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Anti-inflammatory and pharmacokinetic properties of superoxide dismutase derivatized with polyethylene glycol via active esters

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Enzymes used as pharmacological agents for systemic therapy offer promise in the treatment of several diseases, but have considerable limitations because of problems of protein immunogenicity, instability and, often, rapid elimination. One method overcoming these difficulties appears to be the masking of the polypeptide structure by linking polymers to the protein surface (Holcenberg 1982). This technique is still in its early stages concerning choice of polymer, method of coupling and long-term toxicity.

As a contribution to these studies we report here some results obtained on the enzyme superoxide dismutase, which has anti-inflammatory properties (Merberger et al 1973; Lund-Olesen & Menander 1974; Edsmyr et al 1976; Rister et al 1978) but has also a very short half-life in animals (Huber & Saifer 1977). The enzyme was derivatized with monomethoxypolyethyleneglycol (PEG) using a new method that employs active esters as the reactive group, a procedure that appears to have significant advantages over the method employing trichloro-*s*-triazine as the coupling reagent (Abuchowski et al 1977) the disadvantages of which we have discussed (Boccù et al 1982). Moreover, that method cannot be applied to essential -SH containing enzymes since it give stable thioether derivatives (Boccù et al 1983).

The procedure we used (for details see Boccù et al 1983) involves the oxidation of the carbinol function of PEG to carboxylate followed by its activation with *N*-hydroxysuccinimide and dicyclohexylcarbodiimide.

Materials and methods

Preparation of activated PEG. Monomethoxypolyethyleneglycol 5000 (Union Carbide), 5 g, was dissolved in dry dichloromethane; 10 g of activated manganese dioxide was added at room temperature (20 °C) and maintained overnight with stirring. After removal of the insoluble material by filtration, the polymer, oxidized to the aldehyde form, was recovered by evaporation of the solvent, and dissolved in 200 ml of a 3% H_2O_2 solution for oxidation to the carboxylate form. The solution, after standing overnight, was passed through an anionic BIO-RAD AG 1 × 1 column (2·5 × 20 cm) which was rinsed thoroughly with water, to remove the unoxidized form of the polymer such as the starting carbinol or the intermediate aldehyde, while the carboxylate derivative (PEG-COOH) was eluted with 0·02 M HCl and lyophilized. The PEG-COOH, 8 mmol, was dissolved in 100 ml of dry dimethylformamide, cooled at 4 °C, 50 mmol of *N*-hydroxysuccinimide was added and finally 50 mmol of dicyclohexylcarbodiimide was added dropwise. The solution was left overnight to react; urea was removed by filtration and the carboxyl-activated polymer precipitated with dry ether and recrystallized from dichloromethane–ether (1 : 1).

Coupling of activated PEG to superoxide dismutase. To bovine blood superoxide dimutase from Sigma, 2700 U mg^{-1} (10 mg in 5 ml of 0·1 M borate buffer pH 8·5), was added activated PEG at different molar ratios to obtain various degrees of modification, while the pH was maintained with a pH stat. After standing for 6 h at 4 °C, the solution was chromatographed on a Bio-Gel A-0·5 m (3 × 140 cm) equilibrated with 0·1 M phosphate buffer pH 7·2. It was found that when a molar excess of activated PEG over protein of 10 was used in the reaction mixture, 13% of the lysines of the enzyme were modified; with molar ratios of 60 or 800 the lysines modified were 30 and 90% respectively. At these degrees of modification, 3, 6 and 18 moles of PEG were respectively linked to the superoxide dismutase (SD) molecule, corresponding to protein-polymer adducts of mol wt 46 000, 61 000 and 121 000 (SD-PEG-3, SD-PEG-6 and SD-PEG-18). The modification was accompanied by a drop in enzyme activity that for the samples was respectively 90, 80, 72 ± 5% of the native enzyme.

Carrageenan-induced foot oedema test. Female Sprague Dawley rats, 185 ± 11 g were used. The oedema was induced following a modification of the method of Winter et al (1962). Each rat was injected in the plantar aponeurosis of the right hind paw with 0·10 ml of 1% carrageenan (Lambda carrageenan Lot. 60 F-0652, Sigma, St Louis, MO, U.S.A.) suspended in sterile 0·9% NaCl (saline). Foot volume was measured at zero time and 5 h after the injection of the irritant, by means of a water plethysmometer (Mod. 7150—Ugo Basile, Milano, Italy). SD and its derivatives (5000 units) were dissolved in saline (2 ml) and administered intravenously at the same time as the carrageenan injection. Indomethacin was suspended in 5% arabic gum and given orally 1 h before the injection of the irritant. Control rats were i.v. injected with 2 ml kg^{-1} of saline at the same time as the carrageenan injection.

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Table 1. Anti-inflammatory activity of native and derivatized superoxide dismutase and of indomethacin in carrageenan-induced foot oedema test.

	Number of rats	Route of admin.	Dose kg^{-1}	Oedema vol* ml (s.d.)	% of inhibition	P†
Controls	12	i.v.	—	0·82 (0·22)	—	—
Native SD	14	i.v.	5000	0·67 (0·19)	18	>0·10
SD-PEG-3	15	i.v.	5000	0·64 (0·17)	22	<0·05
SD-PEG-18	16	i.v.	5000	0·55 (0·14)	33	<0·01
Indomethacin	17	oral	3·00 mg	0·57 (0·17)	30	<0·01

* 5 h after carrageenan injection.
† Student's *t*-test.

Results and discussion

To study the plasma clearance, the enzyme native superoxide dismutase or the derivatized species, 5000 units in 0·2 ml Ringer solution, was injected into the saphenus vein of Levis male rats (200–250 g) and the blood, collected from the tail vein, was centrifuged and the plasma assayed for enzyme activity according to McCord & Fridovich (1969). The clearance followed first order kinetics, half-lives of 4, 18 and 20 h were found for the derivatives SD-PEG-3, SD-PEG-6 and SD-PEG-18 respectively. The native enzyme, on the other hand under identical conditions, had a half-life of about 5 min in agreement with findings of Wong et al (1980).

Table 1 gives the results obtained by testing the acute anti-inflammatory activity of different SD preparations. A single oral dose of indomethacin was simultaneously tested to ensure the reliability of the in-vivo assay. Native SD was almost inactive as an anti-inflammatory agent, while both SD-PEG preparations caused a statistically significant inhibition of the experimental oedema. However, the activity of the longer-lasting SD-PEG-18 preparation was about 50% higher than of the SD-PEG-3 preparation. In the serum of normal rats, the half-life of the former compound is about 20 h, while the latter molecule has a half-life of about 4 h.

This study shows that the SD-PEG derivatives, obtained with the coupling method based on active esters (Boccù et al 1983) have an increase in half-life concomitant with the increase in mol wt of the derivatized enzyme. The half-life values of the three SD-PEG's prepared are of the same order as the corresponding values obtained with the trichloro-*s*-triazine method (Boccù et al 1982).

There may be in-vivo a direct correlation between the plasma half-life of the molecules studied and their anti-inflammatory activity. Native SD was found to be inactive or scarcely active in the carrageenan-induced foot oedema test when a single dose was administered prophylactically, i.e. before irritant injection (McCord et al 1979; Wong et al 1980). On the other hand, the native enzyme displayed good anti-inflammatory properties if repeated doses were administered both prophylactically and therapeutically, i.e. before and after carrageenan injection (Oyanagui 1976; Huber & Saifer 1977). However, it was found that the conjugation with different macromolecules, such as Ficoll or dextrans, can significantly lengthen the plasma half-life of the SD enzyme and 'unmask' its anti-inflammatory properties. This also applies to the SD-PEG derivatives prepared as described in this paper.

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