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Research Paper

Bis-(3-hydroxyphenyl) diselenide inhibits LPS-stimulated iNOS and COX-2 expression in RAW 264.7 macrophage cells through the NF-κB inactivation

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Abstract

Objectives Previously, we reported that diaryl diselenide compounds have strong inhibitory effects on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in macrophages. In this study, we investigated the molecular mechanisms underlying NO suppression and prostaglandin E_2 (PGE_2) production by diaryl diselenide compounds, bis-(2-hydroxyphenyl) diselenide (DSE-A), bis-(3-hydroxyphenyl) diselenide (DSE-B), bis-(4-hydroxyphenyl) diselenide (DSE-C), dipyridyl diselenide (DSE-D) and diphenyl diselenide (DSE-E).

Methods The effect of these compounds on NO suppression and PGE_2 production was investigated in RAW 264.7 macrophages.

Key findings Our data indicate that of the above, DSE-B most potently inhibits NO and PGE_2 production, and that it also significantly reduces the releases of tumour necrosis factor (TNF)-α, interleukin(IL)-1β and IL-6. Consistent with these observations, DSE-B also reduced the protein levels of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2), and the mRNA levels of iNOS, COX-2, TNF-α, IL-1β and IL-6. Furthermore, DSE-B inhibited LPS-induced nuclear factor-κB (NF-κB) activation, which was associated with the prevention of the inhibitor κB-α (IκB-α) degradation and a subsequent reduction in nuclear p65 protein levels.

Conclusions Taken together, our data suggest that the anti-inflammatory properties of DSE-B are due to reduction in the expression of iNOS, COX-2, TNF-α, IL-1β and IL-6 through the down-regulation of NF-κB binding activity.

Keywords anti-inflammatory activity; diaryl diselenide compounds; NO suppression; PGE_2 production; RAW 264.7 macrophages

Introduction

Chronic inflammation causes the upregulation of pro-inflammatory enzymes and signalling proteins in affected tissues and cells; enzymes and proteins thus upregulated include inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX) family members, which are responsible for elevating nitric oxide (NO) and prostaglandin (PG) levels, respectively. Furthermore, these inflammatory mediators are involved in the pathogenesis of chronic diseases, such as multiple sclerosis, Parkinson's disease, Alzheimer's disease and colon cancer.[1]

NO is produced by iNOS in macrophages, hepatocytes and renal cells when the cells are stimulated by agents such as lipopolysaccharide (LPS).[2] Furthermore, a number of studies have demonstrated that iNOS activity is correlated with the chronic phase of inflammation.[3] The most conclusive evidence, which indicates that NO is a mediator of tissue injury, was obtained during studies on osteoarthritis and rheumatoid arthritis in animal models.[4] COX is the enzyme that converts arachidonic acid to PGs. Of the two known COX isoforms (COX-1 and COX-2), COX-2 is an inducible form, and is responsible for the production of large amounts of pro-inflammatory PGs at sites of inflammation.[5]

Tumour necrosis factor-α (TNF-α) is a potent pro-inflammatory cytokine that plays an important role in immunity and inflammation. TNF-α has many harmful systemic effects, such as fever, systemic oedema and shock.[6] Similarly interleukin(IL)-1β release may lead to cell or tissue damage,[7,8] and conversely, reducing this release of IL-1β by macrophages

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may retard the inflammatory response to lipopolysaccharide (LPS). Finally, the production of pro-inflammatory IL-6 is induced by several factors (e.g., TNF-α, IL-1β and LPS). IL-6 acts as an endogenous pyrogen and it probably has many other effects on the immune system, particularly on haematopoiesis.[9]

Expression of the pro-inflammatory enzymes and cytokines mentioned above may be regulated at the transcriptional, post-transcriptional, translational or post-translational levels.[10,11] Nuclear factor-κB (NF-κB) is one of the most ubiquitous transcription factors and regulates the gene expression required for cellular proliferation, inflammatory response and cell adhesion. Functionally active NF-κB exists mainly as a heterodimer with a Rel family subunit. However, these heterodimers are normally sequestered in the cytosol as inactive complexes due to binding with IκB (inhibitor of κB).[12] NF-κB activation requires IκB phosphorylation at two critical serine residues (Ser^{32} and Ser^{36}) by IκB kinase (IKK) signalosome complex.[13–15] Once IκB is phosphorylated, it is targeted for ubiquitination and subsequent degradation by the 26S proteosome.[16] The resulting free NF-κB is translocated to the nucleus, where it binds to the κB binding sites in the promoter regions of target genes.[17,18]

The selenium-containing compounds ebselen and diphenyl diselenide have been reported to be promising anti-inflammatory drugs.[19,20] Moreover, organic forms of selenium have been suggested to act as antioxidant agents because they exhibit glutathione peroxidase-like activity and oxidise –SH during the reduction of H_2O_2.[19] However, though the mechanism that underlies the anti-inflammatory activity of ebselen is not completely understood, it is known to be linked to the inhibition of NADPH-oxidase, protein kinase C, iNOS and lipoxygenases, probably by interacting with critical thiol/disulfide groups in these enzymes.[20–22]

During our ongoing screening programme designed to identify anti-inflammatory compounds, we found that diaryl diselenide compounds significantly inhibit NO production in LPS-activated RAW 264.7 macrophage cells.[23] This study was undertaken to evaluate the effects of five diaryl diselenides (Figure 1a–e), bis-(2-hydroxyphenyl) diselenide (DSE-A), bis-(3-hydroxyphenyl) diselenide (DSE-B), bis-(4-hydroxyphenyl) diselenide (DSE-C), dipyridyl diselenide (DSE-D) and diphenyl diselenide (DSE-E), on the LPS-induced NO and PGE_2 release. Furthermore, the effects of DSE-B on the inflammatory protein, mRNA, and cytokine levels via NF-κB binding in RAW 264.7 macrophages were investigated to gain a better insight into the mechanism of the anti-inflammatory effects.

Materials and Methods

Materials

bis-(2-Hydroxyphenyl) diselenide (DSE-A), bis-(3-hydroxyphenyl) diselenide (DSE-B), bis-(4-hydroxyphenyl) diselenide (DSE-C), dipyridyl diselenide (DSE-D) and diphenyl diselenide (DSE-E) were synthesised as previously described (Shen *et al.* 2004).[23] These compounds were checked by HPLC and were found to be > 98% pure. Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were obtained from Life Technologies Inc. (NY, US). iNOS, COX-2, p65, IκB-α, β-actin, poly (ADP-ribose) polymerase (PARP) monoclonal antibodies and the peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (CA, US). The enzyme immunoassay (EIA) kits for PGE_2, TNF-α, IL-1β and IL-6 were obtained from R&D Systems (MN, US). iNOS, COX-2, TNF-α, IL-1β, IL-6 and β-actin oligonucleotide primers were purchased from Bioneer (Seoul, Korea). Phenylmethylsulfonylfluoride (PMSF), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), sulfanilamide, aprotinin, leupeptin, dithiothreitol (DTT), L-N^6-(1-iminoethyl)

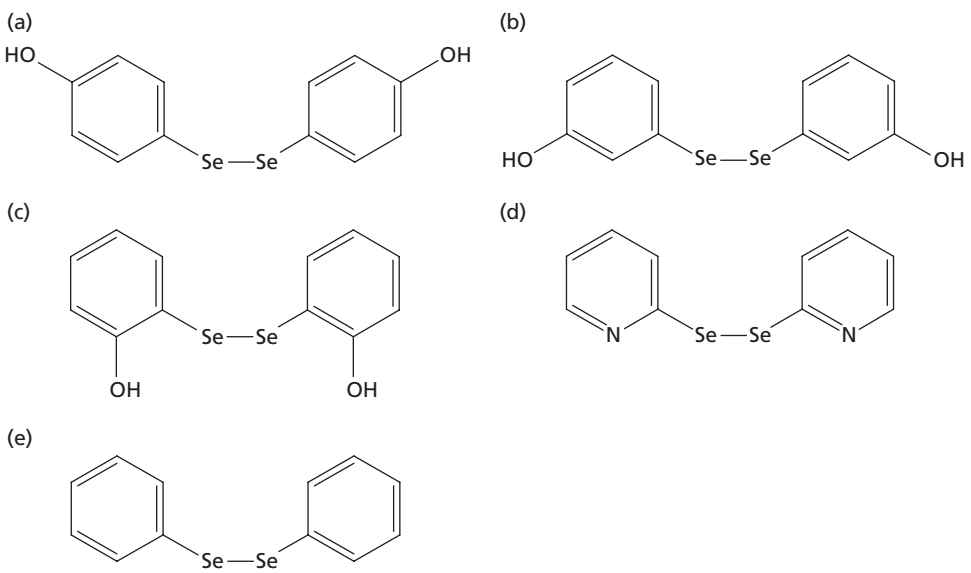

Figure 1 Chemical structures of the diaryl diselenide compounds. (a) bis-(2-Hydroxyphenyl) diselenide (DSE-A); (b) bis-(3-hydroxyphenyl) diselenide (DSE-B); (c) bis-(4-hydroxyphenyl) diselenide (DSE-C); (d) dipyridyl diselenide (DSE-D); (e) diphenyl diselenide (DSE-E).

lysine (L-NIL), 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine (PAPA NONOate), LPS (*Escherichia coli*, serotype 0111:B4), Triton X-100 and all other chemicals were purchased from Sigma Chemical Co. (MO, US).

Cell culture and sample treatment

The RAW 264.7 murine macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). These cells were grown at 37°C in DMEM medium supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin sulfate (100 μg/ml) in a humidified atmosphere of 5% CO_2. Cells were incubated with diaryl diselenide compounds at increasing concentrations (2, 4, 8 μM) or positive chemical and then stimulated with LPS (1 μg/ml) for the indicated time.

MTT assay

Cytotoxicity studies were performed in a 96-well plate. RAW 264.7 cells were mechanically scraped and plated at 1×10^5/well in 96-well plates containing 100 μl of DMEM with 10% heat-inactivated FBS and incubated overnight. Tested compounds were dissolved in dimethyl sulfoxide (DMSO), and the DMSO concentrations in all assays did not exceed 0.01%. After overnight incubation, the test material was added, and the plates were incubated for 24 h. Cells were washed once before adding 50 μl of FBS-free medium containing MTT (5 mg/ml). After 4 h of incubation at 37°C, the medium was discarded and the formazan blue that formed in the cells was dissolved in 100 μl DMSO. The absorbance was measured at 540 nm via a microplate reader.

Nitrite determination

The nitrite accumulated in the culture medium was measured as an indicator of NO production based on the Griess reaction. Briefly, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent (equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamine-HCl), incubated at room temperature for 10 min, and then the absorbance at 540 nm was measured in a microplate reader (Perkin Elmer Cetus, CA, US). Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was measured with the sodium nitrite serial dilution standard curve and nitrite production was measured.

Determination of PGE_2, TNF-α, IL-1β and IL-6 levels

PGE_2, TNF-α, IL-1β and IL-6 levels in the macrophage culture medium were quantified by EIA kits according to the manufacturer's instructions.

Western blot analysis

Cellular proteins were extracted from control and DSE-B-treated RAW 264.7 cells. Cells were collected by centrifugation and washed once with phosphate-buffered saline (PBS). The washed cell pellets were resuspended in extraction lysis buffer (50 mM HEPES pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM PMSF, 0.5 mM DTT, 5 mM sodium fluoride and 0.5 mM sodium orthovanadate) containing 5 μg/ml each of leupeptin and aprotinin and incubated for 20 min at 4°C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. The protein concentration was determined using a Bio-Rad protein assay reagent according to the manufacturer's instructions. Forty micrograms of cellular protein from treated and untreated cell extracts was electroblotted onto a nitrocellulose membrane following separation on a 10% SDS-polyacrylamide gel by electrophoresis. The immunoblot was incubated overnight with blocking solution (5% skimmed milk) at 4°C, followed by incubation for 4 h with a primary antibody. Blots were washed four times with Tween 20/Tris-buffered saline (TTBS) and incubated with a 1 : 1000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Blots were again washed three times and then developed by enhanced chemiluminescence (Amersham Life Science, IL, US).

RNA preparation and reverse transcriptase polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated using an Easy Blue kit (Intron Biotechnology, Seoul, Korea) according to the manufacturer's instructions. From each sample, 1 μg of RNA was reverse-transcribed using MuLV reverse transcriptase, 1 mM dNTP and oligo ($dT_{12\text{-}18}$) 0.5 μg/μl. Then PCR analyses were performed on the samples of the cDNA preparations to detect iNOS, COX-2, TNF-α, IL-1β, IL-6 and β-actin (as an internal standard) gene expression using a thermal cycler (Perkin Elmer Cetus, CA, US). The reactions were carried out in a volume of 25 μl containing (final concentration) 1 unit of Taq DNA polymerase, 0.2 mM dNTP, ×10 reaction buffer and 100 pmol of 5′ and 3′ primers. After initial denaturation for 2 min at 95°C, 30 amplification cycles were performed for iNOS (1 min of 95°C denaturation, 1 min of 60°C annealing and 1.5 min 72°C extension), COX-2 (1 min of 94°C denaturation, 1 min of 60°C annealing and 1 min 72°C extension), TNF-α (1 min of 95°C denaturation, 1 min of 55°C annealing and 1 min 72°C extension), IL-1β (1 min of 94°C denaturation, 1 min of 60°C annealing and 1 min 72°C extension) and IL-6 (1 min of 94°C denaturation, 1 min of 57°C annealing and 1 min 72°C extension). The PCR primers used in this study are listed below and were purchased from Bioneer (Seoul, Korea): sense strand iNOS, 5′-AAT GGC AAC ATC AGG TCG GCC ATC ACT-3′, anti-sense strand iNOS, 5′-GCT GTG TGT CAC AGA AGT CTC GAA CTC-3′; sense strand COX-2, 5′-GGA GAG ACT ATC AAG ATA GT-3′, anti-sense strand COX-2, 5′-ATG GTC AGT AGA CTT TTA CA-3′; sense strand TNF-α, 5′-ATG AGC ACA GAA AGC ATG ATC-3′, anti-sense strand TNF-α, 5′-TAC AGG CTT GTC ACT CGA ATT-3′; sense strand IL-6, 5′-GAG GAT ACC ACT CCC AAC AGA CC-3′, anti-sense strand IL-6, 5′-AAG TGC ATC ATC GTT GTT CAT ACA-3′; sense strand IL-1β, 5′-TGC AGA GTT CCC CAA CTG GTA CAT C-3′, anti-sense strand IL-1β, 5′-GTG CTG CCT AAT GTC CCC TTG AAT C-3′; sense strand β-actin, 5′-TCA TGA AGT GTG ACG TTG ACA TCC GT-3′, anti-sense strand β-actin, 5′-CCT AGA AGC ATT TGC GGT GCA CGA TG-3′. After amplification, portions of the PCR reactions were electrophoresed on 2%

agarose gel and visualised by ethidium bromide staining and UV irradiation.

Nuclear extraction and electrophoretic mobility shift assay

RAW 264.7 macrophages cells were plated in 100-mm dishes (1×10^6 cells/ml). The cells were treated with various DSE-B concentrations (2, 4, 8 μM), stimulated with LPS for 1 h, washed once with PBS, scraped into 1 ml of cold PBS and pelleted by centrifugation. Nuclear extracts were prepared as described previously,[24] with slight modification. The cell pellet was resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT and 10 μg/ml aprotinin) and incubated on ice for 15 min. Then the cells were lysed by the addition of 0.1% Nonidet P-40 and vigorous vortexing for 10 s. The nuclei were pelleted by centrifugation at 12 000 *g* for 1 min at 4°C and resuspended in high-salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM NaF and 1 mM sodium orthovanadate). Nuclear extract 10 μg was mixed with the double-stranded NF-κB oligonucleotide. 5′-AGTTGAGGGGACTTTCC-CAGGC-3′ end-labelled by [γ-^{32}P]dATP (underlining indicates a κB consensus sequence or a binding site for NF-κB/cRel homodimeric and heterodimeric complex). Binding reactions were performed at 37°C for 30 min in 30 μl of reaction buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 4% glycerol, 1 μg of poly (dI-dC) and 1 mM DTT. The specificity of binding was examined by competition with the 80-fold unlabelled oligonucleotide. DNA–protein complexes were separated from the unbound DNA probe on native 5% polyacrylamide gels at 100 V in 0.5 × TBE buffer. The gels were vacuum dried for 1 h at 80°C and exposed to X-ray film at –70°C for 24 h.

Statistical analysis

Results are expressed as means ± SD of at least three experiments performed using different cell preparations *in vitro*. Statistically significant values were compared using a non-parametric multiple comparisons test (Kruskal–Wallis test) followed by Dunn's test. Statistical significance was set at $P < 0.05$.

Results

Effect of diaryl diselenides on NO and PGE_2 production and cell viability

The cytotoxic effects of the five diaryl diselenides (DSE-A, DSE-B, DSE-C, DSE-D and DSE-E) were evaluated in the presence or absence of LPS using MTT assays. Preliminary experiments showed that only DSE-E reduced cell viability (to 48% at 8 μM for 24 h in the presence of LPS); the other four agents had no effect even at 50 μM after 24 h regardless of the presence of LPS (data not shown). Thus, DSE-E was excluded from subsequent studies.

Unstimulated RAW 264.7 cells exhibited a low basal level of NO and PGE_2 production, and the addition of LPS to culture medium dramatically increased this production over the 24-h observation period (Figure 2a, b). To assess the effects of DSE-A, DSE-B, DSE-C and DSE-D on NO production in RAW 264.7 cells, cell culture media were harvested and nitrite production was measured using a Griess-reaction-based method. All four diselenides reduced NO production in a dose-dependent manner with IC50 values of 7.9, 5.0, 5.3 and 15.9 μM, respectively (Figure 2a). L-NIL (10 μM) was used as a positive NO production inhibitor. In addition, we investigated the effects of these diaryl diselenide compounds on PGE_2 production. Accordingly, cells were pre-incubated with each compound at 2, 4 or 8 μM for 1 h, and then activated with 1 μg/ml LPS for 24 h. As shown in Figure 2b, DSE-B also significantly inhibited the production of PGE_2, with an IC50 value of 1.6 μM. We also investigated whether DSE-B scavenges NO radicals released from PAPA NONOate (a water-soluble NO/nucleophile complex), which can spontaneously dissociate to free amine and two NO radicals in a pH-dependent manner following first-order kinetics.[25] In fact, DSE-B (2, 4 or 8 μM) did not scavenge NO radicals (data not shown), whereas caffeic acid 100 μM (positive control) clearly scavenged NO radicals, which reduced from 42.6 ± 1.5 μM to 12.4 ± 0.8 μM.

Effect of DSE-B on LPS-induced TNF-α, IL-1β and IL-6 release and their mRNA expression

Since DES-B was found to most potently inhibit the pro-inflammatory mediators, we further investigated its effect on LPS-induced TNF-α, IL-1β and IL-6 release by enzyme immunoassay and RT-PCR. Pre-treatment of RAW 264.7 cells with DSE-B (2, 4 or 8 μM) for 1 h significantly and dose-dependently reduced TNF-α, IL-1β and IL-6 production (Figure 3a–c) and their mRNA expression (Figure 3d).

Effect of DSE-B on LPS-induced iNOS and COX-2 protein and mRNA expression

Western blot and RT-PCR analysis were performed to determine whether the inhibitory effect of DSE-B on NO and PGE_2 production is related to its modulation of iNOS and COX-2. In unstimulated RAW 264.7 cells, iNOS and COX-2 were undetectable at either the protein or mRNA levels. However, iNOS and COX-2 proteins were obviously induced by LPS, and pre-treatment with DSE-B significantly and dose-dependently inhibited this upregulation (Figure 4a). On the other hand, DSE-B did not affect β-actin expression (the housekeeping gene). In general, these results indicate that the inhibitory effects of DSE-B on LPS-induced NO and PGE_2 production are caused by iNOS and COX-2 suppression. Furthermore, the RT-PCR results obtained suggested that the expression of iNOS and COX-2 mRNA were correlated with their protein levels (Figure 4b).

Effect of DSE-B on LPS-induced p65 nuclear translocation and NF-κB activation

To further investigate the mechanism of the DSE-B-mediated inhibitions of iNOS, COX-2, TNF-α, IL-1β and IL-6 transcription, we focused on NF-κB, a transcription factor known to transactivate iNOS, COX-2, TNF-α and other genes. Thus, we examined whether DSE-B prevents the

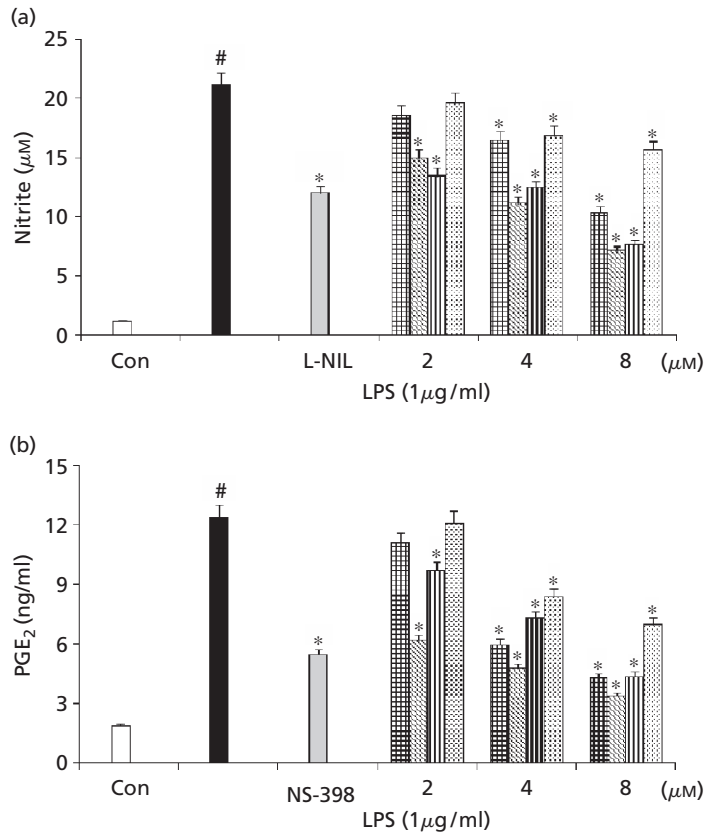

Figure 2 Effect of diaryl diselenide compounds on lipopolysaccharide (LPS)-induced NO (a) and PGE_2 (b) production in RAW 264.7 cells. (a) Cells were treated with different concentrations (2, 4, 8 μM) of the test agents for 1 h, LPS (1 μg/ml) was then added and cells were incubated for 24 h. Control (Con) values were obtained in the absence of LPS or test samples. L-N^6-(1-Iminoethyl) lysine (L-NIL) was used as a positive control at 10 μM. (b) Test samples were treated as described in Figure 2a. NS-398 (10 μM) was used as a positive control. ▥, DSE-A; ▧, DSE-B; ▩, DSE-C; ▨, DSE-D. Values shown are means ± SD of three independent experiments. *$P < 0.05$ vs the group treated with LPS only; $^{\#}P < 0.05$ vs control.

translocation of p65 (the subunit of NF-κB) from the cytosol to the nucleus after p65 release from IκB. Accordingly, RAW 264.7 cells were incubated with LPS (1 μg/ml) in the presence or absence of DSE-B (2, 4 or 8 μM) for 1 h. Western blotting analysis showed that p65 protein levels were negligible in control cell nuclei, but that LPS induced its nuclear translocation, and that DSE-B concentration-dependently attenuated LPS-induced p65 nuclear levels (Figure 5a). PARP was used as an internal control.

Electrophoretic mobility shift assays (EMSA) demonstrated that LPS-induced NF-κB-DNA binding activity in RAW 264.7 macrophages was significantly and dose-dependently reduced by DSE-B (Figure 5b), and that an excess of unlabelled NF-κB oligonucleotide completely inhibited NF-κB-DNA binding activity. These observations suggest that DSE-B inhibited NF-κB binding by preventing the LPS-induced nuclear translocation of p65.

Effect of DSE-B on LPS-induced IκB-α degradation

In unstimulated RAW 264.7 cells, NF-κB is sequestered in the cytosol by its inhibitor IκB, and when stimulated by LPS, IκB is phosphorylated by IκB kinases (IKK), ubiquitinated and rapidly degraded by 26S proteosome, releasing NF-κB.[12] Here, we investigated whether DSE-B (8 μM) inhibits the LPS-stimulated degradation of IκB-α by conducting Western blot assays using anti-IκB-α antibody. Figure 6 shows that LPS induced the degradation of IκB-α at 10–60 min, and that this degradation was substantially blocked by DSE-B pre-treatment.

Discussion

Due to the need for more effective anti-inflammatory compounds with fewer side effects, we investigated whether diaryl diselenide compounds have pharmacological anti-inflammatory activity. Thus, we screened and compared the effects of diaryl diselenides on the levels of LPS-induced pro-inflammatory molecules, including NO and PGE_2. It was found that DSE-B was the most potent inhibitor of these mediators among the tested compounds. Based on the results of the in-vitro structure–activity relationships, it appears that activity is associated with contributions made by the polarity of compounds and the respective Se–Se bond strength due to the delocalisation of ortho-, meta, or para- hydroxyl groups over the molecule. Diaryl diselenide compounds usually can be dissociated to the seleno-radical, which is eventually changed to selenol during metabolism. The resulting selenols

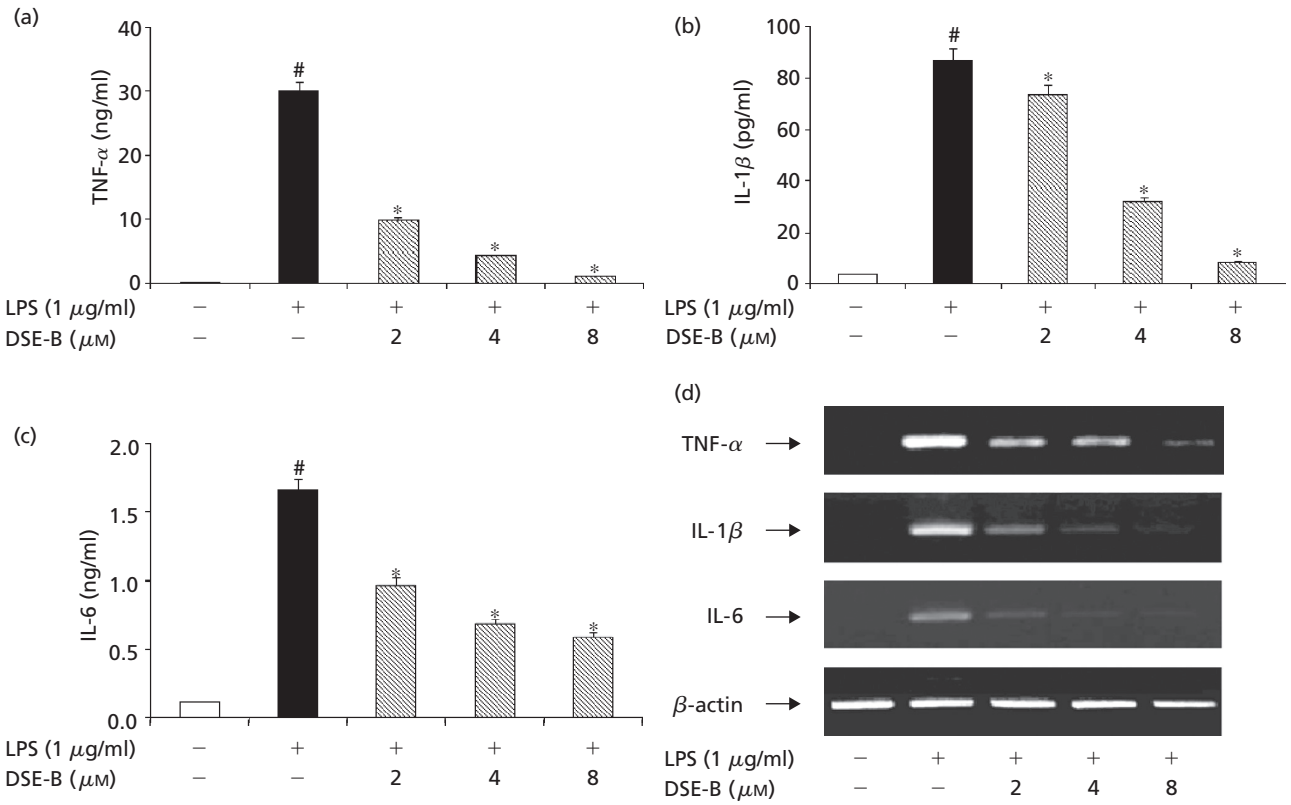

Figure 3 Effect of DSE-B on lipopolysaccharide (LPS)-induced TNF-α (a), IL-1β (b) and IL-6 (c) release and their mRNA expression (d) in RAW 264.7 cells. (a) Cells were treated with different concentrations (2, 4, 8 μM) of DSE-B for 1 h, LPS (1 μg/ml) was then added, and cells were incubated for 24 h. Control (Con) values were obtained in the absence of LPS or DSE-B. Values represent means ± SD of three independent experiments. $^{*}P < 0.05$ vs the LPS-treated group; $^{\#}P < 0.05$ vs control. (b, c) Samples were treated as described for Figure 3a. (d) Total RNA was prepared for the RT-PCR analysis of TNF-α, IL-1β and IL-6 gene expression from RAW 264.7 macrophages pretreated with different concentrations (2, 4, 8 μM) of DSE-B for 1 h, and this was then followed by LPS (1 μg/ml) treatment for 4 h. PCR for β-actin was performed to verify that the initial cDNA content of the samples were similar. This experiment was repeated twice with similar results.

are more polar than diselenide and the availability of these selenols might be reduced. DSE-A and DSE-C can produce very resonance-stabilised seleno-radicals, which means that the Se–Se bond can be broken to give stable radicals. On the other hand, DSE-B cannot make a stable radical, and it is safe to some degree from cleavage of the Se–Se bond. In the case of DSE-D, the dipole momentum of the pyridine moiety means that the Se–Se bond can be easily broken to produce a radical. Of course, this kind of assumption needs further study and should be proved by feedback from in-vivo pharmacological studies in the near future.

To further explore the possible mechanism of the inhibition by DSE-B, we examined iNOS and COX-2 protein levels and iNOS, COX-2, TNF-α, IL-1β and IL-6 mRNA levels. The observed inhibition by DSE-B of the LPS-stimulated expression of these moieties in RAW 264.7 cells was not attributable to cytotoxicity, as assessed by MTT assay and β-actin expression. Furthermore, the inhibition of the iNOS and COX-2 gene expression by DSE-B was demonstrated by concomitant reduction in their mRNA levels, indicating that inhibited NO and PGE_2 release may be due to the downregulation of iNOS and COX-2 transcription followed by protein expression.

The expression of iNOS and COX-2 in murine macrophages has been shown to be dependent on NF-κB activation.[10,11] Thus, we examined the possibility that DSE-B inhibits NF-κB activity. The results obtained indicate that protein levels of iNOS and COX-2, and mRNA levels of iNOS, COX-2, TNF-α, IL-1β and IL-6 inhibition by DSE-B are probably due to NF-κB suppression, which is consistent with the findings of previous reports that found that NF-κB response elements are present in the promoters of iNOS, COX-2, TNF-α, IL-1β and IL-6.[26] NF-κB is primarily composed of two proteins, p50 and p65, which are also referred to as RelA and cRel, respectively.[12] In the resting state, NF-κB is present in cytosol where it is bound to the inhibitory protein, IκB. Following its induction by a variety of agents (e.g. LPS, TNF-α or tissue plasminogen activator), IκB is phosphorylated, which triggers its proteolytic degradation via a 26S proteosome.[16] In so doing, NF-κB is released from IκB and translocated to the nucleus, where it binds to κB binding sites in the promoter regions of target genes. In this study, we found that DSE-B blocked the LPS-induced activation of NF-κB by inhibiting the degradation of IκB. These results suggest that the inhibitory effect of DSE-B on the induction of iNOS and COX-2 expression by LPS is

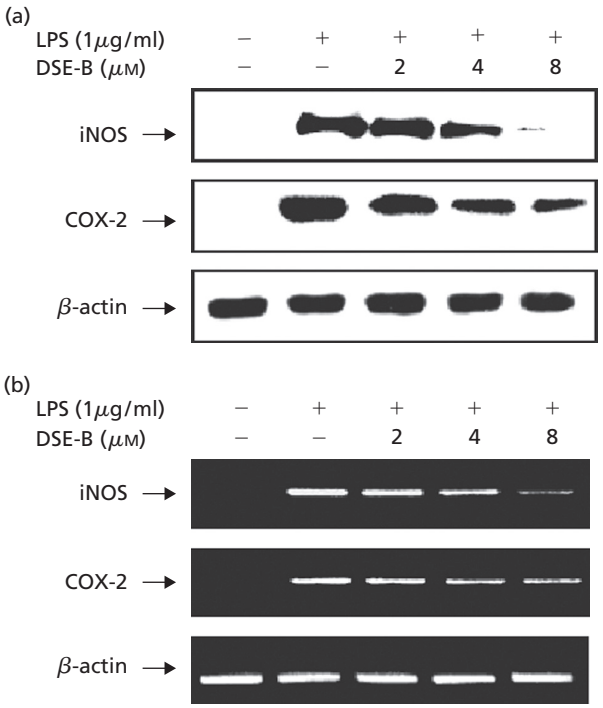

Figure 4 Effect of DSE-B on lipopolysaccharide (LPS)-induced iNOS and COX-2 proteins (a) and their mRNA (b) expression in RAW 264.7 cells. (a) Cells were treated with different concentrations (2, 4, 8 μM) of DSE-B for 1 h, LPS (1 μg/ml) was then added and cells were incubated for 24 h. Total cellular proteins (40 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membranes and detected with specific antibodies as described in Methods. A representative immunoblot of three separate experiments is shown. (b) Samples were treated as described in Figure 3a. Total RNA was prepared using the conditions described in Figure 3b, and RT-PCR was performed to compare the mRNA levels of iNOS and COX-2. iNOS-specific sequences (807 bp) and COX-2-specific sequences (721 bp) were detected by agarose gel electrophoresis, as described in Methods. PCR of β-actin was performed to verify that the initial cDNA contents of samples were similar. The experiment was repeated three times and similar results were obtained.

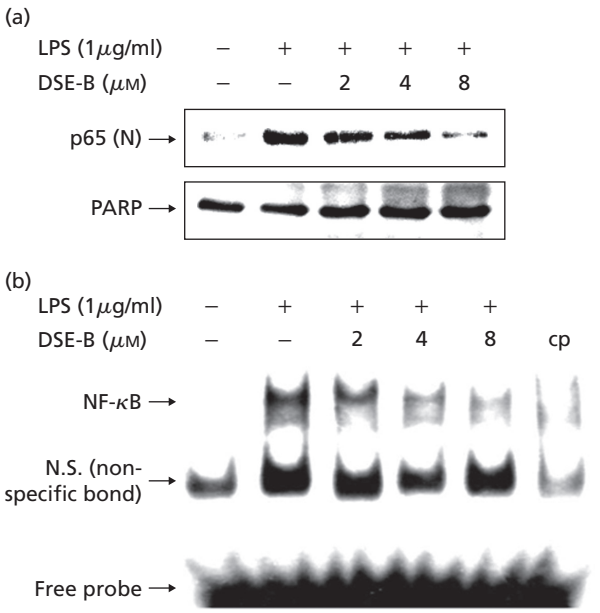

Figure 5 Inhibition of p65 translocation to the nucleus and NF-κB-DNA binding by DSE-B. (a) Nuclear extracts were prepared from untreated control cells or from cells pretreated with different concentrations (2, 4, 8 μM) of DSE-B for 1 h and then treated with lipopolysaccharide (1 μg/ml) for 1 h. Nuclear extracts (40 μg/ml) were prepared for Western blotting of p65 using specific anti-p65 NF-κB monoclonal antibody. (b) Nuclear extracts were analysed for NF-κB binding by electrophoretic mobility shift assay. Specificity of binding was examined by competition with an 80-fold excess of unlabelled NF-κB oligonucleotide (cp). Data are representative of two independent experiments. PARP, poly(ADP-ribose) polymerase.

attributable to its preventing NF-κB activation, although the inhibition of other factors, such as AP-1, interferon response element and γ-activated sites may also be involved. It is well-recognised that activation of IKK complex phosphorylates IκB-α, and the IKK complex may be activated by various upstream kinases, which include protein kinase C and tyrosine kinase family members.[27,28] In addition, mitogen-activated protein (MAP) kinases play a critical role in the regulation of cell growth and differentiation and in the control of cellular responses to cytokines and stresses. Moreover, they are also known to be important for the activation of NF-κB.[29] Therefore, it remains to be established whether DSE-B acts on these upstream kinases and, if so, what is the true pharmacological target of DSE-B.

Diphenyl diselenide, a simple diaryl diselenide, exerts biological actions, including antioxidant,[30] hepatoprotective,[31] anti-ulcer,[32] anti-inflammatory and antinociceptive effects.[33,34] Previous studies have shown that diphenyl diselenide has an anti-inflammatory effect in rat models of inflammation (e.g. carrageenan- and arachidonic acid-induced paw oedema).[33] The anti-inflammatory effect of

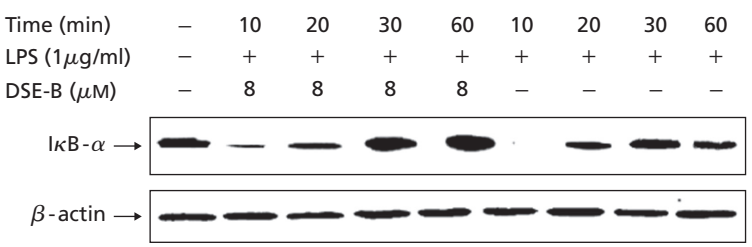

Figure 6 Effect of DSE-B on lipopolysaccharide (LPS)-induced IκB-α degradation. RAW 264.7 macrophages were pretreated or not with the indicated concentrations (2, 4, 8 μM) of DSE-B for 1 h and then incubated with LPS (1 μg/ml) for 15 min. Total cellular proteins were prepared and Western blotted for IκB-α using specific IκB-α antibodies. β-Actin was used as an internal control. Experiments were repeated three times and similar results were obtained.

diphenyl diselenide is known to depend on its ability to modulate NO synthesis and the serotoninergic and glutamatergic systems.[33] However, the results of this study encourage us to propose that the action of bis-(3-hydroxyphenyl) diselenide (DSE-B) depends on its ability to prevent κB degradation and, thus, to reduce the transcriptional activity of NF-κB.

Conclusions

The results of this study indicate that DSE-B is a potent inhibitor of LPS-induced NO, PGE_2, TNF-α, IL-1β and IL-6 production and that it acts at the transcriptional level by blocking NF-κB activation in RAW 264.7 macrophages. We believe that these findings considerably promote the possibility that bis-(3-hydroxyphenyl) diaryl diselenide could be used to treat LPS-induced sepsis syndrome and rheumatoid arthritis. Further investigation is required to find out whether this compound can suppress inflammatory reactions *in vivo*.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to declare.

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References

1. Heiss E *et al.* Nuclear factor kappa B is a molecular target for sulforaphane-mediated anti-inflammatory mechanisms. *J Biol Chem* 1995; 276: 32008–32015.
2. Kuo PC, Schroeder RA. The emerging multifaceted roles of nitric oxide. *Ann Surg* 1995; 221: 220–235.
3. Miller MJ, Grisham MB. Nitric oxide as a mediator of inflammation? You had better believe it! *Mediators Inflamm* 1995; 4: 387–396.
4. Cochran FR *et al.* Insights into the role of nitric oxide in inflammatory arthritis. *Med Rev Res* 1996; 16: 547–563.
5. Weisz A *et al.* Regulation of the mouse inducible-type nitric oxide synthase gene promoter by interferon-gamma, bacterial lipopolysaccharide and NG-monomethyl-L-arginine. *Biochem J* 1996; 316: 209–215.
6. Beutler B, Cerami A. The biology of cachectin/TNF–a primary mediator of the host response. *Annu Rev Immunol* 1989; 7: 625–655.
7. Molloy RG *et al.* Cytokines, sepsis and immunomodulation. *Br J Surg* 1993; 80: 289–297.
8. West MA *et al.* Pre-exposure to hypoxia or septic stimuli differentially regulates endotoxin release of tumor necrosis factor, interleukin-6, interleukin-1, prostaglandin E2, nitric oxide, and superoxide by macrophages. *J Trauma* 1994; 37: 82–89.
9. Van Snick J. Interleukin-6: an overview. *Annu Rev Immunol* 1990; 8: 253–278.
10. Vodovotz Y *et al.* Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor beta. *J Exp Med* 1993; 178: 605–613.
11. Rao KMJ. Molecular mechanisms regulating iNOS expression in various cell types. *Toxicol Environ Health B Crit Rev* 1999; 3: 27–58.
12. Baeuerle PA. IkappaB-NF-kappaB structures: at the interface of inflammation control. *Cell* 1998; 95: 729–731.
13. Brown K *et al*. Control of I kappa B-alpha proteolysis by site-specific, signal-induced phosphorylation. *Science* 1995; 267: 1485–1488.
14. DiDonato J *et al.* Mapping of the inducible IkappaB phosphorylation sites that signal its ubiquitination and degradation. *Mol Cell Biol* 1996; 4: 1295–1304.
15. O'Conell MA *et al.* Role of IKK1 and IKK2 in lipopolysaccharide signaling in human monocytic cells. *J Biol Chem* 1998; 46: 30410–30414.
16. Sanchez-Perez I *et al.* Cell stress and MEKK1-mediated c-Jun activation modulate NFkappaB activity and cell viability. *Mol Biol Cell* 2002; 13: 2933–2945.
17. Baeuerle PA, Baltimore D. NF-kappa B: ten years after. *Cell* 1996; 87: 13–20.
18. Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 1997; 336: 1066–1071.
19. Wendel A *et al.* A novel biologically active seleno-organic compound–II. Activity of PZ 51 in relation to glutathione peroxidase. *Biochem Pharmacol* 1984; 33: 3241–3245.
20. Contreave IA *et al.* Studies on the anti-inflammatory activity of ebselen. Ebselen interferes with granulocyte oxidative burst by dual inhibition of NADPH oxidase and protein kinase C? *Biochem Pharmacol.* 1989; 638: 649–656.
21. Walter M *et al.* The inhibition of mammalian 15-lipoxygenases by the anti-inflammatory drug ebselen: dual-type mechanism involving covalent linkage and alteration of the iron ligand sphere. *J Pharmacol Exp Ther* 1999; 56: 196–203.
22. Mugesh G *et al.* Chemistry of biologically important synthetic organoselenium compounds. *Chem Rev* 2001; 101: 2125–2179.
23. Shen L *et al.* Synthesis of new diselenide compounds as anti-inflammatory agents. *Arch Pharm Res* 2004; 27: 816–819.
24. Won JH *et al.* Anti-inflammatory effect of buddlejasaponin IV through the inhibition of iNOS and COX-2 expression in RAW 264.7 macrophages via the NF-kappaB inactivation. *Br J Pharmacol* 2006; 148: 216–225.
25. Morley D, Keefer LK. Nitric oxide/nucleophile complexes: a unique class of nitric oxide-based vasodilators. *J Cardiovasc Pharmacol* 1993; 22: 3–9.
26. Lappas M *et al.* Nuclear factor kappa B regulation of proinflammatory cytokines in human gestational tissues *in vitro*. *Biol Reprod* 2002; 67: 668–673.
27. Schimedtje JF *et al.* Hypoxia induces cyclooxygenase-2 via the NF-kappaB p65 transcription factor in human vascular endothelial cells. *J Biol Chem* 1997; 272: 601–608.
28. Henkel T *et al.* Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B. *Nature* 1993; 365: 182–185.
29. Vandenberghe W *et al.* p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for nuclear factor-κB p65 transactivation mediated by tumor necrosis factor. *J Biol Chem* 1998; 273: 3285–3290.
30. Meotti FC *et al.* Protective role of aryl and alkyl diselenides on lipid peroxidation. *Environ Res* 2004; 94: 276–282.
31. Borges LP *et al.* Acute liver damage induced by 2-nitropropane in rats: effect of diphenyl diselenide on antioxidant defenses. *Chem Biol Interact* 2006; 160: 99–107.
32. Savegnago L *et al.* Antisecretory and antiulcer effects of diphenyl diselenide. *Environ Toxicol Pharmacol* 2006; 21: 86–92.
33. Nogueira CW *et al.* Anti-inflammatory and antinociceptive activity of diphenyl diselenide. *Inflamm Res* 2003; 52: 56–63.
34. Savegnago L *et al.* Antinociceptive properties of diphenyl diselenide: evidences for the mechanism of action. *Eur J Pharmacol* 2007; 555: 129–138.