Age-related Changes of α -Tocopherol Transfer Protein Expression in Rat Liver

Han-Suk KIM,* Hiroyuki ARAI,¹ Makoto ARITA,¹
Yuji SATO,¹ Tohru OGIHARA, Hiroshi TAMAI,
Keizo INOUE,¹ and Makoto MINO

*Department of Pediatrics, Osaka Medical College,
Takatsuki, Osaka 569, Japan*

*¹Department of Health Chemistry, Faculty of Pharmaceutical Sciences,
The University of Tokyo, Tokyo 113, Japan*

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Summary Developmental changes in expression of α -tocopherol transfer protein (α -TTP) after birth were investigated using rats with respect to plasma changes of tocopherols. The expression of α -TTP in the neonatal rat liver, which was very low immediately after birth, increased steadily during the two weeks of life before weanling and reached the adult level at four weeks. During the suckling period, the plasma ratio of α -tocopherol to γ -tocopherol linearly increased, because plasma α -tocopherol which was low immediately after birth, increased rapidly during the period, while γ -tocopherol remained unchanged. The increase in the ratio seemed to correlate with the developmental expression of α -TTP in the liver during this period. The ratio also reached the adult level after four weeks. The expression of α -TTP was investigated using primary cultured rat hepatocytes. The expression of α -TTP was found to be extremely low after 20 h of culture. The decrease in α -TTP expression was exacerbated by adding epidermal growth factor to the culture medium and was inhibited by adding dexamethasone. These observations suggest that expression of α -TTP may be affected by the state of hepatic differentiation.

Key Words α -tocopherol transfer protein, α -tocopherol, γ -tocopherol, discrimination of tocopherols, aging, primary culture, hepatocyte, cell differentiation

α -Tocopherol, the most active form of vitamin E, is widely distributed in the biomembranes of mammalian tissues (1). It is well known that the cellular concentration of α -tocopherol is about 10-fold greater than that of γ -tocopherol despite the ingestion of a large amount of γ -tocopherol in the diet (2). Recently,

*To whom correspondence should be addressed.

Traber and Kaden demonstrated that the liver is the organ involved in the discrimination of these tocopherols (1,2). Catignani and Bieri reported that rat liver cytosol contains a protein which preferentially binds to α -tocopherol than the other forms of tocopherol (3). Sato et al. recently purified this protein and designated it as α -tocopherol transfer protein (α -TTP) (4). Subsequently, we isolated a cDNA clone encoding α -TTP (5,6) and demonstrated that the α -TTP gene was defective in cases of isolated vitamin E deficiency (7).

We previously noted that newborn infants, especially bottle-fed ones, showed a low plasma α -tocopherol level and relatively high γ -tocopherol level, as compared with adults (8). These findings suggest that the synthesis of α -TTP may be poor in neonates due to inadequate maturation of the liver, resulting in impaired discrimination of α -tocopherol from the other forms of tocopherol. This would result in the absorption of a relatively large amount of γ -tocopherol, explaining why bottle-fed neonates have a relatively high plasma γ -tocopherol level. In the present study, we investigated the changes of α -TTP in the rat liver after birth.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats were divided into five groups depending on age; day 0, day 4, day 14, day 28, and 12-week old rats (the last group were used as adults).

Blood collection. Two milliliters of blood was drawn into a heparinized tube by venipuncture from rats after fasting for at least 6 h overnight. Plasma was separated by centrifugation at $800 \times g$ for 15 min.

Tocopherol assay. Determination and separation of α - and γ -tocopherol were performed by high performance liquid chromatography (HPLC) with an electrochemical detector (an IRIKA Amperometric E-520 detector, IRIKA, Kyoto, Japan) with tocol as the internal standard, as described previously (9). The HPLC system was an IRIKA P-530 (IRIKA) with an IRIKA RP-18 column (4×250 mm). The eluent was methanol/water/ NaClO_4 at a ratio of 1000/2/7 (v/v/w) and a flow rate of 10 ml/min. The retention time was 6.88 min, 9.37 min, and 10.88 min for tocol, γ -tocopherol, and α -tocopherol.

Western blot analysis of α -TTP. Rats were killed by decapitation and their livers were perfused with an ice-cold buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4). Each liver was minced with scissors and homogenized. The cytosolic fractions was separated from the homogenate by ultracentrifugation at $100,000 \times g$ for 60 min. Fifty micrograms of cytosolic protein was determined and carried on SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane using a Bio-Rad blotting apparatus. Western blotting using a monoclonal antibody (AT-R1, see reference (4,6)) was then carried out after blocking of the nitrocellulose membrane with 3% gelatin overnight. Following incubation at 37°C for 1 h, the nitrocellulose membrane was washed and incubated at room temperature for 1 h in a solution containing goat anti-mouse IgG

conjugated to alkaline phosphatase. After washing, the blot was developed by soaking in a buffer (0.1 M NaHCO₃ and 1 mM MgCl₂, pH 9.8) containing 0.3 mg/ml of nitroblue tetrazolium and 0.15 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate. The protein content was determined by the method of Lowry et al. (10).

Primary hepatocyte culture. Hepatocytes were isolated from the livers of 12-week-old male Sprague-Dawley rats by perfusion with a collagenase solution after laparotomy, and then the cells were collected by low-speed centrifugation. The collected cells were transferred to Williams E medium and cultured for 2 h or 20 h before harvesting. Other liver cells were initially cultured for 4 h in the medium. Then 1 μ M of dexamethasone or 20 ng/ml of epidermal growth factor (EGF) was added to the medium and the cells were cultured for 12 h. The harvested cells were lysed in lysis buffer (1% Triton, 2 mM EDTA, 10 mM Tris-HCl, pH 7.4), and immunoprecipitation was performed with anti- α -TTP polyclonal antibody described in reference (5), and the immunoprecipitants were subjected to Western blot analysis using the monoclonal antibody (AT-R1).

RESULTS

Changes of plasma α - and γ -tocopherol level in rats after birth

Figure 1 shows the changes of the plasma α - and γ -tocopherol levels in rats after birth. The plasma α -tocopherol level was low immediately after birth and then increased rapidly during the suckling period, while γ -tocopherol remained constant during this period. After the commencement of weanling at three weeks, the plasma α -tocopherol decreased rapidly. After four weeks of life, plasma tocoph-

Fig. 1. Changes of plasma tocopherols in rats after birth.

Fig. 2. Changes in the discrimination of tocopherols during the suckling period. Discrimination between tocopherols was investigated by calculating the plasma α -tocopherol to γ -tocopherol ratio. The ratio showed linear increase during the suckling period.

erols were fixed at adult level. We examined the ratio of plasma α - to γ -tocopherol during the two weeks when neonatal rats fed only maternal milk. In this period, the ratio showed a linear increase (Fig. 2).

Changes of α -TTP expression in the rat liver after birth

We examined the changes of α -TTP expression level in the rat liver after birth. The α -TTP level was extremely low immediately after birth, and increased progressively during the first four weeks of life. It reached a plateau at the adult level about four weeks after birth (Fig. 3).

Changes of α -TTP expression in primary cultured rat hepatocytes

In primary cultured rat hepatocytes, the expression of α -TTP was extremely low after 20 h of the culture (Fig. 4-a). This decrease in α -TTP expression was exacerbated by adding EGF to the culture medium and was inhibited by adding dexamethasone (Fig. 4-b).

DISCUSSION

In mammalian tissues, α -tocopherol is selectively detected as compared with the other forms, even though the diet predominantly contains the γ -form rather

Fig. 3. Changes of hepatic α -TTP expression in rats after birth. Cytosolic fractions (50 μ g of protein) were obtained from rat liver, on day 0 (lane 1), day 4 (lane 2), day 14 (lane 3), day 28 (lane 4), and week 12 (lane 5) of life. The sample were subjected to SDS-PAGE and transferred to a nitrocellulose filter. Immunoblotting was performed with a monoclonal antibody for α -TTP (AT-R1. see reference (4)).

than the α -form (1). α -TTP has been proposed to play an important role in the liver cytosol in this discrimination of tocopherols after nonselective absorption of tocopherols through the gut, and in the transfer of α -tocopherol to nascent very low density lipoproteins (VLDL) which should be released into the circulation (1). A congenital defect of α -TTP has been demonstrated in patients with isolated vitamin E deficiency (7). These patients have no ability to discriminate α -tocopherol from other tocopherols, resulting in extremely low plasma α -tocopherol levels (11,12).

In neonatal rats, plasma ratio of α - to γ -tocopherol was low at birth, due to a low level of the α -form relative to the γ -form (Fig. 1). After birth, the plasma α -tocopherol level rapidly increased, while the level of γ -form remained unchanged throughout the first two weeks. Also, during this period when neonatal rats took

Fig. 4. a) Expression of α -TTP in rat hepatocyte primary culture. The harvested cells lysed in lysis buffer (1% Triton, 2 mM EDTA, 10 mM Tris-HCl, pH 7.4), and immunoprecipitation using a polyclonal antibody (5) was performed. Then, the immunoprecipitants were subjected to Western blotting analysis with a monoclonal antibody for α -TTP (AT-R1). b) The effect of hormonal factors on the α -TTP expression in cultured rat hepatocytes. Rat hepatocytes were cultured in the absence or presence of 20 ng/ml EGF or 1 μ M of dexamethasone. After 12 h of culture, cells were harvested and lysed. After immunoprecipitation, the immunoprecipitants were subjected to Western blotting analysis.

tocopherols by sucking their maternal milk, the ratio of α - to γ -tocopherol level in the plasma showed a linear increase (Fig. 2). This indicates a selective increase in α -tocopherol in their plasma.

However, at three weeks of life, plasma α -tocopherol declined again. This decline of plasma α -tocopherol was probably due to a decrease in the intake of α -tocopherol from their diets other than maternal milk. As neonatal rats grow rapidly with an about 20-fold increase in their body weight during the first three weeks of life, they need a large amount of milk for their body weight, and the principal form of tocopherol contained in maternal milk is α -form (13). Therefore, it is considered that neonatal rats should take a relatively large amount of α -tocopherol during this period.

With respect to determining the α -tocopherol in plasma and tissues, it is now established that α -TTP is a critical factor (11, 12). The α -TTP level was very low immediately after birth, but constantly increased during the early period of life and reached the adult level at 4 weeks. During this period, the development of α -TTP was simultaneously completed in the liver, and the increase in the plasma ratio of α - to γ -tocopherol seemed to correlate with the increase in the expression of α -TTP. The developmental ability for discrimination of α -tocopherol should also be completed before weanling, because weanling rats would take relative large amount of tocopherol other than the α -form in the diet. However, further investigation is

required to confirm this hypothesis.

On the basis of the low expression of α -TTP in neonatal rat liver immediately after birth when cell differentiation may progress rapidly, the relationship between the expression of α -TTP and the differentiation of liver cells was investigated using primary cultured hepatocytes. As shown in Fig. 4-a, the expression of α -TTP was markedly decreased after 20 h of culture (14). Primary tissue culture is known to induce the dedifferentiation of hepatocytes. We also found that EGF, which induces cell dedifferentiation (15), enhances the reduction of α -TTP expression, while dexamethasone, an inhibitor of cell dedifferentiation (15), suppressed the reduction of α -TTP expression in cultured cells (Fig. 4-b). These findings suggest that the expression of α -TTP may be specifically influenced by the differentiation state of hepatocytes, and showed that developmental changes of α -TTP may be consistent with the differentiation behavior of liver cells. However, the other biological factors which regulate the expression of α -TTP during development remain to be determined.

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