

Review article

Glycative stress and anti-aging: 2. The Evaluation of Glycative Stress: Measurement of blood glucose, glycated proteins and intermediates.

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Abstract

Various substances produced in the course of glycation reaction can be used as markers for the evaluation of glycative stress. The glycative stress markers in the stage before the production and accumulation of advanced glycation end products (AGEs) include blood glucose, glycated protein and intermediates for AGEs. Self-monitoring of blood glucose (SMBG) is a simple method for the measurement of blood glucose. Glycated proteins include hemoglobin A1c (HbA1c) and glycoalbumin (GA), and can be measured by a clinical test organization. The intermediates for AGEs include 3-deoxyglucosone (3DG), glyoxal (GO), methylglyoxal (MG), glyceraldehyde and glycolaldehyde. 3DG can be measured by certain test organizations that can be assigned with special clinical test items. However, for the measurement of other intermediates for AGEs, a measuring system should be structured based on literatures as reference.

KEY WORDS: Glycative stress marker, measurement method, blood glucose, glycated protein, intermediates for AGEs

1. Glycative stress markers

Glycative stress is enhanced by elevated blood glucose and the formations of carbonyl compounds and aldehyde compounds, and consequently leads to the production and accumulation of advanced glycation end products (AGEs) in the body. This series of reactions is accelerated by ultraviolet rays and oxidization. Various substances produced in the course of glycation reaction are used as markers for the evaluation of glycative stress. The measuring methods for blood glucose, glycated protein and intermediates for AGEs are summarized, which are used as glycative stress markers in the previous stage before the production and accumulation of AGEs, as follows.

2. Measurement of blood glucose

Blood glucose can be used as a glycative stress marker because it evaluates elevated after-meal blood glucose condition. Self-monitoring of blood glucose (SMBG) is the simplest method to measure blood glucose. There are two measuring principles for blood glucose, enzymatic electrode method and colorimetry method¹⁾.

Enzymatic electrode method includes two methods, glucose oxidase (GOD) method and glucose dehydrogenase (GDH) method, depending on the difference of enzyme used for dissolving glucose²⁾. In either measuring method, the electrons generated when an enzyme dissolves blood glucose are given to ferricyanide ions, *i.e.* electron mediators, and changes them to ferrioxynide ions (**Fig. 1**). After that, because the electric current generated when a fixed voltage is applied to electrode responds to the concentration of blood glucose, the current value can be measured as glucose concentration.

The enzyme colorimetric method includes two methods, the hexokinase (HX) method and glucose oxidase/peroxidase (GOD/POD) method. In the HX method, after blood glucose is converted to glucose-6-phosphate (G6P), nicotinamide adenine dinucleotide (NADH) and tetrazolium are generated by glucose-6-phosphate dehydrogenase (G6PDH), are activated by diaphorase, and the hormazan dye colored from blue to red or others is colorimetrically determined. In the GOD/POD method, quinone pigment, which is generated when gluconic acid and hydrogen peroxide generated from glucose by the action of GOD react to peroxidase (POD), and the primary color substances of the reaction agent is colorimetrically determined.

In SMBG, when the hematocrit level is near the reference range (20-60%), accurate measuring is possible. However, if the blood hematocrit level is less than 20%, as in the cases of anemia patients and dialysis patients, it shows a higher level. On the other hand, if the hematocrit level is more than 55%, as in the cases of newborn babies and women before a menstrual period, it shows a lower level. In the GOD method, the greater the dissolved oxygen partial pressure in blood is, the lower the level of blood glucose is measured³⁾. In Japan, SMBG instruments with various characteristics are sold by a number of makers. Therefore, it is possible to select an instrument according to necessity⁴⁾. Measuring time differs by each instrument, but the difference is only 5-30 seconds. SMBG is classified into specially controlled medical devices, class 3 by the Pharmaceutical Affairs Act. Therefore, the advertisement of SMBG is not permitted, so general consumers rarely have a chance to know the convenience of blood measurement.

3. Measurement of glycated protein

Glycation reactions can be divided into “early stage reaction,” “intermediate stage reaction” and “late stage reaction.” Amadori rearrangement products produced in early stage reaction include glycated proteins, such as hemoglobin A1c (HbA1c) and glycoalbumin (GA). They are used as the indexes reflecting the past condition of blood glucose for the evaluation of diabetes, and they are useful glycative stress markers, as are blood glucose levels⁵⁾.

Valine, *i.e.* an amino acid of β -chain N-terminal of hemoglobin A, became HbA1c, *i.e.* a glycated protein, through Schiff base caused by a glycation reaction with glucose. As glycation reaction depends on the concentration of blood glucose, the glycation rate of hemoglobin increases, depending upon the level of elevated blood glucose (concentration and time). Therefore, the HbA1c thus formed, exits for 120 days, which is the longevity of red blood cells. As a result, the percentage of HbA1c reflects the blood glucose condition for the past one to two months. There are three methods for measuring HbA1c, the high performance liquid chromatography (HPLC) method, immunization method and enzyme method⁶⁾. In the HPLC method, hemoglobin is separated and analyzed by ion-exchange chromatography, and the percentage of HbA1c is calculated from the peak area of HbA1c and hemoglobin. HPLC method has the merit that it can find fetal hemoglobin (HbF) and abnormal hemoglobin, other than HbA1c. The immunization method is a measuring method using antibodies produced using HbA1c as an antigen, and it can selectively measure only HbA1c. Because it uses a highly specific antibody, it is immune to the influence of hemoglobins other than HbA1c. In the enzyme method, after glycated peptide of β -chain N-terminal of hemoglobin is digested by protease, glycated valine peptide is measured by enzymatic assay using fructosylpeptide oxidase. The enzyme method is suitable for processing a large number of specimens by an automatic analyzer. However, for the immunization method and enzyme method, the total amount of hemoglobin should be measured separately for the purpose of calculating HbA1c. International standardization is applied for the measurement of HbA1c, and the level cited in the National Glycohemoglobin Standardization Program has been

introduced⁷⁾. As a result, the level by the Japan Diabetes Society (JDS), which has been used in routine medical treatment, is required to be adjusted approximately 0.4% higher.

GA is a glycated protein of serum albumin. As the half-life period of serum albumin is about 20 days, GA reflects the average blood glucose level for a period from two weeks to one month in the past. GA can be applied for diabetes patients with a wide fluctuating range of blood glucose level and receiving drug therapy (insulin administration). Four glycated moiety (Lys-199, 281, 439 and 525) have been confirmed in an albumin molecule⁸⁾, and albumin has the characteristic to comparatively respond more swiftly and more largely to the fluctuation of blood glucose level than HbA1c, and continue to transit. The HPLC method and enzyme method are used for the measurement of GA, and the enzyme method is mainly used in clinical examinations at present. In this method, after blood albumin is digested by protease and glycated lysine is released, albumin is measured by enzymatic assay using ketoamine oxidase. The total amount of albumin is specifically measured by the bromocresol purple method (BCP), and the percentage of GA included in albumin is calculated.

HbA1c and GA are among the measurement items of blood test for the examination of lifestyle related diseases and screening test for blood donation, and they can be tested by a clinical test organization.

4. Measurement of intermediates for AGEs

There are 3-deoxyglucosone (3DG), glyoxal (GO), methylglyoxal (MG), glyceraldehyde and glycolaldehyde among the intermediates for AGEs (Fig. 2)⁹⁾. 3DG is an α -dicarbonyl compound formed from glycated protein, has a reactivity 10,000 times higher than glucose, and is involved in the formation of AGEs. If the concentration of 3DG in the blood plasma increases by 100 nmol/L, the risks of diabetic retinopathy and nephropathy increase by about twice as much (Fig. 3)¹⁰⁾. GO is formed by the auto-oxidation of glucose and the peroxidation of lipids. MGO is also formed in the glycolytic pathway, and polyol pathway in cells. Because the glycation pathway of intermediates for AGEs is different depending on each compound, the kinds of AGEs ultimately formed are different. AGEs having glyceraldehydes as intermediate, in particular, are called toxic-AGEs (TAGE) and are deeply involved in the onset and development of complications of diabetes¹¹⁾.

3DG, GO and MG can be measured by the HPLC assay with 2,3-diaminonaphthalene (DAN) pre-column derivatization¹²⁾. For these measurements, after the blood samples are deproteinated, they are added with DAN, and the intermediates for AGEs are derivatized with aminonaphthalene and purified, then are analyzed by the reverse HPLC method. For the detection of intermediates for AGEs converted to aminonaphthalene, ultraviolet absorption (wavelength of 268 nm) or fluorescence (excitation wavelength of 271 nm and fluorescence wavelength of 503 nm) can be applied (Fig. 4). 3DG can be measured by certain test organizations that can be assigned with special items. However, for other intermediates for AGEs, a measuring system should be structured based on literatures as a reference.

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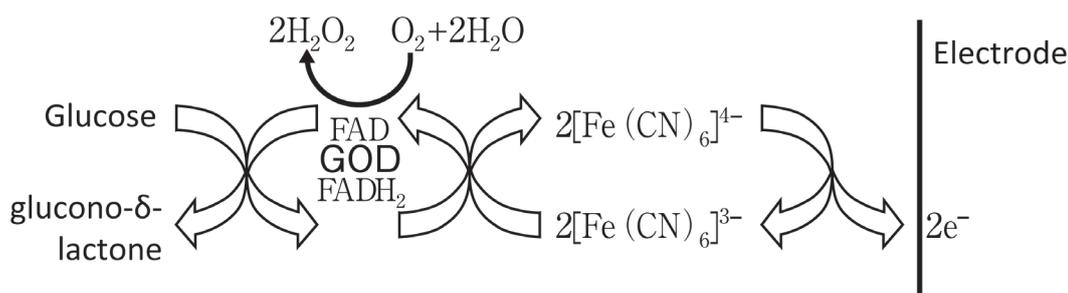
Conflict of interest statement

There are no items deemed to be conflicts of interest in this research.

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Biosensor based on the immobilization of GOD



Biosensor based on the immobilization of GDH

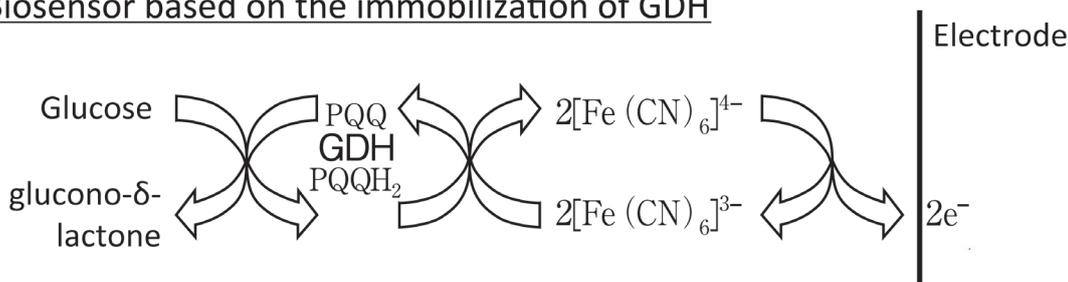


Fig. 1. Reaction scheme of the glucose sensor by an enzymatic electrode method.

GOD, glucose oxidase; GDH, glucose dehydrogenase; FAD, flavin adenine dinucleotide; PQQ, pyrroloquinoline quinone; 2[Fe(CN)₆]⁴⁻, ferrocyanide ion; 2[Fe(CN)₆]³⁻, ferricyanide ion. (adapted from Ref. 2)

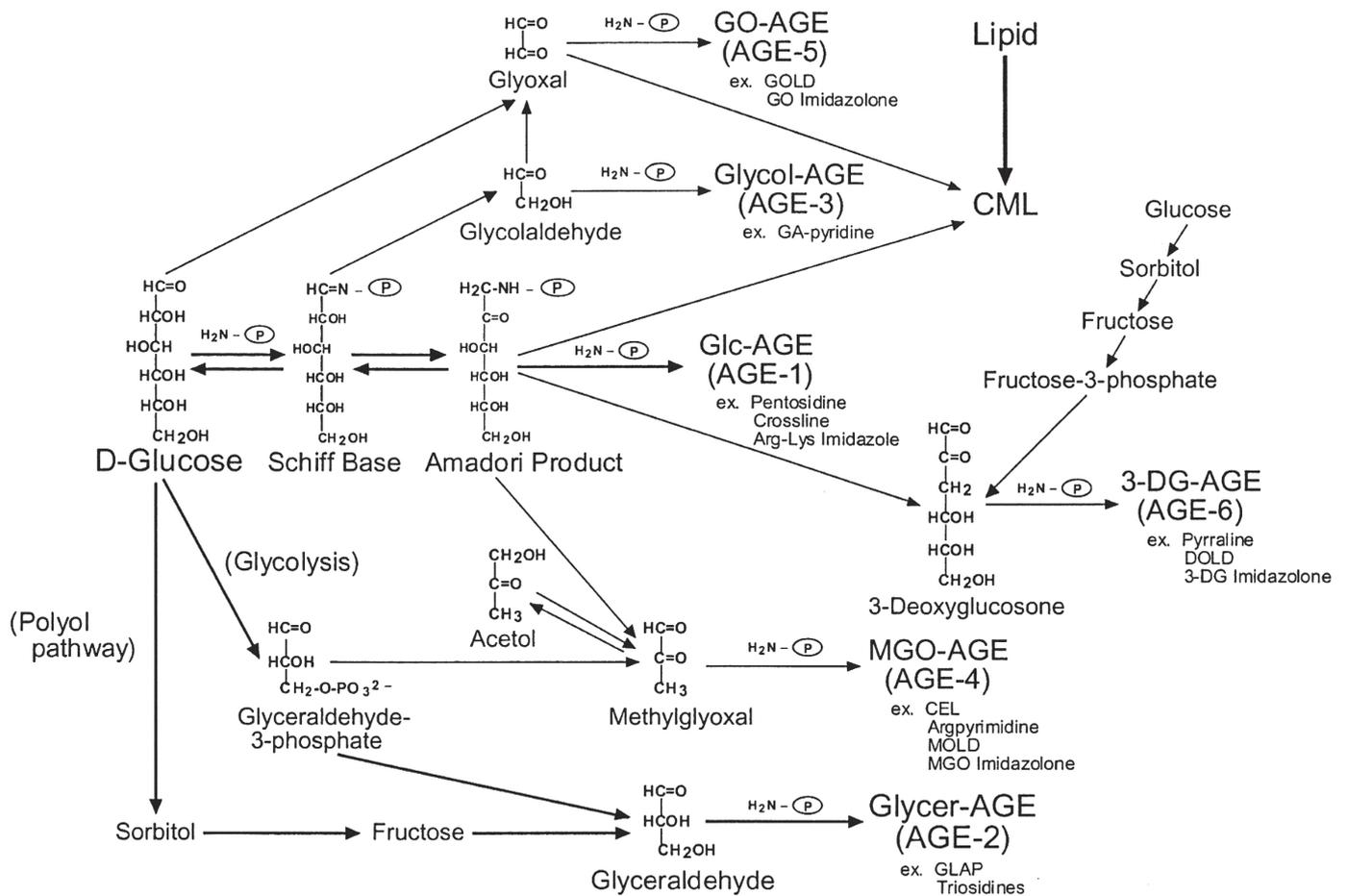


Fig. 2. Alternative routes for the formation of various distinct AGEs *in vivo*.

AGEs, advanced glycation end products; Glc-AGE (AGE-1), glucose-derived AGEs; Glycer-AGE (AGE-2), glyceraldehyde-derived AGEs; Glycol-AGE (AGE-3), glycolaldehyde-derived AGEs; MGO-AGE (AGE-4), methylglyoxal (MGO)-derived AGEs; GO-AGE (AGE-5), glyoxal (GO)-derived AGEs; 3-DG-AGE (AGE-6), 3-deoxyglucosone (3-DG)-derived AGEs; P-NH₂, free amino residue of protein. GOLD, GO-lysine dimer; GA-pyridine, 3-hydroxy-4-hydroxymethyl-1-(5-carboxypentyl) pyridinium cation; CML, N^ε-(carboxymethyl)lysine; DOLD, 3-DG-lysine dimer; CEL, N^ε-(carboxyethyl)lysine; MOLD, MGO-lysine dimer; GLAP, glyceraldehyde derived pyridinium compound. (adapted from Ref. 8)

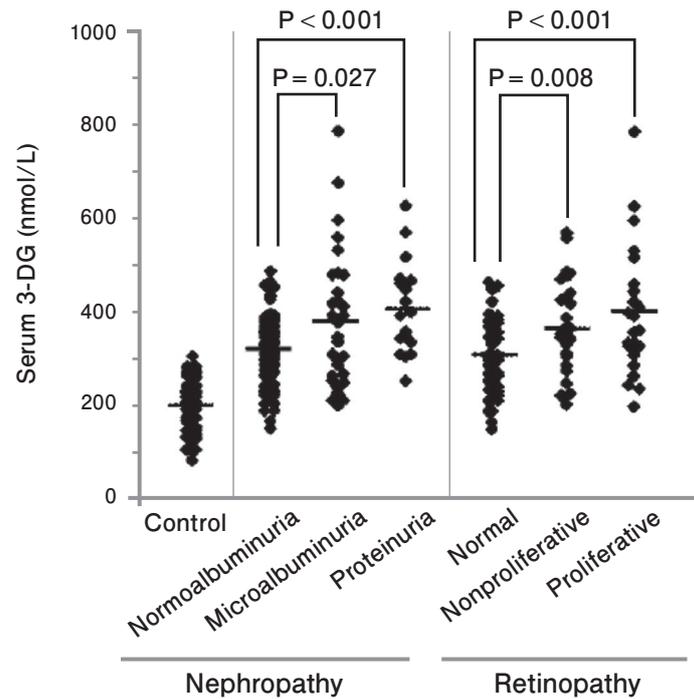


Fig. 3. Serum 3DG concentrations in diabetic patients with various grades of nephropathy and retinopathy.

Control, nondiabetic patients and healthy volunteers; Normal, retinopathy was assessed by ophthalmologists using fundus photography; Benferroni / Dunn multiple comparison test. 3DG, 3-deoxyglucosone. (adapted from Ref. 10)

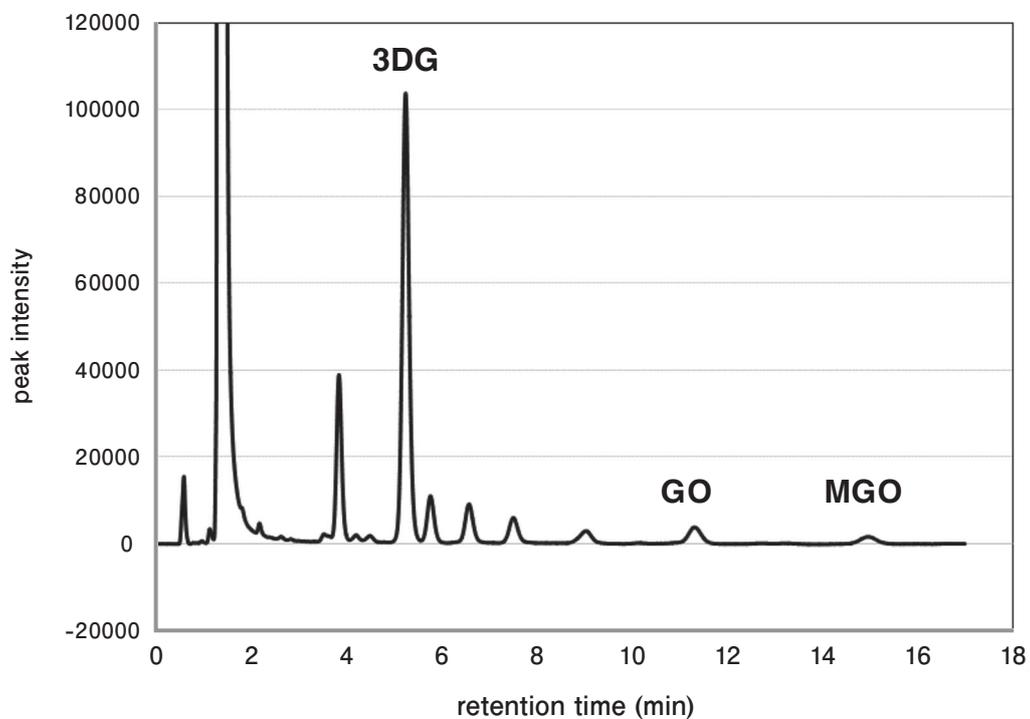


Fig. 4. HPLC chromatogram of intermediate for AGEs.

Human serum albumin (8 mg/mL) and glucose (0.05 mol/L) added into the phosphate buffer (pH 7.4), and incubated in 60°C at 40 hours. HPLC measurement intermediate for AGEs was carried out after pre-label derivatization reaction by 2,3-diaminonaphthalene (DAN). HPLC condition; column, Unison UK-Phenyl (Imtakt), 75 x 3 mm ID; eluent, 0.05 mmol/L phosphoric acid / ACN = 89 / 11; flow rate, 1.0 mL/min; detection, UV 268 nm. AGEs, advanced glycation end products; 3DG, 3-deoxyglucosone; GO, glyoxal; MGO, methylglyoxal; HPLC, high performance liquid chromatography.