Ascorbate synthesis-dependent glutathione consumption in mouse liver

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Abstract Ascorbate synthesis causes glutathione consumption in the liver. Addition of gulonolactone resulted in an increase of ascorbate production in isolated murine hepatocytes. At the same time, a decrease in reduced glutathione (GSH) level was observed. In hepatic microsomal membranes, ascorbate synthesis stimulated by gulonolactone caused an almost equimolar consumption of GSH. This effect could be counteracted by the addition of catalase or mercaptosuccinate, indicating the role of hydrogen peroxide formed during ascorbate synthesis in the depletion of GSH. The observed phenomenon may be one of the reasons why the evolutionary loss of ascorbate synthesis could be advantageous.

Key words: Ascorbic acid; Glutathione; Hydrogen peroxide; Gulonolactone oxidase; Evolution; Mouse liver

1. Introduction

Ascorbic acid and glutathione are involved in the antioxidant defense of the cell. Their connections and interactions have been described from several aspects: they can substitute each other [1], dehydroascorbate can be reduced at the expense of GSH [2] and glutathione depletion results in the stimulation of ascorbate synthesis [3]. In ascorbate-synthesising animals, the formation of ascorbate from gulonolactone catalysed by microsomal gulonolactone oxidase is accompanied by the stoichiometric consumption of O_2 and production of the oxidant hydrogen peroxide [4]. Metabolism of hydrogen peroxide by glutathione peroxidase requires reduced glutathione. Therefore, we supposed that synthesis of ascorbate should decrease the intracellular glutathione level. To prove our hypothesis, experiments were undertaken to investigate the effect of ascorbate synthesis stimulated by the addition of gulonolactone on the oxidation of GSH in isolated mouse hepatocytes and liver microsomal membranes.

2. Experimental

2.1. Materials

Collagenase (type IV), α,α′-dipyridyl, Ellman's reagent, glyoxylic acid, 3-amino-1,2,4-triazole (aminotriazole), mercaptosuccinic acid, *o*-dianisidine and catalase (EC 1.11.1.6) from bovine liver were bought from Sigma (St Louis, MO). Horseradish peroxidase (500 U/mg) was purchased from Reanal (Hungary). All other chemicals were of analytical grade.

2.2. Preparation and incubation of isolated mouse hepatocytes

Isolated murine hepatocytes were prepared from male CFLP mice (30–35 g body weight; LATI, Gödöllõ, Hungary) by the collagenase perfusion method as detailed earlier [5]. Viability of the cells checked by the Trypan blue exclusion test was more than 90%. Cells (2×10^6/ml) were incubated in Krebs–Henseleit bicarbonate buffer 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. Incubations were terminated by the addition of TCA (5% final concentration).

2.3. Microsomal experiments

Hepatic microsomal membranes were prepared and stored according to [6]. Microsomes (usually 1 mg protein/ml) were incubated in 50 mM potassium phosphate buffer pH 7.4 containing 5 mM $MgCl_2$ at 37°C for 30 min. Incubations were terminated by the addition of TCA (5% final concentration).

2.4. Metabolite measurements

Ascorbate and GSH were measured in the trichloroacetic acid soluble supernatant of the cells or microsomes by the method of Omaye [7,8] and Ball [9], respectively. Microsomal oxygen consumption was detected polarographically at 37°C using a Clarke-type oxygen electrode. Microsomal hydrogen peroxide production was measured enzimatically according to [10] in the incubation medium mentioned above supplemented with 40 μg/ml horseradish peroxidase, 66 μg/ml *o*-dianisidine and 0.2 mM sodium azide.

2.5. Miscellaneous

DNA content of isolated hepatocytes was measured by the method of Burton [11]. Protein contents were measured by the biuret method. Statistical analysis was performed using Student's *t*-test.

3. Results

The effect of ascorbate synthesis on reduced glutathione level was studied in mouse liver microsomal membranes. The activity of gulonolactone oxidase, an integral enzyme of hepatic endoplasmic reticulum, was stimulated by the addition of 1 mM gulonolactone. The presence of gulonolactone, in agreement with previous observations [4], resulted in an increased oxygen consumption in microsomes (data not shown). Measurement of hydrogen peroxide in the presence of the catalase inhibitor azide showed a gulonolactone-dependent hydrogen peroxide formation; its rate (2.09 ± 0.13 nmol/min/mg protein, $n=3$) was similar to the rate of ascorbate synthesis (Table 1). In the presence of 1 mM glutathione, ascorbate production was accompanied by an almost equimolar GSH consumption (Table 1). In the absence of microsomes, gulonolactone did not decrease the GSH level of incubates (data not shown). The decrease in GSH was dependent on the initial GSH concentration (Fig. 1a). GSH consumption was linear in time and was dependent on the protein content of incubates (data not shown). The time courses of GSH consumption and ascorbate synthesis showed parallel running down, the decrease of GSH concentration coincided ($r=0.9957$) with the increase of ascorbate (Fig. 1b). The addition of catalase did not affect the rate of ascorbate synthesis but prevented the oxidation of GSH in microsomal membranes (Table 1), indicating a causal role of hydrogen peroxide generated during ascorbate synthesis. The rate of ascorbate synthesis was not dependent on the presence or absence of GSH and/or catalase (Table 1).

Supposing the role of glutathione peroxidase in the ascorbate synthesis-dependent glutathione consumption, we mea-

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Table 1
Effect of microsomal ascorbate synthesis on consumption of reduced glutathione

Addition	Ascorbate synthesis	GSH consumption
	(nmol/min/mg protein)	
1 mM gulonolactone	3.12 ± 0.44	–
1 mM gulonolactone + catalase	3.20 ± 0.21	–
1 mM gulonolactone + 1 mM GSH	3.16 ± 0.33	2.81 ± 0.16
1 mM gulonolactone + 1 mM GSH+catalase	3.31 ± 0.12	0.36 ± 0.66*

Mouse liver microsomes were incubated for 30 min as described in section 2. Ascorbate synthesis was induced by the addition of 1 mM gulonolactone. When indicated, 1 mM GSH and/or 1 mg/ml catalase (2800 U/mg; Sigma) was also added. Ascorbic acid and reduced glutathione content of the microsomal suspensions were measured after 0 and 30 min of incubation. Mean ± S.D. ($n = 4$). Significant difference from the corresponding value is indicated: $^*P < 0.01$.

Table 2
Effect of glutathione peroxidase inhibitor mercaptosuccinate on gulonolactone dependent microsomal glutathione consumption

Addition	GSH consumption (nmol/min/mg protein)
None	–
10 μM mercaptosuccinate	–
1 mM gulonolactone	1.95 ± 0.15
1 mM gulonolactone + 10 μM mercaptosuccinate	1.38 ± 0.02*

Mouse liver microsomes were incubated for 30 min in the presence of 0.2 mM GSH as described in section 2. Ascorbate synthesis was induced by addition of 1 mM gulonolactone. Reduced glutathione content of the microsomal suspensions were measured after 30 min of incubation. Mean ± S.D. ($n = 4$). Significant difference from the corresponding value is indicated: $^*P < 0.01$.

Table 3
Effect of gulonolactone on ascorbate synthesis and reduced glutathione content of isolated mouse hepatocytes

Addition	Ascorbate synthesis (nmol/min/10^6 cells)	GSH content (nmol/10^6 cells)
None	0.28 ± 0.23	29.7 ± 2.7
10 mM gulonolactone	4.03 ± 1.62*	24.6 ± 2.2*
20 mM aminotriazole	0.40 ± 0.20	27.2 ± 1.8
10 mM gulonolactone + 20 mM aminotriazole	4.26 ± 1.77**	19.4 ± 2.3***

Hepatocytes (2×10^6 cells/ml) were incubated for 30 min in the presence of gulonolactone and/or the catalase inhibitor aminotriazole. The ascorbic acid and reduced glutathione content of the hepatocyte suspension were measured after 0 and 30 min of incubation. Means ± S.D. ($n = 8$). Significant differences: $P < 0.01$ *vs. control, **vs. aminotriazole, ***vs. gulonolactone.

sured the microsomal GSH consumption in the presence of glutathione peroxidase inhibitor mercaptosuccinate [12]. Mercaptosuccinate reduced the glutathione consumption caused by gulonolactone (Table 2).

The effect of enhanced ascorbate synthesis was also investigated in isolated mouse hepatocytes. Ascorbate production was stimulated by the addition of 10 mM gulonolactone. The more than 10-fold increase in ascorbate synthesis was accompanied by a decrease of reduced glutathione content of the cells (Table 3). The catalase inhibitor aminotriazole [13] alone did not alter the cellular GSH level and ascorbate synthesis but deepened the GSH depletion caused by gulonolactone addition (Table 3).

4. Discussion

In this paper, a new connection between ascorbate and GSH metabolism is described. Our data show that the synthesis of ascorbate leads to consumption of GSH, the other main intracellular antioxidant (Fig. 1). We suppose that the formation of hydrogen peroxide is underlying the increased GSH consumption. First, oxidation of GSH caused by increased ascorbate synthesis was prevented by the addition of catalase in microsomal membranes (Table 1). Second, inhibition of glutathione peroxidase by mercaptosuccinate moderated the gulonolactone-dependent glutathione consumption in microsomes (Table 2). Third, the inhibition of catalase by aminotriazole deepened the ascorbate synthesis-dependent GSH depletion in isolated hepatocytes (Table 3). This interaction may be one of the causes why primates and some other species have lost their ascorbate-synthesising ability. This event occurred in the ancestors of primates about 70 million years ago, owing to mutation(s) in the gulonolactone oxidase gene [14]. Despite the well-known benefits [15] of ascorbate, the mutation(s) had to be advantageous, as this metabolic error did not remain an enzymopathy affecting only a minority of the population, but spread widely amongst the species (and individuals) of primates and became exclusive [16]. There is no explanation for this unexpected outcome. Based on these analytical data, the following conceptual evolutionary hypothesis can be outlined: in the tropical jungle of the Cretaceous Period, when exogenous ascorbate was abundant [17,18], the loss of gulonolactone oxidase activity could have proved to be advantageous. It saved the reduced GSH, the main defence system against oxidants, while the access to ascorbate was not hindered. Later, the evolutionary gains of these periods allowed the conservation of the genetic disorder manifested in the loss of ascorbate synthesis.

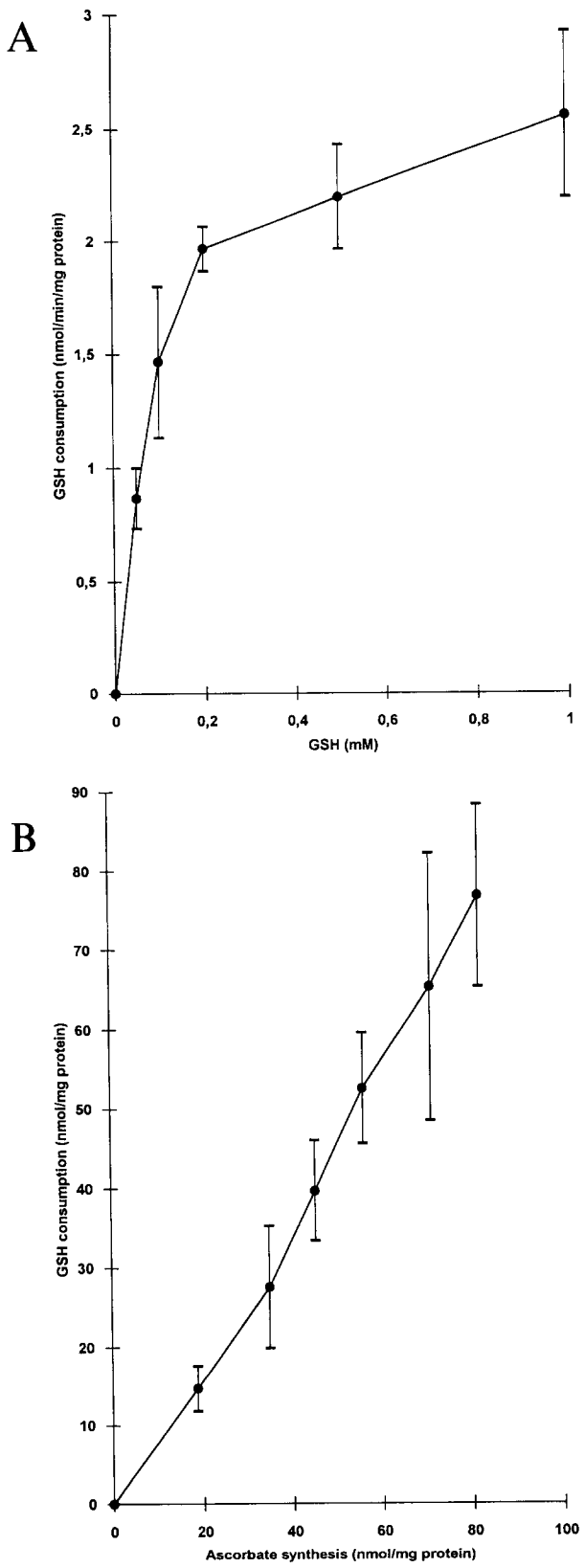

Fig. 1. Glutathione consumption caused by ascorbate synthesis in liver microsomal membranes. Microsomes (1 mg/ml protein) were incubated in the presence of 1 mM gulonolactone and various concentrations of GSH for 30 min (a) or in the presence of 1 mM gulonolactone and GSH for various times (b). Ascorbic acid and reduced glutathione content of the microsomal suspensions were measured. Mean ± S.D. ($n = 6$).

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