Non-enzymic glycation of human extracellular superoxide dismutase

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The secretory enzyme extracellular superoxide dismutase (EC-SOD) is in plasma heterogeneous with regard to heparin-affinity and can be divided into three fractions, A that lacks affinity, B with intermediate affinity and C with high affinity. The C fraction forms an equilibrium between the plasma phase and heparan sulphate proteoglycan on the surface of the endothelium. *In vitro* EC-SOD C could be time-dependently glycosylated. The enzymic activity was not affected in glycosylated EC-SOD, but the high heparin-affinity was lost in about half of the studied glycosylated fraction. Addition of heparin decreased the glycosylation *in vitro*, and EC-SOD C modified with the lysine-specific reagent trinitrobenzenesulphonic acid could not be glycosylated *in vitro*. The findings suggest that the glycosylation sites are localized rather far away from the active site and may occur on lysine residues in the heparin-binding domain in the C-terminal end of the enzyme. The proportion of glycosylated EC-SOD in serum of diabetic patients was considerably higher than in normal subjects. Of the subfractions, EC-SOD B was by far the most highly glycosylated, followed by EC-SOD A. EC-SOD C was glycosylated only to a minor extent. The findings suggest that glycosylation is one of the factors that contribute to the heterogeneity in heparin-affinity of plasma EC-SOD. Since this phenomenon is increased in diabetes, the cell-surface-associated EC-SOD may be decreased in this disease, increasing the susceptibility of cells to superoxide radicals produced in the extracellular space.

INTRODUCTION

Protein glycation, a factor possibly contributing to tissue damage in diabetes mellitus, has been extensively modelled by exposure of proteins to glucose *in vitro* [1]. In glycation, glucose reacts with free amino groups (α -amino groups of terminal amino acid residues and ϵ -amino groups of peptide-bound lysine residues), first forming a reversible Schiff base (aldimine) intermediate, which then undergoes an Amadori re-arrangement to form a stable practically irreversible ketoamine structure [2]. Increased glycation and subsequent reactions of proteins are thought to be involved in not only structural but also functional changes in proteins [3], for example, activation of aldose reductase [4] and inactivation of copper,zinc-superoxide dismutase (Cu,Zn-SOD) [5]. In addition to the above mechanism, it has been reported that glucose can reduce molecular O_2 , yielding ketoaldehyde and H_2O_2 [6]. This 'glucose autooxidation' and/or related metal-ion-catalysed autooxidation processes may also contribute to protein modification [7].

Extracellular superoxide dismutase (EC-SOD, EC 1.15.1.1), a secretory tetrameric Cu- and Zn-containing glycoprotein [8,9], is the major isoenzyme in extracellular fluids such as plasma, lymph [10] and synovial fluid [11], but occurs also in tissues [12,13]. EC-SOD in plasma in man [14] and other mammalian species [15] is heterogeneous with regard to affinity for heparin-Sepharose and can be divided into three fractions, A that lacks affinity, B with weak affinity and C with relatively strong affinity for heparin. Whereas subfractions A and B mainly reside in the plasma phase, EC-SOD C apparently forms an equilibrium between the plasma phase and heparin sulphate proteoglycan of the glycocalyx of endothelial cell surfaces [14–17]. Tissue EC-SOD C is likely to be similarly associated with heparan sulphate proteoglycan on cell surfaces and in the connective-tissue matrix [16]. The molecular background to the heterogeneity of plasma EC-SOD is still unresolved. Studies with

amino acid-specific reagents indicate that both lysine and arginine residues are involved in the binding to heparin and dextran sulphate [18]. With lysine the subtle modification of only a few residues seems to be sufficient for loss of high affinity for heparin [18].

In the present investigation the effect of glycation on enzymic activity and affinity for heparin-Sepharose of EC-SOD are explored. The putative involvement of glycation in the heterogeneity of serum EC-SOD for heparin was also studied.

EXPERIMENTAL

Materials

Human recombinant EC-SOD C (r-EC-SOD C) was prepared as described previously [8] (specific activity: 1300 units/mg by the pyrogallol autooxidation method). Heparin-Sepharose, CNBr-activated Sepharose 4B and Sephadex G-25 were products of Pharmacia LKB Biotechnology, Uppsala, Sweden. Glyco-gel B was purchased from Pierce Chemical Co., Rockford, IL, U.S.A. Alkaline-phosphatase-conjugated goat anti-[mouse IgG (H+L)] antibody was purchased from Zymed Laboratories, San Francisco, CA, U.S.A. Trinitrobenzenesulphonic acid was obtained from Wako Pure Chemical Industries, Osaka, Japan. Immunoplate was purchased from Nunc, Roskilde, Denmark.

Blood samples

Blood samples were tapped into vacuum tubes without anticoagulant. After centrifugation (1500 *g* for 15 min) the serum was kept at -80°C . Both insulin-dependent and insulin-independent diabetes patients were studied. Healthy laboratory personnel served as controls.

Assay of SOD activity

EC-SOD enzymic activity was determined with the pyrogallol autooxidation method as described previously [19,20].

Abbreviations used: EC-SOD, extracellular superoxide dismutase; Cu,Zn-SOD, copper,zinc-superoxide dismutase; r-, recombinant.

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E.i.s.a. of SOD concentration

An 80 μ l portion of 20 μ g/ml rabbit anti-EC-SOD antibody dissolved in 50 mM-sodium carbonate buffer, pH 9.5, containing 0.02 % NaN_3 was added into each well of immunoplates and left to stand overnight at 4 °C. Each well was washed with phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4) containing 0.05 % Tween 20 and 0.05 % merthiolate (washing buffer). The remaining protein-binding sites were blocked with 300 μ l of phosphate-buffered saline containing 1 % BSA, 0.05 % Tween 20 and 0.05 % merthiolate (blocking buffer). The plate was then left to stand at 4 °C until use.

Portions (70 μ l) of sample or standard diluted with blocking buffer were added to the wells. The plate was incubated for 2 h at room temperature and washed three times with the washing buffer. Then 80 μ l of mouse anti-r-EC-SOD monoclonal antibody diluted with blocking buffer was added to each well, and the plate was incubated for 2 h at room temperature and washed three times with the washing buffer. A 90 μ l portion of alkaline-phosphatase-conjugated anti-(mouse IgG) antibody at a 1:1000 dilution in blocking buffer was added to each well and the plate was incubated again for 2 h at room temperature, followed by washing three times with washing buffer. Substrate solution (150 μ l of 0.1 M-diethanolamine hydrochloride, pH 9.8, containing 0.5 mM- MgCl_2 , 0.02 % NaN_3 and 2.7 mM-*p*-nitrophenyl phosphate) was then added to each well and the plate was incubated for 30 min at room temperature. The enzyme reaction was stopped by the addition of 50 μ l of 5 M-NaOH, and the absorbance at 415 nm was measured with a Corona MTP-32 micro-plate reader.

Glycation of EC-SOD *in vitro*

Portions (2 ml) of r-EC-SOD C (40 μ g/ml) were incubated with or without the indicated final concentrations of D-glucose in 0.1 M-potassium phosphate, pH 7.3, at 37 °C for the indicated numbers of days under sterile conditions, followed by extensive dialysis against phosphate-buffered saline with several changes.

Glyco-gel B column chromatography

The samples (50 μ l of serum, 10 μ l of r-EC-SOD C glycated *in vitro* or 1 ml of EC-SOD A, B and C purified with immunoabsorbent column) were applied to a column (volume 0.5 ml or 1 ml) equilibrated with 1 M-sodium acetate buffer, pH 8.5, containing BSA (10 μ g/ml) and washed with the same buffer. The bound fraction was eluted with 1 M-sodium acetate buffer, pH 5.5, containing BSA (10 μ g/ml).

Heparin-Sepharose column chromatography

The samples (6 ml of serum or 1–2 ml of glycated or non-glycated forms of r-EC-SOD C) were dialysed against 25 mM-sodium phosphate buffer, pH 6.5, extensively before application. Dialysed samples were applied to a heparin-Sepharose column (volume as indicated) equilibrated with the above buffer and washed with the same buffer. The bound fraction was then eluted with stepwise additions of buffer containing increasing concentrations of NaCl (0.1 M increments) or a linear gradient of NaCl (0–1 M) in the buffer.

Purification of EC-SOD A, B and C with anti-(r-EC-SOD C)-monoclonal-antibody-conjugated Sepharose 4B

Anti-(r-EC-SOD C) monoclonal antibody was coupled to CNBr-activated Sepharose 4B at a concentration of 3.5 mg/ml of swollen gel, essentially according to the manufacturer's suggestions.

The samples (8–24 ml of EC-SOD A, B and C fractions in serum separated by heparin-Sepharose column chromatography)

were applied to the anti-(r-EC-SOD C)-monoclonal-antibody-conjugated Sepharose 4B (volume 2 ml) equilibrated with coupling buffer and washed continuously with the same buffer until the effluent showed no measurable absorbance at 280 nm. The adsorbed EC-SOD was eluted by 0.5 M-NaCl/0.2 M- Na_2CO_3 containing BSA (100 μ g/ml). The pH of each fraction was neutralized with minimum volume of 2 M-acetic acid. The immunoreactive fractions (assayed by e.i.s.a.) were dialysed against 25 mM-sodium phosphate buffer, pH 7.5.

Trinitrobenzenesulphonic acid modification of EC-SOD

A 2 vol. portion of 3 mM-trinitrobenzenesulphonic acid in 0.2 M- NaHCO_3 was added to 1 vol. of r-EC-SOD C (1 mg/ml) in distilled water and left to stand for 2 h at room temperature. Modified r-EC-SOD C was applied to a Sephadex G-25 column (1 cm $\times$ 60 cm) equilibrated with phosphate-buffered saline to remove excessive trinitrobenzenesulphonic acid.

RESULTS

Percentages of the glycated form of EC-SOD in serum

Samples (50 μ l) of sera from diabetic patients or normal subjects were loaded on a Glyco-gel B column (0.5 ml) equilibrated with 1 M-sodium acetate buffer, pH 8.5, containing BSA (10 μ g/ml). The non-glycated EC-SOD was eluted with this buffer. The glycated EC-SOD was eluted with 1 M-sodium acetate buffer, pH 5.5, containing BSA (10 μ g/ml). The percentages of glycated form of serum EC-SOD in 16 diabetic patients (ten insulin-dependent diabetics and six non-insulin-dependent diabetics, 27–79 years old) was 8.44 ± 3.12 % (mean $\pm$ S.D.), which was significantly higher than that in the normal subjects (3.43 ± 0.516 %, $n = 6$, 25–59 years old), whereas there was no significant difference in serum EC-SOD concentrations between the diabetic patients and normal subjects (Fig. 1). No relation between percentages of glycated EC-SOD and diabetic type or age of the patients was apparent.

Glycation of r-EC-SOD C

Glycation of r-EC-SOD C proceeded time-dependently and also dose-dependently as the glucose concentration was increased (results not shown). After 7 days in 50 mM-glucose about 20 % of the r-EC-SOD C was glycated, but there was no decrease in r-

Fig. 1. Concentrations and percentages of the glycated forms of EC-SOD in serum

EC-SOD concentration in serum was assayed by e.i.s.a. Glycated EC-SOD was determined by means of chromatography on Glyco-gel B columns and assayed by e.i.s.a. as described in the Experimental section. Bars show mean values.

Fig. 2. (a) Glyco-gel B and (b) heparin-Sepharose column chromatography of glycated r-EC-SOD C *in vitro*

(a) A 2 ml portion of r-EC-SOD C (40 µg/ml) was incubated with (ii) or without (i) 50 mM-D-glucose in 0.1 M-potassium phosphate buffer, pH 7.3, at 37 °C for 7 days under sterile conditions, followed by extensive dialysis against phosphate-buffered saline with several changes. Portions (10 µl) were applied to a Glyco-gel B column (volume 0.5 ml) and chromatographed as described in the Experimental section. Elution was started at the point indicated by an arrow. (b) Fractions FR-1 and FR-2 were pooled and dialysed against 25 mM-sodium phosphate buffer, pH 6.5, followed by application on a heparin-Sepharose column (volume 0.5 ml) equilibrated with 25 mM-sodium phosphate buffer, pH 6.5, containing BSA (10 µg/ml) [(i) and (ii) respectively]. Bound EC-SOD was then eluted by stepwise additions of buffer (1 ml) containing increasing concentrations of NaCl (0.1 M increments). EC-SOD concentration in each fraction was assayed by e.l.i.s.a.

EC-SOD C enzymic activity (results not shown). The unbound fraction [FR-1 in Fig. 2(a)(i)] and the bound fraction [FR-2 in Fig. 2(a)(ii)] of r-EC-SOD C, incubated without or with glucose respectively, were dialysed and then applied on a heparin-Sepharose column. All r-EC-SOD C in fraction FR-1 had high affinity for heparin-Sepharose, whereas roughly half of the r-EC-SOD C molecules in fraction FR-2 had lost the high affinity [Fig. 2(b)].

Effects of heparin and trinitrobenzenesulphonic acid on glycation of r-EC-SOD C

The above results suggested that glycation occurred in the heparin-binding domain of r-EC-SOD C. To confirm this notion, the effect of heparin on glycation *in vitro* was studied. In the presence of excess heparin (heparin/r-EC-SOD C subunit ratios 6:1, 20:1 and 60:1) glycation of r-EC-SOD C *in vitro* was inhibited roughly by half (results not shown). The putative

Fig. 3. Heparin-Sepharose column chromatography of human serum

The chromatography was carried out at 4 °C on a heparin-Sepharose column (volume 6 ml) equilibrated with 25 mM-sodium phosphate buffer, pH 6.5. The serum sample (6 ml) was dialysed against the above buffer before application on the column. The sample was applied at 8 ml/h, and when the absorbance at 280 nm approached baseline bound components were eluted with a linear gradient of NaCl in the buffer (0–1 M; total volume 120 ml) at 16 ml/h. ○, A₂₈₀; ●, concentration of EC-SOD assayed by e.l.i.s.a.; ·····, concentration of NaCl in buffer.

involvement of lysine residues in the glycation of r-EC-SOD was tested by exposing r-EC-SOD C to trinitrobenzenesulphonic acid. Trinitrobenzenesulphonic acid-modified r-EC-SOD C that had lost the affinity for heparin-Sepharose completely was not susceptible to glycation *in vitro* (results not shown).

Glyco-gel B column chromatography of EC-SOD A, B and C

Fig. 3 shows a heparin-Sepharose column chromatography of serum from a patient with diabetes. Serum EC-SOD could be separated into three fractions: A without affinity, B with weak affinity and C with relatively strong heparin-affinity, as shown previously [15,16]. Pooled A, B and C fractions were then isolated on anti-(r-EC-SOD C)-monoclonal-antibody-conjugated Sepharose 4B columns as described in the Experimental section. Subsequently the isolated fractions were chromatographed with Glyco-gel B columns. The degree of glycation varied considerably (Fig. 4). A large fraction of EC-SOD B was glycated, EC-SOD A was clearly less so and EC-SOD C was only to a minor extent glycated. On the other hand, EC-SOD A, B and C in serum from a normal control were glycated to much less extent.

DISCUSSION

The present data show that EC-SOD is susceptible to glycation. The glycation of EC-SOD *in vitro* did not result in any change in enzymic activity, whereas it has been reported that the glycation of Cu,Zn-SOD leads to the inactivation of its enzymic activity [5]. The inactivation is suggested to be due to glycation of Lys-122 and Lys-128 located in the active-site liganding loop [21]. In human EC-SOD, the active site of which appears to be very similar to that of the Cu,Zn-SOD's, these residues correspond to Gly-165 and Arg-171 [22]. In fact the sequences of EC-SOD likely to define the active site lack lysine residues, which might explain the glycation-resistance of the enzymic activity of this

Fig. 4. Glyco-gel B column chromatography of purified EC-SOD A, B and C

EC-SOD A, B and C fractions in sera from a diabetic patient and a normal control were pooled and purified with anti-(r-EC-SOD C)-monoclonal-antibody-conjugated Sepharose 4B columns (volume 2 ml) as described in the Experimental section. Dialysed purified EC-SOD A, B and C were applied on a Glyco-gel B column (volume 0.5 ml) as described in the Experimental section. Elution was started at the point indicated by an arrow.

enzyme. However, in the glycosylated EC-SOD fraction studied, half of the molecules had lost their high affinity for heparin, suggesting that the heparin-binding domain of the enzyme should be a main glycation site. This notion was supported by the finding that heparin decreased glycation of this enzyme *in vitro*. Since EC-SOD C carries a net negative charge at neutral pH [23], the strongly negatively charged heparin is likely to interact with a localized cluster of positively charged amino acid residues. Such a cluster exists in the C-terminal end, which contains six arginine, three lysine and one histidine residues among the last 21 amino acid residues. This part of the molecule is highly hydrophilic, forms an α -helix according to computer prediction (S. L. Marklund, unpublished work) and should extend into the solvent and be easily accessible both for binding to heparin and for various modifications. The environments of the two other lysine residues Lys-23 and Lys-74, appear to be less distinctly exteriorized. We have shown before that the lysine-specific reagent trinitrobenzenesulphonic acid causes loss of heparin-affinity without affecting the enzymic activity [18]. We now show that trinitrobenzenesulphonic acid-modified EC-SOD C is resistant to glycation, suggesting that glucose and trinitrobenzenesulphonic acid primarily react with the same lysine residues. The fact that an excess of heparin only decreased glycation of half, however, shows that not all glycation-susceptible lysine residues exist in the heparin-binding domain.

We furthermore show that EC-SOD is glycosylated *in vivo*, and that the glycation is considerably more extensive in diabetics than in controls. No glycation could be demonstrated in the r-EC-SOD C [Fig. 2(a)]. There was no significant difference in serum EC-SOD content between diabetics and controls. Judged

from the studies performed *in vitro* the glycation should not influence the enzymic activity, and this is in accord with previous findings of unaffected plasma cyanide-sensitive SOD activity in diabetes [24]. The main effect of the glycation is instead loss of heparin-affinity and consequently affinity for heparan sulphate proteoglycan on cell surfaces.

The background to the heterogeneity in heparin-affinity of plasma EC-SOD is still unresolved. There is evidence that EC-SOD is primarily synthesized as type C in the body. The EC-SOD cDNA clone that we have isolated encoded EC-SOD of C-type [8]. All nine investigated human cell lines that natively express EC-SOD secreted EC-SOD of C-class to the medium [25], and human tissues, which account for about 99% of the EC-SOD of the body [12], contain almost exclusively EC-SOD of C-class (S. L. Marklund, unpublished work). A-class and B-class EC-SODs are consequently probably the result of secondary modifications. The present findings of high glycation in the A and especially the B fractions indicate that non-enzymic glycation is one such modification. Glycation is apparently a more important contributory factor to formation of EC-SOD A and B in diabetes patients than in controls (Fig. 4). Another likely contributing factor is proteolytic cleavage of the exteriorized lysine- and arginine-rich C-terminal end. The fact that plasma EC-SOD A was less glycosylated than EC-SOD B may be due to more extensive proteolytic cleavage in this fraction.

The binding of EC-SOD C to cellular surfaces might be an especially efficient way of protecting cells against external superoxide anion. It is interesting to note that substitution of Cu,Zn-SOD with polylysine to facilitate association with negatively charged cell membranes highly potentiated the ability of

the enzyme to protect polymorphonuclear leucocytes against self-inactivation [26]. The cell-membrane-associated SOD of *Nocardia asteroides* confers efficient protection of the bacterium against activated polymorphonuclear leucocytes [27,28]. r-EC-SOD C has been shown to be more effective than the non-binding Cu,Zn-SOD in diminishing ischaemia-reperfusion damage in the hamster cheek pouch [29] and in scavenging oxygen radicals in the reperfused rat heart [30]. Furthermore EC-SOD C bound to endothelial cell surfaces may not interfere with putative beneficial effects of superoxide anion produced at the surface of activated neutrophil leucocytes that themselves lack affinity for EC-SOD C [16]. The present investigations suggest that glycation of EC-SOD decreased affinity for heparin without any decrease in its enzymic activity. The loss of affinity for heparin-Sepharose with glycation indicates that *in vivo* the binding of EC-SOD to heparan sulphate in the glycocalyx of endothelial cell surfaces should be weakened in the diabetic state. This phenomenon may result in not only diminished ability to protect the endothelium, but in increased interference with putative useful functions of superoxide anion against external substances in the fluids. One might speculate that the glycation of EC-SOD contributes to the pathogenesis of vascular complications associated with diabetes mellitus.

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