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The antioxidant ESeroS-GS inhibits NO production and prevents oxidative stress in astrocytes

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Abstract

Within the central nervous system uncontrolled production of large amounts of nitric oxide (NO) by activated glial cells might be the common pathogenesis of several neurodegenerative disorders, including Alzheimer's disease and Parkinson's disease. In the present investigation, we measured the effect of a novel antioxidant γ-L-glutamyl-*S*-[2-[[[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2*H*-1-benzopyran-6-yl]oxy]carbonyl]-3-[[2-(1*H*-indol-3-yl)ethyl]amino]-3-oxopropyl]-L-cysteinyl-glycine sodium salt (ESeroS-GS) on NO production in cultured rat astrocytes. Upon stimulation with 1 μg/mL lipopolysaccharide plus 100 U/mL interferon-γ which induced the expression of inducible nitric oxide synthase, cultured astrocytes generated large amounts of NO as measured by nitrite assay and ESR technique. The endogenous NO caused oxidative damage in astrocytes, which was confirmed by the accumulation of both cytosolic and extracellular peroxides, the decrease in the cellular glutathione level, and the formation of thiobarbituric acid reactive substrates. Production of endogenous NO resulted in cell death finally. Pretreatment with the novel antioxidant ESeroS-GS effectively decreased the expression of iNOS gene, inhibited the formation of endogenous NO, and prevented NO-induced oxidative damage and cell death in astrocytes. The results suggest that ESeroS-GS might be used as a potential agent for the prevention and therapy of diseases associated with the overproduction of NO by activated astrocytes.

Keywords: Astrocyte; ESeroS-GS; ESR spin trapping; iNOS; NO; Oxidative stress

1. Introduction

Within the central nervous system (CNS), NO is an important regulatory molecule that is involved in a wide range of physiological and pathophysiological processes [1]. Under normal physiological conditions proper amount of NO can be generated endogenously by a variety of cells, including both neurons and glial cells, and exerts a number of functions, such as neurotransmission, synaptic plasticity, and memory [2]. On the other hand, high concentration of NO is generated endogenously within the CNS under certain pathophysiological conditions, and may cause neuronal injury. However, the underlying mechanisms of the overproduction of NO under these conditions still remain to be elucidated. Astrocytes are one of the most abundant glial cells in the CNS, which play important roles in the maintaining of the normal function of the CNS. Astrocytes regulate the synthesis and release of a variety of neuropeptides and growth factors, which in turn function on neural or glial cells. However, when astrocytes are damaged by certain stimuli, such as oxidative stress, and do not gen-

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Abbreviations: 1400W, *N*-3-aminomethyl benzyl acetamidine; ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DETC, diethyldithiocarbamate; ESeroS-GS, γ-L-glutamyl-*S*[2[[[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2*H*-1-benzopyran-6-yl]oxy]carbonyl]-3-[[2-(1*H*-indol-3-yl)ethyl]amino]-3-oxopropyl]-L-cysteinylglycine sodium salt; HRP, horseradish peroxidase; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO, nitric oxide; TBARS, thiobarbituric acid reactive substances.

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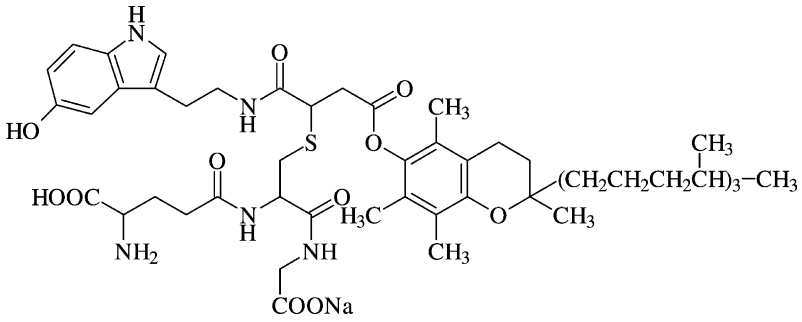

Fig. 1. Molecular structure of ESeroS-GS.

erate sufficient neuropeptides and growth factors, neurons may undergo degenerative changes. Upon stimulation by exposure to the bacterial cell wall components lipopolysaccharide (LPS), various cytokines, such as interferon-γ (IFN-γ), or certain peptides or proteins, such as β-amyloid peptide, astrocytes can be activated and produce large amount of NO *via* the induction of inducible nitric oxide synthase (iNOS) [3] which may damage both astrocytes and neurons. In this regard, developing therapeutic agents that can regulate NO production in astrocytes is very significant.

ESeroS-GS is a novel compound reported by Ogata *et al*. [4]. ESeroS-GS contains α-tocopherol and glutathione (GSH) on succinic acid as the core, the molecular structure of which is shown in Fig. 1. Noda *et al*. [5] have reported that ESeroS-GS is an effective scavenger on free radicals *in vitro*. However, little is known about the effect of ESeroS-GS on cells. In the present investigation, we studied the regulatory effects of ESeroS-GS on the expression of iNOS gene and the subsequent generation of NO in cultured astrocytes stimulated with LPS and IFN-γ. Protection of astrocytes against endogenous NO-induced oxidative stress by ESeroS-GS was also investigated.

2. Materials and methods

2.1. Materials

Sprague–Dawley rats were purchased from Beijing Experimental Animal Center. Cell culture plastic ware was from Corning. Eagle's minimum essential medium (MEM), thiazolyl blue (MTT), LPS (from *Escherichia coli* 026:B6), horseradish peroxidase (HRP), and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) were from Sigma. *N*-3-Aminomethylbenzyl acetamidine (1400W) was from Calbiochem. INF-γ was from Peprotech. Cell culture supplements, fetal bovine serum, and trypsin (1:250) were products of Invitrogen. Monochlorobimane and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were from Molecular Probes. ESeroS-GS was from Senju Pharmaceutical Co Ltd. Monoclonal antibody against iNOS was from BD Transduction Laboratories. Other reagents made in China were of analytical grade.

2.2. Cell culture

Primary cultures of astrocytes were established from neonatal rat cerebra following procedures described previously [6]. Briefly, 1-day-old neonatal Sprague–Dawley rats were decapitated and the forebrains were dissected out. After removal of the meninges and the blood vessels, the cerebral cortices were collected and minced, then dissociated by mild trypsinization. Cells were plated at a density of about 2.5×10^5 cells/cm^2 in 150 cm^2 culture flasks. Culture medium consisted of MEM supplemented with 2 mM L-glutamine, 1 mM L-malate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (v/v). Cells were incubated at 37° under a humidified atmosphere containing 5% CO_2/95% air. For the first 5 days of culture, the MEM was based on D-valine instead of L-valine in order to minimize the growth of fibroblast. Culture medium was changed into fresh MEM based on L-valine on days 5 and 9 after plating. After 13 days in culture, confluent astrocytes were subcultured into 25 cm^2 culture flasks or 24-well multidishes at a density of 1×10^5 cells/cm^2.

2.3. Stimulation with LPS/IFN-γ

Three days after subculture, culture medium was changed into serum-free MEM, and astrocytes were cultured for additional 6 hr. Then, cells were treated with 1 μg/mL LPS and 100 U/mL IFN-γ in serum-free MEM for indicated time [7]. In some experiments cells were preincubated with 50 μM ESeroS-GS or 20 μM iNOS inhibitor 1400W for 3 hr before the addition of LPS/IFN-γ.

2.4. Determination of NO

Synthesis of NO was determined by assay of culture supernatant for nitrite, a stable product of NO with molecular oxygen [8]. Briefly, astrocytes cultured in phenol red-free and serum-free MEM were treated with LPS/IFN-γ for indicated time. Then, 400 μL of the culture supernatant was incubated with 400 μL of Griess reagent at room temperature for 15 min. The optical density at 546 nm was measured. The amount of nitrite in the culture supernatant was calculated from the standard curve. Sodium nitrite was used as an external standard.

Direct evidence for the production of NO in astrocytes was provided by ESR spin trapping with ferrous diethyldithiocarbamate complex [$Fe^{2+}(DETC)_2$] as the spin trap. Although NO by itself is a paramagnetic compound, it is ESR silent at room temperature in solution. However, when NO is trapped with [$Fe^{2+}(DETC)_2$], the resulting complex [$ONFe^{2+}(DETC)_2$] is ESR-detectable at low temperature. Upon enrichment by organic solvents, such as ethyl acetate, hydrophilic [$ONFe^{2+}(DETC)_2$] complex can be detected at room temperature.

The experimental procedure for NO detection was described elsewhere [9]. In brief, cells cultured in 25 cm^2 flasks were treated with LPS/IFN-γ for 12 hr. Then, 1 mM $FeSO_4$, 5 mM diethyldithiocarbamate sodium salt (Na_2DETC), and 5 mM $Na_2S_2O_3$ were added to cells. After incubation at 37° for 3 hr, the cell culture supernatant was collected and extracted with 1 mL of ethyl acetate. After centrifugation at 12,000 *g* for 15 min, the organic layer (upper layer) was collected and transferred into quartz capillary for ESR measurement. ESR spectrum was recorded with a Bruker ER200 spectrometer with conditions described as follows: X-band; sweep width 400 G; microwave power 20 mW; 100 kHz modulation with amplitude 3.2 G; time constant 0.128 s.

2.5. Reverse transcription PCR (RT–PCR)

The levels of iNOS mRNA were determined by RT–PCR. Total RNA from astrocyte cultures was isolated with Uniq-10™ RNA purification kit (Sangon Biotech) according to manufacturer's instructions. RT–PCR was carried out using AccessQuick™ RT–PCR system (Promega), with primers specific for iNOS and GAPDH as a housekeeping gene. For iNOS, the sense primer was 5′-AGAGAGATCCGGTTCACA-3′, and the antisense primer was 5′-CACAGAACTGAGGGTACA-3′, corresponding to positions 88–105 and 446–463, respectively, of the published rat iNOS mRNA sequence (GenBank accession number S71597). PCR products were visualized by electrophoresis through agarose gel stained with ethidium bromide.

2.6. Western blot

The levels of iNOS protein were determined by Western blot. In brief, 10^7 of treated cells were trypsinized, washed twice with PBS, and lysed with 500 μL of lysis buffer (1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 1 μg/mL leupeptin, 1 μg/mL aprotinin) at 4° for 30 min. After centrifugation at 12,000 *g* for 15 min, the supernatant was collected and protein concentration was determined by Bradford method with BSA as standard. Each sample containing 60 μg of cellular protein was subjected to 8% SDS–PAGE and then transferred to PVDF membrane. The membrane was incubated with mouse anti-iNOS or anti-β-actin antibodies, respectively, and then incubated with HRP-conjugated goat anti-mouse IgG antibody. After four washes with 0.05% TBST, the target protein was detected by chemiluminescence.

2.7. Determination of cell viability

Cell viability was assessed by the MTT assay [10]. Astrocytes cultured in 24-well multidishes were treated with LPS/IFN-γ for 12 or 24 hr, respectively. Then, MTT was added to astrocytes (0.5 mg/mL, final concentration). After incubation at 37° for 1 hr, 1 mL of lysis solution (10% SDS, 25% DMF, pH 3.5) was added and the optical density at 570 nm was measured.

Cell injury triggered by the LPS/IFN-γ-treatment was also confirmed by measuring the lactate dehydrogenase (LDH) released from the injured cells into the culture medium [11] using an LDH clinical diagnosis kit (Zhongsheng Bioengineering). The percentage of LDH released into the medium was defined as the ratio of LDH activity in the culture medium to the total LDH activity, where the total LDH activity represents the LDH activity in the cells and the medium. The total LDH activity was measured in sister cultures by freezing/thawing cells rapidly.

2.8. Determination of cytosolic peroxides

The levels of cytosolic peroxides were determined by flow cytometry using DCFH-DA as the peroxide-sensitive fluorescence probe [12]. DCFH-DA is a non-fluorescent compound that can permeate cells freely. When inside cells, it is hydrolyzed to DCFH and is trapped inside cells. Upon oxidation by the cytosolic peroxides, it is converted to the fluorescent compound 2′,7′-dichlorofluorescein (DCF), which can be detected by flow cytometry. Oxidation of DCFH-DA is relatively specific for the intracellular peroxides.

Cells were treated with LPS/IFN-γ for 12 hr, detached by mild trypsination, washed twice with MEM containing 0.2% BSA, and loaded with 5 μM DCFH-DA at 37° for 45 min. After being washed twice, 10,000 cells were analyzed by Coulter EPICS-XL flow cytometer with excitation set at 488 nm and emission at 525 nm.

2.9. Determination of extracellular peroxides

The level of extracellular hydrogen peroxide was determined by HRP/ABTS method [13], which is based on the HRP-catalyzed oxidation of ABTS. Astrocytes cultured in 24-well multidishes were treated with LPS/IFN-γ for 12 or 24 hr, respectively. After drug treatment, 0.4 mL of cell culture supernatant was mixed with 0.4 mL of reaction mixture (5 mM ABTS, 0.2 U HRP, 0.2 M acetate buffer, pH 4.0). After incubation at 37° for 20 min, the absorption at 420 nm was measured immediately. Hydrogen peroxide was used as an external standard.

2.10. Determination of cellular GSH

The cellular GSH level was determined by method described previously [14] with minor modifications. Briefly, 10^7 of treated astrocytes were washed twice with PBS, incubated with 500 μL of 50 μM monochlorobimane at 37° for 10 min, washed twice with PBS, and lysed by 1 mL of 0.2% Triton X-100. The fluorescence intensity with excitation at 400 nm and emission at 480 nm was read by a fluorescence spectrometer. GSH was used as an external standard. The protein concentration was determined by Bradford method.

2.11. Determination of lipid peroxidation

Thiobarbituric acid reactive substances (TBARS), mainly malonydialdehyde, as indicators of lipid peroxidation, were assayed as previously described [10]. Briefly, after exposure to LPS/IFN-γ for 12 hr, 10^7 astrocytes were mixed with 0.4 mL of 2.8% trichloroacetic acid and 0.6 mL of 0.67% thiobarbituric acid, and heated at 95° for 1 hr. After being cooled, 1.5 mL of *n*-butanol was added followed by shaking vigorously. The absorption of the organic layer was determined at 532 nm. The protein concentration was determined by Bradford method.

2.12. Statistical analysis

Each experiment was performed at least three times and the results were presented as mean ± SD. The data were analyzed by one-way ANOVA. A level of $P < 0.05$ was considered significant.

3. Results

3.1. NO production

Exposure of rat cerebral astrocytes to 1 μg/mL LPS plus 100 U/mL IFN-γ induced production of NO as measured by the nitrite assay (Table 1). In astrocytes treated with 1 μg/mL LPS and 100 U/mL IFN-γ for 12 hr, the extracellular nitrite concentration was 518.3 ± 34.5 nmol/mg protein, which was much higher than that of untreated astrocytes (57.8 ± 4.7 nmol/mg protein). In cultures pretreated with 50 μM ESeroS-GS, the NO production was suppressed significantly (189.3 ± 22.4 nmol/mg protein). α-Tocophorol or GSH, two parental compounds of ESeroS-GS, show moderate effect on nitrite production at higher concentration (200 μM). Pretreatment with the selective iNOS inhibitor 1400W dose-dependently inhibited the formation of nitrite (154.6 ± 11.3 nmol/mg protein), suggesting that the nitrite accumulated in the cell culture supernatant was the downstream product of iNOS.

To measure the production of NO directly, ESR spin trapping technique was employed. A three-line ESR spectrum corresponding to the $[ONFe^{2+}(DETC)_2]$ complex at $g = 2.035$ was observed in astrocytes treated with LPS/IFN-γ for 12 hr (Fig. 2A), suggesting that stimulated astrocytes do generate NO. Upon pretreatment with 50 μM of the novel antioxidant ESeroS-GS, the signal intensity of $[ONFe^{2+}(DETC)_2]$ complex decreased significantly as shown in Fig. 2B as well as in Table 2 (relative signal intensity 33.0 ± 3.9% of control cells). Two hundred micromoles of α-tocophorol or GSH shows only moderate inhibition effect on NO production. To elucidate whether the detected NO was generated *via* iNOS, the effect of the selective iNOS inhibitor 1400W, on NO generation was tested. Pretreatment with 20 μM 1400W decreased the signal intensity of $[ONFe^{2+}(DETC)_2]$ complex markedly

Table 1
Effect of ESeroS-GS on nitrite accumulation

	Nitrite (nmol/mg protein)
Untreated cells	57.8 ± 4.7
LPS/IFN-γ	518.3 ± 34.5
LPS/IFN-γ + 5 μM ESeroS-GS	221.8 ± 14.2*
LPS/IFN-γ + 50 μM EseroS-GS	189.3 ± 22.4*
LPS/IFN-γ + 50 μM α-tocopherol	453.1 ± 12.5
LPS/IFN-γ + 200 μM α-tocopherol	387.4 ± 14.6*
LPS/IFN-γ + 50 μM GSH	486.3 ± 20.8
LPS/IFN-γ + 200 μM GSH	404.1 ± 16.3*
LPS/IFN-γ + 2 μM 1400W	259.3 ± 21.5*
LPS/IFN-γ + 20 μM 1400W	154.6 ± 11.3*

Cells were pretreated with different drugs for 3 hr and then stimulated with 1 μg/mL LPS plus 100 U/mL IFN-γ for 12 hr. The production of NO was analyzed by nitrite assay. Data are means ± SD of six different experiments.

* $P < 0.01$ in comparison with cells treated with LPS/IFN-γ.

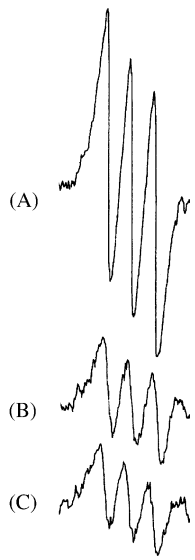

Fig. 2. Direct measurement of NO production by ESR spin trapping. Cells were pretreated with ESeroS-GS (50 μM) or 1400W (20 μM) for 3 hr and then stimulated with 1 μg/mL LPS plus 100 U/mL IFN-γ for 12 hr. The production of NO was detected by ESR spin trapping. (A) Control cells; (B) cells pretreated with 50 μM ESeroS-GS; (C) cells pretreated with 20 μM 1400W.

Table 2
Effect of ESeroS-GS on NO production

	Relative signal intensity (%)
Untreated cells	0
LPS/IFN-γ	100 ± 4.1
LPS/IFN-γ + 50 μM EseroS-GS	33.0 ± 3.9*
LPS/IFN-γ + 200 μM α-tocopherol	68.2 ± 3.6*
LPS/IFN-γ + 200 μM GSH	76.3 ± 0.8*
LPS/IFN-γ + 20 μM 1400W	27.2 ± 4.1*

Cells were pretreated with 50 μM ESeroS-GS or 20 μM 1400W for 3 hr and then stimulated with 1 μg/mL LPS plus 100 U/mL IFN-γ for 12 hr. The production of NO was analyzed by ESR spin trapping. Data are mean ± SD of three different experiments.

* $P < 0.01$ in comparison with cells treated with LPS/IFN-γ.

(Fig. 2C and Table 2; relative signal intensity 27.2 ± 4.1% of stimulated cells), suggesting that LPS/IFN-γ-induced NO production in astrocytes was dependent on iNOS-mediated L-arginine metabolism.

3.2. Induction of iNOS expression

To understand the mechanism of inhibition of NO production in astrocytes by the novel antioxidant ESeroS-GS, we examined the effect of the novel antioxidant ESeroS-GS on the expression of iNOS gene by RT–PCR as well as by Western blot. Upon exposure to 1 μg/mL LPS plus 100 U/mL IFN-γ, the expression level of iNOS gene increased time-dependently as shown in Fig. 3A and B. Twelve hours after LPS/IFN-γ-treatment, the amount of iNOS mRNA and iNOS protein increased more then 10-fold in comparison with untreated cells. Pretreatment with 50 μM ESeroS-GS for 3 hr before LPS/IFN-γ-treatment significantly inhibited the expression of iNOS gene.

3.3. Cell death

Upon treatment with LPS/IFN-γ, astrocytes died gradually as measured by the MTT assay (Table 3). In astrocytes exposed to LPS/IFN-γ for 12 hr, the cell viability

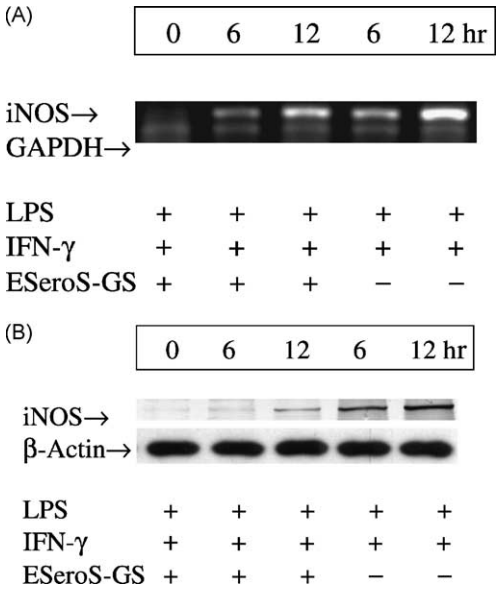

Fig. 3. Detection of iNOS expression. (A) Detection of iNOS mRNA. Cells were preincubated with or without 50 μM ESeroS-GS for 3 hr and then exposed to 1 μg/mL LPS plus 100 U/mL IFN-γ for indicated time. The expression of iNOS was detected by RT–PCR. (B) Detection of iNOS protein. Cells were preincubated with or without 50 μM ESeroS-GS for 3 hr and then exposed to 1 μg/mL LPS plus 100 U/mL IFN-γ for indicated time. The expression of iNOS was detected by Western blot.

decreased slightly (92.4 ± 3.3%). However, the viability of LPS/IFN-γ-treated astrocytes decreased drastically after a further 12-hr culture (52.2 ± 6.8%; $P < 0.01$ in comparison with untreated cells). Cell injury was further confirmed by the LDH efflux assay. LPS/IFN-γ-treatment induced significant leakage of the cytosolic enzyme LDH into the culture medium, a marker of severe cell injury. Twenty-four hours after LPS/IFN-γ-treatment, up to 27.4 ± 3.3% of the total cytosolic LDH released into the cell culture medium. The antioxidant ESeroS-GS showed significant protective effect on LPS/IFN-γ-induced cell death. Preincubation with the iNOS inhibitor 1400W also effectively attenuated cell death induced by LPS/IFN-γ. After exposure to LPS/IFN-γ for 24 hr, the viability of

Table 3
Cell viability of astrocytes

	Cell viability (%)		LDH release (%)	
	12 hr	24 hr	12 hr	24 hr
Untreated cells	100 ± 4.7	101.3 ± 2.8	2.1 ± 0.2	3.3 ± 1.7
LPS/IFN-γ	92.4 ± 3.3	52.2 ± 6.8*	8.3 ± 2.8	27.4 ± 3.3*
LPS/IFN-γ + 50 μM EseroS-GS	94.3 ± 2.1	88.6 ± 3.9**	6.4 ± 2.9	5.3 ± 4.4**
LPS/IFN-γ + 200 μM α-tocopherol	93.3 ± 3.2	70.3 ± 3.3**	5.9 ± 3.6	20.7 ± 3.1
LPS/IFN-γ + 200 μM GSH	92.6 ± 2.8	69.3 ± 6.1	4.7 ± 2.2	23.5 ± 1.7
LPS/IFN-γ + 20 μM 1400W	91.8 ± 3.2	87.2 ± 4.3**	4.6 ± 3.4	7.1 ± 2.2**
50 μM EseroS-GS	97.5 ± 2.1	98.8 ± 3.6	1.7 ± 0.9	2.4 ± 1.0
100 μM EseroS-GS	99.3 ± 4.7	97.8 ± 4.2	2.8 ± 0.6	3.1 ± 0.9

Cells were pretreated with ESeroS-GS, 1400W, α-tocopherol, or GSH for 3 hr and then exposed to 1 μg/mL LPS plus 100 U/mL IFN-γ for 12 or 24 hr. The cell viability was assessed by both MTT assay and LDH efflux assay. Data are mean ± SD of six samples.

* $P < 0.01$ in comparison with untreated cells.

** $P < 0.05$ in comparison with LPS/IFN-γ-treated control cells.

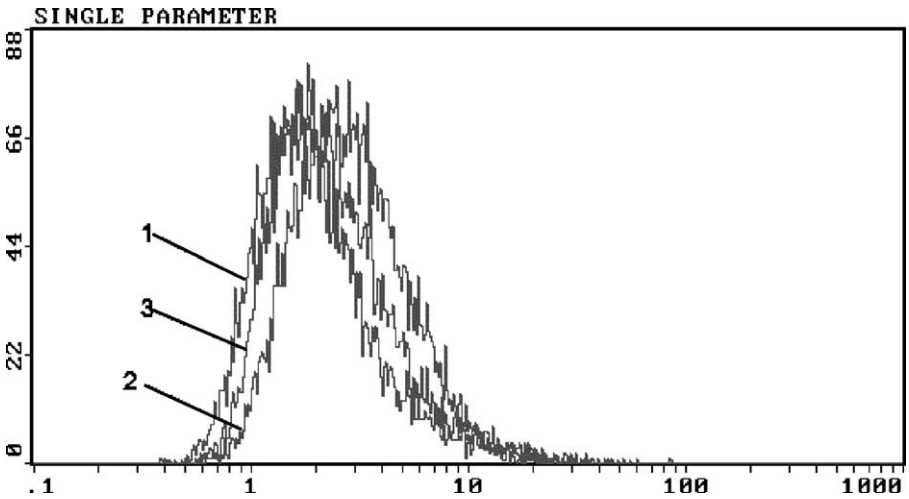

Fig. 4. Cytosolic peroxide formation in astrocytes. After treatment, cells were loaded with 5 μM DCFH-DA for 45 min and analyzed by flow cytometry. (1) Untreated astrocytes; (2) astrocytes exposed to 1 μg/mL LPS plus 100 U/mL IFN-γ for 12 hr; (3) astrocytes pretreated with 50 μM ESeroS-GS for 3 hr and then exposed to LPS/IFN-γ for 12 hr.

astrocytes pretreated with 50 μM ESeroS-GS or 20 μM 1400W was 88.6 ± 3.9% and 87.2 ± 4.3%, respectively. Similarly, LDH efflux induced by LPS/IFN-γ-treatment was also suppressed significantly by both EseroS-GS and 1400W. The parental compounds of ESeroS-GS, α-tocophorol or GSH, show only moderate protective effects on astrocytes at higher concentration (up to 200 μM).

3.4. Increase of cytoslic peroxides

Hydrogen peroxide and some other downstream products of superoxide (such as peroxynitrite and some organic peroxides), but not superoxide itself, could be detected by flow cytometry using DCFH-DA as the oxidative-sensitive fluorescence probe. Upon stimulation with LPS/IFN-γ for 12 hr, the fluorescence intensity of DCF (generated from DCFH-DA upon oxidation) increased significantly as shown in Fig. 4. The average fluorescence intensity of stimulated astrocytes was 2.97 ± 0.31 (Table 4), which was significantly higher then that of untreated cells (2.01 ± 0.12). In cells pretreated with 50 μM EseroS-GS, the cytosolic peroxide level was lower (average fluorescence intensity: 2.31 ± 0.18). Blockading the formation of endogenous NO by pretreatment with 20 μM 1400W effectively prevented the formation of cytosolic peroxides; the average fluorescence intensity of 1400W-treated astrocytes was 2.28 ± 0.15.

Table 4
Effect of ESeroS-GS on cytosolic peroxides

	Fluorescence intensity (arbitrary unit)
Untreated cells	2.01 ± 0.12
LPS/IFN-γ	2.97 ± 0.31*
LPS/IFN-γ + 50 μM ESeroS-GS	2.31 ± 0.18**
LPS/IFN-γ + 20 μM 1400W	2.28 ± 0.15**

Cells were pretreated with 50 μM ESeroS-GS or 20 μM 1400W for 3 hr and then stimulated with 1 μg/mL LPS plus 100 U/mL IFN-γ for 12 hr. The accumulation of cytosolic NO was analyzed by flow cytometry using DCFH-DA as the redox-sensitive probe. Data are mean ± SD of three different experiments.

* $P < 0.01$ in comparison with untreated cells.

** $P < 0.05$ in comparison with cells treated with LPS/IFN-γ.

3.5. Formation of extracellular peroxides

The accumulation of extracellular peroxides was determined by HRP/ABTS method. LPS/IFN-γ-treatment caused the accumulation of extracellular peroxide as shown in Fig. 5. In astrocytes exposed to LPS/IFN-γ for 12 hr, the extracellular peroxide level was 12.6 ± 0.4 nM, which was about 6-fold higher than that of untreated cells (1.8 ± 0.2 nM). The antioxidant ESeroS-GS inhibited the formation of cytosolic peroxide markedly. In cells

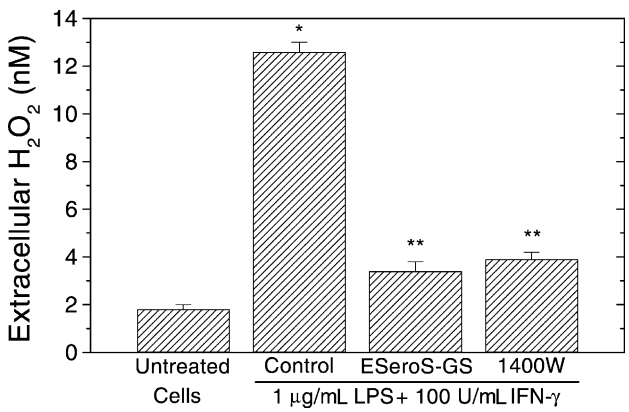

Fig. 5. Extracellular peroxide content in astrocytes. Cells were pretreated with 50 μM ESeroS-GS or 20 μM 1400W for 3 hr and then exposed to 1 μg/mL LPS plus 100 U/mL IFN-γ for 12 hr. The extracellular hydrogen peroxide content was determined spectrometrically. Data are mean ± SD of four samples. *$P < 0.01$ in comparison with untreated cells; **$P < 0.01$ in comparison with LPS/IFN-γ-treated control cells.

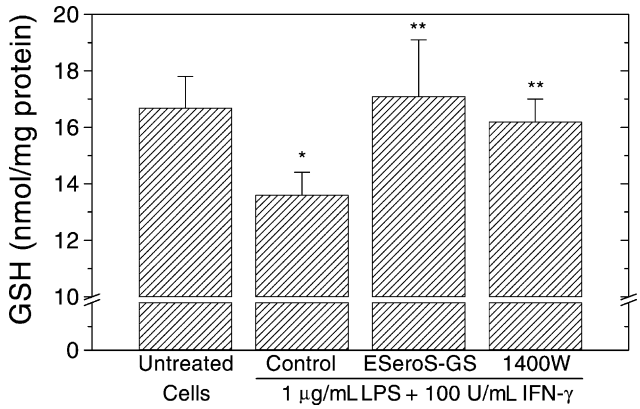

Fig. 6. Cellular GSH contents in astrocytes. Cells were pretreated with 50 μM ESeroS-GS or 20 μM 1400W for 3 hr and then exposed to 1 μg/mL LPS plus 100 U/mL IFN-γ for 12 hr. The cellular GSH content was determined fluorometrically. Data are mean ± SD of six samples. $^{*}P < 0.05$ in comparison with untreated cells; $^{**}P < 0.05$ in comparison with LPS/IFN-γ-treated control cells.

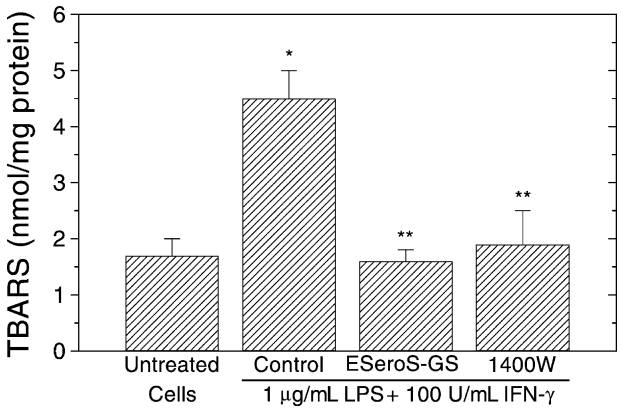

Fig. 7. Lipid peroxidation in astrocytes. Cells were pretreated with 50 μM ESeroS-GS or 20 μM 1400W for 3 hr and then exposed to 1 μg/mL LPS plus 100 U/mL IFN-γ for 12 hr. The lipid peroxidation was determined by TBA assay. Data are mean ± SD of four samples. $^{*}P < 0.01$ in comparison with untreated cells; $^{**}P < 0.01$ in comparison with LPS/IFN-γ-treated control cells.

pretreated with 50 μM ESeroS-GS, the extracellular peroxide level was 3.4 ± 0.4 nM. Pretreatment with 1400W also prevented the formation of extracellular peroxides effectively, suggesting that the accumulation of extracellular peroxide is due to the generation of endogenous NO.

3.6. Decrease of cellular GSH

GSH is one of the most important members of the endogenous non-enzymatic antioxidant defense system and plays important roles in the maintenance of cellular redox homeostasis. The cellular GSH level was determined by fluorescence method. After exposure to LPS/IFN-γ for 12 hr, the cellular GSH level decreased significantly (13.6 ± 0.8 nmol/mg protein vs. 16.7 ± 1.1 nmol/mg protein of untreated astrocytes) as shown in Fig. 6. Pretreating cells with either the antioxidant ESeroS-GS or the iNOS inhibitor 1400W prevented the decrease in the cellular GSH level significantly; the cellular GSH content of astrocytes pretreated with ESeroS-GS or 1400W was 17.1 ± 2.0 nmol/mg protein and 16.2 ± 0.8 nmol/mg protein, respectively.

3.7. Lipid peroxidation

By TBA assay, the lipid peroxidation level in astrocytes was measured. Lipid peroxidation level in astrocytes treated with LPS/IFN-γ increased significantly as indicated by the formation of TBARS (Fig. 7). In astrocytes treated with LPS/IFN-γ for 12 hr, the TBARS level was 4.5 ± 0.5 nmol/mg protein, which was about 1.6-fold higher than that of untreated cells (1.7 ± 0.3 nmol/mg protein). Both ESeroS-GS and 1400W inhibited the formation of TBARS effectively. The TBARS level of astrocytes pretreated with ESeroS-GS or 1400W was 1.6 ± 0.2 nmol/mg protein and 1.9 ± 0.6 nmol/mg protein, respectively.

4. Discussion

In astrocytes, NO is mainly synthesized by calcium/calmodulin-independent iNOS. Cytokines such as IFN-γ and some endogenous peptides or proteins such as β-amyloid peptide and S100β peptide can induce the expression of iNOS gene [15], and trigger the subsequent production of large amounts of NO in astrocytes. NO may cause cell death in both astrocytes and neighboring neurons. It has been proposed that several neuronal diseases, such as Alzheimer's disease and Parkinson's disease, might be related to the high concentration of NO generated by activated astrocytes upon stimulation with cytokines [16,17]. Accordingly, compounds that can regulate the production of NO in astrocytes and antagonize the cytotoxicity induced by endogenous NO might be used as potential drugs for neuronal diseases associated with endogenous NO.

In the present investigation, we used LPS/IFN-γ-induced NO production in astrocytes as the experimental model for the pathophysiological generation of endogenous NO. Exposure of astrocytes to 1 μg/mL LPS plus 100 U/mL IFN-γ for 12 hr induced the expression of iNOS gene, as confirmed by the increase of iNOS mRNA and protein (~10-fold higher then untreated astrocytes). Induction of iNOS gene caused the subsequent production of large amounts of NO as measured by nitrite assay and by ESR spin trapping technique as well. LPS/IFN-γ-treatment resulted in cell death finally in astrocytes as determined by both MTT assay and LDH efflux assay.

In order to elucidate whether cell death induced by LPS/IFN-γ-treatment was due to oxidative stress induced by endogenous NO, we measured the alteration of peroxide levels and cellular GSH levels after exposure of cells to LPS/IFN-γ. LPS/IFN-γ-treatment caused significant increase in the cytosolic peroxides and the accumulation of extracellular peroxides, and induced lipid peroxidation as measured by the increase of cellular TBARS levels. Also

cellular GSH level decreased significantly after exposure to LPS/IFN-γ. Pretreatment with the competitive iNOS inhibitor 1400W, which decreased the formation of NO markedly, inhibited oxidative stress induced by LPS/IFN-γ-treatment, and prevented astrocytes from death. These findings suggest that the oxidative stress and the subsequent cell death induced by LPS/IFN-γ-treatment be due to iNOS-generated endogenous NO. It has been reported that the induction of iNOS causes the inhibition of mitochondrial enzymes in astrocytes and neighboring neurons *via* generation of NO [18]. However, blockade of the mitochondrial electron transport chain by NO may cause the formation of endogenous reactive oxygen species (ROS) as observed in both isolated mitochondria [19] and intact cells [20]. In the present investigation, we directly observed the formation of cytosolic and extracellular ROS in astrocytes stimulated with LPS/IFN-γ for the first time (Figs. 4 and 5 and Table 2). We have reported that exogenous NO generated by NO donors causes oxidative damage and results in cell death in cultured neurons [20]. Comparing that with the findings of present investigation, we may conclude that induction of oxidative stress might be one of the common pathways in NO-induced cytotoxicity in both neuronal and glial cells. In this regard, antioxidants might protect cells from oxidative damage.

We tested the effects of a novel antioxidant ESeroS-GS on the synthesis of NO in astrocytes for the first time. ESeroS-GS contains α-tocopherol and GSH on succinic acid as the core. Its special structure endows it with both hydrophobic and hydrophilic characters. Previous report [5] and our preliminary experiments had revealed that ESeroS-GS is a scavenger on hydrophilic superoxide radicals, hydroxyl radicals, and hydrophobic DPPH radicals as well (data not shown). Also the toxicity of ESeroS-GS is quite low. Up to 100 μM ESeroS-GS showed no influence on the viability of astrocytes. Results of both nitrite assay and ESR spin trapping demonstrated that 50 μM ESeroS-GS, but not its parental compounds α-tocopherol or GSH, significantly inhibited the production of NO in LPS/IFN-γ-treated astrocytes. α-Tocopherol or GSH only show moderate inhibition effects on NO production at higher concentration (200 μM).

To elucidate the mechanism of inhibition of NO production by ESeroS-GS, the effects of ESeroS-GS on iNOS gene expression were measured. In astrocytes preincubated with 50 μM ESeroS-GS for 3 hr, the expression of iNOS induced by LPS/IFN-γ was suppressed significantly as measured by both RT–PCR and by Western blot. Inhibition of iNOS gene expression by ESeroS-GS suggested that the expression of iNOS gene be redox sensitive. Similar with other antioxidant, such as *N*-acetyl cysteine, ESeroS-GS might regulate the expression of iNOS gene by different pathways, especially the NF-κB pathway [8].

ESeroS-GS pretreatment effectively prevented astrocytes from oxidative stress and the subsequent cell death induced by LPS/IFN-γ. However, the two parental compounds of ESeroS-GS, α-tocopherol or GSH, only showed moderate protective effects on astrocytes. Considering ESeroS-GS could inhibit the expression of iNOS gene and could act as potent ROS scavenger, we may conclude that the attenuation effects of ESeroS-GS on endogenous NO-induced oxidative stress might be due to two different pathways. The first pathway is that ESeroS-GS inhibited the expression of iNOS gene, decreased the formation of endogenous NO, and thus prevented NO-induced oxidative stress. Another pathway is that ESeroS-GS, as an effective antioxidant, could scavenge the endogenous ROS induced by NO and prevented oxidative stress. Comparing with the iNOS inhibitor 1400W, which protected astrocytes from LPS/IFN-γ-induced oxidative cell death *via* the first pathway, the efficacy of ESeroS-GS in protecting cells from oxidative damage induced by endogenous NO should be the overall effect of the two different pathways.

In conclusion, induction of the expression of iNOS in astrocytes caused the generation of large amounts of NO, which triggered oxidative stress and resulted in cell death finally. Pretreatment with the novel antioxidant ESeroS-GS effectively inhibited the expression of iNOS, decreased the generation of NO, and prevented astrocytes from oxidative damage. In the present experiment system, ESeroS-GS is more effective then its two parental compounds α-tocopherol or GSH. The results of present investigation suggest that ESeroS-GS might be used as a potential agent for the prevention and therapy of diseases associated with the overproduction of NO by activated astrocytes.

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