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Improved anti-oxidant activity of superoxide dismutase by direct chemical modification

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Abstract

Chemically modified derivatives of superoxide dismutase (SOD), i.e., cationized (Cat-SOD) and mannosylated SOD (Man-SOD), were designed to improve an ability of SOD to suppress reactive oxygen species (ROS)-mediated injury in the alveolar epithelium. To evaluate their effectiveness, an in vitro model of paraquat poisoning was developed with primary cultured rabbit alveolar type II cells. Despite a 5.6-fold higher cellular association than native SOD, Man-SOD did not protect cell injury due to paraquat following evaluation by MTT assay. In contrast, Cat-SOD exhibited a 140-fold higher cellular association than native SOD and greatly suppressed paraquat-induced cell injury, as well as lipid peroxidation. Incubation with 300 U/ml Cat-SOD for 2 h increased intracellular SOD activity 5.3-fold. The increase in intracellular SOD activity was significantly inhibited in the presence of cytochalasin B, an endocytosis inhibitor. Internalization of Cat-SOD was also confirmed by confocal laser scanning fluorescein microscopy. In addition, the protective effect of Cat-SOD against paraquat-induced cell injury was completely abolished by the presence of cytochalasin B. In conclusion, this study demonstrated that cationization of SOD greatly enhances its intracellular delivery and, as a consequence, produces a significant protective effect against ROS-mediated injury of the alveolar epithelium.

Keywords: Superoxide dismutase; Cationization; Mannosylation; Alveolar epithelium; Paraquat

1. Introduction

The lung epithelium is directly exposed to a high concentration of oxygen in the inhaled air, which often also contains many oxidant air pollutants such as ozone, nitrogen oxide, and fuel emissions or tobacco smoke. Exposure to hyperoxia increases the intracellular production of reactive oxygen species (ROS) and damages a variety of biomolecules such as DNA [1,2], lipids [2], and proteins [2], resulting in impairment of cellular functions. As well as intracellular metabolic processes, alveolar macrophages and other inflammatory cells are possible sources of ROS that damage the lung epithelium [3]. Considerable evidence has emerged to support a central role for ROS in many lung diseases.

Superoxide dismutase (SOD), that catalyzes the dismutation of superoxide anion to hydrogen peroxide, is a potential antioxidant therapeutic agent [4]. Although much attention has been paid to inhalation of SOD as a strategy for the treatment of ROS-mediated lung injury, animal and clinical studies demonstrated only a modest protective effect against oxidative stress. To improve the delivery of the enzyme to the sites of action, encapsulation of SOD by liposomes has been often investigated. Liposome encapsulated SOD increases the antioxidant activity in alveolar type II cells [5–7] or lung cancer cells [8] and protects these cells against oxidative stress [5,8]. In addition, intratracheal instillation of liposomal SOD protects against pulmonary oxygen toxicity [9,10].

Direct modification of SOD is an alternative promising approach to improving the delivery of the protein. We have been developing various kinds of Cu/Zn-SOD and catalase derivatives via chemical modification for the treatment of liver or kidney ischemia/reperfusion injuries in vivo [11–13]; these derivatives have included glycosylated, cationic, anionic, and PEGylated proteins. In liver ischemia/reperfusion injury in rats, targeted delivery of SOD and catalase to

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liver non-parenchymal cells appears promising by the use of mannosylated and/or succinylated derivatives: in particular, combination of Man-SOD and succinylated catalase dramatically suppresses an increased in plasma GOT and GPT following ischemia/reperfusion injury, due to independent uptake of both proteins by liver non-parenchymal cells via different receptors, i.e., mannose receptors and scavenger receptors, respectively [12,13]. As for kidney ischemia/reperfusion injury in rats, intravenous injection of Cat-SOD has been found most effective in restoring the normal glomerular filtration rate [11].

The present study was initiated to examine two SOD derivatives, i.e., Man-SOD and Cat-SOD, and their ability to suppress ROS-mediated injury of the alveolar epithelium. We expected that Man-SOD and Cat-SOD might be taken up by type II cells via a surfactant protein-mediated mechanism and an adsorptive endocytosis mechanism, respectively. To focus on the protective effect on cell injury due to ROS produced intracellularly, we used an in vitro model of paraquat poisoning with primary cultured alveolar type II cells. Paraquat produces oxidative stress by redox cycling with a variety of cellular diaphorases, e.g., NADPH-cytochrome P450 reductase, and oxygen to produce O_2^- [14]. In this study, we report that Cat-SOD, following internalization by adsorptive endocytosis, greatly suppresses paraquat-induced injury of cultured alveolar type II cells.

2. Materials and methods

2.1. Materials

Recombinant human SOD (111-Ser) was kindly supplied by Asahi Chemical Industry (Shizuoka, Japan). ^{111}Indium chloride was kindly supplied by Nihon Medi Physics (Takarazuka, Japan). Hexamethylenediamine was obtained from Wako Pure Chemical (Osaka, Japan). Paraquat, cytochalasin B, and fluorescein isothiocyanate (FITC) were purchased from Sigma (St. Louis, MO). Porcine pancreatic elastase and pure Griffonia simplicifolia lectin (GS-I) were obtained from Worthington Biochemical (Freehold, NJ) and EY Laboratories (San Mateo, CO), respectively. Soybean trypsin inhibitor, human recombinant epidermal growth factor (EGF), bovine serum albumin (BSA), hydrocortisone, Dulbecco's modified minimum Eagle's medium nutrient mixture F-12 Ham (DMEM/F12), and Eagle's minimum essential medium Joklik modified for suspension culture (SMEM) were obtained from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Equitech-Bio (Kerrville, TX). ITS+Premix, type I rat tail collagen, and recombinant human fibronectin were obtained from Becton Dickinson Biosciences (Bedford, MA). Other cell culture reagents were obtained from Invitrogen (Grand Island, NY).

2.2. Synthesis and characterization of SOD derivatives

Cat-SOD was obtained by coupling SOD with hexamethylenediamine using carbodiimide as a catalyst [15]. Five hundred milligrams SOD was added to 5 ml 2 M hexamethylenediamine with the pH adjusted to 6.5 using HCl. Thirty minutes and one hour later, 280 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride was added to each. The reaction mixture was agitated overnight with the pH kept at 6.5, followed by extensive dialysis against distilled water. Cat-SOD was purified by chromatofocusing using the Pharmacia polybuffer exchanger 94 resin and the polybuffer 96 elution buffer (Pharmacia, Uppsala, Sweden). The fraction eluted in the void volume (pI of >9.0) was collected. Then the eluent was concentrated by ultrafiltration, with replacement of the solvent with distilled water, and then lyophilized.

Man-SOD was obtained according to the method reported previously [16]. Cyanomethyl 1-thiomannoside (220 mg) prepared by the method of Lee et al. [17], was added to 5.5 ml 0.01 M sodium methoxide in methanol and agitated at room temperature. Twenty-four hours later, the solvent was evaporated in vacuo, and SOD (300 mg) in 15 ml 50 mM borate buffer at pH 10.0 was added to the residual fluid containing 2-imino-2-methoxyethyl-1-thiomannoside. The reaction mixture was agitated at room temperature for 5 h. Man-SOD was purified by extensive dialysis of the reaction mixture against distilled water and the purity of the product was confirmed by affinity chromatography using Con A-Sepharose.

The activities of SOD derivatives were determined by the nitroblue tetrazolium reduction method using an SOD test kit (Wako Pure Chemical, Osaka, Japan). The enzyme activity of native SOD, Man-SOD, and Cat-SOD was 3000, 2730, and 2370 U/mg, respectively. The number of amino groups was determined by trinitrobenzene sulfonic acid using glycine as a standard [18]. The method of Lowry was used to measure the concentration of SOD [19]. The number of modified amino groups was estimated to be 12.0 and 20.2 per SOD molecule for Cat-SOD and Man-SOD, respectively. For cellular uptake studies, native SOD and SOD derivatives were radiolabeled with ^{111}In using the bifunctional chelating agent, diethylenetriaminepentaaceticacid anhydride, according to the method of Hnatowich et al. [20].

2.3. Primary culture of alveolar type II cells

For isolation of alveolar type II cells, three in-house balanced salt solutions were prepared: one balanced salt solution (BSSA') was composed of 137 mM NaCl, 5.0 mM KCl, 0.7 mM Na_2HPO_4, 10 mM HEPES, 5.5 mM glucose at the pH adjusted to 7.4, another balanced salt solution (BSSA) was BSSA' supplemented with 3 mM EDTA, the other balanced salt solution (BSSB) was BSSA' supplemented with 1.8 mM $CaCl_2$ and 1.2 mM $MgSO_4$.

Alveolar type II cells were isolated from the rabbit lung using the method of Shen et al. [21]. Animals were first injected with heparin (1000 U/kg) and then euthanized by rapid injection of sodium pentobarbital (1.5 ml/kg), via a marginal ear vein. The abdominal cavity was opened. While the lung was ventilated manually through a tracheal cannula with a 60 ml syringe, it was perfused with BSSA via the pulmonary vein. The lung was washed several times with