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Enhanced iron availability by protein glycation may explain higher infection rates in diabetics

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Abstract Serum proteins exist in a state of higher glycation among individuals with poor glycemic control, notably diabetics. These non-enzymatic modifications via the Maillard reaction have far reaching effects on metabolism and regulation, and may be responsible for increased infection rates within this population. Here we explore the effects of glycation on iron metabolism and innate immunity by investigating the interaction between siderophores and bovine serum albumin (BSA). Using a quartz crystal microbalance with dissipation monitoring to quantify association rates, glycated BSA exhibited a significantly reduced affinity for apo and holo enterobactin compared to a non-glycated BSA standard. Bacterial growth assays in the presence of BSA and under iron-limited conditions indicated the growth rate of enterobactin-producing *E. coli* increased significantly when the BSA was in a glycated form. The results, in addition to data in the literature, support the hypothesis that glycation of serum proteins may effectively increase the available free iron pool for bacteria in blood serum and weaken our innate immunity. This phenomenon may be partially responsible for higher infection rates in some diabetics, especially those with poor glycemic control.

Keywords Siderophores · Iron homeostasis · Albumin · Diabetes · Glycation · QCM

Introduction

A growing body of evidence indicates that diabetics, which comprise 23.6 million people or 7.8% of the US population, have higher infection risk (Prevention CfDCa 2008). Asymptomatic bacteriuria, lower extremity infections, surgical wound infections, and group B streptococcal infections all tend to be more common in diabetics (Boyko and Lipsky 1995; Peleg et al. 2007; Rayfield et al. 1982). Population-based data also indicate this group exhibits increased influenza and pneumonia mortality rates (Boyko and Lipsky 1995).

Over the past decades, there have been attempts to explain this increased susceptibility, implicating impairment of polymorphonuclear neutrophil function and the effects of glycemic control (Peleg et al. 2007; Rayfield et al. 1982; Rich and Lee 2005). Unfortunately, many of the results from these studies are not reproduced in later experiments or are confounded by differences in experimental methodology (Peleg et al. 2007). It may be inappropriate to reach for a single, exclusive explanation for what heightens infection risk in diabetics; however, studies

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of diabetic mouse models and of human patients have shown that diabetics with carefully controlled blood glucose are no more susceptible to bacterial infections than are healthy individuals (Peleg et al. 2007; Rayfield et al. 1982; Rich and Lee 2005). From this, blood glucose control appears to have a leading role in diabetic complications related to bacterial infection.

An important result of proper glucose control is the prevention of excessive glycation end-products, the formation of which is strongly dependent on the concentration and reactivity of the reducing sugars. Glycation occurs non-enzymatically between free carbonyls of sugars and amino groups of macromolecules by the Maillard reaction (Bierhaus et al. 1998). This reaction occurs naturally, and because the rate is directly dependant on sugar concentration (Suárez et al. 1989), highly glycated proteins will be abundant among those with poor control of their blood sugar levels.

Due to the importance of glycosylation for directing proper protein trafficking and function, it is conceivable that blood proteins and various receptors either lose or gain function and/or specificity with excessive glycation (Huebschmann et al. 2006; Li et al. 1996). Thus, modifications due to glycation likely present a unique opportunity to invading bacteria. The key to a successful bacterial infection is the ability of foreign cells to flourish in a host. The availability of iron, a rate limiting growth nutrient for bacteria, is critical to their survival and growth. Fortunately, endogenous concentrations of free iron are carefully regulated in humans and are as low as 10^{-18} M (Bullen et al. 2006). In response, bacteria have evolved an array of iron acquisition mechanisms, such as siderophores, that scavenge for iron (Raymond et al. 2003). In response to this bacterial adaptation, proteins like serum albumin and lipocalin-2 bind and sequester siderophores, impeding bacterial growth by preventing delivery of iron to bacterial cells. If the specificity of blood proteins for siderophores were diminished or lost, the ability to starve bacteria of iron and stave off infection would be compromised. As a result, normal and easily controlled infections would thrive and propagate even in the body's iron-limited environment.

In this paper we discuss how glycation of bovine serum albumin (BSA) results in a loss of affinity for siderophore scavengers. We propose that a similar change to human serum albumin (HSA), and other serum proteins like lipocalin-2 and transferrin, may disrupt the innate immune system and promote bacterial infection in diabetics by increasing the availability of bacterial growth-limiting micronutrients such as iron.

Materials and methods

Isolation and purification of enterobactin

Escherichia coli strain AN102, which lacks genes for the uptake of enterobactin, was obtained from the CGSC and grown in modified T-medium following the protocol outlined by Payne to produce apo-enterobactin (apo-ent) and ferric-enterobactin (Fe-ent) (Payne 1994). Enterobactin was purified in its apo form from the spent media following the procedure of Neilands (Neilands 1981). Apo-ent was either used immediately or complexed with iron to generate the holo form. UV–Vis spectroscopy was used to identify and quantify the different forms of enterobactin by measuring the absorbance at 495 nm (Harris et al. 1979).

Preparation of glycated BSA

Glycated BSA was prepared following the protocol of Kublashvili et al. then purified following the protocol of Day (Day et al. 1979; Kublashvili and Ugrekhelidze 2005). Because BSA is glycated at the same sites in vitro and in vivo, there was no need to isolate albumin from a live sample (Day et al. 1979). Protein glycation levels were monitored using a thiobarbituric acid assay and measuring 5-hydroxymethylfurfural release upon hydrolysis of ketoamine adducts of the protein at 443 nm (Fluckiger and Winterhalter 1976). BSA had 2.82 $\pm$ 0.03 sites modified, which is consistent with previously reported values (Mohamadi-Nejad et al. 2002).

Siderophore binding to BSA

Frequency and dissipation data were collected using a quartz crystal microbalance with dissipation (QCM-D) monitoring (E4, Q-Sense, Gothenburg, Sweden). QCM-D is a gravimetric technique that is able to measure the change in resonant frequency of a

piezoelectric crystal resonating at 4.95 MHz. By using Sauerbrey's equation, changes in resonant frequency are related to the change in mass on the surface (Sauerbrey 1959). The QCM-D sensor, mounted in a liquid flow cell (40 µl), consisted of an AT-cut piezoelectric quartz crystal disk operated at 4.95 MHz. Crystals were exposed to solution on the active SiO_2 surface (0.2 cm^2 in area, 50 nm thick) and were coated on the opposite side with a gold electrode (100 nm thick). All QCM-D crystals were optically polished with a root-mean-square roughness of less than 3 nm. Crystals were decontaminated by UV-ozonation for 10 min, treated with 2 vol% Hellmanex solution (Hellma GmbH & Co.) for 15–30 min, rinsed with ultrapure water (resistivity > 18 MΩ cm, Milli-Q), blown dry, and finally treated again with UV-ozonation before use. A stable baseline was obtained by flushing the QCM-D flow cell with phosphate-buffered saline (PBS) solution prior to polyelectrolyte layer formation. A peristaltic pump (Ismatec ISM935C) was used to flow solution through the cell at a constant rate of 100 µl/min. A 1 mM polyallylamine hydrochloride (PAH) solution in PBS was flowed through the cell to create a non-denaturing attractive surface onto which the BSA could adsorb. Following polyelectrolyte layer formation, the cell was flushed with PBS. Then glycated or non-glycated BSA was passed over the surface until saturation and rinsed with PBS. Lastly, solutions of apo-ent or Fe-ent were flowed over the sensor surface to measure their binding rate with the different forms of BSA and rinsed to observe their dissociation rate. Details of QCM-D principles and operation can be found elsewhere (Rodahl et al. 1995).

Bacterial growth assays with fetal bovine serum

XL10 *E. coli* were grown overnight at 37°C with 200 rpm shaking in T-medium supplemented with 40 mg/l each of proline, leucine, and tryptophan, 20 mg/l thiamin, 5 mg/l nicotinic acid, 100 mg/ml casamino acids, 17 mg/l $MnSO_4 \cdot H_2O$, and 15 µg/l tetracycline (Payne 1994). Cells were spun down the following morning, resuspended in fresh T-medium to remove siderophore contaminants and diluted to an OD_{600} ~0.5 before inoculating 50 ml cultures of T-medium containing either 10% native or glycated fetal bovine serum (FBS) (Gibco) with either 0.1 of 0.5 ml of cells. FBS was the only iron source added to the media. The final concentrations of essential iron binding proteins were 320 mg/l hemoglobin, 200 mg/l transferrin, and 1.7 g/l albumin. The concentrations of other essential iron metabolism proteins like lipocalin and ferritin were not determined by the manufacturer, but were likely present. Glycated FBS was generated by incubating stock solutions for 2 weeks anaerobically at 37°C with 15 µg/l tetracycline and 1 g/l glucose (the concentration of glucose already present in FBS). The native sample was generated by adding FBS to the T-medium immediately after being defrosted. No visible precipitate was observed after the incubation or defrosting. For each preparation of media, the final glucose concentration was normalized to 0.2 g/l. The growths were carried out at 37°C with 200 rpm shaking and monitored by taking optical density measurements over the course of 10–14 h. Each growth was repeated independently three times. The trends in the growth rates were consistent for each repetition.

Bacterial growth assays with BSA

Growth assays with BSA were conducted as described above with the following differences. T-medium was supplemented with 20 µM or 200 nM $FeCl_3$ and 1.7 g/l glycated or non-glycated BSA. T-medium containing no-BSA was used as a control. Each iron-limited growth was repeated three times. The different iron concentrations were chosen based on data from Wilhelm et al. to observe the prophylactic effect of BSA (Wilhelm et al. 1998).

Results

Bacterial growth with glycated FBS

E. coli was found to grow faster in media containing glycated FBS than native FBS (Fig. 1), which had maximum doubling rates of 0.90 ± 0.05 versus 0.72 ± 0.10 h^{-1}, respectively (unpaired *t*-test, *P*-value < 0.005). Given that FBS was the only iron source in the media, this observation suggested that glycated blood serum components (e.g., albumin, transferrin, and lipocalin) may have weakened affinities for essential micronutrients like iron and heme. The higher carrying capacity of the glycated FBS growth may also reflect a diminished ability of

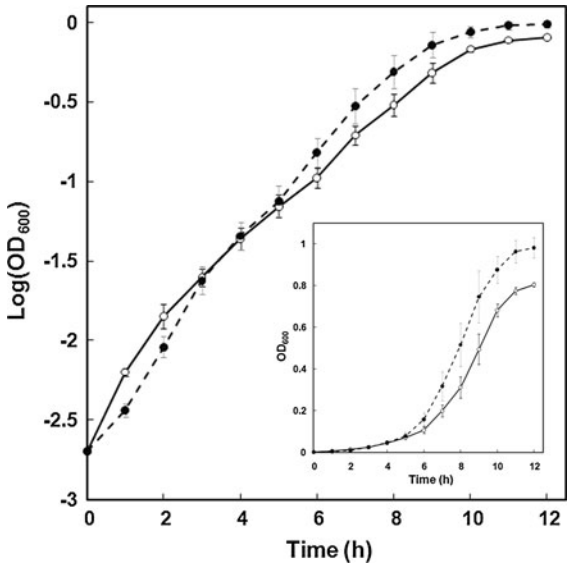

Fig. 1 **a** Semi-log plot of XL10 Gold *E. coli* growth in T-medium containing native FBS (*solid line*) or glycated FBS (*dashed line*). The *inset* depicts the growth as OD versus time. Each *curve* reflects the average of three independent repeats. *Error bars* are ±2 standard error of the mean

glycated FBS to sequester siderophores and hamper bacterial iron-scavenging mechanisms.

Enterobactin binding to BSA

To further explore the effects of glycation on serum proteins, specifically albumin since it is the major component of FBS, enterobactin binding to glycated and non-glycated BSA was measured. Shown in Fig. 2 is the raw QCM-D frequency data from the binding of Fe-ent and apo-ent to both glycated and non-glycated BSA. The first dip in frequency indicates creation of the BSA layer, and the following dip indicates the binding of the different enterobactin forms to BSA. Corresponding increases in dissipation confirm BSA layer formation and enterobactin binding (see supporting information). Subtracting the minimum frequency after binding enterobactin from the maximum frequency at the start of binding, and converting to a mass value using Saurebrey's equation, gives the maximum loading of enterobactin onto BSA, which is reported in Table 1 (Sauerbrey 1959). In both cases of Fe-ent and apo-ent, the decrease in maximum loading due to glycation of BSA was significant (unpaired *t*-test, $P < 0.05$ and $P < 0.005$, respectively).

The binding affinity between enterobactin and BSA was determined by transforming the raw data. Since the concentration of BSA was held constant, pseudo first order kinetics reliant on the concentration of enterobactin described the association step. Concentration independent first order kinetics were assumed to model the dissociation. These constants can be determined by relating the maximum enterobactin loading to the mass changes at each measurement time (Kawakami et al. 1999). Figure 3a, reports a slope that is the negative of the rate of association (M^{-1} s^{-1}) and Fig. 3b reports the rate of dissociation (s^{-1}). Dividing the association rate by the dissociation rate, we found the K_d (M) for the binding of enterobactin to different forms of BSA, reported in Table 1.

Overall, Fe-ent and apo-ent bind tighter to non-glycated BSA (9.81×10^{-6} and 4.06×10^{-5} M, respectively) than to glycated BSA (3.35×10^{-5} and 1.39×10^{-3} M, respectively). The affinities of BSA for Fe-ent and apo-ent as determined by QCM-D are in good agreement with previously reported values of 8.33×10^{-6} and 4.35×10^{-5} M, respectively (Konopka and Neilands 1984).

Effects of glycated and non-glycated BSA on *E. coli* growth

In low-iron media (200 nM $FeCl_3$) *E. coli* grew slower in the presence of non-glycated BSA than glycated BSA (Fig. 4a, b), with maximum doubling rates of 0.49 ± 0.03 and 0.62 ± 0.04 h^{-1}, respectively (unpaired *t*-test, $P < 0.05$). Control growths without BSA grew slightly faster with a maximum doubling time of 0.66 ± 0.01 h^{-1}, which is not significantly different from growths supplemented with glycated BSA (unpaired *t*-test, $P > 0.10$). In media containing 100-fold more iron, these trends disappear (Fig. 4c, d). The no-BSA control, glycated BSA growth, and non-glycated BSA growth each had maximum doubling rates of 0.96 ± 0.05, 1.04 ± 0.03, and 1.02 ± 0.05 h^{-1}, respectfully, which is not a statistically significant difference (ANOVA, $P > 0.05$). Combined, these results indicate *E. coli* growth in the low-iron media is rate-limited by the acquisition of iron through siderophores. Interestingly, glycation incurs a nearly 25% increase in the maximum doubling rate for both the FBS and BSA growths.

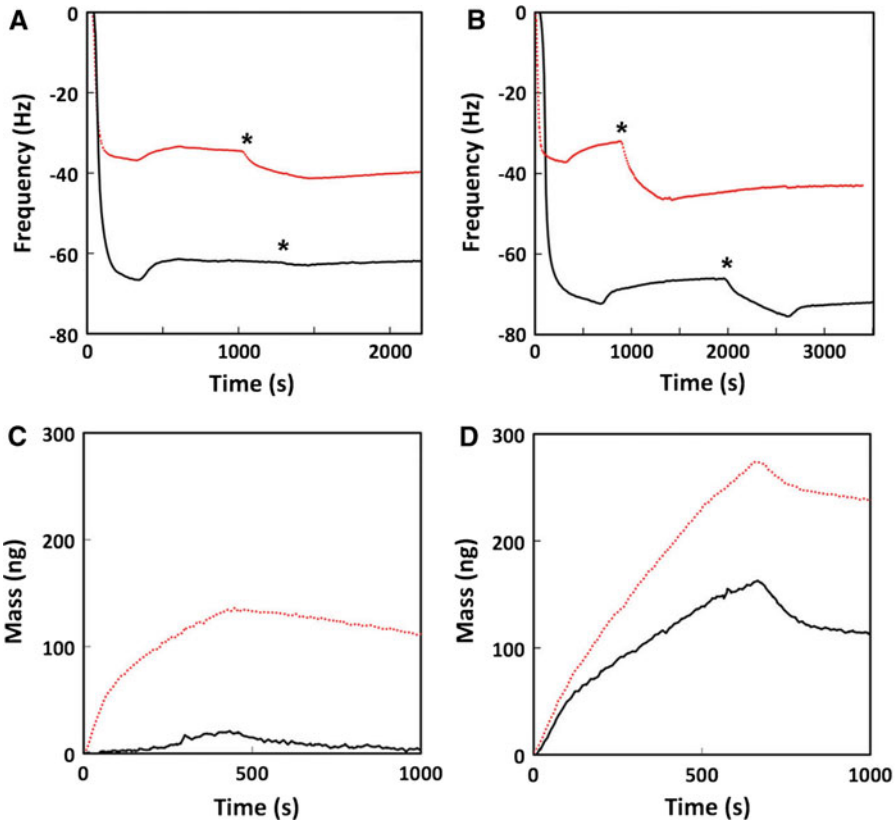

Fig. 2 Binding of Fe-ent (**a**), and apo-ent (**b**) to different forms of BSA. The crystal was pretreated with PAH to create a non-denaturing attractive surface for both forms of BSA. In each panel, the *bottom curve* represents binding with glycated BSA while the *top curve* depicts binding with unmodified BSA. The different forms of BSA were first deposited and rinsed with PBS. Next, Fe-ent or apo-ent were flowed over the surface (*asterisk*) followed by rinsing with PBS. **c** and **d** are the same data as in (**a**) and (**b**) starting from (*asterisk*) and transformed using the Sauerbrey equation to emphasize the mass binding of Fe-ent and apo-ent, respectively

Table 1 Enterobactin binding to BSA

BSA	Maximum loading (ng/cm^2)		K_d (M)	
	Fe-Ent	Apo-Ent	Fe-Ent	Apo-Ent
Non-glycated	280 (±28)	136.9 (±4.1)	9.81 (±1.5) × 10^{-6}	4.06 (±0.13) × 10^{-5}
Glycated	205 (±27)	24.7 (±1.1)	3.35 (±0.32) × 10^{-5}	1.39 (±0.23) × 10^{-3}

± Standard error

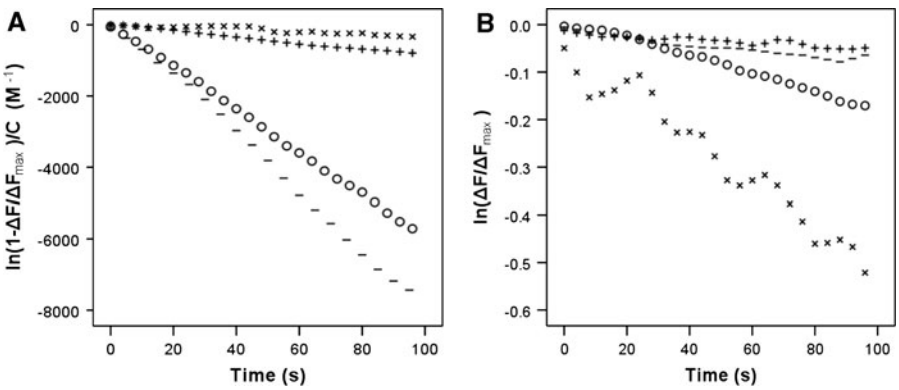

Fig. 3 Rates of association (**a**) and dissociation (**b**) for normal BSA binding to apo-ent (+) and Fe-ent (−) and for glycated BSA to apo-ent (*times symbol*) and Fe-ent (*open circle*)

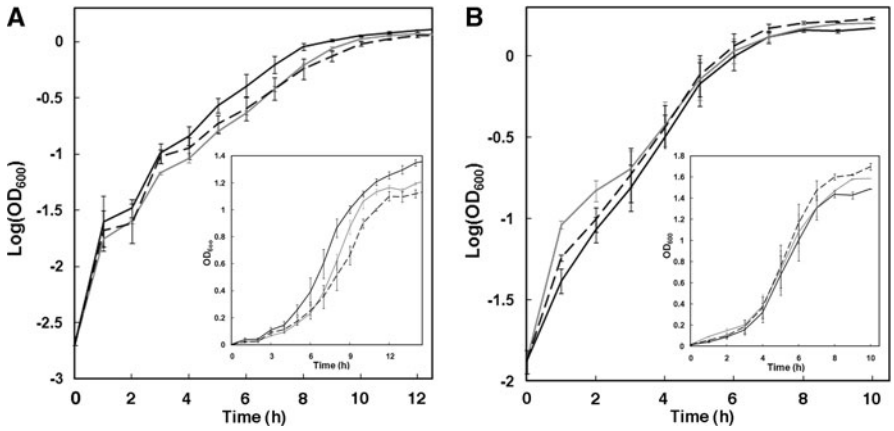

Fig. 4 The semi-log plots of XL10 Gold *E. coli* growth in T-medium containing **a** 200 nM or **b** 20 μM $FeCl_3$. The *dash lines* represent growth curves with medium containing 0.17 mg/ml native BSA, the *gray lines* represent growths with 0.17 mg/ml glycated BSA, and the *solid black line* represents control growths without BSA. *Insets* represent OD versus time plots of the data. Each *curve* reflects the average of three independent repeats. *Error bars* are ±2 standard error of the mean

Discussion

Our results suggest that glycating BSA diminishes its affinity for enterobactin, and allows enterobactin-dependent bacteria to grow more rapidly in iron-limited media. BSA and HSA are 78% identical, glycated at similar positions, and have similar affinities for apo and holo enterobactin (Konopka and Neilands 1984; Barnaby et al. 2011; Hinton and Ames 2006; Garlick and Mazer 1983). Therefore, glycating HSA will likely change its affinity for enterobactin as well. This could significantly impact diabetics, who have higher concentrations of glycated serum proteins. For example, less than 10% of the total HSA in healthy humans is non-enzymatically glycated while more than 20% is glycated in diabetics (Dolhofer and Wieland 1980; Shaklai et al. 1984). It has also been found that the kidneys of diabetics and the elderly lose the ability to selectively excrete glycated serum proteins, which further increases the concentration of glycated HSA in the bloodstream (Bakala et al. 1995). Increased concentrations of glycated HSA, which may have lost the prophylactic ability to bind siderophores, would allow bacteria to more readily acquire iron from its host. Since bacterial growth is normally limited by a lack of available micronutrients in human body, increased iron availability could help explain why some bacteria propagate more rapidly in diabetics. This is consistent with data showing bacteria are more virulent in humans with hereditary hemochromatosis due to the increased availability of iron (Bullen et al. 2006).

Interestingly, one way bacteria have been able to overcome iron starvation is by fashioning more effective siderophores. Salmonella, for example, glycosylate enterobactin to form salmochelins, which are better able to evade neutralization by reducing the compounds affinity for siderocalin and serum albumin (Miethke and Marahiel 2007). Similar to what is evidenced here, these bacteria also have increased growth rates under iron limiting conditions.

Considering the wide variety of siderophore structures, it is likely that different siderophores bind to different sites on HSA—or not at all. Therefore, reduced siderophore affinity for glycated HSA may only be true for some siderophores (Mereish et al. 1982). Ligand binding data with glycated and non-glycated HSA suggests the trend is not isolated to these initial findings. Although, hemin affinity for HSA is unaltered after glycation with glucose, the affinity for bilirubin is nearly halved (Shaklai et al. 1984). Similarly, the affinity of the long chain fatty acid *cis*-parnaric acid is reduced nearly 20-fold for the glycated protein relative to the non-glycated form (Shaklai et al. 1984). Studies also have shown that the binding capacity of albumin for salicylates decreases with glycation (Mereish et al. 1982).

Problems associated with glycation are not limited to serum albumin. Members of the lipocalin super-family, which bind many small molecules including

siderophores, are known to be significantly glycated endogenously (Bonay et al. 1997). Glycation of lipocalin-2 and serum albumin would render these serum proteins less effective at preventing siderophores from scavenging iron and thereby indirectly weaken innate immunity. Glycation can also compromise serum proteins essential for iron homeostasis. Glycated transferrin (Tf) from diabetic rat serum has been shown to have weakened iron binding capacity and more readily generate reactive oxygen species when iron is bound (Fujimoto et al. 1995). Likewise, HbA_{1c}, the major glycated hemoglobin, has weaker heme-globulin affinity than its non-glycated form (Sen et al. 2005). Peroxide-induced iron release from myoglobin is exacerbated by glycation (Roy et al. 2004). Given the available data, we expect other glycated heme and iron-scavenging proteins such as haptoglobin and hemopexin to have their effectiveness disrupted. Complicating matters further, it is possible that glycated serum proteins responsible for facilitating iron uptake by tissues, such as Tf, may not bind to membrane bound receptors as efficiently. These data, together with our findings, suggest that weakened ligand binding due to glycation may explain, in part, why there is an increase in free iron for type 2 diabetics with poor glycemic control but not for those with good glycemic control (Shetty et al. 2008).

If the glycation of proteins, such as HSA, is significant in predisposing diabetics to infection then there should be data showing a relationship between glycemic control and infection. Evidence supporting this relationship has been found when measuring mean plasma glucose but not when measuring HbA_{1c} (Davis et al. 1995; Peleg et al. 2007). This discrepancy can be explained by differences in the lifetimes and relative rates of glycation of physiologically relevant proteins.

The physiological rate of protein glycation is a function of many variables, but relative values can be determined simply by comparing the percent of glycated protein and their lifetimes (Tessier 2010). The lifetime of HbA_{1c} is 90–120 days and its concentration is often used to gauge average blood sugar levels over the preceding 3 months (Davis et al. 1995). A healthy, non-diabetic has HbA_{1c} levels between 4.0 and 5.9% of the total hemoglobin, and the American Diabetes Association recommends that HbA_{1c} levels in diabetics should be controlled to less than 7% (Executive summary 2009). Serum albumin has a lifetime of 12–19 days and over 20% of the total protein has been shown to be glycated in a moderate diabetic, over 94% in a diabetic with critically poor glycemic control, and less than 10% for a non-diabetic (Dolhofer and Wieland 1980; Kisugi et al. 2007; Nguyen et al. 2006; Shaklai et al. 1984). Another serum protein with a short-lifetime, protein HC (human complex-forming glycoprotein heterogeneous in charge), is a member of the lipocalin superfamily that has been found to be glycated around 35% in normal, non-diabetics (Bonay et al. 1997). HSA and protein HC are more glycated than HbA_{1c}, even though they have shorter lifetimes, suggesting their rate of glycation is faster. Therefore, periods of hyperglycemia are likely to have a greater impact on the amount of glycated HSA and protein HC than on HbA_{1c} because differences in the rates of glycation will further increase proportional to sugar concentration.

By contrast, the amount of glycated HbA_{1c} results from averaging months of blood sugar levels and are therefore insensitive to short periods of hyperglycemia since they can be counterbalanced by long periods of good glycemic control or hypoglycemia. This means that periods of hyperglycemia can occur in patients with either high or low levels of HbA_{1c}. In either scenario, measurements of HbA_{1c}, unlike measurements of mean plasma glucose, would not indicate periods of significantly increased amounts of rapidly glycated, short-lifetime proteins (Roohk and Zaidi 2008). That a relationship between glycemic control and infection is present only when accurately taking into account periods of hyperglycemia further supports our hypothesis that the increased glycation of serum proteins important for sequestering iron, such as HSA and Tf, helps to promote bacterial infection in diabetics by increasing the availability of iron to bacteria.

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