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Anti-inflammatory effect of recombinant human superoxide dismutase in rats and mice and its mechanism

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KEY WORDS superoxide dismutase; inflammation; interleukin-1; tumor necrosis factor

ABSTRACT

AIM: To investigate the anti-inflammatory effects and mechanism of recombinant human superoxide dismutase (rhSOD). **METHODS**: Inflammation models such as croton oil-induced ear swelling and carrageenan-induced hind paw edema in mice and rats were prepared. The nitric oxide synthase (NOS) activity was measured by NADPH-diaphoras stain assay, *N*-acetyl-β-*D*-glucosaminidase (β-NAG) activity by spectrophotography, malondialdehyde (MDA) content by thiobarbituric acid (TBA) fluorescence technique, and interleukin-1β (IL-1β), tumor necrosis factor α (TNFα), and IL-8 content by *radioimmunoassay* (RIA). **RESULTS**: rhSOD 20 - 80 mg/kg ip, 40 - 80 mg/kg im, and 80 mg/kg ip significantly inhibited carrageenan-induced paw edema in rats, croton oil-induced ear swelling in mice, and carrageenan-induced hind paw edema in mice, respectively. In the rat arthritis induced by carrageenan, rhSOD 40 mg/kg reduced MDA content in inflamed paws, inhibited NOS activity, and lowered the content of IL-1β and TNFα in exudate significantly. The inhibitory effect of rhSOD 40 mg/kg ip on IL-1β production was more evident than that of dexamethasone 2 mg/kg ip. Also rhSOD obviously inhibited neutrophil infiltration; However, rhSOD had no effect on β-NAG activity in exudate. **CONCLUSION**: rhSOD has anti-inflammatory effect on experimental inflammation in rats and mice, and its mechanisms are relevant to oxygen free radical scavenging, anti-lipid peroxidation, inhibition of neutrophil infiltration, and formation of inflammatory cytokines.

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INTRODUCTION

The superoxide anion appears to be an oxygen free radical possessing high oxidativity. *In general*, it takes the action of body-defense and plays an important role in the inflammation pathogenesis and ischemia-reperfusion injury, *etc*. During the abnormal pathogenesis, many inflammatory factors, such as cytokines, prostaglandin E_2, lysosomal enzyme, and free radicals, take part in the genesis and development of inflammation[1]. Free radical scavenger superoxide dismutase (SOD) is thought unable to entirely arrest the development of inflammation because inflammation is caused by multiple factors. However, a number of data from experiments revealed that SOD possessed potent anti-inflammatory effect. SOD from pig blood inhibited carrageenan-induced foot-edema and croton oil-induced granulation tissue edema of rats. It also inhibited arthritis induced by egg serum and Freund's adjuvant in rats[2]. Treatment of adjuvant arthritis in mice with yeast Cu/Zn superoxide dismutase was shown effective in reducing the paw swelling[3]. And the research on bovine SOD have entered phase Ⅱ trial and obtained encouraging results on Crohn's disease in some reports[2,4]. There were many controversies on the anti-inflammatory effect of SOD. Negative results were also reported in some experiments[5,6]. The contradicted results explain that the understanding for anti-inflammatory action of SOD is superficial as yet, and the anti-inflammatory mechanism of SOD is not only limited to its action of scavenging oxygen free radicals.

There are complex network relationships among superoxide anion, neutrophils, and inflammatory cytokines, and they are more complicated *in vivo*. Neutrophils can be primed and activated by interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα) to immigrate to inflamed sites and release active oxygen species[7,8]; On the other hand, hyperoxia, which produces oxygen free radials, stimulates neutrophils in lungs of rabbits to

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form IL-1β and TNFα[9]. Therefore, it is necessary to study whether anti-inflammatory effect of SOD is related to its regulation to the interaction between superoxide anion and cytokines. This study was to explore the effects of different dosages of rhSOD on experimental inflammation and formation of inflammatory cytokines, especially, IL-1β and TNFα.

MATERIALS AND METHODS

Recombinant human superoxide dismutase (rhSOD) was provided by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China)[10], batch number 20000201, stored at 2 – 8 ℃. Its activity (1 mg = 2529 U) was measured by pyrogallol autoxidation method[11]. Its purity (>98 %) was assayed by SDS-PAGE electrophoresis analysis. Dexamethasone (Dex) was purchased from Lanzhou Pharmaceutical Factory (Batch number 990903). Human serum albumin (HSA) was purchased from Anhui Green Cross Biological Products Company. All above reagents are diluted with normal saline (NS) containing same concentration of HSA before use. Carrageenan (Car) was purchased from Liaoning Institute of Pharmacy, and diluted to 10 g/L with NS. Nitric oxide synthase (NOS) and *N*-acetyl-β-*D*-glucosaminidase (β-NAG) detection kit were obtained from Jiancheng Institute of Biotechnology (Nanjing, China). IL-1β RIA kit was the product of Beijing North Biotechnology Company. TNFα RIA kit was the product of Beijing Bangding Biotechnology Company. All other reagents were of analytical purity grade.

Kunming mice and Wistar rats were obtained from the Experimental Animal Center of Lanzhou Medical College (Grade Ⅱ, Certificate № 14-005 and 14-006, respectively).

Croton oil-induced ear edema in mice Kunming mice (n = 160, 23 g ± 2 g, ♂ ♀) were randomly divided into control (treated with HSA), Dex, and rhSOD treated groups. HSA, Dex, or rhSOD was injected im 30 min before croton oil inducing ear edema. Croton oil mixture 0.2 mL was spread topically in the right ear pinna of mice. The left pinna was served as control. The mice were killed 4 h later. Disks from the pinna were taken with a punch 7 mm in diameter. The edema extent was evaluated as previously[12].

Carrageenan-induced hind paw swelling in mice Kunming mice (n = 60, 22 g ± 2 g, ♂ ♀) were given ip rhSOD 10, 20, 40, and 80 mg/kg, Dex 2 mg/kg, or equal volume of control solvent. The hind paw swelling was induced 30 min after sc administration of 30 μL test agents in the plantar of mice right hind paw. After 5 h sc administration of Car, the mice were sacrificed and both hind paws were cut out symmetrically from ankle joints. The hind paw swelling was obtained by the weight difference between the inflamed and the control hind paw.

Carrageenan-induced arthritis in rats Sixty Wistar rats (140 g ± 20 g, ♂ ♀), used to determine anti-inflammatory effect, were randomly divided into control, Dex 2 mg/kg and rhSOD 10, 20, 40, 80 mg/kg treated groups. HSA, Dex, or rhSOD was injected ip 30 min before carrageenan 0.1 mL was given sc in right hind paw plantar to induce the inflammation. Paw thickness was measured at 1 h, 2 h, and 4 h after challenge with carrageenan. The swelling degree is calculated according to the augment of paw thickness after inducing arthritis.

The other 40 rats were divided into blank, control, Dex 2 mg/kg, and SOD 40 mg/kg treated groups to investigate the effect of rhSOD on formation of inflammatory mediators. Arthritis was induced by carrageenan and first dose of test agents was given as above experiment. But in blank group, right hind foodpad was injected sc NS instead of carrageenan. The second dose of rhSOD was given ip 2 h after the first injection. Correspondingly, HSA was injected ip in control group. The rats were killed 4 h later after inducing inflammation. The skin of inflamed paws was cut open and the entire inflamed paw was washed by extruding with 1 mL ice-cold PBS (50 mmol/L). After the washing, the exudate was harvested and stored at −20 ℃ before use. A piece of tissue was obtained from the center of paw plantar and fixed with 10 % formaldehyde. The slice was embedded with paraffin and dyed with hematoxylin and eosin. Infiltration of inflammatory cells was observed under microscope.

MDA determination in inflamed paws Rats paw was taken off skin and soaked in 2 mL 15 % trichloroacetic acid for 3 h (4 ℃). The supernatant (1.5 mL) was mixed with 2 mL TBA reagent[12]. The reaction mixture was boiled for 25 min and extracted with n-butanol (2 mL × 2 times). The n-butanol layer was assayed by spectrofluorometer with λ_{ex} 520 nm and λ_{em} 550 nm. MDA content was calculated by reference to standards prepared from 1, 1, 3, 3-tetraethoxypropane (Fluka).

Determination of inflammatory factors in

exudate IL-1β and TNFα were measured by RIA. NOS activity by NADPH-diaphoras stain assay and β-NAG activity by colormetric method. The detecting method was according to the protocol enclosed in the detection kit.

Statistics Data were expressed as $\bar{x} \pm s$ and assessed by t-test.

RESULTS

Effect of rhSOD im on ear edema in mice The result showed that rhSOD 40 – 80 mg/kg im obviously inhibited the ear edema induced by croton oil as compared with control. The potency of rhSOD in anti-inflammation was dependent on its dosage ($r = 0.991$, $P < 0.01$) and the effect of rhSOD 80 mg/kg im was close to that of Dex 2 mg/kg im (Tab 1).

Effect of rhSOD ip on hind paw swelling in mice Hind paw swelling induced by carrageenan in mice was lessened significantly by rhSOD 40 – 80 mg/kg ip. The potency of rhSOD 80 mg/kg ip was very near to that of Dex 2 mg/kg ip (Tab 2).

Effect of rhSOD im on arthritis induced by carrageenan in rats The results (Tab 3) showed that rhSOD 20 – 80 mg/kg ip significantly suppressed paw edema in rats induced by carrageenan as compared with control. The potency of anti-inflammation of rhSOD was time-dependent. In the 1st hour after carrageenan administration, the inhibition rate of rhSOD 40 – 80 mg/kg was higher than that of Dex 2 mg/kg. In the 2nd hour, the differences became very little. In the 3rd and 4th hour, the inhibition rate in groups treated with rhSOD was much lower than that of Dex 2 mg/kg.

Effects of rhSOD on formation of MDA in inflamed paws and NOS and β-NAG activity in exudate from carrageenan-induced rats arthritis The content of MDA in inflamed paws and activity ofNOS in rats paw exudates were inhibited by rhSOD and

Tab 1. Effect of rhSOD im on ear edema induced by croton oil in mice. $n = 10$. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* **control.**

Dose/mg·kg^{-1}		Weight of left ear/mg	Weight of right ear/mg	Weight difference between left and right ear/mg	Inhibition rate/%
Control		8.3 ± 1.1	17 ± 3	9 ± 3	–
Dex	2	7.9 ± 0.5	13 ± 2	4.7 ± 2.4^c	47.9
rhSOD	0.25	7.81 ± 0.29	16 ± 4	8 ± 4^a	
	0.5	8.0 ± 1.1	17 ± 4	9 ± 3^a	
	1	7.9 ± 0.8	18.7 ± 2.9	11 ± 3^a	
	2	8.4 ± 0.9	17 ± 3	9 ± 3^a	
	4	8.7 ± 1.2	19 ± 4	10 ± 3^a	
	8	8.1 ± 0.6	18 ± 3	10 ± 3^a	
	16	8.1 ± 0.6	16.2 ± 2.9	8.1 ± 2.5^a	10.7
Control		7.5 ± 0.7	16.7 ± 2.8	9.3 ± 2.7^a	–
Dex	2	7.8 ± 1.0	11.9 ± 1.6	4.1 ± 2.0^c	56
rh SOD	20	7.5 ± 0.6	16 ± 4	8 ± 3^a	12
	40	7.6 ± 0.7	14.8 ± 2.9	7.2 ± 2.9^b	23
	80	7.9 ± 0.8	13 ± 3	4.8 ± 2.4^c	48

Tab 2. Effect of rhSOD ip on hind paw swelling induced by carrageenan in mice. $n = 10$. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* **control.**

Dose/mg·kg^{-1}		Weight of left foot/mg	Weight of right foot/mg	Weight difference between left and right foot/mg	Inhibition rate/%
Control		136 ± 9	205 ± 26	69 ± 19	–
Dex	2	124 ± 12	166 ± 14	41 ± 10^c	40
rhSOD	10	129 ± 11	200 ± 18	71 ± 11^a	–
	20	132 ± 8	199 ± 22	67 ± 20^a	3.5
	40	135 ± 9	193 ± 20	58 ± 17^a	16
	80	133 ± 12	180 ± 30	47 ± 19^b	32

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Tab 3. Effect of rhSOD ip on paw edema induced by carrageenan in rats. $n = 10$. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Dose/ mg·kg^{-1}	0 h	1 h		2 h		3 h		4 h	
	Thickness /mm	Thickness /mm	Swelling degree/%	Thickness /mm	Swelling degree/%	Thickness /mm	Swelling degree/%	Thickness mm	Swelling degree/%
Control	4.55 ± 0.17	6.2 ± 0.5	37 ± 7	7.7 ± 1.0	68 ± 16	8.2 ± 0.8	80 ± 11	8.2 ± 0.5	80 ± 6
Dex 2	4.58 ± 0.26	5.77 ± 0.25	26 ± 6^c	6.2 ± 0.3	36 ± 8^c	6.6 ± 0.4	44 ± 4^c	6.7 ± 0.4	46 ± 6^c
rhSOD 10	4.53 ± 0.09	6.14 ± 0.25	36 ± 6^a	7.5 ± 0.6	66 ± 14^a	7.7 ± 0.9	71 ± 19^a	8.2 ± 0.5	81 ± 11^a
20	4.55 ± 0.13	5.9 ± 0.4	29 ± 7^b	6.7 ± 0.7	48 ± 14^c	7.3 ± 0.8	60 ± 16^c	8.0 ± 0.5	76.0 ± 0.4^a
40	4.52 ± 0.16	5.46 ± 0.26	21 ± 8^c	6.2 ± 0.7	38 ± 16^c	7.2 ± 1.0	58 ± 24^b	7.4 ± 1.2	64 ± 28^a
80	4.54 ± 0.15	5.5 ± 0.3	21 ± 6^c	6.3 ± 0.9	39 ± 20^c	7.5 ± 1.0	64 ± 20^b	8.1 ± 0.7	79 ± 13^a

Dex as compared with control (Tab 4). The inhibition rate of rhSOD and Dex on formation of MDA was 56 % and 37 % respectively. The inhibition rate of rhSOD and Dex on activity of NOS was 26 % and 40 % respectively. But they had no effect on β-NAG activity in exudate.

Tab 4. Effect of rhSOD ip on formation of MDA in inflamed paws, and NOS and β-NAG activity in exudate in carrageenan-induced rats arthritis. $n = 6 - 10$. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.

	MDA/ μmol·L^{-1}	β-NAG/ U·L^{-1}	NOS/ U·L^{-1}
Blank	0.36 ± 0.06^b	10.3 ± 2.6^b	490 ± 226^c
Control	0.70 ± 0.22	15 ± 4	11 535 ± 1 277
Dex 2 mg·kg^{-1}	0.45 ± 0.14^b	15 ± 4^a	6 890 ± 2 382^c
rhSOD 40 mg·kg^{-1}	0.31 ± 0.16^c	15 ± 7^a	8 542 ± 2 053^b

Effect of rhSOD ip on content of inflammatory cytokines in exudate from inflamed paw induced by carrageenan in rats Compared with blank group, the content of IL-1β and TNFα in control group greatly increased in rats paw edema exudate.

rhSOD 40 mg/kg was injected ip 2 times at interval of 2 h. IL-1β level dropped to normal concentration in exudate with inhibition rate near to 100 % in rhSOD treated groups. The inhibition rate of rhSOD on increase of IL-1β level was higher than that of Dex (Tab 5). In the parallel test, rhSOD 40 mg/kg ip lowered TNFα level in exudates with inhibition rate 53 %. The inhibition rate of rhSOD on increase of TNFα level was lower than that of Dex.

Effects of rhSOD ip on inflammatory cell infiltration in inflammatory tissue of carrageenan-induced paw of rats Fusiform shaped fibroblasts and

Tab 5. Effect of rhSOD on level of IL-1β and TNFα in rats paw edema exudate. $n = 7 - 10$. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.

	TNFα/ ng·L^{-1}	Inhibition rate/%	IL-1β/ ng·L^{-1}	Inhibition rate/%
Blank	243 ± 103^c	-	2.6 ± 1.3^c	-
Control	615 ± 238	-	68 ± 19	-
Dex 2 mg·kg^{-1}	151 ± 29^c	75	25 ± 25^c	63
rhSOD 40 mg·kg^{-1}	290 ± 91^c	53	2.0 ± 0.8^c	97

adipose cells were observed in subcutaneous tissue of normal rats paws without inflammatory cell infiltration (Fig 1). In control group, a number of neutrophils infiltrated into subcutaneous tissue of inflamed paw plantar. The extent of neutrophils infiltration in subcutaneous tissue of inflamed paws in Dex-treated group and rhSOD-treated group was obviously lessened as compared with control.

DISCUSSION

When cell membrane lipid was attacked by oxygen free radicals in inflammatory tissue during pathological changes of inflammation, peroxidation took place and lipid peroxidation material was produced, which degraded to stable product MDA[13]. MDA can cross-link to lipids and proteins in cell membrane to make it malfunction. In this experiment, the content of MDA greatly increased in carrageenan-induced inflammatory tissue of rat paw, suggesting that oxidation damage exists in the process. rhSOD inhibited lipid peroxidation of tissue more potently than that of dexamethasone. It is probably one of important anti-inflammation mechanisms of rhSOD.

The recent studies showed that nitric oxide (NO)

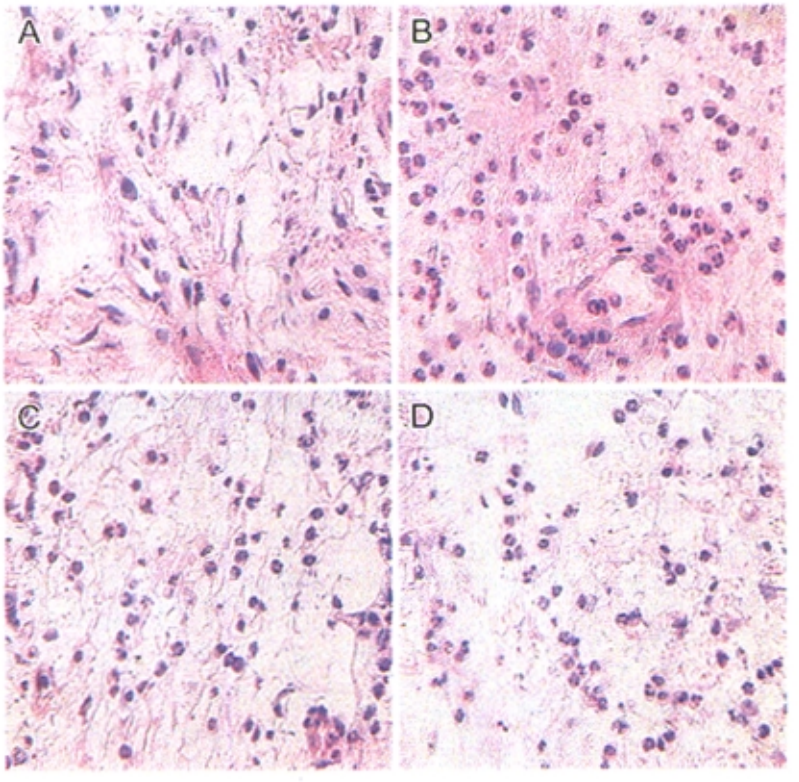

Fig 1. Effects of rhSOD and Dex ip on inflammatory cell infiltration in subcutaneous tissue of rat paws with *carrageenan-arthritis*. A: *Normal group*; B: *Control group*; C: *Dex group*; D: *SOD ip 40 mg/kg group*. ×400.

was a key inflammatory mediator[14]. Peroxynitrite was produced by interaction of NO and superoxide anion, which possessed higher activity of cell damage[15]. This experiment showed that the activity of NOS obviously was higher in exudate of carrageenan-stimulated rat paw than in blank group, and was lessened by rhSOD and dexamethasone. This would reduce formation of NO and prevente cells from damage of peroxynitrite.

The β-NAG activity changes in the inflammatory exudate reflected stability of lysosome membrane[16]. However, there was no significant change in exudate from both rhSOD and dexamethason treated groups, demonstrating that both drugs in the range of dosage in this experiment had no protective effect on lysosome membrane.

IL-1β and TNFα were proinflammatory cytokines[17]. IL-1β can cause fever by inducing generation of PGE_2, promote marrow to release neutrophils, and induce monocytes and neutrophils to migrate to inflammation sites where they release lysosomal enzyme. IL-1β can also stimulate eosinophils and neutrophils to release inflammation mediators. The recent studies reported that IL-1β induced the formation of NO and superoxide anion[18,19]. So exploitation of IL-1β formation inhibitor and its antagonists is a current direction in anti-inflammatory drug research[20]. TNFα can induce formation of IL-1β at low concentration, promote accumulation and activation of neutrophils at inflammatory sites, and strengthen the attack force of macrophages. In common, IL-1β and TNFα take part in the formation and development of rheumatoid arthritis. Many studies found IL-1β and TNFα induced formation of superoxide anion[7,8], but the effects of superoxide anion on formation of IL-1β and TNFα are still unknown. This experiment demonstrated that rhSOD obviously inhibited formation of IL-1β and TNFα, especially, reduced IL-1β concentration in inflammatory exudate to normal level, suggesting that superoxide anion participates in regulation of the formation of inflammatory cytokines. The inhibition of inflammatory cytokines formation is possibly related to the suppression of inflammatory cell immigration by rhSOD as demonstrated in the morphological study; however, the specific mechanism remains to be clarified.

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重组人超氧化物歧化酶在大鼠和小鼠的抗炎作用及机理研究

R96 A

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关键词 超氧化物歧化酶; 炎症; 白介素-1; 肿瘤坏死因子

目的: 研究重组人超氧化物歧化酶(rhSOD)的抗炎作用及其作用机理. **方法**: 采用角叉菜胶诱导的大鼠和小鼠关节炎、巴豆油诱发的小鼠耳肿模型, 研究药物对炎症肿胀度的影响. 大鼠炎症渗出液中 NOS 活性用 NADPH 黄递酶染色法、β-NAG 用对硝基酚比色法、MDA 用 TBA 荧光法测定, IL-1β 和 TNFα 含量用放射免疫法测定. **结果**: rhSOD 20 - 80 mg/kg ip 对大鼠关节炎, 40 - 80 mg/kg im 对小鼠耳肿、80 mg/kg ip 对小鼠足肿有显著的抑制作用. 同时大鼠炎症渗出液中 NOS 活性降低, IL-1β 和 TNFα 含量显著减少. 其抑制 IL-1β 生成的作用明显强于地塞米松 2 mg/kg ip. rhSOD 使炎症组织内中性粒细胞浸润减轻、MDA 生成减少, 但不影响炎症渗出液中 β-NAG 活性. **结论**: rhSOD 对大鼠和小鼠实验性炎症有明显的抗炎作用. 其抗炎作用机理和清除氧自由基、抗脂质过氧化有关, 也和抑制炎症细胞浸润、减少炎症性细胞因子如 IL-1β 和 TNFα 的生成有关.

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