Glutathione depletion induces glycogenolysis dependent ascorbate synthesis in isolated murine hepatocytes

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Abstract The relationship between glutathione deficiency, glycogen metabolism and ascorbate synthesis was investigated in isolated murine hepatocytes. Glutathione deficiency caused by various agents increased ascorbate synthesis with a stimulation of glycogen breakdown. Increased ascorbate synthesis from UDP-glucose or gulonolactone could not be further affected by glutathione depletion. Fructose prevented the stimulated glycogenolysis and ascorbate synthesis caused by glutathione consumption. Reduction of oxidised glutathione by dithiothreitol decreased the elevated glycogenolysis and ascorbate synthesis in diamide or menadione treated hepatocytes. Our results suggest that a change in GSH/GSSG ratio seems to be a sufficient precondition of altering glycogenolysis and a consequent ascorbate synthesis.

Key words: Glutathione; Glycogenolysis; Ascorbate; Mouse hepatocyte

1. Introduction

The liver plays a central role in the antioxidant defence producing the most important water soluble antioxidants, GSH and – in the overwhelming majority of organisms – ascorbate for export. GSH and ascorbate are connected through a redox coupling. They can substitute and save each other in different species [1,2]. Recently it has been described that besides this redox connection there is a biosynthetic link: GSH deficiency produced in mice by in vivo administration of buthionine sulfoximine increases hepatic ascorbate synthesis [3].

In animals with the exception of primates, guinea pig and some other species ascorbate is produced in the uronic acid pathway in the liver or kidney from UDP-glucose through UDP-glucuronic acid. Three microsomal enzymatic steps are involved in this synthesis from UDP-glucuronic acid; glucuronic acid, glucuronolactone and gulonolactone are intermediates in order [4]. The role of liver glycogen in substrate supply of a UDP-glucuronic acid consuming process such as glucuronidation has been shown previously [5]. Recently the dependence of ascorbate synthesis on glycogenolysis has been reported [6]. However, glycogenolysis is also induced by agents, which provoke GSH deficiency [7–9].

GSH depletion and the consequent alteration of the GSH/GSSG ratio can decrease the activity of enzymes involved in glycogen metabolism such as glycogen synthase [10–12] and glycogen phosphorylase phosphatase [13]. Therefore, a direct relationship of GSH depletion, glycogenolysis and ascorbate synthesis was supposed in the liver. Experiments were undertaken to investigate the role of GSH depletion in glycogenolysis and ascorbate synthesis. GSH deficiency was provoked by various agents: the thiol oxidant, diamide [14], the strong NADPH consumer compound, menadione [15], the specific inhibitor of γ-glutamylcysteine synthetase, buthionine sulfoximine [16], the GSH conjugative acetaminophen [17] and dibutyryl cyclic AMP [18]. It was demonstrated that various agents which depleted GSH in hepatocytes, cause an increased ascorbate production via increased glycogenolysis.

2. Materials and methods

2.1. Materials

Collagenase (type IV), α,α′-dipyridyl, dithiothreitol, Ellman's reagent, glyoxylic acid, acetaminophen, L-buthionine-(*S,R*)-sulfoximine, diamide, dibutyryl cyclic AMP, menadione, saponin, UDP-glucose and gulonolactone were purchased from Sigma (St. Louis, MO, USA). Cyclic AMP assay kit was bought from Amersham International plc (Amersham, UK). All other materials were of analytical grade.

2.2. Preparation and incubation of isolated mouse hepatocytes

CFLP male mice (25–35 g body weight, LATI, Gödöllő, Hungary) fed ad libitum were used. Isolated hepatocytes were prepared by the collagenase perfusion method as described earlier [19]. Hepatocytes ($3–5 \times 10^6$ cells/ml) were incubated in Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 2.5 mM $CaCl_2$, 1% albumin, 2.5 mM pyruvate and amino acids (1 mM each) and were stirred by constant bubbling with gas (O_2:CO_2, 95:5 v/v%) at 37°C. Glucose was not added to the medium for determination of glucose production which in this case indicates the rate of glycogen breakdown. Viability of the cells checked by the trypan blue exclusion test was about 90–95%. In a series of experiments hepatocytes were permeabilised by saponin as described earlier [20].

2.3. Measurement of metabolites

Incubations were terminated by the addition of 0.05 volume of 100% trichloroacetic acid for the determination of ascorbate. Ascorbate content was measured by the method of Omaye et al. [21], based on the reduction of Fe^{3+} with the oxidation of ascorbate and the subsequent determination of the Fe^{2+}–α,α′-dipyridyl complex. The samples for the measurement of glycogen were precipitated by adding 10 volumes of 1 N HCl [22]. Glycogen analysis was carried out by hydrolysis [22] and subsequent glucose measurement. Glucose was determined by the *o*-toluidine method [23] to avoid in vitro interference of acetaminophen or dithiothreitol and the glucose oxidase or hexokinase test systems. The GSH content of the suspension was determined according to Ball [24]. Dithiothreitol can react with Ellman's reagent, therefore GSH content was measured by the GSH transferase assay when dithiothreitol was present in the incubations [25]. Intracellular cAMP concentration was measured by radioimmunoassay [26]. Cell numbers were calculated on the basis of DNA content of hepatocytes determined according to [27].

2.4. Statistics

All data were expressed as means ± S.E.M. Statistical analysis was carried out using Student's *t*-test.

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Abbreviations: GSH, glutathione (reduced form); GSSG, glutathione (oxidised form)

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Table 1
Effect of various GSH depleting agents on ascorbate production, intracellular cyclic AMP levels and glycogen breakdown of isolated murine hepatocytes

Treatment	GSH consumption (pmol/min/10^6 cells)	cAMP content (pmol/10^6 cells)	Glycogen breakdown (nmol glucose unit/min/10^6cells)	Ascorbate production (pmol/min/10^6cells)
none	102 ± 5	410	1.57 ± 0.33	55 ± 18
1 mM dibutyryl cyclic AMP	265 ± 21***	N.D.	8.12 ± 0.64***	282 ± 24***
0.1 mM diamide	499 ± 24***	476	7.41 ± 0.82***	271 ± 8***
0.1 mM menadione	502 ± 14***	217	5.87 ± 0.53***	292 ± 3***
5 mM buthionine sulfoximine	585 ± 81***	105	7.55 ± 0.45***	269 ± 15***
5 mM acetaminophen	365 ± 8***	43	10.09 ± 0.56***	257 ± 18***

GSH, glycogen and ascorbate contents were determined in suspensions of isolated murine hepatocytes before and 30 min after administration of various GSH depleting agents. (Initial values were as follows: GSH 28.4 ± 0.8 nmol/10^6 cells, glycogen 279.5 ± 18.8 nmol glucose unit/10^6 cells, ascorbate 3.9 ± 0.8 nmol/10^6 cells; means ± S.E.M. of 27 measurements.) Intracellular cAMP content was measured at 30 min. Values are given as means of 3–15 experiments (cAMP measurement n=2). Statistically significant differences among values gained in treated cells versus corresponding untreated controls: ***$P < 0.01$. N.D., not determined.

3. Results

Different GSH depleting agents were administered to suspensions of isolated murine hepatocytes. These compounds caused a 25–60% decrease in GSH level during 30 min incubations. The GSH consumption was the highest in menadione, diamide or buthionine sulfoximine treated hepatocytes (Table 1). At the same time an about fivefold stimulation of ascorbate synthesis could be observed. Although 1 mM dibutyryl cyclic AMP decreased the cellular GSH less, it also caused a high ascorbate production in hepatocytes (Table 1).

The effects of the various GSH depleting agents on intracellular cAMP levels were also investigated. While diamide moderately increased the cAMP concentration in hepatocytes, the other GSH consuming compounds decreased intracellular cAMP levels (Table 1).

Table 1 shows that all the GSH depleting agents administered enhanced glycogenolysis markedly. Acetaminophen had the strongest glycogen consuming effect, stronger than that of 1 mM dibutyryl cyclic AMP.

To investigate the causal connection between GSH depletion induced glycogenolysis and ascorbate synthesis two different experimental approaches were used. First, fructose was administered to inhibit glycogen breakdown [28] and activate glycogenesis [29]. Addition of 4 mM fructose did not alter the GSH consumption caused by different agents but as expected it prevented their glycogenolytic effect and rather glycogen formation occurred (Table 2). Fructose prevented the stimulation in ascorbate production (Table 2).

Second precursors of the ascorbate synthesis originated directly or indirectly from glycogenolysis were substituted with the addition of UDP-glucose or gulonolactone. Addition of UDP-glucose to permeabilised cells or gulonolactone to intact cells stimulated the synthesis of ascorbate [30] independently of the addition of various GSH depleting agents (Table 3). Under these experimental conditions GSH depletion was detected upon addition of GSH depleting agents (data not shown).

Among the various GSH depleting agents diamide and menadione have strong oxidative effects causing a decrease of the GSH/GSSG ratio. GSSG oxidised enzymes can be reactivated with dithiothreitol [10–12]. Therefore, the effect of dithiothreitol on glycogen metabolism and ascorbate production was investigated in diamide or menadione treated isolated mouse hepatocytes. 2 mM dithiothreitol was added to the suspensions following 30 min incubations with 0.1 mM diamide or 0.1 mM menadione and the incubations were continued further for 20 min. As shown in Table 4 after addition of dithiothreitol the stimulated glycogenolysis and ascorbate production declined both in diamide and in menadione treated hepatocytes.

4. Discussion

This paper demonstrates that GSH deficiencies of various origin result in an increased glycogen breakdown and stimulated ascorbate synthesis (Table 1). The metabolic connection between GSH deficiency and ascorbate synthesis has been

Table 2
Prevention of the stimulation of ascorbate production by administration of fructose in glutathione depleted isolated mouse hepatocytes

Treatment	GSH consumption (pmol/min/10^6cells)	Glycogen breakdown (nmol glucose unit/min/10^6 cells)	Ascorbate production (pmol/min/10^6 cells)
none	98 ± 54	1.57 ± 0.33	55 ± 18
4 mM fructose	128 ± 64	−3.20 ± 1.17***	30 ± 10
4 mM fructose + 1 mM dibutyryl cyclic AMP	305 ± 36**	−2.96 ± 0.39***	64 ± 12
4 mM fructose + 0.1mM diamide	553 ± 36***	−1.72 ± 0.55**	62 ± 24
4 mM fructose + 0.1mM menadione	568 ± 62***	−3.29 ± 0.24***	45 ± 18
4 mM fructose + 5 mM buthionine sulfoximine	596 ± 22***	−3.98 ± 0.82***	76 ± 34
4 mM fructose +5 mM acetaminophen	308 ± 25**	−3.57 ± 0.63***	58 ± 29

Isolated hepatocytes were incubated in the presence of 4 mM fructose and various GSH depleting compounds. For experimental details and initial values see the legend of Table 1. Data are given as means ± S.E.M. of 4 experiments. Statistically significant differences between treated cells versus untreated controls: **$P < 0.02$, ***$P < 0.01$.

Table 3
Ascorbate synthesis from UDP-glucose or gulonolactone in the presence of different glutathione depleting agents

Treatment	Ascorbate production (pmol/min/10^6 cells)	
	permeabilised cells, 5 mM UDP-glucose	intact cells, 5 mM gulonolactone
none	266 ± 20	1844 ± 465
1 mM dibutyryl cyclic AMP	253 ± 33	2107 ± 949
0.1 mM diamide	306 ± 23	2175 ± 929
0.1 mM menadione	266 ± 9	2246 ± 312
5 mM buthionine sulfoximine	261 ± 28	1853 ± 57
5 mM acetaminophen	297 ± 16	1908 ± 810

Isolated mouse hepatocytes were prepared, thereafter half of the cells were permeabilised by 0.005% of saponin for 5 min. After the permeabilisation 5 mM UDP-glucose was added to the cells. In the absence of UDP-glucose ascorbate production of permeabilised hepatocytes was 12 ± 3 pmol/min/10^6cells. In another series of experiments gulonolactone was added to the remaining part of the hepatocyte suspension. For the control values of ascorbate production in intact cells see Table 1. The cells were incubated further in the presence or absence of different GSH depleting compounds. Ascorbate production was determined. Data are given as means ± S.E.M. of 4 experiments.

reported first based on in vivo experiments in livers of GSH deficient mice [3]. Our in vitro data support the assumption that in GSH deficiency there is de novo ascorbate synthesis (Table 1). Previous findings according to which the enhanced glycogenolysis has been responsible for the stimulation of ascorbate production [6] can be further supported by the following observations: (i) inhibition of glycogenolysis by fructose does not modify the GSH depleting effect of agents applied, but prevents their stimulation on ascorbate synthesis (Table 2), (ii) ascorbate production from UDP-glucose or gulonolactone cannot be further increased by GSH depletion (Table 3). It is concluded that the regulated pathway in this sequence of events is glycogenolysis, as the mechanism of GSH depletion does not affect the subsequent process.

Hepatic glycogenolysis is regulated mainly through cAMP and Ca^{2+} dependent mechanisms. Among the GSH depleting agents diamide and menadione have been reported to accumulate cAMP in the cells [31,32]. On the other hand diamide is an inhibitor of cyclic AMP dependent protein kinase A [33]. Under our experimental conditions menadione, like buthionine sulfoximine and acetaminophen, decreases cAMP levels of isolated hepatocytes (Table 1). Thus, it is concluded that cAMP mediation itself cannot be responsible for stimulated glycogenolysis in GSH deficiencies. Another possibility to increase hepatic glycogenolysis is the elevation of cytosolic free Ca^{2+}, a frequent consequence of GSH depletion. The ability of menadione to increase cytoplasmic Ca^{2+} concentration is well known [34,35], like that of acetaminophen. Disruption of Ca^{2+} pumping by acetaminophen leads to higher cytosolic Ca^{2+} levels with the activation of glycogen phosphorylase [36]. However, there is no exact and direct evidence concerning the effect of diamide or buthionine sulfoximine on intracellular Ca^{2+} mobilisation and under our experimental circumstances menadione increased glycogenolysis even in Ca^{2+} depleted hepatocytes (data not shown). It should be noted, however, that oxidation of GSH increases the sensitivity of IP_3 receptors [37]. Thus, a primary role of GSH was supposed in the regulation of glycogenolysis under various GSH deficient conditions.

GSH depletion and/or change of GSH/GSSG ratio can influence glycogen metabolism directly. The increased glycogen breakdown and ascorbate synthesis is diminished after addition of dithiothreitol both in diamide and in menadione treated hepatocytes (Table 4). Mixed disulfide formation between glycogen synthase and GSSG leads to inactivation of the enzyme and causes its dissociation from the glycogen particle [10–12], moreover, stimulation of glycogen phosphorylase via inhibition of phosphorylase phosphatase by GSSG has also been reported [13]. Therefore, in shortage of GSH glycogen metabolism can be shifted to the direction of glycogen breakdown and consequently there is an oversupply of UDP-glucose. Reduction of GSSG with sulfhydryl reagents such as dithiothreitol promotes restoration and reactivation of the GSSG-oxidised glycogen synthase [38–40], therefore it can lower UDP-glucose supply. These findings confirm the suggestion that a change of the GSH/GSSG ratio is a necessary and sufficient precondition for altering glycogenolysis and the consequent glycogenolysis-linked ascorbate synthesis. Other effects of GSH depletion (increased cytosolic [Ca^{2+}] and, in some cases, elevation of cAMP level) may also contribute to the increased glycogenolysis.

In summary, the present study shows that besides the redox connection between GSH and ascorbate another interrelationship also exists: GSH deficiency induces glycogen breakdown

Table 4
Effect of dithiothreitol on glycogen breakdown and ascorbate synthesis of isolated murine hepatocytes treated with menadione or diamide

Treatment	GSH consumption(pmol/min/10^6 cells)		Glycogen breakdown (nmol glucoseunit/min/10^6 cells)		Ascorbate production (pmol/min/10^6 cells)	
	before	after	before 2 mM dithiothreitol	after 2 mM dithiothreitol	before	after
control	91 ± 49	−57 ± 8†	1.63 ± 0.14	1.51 ± 0.07	23 ± 1	28 ± 1
0.1 mM diamide	522 ± 179*	−862 ± 60***†††	9.44 ± 0.52***	4.96 ± 0.22*†††	304 ± 8***	118 ± 5*†††
0.1 mM menadione	593 ± 73***	−730 ± 56***†††	5.29 ± 0.68*	2.25 ± 0.10†††	315 ± 2***	85 ± 4*†††

Isolated hepatocytes were incubated in the presence of 0.1 mM diamide or 0.1 mM menadione for 30 min. 2 mM dithiothreitol was added at 30 min and incubations were continued further for 20 min. GSH consumption, glycogen breakdown and ascorbate production were determined before and after administration of dithiothreitol. Data were expressed as means ± S.E.M. of 3–4 experiments. Significant difference compared to the corresponding controls: $^{*}P < 0.05$, $^{***}P < 0.01$ and between the values before or after addition of dithiothreitol: $^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.01$.

and de novo ascorbate synthesis independently of the origin of GSH depletion. The ascorbate production – stimulated by GSH deficiency – represents a pathway serving a useful balance of the main antioxidants, thus, the liver is able to maintain a continuous antioxidant defence in cases of short-term oxidative challenges.

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