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Research Paper

Chemical Transport Knockout for Oxidized Vitamin C, Dehydroascorbic Acid, Reveals Its Functions *in vivo*

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ABSTRACT

Despite its transport by glucose transporters (GLUTs) *in vitro*, it is unknown whether dehydroascorbic acid (oxidized vitamin C, DHA) has any *in vivo* function. To investigate, we created a chemical transport knockout model using the vitamin C analog 6-bromo-ascorbate. This analog is transported on sodium-dependent vitamin C transporters but its oxidized form, 6-bromo-dehydroascorbic acid, is not transported by GLUTs. Mice (gulo$^{-/-}$) unable to synthesize ascorbate (vitamin C) were raised on 6-bromo-ascorbate. Despite normal survival, centrifugation of blood produced hemolysis secondary to near absence of red blood cell (RBC) ascorbate/6-bromo-ascorbate. Key findings with clinical implications were that RBCs *in vitro* transported dehydroascorbic acid but not bromo-dehydroascorbic acid; RBC ascorbate *in vivo* was obtained only *via* DHA transport; ascorbate *via* DHA transport *in vivo* was necessary for RBC structural integrity; and internal RBC ascorbate was essential to maintain ascorbate plasma concentrations *in vitro*/*in vivo*.

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1. Introduction

Vitamin C (ascorbic acid, ascorbate) entry into cells is essential for all of its functions as a vitamin (Levine et al., 2011; Padayatty and Levine, 2016). Because ascorbate is charged at physiologic pH, it does not diffuse across membranes and requires transporters for cell entry. In cells and in expression systems utilizing *Xenopus* oocytes, two distinct transport mechanisms have been characterized. One is that ascorbate is transported as such, on sodium-dependent vitamin C transporters SVCT1 and SVCT2 (Tsukaguchi et al., 1999; Daruwala et al., 1999). A second mechanism is that ascorbate oxidizes to dehydroascorbic acid (DHA), which is transported on glucose transporters (GLUTs) (Vera et al., 1993; Washko et al., 1993; Rumsey et al., 1997; Corpe et al., 2013). Once intracellular, DHA is rapidly reduced to ascorbate. This mechanism has been termed ascorbate recycling (Washko et al., 1993; May et al., 1995).

It has been unclear what role, if any, DHA transport has in normal physiology and pathophysiology *in vivo*. Possible functions specific to DHA transport are worth understanding due to the structural similarity between glucose and DHA (Tolbert and Ward, 1982). Because DHA transport on GLUTs is competitively inhibited *in vitro* by glucose analogs (Rumsey et al., 1997), knowledge of DHA transport *in vivo* could have clinical implications in diabetes.

In experiments that could determine whether DHA transport was relevant, knockout mice for the sodium-dependent tissue transporter SVCT2 were created (Sotiriou et al., 2002). If mice utilized DHA transport for tissue accumulation, then DHA transport could rescue the absence of SVCT2, tissues of SVCT2 knockout mice could still contain ascorbate, and mice could appear normal. Alternatively, if DHA transport were specific to one or a few cell types, or unimportant *in vivo*, then SVCT2 knockout mice would be expected to be severely ascorbate deficient. These were the observed findings. SVCT2 knockout mice lacked ascorbate in all tissues measured, and did not survive more than minutes after birth.

One reasonable interpretation of SVCT2 knockout mouse experiments is that DHA transport followed by intracellular reduction was not physiologically relevant, at least in mice. If DHA had general physiologic relevance, then DHA transport should have prevented systemic tissue deficiencies and death. However, another explanation is that the cell type or tissue that utilized DHA transport was inadvertently not measured. Despite findings from SVCT2 knockout mice, there are several reasons to pursue DHA transport. DHA has equal or higher affinity than glucose for glucose transporters, such that hyperglycemia could inhibit DHA uptake and thereby create a link to diabetes and its

Abbreviations: bromoAA, 6-deoxy-6-bromo-L-ascorbate; bromoDHA, 6-deoxy-6-bromo-dehydroascorbic acid; DHA, dehydroascorbic acid; GLUTs, glucose transporters; gulo$^{-/-}$ mice, gulonolactone oxidase knockout mice; PBS, phosphate-buffered saline; RBCs, red blood cells; SVCT1, SVCT2, sodium-dependent vitamin C transporter; TCEP, tris(2-carboxyethyl)phosphine; WT, wildtype mice.

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complications (Rumsey et al., 1997; Corpe et al., 2013; Rumsey et al., 2000). Multiple redundant pathways exist in cells that immediately reduce DHA to ascorbate, utilizing both enzymatic and chemical reduction mechanisms (Maellaro et al., 1994; Winkler et al., 1994; Xu et al., 1996; Park and Levine, 1996; May, 2002; Padayatty and Levine, 2016). Existence of redundant pathways implies that DHA acid transport could have functional consequence(s) in as yet unidentified cell(s). In diabetes, if hyperglycemia could create ascorbate deficiency by locally inhibiting DHA transport in a specific cell type, this might uncover a localized heretofore unknown selective cellular deficiency in diabetes.

One possibility to investigate DHA specific transport pathways is to recapitulate the SVCT2 model, by creating knockout mice for DHA transporters. However, because these transporters are glucose transporters, primarily GLUT1 and GLUT3 (Rumsey et al., 1997; Rumsey et al., 2000; Corpe et al., 2013), their elimination would create overwhelming confounding variables. We chose an alternate path, by utilizing a compound that we had previously synthesized that could act as a chemical knockout for DHA transport. The goal was to test *in vivo* an ascorbate analog that was specific only for ascorbate transporters, and not transported by GLUTs. Ascorbate analogs were initially synthesized as 6-halo ascorbates, with 6 deoxy-6-bromo-L-ascorbic acid (bromoAA) as the working compound (Rumsey et al., 1999; Corpe et al., 2005). BromoAA was functionally tested using transporters expressed in microinjected *Xenopus laevis* oocytes and in cell models. BromoAA was transported only by SVCTs, with equal or higher affinity compared to ascorbate. When bromoAA was oxidized, 6-deoxy-6-bromo-dehydroascorbic acid (bromoDHA) formed but was not transported at all *in vitro* by GLUTs, in contrast to controls with DHA. The next step to determine function of dehydroascorbic acid transport, if any, was development of an *in vivo* system. Here, we describe findings in mice ($gulo^{-/-}$ mice) unable to synthesize ascorbate that were provided exclusively with bromoAA.

2. Materials and Methods

2.1. Materials

BromoAA was synthesized as described (Rumsey et al., 1999; Corpe et al., 2005). Ascorbic acid was purchased from Sigma/Aldrich. DHA and BromoDHA were synthesized *de novo* from parent compounds immediately prior to experiments (Corpe et al., 2005; Li et al., 2012; Corpe et al., 2013). All other chemicals were highest purity grade obtainable commercially.

2.2. Mice and Tissue Samples From Mice

Animal experiments were approved by the Animal Care and Use Committee NIDDK, NIH, and were conducted in accordance with NIH guidelines. Mice were fed *ad libitum* on regular chow diet (NIH-07) without detectable ascorbate (detection limit 10 nM). Mice types were C57BL/6 (wildtype, WT) (Charles River Laboratories, Wilmington, MA, USA); gulonolactone oxidase ($gulo^{+/-}$) mice (Mutant Mouse Regional Resource Center, University of California at Davis, USA), bred as described (Maeda et al., 2000). Homozygous $gulo^{-/-}$ mice were bred from heterozygous $gulo^{+/-}$ mice, and confirmed by genotyping using RT-PCR. If not stated otherwise, 8–12-week-old mice were used for experiments. Tissue samples were collected during pathological analysis. Tissue samples (≤100 mg) were harvested from mice and homogenized on ice in 100 μL (adrenal, pituitary) or 1000 μL (all other tissues) in ice-cold 90% methanol containing 1 mM EDTA. Samples were then centrifuged at 25,000*g* at 4 °C for 15 min. Supernatants were collected and diluted in 1:10 (heart) or 1:100 (adrenal gland, pituitary gland, small intestine, brain, liver, lung, and kidney) in 90% methanol containing 1 mM EDTA for ascorbate or bromoAA analyses. Pellets were diluted in 1 mL CHAPS for protein assay (Pierce). Mouse chow was analyzed for ascorbate in the same manner as tissue samples (Corpe et al., 2010).

Plasma and RBCs were collected from whole blood using centrifugation at 200*g* for 5 min at 4 °C to avoid hemolysis. When administered, mice received ascorbate or bromoAA supplements *via* drinking water at a dose of 1 g/L, and water was changed daily.

2.3. Histopathologic Examination

Mice (60–64 weeks old) were anesthetized, euthanized, and organs were then excised. The examined organs were brain, pituitary gland, liver, spleen, kidney, pancreas, heart, stomach, lung, small intestine, large intestine, limbs, spinal cord, eyes, ears, nose, tongue and salivary glands.

2.4. Confocal Microscopy

Confocal microscopy analyses of mouse RBCs were conducted as previously described (Tu et al., 2015). RBCs were fixed using 0.1% glutaraldehyde, stained with Alexa Fluor 488 phalloidin (5 units/mL), and analyzed using confocal microscopy at excitation/emission wavelengths of 489/518 nm. Whole cell and biconcave area diameters were measured using ZEN 2007 software by drawing a horizontal line across the center of the target RBC and calculating the distances between two marginal points.

2.5. Erythrocyte Osmotic Fragility

RBC osmotic fragility is a surrogate for RBC deformability (Clark et al., 1983). RBC osmotic fragility is based on resistance of RBCs to lysis as a function of decreasing NaCl concentrations and the assay was performed as described (Parpart et al., 1947) with modifications for mouse samples. Whole blood (150 μL) from each mouse was collected using heparinized Micro-Hematocrit capillary tubes (Fisher Scientific). NaCl solutions or distilled water (150 μL/well) were added to 12 wells of a 96-well round bottom plate. Concentrations of NaCl solutions were as 0.90%, 0.70%, 0.65%, 0.60%, 0.55%, 0.50%, 0.45%, 0.40%, 0.35%, 0.30% and 0.20%, one concentration per well. One 10 μL aliquot of whole blood was then added to the 12 wells containing NaCl solutions or distilled water. To avoid mechanical hemolysis, each well was gently mixed three times by pipetting up and down. Test plates were incubated for 60 min at room temperature, and subsequently centrifuged at 1740 *g* for 5 min at 4 °C. The resulting supernatant was transferred to a new 96-well flat bottom plate, and then hemoglobin content was determined at 540 nm using μQuant™ Microplate Spectrophotometer (Bio-Tek Instruments, Inc). Values from the well containing RBCs in 0.90% NaCl solution were used as blank. Values from the well containing RBCs in distilled water were used as 100%. Hemolysis status was presented as percentage: % Hemolysis = (O.D. of test well − O.D. of 0.90% NaCl well) / (O.D. of dH_2O well − O.D. of 0.90% NaCl well) × 100%.

2.6. P50 Assay

The P50 value (pO_2 at which 50% of hemoglobin is saturated with O_2), parameter of the hemoglobin − oxygen dissociation rate, was determined by using a HEMOX Analyzer (TCS Scientific Co., New Hope, PA). Oxygen dissociation curves were graphed using dual wavelength spectrophotometry as described (Guarnone et al., 1995). Briefly, mouse whole blood (24 μL) was diluted in 4 mL of Hemox-solution (HS-500, pH 7.4 ± 0.01, TCS Scientific Co.) mixed with 8 μL anti-foam agent (AFA-25, TCS Scientific Co.). The resulting mixture was mixed and heated to 37 °C, and then oxygenated to 100% under air purging. Samples were subsequently deoxygenated under nitrogen purging. The P50 values were determined at the points of 50% oxygen saturation.

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