

Effects of Hydrogen Sulfide-releasing L-DOPA Derivatives on Glial Activation

POTENTIAL FOR TREATING PARKINSON DISEASE^{*[5]}

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The main lesion in Parkinson disease (PD) is loss of substantia nigra dopaminergic neurons. Levodopa (L-DOPA) is the most widely used therapy, but it does not arrest disease progression. Some possible contributing factors to the continuing neuronal loss are oxidative stress, including oxidation of L-DOPA, and neurotoxins generated by locally activated microglia and astrocytes. A possible method of reducing these factors is to produce L-DOPA hybrid compounds that have antioxidant and anti-inflammatory properties. Here we demonstrate the properties of four such L-DOPA hybrids based on coupling L-DOPA to four different hydrogen sulfide-donating compounds. The donors themselves were shown to be capable of conversion by isolated mitochondria to H₂S or equivalent SH⁻ ions. This capability was confirmed by *in vivo* results, showing a large increase in intracerebral dopamine and glutathione after iv administration in rats. When human microglia, astrocytes, and SH-SY5Y neuroblastoma cells were treated with these donating agents, they all accumulated H₂S intracellularly as did their derivatives coupled to L-DOPA. The donating agents and the L-DOPA hybrids reduced the release of tumor necrosis factor- α , interleukin-6, and nitric oxide from stimulated microglia, astrocytes as well as the THP-1 and U373 cell lines. They also demonstrated a neuroprotective effect by reducing the toxicity of supernatants from these stimulated cells to SH-SY5Y cells. L-DOPA itself was without effect in any of these assays. The H₂S-releasing L-DOPA hybrid molecules also inhibited MAO B activity. They may be useful for the treatment of PD because of their significant anti-inflammatory, antioxidant, and neuroprotective properties.

The pathogenesis of Parkinson disease (PD)² results primarily from loss of neurons, especially dopaminergic neurons of the

substantia nigra zona compacta. Levodopa (L-DOPA) therapy is the most widely used treatment because it helps to compensate for the deficiency of dopamine. Whereas L-DOPA treatment is very successful in the early stages of PD, it does not arrest disease progression, and in the long term there are unwanted side effects, such as the development of dyskinesias (1–3). Many previous studies have investigated the reasons for such long term problems. One suggested mechanism is loss of neurons induced by oxidative stress, including the oxidation of L-DOPA (4–6). Another is persisting neuroinflammation caused by activated microglia and astrocytes (7–10). Hybrid molecules which combine L-DOPA with antioxidant and anti-inflammatory moieties might therefore have therapeutic potential.

Hydrogen sulfide (H₂S) has traditionally been regarded as nothing more than a noxious gas with an extremely unpleasant odor. But it is an essential body product. In solution it is a powerful reducing agent with endogenous antioxidant and neuroprotective properties. It is synthesized by the action of two enzymes, cystathione β -synthase (CBS) and cystathione γ -lyase (CGL). CBS is the main enzyme producing H₂S in the brain (11, 12), whereas CGL is the main enzyme producing it in vascular tissue (13). Much attention has been given to this molecule because it has multiple physiological and pathophysiological functions in body organs. A protective role for H₂S in neurons against oxidative stress has been reported (14). It has also been demonstrated that H₂S induces alterations in calcium channels [Ca²⁺]_i in astrocytes and microglia (15, 16) and enhances both NMDA receptor-mediated neurotransmission and long term potentiation (17).

Oxidative stress and neuronal cell death in PD is associated with microglial activation, which involves the release of pro-inflammatory cytokines and free radicals (9, 10). Recently, protective functions of H₂S against LPS-induced inflammation in primary cultured and immortalized microglia have been reported (12, 18, 19). Such a protective effect has been demonstrated *in vivo* in the 6-OHDA rat model of Parkinson disease (20).

To date there are no disease-modifying drugs for the treatment of PD. As an approach to the development of next generation agents, we have prepared hybrid compounds designed to

β -synthase; LPS, lipopolysaccharide; IFN, interferon; LDH, lactate dehydrogenase; MAO, monoamine oxidase.

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S11.

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² The abbreviations used are: PD, Parkinson disease; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IL, interleukin; TNF, tumor necrosis factor; MOPS, 4-morpholinepropanesulfonic acid; OPT, *o*-phthalaldehyde; ANOVA, analysis of variance; CBS, cystathione

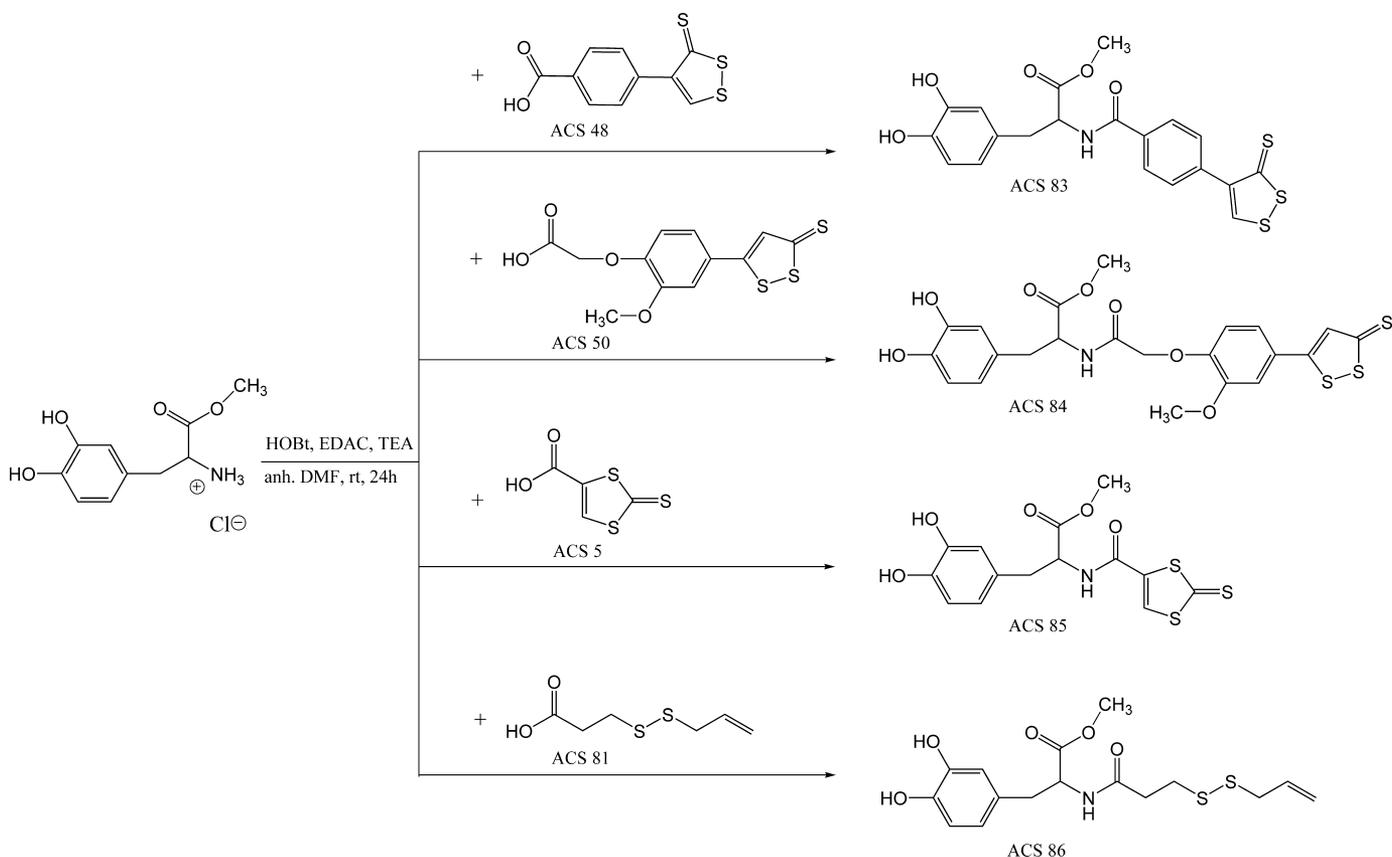


FIGURE 1. The structure and synthetic scheme of H₂S-releasing agents ACS 48, ACS 50, ACS 5, and ACS 81 and the H₂S-releasing L-DOPA derivatives, ACS 83, ACS 84, ACS 85, and ACS 86.

combine the dopamine replacement properties of L-DOPA with the neuroprotective properties of H₂S donors.

In this investigation, we synthesized four H₂S-releasing moieties (ACS48, ACS50, ACS5, and ACS81) and examined whether they release H₂S or equivalent SH⁻ ions in both glia and neurons. The sulfurated moieties were chosen among those resembling the structures of known H₂S-releasing compounds such as the dithiolethione ADT-OH (21) and diallyl-disulfide (22). We coupled these moieties to L-DOPA methyl ester through an amide linkage to create different lipophilic compounds potentially able to release H₂S. The four hybrid molecules were designated ACS83, ACS84, ACS85, and ACS86. The structure of the four donors and the four hybrid molecules are shown in Fig. 1. We made a pilot *in vivo* test with ACS84 to confirm that these types of compounds can reach the brain.

We found that it reached the brain and that it produced an increase of intracerebral dopamine and glutathione. We tested the effects of all these compounds on prevention of neuronal cell death induced by stimulation of four types of cultured human glial cells: astrocytes, microglia, and the THP-1 and U373 cell lines. NaSH was used as the standard H₂S donor for comparative purposes. We found that these hybrid molecules were able to release H₂S or equivalent ions from all cell types tested, and from mitochondria isolated from U373 cells.

EXPERIMENTAL PROCEDURES

Materials—All reagents were purchased from Sigma unless otherwise stated. The following substances were applied to the

cell cultures: bacterial LPS (from *Escherichia coli* 055:B5) and human recombinant interferon- γ (INF γ) (from Bachem California, Torrance, CA). The following substances were used in the assays: diaphorase (EC 1.8.1.4, from *Clostridium kluyveri*, 5.8 units/mg solid), *p*-iodonitrotetrazolium violet (INT), nicotinamide adenine dinucleotide (NAD⁺), and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).

Chemistry—Some of the sulfurated intermediates were already known and were prepared according to the literature. All novel compounds synthesized were characterized by melting point (m.p.; Buchi apparatus), ¹H NMR spectra (Varian Mercuri 300VX spectrometer) and high-resolution mass spectra (HRMS; APEX II ICR-FTMS Bruker Daltonics mass spectrometer, ESI). Log P were calculated by ChemDraw Ultra 9.0 software.

ACS48 (4-(3-thioxo-3H-1,2-dithiol-4-yl)-benzoic acid) was obtained by hydrolysis of the corresponding methyl ester, which was synthesized as previously described by Adelaere (23). ACS50 ([2-methoxy-4-(3-thioxo-3H-1,2-dithiol-5-yl)-phenoxy]acetic acid, m.p. 198–200 °C) was obtained by acidic hydrolysis of the corresponding methyl ester, which was synthesized as described by Lozac'h and Mollier (24). ACS5 (1,3-dithiole-2-thioxo-4-carboxylic acid) was synthesized as previously described by Dartigues *et al.* (25). ACS81 (3-(prop-2-en-1-yl)disulfanyl)propanoic acid) was synthesized as follows: to a stirred solution of diallyl disulfide (2.4g; 13.6 mmol) in a mixture of ether (10 ml) and methanol (20 ml), under nitrogen

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atmosphere at room temperature, was added a solution of 3-mercaptopropanoic acid (0.49 g, 4.6 mmol) in ether (5 ml), followed by a solution of 10 M NaOH (0.46 ml). The reaction mixture was stirred at room temperature for 24 h and, after evaporation of the solvents under reduced pressure, the crude compound was taken up with ether and 1 N HCl. After separation of the organic phase and evaporation of the ether, the residue was purified by column chromatography on silica gel, eluting with CH₂Cl₂/CH₃COOC₂H₅ (60:40). A colorless oil was obtained (520 mg; yield 63%). ¹H NMR (CDCl₃): δ = 5.91–5.77 (m, 1H); 5.24–5.13 (m, 2H); 3.33 (d, *J* = 7.30 Hz, 2H); 2.92 (t, *J* = 6.90 Hz, 2H); 2.80 (t, *J* = 6.90 Hz, 2H). HMRS (ESI) *m/z* calculated for C₆H₁₀O₂S₂Na [M+Na]⁺: 201.00144; found: 201.00147.

Synthesis of Sulfurated Derivatives of L-DOPA Methyl Ester (ACS83, ACS84, ACS85, and ACS86)—L-DOPA methyl ester hydrochloride (200 mg, 0.8 mmol), 1-hydroxybenzotriazole (HOBt, 185 mg; 1.2 mmol) and 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride (EDAC, 232 mg; 0.96 mmol) were added to a solution of the proper sulfurated compounds (ACS48, or ACS50, or ACS5, or ACS81; 0.8 mmol) in anhydrous *N,N*-dimethylformamide (DMF) (4 ml) and, after the addition of triethylamine (0.22 ml; 1.6 mmol), the solution was stirred at room temperature for 24 h under a nitrogen atmosphere. After evaporation of DMF, the residue was dissolved in dichloromethane and the organic solution was washed sequentially with water, 1 N HCl, and water and finally dried with anhydrous Na₂SO₄ and evaporated to dryness. The crude compound was purified by flash chromatography (silica, CH₂Cl₂/MeOH, 98:2).

Methyl 3-(3,4-dihydroxyphenyl)-2-(4-(3-thioxo-3H-1,2-dithiol-4-yl)benzamido)propanoate (ACS83)—Yield 35%. M.p. 160–165 °C. ¹H NMR (DMSO-*d*₆): δ = 9.21 (s, 1H); 8.81 (d, *J* = 7.63 Hz, 1H, collapses with D₂O); 8.74 (s, 1H, collapses with D₂O); 8.66 (s, 1H, collapses with D₂O); 7.85 (d, *J* = 8.21 Hz, 2H); 7.65 (d, *J* = 8.21 Hz, 2H); 6.65 (d, *J* = 1.76 Hz, 1H); 6.59 (d, *J* = 8.21 Hz, 1H); 6.51 (dd, *J* = 1.76, 8.21 Hz, 1H); 4.57–4.50 (m, 1H); 3.62 (s, 3H); 2.99–2.85 (m, 2H). HMRS (ESI) *m/z* calculated for C₂₀H₁₇NO₅S₃Na [M+Na]⁺: 470.01611; found: 470.01620. Calc. log P: 3.58.

Methyl 3-(3,4-dihydroxyphenyl)-2-(2-(2-methoxy-4-(3-thioxo-3H-1,2-dithiol-5-yl)phenoxy)acetoamido)propanoate, (ACS84)—Yield 53%. M.p. 153–155 °C. ¹H NMR (DMSO-*d*₆): δ = 8.79 (s, 1H, collapses with D₂O); 8.75 (s, 1H, collapses with D₂O); 8.29 (d, *J* = 7.91 Hz, 1H, collapses with D₂O); 7.85 (s, 1H); 7.42–7.37 (m, 2H); 6.77 (d, *J* = 8.21 Hz, 1H); 6.61 (d, *J* = 7.91 Hz, 1H); 6.56 (d, *J* = 2.05 Hz, 1H); 6.41 (dd, *J* = 2.05, 7.91 Hz, 1H); 4.65–4.54 (m, 2H); 4.51–4.43 (m, 1H); 3.85 (s, 3H); 3.61 (s, 3H); 2.91–2.72 (m, 2H). HMRS (ESI) *m/z* calculated for C₂₂H₂₁NO₇S₃Na [M+Na]⁺: 530.03724; found: 530.03660. Calc. log P: 3.19.

Methyl 3-(3,4-dihydroxyphenyl)-2-(2-thioxo-1,3-dithiole-4-carboxamido)propanoate, (ACS85)—Yield 56%. M.p. 66–70 °C. ¹H NMR (DMSO-*d*₆): δ = 9.24 (d, 1H, *J* = 7.92 Hz, collapses with D₂O); 8.76 (s, 1H, collapses with D₂O); 8.71 (s, 1H, collapses with D₂O); 8.31 (s, 1H); 6.61–6.58 (m, 2H); 6.46 (dd, *J* = 1.76, 7.92 Hz, 1H); 4.47–4.39 (m, 1H); 3.61 (s, 3H); 2.96–2.76

(m, 2H). HMRS (ESI) *m/z* calculated for C₁₄H₁₃NO₅S₃Na [M+Na]⁺: 393.98481; found: 393.98524. Calc. log P: 1.68.

Methyl 2-(3-(allyldisulfanyl)propanamido)-3-(3,4-dihydroxyphenyl)propanoate, (ACS86)—Yield 50%. Semisolid; ¹H NMR (DMSO-*d*₆): δ = 8.74 (s, 1H, collapses with D₂O); 8.68 (s, 1H, collapses with D₂O); 8.34 (d, *J* = 7.92 Hz, 1H, collapses with D₂O); 6.59 (d, *J* = 7.91 Hz, 1H); 6.54 (d, *J* = 2.06 Hz, 1H); 6.41 (dd, *J* = 2.06, 7.91 Hz, 1H); 5.86–5.72 (m, 1H); 5.20–5.09 (m, 2H); 4.37–4.30 (m, 1H); 3.56 (s, 3H); 3.35 (d, *J* = 7.33 Hz, 2H); 2.82–2.64 (m, 4H); 2.49–2.46 (m, 2H). HMRS (ESI) *m/z* calculated for C₁₆H₂₁NO₅S₂Na [M+Na]⁺: 394.07534; found: 394.07469. Calc. log P: 2.32.

Animal Treatments and Sample Processing—Sprague-Dawley rats (350–400 g) were purchased from Charles River. Rats received administration of ACS84 dissolved in 500 μl of PEG400 via the caudal vein. After 1 h, animals were anesthetized with pentobarbital (60 mg/kg), and blood was collected from the abdominal aorta in tubes containing 50 mg/ml K₃EDTA and immediately processed. Plasma was obtained by centrifugation of blood at 10,000 × *g* for 15 s and immediately deproteinized by addition of 4 volumes of acetonitrile (ACN). The brain was then removed and cut longitudinally to obtain 2 equal halves. One half was homogenized in 5 volumes of 4% (w/v) trichloroacetic acid containing 1 mM K₃EDTA for the analyses of glutathione and dopamine. The other half was homogenized in 5 volumes of a solution of 80% (v/v) acetonitrile to measure ACS84 and its metabolites. All animal manipulations were made in accordance with the European Community guidelines for the use of laboratory animals. The experiments were authorized by the local ethical committee.

HPLC Analyses of ACS84 Metabolites—For the determination of ACS84, ACS84-a, and ACS50, both brain and plasma samples were centrifuged to discard acetonitrile-denatured proteins (10,000 × *g* for 2 min). The clear supernatants were then diluted 1:1 with 0.05% trifluoroacetic acid, loaded onto an HPLC (Zorbax Eclipse XDB-C18 column, 4.6 × 150 mm, 5 μm, Agilent Technologies, Milan, Italy) and separated by the application of a trifluoroacetic acid/acetonitrile solution: 0–9 min, 40% ACN in 0.05% (v/v) trifluoroacetic acid; 9–10 min, 40–90% ACN gradient. Analytes were detected at 435-nm wavelength. The identity of the peak was determined by analyzing plasma samples spiked with the respective authentic compounds. Calibration curves were generated in the 0.2–100 μM range by addition of ACS50, ACS84, or ACS84-a to rat plasma samples obtained from untreated animals.

HPLC Analyses of Dopamine and Glutathione for in Vivo Analysis—For the determination of GSH and dopamine, samples were centrifuged to discard proteins (10,000 × *g* for 2 min). Dopamine was measured on the clear supernatant by UV-HPLC with fluorescence detection (excitation at 270 nm, emission at 320 nm, Zorbax Eclipse XDB-C18 column, 4.6 × 150 mm, 5 μm). Separation was carried out by the application of an isocratic run: 5% (v/v) methanol in 0.05% (v/v) trifluoroacetic acid (26). Another aliquot of the supernatant (0.1 ml) was brought to pH ~8.0 with 15 μl of 2 M Tris, and then 3 μl of 40 mM monobromobimane (mBrB) were added for the analysis of GSH. After 10 min of incubation in the dark, samples were

acidified and analyzed by HPLC (Zorbax Eclipse XDB-C18 column, 4.6 × 150 mm, 5 μm) as previously described (27).

Cell Culture and Experimental Protocols—The human monocyte THP-1 and astrocytoma U373 cell lines were obtained from the American Type Culture Collection (ATCC). The human neuroblastoma SH-SY5Y cell line was a gift from Dr. R. Ross, Fordham University, NY. These cells were grown in DMEM/F12 medium containing 10% fetal bovine serum (FBS), 100 international units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) under humidified 5% CO₂ and 95% air. Human astroglial and microglial cells were isolated from surgically resected temporal lobe tissue. The procedures described previously for obtaining pure cultures of microglia and astrocytes were followed (28).

Experimental Protocol—Human astrocytes, U373 astrocytoma cells and THP-1 cells (5 × 10⁵ cells), as well as human microglial cells (5 × 10⁴ cells) were seeded into 24-well plates in 1 ml of DMEM/F12 medium containing 5% fetal bovine serum. The CBS inhibitor hydroxylamine (1 mM) was added to inhibit endogenous production of H₂S by CBS. L-DOPA, (–)-deprenyl or H₂S-releasing moieties were then added at a concentration of 10 μM. Incubation of the mixtures was carried out for 2, 4, 8, or 12 h. The H₂S-releasing moieties included NaSH, ACS 48, ACS50, ACS5, ACS81, and ADT-OH (21), as well as the hybrid molecules ACS83, ACS84, ACS85, and ACS86. Cells were washed with phosphate-buffered saline (PBS) twice and replated in 800 μl DMEM/F12 medium containing 5% FBS. The cells were then incubated at 37 °C for 2 days in the presence of inflammatory stimulants. For microglia and THP-1 cells, the stimulants were LPS at 1 μg/ml and IFNγ at 333 units/ml. For astrocytes and U373 cells, the stimulant was IFNγ alone at 150 units/ml. A companion set of cells was incubated in medium without inflammatory stimulants. After incubation, the supernatants (400 μl) were transferred to undifferentiated human neuroblastoma SH-SY5Y cells (2 × 10⁵ cells per well). The cells were incubated for a further 72 h, and MTT and LDH assays performed as described below.

SH-SY5Y Cell Viability Assays—The viability of SH-SY5Y cells following incubation with glial cell supernatants was evaluated by the LDH release and MTT assays as previously described in detail (29). For the LDH assay, the amount of LDH released was expressed as a percentage of the value obtained in comparative wells where cells were 100% lysed by 1% Triton X-100. For the MTT assay, data are presented as a percentage of the value obtained from cells incubated in fresh medium only.

Measurement of TNFα and IL-6 Release—Cytokine levels were measured in cell-free supernatants following 48 h of incubation of THP-1 cells, U373 cells, microglial cells, or astrocytes. The cell stimulation protocols were the same as used for measuring H₂S generation. Quantitation was performed with ELISA detection kits (Peprotech, NJ) following protocols described by the manufacturer.

Measurements of Nitrite Release—Accumulation of NO₂[–] as an indicator of NO synthesis was assayed by the standard Griess reaction. After stimulation of cells for 48 h, the media were centrifuged, and the cell-free supernatants mixed with an equal volume of Griess reagent (Sigma). Samples were incubated at room temperature for 15 min, and the fluorescence read at an

excitation of 380 nm and an emission of 540 nm using a plate reader.

Preparation of Mitochondria from U373 Cells—Preparation of functional mitochondria from human U373 cells was performed as described previously (30). Briefly, U373 cells were detached from a tissue culture flask using a cell scraper and were then transferred to 50 ml Falcon tubes for centrifugation (2,000 rpm for 10 min) at 4 °C. After their supernatants were discarded, cells were resuspended in 30 ml of ice-cold mitochondria isolation buffer (10 mM Tris-MOPS, 1 mM EGTA, and 0.2 M sucrose, pH 7.4), and homogenized with a glass/Teflon potter homogenizer. The homogenates were transferred to 50 ml Falcon tubes for centrifugation (2,000 rpm for 10 min) at 4 °C. The supernatants were collected to perform high-speed centrifugation (30,000 rpm, 10 min) at 4 °C. After the supernatants were discarded, the pellets were resuspended with 5 ml of ice-cold mitochondria isolation buffer and centrifuged again (30,000 rpm, 10 min). The pellets including mitochondria were resuspended very gently with 5 ml of PBS for H₂S measurements in the presence of H₂S releasers (ADT-OH, ACS48, ACS50, ACS5, and ACS81).

Measurement of H₂S Levels—H₂S levels were measured using a previously described method (21). To suppress endogenous production of H₂S by CBS, all experiments were done with 1 mM of the specific CBS inhibitor hydroxylamine added to the solutions. Two sets of experiments were conducted: one in which the THP-1 cells and U373 cells were unstimulated and a second where they were stimulated with inflammatory mediators for 48 h. For THP-1 cells, the stimulation was LPS at 1 μg/ml and IFNγ at 333 units/ml, and for U373 cells it was IFNγ at 150 units/ml. The cells in each case were treated with 1 mM hydroxylamine plus NaSH, ADT-OH, ACS83, ACS84, ACS85, or ACS86 (10 μM each) for 2, 4, 8, and 12 h. Following treatment, they were homogenized in 250 μl of ice-cold 100 mM potassium phosphate buffer (pH 7.4) containing trichloroacetic acid (10% w/v). Zinc acetate (1% w/v, 250 μl) was injected to trap the generated H₂S. A solution of *N,N*-dimethyl-*p*-phenylenediamine sulfate (20 μM; 133 μl) in 7.2 M HCl and FeCl₃ (30 μM; 133 μl) in 1.2 M HCl was added. Absorbance at 670 nm of the resulting mixture (300 μl) was determined after 10 min using a 96-well microplate reader (Bio-Rad). The H₂S concentration of each sample was calculated against a calibration curve of NaSH (1–250 μM) and results expressed as μmol/g protein or nmol/ml.

GSH Level in Glial Cells in Vitro—The GSH level was assessed by the method of Hissin and Hilf (31). This assay detects reduced glutathione (GSH) by its reaction with *o*-phthalaldehyde (OPT) at pH 8.0. Cells (10⁶) in 1.5-ml tubes were washed twice with PBS, and treated with 200 μl of 6.5% (w/v) trichloroacetic acid. The mixture was incubated on ice for 10 min and centrifuged (13,000 rpm, 1 min). The supernatant was discarded, and the pellets were resuspended in 200 μl of ice-cold 6.5% (w/v) trichloroacetic acid and centrifuged again (13,000 rpm, 2 min). Supernatants (7.5 μl) were transferred to 96-well plates containing 277.5 μl phosphate-EDTA buffer (pH 8.0) in 1 M NaOH solution. Then 15 μl of OPT (1 mg/ml in methanol) was added. The reaction mixture was incubated in the dark at room temperature for 25 min. The fluorescence at

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TABLE 1

Levels of intact molecule and its metabolites in plasma and brain 1 h after iv rat administration of 40 mg/kg ACS 84

ACS84	Plasma		Brain		
	ACS84-a	ACS50	ACS84	ACS84-a	ACS50
	$\mu\text{M mean} \pm \text{S.E.}$			$\mu\text{M mean} \pm \text{S.E.}$	
1.1137 \pm 0.0867 ^a	6.7267 \pm 0.8933	58.9333 \pm 3.6988	0.1230 \pm 0.0227	0.3497 \pm 0.0568	1.3227 \pm 0.2476

^a Values are mean \pm S.E., $n = 4$.**TABLE 2**

Dopamine, L-DOPA, and GSH levels in brain and/or plasma 1 h after iv rat administration of 40 mg/kg ACS84 or an equimolar dose of L-DOPA

Values are mean \pm S.E., $n = 4$. One-way ANOVA test was carried out to evaluate the significance of differences.

Treatments	Plasma		Brain		
	Dopamine	L-DOPA	Dopamine	L-DOPA	GSH
	μM		μM		
ACS84	2.17 \pm 0.52 ^a	0	13.2 \pm 1.3 ^a	0.277 \pm 0.2775 ^a	2402 \pm 39.3 ^a
L-DOPA	29.2 \pm 3.8 ^b	10.2 \pm 2.8 ^b	9.60 \pm 0.08 ^b	2.00 \pm 0.775 ^b	1479 \pm 34
Vehicle	0	0	5.97 \pm 0.6	0	1670 \pm 60

^a $p < 0.01$ for ACS84 group compared with vehicle group.^b $p < 0.01$ for L-DOPA group compared with ACS84 group.

350 nm excitation/420 nm emission was measured in a multi-well plate reader. The concentration was calculated from a standard curve using a serial dilution of reduced GSH.

Activity of Monoamine Oxidase A and B—Activities of monoamine oxidase A and B (MAO A and MAO B, respectively) after SH-SY5Y cells were exposed to glial-conditioned medium for 1 day were measured using a kit (Amplex Red monoamine oxidase kit, Molecular Probes Inc., Eugene, OR). Experiments were performed as described by the manufacturer.

Data Analysis—The significance of differences between data sets was analyzed by Student's *t* test and one-way or two-way ANOVA. Multiple group comparisons were followed by a post-hoc Bonferroni test.

RESULTS

First, we evaluated the release rate *in vivo* of the H₂S donor from ACS84, a typical L-DOPA hybrid compound, administered intravenously (40 mg/kg) into rats. Table 1 demonstrates the formation of the main metabolites of ACS84. After 1 h, almost all of the ACS84 had disappeared from plasma with the concomitant appearance of both its demethylated derivative (ACS84-a) and of its dithiolethione moiety (ACS50). Measurements performed at earlier times from the treatments indicate that demethylation of ACS84 occurs rapidly, whereas the cleavage of the amide bond between dithiolethione and L-DOPA occurs more slowly (data not shown). Low micromolar or sub-micromolar concentrations of ACS84 and its main metabolites were found in brain 1 h after administration.

Dopamine levels in brain were increased 2.2-fold by treatment with ACS84. Interestingly, GSH, a known antioxidant and neuroprotective agent (32) was increased 1.4-fold by this treatment. Although treatment with an equimolar dose of L-DOPA also increased dopamine levels in brain, the increase was only by 1.6-fold, and there was no increase in GSH (Table 2). These data encouraged us to pursue *in vitro* experiments to determine whether H₂S-releasing L-DOPA hybrid compounds can lead to accumulation of H₂S in both glia and SH-SY5Y cells, and what effects this release might produce.

We commenced by examining the rate of cleavage of the H₂S-releasing moieties (ADT-OH, ACS48, ACS50, ACS5,

and ACS81) under extracellular conditions. We tested release from PBS, the DMEM/F12 culture medium, and human serum. We then compared this with the rate of uptake and cleavage intracellularly in THP-1, U373, and SH-SY-5Y cells (Fig. 2).

In PBS and the DMEM/F12 medium, H₂S was released very slowly, reaching only 10% of the total amount available after 48 h (see Fig. 2B for ACS50 as a representative in DMEM/F12 medium and for ACS50 in PBS). Human serum caused a more rapid release with 50% of the potential being attained in 4.5–18 h. (ADT-OH, 4.5 h; ACS5, 6 h; ACS 48, 6 h; ACS 50, 12 h; and ACS 81, 18 h) indicating the presence of weakly active cleaving enzymes in human serum. In contrast, NaSH, the model H₂S donor, released its full potential immediately. It then showed a slow decay, declining by about 10% over 48 h (see supplemental Fig. S1, B, D, and F), indicating minor decay of SH⁻ ions. These data demonstrate that the H₂S donating molecules are stable extracellularly, and, if administered *in vivo*, should be able to reach their target cells relatively intact.

Fig. 2 also indicates what happens when the H₂S donors do reach their target cells. The data show intracellular levels of H₂S in THP-1, SH-SY5Y, and U373 cells after being exposed to 10 μM of the H₂S donors. H₂S generated from the donor molecules slowly increased in the cytoplasm of THP-1 and SH-SY5Y cells over 48 h. Fig. 2 shows data from ACS50 as a representative of the S-donor compounds. All the H₂S-donor compounds gave highly similar results. For U373 cells, the conversion was more robust than for THP-1 and SY-SY5Y cells with a maximum being reached by 12 h, after which there was a slow decline (Fig. 2A). The intracellular H₂S levels reflect the net effect of at least three mechanisms: uptake of donors into the cells; intracellular cleavage of the molecules; and intracellular metabolism of the H₂S generated. The other H₂S-releasing compounds ACS48, ACS5, and ACS81 showed the same release kinetics in all cells tested (see supplemental Fig. S1), as did the S-DOPA derivative ACS83 (see supplemental Fig. S2).

These data establish that all the moieties are metabolized within each cell type to generate H₂S. To explore a possible mechanism, we purified functional mitochondria from U373

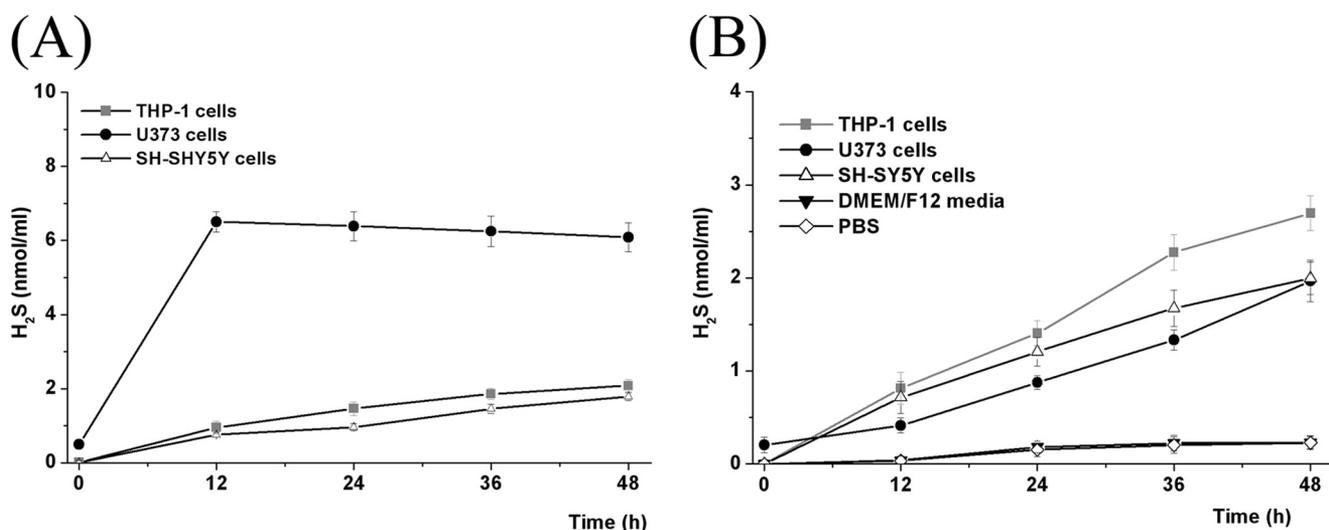


FIGURE 2. Intracellular (A) and extracellular (B) H₂S levels (nmol/ml) after treatment with 10 μM ACS50 in the presence of 1 mM hydroxylamine. Values are mean ± S.E., n = 4.

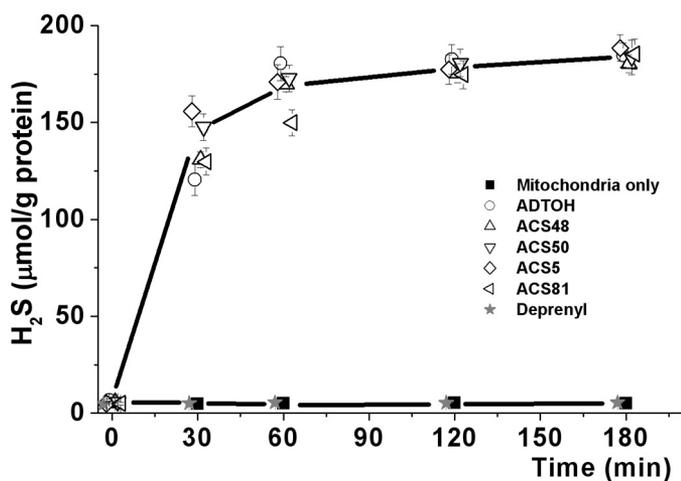


FIGURE 3. H₂S levels (μmol/g protein) generated by isolated functional mitochondria in human U373 cells after treatment with 10 μM ADT-OH, ACS48, ACS50, ACS5, ACS81, or (-)-deprenyl. Values are mean ± S.E., n = 4.

cells. All mitochondria contain molecules which have a high reducing potential such as NADH, FADH₂, and cytochromes.

As shown in Fig. 3, all the donor moieties, but not (-)-deprenyl, a classical inhibitor of monoamine oxidase B, were metabolized to release H₂S within 60 min by the mitochondria. Very similar results were obtained with the four different S-DOPA compounds (see supplemental Fig. S3). The data indicate a potential mechanism by which H₂S is generated intracellularly from the donor moieties.

Within cells, the most important reducing agent is glutathione (GSH). To determine whether the H₂S donors were affecting GSH levels, we measured [GSH]_i in SH-SY5Y cells exposed to the four different S-DOPAs (10 μM each) for 8 h. Equal concentrations of L-DOPA and NaSH were used as negative and positive controls, respectively. The results are shown in Fig. 4A. Treatment with NaSH and the S-DOPAs increased [GSH]_i in SH-SY5Y cells under normal conditions by ~1.5-fold (p < 0.01). We then exposed the cells for 1 day to conditioned medium from stimulated THP-1 cells (LPS/IFNγ

for 2 days) or U373 cells (IFNγ for 2 days). This treatment caused a huge decrease in [GSH]_i (~90% decrease, p < 0.01). NaSH and the four H₂S-releasing S-DOPAs significantly attenuated this decrease (p < 0.01). Nevertheless, the values were still significantly lower than those obtained from control media. These data establish that H₂S generated from the donor compounds is significantly converted into the antioxidant [GSH]_i, and that this [GSH]_i is significantly depleted by exposure to supernatants from glial cells that have received inflammatory stimulation.

Fig. 4, B and C demonstrates the changes in MAO A and B in SH-SY5Y cells in the presence of NaSH, L-DOPA, and the four H₂S-releasing S-DOPAs. SH-SY5Y cells express both MAO A and MAO B, the latter accounting for 75% of the total MAO activity. Treatment with NaSH or four different S-DOPAs (10 μM each) for 8 h reduced the activity of MAO B, but not MAO A. There was no effect of L-DOPA. The decreases were about 85% (p < 0.01) after exposure of SH-SY5Y cells to media from unstimulated THP-1 or U373 cells and about 65% after exposure to medium from THP-1 or U373 cells stimulated for 24 h (p < 0.01). These data establish that all the S-DOPAs are selective MAO B inhibitors.

We next investigated the effects of 1, 3, 10, 30, and 50 μM concentrations of the S-DOPA derivatives, as well as the H₂S donors ADT-OH and NaSH on glial-mediated neurotoxicity. Fig. 5 shows the effect on SH-SY5Y viability of adding NaSH, ADT-OH, L-DOPA, (-)-deprenyl, ACS83, ACS84, ACS85, or ACS86 to LPS/IFNγ-activated THP-1 cells (5A) or IFNγ-activated U373 cells (5B) at a standard concentration of 10 μM. It was found that the H₂S-releasing agents ADT-OH and NaSH, but not L-DOPA or (-)-deprenyl, attenuated the neurotoxicity in an incubation time-dependent manner. This is shown both by the MTT assay (upper panels) and LDH assay (lower panels). The four S-DOPA derivatives ACS83, ACS84, ACS85, and ACS86 were comparably protective to NaSH and ADT-OH. Complete data showing the results at 1, 3, 10, 30, and 50 μM, and 2, 4, 8, and 12 h pretreatment are shown in supplemental Figs. S4 and S5. Again, the results were highly similar for all

H₂S-releasing L-DOPAs and Glial Activation

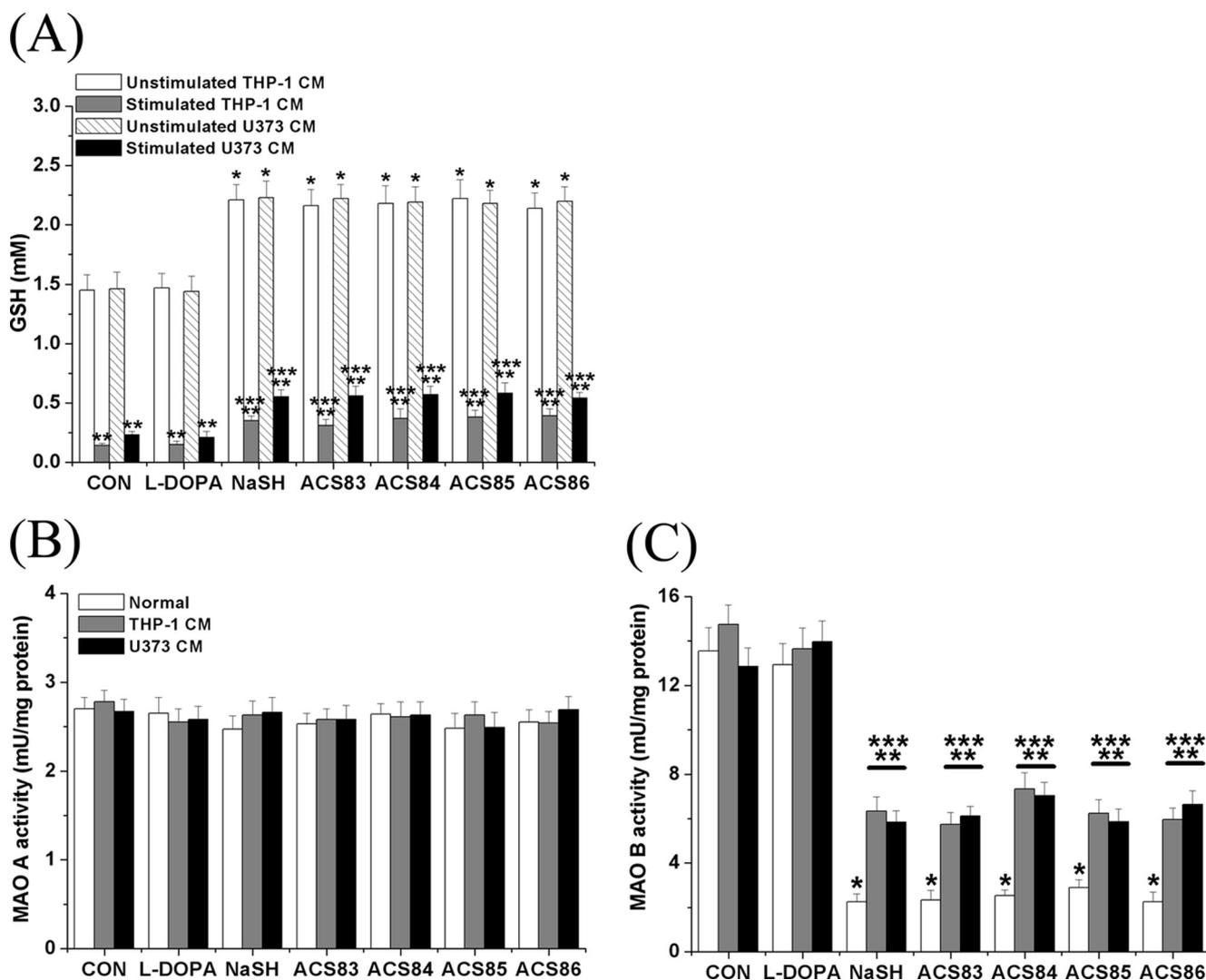


FIGURE 4. A, effect of direct treatment with NaSH, L-DOPA, ACS83, ACS84, ACS85, or ACS86 on GSH levels in SH-SY5Y cells. After SH-SY5Y cells were treated with NaSH, L-DOPA, ACS83, ACS84, ACS85, or ACS86 (10 μ M each) for 8 h and subsequently exposed to THP-1 or U373 conditioned medium (CM) for 1 day, they were extracted to measure GSH levels. B and C, effect of direct treatment with NaSH, ADT-OH (ADT), L-DOPA, ACS83, ACS84, ACS85, or ACS86 on activities of MAO A and MAO B in SH-SY5Y cells exposed to stimulated THP-1 or U373 conditioned medium (CM). After SH-SY5Y cells were treated with NaSH, L-DOPA, ACS83, ACS84, ACS85, or ACS86 (10 μ M each) for 8 h and subsequently exposed to THP-1 or U373 CM for 1 day, they were extracted to measure activities of MAO A (B) and MAO B (C). Notice that none of the compounds inhibited MAO A, but all of them significantly inhibited MAO B except for L-DOPA. Notice also that there was significantly less inhibition when the SH-SY5Y cells were exposed to CM. Values are mean \pm S.E., $n = 4$. Two-way ANOVA was initially carried out to test the significance among the treatment groups. The significance of differences was then adjusted by applying the Bonferroni test for multiple comparisons. *, $p < 0.01$ for NaSH or all the ACS-treated groups compared with untreated group; **, $p < 0.01$ exposed to media from stimulated THP-1 or U373 compared with control media; and ***, $p < 0.01$ exposed to stimulated THP-1 or U373 media treated with NaSH or all the ACS compounds compared with stimulated media only.

the SH-donors and all the S-DOPA derivatives in their concentration and incubation time dependence.

The 8-h time period shown in Fig. 5 demonstrated a robust effect of THP-1 and U373 cells, so it was deemed optimum for showing comparative effects using human microglia and astrocytes. It was found that the protective effect of pretreatment with NaSH and the S-DOPAs (10 μ M, 8 h) was comparable in human microglia and astrocytes to that found for THP-1 and U373 cells (Fig. 6A: microglia and 6B: astrocytes). MTT data are shown in the upper panels and LDH release data in the lower panels. More detailed experimental data (3, 10, and 50 μ M; 8 h of preincubation) showing comparable effects at differing concentrations are shown in supplemental Fig. S6.

Inflammatory stimulation of microglia or THP-1 cells causes them to release the inflammatory cytokines TNF α and IL-6, as well as to generate neurotoxic nitrite ions. Fig. 7 shows the effect of treatment with NaSH, ADT-OH, and the four S-DOPA compounds (10 μ M each, 8 h of preincubation) on THP-1 release of TNF α (Fig. 7A), IL-6 (Fig. 7C), and nitrite ions (Fig. 7E), and on human microglial release of TNF α (Fig. 7B), IL-6 (Fig. 7D) and nitrite ions (Fig. 7F). There was a substantially lower release of nitrite ions by stimulated microglia compared with THP-1 cells, even allowing for the 10-fold lower number of microglial cells that were seeded. This is perhaps related to the reported poor ability of human microglia to express iNOS. Nevertheless there were significant differences between the release of these materials in both types of cells: (1) between stimulated

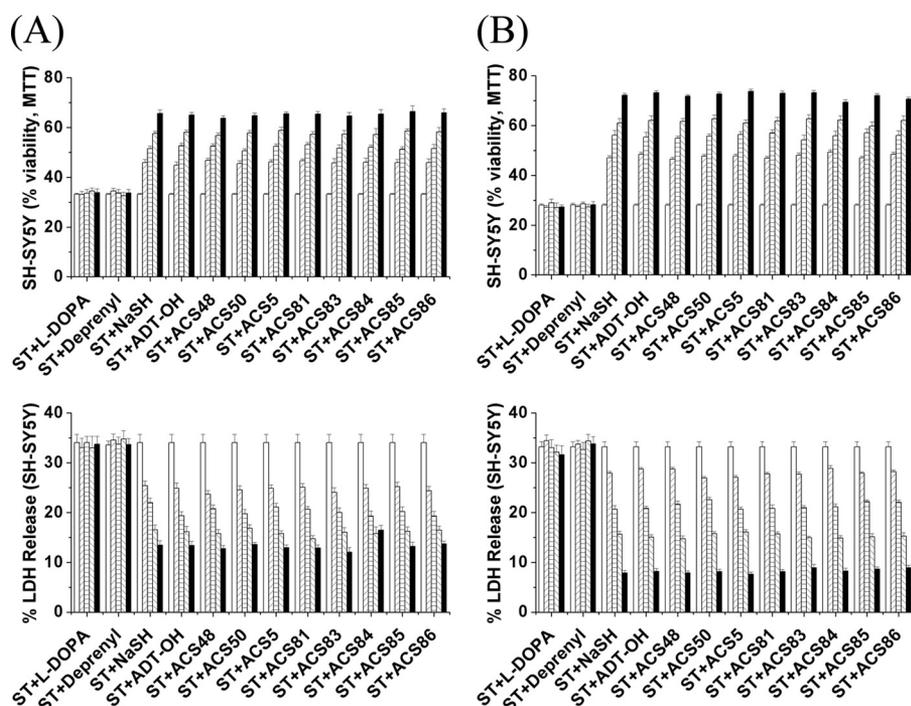


FIGURE 5. Effect on the viability of SH-SY5Y cells following treatment with supernatants from stimulated THP-1 cells (A) or U373 cells (B) that had been exposed to 10 μ M of the neuroprotective moieties as described under "Experimental Procedures." These were NaSH, the H₂S-releasing dithiol-thione moiety ADT-OH, ACS48, ACS50, ACS5, ACS81, ACS83, ACS84, ACS85, or ACS86 pre-administered for (□, 0 h) (▨, 2 h), (▩, 4 h), (▧, 8 h), and (■, 12 h). L-DOPA or (–)-deprenyl, which were without effect, served as the negative control and NaSH as the positive control. Notice that the SH donors were equally protective and were comparable to the standard donor NaSH. Upper panels show MTT results whereas lower panels show LDH results. Values are mean \pm S.E., $n = 4$. Two-way ANOVA was carried out to test the significance of differences. Multiple comparisons were followed with *post-hoc* Bonferroni tests. The time-dependent values were significantly different from control ($p < 0.01$) for NaSH and each of the S-donating compounds.

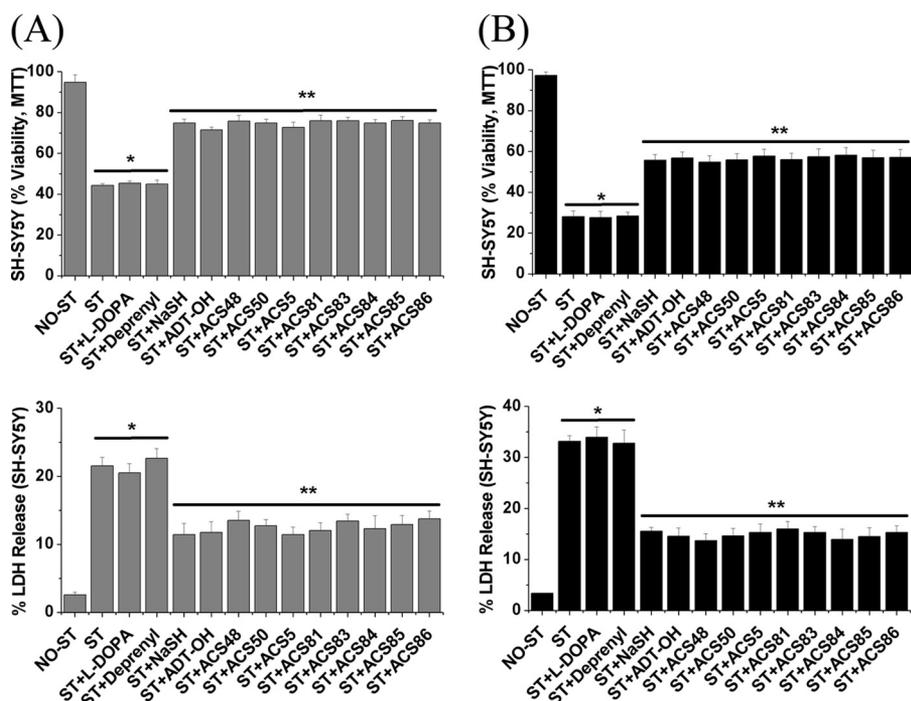


FIGURE 6. Effect of treatment with NaSH, ADT-OH, L-DOPA, (–)-deprenyl, ACS48, ACS50, ACS5, ACS81, ACS83, ACS84, ACS85, or ACS86 (10 μ M each, 8 h of preincubation) on SH-SY5Y cell viability changes induced by activated human microglia (A) or activated astrocytes (B) as followed by MTT (upper panels) and LDH release (lower panels) assays. Values are mean \pm S.E., $n = 4$. One-way ANOVA was carried out to test the significance of differences. Multiple comparisons were followed with *post-hoc* Bonferroni tests where appropriate. *, $p < 0.01$ comparing the stimulated (ST) with the non-stimulated (NO-ST) group and **, $p < 0.01$ comparing stimulated (ST) group with the S-DOPA treatment groups.

and unstimulated cells and (2) between stimulated cells that were untreated and stimulated cells that were treated with NaSH, ADT-OH, or any one of the S-DOPA compounds ($p < 0.01$). However, L-DOPA or (–)-deprenyl did not affect the release of any of these proinflammatory mediators (Fig. 7).

Fig. 8 shows comparable data for IL-6 release from U373 cells (Fig. 8A) and cultured astrocytes (Fig. 8B). Cells were activated with IFN γ as described under "Experimental Procedures" and were then treated similarly to the THP-1 and microglial cells as shown in Fig. 5. In both types of cells significant differences were found: (1) for the release of IL-6 from stimulated compared with unstimulated cells and (2) for stimulated cells treated with NaSH, ADT-OH, or the four S-DOPA compounds compared with stimulated cells that were untreated ($p < 0.01$). Complete data showing a similar concentration dependence of the four donor moieties and the four S-DOPA derivatives on H₂S release (3, 10, and 50 μ M; 8 h pretreatment) are shown in supplemental Figs. S7 and S8. Supplemental Fig. S9 shows that the viability of THP-1, U373, microglia, and astrocytes was unaffected by any of the eight S-donating agents. Supplemental Fig. S10 shows that the neuroprotective effects were additive when there was a combination of two S-DOPA derivatives (*i.e.* ACS 83 + 84, ACS 84 + 85, ACS 84 + 86) compared with the same derivatives alone.

DISCUSSION

In the present study, we examined the antioxidant and antiinflammatory properties of four H₂S donors (ACS48, ACS50, ACS5, and ACS81 as well as ADT-OH), and four L-DOPA hybrid compounds synthesized from the four donors (ACS83, ACS84, ACS85, and ACS86). They all demonstrated therapeutic potential by being taken up by human microglia, and astrocytes, as well as the human THP-1 U373 cell lines and then generating intracellular H₂S. The H₂S they gen-

H₂S-releasing L-DOPAs and Glial Activation

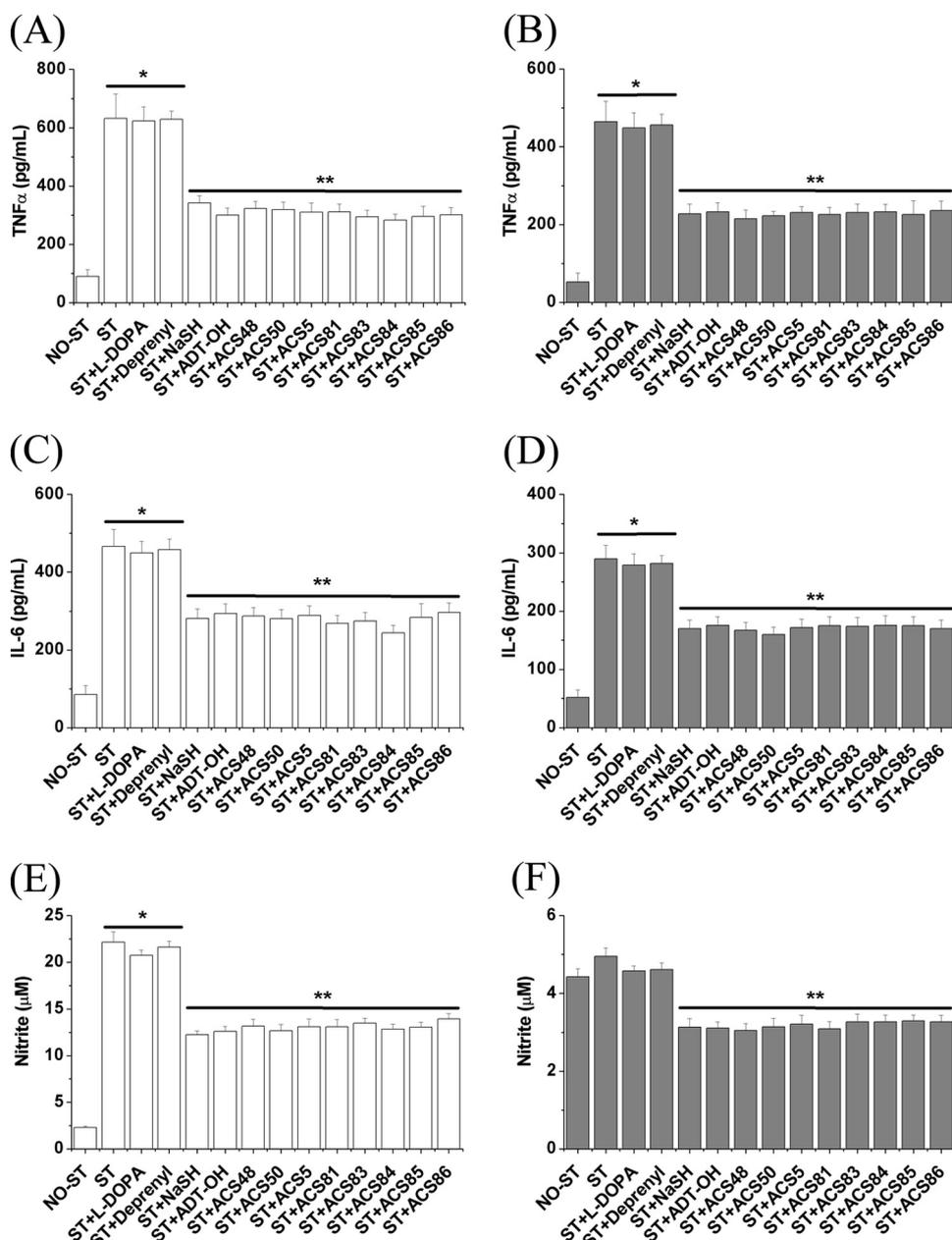


FIGURE 7. Effect of treatment with NaSH, ADT-OH, L-DOPA, (–)-deprenyl, ACS48, ACS50, ACS55, ACS81, ACS83, ACS84, ACS85, or ACS86 (10 μ M each, 8 h of preincubation) on the released levels of TNF α (A, B), IL-6 (C, D) and nitrite ions (E, F) from THP-1 cells (A, C, E) or human microglia (B, D, F). Values are mean \pm S.E., $n = 4$. One-way ANOVA was carried out to test the significance of differences. Multiple comparisons were followed with *post-hoc* Bonferroni tests where appropriate. *, $p < 0.01$ comparing the stimulated group with the non-stimulated group (NO-ST) and **, $p < 0.01$ comparing the stimulated group (ST) with the L-DOPA treatment groups.

erated acted in part to enhance levels of the classical antioxidant GSH. They also inhibited MAO B. Moreover, they acted as antiinflammatory compounds by ameliorating the neurotoxic effects of glial cell supernatants toward SH-SY5Y cells. Additionally, they inhibited release of the proinflammatory mediators TNF α , IL-6, and nitrite ions (Figs. 7 and 8). The compounds were equipotent, but L-DOPA was without effect in all of these assays.

We have previously reported that depleting intracellular GSH by inhibiting its synthetic enzyme γ -glutamylcysteine synthase induces an inflammatory reaction in glial cells with con-

sequent neurotoxic effects (32). The compounds described here could help counteract the consequences of depressed GSH production. Our pilot *in vivo* data with ACS 84 showed that it reached the brain and was substantially metabolized as early as 1 h after iv administration to a rat. There was a more than a 2-fold increase in brain dopamine and a 1.4-fold increase in GSH. This demonstrates that a significant amount of the intact, and very lipophilic ACS84, crosses the blood brain barrier and is hydrolyzed in the brain into its two components L-DOPA and ACS50. Moreover the concomitant inhibition of the dopamine-metabolizing enzyme MAO B, as had previously been shown for dithiolethiones (33), could contribute to sustain the high concentration of dopamine in brain. The increase of GSH presumably results from the H₂S generated by metabolism of the donor moiety. While very preliminary, these data demonstrate that these L-DOPA hybrids may have therapeutic potential by enhancing dopamine and GSH levels.

Earlier studies have indicated that H₂S is a reducing agent that can increase levels of intracellular glutathione (14) thereby inhibiting oxidative stress. It also decreases levels of peroxynitrite-derived nitrated proteins (34). Thus H₂S might block oxidative stress-mediated cell death in PD by directly or indirectly detoxifying free radicals generated from oxidized L-DOPA compounds.

As far as neuroinflammation is concerned, it is well known that areas affected in PD, especially the substantia nigra, are characterized by the presence of activated microglia and activated astrocytes (4, 7–10, 35, 36). There is comparable glial activation in animal models of PD (37). Evidence that these reactive glial cells contribute to the neuronal degeneration comes from epidemiological studies where persons taking NSAIDs are reported to be relatively spared from PD (38, 39) especially if combined with coffee (40). Such protection is also reported for animal models of PD (37, 41).

The MPTP phenomena may be particularly revealing with respect to the potential consequences of inducing SN inflammation. Drug addicts who were originally exposed to MPTP developed a relentlessly progressive parkinsonian syndrome.

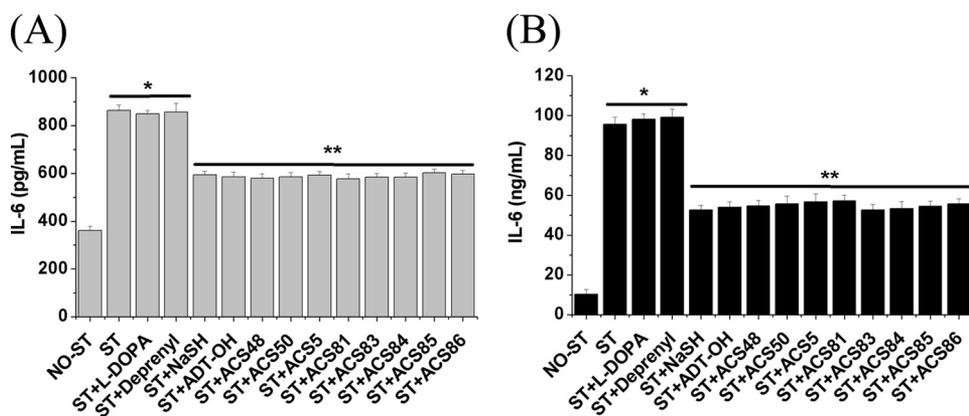


FIGURE 8. Effect of treatment with NaSH, ADT-OH, L-DOPA, (–)Deprenyl, ACS48, ACS50, ACS5, ACS81, ACS83, ACS84, ACS85, or ACS86 (10 μ M each, 8 h of preincubation, protocol 1) on release of IL-6 from U373 cells (A) or human astrocytes (B). Values are mean \pm S.E., $n = 4$. One-way ANOVA was carried out to test the significance of differences. Multiple comparisons were followed with *post-hoc* Bonferroni tests where appropriate. *, $p < 0.01$ comparing the unstimulated (NO-ST) group with the stimulated group. **, $p < 0.01$ comparing the stimulated (ST) group with the S-DOPA-treated groups.

Postmortem studies showed persistent inflammation up to 17 years following their last exposure to the toxin (35). This human experience was duplicated in monkeys where inflammation of the SN was demonstrated up to 14 years after their last MPTP exposure (8). This suggests that inflammation of the SN, once initiated, may be self sustaining. If this is the case, antiinflammatory treatment may be essential to arresting progression of PD.

Our study demonstrates that inflammatory stimulation of glial cells results in a 5–7-fold decrease in intracellular H₂S (see supplemental Fig. S11). This indicates an increase in intracellular consumption as reactive processes are induced. These experiments were performed in the presence of hydroxylamine to suppress endogenous H₂S synthesis by CBS. But in a previous study (12) we showed that such inflammatory stimulation also sharply reduced the expression of CBS. Therefore inflammatory stimulation reduces intra-glial production of H₂S while at the same time increasing consumption. The result is a deprivation of this endogenous anti-inflammatory and neuroprotective agent.

There are studies demonstrating that serum levels of homocysteine, an intermediate of the trans-sulfuration pathway, were increased in PD patients receiving L-DOPA (42–44). One of the main enzymes responsible for hyperhomocysteinemia is CBS, which is mainly expressed in astrocytes in brain (11, 12). So it could be hypothesized that endogenous production of H₂S is reduced in PD brain, thus compounding the potential damage of oxidative stress associated with L-DOPA administration. This provides additional rationale for designing methods of supplementing H₂S availability in PD.

We have previously shown that SH-SY5Y cells express both MAO A and B (45). MAO B is primarily responsible for oxidative degradation of dopamine (46, 47). Treatment with NaSH and H₂S-releasing moieties selectively inhibited MAO B (Fig. 4C). This could be one of the reasons why ACS84-treated rats have higher dopamine levels than vehicle or L-DOPA injected rats (Table 2). However, we could not exclude that dopamine is generated from degradation of ACS84 in rats. MAO B activity in SH-SY5Y cells was less inhibited in cells

exposed to the inflammatory effects of THP-1- or U373 cell-conditioned medium. This could be due to consumption of the generated H₂S in stimulated cells, presumably through oxidative reactions.

In conclusion, our data demonstrate that H₂S not only has anti-inflammatory activity against the glial toxicity which may be associated with the pathogenesis of PD (36), but may also reduce the stress induced by L-DOPA oxidation (14). Furthermore, by inhibiting MAO B, H₂S-releasing L-DOPA compounds could help restore the disease-depleted dopamine levels. The H₂S-releasing L-DOPA derivatives (S-DOPAs) described here represent

compounds that can reach the brain and, in cells under stress, deliver ameliorating SH[–] ions in a time-dependent manner. They have properties that make them candidates for future treatment of PD. However the long term consequences of their use will require much further study.

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