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A novel method to quantify in vivo transferrin glycation: Applications in diabetes mellitus

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Abstract

Background: In vitro glycation of transferrin leads to increased oxidative stress by impairing iron-binding antioxidant capacity. The aim of this study is to develop a method to evaluate in vivo transferrin glycation in diabetes.
Methods: We adapted the nitroblue tetrazolium assay to measure in micro-well plates the fructosamine content of transferrin isolated from serum by immunocomplexation.
Results: Introduction of the immunocomplexation step did not affect the analytical performance of the fructosamine measurement and analytical variability was lower than 7%. The diabetic group ($n=107$) had significantly higher transferrin glycation (1.39±1.12 versus 0.79±1.09 μmol fructosamine/g transferrin in the non-diabetic group, $n=91$, $p<0.0005$) and this was most pronounced in type 1 diabetes (1.95±1.02 versus 1.06±1.04 μmol fructosamine/g transferrin in type 2, $p<0.0005$). Transferrin glycation was associated with parameters of glycaemic control but did not correlate with serum iron or total iron-binding capacity. Total iron-binding capacity was lower in type 1 diabetes (63±9 versus 69±12 μmol/l in type 2, $p<0.05$) and was mainly determined by transferrin concentration.
Conclusions: These results indicate that the adapted nitroblue tetrazolium assay combined with immunocomplexation of serum transferrin is suitable to detect differences in in vivo transferrin glycation between non-diabetic, type 1 and type 2 diabetic subjects.

Keywords: Diabetes mellitus; Transferrin; Glycation; Fructosamine; Iron; Oxidative stress

1. Introduction

Diabetes mellitus is invariably accompanied by oxidative stress [1], which has been postulated to be the unifying pathogenic mechanism mediating the appearance and progression of chronic diabetic complications [2]. The increased levels of lipid and DNA peroxidation products [3,4] found in diabetic patients are the result of an imbalance between pro-oxidants (free radicals) and antioxidants which is directly caused by hyperglycaemia. Acknowledged sources of free radicals in diabetes are auto-oxidation of glucose [5], non-enzymatic glycation of proteins [6], monocyte dysfunction [7], ischemia–reperfusion [8] and mitochondrial dysfunction [9].

It has been proposed that excessive and redox-active iron can be an important and additional source of pro-oxidants in diabetes [10]. This hypothesis is supported by several observations. Firstly, this disease is closely associated with disturbances in iron metabolism ranging from haemochromatosis [11] and excessive iron stores [12,13] to iron-deficiency anemia [14]. Secondly, serum transferrin (Tf) concentration is often diminished in diabetes as a consequence of decreased synthesis and/or increased loss [15]. And lastly, recent experiments have demonstrated that in vitro non-enzymatic glycation of Tf impairs its capacity to bind iron and to prevent

Abbreviations: ANOVA, analysis of variance; AU, absorption units; apoTf, apotransferrin; CRP, C-reactive protein; NBT, nitroblue tetrazolium; Tf, transferrin; TIBC, transferrin iron-binding capacity.

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iron-induced lipid peroxidation in a liposomal model [16]. In vitro glycation also affected the iron-isoform distribution [17]. Consequently, diabetes could interfere with the important antioxidant function of Tf which involves the sequestration of iron in the safe redox-inactive Fe^{3+} form. In this way free, redox-active Fe^{2+} is prevented from participating in the Fenton reaction generating the very reactive hydroxyl radical [18] and in the chain reactions of lipid peroxidation involving alkoxyl and peroxyl radicals.

In order to investigate if this scenario also occurs in vivo and is involved in the increased peroxidation of lipids, proteins and DNA found in diabetes, the degree of glycation of Tf in blood of diabetic patients needs to be quantitated in a precise and reproducible manner. Since Tf has a relatively short half-life of ±7 days [19], Tf glycation will mainly involve the formation of a ketoamine (fructosamine) between the aldehyde group of glucose with the amino group of the protein. A widely used method to measure fructosamine content in serum is based on its reducing ability in alkaline solution [20]. However, the method currently used in diabetic practice does not differentiate between the glycation degree of the individual serum proteins. Since the concentration of albumin exceeds that of Tf by approximately 10–15 times, serum fructosamine measurements will mainly reflect albumin glycation and do not give an insight on the glycation level of Tf.

In this study we developed a new method that makes it possible to specifically quantify the glycation of Tf by adapting the classical fructosamine assay. In a first step the fructosamine assay for total protein glycation was validated for use in a microtiter 96-well plate and applied to compare groups of diabetic and non-diabetic subjects. In a second phase, this approach was further adapted for the measurement of Tf glycation and tested on Tf solutions with increasing degrees of in vitro glycation. Lastly, this newly developed assay was applied to measure in vivo glycation of Tf isolated from serum of patients by immunocomplexation.

2. Material and methods

2.1. Study population

The study was approved by the ethical commission of the University Hospital of Antwerp (ID 3/25/91) and informed consent was obtained from all participants.

Blood was collected from 107 (54 male/53 female) diabetic patients (40 type 1 and 67 type 2 diabetes) with a median age of 57 years, attending the outpatient clinic of the University Hospital for their annual clinical evaluation and 91 (54 males/37 females) healthy volunteers (company employees attending the outpatient clinic for a medical check-up and staff of the hospital and laboratories) with a median age of 46 years. Routine metabolic and iron parameters (glucose, serum iron, transferrin iron-binding capacity (TIBC) and albumin) were measured photometrically on the day of sampling (Vitros 950 AT Ortho Clinical Diagnostic Inc. Rochester-USA). Tf saturation was calculated by the formula: serum iron/ TIBC. HbA_{1C} was measured by ion exchange high performance liquid chromatography (ADAMS HA 8160 Menarini Firenze, Italy). High sensitive C-reactive protein (CRP) and Tf concentration were assayed nephelometrically (BNII Nephelometer, Dade Behring, Marburg, Germany).

To determine total protein glycation and Tf glycation, the blood samples were centrifuged at 1270×*g* for 10 min at 4 °C, the serum separated from the blood clot and frozen at −80 °C until analysis. All serum samples were thawed only once immediately before analysis.

2.2. Measurement of total serum protein glycation

Total serum protein glycation was determined by measuring the fructosamine content in serum using the nitroblue tetrazolium (NBT) colorimetric assay (Fruc, Roche, Mannheim, Germany) [20]. The automated procedure on Cobas Mira (Roche, Basel, Switzerland) was slightly adapted for use on 96-well plates. Briefly 250 µl NBT-reagent mixture was added to 25 µl serum and incubated at 37 °C. Absorption values after 10 and 15 min were registered at 540 nm (Labsystems Multiscan MS 352, Helsinki, Finland). The increase in absorption units (AU) allowed to calculate the fructosamine concentrations from a calibration curve established with a dilution series prepared from human standard serum (441 µmol/l fructosamine, Precimat® Roche, Mannheim, Germany). Precision and accuracy of this adapted method were evaluated using the two commercially available reference sera with fructosamine levels of 258 µmol/l (Precinorm®: range 216–296 µmol/l) and 500 µmol/l (Precipath®: range 424–574 µmol/l, Roche, Mannheim, Germany).

2.3. Preparation of in vitro glycated Tf solutions

Human apotransferrin (apoTf, Sigma-Aldrich, Steinheim, Germany), at a concentration of 5 mg/ml, was dissolved in sodium phosphate buffer (0.1 mol/l, pH 7.4) containing different concentrations (0, 5.6, 11.1, 22.2 or 33.3 mmol/l) of D-glucose (Merck, Darmstadt, Germany) and incubated for 14 days at 37 °C under sterile conditions. Thereafter, remaining free glucose was removed by passing the reaction mixtures through a Sephadex™ PD10 column (Amersham Bioscience, Uppsala, Sweden) equilibrated with sodium phosphate buffer. Protein concentration was determined in each of the collected fractions by the Bradford method [21] using weighed solutions of apoTf for the calibration. Aliquots of 120 µl (3 mg/ml) were stored at −70 °C until further use. Tf integrity after storage was verified nephelometrically (BNII Nephelometer, Dade Behring, Marburg, Germany) using specific antibodies against Tf (OSAX 15, Dade Behring, Marburg, Germany) and calibrated against an international standard (CRM 470).

2.4. Measurement of glycated Tf

The above described fructosamine method for the measurement of total serum protein glycation was adapted for specific quantification of Tf glycation. To eliminate interference from the other serum proteins in the assay, Tf was isolated by