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Effect of encapsulation on the anti-inflammatory properties of superoxide dismutase after oral administration

Corinne Regnault*[a], Monique Roch-Arveiller[b], Michèle Tissot[b], Gilles Sarfati[c], Jean Paul Giroud[b], Eric Postaire[d], Georges Hazebroucq[a]

[a]*Faculté de Pharmacie Paris XI, Service de Pharmacie Clinique, Tour B, rue Jean Baptiste Clément, 92290 Châtenay Malabry, Paris, France*
[b]*CNRS URA 1534, Service de Pharmacologie, Hôpital Cochin, Paris, France*
[c]*Service de Biochimie A, Hôpital Cochin, Paris, France*
[d]*Pharmacie Centrale des Hôpitaux, Service Scientifique, Paris, France*

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Abstract

Anti-inflammatory properties of free superoxide dismutase and superoxide dismutase encapsulated into liposomes, with or without ceramides, have been investigated. Two models were investigated: carrageenan paw oedema and pleurisy. Animals were fed by repeated doses, twice daily from day 1 until day 4. Evaluation consisted of measurement of paw oedema volume with determination of prostaglandin E_2, thromboxane B_2 and 6-keto-prostaglandin F_1 alpha levels. Polymorphonuclear oxidative metabolism was evaluated by measurement of superoxide anion production. Levels of superoxide dismutase were determined in cells and pleural exudates. Higher anti-inflammatory effects were obtained after eight administrations of encapsulated forms (0.5 mg/kg) whereas free superoxide dismutase have shown no effects. Ceramides enhanced the results obtained.

Keywords: Superoxide dismutase; Liposomes; Ceramides; Anti-inflammatory properties; Bioavailability

* Corresponding author.

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1. Introduction

The fact that oxy radicals are generated by many biological processes and play an important role in various diseases has led to the possible therapeutic implications of protective systems. In particular, superoxide dismutase (EC 1.15.1.1; SOD) has been extensively studied following administration by various means. Local instillation, controlled parenteral systemic delivery and/or development of conjugates which enhanced survival features, have been investigated [1]. The efficacy of locally injected SOD in a variety of inflammatory disorders proves the contribution of superoxide anions ($O_2^{\cdot-}$) to clinical inflammation [2]. However, conflicting effects were observed after oral administration [3].

It is often difficult to determine whether discrepancies in the experimental results obtained in vivo are attributable to animal models, to purity of the protein sample or to the short half-life of SOD [4]. In a previous study, we have attempted to measure SOD level in red blood cell pellets (after plasma and buffy coat elimination), collected at various times after administration of SOD by subcutaneous and oral routes, with or without encapsulation into liposomes and ceramides [5].

The importance of the pharmacokinetics is stressed by the substantial, but model-dependent, increase of anti-inflammatory efficacy of SOD [6]. We therefore evaluated this activity in two rat inflammatory models and in exudative cells, after oral administration of bovine Cu-Zn SOD.

2. Materials and methods

2.1. Chemicals

Bovine erythrocyte Cu-Zn SOD was purchased from Allerbio (Varennes-en-Argonne, France). The specific enzymatic activity, measured in duplicate according to the method of Mc Cord and Fridovich [7], was 3685 ± 92 units/mg. Distearoylphosphatidylcholine (DSPC) was obtained from D3F (Paris, France). Cholesterol, stearylamine and sodium chloride were supplied by Pharmacie Centrale des Hôpitaux (Paris, France). Ceramides were extracted from wheat and purified by INOCOSM (Châtenay-Malabry, France). Cytochrome *c* and xanthine oxidase were purchased from Boehringer (Mannheim, Germany). Opsonized zymosan (OZ), phorbol myristate acetate (PMA) were purchased from Sigma (St. Louis, MO, USA). Chemicals were of analytical grade and all other reagents were of the purest grade available.

2.2. Preparation of liposomes

Liposomes entrapping SOD were prepared by a modification of a patented injection method (no. 89401 857-1). Briefly, stearylamine was dissolved at 40°C in 2 ml of chloroform. After evaporation of chloroform, DSPC and cholesterol were added to stearylamine (molar ratio 14:7:4) and dispersed in 20 ml of ethanol. In preparation with ceramides, addition to ethanolic solution has been carried out after dissolution at 80°C. The aqueous phase was prepared by addition of SOD in 40 ml of 0.9% NaCl solution.

The lipid phase placed in a syringe was then admixed to the aqueous phase at 25°C, using an UltraTurrax (IKA, Stauffen, Germany). Resulting liposomes were then dried on a rotary evaporator at 65°C under reduced pressure to eliminate the ethanolic phase. The liposome size, evaluated by a nanosizer (Coultronics), was 250 ± 50 nm.

2.3. Animals and treatments

Male Sprague–Dawley rats weighing 180–200 g (Dépré, Saint Doulchard, France) were used for all experiments.

SOD, encapsulated or not, was administered at various concentrations (0.5–20 mg/kg) by oral administration (by gavage) in a constant volume of 0.5 ml. Administration took place 18 h and 1 h prior to induction of inflammation. Administration was performed twice daily at the same times each day, once in the morning and once in the afternoon.

2.4. Determination of polymorphonuclear leucocyte (PMN) SOD content

SOD activity was measured at 550 nm, according to the technique of Mc Cord and Fridovich [7], based on competition between the reduction of ferricytochrome *c* by $O_2^{\cdot-}$ and the SOD-catalyzed dismutation of superoxide. Under these conditions, the amount of SOD required to inhibit the rate of reduction of cytochrome *c* by 50%, is defined as 1 unit of activity.

Evaluation of SOD activity was performed in pleural exudates diluted (1:10) in saline. Exudative pleural cells were collected in 1 ml of saline and counted prior to SOD measurement. Each sample was diluted with an equal volume of PBS + 1% Triton X-100 in order to lyse liposomes and cell membranes. SOD measurement was performed in this lysate. Results were expressed as units of SOD activity per ml of exudates and per 10^6 cells for cell pellets.

2.5. Carrageenan paw oedema

Oedema was induced in the right hind paw by subplantary injection of 0.1 ml of 1% carrageenan λ (Marine Colloids) suspension in saline. The volume of the paw was measured by means of Hg plethysmometer values, before this injection (for control value), 0.5 h, 1 h... 6 h and 24 h after. Paw oedema was calculated as the difference between each measurement and the control value.

2.6. Pleurisy

Pleurisy was induced by intrapleural injection of 1 ml of isologous decomplemented (1 h 56°C) serum [8]. Polymorphonuclear leukocytes (>95% of pleural cells) were collected 3 h later. Samples for eicosanoid measurements were collected 1 h after induction of the pleurisy, since peaks of these mediators were observed at this time.

2.7. Determination of superoxide generation

Superoxide generation ($O_2^{\cdot-}$) was measured by reduction of ferricytochrome *c* (horse heart type III) as described by Johnson et al. [9]. PMNs (2×10^6 cells/ml)

and 150 μl of a solution of 0.4 mmol/l ferricytochrome *c* were incubated in the presence of OZ (2 mg/ml) or PMA (10^{-7} M) for 15 min at 37°C. Incubations were stopped by placing the tubes in an ice-water bath, after which they were centrifuged at 800 × *g* for 10 min at 4°C. The absorbances of the supernatants were read at 550 nm in a spectrophotometer (Beckman). The results were expressed in nmol of $O_2^{\cdot-}$ released/min per 10^6 cells.

2.8. Prostanoid assays

Thromboxane B_2 (TX B_2) (metabolite of Thromboxane A_2), 6-keto-prostaglandin $F_{1\alpha}$ (6-Keto $PGF_{1\alpha}$) (metabolite of prostaglandin I_2) and prostaglandin E_2 (PGE_2) were measured using enzyme immunoassays (EIA kit Stallergenes, Fresnes, France) [10] in pleural exudates harvested 1 h after induction of a pleurisy by injection of isologous serum and in supernatants of pleural cells resuspended in 0.5 ml of Hank's balanced salt solution for 1 h at 37°C. Results were expressed in ng of eicosanoids/10^6 cells or in ng per rat [11].

2.9. Statistical analysis

Comparisons between the results obtained with the different groups of animals were performed using ANOVA (one way analysis of variance) (Statview II). Student's *t*-test was calculated in each group between treated and non-treated rats.

3. Results

3.1. Evaluation of SOD levels in pleural PMNs

Previous studies have demonstrated that enzymatic activity was present in blood samples collected from animals treated orally by SOD [5]. In this study, we measured SOD activity in pleural exudates and pleural elicited cells, 3 h after injection of isologous serum.

No difference in SOD activity was observed in pleural exudates, whatever the group considered (Fig. 1A). SOD activity was enhanced in cell pellets collected from animals treated by SOD (Fig. 1B). This increase was only significant when animals were treated by encapsulated SOD.

3.2. Effects on paw oedema

High doses of SOD (20 mg/kg), administered 18 h and 1 h before induction of the paw oedema, demonstrated an inhibiting effect on oedema evolution, significant at 4 h when SOD was encapsulated, and at 5 h when it was not (Fig. 2).

Lower doses (0.5 mg/kg) demonstrated a proinflammatory effect after two administrations, and no effect after four administrations (results not shown). However, on continuing the administrations, an inhibitory effect was observed after six and eight administrations. Encapsulation enhanced this effect (Fig. 3).

3.3. Effects on pleural PMNs

$O_2^{\cdot-}$ production by PMNs stimulated by OZ or PMA was reduced by SOD administration whatever the origin of this SOD (Fig. 4).

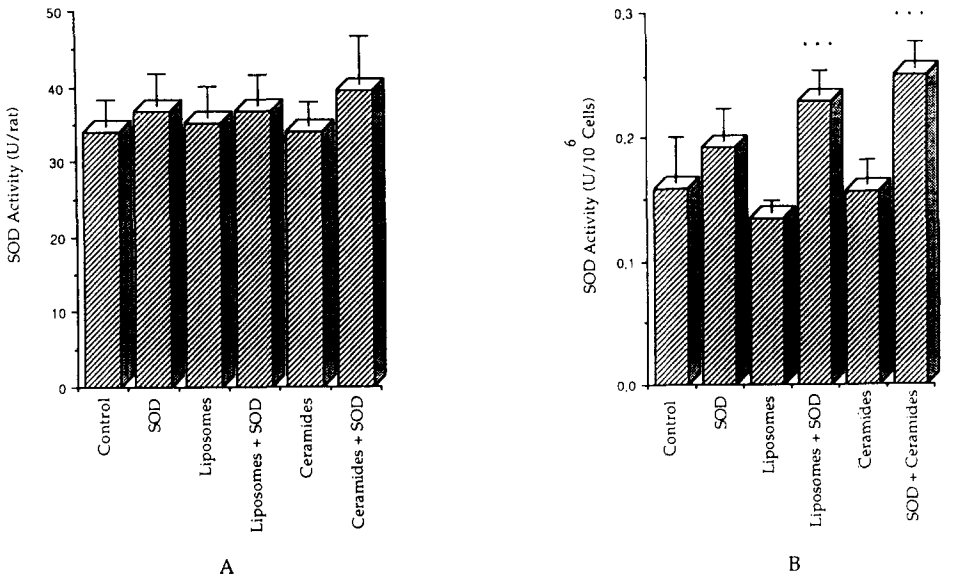

Fig. 1. SOD activity (units per rat ± S.E.M.) in exudates (A) and SOD activity (units/10^6 cells ± S.E.M.) in pleural cells (B) collected from control and treated animals. ***$P < 0.001$.

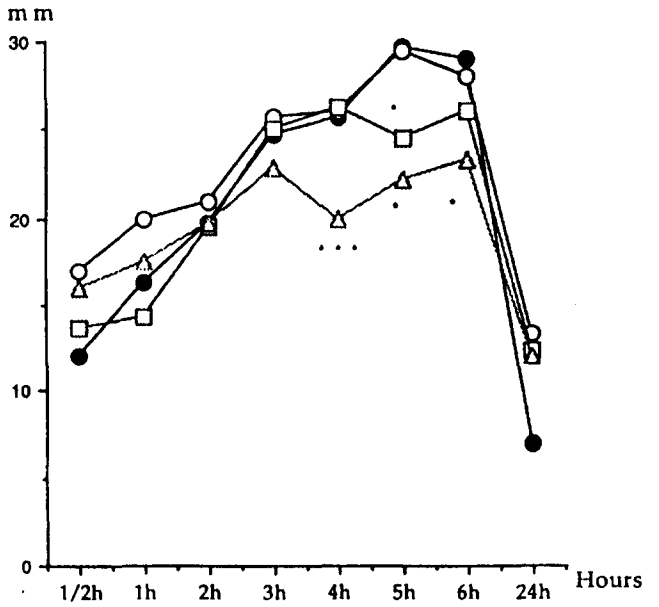

Fig. 2. Time-dependent evolution of carrageenan-induced paw oedema (mmHg) after two treatments with saline (——●——), empty ceramides (——○——), free SOD (20 mg/kg) (——□——) or encapsulated SOD (20 mg/kg) (——△——). *$P < 0.05$; ***$P < 0.001$.

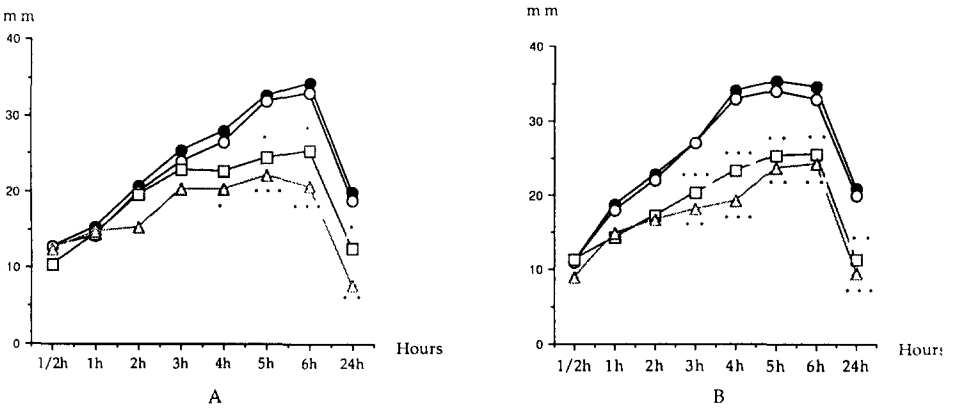

Fig. 3. Time dependant evolution of carrageenan-induced paw oedema (mmHg) after six (A) and eight (B) treatments with 0.5 mg/kg of SOD (——□——) and encapsulated SOD (——Δ——). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

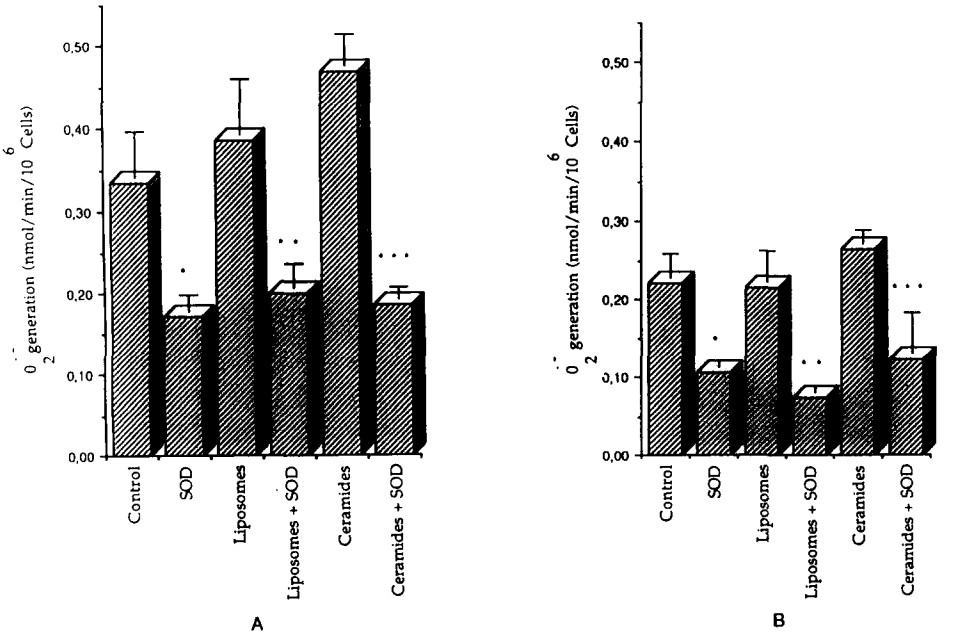

Fig. 4. $O_2^{\cdot-}$ generation (nmol/min per 10^6 cells ± S.E.M.) by PMNs collected from control and treated animals (eight administrations of free or encapsulated SOD, 0.5 mg/kg) after stimulation with OZ (A) and PMA (B). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

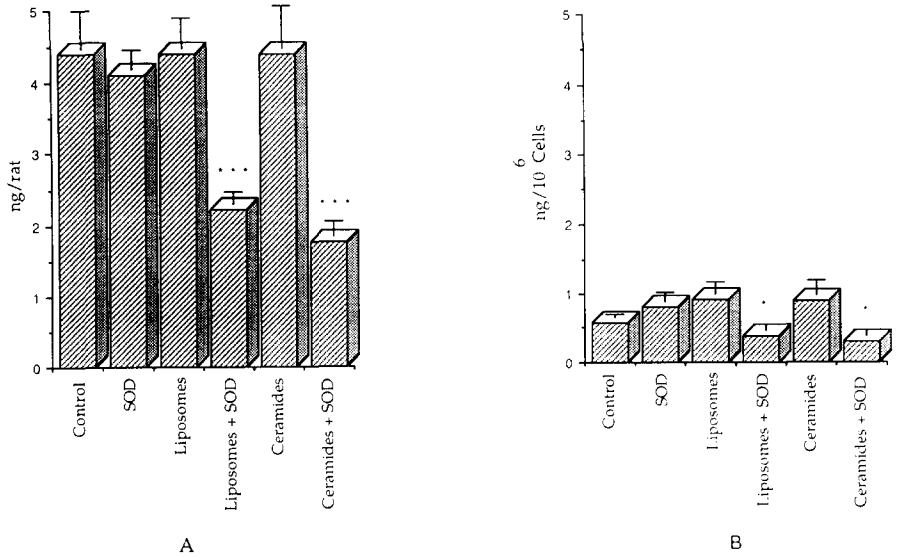

Fig. 5. PGE_2 levels in pleural exudates (A) and supernatants from exudative cells (B) collected 1 h after induction of a pleurisy by isologous serum. $*P < 0.05$; $***P < 0.001$.

3.4. *Effect on prostanoid release*

PGE_2 (Fig. 5) and TX B_2 (Fig. 6) levels decreased in pleural exudates and in supernatants of cells collected 1 h after induction of a pleurisy by isologous serum, only when animals were treated with encapsulated SOD. It should be noted that the presence of ceramides slightly enhanced the phenomenon. Levels of 6-keto $PGF_{1\alpha}$ did not change in the same conditions (results not shown).

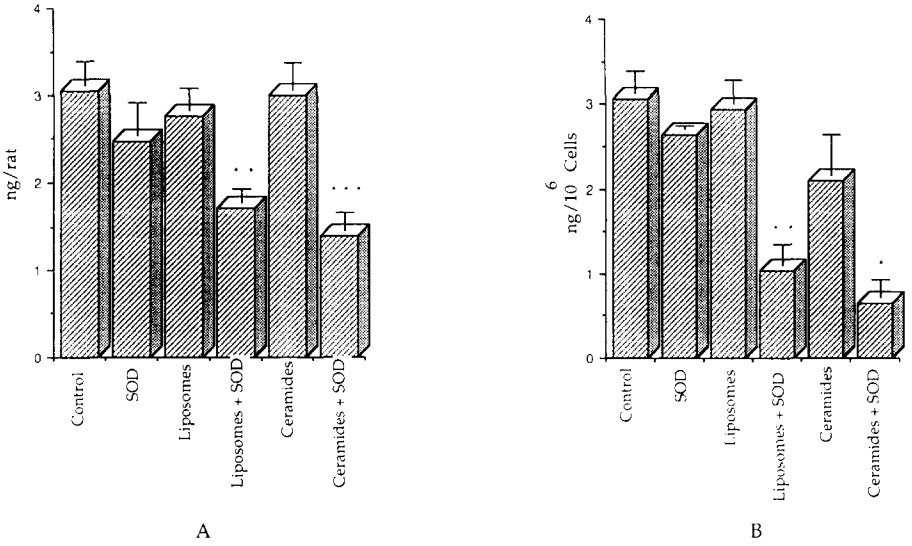

Fig. 6. Thromboxane levels in pleural exudates (A) and supernatants from exudative cells (B) collected 1 h after induction of a pleurisy by isologous serum. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

4. Discussion

According to previous data [12–14], anti-inflammatory activity of SOD has been observed in this study, even after oral administration.

This phenomenon was characterized by a dose-dependent protection against carrageenan paw oedema previously described [15]. It will be noted that small doses may be proinflammatory, but the repetition of oral administration overcomes this effect. It has been previously described that daily topical application [16] improves cutaneous inflammatory reaction. Repetition of administration might also be beneficial in the treatment of radical damage induced by chronic pathological states involving oxygen free radicals.

The pharmacological properties, and the mechanism of action of SOD, may be interpreted as a function of its pharmacokinetic characteristics but poor bioavailability has been previously described after oral administration [17]. It has been suggested that this is attributable to a short half-life [18] and low affinity to cell membranes [19]. Our observations in blood [5] and in inflammatory sites confirm these data. To overcome such disadvantages, SOD incorporation into a drug delivery system was tested. Several authors have tried chemically modified forms, including SOD covalently bound to polyethylene glycol [20], albumin and pyran SOD [21,22], but these forms have not yet been approved for clinical use because of their insufficient pharmacological potency. Encapsulation of the protein into liposomes was then tested [23,24] to determine whether this would allow for better cell penetration and for protection of the enzyme against gastric insult. Our data showed that SOD encapsulation enhanced its bioavailability into blood and exudative cells but SOD levels did not change in pleural exudates whatever the treatment of the rats. These results contradict the hypothesis of Baret et al. [25] and Hardy et al. [26] who suggested that SOD anti-inflammatory activity relies on an increase of its extracellular concentration leading to the scavenging of $O_2^{\cdot-}$ produced by activated phagocytes. For Baret et al. [25], the level of circulating exogenous SOD is not correlated with its anti-inflammatory activity and Vaille et al. [27] add that the probable site of action of SODs is linked to properties of membrane binding. Our results showed a direct effect on cell functions, demonstrated by decrease of some prostanoid and superoxide generation, as observed following the actions of anti-inflammatory drugs. This might be the effect of remodelling of membrane structures.

The release of 6-keto $PGF_{1\alpha}$ was not modified by SOD administration whereas PGE_2 and thromboxane release were inhibited. Such absence of effect on prostacyclin-like material was previously described by Borrelli et al. [28] who, at that time, excluded SOD action on the prostaglandin system. Our results suggest an action on enzymatic systems involving PGE_2- and TXA_2-synthases but not PGI_2-synthase. These observations corroborate those of Ullrich and Hecker [29] who described that the most potent thromboxane synthase inhibitors are inactive for prostacyclin synthase and vice versa, owing to the different orientation of the substrates.

Moreover, this effect was improved by encapsulation. Such observations have been related to other drugs [30,31] which modulate macrophage secretions only after

liposomal encapsulation. The addition of ceramides to liposomal preparations enhanced the phenomenon. These amphipathic molecules which consist of a hydrophobic component linked to a polar moiety of several sugar rings may interact with membrane constituents facilitating drug activity and modifying cellular transduction mechanisms [32]. They are able to orientate membrane receptors such that ligand-induced signal transduction may occur [33] with modulation of cellular oxidant release [34]. Growing evidence suggests that ceramides modulate transmembrane signal transduction by influencing phospholipases and protein kinases associated with receptors inducing cell functions.

The pharmacological activity of liposomal SOD has been shown to depend on its affinity for cell membranes [35] and this work suggests that enhancement of SOD anti-inflammatory properties by drug encapsulation into liposomes is potentiated by addition of amphipathic molecules. The use of such liposomal preparations may increase SOD affinity for tissues and/or activation of receptors linked to cell metabolism which could be important for future SOD-based therapies.

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