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Determinants of outcomes following acute child encephalopathy and encephalitis: pivotal effect of early and delayed cooling

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ABSTRACT

Background Acute encephalopathy/encephalitis is one of the most important causatives of mortality and neurological deficit during childhood. The aim of this retrospective observational study was to investigate clinical variables and therapeutic options associated with the outcome of children with acute encephalopathy/encephalitis.

Methods Relationships between the clinical information at admission and the neurological outcome evaluated using Pediatric Cerebral Performance Category Scale (PCPC) at 12 months after admission were assessed in 43 patients who were treated at 10 Japanese paediatric intensive care units.

Results Sixteen patients were cared for at normothermia, whereas mild hypothermia was applied to 27 children. In univariate analysis, ages ≤18 months, marked elevation in serum lactate dehydrogenase (LD) and aspartate transaminase, diagnosis of either acute necrotising encephalopathy or haemorrhagic shock and encephalopathy syndrome and longer hypothermic periods were associated with increased risks of death or severe neurological deficit, whereas hypothermia showed pivotal effects: the outcome of children cooled after 12 h of diagnosis was statistically invariant with normothermic children, but was significantly worse compared with children cooled ≤12 h. In multivariate analysis, younger ages and elevated serum LD were associated with adverse outcomes, whereas early initiation of cooling was related to favourable outcomes. For normothermic children, PCPC scores were dependent on the computed tomographic findings suggestive of cerebral oedema, serum LD levels and Glasgow Coma Scale at admission. For hypothermic children, PCPC scores depended on longer delays in cooling initiation.

Conclusion Without therapeutic hypothermia, the outcome of children was determined by variables suggestive of the severity of encephalopathy/encephalitis at admission. Hypothermia may have pivotal impacts on the outcome of children according to the timing of cooling initiation following acute encephalopathy/encephalitis.

What is already known on this topic

- Encephalopathy/encephalitis affects more than 1000 Japanese children per annum, with approximately a half of these children resulting in mortality or permanent neurological deficits.
- Hypothermia is neuroprotective following perinatal asphyxia and adult cardiac arrest; however, no randomised controlled trials have been performed for child encephalopathy/encephalitis.

What this study adds

- Therapeutic hypothermia for children with acute encephalopathy/encephalitis may alter the outcome of patients according to the delay in cooling initiation.
- Delayed cooling after 12 h of initial neurological signs of encephalopathy/encephalitis is likely to be deleterious, whereas there remains a possibility that early cooling is neuroprotective.
- Randomised controlled studies of early hypothermia in acute encephalopathy/encephalitis are urgently required.

Acute encephalopathy/encephalitis affects more than 1000 children per year in Japan.1 Of various causatives, flu infection has recently been recognised as one of the commonest triggers in Asian countries.[1 2] According to a recent report on 148 cases of flu-related encephalopathy/encephalitis in childhood, 32% resulted in mortality, whereas 28% led to permanent neurological deficit.[2] There currently is no established therapeutic intervention which ameliorates the outcome of acute child encephalopathy/encephalitis. Therapeutic hypothermia has been demonstrated to be neuroprotective for encephalopathy following perinatal asphyxia[3–5] and adult cardiac arrest.[6 7] Although the neuroprotective effect of therapeutic hypothermia for other pathological conditions has not been demonstrated in the clinical setting,[8–10] several guidelines have already approved careful use of hypothermia for acute cerebral injury in childhood.[11] Consequently, despite the lack of randomised controlled trials which evaluated the protective effect of hypothermia for child encephalopathy/encephalitis, therapeutic

hypothermia for this clinical condition has already become popular among Japanese paediatric intensivists.[12]

We performed a retrospective observational study in children with acute encephalopathy/encephalitis to investigate the dependence of outcomes on clinical backgrounds and therapeutic options. We hypothesised that (1) mild systemic hypothermia initiated shortly after the development of encephalopathy/encephalitis improves the outcome of children without increasing the incidence of adverse events, (2) without hypothermia, the outcome of children would depend on the severity of encephalopathy/encephalitis at admission; however, (3) with therapeutic hypothermia, the outcome would depend on the timing of cooling initiation.

METHODS

This study was performed under the guidance and approval of the local ethical committee.

Study population

Children between 1 month and 14 years old, who developed acute encephalopathy/encephalitis between January 1997 and July 2008 and were cared for at paediatric intensive care units of 10 Japanese tertiary centres, were retrospectively enrolled into the study. Acute encephalopathy/encephalitis was defined as progressive loss or impairment of consciousness with or without other neurological signs such as seizures.[13] Patients with traumatic brain injury, febrile convulsions without prolonged unconsciousness and bacterial meningitis were not included.

Treatment of encephalopathy/encephalitis

For encephalopathic children, six centres routinely provided therapeutic hypothermia throughout the study period; one centre introduced hypothermia only after January 2003; another centre provided cooling only when the routine cranial CT at admission showed significant cerebral oedema; two centres never provided hypothermia. Following the diagnosis of acute encephalopathy/encephalitis, all centres which provided therapeutic hypothermia induced systemic cooling to 33.5–35°C within 48 h of the primary neurological manifestation. Mattresses, through which temperature-adjustable water circulated, were applied over the ventral and/or dorsal trunk of children; hypothermia was initially maintained for 48–72 h according to the protocol of each centre. During cooling, patients were given continuous infusion of thiopental (up to 3 mg/kg/h) or midazolam (up to 1 mg/kg/h) unless contraindicated; in addition, intravenous vecronium or pancronium (up to 0.1 mg/kg/h) was considered when shivering was uncontrollable. At all centres, patients were rewarmed 0.5–1°C per day; rewarming was postponed or slowed down with either the deterioration of neurological findings or signs suggestive of the recurrence of brain oedema with critically increased intracranial pressure. The core temperature was monitored using either rectal or urinary bladder temperature probes; invasive and non-invasive brain temperature monitoring was additionally performed in some children using ventricular, nasopharyngeal or forehead thermo-flux probes. Cranial CT was obtained at timings of admission except for three hypothermic and one normothermic children, of whom MRI was available shortly after admission. CT scans were repeated when the increase in intracranial pressure was suspected; experienced radiologists assessed signs suggestive of cerebral oedema, such as sulcal effacement, size reduction of lateral ventricles and basal cisterns and the loss of grey/white matter differentiation, to assign scores of 0 (up to mild oedema), 1 (moderate oedema) and 2 (severe oedema) (see online supplementary figure 1 for representative CT appearances).

Data collection

Clinical variables were collected for each patient including the age; body weight; sex; type of encephalopathy/encephalitis; preceding infection; Glasgow Coma Scale (GCS) at admission; timing of cooling initiation (hours after the initial neurological manifestation); potential serum markers of tissue damage such as aspartate transaminase (AST), alanine transaminase (ALT) and lactate dehydrogenase (LD) at admission; CT scores at admission; additional treatments (intravenous steroid and/or immunoglobulin) and outcome. Preceding infection by influenza virus, adenovirus, respiratory syncytial virus and rotavirus was confirmed, with at least one positive result of viral culture, antigen test, reverse transcription PCR or significantly raised titres in paired serum samples. Types of encephalopathy/encephalitis were defined according to the definition proposed by Mizuguchi and colleagues[13]; for further analyses, acute necrotising encephalopathy, haemorrhagic shock and encephalopathy syndrome and Reye's syndrome were grouped and discriminated from other types of encephalopathy/encephalitis such as acute encephalopathy with refractory seizures and acute disseminated encephalo-myelitis because of their relationship with unfavourable outcome. Adverse events such as hypotension (defined as systolic blood pressures below average values −2 SD for the patients' age in accordance with the Task Force on Blood Pressure Control in Children),[14] pneumonia, thrombocytopenia (<150 000/μl), coagulation disorders (either prothrombin time >12 s, international normalised ratio of prothrombin time >1.2 s or activated partial thromboplastin time >45 s), arrhythmias (excluding brady cardia) and hypokalaemia (<3.5 mEq/l) were also recorded.

Outcome assessment

The neurological performance at 12 months from the development of encephalopathy/encephalitis was assessed by the Pediatric Cerebral Performance Category Scale (PCPC).[15 16] Additional standard outcome scales, that is, the Glasgow Outcome Scale (GOS) and the Pediatric Overall Performance Category Scale (POPC), were also obtained to enable comparison with other studies. For PCPC and POPC, scores 1–6 represent normal performance, mild disability, moderate disability, severe disability, coma or vegetative state and death, respectively, whereas for GOS, scores 1–5 represent death, persistent vegetative state, severe disability, moderate disability and good recovery, respectively.

Statistical analysis

Clinical variables and therapeutic options associated with adverse acute events (hypotension, pneumonia, thrombocytopenia, coagulation disorders, arrhythmias and hypokalaemia) and unfavourable outcomes (PCPC >3) were examined using the univariate logistic regression analysis: independent variables were dichotomised either at the 75th percentile (AST, ALT and LD) or medically relevant breakpoints (tables 1 and 2). Independent variables with p values less than 0.05 were further tested using multivariate analysis with backward stepwise elimination of non-significant variables.

Eventually, paradoxical dependences of the outcome on early and late hypothermia were observed: to further investigate the types of relationships between clinical factors and

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Table 1 Baseline characteristics, treatments, adverse events and outcome

	Hypothermia (n=27)	Normothermia (n=16)
Baseline characteristics		
Age (month)	45.0 (25.7–64.4)	29.6 (18.8–40.3)
Body weight (kg)	15.3 (11.3–19.3)	12.9 (10.8–15.0)
Sex (female)	14	13
GCS at admission	4.2 (3.2–5.2)	5.6 (3.2–8.1)
Initial CT findings*		
Moderate oedema	5	4
Severe oedema	3	2
Other lesions	3	4
Preceding infection		
Influenza virus	13	4
Adenovirus	0	1
Rotavirus	1	0
Human herpesvirus 6/7	2	1
Others or no preceding infection	11	10
Type of encephalopathy/encephalitis		
Acute necrotising encephalopathy	3	5
Haemorrhagic shock and encephalopathy syndrome	4	1
Acute encephalopathy with refractory seizures	11	5
Others	9	5
Serum markers for tissue damage		
<6 h of admission		
AST (IU/l)	1915 (−534 to 4364)	150 (53–247)
ALT (IU/l)	154 (69–240)	100 (−4 to 209)
LD (IU/l)	2105 (462–3749)	552 (451–652)
48–72 h after admission		
AST (IU/l)	1039 (22–2057)	3003 (874–5132)
ALT (IU/l)	550 (21–1079)	1453 (473–2435)
LD (IU/l)	2725 (910–4539)	3499 (1471–5526)
Treatments		
Steroid	24	10
Immunoglobulin	14	4
Timing of cooling initiation (h)	16.7 (10.3–23.0)	–
Cooling duration (h)	99.7 (78.1–121.3)	–
Adverse events		
Hypotension	11	7
Pneumonia	9	2
Thrombocytopenia (<150 000)	12	9
Coagulation disorder	9	7
Arrhythmias	0	0
Hypokalaemia (<3.5 mEq)	16	10
Outcome		
Mortality	2	4
Death or severe disability	11	7
PCPC	2.9 (2.3–3.6)	3.3 (2.2–4.3)
POPC	3.0 (2.3–3.7)	3.3 (2.3–4.3)
GOS	3.6 (3.0–4.1)	3.3 (2.5–4.2)

ALT, alanine transaminase; AST, aspartate transaminase; GCS, Glasgow Coma Scale; GOS, Glasgow Outcome Scale; LD, lactate dehydrogenase; PCPC, Pediatric Cerebral Performance Category Scale; POPC, Pediatric Overall Performance Category Scale.
Data are shown as number of patients or mean (95% CI).
*Excluding three hypothermic and one normothermic children of whom CT was not obtained at admission.

outcomes; PCPC scores were compared with scale or rank-ordinal clinical variables (GCS and CT scores at admission and variables which were included within the final logistic regression model) using Spearman's rank correlation coefficient for subgroups of normothermic and hypothermic patients, respectively. Intergroup comparisons between normothermic and hypothermic children were also performed: the incidence of adverse events and initial CT scores were compared using either the χ^2 test or Fisher's exact test, whereas scores for GCS and PCPC were compared using the analysis of variance on ranks.

RESULTS

Of 43 children, preceding viral infection was identified in 27 participants, 17 of which were flu. Acute necrotising encephalopathy, haemorrhagic shock and encephalopathy syndrome and acute encephalopathy with refractory seizures were observed in 8, 5 and 16 children, respectively (table 1); however, none developed Reye's syndrome or acute disseminated encephalo-myelitis. No child had hypoxic–ischaemic events before admission; however, one child with preceding flu infection experienced cardiac arrest at admission (hypothermia group). Sixteen children (9–71 months, range) were cared for at normothermia, whereas 27 children (5–213 months) were cooled at 16.7±16.1 h (mean±SD) following the initial neurological signs of encephalopathy/encephalitis. All children were initially cooled to 34–35°C; three children required moderate hypothermia <34°C for the control of severe brain oedema. Seventeen children required intravenous vecronium or pancronium because of shivering resistant to thiopental and/or midazolam. For these children, subsequent duration of cooling ≤35°C was 99.7±37.9 h including the rewarming period.

Intergroup analysis for normothermic and hypothermic patients

There was no difference in the background clinical variables, serum markers of tissue damage, CT findings, incidence of acute adverse events and PCPC scores between normothermic and hypothermic children.

Determinants of outcomes within the overall study population

In the univariate analysis, the incidence of acute adverse events was unrelated with clinical variables and therapeutic options; unfavourable outcomes at 12 months were associated with longer hypothermic periods (p=0.030), serum AST and LD higher than the 75th percentiles (p=0.020 and 0.006, respectively), younger ages ≤18 months (p=0.020) and diagnosis of either acute necrotising encephalopathy or haemorrhagic shock and encephalopathy syndrome (p=0.021), whereas early initiation of hypothermia ≤12 h was related to a reduced risk of adverse outcomes (p=0.004) (table 2). Because of a significant intercorrelation observed between AST and LD (r^2=0.97), only LD but not AST was considered in the multivariate analysis as a representative serum marker of tissue damage. Multivariate analysis identified younger ages (p=0.038) and elevated serum LD levels (p=0.021) as independent variables associated with adverse outcomes, whereas early initiation of cooling was associated with a reduced risk of adverse outcomes (p=0.017).

Intragroup analysis for normothermic and hypothermic patients

In normothermic children, PCPC was dependent on CT scores, GCS and LD levels at admission (p=0.040, 0.004 and 0.002, respectively: figure 1A and online supplementary figures 2 and 3), whereas in hypothermic children, PCPC depended on the timing of cooling initiation (p=0.001: figure 1A–B, see online supplementary figures 4 and 5 for comparisons with other outcome scales).

DISCUSSION

Our results suggested that the age and temperature control are both important factors associated with the outcome of acute encephalopathy/encephalitis in childhood. Younger ages ≤18 months and marked elevation of serum LD greater than the 75th percentile were associated with severe disability or death

Table 2 Determinants of adverse outcomes following acute child encephalopathy/encephalitis

	Outcome							
	Good (PCPC ≤3)		Poor (3 <PCPC)		OR (95% CI)			
	n=25	%	n=18	%	Average	Lower	Upper	p Value
Univariate analysis								
Baseline characteristics								
Age (≤18 months)	5	20.0	10	55.6	5.00	1.30	19.30	0.020
Sex (female)	17	68.0	9	50.0	0.47	0.14	1.64	0.237
Moderate to severe oedema on initial CT*								
	9	40.9	6	35.3	0.79	0.21	2.92	0.721
AST† (190 IU/<)	3	12.5	8	47.1	6.22	1.33	29.01	0.020
ALT† (91 IU/<)	4	16.7	6	35.3	2.73	0.63	11.79	0.179
LD† (835 IU/<)	2	8.3	9	50.0	11.00	1.98	61.26	0.006
Types and severity of encephalopathy/encephalitis								
GCS (≤4)	16	64.0	15	83.3	2.81	0.64	12.41	0.172
Types of encephalopathy/encephalitis associated with adverse outcomes‡								
	4	16.0	9	50.0	5.25	1.28	21.57	0.021
Therapeutic options								
Steroid	19	76.0	15	83.3	1.58	0.34	7.38	0.562
Globulin	11	44.0	7	38.9	0.81	0.24	2.78	0.738
Hypothermia	16	64.0	11	61.1	0.88	0.25	3.09	0.847
Timing of cooling initiation								
Early hypothermia (≤12 h)	14	56.0	3	16.7	0.05	0.01	0.39	0.004
Late hypothermia (12 h <)	2	8.0	8	44.4	1	Reference		
Normothermia	9	36.0	7	38.9	0.19	0.03	1.22	0.112
Target temperature								
Normothermia	9	36.0	7	38.9	1	Reference		
Mild hypothermia (34–35°C)	14	56.0	10	55.6	0.92	0.26	3.30	0.896
Moderate hypothermia (33–34°C)	2	8.0	1	5.6	0.64	0.05	8.62	0.739
Cooling duration (≤35°C)								
None (normothermia)	9	36.0	7	38.9	2.18	0.53	9.02	0.283
≤96 h	14	58.3	5	29.4	1	Reference		
96 h <	1	4.2	5	29.4	14.00	1.30	150.89	0.030
Multivariate analysis								
Age (≤18 months)					7.70	1.12	52.89	0.038
Timing of cooling initiation: early hypothermia (≤12 h)					0.09	0.01	0.65	0.017
LD† (835 IU/l <)					13.66	1.48	126.10	0.021

ALT, alanine transaminase; AST, aspartate transaminase; GCS, Glasgow Coma Scale; LD, lactate dehydrogenase; PCPC, Pediatric Cerebral Performance Category Scale.

*Excluding three hypothermic and one normothermic children of whom CT was not obtained.

†AST, ALT and LD values were dichotomised at the 75th percentile.

‡Including acute necrotising encephalopathy, haemorrhagic shock and encephalopathy syndrome and Reye's syndrome (see the method section and the reference 13 for detail).

after 12 months. In contrast, therapeutic hypothermia might have pivotal effects on the outcome: the incidence of unfavourable outcome for children cooled after 12 h of diagnosis was invariant with normothermic children but was significantly higher compared with children cooled within 12 h. Delayed cooling after 12 h of acute events may be deleterious, whereas early cooling is likely to be neuroprotective. Further prospective studies with larger populations are required to delineate a group of patients who may have the benefit of cooling after acute child encephalopathy/encephalitis.

Limitation of the study

This study was a retrospective observational study based on a limited number of patients from 10 tertiary centres. Because of the variation in the patients' background and the interinstitutional differences in the therapeutic strategy, our study does not have the power to demonstrate the neuroprotective effect of therapeutic hypothermia with the direct comparison between two therapy modes. The therapy mode was determined by the policy of each centre where the children received treatments. Hence, although we did not observe any significant interinstitutional difference in unfavourable outcomes, it is possible that the outcome of children was affected by the quality of intensive care provided at the specific centre and the temperature control. Further, one institution provided hypothermia only for children with evidence of severe brain oedema, which may have led to the underestimation of the benefits of therapeutic hypothermia.

Determinants of outcomes following acute encephalopathy/encephalitis

Univariate analyses in the overall study population identified the eventual cooling duration, age of patients, serum AST and LD at admission, types of encephalopathy/encephalitis and timing of cooling initiation as significant independent variables. It may be counterintuitive that longer cooling periods led to worse outcomes; however, children with more severe cerebral oedema might eventually require slower rewarming compared with peers, presumably leading to adverse outcomes despite special care. Acute necrotising encephalopathy and haemorrhagic shock and encephalopathy syndrome, which had been linked with adverse outcomes in previous studies,[13] were consistently associated with an increased incidence of unfavourable outcomes in our study population. However, these variables were not included within the final multivariate logistic model. In contrast, the age of patients, serum LD and cooling at different timings were all identified as significant determinants of the outcome in the final multivariate model.

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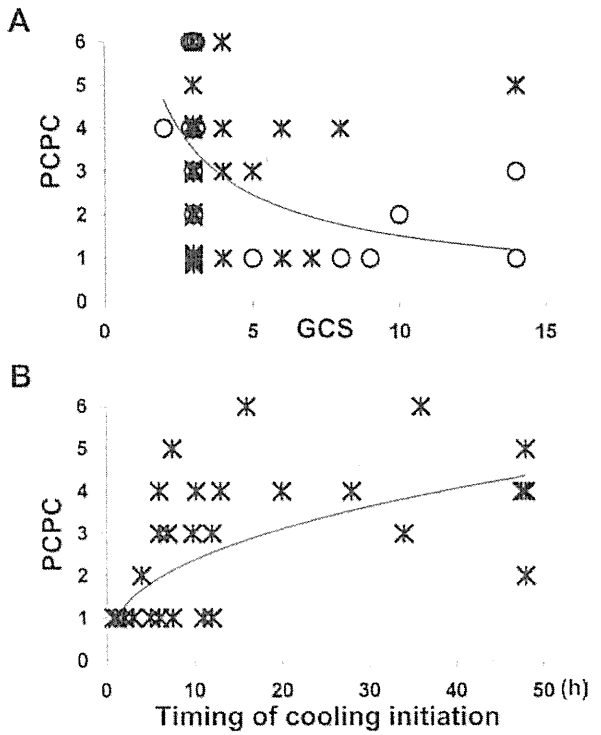

Figure 1 Determinants of outcome in normothermic and hypothermic children. (A) The outcome represented by the Pediatric Cerebral Performance Category Scale (PCPC) after 12 months was dependent on the Glasgow Coma Scale (GCS) at admission in normothermic children (circle, p=0.004, r=−0.72, Spearman's rank correlation coefficient, the regression line independently given based on a logarithmic model) but not in hypothermic children (asterisk). (B) Dependence of outcome on the timing of cooling initiation. In hypothermic children, PCPC after 12 months was depended on the timing of cooling initiation (p=0.001, r=0.60, Spearman's rank correlation coefficient, the regression line independently given based on a logarithmic model).

Special care would be required for young children within 18 months of life. To determine the optimal protocol of therapeutic hypothermia following acute child encephalopathy/encephalitis and also to identify a group of children who are most likely to respond to invasive treatments, future prospective studies should be designed to address the exact associations between these variables and the outcome of children.

Pivotal effect of hypothermia on the outcome of children

It was interesting that hypothermia was paradoxically associated with positive and negative effects according to the timing when cooling was initiated. Early cooling within 12 h of diagnosis was associated with significantly smaller incidence of adverse outcomes compared with delayed cooling after 12 h. Although no difference was recognised between early cooling and normothermia, the lack of the difference between late cooling and normothermia may indirectly suggest the benefit of early cooling. Taken together, early hypothermia is likely to be neuroprotective, whereas delayed cooling may be deleterious. Such a pivotal effect of hypothermia on the outcome may explain the lack of significant difference in PCPC scores between normothermic and hypothermic children.

Further subgroup analyses showed that, with the conventional treatment, the outcome of children following acute encephalopathy/encephalitis was associated with the severity of encephalopathy/encephalitis represented by GCS, serum LD and CT scores at admission. However, with therapeutic hypothermia, the outcome was not related with the severity at admission presumably because cooling significantly altered the outcome of children. PCPC scores rapidly increased with longer delay in cooling initiation, the trend of which was prominent especially after 12 h. The survival without severe neurological deficits (ie, PCPC scores <4) decreased with time, from 100% (six of six) when cooling was initiated before 6 h of the primary neurological events to 67% (six of nine at 6≤ <12 h), 40% (two of five at 12≤ <24 h) and 29% (two of seven thereafter). Such an exponential loss of the protective effect with increasing delay in cooling initiation would not be specific to acute encephalopathy/encephalitis. Studies investigating the cerebral energy metabolism following hypoxia–ischaemia have suggested the variation of the potential therapeutic time window according to the severity of the acute event.[17] Hutchison and colleagues[10] recently reported that therapeutic hypothermia in children with severe head injury was not neuroprotective; the lack of benefit was in part attributed to relatively long delay in cooling initiation ~6.3 h after the acute event. Tools to estimate precise therapeutic time windows for children with different clinical backgrounds have to be developed in the near future to maximise the favourable effect of hypothermia and other treatments.

CONCLUSIONS

Our results suggested that the effect of therapeutic hypothermia for children with acute encephalopathy/encephalitis may be dependent on the timing when cooling was initiated. Early cooling within 12 h of initial neurological signs of encephalopathy/encephalitis may improve the outcome, whereas delayed cooling after 12 h might be deleterious. Our observations may justify a randomised prospective study of early hypothermia in acute encephalopathy/encephalitis in the near future.

Research Network for Acute Encephalopathy in Childhood

Scientific committee TM (chair), GK (co-principal investigator), OI (co-principal investigator), TK, MS and HR.

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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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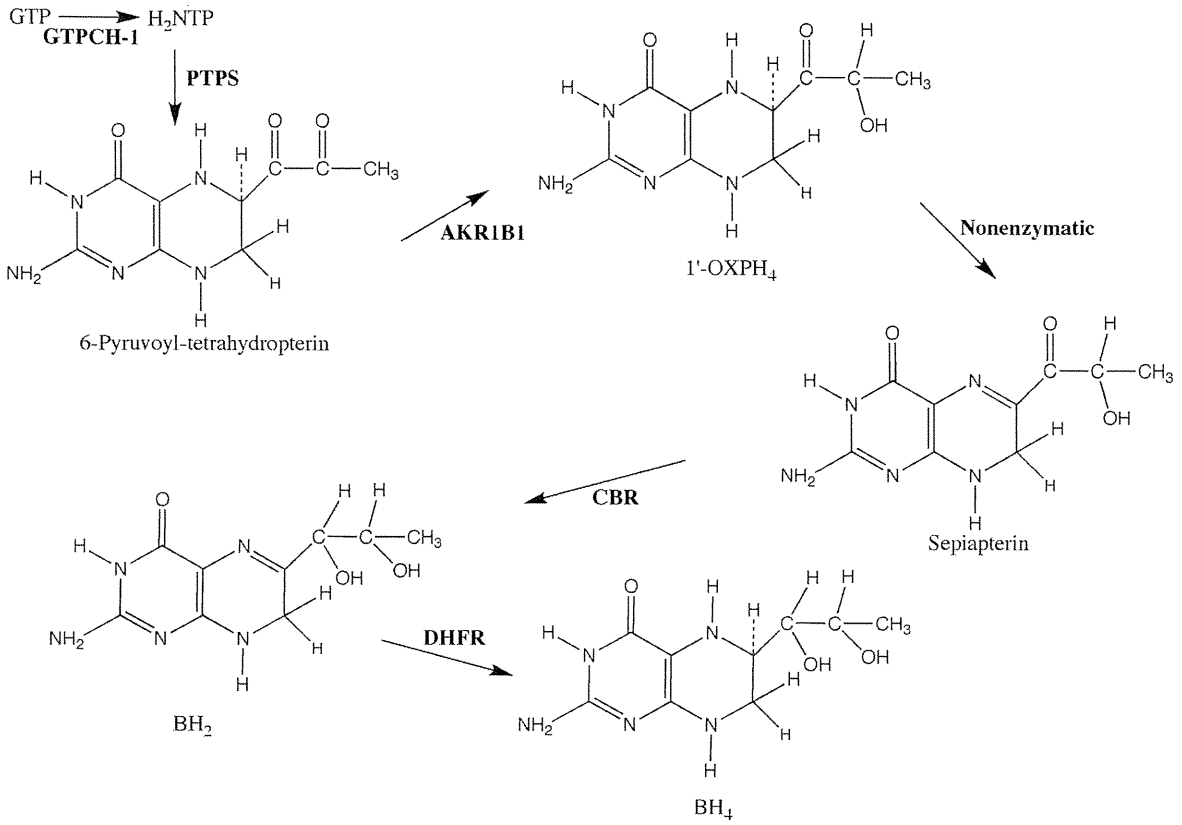


Fig. 1. **The SPR-unrelated BH_4 biosynthetic pathway (salvage pathway I).** In the absence of SPR, 1'-$OXPH_4$ is oxidized non-enzymatically to sepiapterin, which is reduced to BH_4 by CBR and DHFR.

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

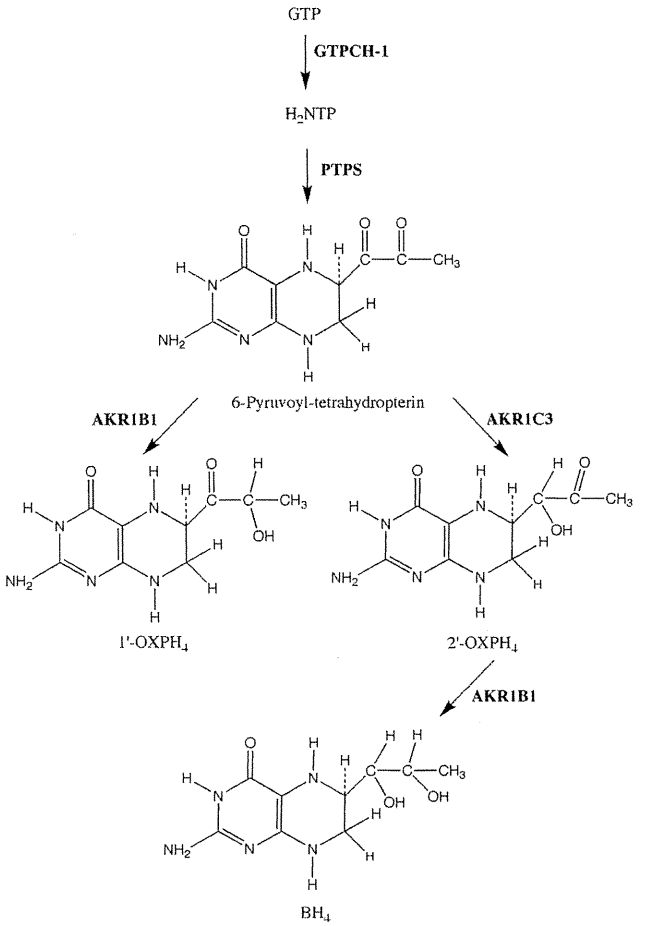


Fig. 2. **The SPR-unrelated BH_4 biosynthetic pathway (salvage pathway II).** BH_4 is synthesized from PPH_4 by AKR1C3 and AKR1B1.

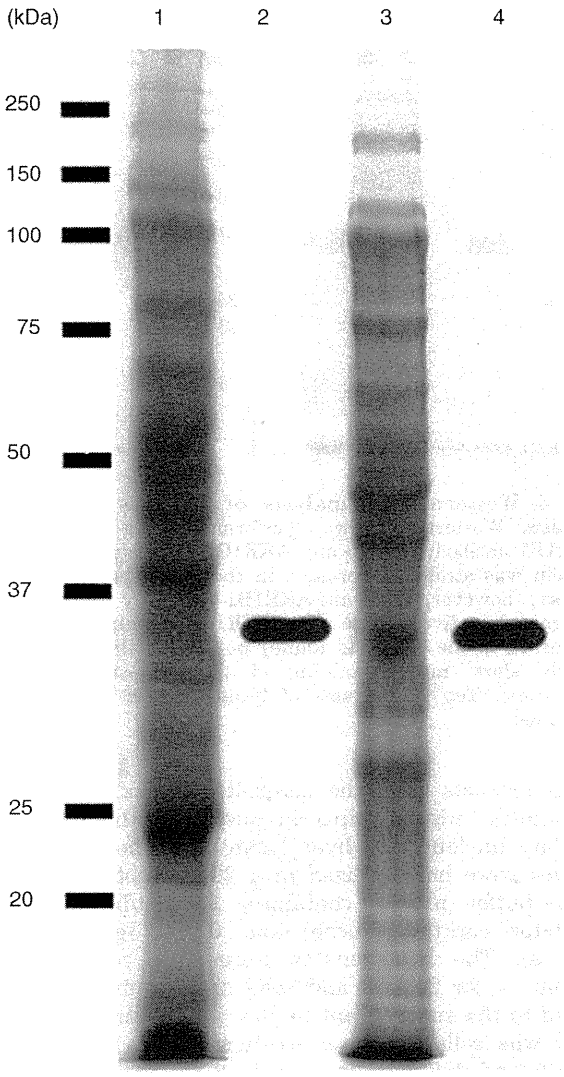


Fig. 3. **Specificity of anti-AKR1B1 and anti-AKR1C3 antibodies.** Lanes 1 and 3 show the SDS/PAGE analysis of the protein from the *E. coli* lysate, which expressed human AKR1B1 and AKR1C3, respectively. The gel was stained with Coomassie Brilliant Blue R-250. Lanes 2 and 4 are immunoblot analyses using 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

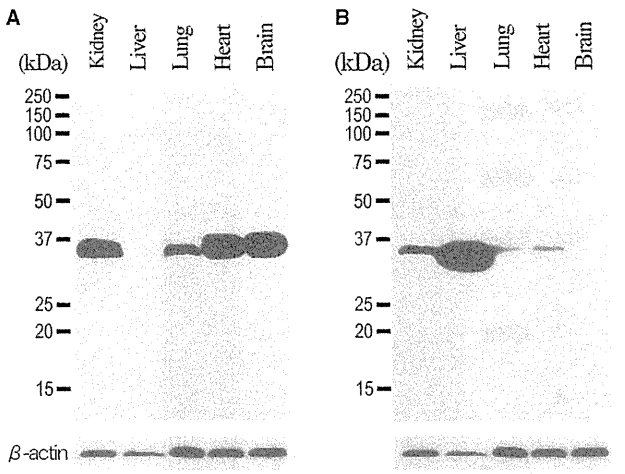


Fig. 4. **Western blot analysis of extracts from mouse tissues.** Western blot was performed using (A), an anti-AKR1B1 antibody; (B) an anti-AKR1C3 antibody. The AKR1B1 protein was strongly expressed in the brain, heart, lung, and kidney; however, the anti-AKR1B1 antibody could not be recognized in liver 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

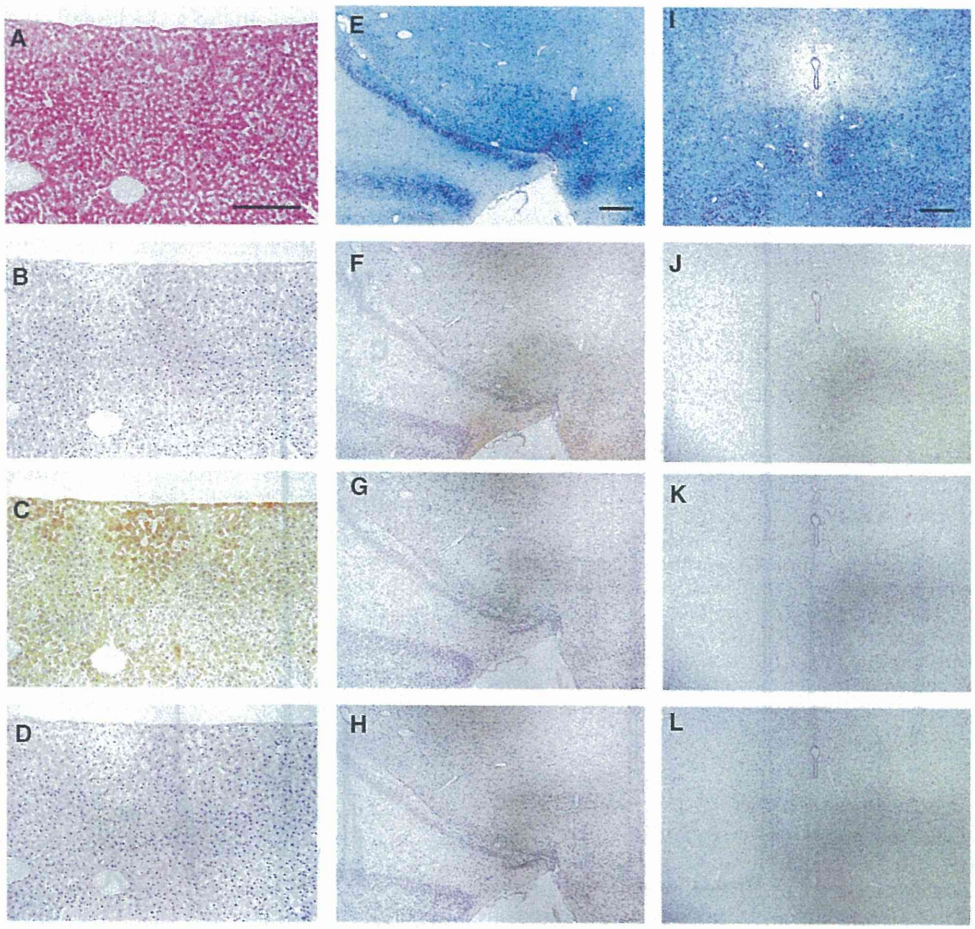
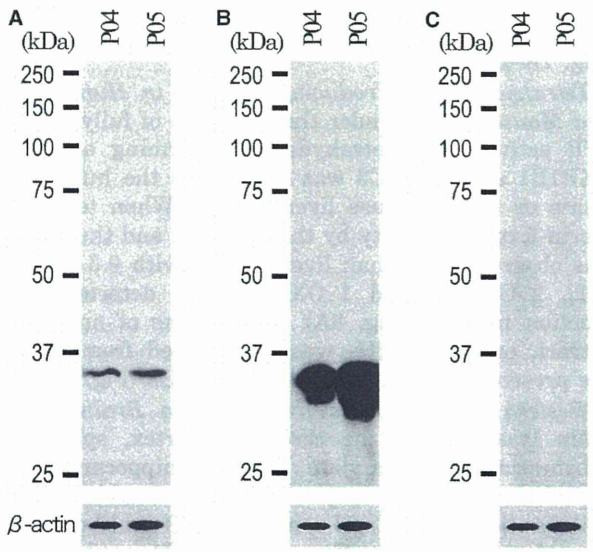


Fig. 5. **Immunohistochemical analysis of AKR1B1 and AKR1C3 in mouse liver and brain.** Paraformaldehyde-fixed paraffin sections of liver were reacted with: (A) hematoxylin-eosin (HE) stain; (B) anti-AKR1B1 serum; (C) anti-AKR1C3 serum; (D) negative control. The substantia nigra (dopaminergic neuron) 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.

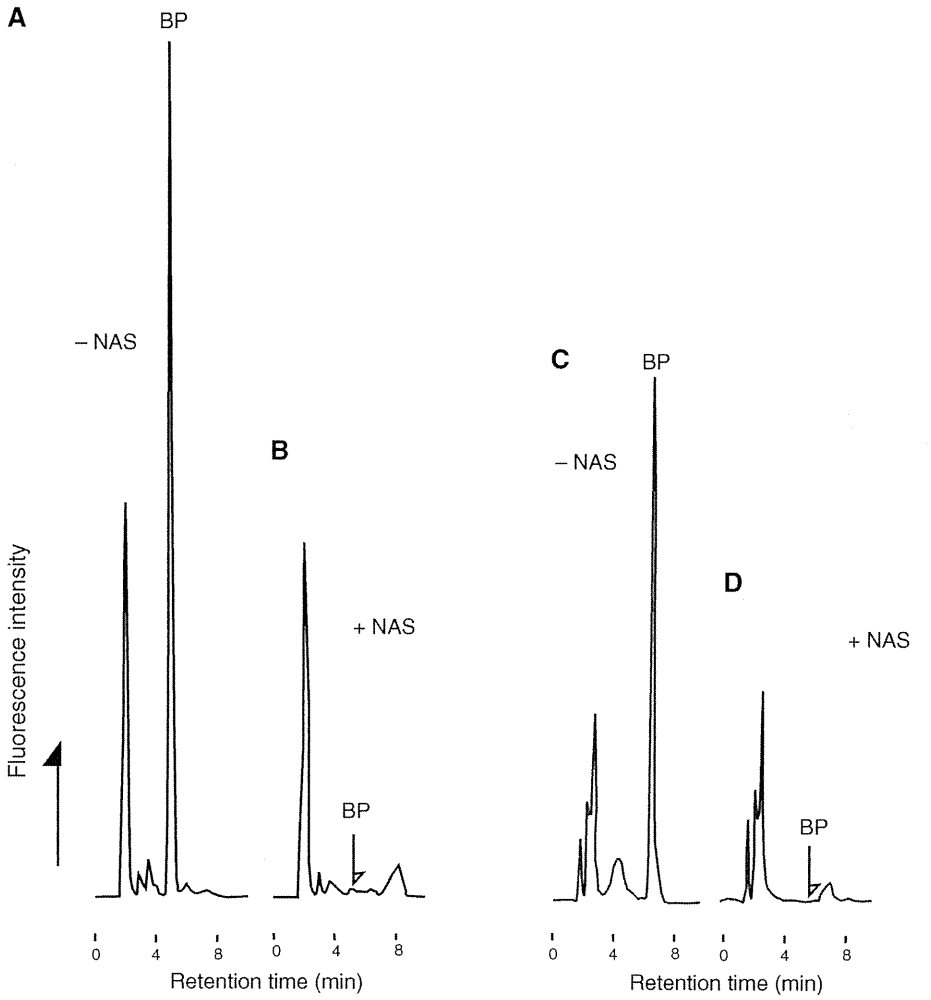


Fig. 7. **Analysis by HPLC of SPR activity.** The reaction mixture contained 15 μM sepiapterin, 1mM NADPH, 0.5 mM NAS or DW, and an appropriate amount of human liver extract from P04 or an appropriate amount of mouse liver extract in a final volume of 100 μl. It was incubated for 20 min at 37°C in darkness.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

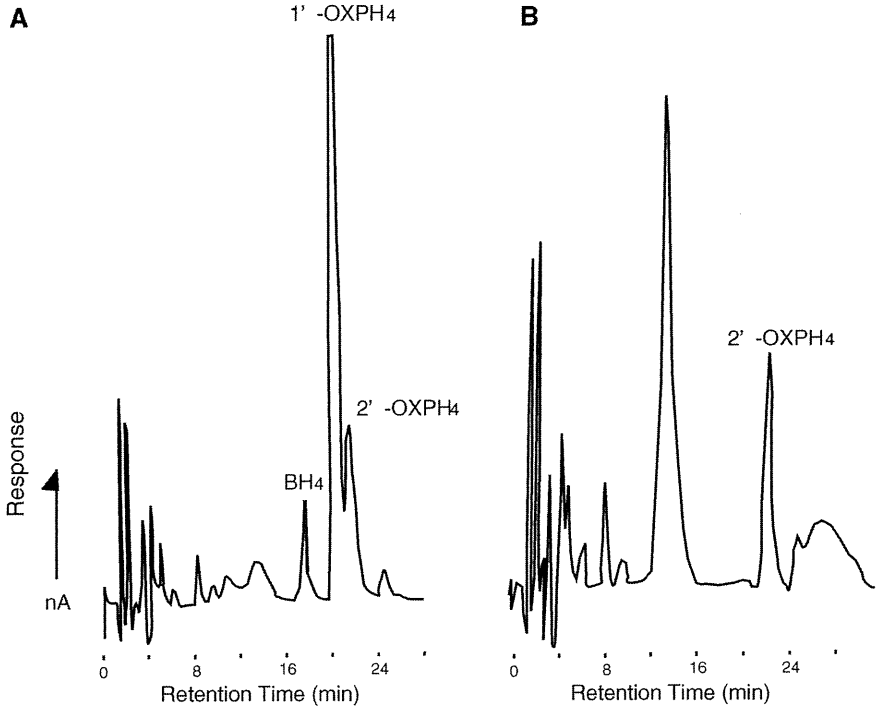


Fig. 8. **Production of BH_4, 2′-$OXPH_4$, and 1′-$OXPH_4$ by human liver or mouse liver extracts.** The reaction mixture contained the following components: a 50 mM potassium phosphate buffer (pH 7.0), 100 μM NADPH, 10 μl of a concentrated solution of PPH_4 synthase, 5 mM dithiothreitol, 8 mM $MgCl_2$, 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

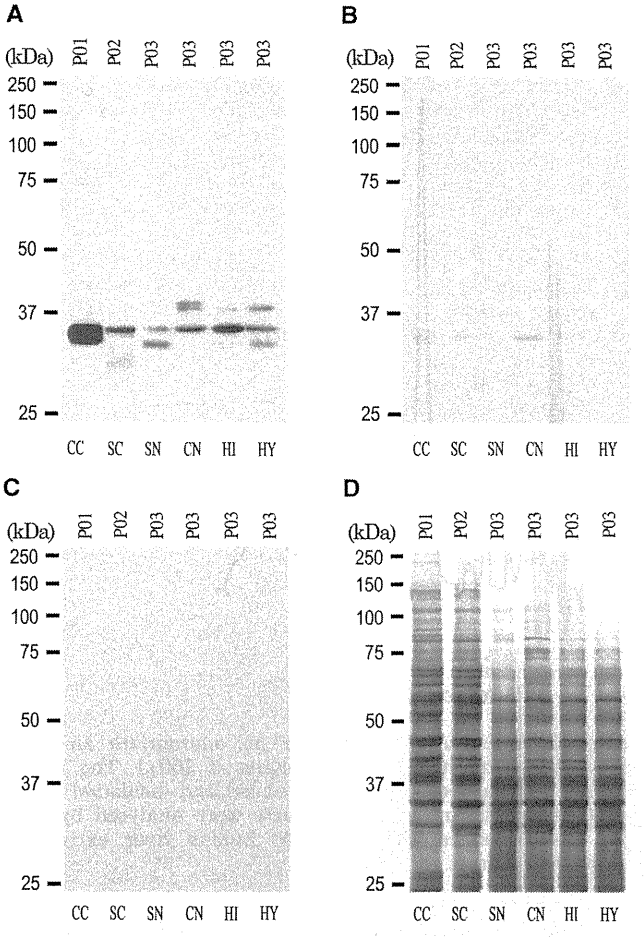


Fig. 9. **Western blot analysis of human brain lysate.** Western blot was performed using (A) an anti-AKR1B1 antibody; (B) an anti-AKR1C3 antibody; and (C) a normal rabbit serum. The gel was stained with (D) Coomassie Brilliant Blue R-250. Ten micrograms of lysate protein of the cerebellar cortex (CC), 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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Urinary metabolic profile of phenylketonuria in patients receiving total parenteral nutrition and medication

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Nutrition and drugs are main environmental factors that affect metabolism. We performed metabolomics of urine from an 8-year-old patient (case 1) with epilepsy and an 11-year-old patient (case 2) with malignant lymphoma who was being treated with methotrexate. Both patients were receiving total parenteral nutrition (TPN). We used our diagnostic procedure consisting of urease pretreatment, partial adoption of stable isotope dilution, gas chromatography/mass spectrometry (GC/MS) measurement and target analysis for 200 analytes including organic acids and amino acids. Surprisingly, their metabolic profiles were identical to that of phenylketonuria. The neopterin level was markedly above normal in case 1, and both neopterin and biopterin were significantly above normal in case 2. Mutation analysis of genomic DNA from case 1 showed neither homozygosity nor heterozygosity for phenylalanine hydroxylase deficiency. The metabolic profiles of both cases were normal when they were not receiving TPN. TPN is presently prohibited for individuals who have inherited disorders that affect amino acid metabolism. Although the Phe content of the TPN was not the sole cause of the PKU profile, its effect, combined with other factors, e.g. specific medication or possibly underlying diseases, led to this metabolic abnormality. The present study suggests that GC/MS-based metabolomics by target analysis could be important for assuring the safety of the treatments for patients receiving both TPN and methotrexate. Metabolomic profiling, both before and during TPN, is useful for determining the optimal nutritional formula not only for neonates, but also for young children who are known heterozygotes for metabolic disorders or whose status is unknown. Copyright © 2009 John Wiley & Sons, Ltd.

Total parenteral nutrition (TPN) is given to patients whose digestive system has been impaired for more than 2 weeks. It provides amino acids directly into a systemic vein, as a source of nitrogen. The amino acid formula follows the recommendation of the Food and Agricultural Organization/World Health Organization (FAO/WHO), which is based on the content of mature human milk. It is generally expected that, in patients on TPN, the blood amino acid level will not exceed twice the control level, and the urinary amino acid profile will be normal. TPN is presently prohibited for individuals who are known to have inherited disorders relating to the metabolism of amino acids but not for those whose status is unknown or who are heterozygous for these disorders. Examination of patient samples by metabolomic screening before beginning TPN could be important for the safety of patients whose status for metabolic disorders is unknown, and could likewise be valuable for heterozygotes for metabolic disorders, both before and during TPN. We have developed a method for noninvasive metabolic profiling using urine samples to measure various classes of compounds including amino acids and organic acids, simultaneously, and we began to use this method to design personalized medicine for patients with inborn errors of metabolism in 1996.[1] This method has allowed us to make chemical diagnoses of many cases of inborn errors of metabolism, including phenylketonuria (PKU) in a single gas chromatography/mass spectrometry (GC/MS) run.

The major metabolic pathway for Phe is hydroxylation to yield tyrosine (Fig. 1). Hyperphenylalaninemia is caused by Phe hydroxylase (PAH) deficiency, reduced supply of tetrahydrobiopterine (BH4), a cofactor for PAH, or impaired regeneration of the BH4. In PKU, due to severe hyperphenylalaninemia, Phe is metabolized via a bypath to phenylpyruvate, 2-hydroxyphenylacetate, phenyllactate and phenylacetate. The levels of these aromatic acids, however, do not increase measurably in mild

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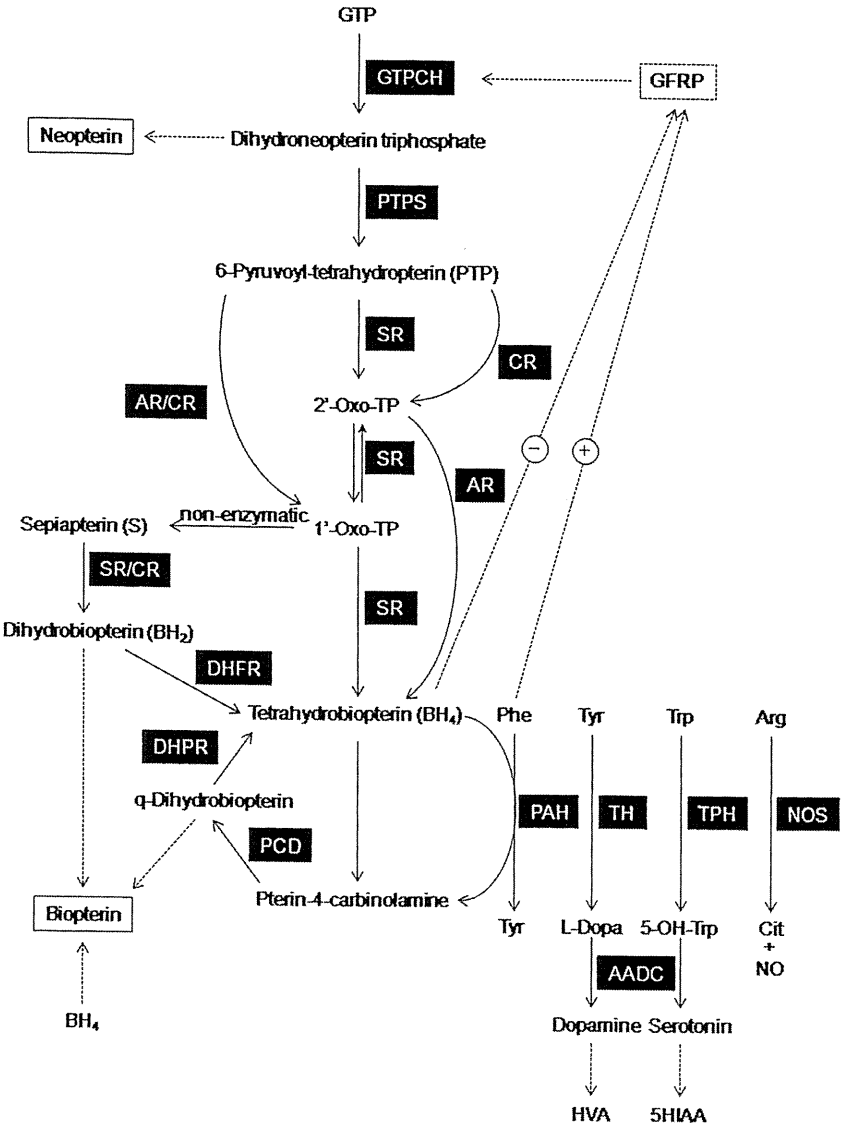

Figure 1. Biosynthesis and recycling of tetrahydrobiopterin. GTPCH, GTP cyclohydrolase I; PTPS, 6-pyruvoyl tetrahydropterin synthase I; SR, sepiapterin reductase; CR, carbonyl reductase; AR, aldose reductase; DHFR, dihydrofolate reductase; GTP, guanosine triphosphate; 1′-oxo-TP, 1′-oxo-2′-hydroxytetrahydropterin (6-lactoyl tetrahydropterin); 2′-oxo-TP, 1′-hydroxy-2′-oxotetrahydropterin; GFRP, GTP cyclohydrolase I feedback regulatory protein; DHPR, dihydropteridin reductase; PCD, pterin-4-carbinolamine dehydratase; PAH, phenylalanine-4-hydroxylase; TH, tyrosine-3-hydroxylase; TPH, tryptophan-5-hydroxylase; 5-OH-Trp, 5-hydroxytryptophan; NO, nitric oxide; NOS, nitric oxide synthase; AADC, aromatic amino acid decarboxylase; HVA, homovanillic acid; 5-HIAA, 5-hydroxyindoleacetic acid.

hyperphenylalaninemia or biopterin-responsive hyperphenylalaninemia.[2,3]

Nutrition and drugs are the main environmental factors that affect metabolism. We found that the metabolic profiles of two patients undergoing TPN were identical with that for PKU, even though these patients did not have PKU and were being treated with different medications. This report describes severe hyperphenylalaninemia during TPN and other factors that may have contributed to this metabolic change.

EXPERIMENTAL

Patients

Case 1

A 4-year-old boy was admitted to the hospital with epilepsy accompanied by pneumonia. Ten months after birth, he had suffered his first seizure. After that, numerous examinations, including a muscle biopsy, had been performed. However, no definite diagnosis had been made. The histopathological findings of the muscle-biopsy specimen suggested myogenic

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disease. The boy had spastic palsy of the extremities accompanied by severe mental retardation.

During this hospital stay, hyperammonemia was noted. A first urine metabolic profiling was conducted based on suspicion of a disturbed amino acid metabolism. The presence of primary and secondary hyperammonemia and other metabolic disorders was ruled out, and the Guthrie blood test done when this patient was a neonate was negative for hyperphenylalaninemia. During this hospitalization, the frequency of the patient's seizures decreased as the infection subsided.

At the age of eight, the frequency of seizures increased again, accompanied by convulsions every few minutes, and the boy was hospitalized. Anesthesia with continuous thiopental drip infusion was attempted to relieve the seizures for 3 days. Because oral intake was impossible, and the risk of aspiration was high, nutritional therapy with TPN containing 2.3 g/L of Phe was initiated, at a rate of 50 mL/h. Brain computer tomography showed brain atrophy, probably due to the continuous thiopental administration. A second urine metabolic profile was conducted. Later, an arachnoid cystectomy was carried out, which alleviated the seizures. Since then, the boy has been staying at another hospital and receiving respiratory management, physical therapy, and other appropriate care. The boy's parents gave their informed consent for us to perform a mutational analysis of the boy's PAH gene.

Case 2

An 11-year-old boy was admitted to the hospital with a subcutaneous mass of the head. His family history was unremarkable. After closer examination, he was given a diagnosis of non-Hodgkin's lymphoma, lymphoblastic type, stage IV. In April of the same year, first-line chemotherapy using methotrexate, vincristine, adriamycin, and prednisolone was started. During the chemotherapy, the patient suffered from severe gastrointestinal toxicity, including nausea and vomiting. TPN was necessary to supply an adequate amount of nutrition. His condition was complicated by steroid-induced diabetes mellitus and renal stones. Insulin therapy was necessary to control his hyperglycemia. To rule out a metabolic disorder, a first metabolic profiling of urine collected during TPN was conducted. At that time, no anti-tumor agent was being used. However, 2 weeks before the sampling, the patient had received high-dose methotrexate therapy.

Two months later, second-line chemotherapy with cyclophosphamide, daunomycin, vincristine, prednisolone, and L-asparaginase was introduced. As the patient's oral intake was much improved, no parenteral nutrition was needed. A second urinary metabolic profiling was conducted during the second-line chemotherapy. Although the boy responded to the second-line chemotherapy, he had repeated cycles of remission and relapse. The patient died of the underlying disease 1 year later.

Materials and methods

The amount of creatinine and creatine in urine was determined on a Beckman CX5 autoanalyzer. The preparation of urine, including the pretreatment with urease and ethanol deproteinization to remove the urease, partial adoption of a stable isotope dilution, trimethylsilylation, and capillary GC/MS analysis were as described.[1,4] A volume of 100 μL of urine was pretreated with type C-3 urease at 37°C for 10 min to decompose and remove excess urea. For quantification, the urine was spiked with fixed amounts of internal standards: 20 nmol each of 2,2-dimethylsuccinate and 2-hydroxyundecanoate; 100 nmol of [$^2H_3$]creatinine; 4 nmol of [$^{15}N_2$]uracil and [$^{15}N_2$]orotate; 10 nmol of [$^2H_3$]methionine, [$^2H_8$]homocystine, [$^2H_4$]cystine, [$^2H_3$]leucine, [$^2H_5$]phenylalanine and [$^2H_4$]tyrosine; 5 nmol of [$^2H_3$]methylcitrate; and 50 nmol [$^2H_5$]glycine and [$^2H_4$]lysine.

Aliquots (0.2 or 1 μL) of the derivatized extracts were injected into a Hewlett-Packard GC-MSD (HP6890/MSD5973) equipped with a fused-silica DB-5 capillary column (30 m × 0.25 mm i.d. with a 0.25 μm film thickness; J&W, Folsom, CA, USA) with a split ratio of 1:10 to 1:50. Electron impact mass spectra were obtained at a scan rate of 2.5 cycles/s from *m/z* 50 to 650.

Two hundred components, from lactate to homocystine, were automatically identified, quantified, or semi-quantified.[5] The levels of most metabolites for age-matched healthy individuals are not normally distributed. Therefore, they were $\log_{10}$-transformed, and the mean and standard deviation (SD) were obtained. For the evaluation of a metabolite in the urine of the patients, an abnormality *n* was defined as *n* in [mean value of age-matched control above *n* × SD], which was obtained automatically.[5]

RESULTS AND DISCUSSION

Analysis of urine metabolome obtained by single 15 min GC/MS measurement enabled detection of the metabolic disorder caused secondarily. A metabolic profile identical with PKU was found for two patients undergoing TPN with 2.3 g/L Phe: an 8-year-old boy under general anesthesia with thiopental, a p-450 inducer, for the control of convulsions (case 1) and an 11-year-old boy with malignant lymphoma receiving methotrexate treatment (case 2). The total ion current (TIC) chromatogram of the trimethylsilyl (TMS) derivatives of metabolites in the first urine sample from case 2 is shown in Fig. 2. A target analysis of more than 200 analytes including amino acids, organic acids, alcohol, sugars, sugar acids, purine and pyrimidine was performed using the intensity of ions of a specific *m/z* at a specific retention time, and the abnormality *n* in [mean above *n* × SD] for the metabolites was calculated automatically.[5] As shown in Table 1, the abnormality of Phe, phenyllactate, 2-hydroxyphenylacetate and phenylacetate was 5.7, 10.2, 7.0 and 4.6, respectively, in case 1, and 7.8, 10.5, 4.9 and 5.7, in case 2. The serum Phe was increased in case 1, and plasma Phe estimated from that in the urine according to Boulos *et al.*[6] was high in both cases. These data indicated that both patients had a metabolic profile identical with PKU. In general, a low Phe diet is recommended to prevent the pathogenesis associated with hyperphenylalaninemia, in classic PKU (higher than 1.2 mM), mild PKU (higher than 0.72 mM), and hyperphenylalaninemia (higher than 0.4 mM), to maintain the blood Phe level below 0.4 mM.[7] When the

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