Analysis of the Metabolic Effects of Diabetes on the Structure and Function of Glycated Human Serum Albumin

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Introduction

Diabetes is a metabolic disease that affects 25.8 million people in the United States and 366 million people worldwide. This disease is related to a number of health disorders, which can result from insulin deficiency or a resistance to insulin. One of the major problems associated with diabetes is the elevated level of glucose that occurs in the bloodstream, which can lead to the non-enzymatic glycation of proteins. Recent studies have indicated that this modification can affect the structure and function of serum transports such as human serum albumin (HSA). During glycation, a nucleophilic reaction between a reducing sugar such as glucose and a free amine group on a protein can occur to form modified proteins such as glycated HSA (gHSA), as shown in **Fig. 1**.

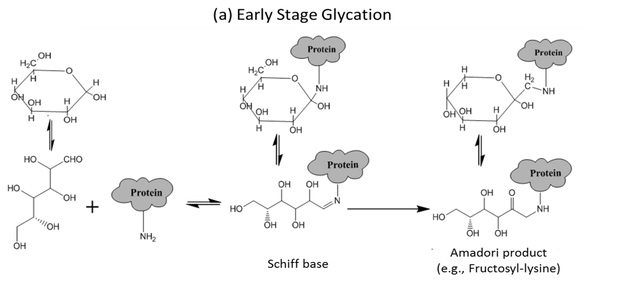

Figure 1: General reactions involved in the glycation of HSA, including both early stage glycaton (e.g., as illustrated through the formation of fructosyl-lysine in the presence of glucose) and advanced stage glycation (e.g., as illustrated in the formation of an advanced glycation end-product through the reaction of α-oxaloaldehyde with HSA)

Goals of Study

The purpose of this study was to characterize the metabolic effects of diabetes on the structure and function of HSA. High-performance affinity chromatography (HPAC) was used to examine the binding of various sulfonylurea drugs to *in vitro* and *in vivo* glycated HSA samples. Mass spectrometry (MS) experiments based on matrix-assisted laser desorption/ionization time-of-flight MS, nano-electrospray ionization time-of-flight MS and MS/MS were used to obtain qualitative and quantitative structural information on the glycation-related modifications that were occurring on the glycated HSA samples. The long-term goal is to use such information to better understand the metabolic effects of diabetes on drug binding with HSA and the effects of glycation on the function of this protein, which could be used in the future to develop improved treatment regimens for patients with diabetes.

Drug Binding Studies by High-Performance Affinity Chromatography

Sulfonylurea drugs are a class of drugs that are commonly used to treat type II diabetes (see **Fig. 2**). These drugs are known to bind tightly to serum proteins and particularly to HSA. High-performance affinity chromatography (HPAC) was used examine the changes in drug-protein-interactions with these drugs caused by glycation (as shown in **Figs. 3-4**) . Zonal elution competition studies were used to profile the binding by each sulfonylurea drug to normal HSA and samples of *in vitro* or *in vivo* glycated HSA. In these experiments, *R*-warfarin was used as a site-selective probe for Sudlow site I of HSA and L-tryptophan was used as a site-selective probe for Sudlow site II. In zonal elution competition studies, a fixed concentration of a probe (e.g., R-warfarin or L-tryptophan) was injected onto a column in the presence of a competing agent (i.e., a sulfonylurea drug) in the mobile phase (see **Fig. 4**). If direct competition took place between the probe and the competing agent at a single site, Eq. 1 was then used to describe this process and to examine binding at the site of competition by using plots like those shown in **Figs. 5(a-b**). The results were then used to find the equilibrium association constants for these agents at the site of competing agent, as summarized in **Tables 1** and **2**.

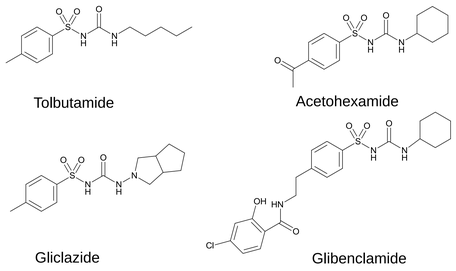

Figure 2: Examples of first-generation sulfonylurea drugs (e.g., tolbutamide and acetohexamide) and second-generation sulfonylurea drugs (e.g., gliclazide and glibenclamide).

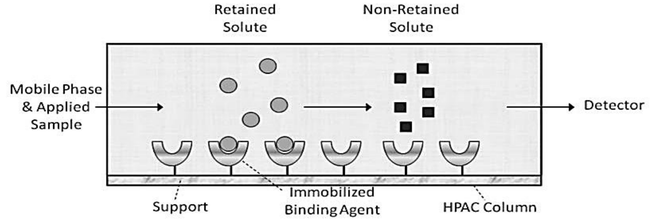

Figure 3: General scheme for using an immobilized binding agent in an HPAC column to recognize and separate a target from other, non-retained sample components.

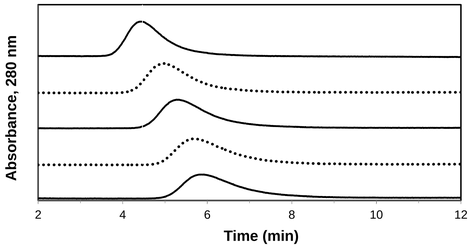

Figure 4: Typical zonal elution competition studies on a normal HSA column using *R*-warfarin as an injected site-specific probe and gliclazide as a mobile phase additive. The results are for gliclazide concentrations of 20, 10, 5, 1 or 0 μM (top to bottom).

Results

The results from the competition studies indicated that each sulfonylurea drug had direct competition with the site-specific probes for Sudlow sites I and II on HSA and glycated HSA samples. The association equilibrium constants determined for the sulfonylurea drugs at Sudlow Sites I and II on normal HSA are shown in **Table 1**. **Table 2** summarizes the trends in binding affinity for these drugs at Sudlow sites I and II for *in vitro* glycated HSA samples with various levels of glycation and for isolated samples of *in vivo* glycated HSA. The binding affinity ranged from a 0.6- to 6- fold change in comparison to normal HSA for the various sulfonylurea drugs during experiments involving the *in vitro* glycated HSA samples. The difference in binding affinity varied from one drug to the next and between the interaction sites. Similar results were seen for the *in vivo* glycated HSA samples, in which up to a 1.8-fold change in binding affinity was seen in some cases. In experiments involving glibenclamide, additional competition studies were performed with digitoxin for normal HSA and the *in vitro* glycated HSA samples; these results may have shown a small decrease in affinity but the values were not significantly different.

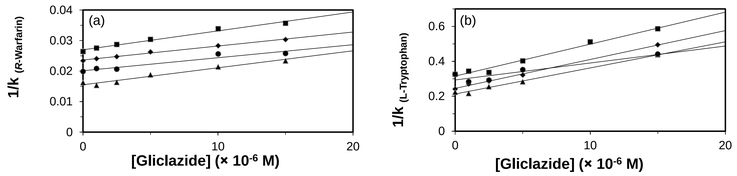

Figure 5: Plots prepared according to Eq. (1), showing how the reciprocal of the retention factor for (a) R-warfarin or (b) L-tryptophan changed on HSA or glycated HSA columns as the concentration of gliclazide was varied in the mobile phase. These results are for normal HSA (♦), and three samples of *in vitro* glycated HSA: gHSA1 (■), gHSA2 (▲), and gHSA3 (●).

Sulfonylurea Drug	Sudlow Site I ($\times 10^4$ M^{-1})	Sudlow Site II ($\times 10^4$ M^{-1})
Acetohexamide	4.2 (± 0.4)	13.0 (± 0.1)
Tolbutamide	5.5 (± 0.2)	5.3 (± 0.2)
Gliclazide	1.9 (± 0.1)	6.1 (± 0.3)
Glibenclamide	2.4(± 0.3)	3.9(± 0.2)

Table 1: Association equilibrium constants measured at pH 7.4 and 37 ºC for sulfonylurea drugs at Sudlow sites I and II of normal HSA

$$\frac{1}{k} = \frac{K_{aI} V_M [I]}{K_{aA} m_L} + \frac{V_M}{K_{aA} m_L}$$

Equation 1: Direct Competition Model

Drug & binding site	gHSA1	gHSA2	gHSA3	CS1	CS2
Sudlow site I					
Acetohexamide	↑ 1.4-fold	N.S. (↓ 10%)	N.S. (< 5%)	↑ 1.1-fold	N.S. (< 5%)
Tolbutamide	↑ 1.3-fold	↑ 1.2-fold	↑ 1.2-fold	↑ 1.2-fold	↑ 1.4-fold
Gliclazide	N.S. (< 10%)	↑ 1.9-fold	N.S. (↑ 10%)	↑ 1.2-fold	↓ 0.7-fold
Glibenclamide	N.S. (< 5%)	↑ 1.7-fold	↑ 1.9-fold	N/A	N/A
Sudlow site II					
Acetohexamide	↓ 0.6-fold	↓ 0.8-fold	N.S. (< 10%)	N.S. (< 5%)	↑ 1.3-fold
Tolbutamide	↑ 1.1-fold	↑ 1.4-fold	↑ 1.2-fold	↑ 1.4-fold	↑ 1.5-fold
Gliclazide	↓ 0.8-fold	↑ 1.3-fold	↓ 0.6-fold	↑ 1.8-fold	↑ 1.5-fold
Glibenclamide	↑ 4.3-fold	↑ 6.0-fold	↑ 4.6-fold	N/A	N/A

Table 2: Changes in the binding of various sulfonylurea drugs at Sudlow sites I and II when comparing *in vitro* glycated HSA (gHSA1-gHSA3) or *in vivo* glycated HSA (CS1-CS2) with normal HSA..

Mass Spectrometry

Mass spectrometry (MS) was used to investigate the structural changes that occurred during the glycation of HSA. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS was used to provide qualitative and quantitative structural information about the glycation-related modifications that occurred on this protein. These results allowed for the identification of possible modifications on HSA as it was glycated. Qualitative analysis was used to determine the location of these modifcations and determine which modifications occurred near Sudlow sites I or II. Quantitative analysis using isotopic labeling was also used to compare the extent to which these various modifications were occurring throughout the structure of HSA. This latter process (shown in **Fig. 6**) allowed for a comparison of all types of modifications by looking at the amount of non-modified peptides that remained versus a control sample. The results from the quantitative analysis showed that significant amounts of modifications were taking place at or near Sudlow sites I and II, as shown in **Fig. 7**. At Sudlow site I, lysine residues K199, K150, K160, K275 and K281 and arginine residues R209 and R257 (AGEs) showed high or moderate susceptibility to glycation-related modifications. Residues K338, K439, and R428 (via AGE formation) showed high or moderate susceptibility to glycation-related modifications at Sudlow site II. Nano-electrospray ionization time-of-flight (Nano-ESI-TOF) MS was then used to provide lower limits of detection and better sequence coverage for quantitative studies for glycated HSA. Preliminary studies using this approach with normal HSA has already provided a 89.2% sequence coverage for an unfractionated HSA sample that was digested with LysC, GluC and trypsin, as shown in **Fig. 8**.

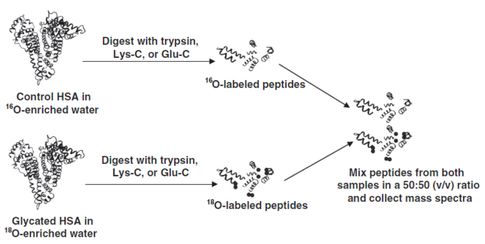

Figure 6: Experimental methods involved in the isotopic labeling of HSA. Normal HSA was reconstituted in ^{16}O enriched water and glycated HSA was reconstituted in ^{18}O enriched water. The samples were then digested using trypsin, Lys-C, or Glu-C. The samples were mixed in a 50:50 (v/v) and a mass spectrum was collected for the mixture .

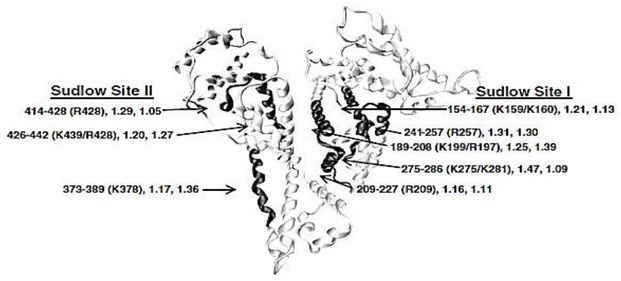

Figure 7: Structure of HSA that shows the locations of Sudlow sites I and II and regions that are highly modified by glycation.

DAHKSEVAHR	FKDLGEENFK	ALVLIAFAQY	LQQCPFEDHV
KLVNEVTEFA	KTCVADESAE	NCDKSLHTLF	GDKLCTVATL
RETYGEMADC	CAKQEPERNE	CFLQHKDDNP	NLPRLVRPEV
DVMCTAFHDN	EETFLKKYLY	EIARRHPYFY	APELLFFAKR
YKAAFTECCQ	AADKAACLLP	KLDELRDEGK	ASSAKQRLKC
ASLQKFGERA	FKAWAVARLS	QRFPKAEFAE	VSKLVTDLTK
VHTECCHGDL	LECADDRADL	AKYICENQDS	ISSKLKECCE
KPLLEKSHCI	AEVENDEMPA	DLPSLAADFV	ESKDVCKNYA
EAKDVFLGMF	LYEYARRHPD	YSVVLLLRLA	KTYETTLEKC
CAAADPHECY	AKVFDEFKPL	VEEPQNLIKQ	NCELFEQLGE
YKFQNALLVR	YTKKVPQVST	PTLVEVSRNL	GKVGSKCCKH
PEAKRMPCAE	DYLSVVLNQL	CVLHEKTPVS	DRVTKCCTES
LVNRRPCFSA	LEVDETYVPK	EFNAETFTFH	ADICTLSEKE
RQIKKQTALV	ELVKHKPKAT	KEQLKAVMDD	FAAFVEKCCK
ADDKETCFAE	EGKKLVAASQ	AALGL	

Figure 8: Nano-ESI-TOF MS was used to obtain the sequence coverage for normal HSA that was digested with Lys-C, Glu-C, and Trypsin

Conclusion

This study examined the metabolic effects of diabetes on the structure and function of glycated HSA. Binding studies by HPAC indicated that there can be significant changes in the site-specific affinities of sulfonylurea drugs with glycated HSA. These binding studies are now being expanded to include the use of free fraction analysis to see how glycation may alter the effective dose of these drugs. Mass spectrometry studies through MALDI-TOF MS were used to investigate glycation-related modifications that may occur on HSA, which included an examination of both types and locations of these modifications. Nano-ESI-TOF MS was used to provide a more detailed analysis of these glycation-related modifications. Future work is planned with MS/MS methods to further investigate these glycation-related modifications.

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