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Superoxide production from nonenzymatically glycated protein

Tamiko Sakurai and Seishi Tsuchiya

Tokyo College of Pharmacy, Horinouchi 1432-1, Hachioji Tokyo 192-03, Japan

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Nonenzymatically glycated human serum albumin and glycated poly-lysine(Lys) in vitro brought about the reduction of nitroblue tetrazolium and ferricytochrome *c* at pH 9.06 and pH 7.8, respectively. This reduction was inhibited partially by superoxide dismutase (SOD). Glycated poly-Lys caused the oxidation of NADH in the presence of LDH at pH 7.0 which was completely inhibited by SOD. Glycated material was found to function both as a reductant and an oxidant. The reactivity of glycated material is discussed and a possible mechanism by which superoxide is produced is proposed. Results may give a clue to diabetic complications.

Nonenzymatic glycation; Superoxide; Poly-lysine; Albumin

1. INTRODUCTION

The determination of glycated protein content is clinically important for glycemic control in diabetes mellitus. Johnson et al. [1] described a new colorimetric assay method for this purpose, based on the ability of the ketoamine (scheme 1) moiety of the glycated protein to reduce nitroblue tetrazolium (NBT) at pH 10. Recently, clinical application of this method has been made [2–7]. Jones et al. [8] found superoxide dismutase (SOD) to inhibit the reducing activity of glycated plasma albumin in diabetic patients; thus possibly, the reduction of NBT may be mediated by superoxide. Diabetic patients show significantly higher levels of lipid peroxidation products in plasma [9]. It is very interesting to know whether glycated proteins have reducing activity under physiological conditions. In this paper, we investigated the possibility of reactivity of glycated proteins, using glycated human serum albumin and glycated poly-lysine in vitro. The results of our experiments indicate that under physiological conditions, glycated material produces superoxide.

Correspondence address: T. Sakurai, Tokyo College of Pharmacy, Horinouchi 1432-1, Hachioji, Tokyo 192-03, Japan

Abbreviations: HSA, human serum albumin; NADH, nicotinamide adenine dinucleotide (reduced); LDH, lactate dehydrogenase

2. MATERIALS AND METHODS

Human serum albumin (Sigma, fatty acid free) was subjected to gel filtration (Sephacryl 200) to obtain its monomer referred to hereafter as HSA. A 4% solution of HSA in a 1/15 M Na-phosphate buffer (pH 7.4) with 1 M glucose was incubated under sterilization for 6 days at 37°C. After dialysis against phosphate buffer at 4°C, a solution of glycated HSA was obtained. The extent of glycation was assessed by the thiobarbituric acid reaction [10] to be 0.32 mM 5-hydroxymethylfurfural (5-HMF) equivalents (approx. 1 mol 5-HMF/mol HSA). 1% of poly-Lys (hydrobromide, M_r 58 000, Sigma) was incubated under the same conditions as HSA. Following dialysis, the extent of glycation was estimated to be 1.2 mM 5-HMF equivalent (approx. 13 mol 5-HMF/mol poly-Lys). In the NBT method [8], 2 ml aliquots of 0.25 mM NBT (Wako) in 50 mM Tris-HCl buffer (pH 7.4, 8.0, 9.06) were titrated by glycated material at a final volume of 3 ml. The reduction rate of NBT was monitored by increase in absorbance at 530 nm for 10 min at 37°C. In the cytochrome *c* (cyt *c*) method [11], a reaction mixture containing 10 μM ferricyt *c* (Sigma, horse heart type III), 100 μM EDTA and 50 mM Na-phosphate buffer (pH 7.8) was titrated by glycated material at a final volume of 3 ml. Reduction rate of ferricyt *c* was monitored by the increase in absorbance at 550 nm for 10 min at 25°C. In the NADH-LDH method [12], a reaction mixture containing 200 μM NADH (Sigma), 0.66 mg of LDH (Boehringer, Mannheim, pig heart), 80 μM EDTA and 28 mM Na-phosphate buffer (pH 7.0)

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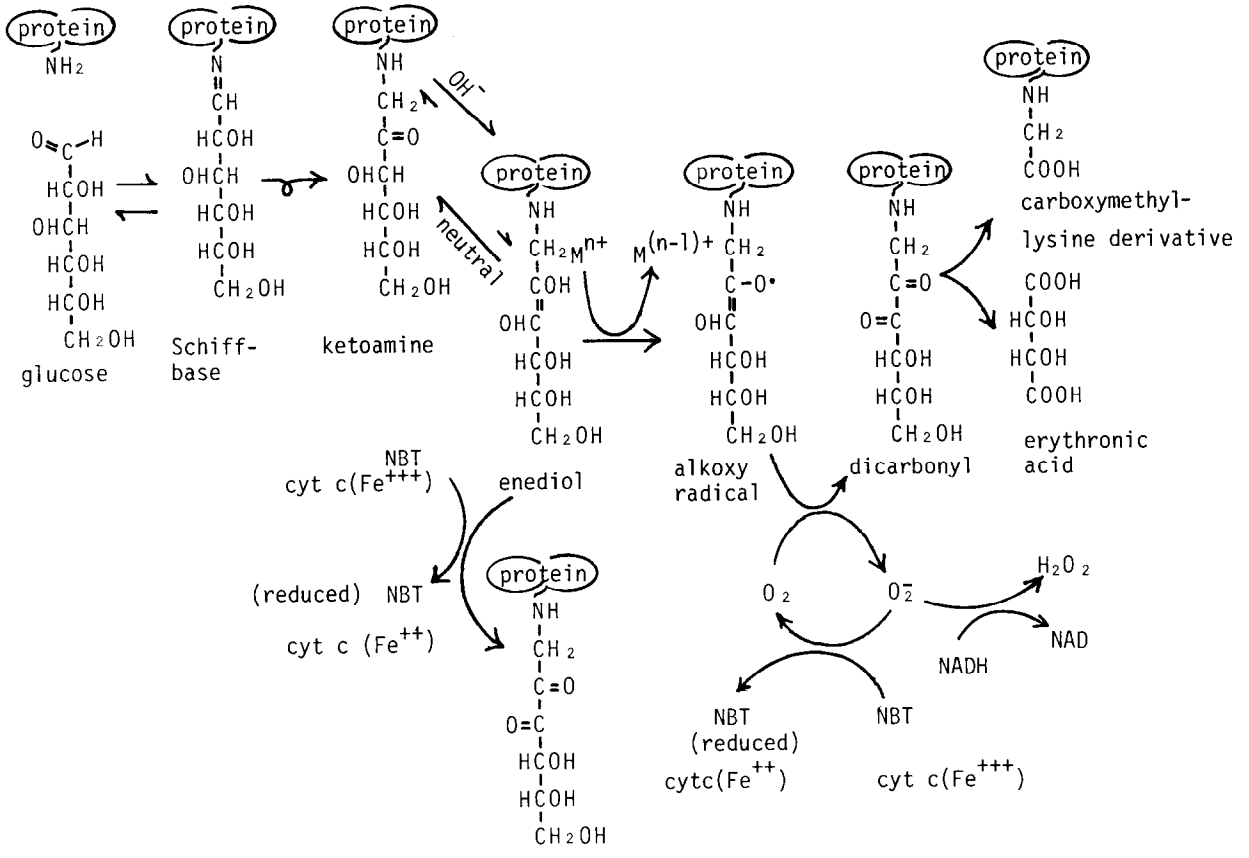

Scheme 1 Possible Mechanism of Superoxide Production

M^{n+} and $M^{(n-1)+}$ indicate metal ions.

was titrated by glycated material at a final volume of 3 ml. NADH oxidation was monitored by decrease in absorbance at 340 nm for 10 min at 37°C. Ultrafiltration was carried out using an ultrafiltration membrane YMT (Amicon). Effects of amino acids on the reduction of NBT or ferricyt *c* and oxidation of NADH were examined in the presence of 0.83 mM L-amino acid; Cys, $(Cys)_2$, Met, Thr, Ser, His, Pro, Arg, Asp, Asn, Glu, Gln, Trp, Tyr and Phe.

3. RESULTS

3.1. *Reduction of NBT and cyt* c

The reduction of NBT by glycated material was studied at pH 9.06 (fig.1a). Glycated HSA and glycated poly-Lys caused the rapid reduction of NBT to a significant degree, the rate of reduction due to the former being twice that of the latter at the same 5-HMF equivalent. Poly-Lys per se did not reduce NBT, though HSA did so, depending on its concentration (200 μl of 2% HSA caused ΔOD 530/10 min = 0.009). Superoxide-scavenging enzyme, 10 μg (0.1 μM) SOD (Sigma, bovine blood, 3250 unit/mg) inhibited this reduction by glycated HSA (200 μl, 0.064 μmol 5-HMF equivalent) and by glycated poly-Lys (200 μl, 0.24 μmol 5-HMF equivalent) to 50% and 64%, respectively (fig.1b). Other active oxygen scavengers such as catalase (50 μg), sodium azide (150 μM), mannitol (17 mM) had no effects. Superoxide is involved in about half the reduction of NBT caused by glycated material. To determine if superoxide is related to the reduction of NBT under physiological conditions, the NBT method was carried out at pH 7.4 and 8.0 also. NBT reduction by glycated HSA was found to decrease sharply with a decrease in pH. At pH 7.4,

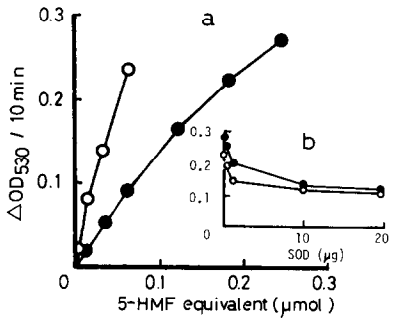

Fig.1. Reduction of NBT by glycated material (pH 9.06) (a) and the effect of SOD (b). (a) 2 ml aliquots of 250 μM NBT were titrated by glycated HSA (○) and glycated poly-Lys (●). (b) After the addition of aliquots of SOD to 2 ml of 250 μM NBT, the reaction was started by the addition of glycated HSA (○, 0.064 μmol 5-HMF equivalent) and glycated poly-Lys (●, 0.24 μmol 5-HMF equivalent).

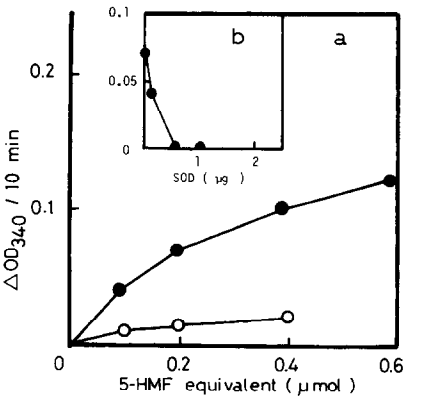

Fig.3. Oxidation of NADH by glycated material (pH 7.0) (a) and the effect of SOD (b). (a) 0.5 ml of 125 mM Na-phosphate buffer, 0.6 ml of LDH (3.3 mg protein/ml) and glycated material (glycated HSA, ○; glycated poly-Lys, ●) were mixed followed by volume adjustment to 2.5 ml. The reaction was started by the addition of 0.5 ml of 1.2 mM NADH. Inset (b) shows the effect of SOD on NADH oxidation by glycated poly-Lys (0.24 μmol 5-HMF equivalent).

no reduction was observed. This dependency on pH was also noted in the reduction by ascorbic acid and HSA, indicating that NBT is non-reducible at physiological pH. Therefore, glycated material cannot be concluded to have no reactivity under physiological conditions.

Ferricyt *c* is reducible to ferrocyt *c* at pH 7.8 by superoxide [11]. The reduction rate of ferricyt *c* increased linearly with the amounts of added glycated poly-Lys and glycated HSA (fig.2a). Poly-Lys per se had no effect on ferricyt *c* and that of

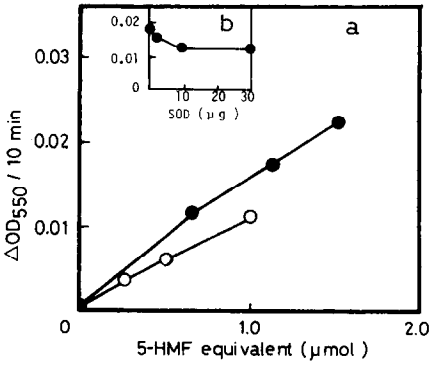

Fig.2. Reduction of ferricyt *c* by glycated material (pH 7.8) (a) and the effect of SOD (b). (a) 10 μM of ferricyt *c* was titrated by glycated HSA (○) and glycated poly-Lys (●). Inset (b) shows the effect of SOD on the reduction of cyt *c* by glycated poly-Lys (1.14 μmol 5-HMF equivalent).

HSA was negligible. SOD (0.1 μM) inhibited the reduction rate of glycated poly-Lys to 30% (fig.2b). These results demonstrate glycated material to function as a reductant.

3.2. *Oxidation of NADH*

Provided glycated material produces superoxide, it can also function as an oxidant because superoxide can serve as an oxidant, depending on the redox potential of a compound. Superoxide can be coupled to oxidation of NADH in the presence of LDH at neutral pH [12]. As shown in fig.3a, oxidation of NADH was brought about by glycated poly-Lys. On the other hand, glycated HSA showed lower levels of oxidation than glycated poly-Lys at the same 5-HMF equivalent. Oxidation of NADH by glycated poly-Lys (0.24 μmol 5-HMF equivalent) was completely inhibited by the addition of 0.5 μg (0.005 μM) SOD (fig.3b).

Should amino acid residues in the HSA molecule be reactive toward superoxide, it follows that NADH must compete with them for superoxide when glycated HSA is added to the NADH (+ LDH) system. Consequently, an examination was made of the effects of amino acids on the oxidation of NADH(+ LDH) by superoxide, produced by the reaction of xanthine with xanthine ox-

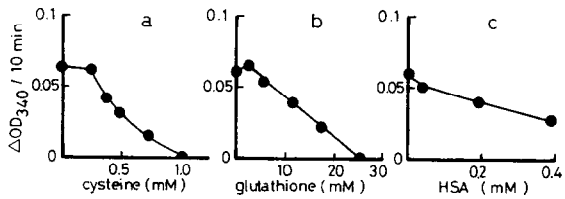

Fig.4. Effects of cyteine (a), glutathione (b) and HSA (c) on the oxidation of NADH by glycated poly-Lys. Cysteine, glutathione and HSA were added to the reaction mixture containing glycated poly-Lys (0.18 μmol 5-HMF equivalent) and after 1 min, the reaction was started by the addition of NADH.

idase. Only Cys was found to decrease the oxidation rate of NADH(+ LDH) (not shown). HSA contains only one free Cys residue [13]. The oxidation of NADH(+ LDH) was observed following the preincubation of glycated poly-Lys with Cys (fig.4a). NADH(+ LDH) oxidation by glycated poly-Lys (0.18 μmol 5-HMF equivalent) failed to occur at all at 1 mM of Cys, as was also noted for glutathione (fig.4b). HSA partially inhibited the oxidation of NADH(+ LDH) (fig.4c), indicating that the Cys residue of HSA is responsible for the low level of oxidation of NADH(+ LDH) by glycated HSA.

3.3. *Chemiluminescence*

Ultraweak chemiluminescence in organs is considered to be due to free radical-mediated reactions [14]. Poly-Lys incubated with glucose showed

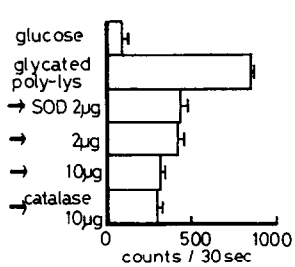

Fig.5. Chemiluminescence in glycated poly-Lys. Poly-Lys (50 mg/ml in a 1/15 M Na-phosphate buffer, pH 7.4) was incubated with 1 M glucose for 6 days at 37°C. Chemiluminescence of 2 ml mixture before and after the addition of enzymes was assayed at 37°C by a single photon-counting chemiluminescence analyzer OX-70 (Tohoku Electronic Industrial Co. Ltd). Mean values were obtained from 10 assays. 1 M glucose was incubated as the control.

significant counts of chemiluminescence which diminished by half following the addition of 2 μg (0.03 μM) of SOD even though further addition of 10 μg of catalase had no effect (fig.5).

4. DISCUSSIONS

The process of nonenzymatic glycation is known as condensation of glucose with the α-amino group of the N-terminus or with the ϵ-amino group of lysine residues via Schiff base intermediates, followed by the rearrangement to a stable ketoamine [15] as shown in the initial stages of scheme 1. However, further reaction of ketoamine is unknown except for a recent report indicating that fructoselysine is oxidatively degraded to carboxymethyllysine and erythronic acid [16]. In our experiment, the production of superoxide under physiological conditions by the glycated material was confirmed by the occurrence of NADH (+ LDH) oxidation by glycated poly-Lys and its complete inhibition by SOD, in addition to the observation of SOD-sensitive ultraweak chemiluminescence. Superoxide produced from glycated material is partially responsible for the reduction of NBT (pH 9.06) or ferricyt *c* (pH 7.8). Superoxide undergoes dismutation to O_2 and H_2O_2. H_2O_2 decomposes to a hydroxy radical under the catalytic influence of base [17]. Addition of H_2O_2 to NBT dissolved in carbonate buffer (pH 10.1) led progressively to development of a pale-blue colored formazan after 1 h. However, possible participation of a hydroxy radical in NBT reduction by glycated material need not be considered, since reduction in this experiment lasted for 10 min and mannitol did not affect the reduction rate of NBT. SOD-insensitive reduction by glycated material suggests the presence of 2,3-enediol with a reducing activity, as the ketoamine tautomer is preferentially present in alkaline medium (scheme 1). The structure of ascorbic acid prompted us to consider the 2,3-enediol form, which may possibly give rise to alkoxy radicals catalytically by the presence of trace amounts of metals in the buffer, reacting with oxygen followed by the production of superoxide and dicarbonyl. The reduction of NBT or ferricyt *c* may occur by two pathways, a superoxide pathway and an enediol pathway, which would explain the SOD-insensitive reduction. Oxidation of NADH occurs only by the superoxide pathway. If dicar-

bonyl were cleaved between C-2 and C-3, the formation of erythronic acid and carboxymethyllysine becomes explicable. We will investigate the possible presence of alkoxy radicals by electron spin resonance.

Why does glycated HSA show a higher reduction rate of NBT than glycated poly-Lys at the same 5-HMF equivalent (fig.1a)? HSA differs from poly-Lys (i) in having amino acid residues in addition to Lys, (ii) in having tertiary structure with three domains consisting of secondary structures with 61% α-helical content and 22% β-sheet content [13] in contrast to the random coil structure of poly-Lys at pH 9.0 [18] and (iii) in being an acidic protein with the p*I* value of 5.0, while poly-Lys has a positive charge at pH 9.0. At pH 9.06, 15 different amino acids were investigated for their effects on NBT. Only Cys was found to cause significant reduction of NBT. NBT has a positive charge due to a quarternary ammonium ion. Repulsion between NBT and poly-Lys molecules may be one of the reasons for the lower reduction of NBT by glycated poly-Lys. HSA is an acidic protein and thus there may be electrostatic interaction between NBT and HSA. HSA is highly capable of binding with a large number of small molecular ligands [13]. The binding of NBT to glycated HSA was confirmed by ultrafiltration. When a mixture of 1 ml of 2% glycated HSA and 0.7 ml of 250 μM NBT was ultrafiltrated, the free form of NBT was found to be only 20%. NBT bound to glycated HSA may possibly be subject to attack by superoxide produced within glycated HSA as free NBT. Thus, the higher reduction rate by glycated HSA may be due to the Cys residue and the binding of NBT to glycated HSA. This consideration regarding the Cys residue in HSA molecule extends to the cyt *c* method. Ferricyt *c* itself was reduced to ferrocyt *c* by only Cys, out of 15 different amino acids. However, the addition of Cys to the reaction mixture during reduction of ferricyt *c* by xanthine + xanthine oxidase system inhibited the reduction of ferricyt *c*, possibly because of the reaction of Cys with superoxide. These facts may make the reaction between ferricyt *c* and glycated HSA complicated. In the NADH method, superoxide produced within the highly structured molecule of glycated HSA may be caught by its own Cys residue or may be less accessible to NADH bound to the active site of LDH, than in the case of superoxide produced by randomly coiled glycated poly-Lys.

Whilst submitting this paper, we read a related study entitled 'Superoxide radical generation by Amadori compound' by Azevedo et al. [19]. The reaction of Amadori compounds with NBT at pH 10.8 in our view may not be comparable to physiological conditions.

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