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Pluronic-Modified Superoxide Dismutase 1 (SOD1) Attenuates Angiotensin II-Induced Increase in Intracellular Superoxide in Neurons

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Abstract

Overexpressing superoxide dismutase 1 (SOD1; also called Cu/ZnSOD), an intracellular superoxide ($O_2^{\bullet-}$) scavenging enzyme, in central neurons inhibits angiotensin II (AngII) intra-neuronal signaling and normalizes cardiovascular dysfunction in diseases associated with enhanced AngII signaling in the brain including hypertension and heart failure. However, the blood-brain barrier (BBB) and neuronal cell membranes impose tremendous impediment for the delivery of SOD1 to central neurons, which hinders the potential therapeutic impact of SOD1 treatment on these diseases. To address this, we developed conjugates of SOD1 with poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) block copolymer (Pluronic) (SOD1-P85 and SOD1-L81), which retained significant SOD1 enzymatic activity. The modified SOD1 effectively scavenged xanthine oxidase/hypoxanthine-derived $O_2^{\bullet-}$, as determined in HPLC and the measurement of 2-hydroxyethidium. Using catecholaminergic (CATH.a) neurons, we observed an increase in neuronal uptake of SOD1-Pluronic after 1, 6, or 24 hrs, compared to neurons treated with pure SOD1 or PEG-SOD1. Importantly, without inducing neuronal toxicity, SOD1-Pluronic conjugates significantly inhibited AngII-induced increases in intra-neuronal $O_2^{\bullet-}$ -levels. These data indicate that SOD1-Pluronic conjugates penetrate neuronal cell membranes, which results in elevated intracellular levels of functional SOD1. Pluronic conjugation may be a new delivery system for SOD1 into central neurons and therapeutically beneficial for AngII-related cardiovascular diseases.

Keywords

Superoxide dismutase 1; Pluronic; Angiotensin II; superoxide; cellular delivery; protein-polymer conjugation; CATH.a neurons; 2-hydroxyethidium

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INTRODUCTION

Elevated levels of reactive oxygen species (ROS) have been observed in many human diseases including both acute (e.g. acute lung injury, hyperoxia, ischemia/reperfusion injury, inflammation) and chronic (e.g. diabetes, hypertension, heart failure) conditions [1–3]. The imbalance of cellular redox environment, due to elevated levels of ROS including superoxide ($O_2^{\bullet-}$), results in oxidative damage to DNA, proteins, and lipids, and ultimately leads to cellular and tissue injury. Therefore, exogenous supplementation of antioxidant enzymes such as superoxide dismutase (SOD) has become a rational therapeutic approach in minimizing ROS-associated damage. For example, recent studies using overexpression of SOD1 (also known as Cu/ZnSOD), which resides primarily in the cytoplasm and scavenges $O_2^{\bullet-}$, clearly show therapeutic efficacy of such treatment in models of brain-related cardiovascular diseases, including stroke, hypertension, and heart failure [4–7].

In cardiovascular disorders, such as hypertension and heart failure, angiotensin II (AngII)-induced increase in $O_2^{\bullet-}$ has been observed in peripheral tissues and in the central nervous system (CNS). Recent investigations indicate that AngII-induced neuronal activation in the CNS involves $O_2^{\bullet-}$ -dependent signaling. In particular, Zimmerman's studies emphasized that an increase in intracellular $O_2^{\bullet-}$, but not extracellular $O_2^{\bullet-}$, in central neurons mediates AngII-induced hypertension [6,8]. Furthermore, in chronic heart failure, increased $O_2^{\bullet-}$ levels in central neurons is associated with an increase in the deleterious sympathoexcitation [9–12]. Together, these studies and others [7] suggest that removal of $O_2^{\bullet-}$ in the CNS, may provide a novel treatment for AngII-associated neuro-cardiovascular diseases.

As such, our particular interest lies in the development of a SOD1-based therapy that will target CNS neurons and attenuate the AngII-induced increase in $O_2^{\bullet-}$ levels. SOD1 delivery has been studied in many aspects to improve its bioavailability; however, there are few studies addressing the targeting of SOD1 to CNS neurons. Perhaps the most widely studied modification of SOD1 is that with polyethyleneglycol (PEG) [13–16]. PEG modification of SOD1, as well as other proteins, has been utilized to improve protein bioavailability and circulation time *in vivo* [16–19]. In addition, modification of SOD1 by PEG appears to enhance its delivery to endothelial cells, as reported in a stretched-injury cell model [20,21]; however, this has not been clearly demonstrated in neurons. Furthermore, PEG cannot permeate cell membranes and there are studies suggesting that it also drastically decreases permeability of SOD1 protein across brain microvessels [22]. Tat-SOD1, another SOD1 modified compound, clearly showed neuronal uptake [23]. When administered intraperitoneally, Tat-SOD1 inhibits neuronal cell death in the hippocampus in response to transient forebrain ischemia; however, the major concern of using Tat-SOD1 clinically is the antigenic properties of tetanus toxin fragment C moiety [24]. More recently, Labhasetwar and colleagues using SOD1-encapsulated poly(d,l-lactide co-glycolide) (PLGA) nanoparticles showed significant reduction in neuronal apoptosis in an ischemia-reperfusion model [28,29]. However, bioavailability of nanoparticle-delivered SOD1 can be quite low due to inefficient release of the enzyme from the nanoparticle as well as enzyme inactivation in PLGA matrix [25]. Altogether, these previous reports strongly support the notion that modified SOD1 may provide a therapeutic benefit to CNS-related diseases. However, it is unclear, from most of these earlier studies, if the modified SOD1 is being delivered into neurons or if its effects are extracellular.

In the present study, we modified SOD1 with Pluronic, an amphiphilic to hydrophobic triblock polymer of poly(ethylene oxide) (PEO, also named PEG) and poly(propylene oxide) (PPO, also named PPG) (Table 1). Such modification was previously shown by us to considerably increase blood-to-brain delivery of horseradish peroxidase, a normally BBB impermeable protein [26]. Herein, we tested the hypothesis that Pluronic modification

delivers active SOD1 into neurons and, in doing so, inhibits the AngII-induced increase in intra-neuronal $O_2^{\bullet-}$ levels. We report the synthesis and characterization of SOD1-Pluronic conjugates and demonstrate that the modified SOD1 penetrates neuronal cell membranes in an active form and attenuates AngII-induced increase in $O_2^{\bullet-}$ in cultured neurons.

EXPERIMENTAL PROCEDURES

Materials and Devices

SOD1 from bovine erythrocyte (s7571, 3870U/mg), superoxide dismutase-polyethylene glycol (PEG-SOD1, s9549, 1,350U/mg), human angiotensin II (A9525), 4-methoxytrityl chloride (MTr-Cl), 1,1'-carbonyldiimidazole (CDI), 1,2-ethylenediamine (EDA), nitroblue tetrazolium (NBT), diethylenetriaminepentaacetic acid (DTPA), 2,4,6-trinitrobenzenesulfonic acid (TNBS), pyrogallol, riboflavin, tetramethylenediamine (TEMED), 2-methoxy ethanol, trifluoroacetic acid (TFA), sinapinic acid, triethylamine, anhydrous acetonitrile, anhydrous pyridine, methanol, dichloromethane, toluene, acetone, ethanol (EtOH), and dimethylformamide (DMF) were purchased from Sigma-Aldrich Co. (St-Louis, MO). Pluronic[®] block copolymers, L81 (lot no. WSOO-25087), P85 (lot no. WPOP-587A) were kindly provided by BASF Corp. (Paris-pany, NJ). Their characteristics are summarized in Table 1. Dithiobis(succinimidyl propionate) (DSP), disuccinimidyl propionate (DSS) and 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) were from Pierce Biotech Co. (Rockford, IL). Alexa Fluor[®] 680 carboxylic acid, succinimidyl ester was from Invitrogen (Carlsbad, CA). Sephadex LH-20 gel and Illustra NAP-25 Columns were from Amersham Biosciences (Pittsburgh, PA). Amicon ultra-15 centrifugal filter, MWCO 10K, membrane NMWL was from Millipore Co. (Billerica, MA). Spectro/Por membrane (MWCO 2,000) was from Spectrum Lab Inc. (New Brunswick, NJ). Flexible thin-layer chromatography (TLC) plates were from Whatman Ltd (Mobile, AL).

Conjugation of SOD1 with Pluronic P85 and L81

The conjugation of SOD1 with Pluronic P85 and L81 included two steps: 1) the generation of mono-amine P85 and mono-amine L81; 2) the attachment of mono-amine Pluronic to SOD1. The generation of mono-amine P85 and L81 was reported previously [26,27]. Briefly, P85 or L81 was reacted with MTr-Cl to protect the hydroxyl group at one end of the polymer chain; the obtained mono-MTr-Pluronic was activated using CDI, followed by a reaction with EDA. The resulting products were further reacted with TFA to remove the protecting group MTr. The resulting mono-amine products were isolated by gel filtration on Sephadex LH-20 column (2.5 × 30 cm). The amino group attachment was identified by presence of blue color after spraying with 1% ninhydrine solution in EtOH (a test for an amino group).

Mono-amine P85 (9.3 mg) was mixed with DSP (4.9mg, 6-fold molar excess) or DSS (4.5mg, 6-fold molar excess) in 0.5ml of DMF and supplemented with 0.1 ml sodium borate buffer (0.1M, pH 8). To attach Pluronic chain to the carboxyl group of SOD1, mono-amine P85 (5 mg) was mixed with EDC (8.5mg, 40-fold molar excess) in solution of 0.4 mL of EtOH and 0.1 mL of sodium phosphate buffer (0.1M, pH 6). After incubating for 30 min at 25°C, the reaction solution was eluted from Illustra NAP-25 columns in 20% aqueous EtOH. About 1.5 mL of fractions containing activated copolymer was collected. DSP or DSS activated copolymer solution was immediately mixed with SOD1 (2 mg, molar ratio to P85 1:5, 1:10, 1:30 or 1:60) in 0.2 ml of 0.1M sodium borate (pH 8). The solutions of EDC activated copolymer were incubated with SOD1 (2mg, molar ratio to P85 1:18) in the presence of 0.2 mL sodium phosphate buffer (0.1M, pH 6). The homogeneous reaction mixture was incubated overnight at 4°C. Modification of SOD1 by mono-amine L81 *via* DSS linker was carried out using a similar procedure. Finally, the conjugates were further

purified by precipitating in cold acetone to remove the excess of non-reacted copolymers as confirmed by TLC.

Characterization of SOD1-Pluronic Conjugates

Standard SDS polyacrylamide gel electrophoresis (SDS-PAGE) was applied. SDS gels (12.5%) were prepared to 1.0mm thickness. All conjugate samples and reference samples were prepared in 5 μ L dd H₂O (2 μ g/ μ L, determined by protein MicroBCA assay) and diluted (1:1) with denaturing solution (3.8 mL of H₂O, 5 mL; 0.5 M Tris HCl (pH 6.8), 8 mL 15% w/v SDS, 4 mL of glycerol, 2 mL of 2-mercaptoethanol, 0.4 mL of bromophenol blue 1% w/v). Exceptionally, no reducing reagent was used in the loading buffer for SOD1-P85 conjugates prepared by DSP linker in order to prevent the degradation of disulfide bond. The mixture of SOD1 and P85 (4:1 by weight) or SOD1 and L81 (16:3 by weight) was prepared based on the weight percentage of SOD1 attached by one Pluronic chain. The samples were heated for 5 min at 100 °C before loading in the gel. After running for 1 h at 200 V, the gel was fixed in 50% methanol/10% acetic acid, stained in SYPRO® Ruby solution (Bio-Rad Lab., CA) and then scanned on a Typhoon gel scanner.

Mass values of SOD1-Pluronic conjugates were determined by matrix-assisted laser desorption/ionization time of fly (MALDI-TOF) spectroscopy using a MALDI-TOF-TOF 4800 (Applied Biosystems Inc., CA), with a laser power of 3000 V, in positive reflector mode. Solution containing saturated sinapinic acid (SA) in 50% acetonitrile with 0.1% TFA was used as matrix for sample preparation. Briefly, 0.5 μ L SA solution was coated on the plate followed by 1) depositing 0.5 μ L solution of salt free SOD1-Pluronic conjugates in water (10⁻⁴M), and, 2) coating with 0.5 μ L SA solution. The mass spectrometer was calibrated against insulin (5729.61Da) and albumin (66429.09Da) (Sigma-Aldrich Co. St-Louis, MO).

The TNBS assay was performed to measure the average number of Pluronic chains attached to each SOD1 macromolecule. Briefly, 10 μ L of SOD1-Pluronic conjugate solutions (protein concentration 0.1–0.6 mg/mL) were mixed with 10 μ L of TNBS solution (1.7 mM) in 80 μ L of sodium borate buffer (0.1 M, pH 9.5) and incubated at 37°C for 2 hrs. The absorbance was measured at 405 nm using the microplate reader SpectraMax M5 (Molecular devices, Sunnyvale, CA). The protein content was measured using MicroBCA kit from Pierce (Rockford, IL). The degree of modification (average number of amino groups modified) was calculated according to the equation:

$$S(\text{modification degree}) = 11 \times \frac{A_{\text{native}}/C_{\text{native}} - A_{\text{modified}}/C_{\text{modified}}}{A_{\text{native}}/C_{\text{native}}}$$

SOD1 Enzymatic Activity Assay Based on Pyrogallol Autoxidation

Pyrogallol autoxidation in the presence of O₂^{•-} occurs rapidly at pH < 9.5 and yields a chromophore that absorbs at 420 nm. SOD1 catalyzes the dismutation of O₂^{•-} and thus inhibits pyrogallol autoxidation. As such, the enzymatic activity of SOD1 or SOD1-Pluronic conjugates can be determined in a kinetic assay by measuring the absorbance of oxidized pyrogallol in the presence of various amounts of enzyme. Briefly, 20 μ L of 0.0002 to 200 μ g/mL SOD1 or SOD1-Pluronic conjugate samples in distilled water were mixed with 20 μ L of a fresh pyrogallol (0.5 mg/mL) in distilled water and then supplemented with Tris/HCl buffer (0.1 M, pH 8) containing 1 mM DTPA to a final volume of 200 μ L. In control experiments we used mixtures of distilled water without enzyme and/or without pyrogallol. The reaction mixtures were added to 96-well plates in triplicate and the rates of autoxidation were measured immediately as slopes by recording increases in absorbance at 420 nm up to

10 min, in the microplate reader SpectraMax M5. The data was interpreted as the inhibition rate (%) using equation: $[(S1-SS)/(S1-S2)] \times 100\%$ where S1 is the slope for enzyme-free water with pyrogallol; S2 is the slope for enzyme-free water without pyrogallol and SS: slope of sample with pyrogallol. One unit of enzyme activity caused 50% inhibition rate in this test. The specific activity of unmodified SOD1 was 12500 units per mg of enzyme. The residual activity of modified SOD1 was expressed on a percentage base of unmodified enzyme.

In-Gel SOD Activity Assay

SOD1-Pluronic conjugates and references samples were prepared similarly as used for SDS-PAGE. The samples were diluted (1:1) in loading buffer containing 0.5 M Tris (pH 6.8), 8.0 mM EDTA, 50% glycerol and 0.1% bromophenol blue. Electrophoresis was carried out at 4°C in a 12% home-made native gel with 1.5 mm thickness. The gel was run in pre-electrophoresis buffer (Tris 0.19 M/EDTA 1.0 mM (pH 8.8)) at 100V for 1 hr. Samples were then loaded into each well and ran first in pre-electrophoresis buffer at 100 V for 1 hr followed by electrophoresis buffer (Tris 8 mM/Glycine 0.3 M/EDTA 1.8 mM (pH 8.3)) at 20 V for 20 hrs. After electrophoresis, the gel was soaked in 25 ml of 2.43 mM NBT, 28 mM TEMED and 28 μ M of riboflavin for 15 min in the dark. The photochemical reaction was initiated by exposing the gel to fluorescent light for 15 min and SOD enzymatic activity was indicated by the appearance of achromatic bands.

Inhibition of Xanthine Oxidase (XO) and Hypoxanthine (HX) Derived $O_2^{\bullet-}$

To examine the ability of SOD1-Pluronic conjugates to scavenge $O_2^{\bullet-}$, we used XO/HX to generate $O_2^{\bullet-}$ in a cell-free system and measured levels of 2-hydroxyethidium (2-OH- E^+) by High Performance Liquid Chromatography (HPLC). For these experiments, we used SOD1-Pluronic conjugates separated from unmodified SOD1 by size exclusion chromatography (SEC) as described in Supplementary data, Figure S1, and thus the influence of residual SOD1 in the conjugate preparations was excluded. Briefly, 25 μ M of dihydroethidium (DHE, 2,7-diamino-10-ethyl-9-phenyl-9,10-dihydrophenanthridine 37291, Sigma-Aldrich Co., St-Louis, MO) was incubated with 5 mU/mL XO and 0.5 mM HX in the presence of purified SOD1-Pluronic conjugates (80 μ g/mL), PEG-SOD1 (110 μ g/mL) (PEG-SOD1 at this concentration displayed the same enzymatic activity as SOD1 at 80 μ g/mL, as determined by pyrogallol kinetic assay), or Pluronic alone (in the amount which was the same as that in 80 μ g/mL of SOD1-Pluronic conjugates) in 20mM Kreps-HEPES buffer (pH 7.4). After 30 min incubation, the obtained reaction products, as well as authentic DHE and pure 2-OH- E^+ were eluted in C18 column and 2-OH- E^+ was analyzed using the HPLC method described previously [28] to specifically measure $O_2^{\bullet-}$ levels.

Neuronal cell cultures

Catecholaminergic (CATH.a) neurons (from ATCC, CRL-11179TM) were seeded in 24-well plates at a density of 100,000 cells/well or 25cm² flask at a density of 3×10^6 in RPMI 1640 media supplemented with 8% horse serum (Gibco, Life Tech., Grand Island, NY), 4% fetal bovine serum (Invitrogen, Carlsbad, California), and 1% penicillin/streptomycin. The cells were cultured at 37°C with 95% humidity and 5% CO₂, and grown for a total of 6 days and differentiated by adding 1 mM of fresh N-6,2'-O-dibutyryl adenosine 3',5'-cyclic-monophosphate (AMP, Sigma-Aldrich Co. St-Louis, MO) to the culture media every 2 days.

Cellular Uptake

CATH.a neurons were serum starved for 24 hrs and then exposed to SOD1 or SOD1-Pluronic conjugates (80 μ g/mL, determined by Pierce MicroBCA assay) in serum-free media for various time intervals at 37°C. The collected CATH.a neuronal cell pellets were

lysed by sonication and total cellular protein was measured using Bio-Rad protein assay. Cell lysates (100 µg) were electrophoresed and SOD activity was assessed as described above.

Confocal Microscopy

Fluorescence labeled SOD1 or SOD1-P85 conjugates were prepared using method recommended from Invitrogen. Briefly, 1 mg of SOD1 or SOD1-P85 conjugates was incubated with Alexa Fluor 680 dye derivative (0.2 mg for SOD1 and 0.5 mg for conjugates) in 0.5 mL of sodium bicarbonate (0.1 M, pH 8.3) at 25°C for 2 hrs. The reaction solutions were eluted from gel filtration Sephadex G25 column (160 mm x 20 mm) in PBS buffer and the fractions containing labeled proteins were further desalted followed by lyophilization. The fluorescence labeling intensity of SOD1 and SOD1-P85 was compared by measuring the fluorescence emission at 702 nm (excitation at 679 nm) of each sample at various concentrations (0.1 ~ 10 µg/mL measured by MicroBCA from pierce). CATH.a neurons in two-well coverglass chamber slides (Fischer Scientific, Waltham, MA) were incubated with 80 µg/mL Alexa Fluor 680 labeled SOD1 or SOD1-P85 conjugates for various time intervals at 37 °C. The medium with the protein was removed and the cells were rinsed twice with PBS. The cells were covered with PBS and confocal fluorescent images were captured using a Zeiss LSM 510 Meta confocal microscope.

Neuronal Toxicity

Neuronal toxicity was determined with a WST-8 based assay using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) (Cell Counting Kit-8; Dojindo Molecular Technologies, Inc., Gaithersburg, MD), according to the manufacturer's directions. Briefly, CATH.a neurons were seeded into 24 well plates and grown as described above. After incubation with SOD1 or SOD1-Pluronic conjugates at 80 µg/mL concentration for various time periods, 30 µL of kit reagent was added to 300 µL media/well and incubated for an additional 1 hr. Cell viability was obtained by scanning with a microplate reader at 450 nm.

Measurement of Intracellular $O_2^{\bullet-}$ Levels by Confocal Microscopy

Levels of intracellular $O_2^{\bullet-}$ in cultured CATH.a neurons were measured using the fluorogenic probe dihydroethidium (DHE, Molecular Probe, Inc). CATH.a neurons exposed to SOD1-P85, SOD1-L81, or native SOD1 (80 µg/mL) for 24 hrs at 37 °C or non-treated control cells were loaded with DHE (5 µM, 20 min). Confocal microscopy (Zeiss 510 Meta confocal microscope) images were captured before and every 2 min for up to 20 min after AngII (100 nM) stimulation. Notably, images were collected using a 405 nm excitation wavelength which, as previously characterized and described by Robinson and colleagues [29], excites 2-OH- E^+ , the $O_2^{\bullet-}$ -specific product of DHE. 2-OH- E^+ fluorescence intensity was quantified using Zeiss LSM 510 analysis software.

Detection of Intracellular $O_2^{\bullet-}$ Using 2-Hydroxyethidium in HPLC-Based Assay

To provide additional evidence that the SOD-Pluronic conjugates do indeed scavenge intracellular $O_2^{\bullet-}$, we measured intracellular $O_2^{\bullet-}$ in AngII-stimulated CATH.a neurons pre-exposed to SOD1 or SEC-purified SOD1-Pluronic conjugates using HPLC analysis of 2-OH- E^+ formation as described previously [28,30]. CATH.a neurons were treated with SOD1 or SOD1-Pluronic conjugates at 80 µg/mL or PEG-SOD1 at 110 µg/mL concentrations. After 24 hrs incubation at 37 °C, media was removed and neurons were exposed to 100 nM AngII for 1 hr followed by incubation with 25 µM DHE in the dark for 20 min. Neurons were then washed by ice-cold Krebs-HEPES buffer and then scraped into 1 mL of ice-cold Krebs-HEPES buffer. Following centrifuging at 1,000 g x 5 min at 4 °C, the cell pellets

were resuspended with 150 μ L of ice-cold 0.1% Triton X-100 in DPBS and lysed using insulin syringe. Samples were centrifuged at $1,000g \times 5$ min at 4 $^{\circ}$ C and 100 μ L of supernatant was transferred to new 1.5-mL tube containing 100 μ L of 0.2 M HClO₄ in methanol. The mixed solution was placed on ice for 1–2 hrs to allow protein precipitation. Meanwhile, the residual supernatant was used for protein quantification by MicroBCA protein assay. The protein precipitates were pellet by centrifuging at $20,000g \times 30$ min at 4 $^{\circ}$ C and then 100 μ L of the supernatant was mixed with 100 μ L of 1 M phosphate buffer pH 2.6. The obtained 200 μ L solution was centrifuged at $20,000g \times 15$ min at 4 $^{\circ}$ C and the supernatant was ready for HPLC analysis. Typically, 100 μ L of sample was injected into HPLC system (Agilent 1200, Agilent Technologies, Palo Alto, CA) with a C18 column (Supelco Nucleosil C18, 250mm $\times$ 4.6mm, 5 μ m, 100 $\text{\AA}$, Sigma-Aldrich, St. Louis, MO) equilibrated with 10% acetonitrile (containing 0.1% TFA (v/v)) in 0.1% TFA aqueous solution and eluted by a linear increase in acetonitrile from 10 to 70% in 46 min at a flow rate of 0.5 mL/min. Fluorescence detection at 500nm (excitation) and 580nm (emission), as well as absorbance at 210, 350, 390, 420nm were used to monitor the elution products. The area under the 2-OH-E⁺ fluorescence peaks (500 nm ex/580 nm em) was measured for each sample, compared with known concentration of the standard and normalized by the cell protein to obtain concentration of 2-OH-E⁺ expressed in nanomoles per mg of cell protein. The standard 2-OH-E⁺ was synthesized and purified as previously described [28,31].

Statistical Analysis

All data are expressed as mean $\pm$ SEM. The statistical analysis was done by one-way ANOVA followed by the Newman-Keuls post-test for multiple comparisons using Prism (GraphPad Software, Inc). P-value less than 0.01 was considered statistically significant for DHE confocal imaging data and p-value less than 0.05 was considered statistically significant for HPLC-based 2-OH-E⁺ measurement.

RESULTS

SOD1-Pluronic Conjugates: Synthesis and Characterization

The generation of SOD1 and Pluronic P85 conjugates using three reagents (DSS, DSP and EDC) is summarized in Scheme 1. The modification of lysine amino acids of SOD1 by mono-amine Pluronic uses similar procedures as described previously for HRP conjugation [26,27]. A homo-bifunctional NHS containing linker, DSS or DSP, was used to activate mono-amine P85 and this was followed by modifying SOD1 in 20% EtOH aqueous solution in alkaline conditions. The reaction proceeded readily upon excess of polymers (5, 10, 30 or 60 $\times$ molar excess) and yielded SOD1 conjugates, which contained on average from about 1 to about 8 Pluronic chains covalently attached to SOD1 aminogroups, as determined by TNBS assay (Table 2). Notably, the reaction was much less efficient when conducted in alkaline aqueous solution in the absence of EtOH, as in such cases the modification degrees were either much less (for DSP linker) or not detectable (for DSS linker) by TNBS assay (Table 2). A similar procedure was utilized to generate SOD1-L81 conjugates. Specifically, two conjugates with an average degrees of modification of 3.3 and 5.5 were obtained using a non-degradable linker DSS in 20% EtOH aqueous solution (Table 2). Considering that elimination of lysine charges, as a result of modification of aminogroups, might decrease SOD1 enzymatic activity [32], we also attempted to modify carboxylic acid groups of SOD1 with amine-P85 in the presence of water-soluble carbodimide reagent EDC under neutral pH conditions and 20% EtOH. One SOD1-LP85 conjugate was synthesized using this method; however, in this case the modification degree cannot be determined by TNBS.

The formation of SOD1-Pluronic conjugates was confirmed using SDS electrophoresis. In the case of SOD1-P85 (DSP linker), the sample was prepared in non-reducing conditions to

maintain the disulfide linkages. As shown in Figure 1, The SOD1-P85 conjugates prepared using DSS and EDC displayed similar profiles suggesting the presence of non-modified SOD1 (monomer) and SOD1-P85 conjugates with various degrees of modification exhibited as relatively well separated bands (indicated by full-length arrows). In comparison, SOD1-L81 conjugate as expected, displayed a slightly lower M.W. band (indicated by arrowhead). Furthermore, presence of protein smears was observed in all lanes containing the SOD1-Pluronic conjugates, but not in lanes with native SOD1 or the mixture (i.e. non-conjugated) of SOD1 and Pluronic. The highly modified SOD1 conjugates (SOD1-P85 with DSP linker, 1:60 molar ratio) showed only a smear of high molecular weight protein and could not be well separated by electrophoresis. Appearance of this smear suggests that the covalent modification of SOD1 with Pluronic chains decreases the protein electrophoretic mobility. Altogether, the decrease of mobility included the separated modified protein bands appeared to be greater than one might expect based on the estimated molecular masses of different conjugates. This may result from increased hydrodynamic diameters of the conjugates due to the presence of Pluronic chains. Furthermore, binding of SDS with Pluronic has also been described [33]. This in the case of SOD1-Pluronic conjugates may result in formation of large and not well defined protein-containing aggregates in the conditions of SDS electrophoresis, which can account for appearance of smears. Notably, the cross-linking of SOD1 is highly unlikely at least when DSP and DSS chemistries are used, because activated Pluronic reagents contain only one reactive group.

To overcome the limitations of SDS electrophoresis we used the mass spectra analysis (Figure 2). Interestingly, upon the deposition/ionization condition, even the unmodified SOD1 sample revealed the presence of the monomer (16 kDa) and dimer (32 kDa) and trimer (48 kDa) forms. Moreover, the mass spectra clearly demonstrated that the SOD1 conjugates contained mixtures of non-modified SOD1 monomer, SOD1 monomers with various numbers of polymer chains attached, as well as various higher molecular mass peaks, which we conditionally ascribed as modified dimers (labeled as (d)SOD1 conjugates). The monomer form of SOD1-P85 conjugates showed average M.W. of 21 kDa ((m)SOD1-P85 1:1 ratio) and 26 kDa ((m)SOD1-P85 1:2 ratio), whereas SOD1-L81 conjugates showed average M.W. of 19 kDa ((m)SOD1-L81 1:1 ratio) and 22 kDa ((m)SOD1-L81 1:2 ratio). Similar mass spectra profiles were observed for SOD1-P85 conjugates using DSS, DSP (Figure 2) and EDC (data not shown). Therefore, our modification procedures produced a range of various modified conjugates. Using size-exclusion chromatography we estimated that the amount of modified protein in two samples SOD1-P85 and SOD1-L81 (marked by asterisk in Table 2) is at least 90% (Supplementary data, Figure S1).

The SOD1-Pluronic conjugates partially retained enzymatic activity, as measured by the pyrogallol autoxidation assay. As the average modification degree of SOD1-Pluronic conjugates increased, their residual activity decreased (Table 2). Specifically, for SOD1-P85 obtained using both DSP and DSS as linkers, the activity decreased from ca. 60 % for low modification degrees (ca. 1 to 3) to ca. 40 to 50 % for intermediate modification degrees (ca. 3 to 4), to less than 30% for high modification degrees (ca. 5 to 8). One highly modified sample of SOD1-P85 conjugated using DSP with ca. 8 Pluronic chains was totally inactive. Likewise the residual activity of SOD1-L81 conjugate obtained using DSS was 47% at intermediate modification degree (3.3), and only 35% at high modification degree (5.6). The use of EDC and carboxylic acid groups of SOD1 for conjugation did not improve the catalytic activity of SOD1 conjugates as expected.

The size-exclusion chromatography of intermediately modified samples SOD1-P85 and SOD1-L81 revealed that the activity was partially decreased in both unmodified and modified SOD1 fractions (Supplementary data, Figure S1). Therefore, we suggested that the

presence of organic solvents (20% EtOH or 20% DMF) during the modification procedure may have also contributed to the decrease in SOD1 activity. Indeed, after incubation of native SOD1 with Pluronic P85, in 20% EtOH or 20% DMF without adding cross linker (i.e. no covalent modification), the enzyme activity decreased to 64% (EtOH) and 44% (DMF).

A native in-gel SOD activity assay reinforced that the SOD1-Pluronic conjugates retained catalytic activity as indicated by the presence of intense achromatic staining (Figure 3A). Notably, among these conjugates, both residual non-modified SOD1 (band at SOD1 dimer position) and modified SOD1 (smear above SOD1 dimer position) exhibit SOD1 activity. In accordance with the previous kinetic assay, a highly modified protein, SOD1-P85 prepared using DSP linker (60 × molar excess) was practically inactive. Interestingly, in the case of SOD1-P85 (DSS) and SOD1-P85 (EDC), achromatic staining was also observed below the SOD1 dimer band, which might suggest that the conjugates also contain active monomeric species. Finally, the biological activity of purified SOD1-Pluronic conjugates was clearly shown by the decrease in XO/HX derived $O_2^{\bullet-}$, using HPLC-based assay for measurement of 2-OH-E⁺, a method that has been referred to as the “gold standard” for measuring $O_2^{\bullet-}$ [34]. In this assay production of 2-OH-E⁺, a specific product of oxidation of DHE by $O_2^{\bullet-}$, was attenuated in the presence of purified SOD1-Pluronic conjugates or PEG-SOD1; however, Pluronic alone failed to inhibit 2-OH-E⁺ generation (Figure 3B). Together with the SOD1 activity data presented in Table 2, these data demonstrate beyond any doubt that our SOD1-Pluronic conjugates, even after removal of residual non-modified SOD1, are active.

Cellular Delivery of SOD1-Pluronic Conjugates to CATH.a Neurons

To begin investigating the ability of our SOD1-Pluronic conjugates to penetrate neuronal cell membrane, we labeled SOD1-P85 conjugates and SOD1 with Alexa Fluor 680 and studied the internalization of these proteins into CATH.a neurons using confocal microscopy. It should be noted that in the following cellular studies, unless specifically mentioned, we focused on two SOD1 conjugates, SOD1-P85 and SOD1-L81 with intermediate modification degrees (3.9 and 3.3, respectively) prepared using DSS linker (marked by asterisk in Table 2). These conjugates maintained 38% and 47% of initial SOD1 activity, respectively. The labeled SOD1-P85 and labeled SOD1 showed identical fluorescence as measured by the emission intensity at 702 nm per $\mu\text{L/mL}$ of proteins (data not shown); thus, allowing us to directly compare the confocal microscopy images of CATH.a neurons treated with SOD1-P85 to those treated with SOD1. As clearly demonstrated in the representative confocal microscopy images (Figure 4A), there was no internalization of native SOD1 after 1 or 6 hrs of incubation and only a modest increase in intracellular fluorescence after 24 hrs. In contrast, intracellular Alexa Fluor 680 fluorescence was observed in neurons exposed to SOD1-P85 for as little as 6 hrs, and this fluorescence was further increased after 24 hrs of incubation (Figure 4A). Next, using the native in-gel SOD activity assay, we determined whether SOD1-Pluronic conjugates delivered to CATH.a neurons were active. As seen by the smear of SOD1 activity, CATH.a neurons treated with SOD1-P85 or SOD1-L81 displayed gradually increasing levels of SOD1 activity after 1, 6, and 24 hrs of incubation (Figure 4B). In contrast, this smear of SOD1 activity was absent in CATH.a neurons incubated with SOD1 alone (Figure 4B). Notably, the smear of SOD1 activity was also absent in CATH.a neurons incubated with a mixture of SOD1 and Pluronic (i.e. without covalent modification) (Supplementary data Figure S2), thus, indicating that conjugation of SOD1 to P85 or L81 is required for neuronal cell uptake. It should also be noted that we observed only a modest smear of SOD1 activity in CATH.a neurons treated with PEG-SOD1, and this was only detected after 24 hrs of incubation (Supplementary data Figure S2). Endogenous SOD2 (MnSOD) and SOD1 were also observed at the top and bottom of each lane respectively. Altogether, these studies

demonstrate that Pluronic modification enhances the ability of SOD1 to enter neurons and that the internalized SOD1-Pluronic conjugates retain SOD1 activity.

SOD1-Pluronic Conjugates Are Nontoxic to Neurons

To determine the safety of our SOD1-Pluronic conjugates, we examined CATH.a neuronal cell toxicity 1, 3, 6, 18, and 24 hrs after incubation with SOD1 protein, SOD1-P85, or SOD1-L81 (80 $\mu\text{g/mL}$). As shown in Figure 5, treating CATH.a neurons with SOD1, SOD1-P85 and SOD1-L81 for these various lengths of time failed to induce any significant neuronal toxicity.

SOD1-Pluronic Conjugates Inhibit AngII-induced Increase in $\text{O}_2^{\bullet-}$

To test the biological activity of our SOD1-Pluronic conjugates, we evaluated the ability of SOD1 conjugates to scavenge intracellular $\text{O}_2^{\bullet-}$ following AngII stimulation of CATH.a neurons, which are known to express AngII receptors [35]. As described in the Methods section, DHE fluorescence confocal microscopy images were captured using an excitation wavelength of 405 nm, which selectively detects 2-OH- E^+ , the $\text{O}_2^{\bullet-}$ -specific product of DHE [29]. As shown in Figure 6A and 6B, the time-dependent increase in fluorescence following AngII stimulation, was significantly attenuated in neurons treated with SOD1-P85 and SOD1-L81 conjugates; whereas such inhibition was not observed in control neurons or native SOD1-treated neurons. Furthermore to relate the observed attenuation in 2-OH- E^+ to the catalytic activity of SOD1-Pluronic conjugates, the SOD1-P85 was irreversibly inactivated by Cu-chelating reagent, diethyldithiocarbamate (DETC). As shown in the Supplementary data (Figure S3) the inactivated conjugate increased the fluorescence in AngII-stimulated neurons, while the active conjugate decreased the fluorescence compared to controls untreated with the conjugate.

To confirm the confocal microscopy data, we utilized the HPLC method and measured 2-OH- E^+ in AngII-stimulated CATH.a neurons pre-treated with SOD1 or purified SOD1-Pluronic conjugates (80 $\mu\text{g/mL}$). There was no detectable peak from control CATH.a neurons without any treatment of AngII or DHE (data not shown). In neurons treated with DHE, the DHE peak (at 22.5 min) was detectable, thus indicating cellular uptake of DHE. In addition, the 2-OH- E^+ peak (at 32 min) was clearly separated from the reaction byproduct ethidium (E^+) peak (at 33 min), as demonstrated in Figure 6C. This HPLC analysis suggested that AngII stimulation significantly increased the formation of 2-OH- E^+ (Figure 6D), and this increase was completely inhibited in cells pretreated for 24 hrs with SOD1-Pluronic conjugates. In contrast, no significant decrease was observed in 2-OH- E^+ formation in AngII-stimulated cells pretreated with native SOD1. Cells incubated with PEG-SOD1 (Figure 6D) showed a slight decrease in 2-OH- E^+ level. However, this was statistically not significantly different from cells treatment with AngII alone. Therefore, the direct measurement of 2-OH- E^+ levels in CATH.a neurons by HPLC method reinforced the results obtained by confocal microscopy (Figure 6A). Notably, in neurons treated with SOD1-Pluronic conjugates the difference in the extent of inhibition of fluorescence detected by confocal microscopy (ca. 5-fold) and the decrease in the amount of 2-OH- E^+ measured by HPLC (ca. 2-fold) may be due to the variation in the cells exposure to AngII (20 min. in confocal experiment vs. 60 min. in HPLC experiment). Alternatively, this difference may be explained by a non-linear dependence of fluorescence of 2-OH- E^+ on its concentration in cells. The HPLC data also suggested that considerable amount of E^+ was produced in the cells, which was not affected by any of the treatments (Supplementary data Figure S4). It is known that upon 405 nm excitation the fluorescence emission of E^+ is much less than that of 2-OH- E^+ [28]. Therefore, its contribution in the overall fluorescence in the confocal study was probably small. Altogether, considering 2-OH- E^+ is the $\text{O}_2^{\bullet-}$ -specific product of DHE [28], these confocal microscopy and HPLC data clearly demonstrate that the enhanced

cellular accumulation of SOD1, as a result of Pluronic modification, provides a significant increase in intracellular $O_2^{\bullet-}$ dismutase activity in AngII-sensitive neurons.

DISCUSSION

In this study, we developed SOD1-Pluronic conjugates and showed that Pluronic modification improves SOD1 intra-neuronal delivery and as a result decreases AngII-induced increase in intra-neuronal $O_2^{\bullet-}$ levels. Two types of modifications strategies were explored for conjugation of SOD1 with amino-derivative of Pluronic. First is the modification of Lys and N-terminal amino groups of SOD1 using NHS-containing bifunctional cross-linkers, DSP and DSS. Second is the modification of Asp and Glu carboxyl groups of SOD1 using water-soluble carbodimide reagent, EDC. Both strategies produced catalytically active modified SOD1 with various amounts of Pluronic chains attached. Such conjugates at least in the case of DSP and DSS modifications retained up to 60% of the activity of the unmodified enzyme. However, the activity drastically decreased as the modification degree was increased above 5 Pluronic chains per the protein molecule. This may be due to a considerable change of global or local conformations of the enzyme, which affects the active center. In particular, native SOD1 exists in a homodimer form (M.W. 32 kDa), which, as suggested by Valentine et al., plays an important role in maintaining SOD1 function [36,37]. One concern with Pluronic modification was a potential for SOD1 dimer dissociation, that may be accompanied by a loss of metals (Cu and Zn), crucial to maintaining the SOD1 activity. Indeed, we observed unmodified SOD1 monomers in SDS-PAGE (Figure 1) and mass spectra (Figure 2) in all SOD1-Pluronic conjugates. Furthermore, the mass spectra clearly suggested presence of various modified SOD1 monomers. However, these data may not truly reflect the dimer dissociation as a result of the modification. In particular, upon denaturing conditions of electrophoresis or sample ionization/dissociation processing in MALDI-TOF the monomeric species can be produced from both non-modified SOD1 dimer or/and modified SOD1 dimer (with Pluronic attached to either one or both SOD1 subunits). Purification of SOD1-Pluronic conjugates in non-denaturing conditions using SEC indicated no detectable monomeric species (Supplementary data Figure S1). Surprisingly, the SOD1 in-gel activity assay, a non-denaturing electrophoresis, showed achromatic staining below the SOD1 dimer band. This indicated that the conjugation reaction might generate a small portion of Pluronic-modified monomeric SOD1, which cannot be determined using SEC but which is active. The catalytic activity displayed by such modified SOD1 may be due some stabilization effect of Pluronic chains that can shield the surface of the monomeric protein globule and stabilize the incorporated metals.

In addition to potential conformational changes and inactivation as a result of modification of the protein aminogroups by Pluronic the activity loss may be partially due to exposure of the enzyme to the organic solvents during modification. Such solvents include the aqueous-EtOH solution to increase the modification yield and cold acetone to purify the conjugates by acetone precipitation. As shown in our SEC purification experiment at least the latter step of acetone precipitation can be eliminated, because SEC can remove excess of unreacted Pluronic amine in non-denaturing conditions. This may be used in future to further increase the yield of active conjugate. However, the use of aqueous-organic solution during SOD1 and Pluronic conjugation appears to be necessary as it is likely to prevent association of polymer chains and, as such, increase the reaction yield. Yet, alternative aqueous-organic solutions can be also used for modifications that can further preserve the enzyme activity. In particular, we found that 20% 1,4-butanediol or 20% DMSO aqueous solutions resulted in less SOD1 activity loss and comparable reaction efficiency compared to aqueous-EtOH or aqueous-DMF solutions (data not shown).

The major result of this study is a clear demonstration that Pluronic-modified SOD1 can penetrate into neurons and display intra-neuronal enzymatic activity. Furthermore, we provide evidence that once inside the cell the SOD1-Pluronic is biologically functional. In particular, the SOD1-Pluronic conjugates attenuated the increase in 2-OH-E⁺ fluorescence in neurons loaded with DHE and stimulated with AngII. Although we assessed 2-OH-E⁺ fluorescence using an excitation of wavelength of 405 nm and confocal microscopy to specifically measure intracellular O₂^{•-}, as previously described [29], we understand the limitation of this method and thus performed similar experiments and measured 2-OH-E⁺ levels with HPLC. The use of HPLC to measure the oxidation products of DHE, both 2-OH-E⁺ and ethidium, in biological samples is now considered by some to be the “gold standard” [34]. Importantly, our HPLC data corroborate our confocal microscopy data, and convincingly demonstrate that SOD1-Pluronic conjugates attenuate the AngII-induced increase in intra-neuronal O₂^{•-} levels. As the SOD1 conjugates obtained and used in some of the cellular studies are a mixture of non-modified SOD1 and SOD1 conjugates, one potential concern was that non-modified SOD1 interfered with the interpretation of the results. To address this, we characterized the mixture obtained after SEC and showed that about 10% of non-modified SOD1 was present (based on area percentage in HPLC profile), and each fraction (non-modified SOD1, mono-Pluronic SOD1, and SOD1 modified by multiple Pluronic chains) showed similar activity as determined by pyrogallol kinetic assay (Supplementary data Figure S1). Thus, we conclude that the majority (90%) of the mixture was modified SOD1, and the measured activity of conjugates and obtained cellular responses can be mainly attributed to the SOD1-Pluronic conjugates. Furthermore, taking advantage of the SEC purification method, the purified SOD1-Pluronic fractions were collected and used in our HPLC experiments (Figure 6C and D). These studies clearly show that even without the influence of non-modified SOD1, SOD1-Pluronic conjugates attenuate AngII-induced increase in intracellular O₂^{•-}.

Numerous investigations have clearly shown that O₂^{•-} in neurons is an important target for the improved treatment of AngII-dependent neuro-cardiovascular diseases. For example, injection of adenoviral vectors encoding SOD1 directly into the brain attenuates O₂^{•-} levels in the brain and the elevated blood pressure in a mouse model of AngII-dependent hypertension, whereas gene transfer of extracellular SOD had no effect [6]. Similarly in a heart failure model, which is associated with increased AngII signaling in the brain, overexpression of SOD1 in the brain attenuates sympathetic output and improves cardiac function [38]. Although these previous studies clearly demonstrate a therapeutic benefit of overexpressing SOD1 in the brain in the pathogenesis of brain-related cardiovascular diseases, the potential toxicity associated with viral vectors and the inability of viral vectors to penetrate the BBB calls for the development of novel SOD1 delivery systems.

To address this concern, other investigators have developed PEG modified SOD1 and evaluated PEG-SOD1 antioxidant therapy in many disease models. For example, PEG modification has shown to stabilize the enzyme against degradation; however, little evidence indicates that PEG modification increases SOD1 penetration of neuronal cell membranes *in vitro* or transport across the BBB in both normal and hypertension animals [20,22]. In addition, the therapeutic effectiveness of intravenously injected PEG-SOD1 in hypertensive brain injury was suggested to be due to its action in the vascular wall or its extracellular activity [22]. In the present study, PEG-SOD1 failed to significantly increase intra-neuronal SOD1 activity and did not inhibit that AngII-induced increase in intra-neuronal O₂^{•-} levels. In comparison, SOD1-Pluronic does increase SOD1 activity in neurons and does scavenge elevated levels of O₂^{•-}. Considering Pluronic modification of HRP enhances its BBB permeability *in vitro* and *in vivo*, as we previously reported [26,27], it is tempting to speculate that Pluronic-modified SOD1 might also penetrate the BBB to exert its activity in the brain. Furthermore, similar to what we observed for Pluronic modified leptin [39],

Pluronic modification may increase SOD1 half-life and stability in circulation and provide an independent transportation route to cross the BBB.

The enhanced cellular uptake of the conjugates may be due to hydrophobic interactions of amphiphilic Pluronic chains with the neuronal cell membrane. Pluronics are neutral block copolymers consisting of hydrophobic PPO and hydrophilic PEO blocks. It was previously shown with HRP, a model protein, that the optimal Pluronic modifications for cellular uptake are Pluronic P85 and L81 (PPO₄₀) vs. Pluronic L121 and P123 (PPO₇₀) [27]. In the current study, we further demonstrate that Pluronics can serve as synthetic transduction agents capable of facilitating neuronal cell penetration of a potential therapeutic protein. Modification of SOD1 by both Pluronic P85 (having intermediate hydrophobicity) and Pluronic L81 (which is more hydrophobic) resulted in enhanced cell membrane penetration without inducing cellular toxicity. A recent study has shown that Pluronic P85 employs a pathogen like mechanism for the cellular entry, which mirrors that of cholera toxin B [40,41]. First, the copolymer chains bind with the cholesterol-rich domains in the cell membranes. Second, they enter cells through caveolae-mediated endocytosis or a caveolae- and clathrin-independent pathway. The efficient transport of Pluronic P85 in brain microvessel endothelial cells and primary neurons has also been demonstrated. Interestingly, in neurons the entry of Pluronic starts from accumulation in the cell body followed by anterograde trafficking towards axons/dendrites[41]. Further studies are required to understand the mechanism of cellular entry and subsequent trafficking of SOD1-Pluronic conjugates.

In summary, the data presented herein demonstrate that Pluronic P85 and L81 modified SOD1 penetrate neuronal cell membranes resulting in an increase intracellular SOD1 activity. In addition, SOD1-Pluronic conjugates inhibit AngII-induced increase in intraneuronal O₂^{•-} levels. These data suggest that Pluronic modification may be a new delivery system for SOD1 into neurons of the CNS, and may have therapeutic effects in cardiovascular diseases associated with increased AngII and O₂^{•-} levels signaling in the brain. Future studies, which are currently underway in our laboratory, are needed to investigate the BBB permeability of SOD1-Pluronics and to test their therapeutic effect in neuro-cardiovascular disease models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Representative SDS-PAGE gel electrophoresis of SOD1 conjugates

All conjugates were prepared in 20% aqueous EtOH supplemented by borate buffer (0.1M, pH8.0). The reaction molar ratio of Pluronic to SOD1 in SOD1-P85 (DSS), SOD1-L81 (DSS), SOD1-P85 (EDC) and SOD1-P85 (DSP) were 10, 5, 18 and 60 respectively. The mixture (i.e. no covalent modification) of SOD1 and P85 (4:1 by weight) or SOD1 and L81 (16:3 by weight) is prepared based on the weight percentage of SOD1 attached by one Pluronic chain.

Fig. 2. Representative mass spectra of SOD1-Pluronic conjugates obtained by MALDI-TOF
 The conjugates were obtained using the reaction conditions described in Fig. 1. (m)SOD1 and (d)SOD1 represent monomer (16 kDa) and dimer (32 kDa) respectively. The average molecular weight at the centroid of the peak was labeled for (m)SOD1 (16 kDa) and its conjugate form: (m)SOD1-P85(1:1), 21 kDa; (m)SOD1-P85(1:2), 26 kDa; (m)SOD1-L81(1:1), 19 kDa; (m)SOD1-L81(1:2), 22 kDa.

Fig. 3. SOD1-Pluronic conjugates retain SOD1 activity

(A) Representative in-gel SOD activity assay. The conjugates and mixtures of SOD1 and Pluronic were prepared as described in Fig. 1. SOD activity is indicated by the appearance of achromatic bands. (B) HPLC profile of authentic DHE, pure 2-hydroxyethidium (2-OH-E⁺) and DHE incubated with xanthine oxidase (XO) and hypoxanthine (HX) in the presence of PEG-SOD1, purified SOD1-Pluronic conjugates, or Pluronic alone. The amount of Pluronic used was calculated based on the weight percentage of Pluronic in the SOD1-Pluronic conjugates.

Fig. 4. Cellular uptake of SOD1-Pluronic conjugates in CATH.a neurons
(A) Cellular internalization of SOD1 and SOD1-P85 labeled by Alex Fluor 680 within CATH.a neuronal cells at various time intervals. **(B)** Representative SOD1 activity assay showing SOD1 activity in CATH.a neurons exposed to SOD1-P85, SOD1-L81, or native SOD1 at 37°C for 1, 6, and 24 hrs.

Fig. 5. Viability of CATH.a neurons treated with **SOD1-P85 or SOD1-L81 conjugates**. CATH.a neurons were exposed to SOD1-P85, SOD1-L81, or native SOD1 (80 $\mu\text{g/mL}$) at 37°C for 1, 3, 6, 18 and 24 hrs. Data were obtained in triplicate and present as means $\pm$ SEM. There is no statistical significance between groups.

Fig. 6. SOD1-P85 and SOD1-L81 conjugates Inhibit Ang-II induced $O_2^{\bullet-}$ level in CATH.a neurons

(A) Representative confocal images of DHE-loaded CATH.a neurons, showing the effects of AngII (100 nM) treatment after 10 min in cells pre-exposed to SOD1-P85, SOD1-L81, SOD1 alone or media for 24 hrs. (B) Summary data of the fluorescence in DHE-loaded CATH.a neurons recorded for 20 min after AngII treatment. Images were analyzed for fluorescence intensity per cell in equal numbers of cells (10 cells from each field, 5 fields total). Data are means $\pm$ SEM and expressed relative to the cell fluorescence before AngII treatment. * $P < 0.01$ vs control cells; # $P < 0.01$ vs cells treated with AngII alone at 10, 12, 14, 16 and 18 min; NS not significant between SOD1 treated cells and cells treated with AngII alone. (C) Representative HPLC profile of the cell extract from CATH.a neurons pre-treated with SOD1 samples for 24 hrs followed by AngII stimulation (100 nM) for 1 hrs and DHE (25 μ M) incubation for 20 min. (D) Quantification of actual concentrations of 2-OH-E⁺ generated under the conditions described in (C) using 2-OH-E⁺ as standard. Data are present

as the amount of 2-OH-E⁺ normalized by cell protein content obtained by MicroBCA assay (n=3). Data are mean $\pm$ SEM. * P < 0.05 vs. Control. # P < 0.05 vs. AngII.

Sch. 1.

Conjugation of SOD1 with mono-amine P85 through biodegradable linker, DSP and non-degradable linkers, DSS and EDC.

Table 1

Structure and properties of Pluronic copolymers, which contain ethylene oxide (EO) and propylene oxide (PO) repeating units.

Pluronic®	Structure	M.W.	HLB ^a	CMC (%) ^b
L81	EO ₃ -PO ₄₃ -EO ₃	2750	2	0.006
P85	EO ₂₆ -PO ₄₀ -EO ₂₆	4600	16	0.03

^aHydrophilic-lipophilic balance (HLB);

^bcritical micelle concentration (CMC) in aqueous solution values at 37°C as determined using pyrene probe.

Table 2

Characteristics of SOD1-Pluronic Conjugates

Pluronic	Linker	Pluronic:SOD1 molar ratio during the reaction	Reaction buffer ^a	Modification degree ^b	Residual activity ^c
P85	DSP	5	20% EtOH/pH8.0	1.26	62
P85	DSP	5	H ₂ O/pH8.0	0.83	61
P85	DSP	10	20% EtOH/pH8.0	2.92	57
P85	DSP	30	20% EtOH/pH8.0	4.11	44
P85	DSP	60	20% EtOH/pH8.0	7.99	NN
P85	DSS	5	20% EtOH/pH8.0	3.9	38[*]
P85	DSS	5	H ₂ O/pH8.0	NN	60
P85	DSS	10	20% EtOH/pH8.0	5.41	27
P85	DSS	30	20% EtOH/pH8.0	7.44	25
P85	EDC	18	20% EtOH/pH8.0	ND	34
L81	DSS	5	20% EtOH/pH8.0	3.3	47[*]
L81	DSS	10	20% EtOH/pH8.0	5.6	35
P85	none	5	20% EtOH/pH8.0	NN	64 [#]
P85	none	5	20% DMF/pH8.0	NN	44

^a Last stage of conjugation of SOD1 with activated derivative;

^b Number of copolymer chains per SOD1 as determined by TNBS titration of amino groups;

^c Residual activity was determined by pyrogallol autooxidation kinetic assay and calculated based on the percentage of the activity of the native SOD1. NN: no modification or no activity; ND: not determined;

* These SOD1-Pluronic conjugates were used in the cellular studies reported herein;

In this reaction, SOD1 was incubated with mono-amine Pluronic P85 in the absence of cross linker DSP or DSS in 20% ethanol, thus no covalent modification occurred.