

The Suppressive Effects of Bursopentine (BP5) on Oxidative Stress and NF- κ B Activation in Lipopolysaccharide-activated Murine Peritoneal Macrophages

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Key Words

Bursopentine (BP5) • Oxidative stress • Reactive oxygen species (ROS) • Glutathione (GSH) • Nuclear factor kappa B (NF- κ B)

Abstract

Background/Aim: Bursopentine (BP5) is a novel thiol-containing pentapeptide isolated from chicken bursa of Fabricius, and is reported to exert immunomodulatory effects on B and T lymphocytes. It has been found that some thiol compounds, such as glutathione (GSH) and N-acetylcysteine (NAC) protect living cells from oxidative stress. This led us to investigate whether BP5 had any ability to protect macrophages from oxidative stress as well as any mechanism that might underlie this process. **Methods:** Murine peritoneal macrophages activated by lipopolysaccharide (LPS) (2 μ g/ml) were treated with single bouts (0, 25, 50, and 100 μ M) of BP5. **Results:** BP5 potently suppressed the markers for oxidative stress, including nitric oxide (NO), reactive oxygen species (ROS), lipid peroxidation, and protein oxidation. It also decreased the expression and activity of inducible nitric oxide synthase (iNOS) and promoted a protective antioxidant state by elevating GSH content and by activating the expression and

activity of certain key antioxidant and redox enzymes, including glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD) and catalase (CAT). This suppressive effect on oxidative stress was accompanied by down-regulated expression and activity of nuclear factor kappa B (NF- κ B). **Conclusion:** These findings demonstrate that BP5 can protect LPS-activated murine peritoneal macrophages from oxidative stress. BP5 may have applications as an anti-oxidative stress reagent.

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Introduction

The bursa of Fabricius (BF), a primary immune organ unique to birds, is the site of early B lymphocyte proliferation and differentiation. Studies with BF extracts showed that it contains multiple biologically active factors [1, 2]. A previous study in our laboratory has demonstrated that a new pentapeptide, Bursopentine (BP5, Cys-Lys-Arg-Val-Tyr) is isolated from BF by reverse-phase high

performance liquid chromatography (RP-HPLC). It exerts immunomodulatory effects on B and T lymphocytes [3]. It has been reported that some thiol compounds, such as glutathione (GSH) and N-acetylcysteine (NAC), can protect cells from oxidative stress by scavenging free radicals and by enzymatic reactions. Glutathione is the most important cellular thiols, modulating redox-regulated signal transduction, acting as a substrate for several transferases, peroxidases, and other enzymes that prevent or mitigate the deleterious effects of ROS [4, 5]. N-acetylcysteine (NAC) is a well-established thiol antioxidant, which acts as a precursor of intracellular reduced glutathione, and functions as a ROS scavenger in conjunction with glutathione peroxidase [6, 7]. Because BP5 is also a novel thiol-containing peptide, we tested the hypothesis that BP5 might protect living cells from oxidative stress.

Macrophages play a pivotal role in innate and adaptive immunity by killing foreign organisms, presenting antigens, secreting chemical mediators, phagocytizing foreign invaders, and undergoing oxidative stress. They interact with cells such as T cells, B cells, natural killer cells, dendritic cells, neutrophils, and fibroblasts. Activated macrophages produce a number of multifunctional compounds including cytokines, reactive nitrogen species (RNS, e.g. nitric oxide, NO), and reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen (1O_2) and hydroxyl radical (OH) [8-11]. It is believed that these free radical species play an important role in the defense against infectious agents at low and moderate concentrations, functioning in a number of cellular signaling pathways and in the induction of mitogenic responses. However, overproduction of free radical species results in oxidative stress, a deleterious process that can be an important mediator of damage to cell structures, including lipids and membranes, proteins, and DNA [12]. To protect themselves from constant oxidative stress, macrophages have developed defense mechanisms that ensure the proper balance between peroxidant and antioxidant molecules [13]. In addition to enzymes that remove ROS, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR), glutathione is also a key player in the maintenance of redox status in macrophages. This state defined as the ratio of the concentration of oxidizing equivalents to that of reducing equivalents (GSH/GSSG). Thus, the redox balance is an important determinant of macrophage activity. Previous reports have established that LPS can induce oxidative stress in murine peritoneal macrophages

[14-16]. We used a living cell system of LPS-stimulated murine peritoneal macrophages as an oxidative stress model to find out whether BP5 can modulate macrophage activity.

Considering that activated macrophages are potent producers of free radicals and continuing oxidative stress, we investigated possible effects of BP5 on the markers of cellular oxidative stress, such as levels of NO, intracellular ROS, lipid peroxidation, and protein peroxidation in LPS-activated macrophages. It has been reported that increased NO production in activated macrophages can be due to increased levels of inducible iNOS expression, which in turn increase the transformation of L-arginine to NO [17]. This can be used to evaluate the effects of BP5 on iNOS expression in LPS-activated macrophages. To further determine the effects of BP5 on cell redox status, GSH, glutathione disulfide (GSSG), glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), and catalase (CAT) were assayed in LPS-activated macrophages. Numerous reports have indicated that nuclear factor kappa B (NF- κ B) transcription is a redox-sensitive process [18, 19]. LPS stimulation triggers signaling pathways that activate transcription factor kappa B (NF- κ B) [20, 21]. For this reason, we also investigated whether BP5 could affect NF- κ B activity in LPS-activated macrophages.

Materials and Methods

Preparation of BP5

Natural BP5 was isolated as described previously from chicken bursae of Fabricius [3]. Synthetic BP5 was purchased from Shanghai Biotech Bioscience and Technology Co., Ltd (P. R. China). The sequence of the isolated BP5 and the synthetic peptide was confirmed by electrospray ionisation tandem mass spectrometry (ESI-MS/MS). The purity of the isolated BP5 and the synthetic peptide was >98% by RP-HPLC. To rule out LPS contamination, BP5 was tested using the E-Toxate Limulus LPS detection kit (Sigma Chemical Co), which is sensitive to 0.05 to 0.1 endotoxin units/ml. The kit was used according to the manufacturer's instructions. If the peptide preparations showed a negative result with this test, they were considered uncontaminated. Only uncontaminated preparations were used.

Preparation and treatment of peritoneal macrophages

Macrophages were harvested from 6-8-week old BALB/c mice as described previously [22]. Mice were injected i.p. with 3 ml of 2.5% thioglycolate, and peritoneal cells were harvested 3 days later. The collected cells were cultured and allowed to adhere for 3 h at 37°C in a 5% CO₂ incubator. Nonadherent cells

were removed and adherent cells were subcultured in tissue culture plates at 5×10^6 cells/plate/15 ml culture medium for 24 h to form an adherent monolayer.

The viability of the adherent cells used in experiments was always >95% as determined by trypan blue exclusion. For this study, we determined the nontoxic concentration of BP5 based on its ability to protect macrophages from lipopolysaccharide-induced (LPS-induced) oxidative stress: Macrophages (2×10^6 cells/ml) and various concentrations of BP5 (1-1000 μ M) were mixed with 1 ml of RPMI-1640 medium with 10% FBS, and incubated for 24 h. The viability of these cells remained >95% when they were evaluated with trypan blue exclusion, ruling out the possibility that the suppression might be due to BP5 toxicity. We conservatively selected nontoxic concentrations of BP5 for subsequent experiments. In the following assays, macrophages (2×10^6 cells/ml) were incubated with varying concentrations of BP5 (25, 50, 100 μ M) for 2 h and then incubated with LPS (2 μ g/ml) for another 18 h. After the experiments, the cells were harvested and sonicated on ice. Then cell lysate was immediately centrifuged at $10,000 \times g$ for 10 min at 4°C. The supernatant of cell lysate was collected for the following different assays.

NO production

As an indicator of NO synthesis, the concentrations of nitrite in the macrophage culture medium were estimated using the Griess reagent [23]. Equal volumes of culture supernatant and Griess reagent (0.1% N-1-naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 2.5% phosphoric acid) were mixed and incubated for 10 min in darkness at room temperature. The absorbance was measured at 540 nm and calibrated with a nitrite standard curve to determine the nitrate concentration in supernatants.

Intracellular ROS

Intracellular ROS was measured as described previously with some modifications [24]. Macrophages (2×10^6 cells/well) were seeded in 24-well flat-bottom plates, then preincubated with BP5 (0, 25, 50, 100 μ M) for 2 h, followed by 18 h with or without LPS (2 μ g/ml). They were then treated with 5 μ M carboxy-2', 7'-dichlorofluorescein diacetate for 1 h. Fluorescence intensity was measured using a microplate reader with a 485/52 nm filter set. Phorbol 12-myristate 13-acetate (PMA, 1 μ M, Sigma-Aldrich) was used as a positive control for ROS induction. The conversion of DCF-DA to DCF was determined by reference to a DCF standard (Sigma-Aldrich). It was calculated as nmol/ 2×10^6 cells (well)/h.

Lipid peroxidation and protein carbonyls contents (PC)

Lipid peroxidation was examined using a modified thiobarbituric acid reactive substance (TBARS) assay [25]. Two hundred microliters of each cell lysate supernatant was added with 400 μ l of thiobarbituric acid (TBA) reagent. The reaction mixture was incubated at 37°C in a shaking water bath for 1 h and then boiled at 95°C for 30 min. Then the tubes were shifted to a crushed ice bath and centrifuged at $2500 g$ for 10 min. The amount of malonaldehyde (MDA) formed in each sample was measured with a 1,1,3,3-tetraethoxypropane serial dilution

standard curve by measuring the optical density of the supernatant at 532 nm using a spectrophotometer against a reagent blank. TBARS values are expressed as nmol MDA/h/mg protein.

Protein oxidative modification was measured using 2, 4-dinitrophenylhydrazine (DNPH) derivatization of oxidized proteins according to the methods described by Reznick and Packer and Levine et al. [26, 27]. In general, cell lysate proteins in 50 mM potassium phosphate buffer, pH 7.4, were derivatized with DNPH (21% in 2 N HCl). Blank samples were mixed with 2 N HCl incubated at 1 h in the dark; protein was precipitated with 20% trichloro acetic acid (TCA). Underivatized proteins were washed with an ethanol:ethyl acetate mixture (1:1). Final pellets of protein were dissolved in 6N guanidine hydrochloride and absorbance was measured at 370 nm. Protein carbonyl content is expressed in terms of μ mol/mg protein.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was used to analyze the mRNA expressions of iNOS, antioxidative enzymes (GPx, GR, SOD, and CAT) and NF- κ B. Macrophages (2×10^6 cells/well) were seeded in 24-well flat-bottom plates then preincubated with BP5 (25, 50, 100 μ M) for 2 h, followed by 18 h with or without LPS (2 μ g/ml). Isolation of RNA was achieved with an RNeasy RNA® extraction kit based on the procedure provided by the manufacturer (Invitrogen, Norway). The RNA was reverse-transcribed to cDNA from 2 μ g total RNA using a reverse transcription system from Promega (Germany). Separate but simultaneous PCR amplifications were performed with aliquots of cDNA (10 μ l) at a final concentration of $1 \times$ PCR buffer, 4 mM $MgCl_2$, 400 μ M dNTPs, and 2 U *Taq* DNA polymerase (TaKaRa, Dalian, China) in a total volume of 20 μ l using 1 μ l (20 μ M) each of forward and reverse primers. The primer sequences of iNOS were selected according to Park et al. [28]. The sequences of GPx, GR, Cu/Zn SOD, Mn SOD, and CAT were selected according to Ferret et al. [29]. The sequences of NF- κ B-p65 and β -actin were selected according to Cohen-Lahav et al. [30]. The cycle number was optimized to ensure product accumulation in the exponential range. The PCR products specific to each cDNA were analyzed by electrophoresis on a 2% agarose gel and documented using the Gel Doc EQ System (Bio-Rad). All signals were normalized to mRNA levels of the house keeping gene, β -actin, and expressed as a ratio.

iNOS activity

iNOS activity was analyzed using a commercial assay kit (Jiancheng Bioengineering Institute, Nanjing, China). The assay for iNOS activity depends on the ability of the synthase to catalyze arginine (Arg) to form NO, which can further react with nucleophilic substances to produce chromophoric compound, which has a peak absorbance at 530 nm. iNOS activity can be determined based on fact that nNOS (neuronal nitric oxide synthase) and eNOS (endothelial nitric oxide synthase) are Ca^{2+} -dependent, while iNOS is Ca^{2+} -independent [31, 32]. One unit of iNOS activity was here defined as the amount capable of forming 1 nmol NO in 1 min per milliliter of medium.

GSH content and antioxidative enzyme activities

Total glutathione (T-GSH), GSH and GSSG were measured spectrophotometrically according to the commercial assay kit procedure (Beyotime Institute of Biotechnology, Nanjing, China). Briefly, T-GSH was assayed using the 5, 5'-dithio-bis (2-nitrobenzoic) acid (DTNB)-GSSG reductase recycling. The concentration of reduced GSH in the sample was obtained by subtracting GSSG from T-GSH [33]. Both concentrations of reduced GSH and GSSG were calculated from formula and expressed as nmol/mg protein.

The activities of GPx, GR, SOD, and CAT in cell lysate were measured by standard chemical colorimetric assays using commercial kits (Jiancheng Bioengineering Institute, Nantong, China).

GPx activity was determined by quantifying the rate of oxidation of the reduced glutathione to the oxidized glutathione on the base of its catalysis. GSH reacts with 5, 5' dithiobis-p-nitrobenzoic acid (DTNB) and produces yellow compounds that can be detected at 412 nm using a spectrophotometer. This was taken to represent the reduction of GSH [34]. One unit of the enzymatic activity was here defined as reduction of 1 μ mol GSH per min per 1 mg protein after the reduction of GSH of the nonenzymatic reaction has been subtracted. Enzyme activity was expressed as U/mg protein.

GR activity was measured using the method described by Carlberg and Mannervik [35]. Glutathione reductase is required for the NADPH-dependent conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH). The exponential decrease of NADPH was detected spectrophotometrically at 340 nm. One unit of enzymatic activity was here defined as the oxidation of 1 μ mol/L NADPH per min for 1 mg protein. Enzyme activity is expressed as U/mg protein.

SOD activity was measured by its ability to inhibit the reduction of nitroblue tetrazolium (NBT) via superoxide ions generated by the xanthine/xanthine oxidase system [36]. The extent of NBT reduction was followed spectrophotometrically by measuring the absorbance at 560 nm. One unit of SOD activity was defined as the amount of enzyme capable of causing 50% inhibition in 1 mL reaction solution per milligram protein. Enzyme activity was expressed as U/mg protein.

CAT activity was detected by measuring the intensity of a yellow complex formed by molybdate and H_2O_2 at 405 nm, after ammonium molybdate was added to terminate the H_2O_2 degradation reaction catalyzed by CAT [37]. One unit of enzymatic activity was here defined as the degradation of 1 μ mol H_2O_2 per second per mg protein. Enzyme activity was expressed as U/mg protein.

The protein content of cell lysate was determined by standard methods.

NF- κ B activity

NF- κ B activity was examined using a TransAMTM NF- κ B p65 transcription factor assay kit (Active Motif, Carlsbad, CA, U.S.) according to the manufacturer's instructions. The kit contains 96-well plates to which oligonucleotides containing an NF- κ B consensus binding site. The activated NF- κ B contained in nuclear extracts was able to bind specifically to these oligonucleotides. It was detected using specific

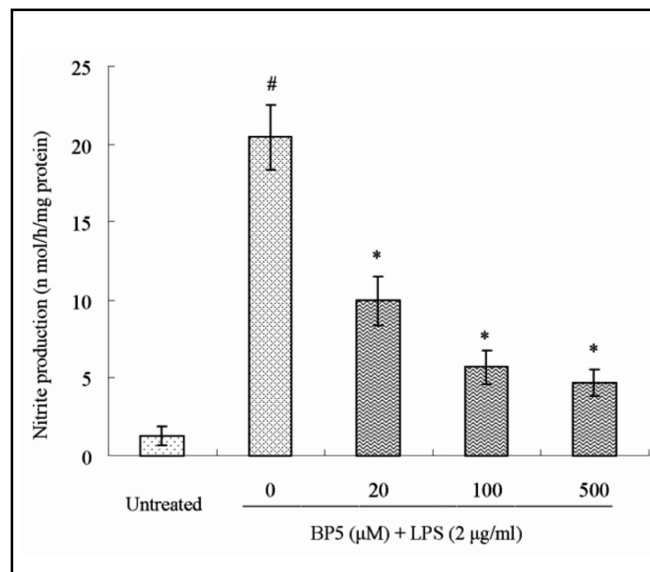


Fig. 1. Inhibitive effects of BP5 on NO in cultured murine peritoneal macrophages. Macrophages (2×10^6 cells/well) were incubated with the indicated concentrations of BP5 (0, 25, 50, 100 μ M) for 2 h, then incubated with or without LPS (2 μ g/ml) for 18 h. Levels of nitrite in the culture medium were estimated using the Griess reagent. "Untreated" is the negative control without LPS and BP5 treatment. Data represent the mean \pm S.D. of triplicate experiments. * $P < 0.05$ relative to the LPS-only group; # $P < 0.05$ relative to the untreated group.

antibodies. Five micromoles of Bay 117082 (an inhibitor of NF- κ B) was used as a positive control for NF- κ B inhibition.

Statistical analysis

The results were recorded as mean \pm SD. Biochemical and physiological parameters were analyzed statistically using one way analysis of variance (ANOVA) followed by Dunnett-t-testing to evaluate variations between groups. $P < 0.05$ was considered significant and $P < 0.01$ was considered highly significant.

Results

BP5 exhibits inhibitive effects on NO production

To determine whether the protective effects of BP5 were exerted via a NO-mediated mechanism, the amount of NO produced by the macrophages was measured. LPS significantly induced the production of NO in the LPS-only group when compared with that in the untreated group (* $P < 0.05$). LPS-induced NO production was markedly attenuated by BP5 (20, 100, and 500 μ M) treatment (* $P < 0.05$) (Fig. 1).

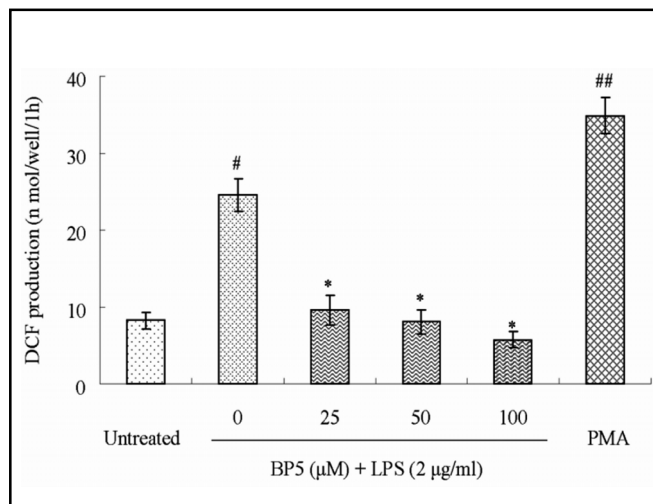


Fig. 2. Inhibitory effect of BP5 on ROS generation in cultured murine peritoneal macrophages. Macrophages (2×10^6 cells/well) were incubated without graded concentrations of BP5 (0, 25, 50, 100 μ M) for 2 h, followed by LPS (2 μ g/ml) for 18 h, and then treated with 5 μ M carboxy-2', 7'-dichlorofluorescein diacetate (DCF-DA) for 1 h. Phorbol 12-myristate 13-acetate (PMA) (1 μ M) was used as a positive control for ROS production. ROS levels are expressed as DCF production (nmol per well/1 h). Results are expressed as mean \pm S.D. of five separate experiments. ^{*} $P < 0.05$, compared with LPS alone group; [#] $P < 0.05$, ^{##} $P < 0.01$, relative to the untreated group.

BP5 exhibits suppressive effects on the intracellular generation of ROS

To investigate whether BP5 could suppress intracellular ROS production, the ROS levels in the cultures of LPS-activated macrophages were measured using the redox sensitive fluorescent dye carboxy-2', 7'-dichlorofluorescein diacetate (DCF-DA, a reliable fluorogenic marker for ROS, Invitrogen). The intracellular hydrolyzed DCF-DA, the active probe, reacts with ROS to form the fluorescent oxidation product DCF. The intensity of fluorescence was measured using a microplate reader and a 485/52 nm filter set. As shown in Fig. 2, ROS production induced by LPS in LPS-activated macrophages was significantly decreased by BP5 (25-100 μ M) relative to the LPS-only group (^{*} $P < 0.05$).

BP5 exhibits anti-lipid peroxidation activities

We used thiobarbituric acid reactive substance formation (TBARS formation) as an indicator of lipid peroxidation. Compared with in the control group, the TBARS level was significantly elevated in the LPS-only group ([#] $P < 0.05$). However, incubation with 25-100 μ M

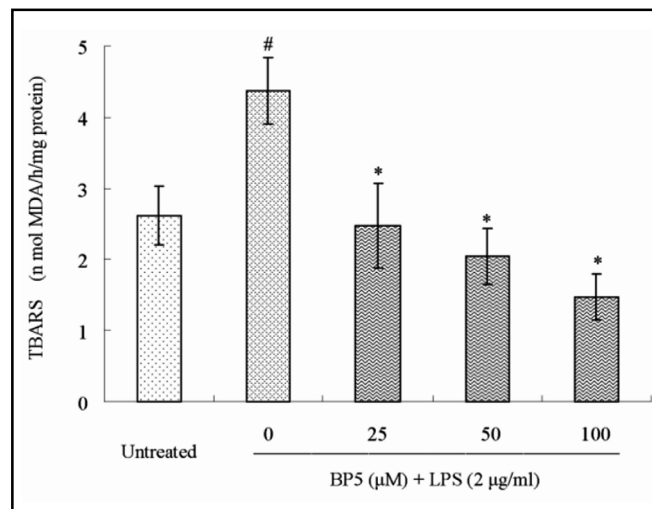


Fig. 3. Suppressive effect of BP5 on LPS-induced lipid peroxidation in cultured murine macrophages. Macrophages (2×10^6 cells/well) were incubated without graded concentrations of BP5 (0, 25, 50, 100 μ M) for 2 h, and then incubated in the presence or absence of LPS (2 μ g/ml) for 18 h. Lipid peroxidation was assayed as described in the text. Data, expressed as nanomolar malondialdehyde (MDA) per hour per milligram protein of cell lysate, are given as the mean \pm S.D. of three separate experiments. ^{*} $P < 0.05$ relative to the LPS-only group; [#] $P < 0.05$ relative to the untreated group.

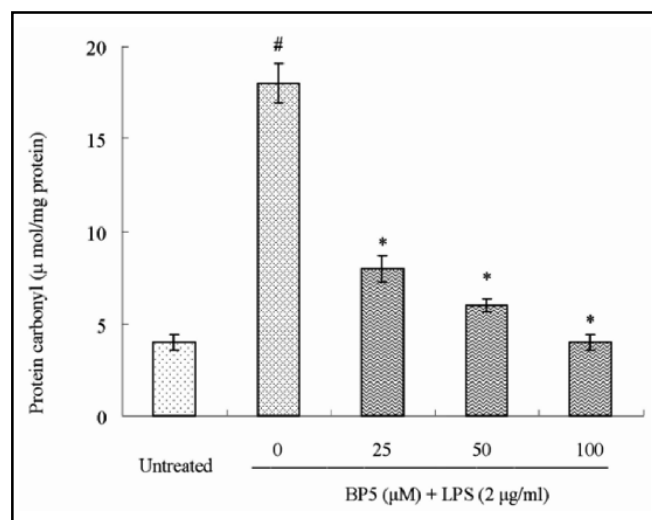


Fig. 4. Inhibitory effects of BP5 on LPS-induced protein carbonyls in cultured murine peritoneal macrophages. Macrophages (2×10^6 cells/well) were incubated with the indicated concentrations of BP5 (0, 25, 50, 100 μ M) for 2 h, then with or without LPS (2 μ g/ml) for 18 h. A protein oxidative carbonylation assay was based on the coupling of BP5-treated cellular fractions with 2, 4-dinitrophenylhydrazine (DNPH). The levels of derivatized protein were measured spectrophotometrically at 270 nm. "Untreated" is the negative control without LPS and BP5 treatment. Data represent the mean \pm S.D. of triplicate experiments. ^{*} $P < 0.05$ relative to the LPS-only group; [#] $P < 0.05$ relative to the untreated group.

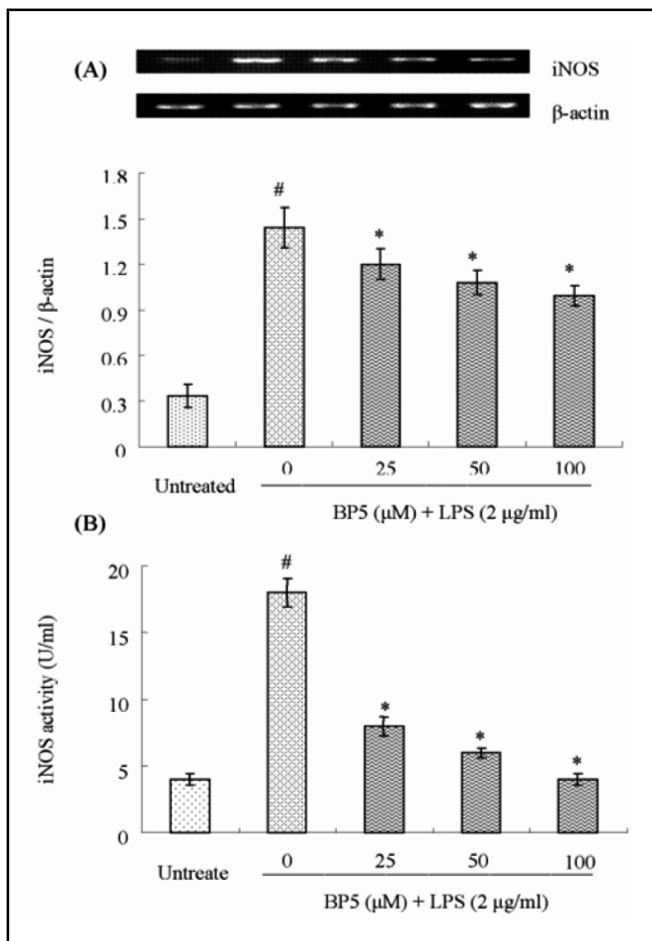


Fig. 5. Inhibitory effects of BP5 on iNOS mRNA expression and iNOS activity in cultured murine peritoneal macrophages. Macrophages (2×10^6 cells/well) were incubated with the indicated concentrations of BP5 (0, 25, 50, 100 μ M) for 2 h, then incubated with or without LPS (2 μ g/ml) for 18 h. (A) iNOS mRNA expression was quantified using RT-PCR. All signals were normalized to mRNA levels of β -actin and expressed as ratios. The results of a typical experiment are expressed above as the mean \pm S. D. of triplicate experiments. * $P < 0.05$ relative to the LPS-only group; # $P < 0.05$ relative to the untreated group. (B) iNOS activity was measured using a commercial assay kit. "Untreated" is the negative control without LPS and BP5 treatment. Data represent the mean \pm S.D. of triplicate experiments. * $P < 0.05$ relative to the LPS-only group; # $P < 0.05$ compared with relative to the untreated group.

BP5 significantly decreased TBARS levels (* $P < 0.05$) (Fig. 3). This suggested that lipid peroxidation was attenuated in LPS-activated macrophages by BP5 treatment.

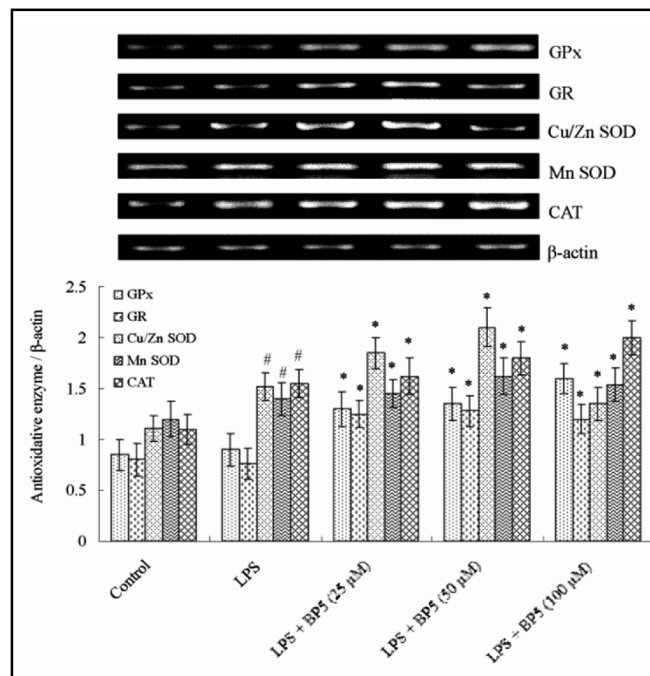


Fig. 6. Expression levels of key antioxidant and redox enzymes. Macrophages (2×10^6 cells/well) were incubated either without BP5 or with the indicated concentrations of BP5 for 2 h and then incubated with or without LPS (2 μ g/ml) for 18 h. The levels of expression of enzyme genes were measured using RT-PCR. All signals were normalized to mRNA levels of β -actin and expressed as ratios. Each value represents the mean \pm S.D. of three experiments performed in parallel. * $P < 0.05$ relative to the LPS-only group; # $P < 0.05$ relative to the untreated group.

BP5 exhibits anti-protein peroxidation activities

Protein peroxidation is another important indicator of cellular oxidative stress. In this study, protein peroxidation in peritoneal macrophages was measured in terms of protein carbonyls (PC). As shown in Fig. 4, the levels of protein carbonyls were significantly elevated in the LPS-only group relative to the control group (* $P < 0.05$). However, exposure of macrophages to BP5 (25–100 μ M) significantly decreased protein carbonyl (PC) levels (* $P < 0.05$). This suggested BP5 treatment attenuated protein peroxidation in LPS-activated macrophages.

BP5 exhibits suppressive effects on iNOS

As shown in Fig. 5, iNOS mRNA expression was hardly detectable in the untreated group, but it was highly up-regulated in the LPS-only group (* $P < 0.05$ relative to the control group, Fig. 5A). BP5 treatment significantly

Bp5 ($\mu\text{g/ml}$)	GSH (nmol/mg protein)	GSSG (nmol/mg protein)	GSH/GSSG (ratio)
Untreated	31.06 ± 1.53	0.48 ± 0.07	64.71 ± 2.45
LPS	$41.18 \pm 2.48^{\#}$	$0.91 \pm 0.08^{\#}$	$45.25 \pm 5.45^{\#}$
LPS + BP5 (25 μM)	$52.68 \pm 3.27^*$	$0.67 \pm 0.05^*$	$82.43 \pm 7.41^*$
LPS + BP5 (50 μM)	$60.45 \pm 3.45^*$	$0.65 \pm 0.09^*$	$93.05 \pm 8.16^*$
LPS + BP5 (100 μM)	$65.87 \pm 3.87^*$	$0.68 \pm 0.06^*$	$96.88 \pm 8.86^*$

Table 1. Effect of BP5 on glutathione redox couple (GSH/GSSG) in cultured murine peritoneal macrophages. Macrophages (2×10^6 cells/ml) were incubated with varying concentrations of BP5 (25, 50, 100 μM) for 2 h. Then the cells were incubate with LPS (2 $\mu\text{g/ml}$) for another 18 h. The levels of GSH and GSSG, and the ratio of GSH/GSSG in the cells were measured using commercial kits. “Untreated” is the negative control without LPS or BP5 treatment. The data presented are mean \pm S.D. values of four replicates. $^{\#}P < 0.05$ relative to the control group; $^*P < 0.05$ relative to the LPS-only group.

BP5 ($\mu\text{g/ml}$)	GPx (U/mg protein)	GR (U/mg protein)	SOD (U/mg protein)	CAT (U/mgprotein)
Untreated	2.03 ± 0.05	0.41 ± 0.05	0.85 ± 0.55	215 ± 14.35
LPS	2.42 ± 0.08	0.34 ± 0.08	$1.69 \pm 0.65^{\#}$	$288 \pm 16.44^{\#}$
LPS + BP5 (25 μM)	$3.78 \pm 0.25^*$	$0.46 \pm 0.03^*$	$2.56 \pm 0.75^*$	$369 \pm 22.75^*$
LPS + BP5 (50 μM)	$3.59 \pm 0.38^*$	$0.45 \pm 0.07^*$	$2.73 \pm 0.96^*$	$396 \pm 32.08^*$
LPS + BP5 (100 μM)	$3.82 \pm 0.26^*$	$0.44 \pm 0.06^*$	$2.87 \pm 0.05^*$	$448 \pm 29.75^*$

Table 2. Effects of BP5 on the specific activities of antioxidant defense enzymes in cultured murine peritoneal macrophages. Macrophages (2×10^6 cells/ml) were incubated with varying concentrations of BP5 (25, 50, 100 μM) for 2 h. Then the cells were incubated with LPS (2 $\mu\text{g/ml}$) for another 18 h. Intracellular levels of GPx, GR, SOD, and CAT were measured using commercial kits. The units of enzyme activity are given in the Materials and Methods section. “Untreated” is the negative control without LPS or BP5 treatment. The data presented are mean \pm S.D. of triplicate experiments. $^{\#}P < 0.05$ relative to the control group; $^*P < 0.05$ relative to the control group or the LPS-only group.

reversed the elevated levels of iNOS mRNA expression in a dose-dependent manner ($^*P < 0.05$ relative to the LPS-only group, Fig. 5A). BP5 also reversed increased iNOS activity (induced by LPS) significantly ($^*P < 0.05$) in a dose-dependent manner (Fig. 5B).

BP5 exhibits modulating effects on intracellular GSH, GSSG, and the GSH/GSSG ratio

We investigated the effects of BP5 on cellular levels of the reduced and oxidized forms of glutathione (GSH and GSSG). As shown in Table 1, exposure to

LPS induced a significant increase in the GSH levels in the macrophages ($^{\#}P < 0.05$). GSH levels were further increased in LPS-treated macrophages that were also given BP5 (25, 50, 100 μM) relative to cells given LPS alone ($^*P < 0.05$). The GSH/GSSG ratio take into account of the accumulation of GSSG, thus it is a more reliable indicator of cellular redox status than raw GSH level. As shown in Table 1, the GSH/GSSG ratio was also significantly increased in activated macrophages given BP5 ($^*P < 0.05$) relative to the LPS-only group.

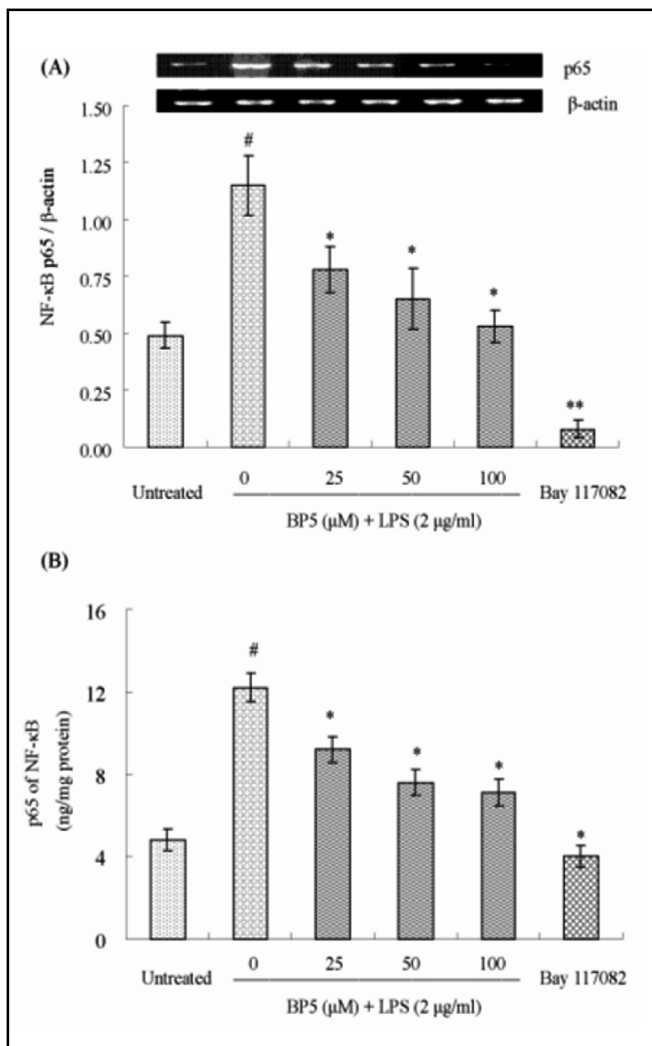


Fig. 7. Suppressive effects of BP5 on gene expression and activity of LPS-induced NF-κB in macrophages. Macrophages (2×10^6 cells/well) were incubated either without BP5 or with the indicated concentrations of BP5 for 2 h, then incubated with or without LPS ($2 \mu\text{g/ml}$) for 18 h. (A) NF-κB gene expression was measured using RT-PCR. Each value represents the mean \pm S.D. of three experiments performed in parallel using β -actin as the PCR control. $^*P < 0.05$ relative to the LPS-only group; $^{\#}P < 0.05$ relative to the untreated group. (B) NF-κB activity was measured using the TransAM™ NF-κB p65 transcription factor assay kit. “Untreated” is the negative control without LPS and BP5 treatment (0, 25, 50, 100 μM). Bay 117082 (an inhibitor of NF-κB) was used as a positive control for NF-κB inhibition. Data represent the mean \pm S.D. of triplicate experiments. $^*P < 0.05$ relative to the LPS-only group; $^{\#}P < 0.05$ relative to the untreated group.

BP5 exhibits enhancing effects on antioxidative enzymes

The effects of BP5 on the expression levels of antioxidative enzymes (GPx, GR, Cu/Zn SOD, Mn SOD,

and CAT) were assessed using RT-PCR. As shown in Fig. 6, in LPS-activated macrophages, the mRNA expression of GPx and GR genes were up-regulated to a negligible extent, but expression of Cu/Zn SOD, Mn SOD and CAT genes were significantly up-regulated relative to the control ($^{\#}P < 0.05$). BP5 pre-administration up-regulated the levels of GPx, GR, Cu/Zn SOD, Mn SOD, and CAT mRNA expression as relative to the LPS-only group ($^*P < 0.05$).

The effects of BP5 on the specific activities of antioxidant defense enzymes in LPS-stimulated macrophages are shown in Table 2. Exposure of LPS-activated macrophages to BP5 (25, 50, 100 μM) significantly elevated the activities of the antioxidant defense enzymes GPx, GR, SOD, and CAT ($P < 0.05$ relative to the control and LPS-only groups).

BP5 exhibits suppressive effects on NF-κB

To determine whether the protective effects of BP5 in LPS activated macrophages was due to the attenuation of NF-κB, the levels of gene expression and activity of NF-κB were assayed by RT-PCR and an ELISA kit, respectively. The RT-PCR assay showed that LPS-treatment was accompanied by an increase in levels of NF-κB (p65) mRNA expression ($^{\#}P < 0.05$ relative to the control group, Fig. 7A). The addition of BP5 (25, 50, 100 μM) reduced the expression of the LPS-induced mRNA after 20 h of treatment ($^*P < 0.05$ relative to the LPS-only group, Fig. 7A).

As shown in Fig. 7B, relative to the LPS-only group, BP5 significantly inhibited the NF-κB activity, and it did so in a dose-dependent manner ($^*P < 0.05$). As a positive control, LPS-induced NF-κB activation was also inhibited by NF-κB inhibitor Bay 117082 ($^*P < 0.05$ relative to the LPS-only group).

Discussion

Natural products are known to be sources of bioactive components exerting anti-oxidative activities. Previous studies have shown that secreted peptide hormones, such as luteinizing hormone-releasing hormone, enkephalin, angiotensin and oxytocin, act as biochemical antioxidants [38]. This suggests that some peptides may have multiple functions *in vivo*. Some thiol-containing products, such as glutathione (GSH) and N-acetylcysteine (NAC), have been clinically used. Glutathione, a well-known tripeptide ubiquitously distributed in most living cells, has immune-stimulating and antioxidant activities

and plays a role in immunity against chronic diseases, such as heart disease, arthritis, and diabetes [39-41]. It is the main cellular defense against oxidative related diseases [42]. Studies have also shown NAC to be an antioxidant drug largely used in different clinical situations [43, 44]. Bursopentine (BP5) is a novel natural thiol-containing peptide and has been reported to exert immunomodulatory effects on the immune system [3]. Whether this interesting peptide has other molecular effects that are potentially therapeutic is unknown. In this study, BP5 was investigated in a living cell model. Our approach was to use LPS-stimulated murine peritoneal macrophages as a vitro model to evaluate the effects of BP5 on oxidative stress in living cells. Numerous experimental studies show that macrophages are sensitive to LPS derived from gram-negative bacteria and thus provide an inducible form of macrophage that undergoes oxidative stress and secretes lysozyme upon activation [14-16]. This allows the evaluation of BP5 on intercellular redox status, gene profiles, and functional characteristics of macrophages in both their activated and non-activated forms.

NO acts as an intracellular messenger and regulates cellular functions such as inflammation and the elimination of pathogens. However, excess NO can combine with O_2^- to form $ONOO^-$, causing oxidative stress and cell damage [45]. In this study, the results showed that LPS significantly induced NO production in macrophages and that this effect could be markedly reduced by BP5 treatment. ROS can induce changes in the redox balance to regulate cellular activity [46]. However, overproduction of ROS has most commonly been associated with oxidative stress, which is characterized by a major shift in the cellular redox balance and usually accompanied by ROS-mediated damage [47]. In this study, we found that the exposure of macrophages to LPS (2 μ g/ml) can cause significant increases in the levels of ROS, and these increases can be attenuated by BP5. This suggests that BP5 exerts powerful suppressive effects on intracellular ROS. In LPS-activated macrophages, ROS initiates the lipid peroxidation process and protein oxidation process. Malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acid peroxidation. An increase in ROS causes overproduction of MDA, which is commonly known as a marker of oxidative stress and antioxidant status in the cells [48]. Among the various oxidative modifications in proteins, carbonyl formation may be an early marker of protein oxidation [49]. In this study, we have found that exposure of macrophages to LPS (2 μ g/ml) can result in significant increases in the levels of MDA and protein carbonyls, both of which can be attenuated

by BP5. These results suggested that BP5 exerts powerful suppressive effects on lipid peroxidation and protein oxidation. The fact that BP5 can ameliorate lipid peroxidation and protein oxidation further supports the conclusion that BP5 is capable of scavenging ROS.

To determine the mechanisms involved in BP5 suppression of oxidative stress, we evaluated inducible nitric oxide synthase (iNOS) and the free radical scavenging systems, focusing on the glutathione (GSH) redox cycle and antioxidant enzymes. NO is synthesized from L-arginine by the 3 major NOS isoforms, namely, neuronal NOS (nNOS), endothelial NOS (eNOS), and iNOS. nNOS and eNOS are controlled by Ca^{2+} /calmodulin [31, 32]. There is considerable evidence that inhibition of excessive iNOS-derived NO production acts against oxidative stress. In this study, the molecular mechanisms of BP5-induced NO suppression were examined. We found that iNOS gene expression and iNOS activity were down-regulated following treatment with BP5. This is in accordance with the observed suppression of NO production. These data suggest that the protective effects of BP5 against LPS-induced oxidative stress in macrophages may include attenuating NO production via down-regulation of iNOS activity.

Previous studies have found that high concentrations of glutathione within cells protect them from different ROS. The GSH/GSSG ratio in the plasma can reflect changes in the stability of the redox status of an organism [50]. The GSH redox cycle begins when GSH converts the highly ROS to more stable molecules. GSH-Px catalyzes GSH-dependent reduction of hydrogen peroxide. In this reaction, GSH is oxidized to its disulfide form, GSSG, which is quickly reduced back to GSH by GR with reduced NADPH. Our results showed that BP5 promotes the GSH redox cycle by raising the intracellular GSH content and GSH/GSSG ratio and by enhancing the activities of GPx and GR. Therefore, the increase in GSH content caused by BP5 treatment may lead to decreased intracellular oxidative stress. In this way, it is part of the mechanism behind the anti-oxidative effects of BP5.

In addition to glutathione system, SOD and CAT are also two important antioxidant pathways in the removal of ROS. SOD catalyzes the dismutation of the superoxide anion (O_2^-) into H_2O_2 . H_2O_2 can be transformed into H_2O and O_2 by CAT [51]. In this study, we examined the activities of SOD and CAT to determine the protective effects of BP5 on oxidative stress induced by LPS in macrophages. Our results showed that the activities of SOD (Cu/Zn SOD and Mn SOD) and CAT were also enhanced by BP5. Taken together, the elevation in GSH

and antioxidative enzyme activity may be responsible for the suppression of oxidative stress in LPS-activated murine peritoneal macrophages.

To further clarify the mechanistic basis involved in BP5-mediated suppression of oxidative stress, the effect of BP5 on the levels of NF- κ B gene expression and NF- κ B activity in LPS activated macrophages was also examined. NF- κ B is a transcription factor that modulates the expression of variety of genes involved in immune and inflammatory responses, including iNOS and tumor necrosis factor (TNF- α) [52, 53]. It has been reported that ROS is the central pathway to NF- κ B activation [54, 55]. The inhibition mechanisms of many ROS scavengers are based on their abilities to inhibit the activation of NF- κ B. Therefore, this study was also designed to investigate the potential effects of BP5 on NF- κ B activation in LPS-activated macrophages. As shown in Fig. 7, incubation with BP5 at the indicated concentrations significantly inhibited the NF- κ B activation induced by LPS, including gene expression and activity levels. Based on the results of our experiments, the suppressive effect of BP5 on NF- κ B activation induced by LPS can be explained by ameliorated intracellular oxidative stress in the signaling pathway leading from LPS to NF- κ B activation. The inhibitive effect of BP5 on NF- κ B activation coincides with attenuated NO, ROS, TBARS, and protein carbonyls, and these attenuations is

occur via down-regulation of iNOS gene expression and iNOS activity and via up-regulation of GSH content and antioxidative enzyme activities. Considering that NF- κ B is a redox sensitive factor and a key regulator of antioxidant enzymes [56], we conclude that the mechanism by which BP5 decreases intracellular oxidative stress is also associated with the suppression of NF- κ B activation.

In summary, BP5 protects LPS-activated murine peritoneal macrophages from oxidative stress by reducing the levels of NO production, ROS generation, lipid peroxidation, and protein oxidation. This protective effect is due to BP5's ability to inhibit iNOS expression and activity and to modulate the glutathione (GSH) redox cycle and antioxidative enzymes. The suppressive effect of BP5 on oxidative stress takes place via down-regulation of expression and of the activity of nuclear factor kappa B (NF- κ B). This study indicates that BP5 may have applications as a new anti-oxidative stress reagent.

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