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Synthesis of acylated SOD derivatives which bind to the biomembrane lipid surface and dismutate extracellular superoxide radicals

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Involvement of oxygen radicals in the pathogenesis of various inflammatory diseases has been the focus of recent attention. Since lipid peroxidation of cell membranes is postulated to be one of the major reasons for radical-induced tissue injury, inhibition of oxygen toxicity at or near plasma membranes is important. To metabolize extracellular superoxide radicals effectively at or near cell membranes, we synthesized amphipathic superoxide dismutase (SOD) derivatives (AC-SOD) by covalently linking hydrophobic fatty acids with different chain lengths, such as caprylic acid, capric acid, lauric acid and myristic acid, to the lysyl amino groups of the enzyme. When incubated with erythrocytes or polymorphonuclear leukocytes (PMNs), AC-SOD, but not SOD, bound to plasma membranes of these cells. When topically instilled to the eye, AC-SOD also bound to corneal epithelial cell surface. Upon activation by phorbolmyristyl acetate, extracellular cytochrome *c* was rapidly reduced by PMNs which were pretreated with SOD. In contrast, PMNs preincubated with AC-SOD failed to catalyze the reduction of cytochrome *c* under the same experimental conditions. These results suggested that AC-SOD bound to cell membranes and effectively dismutated superoxide radicals at or on the outer surface of plasma membranes.

Acylated superoxide dismutase derivative; Superoxide dismutase; Oxygen toxicity; Biomembrane surface

1. INTRODUCTION

Reactive oxygen species, such as superoxide radicals, play an important role in cellular defense mechanisms including bactericidal action of macrophages and PMNs [1,2]. However, these reactive oxygens also result in oxidative damage of organisms as observed in various inflammatory diseases. Since both eye and skin are surrounded by a high concentration of air oxygen, their incidence to be exposed to oxidative stress would be high particularly when these tissues were challenged with phrogogenic agents. Hence, the oxidative injury of these tissues would be prevented by anti-oxidants and some enzymes, including SOD [3,4]. However, clinical use of these enzymes in eye and skin inflammation has been seriously limited predominantly because topically administered enzymes are easily eliminated from the surface of these tissues.

To tie over this frustrating situation, we synthesized SOD derivatives (AC-SOD) by covalently linking fatty acids with various carbon chain lengths to the lysyl residues of SOD. Because of their amphipathic nature, AC-SOD bound to plasma membrane surface of various cells and effectively dismutated superoxide radicals on the outer surface of plasma membranes. The present work demonstrates the physicochemical properties of AC-SOD.

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Abbreviations: SOD, superoxide dismutase; PMN, polymorphonuclear leukocyte; TNBS, 2,4,6-trinitro-benzene sulfonic acid; KRP, Krebs-Ringer phosphate buffer; PMA, phorbolmyristyl acetate

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2. MATERIALS AND METHODS

2.1. *Materials*

Xanthine, nitroblue tetrazolium, xanthine oxidase and cytochrome *c* were purchased from Sigma Chemical Co. (St. Louis). Human erythrocyte-type SOD was purified by the method of Gartner et al. [5]. 2,4,6-Trinitro-benzene sulfonic acid and various fatty acids, such as caprylic acid (C_8), capric acid (C_{10}), lauric acid (C_{12}) and myristic acid (C_{14}), were obtained from Wako Pure Chemical Co. (Osaka). ^{125}I-labeled Bolton-Hunter reagent (2200 Ci/mmol) was from New England Nuclear Co. (Boston). SOD samples were labeled by ^{125}I-Bolton-Hunter reagent as described [6]. *N*-Hydroxysuccinimide esters of various fatty acids with different carbon chain lengths were synthesized as described [7].

2.2. *Synthesis of AC-SOD*

Incubation medium contained, in a final volume of 1 ml, 0.5 M sodium bicarbonate buffer, pH 8.0, and varying concentrations of the active fatty acid ester and SOD. The reaction was started by adding each of the active esters dissolved in 0.1 ml of dimethyl sulfoxide. During incubation at 37°C, aliquots of 20 μl were withdrawn and determined for the enzyme activity and the number of TNBS-titratable amino groups of the enzyme as described previously [8]. Extensive acylation of SOD by these esters did not appreciably inactivate the enzyme: more than 80% of its catalytic activity remained unaffected after linking 6 mol of each fatty acid per mol of SOD. Isoelectric focusing of SOD and AC-SOD was carried out as described previously [9].

2.3. *In vivo experiments*

Male Sprague-Dawley rats, 200 g, were fed laboratory chow and water ad libitum, and used for experiments after fasting for 16 h.

2.4. *Preparation of PMNs*

PMNs were obtained from rats 16 h after intraperitoneal injection of 20 ml of 2% casein as described previously [10]. The cells were washed 3 times by centrifugation and suspended in a Ca^{2+}-free Krebs-Ringer phosphate buffer solution (KRP).

2.5. *Binding of AC-SOD to cell surface membranes*

Radioactive SOD or AC-SOD samples (64 μg/ml) were added to 1 ml suspension of rat erythrocytes (1×10^9) or PMNs (1×10^8) in KRP. Superoxide formation by PMNs was monitored in a Shimazu UV-300 spectrophotometer by the cytochrome *c* reduction method [11] in the presence of phorbol-myristyl acetate. After incubation for 20 min at 37°C, cells were washed three times with 3 ml of ice-cold KRP to remove unbound enzymes. The radioactivity associated with the cells and tissues was determined in a Packard autogamma-scintillation spectrophotometer, model 5130.

3. RESULTS AND DISCUSSION

Human erythrocyte-type SOD possesses 22 lysyl ϵ-amino groups per mol of dimeric enzyme while the two α-amino groups of its N-terminal alanyl residues are acetylated [12]. Fig.1 shows the time course of acylation of ϵ-amino groups of the enzyme by the activated capric acid ester. Upon incubation at 37°C, the number of TNBS-reactive amino groups decreased rapidly during the initial 45 min; no significant reaction occurred thereafter. About 6 mol of amino groups were acylated per mol of SOD after 1 h of incubation. More than 80% of the catalytic activity of SOD was found to remain after acylation of 6 mol of amino groups. The number of TNBS-titratable amino groups decreased even further after 1 h of incubation by adding the active ester (not shown), suggesting that the initially added active ester would have reacted with amino groups of SOD and with water (hydrolysis) within 45 min. Similar results were obtained with active esters of caprylic acid, lauric acid and myristic acid. Isoelectric focusing revealed that p*I* values for SOD and AC-SOD type that linked 6 mol of capric acid per mol of the enzyme were 5.1 and 4.4, respectively.

SOD is highly stable against heat treatment [13].

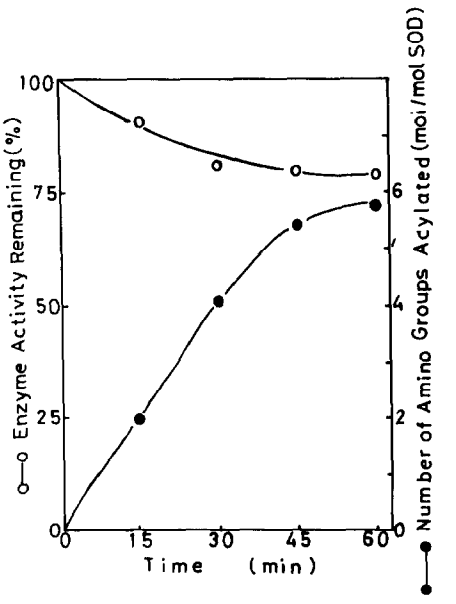

Fig.1. Acylation of SOD. Incubation mixtures contained, in a final volume of 1 ml, 3.2 mg of SOD, 0.1 M sodium bicarbonate buffer, pH 8.0, 1 mM of capric acid active ester and 5% dimethyl sulfoxide. The reaction was started by adding the active ester dissolved in dimethyl sulfoxide and the incubation was carried out at 37°C. At the indicated times, aliquots of 0.02 ml were removed and determined for the TNBS-titratable amino groups and the catalytic activity of the enzyme (open circles) as described in the text. Closed circles, number of acylated amino groups per mol of SOD.

To know the effect of acylation on the heat stability of the enzyme, catalytic activity of SOD and AC-SOD was determined before and after incubation of the enzyme samples at 70°C for 10 min. More than 90% of the catalytic activity of both samples remained unaffected after this treatment. Thus, acylation of SOD by these fatty acids had no appreciable effect on the heat stability of the enzyme. Since proteins having lysyl and/or arginyl residues could be hydrolyzed by trypsin-like proteases, acylation of the lysyl groups might increase the stability of the enzyme against such proteases. In contrast, it may also be possible that chemical modification affects the tertiary structure of an enzyme and increases its susceptibility to proteases. To test this possibility, the effect of various proteases was examined on the catalytic activity of SOD and AC-SOD. Kinetic analysis revealed no significant decrease in the catalytic activity of both enzyme samples during incubation with trypsin and chymotrypsin at 37°C for 12 h (fig.2). Papain decreased the catalytic activity of both samples by 20–25% under these conditions. Thus, AC-SOD and SOD were equally resistant to these proteases. Similar results were also obtained with other AC-SOD samples prepared by linking with 6 mol of other fatty acids (not shown). Since a hydrophobic fatty acid is soluble in organic solvent, AC-SOD may have an amphipathic nature and a high affinity for membrane lipid bilayers. To test this possibility, the binding of radioactive AC-SOD to erythrocyte membranes was compared with that of SOD. Upon incubation at 37°C for 20 min, a significant amount of AC-SOD bound to erythrocyte membranes while only very little binding of SOD occurred under the same experimental conditions (fig.3). Due to such a high affinity of AC-SOD for the membrane surface, AC-SOD also remained bound to PMN membranes even after extensive washing with KRP (fig.4). When challenged with phorbolmyristyl acetate, a marked reduction of cytochrome *c* was observed with PMNs which were preincubated with either saline or SOD (fig.5). In contrast, little reduction of cytochrome *c* occurred with PMNs preincubated with AC-SOD. When AC-SOD was inactivated by boiling for 30 min, no such inhibitory action was observed. Supravital staining with 0.5% eosin and phase-contrast microscopic observation revealed that intact AC-SOD did not show any cytotoxic ef-

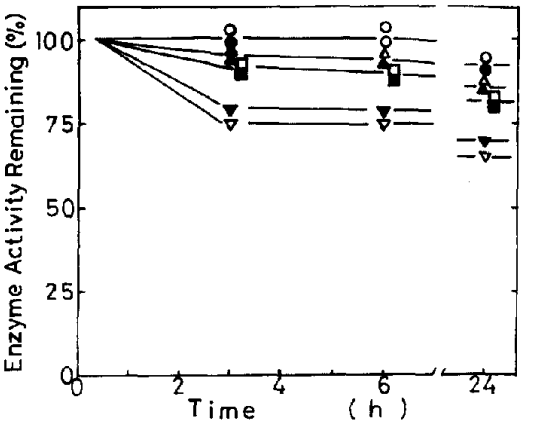

Fig.2. Effect of various proteases on the enzyme activity. Incubation mixtures contained, in a final volume of 1 ml, 50 mM phosphate buffer, pH 7.4, 0.15 M NaCl, 5 μg of SOD or AC-SOD linked with 6 mol of capric acid and 0.1 mg of either trypsin (□,■), chymotrypsin (△,▲) or papain (▽,▼). At the indicated times after incubation at 37°C, the enzyme activity was determined as described in the text. Incubation was also carried out in the absence of proteases (circles). Open symbols, SOD; closed symbols, AC-SOD.

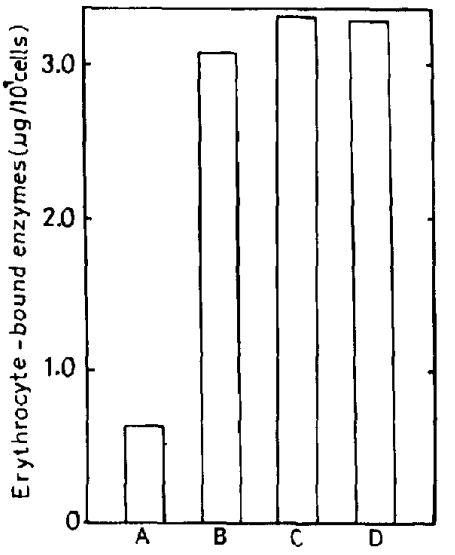

Fig.3. Binding of AC-SOD to surface membranes of erythrocytes. To the erythrocyte suspensions (1×10^9 cells/ml) were added 64 μg of radioactive SOD or AC-SOD samples which were prepared by linking with 6 mol of either caprylic acid (C_8), capric acid (C_{10}) or lauric acid (C_{12}). After incubation at 37°C for 20 min, erythrocytes were repeatedly washed with 5 ml of ice-cold saline and cell-associated radioactivity was determined. Values show the amounts of enzyme samples bound to erythrocytes. A, SOD; B, C_8-AC-SOD; C, C_{10}-AC-SOD; D, C_{12}-AC-SOD.

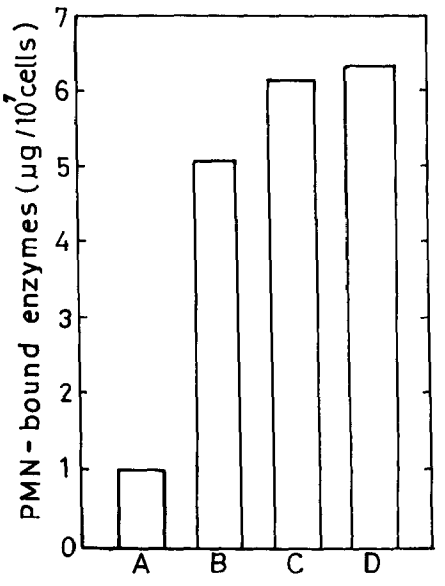

Fig.4. Binding of AC-SOD to surface membranes of PMNs. To the PMN suspension (1×10^8 cells/ml) were added 64 μg of ^{125}I-labeled SOD or AC-SOD containing 6 mol of either caprylic acid (C_8), capric acid (C_{10}) or lauric acid (C_{12}). After incubation at 37°C for 20 min, cells were washed with 5 ml of ice-cold KRP for three times. Then, cell-associated radioactivity was determined. Values show the amounts of enzyme samples remained bound to PMNs. A, SOD; B, C_8-AC-SOD; C, C_{10}-AC-SOD; D, C_{12}-AC-SOD.

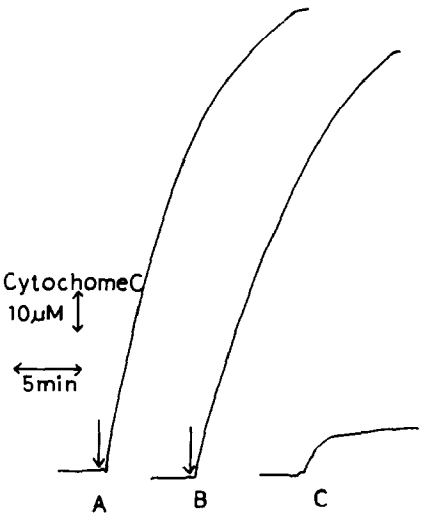

Fig.5. Effect of AC-SOD on cytochrome *c* reduction by activated PMNs. After incubation of PMNs (2×10^7 cells/ml) with 1300 units of either SOD or AC-SOD at 37°C for 20 min, cells were washed three times with 5 ml of ice-cold KRP. The washed PMNs were resuspended in KRP (2×10^7 cells/ml) at 37°C. In the presence of 100 nmol of cytochrome *c*, 1 ml of PMN suspension was added with 10 pmol of PMA dissolved in 10 μl of ethanol. Then, time-dependent reduction of cytochrome *c* was measured as described in the text. AC-SOD used for the experiments contained 5 mol of capric acid per mol of SOD. (A) Control PMNs; (B) PMNs preincubated with SOD; (C) PMNs preincubated with AC-SOD.

fect on PMNs under the present experimental conditions (not shown). Pretreatment of PMNs with free fatty acids at concentrations corresponding to those incorporated into AC-SOD samples had no appreciable effect on cytochrome *c* reduction. These results suggested that AC-SOD bound to PMN membranes and efficiently dismutated superoxide radicals on the outer surface of cell membrane without affecting cellular viability and NADPH-oxidase activity. Similar binding to the membranes and inhibition of the PMN-catalyzed cytochrome *c* reduction were also observed with AC-SOD samples prepared by linking with other fatty acids having longer carbon chain length than caprylic acid.

To test whether a similar binding of AC-SOD to the biomembrane surface would occur also in vivo, radioactive enzyme samples were topically instilled to the eye and determined for radioactivity remained bound to its epithelial cell surface. After 20 min

Table 1

Binding of AC-SOD to cornea and conjunctiva

Sites	Native SOD (ng/tissue)	AC-SOD (ng/tissue)		
		C_8	C_{10}	C_{12}
Cornea	96	1024	992	1088
	(0.6)	(6.4)	(6.2)	(6.8)
Conjunctiva	240	1000	848	1520
	(1.5)	(6.3)	(6.2)	(9.5)
Unbound[a]	320	928	904	712
	(2.0)	(5.8)	(5.7)	(4.5)

[a] Amounts of radioactivity in washing solution

Under light ether anesthesia, 20 μg of radioactive SOD or AC-SOD (100 000 cpm/eye) was topically instilled to rat eye. After 20 min of administration, cornea and conjunctiva were excised and washed with 5 ml of ice-cold saline solution for 1 min. Then, tissue-associated radioactivity was determined. AC-SOD used in the experiments were prepared by linking with 6 mol of either caprylic acid (C_8), capric acid (C_{10}) or lauric acid (C_{12}). Specific radioactivity of AC-SOD was 5000 cpm/μg of the enzyme. Values show the amounts of radioactive enzyme found to associate with each tissue. Numbers in parentheses show % of dose

of instillation, significantly larger amounts of AC-SOD were found to remain associated with cornea and conjunctiva than SOD (table 1).

Since acylation of these amino groups led to only 20% loss of enzyme activity, the lysyl residues of acylated SOD under the present condition appear not to be essential for the catalytic activity [14]. Similar phenomena were also observed with SOD samples from bovine erythrocytes and yeasts (not shown).

Since the topically instilled AC-SOD remained bound to surface membranes of cornea and conjunctiva for a fairly long time, they would efficiently dismutate superoxide radicals in situ and protect epithelial cells of cornea and conjunctiva from oxygen toxicity. In fact, preliminary experiments revealed that topically instilled AC-SOD markedly inhibited the occurrence of endotoxin-induced keratitis in both rabbits and guinea pigs [15]. These results suggest that AC-SOD may be useful for decreasing oxidative stress in the eye and other tissues of body surface during the course of inflammation.

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