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Archives of Medical Research 30 (1999) 69–73

Archives of Medical Research

RAPID COMMUNICATION

Anti-Inflammatory Activity of *Debaryomyces hansenii* Cu,Zn-SOD

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Received for publication March 4, 1998; accepted August 7, 1998 (98/025).

Background. Cu,Zn-superoxide-dismutase, Cu,Zn-SOD, can be obtained from different sources with different anti-inflammatory activities. In this study we compared the anti-inflammatory capacity of the marine yeast *Debaryomyces hanseii* Cu,Zn-SOD (Dh-SOD) with that of bovine erythrocytes (Be-SOD) in a preventive and a therapeutic fashion.

Methods. Edema was induced by carrageenan injection into the rat hind paw and was evaluated using a mercury plethysmograph. Development of the inflammatory process was followed by volume displacement at time 0 (carrageenan injection), 1, 2, 3, 4, 5, 6, 9, 12, and 24 h thereafter. Three different SOD doses were used in preliminary experiments to prevent edema: 10, 100, and 1,000 U/kg.

Results. The results indicate that, at the lowest dose (10 U/kg), both SOD samples are effective in reducing inflammation in both the prostaglandin and amplification phases (−24.8% and −17.5% in the case of Be-SOD, and 11.8% and −18.7% in the case of Dh-SOD, respectively) ($p < 0.05$). At 100 U/kg, Be-SOD also shows good anti-inflammatory activity in all edema phases (−27.1% in the serotonin phase; −19.4% in the prostaglandin phase; and −20% in the amplification phase) ($p < 0.05$), but Dh-SOD was less effective (−10.9%, −9.1%, and −5.7%). At the highest dose tested (1000 U/kg), Dh-SOD was, again, more effective than Be-SOD in all three edema phases (−33.1% and −1.5%; −17.9% and −2.6%; and −13.8% and 6.7%, respectively) ($p < 0.05$). When evaluated as a therapeutic alternative, single doses of Dh-SOD at 1,000 U/kg, and Be-SOD at 100 U/kg, both showed good anti-inflammatory activities (−31.7% and −23.5%, respectively) ($p < 0.05$).

Conclusion. For therapy purposes alone, Dh-SOD appears to be a better anti-inflammatory agent than Be-SOD in carrageenan-induced edema.

Key Words: Carrageenan, Superoxide dismutase, Rats, *Debaryomyces hansenii*, Inflammation.

Introduction

Free radicals participate in both initiation and perpetuation of the inflammatory process observed in many diseases, trauma, and/or infection (1). Superoxide dismutase (SOD) is an enzyme that has anti-inflammatory capacity because of its ability to scavenge the superoxide free-radical (2), but its biological activity in rats and humans is poorly understood (3). SOD comprises a family of isoenzymes which can be obtained from different sources with distinct anti-inflammatory efficacy (4). In addition, they seem to influence in a different way the various phases of the carrageenan-induced edema (CIE) model, in spite of having the same specific activity, metal at the active center, circulating half life, or molecular weight. Because the Cu,Zn-SOD from bovine erythrocytes (Be-SOD) was the first clinically used (5), and has been the most pharmacologically studied (6), we decided to compare its anti-inflammatory activity against a new Cu, Zn-SOD obtained from the marine yeast *Debaryomyces hanseii* (Dh-SOD) (7). As reported elsewhere (8), the physi-

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0188-0128/99 $–see front matter.
PII S0188-0128(98)00005-0

cochemical and molecular characteristics of Dh-SOD indicate important similarities and differences with Be-SOD and other SOD sources, which may be reflected in different anti-inflammatory activity.

Several experimental animal models have been developed in order to study the anti-inflammatory activity of drugs. Among the most commonly used is the carrageenan-induced edema (CIE) model described originally by Winter et al. (9). Based on this test, drugs can either affect the swelling to the early serotonin phase, or the late prostaglandin phase, or both. In addition, a very late amplification period is now being evaluated for the long-term effect of anti-inflammatory drugs. To our knowledge, no experimental work has been conducted to study the anti-inflammatory activity of SOD during the late amplification period, and has not been evaluated in animal experimental models once the lesion has been established (therapeutic mode).

Materials and Methods

SOD source. The enzyme Dh-SOD was obtained according to Reference 7. For this, the yeast biomass was produced in a sea-water formulated medium containing glucose 20 g/l, peptone 10 g/l, yeast extract 5 g/l and the pH adjusted to 5.0 with 0.1 N HCl. The culture was done in 60 l nalgene carboy sterilized with several washes of 70% ethanol (3 × 3 l), 1% NaHClO (1 l) for 30 min, sterile acid water (5% HCl) and finally rinsed with distilled water. A 7% chloride dioxide (HALOX E-100™, The Halox Co., Burlingame, CA, USA) solution was added (2 ml/l) to 30 l of culture medium to prevent the growth of contaminant and 10% of FG10 antifoam agent (Dow Corning) to prevent foaming. The inoculum was prepared in three 2-l flasks containing 500 ml of sterile culture medium by incubating at room temperature for 18 h at 100 rpm. The inoculum (1.5 l) was added to the fermentation bottle and the yeast allowed to grow under an air-flow of 3.3 l/min at room temperature during 48 h. After this incubation the cell biomass was removed by continuous centrifugation and the Dh-SOD enzyme obtained by disruption in a Bead-Beater as described previously (7). Be-SOD was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Animals. The assays reported here were approved by the Animal Ethics Committee of CIBNOR. The female 130–170 g Wistar rats were purchased from a commercial supplier (Bioterio México, Mexico City). The animals were allowed to adapt for a 4-day period at the animal facility with water and food *ad libitum.* Only during the evaluation period that lasted 24 h were the animals restricted in food supply, but again water was provided *ad libitum.*

Induction of edema. Type IV carrageenan (1 type) was purchased from Sigma (St. Louis, MO, USA). A 1% solution in 0.9% saline was prepared 1 h before the experiment. One hundred milliliters of the carrageenan solution was administered intraplantary into the right hind paw of each animal (time 0).

Test groups. Ten groups of seven animals each were used to evaluate the preventive and therapeutic effect of Be-SOD and Dh-SOD. Their corresponding specific activity, by the nitroblue tetrazolium (NBT) method, was 4,400 U/mg and 8,328 U/mg, respectively. The enzymes were administered intraperitoneally in a 1 ml single dose 30 min before edema induction in the preventive study, and 90 min after carrageenan injection in the therapeutic evaluation.

Evaluation of edema. The volume of edema was evaluated using a mercury plethysmograph constructed by the Division of Technology of this Center. For this, the hind paw of the rat was marked with ink up to the tibiotarsalis joint and immersed into a mercury-filled cuvette which was connected to a transducer that conveyed an electrical signal to an ink recorder. The determinations of edema volume were performed at time 0 (carrageenan injection), 1, 2, 3, 4, 5, 6, 9, 12, and 24 h thereafter. Changes in edema volume of the hind paw are expressed in percentage of variation from time 0. For data analysis, the results of the serotonin phase are considered as the average of hours 1 and 2, while results of the prostaglandin phase as average of hours 3, 4, and 5; the amplification phase represents the average of hours 6 and 9.

Statistical analysis. Two-tail *t* test was used for comparison among groups in the therapeutic study protocol. Analysis of variance was used for the groups in the prophylactic protocol. The level of significance was 0.05%. All determinations were done by Quattro-Pro and STATISTICS computational programs.

Table 1. Preventive effect of Be-SOD and Dh-SOD in collagen-induced edema (CIE) in rats

	Serotonin 1 + 2 h	Prostaglandin 3 + 4 + 5 h	Amplification 6 + 9 h	End 24 h
Dh-SOD				
10 U/kg	−10.5	−11.8[a]	−18.7[a]	−5.2
100 U/kg	−10.9	−9.1	−5.7	+9.1
1000 U/kg	−33.1[a]	−17.9	−13.8[a]	+11.1
Be-SOD				
10 U/kg	−13.3	−24.8	−17.5[a]	−12.27
100 U/kg	−27.1[a]	−19.4[a]	−20.0[a]	−13.51
1000 U/kg	−1.5	−2.6	−6.7	−3.5

Note: Values correspond to percentage deviation from control (placebo group).

[a]Values with a significance of $p < 0.05$.

Table 2. Therapeutic effect of Be-SOD and Dh-SOD in collagen-induced edema (CIE) in rats

	Percentage deviation (%)
Marine yeast SOD 1000 U/kg	−31.7[a]
Bovine erythrocyte SOD 100 U/kg	−23.5[a]

[a]Values correspond to inflammation averages from 1.5 to 10.5 h after SOD administration. In both cases the values were statistically different from those of control groups ($p < 0.05$).

Results

In our case, when 1000 U/kg of either Dh- or Be-SOD were administered 30 min before the carrageenan injection, only the marine yeast SOD was able to inhibit edema development in all three phases (Table 1). In contrast, when doses of 100 U/kg were used, Be-SOD was more effective than Dh-SOD in all three phases (Table 1); at doses of 10 U/kg, however, both types of SOD showed a similar pattern of anti-inflammatory activity, being less effective at the early phase of CIE, but with good anti-inflammatory activity during the prostaglandin and the amplification periods.

The dose response at 100 U/kg during the first 1–5 h interval has been used by Jadot et al. (4) to compare the anti-inflammatory activity among different SODs. In our case, Be-SOD and Dh-SOD showed a swelling reduction (23.2 and 10%, respectively) but when the dose was increased to 1000 U/kg, the anti-inflammatory efficacy of such compounds was reversed, edema reduction being only 2.0% in the case of Be-SOD, while Dh-SOD showed a 25.5% decrease in edema volume. Therefore, not only the nature, or origin, of the SOD may determine its biological properties, but also the dose applied, and this is probably the reason why the application of SOD as an anti-inflammatory agent in clinics has been hampered.

Until now, the different available SODs have not been tested as therapeutic agents in the rat CIE model. In our study, the doses chosen for this experiment were selected on the basis of the anti-inflammatory efficacy observed during the preventive treatment for Be-SOD and Dh-SOD. Thus, the marine yeast SOD at 1000 U/kg, or the bovine erythrocyte SOD at 100 U/kg, was injected intraperitoneally 90 min after carrageenan administration. The percentage of edema volume reduction after SOD application is depicted in Table 2. As can be observed, volume reduction was −23.5 and −31.7% ($p < 0.05$) with Be-SOD and Dh-SOD, respectively. Therefore, the marine yeast SOD seems to be significantly ($p < 0.05$) superior to bovine erythrocyte SOD as an anti-inflammatory agent.

Interestingly, when the experiment was continued for 22.5 h after SOD application, the animal group treated with Dh-SOD still showed anti-inflammatory activity ($p < 0.05$), while the Be-SOD group had lost such an effect. Either treatment was most effective from 2.5 to 4.5 h after applica-

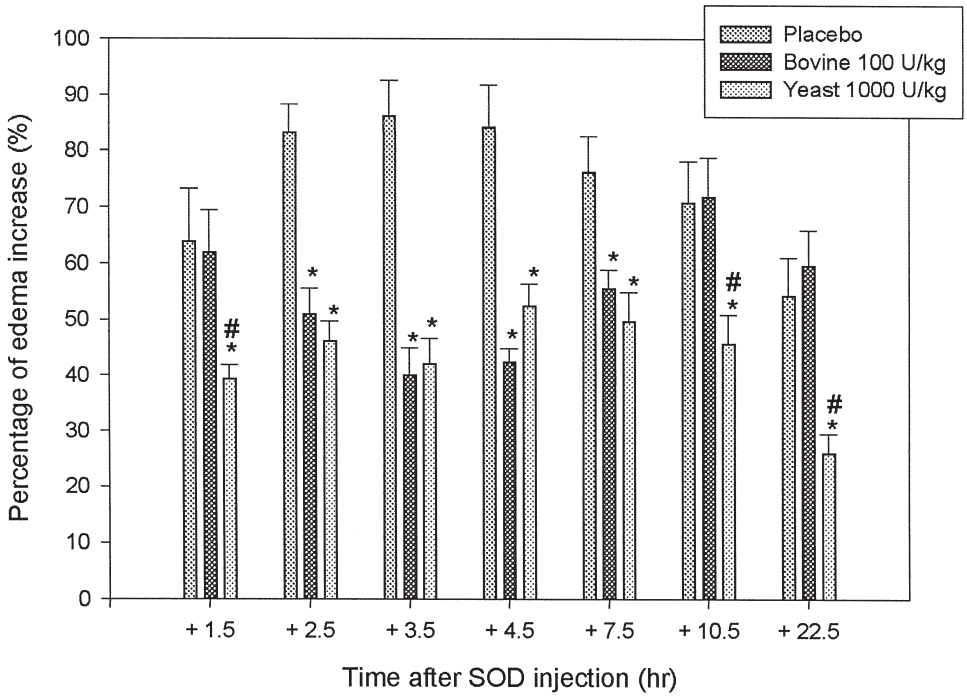

Figure 1. Carrageenan-induced edema development. Effect of Be-SOD and Dh-SOD as anti-inflammatory drugs in a therapeutic protocol. Bar height represents mean values with standard deviation indicated for each group. Drugs were administered intraperitoneally 90 min after hind paw edema induction with carrageenan. *$p < 0.05$ when compared to control (placebo) group. #$p < 0.05$ when Dh-SOD group vs. Be-SOD group.

tion, but the marine yeast SOD showed an earlier and longer lasting anti-inflammatory activity (Figure 1).

In our work we also noticed that the injection of either Be-SOD or Dh-SOD showed a very good anti-inflammatory activity that lasted up to 9 h. Since, in the case of Be-SOD, the maximum serum concentration is achieved 1 h after injection, with an average half-life of 3 h (4), this observation suggests that the mechanism of action of exogenous superoxide dismutase is not exclusively related to its half-life in circulation (11).

Discussion

The possibility of employing SOD as a therapeutic agent in inflammatory diseases, such as arthritis, is certainly a much desired goal (10). Unfortunately, clinical studies with humans are still insufficient in spite of the many promising results obtained with animal studies (4,11). We also decided to employ the carrageenan-induced edema (CIE) model in rats to study the anti-inflammatory effect of Dh-SOD because it reflects a local inflammatory response. As is known, carrageenan is not antigenic and therefore cannot produce a systemic effect and, in addition, the CIE test is known to be highly reproducible (9).

The CIE phenomenon is considered to be limited to the primary response of vasodilatation, plasmatic exudation, migration of neutrophils and monocytes, and the production or activation of proteases (12). As previously reported, this edema process is carried out in two phases: the initial phase, within the first 2 h, represents the early stage of the inflammatory process and is characterized by the presence of serotonin, histamine, kinins, and a negative interstitial fluid pressure (13). The second phase develops within 3 to 6 h after edema induction, in which mainly kinins and prostaglandins appear to be involved. Although paw swelling reaches a peak within 3 to 5 h, the edema continues to be present up to 12 h after carrageenan injection. This is why the administration of anti-inflammatory drugs in some pharmacological studies is evaluated over an interval of 24 h (14). The physiological events occurring during the very late period seem to be connected with an amplification response to leukocyte and complement activation, and with leukotriene synthesis (15).

Differences in anti-inflammatory activity with different SOD sources have been reported before (11). For example, *E. coli* Mn-SOD and Be-SOD show good inhibitory properties in the rat CIE model, but human Cu,Zn-SOD, and the homologous rat Cu,Zn-SOD were proven to be pro-inflammatory in the same assay. These findings suggest that every new SOD source thought to be used for clinical application as an anti-inflammatory needs to be evaluated.

Oyanagui (16), however, has suggested that SOD may also have a steroid-like anti-inflammatory effect. He demonstrated that when dexamethasone was injected subcutaneously, an hour time lag was necessary to inhibit serotonin-induced mice foot edema. In contrast, SOD showed a very short lag time when applied either subcutaneously or intravenously. When both dexamethasone and SOD were simultaneously administered, they appeared to have additive anti-inflammatory activities. Interestingly, the protein synthesis inhibitor cyclohexamide reversed the anti-inflammatory effect of both dexamethasone and SOD. Therefore, it was concluded that SOD might work through the stimulation of a natural anti-inflammatory protein (protein-X) production by endothelial cells.

The doses used in our protocol were chosen in agreement with Vaille et al. (17) who observed a clear dose-response relationship for Be-SOD and its anti-inflammatory activity in the range of 20 to 500 U/kg in animals. Similar or higher doses have been used in ischemia studies with good results (18,19). Yet, the negative effects of the application in excess of external SOD observed *in vivo* experiments should be borne in mind (18).

Jadot et al. (4) found that in a preventive treatment the more potent anti-inflammatory SOD preparation was that of *E. coli* Mn-SOD, with an average edema decrease of 82.5% in a low-dose treatment. A medium anti-inflammatory effect (from 17.0 to 40%) was observed with human, yeast, leek and turkey SOD, which compare well with our Dh-SOD. On the other hand, it has been shown that during the prostaglandin phase of the inflammatory response induced by carrageenan there is an activation of proteases, e.g., gelatinases and collagenases (20,21). Also, it has been demonstrated that SOD influences the rapid inactivation of a 1-Protease Inhibitor (PI) following the liberation of its Cu^{2+} on exposure to hydrogen peroxide (the reaction product of SOD plus superoxide) (22). Because of this, it is possible to assume that the therapeutic anti-inflammatory activity of our Dh-SOD stems from the prevention of PI inactivation. Additional work is necessary to fully elucidate the different roles that exogenous SOD may have in anti-inflammatory assays, yet we believe that our work is an important contribution in such a direction.

Acknowledgments

We thank Adriana Greene and Roberto Carlos Morales for their technical assistance and Martin Ramírez Orozco and Norma Hernández-Saavedra for providing the biological material. This work is part of the Ph.D. thesis of Adolfo García.

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