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Targeted delivery of catalase and superoxide dismutase to macrophages using folate

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Abstract

Reactive oxygen species (ROS) secreted by activated macrophages play a central role in causing rheumatoid arthritis, and therapeutics that can inhibit the production of ROS by macrophages have great clinical potential. Superoxide dismutase (SOD) and catalase (CAT) are two enzymes that scavenge ROS and have great potential for treating rheumatoid arthritis. However, clinical trials with these enzymes have been ineffective, due to drug delivery problems, and effective SOD and CAT delivery vehicles are greatly needed. In this communication, we demonstrate that CAT and SOD can be effectively targeted to activated macrophages, via the folate receptor. Folate was conjugated to CAT and SOD using NHS/EDC chemistry with high efficiency. Cell culture experiments demonstrated that folate conjugation increased their ability to scavenge ROS, produced by the macrophages, and also enhanced their uptake into activated macrophages. We anticipate numerous applications of folate-conjugated CAT and SOD in treating inflammatory diseases, based on their efficacy and straightforward synthesis.

Keywords: Folate; Catalase; Superoxide dismutase; Conjugation; Macrophages; Inflammatory diseases

Rheumatoid arthritis is a painful inflammatory disorder that affects approximately 2.5 million people in the US. Reactive oxygen species (ROS) produced by macrophages play a central role in causing rheumatoid arthritis, and therapeutics that can inhibit the production of ROS from macrophages have great clinical potential [1,2]. Superoxide dismutase (SOD) and catalase (CAT) are two antioxidant enzymes that scavenge superoxide and hydrogen peroxide, respectively. They have the potential to suppress ROS production by macrophages [3,4] and are being considered as treatments for arthritis [5–7]. However, clinical trials with these enzymes have provided little therapeutic benefits, due to drug delivery problems, and delivery vehicles are greatly needed that can enhance their efficacy. Liposome-based SOD and CAT delivery vehicles have shown promise for enhancing the delivery of SOD and CAT in animal models [8,9]; however their poor shelf-life has limited their progress into clinical trials [10]. Polymeric microparticles based on polyesters are also being investigated for the delivery of SOD and CAT. Although polyester-based microparticles have an excellent shelf life and well-characterized degradation products [11,12], their application for the treatment of arthritis could be problematic because their acidic degradation products can themselves cause inflammation [13]. Therefore alternative methods for targeting CAT and SOD to macrophages are greatly needed.

Recently, the Low laboratory and others have demonstrated that the folate receptor is overexpressed on activated macrophages in arthritic tissue, [14,15] and that folate-conjugated proteins can be delivered to the cytoplasm of target cells via folate receptor-mediated endocytosis [16,17]. Based on this evidence, conjugation of folate to SOD and CAT could potentially target these enzymes to activated macrophages and enhance their efficacy in treating inflammatory diseases. In this communication, we demonstrate that folate can be readily conjugated to CAT and

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SOD with high efficiency, and that conjugation of folate does not lower the activity of these enzymes. We also demonstrate that conjugation of folate enhances the ability of CAT and SOD to scavenge ROS produced by activated macrophages. Based on these observations, we anticipate that folate-conjugated CAT and SOD will have numerous applications for the treatment of rheumatoid arthritis and other inflammatory diseases.

Materials and methods

Materials. Superoxide dismutase (EC 1.15.1.1), folic acid, cytochrome *c*, and lipopolysaccharide (LPS) were purchased from Sigma–Aldrich (St. Louis, MO). Bovine liver catalase (EC 1.11.16.1, MW 247,000) was obtained from EMD chemicals Inc. (Gibbstown, NJ). The RAW 264.7 macrophage cell line (ATCC No: TIB-71) was purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Cell culture reagents and the Amplex Red assay kit were purchased from Invitrogen Life Technologies (Carlsbad, CA). PD-10 desalting columns were purchased from GE Healthcare Life Science (Piscataway, NJ). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO) and used as received unless otherwise specified.

Folate conjugation with CAT and SOD. Folic acid (10 mM) was completely dissolved in a pH 9.0 buffer solution (100 mM, carbonate buffer) and then an equimolar quantity of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (10 mM) was added. The mixture was stirred for 40 min at room temperature. The activated folate (1 mL) was added to 10 μM CAT or 5 μM SOD solutions (1 mL, pH 9.0) and incubated for 18 h at room temperature. The folate-conjugated proteins were separated from unreacted folate using a PD-10 desalting column equilibrated in PBS (pH 7.4). The degree of folate conjugation was determined spectrometrically by measuring the difference in absorbance between the conjugated proteins and free proteins at 363 nm (folate, $\varepsilon = 6197\ M^{-1}\ cm^{-1}$ in PBS (pH 7.4)).

Determination of folate-conjugated enzyme activity in vitro. The activity of folate-conjugated CAT was determined by measuring the decomposition rate of H_2O_2. A solution containing either folate-conjugated CAT or free CAT (0.1 mL, either 16 or 3 μg/mL) was mixed with 2.9 mL of 10 μM H_2O_2 in a pH 7.4 buffer solution (50 mM, phosphate buffer) and the absorbance of H_2O_2 was monitored at 240 nm. The activity of folate-conjugated SOD was determined by the SOD assay kit (SOD determination kit 19160, Fluka, St. Louis, MO) following the instructions in the kit.

Delivery of folate-conjugated CAT to activated macrophages. RAW-264.7 macrophages (1×10^6 cells/well, 12-well plate) were grown in folate-deficient RPMI-1640 medium supplemented with 10% FBS. Folate receptor expression, in RAW-264.7 macrophages, was induced by 5 μg/mL LPS for 4 h [18]. The activated cells were incubated with either 166.7 μg/mL CAT or an equivalent CAT concentration of folate-conjugated CAT for 4 h. The cells were washed three times and then stimulated with 5 μg/mL LPS for 2 h to generate ROS. The extracellular hydrogen peroxide from these macrophages was then measured using Amplex Red. Briefly, 100 μL of a Krebs–Ringer bicarbonate buffer (pH 7.4) solution, containing 50 μM 10-acetyl-3,7-dihydroxyphenoxazine and 0.1 U/mL horseradish peroxidase (HRP), pre-warmed at 37 °C, was added to the cells (grown in a 96-well plate). After a 2-h incubation period at 37 °C, the fluorescence of the cells was measured using a microplate reader, with excitation at 530 nm and emission at 590 nm.

Delivery of folate-conjugated SOD to activated macrophages. RAW-264.7 macrophages (1×10^6 cells/well, 12-well plate) were grown in folate-deficient RPMI-1640 medium supplemented with 10% FBS. Folate receptor expression in RAW-264.7 macrophages was induced by 5 μg/mL LPS for 4 h and the activated cells were incubated with either 2.78 μg/mL SOD or an equivalent SOD concentration of folate-conjugated SOD for 4 h. The cells were washed three times and then stimulated with 5 μg/mL LPS for 2 h to generate ROS. The extracellular superoxide production from these macrophages was then measured using a cytochrome *c*-based assay. Briefly, 100-μL Krebs–Ringer bicarbonate buffer solution, containing 20 nM cytochrome *c*, pre-warmed at 37 °C, was added to the cells (grown in a 96-well plate). After a 30-min incubation period, at 37 °C, the absorbance of the cells was measured at 550 nm using a microplate reader.

Cellular uptake of folate-conjugated CAT. The uptake of folate-conjugated CAT was determined by flow cytometry (BD FACS Vantage™, BD Biosciences) (San Jose, CA) using fluorescein isothiocyanate (FITC)-labeled FOL–CAT (FITC–CAT–FOL). FITC–CAT–FOL was synthesized in two steps, first, folate was conjugated to CAT (FOL–CAT), as described above, and then labeled with FITC. Briefly, FOL–CAT was dissolved in a pH 9.0 buffer solution (100 mM, carbonate buffer) and 50 μL of a 10-mg/mL FITC solution, in DMSO, was added to it and mixed for 2 h at room temperature. The FITC–CAT–FOL was separated from unreacted FITC using a PD-10 desalting column, equilibrated in PBS (pH 7.4). RAW-264.7 macrophages (1×10^6 cells/well, 12-well plate) were grown in a folate-deficient RPMI-1640 medium supplemented with 10% FBS. Folate receptor expression in the macrophages was induced by 5 μg/mL LPS, in folate-deficient medium, for 4 h. The activated cells were incubated with either 166.7 μg/mL FITC-labeled CAT or the same CAT concentration of FITC–CAT–FOL for 4 h. The cells were washed three times and analyzed by flow cytometry.

Results and discussion

Folate can be efficiently conjugated to CAT and SOD

Folate was efficiently conjugated to CAT and SOD using NHS/EDC chemistry, reacting the lysine residues of CAT and SOD with the carboxyl groups of folic acid (Table 1 and Scheme 1). For example, each CAT, composed of four homo-subunits, was conjugated with 84 folic acids, which represents 75% of its total lysines. Similarly SOD, composed of two subunits, was conjugated with 19 folic acids, which represents 95% of its total lysines. Although many lysine residues in CAT and SOD were reacted with folate, Fig. 1 demonstrates that folate-conjugated CAT and SOD still retained their enzymatic activities. This can be explained based on the catalytic mechanism of the two enzymes. CAT has no lysine residues directly involved in its catalytic activity, and SOD only uses lysines for the electrostatic attraction of substrates. These activity results are in accordance with other reports, for example, extensive modification of the lysine residues of CAT and SOD with either PEG, succinic anhydride, galactose or mannose also did not affect the activities of CAT and SOD significantly [19–21].

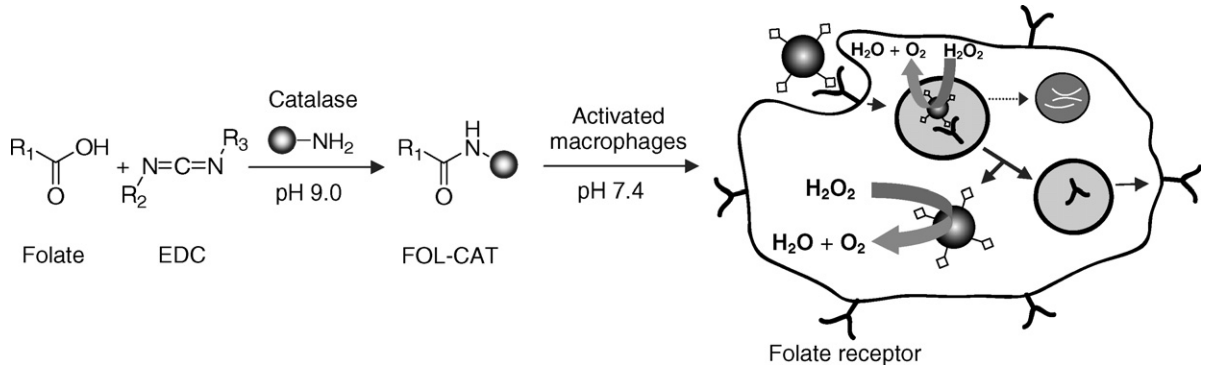
Folate conjugation enhances the delivery of SOD and CAT to macrophages

In this section, we investigated the ability of folate-conjugated CAT and SOD to scavenge hydrogen peroxide and

Table 1
Folate can be effectively conjugated to CAT and SOD

	Catalase	Superoxide dismutase
Total number of lysines	112	20
Folates conjugated with lysines	84	19

The total number of lysine residues was determined based on four subunits of CAT and two subunits of SOD.



Scheme 1. Folate-conjugated antioxidant enzymes are endocytosed by activated macrophages and scavenge ROS produced by macrophages. Folate is conjugated to CAT using NHS/EDC chemistry. Folate-conjugated enzymes are endocytosed by activated macrophages via receptor mediated-endocytosis. The enzymes scavenge ROS in the endosome or cytoplasm of the macrophages, leading to reduced inflammation.

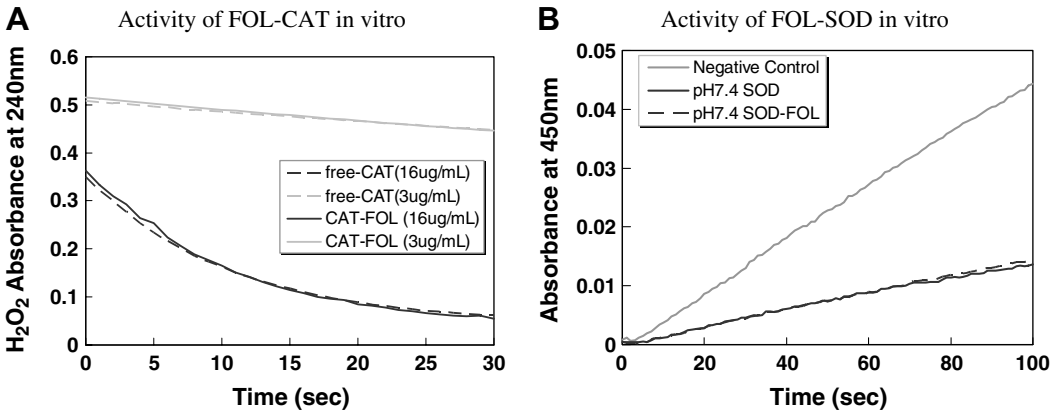


Fig. 1. Folate-conjugated CAT and SOD retain their enzymatic activity. (A) The effect of folate conjugation on CAT activity. CAT activity was determined by measuring the reduction rate of H_2O_2-free CAT (broken lines) and folate conjugated enzyme (solid lines), 3 μg/mL (gray lines) and 16 μg/mL (black lines). (B) The effect of the folate conjugation on SOD activity. SOD activity was determined by measuring the inhibition of WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt) oxidation—negative controls (gray line), free SOD (black broken line), and folate-conjugated SOD (black solid line).

superoxide produced by macrophages. RAW-264.7 macrophages were activated with LPS to overexpress the folate receptor, and incubated with either free enzymes or the same concentration of folate-conjugated enzymes, and then stimulated with LPS again to induce ROS production. As shown in Fig. 2, folate conjugation significantly enhanced the ability of CAT and SOD to scavenge ROS produced by macrophages. For example, as shown in Fig. 2(A), folate-conjugated CAT scavenged 78% of hydrogen peroxide produced from LPS-treated macrophages, whereas free CAT scavenged only 20% of hydrogen peroxide produced by LPS-treated macrophages. Similarly, Fig. 2(B) demonstrates that folate-conjugated SOD scavenged 51% of superoxide produced by LPS-treated macrophages; in contrast free SOD had no effect on superoxide levels. In order to explain the enhanced effectiveness of folate-conjugated proteins, we investigated the uptake of fluorescein-labeled FOL–CAT by activated macrophages. Macrophages were activated with LPS to overexpress the folate receptor, and then incubated with either fluorescein-labeled FOL–CAT or fluorescein-labeled CAT, the uptake of these enzymes was then measured by flow cytometry. As shown in Fig. 3, the uptake of folate-conjugated CAT was 4-fold greater than free CAT, suggesting that enhanced endocytosis of FOL–CAT is partially responsible for its greater efficacy.

Summary

In this communication, we investigated the effects of folate conjugation on CAT and SOD. Folate was conjugated to CAT and SOD using NHS/EDC chemistry with high efficiency. Folate-conjugated enzymes had the same activity as free enzymes *in vitro*. In cell culture experiments, folate conjugation dramatically enhanced the ability of CAT and SOD to scavenge reactive oxygen species produced by activated macrophages. Flow cytometry experiments demonstrated that folate conjugation increased the uptake of CAT by activated macrophages. Based on these results, we anticipate numerous applications of folate-

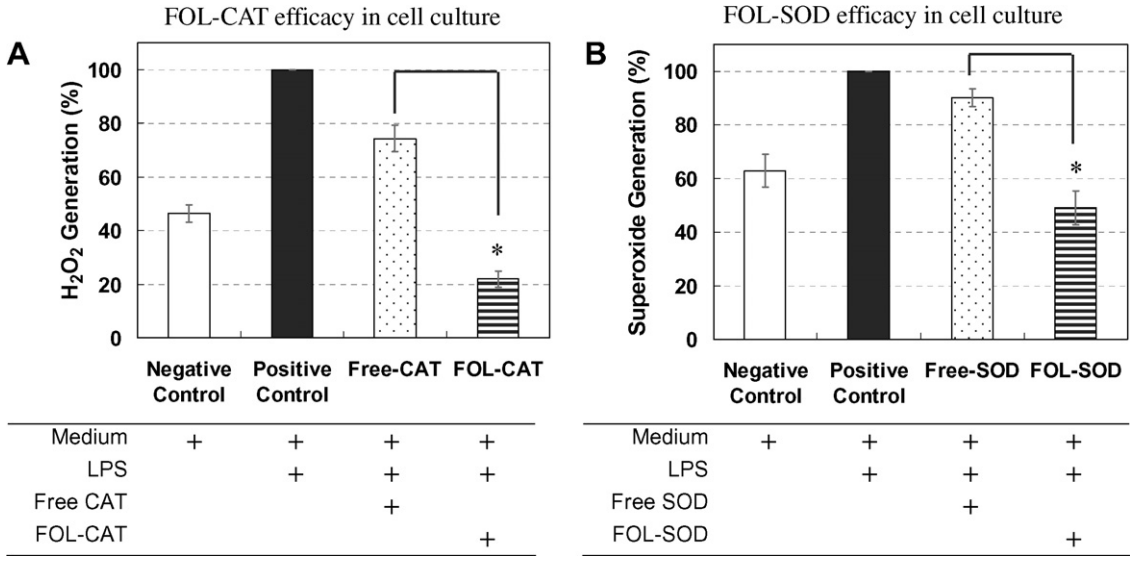


Fig. 2. Folate-conjugated CAT and SOD efficiently scavenge ROS produced by activated macrophages. Macrophages were activated with LPS, to overexpress the folate receptor, and incubated with either free enzymes, or the same concentration of folate-conjugated enzymes, ROS production was then induced by LPS. (A) Extracellular hydrogen peroxide, from activated macrophages, was measured using the Amplex Red assay, (B) Extracellular superoxide, from activated macrophages, was measured using a cytochrome *c*-based assay. Negative controls (white bars), positive control (black bars), free enzymes (dotted bars), and folate-conjugated enzymes (horizontal stripes). For statistical analysis, three independent wells were measured for each sample. Significance of results was determined via the paired *t*-test between folate-conjugated enzyme and free enzyme with $p < 0.05$.

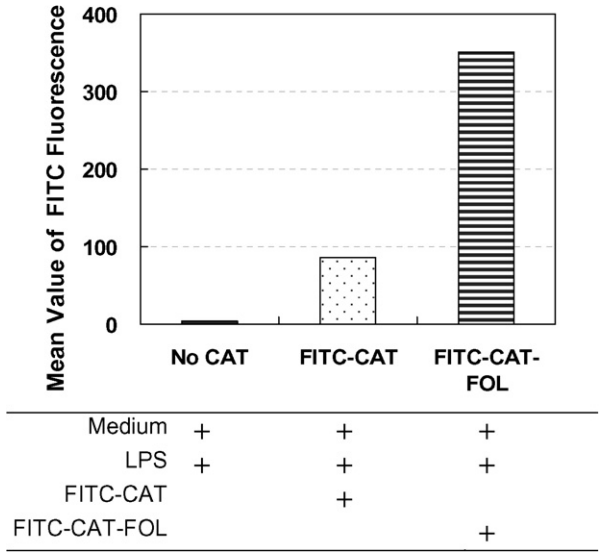


Fig. 3. Folate-conjugated CAT is taken up by activated macrophages via folate receptor-mediated endocytosis. LPS-activated cells were incubated with either FITC-CAT or FITC-CAT–FOL, and their uptake was determined by flow cytometry. No CAT (black bars), free CAT (dotted bars), and folate-conjugated CAT (horizontal stripes).

conjugated CAT and SOD for the treatment of rheumatoid arthritis and other inflammatory diseases.

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