The Regulatory Effect of Ascorbate on the Carnitine Synthesis in Primary Cultured Guinea Pig Hepatocytes

Tae YOUL HA, Megumi OTSUKA, and Nobuhiko ARAKAWA

Department of Food and Nutrition, Ochanomizu University, Otsuka, Bunkyo-ku, Tokyo 112, Japan

(Received March 13, 1991)

Summary The effect of ascorbate (AsA) on the synthesis of carnitine from γ-butyrobetaine (BB) in primary cultured guinea pig hepatocytes was investigated. The hepatocyte monolayers preloaded with AsA were incubated for 4 h in medium with various concentrations of BB as the precursor of carnitine. The accumulation of carnitine reached a maximum when the cells were incubated with 0.05–1.0 mM BB and significantly decreased with excess BB (5 mM). In contrast, increasing concentrations of AsA supplemented to medium led to an increase in carnitine content, but AsA and total AsA contents in cells decreased by BB supplementation. Regarding the enhancement of hydroxylation of BB in the hepatocytes, AsA was the most effective among such other reducing agents as glutathione and dithiothreitol. Although erythorbate (ErA) also stimulated the hydroxylation of BB, carnitine content in cells preloaded with ErA was only 60% of that with AsA. These results suggest that AsA is specifically required for the hydroxylation of BB. Furthermore, AsA can regulate carnitine synthesis in the primary cultured guinea pig hepatocytes.

Key Words ascorbate, carnitine synthesis, primary cultured guinea pig hepatocyte

It has been well documented that carnitine, which is required for the β-oxidation of long-chain fatty acid (1, 2), is synthesized from lysine and methionine in vivo (3). Ascorbate (AsA) is a cofactor for the enzyme catalyzing the hydroxylations of trimethyllysine to hydroxytrimethyllysine and of γ-butyrobetaine (BB) to carnitine (4, 5). In our previous paper (6), we observed that a deficiency of tissue AsA in guinea pigs led to a decrease in tissue carnitine levels.

Sandor and Hoppel (7) reported that the biosynthesis of carnitine in rats was regulated by the availability of BB. The hydroxylation of BB, which takes place exclusively in the liver, was proposed as a rate-limiting step on the carnitine biosynthesis pathway (8, 9). Christiansen and Bremer (10) showed that the rate of
BB hydroxylation in freshly isolated liver cells was sufficient to cover the turnover of carnitine in the whole rat. They also observed that liver cells lost 80% of their carnitine content during the isolation procedure using the collagenase perfusion method.

Cultured liver cells are widely used for metabolic studies (11). Therefore, primary cultured hepatocytes from the guinea pig, which cannot synthesize AsA, are considered to present a very successful model for studying the effect of AsA on the hydroxylation of BB. However, little study of AsA on carnitine synthesis in primary cultured hepatocytes has been reported.

The present study is designed to clarify the role of AsA on the hydroxylation of BB, which is the last step of the carnitine biosynthesis pathway, in primary cultured guinea pig hepatocytes. We determined carnitine content in AsA-preloaded hepatocytes incubated in medium with BB. We also investigated the effect of various reducing agents on the hydroxylation reaction of BB.

**MATERIALS AND METHODS**

Carnitine, BB, trypsin inhibitor, and acetyl CoA was obtained from Sigma Chemical Co. (St. Louis, MO). Carnitine acetyltransferase was purchased from Boehringer Mannheim GmbH (West Germany). Collagenase, sodium L-ascorbate, and sodium erythorbate were obtained from Wako Pure Chemical Ind., Ltd. All the other reagents were of the highest purity available from commercial sources.

**Hepatocyte isolation and culture.** Parenchymal hepatocytes were isolated from male albino guinea pigs (150–200 g) by the recirculation collagenase perfusion method as described by Tanaka et al. (12). The viability of isolated hepatocytes was tested by trypan blue (0.4% w/v) exclusion. The hepatocytes were suspended in Dulbecco's Modified Eagle's Medium (DMEM; Nissui Pharmaceutical Co., Ltd.) supplemented with 10% (v/v) heat-denatured fetal bovine serum (FBS) and placed in 60 mm dishes (2 × 10⁶ cells). The dishes were equilibrated with 5% CO₂/air and incubated for 4 h at 37°C. The unattached and non-viable cells were removed with the medium. The cells were then incubated for 21 h in fresh DMEM. After removing the medium, the cells were preincubated for 4 h in the presence of 0.3–1 mM AsA.

The medium were replaced with the fresh DMEM containing 0.05–5 mM BB. After various time periods of incubation at 37°C, an aliquot of the medium was extracted with the equal volume of 6% HClO₄ for carnitine assay. The cell layers were rapidly rinsed three times with cold calcium-magnesium-free Dulbecco's phosphate-buffered saline (PBS (-); Nissui Pharmaceutical Co., Ltd.), and the carnitine in the cells was extracted with 3% HClO₄. The perchloric acid extracts of cell layers and medium were centrifuged at 5,000 g for 10 min, and the supernatants were stored at −80°C until analysis.

**Carnitine analysis.** The carnitine contents in the perchloric acid extracts of the cells and medium were determined by HPLC method described in our previous
EFFECT OF ASCORBATE ON CARNITINE SYNTHESIS

AsA and total AsA analysis. After extraction of AsA in the cells with 3% metaphosphoric acid solution, the concentrations of AsA and total AsA were measured by HPLC method as described previously (14).

The content of carnitine in cells is expressed as nmol/mg of cell DNA. The DNA contents of cells were determined using the fluorescence enhancement of 4,6-diamidino-2-phenylindole complexed with DNA as described by Brunk (15).

RESULTS

Table 1 shows the effect of BB concentrations supplemented to medium on the carnitine synthesis in hepatocytes monolayers. The accumulation of carnitine converted from BB reached a maximum when AsA-preloaded cells were incubated in medium with 0.05-1.0 mM BB and significantly decreased with excess BB (5 mM).

The effects of AsA on the synthesis of carnitine is shown in Table 2.

Table 1. The effect of γ-butyrobetaine concentrations on the synthesis of carnitine in cells.

| γ-Butyrobetaine (mM) | Carnitine (nmol/mg DNA) |
|----------------------|-------------------------|
| 0                    | 11.8±1.3 a               |
| 0.05                 | 106.5±11.1 b             |
| 0.5                  | 104.7±15.9 b             |
| 1.0                  | 104.9±18.1 b             |
| 5.0                  | 66.0±3.2 c              |

Hepatocyte monolayers preincubated for 4 h in DMEM with 0.3 mM AsA were incubated for a further 4 h in medium with various concentrations of BB. Values are means±SD for six cultures. The significant difference (p<0.01) between the values with different superscript letters is observed.

Table 2. The effect of ascorbate concentrations on the synthesis of carnitine in cells.

| Ascorbate (mM) | Carnitine (nmol/mg DNA) |
|----------------|-------------------------|
| 0              | 24.3±4.9 a               |
| 0.005          | 49.5±15.0 ab             |
| 0.05           | 68.3±15.0 b              |
| 0.1            | 75.9±21.0 bc             |
| 0.3            | 104.9±18.1 c             |
| 3.0            | 120.6±15.4 d             |

Hepatocyte monolayers preincubated for 4 h in DMEM with various concentrations of AsA were incubated for a further 4 h in medium with 1 mM BB. Values are means±SD for six cultures. The significant difference (p<0.01) between the values with different superscript letters is observed.

Vol. 37, No. 4, 1991
increasing concentrations of AsA led to an increase in the carnitine content. The cells preincubated with 3 mM AsA had fivefold the carnitine content of those without AsA.

Figure 1 illustrates carnitine content in cells and medium at different incubation times in the presence of 1 mM BB. The carnitine content in cells preloaded with 0.3 mM AsA increased quickly with incubation time, reached a maximum at about 4 h of incubation with BB, and then decreased slightly. The carnitine contents in the cells without AsA, however, did not change with an increase in time at least to 24 h from 4 h incubation although only a slight increase was shown at 2 h incubation. In contrast, carnitine concentrations in medium gradually increased with incubation time.

*J. Nutr. Sci. Vitaminol.*
Table 3. The effect of various reducing agents on the synthesis of carnitine in cells.

| Reducing agents (mM) | Carnitine concentrations (nmol/mg DNA) |
|----------------------|---------------------------------------|
| None                 | 25.5 ± 9.3                            |
| Ascorbate (3.0)      | 121.4 ± 22.6**a                       |
| Ascorbate (0.3)      | 102.7 ± 18.8**a                       |
| Erythorbate (3.0)    | 78.8 ± 14.8**b                       |
| Erythorbate (0.3)    | 65.1 ± 4.7**b                        |
| Glutathione (3.0)    | 30.8 ± 17.5c                          |
| Glutathione (0.3)    | 34.9 ± 14.1c                          |
| Dithiothreitol (3.0) | 31.7 ± 4.9g                           |
| Dithiothreitol (0.3) | 27.4 ± 11.8g                          |

Hepatocyte monolayers preincubated for 4 h in DMEM with various reducing agents were incubated for a further 4 h in medium with 1 mM BB. Values are means ± SD for six cultures. *Significantly different from none, p < 0.01. The significant difference (p < 0.01) between the values with different superscript letters in the same concentration is observed.

Table 4. The consumption of ascorbic acid on the synthesis of carnitine in cells.

|                        | AsA µg/2 × 10^6 cells (%) | Total AsA µg/2 × 10^6 cells (%) |
|------------------------|----------------------------|---------------------------------|
| Initial contents       | 5.7 (100)                 | 6.1 (100)                       |
| Final contents (−BB)   | 1.6 (28)                  | 2.4 (39)                        |
| (± BB)                 | 1.1 (19)                  | 1.3 (21)                        |

Hepatocyte monolayers preincubated for 4 h in DMEM with 0.3 mM AsA were incubated for a further 4 h in medium with 0.5 mM BB (+ BB) or without BB (− BB). Values are means for three cultures. Figures in parentheses are percentage of final contents against initial contents.

Table 3 shows the effect of various reducing agents on the synthesis of carnitine in hepatocytes. The highest carnitine content was obtained in the cells preincubated with AsA. Although erythorbate (ErA) instead of AsA also stimulated the synthesis of carnitine from BB, the accumulation of carnitine in cells with ErA was only 60% of that with AsA. The formation of carnitine in cells with dithiothreitol as well as glutathione were similar to those without reducing agents.

The changes of AsA and total AsA contents in cells during incubation are shown in Table 4. The cells preincubated with AsA for 4 h contained 5.7 µg/dish AsA and 6.1 µg/dish total AsA. After 4 h incubation without AsA supplementation, AsA and total AsA contents decreased to 28% (AsA) and 39% (total AsA), respectively, of the initial contents, while the supplementation of 0.5 mM BB caused further decreases of AsA and total AsA, that is, only 19% (AsA) and 21% (total AsA) of the initial contents remained.
DISCUSSION

In the previous study, we found that carnitine contents in tissues of guinea pigs were greatly influenced by tissue AsA levels. The present study showed that in guinea pig hepatocytes monolayer cultures, AsA enhanced the carnitine synthesis. Furthermore, there was no increase in carnitine contents with an increase in time to 24 h from 4 h incubation without AsA preincubation. A slight increase of carnitine contents in cells without AsA preincubation shown in culture at 2 h may be due to the stimulation of carnitine synthesis by trace amounts of AsA in FBS and intact liver. These results suggested that AsA was essential to the carnitine synthesis in the guinea pig hepatocytes.

On the other hand, BB hydroxylase seemed to be apparently inhibited by the excess of the substrate since the amounts of carnitine significantly decreased at 5 mM concentrations of BB in medium. The result is similar to that of Christiansen and Bremer (10), who reported that the hydroxylation rate of BB in the isolated rat liver cells reached a maximum with 0.05 mM BB and markedly decreased with more than 1 mM BB. Daveluy et al. (16) reported that for optimal BB hydroxylase activity in vitro, BB concentrations should be near 0.1 mM and not more than 1 mM.

When the cells were incubated with trimethyllysine instead of BB as a substrate, carnitine contents were similar to those without BB (data not shown). Haigler and Broquist (17) reported that liver slices converted only 7% of trimethyllysine to BB, and this low rate of conversion might be caused by the impermeability of trimethyllysine to cell membranes. Nelson et al. (18) reported that less than 2% of the administered radioactive trimethyllysine was absorbed by the liver as compared with approximately 20% by the kidneys in the 1 h duration of experiment, whereas labelled BB was readily taken up by the liver. We considered that the absence of increase of carnitine contents in guinea pig hepatocytes incubated in medium containing trimethyllysine was due to the fact that it was not taken up by hepatocytes.

We observed the decrease of carnitine contents in hepatocytes and the increase in medium. This indicated that carnitine was synthesized in AsA-preloaded hepatocytes incubated in medium containing BB, and then the synthesized carnitine was released to the medium. It was reported that carnitine and acetylcarnitine synthesized from BB were released from isolated rat liver cells to the medium. In the intact animal, extrahepatically synthesized BB is hydroxylated to carnitine only in the liver, and subsequently transported to other tissues (19). Therefore, the release of carnitine synthesized from hepatocytes to the medium suggests that carnitine is transported from liver to other tissues, such as muscle and heart, which cannot synthesize carnitine.

We also observed that AsA and total AsA contents in cells decreased by BB supplementation, which suggested that AsA was consumed during hydroxylation of BB.

J. Nutr. Sci. Vitaminol.
In primary cultured guinea pig hepatocytes, AsA was the most effective among other reducing agents. Glutathione and dithiothreitol could not stimulate the hydroxylation reaction of BB and could not replace AsA. On the other hand, ErA led to lower carnitine contents in cells than AsA. Arakawa et al. (20) observed that when guinea pigs were orally supplemented with ErA at 20 times the AsA dose, the contents of ErA in liver were only about 20% of AsA. The rate of loss of ErA in tissues of the guinea pig was reported to be more rapid than that of AsA (21). These observations indicate that AsA seems to be retained in tissues more selectively than ErA, and the transport system of ErA to cell membranes may be different from that of AsA. The difference of carnitine contents between AsA and ErA in our study may be due to their storage amounts in hepatocytes rather than their effects on the hydroxylation reaction.

From these results, we suggest that in primary cultured guinea pig hepatocytes, AsA is specifically required for the hydroxylation of BB, and that, furthermore, AsA may regulate carnitine synthesis.

REFERENCES

1) Fritz, I. B. (1963): Carnitine and its role in fatty acid metabolism. *Adv. Lipid Res.*, 1, 285–334.
2) Fritz, I. B., and Yue, K. T. N. (1963): Long-chain carnitine acyltransferase and the role of acylcarnitine derivatives in the catalytic increase of fatty acid oxidation induced by carnitine. *J. Lipid Res.*, 4, 279–288.
3) Hulse, J. D., Ellis, S. R., and Henderson, L. M. (1978): Carnitine biosynthesis. β-Hydroxylation of trimethyllysine by α-ketoglutarate dependent mitochondrial dioxygenase. *J. Biol. Chem.*, 253, 1654–1659.
4) Englard, S., and Seifter, S. (1986): The biochemical functions of ascorbic acid. *Ann. Rev. Nutr.*, 6, 365–406.
5) Lindstedt, G., and Lindstedt, S. (1970): Cofactor requirements of γ-butyrobetaine hydroxylase from rat liver. *J. Biol. Chem.*, 245, 4178–4186.
6) Ha, T.-Y., Otsuka, M., and Arakawa, N. (1990): The effect of graded doses of ascorbic acid on the tissue carnitine and plasma lipid concentrations. *J. Nutr. Sci. Vitaminol.*, 36, 227–234.
7) Sandor, A., and Hoppel, C. L. (1989): Butyrobetaine availability in liver is a regulatory factor for carnitine biosynthesis in rat. *Eur. J. Biochem.*, 185, 671–675.
8) Shenai, J. P., and Borum, P. R. (1984): Tissue carnitine reserves of newborn infants. *Pediatr. Res.*, 18, 679–681.
9) Cederblad, G., Fahraeus, L., and Lindgren, K. (1986): Plasma carnitine and renal-carnitine clearance during pregnancy. *Am. J. Clin. Nutr.*, 44, 379–383.
10) Christiansen, R. Z., and Bremer, J. (1976): Active transport of butyrobetaine and carnitine into isolated liver cells. *Biochim. Biophys. Acta*, 448, 562–577.
11) Seglen, P. O. (1976): Preparation of isolated rat liver cells. *Methods Cell Biol.*, 13, 29–83.
12) Tanaka, K., Sato, M., Tomita, Y., and Ichihara, A. (1978): Biochemical studies on liver functions in primary cultured hepatocytes of adult rats. *J. Biochem.*, 84, 937–946.
13) Arakawa, N., Ha, T.-Y., and Otsuka, M. (1989): An improved high-performance liquid chromatographic assay for the determination of free and esterified carnitine in animal tissues. J. Nutr. Sci. Vitaminol., 35, 475–479.

14) Otsuka, M., Kurata, T., Suzuki, E., Arakawa, N., and Inagaki, C. (1981): Separative determination of ascorbic acid and erythorbic acid in animal tissues by high-performance liquid chromatography. J. Nutr. Sci. Vitaminol., 27, 9–15.

15) Brunk, C. F. (1979): Assay for nanogram quantities of DNA in cellular homogenates. Anal. Biochem., 92, 497–500.

16) Daveluy, A., Parvin, R., and Pand, S. V. (1982): Enzymatic synthesis of radioactive carnitine from γ-butyrobetaine prepared by the methylation of γ-aminobutyric acid. Anal. Biochem., 119, 286–292.

17) Haigler, H. T., and Broquist, H. P. (1974): Carnitine synthesis in rat tissue slices. Biochem. Biophys. Res. Commun., 56, 676–681.

18) Nelson, P. J., Pruitt, R. E., Henderson, L. L., Jenness, R., and Henderson, L. M. (1981): Effect of ascorbic acid deficiency on the in vivo synthesis of carnitine. Biochim. Biophys. Acta, 672, 123–127.

19) Bohmer, T. (1974): Conversion of butyrobetaine to carnitine in the rat in vivo. Biochim. Biophys. Acta, 343, 551–557.

20) Arakawa, N., Suzuki, E., Kurata, T., Otsuka, M., and Inagaki, C. (1986): Effect of erythorbic acid administration on ascorbic acid contents in guinea pig tissues. J. Nutr. Sci. Vitaminol., 32, 171–181.

21) Hughes, R. E., Hurley, R. J., and Jone, P. R. (1971): The retention of ascorbic acid by guinea-pig tissues. Br. J. Nutr., 26, 433–438.