EBioMedicine 2 (2015) 1735–1750

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Article

Low Red Blood Cell Vitamin C Concentrations Induce Red Blood Cell Fragility: A Link to Diabetes Via Glucose, Glucose Transporters, and Dehydroascorbic Acid

Hongbin Tu [1], Hongyan Li [1], Yu Wang [1], Mahtab Niyyati, Yaohui Wang, Jonathan Leshin, Mark Levine *

Molecular and Clinical Nutrition Section, Digestive Diseases Branch, Intramural Research Program, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (NIDDK, NIH)

ARTICLE INFO

Article history:
Received 13 March 2015
Received in revised form 25 September 2015
Accepted 28 September 2015
Available online 3 October 2015

Keywords:
Ascorbic Acid
Dehydroascorbic Acid
Red Blood Cells
Diabetes
Glucose Transport
β-Spectrin

ABSTRACT

Strategies to prevent diabetic microvascular angiopathy focus on the vascular endothelium. Because red blood cells (RBCs) are less deformable in diabetes, we explored an original concept linking decreased RBC deformability to RBC ascorbate and hyperglycemia. We characterized ascorbate concentrations from human and mouse RBCs and plasma, and showed an inverse relationship between RBC ascorbate concentrations and deformability, measured by osmotic fragility. RBCs from ascorbate deficient mice were osmotically sensitive, appeared as spherocytes, and had decreased β-spectrin. These aberrancies reversed with ascorbate repletion in vivo. Under physiologic conditions, only ascorbate's oxidation product dehydroascorbic acid (DHA), a substrate for facilitated glucose transporters, was transported into mouse and human RBCs, with immediate intracellular reduction to ascorbate. *In vitro*, glucose inhibited entry of physiologic concentrations of dehydroascorbic acid into mouse and human RBCs. *In vivo*, plasma glucose concentrations in normal and diabetic mice and humans were inversely related to respective RBC ascorbate concentrations, as was osmotic fragility. Human RBC β-spectrin declined as diabetes worsened. Taken together, hyperglycemia in diabetes produced lower RBC ascorbate with increased RBC rigidity, a candidate to drive microvascular angiopathy. Because glucose transporter expression, DHA transport, and its inhibition by glucose differed for mouse versus human RBCs, human experimentation is indicated.

1. Introduction

Diabetes type 2 is an epidemic public health problem. Poorly controlled diabetes results in accelerated microvascular disease and chronic debilitating morbidities and mortality (Beckman et al., 2002). Diabetic microvascular angiopathy is the leading cause of blindness, end stage renal disease and amputations worldwide, as well as myocardial infarction, stroke and peripheral arterial disease.

Preventing or delaying microvascular disease could improve the lives of millions, prevent catastrophic illness, and save billions of dollars. The pathogenesis of microangiopathy in diabetes is unknown. Clinical efforts are based on glycemic control. The research focus of some prevention efforts is the endothelium and its role in protecting blood vessels (Fioretto et al., 2010; Wong et al., 2010). Vascular smooth muscle abnormalities, platelet dysfunction, abnormal coagulation and impaired vascular repair are other pathologies proposed to lead to diabetic vasculopathy (Beckman et al., 2002; Cubbon et al., 2013). Oxidants generated by hyperglycemia within endothelial cells (Nishikawa et al., 2000) or other vascular cells may initiate endothelial cell damage (Yu et al., 2006; Wang et al., 2012).

Endothelial cell damage secondary to hyperglycemia can be one initiator of diabetic microangiopathy by impairing oxygen delivery, resulting in microvascular hypoxia. Another plausible pathway is that oxidants generated by hyperglycemia, from endothelial cells or others, impair oxygen delivery by affecting the delivery system itself: red blood cells (RBCs). In fact, substantial evidence indicates that diabetes induces changes in RBC structure and function through a progressive decline in RBC deformability (Peterson et al., 1977; McMillan et al., 1978; Kamada et al., 1992; Virtue et al., 2004; Diamantopoulos et al., 2004; Brown et al., 2005; Shin et al., 2007; Kung et al., 2009; Keymel et al., 2011; Buys et al., 2013). RBC deformability is vital to RBC function, and plays a major role in microvascular flow. Impaired deformability adversely affects capillary perfusion (Simchon et al., 1987; Parthasarathi and Lipowsky, 1999). Consistent with decreased deformability, diabetes is associated with increased RBC fragility and decreased RBC survival (Peterson et al., 1977; Parthasarathi and Lipowsky, 1999; Virtue et al., 2004; Kung et al., 2009). Because stiffer RBCs may compromise the microcirculation and oxygen delivery,

Abbreviations: AA, ascorbic acid; DHA, dehydroascorbic acid; *GLUT*, facilitated glucose transporter; *Gulo*$^{-/-}$, gulonolactone oxidase knockout mouse unable to synthesize ascorbate; PBS, phosphate buffered saline; RBCs, red blood cells; RIPA, Western blot cell lysis buffer; *SVCT*, sodium-dependent vitamin C transporter; TCEP, Tris(2-carboxyethyl)phosphine; 3-O-MG, 3-O-methylglucose; WT, wildtype mouse.

* Corresponding author.

[1] Hongbin Tu and Hongyan Li and Yu Wang contributed equally to this work.

http://dx.doi.org/10.1016/j.ebiom.2015.09.049

strategies to improve RBC deformability could modify microvascular hypoxia, with direct clinical implications.

In this regard, there are underappreciated links between RBCs, vitamin C, and diabetes. Multiple reports describe lower vitamin C concentrations in diabetic subjects, especially those with microvascular complications such as retinopathy and nephropathy (Som et al., 1981; Ali and Chakraborty, 1989; Sinclair et al., 1991; Will and Byers, 1996; Lindsay et al., 1998; Cunningham, 1998; Chen et al., 2006). However, many datasets utilized unreliable vitamin C assays, making it difficult to interpret findings (Will and Byers, 1996; Padayatty et al., 2003). Diabetic vascular disease and vitamin C deficiency were tied together in an early hypothesis (Mann and Newton, 1975), but it lacked mechanism and supportive evidence. Consistent with a hypothesized role for a RBC deformability defect in diabetes, anemia and hemolysis are manifestations of vitamin C deficiency in humans, and in mice (*Gulo*$^{-/-}$) unable to synthesize the vitamin (Chazan and Mistilis, 1963; Hart et al., 1964; Cox, 1968; Maeda et al., 2000). Unfortunately, deformability measures were not described in vitamin C deficient patients, and their clinical data are confounded by co-existent vitamin deficiencies.

Here we couple original links between diabetes and vitamin C with RBCs as a key cell type; oxidized vitamin C (dehydroascorbic acid, DHA) as a key transported substrate; and the chemical structure similarity between DHA and glucose (Vera et al., 1993; Rumsey et al., 1997; Corpe et al., 2013). For nearly all tissues, ascorbate transport is mediated by sodium-dependent vitamin C transporter *SVCT2* (Sotiriou et al., 2002). However, *SVCT2* is absent from RBCs (May et al., 2007). Because RBCs contain ascorbate (Li et al., 2012), another transport mechanism exists. It is likely that the product of ascorbate oxidation, dehydroascorbic acid, is transported on facilitated glucose transporters (*GLUTs*) and immediately reduced to ascorbate within RBCs (Hughes and Maton, 1968; Bianchi and Rose, 1986; Mendiratta et al., 1998). Based on expressed transporter data, hyperglycemia from diabetes could inhibit dehydroascorbic acid entry into RBCs (Vera et al., 1993; Rumsey et al., 1997). Some data do not support this rationale, but experiments were performed using DHA concentrations 2–3 orders of magnitude above physiological concentrations, and indirect assays that did not account for substrate degradation (Montel-Hagen et al., 2008a; Sage and Carruthers, 2014). Lower DHA concentrations could not be investigated due to assay limitations, also precluding accurate RBC ascorbate measurements (May et al., 2001; Montel-Hagen et al., 2008a; Li et al., 2012).

Utilizing a recently developed ultrasensitive assay for vitamin C in RBCs and physiologic transport conditions (Li et al., 2012), we propose an original multicomponent hypothesis linking vitamin C to diabetes. The parts of the hypothesis investigated here are whether low ascorbate concentrations occur in RBCs in comparison with other cells; whether low ascorbate concentrations in RBCs have a consequence; and whether and how low ascorbate RBC concentrations can be coupled to hyperglycemia in vitro and in vivo.

2. Methods

2.1. Materials

Ascorbic acid was purchased from Sigma/Aldrich. Dehydroascorbic acid was synthesized *de novo* from ascorbate immediately before each experiment (Li et al., 2012; Corpe et al., 2013). Antibodies were obtained from Abcam (Cambridge MA), Santa Cruz Biotechnology (Dallas TX), and Novus Biologicals (Littleton, CO). Antibodies from Abcam: anti-GLUT 1 antibody (ab652), anti-GLUT 2 antibody(ab54460), anti-GLUT 3 antibody (ab41525), anti-GLUT 4 antibody (ab654), anti-β actin antibody (ab6276), anti-β 1 spectrin (ab2808), and anti-(α + β) spectrin (ab11182). Antibodies from Santa Cruz Biotechnology: anti-Ankyrin-1 (sc-12,733). Antibodies from Novus Biologicals: anti-protein 4.2 (NBP1-56,647). All other chemicals were highest purity grade available commercially.

2.2. Mice and Blood Samples from Mice

Animal experiments were approved by the Animal Care and Use Committee NIDDK, NIH, and were conducted in accordance with NIH guidelines. Unless otherwise indicated, mice were 10–14 week old males with free access to food and water, and were maintained on regular chow diet (NIH-07) without detectable ascorbate (detection limit 10 nM). Mice were type C57BL/6 (wildtype, WT) (Charles River Laboratories, Wilmington, MA, USA); transgenic AZIP mice (original FVB/N A-ZIP/F-1 line) Moitra et al., 1998); and gulonolactone oxidase (*Gulo*$^{+/-}$) mice (Mutant Mouse Regional Resource Center, University of California at Davis, USA), bred as described (Maeda et al., 2000). Plasma and RBCs were obtained from mouse whole blood as described (Li et al., 2012), with the modification that samples were centrifuged at 200 x g for 5 min due to hemolysis (see Fig. 1 and results). When ascorbate was provided, mice received it via drinking water, which was changed daily, or via gavage where indicated. See supplementary methods for additional details.

2.3. Human Subjects and Samples

Clinical research was approved by the Institutional Review Board, NIDDK/NIAMS, NIH, and conducted in accordance with NIH guidelines. Blood and cell samples from healthy subjects (NIH Protocols 04-DK-0021; 99-CC-0168; and 92-DK-0033) and diabetic subjects (NIH Protocol 04-DK-0021) were obtained and processed as described (Levine et al., 1996) (Li et al., 2012).

2.4. Erythrocyte Osmotic Fragility

RBC deformability is related to RBC osmotic fragility (Clark et al., 1983). RBC osmotic fragility based on resistance of RBCs to lysis as a function of decreasing NaCl concentration was performed as described (Parpart et al., 1947) with modifications (see supplementary methods).

2.5. Dehydroascorbic Acid and Ascorbate Transport

2.5.1. Preparation of RBCs

RBCs were prepared as described (Li et al., 2012), with centrifugation modifications above.

2.5.2. Transport of Dehydroascorbic Acid and Ascorbate into Mouse and Human RBCs

Human (50 μL) or mouse (30 μL) RBCs were added to PBS (450 μL for human RBCs, 270 μL for mouse RBCs) containing 5 mM glucose and freshly prepared ascorbate, [^{14}C]ascorbate, dehydroascorbic acid, or [^{14}C]dehydroascorbic acid. RBCs and supernatants were prepared and analyzed as described previously (Li et al., 2012).

2.6. Inhibition of 3-O-[^{3}H]MG and [^{14}C]DHA Uptake into Mouse and Human RBCs

2.6.1. Inhibition of 3-O-[^{3}H]MG and [^{14}C]DHA Uptake into Mouse and Human RBCs by Unlabeled 3-O-MG

Human RBCs 2 mL prepared as above were incubated with 20 mM 3-O-MG in PBS (final volume 20 mL) for 20 min at 37 °C (See supplementary methods). After centrifugation at 500 x g for 5 min, the supernatant was removed and preloaded RBCs placed on ice until use. For competition between unlabeled and 3-O-[^{3}H]MG, RBC 50 μL were added to 450 μL PBS on ice containing 1 μCi/mL 3-O-[^{3}H]MG and one of the following concentrations of unlabeled 3-O-MG: 0.005, 1, 2.5, 5, 10, 20, 30 mM. For competition between unlabeled 3-O-MG, and [^{14}C]DHA, RBC 50 μL were added to 450 μl PBS on ice containing freshly prepared 5 μM [^{14}C]DHA and one of the following concentrations of unlabeled 3-O-MG: 0.005, 1, 2.5, 5, 10, 20, 30 mM. After incubation on ice for 1 min, 1 mL ice-cold stop buffer (10 μM cytochalasin B in PBS) was added, and the mixture centrifuged at