and Mouse Liver—Under the condition of fully inhibited SPR activity, the tetrahydropterin-forming activity by AKR1B1 and AKR1C3 was assayed in the human liver lysate or in the mouse liver extract. When tetrahydropterin-forming activity by the AKR1B1 and the AKR1C3 was observed in human liver extract with 0.5 mM NAS, BH_4, 2′-$OXPH_4$, and 1′-$OXPH_4$ were detected in the reaction mixture (Fig. 8A). In the case of mouse liver extract, only 2′-$OXPH_4$ was synthesized from PPH_4 in the presence of 0.5 mM NAS by AKR1C3 (Fig. 8B).

Western Blot Analysis from Human Brain—Human brain lysates from the cerebellar cortex, spinal cord, substantia nigra, caudate nucleus, hippocampus and hypothalamus were subjected to western blot analysis with anti-AKR1B1 and anti-AKR1C3 antibodies and normal rabbit serum. The anti-AKR1B1 antibody could

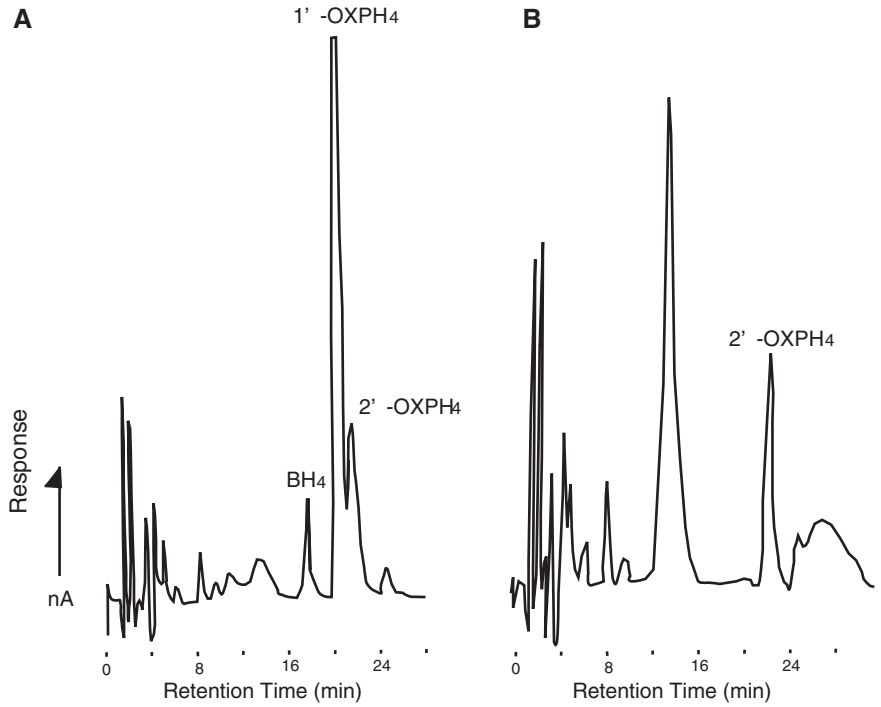


Fig. 8. **Production of BH_4, 2′-$OXPH_4$, and 1′-$OXPH_4$ by human liver or mouse liver extracts.** The reaction mixture contained the following components: a 50 mM potassium phosphate buffer (pH 7.0), 100 μM NADPH, 10 μl of a concentrated solution of PPH_4 synthase, 5 mM dithiothreitol, 8 mM $MgCl_2$, 14 μM NH_2TP, 0.5 mM NAS and an appropriate amount of human liver extract from P04 or an appropriate amount of mouse liver extract in a final volume of 100 μl. The reaction mixture was flushed with N_2 gas, sealed and incubated at 37°C for 30 min in darkness. The products were analysed by HPLC with electrochemical detection. (A) human liver extract; (B) mouse liver extract.

detect a major band of ~36 kDa in the extract from the cerebellar cortex, spinal cord, substantia nigra, caudate nucleus, hippocampus and hypothalamus (Fig. 9A). The AKR1B1 protein was widely detected in the human brain; however, the AKR1C3 protein was scarcely detected in the brain, in contrast to the case of the negative control (Fig. 9B and C).

DISCUSSION

In 2001, SPR deficiency was first discovered from a patient with progressive psychomotor retardation and dystonia. However, the patient showed normal urinary pterins without HPA (*8*). To explain this observation, Blau *et al*. (*9*) proposed that BH_4 was synthesized through salvage pathway I in the case of SPR deficiency (Fig. 1).

Recently, Yang *et al*. (*16*) indicated that SPR knockout mice exhibited HPA, dwarfism and impaired bodily movement. Furthermore, $Spr^{-/-}$ null mice, as reported by Takazawa *et al*. (*17*), also showed HPA. In spite of the fact that adequate activity of CBR and DHFR exists in mouse liver, SPR knockout mouse showed HPA. These results suggested that salvage pathway I may not be at work in mouse liver.

We have reported on a novel SPR-unrelated biosynthetic pathway (salvage pathway II) from PPH_4 to BH_4, in which AKR1C3 and AKR1B1 work in concert (Fig. 2) (*15*). We believe that salvage pathway II works in human liver but not in wild-type mouse liver, since the SPR knockout mouse showed HPA and a patient with SPR deficiency did not. Therefore, we prepared specific antibodies against AKR1B1 and AKR1C3 proteins (Fig. 3, lanes 2 and 4).

Western blot analysis from mouse tissue lysates using specific antibodies showed that the AKR1B1 protein was strongly expressed in the brain but not in the liver. The AKR1C3 protein existed in large amounts in the liver; however, it could not be detected in the brain (Fig. 4A and B). The immunohistochemical analysis of AKR1B1 and AKR1C3 in mouse liver and brain showed similar results to those of western blot analysis. AKR1C3 immunoreactivity was shown in the liver but not in monoaminergic neurons. In the case of AKR1B1, weak immunoreactivity was shown in the substantia nigra and the dorsal nucleus raphe, but not in the liver (Fig. 5). These results suggested that salvage pathway II, which was the SPR-unrelated BH_4 formation pathway from PPH_4, does not act in mouse liver and brain.

On the other hand, both AKR1B1 and AKR1C3 proteins were detected with each antibody from the human liver lysate (Fig. 6A–C). Although AKR1B1 can reduce the 2′-keto group of both PPH_4 and 2′-$OXPH_4$, AKR1C3 specifically reduces the 1′-keto group of PPH_4 but not the 1′-keto group of 1′-$OXPH_4$ (Fig. 2). If the PPH_4 is immediately reduced to 1′-$OXPH_4$ by AKR1B1, the formation of BH_4 does not occur in human liver because AKR1C3 cannot reduce 1′-$OXPH_4$ to BH_4. To demonstrate the BH_4-forming activity by salvage pathway II, the tetrahydropterin-producing activity in

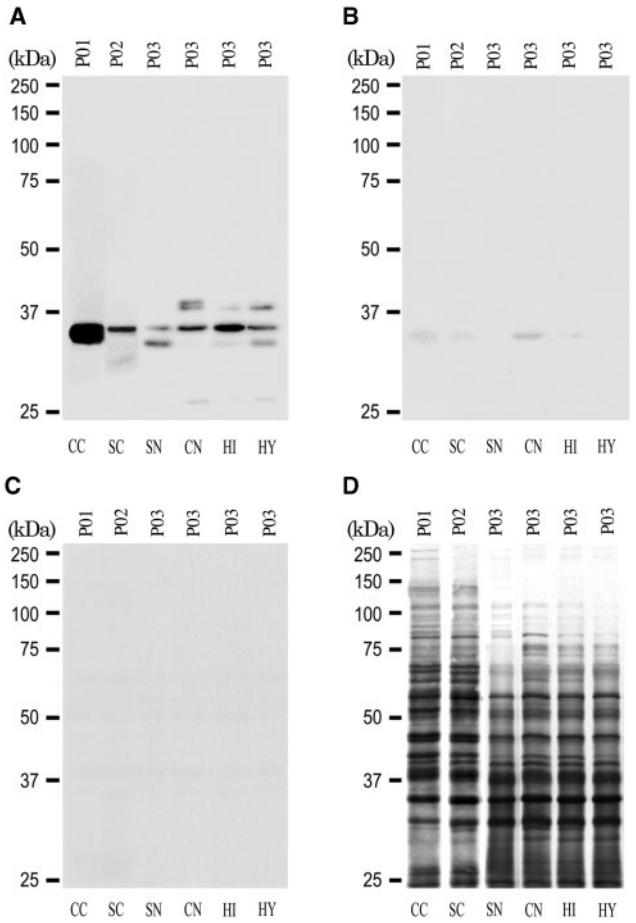


Fig. 9. **Western blot analysis of human brain lysate.** Western blot was performed using (A) an anti-AKR1B1 antibody; (B) an anti-AKR1C3 antibody; and (C) a normal rabbit serum. The gel was stained with (D) Coomassie Brilliant Blue R-250. Ten micrograms of lysate protein of the cerebellar cortex (CC), spinal cord (SC), substantia nigra (SN), caudate nucleus (CN), hippocampus (HI) and hypothalamus (HY) was used. The AKR1B1 protein was widely detected in the human brain; however, the AKR1C3 protein was scarcely detected in the brain, which contrasts the case of the negative control.

human liver and mouse liver extracts was assessed with PPH_4 as a substrate. First, we tried to determine the concentration on NAS, which completely inhibited SPR activity, because SPR has strong tetrahydropterin-producing activity from PPH_4. In the presence of 0.5 mM NAS, SPR activity was fully suppressed in human liver and mouse liver extracts (Fig. 7A–D). When tetrahydropterin-producing activity by the AKR1B1 and the AKR1C3 was observed in human liver extract with 0.5 mM NAS, BH_4, 2′-$OXPH_4$ and 1′-$OXPH_4$ were synthesized in the reaction mixture (Fig. 8A). This means that 2′-$OXPH_4$, which is synthesized from PPH_4 by AKR1C3, was reduced to BH_4 by AKR1B1 in human liver extract. In the case of mouse liver extract, only 2′-$OXPH_4$ was synthesized from PPH_4 in the presence of 0.5 mM NAS by AKR1C3 (Fig. 8B). This suggests that AKR1B1, which reduces 2′-$OXPH_4$ to BH_4, does not act in mouse liver. The results of this experiment suggest that salvage pathway II, which is relevant to AKR1B1 and AKR1C3, works in human liver but not in mouse liver. In spite of adequate activity of CBR and DHFR in mouse and human liver, SPR knockout mice show HPA, and *SPR*-deficient patients do not. We have reported that the formation rate of sepiapterin from the non-enzymatic degradation of 1′-$OXPH_4$ was very slow (*26*) and, thus, salvage pathway I would not advance even if CBR and DHFR existed in the human and mouse liver. An *SPR*-deficient patient does not show HPA; in other words, salvage pathway II acts in the liver of the patient.

SPR-deficient patients displayed abnormal responses in the phenylalanine loading test, indicating that the phenylalanine hydroxylase (PAH, EC 1.14.16.1) function was somewhat impaired although the phenylalanine levels in these patients appeared to be normal (*8*); on the other hand, the $Spr^{-/-}$ mouse serum contained a high level of phenylalanine (*16*, *17*). One interesting explanation for this discrepancy between the phenylalanine levels of *SPR*-deficient patients and those of $Spr^{-/-}$ mice was proposed by Yang *et al*. (*16*). These researchers contend that mice and humans have different levels of alternative enzyme activities that compensate for the loss of SPR; thus, a higher level of BH_4 (a level sufficient for the function of PAH) is produced in human liver than in mouse liver. Therefore, salvage pathway II in human liver, which is relevant to AKR1B1 and AKR1C3, is an alternative BH_4 formation route that compensates for the loss of SPR.

The results of western blot analysis showed that a large amount of the AKR1B1 protein was detected in human brain but the amount of the AKR1C3 protein was extremely scarce in it (Fig. 9). Penning *et al*. (*27*) have reported that the mRNA for AKR1C3 was expressed in many human tissues; however, the expression level of AKR1C3 mRNA in the brain was very low compared to that in other tissues.

This suggests that salvage pathway II cannot progress in human and mouse brain and that a large amount of 1′-$OXPH_4$ synthesized from PPH_4 by AKR1B1 accumulates in the entire brain region. These results of the expression analysis of salvage pathway II in humans and mice can explain why a patient with SPR deficiency shows progressive neurological deterioration without HPA and SPR knockout mice exhibit abnormal locomotion activity with HPA. SPR deficiency can be diagnosed by investigating the pteridine metabolites in CSF, in which the sepiapterin level is high, biopterin is mildly increased, and neopterin is normal [sepiapterin: 5–20 nmol/l, (SPR deficiency), not detectable, (normal); biopterin: 24–60 nmol/l, (SPR deficiency), 10–40 nmol/l, (normal); neopterin: 11–25 nmol/l, (SPR deficiency), 10–30 nmol/l, (normal), BIODEF database www.bh4.org]. It has been reported that the sepiapterin level was significantly elevated in the brain of $Spr^{-/-}$ mice (*16*). We have reported that a small amount of sepiapterin was formed in the nonenzymatic degradation of 1′-$OXPH_4$ and the rate of the nonenzymatic formation of sepiapterin from 1′-$OXPH_4$ was quite slow (*15*, *26*). However, sepiapterin is a stable molecule in the dihydro form of pteridine

derivatives and may accumulate in the brain of $Spr^{-/-}$ mice and in the CSF of *SPR*-deficient patients over a long period of time. Therefore, the sepiapterin level may be elevated in the CSF of a patient with SPR deficiency and in the brain of $Spr^{-/-}$ mice. In the case of mutant mice, the amount of neopterin increased (to five times of that found in $Spr^{+/+}$ mice) in the brain (*16*). These results suggest that PTPS, which synthesizes PPH_4 from NH_2TP in the brain of mutant mice, may be inhibited by the large amounts of 1′-$OXPH_4$ synthesized from PPH_4 by AKR1B1. In consequence, the amount of neopterin, an NH_2TP metabolite, may increase in the brain of $Spr^{-/-}$ mice. Despite the large amount of 1′-$OXPH_4$ synthesized in the brain of a patient with SPR deficiency, the neopterin level was normal in the CSF of such a patient. It is not clear why the amounts of neopterin do not increase in the CSF of a patient with SPR deficiency. Furthermore, the fact that the level of biopterin, a BH_4 metabolite, moderately increases in the CSF of an *SPR*-deficient patient cannot be explained by the results of our experiment. Yang *et al*. (*16*) reported that the BH_4 level in the liver was more dramatically reduced (to 1.1% of that of the wild type) in $Spr^{-/-}$ mice than in the control, whereas a relatively mild decrease (40.5% of that of the wild type) was detected in the brain of SPR knockout mice. Similar results have been reported by Takazawa *et al*. (*17*). These findings suggest that an unknown quantity of SPR-unrelated BH_4 production may occur in the brain; therefore, the biopterin level mildly increases in the CSF of patients with SPR deficiency. Further studies on the SPR-unrelated BH_4 formation route will be necessary to understand the differences in the levels of pteridine metabolites in the CSF of patients with SPR deficiency and in the brain of SPR knockout mouse.

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CONFLICT OF INTEREST

None declared.

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