Influence of Nicotinic Acid on Cerebroside Synthesis in the Brain of Developing Rats

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Summary The effect of nicotinic acid on synthesis of cerebroside was studied during brain development. Nicotinic acid concentration in the whole brain and liver of rats fed on nicotinic acid-deficient diet for 10 days after weaning was lower than that of animals fed on nicotinic acid-supplemented diet. The cerebroside concentration was markedly lower and the total lipid concentration was slightly lower in the brain of nicotinic acid-deficient animals than in those receiving nicotinic acid-supplemented diet. Therefore, the ratio of cerebrosides to total lipids of nicotinic acid-deficient rats was significantly lower than that of nicotinic acid-supplemented rats. In nicotinic acid-deficient rats, the ratio of long-chain to short-chain fatty acid \((C_{20-24}/C_{14-18})\) was decreased in the nonhydroxy fatty acid fraction. Moreover, the ratio of synthesis of cerebrosides with hydroxy fatty acid to nonhydroxy fatty acid of nicotinic acid-deficient rats was higher than that of rats fed on nicotinic acid-supplemented diet. These observations suggest that nicotinic acid affects the synthesis of cerebrosides with nonhydroxy fatty acid.

Key Words nicotinic acid, cerebroside, long-chain fatty acid, hydroxy fatty acid, nonhydroxy fatty acid

Morphological studies indicate that the myelin sheath is an extension of the oligodendroglial cell plasma membrane which wraps around axons and insulates nerve conduction in axons (1). Cerebrosides are one of the major lipid components of myelin membranes and are also present in low concentration in other subcellular membranes of the brain (2–5). They are almost nonexistent until about 10 days after birth, but increase sharply from the second to the third postnatal week. This age of maximum biosynthesis correlates well with the period of most active myelination (6).

Previous studies in this laboratory have shown that myelin yield and cerebroside level in the brain of rats fed on nicotinic acid-deficient diet show a decrease

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compared to rats fed on nicotinic acid-supplemented diet (7–10). Moreover, deposition of cerebrosides in the brain of nicotinic acid-deficient rats is delayed due to the decrease of biosynthesis of cerebrosides which contain high levels of long chain nonhydroxy and hydroxy fatty acid (9).

The most abundant fatty acids in cerebrosides are those with 24 carbon atoms such as nervonic acid, cerebronic acid and lignoceric acid (11). Synthesis of these long chain fatty acids dramatically increases at the onset of myelination (12–15). Since pyridine nucleotide is the preferred cofactor for fatty acid elongation, it is assumed that elongation of fatty acid decreases in the brain of rats fed on nicotinic acid-deficient diet.

Moreover, pyridine nucleotide is the preferred cofactor for fatty acid α-hydroxylating enzyme (16,17). Therefore, the diversity of α-hydroxylation and of the balance of chain length in the fatty acid contributes to the synthesis of cerebrosides. The profound change in fatty acid composition induced by nicotinic acid deficiency may alter the integrity and function of myelin. The present paper describes the change of fatty acid composition of cerebrosides and synthesis of cerebrosides with nonhydroxy and hydroxy fatty acid in the brain of rats fed on nicotinic acid-deficient diet and nicotinic acid-supplemented diet.

METHODS

**Animals and diet.** Animals used were rats of the Sprague-Dawley strain. Dams of suckling animals were fed on commercial diet. Litters were reduced to ten each at birth. Offspring were weaned 12 days after birth. The weaned animals were placed on either nicotinic acid-deficient diet or nicotinic acid-supplemented diet, given ad libitum or by pair-feeding according to the quantity consumed by the deficient group on the previous day. They were weighed at least twice weekly during the experimental period.

| Component          | g/kg |
|--------------------|------|
| Sucrose            | 800  |
| Casein             | 70   |
| Amino acid mix*    | 10   |
| Soybean oil        | 40   |
| Cod-liver oil      | 10   |
| Mineral mix*       | 40   |
| Cellulose          | 20   |
| Vitamin mix*       | 10   |

*Amino acid mix, mineral mix and vitamin mix were prepared as described previously (7).*

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Nicotinic acid-deficient diet was prepared as described by Nakashima et al. (18). In order to limit the amount of tryptophan in this diet, we used a nicotinic acid-free low-casein diet to which a small amount of both methionine and threonine was added (tryptophan-imbalanced diet) (18, 19), because pyridine nucleotides are synthesized from tryptophan in the rat (20). To the control group, 10 mg of nicotinic acid was added per 100 g of this nicotinic acid-deficient diet.

Measurement of synthesis of cerebrosides with nonhydroxy and hydroxy fatty acid. Weanling rats (weaned 12 days after birth) were fed on nicotinic acid-deficient diet or nicotinic acid-supplemented diet for 10 days. The rats anesthetized with ether were given intraventricular injection of 1 µCi of L-[U-14C]serine (0.1 µmol) in a 10 µl volume, as described by Haynes and Jungalwala (21). Forty-eight hours after [14C]serine injection, the rats were decapitated and the whole brain was removed, weighed and homogenized in 10 ml of ice-cold 0.32 M sucrose in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 105,000 x g for 60 min and the supernatant fluid was carefully removed. The pellet was suspended in 2 ml of 0.32 M sucrose and extracted with 20 vol of chloroform–methanol (2:1, v/v) by the methods of Folch et al. (22). Total lipid was determined gravimetrically. Total lipid was fractionated by silicic acid (Wako gel Q-23, 100–200 mesh) column chromatography. The cerebroside fraction was evaporated to dryness and dissolved in 0.5 ml of chloroform–methanol (2:1, v/v) and an aliquot was chromatographed on silica-gel G thin-layer plate (Merk) as described by Norton and Poduslo (5). The band corresponding to the cerebroside fraction was purified by thin-layer chromatography in the same way and used for quantitative determination of cerebroside as described previously (7, 8).

Gas-liquid chromatographic analysis of fatty acids in cerebroside. A fraction of cerebroside was purified by silicic acid column chromatography and thin-layer chromatography on silica-gel as described previously (23, 24). For transmethylation of fatty acids of cerebroside, 0.5 ml of 14% boron trifluoride-methanol complex was added to about 1 mg of cerebroside contained in individual tubes. The latter were filled with nitrogen, sealed and heated at 100 °C for 1 h according to the methods of Morrison and Smith (25). They were cooled and 1 ml of water was added to each. The fatty acid methyl esters of cerebroside were extracted 3 times with 2 ml portions of hexane. The combined extracts were evaporated to dryness in vacuo, and the esters were redissolved in chloroform–hexane (1:1). Methyl esters of nonhydroxy and hydroxy fatty acids were separated by chromatography on silicic acid according to the methods of O’Brien and Rouser (26). Methyl esters of nonhydroxy fatty acids were analyzed by gas-liquid chromatography (Ohkura gas chromatograph, Model 103). Hydroxy fatty acids were acetylated and analyzed by gas-liquid chromatography according to the method of O’Brien and Rouser (26). Identification of nonhydroxy and hydroxy fatty acids was made using the standard mixture of GC Mix-H-A and GC Mix-H-C (Applied Science Laboratories, Inc.).

Assay of nicotinic acid. A volume of 0.1 ml of the brain homogenate in 0.32 M sucrose was used for analysis of nicotinic acid. Nicotinic acid in tissue was extracted
as described previously (18). The nicotinic acid content of the extract was determined microbiologically by the methods of Snell and Wright using Lactobacillus arabinosus, strain 17-5 ATCC 8014 (27).

RESULTS

Effect of nicotinic acid on body and brain weight and nicotinic acid concentration in the brain and liver

The final body weight of animals fed on nicotinic acid-deficient diet was significantly reduced for the groups given nicotinic acid-supplemented diet ad libitum (Table 2). In the pair-fed group given nicotinic acid-supplemented diet, body weight was remarkably lower than that for the group fed on nicotinic acid-supplemented diet ad libitum. No remarkable difference in brain weight was observed between the nicotinic acid-deficient group and nicotinic acid-supplemented group (pair fed and ad libitum groups). Nicotinic acid concentration in the whole brain of rats fed on nicotinic acid-deficient diet was significantly lower than with the animals fed on nicotinic acid-supplemented diet (pair-fed and ad libitum groups).

Concentration of total lipids and cerebroside in the brain of rats fed on nicotinic acid-deficient diet and nicotinic acid-supplemented diet

Total lipid concentration in the brain was lower in rats fed on nicotinic acid-deficient diet than in those receiving nicotinic acid-supplemented diet ad libitum (Table 3). However, total lipid concentration did not differ between the groups fed on nicotinic acid-deficient diet and nicotinic acid-supplemented diet by pair-feeding.

Table 2. Effect of nicotinic acid on body and brain weight, and concentration of nicotinic acid in the brain and liver of rat.

Weanling rats (weaned 12 days after birth) were fed on nicotinic acid-deficient diet or nicotinic acid-supplemented diet for 10 days. (P): The group was fed on nicotinic acid-supplemented diet by pair feeding (the amount given was equivalent to the quantity consumed by the deficient group of the previous day). The rats were killed by decapitation and the brain and liver were removed. The concentration of nicotinic acid was assayed as described under METHODS. All values represent the mean of 6 rats ± SE.

| Nicotinic acid in diet | Body weight (g) | Brain weight (g) | Nicotinic acid content |
|-----------------------|----------------|-----------------|-----------------------|
|                       |                |                 | Brain (µg/g brain)    | Liver (µg/g liver) |
| −                     | 26 ± 3         | 1.1 ± 0.1       | 29.9 ± 3.9            | 118 ± 4.5         |
| +(P)                  | 27 ± 2         | 1.1 ± 0.1       | 36.4 ± 4.2            | 163 ± 5.5         |
| +                     | 38 ± 3         | 1.2 ± 0.1       | 36.8 ± 4.4            | 179 ± 5.8         |

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Table 3. Composition of total lipid and cerebroside in the brain of rats fed on nicotinic acid-deficient diet and nicotinic acid-supplemented diet.
Treatment of groups is explained in the footnote to Table 2. Lipid were extracted according to the methods of Folch et al. and assayed for total lipids and cerebroside as described under METHODS. All values represent the mean of 6 rats ± SE.

| Nicotinic acid in diet | Total lipid (mg/g brain) | Cerebroside (mg/g brain) | Cerebroside/Total lipid |
|-----------------------|--------------------------|--------------------------|------------------------|
| -                     | 40.1 ± 5.3               | 2.9 ± 0.4                | 0.072                  |
| +(P)                  | 40.6 ± 6.4               | 4.3 ± 0.4                | 0.106                  |
| +                     | 45.2 ± 5.8               | 4.9 ± 0.5                | 0.108                  |

The concentration of cerebroside in the brain of rats fed on nicotinic acid-deficient diet was markedly lower than in the brain of those receiving nicotinic acid-supplemented diet (pair-fed and ad libitum groups). Therefore, the ratio of cerebroside to total lipids of rats fed on nicotinic acid-deficient diet was markedly lower than that of nicotinic acid-supplemented rats. There was no difference in the ratio of cerebroside to total lipids between the pair-fed group and the ad libitum group.

Effect of nicotinic acid on the composition of fatty acids of cerebroside

Pyridine nucleotide is the preferred cofactor for fatty acid synthetase, fatty acid elongating enzyme and fatty acid α-hydroxylating enzyme (15–17). Therefore, it is assumed that nicotinic acid influences the composition of fatty acid of cerebroside and the proportion of cerebroside with nonhydroxy and hydroxy fatty acids. The fatty acid composition of cerebroside from rats fed on either nicotinic acid-deficient diet or nicotinic acid-supplemented diet is shown in Table 4. In the nonhydroxy fatty acid fraction, there was a decrease of 22:0, 24:0 and 24:1 in rats fed on nicotinic acid-deficient diet, while 16:0, 18:0 and 18:1 increased. The ratio of long-chain to short-chain fatty acid (C20-24/C14-18) was then calculated to be 0.9 in the nicotinic acid-supplemented diet group fed ad libitum and 2.0 in the rats fed on nicotinic acid-supplemented diet by pair-feeding. Therefore, the ratio of long-chain nonhydroxy fatty acid was markedly decreased in rats fed on nicotinic acid-deficient diet.

In the hydroxy fatty acid fraction, the percentage of 24:0, which are the typical characteristic fatty acids in cerebroside, decreased in rats fed on nicotinic acid-deficient diet, whereas the percentage of 22:0 increased.

Effect of nicotinic acid on synthesis of cerebroside with nonhydroxy and hydroxy fatty acid

Table 5 presents the content and synthesis of cerebroside with nonhydroxy and
Table 4. Effect of nicotinic acid on fatty acid composition of cerebroside.
Weanling rats were fed on either nicotinic acid-deficient diet or nicotinic acid-supplemented diet for 3 weeks. (P): The group was fed on nicotinic acid-supplemented diet by pair feeding. The fatty acid composition of cerebroside was determined by gas-liquid chromatography as described under METHODS. Values are percentages of total nonhydroxy and hydroxy fatty acids. All values represent the mean of 6 rats.

| Nicotinic acid in diet | + (%) | + (P) (%) | - (%) |
|-----------------------|-------|-----------|-------|
| Nonhydroxy fatty acid |       |           |       |
| 16:0                  | 9.0   | 9.2       | 13.4  |
| 18:0                  | 11.5  | 11.8      | 18.6  |
| 18:1                  | 10.3  | 9.9       | 16.4  |
| 20:1                  | 2.1   | 2.1       | 2.5   |
| 22:0                  | 13.3  | 12.6      | 10.7  |
| 24:0                  | 40.6  | 38.8      | 27.9  |
| 24:1                  | 9.1   | 8.8       | 5.3   |
| Unidentified          | 4.1   | 6.8       | 5.2   |
| 14–18                 | 30.8  | 30.9      | 48.4  |
| 20–24                 | 65.1  | 62.3      | 46.4  |
| Ratio (20–24/14–18)   | 2.1   | 2.0       | 0.9   |
| a-Hydroxy fatty acid  |       |           |       |
| 22h:0                 | 20.3  | 21.0      | 26.2  |
| 23h:0                 | 6.7   | 7.2       | 9.0   |
| 24h:0                 | 56.6  | 55.4      | 49.3  |
| Unidentified          | 6.4   | 6.4       | 5.5   |

The content of cerebroside with nonhydroxy fatty acid was significantly decreased in the brain of rats fed on nicotinic acid-deficient diet. However, the content of cerebroside with hydroxy fatty acid showed no difference among these three groups. Therefore, the ratio of the content of cerebroside with hydroxy fatty acid to nonhydroxy fatty acid of nicotinic acid-deficient rats was higher than that of rats fed on nicotinic acid-supplemented diet.

The total amount of radioactivity incorporated into the fraction of cerebroside with nonhydroxy fatty acid of nicotinic acid-deficient rats was smaller than that of rats fed on nicotinic acid-supplemented diet (pair-feeding and ad libitum). These was a small but significant difference in the radioactivity incorporated into the fraction of cerebroside with hydroxy fatty acid in the brain of nicotinic acid-deficient rats. Therefore, the ratio of synthesis of cerebroside with hydroxy fatty acid to nonhydroxy fatty acid of nicotinic acid-deficient rats was higher than that of rats fed on nicotinic acid-supplemented diet.
Table 5. Effect of nicotinic acid on synthesis of cerebroside with nonhydroxy and hydroxy fatty acids.

Treatment of groups is explained in the footnote to Table 2. Cerebrosides were separated, and amount and synthesis of cerebroside were assayed as described under METHODS. All values represent the mean of 6 rats ± SE.

| Nicotinic Acid in Diet | Cerebroside Content | Synthesis |
|-----------------------|---------------------|-----------|
|                       | NFA (mg/brain)      | HFA (mg/brain) | HFA NFA (dpm/brain) | HFA NFA (dpm/brain) | HFA NFA |
| -                     | 0.6 ± 0.1           | 2.0 ± 0.3    | 3.3               | 650 ± 200            | 2,290 ± 200 | 3.5   |
| + (P)                 | 1.2 ± 0.1           | 2.5 ± 0.1    | 2.1               | 1,340 ± 150          | 2,800 ± 210 | 2.1   |
| +                     | 1.4 ± 0.2           | 2.6 ± 0.3    | 1.9               | 1,600 ± 350          | 3,420 ± 400 | 2.1   |

DISCUSSION

In the present and previous studies we have shown that the content of myelin and cerebroside in the brain of rats fed on nicotinic acid-deficient diet is lower than in the brain of those on nicotinic acid-supplemented diet (7–10). Deposition of cerebrosides in the brain of nicotinic acid-deficient rats was delayed due to the decline of biosynthesis of cerebroside which contains high levels of long chain fatty acids (8, 9). In these experiments, we used a nicotinic acid-free low-casein diet to which a small amount of both methionine and threonine was added and from which tryptophan was excluded (tryptophan-imbalanced diet) (19), because pyridine nucleotides are synthesized from tryptophan in rats (20). Therefore, it was considered that the inadequate amount of tryptophan in the tryptophan-imbalanced diet was implicated in the formation of myelin. However, the yield of myelin and content of cerebroside in the brain of rats fed on a diet which contains an adequate amount of tryptophan (AIN-76 diet) and successively given 3-acetylpyridine which was an antagonistic agent of nicotinic acid was lower than those of the controls. Therefore, it was considered that nicotinic acid (not tryptophan) in the diet influenced myelination associated with synthesis of cerebrosides.

Previous studies from our laboratory have shown that the yield of myelin in the whole brain of nicotinic acid-deficient rats is significantly lower than that of animals fed on nicotinic acid-supplemented diet (9). However, no significant difference was observed regarding the percentage of the gross composition of myelin; total lipid: protein and the ratio of cholesterol, galactolipids and phospholipids (9, 23). Galactolipids, especially cerebrosides, are only detectable in a significant amount at the onset of myelination and possibly are of much importance in this process (28, 29). Other lipid compounds of myelin have been shown to occur in other
membranes and are already present in large quantities at the onset of myelination (30). Therefore, it has been suggested that the rate of myelin synthesis depends upon cerebroside production. Moreover, as shown previously, it was considered that the marked reduction of myelin in nicotinic acid-deficient rats is due to the decrease of synthesis of cerebroside (8).

As shown in this experiment, there was a slight decrease of synthesis of cerebroside with hydroxy fatty acid in the brain of rats fed on nicotinic acid-deficient diet. However, synthesis of cerebroside with nonhydroxy fatty acid in the brain of nicotinic acid-deficient rats was significantly smaller than that of nicotinic acid-supplemented rats. Cerebroside of the nervous system is characterized by a relatively high content of long-chain fatty acids which dramatically increase at the onset of myelination (31–33). As shown in Table 4, in the nonhydroxy fatty acid fraction, there was a decrease of long-chain fatty acids (C_{20–24}) in nicotinic acid-deficient rats, whereas in the hydroxy fatty acid fraction, a slight decrease was observed in the percentage of 24:0. Therefore, the fatty acid profile of myelin cerebroside of nicotinic acid-deficient rats differed significantly from that of nicotinic acid-supplemented rats. The fatty acid alterations of myelin cerebroside induced by nicotinic acid deficiency may alter the integrity and function of the membrane structure. Nicotinic acid has been recognized as a factor responsible for pellagra, which was frequently observed with disturbances of the central nervous system, leading to dementia. It is not clear whether the changes of fatty acid composition of myelin in the brain of rats fed on nicotinic acid-deficient diet is directly accompanied by the mental symptoms of pellagra.

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