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Research Paper

D-Ribose as a Contributor to Glycated Haemoglobin

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ABSTRACT

Glycated haemoglobin (HbA1c) is the most important marker of hyperglycaemia in diabetes mellitus. We show that D-ribose reacts with haemoglobin, thus yielding HbA1c. Using mass spectrometry, we detected glycation of haemoglobin with D-ribose produces 10 carboxylmethyllysines (CMLs). The first-order rate constant of fructosamine formation for D-ribose was approximately 60 times higher than that for D-glucose at the initial stage. Zucker Diabetic Fatty (ZDF) rat, a common model for type 2 diabetes mellitus (T2DM), had high levels of D-ribose and HbA1c, accompanied by a decrease of transketolase (TK) in the liver. The administration of benfotiamine, an activator of TK, significantly decreased D-ribose followed by a decline in HbA1c. In clinical investigation, T2DM patients with high HbA1c had a high level of urine D-ribose, though the level of their urine D-glucose was low. That is, D-ribose contributes to HbA1c, which prompts future studies to further explore whether D-ribose plays a role in the pathophysiological mechanism of T2DM.

1. Introduction

Type 2 diabetes mellitus (T2DM) is the most common type of diabetes mellitus and is characterized by hyperglycaemia (Trujillo et al., 2013) and insulin resistance (Reaven, 1988). Many diabetic patients develop acute or chronic complications, including blood vessels, brain, kidney, and liver damage (Nathan, 1993). Glycated haemoglobin A1c (HbA1c), resulting from an abnormally high level of reduced monosaccharides (such as D-glucose and D-ribose), is a factor in these complications (Chou et al., 2009; Huang et al., 2015; Sherwani et al., 2016). Both D-ribose and D-glucose react with haemoglobin (Hb), thus yielding HbA1c (Huisman et al., 1958; Koenig et al., 1976), which is the most important biomarker for chronic hyperglycaemia (Berg, 2013). As an active reducing monosaccharide, D-ribose reacts with amino acids, peptides and proteins, and produces glycated derivatives much more rapidly than D-glucose (Chen et al., 2009; Wei et al., 2009). The link between blood D-glucose and HbA1c has been intensively studied (Makris and Spanou, 2011); however, whether D-ribose is involved in the glycation of Hb and the subsequent production of HbA1c in diabetic patients is still not fully clarified and therefore requires further investigation.

2. Materials and Methods

2.1. Materials

D-ribose, D-glucose and benfotiamine (benzenecarbothioic acid, S-[2-[[(4-amino-2-methyl-5-pyrimidinyl) methyl] formylamino]-1-[2-(phosphonooxy)ethyl]-1-propen-1-yl]ester) were purchased from Sigma (St. Louis, Missouri). 4-(3-methyl-5-oxo-2-pyrazolin-1-yl) benzoic acid was purchased from J&K Scientific (Beijing, China).

2.2. Subject Enrolment

Patients with T2DM (n = 82, between 50 and 83 years old) were recruited from the Jianheng Diabetes Hospital, Beijing. Age-matched community-dewelling healthy subjects (n = 41) were used as controls, and their physical examinations were performed by the Medical Examination Center of the Third Hospital of Peking University. Informed consents were obtained from all participants. Subjects with T2DM conformed to the classification scheme and diagnostic criteria for DM as published in a report from an international expert committee. Their personal information and medical history were recorded in details. According to the diagnosis of diabetes recommended by the WHO, the patients were divided into two groups on the basis of their HbA1c levels: group 1 (6.5% (48 mmol/mol) ≤ [HbA1c] < 8.0% (64 mmol/mol)), and group 2 (HbA1c ≥ 8.0% (64 mmol/mol)). The patients were excluded if they were tested positive for urine proteins. To ensure the accuracy of sample analysis and to avoid protein interference, middle stream

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morning urine was collected from subjects before their breakfast. The participants were instructed to avoid consuming high-fat diets and sugar one week before the samplings. Beverages such as wine and alcohol were forbidden the day before sampling. Prior to analysis, the samples were stored in a sealed sterile container at −80 °C. This study was approved by the ethics committee of the Institute of Biophysics, Chinese Academy of Sciences (2014-HRQ-1).

2.3. Data Availability

This trial was registered with the Chinese Clinical Trial Registry (ChiCTR), which was granted by the WHO International Clinical Trial Registration Platform (WHO ICTRP). The trial number is ChiCTR-RCS-14004437 (http://www.chictr.org/cn/).

2.4. Measurements of Urine D-ribose and D-glucose by UV-HPLC

Analyses of urine D-ribose and D-glucose were performed in a double-blind manner by the biochemical laboratory and the clinic (Jianheng Diabetic Hospital, Beijing, China). Urine D-ribose and D-glucose were measured as described previously (Su et al., 2013). A 1.0 ml urine sample (thawed at 4 °C) was pipetted into 1.5 ml Eppendorf tube and centrifuged (12,000 rpm, 4 °C, and 30 min). Serum proteins were then precipitated by addition of three-fold acetonitrile and were centrifuged (12,000 rpm, 4 °C, and 10 min). 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). Samples were vortexed vigorously for 30 s before centrifugation (12,000 rpm, 4 °C, and 10 min) and then heated in a 70 °C water bath for 90 min; this was followed by additional centrifugation (12,000 rpm, 4 °C, and 10 min). The mixture was acidified by addition of 150 μl of aqueous 2 M HCl solution to precipitate the excess MOPBA. The mixture was vigorously vortexed and centrifuged (12,000 rpm, 4 °C, and 10 min) and then filtered (0.22 μm). Twenty microliters of the solution was then subjected to high-performance liquid chromatography (HPLC).

The HPLC system (LC-20A, Shimadzu, Japan) was equipped with an ultraviolet detector. The MOPBA-sugar derivative was collected from the C18 column with a binary mobile phase gradient. Mobile phase A was 10 mM of sodium 1-hexanesulfonate; the pH was stabilized at 2.5 by phosphoric acid. Mobile phase B was 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 40 °C. The procedure for D-ribose analysis was identical to the procedure for detecting D-glucose, except in the latter 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 curve.

2.5. In Vitro Studies

Blood samples were obtained from healthy volunteers from 20 to 40 years of age. Whole blood was treated with EDTA anticoagulant and incubated with 0.2 mM D-ribose, 7 mM D-ribose or 7 mM D-glucose in a rigorous sterile operation; as in a previous study, the urine D-ribose level was approximately 0.2 mM (Su et al., 2013a). The samples were warmed in a 37 °C water bath for 7 days. To observe the effects of different concentrations of D-ribose and D-glucose on red blood cells, blood films were used, as described in a previous study (Warhurst and Williams, 1996). The blood cell numbers were counted with a haemacytometer (Hamaker, 1958).

D-ribose (0.5 mM) was added to foetal calf serum or human urine at 37 °C, and aliquots were collected and used for measurement of D-ribose at different time intervals (0, 2, 4, 6, 8, 24, 36, 48, and 72 h). Haemoglobin (10 mg/ml) was incubated with different concentrations of D-ribose (0, 1, 20, 50, 100, and 200 mM) for 5 days, and aliquots were collected and used for the detection of HbA1c each day. Haemoglobin (10 mg/ml) was incubated with 0.2 mM D-ribose or 7 mM D-glucose (0, 6, 12, 24, 36, 48, 72, and 96 h), and aliquots were collected and used for detection of HbA1c at each interval. HbA1c was determined with an ELISA kit for human HbA1c. All data were from 3 separate experiments.

2.6. Determination of HbA1c

1) Levels of HbA1c (clinical samples) were measured using the ion-exchange HPLC method, as certified by NGSP (Weykamp et al., 2011; Kashiwagi et al., 2012), with an HbA1c detective system MQ-2000PT, according to the manufacturer's instructions (Medconn Corporation, Shanghai, China).

To verify that the ribosylation of Hb produced HbA1c, haemoglobin (final concentration 10 mg/ml, Sigma, USA) was incubated with 100 mM D-ribose or D-glucose for 3 days, and aliquots were collected for measurements with a Human HbA1c Kit (Catalogue #80099, Crystal Chem, USA).

To determine whether D-ribose could enter RBCs, fresh blood (2 ml) was incubated with D-ribose (0.2 mM or 7 mM) or D-glucose for 7 days and centrifuged to isolate blood cells. The cells were treated with lysis buffer, and HbA1c was determined according to the manufacturer's instructions.

2) Mouse and rat HbA1c levels were determined with a Mouse HbA1c Kit (Catalogue #80310, Crystal Chem, USA) and Rat HbA1c kit (Catalogue #80300, Crystal Chem, USA), according to the manufacturer's instructions.
3) To monitor the kinetics of the ribosylation of Hb, the time course of fructosamine formation was determined with an NBT assay Kit (Huili Biotech, China) during the incubation of Hb with D-ribose. First, 500 μl of human haemoglobin (final concentration 10 mg/ml, Sigma, USA) was dissolved in 500 μl of D-ribose or D-glucose (final concentration 100 mM) at 37 °C for 7 days. Then, 100 μl of pre-warmed NBT reagent (Somani et al., 1989; Xu et al., 2002) was added to the 100 μl mixture, and the absorbance was measured at 530 nm in the 96-well plates for a time interval between 5 min (A1) and 10 min (A2) on a multiscan spectrum (SpectrMax Para4digm, Molecular Devices, USA). The $\Delta A = A2 - A1$ was measured, and the data were expressed as ΔA/min.
4) To investigate the potential contribution of D-ribose to HbA1c formation, different concentrations of D-ribose (0, 1, 20, 50, 100, and 200 mM) were incubated with human haemoglobin (10 mg/ml) (H7379, Sigma, USA) at 37 °C for five days. The levels of HbA1c were detected with an Enzyme-linked Immunosorbent Assay kit (CEA190Hu, Cloud-Clone Corp., China) for human HbA1c each day.

2.7. Analysis of Carboxymethyl-L-lysine in HbA1c by LC-MS/MS

Human haemoglobin (final concentration 10 mg/ml) was incubated with D-ribose (1 M) for four days, and aliquots (containing 10 μg protein) were collected daily and used for analysis by 15% SDS-PAGE. The bands were excised from the polyacrylamide gel, washed twice with double-distilled water, and destained with 40% acetonitrile/50 mM NH_4HCO_3 for CBB staining. The gel pieces were dehydrated with 100% acetonitrile and dried for 5 min with a SpeedVac. Disulfide bonds were reduced with DTT (10 mM, 56 °C, 45 min), and free sulfhydryl groups were alkylated with iodoacetamide (55 mM, 25 °C, 60 min in the dark). Gel pieces were washed with 50 mM NH_4HCO_3, then 50% acetonitrile/50 mM NH_4HCO_3, and dehydrated with 100% acetonitrile. After being dried with a SpeedVac, the gel was rehydrated with 100 ng/μl chymotrypsin (50 mM NH_4HCO_3, pH = 8.3) on ice for 30 min, and the digestion was carried out at 37 °C for 60 min and then quenched with

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