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D-Ribose contributes to the glycation of serum protein

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ABSTRACT

D-Ribose is active in glycation and rapidly produces advanced glycation end products, leading to cell death and to cognitive impairment in mice. Glycated serum protein (GSP) is a relatively short-term biomarker for glycemic control in diabetes mellitus. However, whether D-ribose is related to GSP is unclear. The aim of this work was to identify the contribution of D-ribose to GSP compared to D-glucose. Here, we showed that the yield of glycated human serum albumin with D-ribose was at least two-fold higher than that with D-glucose in a 2-week incubation. The glycation of human serum albumin (HSA) with D-ribose was much faster than that with D-glucose, as determined by monitoring changes in the fluorescent intensity of glycation products with time. Liquid chromatography-mass spectrometry/mass spectrometry revealed that 17 and 7 lysine residues on HSA were glycated in the presence of D-ribose and D-glucose, respectively, even when the concentration ratio [D-ribose]/[D-glucose] was 1/50. The intraperitoneal injection of D-ribose significantly increased the GSP levels in Sprague Dawley rats, but the injection of D-glucose did not. The level of D-ribose was more positively associated with GSP than the level of D-glucose in streptozotocin-treated rats. In diabetic patients, the levels of both D-ribose and D-glucose were closely related to the level of GSP. Together, these in vitro and in vivo findings indicated that D-ribose is an important contributor to the glycation of serum protein, compared to D-glucose. To assess GSP levels in diabetes mellitus, we should consider the contribution from D-ribose, which plays a nonnegligible role.

1. Introduction

Diabetes is characterized by hyperglycemia resulting from deficiency in insulin secretion and/or insulin activity [1]. Many diabetic patients develop acute or chronic complications, including damage to the blood vessels, brain, and kidney [2,3]. Diabetic complications are partly due to the role of hyperglycemia in the nonenzymatic glycation of proteins, which leads to increased molecular oxidation in diabetes mellitus [4]. Glycation occurs at the side chains (amino groups) of a protein, such as the lysine residue [5], and produces advanced glycation end products (AGEs) that include a series of heterogeneous compounds such as carboxymethyllysine (CML), carboxyethyllysine (CEL) and pyrraline [6]. AGEs in vivo act as an aging factor and contribute to the pathophysiology of degenerative diseases, such as chronic kidney disease [7] and Alzheimer's disease [8].

Although AGEs result from the reaction of D-glucose with proteins, Duckworth and colleagues observed that intensive D-glucose control in veterans with poorly controlled type 2 diabetes had no significant effect on the rates of major cardiovascular events, death and microvascular complications [9]. After nearly 10 years of follow-up, Hayward and colleagues reported that patients with type 2 diabetes who had been randomly assigned to intensive glucose control for 5.6 years had 8.6 fewer major cardiovascular events per 1000 person-years than those assigned to standard therapy, but no improvement was observed in the rate of overall survival [10]. A meta-analysis of data from 58,160 patients in 13 randomized controlled trials showed that intensive D-glucose-lowering therapy had no significant influence on the risk of total mortality, cardiac death, stroke, and congestive heart failure in diabetic patients [11]. As we know, the accumulation of AGEs is thought to play an important role in the pathogenesis of diabetic complications [12]. However, no significant correlations were reported to exist for glycated albumin and postprandial markers of blood glucose control in

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euglycemic adults [13]. It appears that, in addition to D-glucose, there may be some other etiology, for instance D-ribose, involved in protein glycation. Therefore, clarifying whether D-ribose contributes to glycated serum protein (GSP) will help us to understand more about diabetes mellitus.

D-Ribose is a naturally occurring pentose monosaccharide present in all living cells and their microenvironments and a key component of numerous biomolecules involved in many metabolic pathways [14,15]. It is much more active in protein glycation than D-glucose [16]. More AGEs were formed in D-ribose-glycation than in D-glucose-glycation under the same in vitro conditions [17]. AGEs produced from D-ribose-glycated proteins had severe cytotoxicity, leading to cell death [18] and to cognitive impairment in mice [19]. The concentration of D-ribose in serum is 0.01–0.1 mM in normal individuals [20], but was abnormally increased in urine of diabetic patients [21,22]. A recent study showed that D-ribose plays an important role in the formation of glycated hemoglobin (HbA1c) [23]. As described by Siddiqui and colleagues, glycation of hemoglobin with D-ribose leads to the immunogenicity as a result of neoepitope generation [24]. Their further work showed prevalence of auto-antibodies against D-ribose-glycated-hemoglobin in diabetes mellitus [25]. Therefore, the extent of the involvement of D-ribose in glycation is important to clarify.

The glycation of serum proteins and hemoglobin has been used to estimate the glycemic status of diabetic patients. GSP provides a criterion for short-term glycemic status in the last 1 to 3 weeks and is a cause of tissue damage during development [26], whereas HbA1c reflects the average plasma D-glucose over the previous 8 to 12 weeks, according to the WHO [27]. A number of studies have recommended GSP as a possible diagnostic biomarker in diabetes-related complications [28], including cardiovascular disease [29] and Cushing's syndrome [30]. GSP is also useful for the assessment of glycemic control in situations where HbA1c may not be appropriate, such as hemodialysis [31], hemolytic anemia [32] and gestational diabetes [33]. Since GSP has attracted increasing attention in recent years [34], the relationship between D-ribose and GSP needs to be investigated.

The aim of this work was to identify the contribution of D-ribose to the glycation of serum protein compared to the contribution of D-glucose. For this purpose, we analyzed the glycation of human serum albumin (HSA) by fluorescence and kinetic methods. Using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS), the glycated sites on HSA were determined. We also investigated the relationship between D-ribose and GSP in both healthy Sprague Dawley (SD) rats and a diabetic rat model, and the correlativity of D-ribose with GSP in type 2 diabetic patients in order to clarify whether D-ribose plays a nonnegligible role in nonenzymatic glycation in diabetes mellitus.

2. Material and methods

2.1. Preparation of glycated products

HSA (final concentration 150 μM) (Sigma Aldrich, USA) was incubated with D-ribose (final concentration 20 mM) (Amresco, USA) or different concentrations of D-glucose (final concentrations 20 mM and 1.0 M) (Amresco, USA) in Tris-HCl buffer (20 mM, pH = 7.4, 37 °C) for different durations. HSA, D-ribose, and D-glucose alone in Tris-HCl buffer were used as controls. All solutions were filtered through 0.22 μm membranes (Millipore, USA). Aliquots of the reaction mixtures were subjected to fluorescence measurements at different time intervals and stored at −20 °C for LC-MS/MS detection.

Different concentrations of L-lysine (final concentrations 50 mM and 100 mM) (Sigma Aldrich, USA) were incubated with D-ribose (final concentration 20 mM) or D-glucose (final concentration 2.0 M) in Tris-HCl buffer (20 mM, pH = 7.4, 37 °C) for 2 weeks. The reaction mixtures were stored at −20 °C for high performance liquid chromatography-mass spectrometry measurement. The other conditions were as described above for the incubation of HSA.

2.2. Fluorescence detection

The fluorescence of AGEs was monitored on a FluoroMax-4 Spectrofluorometer (Horiba, Japan), turned on 30 min earlier, maintained at 20 °C in a 5 nm slit. The wavelengths λ_{em} = 410 nm/λ_{ex} = 320 nm and λ_{em} = 460 nm/λ_{ex} = 355 nm were employed [16,35]. The first-order rate constants of kinetic reaction of D-ribose and D-glucose were calculated by Tsou's method on the basis of fluorescence detection [36].

2.3. Analysis of CML, CEL and pyrraline in glycated HSA by LC-MS/MS

CML, CEL and pyrraline in glycated HSA were identified by LC-MS/MS as described [37]. Briefly, glycated products were collected and subjected to 12% SDS-PAGE. After successive decolorization, reduction by dithiothreitol (Sigma Aldrich, USA) and alkylation by iodoacetamide (Sigma Aldrich, USA), the protein bands were digested with trypsin overnight followed by an addition of 5% formic acid solution to stop the digestion reaction. Collecting the whole supernatant, vacuum drying and keep until redissolving the trypsin-digested peptides using 0.1% formic acid before analyzed by nanoLC-LTQ-Orbitrap XL (Thermo, San Jose, CA).

The trypsin-digested peptides were loaded on a C18 reversed-phase column (150 μm × 3 cm, filled with 5-μm ReproSil-Pur C18-AQ from Dr. Maisch GmbH, Ammerbuch, Germany) and separated with a C18 reversed-phase column (75 μm × 15 cm, filled with 3-μm ReproSil-Pur C18-AQ from Dr. Maisch GmbH, Ammerbuch, Germany) in an LTQ-Orbitrap MS/MS system. Mobile phase A was a 0.1% formaldehyde-water solution, and mobile phase B was a 0.1% formaldehyde and 80% acetonitrile-water solution. The flow rate was 300 nL/min. A 90 minute gradient was employed. Detection was performed in the electrospray ionization mode, and the source parameters were set as follows: capillary temperature of 225 °C and nozzle voltage of 2.1 kV.

Data were analyzed by Proteome Discoverer Software (version 1.4.0.288, Thermo Fischer Scientific). The second MS spectra were searched against the Uniprot human 2017 HSA database by using the SEQUEST search engine. The mass accuracy was set at 20 ppm for MS mode and at 0.6 Da for MS/MS mode, and two missed chymotryptic cleavage sites were allowed in the search. CML, CEL, pyrraline and the oxidation of methionine were set as variable modifications, and the alkylation of cysteine was set as fixed modification. The matching of the searched peptide and MS spectrum was filtered by the Percolator algorithm. The raw MS data are available online (https://pan.baidu.com/s/1XNYb7ORWKNN4njX-45cTdw).

2.4. Analysis of CML, CEL and pyrraline in the glycation of L-lysine by HPLC-MS

CML, CEL and pyrraline in incubated L-lysine mixtures were detected by high-performance liquid chromatography (HPLC) (Agilent 1290, USA) coupled with mass spectrometry (Agilent 6460, USA). The HPLC system was equipped with a LiChrospher 100RP-18 (250 mm × 4.6 mm × 5 μm) (Merck, Germany) as the phase column. The mobile phase A was 0.1% tridecafluoroheptanoic acid (Tokyo Chemical Industry, Japan)-water solution, and the mobile phase B was 0.1% tridecafluoroheptanoic acid-acetonitrile solution. The elution conditions are shown in Supplementary Table 1. The flow rate was 1 mL/min, the column temperature was 35 °C, and 10 μL of the solution was injected into the analytical column. The MS conditions included electrospray ionization mode and multiple-reaction monitoring. The charge ratios of the parent ions and product ions of the CML, CEL and pyrraline standards and the collision voltage and collision energy of the product ions are shown in Supplementary Table 2. The yields of different glycated residues in the incubation of L-lysine were determined according to the standard curves for CML (Toronto Research Chemicals, Canada), CEL (Toronto Research Chemicals, Canada) and pyrraline

(Toronto Research Chemicals, Canada) resulting from the chromatographic peak area of the quantitative product ions.

2.5. Animal administrations and ethics

Male SD rats (aged 8 weeks, weighing 180–200 g) were obtained from Vital River Laboratory Animal Technology Co. Ltd. (China). After 3 days of acclimation, 21 SD rats were randomly divided into 3 groups that received intraperitoneal injections of D-ribose (4 g/kg) (n = 7), D-glucose (4.8 g/kg) (n = 7) or saline (0.9% NaCl) (n = 7) once daily for 2 weeks [37]. Weight was monitored during administration to adjust the injection dosage. After the administration, their serum was collected and stored at −80 °C for GSP detection.

A single injection of streptozotocin (STZ, 70 mg/kg) (Sigma Aldrich, USA) was given to SD rats to create the diabetic rat model, and saline was given to controls (n = 17). STZ-induced rats were fed for 12 weeks [38]. Following the experimental period, 10% chloral hydrate was intraperitoneally injected into the animals for adequate anesthesia. When they were sacrificed, their blood was stored at room temperature for 60 min and then centrifuged (3000*g*, RT, 30 min). The serum was extracted and stored at −80 °C for D-ribose and GSP detections.

The two animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Biological Research Ethics Committee of the Institute of Biophysics, Chinese Academy of Sciences (Permit Number: SYXK2016-32). All rats were maintained in animal facilities under pathogen-free conditions. They were raised in normal cages at 20–26 °C, with light and dark alternating for 12/12 h, and were freely fed sterile drink and food.

2.6. Sample collection

Urine and fasting blood samples were collected from type 2 diabetic patients (n = 134) and control participants without diabetes (n = 70) for the determination of D-ribose, D-glucose and GSP. The background of participants was shown in Supplementary Table 3. The exclusion criteria included histories of nephropathy (only controls), cancer or any other serious systemic diseases. One day before the morning urine and fasting blood samples were collected, the participants had not performed the following activities: heavy labor, overeating, drinking alcohol, taking diuretics or analgesics, or taking D-ribose as an energy supplement. They had also not undergone any surgery within 3 months. This clinical investigation was strictly conformed to the regulations of the ethics committee of the Affiliated Hospital of Southwest Medical University (No. KY2017011), and registered with the Chinese Clinical Trial Registry (ChiCTR), which is supported by the WHO International Clinical Trial Registration Platform (No. ChiCTR-DDD-17010722).

The serum biochemical indicators were examined by a fully automatic biochemical analyzer (ADVIA2400 Siemens, Germany). Total protein (Mindray, China) and albumin (Mindray, China) were tested by the biuret assay and bromocresol green method, respectively, according to the instruction of the Mindray manufacture. Briefly, for total protein, serum samples were mixed with biuret reagent and measured the absorbance at 540 nm, for albumin, serum samples were mixed with bromocresol green reagent and measured the absorbance at 630 nm, and then calculated the concentrations according to their own standard substances. Globulin was gain from total protein minus albumin.

2.7. Measurements of D-ribose and D-glucose by UV-HPLC

D-Ribose and D-glucose in urine were measured as described previously [21]. The urine samples and serum samples were centrifuged (12,000 rpm, 4 °C, 10 min) after the precipitation of serum proteins by the addition of three-fold acetonitrile (Fisher Chemical, USA). A 0.4 mL aliquot of the supernatant was mixed with 0.6 mL 4-(3-methyl-5-oxo-2-pyrazolin-1-yl) benzoic acid (MOPBA, final concentration 150 mM, in 250 mM NaOH in 50% methanol-water solution) (Sigma Aldrich, USA) and then heated in a 70 °C water bath for 90 min followed by an additional centrifugation (12,000 rpm, 4 °C, 10 min). The mixture was acidified by the addition of 150 μL of 2 M HCl solution to precipitate the excess MOPBA and centrifuged (12,000 rpm, 4 °C, 10 min), then filtered through 0.22 μm membranes. Twenty microliters of the solution was then collected from the C18 column with a binary mobile phase gradient and subjected to HPLC (LC-20A, Shimadzu, Japan) with an ultraviolet detector at 271 nm wavelength. Mobile phase A was 10 mM sodium 1-hexanesulfonate (Tokyo Chemical Industry, Japan), and mobile phase B was a 50% acetonitrile solution. The elution conditions were 38%–60% B for 15 min, 100% B for 5 min, and 38% B for 5 min. The flow rate was 1 mL/min, and the column temperature was 45 °C. The procedure for D-ribose analysis was identical to the procedure for D-glucose detection except for the ratio of the binary mobile phase, in which the elution conditions were 42%–60% B for 15 min, 100% B for 5 min, and 42% B for 5 min, and 2 μL of the solution was injected into the analytical column. The reference concentrations of D-ribose and D-glucose were determined according to the standard curves.

2.8. Nitroblue tetrazolium assay

The nitroblue tetrazolium assay was commonly used to detect GSP formation in serum [39,40]. The samples were mixed with nitroblue tetrazolium dye, and the absorbance was measured at 540 nm. The final concentration was calculated according to the manufacturer's instructions (Nanjing Jiancheng, China).

2.9. Statistics

The nonhuman values were reported as the mean ± S.E.M., and the human data were expressed as the median and interquartile range. The significance of the differences between the two nonhuman groups and the two human groups were calculated with the two-sided unpaired Student's *t*-test and the Mann-Whitney *U* test, respectively. The Pearson correlation method was used to assess the correlation of D-ribose with GSP and of D-glucose with GSP. Differences with a probability level of 95% ($P \leq 0.05$) in the two-tailed test were considered significant. The statistical analyses were performed in SPSS 17.0 (International Business Machines Corporation, USA). The figures were created in Origin 9.0 (OriginLab Corporation, USA).

3. Results

3.1. D-Ribose glycates HSA more rapidly than D-glucose in vitro

To investigate the glycation reaction of D-ribose and serum protein, HSA (150 μM) was incubated with 20 mM D-ribose in Tris-HCl buffer (pH = 7.4, 37 °C) for 0 to 14 days. Changes in the intensity of fluorescence (λ_{em} = 410 nm/λ_{ex} = 320 nm; λ_{em} = 460 nm/λ_{ex} = 355 nm) that are commonly used to detect the formation of AGEs were monitored. The emission intensity at 410 nm of the glycation of HSA with D-ribose increased with incubation time (Fig. 1A). The fluorescence of HSA incubated with D-glucose did not markedly increase under the same experimental conditions. Even when 1.0 M D-glucose was used, no clear increase in fluorescent intensity could be detected. Similar results were observed when the emission wavelength at 460 nm was monitored (Fig. 1B). HSA, D-ribose and D-glucose alone incubated as controls showed no marked increase in AGEs fluorescence.

To compare whether glycation with D-ribose is faster than glycation with D-glucose, Tsou's method was applied to analyze the first-order rate constant of the kinetic changes in AGEs fluorescence intensity [36]. Changes in the intensities of the emission at either 410 nm (Fig. 1A′) or 460 nm (Fig. 1B′) of HSA incubated with 20 mM D-ribose or 1.0 M D-glucose processed biphasically, with a slow phase in the initial reaction followed by a fast phase. In 20 mM D-glucose and the other controls, the

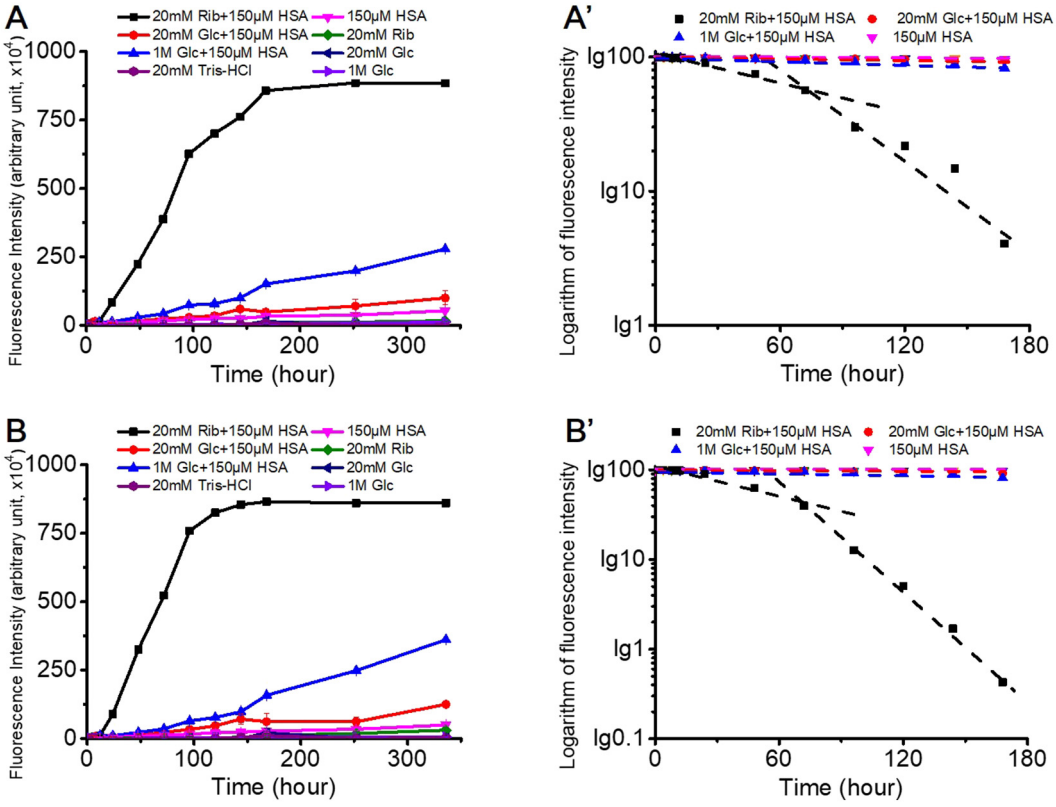

Fig. 1. Changes in the fluorescence of glycated HSA in the presence of D-ribose and D-glucose over time. HSA (150 μM) was incubated with D-ribose (20 mM) or different concentrations of D-glucose (20 mM and 1.0 M) in 20 mM Tris-HCl buffer (pH = 7.4, 37 °C) for 0 to 14 days. HSA, D-ribose and D-glucose alone in Tris-HCl buffer were used as controls. Aliquots were taken for the measurement of fluorescence intensity at different time intervals. The maximal fluorescence intensity of glycated HSA (λ_{em} = 410 nm/λ_{ex} = 320 nm, A) (λ_{em} = 460 nm/λ_{ex} = 355 nm, B) was employed. The same data shown in A and B were plotted in semilogarithmic form to calculate the first-order rate constants of the fluorescent changes (A′ and B′, respectively) by Tsou's method. Rib, D-ribose; Glc, D-glucose. The values are expressed as the mean ± S.E.M., n = 3.

fluorescent changes showed monophase behavior during the incubation.

As shown in Table 1, the first-order rate constant of the glycation of HSA by 20 mM D-ribose (K_R) was much greater than that obtained for glycation by 20 mM D-glucose (K_{G1}) and even by 1.0 M D-glucose (K_{G2}), as monitored by fluorescence detection either at 410 nm or 460 nm and in either the fast phase or the slow phase. According to the changes in the intensity of fluorescence at 410 nm, the first-order rate constant K_R was ~28 and ~68 times as fast as K_{G1} in the slow and fast phases, respectively, and was ~12 and ~11 times as fast as K_{G2}. Similarly, for the emission at 460 nm in the slow and fast phases, K_R was at least ~9 and ~27 times as fast as K_{G1} and ~8 and ~3 times as fast as K_{G2}, respectively. These data demonstrated that the glycation of HSA with D-ribose is faster than that with D-glucose at the same or even 50 times higher concentration than that of D-ribose.

Table 1
The first-order rate constants of changes in the fluorescence of glycation of HSA in the presence of D-ribose and D-glucose.

Rate constants# (10^{-6} s^{-1})	λ_{em} = 410 nm		λ_{em} = 460 nm	
	Slow	Fast	Slow	Fast
K_R	2.773	6.710	1.290	3.742
K_{G1}	0.098	0.098	0.140	0.140
K_{G2}	0.223	0.602	0.155	1.308
K_{HSA}	0.049	0.049	0.065	0.065
K_R/K_{G1}	28.296	68.469	9.214	26.729
K_R/K_{G2}	12.435	11.146	8.323	2.861

"#" were analyzed by Tsou's method and shown in $10^6 \cdot s^{-1}$, including the slow and fast phase. K_R, K_{G1}, K_{G2} and K_{HSA} are the constants of 20 mM D-ribose, 20 mM D-glucose and 1.0 M D-glucose incubated with human serum albumin, and the native albumin incubated without any sugar, respectively. K_R/K_{G1} and K_R/K_{G2} are the fold of K_R to K_{G1} and K_R to K_{G2}. "λ_{em}" is the wavelength of emission light. Data were from three independent fluorescence detections.

3.2. D-Ribose modifies a higher number of lysine residues on HSA

To reveal the difference between D-ribose and D-glucose in the modification of amino acid residues, we incubated HSA with D-ribose for 2 weeks and then identified the glycated lysine residues by LC-MS/MS. First, we observed that the native HSA incubated without sugars had four glycated lysine residues: CML-214, CML-223, CML-375, and CML-549 (Fig. 2). All samples of HSA incubated with or without any sugar had the same four modified sites. Thus, we regarded them as background modifications. The incubation of HSA with 20 mM D-ribose produced one CEL (CEL-437) and 16 CMLs in addition to the background. HSA incubated with 20 mM D-glucose yielded only one CML. HSA incubation with 1.0 M D-glucose produced one pyrraline (pyrraline-300) and 6 CMLs under the same experimental conditions. More than twice as many lysine residues were glycated by 20 mM D-ribose as by 1.0 M D-glucose. This notable difference in modification ability between D-ribose and D-glucose suggested that the contribution of D-ribose to protein glycation cannot be ignored.

To compare the ability of glycation between D-ribose and D-glucose, we incubated L-lysine (containing α- and ε-amino group) with 20 mM D-ribose and 2.0 M D-glucose. Then, we detected CML, CEL and pyrraline at different intervals by HPLC-MS according to their standard curves (Supplementary Fig. 1) [41,42]. The yield of glycation products (CML and CEL) in the glycation of L-lysine with D-ribose was far more than

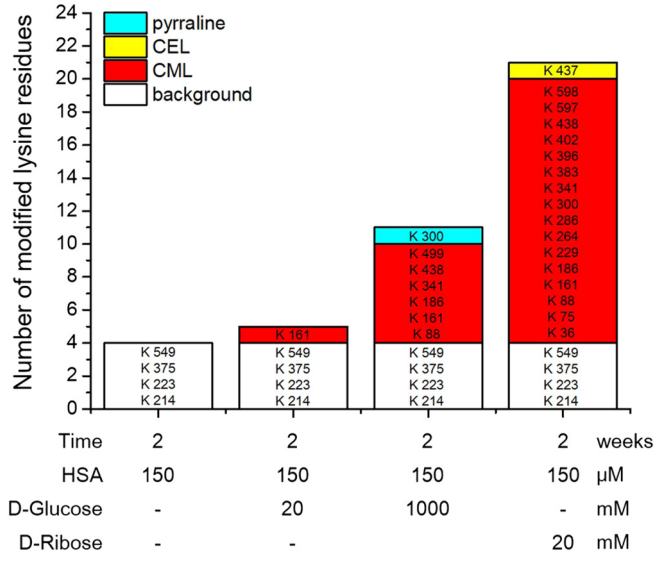

Fig. 2. Glycation sites on HSA in the presence of D-ribose and D-glucose analyzed by LC-MS/MS. The conditions of HSA glycation are as shown in Fig. 1, except that 2-week glycated HSA was employed for mass spectrometry. The glycated samples were processed in 12% SDS-PAGE and sequentially decolorized, reduced and alkylated. The protein bands were digested with trypsin overnight for peptide fragments. Those peptides were then analyzed by LC-MS/MS. Pyrraline (blue); CEL, carboxyethyllysine (yellow); CML, carboxymethyllysine (red); background (CML in white); K, lysine residue.

that with D-glucose (Fig. 3A, B). The glycation products increased with the L-lysine concentration and reaction time. Pyrraline, however, was significantly yielded from the incubation of L-lysine with D-glucose but not with D-ribose (Fig. 3C).

3.3. GSP is linked with D-ribose in vivo

To determine the contribution of D-ribose to the glycation of serum protein in vivo, we intraperitoneally injected (once daily, 2 weeks) SD rats (n = 21) with D-ribose (4 g/kg) (n = 7), D-glucose (4.8 g/kg) (n = 7) and saline (0.9% NaCl) (n = 7) as controls. As shown in Fig. 4A, GSP levels were significantly increased in D-ribose-injected rats but not in D-glucose-injected rats compared to those in saline-injected rats. This result indicated that D-ribose plays a role in the glycation of serum protein in vivo.

To confirm the contribution of D-ribose to GSP in vivo, we injected SD rats with STZ (70 mg/kg) singly to establish a diabetic rat model, and gave saline to controls (n = 17). After 12 weeks, the GSP and D-ribose levels were measured. As shown in Fig. 4B, the GSP levels in the STZ rats were significantly elevated. The concentrations of both D-ribose and D-glucose were significantly correlated with the concentration of GSP. However, the correlation coefficient of D-ribose ($r = 0.771$, $P < 0.001$) (Fig. 4C) was greater than that of D-glucose ($r = 0.610$, $P = 0.009$) (Fig. 4D). These results demonstrated that D-ribose is an important contributor to GSP in the diabetic animal model.

3.4. Correlation between D-ribose and GSP in patients with type 2 diabetes

To clarify whether D-ribose contributes to the glycation of serum protein, the urine and blood samples of patients with type 2 diabetes (n = 134) and controls (n = 70) were collected. We determined the GSP and D-ribose levels in both urine and serum. As shown in Fig. 5A, the urine D-ribose levels of diabetic patients were significantly higher than those of the controls ($P < 0.001$), as were their urine D-glucose levels ($P < 0.001$, Fig. 5B). Both D-ribose (Fig. 5C) and D-glucose (Fig. 5D) in the serum were significantly ($P < 0.001$) increased in the patients. The GSP levels in diabetic patients were also clearly higher than those in the controls ($P < 0.001$, Fig. 5E). At the same time, the levels of albumin, total protein and globulin in diabetic patients were lower ($P < 0.001$, Fig. 5F–H). That is, GSP levels were elevated and serum proteins reduced in diabetic patients compared to normal participants. This finding verified that both D-ribose and D-glucose levels are elevated, and thus, both are important contributors to the glycation of serum protein.

We further analyzed the relationship between GSP and D-ribose. A positive correlation was observed not only between urine D-ribose and GSP ($r = 0.448$, $P < 0.001$) (Fig. 5I) but also between serum D-ribose and GSP ($r = 0.429$, $P < 0.001$) (Fig. 5J). Nonetheless, the correlation coefficients of urine D-glucose ($r = 0.401$, $P < 0.001$) and serum D-glucose ($r = 0.442$, $P < 0.001$) with GSP (Fig. 5K, L) were both lower than those of urine D-ribose. In the control participants, the urine and serum D-ribose were not significantly related to GSP, similar to D-glucose ($P > 0.05$) (Supplementary Fig. 2). The intimate relationship of D-ribose with GSP indicated that D-ribose is also an important contributor to GSP in diabetes mellitus, in addition to D-glucose.

4. Discussion

This study shows that D-ribose contributes more than D-glucose to the glycation of serum protein. This viewpoint is based on the following data. 1) The yield of glycated HSA with D-ribose was at least two-fold that with D-glucose for a 2-week incubation reaction. 2) In the kinetics study, the glycation of HSA with D-ribose was much faster than that with D-glucose, as measured by fluorescence intensity at 410 nm and 460 nm. 3) More than twice as many glycated lysine residues were detected by LC-MS/MS in the presence of D-ribose (17 lysine residues) as with D-glucose (7 lysine residues), even when the concentration ratio [D-ribose]/[D-glucose] equaled to 1/50. 4) The intraperitoneal injection of D-ribose increased GSP levels in SD rats, but D-glucose did not cause a significant change. 5) D-Ribose was more positively associated with GSP than was D-glucose in STZ-injected diabetic rats. Finally, 6) like D-

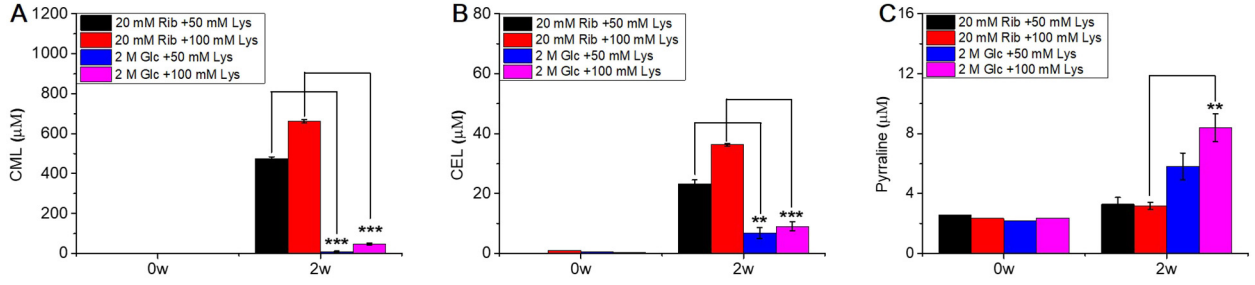

Fig. 3. Changes in AGEs in the presence of L-lysine and D-glucose. L-Lysine (50 mM or 100 mM) was incubated with D-ribose (20 mM) or D-glucose (2.0 M) in 20 mM Tris-HCl buffer (pH = 7.4, 37 °C) for 0 week (L-lysine incubated without sugar) and 2 weeks. Aliquots were taken for the measurement of CML (A), CEL (B) and pyrraline (C) by HPLC-MS. Rib, D-ribose; Glc, D-glucose; w, week. All values are expressed as the mean ± S.E.M. Two-tailed unpaired *t*-test, **P < 0.01, ***P < 0.001, n = 3.

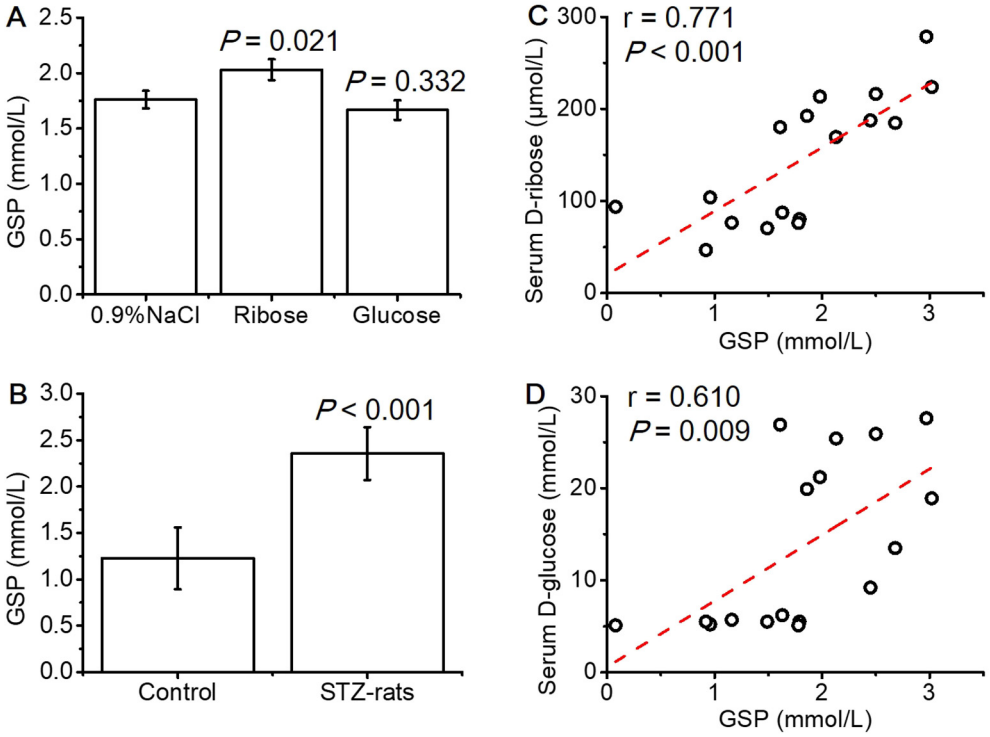

Fig. 4. Correlation of D-ribose/D-glucose with GSP in vivo. SD rats (n = 21) were intraperitoneally injected (once daily, 2 weeks) with D-ribose (4 g/kg), D-glucose (4.8 g/kg) or saline followed by the determination of GSP (A) with a GSP kit. STZ (70 mg/kg) was singly injected to establish the diabetic rat model, and the control rats were injected with saline (n = 17), followed by the detection of GSP at 12th week (B). Data are expressed as the mean ± S.E.M., and P values were calculated by a two-tailed unpaired *t*-test. The correlations of serum D-ribose (C) and D-glucose (D) with GSP in STZ rats and controls were analyzed by the Pearson correlation method (r) (n = 17).

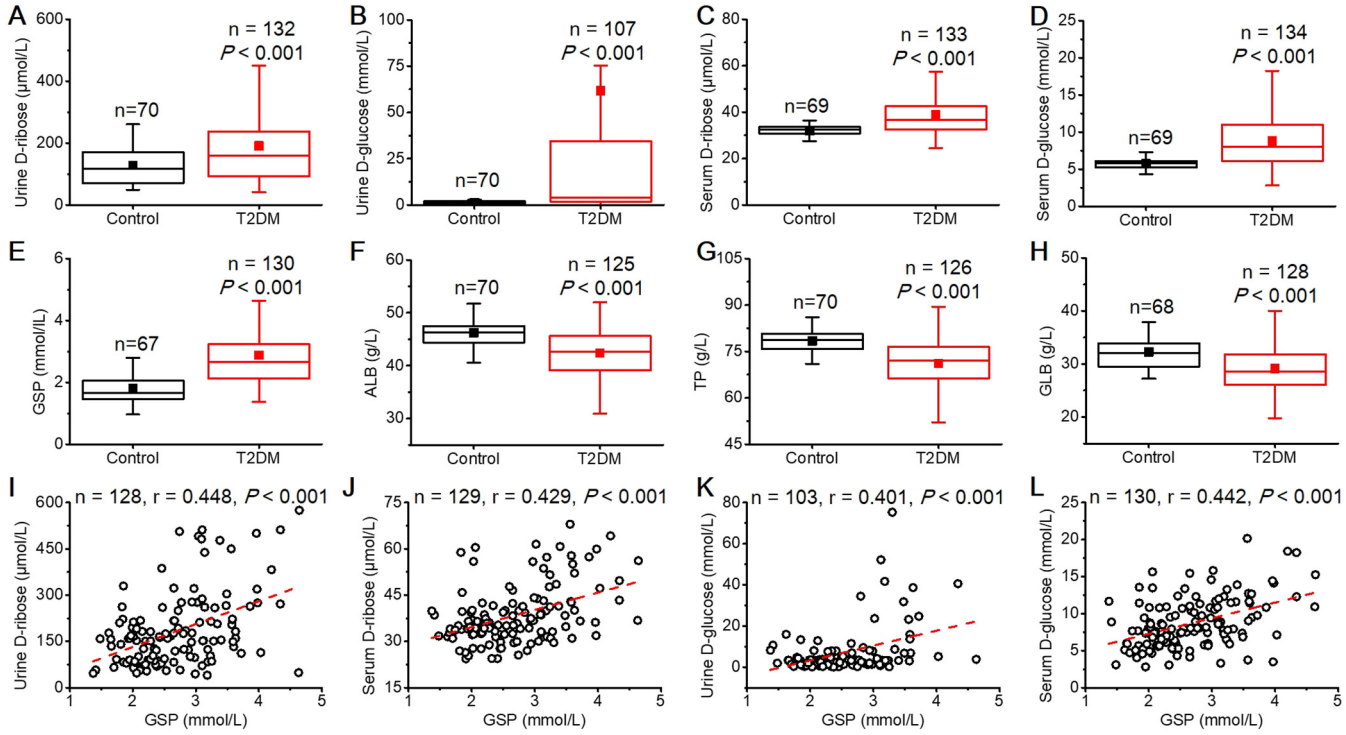

Fig. 5. Correlation between D-ribose/D-glucose and GSP in patients with type 2 diabetes. Morning urine and fasting blood samples of patients with type 2 diabetes (n = 134) and control participants (n = 70) were collected for the measurement of urine D-ribose (A), urine D-glucose (B), serum D-ribose (C), serum D-glucose (D), GSP (E), ALB (F), TP (G) and GLB (H). Data are expressed as the median and interquartile range, and the solid square is the mean. P values were calculated using a two-tailed Mann-Whitney *U* test. The Pearson correlation (r) method was used to analyze the correlation of GSP with urine D-ribose (I), serum D-ribose (J), urine D-glucose (K) and serum D-glucose (L) in diabetic patients. The exact numbers (n) (excluded missing data) and P values (P) are shown in the figures.

glucose, D-ribose was closely related to GSP in diabetic patients. These in vitro and in vivo experimental data demonstrated that D-ribose plays a nonnegligible role in the nonenzymatic glycation of serum protein.

CML, CEL and pyrraline are three of the most common AGEs compounds produced from protein glycation [6,43]. This modification often occurs on lysine residues, and its levels reflect the activity of the reducing sugar and the degree of Maillard reaction [44,45]. As mentioned above, glycation with D-ribose yields more AGEs compounds, for example, CML, with a faster kinetic reaction than that of D-glucose (as monitored by AGEs fluorescence). Why did the glycation of albumin

with D-ribose proceed faster and yield more AGEs? D-Ribose is a pentose sugar (five carbons), while D-glucose is a hexose sugar (six carbons). The pentose ring is not planar but occurs in one of a variety of conformations generally described as "puckered", which makes the unstable aldofuranose ring vulnerable to reactions with amino groups, making the glycation ability of aldofuranose stronger than that of pyranose [16,46]. The linear and ring forms of both D-ribose and D-glucose are in equilibrium in aqueous solution: the forms of D-ribose include furanose (20%), pyranose (79%) and the open chain form, while > 99% of glucose molecules are pyranose and furanose appears in negligible amounts [47,48]. That is, D-ribose is more active in reaction with protein and produces more AGEs.

In diabetic patients, the correlation coefficient between GSP and urine D-ribose was greater than that between GSP and urine D-glucose. However, the correlation coefficient of GSP with serum D-ribose was somewhat less than that with serum D-glucose. The reason may also be the highly reactive characteristics of D-aldofuranose. D-Ribose reacts with proteins in human blood. The inevitable and effective glycation reaction with serum protein consumes and decreases the concentration of serum D-ribose. In addition, urine contains only trace amounts of protein, and glycation is not sufficient to interfere with our determination of urine D-ribose. Therefore, the correlation coefficient of GSP with urine D-ribose is greater than that with serum D-ribose.

D-Ribose should also play an important role in diabetes mellitus, in addition to D-glucose. This conclusion is supported by these observations. 1) The levels of both urine and serum D-ribose in diabetic patients were significantly higher than those in age-matched normal participants [21]. 2) The current work shows that the glycation of serum protein is closely related to both serum and urine D-ribose in diabetic patients. 3) Animal models of diabetes, such as STZ-treated rats and Goto-Kakizaki rats (result not shown), exhibited high levels of D-ribose and GSP. 4) The glycation ability of D-ribose is stronger than that of D-glucose because the unstable aldofuranose ring is vulnerable to reaction with amino groups [16], even though the concentration of blood D-ribose [20] is lower than that of blood D-glucose [49]. 5) D-Ribose also plays an important role in HbA1c compared to D-glucose [37]. 6) D-Ribose-glycation generates the immunogenic neoepitopes on low density lipoproteins in vitro [50,51], and administration of D-ribose can increase hepatic triglyceride in SD rats [52]. Finally, 7) the neoepitopes induced by ribosylation have been shown a higher affinity and autoantibody prevalence level in type 2 diabetes mellitus [53]. All these data demonstrate that not only D-glucose dysmetabolism but also D-ribose dysmetabolism is involved in diabetes. Thus, D-ribose may be used as a potential biomarker to assist in diagnosing diabetes and monitoring disease progression.

Unfortunately, the HSA used in this study has four glycated lysine residues (CML-214, CML-223, CML-375 and CML-549) as a background. The HSA is a qualified and guaranteed product purchased from Sigma Aldrich Company (USA). This protein was recombined and expressed in rice. Understandably, HSA encounters some reducing sugars when expressed in a rice system [54]. We treat these four glycated lysine residues as the experimental background. This background did not disturb the LC-MS/MS analysis because we compared the glycation of HSA with D-ribose only to that with D-glucose.

As described previously, D-ribose rapidly glycates proteins and produces AGEs both in vitro and in vivo [16]. The aggregated protein (monomer) with D-ribose had high cytotoxicity, leading to cell dysfunction and subsequent cognitive impairments [55,56]. Su and colleagues found D-ribose dysmetabolism in patients with type 2 diabetes [21]. D-Ribose also may be involved in the progression of some diseases such as nephropathy [57] and retinopathy [22]. Furthermore, GSP is not only a possible future diagnostic biomarker of diabetic complications [28], such as cardiovascular disease [29] and Cushing's syndrome [30], but is also used to assess the glycemic control of hemodialysis [31], hemolytic anemia [32] and gestational diabetes [33]. Hence, D-ribose probably plays a role in diabetic complications, such as age-related cognitive impairment [19]. However, whether glycation with D-ribose is linked to diabetic complications such as age-related dementia needs further investigation.

5. Conclusions

In conclusion, the glycation of HSA with D-ribose was faster than that with D-glucose. Glycated lysine residues by D-ribose on HSA were more than that by D-glucose, even when the concentration ratio [D-ribose]/[D-glucose] was 1/50. D-Ribose was more strongly positively associated with GSP than D-glucose in STZ-treated rats. In diabetics, levels of both D-ribose and D-glucose were closely related to GSP levels. That is, D-ribose is also an important contributor to the glycation of serum protein, in addition to D-glucose. To assess GSP levels in diabetes mellitus, we should consider the contribution from D-ribose, which plays a nonnegligible role.

Abbreviations

GSP	glycated serum protein
HSA	human serum albumin
AGEs	advanced glycation end products
CML	carboxymethyllysine
CEL	carboxyethyllysine
HbA1c	glycated hemoglobin
STZ	streptozotocin
LC-MS	liquid chromatography-mass spectrometry
MS	mass spectrometry
HPLC	high-performance liquid chromatography

Transparency document

The Transparency document associated this article can be found, in online version.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2019.05.005.

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