Chicken albumin exhibits natural resistance to glycation

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Glycation of proteins and subsequent production of advanced glycation end products (AGEs) is a major contributor to the pathophysiology of diabetes. The objective of the present study was to compare the glycation of avian and human serum albumin to elucidate the mechanisms by which protein glycation in birds is prevented in the presence of naturally high plasma glucose concentrations. Solutions of purified chicken and human serum albumin (CSA and HSA) were prepared with four different glucose concentrations (0, 5.56, 11.1, and 22.2 mM) and incubated at three temperatures (37.0, 39.8, and 41.4 °C) for seven days. The solutions were sampled on Days 0, 3, and 7 and analyzed by liquid chromatography-electrospray ionization-mass spectrometry for the presence of glycated albumin. Four-way repeated measures ANOVA ($p = 0.032$) indicate that all independent variables (albumin type, glucose concentration, temperature and time) interacted to affect the degree of glycation. With increasing glucose concentration, the glycation of both HSA and CSA increased with time at all temperatures. In addition, HSA was glycated to a greater extent than CSA at the two higher glucose concentrations for all conditions. Glycation was elevated with increasing temperatures for HSA but not CSA. The results suggest an inherent difference between human and chicken albumin that contributes to the observed differences in glycation. Further research is needed to characterize this inherent difference in an effort to elucidate mechanisms by which avian plasma protein is glycated to a lesser degree than that of mammals (humans).

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concentrations of all vertebrates measured (300–756 mg/dl in the fasted and fed states, respectively), were found to have glycated hemoglobin levels of 3.7–4.6% (Beuchat and Chong, 1998). Although higher than other avian species, the percent glycation is lower than what the World Health Organization has established as a guideline for diagnosing diabetes in humans, 6.5% (WHO, 2011).

Iqbal et al. (1999) concluded that protein glycation in chickens is not likely the main cause of glucose-derived crosslinks and that the accumulation of glycated products is less in birds than mammals. In fact, recent studies have shown that the receptor for AGEs (i.e. RAGE) first appeared in mammals and is not present in the avian genome (Sessa et al., 2014). The lack of RAGE in birds could also explain why high blood glucose concentrations do not cause the same complications as observed in humans with hyperglycemia. However, this does not explain the protective mechanisms that exist in birds to reduce glycation of plasma proteins. Considering these remarkable characteristics of birds, it is perhaps not surprising that our outline mammals of comparable body size (Braun and Sweazea, 2008; Holmes et al., 2001; Speakman, 2005). Moreover, these data suggest that the production of glycated proteins as well as hyperglycemia-mediated inflammation and oxidative stress, as opposed to elevated plasma glucose concentrations per se, are what promotes pathologies.

The goal of the present study was to identify factors that contribute to lower levels of protein glycation in avian species, despite their naturally high glucose concentrations. As a prior study described high protein turnover in birds (Jaensch, 2013), the present study utilized isolated albumin to eliminate this factor. Moreover, other data have described a lower concentration of plasma proteins in birds, which would result in less overall glycation (Bartholomew and Dawson, 1954). Thus, we examined protein glycation in HSA and CSA samples matched for protein concentration. The reversible exothermic nature of the protein glycation reaction suggests that temperature may impact the reaction. Therefore, all experiments were carried out at temperatures relevant for humans and chickens. Normal body temperature of birds is approximately 39–42 °C (Whittow, 1999), slightly higher than the normal body temperature of humans (Baumann and Baumann, 1977). Urbanowski et al. (1982) incubated purified human serum albumin in vitro for 24 h at temperatures ranging from 5 to 55 °C in the presence of constant concentrations of either p-galactose or d-glucose. The authors observed a positive correlation between incubation temperature and protein glycation with both sugars (Urbanowski et al., 1982). Considering the naturally low levels of protein glycation in birds, we hypothesized that chicken serum albumin would be more resistant to glycation compared to human serum albumin at any temperature, time or glucose concentration.

Albumin was chosen as it is the most abundant circulating protein (Kim and Lee, 2012; Singh et al., 2014) and it exhibits a relatively high degree of glycation when exposed to glycemic conditions over time (Austin et al., 1987). In addition, previous work has shown that the susceptibility of albumin to glycation does not differ greatly across mammalian species as it does for hemoglobin (Rendell et al., 1985). Albumin has a half-life of 12 to 21 days in humans (Rondeau and Bourdon, 2011). This is significantly shorter than the half-life of hemoglobin (Kim and Lee, 2012). Data show a strong correlation between the glycation of albumin over a 3-week period and that of hemoglobin over a 3-month period (Kim and Lee, 2012). Therefore, the use of albumin as a marker of protein glycation allows an assessment of blood glucose control over a shorter-period of time (Kim and Lee, 2012; Rondeau and Bourdon, 2011). The use of liquid chromatography (LC) and mass spectrometry (MS), known as LC-MS, to analyze protein glycation (Borges et al., 2011 & 2014) allows for higher throughput and greater sensitivity than prior analyses (Wuhrer et al., 2005) and has been widely implemented to measure percent glycated albumin (Borges et al., 2011; Brede et al., 2016; Dong et al., 2014; Rabban et al., 2016). Another advantage to this technique is the ability to pinpoint and quantify individual albumin molecules with two (i.e. doubly-glycated), rather than just one, glucose molecules attached. This level of precision in measurement allows for a more thorough and detailed examination of the extent of protein glycation in a sample that is not available using other methods (Wuhrer et al., 2005).

2. Material and methods

2.1. Materials

Purified albums were purchased from commercial suppliers. Chicken serum albumin (CSA) from Equitech-Bio, Inc., Kerrville, TX (Cat No. CSA62 Lot #CSA62-1254) and human serum albumin (HSA) from Sigma Aldrich, St. Louis, MO (Cat No. A3782 Lot #SLBD7204V). The reported purity was ≥96% for CSA and ≥99.9% for HSA. HEPES-buffered saline line solution prepared in deionized water was used as vehicle (134.3 mM NaCl (Cat. No. S9888, Sigma-Aldrich, St. Louis, MO), 6 mM KCl (Cat. No. P4504, Sigma, St. Louis, MO), 1.0 mM MgCl2 (Cat. No. M2670, Sigma-Aldrich, St. Louis, MO), 1.8 mM CaCl2 (Cat. No. C3881, Sigma-Aldrich, St. Louis, MO), and 9.14 mM NaHEPES (Cat. No. H3784, Sigma, St. Louis, MO)). A stock solution of 27.8 mM (0.5 mg/μL) d-glucose (Cat. No. G8270, Sigma-Aldrich, St. Louis, MO) was also prepared.

2.2. In vitro incubations

Following the preparation of 8.4 mg/mL HSA or CSA in HEPES-buffered saline, the pH was adjusted to 7.4 (VWR Symphony pH meter, Ross Electrode, Radnor, PA) and the solutions were sterilized using a syringe filter. Glucose stock (0.5 mg/μL) was then added to the albumin-HEPES solutions to achieve the following final concentrations of glucose (in mM): 0, 5.56 (normal human), 11.11 (normal avian), and 22.22. Aliquots (100 μL) of each solution were then transferred to PCR tubes and incubated in triplicate at 37.0 °C (normal human), 39.8 °C (normal avian, high human), and 41.4 °C (high avian) using a thermal cycler (MyCycler; BioRad, Hercules, CA) to set each temperature indefinitely. Aliquots (2 μL) were extracted from all samples after vortexing at baseline (Day 0, prior to incubation), Day 3, and Day 7 and were stored at −80 °C until analyses.

2.3. Measurement of percent glycated albumin by LC-ESI-MS

All aliquots were diluted 30:1 with 0.1% trifluoroacetic acid (TFA) and analyzed intact by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) on a Dionex Ultimate 3000 HPLC (Thermo Scientific, Sunnyvale, CA) equipped with a 1:100 flow splitter connected to a Bruker Maxis 4G quadrupole-time-of-flight (Q-TOF) mass spectrometer (Billericia, MA). A trap-and-elute form of LC-MS was carried out in which 15 μL solutions were loaded at 10 μL/min in 80/20 water/acetonitrile containing 0.1% formic acid (loading solvent) onto a Bruker-Michrom protein captrap configured for bi-directional flow on a 6-port diverter valve. The flow over the captrap was then switched to the micropump, set at 2 μL/min, and ramped over 5 min from 80% water containing 0.1% formic acid (Solvent A) / 20% acetonitrile (Solvent B) to 90% acetonitrile and held for 3 min. The captrap eluent was directed to the mass spectrometer operating in positive ion, TOF-only mode, acquiring spectra in the m/z range of 300 to 3000 with a nominal resolving power of ~60,000 m/Δm FWHM. ESI settings for the Agilent G1385A capillary microflow nebulizer ion source were as follows: End plate offset ~500 V, capillary ~3500 V, nebulizer nitrogen 2 bar, dry gas nitrogen 3.0 L/min at 225 °C. Data were acquired in profile mode at a digitizer sampling rate of 4 GHz. Spectra rate control was by summation at 1 Hz. HSA eluted over a period of about 1 min; under the above conditions, HSA ranged in charge state from +32 to +71. Raw mass spectra were averaged across this timeframe, smoothed 0.0482 Da, baseline subtracted 0.85, charge deconvoluted and baseline subtracted 0.85.
with Bruker DataAnalysis 4.1 charge deconvolution software to a mass range of 1000 Da on either side of any identified peak.

CSA eluted over a period of about 0.5 min; under the above conditions, CSA ranged in charge state from +32 to +64. Raw mass spectra were averaged across this timeframe, smoothed 0.0482 Da, baseline subtracted 0.85, charge deconvoluted and baseline subtracted 0.85 with Bruker DataAnalysis 4.1 charge deconvolution software to a mass range of 1000 Da on either side of any identified peak.

2.4. Percent glycated albumin calculation

The mass spectra obtained from the LC-ESI-MS analysis of each sample were used to calculate percent glycated serum albumin. Examples of the spectra for HSA and CSA examined under physiological conditions at Day 3 are shown in Fig. 1.

For HSA samples, the peak counts corresponding to the peaks for S-cysteinylated albumin, glycated S-cysteinylated albumin, and doubly glycated S-cysteinylated albumin obtained from the mass spectra (Fig. 1A) were used in the following formula to calculate percent glycated albumin: Percent Glycated Human Serum Albumin = [(glycated S-cysteinylated albumin + 2(doubly glycated S-cysteinylated albumin)) / (S-cysteinylated albumin + glycated S-cysteinylated albumin + doubly glycated S-cysteinylated albumin)] * 100.

For CSA samples, the peak counts corresponding to the peaks for native albumin, glycated albumin, and doubly glycated albumin obtained from the mass spectra (Fig. 1B) were used in the following formula to calculate percent glycated albumin: Percent Glycated Chicken Serum Albumin = [(glycated albumin + 2(doubly glycated albumin)) / (native albumin + glycated albumin + doubly glycated albumin)] * 100.

Fig. 1. Examples of spectra for HSA (A) and CSA (B) measured at Day 3 under physiological conditions.

Slightly different formulae were used for the calculation of percent glycation of HSA and CSA. This was because, unlike HSA, CSA does not have a S-cysteinylated form. The S-cysteinylated albumin form was used for the percent glycation of HSA calculation in lieu of the native albumin because the intensity of the S-cysteinylated peak in the samples tended to be stronger than that of the native peak, thus the S-cysteinylated peaks gave a more accurate reading. The underlying assumption was made that glycation of HSA occurs in equal proportion in native and S-cysteinylated forms.

2.5. Statistical analyses

Percent glycated albumin was measured on a total of 12 unique treatment conditions at three distinct time points for both HSA and CSA. All statistical analyses were performed using SPSS Statistics version 22. All data are expressed as mean ± SEM. Data was arcsine transformed prior to analyses to approximate normality. Data were normally distributed within each day for all albumin type, temperature, glucose concentration group based on results of the Shapiro-Wilk test of normality. All data were analyzed using 4-way repeated-measures analysis of variance (ANOVA) to examine the change in percent glycation of human and chicken serum albumin over time with each treatment and to identify significant interactions among all independent variables. Two-way repeated measures ANOVA tests were run to explore confirmed interactions and to confirm between group variations within specific time points. A Bonferroni procedure was used for all post hoc analyses. The sphericity assumption of all repeated measures ANOVA tests was met if the Greenhouse-Geisser Epsilon value was greater than or equal to 0.75; if the assumption was not met, then the Greenhouse-Geisser correction factor was used to adjust the degrees of freedom. A p-value ≤ 0.05 (alpha set to 0.05) was considered significant.

3. Results

3.1. Main effects of temperature, glucose concentration, and albumin type on percent glycated albumin across time

The results of the four-way repeated measures ANOVA test are summarized below in Table 1. There was no significant difference between albumin type (Human Serum Albumin and Chicken Serum Albumin) group means (p = 0.095). There was a statistically significant difference in temperature group means (p < 0.001); the mean percent glycated albumin for both chicken and human serum albumin at all glucose concentrations and on all days is significantly higher at 37.0 °C (p = 0.001) and at 39.8 °C (p = 0.038). With respect to glucose concentration group means, there was a statistically significant difference (p < 0.001). Specifically, the mean percent glycated albumin for both chicken and human serum albumin at all temperatures significantly increased as glucose concentration increased (p < 0.001 for all comparisons). Also, there was a statistically significant difference in means across time (p < 0.001). The mean percent glycated albumin for both chicken and human serum albumin at all temperatures and all glucose concentrations significantly increased across time points (p < 0.001 for all comparisons).

3.2. Interaction effects of temperature, glucose concentration, and albumin type on percent glycated albumin across time

All possible interactions, except for the interaction of albumin type x temperature x glucose concentration, were significant based on the results of the four-way repeated measures ANOVA test (Table 1). The statistically significant interaction between glucose concentration, temperature, albumin type, and time (p = 0.032) indicates that all independent variables interact to affect the mean percent glycation of albumin. Two-way repeated measures ANOVA were used to characterize
these interactions. Figs. 2, 3, and 4 directly compare the effect of varying glucose concentrations on the percent glycation of HSA and CSA at 37.0 °C, 39.8 °C, and 41.4 °C, respectively. As shown in the figures, as glucose concentration increased, the percent glycation of HSA and CSA increased over time at all temperatures. In addition, HSA was glycated to a greater extent in comparison to CSA at the two higher glucose concentrations examined for all temperature conditions.

As expected, percent glycation of HSA and CSA were not differentially affected by the absence of glucose (0 mM) in the solution at any temperature examined (p = 0.230 for 37.0 °C, p = 0.215 for 39.8 °C, and p = 0.065 for 41.4 °C). Both CSA and HSA decreased in percent glycation over time at all temperatures examined at the 0 mM glucose concentration (see Figs. 2A, 3A, and 4A). The percent glycation of HSA and CSA were differentially affected by the 5.56 mM glucose concentration at the two highest temperatures examined (p = 0.039 for 39.8 °C, and p = 0.002 for 41.4 °C). At both of these temperatures, CSA decreased in percent glycation over time while the percent glycation of HSA remained constant over time (see Figs. 2B, 3B, and 4B). The 11.11 mM glucose concentration differentially affected percent glycation of HSA and CSA at all temperatures (p < 0.001 for 37.0 °C, p < 0.001 for

### Table 1

Summary of four-way repeated measures ANOVA.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares (SS)</th>
<th>Df</th>
<th>Mean squares (MS)</th>
<th>F</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between subjects</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin type</td>
<td>0.006</td>
<td>1.000</td>
<td>0.006</td>
<td>1.901</td>
<td>0.174</td>
</tr>
<tr>
<td>Temperature</td>
<td>0.046</td>
<td>2.000</td>
<td>0.023</td>
<td>7.509</td>
<td>0.001</td>
</tr>
<tr>
<td>Glucose concentration</td>
<td>1.835</td>
<td>3.000</td>
<td>0.612</td>
<td>199.338</td>
<td>0.001</td>
</tr>
<tr>
<td>Albumin type × glucose concentration</td>
<td>0.294</td>
<td>3.000</td>
<td>0.098</td>
<td>31.972</td>
<td>0.001</td>
</tr>
<tr>
<td>Temperature × glucose concentration</td>
<td>0.125</td>
<td>6.000</td>
<td>0.021</td>
<td>6.764</td>
<td>0.001</td>
</tr>
<tr>
<td>Albumin type × temperature × glucose concen-</td>
<td>0.029</td>
<td>6.000</td>
<td>0.005</td>
<td>1.563</td>
<td>0.179</td>
</tr>
<tr>
<td>Error</td>
<td>0.147</td>
<td>48.000</td>
<td>0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within subjects</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>0.231</td>
<td>1.409</td>
<td>0.164</td>
<td>93.382</td>
<td>0.001</td>
</tr>
<tr>
<td>Time × albumin type</td>
<td>0.397</td>
<td>1.409</td>
<td>0.282</td>
<td>160.786</td>
<td>0.001</td>
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<tr>
<td>Time × temperature</td>
<td>0.023</td>
<td>2.818</td>
<td>0.008</td>
<td>4.608</td>
<td>0.006</td>
</tr>
<tr>
<td>Time × glucose concentration</td>
<td>1.294</td>
<td>4.227</td>
<td>0.306</td>
<td>174.583</td>
<td>0.001</td>
</tr>
<tr>
<td>Time × Albumin Type × temperature</td>
<td>0.022</td>
<td>2.818</td>
<td>0.008</td>
<td>4.444</td>
<td>0.008</td>
</tr>
<tr>
<td>Time × albumin type × glucose concentration</td>
<td>0.223</td>
<td>4.227</td>
<td>0.053</td>
<td>30.808</td>
<td>0.001</td>
</tr>
<tr>
<td>Time × temperature × glucose concentration</td>
<td>0.071</td>
<td>8.455</td>
<td>0.008</td>
<td>4.757</td>
<td>0.001</td>
</tr>
<tr>
<td>Time × albumin type × temperature × glucose</td>
<td>0.039</td>
<td>8.455</td>
<td>0.005</td>
<td>2.600</td>
<td>0.014</td>
</tr>
<tr>
<td>Error</td>
<td>0.119</td>
<td>67.639</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sphericity assumption was not met, thus the Greenhouse-Geisser correction factor was used to obtain the values presented for within subjects. Albumin type, human or chicken serum albumin. Temperature, 37 °C or 39.8 °C or 41.4 °C. Glucose concentration, 0 mM or 5.56 mM or 11.11 mM or 22.22 mM. Time, Day 0 or Day 3 or Day 7. *p ≤ 0.05.
Fig. 3. Percent glycation in response to varying glucose concentrations at 39.8 °C. Time 0 was measured immediately after preparing the samples. A: Changes in percent glycation of HSA (closed squares) and CSA (open squares) in the presence of 0 mM glucose (time, \( p < 0.001 \); albumin type, \( p = 0.002 \); interaction, \( p = 0.215 \)). B: Changes in percent glycation of HSA and CSA in the presence of 5.56 mM glucose (time, \( p = 0.294 \); albumin type, \( p = 0.011 \); interaction, \( p = 0.039 \)). C: Changes in percent glycation of HSA and CSA in the presence of 11.11 mM glucose (time, \( p = 0.004 \); albumin type, \( p = 0.325 \); interaction, \( p < 0.001 \)). D: Changes in percent glycation of HSA and CSA in the presence of 22.22 mM glucose (time, \( p < 0.001 \); albumin type, \( p = 0.226 \); interaction, \( p = 0.004 \)). Between group variations were confirmed with two-way repeated measures ANOVA and Bonferroni Post Hoc Analyses. *Indicates significant difference between mean percent glycation of HSA and CSA (\( p \leq 0.05 \)). All data are displayed as mean ± SEM with trendline. \( n = 3 \) per group.

Fig. 4. Percent glycation in response to varying glucose concentrations at 41.4 °C. Time 0 was measured immediately after preparing the samples. A: Changes in percent glycation of HSA (closed triangles) and CSA (open triangles) in the presence of 0 mM glucose (time, \( p < 0.001 \); albumin type, \( p < 0.001 \); interaction, \( p = 0.065 \)). B: Changes in percent glycation of HSA and CSA in the presence of 5.56 mM glucose (time, \( p = 0.026 \); albumin type, \( p = 0.009 \); interaction, \( p = 0.002 \)). C: Changes in percent glycation of HSA and CSA in the presence of 11.11 mM glucose (time, \( p = 0.002 \); albumin type, \( p = 0.096 \); interaction, \( p = 0.001 \)). D: Changes in percent glycation of HSA and CSA in the presence of 22.22 mM glucose (time, \( p = 0.001 \); albumin type, \( p = 0.029 \); interaction, \( p = 0.004 \)). Between group variations were confirmed with two-way repeated measures ANOVA and Bonferroni Post Hoc Analyses. *Indicates significant difference between mean percent glycation of HSA and CSA at the time point (\( p \leq 0.05 \)). All data are displayed as mean ± SEM with trendline. \( n = 3 \) per group.
39.8 °C, and p = 0.001 for 41.4 °C). At all temperatures examined the percent glycation of CSA remained constant over time, however, the percent glycation of HSA increased moderately over time (see Figs. 2C, 3C, and 4C). Finally, the 22.22 mM glucose concentration also differentially affected percent glycation of HSA and CSA at all temperatures (p = 0.001 for 37.0 °C, p = 0.004 for 39.8 °C, and p = 0.004 for 41.4 °C). The percent glycation of HSA and CSA both increased over time at all temperatures, however, the percent glycation of HSA increased to a greater extent (see Figs. 2D, 3D, and 4D).

Temperature had a differential effect on the percent glycation of HSA and CSA. The increase in percent glycation of HSA over time at the 11.11 mM glucose concentration and the 22.22 mM glucose concentration was significantly greater at higher temperatures compared to lower temperatures (see Figs. 2C, 3C, and 4C for 11.11 mM glucose and Figs. 2D, 3D, and 4D for 22.22 mM glucose). This differential effect of temperature at the two highest glucose concentrations examined was not significant for CSA.

4. Discussion

This study was designed to directly compare the effects of temperature, time and glucose concentrations on the glycation of HSA and CSA in an effort to elucidate factors that may protect avian albumin from glycation. Similar to other reports, the present study supports a positive relationship between temperature and percent glycation of HSA over time (Urbanowski et al., 1982). A positive relationship was also found for percent glycation of CSA with time with increasing temperatures. As expected, percent glycation also increased for both HSA and CSA with increasing glucose concentrations. Although HSA and CSA displayed these positive relationships with temperature and glucose, it is important to note that the degree of glycation observed between HSA and CSA under these conditions differed significantly.

Only a few studies have specifically examined the percent glycation of albumin in avian species. An early study by Rendell et al. (1985) found percent glycation of albumin was higher in several species of birds compared to humans. However, a more recent examination of the percent glycation of plasma albumin using more precise techniques found significantly lower levels in budgerigars (parakeets) compared to human plasma albumin (Holmes et al., 2001). Similarly, the present findings suggest that improved methods for quantification of percent glycation, including the ability to detect double and triple-glycated albumin, provides more accurate measurements of the true level of albumin glycation in humans and birds.

Our results reinforce the current understanding of protein glycation at physiological conditions in both humans and chickens (Brownlee et al., 1984; Ulrich and Cerami, 2001). When incubated at physiologically relevant conditions for human albumin (5.56 mM glucose at 37.0 °C), the percent glycation was at equilibrium, as levels remained constant over time. The percent glycation of HSA increased over time at glucose concentrations higher than physiological (11.11 mM and 22.22 mM glucose) while the percent glycation of CSA decreased over time at glucose concentrations lower than physiological (0.00 mM glucose). The same was true for the glycation of CSA. When incubated at physiologically relevant conditions for chicken albumin (11.11 mM glucose and 39.8 °C), the percent glycation of CSA was at equilibrium. The percent glycation of CSA increased over time at glucose concentrations higher than physiological (22.22 mM glucose) while the percent glycation of CSA decreased over time at glucose concentrations lower than physiological (0.00 mM glucose and 5.56 mM glucose).

The observed decrease in percent glycation from Day 0 to Day 7 at 0 mM glucose for both CSA and HSA, and at 5.56 mM glucose for CSA demonstrates the reversibility of the Schiff base formation reaction (Brownlee et al., 1984; Ulrich and Cerami, 2001). This de-glycation of HSA and CSA occurred at a similar rate at all temperatures examined.

Degradation of the proteins was observed over time during all incubations. Samples were diluted 30:1 for the LC-ESI-MS analyses, as described in the methods, thus the total signal count obtained from the mass spectrum for each sample is indicative of the quantity of albumin in the sample. The average total counts for HSA (S-cysteinylated albumin + glycated S-cysteinylated albumin + doubly glycated S-cysteinylated albumin) was 68,738 on Day 0, 53,797 on Day 3, and 30,392 on Day 7. The average total counts for CSA (native albumin + glycated albumin + doubly glycated albumin) was 85,295 on Day 0, 56,571 on Day 3, and 28,888 on Day 7. This decrease in total albumin is not due to loss to the glycated albumin pool as the average total counts for HSA and CSA include the glycated albumin pool. Based on the average total counts obtained from the mass spectrum of each sample, CSA degraded at a faster rate than HSA. Regardless, the percent glycation is a reflection of the amount of glycated albumin as a ratio of the total albumin present in each sample at each time point. The disparity in elution time observed between CSA and HSA, is likely due to differences in the relative affinity of the different albumin molecules to the hydrophobic protein captrap that was used; however, this is merely speculation and further research would be necessary to address this difference.

Unlike HSA, temperature was not found to have a significant effect on the percent glycation of CSA. This finding indicates that the reactant-favored chemical environment (i.e. higher body temperature of avian species compared to humans) is not the mechanism by which birds resist protein glycation. In fact, lack of effect of temperature on the percent glycation of CSA suggests that birds also resist the increase in protein glycation at higher temperatures than those seen in humans.

4.1. Strengths and limitations

The in vitro manipulations were conducted in a highly controlled environment and thus equalized the background matrix between the two types of albumin. This allowed for the direct comparison of the purified human and chicken albumin molecules at a range of physiologically relevant temperatures and glucose concentrations. The commercially purchased CSA and HSA are pooled samples from numerous individual animals, thus individual variation in the protein molecule is accounted for. Both HSA and CSA were highly purified reducing any potential influence of impurities. In addition, the use of LC-ESI-MS as the method for the quantification and calculation of percent glycation of both HSA and CSA is both precise and reliable. The ability to differentiate between singly and doubly glycated albumin is unique to this method and strengthens the accuracy of the data as compared to prior studies that only measured singly glycated albumin.

Despite the strengths associated with an in vitro experiment, there are inherent limitations as well. The in vitro environment does not adequately simulate in vivo conditions. Thus, there could be other factors that play a role in the in vivo setting that are not accounted for in an in vitro study. For example, other serum factors may affect protein glycation. In addition, the results of this study are not generalizable to the whole animal (either human or chicken) nor are they generalizable to any species not included in this study.

4.2. Conclusions

This study revealed that higher body temperature is not a mechanism by which birds protect against protein glycation. Specifically, temperature did not impact percent glycation of CSA, but temperature did impact percent glycation of HSA at the highest glucose concentrations. It was also shown that as glucose concentration increased, the percent glycation of both HSA and CSA increased. HSA was glycated to a greater extent when compared to CSA in the presence of high glucose concentrations.

The results of this study suggest there is an inherent difference between human and chicken albumin that contributes to the differential glycation. One possible explanation for this inherent difference is a structural variation in the chicken and human albumin proteins. The
chicken albumin protein may be folded in such a way that the amino acid residues (i.e. lysine) that glucose binds to are protected or shielded, thus decreasing the susceptibility of the protein to glycation. In line with the reported absence of the RAGE protein in birds, the current findings help to further elucidate how birds resist some of the major complications associated with high circulating glucose concentrations, such as oxidative stress and aging.

Declaration of interest

The authors have no conflicts of interest to report.

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