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Ascorbic acid synthesis is stimulated by enhanced glycogenolysis in murine liver

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Abstract Ascorbic acid synthesis was stimulated by glucagon, dibutyryl cyclic AMP, as well as phenylephrine, vasopressin or okadaic acid, in hepatocytes prepared from fed mice. However, no such effect was observed in glycogen-depleted cells from starved animals, either in the presence or absence of glucose. The rate of ascorbate synthesis showed close correlation with the glucose release by hepatocytes. In mice the injection of glucagon increased plasma ascorbate concentration fifteenfold, and caused a sixfold elevation of the ascorbate content of the liver. These results show that hepatic ascorbate synthesis is dependent on glycogenolysis, and indicate a regulatory role of ascorbate released by the liver.

Key words: Ascorbic acid; Glycogen; Glycogenolysis; Glucagon; Mouse liver

1. Introduction

Ascorbic acid is involved in the antioxidant defence of the cell: it is connected to glutathione reduction, participates in free radical scavenging as an antioxidant, and also plays an important role in biosynthetic processes, such as collagen and catecholamine synthesis [1–3]. Despite the important functions of ascorbic acid, relatively few data are available on the regulation of its biosynthesis. Ascorbate is produced in many plants, microorganisms and animals, except for primates and guinea pig. In animals ascorbic acid is synthesized from UDP-glucuronic acid in the uronic acid pathway in the liver and kidney. Previously we have shown that the substrate supply of another UDP-glucuronic acid-consuming process, glucuronidation, is furnished by glycogenolysis and not gluconeogenesis [4,5]. Furthermore, we have suggested a coordinated regulation of glycogenolysis and glucuronide conjugation: the stimulation of glycogenolysis has been accompanied with the inhibition of glucuronide conjugation by various agents acting via cAMP or Ca^{2+}-dependent mechanisms [6–9]. Knowing all this, experiments were undertaken to investigate the connection between glycogen metabolism and ascorbate formation. Our results show that in mouse liver, ascorbate production can be enhanced via stimulated glycogenolysis in both in vivo and in vitro in isolated hepatocytes.

2. Experimental

2.1. Materials

Collagenase (type IV), α,α′-dipyridyl, *o*-dianisidine, glucose oxidase, okadaic acid, phenylephrine [Arg^8]-vasopressin and dibutyryl cyclic AMP were bought from Sigma, St. Louis, MO, USA. Horseradish peroxidase was purchased from Reanal Fine Chemicals, Budapest, Hungary. Glucagon was received from Novo Nordisk A/S 2880 Bagsvaerd, Denmark. All other chemicals were of analytical grade.

2.2. Blood samples and liver tissue preparation from in vivo glucagon-treated mice

Male CFLP mice (30–40 g body weight) fed ad libitum were used. 100 μg/kg glucagon was administered to animals intraperitoneally in a final volume of 1 ml isotonic saline. The control group received 1 ml of isotonic saline. The mice were sacrificed 15 min after the injection. Blood samples for determination of blood glucose and plasma ascorbic acid were obtained after Nembutal anaesthesia by puncture of the inferior vena cava. The weight of the livers was measured following their excision. The livers then were homogenized in cold 5% TCA as described by Zannoni et al. [10]. The final concentration of the liver tissue homogenate was about 5%.

2.3. Preparation and incubation of isolated mouse hepatocytes

Isolated hepatocytes were prepared with the collagenase perfusion method as detailed earlier [11] from male CFLP mice fed ad libitum or starved for 48 h. Viability of the cells checked by the Trypan blue exclusion test was more than 90%. Cells were incubated in Krebs–Henseleit bicarbonate buffer (pH 7.4) containing 2.5 mM Ca^{2+}, 1% albumin, 8.5 mM glucose, 5 mM pyruvate and amino acids (1 mM of each) under constant bubbling of gas (O_2/CO_2, 95:5, v/v) at 37°C. In a series of experiments glucose, pyruvate and amino acids were omitted from the incubation medium. Incubations were terminated by the addition of two vols. of cold 5% TCA.

2.4. Metabolite measurements

Ascorbic acid (reduced form) contents of plasma, liver tissue homogenate and hepatocyte suspension were determined by the dipyridyl reaction according to Omaya et al., which relies on the reduction of ferric iron by ascorbic acid followed by formation of a complex of the ferrous iron product and α,α′-dipyridyl [12]. Blood glucose and the glucose production of hepatocytes were determined by the method of Bergmeyer and Bernt [13].

2.5. Miscellaneous

DNA content of isolated hepatocytes was measured by the method of Burton [14]. Data are expressed as mean ± S.E.M.; statistical analysis was performed using Student's *t*-test.

3. Results

3.1. Ascorbic acid synthesis in isolated mouse hepatocytes: effect of agents stimulating glucogenolysis

The role of the hepatic glycogen content in ascorbic acid synthesis was investigated in isolated mouse hepatocytes. The cells were prepared from fed or 48 h-starved mice and the ascorbic acid content was measured in the suspension (cells+medium). After 48 h starvation hepatocytes did not contain measurable amounts of glycogen. The initial concentration of ascorbic acid was lower in the suspension of glycogen-depleted hepatocytes compared to the fed controls (Fig. 1) and only a moderate synthesis could be observed under both nutritional conditions. The effects of dibutyryl cAMP and glucagon on ascorbate synthesis were examined. Glucagon or dibutyryl

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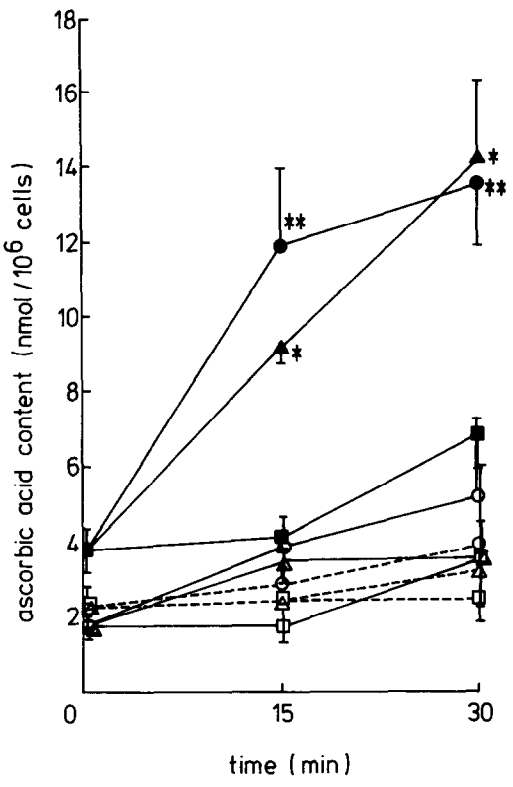

Fig. 1. Effect of glucagon and dibutyryl cyclic AMP on the ascorbic acid production of isolated mouse hepatocytes. Hepatocytes (2×10^6 cells/ml) prepared from fed (filled symbols) or 48 h-starved (open symbols) animals were incubated in the presence (continuous lines) or in the absence (dashed lines) of glucose, pyruvate and amino acids. The ascorbic acid content of cell suspensions was measured. (■, □) Control, (●, ○) 1 mM dibutyryl cyclic AMP, (▲, △) 100 nM glucagon. Data are means ± S.E.M., $n = 4$–9.

cyclic AMP caused a stimulation of ascorbic acid synthesis in hepatocytes from fed mice, while in hepatocytes from 48 h-starved animals ascorbic acid production was not increased significantly by the two agents (Fig. 1). The addition of glucose and gluconeogenic precursors to the incubation medium did not result in a significant increase in ascorbic acid production (Fig. 1).

In another series of experiments glucose and ascorbic acid production of the cells was measured simultaneously. The rate of glucose production (in the absence of gluconeogenic precursors mainly via glucogenolysis) and ascorbic acid synthesis showed a close correlation ($r = 0.9091$) (Fig. 2).

As ascorbic acid synthesis and glycogenolysis seemed to be connected, we examined the effect on ascorbic acid synthesis of various agents known to increase glycogenolysis. The α1 agonist phenylephrine, the protein phosphatase inhibitor okadaic acid and vasopressin all increased the rate of ascorbic acid production in isolated hepatocytes prepared from fed mice similarly to glucagon (Table 1).

3.2. Effect of glucagon on ascorbic acid synthesis in vivo

Glycogenolysis was stimulated by the in vivo addition of glucagon. Glucagon elevated the blood glucose level of mice by 50%; at the same time a more than fifteenfold increase of plasma ascorbic acid concentration could be observed (Table 2). The concentration of ascorbic acid in the liver was also increased, indicating a stimulated hepatic synthesis (Table 2).

4. Discussion

Glycogen content is considered to be a sensitive marker showing the actual metabolic state of the liver. Observations described in this paper suggest that ascorbic acid synthesis in murine liver is tightly connected with the glycogen pool; the source of ascorbic acid is glycogen. The following results gained in isolated hepatocytes support this assumption: first, in hepatocytes isolated from glycogen-depleted animals the ascorbic acid level as well as the rate of synthesis is lower than that in hepatocytes from control fed mice (Fig. 1); second, different glycogen-mobilizing agents acting via different mechanisms enhance ascorbic acid production in hepatocytes from fed but not from fasted animals (Fig. 1, Table 1); third, addition of glucose to hepatocytes prepared from glycogen-depleted mice failed to increase the formation of ascorbic acid (Fig. 1). The results gained under in vitro conditions in isolated hepatocytes were confirmed by in vivo experiments: a single i.p. injection of glucagon elevated both the plasma and liver ascorbic acid levels within 15 min (Table 2).

Hepatic regulation of glycogen metabolism affects not only the metabolism of glucose, but the availability of several glucose derivatives as well. Previously it has been shown that different conditions in glycogen metabolism, but not that of gluconeogenesis, alter the rate of glucuronidation, a UDP-glucuronic acid (thus finally glucose) consuming process. It has been suggested that in hepatocytes there are two separate glucose-6-phosphate pools: one is connected with glycogenolysis and another related to gluconeogenesis and glucose uptake [15,16]. Our data indicate that (similarly to glucuronidation) ascorbic acid production is ascribable to the former pool, and in mouse liver it is controlled through the regulation of glycogenolysis. It is noted that besides glycogenolysis a second regulated step of ascorbate synthesis cannot be excluded [17].

The finding that the source of ascorbate production is glycogenolysis is in according with the fact that liver and kidney –the main sites of glycogen storage – are responsible for the ascorbic acid supply in most animal species [2]. The increased hepatic ascorbic acid production after glucagon administration can be explained as a compensatory mechanism of the missing intake of ascorbate, i.e. adaptation of ascorbic acid supply from exter-

Table 1
Ascorbic acid production of isolated mouse hepatocytes after the treatment with different glycogen-mobilizing agents

Treatment	Ascorbic acid production (pmol/min per 10^6 cells)
Control	140 ± 45
1 mM dibutyryl cAMP	562 ± 96***
0.1 μM glucagon	496 ± 39**
1 μM okadaic acid	889 ± 157***
200 nM vasopressin	543 ± 46***
100 nM phenylephrine	580 ± 63***

Hepatocytes (2×10^6 cells/ml) prepared from fed animals were incubated in the presence of different glycogen-mobilizing agents. The ascorbic acid content of the hepatocyte suspension was measured after 0 and 15 min of incubation. Values are given as means ± S.E.M. of 4 experiments. Significant differences from the control value are indicated: $^{**}P < 0.02$, $^{***}P < 0.01$.

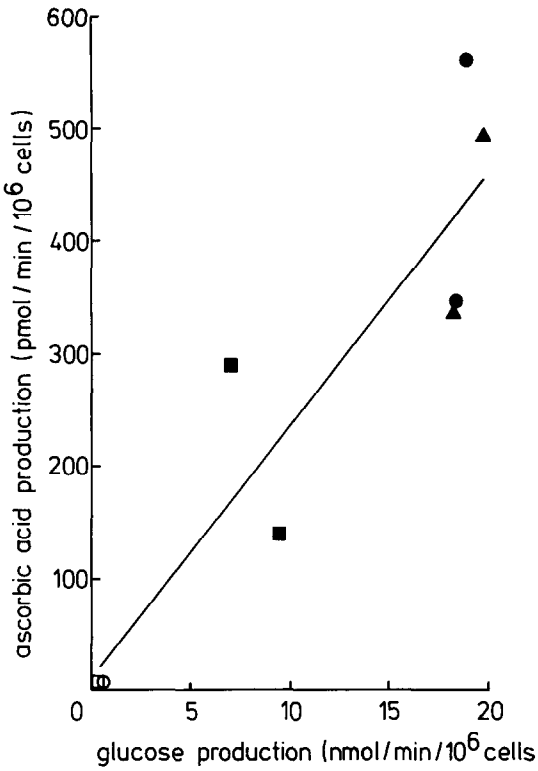

Fig. 2. The relationship between glucose and ascorbic acid production of isolated mouse hepatocytes. Cells (2×10^6 cells/ml) prepared from fed (filled symbols) or 48 h-starved (open symbols) animals were incubated in the absence of glucose, pyruvate and amino acids. Glucose and ascorbic acid production of the cells were measured. Data are partially taken from Fig. 1 and Table 1. (■, □) Control, (●, ○) 1 mM dibutyryl cyclic AMP, (▲) 100 nM glucagon. Data are means ± S.E.M., $n = 4$–9.

nal to internal sources. Considering the fifteenfold elevation of plasma ascorbate levels, in the light of recent findings concerning the effect of ascorbate on insulin secretion [18] and on the calcium channels in pancreatic beta cells [19] it might be also regarded as a possible intercellular messenger.

Liver plays a central role in the antioxidant defence of the organism, as well. The hepatic glutathione synthesis is essential for maintenance of the inter-organ cycle of this compound. Our results suggest that a similar situation exists in the case of ascorbate; the ascorbic acid claim of different cells (adrenal medulla [3], neutrophil granulocyte [20,21] etc.) is satisfied by the hepatic synthesis. The observation that insulin-induced hypoglycemia in vivo causes a rapid depletion of ascorbate in the adrenal medulla of rats [3] supports this assumption. The elevated hepatic ascorbic acid formation upon addition of the ancient hunger signal cAMP (Fig. 1) or in glutathione deficiency induced by the administering buthionine sulfoximine [22] or by starvation [23] can be a compensatory mechanism. Considering the effect of ascorbate on the glutathione cycle in the liver, the protective effect of ascorbate [24] indicates that it can act as an essential antioxidant in glutathione deficiency.

Table 2
Effect of glucagon on plasma and hepatic ascorbic acid concentrations in mice

	Control	100 μg/kg glucagon i.p.
Blood glucose concentration (mM)	6.67 ± 0.11	10.14 ± 0.23***
Plasma ascorbic acid concentration (μM)	3.45 ± 1.21	53.39 ± 6.25***
Ascorbic acid content of the liver (μmol/g)	0.58 ± 0.15	3.83 ± 0.16***

100 μg/kg glucagon was administered to mice intraperitoneally in a final volume of 1 ml isotonic saline. The control group received 1 ml of isotonic saline. The animals were sacrificed 15 min after the injection. Blood samples and liver tissue preparation were executed as detailed in section 2. The ascorbic acid content of plasma, liver tissue homogenate and blood glucose concentration were determined. Data are means ± S.E.M. of 4 experiments. ***Statistically significant difference: $P < 0.01$.

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References

[1] Szent-Györgyi, A. (1928) Biochem. J. 22, 1387–1409.
[2] Rose, R.C. and Bode, A.M. (1993) FASEB J. 7, 1135–1142.
[3] Diliberto Jr., E.J., Daniels, A.J. and Viveros, O.H. (1991) Am. J. Clin. Nutr. 54, 1163S–1172S.
[4] Bánhegyi, G., Garzó, T., Antoni, F. and Mandl, J. (1988) Biochim. Biophys. Acta 967, 429–435.
[5] Bánhegyi, G., Puskás, R., Garzó, T., Antoni, F. and Mandl, J. (1992) Biochem. Pharmacol. 42, 1299–1302.
[6] Shipley, L.A., Eacho, P.I., Sweeny, D.J. and Weiner, M. (1986) Drug Metab. Dispos. 14, 526–531.
[7] Bánhegyi, G., Garzó, T., Mészáros, Gy., Faragó, A., Antoni, F. and Mandl, J. (1988) Biochem. Pharmacol. 37, 849–854.
[8] Beuers, U., Pogonka, T., Esterline, R., Ji, S. and Jungermann, K. (1986) Toxicol. Lett. 34, 849–854.
[9] Bánhegyi, G., Bellomo, G., Fulceri, R., Mandl, J. and Benedetti, A. (1993) Biochem. J. 292, 99–104.
[10] Zannoni, V., Lynch, M., Goldstein, S. and Sato, P. (1974) Biochem. Med. 11, 41–48.
[11] Mandl, J., Garzó, T., Mészáros, K. and Antoni, F. (1979) Biochim. Biophys. Acta 586, 560–567.
[12] Omaye, S.T., Turnbull, J.D. and Sauberlich, H.E. (1979) Methods Enzymol. 62, 3–7.
[13] Bergmeyer, H.U. and Bernt, E. (1963) in: Methods in Enzymic Analysis (Bergmeyer, H.U., ed.) pp. 123–130, Academic Press, New York.
[14] Burton, K. (1956) Biochem. J. 62, 315–323.
[15] Christ, B. and Jungermann, K. (1987) FEBS Lett. 221, 375–380.
[16] Kalant, N., Parniak, M. and Lemieux, M. (1987) Biochem. J. 248, 927–931.
[17] Horio, F., Shibata, T., Naito, Y., Nishikimi, M., Yagi, K. and Yoshida, A. (1993) J. Nutr. Sci. Vitaminol. 39, 1–9.
[18] Bergsten, P., Moura, A.S., Atwater, I. and Levine, M. (1994) J. Biol. Chem. 269, 1041–1045.
[19] Parsey, R.V. and Matteson, D.R. (1993) J. Gen. Physiol. 102, 503–523.
[20] Washko, P., Rotrosen, D. and Levine, M. (1989) J. Biol. Chem. 264, 18996–19002.
[21] Washko, P.W., Wang, Y. and Levine, M. (1993) J. Biol. Chem. 268, 15531–15535.
[22] Mårtensson, J. and Meister, A. (1992) Proc. Natl. Acad. Sci. USA 89, 11566–11568.
[23] Kalapos, M.P., Garzó, T., Antoni, F. and Mandl, J. (1991) Biochim. Biophys. Acta 1092, 284–290.
[24] Mårtensson, J. and Meister, A. (1991) Proc. Natl. Acad. Sci. USA 88, 4656–4660.