Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 317 (2004) 149–156
BBRC
www.elsevier.com/locate/ybbrc

Glycogenolysis is directed towards ascorbate synthesis by glutathione conjugation

Tom S. Chan,[a] John X. Wilson,[b] and Peter J. O'Brien[a,*]

[a] *Faculty of Pharmacy, University of Toronto, 19 Russell St. Rm 522, Toronto, Ont., Canada M5S 2S2*
[b] *Department of Physiology and Pharmacology, University of Western Ontario, Medical Science Bldg. Rm 229, London, Ont., Canada N6A 5C1*

Received 3 March 2004

Abstract

Using isolated rat hepatocytes we have shown that glutathione (GSH) depletion by glutathione-*S*-transferase (GST)-catalyzed conjugation with 1-bromoheptane or phorone was accompanied by a significant elevation in ascorbate synthesis. Glycogenolysis was also stimulated without a significant rise in glucose synthesis. Furthermore, when glycogenolysis was stimulated in control hepatocytes by increasing intracellular cAMP levels (with glucagon or dibutyryl cAMP), cellular glucose levels, but not ascorbate levels, increased. These data suggest that GSH depletion can stimulate ascorbate synthesis independently of glucose synthesis and that hepatocytes can direct glycogenolysis towards ascorbate synthesis during GSH conjugation.

Keywords: Ascorbate; Vitamin C synthesis; Glutathione; Glutathione conjugation; Glycogen; Glucose; Glycogenesis; cAMP; Glucuronic acid; Glucuronate pathway

Vitamin C (ascorbate) is an important enzyme cofactor and antioxidant. Although most animals can synthesize vitamin C, several including humans, some birds, some monkeys, and the guinea pig are unable. Ascorbate synthesis has been shown to be stimulated in murine liver after the administration of buthionine sulfoximine (BSO—an inhibitor of glutathione [GSH] synthesis), suggesting a close relationship between ascorbate and GSH whereby ascorbate could replace GSH during oxidative stress [1]. In murine hepatocytes both an increase in the level of cAMP [2] and GSH depletion using various agents including diamide, BSO, and menadione triggered an increase in ascorbate synthesis [3]. GSH depletion-dependent ascorbate synthesis was found to be decreased by the addition of dithiothreitol (DTT), suggesting that mixed protein disulfides that triggered glycogenolysis were responsible for the increased ascorbate synthesis [3].

The present view suggests that GSH depletion-dependent ascorbate synthesis occurs as a redundant offshoot from the process of glycogenolysis. Considering that ascorbate synthesis, glucose synthesis, and GSH recycling from GSSG rely heavily on phosphorylated hexose units from glycogenolysis, a gain in the activity of one pathway would imply a loss in the activity of the other two. This implies that activation of the pentose phosphate pathway may inhibit ascorbate synthesis and vice versa. Therefore, we hypothesized that stimulation of ascorbate synthesis by depleting GSH is not solely due to the oxidation of GSH [4]. To test this hypothesis we compared the ascorbate synthesizing, glutathione depleting, and glycogenolysis-inducing ability of the GSH oxidant, diamide with the GSH conjugators, 1-bromoheptane, and phorone (which do not oxidize GSH), in isolated rat hepatocytes. To address the effect of glycogenesis on ascorbate synthesis we compared these GSH depletors with cAMP inducers, glucagon and dibutyryl cAMP (known to stimulate glycogenolysis and glucose synthesis), for their ability to stimulate ascorbate synthesis and glycogenolysis.

Materials and methods

Materials. Dibutyryl cAMP, glucagon, amyloglucosidase, glucose, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)

* Corresponding author. Fax: +1-416-978-8511.
E-mail address: peter.obrien@utoronto.ca (P.J. O'Brien).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.03.022

diammonium salt, metaphosphoric acid, glucose oxidase, peroxidase (type IV), 1-bromoheptane, phorone, and diamide were purchased from Sigma–Aldrich Chemicals (St. Louis, MO). Sorbinil–HCl was generously donated by Pfizer (New York, NY). HPLC solvents and chemical vehicles were purchased locally from the University of Toronto supplier, Medstore (Toronto, ON) and Fischer Scientific (Fair Lawn, NJ).

Animals. Male Sprague–Dawley rats (Charles Rivers, Constance QC) weighing approximately 250–275 g were fed ad libitum for 1 week on standard lab chow before being sedated with 52 mg sodium pentobarbital i.p. (Somnitol).

Isolation of rat hepatocytes. Hepatocytes were isolated under an atmosphere of 95% O_2/5% CO_2 by perfusion of the rat liver through the portal vein with Hanks' balanced salt solution containing bovine collagenase (bovine) (0.7 mg/mL) [5]. Kupffer cells were aspirated away and the cells were washed several times before they were resuspended in Krebs–Henseleit buffer with 2.3 mg/mL bicarbonate [6].

Ascorbate measurement. Four hundred and fifty microliters of cell suspension was added to 50 μL metaphosphoric acid (8.5%) containing 10 mM DTT. The samples were immediately placed in dry ice until the end of the experiment. Samples were then stored in a −70 °C freezer until analysis took place. Ascorbate detection was performed by HPLC with electrochemical detection. The mobile phase consisted of 80 mM sodium acetate, 0.015% metaphosphoric acid, 1 mM *N*-octylamine, and 15% methanol. A Resolve C18 90 A silica 3.9 × 150 mm column was used with a flow rate of 0.5 mL/min. The eluted peaks were compared to authentic standards. The sensitivity of the assay was 5 pmol/injection [7].

GSH measurement. GSH was detected by extracting an 800 μL aliquot of cell suspension with 200 μL of 25% metaphosphoric acid. GSH thiol groups were conjugated with iodoacetic acid and the primary amines of GSH and GSSG were chemically tagged with 2,4-dinitrofluorobenzene. Gradient HPLC analysis using a μ-Bondapak NH_2 column (300 mm × 3.9 μm) connected to 2 Waters 501 HPLC pumps, Waters Associates 710B autosampler and 1 Lamda-Max Model 481 spectrophotometer was used to detect conjugated GSH and GSSG. A linear gradient was used consisting of 90% Mobile A (MeOH:H_2O (5:1)) and 10% Mobile B (MeOH:4 M sodium acetate in 13.2 M acetic acid) to 10% A and 90% B over 25 min.

Glucose and glycogen measurements. Glucose levels were monitored by extracting a 500 μL aliquot of cell suspension with 50 μL of 30% metaphosphoric acid. Glucose was measured by detecting the generation of the ABTS radical cation (which absorbs intensely at 734 nm) formed by the oxidation of ABTS by peroxidase in the presence of glucose oxidase. Glycogen was determined by removing the media prior to analysis. Cell pellets were sonicated for 5 min before the addition of sodium acetate (0.1 M, pH 4.8) containing amyloglucosidase (1 U/mL). Glycogen was degraded by amyloglucosidase for 2 h at 37 °C before being measured spectrophotometrically in the same manner as glucose. The glycogen was then expressed in terms of nanomoles of liberated glucose/10^6 cells [8].

Results

Stimulation of ascorbate synthesis by GSH depletion

The addition of 1-bromoheptane (200 μM), phorone (500 μM), and diamide (1 mM) caused rapid and near complete depletion of GSH without exhibiting significant cytotoxicity (Fig. 1A). Only diamide was able to transiently stimulate GSSG formation, while 1-bromoheptane and phorone appeared to decrease intracellular GSSG although the results were not statistically significant (Fig. 1B). Ascorbate content increased significantly approximately 2 h following the addition of 1-bromoheptane and phorone (Fig. 2). By varying the concentration of 1-bromoheptane, ascorbate synthesis was found to be inversely associated with GSH content in the cell (Fig. 3). Contrary to this effect, the GSH oxidizer, diamide, decreased cellular ascorbate levels. Glucagon or dibutyryl cAMP was unable to stimulate ascorbate synthesis compared to the untreated control (Fig. 2). GSH depletion was not observed with glucagon or dibutyryl cAMP at the doses administered (results not shown). Also, the addition of DTT to GSH-depleted hepatocytes did not affect the amount of ascorbate synthesized. Ascorbate synthesis stimulation could, however, be completely abrogated by the addition of 100 μM sorbinil, a potent aldose/glucuronate reductase inhibitor and by fructose 10 mM, an inhibitor of glycogenolysis (Table 1). Cellular ascorbate levels from 18 h fasted rats did not increase in response to treatment with phorone or 1-bromoheptane.

Stimulation of glycogenolysis

To determine whether enhanced ascorbate synthesis was derived from glycogenolysis we measured the loss of glycogen over time in the presence of 1-bromoheptane, phorone, and diamide vs cAMP elevation (Fig. 4). All treatments decreased cellular glycogen content compared to control (Fig. 4A), however, dibutyryl cAMP treatment was significantly more effective than 1-bromoheptane, phorone, diamide, and glucagon in causing glycogenolysis. The order of glycogen depletion from greatest to least occurred as follows: dibutyryl cAMP > glucagon = phorone = diamide > 1-bromoheptane > no treatment control.

Stimulation of glucose synthesis

The rate of cellular glucose production increased significantly in the presence of dibutyryl cAMP and glucagon (Fig. 4B). Dibutyryl cAMP was the most effective agent for stimulating glucose production. Diamide treatment was also able to stimulate glucose production although to a lesser extent than either dibutyryl cAMP or glucagon. Contrarily, neither 1-bromoheptane or phorone was able to elevate the rate of glucose production despite the apparent loss in glycogen.

Discussion

Ascorbate synthesis has been hypothesized to occur through two different pathways that both originate from UDP-glucuronic acid (UDPGA) and converge at the production of glucuronic acid [9] (Scheme 1). UDPGA formed from glucose-1-phosphate has been hypothesized to be converted to glucuronate either through

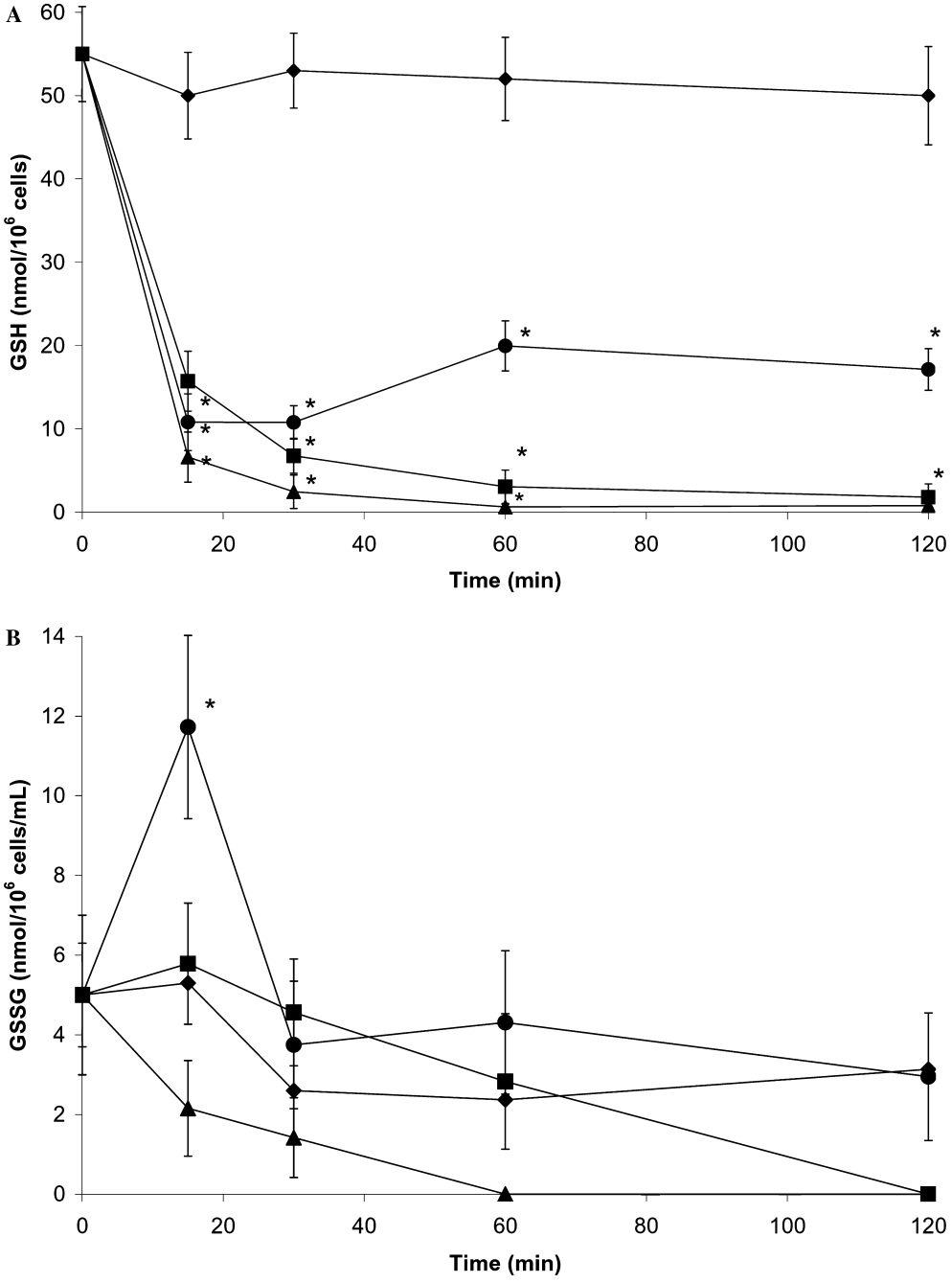


Fig. 1. Effects on intracellular (A) GSH and (B) GSSG content by phorone, 1-bromoheptane, and diamide. GSH and GSSG were determined by HPLC–UV detection as described in Materials and methods. Isolated rat hepatocytes were incubated in round-bottomed flasks and rotated in a water bath under an atmosphere of 95% O_2 and 5% CO_2 at 37 °C. The data are represented as follows: control (◆), phorone 500 μM (▲), 1-bromoheptane 200 μM (■), and diamide 100 μM (●). Neither the methanolic vehicle used to dissolve 1-bromoheptane (0.01%) nor DMSO (0.01%) affected GSH or GSSG content in the cell. Values are expressed as means ± SEM for at least three separate experiments. *Significantly different from the untreated control ($p < 0.05$).

a series of dephosphorylation steps [9] or through the obligatory formation of a xenobiotic glucuronide prior to its chemically induced or β-glucuronidase-catalyzed deglucuronidation [10]. The latter pathway would be most pertinent to labile acyl glucuronides such as the non-steroidal anti-inflammatory drug, diflunisal, which has been shown to undergo β-glucuronidase-mediated hydrolysis rapidly, but would be an unlikely pathway for chemicals with primary amines or hydroxyl groups. [11]. Ascorbate synthesis caused by GSH depletion in our system was indeed derived from glycogenolysis because both hepatocytes from fasted rats and fructose treated hepatocytes were unable to synthesize ascorbate in response to GSH conjugation. The inhibitory effect of

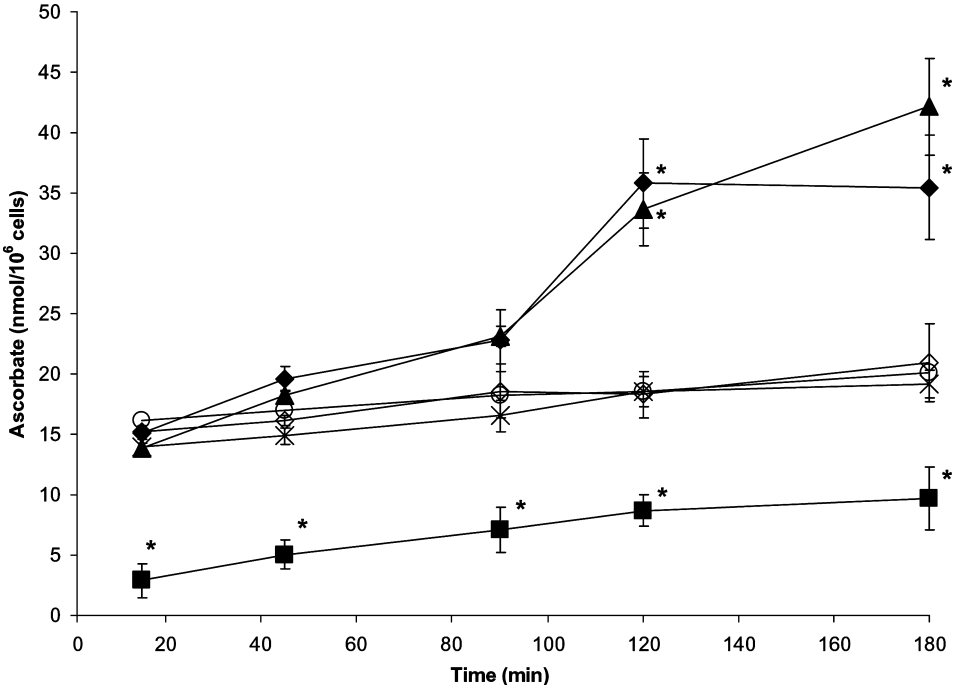


Fig. 2. Ascorbate synthesis in the presence of GST substrates vs dibutyryl cAMP and glucagon. Control (×), phorone 500 μM (▲), 1-bromoheptane 200 μM (◆), diamide 1 mM (■), dibutyryl cAMP 1 mM (○), and glucagon 2 μM (◊). Ascorbate was determined by HPLC-ECD. Isolated rat hepatocytes (10^6 cells/mL) were incubated in an atmosphere of 95% O_2, 5% CO_2 at 37 °C. Values are expressed as means ± SEM for between three and six separate experiments. None of the vehicles used (DMSO 0.01% nor methanol 0.01%) affected ascorbate content in the cell. Values are expressed as means ± SEM for three separate experiments. *Significance compared to the no treatment control at individual time points was assessed using one-way ANOVA followed by Tukey's post hoc test.

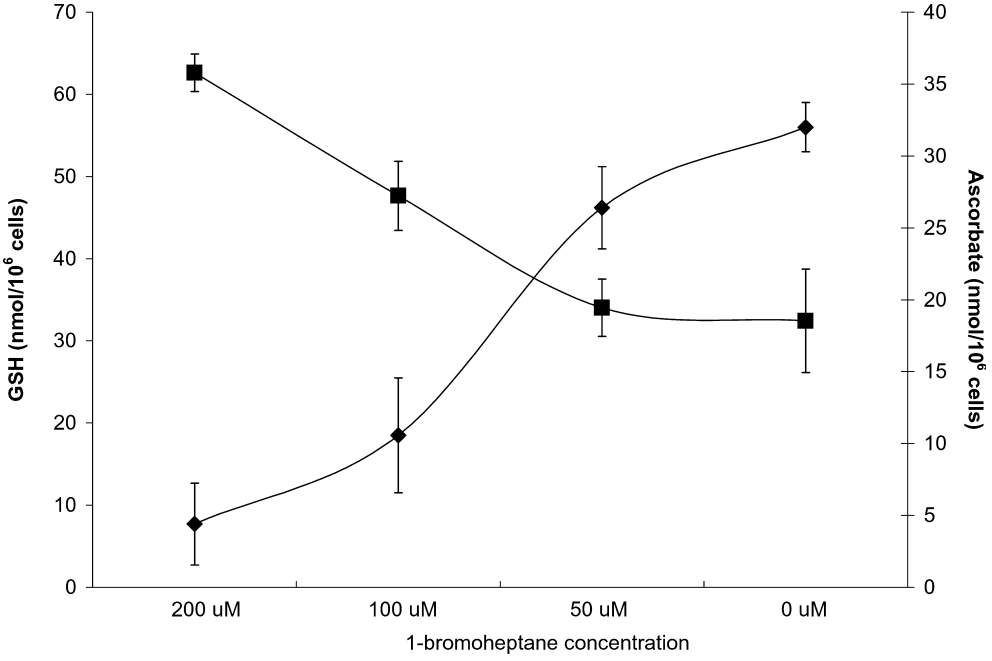


Fig. 3. Ascorbate content (■) in hepatocytes treated with varying concentrations of 1-bromoheptane for 2 h is inversely associated with GSH content (◆) in the cell. 10^6 hepatocytes/mL were incubated in round-bottomed flasks under an atmosphere of 95% O_2 and 5% CO_2 at 37 °C. Ascorbate and GSH were determined as described in Materials and methods. Values are expressed as means ± SEM for between three and six separate experiments.

sorbinil on ascorbate synthesis caused by 1-bromoheptane and phorone suggested that GSH conjugation gave rise to the rapid synthesis and reduction of glucuronic acid which proceeded towards ascorbate synthesis. Despite previous reports of ascorbate synthesis induced by diamide [3], we consistently found that this compound

Table 1
Effect of dithiothreitol, fructose, and sorbinil on ascorbate synthesis caused by GSH-depleting agents, 1-bromoheptane, phorone or diamide

Treatment	Ascorbate (nmol/10^6 cells: 2 h following treatment)				
	No cotreatment	DTT (10 mM)	Fructose (10 mM)	Fasted cells[a]	Sorbinil (100 μM)
Untreated control	18.5 ± 1.3	17.2 ± 1.6	17.5 ± 1.3	12 ± 2.3	16.7 ± 2.1
+ 1-Bromoheptane 200 μM	35.8 ± 3.6*	31.5 ± 2.5*	17.9 ± 2.5	5 ± 2*	21.4 ± 2.4
+ Phorone 500 μM	33.3 ± 2.2*	32.8 ± 2.8*	19.8 ± 1.6	4 ± 1*	19.6 ± 3.1
+ Diamide 1mM	8.65 ± 1.54*	N.D.	N.D.	N.D.	N.D.

Rat hepatocytes were isolated from fed male Sprague–Dawley rats. 1-Bromoheptane was dissolved in methanolic vehicle and phorone was dissolved in DMSO. Ascorbate induction was not initiated by either vehicle. Control intracellular concentration at 2 h after treatment with vehicle was 18.53 ± 1.29. N.D., not determined.

[a] Glycogen free cells were isolated from male Sprague–Dawley rats that were fasted for 18 h prior to sacrificing. Ascorbate was measured by HPLC-ECD as described in Materials and methods. Values are represented as means ± SEM for least three to seven separate experiments.

* Significantly different from the untreated control ($p < 0.05$).

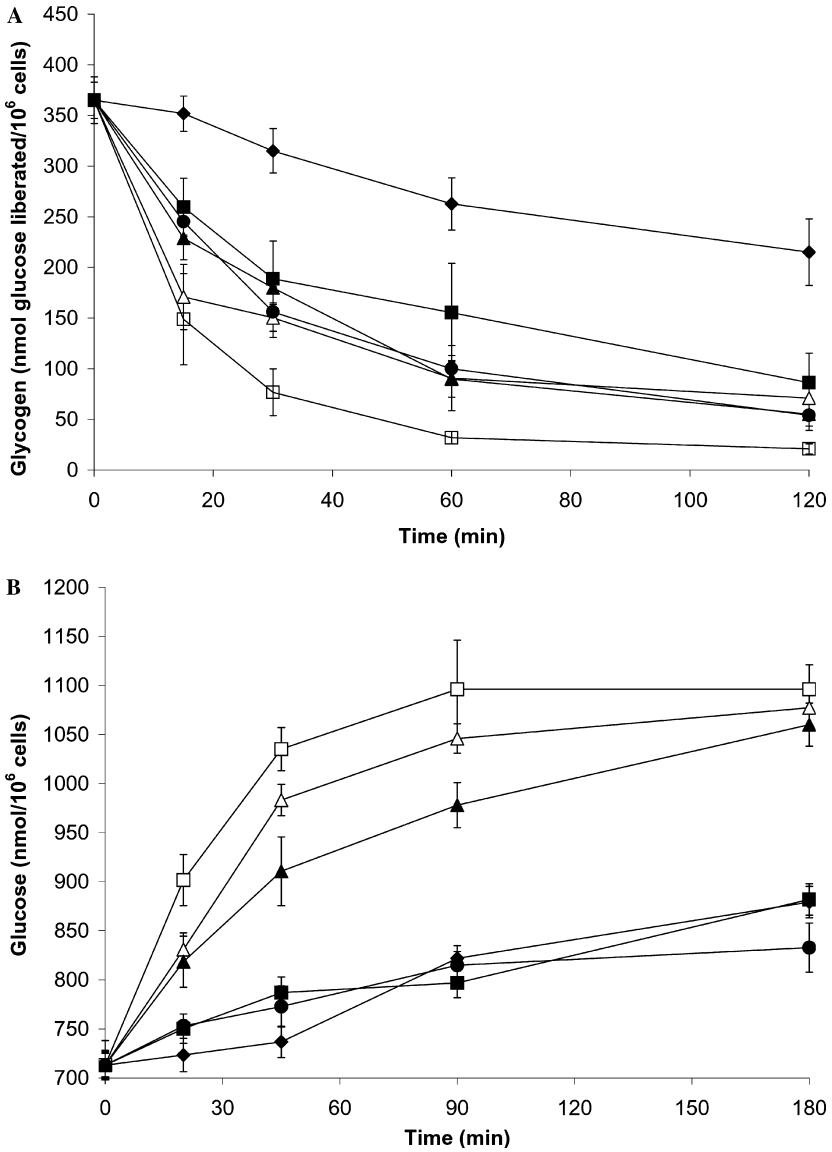
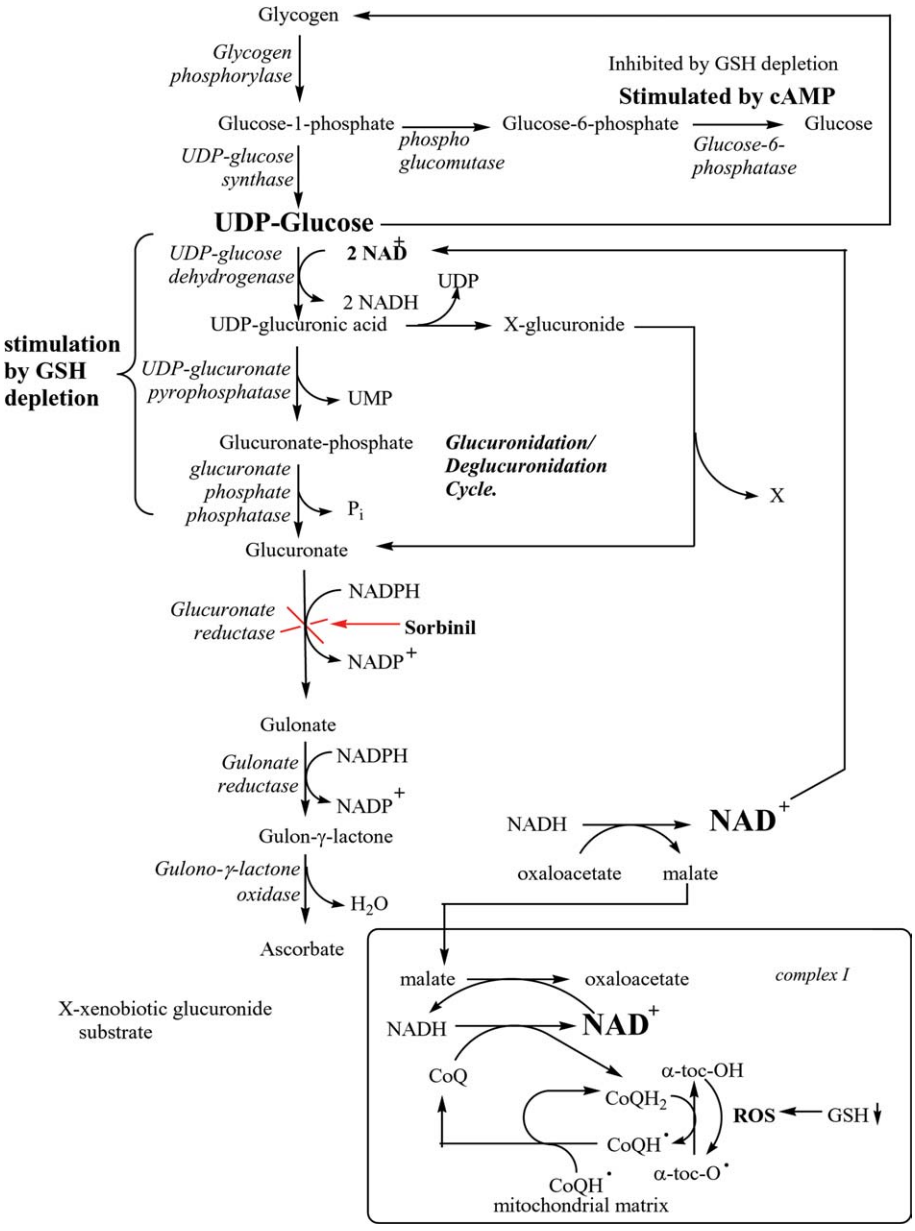


Fig. 4. (A) Glycogenolysis caused by 1-bromoheptane and phorone is not associated with glucose synthesis. (B) Glucose production in the presence of cAMP inducers vs GSH depletion. Control (◆), phorone 500 μM (●), 1-bromoheptane 200 μM (■), diamide 1 mM (▲), glucagon 2 μM (△), and dibutyryl cAMP 1 mM (□). Glucose content in cell suspension was determined as described in Materials and methods. Isolated rat hepatocytes were incubated in round-bottomed flasks and rotated in a water bath under an atmosphere of 95% O_2 and 5% CO_2 at 37 °C. Values are expressed as means ± SEM for between three and six separate experiments.



Scheme 1. Stimulation of UDP-glucose conversion to glucuronic acid resulting in enhanced ascorbate synthesis. Possible stimulation by a change in cellular NADH:NAD^+ ratio caused by mitochondrial GSH depletion.

decreased cellular ascorbate levels. Furthermore, diamide caused only a transient increase in GSSG content in the cell which was likely a result of the combined activity of the NAD(P)H consuming pentose phosphate pathway and GSSG efflux from the cell. This could explain why diamide decreased cellular ascorbate content as glycogen derived hexose units were consumed in the form of glucose-6-phosphate for the purpose of reducing GSSG via the pentose phosphate pathway. For the same reason the lack of GSSG formation from 1-bromoheptane and phorone treatment may contribute to ascorbate synthesis.

Previously, it was suggested that ascorbate synthesis in murine hepatocytes occurred as a result of the induction of glycogenolysis by either cAMP elevation or GSSG production [2,3]. Contrary to these observations, we demonstrated that although cAMP-inducing agents significantly stimulated glycogenolysis they failed to increase cellular ascorbate content. Furthermore, we and others have found that the GSH oxidant diamide could not stimulate hepatocyte ascorbate synthesis, but could stimulate GSSG formation [12].

GSH conjugation caused glycogenolysis leading to elevated levels of ascorbate and not glucose. This suggests that GSH conjugation caused the redirection of glycogenolysis towards ascorbate generation rather than glucose generation. The observed levels of intracellular ascorbate caused by GSH conjugation could only

account for approximately 5–10% of the glycogen consumed, suggesting that glucose units derived from glycogenolysis were shared by other processes such as glycolysis and the pentose phosphate shunt.

Hypotheses which might explain why this redirection of glycogenolytic metabolites occurs could involve the inhibition of glucose synthesizing enzymes such as glucose-6-phosphatase by lipid peroxidation products [13] or stimulation of the glucuronic acid pathway. The latter hypothesis is more plausible given that GSH conjugation itself does not decrease the normal production of glucose from the cell.

Recently, various chemicals capable of stimulating ascorbate synthesis were found to stimulate the conversion of UDP-glucose to glucuronic acid [12]. We hypothesize that GSH conjugation increases the rate of UDP-glucose conversion to glucuronate thereby stimulating the degradation of glycogen without the production of glucose (Scheme 1). This may partially explain why hepatocytes depleted of GSH using 1-bromoheptane was more susceptible to cyanide-mediated cell death as glycogenolysis may be unable to generate adequate glucose levels for compensatory glycolytic metabolism [14].

The rate of glycogenolysis could also be stimulated by elevated levels of reactive oxygen species, causing calcium influx from the extracellular space or release from intracellular stores [15]. Although glycogenolysis could be elevated independent of cAMP levels through the generation of mixed protein disulfides (for example, bovine cardiac glycogen synthase I and rabbit liver glycogen phosphorylase phosphatase were inhibited by the formation of their mixed protein disulfide [16,17]), the failure of the thiol reducing agent, DTT to inhibit ascorbate synthesis suggested that mixed protein disulfides were not involved in the stimulation of ascorbate synthesis.

Phorone has been found to deplete GSH in rat liver mitochondria and increase both hepatic lipid peroxidation and ascorbate content [18]. A decrease in mitochondrial GSH would lead to elevated reactive oxygen species accumulation in the mitochondria. Since, coenzyme Q and vitamin E have been shown to protect mitochondria against lipid peroxidation [19], the elevated reduction of coenzyme Q through mitochondrial NADH:coenzyme Q oxidoreductase (complex I), succinate dehydrogenase or glycerol-3-phosphate dehydrogenase may decrease the NADH:NAD^+ ratio. This would increase the activity of both glycolysis and the glucuronic acid pathway as NAD^+ is a necessary cofactor for UDP-glucose dehydrogenase [9]. UDP-glucose dehydrogenase could therefore maintain NADH concentrations in the cell for use in mitochondrial respiration.

cAMP is a secondary messenger responsible for activating glycogenolysis for glucose synthesis. The unrestricted flux of carbohydrates derived from glycogenolysis through the D-glucuronic acid pathway could constitute a drain on energy supply. Furthermore, since both ascorbate synthesis and GSH recycling are NADPH-dependent processes, ascorbate synthesis could hinder GSH recycling in the liver. Considering this, the loss in ascorbate synthesizing activity in humans and other animals may constitute an evolutionary advantage [20].

We speculate that a possible alternative regulatory mechanism involving the mitochondrial GSH pool could be responsible for activating ascorbate synthesis in response to GSH depletion and deactivating ascorbate synthesis in response to cAMP stimulation. Since it is hypothesized that the majority of reactive oxygen species in the cell is generated by the mitochondria [21], GSH depletion would contribute to the oxidation of endogenous coenzyme Q. In order to maintain functional respiration, the oxidation of NADH to NAD^+ would increase. The elevated NAD^+ generated would then stimulate the glucuronic acid pathway via UDP-glucose dehydrogenase. Glycogenolysis could be stimulated as a secondary response to decreased UDP-glucose levels.

Acknowledgments

We thank Pfizer Inc. for their generous donation of Sorbinil and Ewa Jaworski and Magdelena Dragan for analyzing ascorbate content. This research was funded by an operating grant from the Natural Science and Engineering Research Council of Canada (NSERC). Tom Chan is also funded by an NSERC postgraduate scholarship.

References

[1] J. Martensson, A. Meister, Glutathione deficiency increases hepatic ascorbic acid synthesis in adult mice, Proc. Natl. Acad. Sci. USA 89 (1992) 11566–11568.
[2] L. Braun, T. Garzo, J. Mandl, G. Banhegyi, Ascorbic acid synthesis is stimulated by enhanced glycogenolysis in murine liver, FEBS Lett. 352 (1994) 4–6.
[3] L. Braun, M. Csala, A. Poussu, T. Garzo, J. Mandl, G. Banhegyi, Glutathione depletion induces glycogenolysis dependent ascorbate synthesis in isolated murine hepatocytes, FEBS Lett. 388 (1996) 173–176.
[4] P. Buc-Calderon, I. Latour, M. Roberfroid, Biochemical changes in isolated hepatocytes exposed to tert-butyl hydroperoxide. Implications for its cytotoxicity, Cell Biol. Toxicol. 7 (1991) 129–143.
[5] P.O. Seglen, Preparation of isolated rat liver cells, Methods Cell Biol. 13 (1976) 29–83.
[6] T. Yoshida, Determination of reduced and oxidized glutathione in erythrocytes by high-performance liquid chromatography with ultraviolet absorbance detection, J. Chromatogr. B Biomed. Appl. 678 (1996) 157–164.
[7] R. Siushansian, J.X. Wilson, Ascorbate transport and intracellular concentration in cerebral astrocytes, J. Neurochem. 65 (1995) 41–49.

[8] M.J. Gomez-Lechon, X. Ponsoda, J.V. Castell, A microassay for measuring glycogen in 96-well-cultured cells, Anal. Biochem. 236 (1996) 296–301.
[9] N. Smirnoff, L-Ascorbic acid biosynthesis, Vitam. Horm. 61 (2001) 241–266.
[10] F. Horio, T. Shibata, S. Makino, S. Machino, Y. Hayashi, T. Hattori, A. Yoshida, UDP glucuronosyltransferase gene expression is involved in the stimulation of ascorbic acid biosynthesis by xenobiotics in rats, J. Nutr. 123 (1993) 2075–2084.
[11] F.M. Brunelle, R.K. Verbeeck, Conjugation–deconjugation cycling of diflunisal via beta-glucuronidase catalyzed hydrolysis of its acyl glucuronide in the rat, Life Sci. 60 (1997) 2013–2021.
[12] C.L. Linster, E. Van Schaftingen, Rapid stimulation of free glucuronate formation by non-glucuronidable xenobiotics in isolated rat hepatocytes, J. Biol. Chem. 278 (2003) 36328–36333.
[13] M.L. Hu, A.L. Tappel, Glutathione and antioxidants protect microsomes against lipid peroxidation and enzyme inactivation, Lipids 27 (1992) 42–45.
[14] H. Niknahad, S. Khan, C. Sood, P.J. O'Brien, Prevention of cyanide-induced cytotoxicity by nutrients in isolated rat hepatocytes, Toxicol. Appl. Pharmacol. 128 (1994) 271–279.
[15] M. Younes, O. Strubelt, The involvement of reactive oxygen species in hypoxic injury to rat liver, Res. Commun. Chem. Pathol. Pharmacol. 59 (1988) 369–381.
[16] K.H. Lau, J.A. Thomas, Specific mixed disulfide formation with purified bovine cardiac glycogen synthase I and glutathione, J. Biol. Chem. 258 (1983) 2321–2326.
[17] T. Shimazu, S. Tokutake, M. Usami, Inactivation of phosphorylase phosphatase by a factor from rabbit liver and its chemical characterization as glutathione disulfide, J. Biol. Chem. 253 (1978) 7376–7382.
[18] G. Mehmetcik, N. Alptekin, G. Toker, M. Uysal, Mitochondrial lipid peroxides and antioxidant enzymes in the liver following phorone-induced glutathione depletion, Res. Commun. Mol. Pathol. Pharmacol. 96 (1997) 353–356.
[19] A. Lass, R.S. Sohal, Electron transport-linked ubiquinone-dependent recycling of alpha-tocopherol inhibits autooxidation of mitochondrial membranes, Arch. Biochem. Biophys. 352 (1998) 229–236.
[20] I.F. Benzie, Evolution of dietary antioxidants, Comp. Biochem. Physiol. A Mol. Integr. Physiol. 136 (2003) 113–126.
[21] G. Lenaz, The mitochondrial production of reactive oxygen species: mechanisms and implications in human pathology, IUBMB Life 52 (2001) 159–164.