

Non-Protein-Bound Transition Metals and Hydroxyl Radical Generation in Cerebrospinal Fluid of Newborn Infants with Hypoxic Ischemic Encephalopathy

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ABSTRACT

Among various hypothetical mechanisms for the *in vivo* production of reactive oxygen species, transition metal-catalyzed reactions in cooperation with a biologic reducing agent like ascorbic acid or superoxide may be some of the most important. In the present study, we retrospectively examined the existence of non-protein-bound metal ions, an essentially hazardous pro-oxidant form of various transition metals, and the occurrence of metal-catalyzed reactive oxygen species production in cerebrospinal fluid (CSF) of 10 infants with hypoxic ischemic encephalopathy (HIE) subsequent to perinatal asphyxia and 12 control infants within 72 h of birth. Non-protein-bound iron was detected in eight out of 10 CSF samples from the HIE infants and its level was significantly correlated with Sarnat's clinical stage, whereas none of the control infants had detectable non-protein-bound iron levels. Non-protein-bound copper was below the detection limit in all CSF samples from both groups. Ascorbic acid was significantly increased in the CSF of HIE infants when compared with that of controls (means, 664.9 versus 449.4 μM , $p = 0.008$). *ortho*-Tyrosine and *meta*-tyrosine, which are highly specific and sensitive markers of protein oxidation induced by hydroxyl radicals, were significantly higher in HIE infants than in controls when evaluated by the ratio relative to their source amino acid, phenylalanine [means, 110.5 versus

75.4, $p = 0.018$ for *ortho*-tyrosine/phenylalanine; 104.6 versus 67.7 ($\text{nM}/\mu\text{M} \times 10^2$), $p = 0.048$ for *meta*-tyrosine/phenylalanine]. Both ratios were significantly correlated with non-protein-bound iron, but not with ascorbic acid. Our preliminary observations provide direct evidence that hydroxyl radicals are generated in the CNS during asphyxiation. Iron chelation therapy could be worth developing as a neuroprotective strategy for perinatal asphyxia. (*Pediatr Res* 53: 594–599, 2003)

Abbreviations

ROS, reactive oxygen species
HIE, hypoxic ischemic encephalopathy
CSF, cerebrospinal fluid
NPBI, non-protein-bound iron
NPBC, non-protein-bound copper
***o*-Tyr**, *ortho*-tyrosine
***m*-Tyr**, *meta*-tyrosine
Phe, phenylalanine
AA, ascorbic acid
DHAA, dehydroascorbic acid
UA, uric acid
GC/MS, gas chromatography-mass spectrometry

Advanced perinatal and obstetric management cannot yet prevent brain damage in newborn infants after perinatal asphyxia. Current therapeutic strategies for HIE occurring after perinatal asphyxia are not specific and are only supportive. Numerous studies have suggested that free radicals could have a key role in causing hypoxic ischemic damage to the brain, especially during the reoxygenation/reperfusion phase (1, 2).

Among various hypothetical mechanisms for the production of ROS in living organisms, transition metal-catalyzed reactions may be some of the most important (3). When transition metals like iron or copper exist in their non-protein-bound "free" form *in vivo*, these metals can convert less reactive radicals to more reactive species. Thus, the human body is normally very careful to sequester these metal ions to be incorporated into specific metal-binding proteins. However, it is known that iron can be liberated from iron-storage ferritin when environmental pH decreases sufficiently, which may occur during perinatal asphyxia (4).

Several studies have detected NPBI in the plasma of newborn infants, who show immature iron metabolism (5–7). In

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infants with severe perinatal asphyxia, a close association has been reported between an adverse outcome and the plasma concentration of NPBI (8). In addition, CSF essentially has no significant binding capacity for iron or copper because its content of transferrin and ceruloplasmin is very low (9), and it was recently reported that NPBI is elevated in the CSF of preterm infants with posthemorrhagic ventricular dilatation (10). In this context, several animal studies have shown the effectiveness of iron chelation therapy for post hypoxia/ischemia-reperfusion injury of the brain (11, 12).

In the brain, various nonradical compounds, such as catecholamines, ascorbic acid, thiols, and lipid peroxides, can produce reactive radical species in the presence of transition metal ions (9). Among them, AA is known to be strongly concentrated in the brain tissue and CSF by active transport mechanisms (13). In neonates with birth asphyxia, the AA concentration of CSF is known to increase markedly above the normal nonasphyxia level (14). AA, together with UA, is an integral water-soluble antioxidant that seems to serve as the main defense against oxidizing species in the aqueous phase of human body fluids. However, it has also been well confirmed that AA can contribute to the formation of highly reactive hydroxyl radicals in cooperation with redox active metal ions (15). Thus, we hypothesized that ROS formation catalyzed by redox-active transition metals may take place when HIE occurs after perinatal asphyxia.

In the present study, we measured the concentrations of NPBI, NPBC, and AA in the CSF of infants with HIE and matched controls, together with the oxidation products of AA and UA (DHAA and allantoin) as markers of ROS generation (16), $F_{2\alpha}$ -isoprostane as a marker of lipid peroxidation (17), and the levels of *o*-Tyr and *m*-Tyr as highly specific and sensitive markers of protein oxidation induced by hydroxyl radicals (18, 19). We also analyzed whether these parameters were correlated with other well-established markers of HIE, including hypoxanthine (a marker of brain ischemia/hypoxia) (20), neuron-specific enolase (a marker of neuronal damage) (21), and excitatory amino acids (22).

MATERIALS AND METHODS

Patients. Among the infants admitted to the Neonatal Intensive Care Unit of Osaka Medical College Hospital between April 1999 and March 2001, term infants (gestational age ≥ 37 wk) who had abnormal neurologic signs as described by Sarnat and Sarnat (23), such as increased irritability and jitteriness, abnormal tone, abnormal primitive reflexes, altered consciousness, or convulsions, within the first 24 h of life and underwent lumbar puncture within 72 h of birth to rule out meningitis or subarachnoid hemorrhage associated with at least three of the following criteria were enrolled in this study: 1) signs of fetal distress, defined as an abnormal cardiotocogram (late deceleration, absent variability, or bradycardia) with thick meconium staining of amniotic fluid; 2) an Apgar score ≤ 6 at 5 min after birth; 3) a requirement for more than 3 min of positive pressure resuscitation immediately after birth; and 4) umbilical cord blood pH < 7.00 or a base deficit > 20 mM.

Neurologically normal term infants without a history of perinatal asphyxia (no signs of fetal distress and an Apgar score ≥ 9 at 5 min after birth), in whom lumbar puncture was performed within the first 72 h of life to exclude meningitis as part of a septic workup for suspected systemic infection, were used as controls.

Exclusion criteria for both groups were as follows: 1) congenital heart disease; 2) multiple malformations; 3) documented chromosomal abnormality; 4) an increase of leukocytes and/or erythrocytes in CSF; 5) macroscopic hemolysis in CSF supernatant; and 6) positive bacterial cultures of CSF or blood. During the study period, 10 HIE and 12 control infants fulfilled the above criteria.

The study protocol was approved by the ethics committee of our hospital and investigations were only performed after informed consent had been obtained from the parents of the subjects.

Methods. CSF samples were immediately centrifuged to remove cell components and the supernatant was stored in plastic metal-free containers under nitrogen gas at -80°C until being thawed once for analysis.

NPBI was measured by the method described previously based on bleomycin-iron complex dependent degradation of DNA (24). This assay only detects "free" iron and not iron bound to specific iron binding and storage proteins such as transferrin, ferritin, and Hb. NPBC was measured by a similar method, using phenanthroline as a chelator instead of bleomycin (25). For both methods, the detection limit was $0.2 \mu\text{M}$ and values of $< 0.2 \mu\text{M}$ were expressed as zero.

o-Tyr and *m*-Tyr were quantified by isotope dilution negative-ion chemical ionization GC/MS as their *n*-propyl heptafluorobutyryl derivatives (26) using a Shimadzu QP-5050A GC/MS system (Shimadzu, Kyoto, Japan). Three excitatory amino acids (glycine, aspartate, and glutamate) and phenylalanine were measured by electron-impact ionization GC/MS as their *tert*-butyldimethylsilyl derivatives (27) using a Shimadzu QP-5000 ver. 2 GC/MS system. $F_{2\alpha}$ -Isoprostane was measured with a competitive enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, U.S.A.). For the GC/MS analyses and the isoprostane assay, precipitation of protein with two volumes of ethanol was done before treatment of the sample to investigate only the non-protein-bound free form of each substance. Addition of FeSO_4 at the final concentration of $100 \mu\text{M}$ to the CSF samples before analysis did not influence the results of either measurement (data not shown).

AA, UA, and their oxidation products (DHAA and allantoin) were measured by HPLC according to the method of our previous report (16, 28). Hypoxanthine was also determined by HPLC (29) and the concentration of neuron-specific enolase was measured using an immunoradiometric assay kit (Eiken Chemical, Tokyo, Japan).

Isoprostane could not be assayed in one of the HIE infants and four of the control infants because CSF samples were insufficient.

Statistical analysis. Comparison of characteristic variables between groups was performed using the Mann-Whitney *U* test for continuous variables and Fisher's exact test for categorical data. Differences between the two groups were assessed by

Mann-Whitney *U* test. For correlation analysis, Spearman's correlation coefficients were calculated. A *p* value < 0.05 was considered to be significant. Data were analyzed using SPSS statistical software (SPSS Inc., Chicago, IL, U.S.A.).

RESULTS

Clinical characteristics were comparable between the HIE and control groups, with the exception of the Apgar scores. Although the age at lumbar puncture varied widely because this examination was only performed for clinical indications, there was no statistical difference between the two groups (Table 1).

According to Sarnat and Sarnat (23), HIE was classified as stage 1 in four infants, stage 2 in three infants, and stage 3 in three infants. The outcome was normal for stage 1 and 2 infants. In contrast, all stage 3 infants had severe motor and mental disabilities as well as multicystic encephalomalacia with severe cortical atrophy on brain MRI at the age of 3–6 mo.

As shown in Figure 1, NPBI was detected in eight out of 10 CSF samples from the HIE infants, but none of the control infants had detectable NPBI levels in their CSF. Although the number of subjects was small, the concentration of NPBI in the CSF was strongly correlated with the clinical stage ($r = 0.857$, $p = 0.010$) of the HIE infants. NPBC was below the detection limit in all samples from both groups (Table 2).

As shown in Table 2, AA and UA levels were significantly increased in the HIE infants when compared with the controls. The oxidation products of AA and UA, *i.e.* DHAA and allantoin, respectively, and the ratio of DHAA to AA were also significantly higher in the HIE infants, but no such difference was noted for the allantoin/UA ratio. CSF hypoxanthine and neuron-specific enolase levels were significantly higher in the HIE infants than in the controls. Excitatory amino acids, such as glycine, aspartate, and glutamate, did not differ significantly between the two groups, although the mean CSF level of each amino acid was about 2-fold higher in the HIE infants when compared with that in the control infants.

Figure 2 shows excellent chromatograms and resolution of *o*-Tyr and *m*-Tyr peaks obtained by selected ion monitoring of an *n*-propyl heptafluorobutyryl derivative from a CSF sample. The HIE infants had significantly higher *o*-Tyr and *m*-Tyr levels in the CSF when compared with the control infants (mean \pm SD, 20.6 ± 18.6 versus 8.7 ± 2.6 nM, $p = 0.002$ for

Table 1. Clinical characteristics of study neonates

	HIE (<i>n</i> = 10)	Control (<i>n</i> = 12)
Gestational age (wk)	39 (37–41)	39 (37–41)
Birth weight (g)	2669 (2280–4262)	2772 (2120–3840)
Gender (M/F)	7/3	5/7
Cesarean section (<i>n</i>)	3/10	4/12
Apgar score		
at 1 min	3 (0–4)*	9 (6–10)
at 5 min	5 (1–6)*	10 (9–10)
Age at lumbar puncture (h)	25 (5–69)	48 (8–72)

Values are expressed as the median (range). Differences between groups were assessed by Mann-Whitney *U* test or Fisher's exact test as appropriate.

* $p < 0.05$ vs control infants.

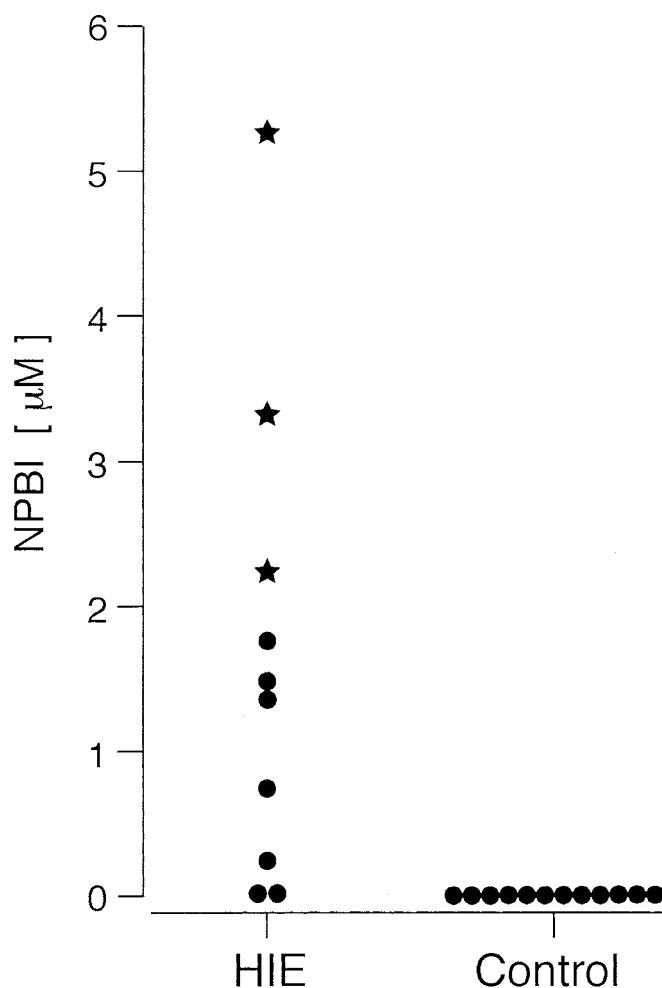


Figure 1. Concentration of NPBI in the CSF of newborn infants with HIE or control infants. Stars represent stage 3 HIE according to Sarnat and Sarnat (23).

Table 2. Non-protein-bound metal ions, major water-soluble antioxidants, and other biochemical parameters in the newborn infants with HIE and the control infants

	HIE	Control	<i>p</i> Value
NPBI	1.6 ± 1.6	0.0 ± 0.0	0.002
NPBC	0.0 ± 0.0	0.0 ± 0.0	—
AA	664.9 ± 213.6	449.4 ± 121.5	0.008
DHAA	10.6 ± 5.0	4.0 ± 4.2	0.002
DHAA/AA (%)	1.8 ± 1.3	0.9 ± 1.0	0.007
UA	136.5 ± 176.1	29.3 ± 13.9	<0.001
Allantoin	2.2 ± 0.8	1.3 ± 0.4	0.014
Allantoin/UA (%)	3.0 ± 1.9	5.1 ± 2.5	0.057
Hypoxanthine	11.7 ± 10.7	6.5 ± 1.5	0.032
NSE (ng/mL)	107.9 ± 164.0	16.7 ± 4.1	0.018
Glycine	19.4 ± 17.7	11.5 ± 2.5	0.129
Aspartate	1.9 ± 2.6	0.9 ± 0.3	0.391
Glutamate	5.1 ± 4.3	3.1 ± 0.6	0.510

Values are expressed as the mean \pm SD. The unit of concentration was micromolar, unless otherwise stated in parentheses. Differences between groups were assessed by Mann-Whitney *U* test. NSE, neuron-specific enolase.

o-Tyr and 20.5 ± 24.9 versus 8.0 ± 3.1 , $p = 0.021$ for *m*-Tyr) (Fig. 3A). The ratios of both tyrosine molecules to phenylalanine (the source amino acid) were also higher in the HIE

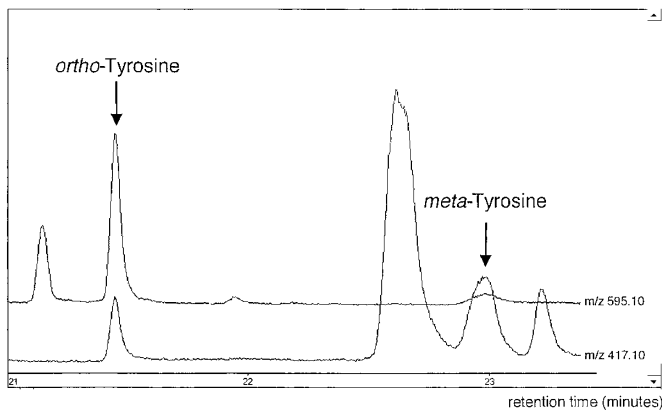


Figure 2. Typical selected-ion monitoring chromatograms of *o*-Tyr and *m*-Tyr at *m/z* 417.10 and 595.10 obtained from an *n*-propyl heptafluorobutyl derivative of CSF by isotope dilution negative ion chemical ionization GC/MS analysis.

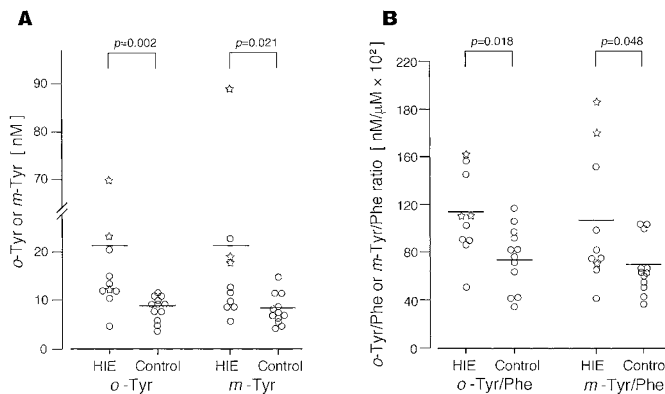


Figure 3. (A) *o*-Tyr and *m*-Tyr concentrations in the CSF of newborn infants with HIE or control infants. (B) The ratios of these tyrosines to Phe. Stars represent stage 3 HIE according to Sarnat and Sarnat (23).

infants [110.5 ± 35.0 versus 75.4 ± 25.5 ($\text{nM}/\mu\text{M} \times 10^2$), $p = 0.018$ for the *o*-Tyr/Phe ratio and 104.6 ± 55.0 versus 67.7 ± 23.5 , $p = 0.048$ for the *m*-Tyr/Phe ratio] (Fig. 3B). In the HIE infants, the CSF levels of *o*-Tyr and *m*-Tyr were significantly correlated with the clinical stage ($r = 0.642$, $p = 0.048$ for *o*-Tyr and $r = 0.779$, $p = 0.019$ for *m*-Tyr), although the correlations of either ratio with stage were not statistically significant ($r = 0.565$, $p = 0.090$ for the *o*-Tyr/Phe ratio and $r = 0.578$, $p = 0.083$ for the *m*-Tyr/Phe ratio).

Correlations between the *o*-Tyr/Phe or *m*-Tyr/Phe ratio and the other biochemical parameters were assessed in 22 study subjects from both groups. These ratios showed a significant correlation with NPBI ($r = 0.654$, $p = 0.003$ for the *o*-Tyr/Phe ratio and $r = 0.507$, $p = 0.020$ for the *m*-Tyr/Phe ratio), but not with the level of AA or the DHAA/AA ratio. Other parameters were also not significantly correlated with these ratios, except for hypoxanthine with the *o*-Tyr/Phe ratio ($r = 0.500$, $p = 0.026$) and neuron-specific enolase with the *o*-Tyr/Phe ratio ($r = 0.648$, $p = 0.004$).

The CSF concentration of $F_{2\alpha}$ -isoprostane was significantly increased in the HIE infants when compared with the control infants (177.3 ± 67.1 versus 65.9 ± 28.0 pM, $p = 0.001$) (Fig.

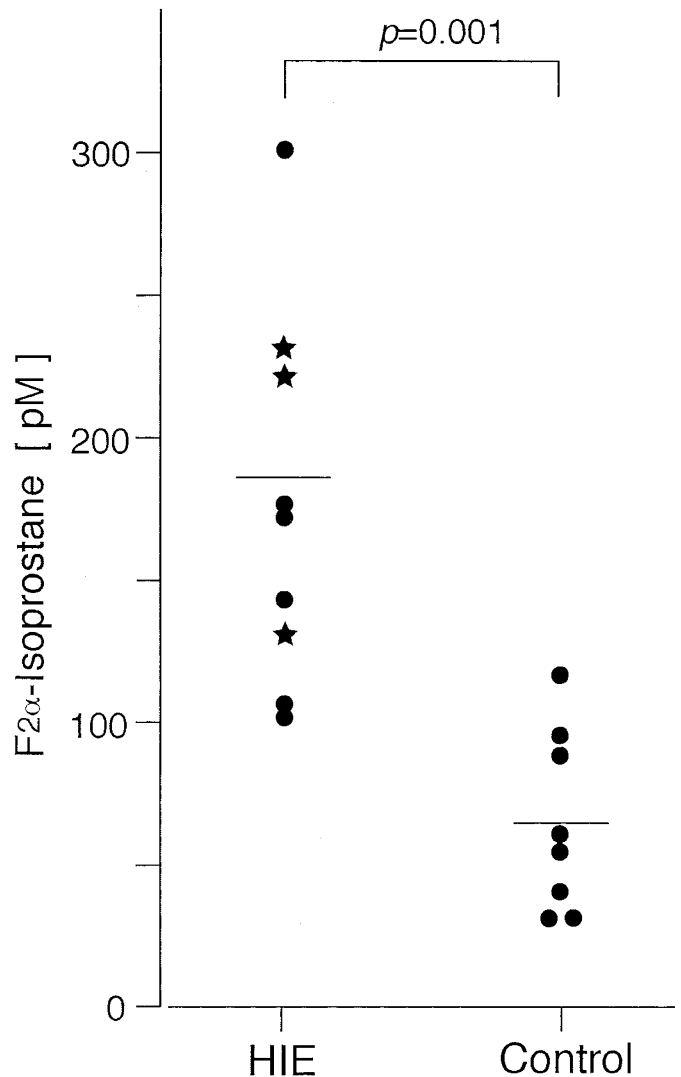


Figure 4. Concentration of $F_{2\alpha}$ -isoprostane in the CSF of newborn infants with HIE or control infants. Stars represent stage 3 HIE according to Sarnat and Sarnat (23); horizontal bar = mean.

4), and it was significantly correlated with NPBI ($r = 0.836$, $p = 0.008$), AA ($r = 0.564$, $p = 0.024$), hypoxanthine ($r = 0.695$, $p = 0.007$), *o*-Tyr/Phe ($r = 0.598$, $p = 0.017$), or *m*-Tyr/Phe ($r = 0.56$, $p = 0.025$) in all measurable samples, but was not significantly correlated with clinical stage in the HIE group ($r = 0.58$, $p = 0.1$).

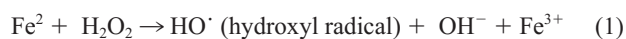
DISCUSSION

The most striking finding of the present study was the presence of NPBI in the CSF of HIE infants and not in any of the control infants. This is the first report of such a finding to the best of our knowledge. Newborn infants are essentially predisposed to releasing free iron due to their low iron-binding capacity because of the high iron saturation of transferrin and a decreased ferroxidase activity due to the low level of ceruloplasmin (7). Ferroxidase catalyzes the oxidation of ferrous iron to ferric iron and this process is essential for ionic forms of iron to be incorporated into transferrin.

The CSF itself has no significant iron-binding capacity because its content of transferrin is very low and CSF transferrin is often close to iron saturation (9). The concentration of ceruloplasmin in CSF is also known to be quite low. In addition, the high AA level in HIE infants may possibly inhibit the ferroxidase activity of ceruloplasmin (30). Therefore, NPBI in CSF is likely to exist in the reduced ferrous form, which is thought to be an essentially pro-oxidant form of iron because of its ability to catalyze the formation of free radicals.

If the oxidized ferric form of NPBI comes into existence in CSF, it can be reduced to the ferrous state by some reductants with a lower redox potential than that of NPBI-Fe(III)/Fe(II) couple. Theoretically, the main candidates for such a role in CSF are glutathione (one-electron redox potential at pH 7.0 of GSSG/GSSG \cdot = -1500 mV *versus* a standard hydrogen electrode), superoxide (O $_2$ /O $_2^{\cdot-}$ = -330 mV), and AA (DHAA/ascorbate \cdot^- = -174 mV), because the redox potential of low molecular mass iron, Fe(III)/Fe(II), is around +100 mV (31). Among them, it is unlikely that glutathione plays an important role because its concentration in CSF is very low (32). The higher DHAA/AA ratio in our HIE infants than in control infants suggested a possible role for AA in the reduction of ferric iron to ferrous iron in CSF, although it is also possible that the increased ratio may have resulted from the oxidation of AA acting as a sacrificial antioxidant to scavenge free radicals. In fact, it has been shown that, in the absence of ferroxidase activity, iron in the CSF remains in the ferrous state until AA is completely consumed during the incubation of CSF with ascorbate oxidase (33).

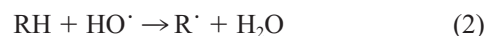
Non-protein-bound ferrous iron catalyzes the following reaction with hydrogen peroxide to yield hydroxyl radicals through Fenton chemistry (Eq. 1).



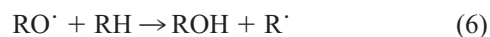
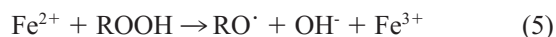
Hydroxyl radicals can react with the aromatic ring of phenylalanine to generate the unnatural isomers *o*-Tyr and *m*-Tyr, instead of natural *para*-(*p*-) tyrosine, after processing by phenylalanine hydroxylase (18). Because *o*-Tyr and *m*-Tyr are stable molecules and are not normally present in the body, they may serve as useful markers of oxidative protein damage induced by hydroxyl radicals (19). Although the precise and reliable quantification of these molecules is difficult because their concentrations in biologic fluids are usually extremely low, highly sensitive GC/MS analysis makes it possible. Significant elevation of *o*-Tyr and *m*-Tyr levels and their ratios to Phe in CSF from our HIE infants strongly supported the possible generation of hydroxyl radicals in the CNS during asphyxiation. As far as we are aware, this is also the first report of such an observation. Elevation of free iron and AA in CSF together with DHAA/AA ratio raises the possibility that hydroxyl radical production may take place *via* Fenton chemistry (Eq. 1) after NPBI reduction from the ferric to ferrous state by two-electron oxidation of AA. The good correlation of *o*-Tyr/Phe and *m*-Tyr/Phe ratios with NPBI further supports this hypothesis. However, the lack of any association between these ratios and AA level or DHAA/AA ratio suggested that the absolute level of AA might not be directly related to hydroxyl radical generation. Although CSF concentration of AA was

markedly raised in the HIE infants, being over four times that previously reported in adults (about 150 μM), the level in our control infants was also about three times the adult value. Therefore, if NPBI is not present, AA might not act as a pro-oxidant in CSF irrespective of its level. The possibility of hydroxylation of phenylalanine by peroxynitrite itself or peroxynitrite-derived hydroxyl radicals (34) could not be excluded because we did not estimate the contribution of nitric oxide by methods such as measurement of nitrotyrosine.

Once hydroxyl radicals have been formed, they attack biologic molecules (RH) at almost a diffusion-controlled rate and steal an electron, after which the radical chain reaction proceeds *via* the following sequence (Eq. 2-4) (35, 36).



Ferrous iron also catalyzes the production of alkoxy radical (RO \cdot) in the presence of an organic hydroperoxide (ROOH) (Eq. 5), after which the radical chain reaction occurs (Eq. 6 and Eqs. 3 and 4) (36).



Brain tissue is very rich in polyunsaturated fatty acids, which are especially sensitive to free radical attack (9). An increased CSF concentration of arachidonic acid metabolites has been reported in HIE infants (37). When RH is a polyunsaturated fatty acid, radical chain lipid peroxidation occurs, leading to the oxidative destruction of lipids. Significant elevation of isoprostane and its good correlation with NPBI support the occurrence of such processes.

Although the age of the two groups at lumbar puncture was quite different, the difference did not reach statistical significance ($p = 0.065$, Mann-Whitney U test), and the variations in sampling time also showed no significant difference ($p = 0.088$, one-way ANOVA). In addition, since the study ended, four other control babies were studied within the first 24 h of life and the values of NPBI, AA, excitatory amino acids, and specific markers of oxidative damage did not differ significantly from the values for the control group (data not shown). Therefore, the differences in biochemical parameters between the asphyxiated infants and controls might not have originated from the difference in timing of the spinal tap.

Our results are strongly consistent with a previous report of a close association between an elevated plasma concentration of NPBI within 8 h after birth and an adverse outcome of severe birth asphyxia (8). Our findings also suggest that the marked effectiveness of iron chelators for ischemia-reperfusion injury in animal models (11, 12) or cultured cells may also be applicable for human birth asphyxia. Moreover, because NPBC was never detected in the CSF of HIE infants, it may be sufficient for only iron to be targeted as the metal for chelation. Although the clinical significance of our preliminary findings

was not confirmed because of the small number of severely affected infants, we encourage the addition of iron chelation therapy to the list of neuroprotective strategies to be developed for perinatal asphyxia in the future.

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This article is dedicated to Dr. Makoto Mino.

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