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Effect of Active Oxygen Radicals on Protein and Carbohydrate Moieties of Recombinant Human Erythropoietin

ERIKO UCHIDA*, KAZUSHIGE MORIMOTO, NANA KAWASAKI, YOKO IZAKI, AHMED ABDU SAID† and TAKAO HAYAKAWA

Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158, Japan

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Our previous study showed that active oxygen radicals generated from a Fenton system and a xanthine plus xanthine oxidase system caused serious loss of *in vivo* bioactivity of recombinant human erythropoietin (EPO), a highly glycosylated protein. In the present study, we characterized the oxidative modifications to the protein and carbohydrate moiety of EPO, which lead to a reduction of its bioactivity. *In vitro* bioactivity was reduced when EPO was treated with oxygen radicals generated from a Fenton system in the presence of 0.016 mM H_2O_2, and the reduction was directly proportional to the loss of *in vivo* bioactivity. SDS-PAGE analysis showed that dimer formation and degradation was observed under more severe conditions (Fenton reaction with 0.16 mM H_2O_2). The tryptophan destruction was detected at 0.016 mM H_2O_2 and well correlated with the loss of *in vitro* bioactivity, whereas loss of other amino acids were occurred under more severe conditions. Treatment with the Fenton system did not result in any specific damage on the carbohydrate moiety of EPO, except a reduction of sialic acid content under severe condition. These results suggest that active oxygen radicals mainly react with the protein moiety rather than the carbohydrate moiety of EPO. Destruction of tryptophan residues is the most sensitive marker of oxidative damage to EPO, suggesting the importance of tryptophan in the active EPO structure. Deglycosylation of EPO caused an increase of susceptibility to oxygen radicals compared to intact EPO. The role of oligosaccharides in EPO may be to protect the protein structure from active oxygen radicals.

Keywords: Erythropoietin, oxygen radicals, Fenton, glycoprotein, carbohydrate

INTRODUCTION

Oxygen free radicals are known to cause damaging effects on biomolecules such as unsaturated lipids, nucleic acids, carbohydrates and proteins. *In vivo*, active oxygen species are generated from cells in normal and pathological conditions, as well as from xenobiotics including drugs for therapeutic use[1] and environmental agents.[2] Oxidative

* Corresponding author.
† Present address: Department of Pharmacology, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt.

damages in the cells and biomolecules are implicated in the development of pathophysiological states such as ageing, cancer, heart disease, muscular dystrophy and so on.[3] Many studies have done on the damaging effect of active oxygen species on proteins which result in modifications of amino acids or alteration of structures and functions of proteins.[4–7] However, little work has focused on the effect of oxygen free radicals on glycoproteins, especially on the carbohydrate moiety present on glycoproteins, although it appears to be essential for their function.

Erythropoietin (EPO) is a hematopoietic hormone produced in the kidney and secreted into the blood stream to stimulate the proliferation and differentiation of erythroid progenitor cells to erythrocytes. EPO is a heavily glycosylated protein. Its molecular weight is about 35 kDa, and carbohydrate moiety is about 40% of the molecular mass. Human EPO has three N-linked and one O-linked oligosaccharides. N-linked oligosaccharides with terminal sialic acids are important for *in vivo* bioactivity of EPO.[8–11] Recombinant EPO has been successfully used for the treatment of dialysis patients with anemia. It is reported that phagocyte activation was occurred on the dialysis membrane.[12] Since activated phagocytes are capable of producing large amounts of active oxygen radicals on their surface, it is possible that EPO might be exposed to active oxygen radicals in the blood stream.

We have previously tested the effect of oxygen radicals on EPO, a model glycoprotein. We demonstrated that active oxygen radicals generated from both the Fenton and the xanthine plus xanthine oxidase systems have a potential to react with EPO, and lead to a reduction of its *in vivo* bioactivity.[13] In the present study, we have analyzed the effects of oxygen radicals on the protein and carbohydrate moieties of EPO, as well as the effect on its *in vitro* bioactivity, and studied the mechanism of loss of bioactivity. Changes of protein size such as fragmentation and aggregation were analyzed by SDS-PAGE. Modifications of protein moiety were assessed by tryptophan oxidation, bityrosine formation, amino acid analysis, and peptide mapping. Effects on carbohydrate moiety were examined by oligosaccharide profile analyzed on RP-HPLC and monosaccharide analysis. Role of N-linked oligosaccharides of EPO on the damaging effects of active oxygen radicals was also studied in this paper.

MATERIALS AND METHODS

Materials

Recombinant human Erythropoietin (EPO; specific activity 2.2×10^5 IU/mg protein) produced from Chinese hamster ovary cells was kindly provided by Kirin Brewery, Japan. Water soluble tetrazolium salt, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5H-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt[14] (WST-1) and 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) were purchased from Dojindo Lab. (Kumamoto, Japan). An murine myeloid cell line, EP-FDC-P2[15] cells were a kind gift from Snow Brand Milk Products Co. (Tochigi, Japan).

Treatment by Fenton System

EPO (59 μg) was mixed in a reaction mixture of 600 μl of 0.24 mM EDTA, 0.16 mM ammonium ferrous sulfate and 0.0016–0.16 mM H_2O_2 in 30 mM NaCl (pH 7.4), and incubated at 37°C for 30 min. For *in vivo* and *in vitro* bioassay, aliquots of the mixture were directly diluted with the appropriate buffer after incubation. For analytical studies, the reaction mixture was changed to H_2O or 10 mM phosphate buffer (pH 7.4) by Ultrafree C3LCC (MW 5,000 cut; Millipore) and lyophilized.

In vivo Bioassay

In vivo bioactivity of EPO were estimated as described previously.[13] Briefly, EPO samples were diluted with a solution consisting of 0.9%

NaCl, 5% mannitol and 0.05% bovine serum albumin, and were injected subcutaneously into female ICR mice for 3 consecutive days. On day 4, blood was collected and haemolizer resistant cells were counted using an automated microcell counter (Sysmex F-300) set on leukocyte mode.

In vitro Bioassay

In vitro bioactivity of EPO was tested by the stimulation of cell proliferation of the EPO-dependent murine myeloid cell line, EP-FDC-P2, as described previously.[16] Briefly, cells were cultured with various concentrations of EPO for 2 days in 96 well plate. Cell proliferation was measured by colorimetry using WST-1 with 1-methoxy PMS. Relative biological activity was calculated by means of the parallel line assay from the dose-response curve.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

EPO samples were incubated in the presence of 2-mercaptoethanol prior to electrophoresis and analyzed on 12.5% SDS-polyacrylamide gels as described by Laemmli.[17] Gels were stained with Coomassie Brilliant blue R-250. Protein concentrations of the stained bands were analyzed with a video densitometer system Densitograph (Atto, Japan).

Fluorescence Measurements[6,7]

Tryptophan oxidation and bityrosine production of EPO samples were measured by means of fluorometry. Fluorescence spectra were recorded on a Hitachi F-3010 Fluorescence Spectrophotometer. Aliquot (10 µl) of EPO samples was denatured in 1 ml of 10 mM phosphate buffer (pH 7.4) containing 6 M guanidine hydrochloride and 30 mM 2-mercaptoethanol for 20 min. Tryptophan content was measured at the maximum of the fluorescence spectrum emission at 350 nm after excitation at 290 nm. Bityrosine production of the sample was assessed at 315 nm excitation and 340 nm emission after denaturation in 6 M urea solution. Calibration was done with 2,2'-biphenol in 6 M urea.

Amino Acid Analysis

EPO samples were lyophilized and reconstituted in 50 µl of distilled water. 40 µl of these samples were combined with 500 µl of 6 N constant boiling HCl (Pierce Chemical Co., USA) and transferred to glass hydrolysis ampoules. Ampoules were evacuated and hydrolysis was performed for 24 h at 110°C. After cooling, samples were reconstituted with 500 µl 0.02 N HCl, and analyzed on a Hitachi Model 835 Amino Acid Analyzer.

Peptide Mapping

EPO (236 µg) was treated with the Fenton system and then lyophilized. Samples were reduced and pyridylethylated as described.[18] Pyridylethylated EPO was digested with endoproteinase Lys-C (*Lysobacter enzymogenes*, Boehringer mannheim) and the resulting peptides were separated by RP-HPLC using a Bio-Rad Hi-Pore RP 318 column (4.6 mm × 250 mm) combined with a Bio-Rad Hi-Pore Guard column as described.[18] Peptides were detected by their absorbance at 214 nm and identified by comparison with the peptide map of EPO reported in the literature.[19] Peptide contents were compared by peak area.

Analysis of N-linked Oligosaccharides

N-linked oligosaccharides in EPO samples were released by hydrazinolysis with Hydraclub S-204 (Honen Corporation, Japan). The released N-linked oligosaccharides were N-acetylated and pyridylaminated with 2-aminopyridine.[20] The pyridylamino (PA) derivatives were purified by gel filtration on Sephadex G-15 column and dried. The PA-labelled oligosaccharide were desialylated with 30 mU neuraminidase from

Streptococcus sp. (Seikagaku Co., Japan) for 3 h at 37°C in 100 µl sodium acetate buffer 100 mM (pH 6.5) containing 10 mM $CaCl_2$ and analyzed on a Shimpak CLC-ODS column (0.6 × 15 cm) as described previously.[13]

Monosaccharide Analysis

Monosaccharide analysis of EPO was performed using a Dionex system DX-300 High Performance Anion Exchange Chromatography (HPAEC) equipped with a pulsed amperometric detector (PAD, Dionex) according to Hardy *et al.*.[21] Briefly, Fenton-treated EPO was hydrolyzed with 2 M TFA at 100°C for 4 h, evaporated and filtered with Ultrafree C3LGC. Neutral and amino sugars were analyzed on an anion exchange PA-1 column (0.47 × 25 cm, Dionex) equilibrated and eluted with 15 mM NaOH at a flow rate of 1.0 ml/min.

Sialic Acid Analysis

Sialic acid contents were measured by three methods.

1) HPAEC-PAD method:[22] Fenton-treated EPO was filtered with Ultrafree C3LCC (MW 5,000 cut) and digested with 10 mU neuraminidase from *Arthrobacter ureafaciens* (Nakarai Tesque, Kyoto, Japan) at 37°C for 3 h in 10 mM sodium acetate (pH 5.0). After filtration with Ultrafree C3LGC (M.W. 10,000 cut), released sialic acids were lyophilized, reconstituted with 200 µl H_2O, and an aliquot (25 µl) was analyzed on HPAEC-PAD. PA-1 column was equilibrated and eluted with 100 mM NaOH-100 mM sodium acetate at a flow rate of 1.0 ml/min.
2) Resorcinol method: Fenton-treated EPO was mixed with 0.5 M arginine/200 mM phosphate buffer (pH 7.2). Resorcinol (20 mM) in 28 M HCl and 0.8 mM $CuSO_4$ solution were added to the sample and boiled for 15 min. After cooling, sialic acid-dye materials were extracted with 300 µl of n-butyl acetic acid/butanol (17/3, V/V). After centrifugation at 10,000 rpm for 5 min, the absorbance of the top fraction was measured at 580 nm.
3) DBM method:[23] EPO samples treated with Fenton system were acid hydrolyzed with 50 mM HCl at 80°C for 1 h. Released sialic acids were labeled with 1,2-diamino-4,5-methylenedioxybenzene (DMB) according to the manual of Sialic acid labeling kit (Takara Shuzo Co., Kyoto, Japan). DMB derivatives of neuraminic acids were analyzed on HPLC using a PALPAK Type R column (4.6 × 250 mm, Takara Shuzo Co.) equilibrated with acetonitrile/ methanol/H_2O (9/7/84, V/V) at a flow rate of 1.0 ml/min, and detected at the maximum emission wavelength of 448 nm (excitation at 373 nm).

Preparation of Fully De-N-glycosylated EPO

Recombinant human EPO (650 µg) was digested with 10 U of recombinant N-Glycanase (peptide: N-glycosidase F; EC 3.5.1.52, Seikagaku Co., Tokyo Japan) from *Flavobacterium meningosepticum* in 200 mM phosphate buffer (pH 8.6) containing 20 mM EDTA for 26 h at 37°C. The digest was applied to a *Datura stramonium* agglutinin (DSA)-agarose column (Seikagaku Co., Tokyo, Japan) equilibrated with 10 mM PBS (pH 7.2). Fully de-N-glycosylated EPO was collected as the unbound fraction.

RESULTS

Biological Activity of EPO is Reduced by Active Oxygen Radicals

The effect of oxygen radicals on *in vitro* bioactivity of EPO was determined by the proliferation of EPO dependent EP-FDC-P2 cells. When EPO was treated with the Fenton system with H_2O_2 the *in vitro* bioactivity was lost in a dose dependent manner relative to an increase of H_2O_2 concentra-

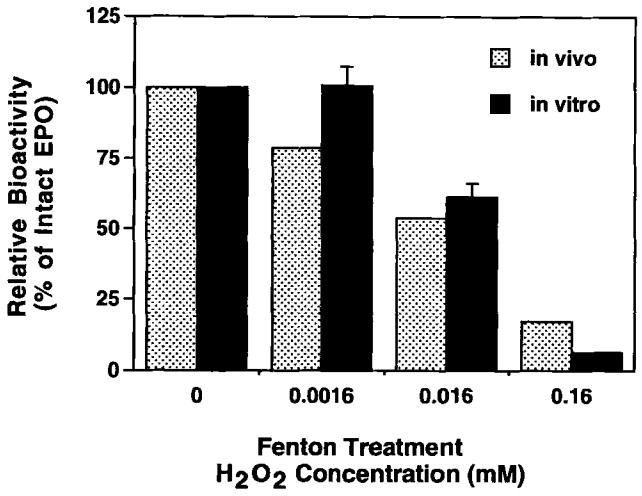

FIGURE 1 Effects of Fenton system on the *in vivo* and *in vitro* bioactivity of human recombinant erythropoietin (EPO). Intact EPO was treated with Fenton reaction mixtures of the following composition: 0.24 mM EDTA, 0.16 mM ammonium ferrous sulfate without or with H_2O_2 of 0.0016, 0.016 and 0.16 mM in 30 mM NaCl (pH 7.4) at 37 °C for 30 min. The relative *in vivo* bioactivity of EPO treated with Fenton system was analyzed by the growth of haemolyser-resistant cells as described in the Materials and Methods and expressed as the percentage of intact EPO. The relative *in vitro* bioactivity of Fenton-treated EPO was analyzed by the growth of EPO-dependent EP-FDC-P2 cells as described in the Materials and Methods. Values are the mean ± S.E. of 4 separate experiments.

tion (Fig. 1). By the Fenton system in the presence of 0.016 and 0.16 mM H_2O_2, *in vitro* bioactivity was reduced to 61% and 6% of intact EPO, respectively. Loss of *in vitro* bioactivity was shown in parallel with the reduction of *in vivo* bioactivity (Fig. 1). In case of EPO, *in vivo* bio-activity reflects on both the interaction with target cells and the survival in the circulation, which is depend on the carbohydrate moiety of EPO, whereas *in vitro* bioactivity reflects only on the reactivity with the receptor on the target cells. Thus, the active structure recognized by the EPO receptor is suggested to be damaged by oxygen radicals.

Analysis of Protein Moiety of EPO Treated with Active Oxygen Radicals

In order to examine the damaging effect of Fenton system on the structure of EPO molecule, SDS-PAGE was performed (Fig. 2A) and the protein bands around 35 kDa and 64 kDa were quantified by video densitometer (Fig. 2B). Intact EPO was detected as the broad band around 35 kDa, and no change was observed by treatment of Fenton system with less than 0.016 mM of H_2O_2. In contrast, Fenton system with 0.16 mM H_2O_2 caused a drastic effect on the EPO molecule. Only less than 30% of the 35 kDa band was retained intact, and both the higher molecular weight 64 kDa band and dispersed lower molecular weight substances were generated. The 64 kDa band is considered as the dimerized form of EPO. Lower molecular weight substances were considered as the degradative products of EPO. Thus, the cause of loss of bioactivity seems quite different between 0.16 mM and 0.016 mM H_2O_2. It is no wonder that seriously damaged EPO molecules that were treated with 0.16 mM H_2O_2 lost their bioactivity. Modifications by Fenton treatment with the lowest concentration of 0.016 mM H_2O_2 appear to be interesting to study the mechanism of loss of bioactivity.

The effects of the Fenton system on tryptophan and tyrosine residues were analyzed by fluorescence spectra. There are three tryptophan residues (Trp51, Trp64, Trp88) and tyrosine residues (Tyr15,

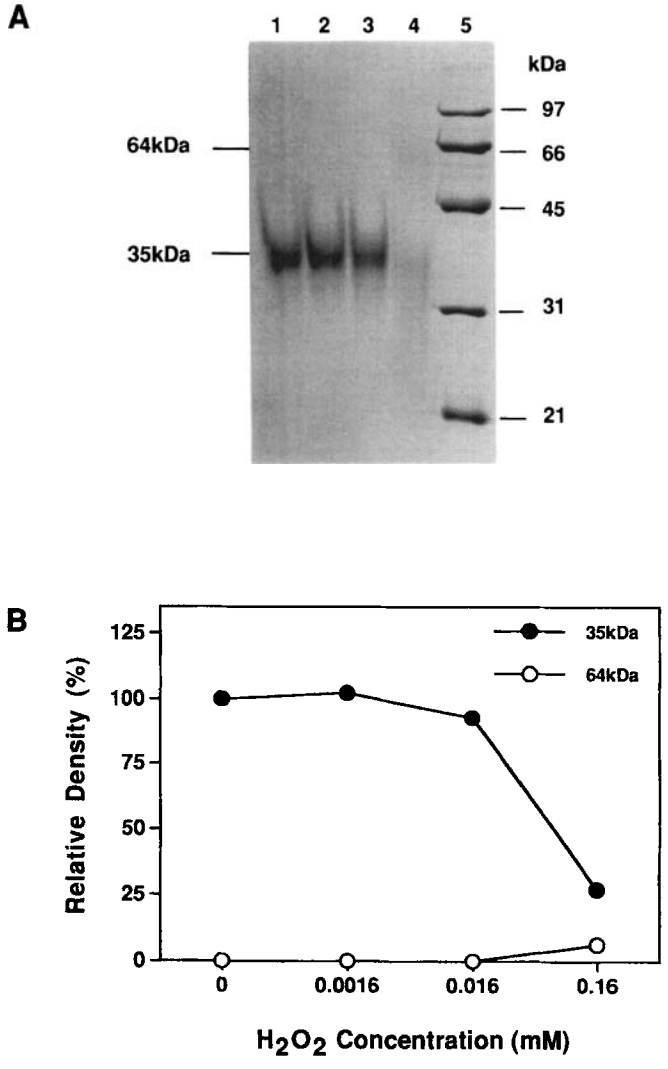

FIGURE 2 SDS-PAGE analysis of EPO treated with Fenton system. **(A)** EPO samples were analyzed on 12.5% SDS-PAGE gels and stained with Coomassie brilliant blue R-250. Lane 1 is intact EPO. Lanes 2–4 are EPO treated with Fenton system with H_2O_2 of 0.0016, 0.016 and 0.16 mM, respectively. Lane 5 is molecular weight standard. **(B)** Relative concentration of protein band of 35 kDa (●) and 64 kDa (○) is calculated with video densitometer and expressed as the percentage of 35 kDa band of intact EPO.

Tyr145, Tyr156) in the EPO molecule. When EPO was treated with Fenton reagent with 0.016 mM and 0.16 mM H_2O_2, tryptophan fluorescence was reduced to 76% and less than 40% of the intact EPO, respectively (Fig. 3). The loss of tryptophan residues coincided well with the loss of *in vitro* bioactivity.

Bityrosine fluorescence was detected but very weak (Fig. 3). By Fenton treatment with 0.16 mM H_2O_2, 0.4 mmol of bityrosine were produced in 1 mol EPO. This may be one of the possible mechanisms for dimer production.

Modifications of other amino acid residues were estimated by amino acid analysis (Table I). Amino acid contents of EPO did not change by Fenton treatment with less than 0.016 mM H_2O_2. EPO exposed to the Fenton system with 0.16 mM H_2O_2 resulted in a reduction of all amino acid

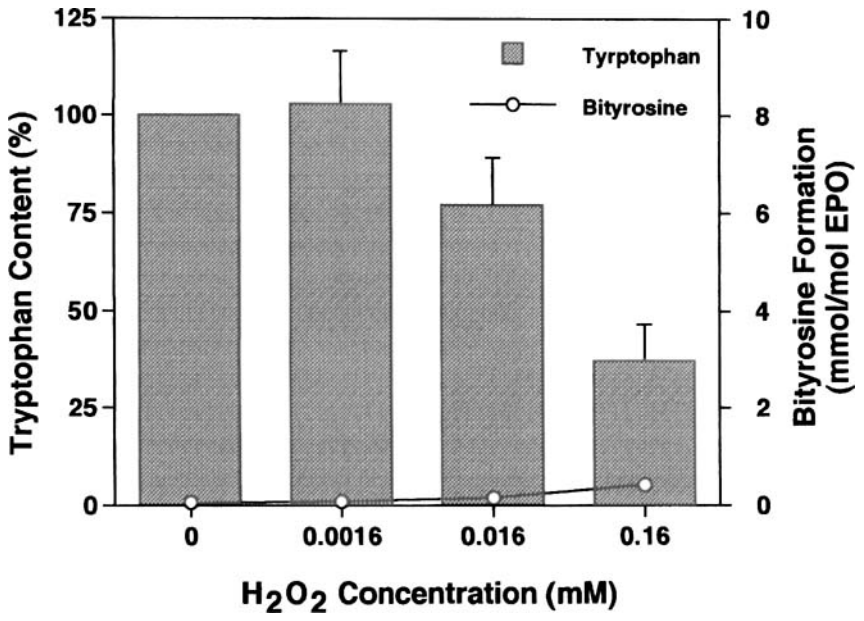

FIGURE 3 Tryptophan destruction and bityrosine production of EPO treated with the Fenton system. Fluorescence of tryptophan (■) and bityrosine production (○) of EPO exposed to Fenton reagent with various concentration of H_2O_2 were measured as described in Materials and Methods. Each value represents the mean ± S.E. of 5 experiments (tryptophan) and the average of 2 experiments (bityrosine).

residues because of the reduction of the recovery. Although the average loss was 20% of intact EPO, the recoveries of cysteine and histidine residues were lower than those of other amino acid residues.

In order to specify the sites of the EPO molecule that reacted with oxygen radicals, peptide maps of EPO untreated and treated with Fenton system were compared by RP-HPLC (Fig. 4 and Table II). Endoproteinase Lys-C digest of EPO produced 9 peptides, and eight peaks were detected by HPLC analysis. The peptide map obtained from intact EPO was well in agreement with the peptide map reported in the literature.[19] Each peak was assigned as follows: peak 1:K9 peptide (residues 155–165); 2:K2 (21–45); 3:K3 (46–52); 4:K6 (117–140); 5:K7 (141–152); 6:K1 (1–20); 7:K5 (98–116); 8:K4 (53–97). Although the recovery of each peptide was reduced by Fenton treatment, peak 8 (K4 peptide; containing Asn83, Trp64 and Trp88) and peak 2 (K2 peptide; containing Asn 24

TABLE I Effect of Fenton treatment on amino acid content of erythropoietin

	Amino Acid Content (%)		
	Fenton Treatment (H_2O_2 Concentration, mM)		
Amino Acid	0.0016	0.016	0.16
Asp	100.2	100.2	84.3
Thr	99.2	100.5	82.8
Ser	101.2	97.0	85.8
Glu	100.1	99.7	84.2
Gly	103.5	96.7	84.7
Ala	98.5	101.0	81.0
Cys	97.6	97.2	68.3
Val	101.5	103.0	83.5
Met	98.3	98.5	76.8
Ile	96.0	104.3	90.7
Leu	94.1	96.9	75.2
Tyr	105.3	98.4	76.5
Phe	100.2	102.2	79.2
Lys	99.6	100.0	78.0
His	103.0	97.2	69.0
Arg	98.0	99.3	80.6
Pro	97.9	100.0	79.5

Values are the relative amino acid content of intact EPO.

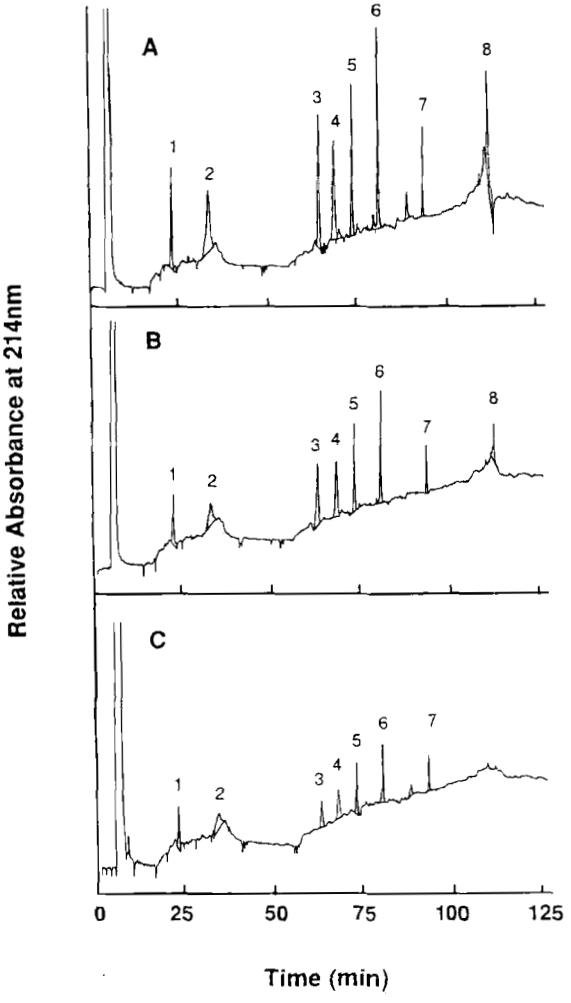

FIGURE 4 Comparison of endoproteinase Lys-C peptide maps of Fenton-treated and untreated EPO. Intact EPO **(A)**, EPO treated with Fenton system with 0.016 mM H_2O_2 **(B)**, and EPO treated with Fenton system with 0.16 mM H_2O_2 **(C)** were pyridylethylated and digested with endoproteinase Lys-C, and analyzed on RP-HPLC as described in materials and methods. Peptides were numbered according to elution position.

and Asn38) were clearly reduced in comparison with other peaks at 0.016 mM H_2O_2. Furthermore, K4 peptide was completely lost at 0.16 mM H_2O_2. These results indicated that K4 was the most susceptible peptide to Fenton treatment. We could not detect any novel peaks nor shifts of the peaks of K2 and K4 corresponding to the deglycosylated forms of these peptides,[10] suggesting that neither the deglycosylation nor the change of the carbohydrate moiety of these peptides by Fenton treatment was the cause of loss of K2 and K4 peptides.

Effect of Active Oxygen Radicals on Carbohydrate Moiety of EPO

To determine the effect of Fenton system on carbohydrate structure, asialo-oligosaccharides released from EPO were analyzed on ODS-silica column. Figure 5 shows the representative

TABLE II Peptide mapping of EPO treated with Fenton system

		Peptide Content (% of intact EPO)		
		H_2O_2 Concentration (mM)		
Peak No.	Peptide	0.0016	0.016	0.16
1	K9 (155–165)	70.8	53.5	38.9
2	K2 (21–45)	84.3	35.0	31.9
3	K3 (46–52)	73.3	61.3	23.0
4	K6 (117–140)	72.0	56.1	33.0
5	K7 (141–152)	73.1	60.5	35.2
6	K1 (1–20)	68.8	55.6	32.2
7	K5 (98–116)	74.1	60.1	42.0
8	K4 (53–97)	116.1	29.4	0.0

oligosaccharide profiles on RP-HPLC obtained from intact EPO and Fenton treated EPO with 0.16 mM H_2O_2. Main peak around 40 min was identified as the tetraantennary sugar chain with fucose, which is the main oligosaccharide of EPO. Because of the low recovery of the EPO molecule, peak heights were decreased after Fenton treatment. However, the pattern of the oligosaccharide profiles which reflect the heterogeneity of carbohydrate structure did not change by Fenton treatment. This result suggests that Fenton treatment with 0.16 mM H_2O_2 did not cause selective damage of the oligosaccharide structure.

Sugar compositions of untreated and treated EPO were compared by HPAEC-PAD system. Neutral sugars and amino sugars were separated as 5 peaks. As shown in Figure 6, monosaccharide profiles obtained from intact EPO were consistent with those from EPO treated with Fenton system in the presence of 0.16 mM H_2O_2. The composition of neutral and amino sugars of EPO did not change by Fenton treatment.

Since *in vivo* bioactivity of EPO is depend on the number of sialic acids,[19] we measured the sialic acid contents in EPO by three methods. As shown in Table III, sialic acids were almost retained by Fenton treatment with less than 0.016 mM H_2O_2. Only at the concentration of 0.16 mM H_2O_2, 20% losses of sialic acid content were observed by DMB method. This reduction of sialic acid content is not sufficient to explain the serious loss of *in vivo* bioactivity under the same condition.

Role of N-glycosylation of EPO on The Effect of Active Oxygen Radicals

To study the role of oligosaccharide on the damaging effect of oxygen radicals, intact EPO were digested with N-glycanase and the fully deglycosylated EPO (N(0)-EPO) was purified by DSA-agarose column. The effects of Fenton treatment on the *in vitro* bioactivity were compared between intact EPO and N(0)-EPO. As shown in Figure 7, N(0)-EPO was more sensitive to the damaging effect of radicals than intact EPO and easily lost its *in vitro* bioactivity. This result suggests that N-linked sugar chains contribute to the protection from the active oxygen radicals.

DISCUSSION

Our previous study[13] showed that *in vivo* bioactivity of EPO was lost by treatment of Fenton system which generate the hydroxyl radical, a highly-reactive oxygen radical. In the present paper, to study the mechanism of loss of bioactivity by oxygen radicals, we characterized the effect of Fenton treatment on *in vitro* bioactivity and damage of protein moiety and carbohydrate moiety of EPO.

When EPO was treated with Fenton system at a concentration of 0.16 mM H_2O_2, the structure of the EPO molecule was seriously damaged. Degradation and oligomerization were clearly observed on SDS-PAGE analysis (Fig. 2). A significant low recovery of amino acids (Table I) and sialic acid (Table III) were also observed. Thus, the loss of *in vivo* and *in vitro* bioactivity appears to be caused by damage to the whole structure of the EPO molecule.

In contrast, when EPO was treated with Fenton system with 0.016 mM H_2O_2, the protein structure analyzed by SDS-PAGE was almost retained

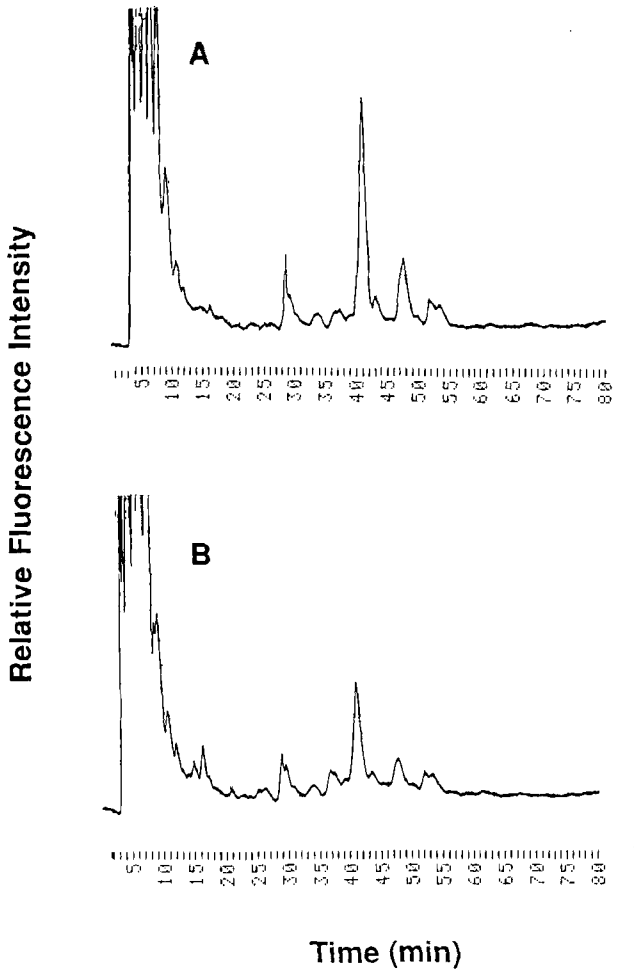

FIGURE 5 A Comparison of RP-HPLC profiles for PA-asialo-pyridylaminated oligosaccharide from Fenton-treated and untreated EPO. Oligosaccharide derivatives were obtained from intact EPO **(A)** and EPO treated with Fenton reagent with 0.16 mM H_2O_2 **(B)**. HPLC on ODS column were performed as described in Materials and Methods.

(Fig. 2), although the *in vivo* and *in vitro* bio-activity was reduced to 40–50% (Fig. 1). Therefore the oxidative damage under this condition is completely different from that which occurred at 0.16 mM H_2O_2. The damage must be occurred only partially or in specific parts of the EPO molecule. We found that the tryptophan oxidation is the most sensitive marker for the damage of oxygen radicals. The destruction of tryptophan residues were detected at 0.016 mM H_2O_2 (Fig. 3). On the other hand, we could not detect any modification of other amino acids or the carbohydrate moiety under this condition (Fig. 5, Fig. 6, Table III). Thus, the modification of tryptophan residues may be related to the loss of bioactivity. Davies *et al.*[5] reported that tryptophan, tyrosine, histidine, and cysteine were more sensitive to the hydroxyl radical than other residues in model protein BSA. These modifications of amino acids were also observed in the case of EPO, a glycosylated model protein (Fig. 3, Table I).

Tertiary structure and functionally important sites of EPO molecule were predicted by immunochemical studies[24–26] and mutagenesis studies.[27–30] The predicted three dimensional

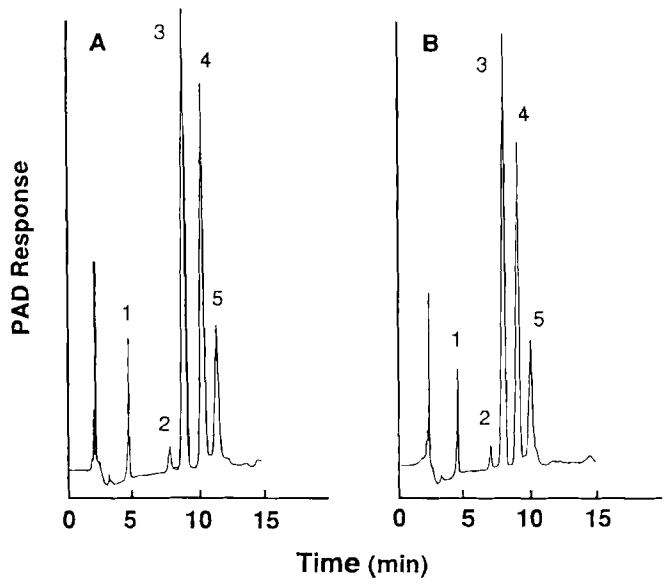

FIGURE 6 Monosaccharide analysis of Fenton-treated and untreated EPO by High-Performance Anion-Exchange Chromatography. Monosaccharides prepared from intact EPO **(A)** and EPO treated with 0.16 mM H_2O_2 **(B)** were analyzed on HPAEC-PAD as described in Materials and Methods. Monosaccharide peaks were identified as follows: 1, Fucose; 2, Galactosamine; 3, Glucosamine; 4, Galactose; 5, Mannose.

model of EPO is an anti-parallel four helix bundle structure.[25,26,29] The disulfide bond between Cys7-Cys161,[26] Arg14,[30] several amino acid residues in 99–110[28–30] and C-terminal 152–166[25] were pointed out as the critical residues for bioactivity. Elliott *et al.*[26] recently reported a neutralizing monoclonal antibody which recognized a conformational epitope of EPO that includes amino acids 64–78 and 99–110. These amino acid residues are suggested to be exposed on the surface of the EPO molecule and are located in the receptor binding sites. This is consistent with our results that K4 peptide (amino acid 53–97) which contains amino acids 64–78 is the most susceptible peptide to Fenton treatment (Fig. 4, Table II). Although alanine mutation of Trp64 did not cause any effect on the bioactivity,[30] tryptophan is the most sensitive amino acid to Fenton treatment (Fig. 3) and its destruction occurred in parallel with loss of bioactivity, hence tryptophan residues might be important for the active EPO structure. At least, it is possible that modification of the tryptophan residue may affect the receptor recognition structure of EPO.

TABLE III Sialic acid contents of Fenton-treated EPO

Fenton Treatment H_2O_2 Conc. (mM)	Sialic Acid Contents (% of intact EPO)		
	HPAEC	Resorcinol	DMB
0.0	100.0	100.0	100.0
0.0016	104.1	102.5	107.6
0.016	97.5	98.4	98.8
0.16	96.9	90.3	81.8

Since EPO is a highly glycosylated protein, sugar chains are supposed to mask a considerable part of the surface of the EPO molecule. It is reported that hydroxyl radical generated by gamma irradiation of more than 1000 Gy were found to destroy terminal galactose on the carbohydrate moiety of IgG.[31] However, we could not detect any selective and specific damage on carbohydrate structure of EPO in the present study, except the loss of sialic acid residues at 0.16 mM H_2O_2. Thus it is suggested that carbohydrate moiety is less sensitive to active oxygen radicals than protein moiety, especially aromatic groups of EPO.

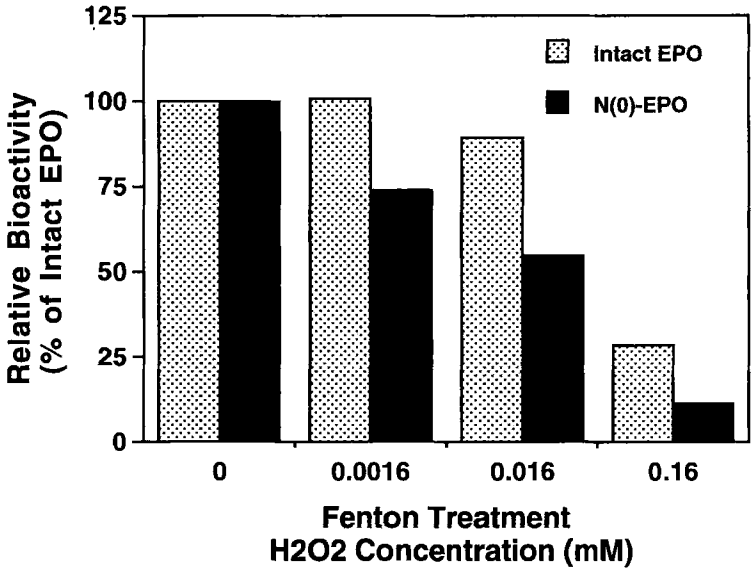

FIGURE 7 *In vitro* bioactivity of intact and N(0)-EPO after treatment with Fenton reagent, Intact EPO and N(0)-EPO were treated with Fenton reagent and the *in vitro* bioactivity was measured as described in materials and methods using EP-FDC-P2 cells.

The oligosaccharides and terminal sialic acid of EPO are reported to contribute for stability to heat,[32] pH and guanidine-HCl.[11,33,34] Our data showed that deletion of all N-linked sugar chains from EPO increased susceptibility to oxygen free radicals (Fig. 7). These data provide the evidence that N-linked sugar chains are also important for the protection from active oxygen radicals. Since oxygen radicals scarcely affected to the carbohydrate moiety of EPO, it is reasonable to imagine that the carbohydrate moiety might protect EPO by the masking effect on the active site of EPO. Although sugar chains are supposed to mask the protein moiety of EPO flexibly, it might be still difficult for oxygen radicals to attack on the protein moiety when covered by sugar chains, since oxygen radical species are very unstable in aqueous solution.

In conclusion, active oxygen radicals generated from the Fenton system mainly react with the protein moiety of EPO, especially with tryptophan residues, rather than the carbohydrate moiety, and cause loss of bioactivity. The glycosylation of EPO contributes to the protection from active oxygen radicals.

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