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Role of oxidized Met111 of human serum albumin for an oxidative stress biomarker in diabetes

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Background: Different types of oxidative stress biomarkers have been identified, and yet very few have made it into routine clinical use. We recently found that oxidized methionine (Met) residues of human serum albumin can be used as clinical markers that reflect the intravascular oxidative stress status.
Methods: We quantified oxidized and non-oxidized Met at position 111 of human serum albumin (Alb[Met^{111}]) by liquid chromatography-high resolution mass spectrometry using stable isotope-labeled peptides and evaluated their correlation with clinical parameters in diabetes theoretically known to induce oxidative stress.
Results: A group of patients with diabetes showed enhanced levels of Alb[Met^{111}] oxidation. Glycated albumin/glycated hemoglobin ratio, known to reflect glycemic fluctuations, independently influenced Alb[Met^{111}] oxidation. Continuous glucose monitoring revealed that Alb[Met^{111}] oxidation correlated with the standard deviation of sensor glucose concentrations and the time spent with hyperglycemia each day. However, none of the serum biochemical parameters known to affect oxidative stress status, such as creatinine, high-density lipoprotein cholesterol, or serum bilirubin, were associated with the oxidation status of Alb[Met^{111}].
Conclusions: Oxidized Alb[Met^{111}] was closely associated with the glycemic variability of diabetic patients; however, it may have limited power to detect the degree of intravascular oxidative stress induced by endogenous antioxidants or renal failure.

Key words: oxidative stress, diabetes mellitus, liquid chromatography-mass spectrometry, biomarker, glycemic fluctuation

Introduction

Oxidative stress is now regarded as a prominent feature of a number of major human diseases[1-7] and of the normal aging process.[8,9] A plethora of markers and methods have been developed and applied to a variety of human diseases in an attempt to measure the extent and nature of oxidative stress.[10,11] However, demonstration of definitive evidence for the association remained unattainable, because many of these are not specific and do not reflect a state of oxidative stress or correlate well with each other.[10,11]

Methionine (Met) is vulnerable to oxidation, resulting in formation of Met sulfoxide (MetO) under conditions of oxidative stress.[12] Oxidation of Met to MetO is a reversible reaction *in vitro*, which is catalyzed by MetO reductases.[13-15] Because Met is one of the most easily oxidized amino acids, and the reduction of MetO is also a very common reaction, their quantification has long been regarded as an inefficient strategy to assess the degree of oxidative stress.[16] However, we found that quantification of serum tryptic peptides containing oxidized and non-oxidized Met residues can be very stably and reproducibly measured using liquid chromatography-high resolution mass spectrometry (LC-MS).[17] This may be because of the presence of MetO reductase inhibitors in human blood,[18] although exact proteins inhibiting MetO reductases have not been identified to date.

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We recently succeeded to develop a novel technique to accurately quantify serum tryptic peptides containing Met and MetO using a single drop of human serum prespiked with stable isotope labeled peptides and demonstrated that quantification of oxidized and non-oxidized Met at position 147 of human serum albumin (Met^{147}) can detect intravascular oxidative stress status elicited by blood glucose fluctuations, hypoglycemia, and hyperglycemia in diabetes, as well as that affected by endogenous factors such as serum bilirubin and high-density lipoprotein (HDL) cholesterol.[19] In the present study, we assessed whether or not Met at position 111 of human serum albumin (Met^{111}) can also be used to detect the intravascular redox status of patients with diabetes and be used as a clinical biomarker.

Patients and Methods

Study design

We reanalysed all the clinical serum samples that we used in our previous study, in which we quantified oxidized Alb[Met^{147}] using stable isotope-labeled peptide by pretreating with excess L-cysteine (L-Cys) and L-methionine (L-Met).[19] Briefly, the study population consisted of 40 healthy volunteers (25 males and 15 females, 53.2 ± 16.4 years), and 124 diabetic patients (70 males and 54 females, 54.3 ± 13.9 years, 94 with type 2 diabetes and 30 with type 1 diabetes). Seven non-diabetic volunteers and 28 diabetic patients underwent continuous glucose monitoring (CGM) using the iPro 2 CGM system (Medtronic Minimed, Northridge, CA, USA)[20,21] for 4－7 days and provided an overnight-fasted blood sample during the period. The complete sensor glucose level (SGL) data were used to assess each glucose profile. The standard deviation (SD) and % coefficient of variation (%CV) were used to assess glycemic fluctuations, and the maximum, minimum and mean SGL were used to evaluate glycemic control. The protocol was approved by the Kitasato University Medical School/Hospital Ethics Committee (B17-040, B15-181) and written informed consent was obtained from all participants. All study protocols were performed in accordance with the relevant guidelines and regulations of Kitasato University Medical School as well as the Ethical Guidelines for Medical and Health Research Involving Human Subjects in Japan and under the Code of Ethics of the Helsinki Declaration.

Spiking stable isotope-labeled peptides, trypsin digestion, and LC-MS analyses

The following amino acids containing ^{13}C and ^{15}N were uniformly labeled with 9-fluorenylmethoxycarbonyl (FMOC) and used to synthesize two stable isotope-labeled peptides at Scrum (Tokyo): L-Alanine-N-FMOC ($^{13}C_3$, 98%; ^{15}N, 98%); and L-Lysine-N-FMOC ($^{13}C_6$, 98%; $^{15}N_2$, 98%). The amino acid sequences of the two stable isotope-labeled peptides were ETYGEMADC (Carbamidomethyl) C (Carbamidomethyl) A<u>K</u> (SI-Alb[Met^{111}]), ETYGEM (Oxidation) ADC (Carbamidomethyl) C (Carbamidomethyl) <u>AK</u> (SI-Alb[Met^{111}O]), with the underlined amino acids containing stable isotope. All serum samples were digested with trypsin by prespiking SI-Alb[Met^{111}] and SI-Alb[Met^{111}O] and by pretreatment with excess L-Cys to prevent the carbamidomethylation of N-terminal amino acid residues and completed by the addition of L-Met immediately after the enzyme digestion reaction to inhibit the spontaneous oxidization of Met.[19,22] Trypsin-digested serum samples were analyzed using LC-MS to quantify Alb[Met^{111}] and Alb[Met^{111}O] as described.[19] The extracted ion chromatogram (XIC) intensities for the two endogenous tryptic peptides, Alb[Met^{111}] and Alb[Met^{111}O], and the corresponding stable-isotope-labeled peptides, SI-Alb[Met^{111}] and SI-Alb[Met^{111}O] were used to extrapolate the serum concentrations of Alb[Met^{111}] ($C_{Alb(Met^{111})}$) and Alb[Met^{111}O] ($C_{Alb(Met^{111}O)}$) from the XICs generated using the respective endogenous peptides and the corresponding spiked stable isotope-labeled peptides as described previously.[19] The oxidation ratio for Met^{111} was obtained by dividing $C_{Alb(Met^{111}O)}$ by $C_{Alb(Met^{111})}$.

Statistical analyses

Data are expressed as mean ± SD. The Mann-Whitney U test was used to compare $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$ values between diabetic and non-diabetic groups. The comparison, assessing the effects of treatment on the $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$ level, was performed using the Wilcoxon signed-rank test for paired data. Linear regression models were used to compare the $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$ values and to determine the correlations with age, sex, body mass index (BMI), glycated albumin (GA), glycated hemoglobin (HbA_{1c}), GA/HbA_{1c} and biochemical parameters. Multivariate analyses were performed essentially as described,[19] except that age, sex, BMI, GA/HbA_{1c}, eGFR, uric acid, total bilirubin, HDL cholesterol and the use of metformin, a statin or an angiotensin-converting enzyme inhibitor/angiotensin II receptor blocker were used as explanatory variables, and $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$ was used as the objective variable. All analyses were performed using GraphPad Prism 5.02 software (GraphPad Software Inc. San Diego, CA, USA)

and/or JMP ver. 5.0.1a (SAS, Cary, NC, USA). $P < 0.05$ was considered to represent statistical significance.

Results

We first measured $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$ in 40 healthy volunteers and compared the results with 124 diabetic patients. There was no statistically significant difference in $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$ levels between diabetic and non-diabetic populations although some of the patients with diabetes showed distinctly high levels (Figure 1). Single regression analysis using the results of all the 164 participants revealed that GA/HbA_{1c}, uric acid, HDL cholesterol positively correlated with $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$ levels. BMI and triglyceride negatively correlated with $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$ (Table 1). Least square multivariate analysis was undertaken using these statistically significant parameters as explanatory variables, as well as those reported to have an antioxidant activity, and revealed that sex and GA/HbA_{1c} significantly and independently influenced $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$ level (Table 2).

Of all study participants, 35 (17 men and 18 women; 28 diabetic and 7 non-diabetic participants; 47.2 ± 15.5 years) had their $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$ measured while undergoing CGM. The SD, %CV and the mean SGL were calculated over 4−7-day monitoring periods. The periods of time during each day the participant was hypoglycemic (SGL <70 mg/dl), normoglycemic (70 mg/dl < SGL <140 mg/dl) and hyperglycemic (140 mg/dl < SGL) were also calculated exactly as we reported previously with $C_{Alb(Met^{147}O)}/C_{Alb(Met^{147})}$.[19] The SD significantly correlated with $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$ (SD: $P = 0.0452$, $r = 0.3407$) (Figure 2A) and the length of the hyperglycemic periods positively correlated with

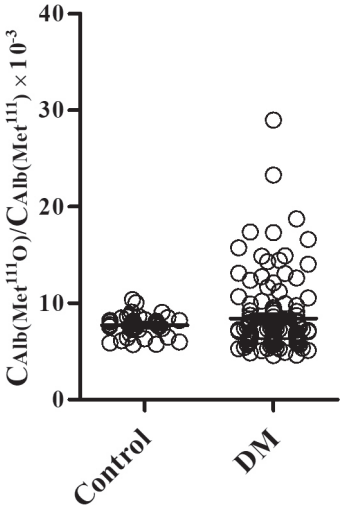

Figure 1. Levels of Met oxidation in diabetic and non-diabetic participants

The serum concentrations of Alb(Met^{111}) ($CAlb(Met^{111})$) and Alb($Met^{111}O$) ($C_{Alb(Met^{111}O)}$) were determined using the XICs generated by LC-MS analyses of Alb(Met^{111}), Alb($Met^{111}O$), SI-Alb(Met^{111}), and SI-Alb($Met^{111}O$) peptides. $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$ was determined in 40 healthy volunteers (Control) and 124 diabetic participants (DM).

Table 1. Correlations between the serum level of methionine oxidation and other parameters (univariate analyses)

Parameters	r	P
Age (years)	-0.0344	0.6623
Male: Female		0.0017
BMI	-0.3961	<0.0001
HbA1c (%)	0.0219	0.7976
GA (%)	0.1480	0.1318
GA/HbA1c	0.3068	0.0015
BUN (mg/dl)	0.0764	0.3323
Cr (mg/dl)	0.1031	0.1904
eGFR (ml/min/1.73m^2)	-0.0564	0.4748
Uric acid (mg/dl)	0.1794	0.0250
Total bilirubin (mg/dl)	-0.0600	0.4627
Triglyceride (mg/dl)	-0.2493	0.0015
HDL-cholesterol (mg/dl)	0.2336	0.0030
LDL-cholesterol (mg/dl)	-0.0233	0.7709

BMI, body mass index; HbA_{1c}, glycated hemoglobin; GA, glycated albumin; BUN, blood urea nitrogen; Cr, creatinine; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein

Table 2. Multivariate analysis of the relationship between the serum level of methionine oxidation and other participant characteristics

Parameters	β	F	P
Age	-0.2575	2.4028	0.1265
Male/Female	0.2476	4.5562	0.0370
BMI	-0.3320	3.4777	0.0672
GA/HbA1c	0.3543	5.4008	0.0236
eGFR (ml/min/1.73m^2)	-0.2107	2.3141	0.1335
Uric acid (mg/dl)	0.0206	0.0154	0.9017
Total bilirubin (mg/dl)	-0.0932	0.6427	0.4259
HDL-cholesterol (mg/dl)	0.0495	0.1585	0.6920
Metformin (+/-)	-0.0373	0.0890	0.7665
Statin (+/-)	0.0029	0.0006	0.9803
ACEI/ARB (+/-)	-0.2107	2.8501	0.0966

ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blocker

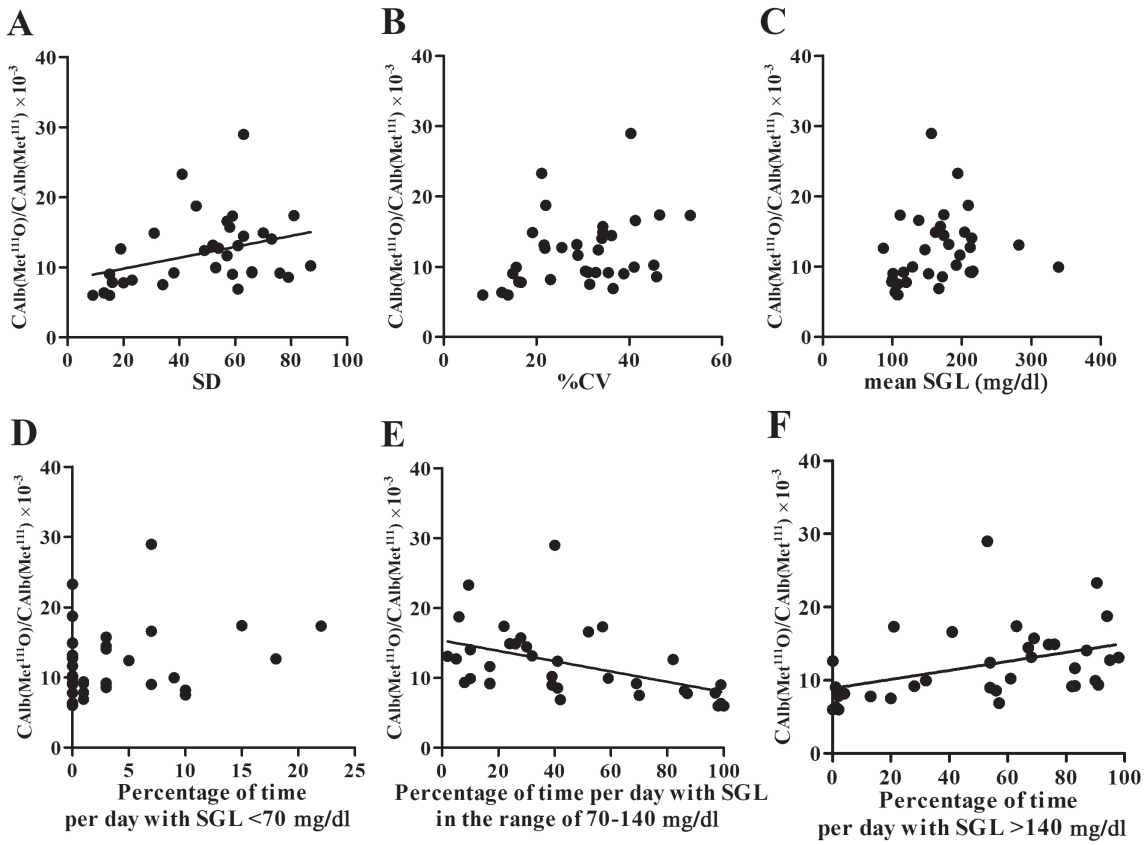

Figure 2. The relationship between Met oxidation and blood glucose profiles, evaluated using continuous glucose monitoring

Continuous glucose monitoring was performed in 35 participants for 4－7 days, and the sensor glucose levels (SGL) over the entire monitoring period were used to calculate the standard deviation (SD) (**A**), % coefficient of variation (%CV) (**B**), and the mean SGL (**C**) values. The relative lengths of time with SGL <70 mg/dl (**D**), 70－140 mg/dl (**E**), and >140 mg/dl (**F**) were plotted against $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$, and the corresponding significant regression lines are shown.

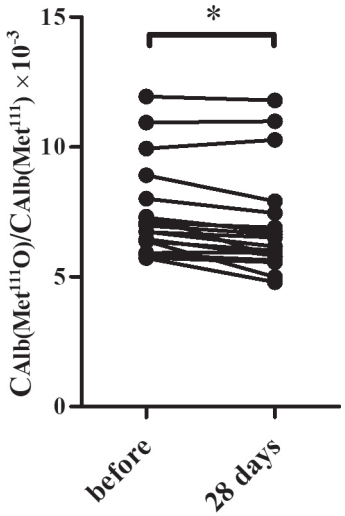

Figure 3. Effect of sodium glucose co-transporter 2 inhibitor treatment on the Met oxidation status of serum albumin

$C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$ was determined in 18 types of 2 diabetic participants before and after a 28-day oral administration of a sodium glucose co-transporter 2 inhibitor.
*P < 0.005, Wilcoxon signed-rank test

$C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$ (P = 0.0134, r = 0.414) (Figure 2F). In contrast, the time spent in the normoglycemic range negatively correlated with $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$ (P = 0.0040, r = -0.475) (Figure 2E).

Finally, we determined the effect of a sodium glucose cotransporter 2 inhibitor, which suppresses glycemic fluctuations, on $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$. $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$ in 18 diabetic participants (9 men and 9 women; 54.3 ± 9.5 years; HbA_{1c} 9.3 ± 1.7%, BMI 32.1 ± 5.6 kg/m^2). $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$ significantly decreased after either canagliflozin, luseogliflozin, or empagliflozin was administered for 28 days (Figure 3).

Discussion

Using our recently established methodology to quantify the oxidation rate of Met residues in blood proteins using LC-MS, we demonstrated that Met and MetO residues of human serum albumin can be accurately and reproducibly quantified with stable isotope-labeled peptides and further showed that $C_{Alb(Met^{147}O)}/C_{Alb(Met^{147})}$ can detect intravascular oxidative stress elicited by blood glucose fluctuations, hypoglycemia, and hyperglycemia in diabetes, as well as that affected by endogenous factors such as serum bilirubin and HDL cholesterol.[19] In the present study, we used exactly the same samples we used in the study and quantified the oxidation levels of another Met residue at position 111 of human serum albumin, $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$, in order to determine whether or not Met^{111} can also be used as a useful clinical biomarker that detects oxidative stress in human disease pathophysiology.

In contrast to $C_{Alb(Met^{147}O)}/C_{Alb(Met^{147})}$, $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$ did not show significantly high levels in diabetic groups than the controls. $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$ level was associated with the GA/HbA_{1c} as analysed by multiple regression analyses, but unlike $C_{Alb(Met^{147}O)}/C_{Alb(Met^{147})}$, it did not correlate with endogenous factors theoretically influencing oxidative stress, such as HDL cholesterol or serum bilirubin. In patients undergoing CGM, $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$ showed a positive correlation with SD and length of time spent with hyperglycemia, but did not reveal any significant correlation with the %CV, nor the percentage of time spent with hypoglycemia (Figure 2). These results demonstrate that $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$ may not be as sensitive as to detect redox status changes with $C_{Alb(Met^{147}O)}/C_{Alb(Met^{147})}$. However, $C_{Alb(Met^{111}O)}/C_{Alb(Met^{111})}$ still reflected the effects by hyperglycemia and glycemic excursion in diabetes and, unlike $C_{Alb(Met^{147}O)}/C_{Alb(Met^{147})}$, showed higher values in females than in males.

Of the 6 Met residues in human serum albumin, Met^{111} and Met^{147} had the lowest baseline oxidized ratio in comparison to the remaining residues.[17] Met residues with elevated baseline oxidation levels are located on the surface of human serum albumin molecules. It is speculated that susceptibility to oxidation in response to oxidative stress may be dependent upon the position of each residue in the three-dimensional structure of the protein molecule, and therefore each residue may reflect a distinct oxidative stress-related condition.

In conclusion, the quantification of the oxidized ratio of Met^{111} may be less sensitive than that of Met^{147} to reflect oxidative stress elicited by diabetic disease conditions, such as hypoglycemia, renal disfunction, and endogenous oxidative stress factors, e.g., serum bilirubin and HDL cholesterol. It is speculated that respective Met residues of human serum proteins could reflect differential aspects of intravascular redox status depending upon their locations in the three-dimensional structure. Further studies should clarify whether a variety of Met residues of human serum proteins reflect oxidative stress induced by differential human disease pathophysiology.

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Conflicts of Interest: None

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