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Original Contribution

The biosynthesis of ascorbate protects isolated rat hepatocytes from cumene hydroperoxide-mediated oxidative stress

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Abstract

Most animals synthesize ascorbate. It is an essential enzymatic cofactor for the synthesis of a variety of biological molecules and also a powerful antioxidant. There is, however, little direct evidence supporting an antioxidant role for endogenously produced ascorbate. Recently, we demonstrated that incubation of rat hepatocytes with 1-bromoheptane or phorone simultaneously depleted glutathione (GSH) and triggered rapid ascorbate synthesis. The present study investigates the hypothesis that endogenous ascorbate synthesis can confer protection against oxidative stress. Rat and guinea pig hepatocytes were depleted of GSH with 1-bromoheptane and subsequently treated with the oxidative stressor cumene hydroperoxide (CHP) in the presence or absence of the ascorbate synthesis inhibitor sorbinil. In rat hepatocytes, ascorbate content increased linearly (from 15.1 to 35.8 nmol/10^6 cells) over a 105-min incubation. Prior depletion of GSH increased CHP-induced cellular reactive oxygen species (ROS) production, lipid peroxidation, and cell death in rat and guinea pig hepatocytes. Inhibiting ascorbate synthesis, however, further elevated ROS production (2-fold), lipid peroxidation (1.5-fold), and cell death (2-fold) in rat hepatocytes only. This is the first time that endogenous ascorbate synthesis has been shown to decrease cellular susceptibility to oxidative stress. Protection by endogenously produced ascorbate may therefore need to be addressed when extrapolating data to humans from experiments using rodents capable of synthesizing ascorbate.

Keywords: Free radicals; Vitamin C; Ascorbate; Antioxidant; Synthesis; Guinea pigs; Gulonolactone oxidase; Hepatocytes; Oxidative stress

Introduction

Most animal species can synthesize ascorbate in either the liver or the kidney or both; however, a few (including humans and guinea pigs) have lost this ability due to a mutation in the gene encoding L-gulono-γ-lactone oxidase (GLO), the terminal enzyme in the ascorbate biosynthetic pathway [1]. It is clear that ascorbate functions as an enzyme cofactor but its importance for antioxidant defense is uncertain. Exogenous ascorbate has been shown to have both antioxidant and pro-oxidant activities. As an example of the latter, the simultaneous addition of ascorbate and cumene hydroperoxide to rat liver microsomes resulted in enhanced lipid peroxidation relative to incubation of the organic hydroperoxide with microsomes alone. This effect of exogenous ascorbate was evidently due to metals present in the test system because it was abolished by the divalent metal chelator EDTA [2].

Although many studies have demonstrated the antioxidant and ROS scavenging activity of glutathione (GSH) and ascorbate [3–5], it is not known if endogenously synthesized ascorbate can protect against oxidative stress.

Abbreviations: CHP, cumene hydroperoxide; DCF, 2′,7′-dichlorofluorescein; DCFDA, 2′,7′-dichlorofluorescin diacetate; DMSO, dimethyl sulfoxide; DNPH, 2,4-dinitrophenylhydrazine; DTT, dithiothreitol; GLO, gulono-γ-lactone oxidase; GSH, glutathione; 3-*O*-MG, 3-*O*-methylglucose; ROS, reactive oxygen species.

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Depletion of ascorbate through dietary restriction or "loss-of-function" mutations in GLO gives rise to scorbutic animals in which metabolic complications confound assessment of the direct effects of ascorbate [6,7]. Furthermore the high cellular concentration of GSH, another low-molecular-weight antioxidant, makes it difficult to isolate the antioxidant role of ascorbate, especially because GSH has been shown to recycle ascorbate from its oxidized form, dehydroascorbate.

Incubation of rat hepatocytes with 1-bromoheptane or phorone extensively lowered intracellular GSH concentration but stimulated ascorbate synthesis. Depletion of GSH in 30 min using 200 μM 1-bromoheptane was accompanied by rapid ascorbate synthesis over a period of 2 h. Sorbinil abolished the 1-bromoheptane-mediated production of ascorbate without affecting the depletion of GSH. This effect of sorbinil has been attributed to the inhibition of an enzyme catalyzing glucuronic acid oxidation (glucuronate reductase) that is similar or identical to aldehyde reductase [8,9].

The present study evaluated the hypothesis that endogenous ascorbate synthesis confers protection against oxidative stress. Cumene hydroperoxide-mediated lipid peroxidation, reactive oxygen species formation, and cell death were compared between GSH-depleted hepatocytes from rats, which can synthesize ascorbate, and from guinea pig hepatocytes, which cannot.

Materials and methods

Materials

1-Bromoheptane, cumene hydroperoxide (CHP), 2′,7′-dichlorofluorescin diacetate (DCFDA), dithiothreitol (DTT), 3-*O*-methyl pyranose, octylamine, sodium acetate, and malondialdehyde bis-acetal were purchased form Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Sorbinil was generously donated by Pfizer (Groton, CT, USA). Collagenase (type IV, from Worthington Biochemicals, Inc.) was a generous gift from Dr. Raymond Poon of Health Canada. 2,4-Dinitrophenylhydrazine (DNPH) was purchased from AlfaAesar (Ward Hill, MA, USA). Dimethyl sulfoxide (DMSO; EMD Chemicals, Gibbstown, NJ, USA) was purchased from the University of Toronto supplier Medstore (Toronto, ON, Canada). HPLC-grade methanol was purchased from Fischer Scientific (Fair Lawn, NJ, USA). Tritiated 3-*O*-methyl-D-glucose (1 μCi/μl) was purchased from Perkin-Elmer/NEN (Boston, MA, USA).

Isolation of rat and guinea pig hepatocytes

Male Sprague-Dawley rats weighing approximately 275–350 g and male Hartley albino guinea pigs weighing 300–350 g were fed standard chows for rats and guinea pigs, respectively, and were kept in a regular light and dark cycle. Hepatocytes were isolated under an atmosphere of 95% O_2/5% CO_2 by perfusion of the liver through a portal vein cannula with Hanks' balanced salt solution containing bovine collagenase (0.7 mg/ml). Nonparenchymal cells were aspirated away and the cells were washed several times before they were resuspended in Krebs Henseleit buffer (pH 7.4) containing Hepes (3 mg/ml). Cells were maintained under an atmosphere of 95% O_2 and 5% CO_2 throughout the entire experiment. The hepatocytes used were at least 90–95% viable immediately after isolation [10].

Time course of measurements

All markers of oxidative stress measurements including viability, ROS formation, and lipid peroxidation were assayed after a preincubation period consisting of: (1) 30 min under incubation atmosphere and temperature (95% O_2, 5% CO_2, 37°C), (2) addition of sorbinil (100 μM) or vehicle (20 min), (3) addition of 1-bromoheptane (200 μM) or vehicle (60 min), and (4) addition of CHP (80 μM) or vehicle (15 min) unless stated otherwise. Ascorbate analysis took place for up to 180 min after the addition of 1-bromoheptane. GSH measurements were obtained 60 min after the addition of 1-bromoheptane.

Cell viability

Viability was determined by assessing loss in plasma membrane integrity by the cellular uptake of trypan blue. Hepatocytes were pretreated with sorbinil or vehicle DMSO for 20 min followed by an additional 60 min incubation with 1-bromoheptane. The hepatocytes were then treated with 80 μM CHP and viability was assessed by monitoring trypan blue uptake at 15, 30, and 60 min [11].

Determination of intracellular water space

Intracellular water space was determined by the radioisotope dilution method employing tritiated 3-*O*-methyl-D-glucose which was modified from a previously described technique [12]. Control cells treated with vehicle (DMSO) were incubated for 20 min in parallel with the incubation time described for sorbinil treatment and a further 30 min with another aliquot of DMSO as described for the addition of 1-bromoheptane. Cells were then treated with 3-*O*-MG (final concentration 1 mM) spiked with 3-*O*-[^{3}H]MG (final concentration 1 μCi/ml) and incubated for an additional 30 min. A 1-ml aliquot of cell suspension was extracted and washed twice with an ice-cold, isotonic, 0.2 M sucrose-tris-nitrate solution (pH 7.4) containing 1 mM phloretin to block 3-*O*-MG efflux. Cells were then air-dried for 3 h and digested in 1 M NaOH. The concentration of intracellular 3-*O*-[^{3}H]MG was determined by assuming that the distribution of 3-*O*-MG was equal in both the medium and the intracellular water space. Radioactivity was counted using a Beckman LS 5000TD liquid scintillation counter.