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Bromosulphophthalein Suppresses Inflammatory Effects in Lipopolysaccharide-Stimulated RAW264.7 Macrophages

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Abstract

Objective: It has been reported that glutathione (GSH), the most abundant cellular antioxidant, can inhibit production of pro-inflammatory cytokines by activated macrophages. Bromosulphophthalein (BSP) has been recognized as an inhibitor of the efflux of reduced GSH from cells, leading to an increase in the intracellular GSH level. In this study, we evaluated, for the first time, whether BSP possessed anti-inflammatory effects in lipopolysaccharide (LPS)-stimulated macrophages.

Materials and Methods: RAW 264.7 cells were treated with BSP and the levels of proinflammatory cytokines, GSH, and nitrite were assessed. Gene expression of inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF α), interleukin-1 β (IL-1 β) and interleukin-6 (IL-6) was analyzed via quantitative RT-PCR. We also examined various inflammatory signaling pathways including Akt/forkhead box protein O1 (FoxO1)/ toll-like receptor 4 (TLR4), mitogen-activated protein kinases (MAPKs) and Fas proteins by Western blot and flow cytometry analysis.

Results: Our study demonstrated that BSP induced an increase in intracellular GSH levels in LPS-stimulated macrophages. BSP inhibited production of nitric oxide and proinflammatory cytokines. BSP increased phosphorylation of Akt and nuclear exclusion of FoxO1 and suppressed TLR4 expression. Additionally, BSP decreased MAPKs activation and Fas expression.

Discussion and Conclusions: Taken together, these data suggest that BSP can attenuate inflammation through multiple signaling pathways. These findings highlight the potential of BSP as a new anti-inflammatory agent.

Keywords

bromosulphophthalein; glutathione; macrophages; inflammatory responses; signal pathways

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Conflict of Interest statement

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1. Introduction

Lipopolysaccharide (LPS) is an important structural component of the outer membrane of Gram-negative bacteria. LPS can activate macrophage and induce the production of pro-inflammatory cytokines including tumor necrosis factor alpha (TNF α), interleukin-1beta (IL-1 β) and interleukin-6 (IL-6), as well as nitric oxide(NO) which is catalyzed by inducible NO synthase (iNOS) [1]. These events are commonly initiated by the binding of LPS to its specific cell receptor toll-like receptor 4 (TLR4), and mediated by cytoplasmic molecules such as reactive oxygen species (ROS), mitogen activated protein kinases (MAPKs) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB or Akt). These molecules interact closely with each other and further modulate the activity of several nuclear transcription factors including Forkhead box protein O1 (FoxO1), leading to changes in the transcription of specific genes involved in the inflammatory response, such as TLR4 [2]. Another possible pathway regulating LPS-induced pro-inflammatory cytokines in macrophages involves Fas, also called CD95 or Apo-1 [3].

Bromosulphophthalein (BSP) is a triphenylmethane dye with two sulfonic acid groups. BSP has been frequently employed in studying the membrane transporters that mediate the transport of reduced glutathione (GSH) and organic anions across the cell membrane [4–6]. In this regard, BSP was able to function as an inhibitor of GSH efflux transporters on the cell membranes, leading to an increase in intracellular GSH level[5,6].

GSH is a tripeptide enzymatically synthesized by glycine, cysteine and glutamate in living organisms. It is the most abundant non-protein thiol and, probably, the most important cellular antioxidant with a crucial role in eliminating cellular ROS and thus maintaining a normal redox status in mammals [7]. The intracellular GSH level is dynamically controlled by several cellular activities, including biosynthesis, oxidation to form glutathione disulfide and efflux from the cells [5,8]. We previously reported that exogenous GSH could modulate the diverse cellular activities via ROS scavenging, including inhibition of adipogenesis in mouse bone marrow stem cells and protection of apoptosis and matrix synthesis in human intervertebral disc cells [9, 10]. With regard to its relevance to inflammation and macrophage activation, results from an *in vitro* experiment using a murine macrophage cell line J774 and primary mouse alveolar macrophage showed that LPS-stimulated TNF α release could be remarkably inhibited by GSH added to culture medium [7].

We are interested in investigating whether BSP can affect intracellular GSH level in macrophages and, more importantly, if it can inhibit macrophage activation. The present study compared levels of intracellular GSH and inflammatory biomarkers in BSP treated *vs* control groups using mouse macrophage cell line RAW264.7, a well-established *in vitro* model for inflammation and macrophage activation [1]. Furthermore, several signal molecules related to inflammatory pathways were also evaluated, including Akt, FoxO1, MAPKs and Fas.

2. Materials and methods

2.1. Cell culture and treatment

RAW264.7 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells at passage 4–6 were cultured in low glucose Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, VT, USA), 1% penicillin/streptomycin solution (Life Technologies, Grand Island, NY, USA) at 37 °C with 5% CO₂ in a humidified incubator. In all experiments except those specifically indicated, 200 µM BSP (Thermo Scientific, Waltham, MA, USA) was added to the culture 0.5 h prior to addition of 100 ng/mL LPS (Sigma-Aldrich Co., St Louis, MO, USA) and then cells were co-treated with LPS for 4 h.

2.2. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay

Cells were seeded on a 96-well plate (5000 cells/well). MTS assay kit (Promega, Madison, WI, USA) was used to test cytotoxicity according to the manufacturer's manual. Briefly, the cells were incubated in the normal culture medium for 24 hr after removal of drugs, and 20 µL of the assay solution was added into each well of the plate containing 100 µL of the culture medium. After 2 hr incubation, absorbance at 490 nm of the resultant product was obtained by a microplate reader.

2.3. Intracellular levels of GSH and NAD(P)H, production of nitrite and TNFα, and expression of CD11b and Fas

The amounts of GSH, nitrite and TNFα for cells growing on a 96-well plate were measured by the GSH assay kit (Sigma-Aldrich), the Griess reagent (Promega) and the ELISA kit (Sigma-Aldrich), respectively, following the instructions provided by the manufacturers. The culture medium was first collected for measuring nitrite and TNFα production. To prepare samples for intracellular GSH level, 50 µL of 5% 5-sulfosalicylic acid solution was added to each well after removal of the culture medium. The cells were then frozen under –80 °C and thawed at room temperature (RT) and left at 4 °C for 5 min. The cell lysis was centrifuged at 10,000 g for 10 min. The supernatants were collected and GSH values were determined by a specific color reaction as instructed. All GSH, nitrite and TNFα were normalized by cell number obtained from the MTS assay. The autofluorescence of NAD(P)H, including hydrated nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH), was determined on a BD LSRFortessa™ cell analyzer (San Jose, CA) with UV (351–365 nm) for excitation and a 450/50 nm BP filter for emission detection [11]. The same cell analyzer was also used for assessing expression of CD11b and Fas using a FITC-labeled rat anti-mouse CD11b (Clone: M1/70) and hamster anti-mouse Fas (Clone: JO2) antibodies (Fisher Scientific, Hampton, NH), respectively. A total of 10,000 cells were harvested per sample, and FCS Express 6 Plus software was used for data analysis.

2.4. Quantitative RT-PCR

Isolation of total cellular RNA and synthesis of cDNA were performed with the RNeasy® Mini kit (Qiagen, Valencia, CA, USA) and the Reverse Transcription System kit (Promega) following the manufacturers' instructions, respectively. Real-time PCR was run with the QuantiTect® SYBR Green PCR master mix (Qiagen). The threshold cycle (CT) value was calculated from amplification plots. Data was analyzed using the 2^{-CT} method with 18s rRNA as the reference [12]. Gene expression was normalized to the control group in each experiment and represented as fold of change. The specific primers were as follows: 5'-CATCTTCTCAAAATTCGAGTGACAA-3' (forward), 5'-TGGGAGTAGACAAGGTACAACCC -3' (reverse) for TNF α , 5'-GAGGGATGCCTTCCGCAGCTG -3' (forward), 5'-CATTGGAAGTGAAGCGTTTCG -3' (reverse) for iNOS, 5'-GAGTCCTTCAGAGAGATACAG-3' (forward), 5'-TGGTCTTGGTCCTTAGCC -3' (reverse) for IL-6, 5'-CAACCAACAAGTGATATTCTCCATG -3' (forward), 5'-GATCCACACTCTCCAGCTGCA -3' (reverse) for IL-1 β and 5'-GTGACCAGTTCAGTCTTGGT -3' (forward), 5'-GAATCGAACCCCTGATTCCCCGTC -3' (reverse) for 18s rRNA.

2.5. Western blot

The cytoplasmic and nuclear proteins were prepared from cells growing on a 6-well plate (approximately 500,000/well) using the Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific). Then the protein concentration was determined using the Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). The samples containing 100 μ g proteins were run on 10 % SDS-polyacrylamide gels at constant current of 80 V and electro-transferred to nitrocellulose membranes (Thermo Scientific) at constant voltage of 10 V overnight. Membranes were blocked with 5% fatty acid free bovine serum albumin (BSA) fraction V (Roche Diagnostics, Indianapolis, IN, USA) in Tris-buffered saline containing 0.1% Tween 20 (TBST) solution for 1 hr at RT, washed, and incubated overnight at 4°C in 5% BSA in TBST solution containing each of the specific primary antibodies against p-p38, p-Akt1/2/3, and TLR4 (these three antibodies were from Santa Cruz Biotechnology), p-ERK1/2 (BD Biosciences, San Jose, CA, USA), FoxO1 (Cell Signaling), and GAPDH (Sigma-Aldrich). The membranes were then incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling) for 1 hr at RT followed by chemiluminescent substrate for HRP antibody and enhancer solution (Thermo Scientific) mixed in a 1:1 ratio. The membranes were incubated in the dark with autoradiography films (Genesee Scientific, San Diego, CA, USA), and the films were developed to visualize the bands. Then the grayscale images (300–400 dpi) on films were scanned using a flat-bed scanner. Densitometry was carried out in Photoshop. Using the magic wand tool from the tool palette, the area of each band was selected, and the mean histogram was recorded and then charted. All Western blots were conducted in triplicates or duplicates.

2.6. Statistical analysis

Data were expressed as mean $\pm$ SD. Statistical evaluation was performed by the Analysis of Variance (ANOVA) using the SPSS 15.0 software. A *p* value of 0.05 or less was considered

as significant difference. A student t-test (two-tailed) was performed to compare the difference between two groups, and $p < 0.05$ was considered significant.

3. Results

3.1. Effects of BSP on cellular levels of antioxidants GSH and NAD(P)H

BSP at a concentration of 200 μM for 24 h did not cause significant cell cytotoxicity, neither alone nor together with 100 ng/mL of LPS (Fig. 1a). Treatment of BSP (200 μM /24 h) significantly increased intracellular GSH level, while a GSH depleting agent buthionine sulfoximine (BSO) induced a remarkable decrease in GSH level. Similar results were obtained in the presence of LPS (Fig. 1b). In addition, it was reported that RAW264.7 cells displayed two subpopulations with distinct forward scatters (FSC) by flow cytometry [13,14]. These two subpopulations with distinct cell sizes probably represented macrophage-like and dendritic-like cells occurring after multiple subculture [15]. Our experiments revealed that BSP could tremendously increase the NAD(P)H levels of both subpopulations I & II (Fig. 2). The mean fluorescence intensities (MFIs) of the NT group were 73 for subpopulation I and 93 for subpopulation II, while the MFIs of the BSP group were 180 for subpopulation I and 222 for subpopulation II.

3.2. Effects of BSP on inflammatory responses stimulated by LPS

BSP remarkably suppressed production of NO in the presence of LPS (Fig. 3a), and LPS-stimulated mRNA level of iNOS (Fig. 3b). It also significantly inhibited production of TNF α (Fig. 3c), as well as mRNA levels of TNF α , IL-6 (Fig. 3d) and IL-1 β (Fig. 3e). When different doses were applied, BSP was more effective at the high dose of 200 μM than the low dose of 50 μM (Fig. 3a and Fig. 3b), while the efficiency of BSP at 50 μM was equivalent to that of dexamethasone (DEX) in preventing LPS-induced NO production (Fig. 3a). Increase in expression of CD11b has been reported as a typical response to LPS stimulation in RAW264.7 cells [16]. It was found in the present experiment that BSP could suppress this inflammatory response with similar effects to that of DEX (Fig. 4). The CD11b + ratios were 1.31% for NT group, 1.33% for BSP group, 11.57% for LPS group, 7.93% for LPS+BSP group and 7.05% for LPS+DEX group, respectively.

3.3. Effects of BSP on TLR4 expression via modulating Akt/FoxO1/TLR4 pathway

The Akt/FoxO1/TLR4 pathway has been reported as a mechanism of negative feedback regulation on the inflammatory response to LPS stimulation [2]. Indeed, in the present experiments LPS reduced the nuclear quantities of FoxO1 determined by Western blot (Fig. 5a). Nevertheless, it stimulated the level of phosphorylated Akt (Fig. 5b) and suppressed expression of TLR4 (Fig. 5c). Importantly, combined treatment of LPS with BSP caused stronger effects on Akt, FoxO1 and TLR4 than LPS alone (Fig. 5a–5c). Based on these data, we propose a mechanism of the anti-inflammatory activities of BSP underlying the Akt/FoxO1/TLR4 pathway. Briefly, BSP caused activation of Akt kinase, stimulating phosphorylation of FoxO1 which prevented nuclear accumulation of FoxO1. The decrease in nuclear FoxO1 resulted in a decrease in TLR4 expression which is a direct target gene of FoxO1 [2]. Consequently, the inflammatory responses were suppressed due to reduced TLR4 availability for LPS binding (Fig. 5d).

3.4. Effects of BSP on MAPKs activation and Fas expression

The MAPK signal pathway played a crucial role in modulating the cellular responses to inflammatory stimulus. In the current experiments, it was found that BSP inhibited LPS-stimulated phosphorylation of P38 (Fig. 6a). Similar data was obtained on phosphorylation of ERK (Fig. 6b). Based on these data, we proposed the GSH/ROS/MAPKs pathway underlying that BSP regulated LPS-stimulated inflammatory response (Fig. 6c). MAPKs were important mediators of LPS-induced inflammatory responses, especially by increasing intracellular ROS which further promoted phosphorylation of P38 and ERK [17]. In the presence of BSP, intracellular GSH was elevated due to blockage of its efflux. Then increased GSH caused reduction of ROS, which prevented activation of MAPKs. As a result, the inflammatory response was reduced. It is worth noting that GSH induced decrease in ROS level might be reflected by an increase in NAD(P)H levels shown in Fig. 2. In addition, flow cytometric measurement revealed that LPS-stimulated Fas expression was effectively blocked by BSP, using a FITC-labeled antibody against mouse Fas protein. The MFIs were 4347 for NT group, 4748 for BSP group, 13239 for LPS group and 10875 for LPS+BSP group, respectively (Fig. 7).

4. Discussion

In the present study, we discovered that BSP increased intracellular GSH (Fig. 1) and NADPH concentrations (Fig. 2). Additionally, we found that BSP suppresses LPS-induced activation of RAW264.7 macrophages by suppressing nitric oxide and iNOS mRNA levels (Fig. 3). BSP also reduced expression of pro-inflammatory cytokines including TNF α , IL-1 β and IL-6 (Fig. 3), as well as expression of CD11b (Fig. 4). We demonstrated that BSP stimulated phosphorylation of Akt and reduced nuclear FoxO1 and cellular TLR4 (Fig. 5), as well as inhibited LPS-induced phosphorylation of MAPK P38 and ERK (Fig. 6) and expression of Fas (Fig. 7). Put together, we demonstrated for the first time that as an inhibitor of GSH efflux transporters, BSP suppressed inflammation through the Akt/FoxO1/TLR4, GSH/ROS/MAPKs and Fas pathways. The current findings are truly of importance with regard to the establishment of BSP as a new anti-inflammatory agent.

TNF α has been implicated in the regulation of cell death, survival, differentiation, and migration, as well as leukocyte adhesion, vascular leak and upregulating inflammatory markers [18]. Clinically, TNF α has been implicated in the pathogenesis of many inflammatory disorders including psoriasis, sepsis, diabetes, and others. IL-1 β is also involved in a host of inflammatory pathways, specifically responsible for fever during bacterial infection and, on a cellular level, neutrophil recruitment to the site of injury [19]. Finally, IL-6 is responsible for the induction of several acute phase reactants including C-reactive protein, serum amyloid A, haptoglobin, and others [20]. BSP's ability to suppress the expression of these three inflammatory cytokines demonstrates its inherent anti-inflammatory effects during bacterial infection and tissue injury.

The Akt-FoxO1 pathway is another signal transduction pathway involved in inflammatory pathways, but it has predominantly been implicated in the modulation and suppression of inflammation [21]. FoxO1, a downstream constituent of the Akt-PI3K pathway, is a transcriptional regulatory element, capable of modulating enhancer elements within the

TLR4 gene and increasing the pro-inflammatory cytokine profile of the cell [2]. However, by phosphorylation of Akt via TLR4 modulation, FoxO1 is exported from the nucleus, thereby unable to exert its pro-inflammatory effects [2]. Our present study revealed that BSP might inhibit the inflammatory responses through the Akt/FoxO1/TLR4 pathway in RAW264.7 macrophages stimulated by LPS (Fig. 3–5). Indeed, natural agents such as baicalin have been found to attenuate the inflammatory expression of depression via the regulation of TLR4 by increasing phosphorylation of Akt and nuclear exclusion of FoxO1 [22].

The p38 and ERK MAPKs have been shown previously to be upregulated in response to a variety of tissue insults, namely osmotic shock, heat shock, irradiation, as well as pro-inflammatory cytokines [23]. Furthermore, Fas is a member of the TNF receptor family, which is ligated and oligomerized by Fas ligand (FasL). Although Fas is best known for its involvement in the regulation of apoptosis, Fas ligation may also contribute to nonapoptotic signaling, for example, pro-inflammatory signaling in LPS-stimulated macrophages [3]. In the present study, we found that BSP prevented MAPKs activation and Fas expression (Fig. 6& Fig. 7), thereby underlying different mechanisms by which BSP can invoke anti-inflammatory effects. While a possible pathway involving GSH and ROS was proposed with regard to regulation of MAPKs by BSP (Fig. 6c), there is currently no clue to inhibition of Fas expression by BSP.

Though this study has many strengths, it also has several limitations. First, it is necessary to investigate if BSP has the same impacts on more macrophages, especially primary cultured human macrophages. Additionally, more characterization of Akt/FoxO1/TLR4, MAPKs and Fas pathways, together with their possible cross-talks should be further elucidated. Furthermore, it will become critical to evaluate the anti-inflammatory activities of BSP using suitable animal models involving macrophage activation, such as experimental animal models of osteoarthritis [24]. Last, LaminA, LaminB, PCNA or Histone H3 commonly serves as the internal control for nuclear proteins for western blot assay, and the use of GAPDH for expression of the nuclear protein FoxO1 was not an optimal choice in the current experiment. Due to the presence of GAPDH in both the cytoplasm and the nucleus, it is not sure if there was contamination of cytoplasmic proteins during the extraction of FoxO1. Future investigation is needed to support the current findings using a more appropriate internal control for nuclear proteins. After these additional *in vivo* and *in vitro* studies of BSP have been performed, more experimental evidence is necessary to clarify its mechanisms and quantify its effects of inflammatory attenuation in order to further develop a new anti-inflammation medicine.

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Figure 1. BSP (200 μ M, 24 h) increased GSH level in RAW264.7 cells.

(a) BSP was not toxic against cells in the absence or presence of LPS (100 ng/mL) by the MTS assay. (b) BSP enhanced cellular GSH by a GSH assay kit. NT, Non-treatment; BSO, buthionine sulfoximine, 50 μ M. * p <0.05, n =4.

Figure 2. BSP elevated cellular NADPH level in RAW264.7 cells measured by flow cytometry. The experiment was duplicated, and a typical result was shown. (a) Representative histograms. (b) Chart of mean fluorescence intensities from (a).

Figure 3. BSP reduced macrophage activation in RAW264.7 cells.

(a) Cells were treated with different reagents in the presence of LPS for 24 h and the nitrite amounts of cell medium were measured using the Griess reagent. (b & d & e) Cellular mRNA was isolated from cells at 24 h after different treatments and mRNA expression of various genes iNOS (b), TNF α & IL-6 (d) and IL-1 β (e) was determined by quantitative real-time PCR. (c) Cell medium was harvested at 24 h after different treatments, and the TNF α amounts were measured using an ELISA assay kit. For charts a–b, NT, Non-treatment; BSP, 200 μ M; BSPI, 50 μ M; LPS, 100 ng/mL; DEX, 25 μ M. * p <0.05, n =4; For charts c–e, NT, Non-treatment; BSP, 200 μ M; LPS, 100 ng/mL. # p <0.05 vs NT, ## p <0.05 vs LPS, n =4.

Figure 4. BSP reduced CD11b expression measured by flow cytometry.

CD11b+ cells were probed with a FITC-labeled antibody specifically against CD11b protein and ratios of CD11b+ cells with different treatments were calculated. (a) NT; (b) BSP; (c) LPS; (d) LPS+BSP; (e) LPS+DEX. The experiment was duplicated, and a typical result was shown. NT, Non-treatment; BSP, 200 μ M; LPS, 100 ng/mL; DEX, 25 μ M.

Figure 5. BSP suppressed inflammatory responses via Akt/FoxO1/TLR4 pathway in RAW264.7 cells. [5]

(a) BSP reduced nuclear FoxO1. The nuclear proteins were isolated after cells were treated by LPS for different periods, and the FoxO1 level was determined by western blot using a specific antibody against FoxO1. (b) BSP stimulated phosphorylation of Akt protein kinase. The cytosol proteins were isolated after 4 h of treatment with various reagents and western blot was performed using a specific antibody against phosphorylated Akt. (c) BSP decreased TLR4 expression. After 4 h of treatment, cellular TLR4 was measured by western blot. NT, Non-treatment; BSP, 200 μ M; LPS, 100 ng/mL. * $p < 0.05$, $n = 4$. (d) A proposed pathway delineating a central role of transcriptional factor FoxO1 in modulation of BSP on TLR4 expression.

Figure 6. BSP prevented LPS-induced MAPKs activation in RAW264.7 macrophages.

(a, b) The cytosol proteins were isolated after 4 h of treatment and immunoblotted with the specific antibodies against phosphorylated P38 (a) and ERK (b). NT, Non-treatment; BSP, 200 μ M; LPS, 100 ng/mL. * $p < 0.05$, $n = 4$. (c) The possible pathway of GSH/ROS/MAPKs was illustrated with regard to regulation of inflammatory response by BSP.

Figure 7. BSP prevented LPS-induced Fas expression in RAW264.7 macrophages.

Cells were treated for 24 h with different reagents and the cellular Fas level was represented as mean fluorescence intensity (MFI) by flow cytometry. Chart (a) was the NT/unstained sample and other charts were samples of different treatments (b, NT; c, BSP; d, LPS; e, LPS +BSP) stained with a FITC-labeled antibody against FAS protein. The experiment was duplicated and a typical result was shown. NT, Non-treatment; BSP, 200 μ M; LPS, 100 ng/mL.