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Comparison of Cu(II)-DIPS and Human Recombinant Superoxide Dismutase, an Antioxidant

Chul Soon Yong†, Doo Hyun Nam, Keun Huh

College of Pharmacy, Yeungnam University, Gyongsan 712-749, Korea

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항산화제인 Cu(II)-DIPS와 재조합 인간 수퍼옥사이드 디스뮤타제의 비교

영남대학교 약학대학

용철순† · 남두현 · 허 근

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The superoxide dismutase (SOD) mimetic activity of copper complex of 3, 5-disopropylsalicylic acid (Cu(II)-DIPS) was tested and compared to those of human recombinant SOD (hrSOD) and its conjugate form with polyethyleneglycol (PEG) using fer- ricytochrome c reduction assay. Stability constant of Cu(II)-DIPS was measured po- tentiometrically using SCOGS2 program. In the presence of 10 g/L albumin, Cu(II)-DIPS lost most of its SOD mimetic activity. HrSOD was modified with polyethylene glycol (PEG) of M.W. 5000. These conjugates have markedly prolonged plasma half-lives of enzymatic activity (15.5 hr) compared to native hrSOD (5 min). In summary, efficient SOD mimetics should be stable enough not to dissociate in blood by serum protein. HrSOD could have longer half-life by conjugation with inert PEG for sustained SOD effect.

Keywords—Polyethylene glycol, Human recombinant superoxide dismutase, Copper complex of 3, 5-didsopropylsalicylic acid, Superoxide dismutase-mimetic activity.

The theory of oxygen toxicity proposed by Fridovich and coworkers,[1] stating that superoxide anion (O_2^-) is the toxic species in oxygen toxicity and SOD provides marked protection against oxygen toxicity by destroying potentially harmful O_2,$^-$ has received general acceptance. Oxygen free radicals have been linked to the pathophysiology of various disease states[2] and clinical applications of SOD for these diseases have been reported.[3,4]

Several authors have observed possible use of SOD as an antiinflammatory drug.[5,6] It may find applications in radioprotection,[7] antiischemia/reperfusion,[8,9] protective agent for transplantation[10,11] and diabetes.[12,13] It, however, has some drawbacks due to its proteinacious character. First, it has a very short circulating half-life due to rapid glomerular filtration following I.V. injection.[14] Secondly, SOD can not pass through cell membrane because of the large molecular size and its anionic character.[15] Finally, instability, potential immunogenicity and short supplies are other factors associated with its protein nature. Some of these problems can be circumvented to some degree by special techniques such as liposome-entrapment,[16] scrape-loading[17] and conjugation with polymer.[18,19]

Various shortcomings intrinsic to SOD coupled with the fact that Cu, Fe and Mn exist at the active sites of SOD[20,21] have led to the screening and synthesizing of low molecular

†To whom correspondence should be addressed

weight metal complexes with SOD mimetic activity. Ample evidence has been accumulated in the literature that copper complexes have a variety of effects in biological systems.[22] The area of clinical and experimental applications of copper complexes has shown a degree of similarity with those of SOD, probably because copper complexes have a variety of effects in which copper-dependent enzymes take an important part.[23] One of the main functions of copper complexes suggested by Sorenson[24] is that copper complexes manifest their activity via their own chemical reactivities such as superoxide dismutation. Thus, it is worthwhile using copper complexes to substitute for native SOD. Several studies have been performed to prepare efficient SOD mimetics on the basis of structure difference of chelating ligand.[25] Copper chelates display marked scavenging effects on superoxide anion at physiological pH values and have been extensively studied as potential SOD mimetics.[26, 27]

The surface modification of therapeutically useful enzymes by covalent linkage of nontoxic, nonimmunogenic, and biocompatible polymers is a technique now widely investigated to overcome several disadvantages of the use of enzymes in their native forms. These disadvantages often include rapid clearance from circulation, immunogenicity and antigenicity, degradation by proteolytic enzymes, and finally low stability in handling and conservation.[28] The renal clearance of SOD can be prevented by increasing their molecular weight through covalent attachment of the inert polymer. SOD has been used as a novel anti-inflammatory matalloprotein drug useful in the treatment of many pathologies related to abnormal production of the free superoxide radical.[29]

We prepared copper complex of 3, 5-diisopropylsalicylic acid(DIPS) and hrSOD-PEG conjugate, and compared their SOD activity with hrSOD *in vitro* to establish their possible applications in clinical situations. These results might aid in the better understanding of the biochemical basis for the SOD mimetic reaction mechanism of copper complex and hrSOD-conjugate *in vivo* as well as in designing more suitable SOD-mimetics.

EXPERIMENTAL

Materials and Animals

Male Sprague-Dawley rats weighing 200~250 g were used and allowed to drink water *ad libitum* through the experimemt. HrSOD was generously provided by Lucky Biotech. PEG 5000 was purchased from Sigma Chem. Co.. SOD from bovine erythrocytes, lyophilized powder, 98% protein, 3570 units/mg protein were obtained from Sigma Chemical (St. Louis, MO). Commercially available 3, 5-diisopropylsalicylic acid (DIPS) was obtained as a tan solid and purified by extracting an aqueous solution of its sodium salt, formed with sodium bicarbonate, three times with benzene. The aqueous layer was then acidified with concentrated hydrochloric acid to obtain the light tan DIPS. All the chemicals mentioned above as well as hydrochloric acid (volumetric standard, 1.0 N), potassium hydroxide (volumetric standard, 0.1 N), sulfuric acid, cupric chloride, cytrochrome c from horse heart muscle, brilliant blue G, acetaldehyde, and allopurinol were purchased from Aldrich Chemical Co. (Milwaukee, WI). Methyl alcohol and ethyl alcohol were HPLC grade and were used as supplied by Fisher Scientific Co. (Fair Lawn,NJ). Sodium monophosphate, sodium diphosphate, sodium hydroxide and potassium chloride, all reagent grade, were also from Fisher. Xanthine oxidase (XOD) from buttermilk, grade I, No. X-1875, suspension in 2.3 M ammonium sulfate, 13.2 mg protein/ml, activity 0.59 units/mg protein, xanthine, bovine albumin, No.A 4378, crystallized and lyophilized. All other che-

micals were also of analytical reagent grade.

Preparation of Cu(II)-DIPS

Cu(II)-DIPS was prepared according to Sorenson[30] with minor modifications. Briefly, 0.025 mole of DIPS was dissolved in 100 ml of water with a solution of NaOH (50%), filtered and back-titrated if necessary with a solution of HCl (10%) until pH meter shows the solution to be weakly basic. This solution was dropped into 100 ml of water containing 0.05 mole of $CuCl_2$. The resulting blue precipitate was removed by filtration and dissolved again in 50 ml of boiling water. Recooling resulted in renewed crystallization of the complex. The complex was washed with cold water and then dried at 50~80℃ and 15 mmHg vacuum for 3 days prior to submission to elemental analysis.

Determination of SOD Mimetic Activity of Cu (II)-DIPS

The ferricytochrome c reduction assay was performed accordidng to Crapo *et al.*[31] with a slight modification. Our standard assay was as follows: cytochrome c solution (0.05 mM) and xanthine solution (0.5 mM) were prepared in 0.05 M potassium phosphate buffer solution (PBS, pH 7.4). XOD (stock enzyme) was diluted with the same PBS so that a 10 μl sample would catalyze an absorbance change of 0.025 per min at 550 nm in the absence of Cu (II)-DIPS. Cytochrome c solution (0.6 m*l*), xanthine solution (0.6 m*l*) and 1.8 m*l* of the PBS were placed in a 5 m*l* cuvette with a light path of 1.0 cm, in a thermostated cell compartment at 25℃. Solutions to be assayed were saturated with oxygen by bubbling the gas through the solutions for 30 min. The final reaction mixture contained 10 M ferricytochrome c and 0.1 mM xanthine. Reactions were initiated by adding 10 μl of XOD (0.008 U) and a stirrer was employed. In the initial assay, the course of the reaction was followed by the increase in absorbance at 550 nm as ferricytochrome c were converted to ferrocytochrome c. The rate of increase in absorbance in the absence of Cu (II)-DIPS was taken as the value 100% activity. Other reaction mixture were then prepared in which Cu (II)-DIPS solutions at varying concentrations were added and the resulting mixtures were allowed to be stirred at 25℃ for 5 min. The rate was again monitored after adding XOD to generate $O^{\cdot}$. The rate of increase in absorbance (ΔA550/min) in the presence of different amounts of Cu(II)-DIPS was calculated in percent of the rate of increase in absorbance in the absence of Cu (II)-DIPS and plotted against the negative logarithm of Cu (II)-DIPS concentration. The plots obtained were linear over the experimental concentration range studied allowing a determination of EC_{50}, the concentration of Cu (II)-DIPS required to inhibit the rate of reduction of ferricytochrome c by 50% under these specified conditions. Inhibition of the reduction of ferricytochrome c by native hrSOD and hrSOD-PEG was used for comparative purposes. Each determination was performed in triplicate.

Potentiometric Measurement for Stability Constant

Stability constant of Cu (II)-DIPS was measured potentiometrically based on alkaline titration of DIPS solution in the presence of copper ion. Carbon dioxide was excluded by bubbling with nitrogen gas. Titration was performed by standard CO_2-free 0.1 N KOH traced by Corning pH/ion analyzer 250 (Corning, New York). To obtain useful stoichiometric quantities directly from potentiometric measurements, the pH scale of $[H^+]$ ion concentration rather than (H^+) ion activity was used. The mean ionic activity coefficient, $f_{\pm}$, was need for the conversion of hydrogen ion activity to concentration ($[H^+]=(H^+)/f_{\pm}$). Value of 0.81564 for $f_{\pm}$ was calculated from the Davies equation[32] when I=0.1 and 13.9965[33] was taken for pKw of water at 25℃. The following traditional stability constants for complex

Table I—*The Stability Constants of the Copper(II) Complex of DIPS at I=0.1 (KCl) and 25℃*

Ligand	$\log k_2$	$\log k_1$	$\log \beta_2$
DIPS	10.78	7.20	17.98

$K_n = K(CuL_{n-1} + L = CuL_n)$ and $\beta_n = K(Cu + nL = CuL_n)$. Calculated with the computer program SCOGS2.

Table II—*The Cytochrome c Assay of HrSOD*

hrSOD	$\triangle A_{550}$/min	%inhibition
0	0.024	0
1.33	0.0164	31.67
3.33	0.0142	40.83
6.67	0.0104	56.67
13.33	0.0074	69.17
33.33	0.0033	86.25

$\triangle A_{550}$/min was adjusted to about 0.024 in the absence of hrSOD by adding 0.008 unit of XOD. EC_{50} was found to be 4.49 nM.

have been used according to equations below:

$$k_n = [CuL_n]/\{[CuL_{n-1}][L]\} \quad (1)$$

$$\beta_n = [CuL_n]/\{[Cu][L]^n\} \quad (2)$$

The two stepwise equilibria between the copper (II) ion and DIPS may be written as:

$$Cu + L = CuL;\ k_1 = [CuL]/\{[Cu][L]\} \quad (3)$$

$$CuL + L = CuL_2;\ k_2 = [CuL_2]/\{[CuL][L]\} \quad (4)$$

and the overall reaction is:

$$Cu + 2L = CuL_2;\ \beta_2 = k_1k_2 = [CuL_2]/\{[Cu][L]^2\} \quad (5)$$

$$\beta_1 = k_1$$

In these equations, [Cu] and [L] are the total molar concentrations of copper ion and ligand(DIPS) respectively. Numerical evaluation of all the potentiometric data was carried out with the computer program SCOGS2[34] on VAX.

Preparation of HrSOD-PEG Conjugates

To 45.5 mg of hrSOD in 8 ml of 0.1 M borate buffer, pH 9.8, was added with stirring 142.2 mg of activated PEG, an amount 20-fold in molar excess. After 2 hr at 4℃, unattached PEG was removed by ultrafiltration using Amicon Centricon-10 and the concentrated solution was loaded on a Sephadex G-200 (1.5×100 cm) column which was eluted with 0.05 M potassium phosphate buffer pH 6.8 at a flow rate of 0.2 ml/min. The fractions were analyzed at 258 nm and the enzymatic activity was determined. The eluate of the pooled peak corresponding to the hrSOD-PEG conjugates was dialysed against distilled water to remove salt for 24 hr at 4℃ and lyophilized. The extent of conjugation, measured by the number of reactive amino groups, was determined by titration with trinitrobenzenesulfonic acid,[35] and protein concentration were measured by the Bradford method using BSA as the calibration standard.[35]

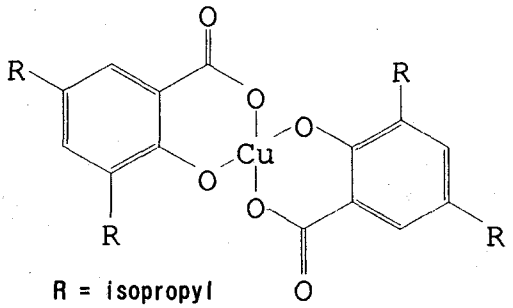

Figure 1—Structure of Cu (II)-DIPS

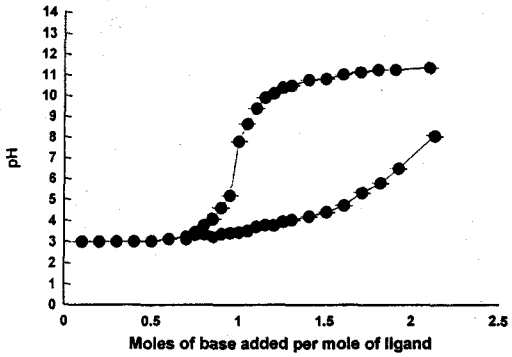

Figure 2—Titration curves of Cu (II) and DIPS at 25℃ with I = 0.1 (KCl). Upper line: DIPS (1.2×10^{-4} M); Lower line DIPS(1.2×10^{-4} M) + $CuCl_2$ (0.6×10^{-4} M).

SOD Activity in Plasma

Blood samples were previously centrifuged and the plasma was used to evaluate the enzymatic activity. One unit of SOD was defined as the amount that caused 50% inhibition of

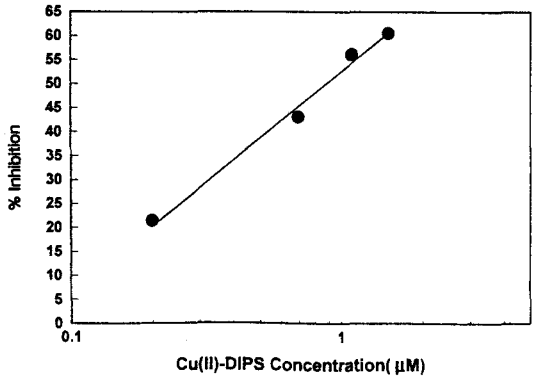

Figure 3—Estimation of EC_{50} for Cu (II)-DIPS.

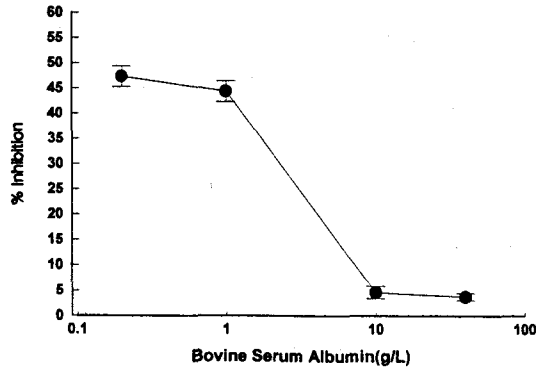

Figure 4—SOD activity of Cu (II)-DIPS in the presence of BSA.

the rate of reduction of ferricytochrome c under specified conditions.[31]

Half-lives of HrSOD, HrSOD-PEG Conjugates in Rats

The *in vivo* half-lives of the native and modified hrSOD preparations were measured in rats (n=6). They were injected with 5 mg/kg of hrSOD or equimolecular amount of hrSOD-PEG conjugates in 0.2 m*l* dissolved in normal saline into the femoral vein. The blood was collected in heparinized tubes from the femoral artery. After centrifugation it was assayed for enzymatic activity according to the above method. The half-lives of SOD species were determined using the RSTRIP program.[37]

RESULTS AND DISCUSSION

Fig. 1 shows the structure of Cu (II)-DIPS prepared. Stability of Cu (II)-DIPS was obtained potentiometrically using SCOGS2 program on VAX (Table I) with titration curves shown on Fig. 2. Cytochrome c is reduced by superoxide radicals generated by the action of XOD on xanthine. SOD catalyzes the dismutation of superoxide radicals in buffer solutions as shown by inhibition of cytochrome c reduction. Based on this, SOD activity of hrSOD (Table II) and Cu(II)-DIPS was measured. Plotting % inhibition against negative logarithm of Cu(II)-DIPS concentration (Fig. 3) showed straight line from which EC_{50} value was then determined to be 1.10 μM by interpolation. The EC_{50} value for native hrSOD was 4.49 nM. However comparing the molecular weight of hrSOD (32 kDa) and that of Cu (II)-DIPS (524.1), Cu (II)-DIPS can function as effectively as hrSOD on weight per weight basis.

As shown in Fig. 4, in contrast to native SOD, Cu (II)-DIPS showed a pattern of losing their SOD mimetic activities in the presence of bovine serum albumin (BSA) around 10 g/L, even less than the physiological concentration 40 g/L. This result indicated that even though Cu(II)-DIPS showed efficient SOD mimetic activity *in vitro*, it may not survive chelating biomolecules such as serum albumin *in vivo*. However the exact nature of active therapeutic components after they enter in biological systems still remains in doubt. This result was consistent with the observation of Miesel *et al.*.[38]

James and Williams[39] reported the oxidation-reduction potentials of some copper complexes. They showed that the chelating ligand affects the redox potential of cupric and cuprous complex couple. In light of this, Bijiloo *et al.*[40] suggested that the effect of steric factor (E_s) may be explained in terms of alteration in redox po-

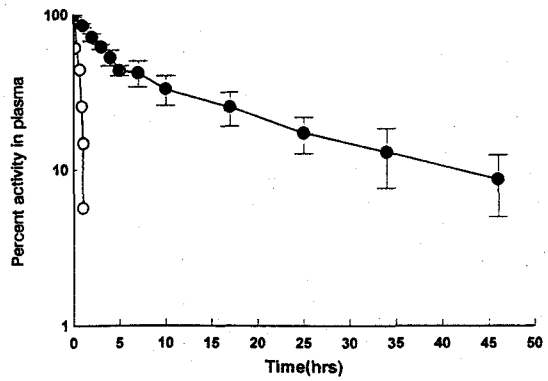

Figure 5—Plasma clearance of SOD activity following i.v. injection of hrSOD-PEG conjugate and hrSOD into rats. Key : (○); hrSOD, (●); hrSOD-PEG conjugate

tential of Cu (I) complex/Cu (II) complex. Considering these together, further ex- periments on redox potential of Cu (II)-DIPS are required for better understanding of the relative role of E_s in SOD mimetic activity, which reflects changes in the acid dissociation constants, and consequent changes in stability constants.

The experimental and therapeutic applications of SOD are governed by a number of factors. First, SOD has a very short circulating half-life due to rapid glomerular filtration following i.v. injection.[14] Secondly, exogenous SOD can not pass through cell membrane because of the large molecular size of SOD and its anionic character.[15] Thus SOD can not readily reach intracellular sites of hazardous oxygen species generation to exert pharmacologic activity effectively unless special techniques such as liposome-fusion[41] or scrape-loading are used. As a result, its protective effect can only be achieved in the extracellular spaces. To overcome these drawbacks, the free hydroxyl of PEG activated by trichloro-s-triazine was conjugated to ε-amino groups of lysine on hrSOD. PEG was selected as the modifier as it was already known to be a non-toxic polymer. PEG has both hydrophilic and hydrophobic properties so that PEG-enzymes may be enzymatically active in both aqueous and lipophilic environments and could interact with cell membranes owing to the amphipathic properties. Modification by PEG blocks renal clearance and increase the circulating enzyme half-life from 5 min to 15.5 hr in the rats, as shown in Fig. 5. The inert nature of PEG also reduces the antigenicity of the native protein and inhibits the hydrolysis of protease-sensitive proteins.[42] HrSOD retained a high percentage of its enzymatic activity after PEG modifications, i. e., 90% of native hrSOD. PEG-modification of the enzyme might be useful to increase the half-life of the native enzyme and pharmacological effect. This compares to only 51% retention of enzymatic activity upon derivatization of SOD with cyanuric chloride activated PEG.[43]

Fig. 5 shows the pharmacokinetic behavior of PEG-derivatized hrSOD after intravenous administration at a dose of 5 mg/kg. The time-concentration profile was not linear on a semi-log scale. The plasma concentration decay of the free hrSOD was very rapid(5 min) which was very close to that found in rats by Huber and Saifer[44] and Wong *et al.*.[45] HrSOD-PEG, however, could be described as two semilog phases with 1.4 hr and 15.4 hr half-lives respectively.

The present study demonstrates that problems of rapid clearance of hrSOD can be largely overcome by conjugation with PEG. The development of conjugates of hrSOD with long plasma half-lives and low immunogenicity provides new possibilities for potential applications in biology and medicine, especially extracellular protection against oxygen toxicity. Cu (II)-DIPS, however, might find applications as an intracellular antioxidant due to its lipid solubility.[46]

Lyophilyzed conjugates, which can be recon-

stituted without loss of activity, may provide a formulation suitable for prolonged storage prior to use.

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