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Original Contribution

Essential role of intracellular glutathione in controlling ascorbic acid transporter expression and function in rat hepatocytes and hepatoma cells

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ABSTRACT

Although there is *in vivo* evidence suggesting a role for glutathione in the metabolism and tissue distribution of vitamin C, no connection with the vitamin C transport systems has been reported. We show here that disruption of glutathione metabolism with buthionine-(*S*,*R*)-sulfoximine (BSO) produced a sustained blockade of ascorbic acid transport in rat hepatocytes and rat hepatoma cells. Rat hepatocytes expressed the Na^+-coupled ascorbic acid transporter-1 (SVCT1), while hepatoma cells expressed the transporters SVCT1 and SVCT2. BSO-treated rat hepatoma cells showed a two order of magnitude decrease in SVCT1 and SVCT2 mRNA levels, undetectable SVCT1 and SVCT2 protein expression, and lacked the capacity to transport ascorbic acid, effects that were fully reversible on glutathione repletion. Interestingly, although SVCT1 mRNA levels remained unchanged in rat hepatocytes made glutathione deficient by *in vivo* BSO treatment, SVCT1 protein was absent from the plasma membrane and the cells lacked the capacity to transport ascorbic acid. The specificity of the BSO treatment was indicated by the finding that transport of oxidized vitamin C (dehydroascorbic acid) and glucose transporter expression were unaffected by BSO treatment. Moreover, glutathione depletion failed to affect ascorbic acid transport, and SVCT1 and SVCT2 expression in human hepatoma cells. Therefore, our data indicate an essential role for glutathione in controlling vitamin C metabolism in rat hepatocytes and rat hepatoma cells, two cell types capable of synthesizing ascorbic acid, by regulating the expression and subcellular localization of the transporters involved in the acquisition of ascorbic acid from extracellular sources, an effect not observed in human cells incapable of synthesizing ascorbic acid.
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Introduction

Vitamin C and glutathione (L-g-glutamyl-L-cysteinyl-glycine) are two water-soluble antioxidants fundamental for the physiology of cells [1]. Vitamin C is required for the synthesis of collagen and carnitine, as a cofactor in enzymes to maintain metal ions in their reduced form, and as a potent antioxidant and free radical scavenger [2,3]. Glutathione is the most abundant nonprotein thiol in mammalian cells, acting as a reducing agent, antioxidant, and free-radical scavenger and is involved in the metabolism and detoxification of xenobiotics [4]. Glutathione is found in cells predominantly as reduced glutathione (GSH), with very low levels of oxidized glutathione (GSSG) present under physiological conditions. Mammalian cells synthesize glutathione from its constitutive amino acids with participation of the enzymes γ-glutamylcysteine synthetase and glutathione synthetase, and oxidized glutathione can be recycled back to reduced glutathione by enzymes with glutathione reductase activity [4]. On the other hand, humans lack the capacity to synthesize vitamin C and therefore obtain this vitamin from external sources through the diet [5,6]. Vitamin C exists in two chemically distinct forms in solution, the reduced ionizable form known as L-ascorbic acid (ascorbic acid, AA), and the oxidized non-ionic form known as dehydro-L-ascorbic acid (dehydroascorbic acid, DHA). The plasma concentration of vitamin C (in the form of AA) is approximately 50 μM, with intracellular and tissue levels several orders of magnitude higher, indicating that vitamin C is concentrated in the cellular compartment of target tissues [5]. Cells acquire vitamin C by transporting both chemical forms across cell membranes with the participation of two complementary transporter systems that show absolute specificity for each form of the vitamin.

Abbreviations: AA, ascorbic acid; BSO, buthionine-(*S*,*R*)-sulfoximine; DEM, diethylmaleate; DHA, dehydroascorbic acid; qPCR, quantitative real-time RT-PCR.

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One transporter system is a low affinity, high capacity system that includes four members (GLUT1, GLUT2, GLUT3, and GLUT4) of the facilitative glucose transporter family that transport dehydroascorbic acid down its concentration gradient [7–11]. Importantly, the glucose transporters allow the recycling of dehydroascorbic acid generated in oxidative reactions associated with the normal metabolism of cells, and may be central to the low daily requirements of vitamin C in humans [12,13]. A second transport system for vitamin C is a high affinity, low capacity system composed of two members, SVCT1 and SVCT2 [14]. These transporters show absolute specificity for reduced vitamin C, are activated by sodium ions, and transport ascorbic acid down the electrochemical sodium gradient [15–20].

Glutathione has been implicated in the cellular accumulation of vitamin C and the intracellular maintenance of the vitamin in its reduced state [1,5]. Mammalian cells express a number of glutathione-dependent dehydroascorbate reductases involved in vitamin C metabolism [5]. Glutathione and vitamin C show a strong functional interdependence *in vivo*. Disruption of glutathione metabolism *in vivo* in rats and guinea pigs by treatment with buthionine-(*S,R*)-sulfoximine (BSO), a potent and specific glutathione synthesis inhibitor, revealed that the dysfunction and mortality associated with glutathione deficiency can be ameliorated by vitamin C supplementation [21]. Aside from the expected rise in the tissue ascorbate levels, there was also a remarkable increase in cellular glutathione, suggesting that ascorbic acid has an important role in the maintenance of adequate cellular levels of glutathione. Inversely, glutathione ester supplementation delayed the effects of a vitamin C-free diet in newborn rats and guinea pigs unable to synthesize vitamin C [22].

The limiting step for vitamin C bioavailability in humans is the transcellular transport across the intestinal barrier [13,23–25]. In contrast, species such as the rat obtain vitamin C from two complementary sources, from the *de novo* synthesis of ascorbic acid in the hepatocyte and from dietary sources [5]. Rat hepatocytes and hepatoma cells take up dehydroascorbic acid, but the identity of the transporters involved is still pending [26], and there is currently no clarity about the mechanism by which hepatocytes acquire ascorbic acid. Although *in situ* hybridization studies indicated abundant expression of SVCT1 mRNA in rat hepatocytes [14], uptake studies suggested the expression of a transporter with kinetic properties different from SVCT1 [27]. Adult rats made glutathione deficient by treatment with BSO showed increased hepatocyte synthesis of ascorbic acid followed by a marked decrease in the tissue levels of ascorbic acid [28], indicating a role for glutathione in the metabolism and tissue distribution of vitamin C that may include an effect on the expression and function of the vitamin C transporters

We used *in vitro* cultured, freshly isolated rat hepatocytes and the rat-derived hepatoma cell line H4IIE as models to study the mechanism by which cells with the capacity to synthesize ascorbic acid obtain vitamin from extracellular sources and the role of glutathione in the overall process. Pharmacological disruption of normal glutathione metabolism rendered the cells unable of transporting ascorbic acid by selectively affecting the expression or the membrane localization of the ascorbic acid transporters, indicating that blockade of ascorbic acid transport is the primary response of rat hepatocytes and hepatoma cells to decreased glutathione levels. Importantly, transport of dehydroascorbic acid was unaffected in the glutathione-deficient cells, and moreover, glutathione depletion failed to affect ascorbic acid transport in human-derived HepG2 hepatoma cells.

Materials and methods

Rat hepatocytes isolation

All experiments were performed according to the National Council for Science and Technology Research (CONICYT, Chile) Guide for the Care and Use of Laboratory Animals in accordance with the Institutional Ethics Review Committee of the Universidad de Concepción. Hepatocytes were isolated from female Sprague-Dawley rats by a recirculating collagenase technique [29], resuspended in Krebs-Ringer-Hepes, and used immediately. Rat (H4IIE) and human (HepG2) hepatoma cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% L-glutamine, penicillin-streptomycin, and fungizone [26]. Cell viability was always greater than 95% as assessed by trypan blue exclusion.

Glutathione depletion

For *in vivo* glutathione depletion, Sprague-Dawley rats were injected ip twice daily with an isosmolar solution of 3 mmol/kg L-buthionine-[*S,R*]-sulfoximine [21]. After 24, 48, and 72 h treatment, rat hepatocytes were isolated and used immediately. For *in vitro* glutathione depletion, hepatoma cells were incubated for 24 h with 2 mM BSO and 1 h with 1 mM diethylmaleate (DEM). For long-term depletion, cells were cultured in medium containing 2 mM BSO (changed on a daily basis) for up to 15 days. For total glutathione determination, cells were washed twice with normal saline and lysed with 0.25 ml of 0.4% Triton X-100, and the supernatant was processed using the recycling procedure using 5,5′-dithio-bis-(2-nitrobenzoic acid) [30].

Transport assays

Cells were deprived of fetal bovine serum, growth factors, and other media supplements by replacing the culture media with incubation buffer (15 mM Hepes, pH 7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, and 0.8 mM $MgCl_2$) 30 min before performing the assays [31,32]. Ascorbic acid transport assays were performed in incubation buffer containing 0.05 μCi L-[^{14}C]ascorbic acid (specific activity 8.2 mCi/mmol, NEN-DuPont) and a final concentration of 5–500 μM ascorbic acid in the presence of 0.1 mM DTT. Transport was finished with cold stopping solution and the cells were washed and lysed and the incorporated radioactivity was determined by scintillation spectrometry [31,32]. For transport in the absence of sodium ions, NaCl in the incubation media was replaced with 135 mM choline chloride, lithium chloride, or cesium chloride, or 270 mM sucrose. Uptake assays of the nonmetabolizable glucose analog 3-*O*-methyl-D-glucose (methylglucose) were similarly performed using 1 μCi of 3-*O*-[methyl-^{3}H]-D-glucose (specific activity 10 Ci/mmol, NEN-DuPont) and 1 mM 3-*O*-methyl-D-glucose [31,32]. Time-course experiments measuring the transport of methylglucose showed that the rate of transport for 1 mM substrate was linear during the first 60 s and equilibrium was reached in about 30 min, allowing us to estimate an intracellular water exchangeable volume of 2 μl/10^6 cells for hepatocytes and 0.4 μl/10^6 cells for H4IIE cells. Transport data are presented as the average ± the standard deviation and correspond to a minimum of three assays performed independently in triplicate. Kinetic parameters were calculated with the program IGOR Pro (WaveMetrics.Inc) using nonlinear fitting to the Michaelis-Menten equation and the linear transformation of Eadie-Hofstee.

RT-PCR

Total RNA was prepared from freshly isolated hepatocytes (~5×10^6 cells) and cultured H4IIE (~2×10^6) or HepG2 (~2×10^6) cells with Absolutely RNA miniprep kit (Stratagene) and cDNA synthesis was performed with StrataScrip First-Strand Synthesis System (Stratagene). Oligonucleotide primer pairs for SVCT1 (forward primer, 5′-act ctc ctc cgc atc cag at-3′; reverse primer, 5′-cca ggc ggg cac agg cgt ag-3′) and SVCT2 (forward primer, 5′-agt atg gct tct atg ctc gc-3′; reverse primer, 5′-ttc cgg atc ctg tgc tgg a-3′) were designed

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