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ASSESSMENT OF A HUMAN RECOMBINANT MANGANESE SUPEROXIDE DISMUTASE IN MODELS OF INFLAMMATION

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We evaluated a novel human recombinant preparation of manganese superoxide dismutase (MnSOD) for anti-inflammatory and anti-oxidant activity compared with a copper zinc (CuZn) SOD preparation. The results showed that administration of MnSOD (50,100 and 200 μgkg^{-1}) in the Freund's Complete Adjuvant (FCA) mediated paw oedema model suppressed the inflammation at 4 hours by 43, 25 and 43% ($P < 0.001$, $P < 0.01$ and $P < 0.001$ at respective doses). However, 24 hours post-challenge, MnSOD (50 and 100 μgkg^{-1}) suppressed the inflammation by 19% ($P < 0.001$). In contrast, Mn SOD at higher doses (400-800 μgkg^{-1}; 2mgkg^{-1}) exacerbated the inflammatory response at 4 hours. This pro-inflammatory response declined progressively by 24 hours. Furthermore, CuZn SOD produced no significant effects on the inflammatory response. In the carrageenan-induced synovitis model, Mn SOD (25 and 50 μg; intra-articular administration) exacerbated the inflammation at 48 hours. In contrast, Mn SOD at 5 μg produced a significant suppression (44%, $P < 0.05$) in knee joint swelling at 24 hours. The CuZn SOD preparation produced marked pro-inflammatory effects in the joints whilst it lacked activity in the FCA-mediated paw oedema model. These findings support a therapeutic potential of MnSOD in inflammatory disorders, however the compound has a complex pharmaco-dynamic profile.

KEY WORDS: Manganese superoxide dismutase, inflammation, nitric oxide, reactive oxygen species

INTRODUCTION

The role of antioxidant enzymes (especially superoxide dismutase; SOD) as potential therapeutic agents in inflammatory diseases has been the focus of intense debate in recent years[1]. This debate originates from the numerous contradictory studies (*in vitro* and *in vivo*) investigating their anti-inflammatory effects in both clinical and experimental inflammation[2]. These discrepancies have been attributed to the pharmacokinetic properties of the protein including type of SOD preparations utilised, bioavailability, cell penetration, half life and of paramount importance the dosing regimen used. Indeed, numerous reports have shown SOD to display negative effects or attenuated efficacy at high doses of SOD in both *in vitro*[3] and *in vivo*[4] studies. In contrast, lower doses of SOD elicit anti-inflammatory activity. Thus the aims of this present study were two fold; firstly to assess the anti-inflammatory activity of a novel human recombinant manganese superoxide dismutase (rh MnSOD) in two models of experimental inflammation and secondly, to perform a dose response study thereby allowing us to predict a potential therapeutic dose index for future clinical trials.

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MATERIALS AND METHODS

Adjuvant-induced Paw Oedema in the Rat

Foot pad oedema was induced in a series of male Wistar rats (150–200 g, $n = 6$) by a single subplantar injection (50 μl) of Freund's Complete Adjuvant (FCA) (heat-killed *Mycobacterium tuberculosis*, human strains C, DT, and PN mixed and suspended in liquid paraffin BP/10 $mgml^{-1}$) previously immunised with FCA (75 μl) in the scruff 6 days earlier. This model is an example of a cell-mediated hypersensitivity reaction[5]. Human recombinant manganese superoxide dismutase (rh MnSOD), specific activity: 3625 Umg^{-1}) was dissolved in phosphate buffer (0.06 M, pH 7.8) at dosage levels of 50, 100, 200, 400, 800 μgkg^{-1} and 2 $mgkg^{-1}$ and administered intra-peritoneally one hour before FCA challenge in one paw. An additional group of animals received CuZn SOD dissolved in phosphate buffer (100 μgkg^{-1}) 1 hour before FCA challenge. Paw oedema was assessed by measurement of paw circumference (mm) at 4 and 24 hours post challenge and expressed as the difference in size of inoculated compared with non-inoculated paws.

Carrageenan-induced Synovitis Model in the Rat

Inflammation was induced in a series of male Wistar rats (150–200 g; n = 6/7) by a single intra-articular injection (50 μl), in one knee, of carrageenan (1% w/v in sterile physiological saline). At specific time points after challenge (0, 4, 24, 48 hours) joint oedema was assessed by measurement of knee width (mm) and expressed as the difference between injected and contra-lateral non-injected knee joints. Groups of rats (n = 6/7) were given a single intra-articular injection (50 μl) in one knee joint of either a) carrageenan, b) carrageenan + rh MnSOD (5, 12.5, 25, 50 μg), c) rh MnSOD (50 μg), d) carrageenan + CuZn SOD (5, 50 μg) or e) saline.

Drugs

Human recombinant manganese superoxide dismutase (specific activity: 3625 units per mg protein, assayed per method of McCord and Fridovich[6]) was generously provided by Bender & Co. GesmbH, Vienna, Austria. Copper zinc superoxide dismustase (from human–erthyrocytes; specific activity; 3610 Umg^{-1} protein) was purchased from the Sigma Chemical Company, U.K. *Mycobacterium tuberculosis* human strains C, DT, PN mixed, freeze dried, 1969 was supplied by Central Veterinary Laboratories, MAFF, Weybridge, Surrey, U.K. Carrageenan Viscarin 402 was purchased from Marine Colloids Inc., Springfield, U.S.A.

Statistical Analysis

All results are reported as the mean ± standard error of the mean (sem) where n is the number of samples within each group. Analysis of significance was calculated using the unpaired Student 't' test. A probability of 0.05 was set as the minimum level of significance.

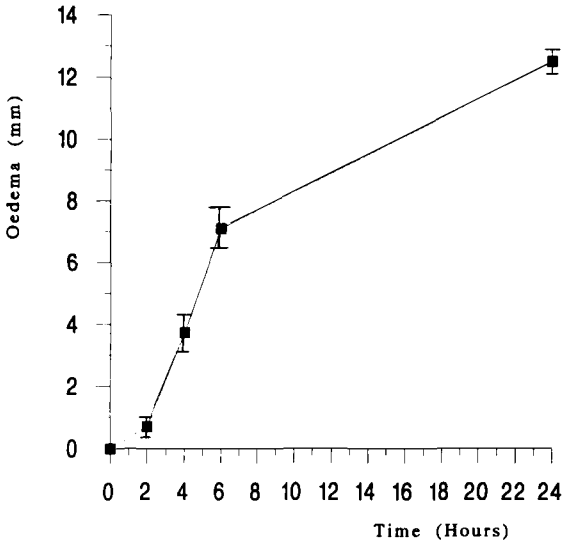

FIGURE 1 Time course of oedema development in a FCA-mediated foot pad model. Paw oedema was assessed by measurement of paw circumference (mm) at 4 and 24 hours post challenge and expressed as the difference in size of inoculated compared with non-inoculated paws. Each point represents mean ± s.e.m. (n = 6).

RESULTS

Dose Response Study of rh MnSOD on Oedema Development in an FCA-mediated Paw Oedema Model

A series of experiments were undertaken to examine the dose response effect of rh MnSOD on oedema development at 4 and 24 hours post challenge. Figure 1. describes a typical response following FCA challenge where paw oedema developed rapidly over the initial 4 hours reaching maximum levels by 24 hours. Figure 2. summarises the effects of pooled data from 3 individual experiments where the effects were calculated as a % change of vehicle treated groups (which has been designated as zero percent; statistical analysis was performed on the raw data). The results showed that adminstration of rh MnSOD (50, 100 and 200 μgkg^{-}) in the FCA-mediated paw oedema model suppressed the inflammation at 4 hours by 43, 25 and 43% ($P < 0.001$, $P < 0.01$ and $P < 0.001$ at respective doses). However, 24 hours post-challenge, rh MnSOD (50 and 100 μgkg^{-1}) suppressed the inflammation by 19% ($P < 0.001$). In contrast, rh MnSOD at higher doses (400, 800 μgkg^{-1}; 2 $mgkg^{-1}$) exacerbated the inflammatory response at 4 hours. This pro-inflammatory response declined progressively by 24 hours. Furthermore, CuZn SOD (100 μgkg^{-1}) produced no significant effects on the inflammatory response. These data are summarised in Figure 3.

Carrageenan-induced Synovitis Model

lntra-articular injection (50 μl) of 1% w/v carrageenan into one knee joint of rats produced a steady increase in joint oedema which reached maximal levels by 24

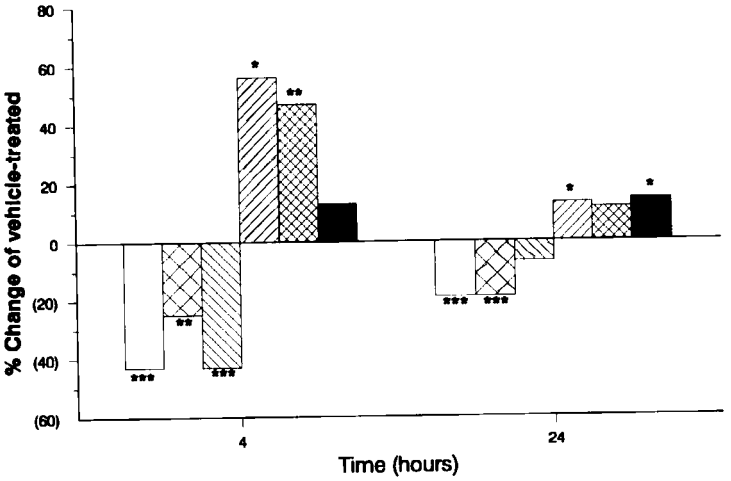

FIGURE 2 Dose-response of recombinant human Manganese SOD on oedema in a FCA-mediated foot pad model. Symbols represent animals treated with rhMnSOD at 50 (□), 100 (▨), 200 (▧), 400 (▨) or 800 μg kg^{-1} (▩) and (■) 2 mgkg^{-1}, intraperitoneally: see methods for details. Each point represents mean data from 6 animals and is expressed as % change from vehicle -treated groups (0%). **$p < 0.01$, ***$p < 0.001$ significantly different from vehicle -treated groups (Unpaired Student t test).

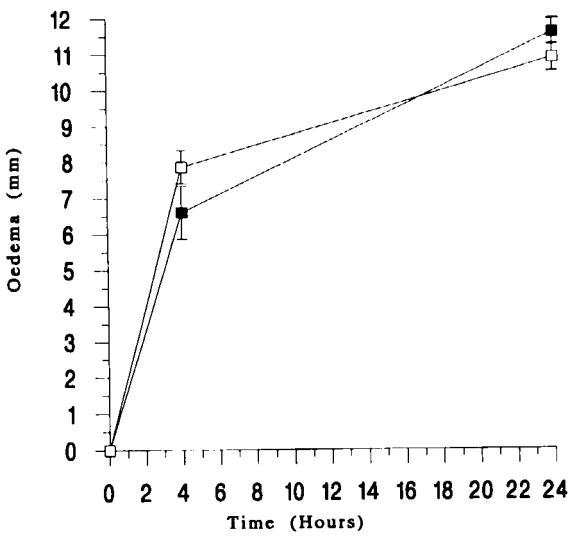

FIGURE 3 Effects of CuZn SOD on oedema in a FCA-mediated foot pad model. Symbols represent animals treated with vehicle (■) or CuZn SOD at 100 μgkg^{-1} (□) intraperitoneally; see methods for details. Each point represents mean ± s.e.m. (n = 6).

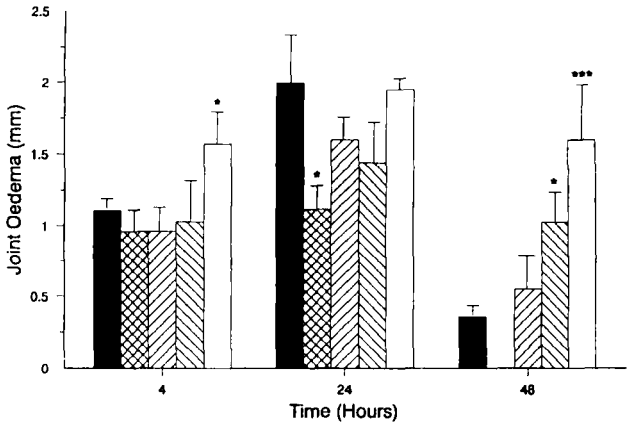

FIGURE 4 Dose response of recombinant human Manganese SOD on joint oedema in a model of carrageenan-induced synovitis. The histogram represents animals treated with vehicle (■) or rh MnSOD at 5 μg (▩), 12.5 μg (▨), 25 μg (▧) or 50 μg (□), i.a; see methods for details. Each point represents mean data from 6 or 7 animals; vertical bars show s.e.mean. *$p < 0.05$, ***$p < 0.001$ significantly different from vehicle-injected animals (Unpaired Student t test).

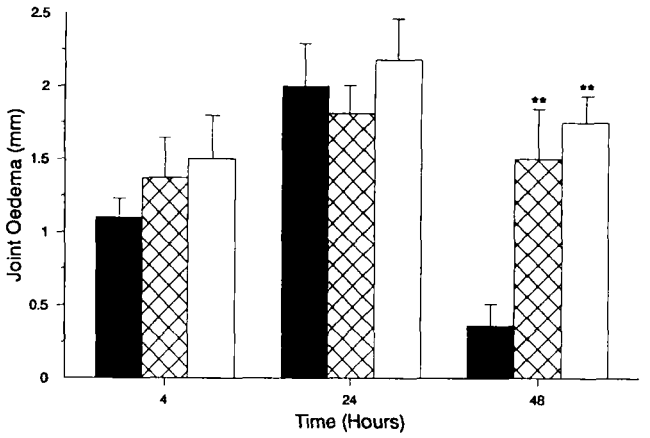

FIGURE 5 Effect of CuZn SOD on joint oedema in a model of carrageenan-induced synovitis. The histogram represents animals treated with vehicle (■) or CuZnSOD at 5 μg (▩) or 50 μg (□), i.a; see methods for details. Each point represents mean data from 5 or 6 animals; vertical bars show s.e.mean. **$p < 0.01$ significantly different from vehicle-injected animals (Unpaired Student t test).

hours. Treatment with rh MnSOD at doses of 25 and 50 μg (intra-articular route) resulted in a marked exacerbation of the oedema response by 48 hours (Figure 4). A significant reduction (44% $p < 0.05$) in oedema was only noted with the lowest dose (rh MnSOD 5 μg) at 24 hours. There were no untoward effects on joint size following challenge with either rh MnSOD (50 μg) or saline alone (data not shown). However, a pro-inflammatory effect was seen in the CuZn SOD (5 and 50) treated groups by 48 hours, as illustrated in Figure 5.

DISCUSSION

Oxygen radical scavengers are responsible for modulating the intra-cellular and interstitial levels of reactive oxygen species (ROS) thereby maintaining a physiological environment. In numerous pathological conditions eg. rheumatoid arthritis[7] and ulcerative colitis[8] the balance between ROS production and sequestration is affected leading to an elevation of ROS. One of the key regulatory mechanism(s) involves the dismutation of the superoxide anion radical ($O_2^{\cdot -}$) via the enzyme superoxide dismutase (SOD) the activity of which is curtailed in some inflammatory disorders. Indeed, studies have demonstrated that SOD therapy leads to some degree of protection against the deleterious nature of excess ROS release in both experimental models of inflammation and clinical studies[1]. The pharmacological efficacy of SOD preparations is governed by a range of factors including bioavailability, rate of cellular transport and plasma half life.

SODs are a group of enzymes which catalytically dismutate the superoxide anion radical to hydrogen peroxide (H_2O_2)[6]. In mammals three types of SOD have been identified, cytosolic (CuZn SOD, molecular weight 33,000), mitochondrial (MnSOD, molecular weight 80,000) and the third, although present in low concentration found in extracelluar fluid, (EC SOD, molecular weight 135,000). In this study we assessed the anti-inflammatory activity of a novel human recombinant manganese SOD in two models of experimental inflammation. MnSOD was used as a consequence of numerous reports in the literature describing its biochemical properties which would enhance its biological effects compared with the widely utilised CuZn SOD preparation. As well as having a plasma half life of 6–8 hours[4], MnSOD, has been shown to exist in a cationic form at plasma pH, thus facilitating its rapid equilibration with interstitial fluid. The latter function is reported to be significant in promoting cardioprotection i.e. myocardial cells are protected during the events of hypoxia/reperfusion injury[9]. The pivotal biochemical property of MnSOD that delineates its biological effects from CuZn SOD is its ability to resist inactivation by H_2O_2 whilst CuZn SOD is rapidly inactivated thereby limiting its efficacy[10]. This intrinsic MnSOD property is demonstrable in experimental models.

In the adjuvant-induced foot pad oedema model, pre-treatment with MnSOD (dose range of 50 μgkg^{-1}–2 $mgkg^{-1}$) resulted in a bell shaped curve in which low doses of MnSOD (50–200 μgkg^{-1}) were protective, but increase in dose (400 μg–2 $mgkg^{-1}$) led to a loss of protection. The presence of a bell shaped curve has previously been described in other models for eg., SOD treatment prevented the development of reperfusion-induced arrhythmias in both isolated heart preparations *in vitro*[11] and *in vivo*[12]. The bell-shaped curve in our studies shows an exacerbated oedematous response with higher doses of SOD. This was well substantiated in the foot pad model and moreover in the carrageenan-induced synovitis model; where only the lowest dose (5 μg) given intra-articularly at the time of challenge afforded protection during the course of the inflammatory response. All the other doses (12.5 μg–50 μg) resulted in either no effect or a marked worsening of the oedematous response by 48 hours. Furthermore, it is noteworthy that treatment with CuZn SOD was either non-effective or pro-inflammatory at all doses tested in both models.

Considerable evidence has now accumulated where a dose range of SODs have been employed in experimental studies with varying degrees of efficacy. Nimrod *et al.*[13] found human recombinant MnSOD to be effective using doses of 5–50 $mgkg^{-1}$ in *in vivo*; whereas other reports have demonstrated anti-inflammatory effects using substantially lower doses of enzyme (6.6 μg–166 μgkg^{-1})[4,2].

Several theories have been forwarded to explain the exacerbation of damage observed at high SOD doses. Increased H_2O_2 formation, the product of the dismutation reaction may partially explain the toxicity of the enzyme. H_2O_2 can easily penetrate the membranes of adjacent cells and in the presence of ions of an adequate transition metal (eg. iron), H_2O_2 can interact with the reduced form of the metal ion to form several highly oxidising species, the most important of which is probably the hydroxyl radical ($^{\cdot}OH$). This reaction is referred to as the Fenton reaction. The hydroxyl radical is highly reactive and thus will combine immediately with molecules that are present in close proximity to its site of formation[14].

Recently, studies have focused on a novel mechanism for hydroxyl radical production which is independent of the presence of transition metal ions[15]. It involves the interaction of O_2 with nitric oxide (NO). NO and NO-generated compounds are responsible for the biological actions of endothelium-derivedrelaxing factor (EDRF), a potent vasodilator produced by vascular endothelial cells[16]. The reaction of $O_2^{\cdot -}$, with NO results in the formation of a peroxynitrite which further decomposes to form $^{\cdot}OH$ as outlined below.

$$NO + O_2^{\cdot -} \longrightarrow ONOO^-$$

$$ONOO^- + H^+ \longrightarrow ONOOH$$

$$ONOOH \longrightarrow {}^{\cdot}OH + NO_2^{\cdot}$$

This reaction has been confirmed by Hogg *et al.*[17]. The net result suggests, therefore, that overscavenging of $O_2^{\cdot -}$, may prolong the vasorelaxant effects of NO leading to extravasation and oedema which could partially account for the inflammatory effects seen with high doses of MnSOD in our studies. Indeed it has been shown that SOD increased the half life of endothelial derived NO in preparations of aortic rings[18]. In addition, Murphy and Sies[19] recently proposed that SOD may support a reversible reduction of NO to the nitroxyl anion (NO^-) which has been suggested to be an additional form of EDRF.

Another possible explanation for the toxicity of high levels of SOD has been put forward by McCord and his workers[20]. They have suggested that the hydroperoxyl radical ($HO_2^{\cdot}$), the protonated lipophilic form of $O_2^{\cdot -}$, may be important for the termination of lipid (L) peroxidation as follows:

$$LOO^{\cdot} + HO_2^{\cdot -} \longrightarrow LOOH + O_2$$

Thus overscavenging of superoxide by excess SOD would eliminate an important step of lipid peroxidation and thus exacerbate the inflammatory response. In conclusion, findings demonstrate that rh MnSOD at low doses is an effective anti-inflammatory agent by suppressing oedema in the models described. Indeed, Mn SOD has proved to be more efficacious than the traditionally used CuZn SOD. Furthermore, we have described the existence of a bell-shaped curve where high doses of rh MnSOD and CuZn SOD were either ineffective or exacerbatory. It is noteworthy, that the pro-inflammatory effects of both CuZn and Mn SOD in our studies are not due to the presence of endotoxin or free copper. This is based on our preliminary studies in which intradermal (abdominal skin) and intra-articular administrations alone had no effects (data not shown). With the growing appreciation of the role of reactive oxygen species in both acute and chronic disorders, the results from this study have confirmed a therapeutic potential of rh MnSOD in inflammation and suggest a new class of compound with potential appreciation to synovitides. However, it is important to note that the dose of SOD is clearly critical and affects the therapeutic index of this drug.

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