Article

Albumin Glycation Affects the Delivery of C-Peptide to the Red Blood Cells

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ABSTRACT: Serum albumin is a prominent plasma protein that becomes modified in hyperglycemic conditions. In a process known as glycation, these modifications can change the structure and function of proteins, which decrease ligand binding capabilities and alter the bioavailability of ligands. C-peptide is a molecule that binds to the red blood cell (RBC) and stimulates the release of adenosine triphosphate (ATP), which is known to participate in the regulation of blood flow. C-peptide binding to the RBC only occurs in the presence of albumin, and downstream signaling cascades only occur when the albumin and C-peptide complex contains Zn^{2+}. Here, we measure the binding of glycated bovine serum albumin (gBSA) to the RBC in conditions with or without C-peptide and Zn^{2+}. Key to these studies is the analytical sample preparation involving separation of BSA fractions with boronate affinity chromatography and characterization of the varying glycation levels with mass spectrometry. Results from this study show an increase in binding for higher % glycation of gBSA to the RBCs, but a decrease in ability to deliver C-peptide (0.75 ± 0.11 nM for 22% gBSA) compared to samples with less glycation (1.22 ± 0.16 nM for 13% gBSA). A similar trend was measured for Zn^{2+} delivery to the RBC as a function of glycation percentage. When 15% gBSA or 18% gBSA was combined with C-peptide/Zn^{2+}, the derived ATP release from the RBCs significantly increased to 113% or 36%, respectively. However, 26% gBSA with C-peptide/Zn^{2+} had no significant increase in ATP release from RBCs. These results indicate that glycation of BSA interferes in C-peptide and Zn^{2+} binding to the RBC and subsequent RBC ATP release, which may have implications in C-peptide therapy for people with type 1 diabetes.

KEYWORDS: C-peptide, red blood cell, albumin, glycation
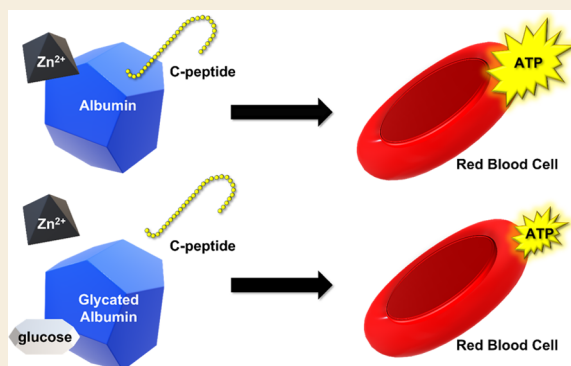
INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of the pancreatic β-cells; thus, insulin and other hormones secreted from these pancreatic cells are depleted. In 2018, diabetes was listed as the seventh leading cause of death in the United States.[1] The Centers for Disease Control and Prevention reported that 34.2 million people, or 10.2% of the U.S. population, have diabetes; of these cases, approximately 10% are T1D.[2] Individuals with T1D often have complications with the kidneys (nephropathy),[3,4] nerves (neuropathy), and eyes (retinopathy),[5,6] and these complications are often attributed to poor microvascular blood flow.[7] Interestingly, most cells in the circulation are not insulin-dependent, thereby suggesting a missing additional therapy to alleviate these complications.[8]

C-peptide is a molecule that is released from the pancreatic β-cells in equimolar amounts with insulin and Zn^{2+} after being cleaved from the proinsulin molecule. C-peptide stimulates the release of adenosine triphosphate (ATP) from red blood cells

(RBCs).[9,10] The ATP can diffuse to the endothelial cells lining the blood vessels, resulting in release of endothelial nitric oxide (NO), a known vasodilator and regulator of blood flow.[11] Our group has shown that C-peptide binding to RBCs requires the presence of albumin, and C-peptide requires both albumin and Zn^{2+} to elicit ATP release and other downstream effects such as an increase in measurable membrane-bound GLUT1 (the main glucose transporter in the RBC).[12] Research has also shown that C-peptide alone binds to albumin with a K_d of $5.7 \pm 1.6 \times 10^{-6}$ M,[12] and Zn^{2+} alone binds to albumin with a K_d of $2.1 \pm 0.5 \times 10^{-7}$ M.[13] Importantly, insulin does not affect C-peptide uptake or RBC-derived ATP release, thus suggesting

a novel role for C-peptide in the circulation but a role that requires albumin.[14]

Serum albumin is the most prominent carrier protein in the body, transporting metals, fatty acids, drugs, and hormones, thus regulating the bioavailability of these important species *in vivo*.[15−22] Unfortunately, albumin is prone to glycation, a non-enzymatic mechanism involving glucose binding to proteins, due to the high content of lysine and arginine residues.[23] In non-enzymatic glycation, or the Maillard reaction, sugar molecules, such as glucose or fructose, spontaneously interact with these amine-containing amino acids on albumin, possibly resulting in irreversible advanced glycation end (AGE) products.[23−26] The persistent hyperglycemic conditions in T1D result in the acceleration of AGE formation.[7] Glycation has been found to have effects on albumin's structure and function.[24,27,28]

Measuring glycated human serum albumin (gHSA) can estimate the mean bloodstream glycemic levels over periods of 2 to 3 weeks.[23,26] The gHSA can be measured by an enzymatic assay or by comparing the ratio of the gHSA peak area to the total HSA peak area with common methods such as affinity chromatography, liquid chromatography (LC), and ion exchange chromatography, followed by mass spectrometry (MS).[13,23,24] In healthy individuals, a normal range of gHSA is below 3%, as reported by over half of scientific and clinical reports.[15,24,29] However, healthy levels have been closer to 17% in other studies.[26] The level of gHSA can reach the low 20% in T1D due to persistent higher blood glucose levels,[30] although values differ depending on the methods employed for the separation of glycation species and detection methods.[30] We recently reported that glycation levels for healthy individuals are 13.0 ± 0.8%, whereas people with T1D had a glycated albumin value that was statistically doubled to 27 ± 7% ($p <$ 0.05).[13] While absolute values of glycation differ for healthy and T1D individuals, most reports show that gHSA levels in patients with T1D are 2−5 times higher than those of a healthy non-T1D individual.[29] Here, for clarity, glycation levels of 15% and lower are considered healthy control levels of glycated bovine serum albumin (gBSA), while anything above that level of glycation is considered highly glycated (such as those levels in most people with diabetes).

We previously reported decreased binding of Zn^{2+} to gHSA[13] and statistically equal binding of C-peptide to gHSA,[31] which may have implications on the biological effect of C-peptide in the bloodstream of people with T1D. Here, we report quantitative binding values for C-peptide, Zn^{2+}, and gBSA to the RBC. Key to these measurements was a comprehensive fractionation of albumin samples into various glycation ranges using affinity chromatography, followed by MS detection. These quantified fractions were then combined in various ratios to provide buffer samples containing gBSA with exquisite control. In addition to ligand binding to the RBC, we also report quantitative measurements of RBC-derived ATP using the fractionated gBSA samples.

■ METHODS

Plasma/RBC Collection

The procedure and consent form were approved by the Michigan State University Biomedical and Health Institutional Review Board (IRB). Whole blood was collected from healthy control donors via venipuncture into heparinized tubes. After being centrifuged for 10 min at 500*g*, the buffy coat and plasma were removed by aspiration, and the remaining RBCs were suspended and washed in a

physiological salt solution [PSS; 4.7 mM KCl (Fisher Scientific, Waltham, MA), 2.0 mM $CaCl_2$ (Fisher Scientific), 140.5 mM NaCl (Sigma Aldrich, St. Louis, MO), 12.0 mM $MgSO_4$ (Fisher Scientific), 21.0 mM tris(hydroxymethyl)aminomethane (Invitrogen, Carlsbad, CA), 5.5 mM dextrose (Sigma-Aldrich), and 0.5% BSA (Sigma-Aldrich; ≥98% by heat shock fraction) at pH 7.40]. An albumin-free version of the PSS (AF-PSS) was also utilized to wash the RBCs. A StatSpin MP microhematocrit centrifuge (Beckman Coulter, Brea, CA) and a digital hematocrit reader (StatSpin CritSpin; Beckman Coulter) were used to determine the RBC hematocrit. RBCs were prepared on the day of the experiment and used within 8 h of collection.

Separation of Control and Enriched gBSA Using Boronate Affinity Chromatography

Boronate affinity LC was used to isolate a control glycation fraction of BSA (avg. 13% gBSA) and an enriched glycation fraction of BSA (avg. 50% gBSA). Wash buffer [50 mM HEPES (Fisher Scientific), 0.5 M NaCl, pH 8.0−8.5] and elution buffer [100 mM sorbitol (Sigma-Aldrich), 50 mM HEPES, 0.5 M NaCl, pH 8.0−8.5] were prepared on the day of the experiment. A gravity flow column (Takarta, Mountain View, CA) was placed in the upright position, and a glycoprotein enrichment resin (Takarta) was added to the column.[21] A wash buffer/BSA solution was added to the column and incubated at 25 °C on an orbital shaker (260 rpm, Talboys Professional, Thorofare, NJ). Subsequently, the column was placed in the upright position, and the ∼13% gBSA fraction was collected into a tube. The column was washed four times with wash buffer. Five separate aliquots of the elution buffer were then added to the column to elute ∼50% gBSA off the column and collect into a tube. Both ∼13% gBSA and ∼50% gBSA samples were further purified using Amicon Ultra-15 centrifugal filters (10 kDa, MilliporeSigma, Burlington, MA) in seven washing steps (this number of steps was confirmed) to ensure that all contaminates were sub-nanomolar. Purified gBSA was lyophilized (Labconco Corporation, Kansas City, MO) and stored at −20 °C until further experimentation.

MS Identification of Isolated BSA

To determine the percent glycation of BSA, the mass of purified intact protein was analyzed by LC/MS using a Waters Xevo G2-XS QTOF interfaced with a Waters Acquity UPLC system. 10 μL of the sample was injected onto a short online desalting column (1.0 × 10 mm, HyperSil Gold CN, Thermo Scientific, Rockford, IL), and protein was eluted using the following gradient: initial conditions were 98% A (0.1% formic acid in water) and 2% B (acetonitrile) and were held until 5 min with the first 3 min diverted to waste, ramped to 75% B at 10 min, held at 75% B until 12 min, returned to 98% A at 12.01 min, and held until 15 min. The column was held at 30 °C, and the flow rate was 0.1 mL/min. Proteins were ionized by an electrospray operating in positive ion mode with capillary voltage at 3 kV, cone voltage at 35 V, source temp at 100 °C, desolvation temp at 350 °C, a desolvation gas flow of 600 L/h, and a cone gas flow of 25 L/h. Mass spectra were acquired in continuum mode with a 1 s scan time across the m/z range of 200−2000. Protein mass spectra were deconvoluted to give a neutral mass of the intact proteins using the MaxEnt I algorithm in MassLynx software. Albumin isoforms were determined as shifted from the base mass peak: glucose (+162 Da), cysteinylated-BSA (+119 Da), and potassium-BSA (+39 Da). The ion counts of the glucose peaks were summed and divided by the total ion counts to obtain a percentage. Each boronate affinity isolation results in varying glycation percentages, and these values are averaged over the course of each experiment. This results in each experiment having a different glycation percentage for the same ratios of gBSA, and these are each reported in their respective section for the upcoming experiments. It is important to note that the glycation percentage of the albumin corresponds to the total albumin molecules in the sample that contain bound glucose molecules rather than the concentration of albumin in the sample.

Radiolabeling BSA

Isolated albumins (11 and 48% gBSA) were incubated with 6:1 molar ratios of succinimidyl 6-hydrazinonicotinate/dimethylformamide (HYNIC; courtesy of Dr. Gary Bridger, AnorMED, Inc., Langley, British Columbia, Canada) for 1 h to allow conjugation through the 1′ amine groups on the BSA. The gBSAs were placed into separate Slide-A-Lyzer 10K MWCO dialysis cassettes (Thermo Scientific) to remove excess HYNIC. The dialysis cassettes were placed into phosphate buffered saline (PBS) at 4 °C for 2 h, at which point PBS was replaced with fresh PBS and left at 4 °C overnight. PBS was previously prepared [10.1 mM Na_2HPO_4 (Sigma-Aldrich), 2.7 mM KCl, 136.9 mM NaCl, and 1.8 mM KH_2PO_4 (Sigma-Aldrich) at pH 7.40]. The following day, sodium pertechnetate (TcO_4^-; 50 mCi; Cardinal Health, Swartz Creek, MI) was incubated for 15 min in a 0.25 mM tin chloride (Acros Organics, Geel, Belgium)/0.21 M tricine (Sigma-Aldrich) kit. (Note: Previous experiments indicated that ^{99m}Tc did not affect BSA binding to C-peptide,[32] and other groups have found no effects on biological activity once the protein is radiolabeled with ^{99m}Tc through HYNIC conjugation.)[33−36] The tin reduced TcO_4^-, and HYNIC was stabilized by the tricine acting as a coligand reagent.[37] Next, 150 μL of this solution was then added to the gBSA-HYNIC complexes. After 30 min incubation, 1 mL of each solution was added to separate 10 mL 6K Pierce polyacrylamide desalting columns (Thermo Scientific) to isolate the BSA-^{99m}Tc from the free ^{99m}Tc. The columns were washed with 1 mL of PBS eight times, and the fractions were collected. A CRC-25R dose calibrator (Capintec Inc., Florham Park, NJ) was utilized to determine the activity of each fraction. The first fractions with the highest activity, for both 11 and 48% gBSAs, were tested for free and colloidal ^{99m}Tc by thin layer chromatography (TLC) using Tec-Control ^{99m}Tc chromatography strips (Biodex, Shirley, NY). The 11% gBSA-^{99m}Tc fraction was dotted (2 μL) onto two Tec-Control dark green chromatography strips and placed into either PBS or methyl ethyl ketone (MEK; Acros Organics, Geel, Belgium). The top portion of the strip was cut off, and both portions were analyzed using a 2480 WIZARD^2 automatic gamma counter (Perkin Elmer, Waltham, MA). The PBS TLC strip provided the free ^{99m}Tc percentage, and the MEK strip provided the colloidal ^{99m}Tc percentage because only TcO_4^- traveled with the MEK solvent front.[38] The TLC experiment was then repeated for the 48% gBSA-^{99m}Tc. A Lowry assay was completed for each selected fraction to determine the concentration of BSA in the solutions.

gBSA Sample Preparation

Stock solutions were created for both 11% gBSA and 48% gBSA to be 3200 nM in either AF-PSS or PSS (depending on the sample set). Samples were then created with the desired amount of 11% gBSA-^{99m}Tc or 48% gBSA-^{99m}Tc (0, 50, 100, 340, 775, 1000, 1600, and 2700 nM), 7% RBCs, C-peptide, Zn^{2+}, and PSS (or AF-PSS). (Note: 7% RBCs will be utilized throughout the course of this study to mimic the hematocrit of the microvasculature.) Half of the samples (for both 11% gBSA and 48% gBSA) were created with excess unlabeled BSA (PSS) to block the BSA-^{99m}Tc-specific binding and demonstrate nonspecific binding, while the other samples were created without excess unlabeled BSA (AF-PSS) to determine the total binding. The specific binding of BSA to RBCs was calculated by subtracting the BSA nonspecific binding from the BSA total binding. Samples were incubated for 2 h at 37 °C. The samples were centrifuged for 1 min at 750g, and the supernatant was removed before adding AF-PSS to wash the cells. The RBCs were again centrifuged and washed an additional four times with AF-PSS to remove the loosely adsorbed proteins. RBC samples were analyzed using a 2480 Wizard2 automatic gamma counter (PerkinElmer, Waltham, MA) using a 20 s run time ^{99m}Tc method. Samples were then repeated with gBSA (containing physiologically relevant percentages of glycation) with and without C-peptide and Zn^{2+}.

The gamma counter sample counts per minute were converted to micrograms through a calibration curve and further calculated to BSA molecules/RBC. Values, such as K_d and B_{max}, were calculated utilizing the Sigma Plot (one site saturation ligand binding). Analysis of means was calculated using a one-tailed t-test: two samples assuming equal variances. The significant difference was set at a p-value less than 0.05 for this experiment and others in this paper.

C-Peptide Binding to RBCs

Samples were prepared containing 20 nM C-peptide and Zn^{2+} (or DDI H_2O for samples in absence of these species), 2 μM gBSA at varying glycation percentages, and 7% RBCs. (Note: 20 nM C-peptide was selected based on previous studies demonstrating RBC saturation with 20 nM C-peptide.)[12] These samples were incubated at 37 °C for 2 h on an orbital shaker (260 rpm). After incubation, the samples were centrifuged at 500g for 5 min, and the supernatant was removed. The supernatant was diluted 1:50 in the appropriate solution, and the concentration of free C-peptide was determined using a C-peptide enzyme-linked immunosorbent assay (ELISA; ALPCO, Salem, NJ). C-peptide binding to RBCs was calculated using a standard curve to determine the concentration of free C-peptide in each sample and subtracted from the total C-peptide added.

Zinc Binding to RBCs

Radioactive $^{65}ZnCl_2$ (Perkin Elmer, Waltham, MA) was used in the preparation of samples. The samples were prepared containing 20 nM C-peptide and $^{65}Zn^{2+}$ (or DDI H_2O), 75 μM gBSA at varying glycation percentages, and 7% RBCs. These samples were incubated at 37 °C for 2 h on an orbital shaker (260 rpm). The samples were centrifuged at 500g for 5 min, and the supernatant was removed. The concentration of bound $^{65}Zn^{2+}$ was quantified using a 2480 Wizard^2 automatic gamma counter (PerkinElmer) using a 5 min protocol to specifically measure $^{65}Zn^{2+}$. Zn^{2+} binding to RBCs was then calculated by using a standard curve to determine the concentration of bound Zn^{2+} in each sample.

Sample Preparation and ATP Release

RBC-derived ATP release was quantified via the luciferin/luciferase assay. A solution of 5 mg potassium luciferin (Gold Biotechnology, Inc., Olivette, MO) was added to 100 mg of firefly lantern extract (Sigma-Aldrich) and placed into a 15 mL tube. The samples were prepared to contain 20 nM C-peptide and $^{65}Zn^{2+}$ (or DDI H_2O), 20 μM gBSA at varying glycation percentages, and 7% RBCs. The samples were incubated at 37 °C for 3 h. Following incubation, samples were centrifuged at 500g for 5 min, and the supernatant was removed to be placed into another 1.7 mL tube. Subsequently, 100 μL of the supernatant was added to the well of a black 96-well plate, and 50 μL of luciferin/luciferase was manually injected into this well. As the luciferin/luciferase was injected, a timer was started, and at 20 s, the plate reader was programmed to measure the chemiluminescence of the sample. This process was repeated three additional times per sample and averaged to compute the concentration of ATP from a standard curve.
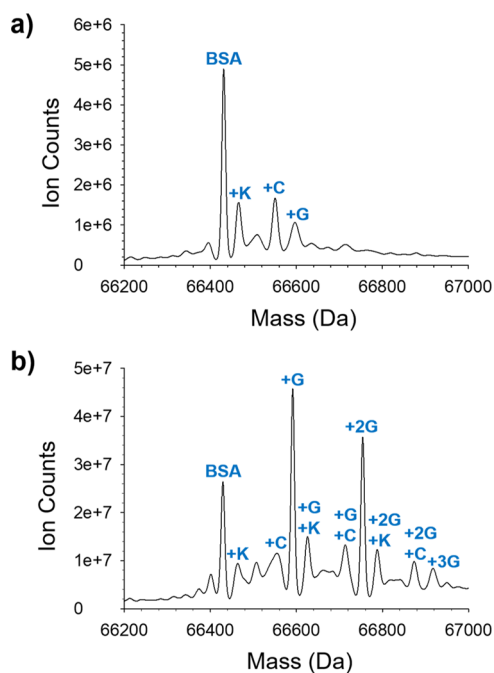
Safety

No unexpected, new, and/or significant hazards were associated with this work. Human subject research was conducted under Michigan State University IRB approval LEGACY17-826.

■ RESULTS

MS Spectra for Separated gBSA

The BSA samples were analyzed using MS to determine the percentage of glycation in each sample. Mass spectra for the two extreme fractions of gBSA (control gBSA and enriched gBSA), separated using boronate affinity chromatography, are shown in Figure 1. After determining the main peak, the glycation peaks (+G) were identified along with cysteine (+C) and potassium (+K) peaks. The peak ion counts were divided by total ion counts to obtain a percentage. The glycation percentage of the control gBSA in Figure 1a was determined to be 11% glycated, whereas the percentage of glycation in the enriched gBSA, depicted in Figure 1b, was 48% glycated. For all subsequent experiments (i.e., BSA binding, C-peptide binding, Zn^{2+} binding, and ATP release), the average glycation
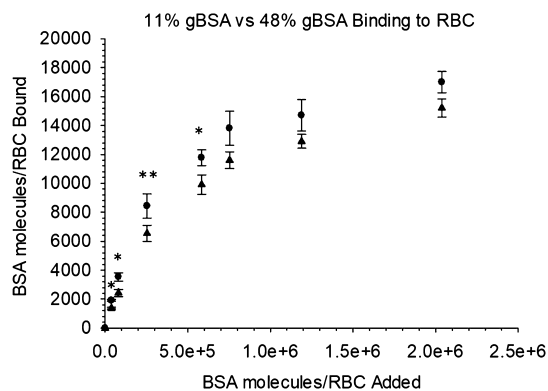
Figure 1. Mass spectra of albumin. Examples of control gBSA and hyperglycemic gBSA mass spectra to determine the glycation percentage. (a) Healthy control gBSA sample at 11% glycation and (b) enriched gBSA sample at 48% glycation. Labeled peaks include BSA, +G (glucose), +K (potassium), and +C (cysteine).

Figure 2. Binding of the glycated albumins to RBCs with extreme glycation percentages. The specific binding of 11% gBSA (triangles) and 48% gBSA (circles) to RBCs in the presence of C-peptide and Zn^{2+} ($n \geq 4$, error = SEM, *$p < 0.05$).

receptor molecules/RBC. The K_d and B_{max} between these two curves were not statistically different ($p > 0.05$).

Binding of gBSA to RBCs with and without C-Peptide and Zn^{2+} at Varying Glycation Percentages
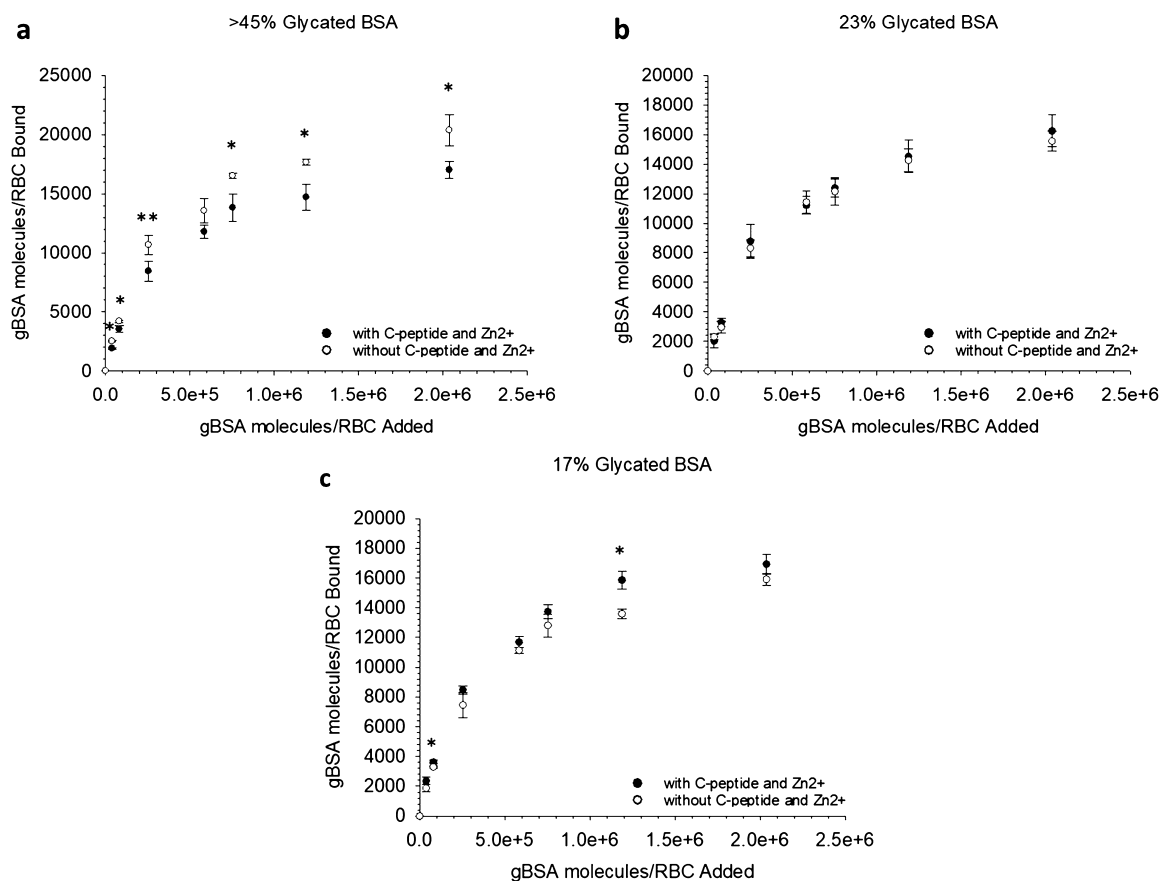
Samples were prepared with varying BSA glycation percentages (17–48%) in the presence and absence of C-peptide and Zn^{2+}. The specific binding curves of 45–48% gBSA in the presence (from Figure 2) and absence of C-peptide and Zn^{2+} are shown in Figure 3a. The 45% gBSA samples without C-peptide and Zn^{2+} saturated at an average of 20,372 ± 1315 BSA molecules/RBC. The equilibrium dissociation constant for this 45% gBSA sample was $4.2 \pm 0.3 \times 10^{-7}$ M, with a B_{max} of $3.21 \pm 0.06 \times 10^{-8}$ M, or approximately 23,000 receptor molecules/RBC. The results for 48% gBSA samples containing C-peptide and Zn^{2+} were presented previously in the article. The K_d and B_{max} between these two curves were not statistically different ($p > 0.05$).

When the BSA glycation percentage was decreased to 23% (Figure 3b), the BSA binding saturated at an average of 15,565 ± 591 BSA molecules/RBC with a K_d of $4.1 \pm 0.2 \times 10^{-7}$ M and a B_{max} of $2.49 \pm 0.03 \times 10^{-8}$ M (approximately 17,800 molecules/RBC). When C-peptide and Zn^{2+} were present, 23% gBSA specific binding saturated at 16,249 ± 926 BSA molecules/RBC with a K_d of $4.2 \pm 0.2 \times 10^{-7}$ M and a B_{max} of $2.57 \pm 0.04 \times 10^{-8}$ M (approximately 18,300 receptor molecules/RBC). The K_d and B_{max} between these two curves were not statistically different ($p > 0.05$).

A further decrease in BSA glycation percentage (17%; Figure 3c) resulted in the BSA binding saturating at an average of 15,910 ± 382 BSA molecules/RBC. The resulting K_d was $4.7 \pm 0.2 \times 10^{-7}$ M, and the B_{max} was $2.57 \pm 0.03 \times 10^{-8}$ M or approximately 18,300 receptor molecules/RBC. When C-peptide and Zn^{2+} were added, 17% gBSA binding specifically saturated at 16,926 ± 657 BSA molecules/RBC binding. The resulting equilibrium dissociation constant was $4.5 \pm 0.2 \times 10^{-7}$ M, and the B_{max} was $2.78 \pm 0.04 \times 10^{-8}$ M or approximately 19,800 receptor molecules/RBC. The K_d and B_{max} between these two curves were not statistically different ($p > 0.05$).

C-Peptide Uptake by RBCs with Varying BSA Glycation Percentages

The binding of C-peptide to RBCs at varying gBSA conditions was analyzed by incubating different percent gBSA samples (13–50%) with C-peptide, Zn^{2+}, and 7% RBCs. C-peptide

percentages for the varying glycated BSA stocks were 13, 18, 24, and 50% gBSA, respectively.

Binding of 11 and 48% gBSAs to RBCs

BSA was separated using boronate affinity chromatography to collect 11% gBSA and 48% enriched gBSA. The 11% gBSA and 48% gBSA samples were used to prepare stocks at different glycation percentages that correlate to the values of control and diabetic albumin seen in other studies.[13] Technetium-labeled BSA (BSA-^{99m}Tc) was utilized to determine the binding of gBSA to RBCs. Increasing concentrations of 11% gBSA-^{99m}Tc and 48% gBSA-^{99m}Tc (ranging from 0 to 2700 nM) were added to the RBCs in the presence of C-peptide and Zn^{2+}. To demonstrate the total binding, 11% gBSA-^{99m}Tc and 48% gBSA-^{99m}Tc were added to AF-PSS. A second sample set was prepared by adding 11% gBSA-^{99m}Tc and 48% gBSA-^{99m}Tc to albumin-containing PSS, which acted as a blocking experiment to determine the nonspecific binding of albumin to RBCs. The specific binding of albumin to RBCs was calculated by subtracting the nonspecific binding from the total binding. The specific binding curves of 11% gBSA and 48% gBSA are shown in Figure 2. 11% gBSA-^{99m}Tc specifically bound to the RBCs and saturated at an average of 15,222 ± 627 BSA molecules/RBC in the presence of binding ligands (C-peptide and Zn^{2+}). The equilibrium dissociation constant was $6.3 \pm 0.2 \times 10^{-7}$ M with a B_{max} of $2.60 \pm 0.03 \times 10^{-8}$ M or approximately 18,500 receptor molecules/RBC. gBSA-^{99m}Tc also specifically bound to the RBCs and saturated at an average that was higher than 11% gBSA in the presence of binding ligands (C-peptide and Zn^{2+}). The 48% gBSA binding saturated at 17,011 ± 732 BSA molecules/RBC. The equilibrium dissociation constant was $4.4 \pm 0.2 \times 10^{-7}$ M with a B_{max} of $2.73 \pm 0.04 \times 10^{-8}$ M or approximately 19,500

Figure 3. Binding of the glycated albumin to RBCs in the presence or absence of C-peptide and Zn^{2+}. The specific binding of >45% gBSA (a), 23% gBSA (b), and 17% gBSA (c) to RBCs in the presence (closed) and absence (open) of C-peptide and Zn^{2+} ($n = 4$, error = SEM, $*p < 0.05$, $**p = 0.05$).

concentration was quantified using a C-peptide ELISA. As shown in Figure 4, samples signifying healthy control
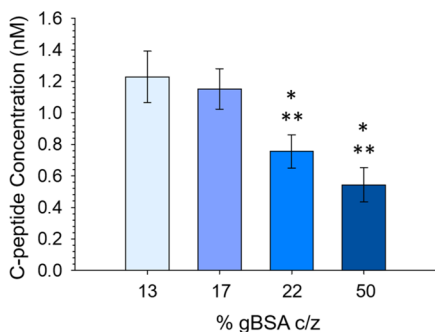

Figure 4. Binding of C-peptide to RBCs with different percent gBSA samples. In samples containing 13% gBSA and 17% gBSA, there was statistically the same amount of C-peptide bound to RBCs. There was a significant decrease in C-peptide binding when 22% gBSA and 50% gBSA were present in comparison with both 13% gBSA and 17% gBSA ($n = 4-6$, error = SEM, $*p < 0.05$ to 13% gBSA, $**p < 0.05$ to 17% gBSA).

conditions had a glycation percentage of 13%. The total amount of C-peptide that bound to RBCs in these control conditions was 1.2 ± 0.2 nM C-peptide or 1148 ± 126 C-peptide molecules/RBC. There is a significant decrease in C-peptide uptake as the percent glycation of BSA increases ($p < 0.05$). RBCs in the presence of 50% gBSA had 0.5 ± 0.1 nM C-

peptide or 421 ± 74 molecules of C-peptide bound to the RBC, resulting in a 56% decrease or a decrease of 727 C-peptide molecules bound compared to those in the 13% gBSA control ($p < 0.05$). In samples containing 17% gBSA, there was 1.15 ± 0.1 nM C-peptide or 893 ± 99 molecules of C-peptide bound to the RBC, which was statistically the same as those in the control conditions at 13% gBSA ($p > 0.05$). There was a significant decrease in C-peptide binding in the 22% gBSA samples at 0.75 ± 0.1 nM C-peptide or 598 ± 76 molecules of C-peptide bound when compared to both 13% and 17% gBSA samples ($p < 0.05$). Results indicate that C-peptide binding to the RBCs is inhibited as glycation increases from 17% gBSA to 22% gBSA.

Zn^{2+} Uptake by the RBC with Varying BSA Glycation Percentages

Zn^{2+} samples were prepared as stated in C-peptide binding studies; however, radioactive $^{65}Zn^{2+}$ was used in place of Zn^{2+}. Gamma emission detection using a gamma counter was utilized to quantify the amount of bound Zn^{2+} on the RBCs. In Figure 5, there is a significant increase in Zn^{2+} binding to the RBCs (3.09 ± 0.06 nM, or 2395 ± 47 molecules/RBC) with healthy control gBSA at 14% glycated ($p < 0.05$). Samples containing 49% gBSA resulted in a 38% decrease or a decrease in approximately 921 Zn^{2+} molecules bound to the RBCs (1.90 ± 0.14 nM or 1474 ± 110 molecules/RBC) when compared to those in 14% gBSA ($p < 0.05$). There was also a significant decrease in Zn^{2+} binding to RBCs in the 18% gBSA (2.56 ± 0.14 nM or 1981 ± 108 molecules/RBC) and the 23% gBSA
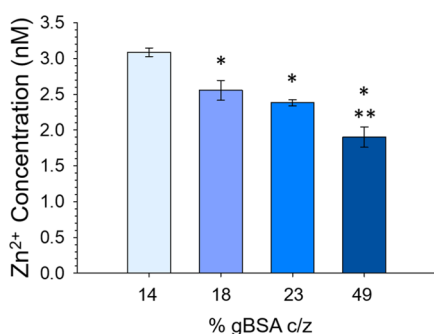
Figure 5. Binding of Zn^{2+} to RBCs with different percent gBSA samples. Samples signifying controls at 14% gBSA had a significant increase in Zn^{2+} binding when compared to all other glycation percentages. There was a significant increase in Zn^{2+} binding in 18% and 23% gBSA samples compared to the 49% gBSA sample. However, 18% gBSA and 23% gBSA had statistically the same amount of Zn^{2+} binding ($n = 4$, error = SEM, *$p < 0.05$ from 14% gBSA, $p < 0.05$ from 18 and 23% gBSA).

(2.38 ± 0.04 nM or 1847 ± 34 molecules/RBC) samples when compared to the control gBSA conditions. Results indicate that Zn^{2+} binding decreases as glycation percentage of the BSA increased from 14% to 18% gBSA.

ATP Release from RBCs with Varying BSA Glycation Percentages

ATP release from 7% RBCs in varying glycated conditions was measured in the presence or absence of C-peptide and Zn^{2+}. In Figure 6, there is a significant increase in ATP release (113%)
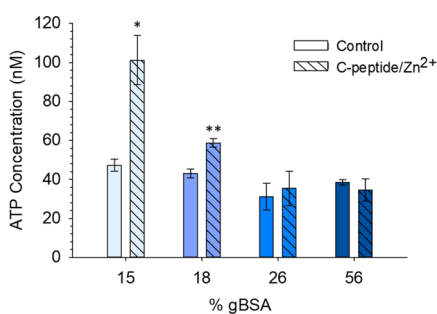
Figure 6. ATP release from the RBCs with various gBSA percentages with and without C-peptide and Zn^{2+}. The RBC-derived ATP release was significantly higher in the control conditions with the 15% gBSA samples when C-peptide and Zn^{2+} were present. There was a significant increase in ATP from RBCs and18% gBSA with C-peptide and Zn^{2+} compared to those without C-peptide and Zn^{2+}. There was no statistical increase in ATP in 26% gBSA and 56% gBSA samples with and without C-peptide and Zn^{2+} ($n = 3-6$, error = SEM, striped bars denote the samples containing C-peptide and Zn^{2+}, *$p < 0.05$ for all gBSA samples, **$p < 0.05$ for all samples except 15% gBSA c/z).

in the 15% gBSA samples, depicting healthy control gBSA conditions, containing C-peptide and Zn^{2+} (101 ± 13 nM) compared to the samples without C-peptide and Zn^{2+} (47.3 ± 3.1 nM; $p < 0.05$). There was also a significant increase in the amount of ATP released (36%) from the samples containing 18% gBSA with C-peptide and Zn^{2+} (58.7 ± 2.3 nM) compared to those without C-peptide and Zn^{2+} (43.1 ± 2.3 nM; $p < 0.05$). In the 26% gBSA samples, there was no statistical difference between samples with or without C-peptide and Zn^{2+} (35.4 ± 8.8 nM vs 31.1 ± 6.9 nM, respectively, $p > 0.05$). In addition, in 56% gBSA samples,

there was no statistical difference between samples containing C-peptide and Zn^{2+} (34.5 ± 5.7 nM) and those without (38.4 ± 1.3 nM; $p > 0.05$). The results indicate that as the glycation percentage increases to 26% gBSA, there was no statistical increase in C-peptide-derived ATP release from the RBCs when compared to that under control conditions without C-peptide and Zn^{2+} ($p > 0.05$).

■ DISCUSSION

While previous studies disagree on the exact cutoff point for albumin glycation between healthy individuals and those with diabetes in absolute numbers, there is agreement that albumin is 2−5 times more glycated in patients with diabetes than in healthy controls.[29] Previous data in our group indicate that healthy individuals have an average glycated albumin of 13.0 ± 0.8%, whereas people with diabetes have 27 ± 7% glycated albumin on average.[13] In these studies, BSA was isolated into two fractions utilizing boronate affinity chromatography, specifically, control gBSA (with an average glycation percentage of ∼13%) and enriched gBSA (with an average glycation percentage of ∼50%). However, because albumin in healthy plasma and plasma from people with diabetes commonly do not have glycation percentages as high as 50%, ∼13% gBSA and ∼50% gBSA were mixed to obtain physiologically relevant glycation percentages. The glycation percentages that were created represented individuals with T1D that have well-controlled blood glucose levels (with an average glycation percentage of ∼18%) and individuals with T1D that have average blood glucose levels (with an average glycation percentage of ∼24%). The range of gBSA used in these studies correlate to actual glycation percentages of albumin found in people with T1D and healthy controls.

Previous research in our group analyzing the specific binding of BSA to RBCs in the presence of C-peptide and Zn^{2+} reported a K_d of $2.00 \pm 0.05 \times 10^{-7}$ M and a B_{max} of $2.50 \pm 0.01 \times 10^{-8}$ M.[32] This study found a K_d of $6.3 \pm 0.2 \times 10^{-7}$ M, and the B_{max} was $2.60 \pm 0.03 \times 10^{-8}$ M for BSA binding to RBCs in the presence of C-peptide and Zn^{2+}. There was also a difference in the BSA molecules/RBC bound at saturation in this study ($15,222 \pm 627$) and in the previous study ($16,695 \pm 1479$).[32] However, the insignificant differences between prior data and data shown here could be due to sample preparation prior to the analysis. Previously, the BSA used in the samples was a commercially available form used without further purification. Here, the control 11% gBSA and 45−48% gBSA were separated through boronate affinity chromatography prior to MS characterization of the glycation percentage. BSA from the previous studies was later determined to be 14% glycated after improved sample preparation methodologies were employed. Since the glycation was not determined on an experimental basis (in contrast to our studies reported here), the option to determine percent glycation was to analyze the commercial product directly. Not only was there a difference in the BSA percent glycation, but also a variation in how the percentage was determined. The fact that less BSA molecules bound per RBC in this study (11% glycation) compared to the previous study (14% glycation) is consistent with the observation here that more gBSA molecules bind per cell as the glycation percentage increases.

When comparing BSA binding with or without C-peptide and Zn^{2+} in varying glycation percentages, we measured the differences between samples containing C-peptide and Zn^{2+} and those without C-peptide and Zn^{2+}. Interestingly, at the

higher glycation levels, there was more BSA binding without C-peptide or Zn^{2+}. As the glycation percentages approach physiological levels in patients with T1D that have well-controlled glucose levels (17%), we saw a decrease in the difference of albumin binding with or without C-peptide and Zn^{2+}. This could indicate that the 45−48% gBSA carrying the C-peptide and Zn^{2+} is not as effective at binding to the RBCs (evident by the reduction in a binding increase) as the 11% gBSA. Also, the gBSA could be binding to glycoproteins within the RBC membrane[39] as opposed to the albumin/C-peptide complex receptor.

C-peptide binding to the RBCs in the presence of healthy control 13% gBSA was statistically the same as in individuals with T1D that have well-controlled blood glucose levels at 17% gBSA. As the BSA glycation percentage increased from 17% gBSA to 22% gBSA, a glycation percentage representing average T1D blood glucose levels, C-peptide binding to the RBCs significantly decreased. This indicates that as glycation percentages of BSA reach average glycation levels often reported in T1D (and above), gBSA does not carry C-peptide to the RBCs in the same manner as lower gBSA samples. In addition, Zn^{2+} binding to the RBCs was measured using similar glycation percentages of BSA. There was a significant decrease in Zn^{2+} binding to the RBCs in all BSA glycation percentages (18% and above) when compared to healthy control 14% gBSA. These results indicate that even at levels of glycation comparable to individuals with T1D that have well-controlled glucose levels, Zn^{2+} binding to the RBC is decreased.

Perhaps most importantly, ATP release from the RBCs was measured using similar gBSA percentages as those depicted in BSA, C-peptide, and Zn^{2+} binding experiments. The samples were analyzed to determine the downstream effects that albumin glycation has on the RBCs. There was a significant increase in ATP release from the RBCs when using 15% gBSA and 18% gBSA with C-peptide and Zn^{2+} when compared to those without. This shows that although there is a 36% increase in ATP release with 18% gBSA with C-peptide and Zn^{2+}, there is a further 113% increase in ATP release when 15% gBSA is under the same conditions. There was a significant decrease in ATP release in the 26% gBSA and 56% gBSA samples with and without C-peptide and Zn^{2+}, showing that as glycation increases from 18% to 26% and above, the ability of C-peptide to increase ATP from the RBCs is inhibited. These results hold immense importance for the indirect role of RBC-derived ATP release in the bloodstream and could further provide evidence for microvascular complications in T1D associated with decreased ATP release. These results confirm that as the glycation of BSA increases, C-peptide-derived ATP release from the RBCs decreases.

CONCLUSIONS

While insulin has been used in humans for a century with great success, the use of C-peptide as an auxiliary exogenous therapy to reduce diabetes-related complications has been less successful.[40−43] While we recently reported that albumin binds C-peptide with affinities that are independent of albumin's glycation level,[31] the results obtained from our current studies enable us to conclude that an increase in the BSA glycation level, especially those approaching 20%, results in reduced C-peptide delivery to the RBCs. Furthermore, Zn^{2+} binding to albumin is reduced as the percent glycation of albumin is increased.[13] Zn^{2+} delivery to RBCs was decreased as the albumin glycation level increased, thus following a similar

trend to C-peptide. Based on previous reports, a decrease in C-peptide and Zn^{2+} delivery to the RBCs would be expected to result in decreased RBC-derived ATP. Collectively, as the extent of glycation of albumin used in the albumin/C-peptide/Zn^{2+} formulation increased, a decrease in ATP release from the RBCs would be anticipated. The data presented here confirm this theory. These new findings may provide insight on C-peptide's lack of efficacy in clinical trials involving people with T1D. Specifically, the increased glycated albumin in the T1D bloodstream is not delivering C-peptide and Zn^{2+} to the RBCs as well as albumin in the control bloodstream. In turn, this is negatively affecting the ability of the RBCs to release ATP, a known stimulus of vessel relaxation and blood flow in the circulation. The addition of C-peptide (alone) to the bloodstream of a patient with T1D may have minimal effect due to the high percentage of glycation of albumin, thus explaining, in part, the failed C-peptide clinical trials in the past.[40] Future studies need to consider the effect of glycation on the albumin's ability to carry C-peptide and Zn^{2+} to the RBCs for C-peptide therapeutic development. It is important to include not only C-peptide in a new T1D therapy regime but also healthy, low glycated albumin to allow for effective transport and RBC signaling.

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Notes

The authors declare the following competing financial interest(s): DMS has a financial interest in LifeBlood, which has a diagnostic based on C-peptide binding to cells from people with Multiple Sclerosis.

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