1,3-Selenazol-4-one Derivatives Inhibit Inducible Nitric Oxide-Mediated Nitric Oxide Production in Lipopolysaccharide-Induced BV-2 Cells

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Activated microglia extensively produce nitric oxide (NO) by inducing expression of inducible NO synthase (iNOS). NO plays a deleterious role in brain inflammation and neuronal death. In the present study, we investigated the effects of 1,3-selenazol-4-one derivatives (Sz-A, B, C, D and E) on NO production and iNOS expression in lipopolysaccharide (LPS)-induced BV-2 cells, a murine microglia cell line. Among these compounds, Sz-B and C remarkably inhibited LPS-induced NO production relative to that of Sz-A, D, and E at 5 μM in BV-2 cells. Sz-B and C dose-dependently inhibited NO production at 1, 5, and 10 μM without toxicity to BV-2 cells. Sz-B and C also dose-dependently suppressed iNOS expression at the same concentrations in LPS-induced BV-2 cells. This result suggests that Sz-B and C inhibit iNOS-mediated NO production in LPS-induced BV-2 cells. Structurally, Sz-B and C bear an ethyl or methyl group at the 5 positions of the 4-selenazolone skeletons, which could play an important role in inhibiting iNOS-mediated NO production.

Key words 1,3-selenazol-4-one derivative; microglia; BV-2 cell; nitric oxide; inducible nitric oxide synthase (iNOS)

Microglia, resident brain inflammatory cells, become activated in injured brain conditions and release various inflammatory mediators such as nitric oxide (NO), tumor necrosis factor-α, interleukin-1β, and glutamate.[1—3] Microglia activation aggravates brain injury and leads to brain inflammation, which is considered to be a risk factor of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and ischemia.[4—6] Among these inflammatory substances, NO is a major toxic substance released from activated microglia.[7,8] NO rapidly and spontaneously reacts with a superoxide anion (O_2^-) to form a peroxynitrite anion ($ONOO^-$) and its conjugated acid, peroxynitrous acid (ONOOH), which is more toxic to biological systems than O_2^- or NO alone. NO is biosynthesized by the oxidation of L-arginine to NO and citrulline *via* the intermediate N_ω-hydroxy-L-arginine. The reaction is a five-electron oxidation of L-arginine using nicotinamide adenine dinucleotide phosphate (NADPH) as the source of electrons. All processes are catalyzed by the family of NO synthase (NOS) isoenzymes; neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). Generally, the eNOS and nNOS isoforms are constitutively expressed and synthesize NO only when the intracellular Ca^{2+} concentration is elevated and calmodulin is bound to the enzyme. By contrast, iNOS is not normally expressed but can be induced by selected immunological stimuli such as lipopolysaccharide (LPS), interferon-γ, tumor necrosis factor-α, and interleukin-1β, and produces NO continuously and independently of intracellular Ca^{2+}.[9] In the brain, activated microglia extensively produce NO by inducing expression of iNOS. Therefore, a potential therapeutic strategy for reducing neuronal cell injury or death in neurodegenerative diseases would be to identify inhibitors of iNOS induction resulting in decreased NO production.[10]

Recently, the biological activities of selenium-containing heterocyclic compounds have been reported,[11—14] and their use as nutritional supplements has been popularized recently because of their potential roles as an antioxidant at low concentrations and as an anticancer agent at higher concentrations.[15,16] 1,3-Selenazol-4-one derivatives synthesized by the reaction of primary selenoamides with haloacyl halides in the presence of pyridine were prepared by our research group.[17] In this study, the effects of 1,3-selenazol-4-one derivatives (Sz-A, B, C, D and E) on LPS-induced NO production and iNOS expression were investigated.

MATERIALS AND METHODS

Preparation of 1,3-Selenazol-4-one Derivatives 1,3-Selenazol-4-one derivatives were prepared according to a procedure previously reported.[17] For example, 2-(4-methylphenyl)-1,3-selenazol-4-one (Sz-A) was synthesized as followed: chloroacetyl chloride (0.12 g, 1.0 mmol) in dry dichloromethane (5 ml) was added dropwise to a stirred solution of 4-methylbenzeneselenoamide (0.20 g, 1.0 mmol) in dry dichloromethane (5 ml) at 0 °C under an argon atmosphere. The reaction mixture was stirred for 1 h at room temperature. Dry pyridine (0.16 g, 2.0 mmol) in dry dichloromethane (5 ml) was then added dropwise into the mixture at 0 °C. The reaction mixture was stirred for 2 h at 0 °C, extracted with dichloromethane (100 ml), and washed with water (30 ml). The organic layer was dried over sodium sulfate and evaporated to dryness. The residue was purified by flash chromatography on silica gel with dichloromethane to give Sz-A (0.19 g, yield: 80%). mp: 109.5—111.5 °C. IR (KBr) cm^{-1}: 1707, 1609. ^{1}H-NMR ($CDCl_3$): δ 2.45 (3H, s, CH_3Ar), 4.26 (2H, s, CH_2), 7.32 (2H, d, J=7.6 Hz, Ar), 7.96 (2H, d, J=7.6 Hz, Ar). ^{13}C-NMR ($CDCl_3$): δ 21.9, 34.8, 129.2, 129.8, 131.7, 146.6, 193.1, 195.9. ^{77}Se-NMR ($CDCl_3$): δ 458.1. MS (CI): m/z=240 [M^++1]. *Anal.* Calcd for $C_{10}H_9NOSe$: C, 50.43; H, 3.81; N, 5.88. Found: C, 50.23; H, 3.92; N, 5.88. Sz-B (yield: 47%). mp: 69.5—71.0 °C. IR (KBr) cm^{-1}: 1705, 1608. ^{1}H-NMR ($CDCl_3$): δ 1.10 (3H, t,

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J=7.2 Hz, CH_3), 2.04 (1H, m, CH_2), 2.37 (1H, m, CH_2), 2.44 (3H, s, CH_3Ar). 4.64 (1H, dd, J=4.0, 8.8 Hz, CH), 7.31 (2H, d, J=8.4 Hz, Ar), 7.96 (2H, d, J=8.4 Hz, Ar) ^{13}C-NMR ($CDCl_3$): δ 13.4, 21.9, 26.9, 56.9, 129.2, 132.0, 146.4, 195.1. ^{77}Se-NMR ($CDCl_3$): δ 521.5. MS (CI): m/z=268 [M^++1]. High resolution (HR)-MS: m/z Calcd for $C_{12}H_{13}NOSe$: 267.0162. Found: 267.0142. Sz-C (yield: 41%). mp: 83.0—85.0 °C. IR (KBr) cm^{-1}: 1702, 1610. ^{1}H-NMR ($CDCl_3$): δ 1.86 (6H, s, C $(CH_3)_2$), 2.44 (3H, s, CH_3Ar), 7.30 (2H, d, J=8.0 Hz, Ar), 7.95 (2H, d, J=8.0 Hz, Ar). ^{13}C-NMR ($CDCl_3$): δ 21.8, 28.6, 60.6, 129.1, 129.7, 132.1, 146.2, 193.7. 197.6. ^{77}Se-NMR ($CDCl_3$): δ 658.7. MS (CI): m/z=268 [M^++1]. HR-MS: m/z Calcd for $C_{12}H_{13}NOSe$: 267.0162. Found: 267.0164. Sz-D (yield: 76%). mp: 80.0—81.0 °C. IR (KBr) cm^{-1}: 1711, 1595. ^{1}H-NMR ($CDCl_3$): δ 4.27 (2H, s, CH_2), 7.51 (2H, t, J=8.0 Hz, Ar) 7.67 (1H, t, J=8.0 Hz, Ar), 8.05 (2H, d, J=8.0 Hz, Ar). ^{13}C-NMR ($CDCl_3$): δ 34.9, 128.99, 129.0, 134.2, 134.9, 192.8, 196.1. ^{77}Se-NMR ($CDCl_3$): δ 464.5. MS (CI): m/z=226 [M^++1]. HR-MS: m/z Calcd for C_9H_7NOSe: 224.9692. Found: 224.9684. Sz-E (yield: 62%). mp: 147.5—150.0 °C. IR (KBr) cm^{-1}: 1702, 1591. ^{1}H-NMR ($CDCl_3$): δ 4.30 (2H, s, CH_2), 7.49 (2H, d, J=8.8 Hz, Ar) 7.99 (2H, d, J=8.8 Hz, Ar). ^{13}C-NMR ($CDCl_3$): δ 35.3, 129.4, 130.2, 132.7, 141.5, 192.6, 194.5. ^{77}Se-NMR ($CDCl_3$): δ 465.8. MS (CI): m/z=260 [M^++1]. HR-MS: m/z Calcd for $C_9H_6NOSeCl$: 258.9303. Found: 258.9289.

Cell Culture BV-2 cells, a murine microglia cell line obtained from Dr. E. J. Choi of Korea University, were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, U.K.) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% antibiotic-antimycotic (Gibco).

Measurement of Nitrite Production To measure NO production, BV-2 cells were plated into 96-well plate (3×10^4 cells/well) and were untreated (Control), treated with 100 ng/ml of LPS (from *Salmonella enteritidis*, Sigma, U.S.A.) (LPS) or cotreated with LPS (100 ng/ml) and 1,3-selenazol-4-one derivatives for 24 h. Nitrite, a soluble oxidation product of NO, in the culture media was determined using the Griess reaction. Supernatant (50 μl) was harvested and mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% *N*-1-napthylethylenediamine dihydrochloride in 5% phosphoric acid). After 10 min, the absorbance at 540 nm was measured using a microplate reader (Emax, Molecular Device, U.S.A.). Sodium nitrite was used as a standard to calculate the concentration of NO_2^-.

Cell Viability Cell respiration, an indicator of cell viability, was determined by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT, Sigma) to formazan. MTT solution (50 μl, 0.5 mg/ml) was added to BV-2 cell cultures and incubated for 1 h. The conversion of MTT to formazan by metabolically visible cells was measured with a microtiter plate reader (Molecular Devices, U.S.A.) at 595 nm [12].

Western Blot Analysis iNOS expression was analyzed using Western blot. BV-2 cells were seeded into 6-well plates (4×10^5 cells/well) and exposed to LPS (100 ng/ml) in the presence or absence of Sz-B and C for 6 h. Protein samples (50 μg for each) from BV-2 cell extracts were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Germany). The membrane was blocked with 5% skim milk and then incubated with primary antibody (rabbit anti-iNOS; BD Transduction Laboratories, U.S.A.) and peroxidase-conjugated secondary antibody (goat anti-rabbit IgG; Amersham Lifescience, Germany). The blots were detected by ECL Western Blotting Detection Reagents (Amersham Lifescience, Germany).

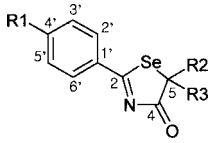
Statistical Analysis Data are reported as the mean± S.E.M. values of three independent determinations. All experiments were done at least three times, each time with three or more independent observations. Statistical analysis was performed by analysis of variance (ANOVA) followed by Duncan's test.

RESULTS

Effect of 1,3-Selenazol-4-one Derivatives on LPS-Induced NO Production To study the effect of 1,3-selenazol-4-one derivatives (Sz-A, B, C, D, and E) on LPS-induced NO production, BV-2 cells were treated with LPS (100 ng/ml) in the absence or presence of 5 μM concentration of Sz-A, B, C, D, and E. Among these compounds, LPS-induced NO production was reduced to 74.48±2.1%, and 70.13±5.6% of that produced by LPS alone. These inhibitory effects of Sz-B and C on NO production were unlikely to be due to their toxicity based on the results of the cell viability experiments (data not shown). In contrast, the other compounds did not markedly reduce NO production (Table 1).

Effects of Sz-B and C on iNOS-Mediated NO Production Sz-B and C dose-dependently inhibited LPS-induced NO production at 1, 5, and 10 μM in BV-2 cells without exhibiting any toxicity toward the cells (Fig. 1). Western blot analysis was performed to determine if the inhibitory effect of Sz-B and C on NO production was related to iNOS induction. Sz-B and C also dose-dependently suppressed iNOS expression at the same concentrations (Fig. 2). These results suggest that they inhibit iNOS-mediated NO production in LPS-induced BV-2 cells.

Table 1. Effect of 1,3-Selenazol-4-one Derivatives on LPS-Induced NO Production



Compound	Substituent			Inhibition of NO release at 5 μM (%)
	R1	R2	R3	
Sz-A	CH_3	H	H	23.1±1.9*
Sz-B	CH_3	CH_2CH_3	H	74.5±2.1*
Sz-C	CH_3	CH_3	CH_3	70.1±5.6*
Sz-D	H	H	H	26.3±3.6
Sz-E	Cl	H	H	25.3±2.1*

BV-2 cells were untreated (control), stimulated with LPS (100 ng/ml) (LPS) or cotreated with LPS (100 ng/ml) and 1,3-selenazol-4-one derivatives (Sz-A, B, C, D and E) at 5 μM for 24 h. Nitrite level in supernatant was quantified by Greiss reaction and sodium nitrite was used as standard. Values of each group are expressed as the mean±S.E.M. * $p<0.05$; significantly different from LPS-treated alone group.

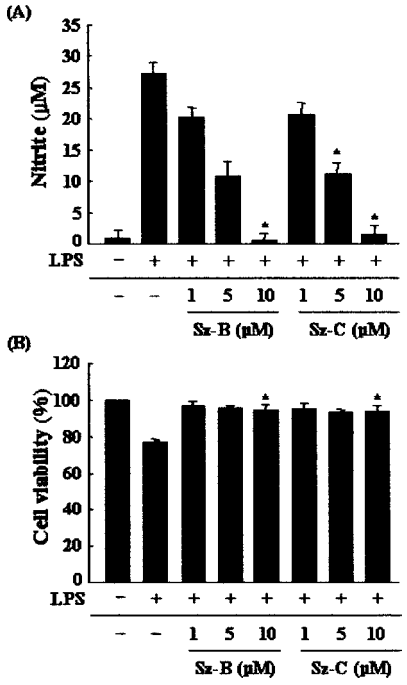


Fig. 1. Effect of Sz-B and C on LPS-Induced NO Production (A) and Cell Viability (B)

BV-2 cells were untreated (control), stimulated with LPS (100 ng/ml) (LPS) or cotreated with LPS (100 ng/ml) and Sz-B and C at 1, 5, and 10 μM for 24 h. All data of each group are expressed as the mean±S.E.M. $*p<0.05$; significantly different from LPS-treated alone group (A) and untreated group (B). Cell viability was expressed relative to untreated (control) culture (100%).

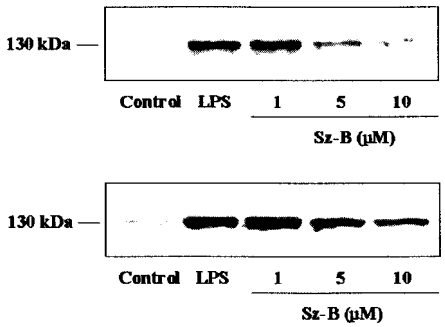


Fig. 2. Effect of Sz-B and C on LPS-Induced iNOS Expression

Expression of iNOS was determined by western blot analysis (50 μg total cell protein per lane). BV-2 cells were treated LPS (100 ng/ml) and in the absence or presence of Sz-B and C for 6 h. A representative picture from one of three different experiments is shown.

DISCUSSION

Activated microglia extensively produce nitric oxide (NO) by inducing the expression of inducible NO synthase (iNOS). NO is a major toxic substance released from activated microglia and plays a deleterious role in brain inflammation and neuronal death. Although NO has been particularly well studied because of its neurotoxicity in neuronal cells, the roles of NO have not been definitively elucidated. It was reported that NO demonstrated a neuroprotective effect through the S-nitrosylation of cysteine in caspase, which plays an important role in neuronal cell apoptosis.[18] On the other hand, NO was reported to induce neuronal cell death following DNA damage and disruption of mitochondrial function. NO reacts with superoxide anion to yield peroxynitrite, which is a strong oxidative stress agent.[19] The neuroprotective or neurotoxic action of NO may depend on several factors, including the amount of NO production and the microenvironment surrounding the NO. However, excessive NO produced in pathological conditions may contribute to neuronal cell death.[1,7,20] Therefore, NO production by iNOS provides a measure with which to assess the effects of drugs on brain inflammation.

In the present study, the effects of 1,3-selenazol-4-one derivatives on LPS-induced NO production and iNOS expression were examined. Among these compounds, Sz-B and Sz-C dose-dependently inhibited not only NO production but also iNOS expression in LPS-induced BV-2 cells. 1,3-Selenazol-4-one derivatives (Sz-A, B, C, D, and E) have a structural similarity but have slightly different functional groups at the 4′ and 5 positions. The five 1,3-selenazol-4-one derivatives were observed to have highly specific structurally-dependent, effects on LPS-induced NO production and iNOS expression in BV-2 cells. Sz-B and Sz-C have been found to be very potent inhibitors of NO production. On the other hand, the other three, Sz-A, D, and E, did not have any inhibitory effects on NO production. Compounds Sz-A and Sz-E bear the same 4-selenazolone skeleton but they have different R1 groups *i.e.*, a methyl group (Sz-A) and a chloride group (Sz-E), in the phenyl ring at the 4′ position. Although they have different R_1 groups, this is unlikely to affect NO production. Compounds Sz-B and C bearing ethyl or two-methyl groups, respectively, exhibited the strongest inhibitory effects on NO production. Consequently, an ethyl or two methyl groups at the 5 position of the 4-selenazolone skeleton are the major functional groups that play important roles in inhibiting NO production and iNOS expression.

Acknowledgments This work was supported by grants from the Brain Korea 21 projects (Ministry of Education, Korea) and Biogreen 21 (RDA, Korea, 02-N-I-02).

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