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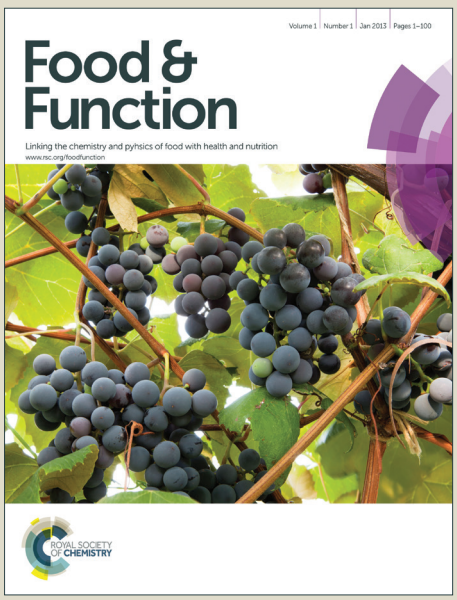

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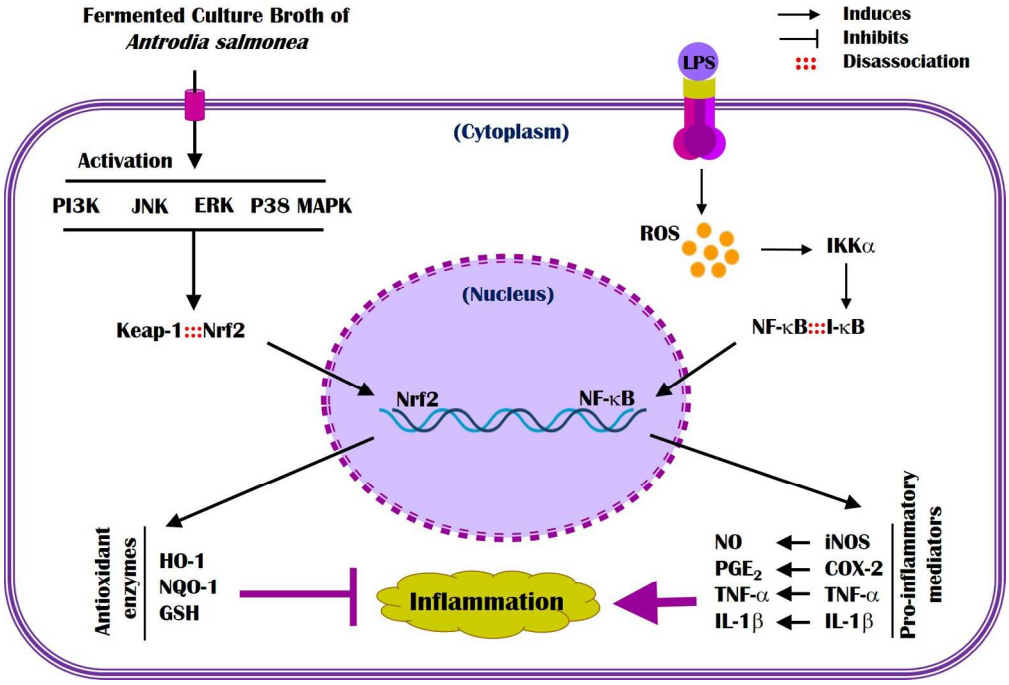

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Research Article:

Induction of Nrf2-mediated genes by *Antrodia salmonea* inhibits ROS generation and inflammatory effects in lipopolysaccharide-stimulated RAW264.7 macrophages

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Abbreviations

AS, *Antrodia salmonea*; LPS, lipopolysaccharide; ROS, reactive oxygen species; Nrf2, NF-E2-related factor-2; ARE, antioxidant response element; HO-1, heme oxygenase-1; NQO-1, NAD(P)H: quinone acceptor oxidoreductase 1; γ-GCLC, γ-glutamate-cysteine ligase catalytic subunit; GSH, glutathione; NO, nitric oxide; PGE_2, prostaglandin E_2; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; PI3K, phosphatidylinositol 3-kinase; JNK, c-JUN N-terminal kinase; ERK, extracellular signal-regulated kinase; ICAM-1, intercellular adhesion molecule-1.

ABSTRACT

Antrodia salmonea (AS), a well-known medicinal mushroom in Taiwan, has been reported to exhibit anti-oxidant, anti-angiogenic, anti-atherogenic, and anti-inflammatory effects. In the present study, we investigated the activation of Nrf2-mediated antioxidant genes in RAW264.7 macrophages by the fermented culture broth of AS, studied the resulting protection against lipopolysaccharide (LPS)-stimulated inflammation, and revealed the molecular mechanisms underlying these protective effects. We found that non-cytotoxic concentrations of AS (25-100 μg/mL) protected macrophages from LPS-induced cell death and ROS generation in a dose-dependent manner. The antioxidant potential of AS was directly correlated with the increased expression of the antioxidant genes HO-1, NQO-1, and γ-GCLC, as well as the level of intracellular GSH followed by an increase in the nuclear translocation and transcriptional activation of the Nrf2-ARE pathway. Furthermore, Nrf2 knockdown diminished the protective effects of AS, as evidenced by the increased production of pro-inflammatory cytokines and chemokines, including PGE_2, NO, TNF-α, and IL-1β, in LPS-stimulated macrophages. Notably, AS treatment significantly inhibited the LPS-induced ICAM-1 expression in macrophages. Our data suggest that the anti-inflammatory potential of *Antrodia salmonea* is mediated by the activation of Nrf2-dependent antioxidant defense mechanisms. Results support the traditional usage of this beneficial mushroom for the treatment of free radical-related diseases and inflammation.

Keywords: *Antrodia salmonea*, Nrf2, antioxidants, siRNA

Introduction

Macrophages play a pivotal role in the innate immune response and serve as the first line of defense against invading pathogens by facilitating cytoprotection and repair processes.[1] Despite its beneficial role in host defence, aberrant macrophage activation by endotoxins induces a number of major cellular responses that play roles in the pathogenesis and progression of inflammatory responses.[2] Lipopolysaccharide (LPS), which is a component of the cell walls of gram-negative bacteria, can activate a number of signals within macrophages during inflammation and infection.[3] Activated macrophages produce a variety of pro-inflammatory molecules, including nitric oxide (NO), prostaglandin E_2 (PGE_2), tumor necrosis factor-α (TNF-α), and interleukins, which are involved in the development and progression of inflammatory diseases and cancer.[4,5] Macrophage activation by LPS can initiate an increase in oxygen uptake that gives rise to be a variety of reactive oxygen species (ROS), which are the major factors driving oxidative stress-induced inflammation in immune cells.[6]

A number of lines of evidence suggest that nuclear factor E2-related factor-2 (Nrf2) plays an important role in protecting macrophages from LPS-stimulated inflammation.[7] Nrf2 belongs to the basic-leucine zipper (bZIP) family of transcriptional activator proteins and can be activated by endogenous products of oxidative stress.[8] The expression levels of many cytoprotective enzymes that are induced in response to oxidative stress are regulated primarily at the transcriptional level. The activation of the *cis*-acting anti-oxidant response element (ARE) is dependent on the transcription factor Nrf2. Nrf2 activates several antioxidant genes that are important for the elimination of oxidative stress in cellular systems, hemeoxygenase-1 (HO-1), NAD(P)H-quinone oxidoreductase-1(NQO-1), γ-glutamylcysteine synthetase (γ-GCLC), glutathione (GSH), and glutathione S-transferase A2 (GST-A2).[9,10] These enzymes exert cytoprotective, anti-oxidant, and anti-inflammatory effects in endotoxin-induced macrophages.[11]

The medical mushroom *Antrodia salmonea* (or *Taiwanofungus salmoneus*), a newly identified medicinal fungus species, belongs to the genus *Taiwanofungus*. The fruiting body of *Antrodia salmonea* has long been used in Taiwanese folk medicine for the treatment of diarrhea, abdominal pain, hypertension, itchy skin, and liver cancer. It has also been used as a food and drug detoxicant.[12] In our previous study, *Antrodia salmonea* in submerged culture exhibited antioxidant activities *in vitro* and protected human erythrocytes and low-density lipoproteins from oxidative modification.[13] Moreover, the safety levels and nontoxic characteristics of the fermented culture broth of *Antrodia salmonea* were evaluated using acute toxicity studies in a mouse model.[14] To date, several novel compounds have been isolated from the basidiomata of *Antrodia salmonea*, and *in vitro* studies have shown that these compounds display anti-oxidative and anti-inflammatory activities.[15,16,17] However, the possible mechanisms underlying the protective activity of *Antrodia salmonea* have yet not been fully elucidated. Recently, several naturally occurring compounds and crude extracts of medicinal herbs have been reported to induce the expression of phase II detoxifying enzymes in different cell types; in some of these studies, Nrf2-mediated genes were shown to exert anti-oxidant and anti-inflammatory activities.[18] Therefore, the present study was designed to investigate whether the anti-oxidant and anti-inflammatory potential of *Antrodia salmonea* is mediated by the activation of Nrf2-stimulated defense mechanisms. This study may help us to further evaluate the clinical therapeutic food detoxifying potential of *Antrodia salmonea* with respect to its anti-oxidant and anti-inflammatory properties.

Materials and Methods

Chemicals

Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), glutamine, and penicillin-streptomycin were purchased from GIBCO BRL (Grand Island, NY). LPS (from

Escherichia coli 055:B5), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2′,7′-dihydrofluorescein-diacetate ($DCFH_2$-DA) were purchased from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal antibodies against TNF-α and HO-1 were purchased from Abcam (Cambridge, UK). Nrf2, NQO-1, and IL-1β antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-γ-GCLC antibody was obtained from GeneTex Inc. (Irvine, CA). Antibodies against p-JNK1/2, p-ERK1/2, p-p38 MAPK, and p-PI3K were obtained from Cell Signaling Technology Inc. (Danvers, MA). 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) was obtained from Calbiochem (La Jolla, CA). All other chemicals were of the highest commercially available grade and were supplied by either Merck (Darmstadt, Germany) or Sigma.

***Antrodia salmonea* from submerged cultures**

The strain of the fungus *Antrodia salmonea* was collected in Nantou County in middle Taiwan. The plant was identified by Dr. **Shy-Yuan Hwang of Endemic Species Research Institute,** Nantou, Taiwan. Voucher specimens (No. AS001) have been deposited in China Medical University, Taichung, Taiwan. The hyphae of *Antrodia salmonea* were separated from the fruiting bodies. The whole colony was cut and placed into a flask with 50 mL of sterile water. After homogenization, the fragmented mycelial suspension was then inoculated into a culture medium comprising 2.0% glucose, 0.1% wheat powder, and 0.1% peptone in distilled water. The medium was adjusted to an initial pH of 5.0. Each culture was grown in a 2 L Erlenmeyer flask (containing 1 L of medium) and incubated at 25°C for 10 days with shaking at 120 rpm. Thereafter, 3.5 L of the culture was inoculated into a 500 L fermenting tank containing 300 L of culture medium. The resulting mixtures were then cultured at 25°C for 30 days. The fermentation conditions were the same as those used for the seed fermentation but with an aeration rate of 0.075 vvm, to produce a mucilaginous medium containing the mycelia. The

experiments were performed using 2~4 different batches of whole fermented culture of *Antrodia salmonea*.

Sample preparation

The fermentation product was poured through a non-woven fabric on a 20 mesh sieve to separate the deep yellow fermented culture into filtrate and mycelia and subsequently concentrated under a vacuum and freeze dried. The dry matter yield of the fermented culture was approximately 15 g/L. The freeze-dried samples were ground, shaken with distilled water, and then centrifuged at 3000 × *g* for 5 min, followed by passage through a 0.2 μm filter. The aqueous extracts were concentrated in a vacuum and freeze dried to form a powder. The yield of the fermented culture broth (AS) (1 g) was approximately 0.375 g. To prepare the stock solution, the powdered AS samples were dissolved in 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl (PBS) at 25°C. The solution was stored at -20°C before its anti-oxidant and anti-inflammatory potential were analyzed.

Cell culture

The murine macrophage (RAW264.7) cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD) and cultured in DMEM containing 2 mM glutamine, 1% penicillin-streptomycin, and 10% heat-inactivated FBS at 37°C in a humidified atmosphere containing 5% CO_2. For all experiments, the supernatant was removed 1 h after AS supplementation, the cells were washed with PBS, and the culture medium was replaced with new medium with or without LPS (1 μg/mL) for the indicated time. The LPS was dissolved in PBS (pH 7.2).

MTT assay

The effect of AS on cell viability was monitored by the MTT colorimetric assay. RAW264.7 cells (4×10^5 cells/well) were grown to confluence on 12-well cell culture plates, incubated with AS (25-100 μg/mL) in the absence or presence of LPS (1 μg/mL), and allowed to proliferate for 24 h. After treatment, the cells were incubated with 400 μL of 0.5 mg/mL MTT in PBS for 2 h. The culture supernatant was removed, the remaining MTT formazan was dissolved in 400 μL of isopropanol, and the absorbance was measured at 570 nm using an ELISA reader (Bio-Tek Instruments, Winooski, VT). The effect of AS on cell viability was assessed as the percentage of viable cells compared with the vehicle-treated control cells, which were arbitrarily designated as 100%. The assay was performed in triplicate at each concentration.

Measurement of intracellular ROS generation

The production of intracellular ROS was detected by fluorescence microscopy using $DCFH_2$-DA as described previously.[19] Briefly, RAW264.7 cells at a density of 4×10^5 cells/well in 12-well plate were incubated with AS (25-100 μg/mL) in the presence or absence of LPS (1 μg/mL) for 30 min. Then, 10 μM $DCFH_2$-DA was added to the culture medium, and the cells were incubated at 37°C for further 30 min. After incubation, the cells were washed with warm PBS, and the ROS production was measured by changes in fluorescence due to the intracellular production of DCF caused by the oxidation of $DCFH_2$. The DCF fluorescence was measured via fluorescence microscopy (200 × magnification) (Olympus, Center Valley, PA). The fold increase of intracellular ROS compared with the control was quantified by measuring the DCF fluorescence intensity in the microscope field.

Preparation of cell extracts and western blot analysis

RAW264.7 cells (4×10^6 cells/dish in 10 cm dish) were incubated with AS (50 μg/mL), after

which they were incubated with LPS for the indicated time. After treatment, the cells were detached, washed once in cold PBS, and suspended in 100 μL lysis buffer (10 mM Tris-HCl [pH 8.0], 0.32 M sucrose, 1% Triton X-100, 5 mM EDTA, 2 mM DTT, and 1 mM phenylmethyl sulfonyl fluoride). The suspension was kept on ice for 20 min and then centrifuged at 16000 × *g* for 20 min at 4°C. The total protein content was determined using a Bio-Rad protein assay reagent, with bovine serum albumin (BSA) as the standard. Protein extracts were reconstituted in sample buffer (0.062 M Tris-HCl [pH 6.8], 2% SDS, 10% glycerol and 5% β-mercaptoethanol), and the mixture was boiled for 5 min. Equal amounts (50 μg) of denatured protein were loaded into each lane and separated on 8-15% SDS-PAGE, followed by transfer onto PVDF membranes overnight. The membranes were blocked with 0.1% Tween-20 in Tris-buffered saline containing 5% non-fat dry milk for 30 min at room temperature and then reacted with primary antibodies for 2 h. They were then incubated with a horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody for 2 h and developed using the enhanced chemiluminescence substrate (Pierce Biotechnology, Rockford, IL). A densitometric graph of band intensities was generated using commercially available software (AlphaEase, Genetic Technology Inc. Miami, FL), with the control representing 100% or 1.0-fold, as shown below the gel data.

Immunofluorescence staining

RAW264.7 cells at a density of 1×10^4 cells/well in 8-well plates were cultured in DMEM containing 10% FBS in an eight-well glass Tek chamber and treated with AS (50 μg/mL for 1 h). The cells were then fixed in 2% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 10 min, washed, blocked with 10% FBS in PBS, and then incubated with anti-Nrf2 primary antibody in 1.5% FBS for 2 h, followed by incubation with FITC (488 nm)-conjugated secondary antibody for 1 h in 6% BSA. Cells were stained with 1 μg/mL of

4′,6-diamidino-2-phenylindole (DAPI) for 5 min. The stained cells were washed with PBS and visualized using a confocal microscope at 630 × magnification.

RNA extraction and RT-PCR analysis

RAW264.7 cells were seeded at a density of 4×10^6 cells/dish in 10 cm dish. After reaching 90% confluence, the cells were incubated with AS (50 μg/mL) for various times (1-12 h). Total RNA from cultured cells was prepared using the TRIzol reagent (Invitrogen, Carlsbad, CA). A 1 μg sample of total RNA was subjected to RT-PCR using a BioRad iCycler PCR instrument (Bio-Rad, Hercules, CA) and the SuperScript-III® One-Step RT-PCR Platinum *taq*® Kit (Invitrogen); amplification was performed in 30-38 cycles of 94°C for 45 s (denaturing), 60-65°C for 45 s (annealing), and 72°C for 1 min (primer extension). The sequences of the primers used were as follows: HO-1 forward, 5′-TTACCTTCCCGAACATCGAC-3′, reverse: 5′-GCATAAATTCCCACTGCCAC-3′; NQO-1 forward, 5′-CAGATCCTGGAAGGATGGAA-3′, reverse, 5′-AAGTTAGTCCCTCGGCCATT-3′; and Nrf2 forward, 5′-AGCAGGACATGGAGCAAGTT-3′, reverse, 5′-TTCTTTTTCCAGCGAGGAGA-3′. The PCR products were electrophoresed in a 1% agarose gel and stained with ethidium bromide.

ARE promoter activity

To examine promoter activity, we used a dual-luciferase reporter assay system (Promega, Madison, WI). Briefly, RAW264.7 cells were cultured in 24-well plates until reaching ~80% confluence and then incubated for 5 h in serum-free DMEM that did not contain antibiotics. The cells were then transfected with the pcDNA vector or ARE plasmid with β-gal and GFP using Lipofectamine 2000 (Invitrogen). After plasmid transfection, cells were treated with AS (50 μg/mL) for 24 h. After incubation, the cells were lysed, and the luciferase activity was

measured using a luminometer (Bio-Tek instruments Inc., Winooski, VT). The luciferase activity was normalized to the β-galactosidase activity in the cell lysates, and data were expressed as the averages of three independent experiments.

Determination of intracellular GSH

RAW264.7 cells were seeded at a density of 4×10^6 cells/dish in 10 cm dish. After reaching 90% confluence, the cells were incubated with AS (50 μg/mL) for various times (1-12 h). The total GSH content of macrophages was determined using the Cayman's GSH assay kit (Cayman Chemical Co, Ann Arbor, MI). The kit uses an enzymatic recycling method involving glutathione reductase to quantify glutathione (GSH). The GSH produced by the interaction of the sulfhydryl group of GSH and 5,5'–dithiobis-2-nitrobenzoic acid (DTNB) and Ellman's reagent to produce a yellow-colored compound, 5-thio-2-nitrobenzoic acid (TNB). The rate of TNB production is directly proportional to the rate of this recycling reaction, which is in turn directly proportional to the concentration of GSH in the sample. Thus, the net absorbance at 405 nm yields an accurate estimate of GSH in the sample. The GSH values were extrapolated from the absorbance values based on the standard curve provided with the assay kit.

Transient transfection of siRNA targeting Nrf2

RAW264.7 cells were transfected with Nrf2 siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. For the transfections, RAW264.7 cells were grown in DMEM containing 10% FBS and plated in 6-well plates to 60% confluence at the time of transfection. On the next day, the culture medium was replaced with 500 μL of Opti-MEM (Invitrogen), and the cells were transfected using the RNAiMAX transfection reagent (Invitrogen). For each transfection, 5 μL RNAiMAX was mixed with 250 μL of Opti-MEM and incubated for 5 min at room temperature. In a separate tube, siRNA (100 pM,

for a final concentration of 100 nM in 1 mL of Opti-MEM) was added to 250 μL of Opti-MEM, and the siRNA solution was added to the diluted RNAiMAX reagent. The resulting siRNA/RNAiMAX mixture (500 μL) was incubated for an additional 25 min at room temperature to allow complex formation. Subsequently, the solution was added to the cells in the 6-well plates, for a final transfection volume of 1 mL. After incubation for 6 h, the transfection medium was replaced with 2 mL of standard growth medium, and the cells were cultured at 37°C. Then, the cells were co-incubated with or without AS (50 and 100 μg/mL) in the presence or absence of LPS (1 μg/mL) for 1-24 h. The protein expression levels of Nrf2 and β-actin and the production of NO, PGE_2, TNF-α, and IL-1β were quantified.

Determination of nitric oxide production

The concentration of NO in the culture supernatant was determined based on the accumulation of nitrite, a major stable product of NO, using a Griess reagent-based colorimetric assay. RAW264.7 cells were seeded at a density of 4×10^5 cells/well in 12-well plates. After transfection with Nrf2 siRNA, cells were pre-treated with or without AS (50 and 100 μg/mL) prior to incubation with LPS (1 μg/mL) for 18 h. Culture supernatants (100 μL) were mixed with an equivalent volume of Griess reagents, and the absorbance of the mixture at 540 nm was measured using an ELISA microplate reader. A standard curve was constructed using known concentrations of sodium nitrate.

Determination of PGE_2 production

The PGE_2 concentration in the culture media was determined using an EIA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. RAW264.7 cells were seeded at a density of 4×10^5 cells/well in 12-well plates. After Nrf2 siRNA transfection, cells were pre-treated with or without AS (50 and 100 μg/mL) prior to incubation with LPS (1 μg/mL) for

18 h. The culture supernatants (100 μL) were collected, and the PGE_2 concentrations were determined using an ELISA microplate reader.

Quantification of TNF-α and IL-1β

The TNF-α and IL-1β concentrations in the cell culture media were determined using ELISA kits (R&D systems), as described previously.[20] Briefly, the Nrf2 siRNA-transfected cells were seeded at a density of 4×10^5 cells/well in 12-well plates and incubated with or without AS (50 and 100 μg/mL) in the presence or absence of LPS (1 μg/mL) for 18 h. Culture media were collected and transferred into 96-well plates that were coated with anti-mouse TNF-α or IL-β monoclonal antibodies, and the plates were incubated at room temperature for 2 h. After five washes, biotinylated anti-mouse TNF-α or IL-β monoclonal antibodies and avidin-horseradish peroxidase were added, and the plates were incubated for 2 h at room temperature. After a final wash, the chromogenic substrate (tetramethylbenzidine and hydrogen peroxide) was added to each well, and the plates were incubated for 30 min at room temperature in the dark. The absorbance at 405 nm was read in an ELISA plate reader after the addition of a stop solution.

Statistical analysis

The experimental results are presented as the mean ± standard deviation (mean ± SD). All study data were analyzed using an analysis of variance (ANOVA), followed by Dunnett's test for pair-wise comparisons. Statistical significance was defined as $p < 0.05$ for all tests.

Results

The effects of AS on cell viability in RAW264.7 macrophages

Prior to the *in vitro* anti-oxidant and anti-inflammatory study, we examined whether AS (25-100 μg/mL) affects RAW264.7 cell viability using an MTT assay. The macrophages were

treated with various concentrations of AS for 24 h, and the cell viability was unaffected by AS at concentrations of up to 100 μg/mL (Fig. 1A). As shown in Fig. 1B, treatment with LPS (1 μg/mL) slightly reduced cell viability to 89 ± 3%, and this LPS-induced reduction of cell viability was significantly prevented by AS at 50 or 100 μg/mL ($p < 0.05$). Based on these data, we used the non-cytotoxic concentrations of AS (i.e., ≤ 100 μg/mL) to evaluate its antioxidant/anti-inflammatory effects in RAW264.7 cells.

AS inhibits LPS-induced ROS generation in LPS-stimulated macrophages

The LPS-induced intracellular ROS accumulation was monitored within cells *in vitro* using $DCFH_2$-DA fluorescence microscopic analysis. As shown in Fig. 2A and B, the incubation of RAW264.7 cells with LPS (1 μg/mL) caused a significant increase in the intracellular ROS in RAW264.7 cells (24-fold), whereas co-treatment with AS (25-100 μg/mL) resulted in a significant ($p < 0.05$) dose-dependent reduction (<10-fold) of ROS accumulation in LPS-induced RAW264.7 cells. In addition, when the cells were incubated with AS alone for the same length of time, the concentration of ROS was maintained at a background level similar to that in unstimulated cells (data not shown). Therefore, we concluded that AS could suppress LPS-induced ROS generation in macrophages.

AS activates the antioxidant gene Nrf2 in macrophages

Nrf2 is a transcription factor that serves as a sensor for oxidative stress and coordinates the expression of antioxidant stress response genes upon exposure to oxidative stimulation. We hypothesized that the protective effects of AS against LPS-induced oxidative stress (intracellular ROS accumulation) were mediated by the induction of antioxidant genes through Nrf2. As we predicted, western blotting showed that AS (50 μg/mL) significantly increased the nuclear translocation of Nrf2, as evidenced by the accumulation of Nrf2 in the nucleus. AS

treatment also significantly increased the total Nrf2 expression in LPS-induced macrophages (Fig. 3A). Aberrant Nrf2 activation by AS was observed within 2 h, whereas the increase in Nrf2 expression was gradually decreased when AS was applied at later time points (Fig. 3A). To further confirm this protective effect, we monitored the nuclear translocation of Nrf2 by immunofluorescence assay. As shown in (Fig. 3B), the nuclear accumulation of Nrf2 was significantly increased by AS compared with the control cells. More precisely, the Nrf2 expression in control cells was observed in the cytoplasm, whereas Nrf2 accumulation in the nucleus was observed after 1 h of treatment with AS (Fig. 3B). These results strongly suggest that AS promotes the activation and nuclear translocation of Nrf2 in macrophages.

AS activates ARE activity in macrophages

To further test the hypothesis that AS may promote the transcriptional activation of Nrf2 in RAW264.7 cells, the promoter activity of Nrf2 (ARE) was measured using a luciferase reporter assay. An Nrf2 promoter construct in a pcDNA vector was transiently transfected into RAW264.7 cells, and the induced luciferase activity was measured after treatment with various concentrations of AS (25-100 μg/mL) for 1 h. The luciferase activity was normalized to the β-galactosidase activity in the cell lysate and considered the basal level (1-fold). The luciferase activity derived from the ARE promoter was consistently increased after treatment with AS in a dose-dependent manner from 1-fold to 1.5-, 1.7-, and 2.5-fold by 25, 50, and 100 μg/mL, respectively (Fig. 3C). The luciferase activity of blank plasmid pcDNA in RAW264.7 cells was not affected by AS treatment (data not shown). This result confirms that AS treatment not only induces the nuclear translocation of Nrf2 but also promotes ARE-driven transcriptional activity in RAW264.7 cells.

AS upregulates HO-1 and NQO-1 expression in macrophages

The activated Nrf2 might induce various proteins with antioxidant and anti-inflammatory activities. HO-1 and NQO-1 are the anti-oxidant/anti-inflammatory molecules that are most strongly upregulated by Nrf2 under various stress conditions or in response to oxidants. Therefore, we hypothesized that the nuclear translocation and transcriptional activation of Nrf2 by AS might induce HO-1 and NQO-1 gene expression. As shown in Fig. 4A, compared with untreated control cells, AS (50 μg/mL) significantly increased the HO-1 mRNA expression in a time-dependent manner. In particular, higher HO-1 expression was observed at 9 h after AS treatment in RAW264.7 cells (245 ± 4%). Furthermore, the mRNA expression of NQO-1 was also significantly increased by AS (50 μg/mL) in a time-dependent manner; the highest expression was observed after 2 h (228 ± 6%), after which the NQO-1 level gradually declined (Fig. 4A). Concomitant with the mRNA expression, we also found that the protein levels of HO-1 were significantly increased by AS in a time-dependent manner, and the highest expression was observed after 8 h (210 ± 3%) (Fig. 4B). Similarly, the NQO-1 protein expression was significantly increased by AS. The highest level of NQO-1 expression was observed after 2-4 h treatment (243 ± 5%), after which the expression gradually declined (Fig. 4B). These data support our hypothesis that AS-induced Nrf2 activation promotes the transcription of antioxidant genes, including HO-1 and NQO-1.

AS increased intracellular GSH and γ-GCLC expression in macrophages

GSH is the predominant low-molecular weight thiol and the most important non-enzyme antioxidant in mammalian cells. GSH effectively protects cells from various oxidative stresses caused by scavenging free radicals, suppressing lipid peroxidation and removing hydrogen peroxides in the cellular system.[21] Therefore, we hypothesized that the antioxidant potential of AS may be mediated through the induction of intracellular GSH, and the levels of intracellular GSH were determined. As shown in Fig. 5A, the untreated control cells showed a basal level of

280 ± 18 μM GSH, and AS (50 μg/mL) treatment consistently increased intracellular GSH to 343 ± 35 μM, 368 ± 14 μM, and 423 ± 7 μM after 4, 8, and 12 h, respectively. To further confirm this protective effect, the level of γ-GCLC protein expression was monitored by Western blotting. GSH is sequentially synthesized from glutamate, cysteine, and glycine, and this synthesis is controlled by the rate limiting enzymes of these reactions, including γ-GCLC. We found that AS (50 μg/mL) treatment significantly increased γ-GCLC expression in a time-dependent manner. Compared to the untreated control cells (100%), the AS-treated macrophages exhibited a more than 2-fold (200 ± 20%) increase in γ-GCLC expression (Fig. 5B). These data may suggest that AS eliminates LPS-induced oxidative stress by inducing the expression of γ-GCLC, leading to the elevation of intracellular GSH.

Nrf2 knockdown diminishes the protective effect of AS in LPS-stimulated macrophages

To demonstrate the importance of Nrf2 regulation, we developed an Nrf2 knockdown model in RAW264.7 cells using siRNA transfection. The efficacy of the Nrf2 gene knockdown was confirmed by Western blot analysis, which showed that the transfection of siNrf2 (100 pM) led to a reduction in the Nrf2 protein level (Fig. 6A). However, the siRNA-induced reduction in Nrf2 was not altered by AS treatment (Fig. 6A), even after 18 h of AS treatment; that is, the transfection with siNrf2 abrogated the protective effect of AS on the LPS-induced production of NO, PGE_2, TNF-α, and IL-1β in RAW264.7 cells (Fig. 6 B-E). This finding is evidence that the augmentation of Nrf2 is directly involved in the AS-mediated anti-inflammatory effects in macrophages.

AS upregulates the AKT and MAPK signaling pathways in macrophages

The role of PI3K/AKT activation in the transcriptional regulation of Nrf2 has been studied in

detail.[11] Based on these reports, we used Western blot analysis to monitor whether AS could induce PI3K activation in macrophages. As shown in Fig. 7, AS treatment caused a time-dependent increase in PI3K phosphorylation in macrophages. The phosphorylation levels peaked within 45 min and were sustained for 60 min.

MAPK family proteins, such as JNK1/2, ERK1/2, and p38 MAPK, have been shown to play important roles in the activation of Nrf2 [11]. Therefore, the effects of AS on MAPK activation were examined. Macrophages were treated with AS (50 μg/mL) for 5-60 min, and the resulting phosphorylation levels of JNK1/2, ERK1/2, and p38 MAPK were assessed by immunoblotting. As shown in Fig. 7, AS treatment caused a marked increase in JNK1/2 phosphorylation after 5-10 min, after which the phosphorylation gradually declined to the basal level. AS treatment also increased the phosphorylation of ERK1/2 in a time-dependent manner. The highest level of ERK1/2 phosphorylation was observed after 45 min and was sustained thereafter (Fig. 7). Furthermore, the phosphorylation of p38 MAPK was observed after 5 min of AS treatment and peaked after 45 min of treatment (Fig. 7). Taken together, these results suggest that the AS-mediated induction of Nrf2 is driven by the activation of up-stream kinases including PI3K/AKT, JNK1/2, ERK1/2, and p38 MAPK.

AS inhibits LPS-induced ICAM-1 expression in macrophages

Moreover, during inflammation, macrophages adhesion to endothelial cells is highly influenced by ICAM-1. Also, the expression of ICAM-1 by macrophage cells in induced by various cytokines including IL-1β, TNF-α, and LPS.[22] Therefore, next we sought to investigate the effect of AS on LPS-induced ICAM-1 expression in macrophage cells. AS shown in Fig. 8, compared with un-stimulated control cells, LPS treatment significantly increased ICAM-1 expression (7.1-fold), whereas, AS significantly reduced the LPS-induced ICAM-1 expression.

Discussion

The results of this study clearly indicate that the antioxidant potential of AS was directly correlated with the increased expression of the antioxidant genes HO-1, NQO-1, and γ-GCLC and the level of intracellular GSH, which was followed by the augmentation of nuclear translocation and the transcriptional activation of Nrf2-mediated antioxidant gene pathways. Nrf2 knockdown diminished the protective effects of AS, as evidenced by the increased production of pro-inflammatory cytokines and chemokines including PGE_2, NO, TNF-α, and IL-1β in LPS-stimulated RAW264.7 cells. These results suggest that the activation of Nrf2-mediated antioxidant defense mechanisms by *Antrodia salmonea* protect macrophages from LPS-stimulated inflammation.

ROS are created by variety of cellular processes as part of cellular signaling events. A number of lines of evidence suggest that ROS participate in inflammation, and LPS-induced ROS generation has been widely studied in various *in vitro* and *in vivo* systems.[23,24] In particular, ROS are major oxidative products that are mainly released by the mitochondria, cytochrome p450 metabolism, peroxysomes, and inflammatory cell activation by endotoxins in macrophages.[25] Moreover, ROS production is an important component of the initiation and enhancement of cell death via apoptosis or authophagy.[26] In this study, we observed that LPS treatment enhanced intracellular ROS accumulation in RAW264.7 macrophages and that the elevated ROS generation was significantly inhibited by AS.

It has been reported that the Nrf2/ARE-mediated antioxidant genes are induced by various external stimuli and plant-derived natural products.[11,27,28] Activated Nrf2 translocates from the cytoplasm to the nucleus and binds DNA at ARE-binding motifs to activate the transcription of various detoxifying (phase II) enzymes including HO-1, NQO-1, and γ-GCLC.[5] This induction of these anti-oxidant genes has been assumed to be the mechanism through which Nrf2 inhibits LPS-induced inflammation. Several studies revealed that increases in the expression of

antioxidant enzymes (HO-1, NQO-1, and γ-GCLC) can reduce ROS levels in cells, eventually creating a reducing intracellular environment and maintaining Nrf2 in its augmented configuration.[29] In this study, we found that the nuclear translocation of Nrf2 in macrophages significantly increased in the presence of AS, suggesting that the elimination of intercellular ROS may be due to the transcriptional activation of Nrf2 by AS. This is consistent with previous reports that the extracts of many plants contain anti-inflammatory agents that inhibit ROS formation *via* the activation of Nrf2 cascades in macrophages.[11]

The discovery of RNA interference has revolutionized the study of specific gene functions and pathways.[30] Importantly, siRNA-directed transcriptional silencing is conserved in mammalian cells, providing a means to inhibit the functions of specific mammalian genes.[30] Our data show that the siRNA-mediated knockdown of Nrf2 expression enhances the production of NO, PGE_2, TNF-α, and IL-β in macrophages. AS failed to protect the Nrf2-knockdown macrophages from LPS-induced NO, PGE_2, TNF-α, and IL-β production. These data demonstrate that the protective effect of AS is mediated mainly by Nrf2 activation.

HO-1 is a well-known antioxidant enzyme that plays an important role in the defense against LPS-induced ROS generation and inflammation in macrophages.[31] Several lines of evidence have demonstrated that HO-1 expression, driven by Nrf2 and, the products of HO-1 activity are likely to inhibit NO production.[32] The genes regulated by Nrf2 include phase II enzymes such as NQO-1, which detoxify endogenous and exogenous chemicals through reduction and conjugation reactions.[33] A previous study showed that NQO-1 knockdown led to the increased expression of LPS-induced inflammatory cytokines including TNF-α and IL-1β in cultured monocytes.[34] The regulation of GSH/GCLC by Nrf2 also protects cells from LPS-induced inflammatory responses.[35] These antioxidant enzymes are vital for protecting cells from oxidative stress and electrophile toxicity as well as preventing carcinogenesis. Thus, AS protects the macrophages from LPS-induced inflammation mainly by elevating the intracellular

anti-oxidative enzymes by enhancing the accumulation of Nrf2, a transcription factor for antioxidant genes, and thus dramatically inducing the expression of the antioxidant genes HO-1, NQO-1, and γ-GCLC in response to LPS stimulation.

Moreover, cross-talk between the transcription factors NF-κB and Nrf2 is involved in inflammatory gene expression.[28] The over-expression of Nrf2 suppressed the expression of pro-inflammatory genes in human arotic endothelial cells.[36] Paur et al. (2010) reported that extracts of oregano, coffee, thyme, clove, and walnut inhibited LPS-induced NF-κB activation via the up-regulation of Nrf2-mediated antioxidant genes in cultured monocytes.[37] Furthermore, the pre-treatment of peritonial macrophage cells with the Nrf2 inducer sulforaphane appeared to inhibit the LPS-induced induction of iNOS, COX-2, TNF-α, and IL-1β expression.[38] Thus, it is reasonable to assume that there are at least two possible explanations for the mechanism underlying the anti-inflammatory effect of AS: the inactivation of NF-κB and activation of Nrf2, which stimulates anti-inflammatory HO-1 gene expression. In that regard, controlling macrophage activation may have potential therapeutic implications for the treatment of various inflammatory diseases.

The PI3K/AKT and MAPK signaling pathways, including the JNK, ERK, and MAPK p38 pathways, and their downstream transcription factors play critical roles in cell fate determination. In addition, previous studies have demonstrated that the transduction of oxidative stress signals to ARE-mediated gene expression involve three main pathways, i.e., the activation of the PI3K/AKT and MAPKs cascades.[11] Therefore, to further identify the upstream regulatory mechanisms involved in AS-induced Nrf2 signaling, the PI3K/AKT and MAPK pathways were examined in the present study. We found that AS significantly induced the phosphorylation of PI3K, JNK, ERK, and p38 MAPK in LPS-challenged RAW264.7 cells. One possible explanation for this observation is that the activation of the Nrf2/ARE pathway may be associated with the activation of the PI3K/AKT and MAPKs cascades.

Recent studies have shown that certain bioactive compounds, such as ergostanes, lanostanes, and naphthoquinones, are present in AS and exhibit potential anti-oxidative and anti-inflammatory effects *in vitro*.[15,17] The three new ergostanes exhibited anti-oxidative effects by inhibiting NADPH oxidase activity but did not exhibit free radical scavenging activity. In addition, the ergostanes strongly inhibited nitric oxide production in activated neutrophils and microglial cells. A previous study also showed that ethanolic extracts of AS significantly reduced serum levels of NO and TNF-α as well as the expression of the iNOS protein, both *in vitro* and *in vivo*, which might be mediated through the pharmacological effect of adenosine.[16] Huang et al. (2012) also reported that two bioactive compounds, adenosine and zhankuic acid A, have been identified in the ethanolic extract of the fruiting body of AS.[16] During the inflammation, adenosine levels rise and modulate inflammatory responses by interacting with their receptors.[39] In other experimental studies also observed that *Antrodia salmonea* contains an enormous amount of polyphenols, which are believed to be the molecules responsible for its anti-oxidative effects.[15,17] Moreover, our recent study with HPLC analysis shown that AS was composed ~11.6% of 2,4-dimethoxy-6-methylbenzene-1,3-diol.[13] Taken together, these studies demonstrate that different extraction conditions produced compounds with different biological activities. Our previous studies revealed that the fermented culture broths of *Antrodia camphorata*, a similar medicinal fungus, exhibited various biological activities.[40-42] Therefore, we believe that the anti-oxidant and anti-inflammatory effects of AS may be attributable to the pharmacological activities of its major bioactive compounds. However, further studies of these bioactive compounds are highly warranted.

Our finding that *Antrodia salmonea* can induce the expression of antioxidant genes through the activation of the Nrf2 signaling pathway is novel and important. The delayed and increased induction of HO-1, NQO-1, γ-GCLC, and the elevation of GSH likely tilts the balance in favor of an anti-oxidative and anti-inflammatory environment. Based on the above reported

bioactivities, it is clear that *Antrodia salmonea* exerted an anti-inflammatory effect in LPS-stimulated macrophages through the up-regulation of Nrf2-mediated antioxidant defense mechanisms. This antioxidant and anti-inflammatory potential may therefore indicate the presence of a novel mechanism of action underlying the apparent efficacy of this beneficial mushroom. However, further *in vivo* investigation of this activity is necessary to elaborate the underlying mechanism(s) and permit the full exploitation of its promise.

Acknowledgments

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Figure Legends

Fig. 1 The effects of the fermented culture broth of *Antrodia salmonea* (AS) on murine macrophage cell viability. RAW264.7 cells were treated with various concentrations of AS (25, 50, and 100 μg/mL) in the absence (A) or presence (B) of LPS (1 μg/mL) for 24 h. After treatment, cells were incubated with MTT for 2 h. The culture supernatant was removed and re-suspended with isopropanol to dissolve the MTT formazan, and the absorbance was measured at 570 nm using an ELISA micro-plate reader. The results are the mean ± SD of three assays. * indicates a significant difference from the LPS-activated group ($p < 0.05$).

Fig. 2 AS suppresses ROS generation in LPS-stimulated macrophages. (A) RAW264.7 cells were treated with various concentrations of AS (25, 50, and 100 μg/mL) in the presence or absence of LPS (1 μg/mL) for 30 min. The non-fluorescent cell membrane-permeable probe $DCFH_2$-DA was added to the culture medium at a final concentration of 10 μM. $DCFH_2$ was reacted with cellular ROS and metabolized to fluorescent DCF. DCF fluorescence was imaged under a fluorescence microscope (200 × magnification). (B) The fold increase of intracellular ROS, compared to control (1-fold), was quantified by measuring the DCF fluorescence intensity in the microscopic field. The results shown are the mean ± SD of three assays. * indicates a significant difference from the LPS-activated group ($p < 0.05$).

Fig. 3 AS up-regulates Nrf2 transcriptional activation in macrophages. (A) RAW264.7 cells were treated with AS (50 μg/mL) for 1-4 h. Western blotting was performed to analyze the overall and nuclear Nrf2 expression using specific whole-cell and nuclear extracts. Equal amounts (50 μg) of cell lysate from each sample were resolved by 8-15% SDS-PAGE. β-actin and histone served as internal controls for the total and nuclear fractions, respectively. The relative changes in the intensities of the protein bands were measured by densitometry. (B)

Cells grown in chamber slides were pretreated with 50 μg/mL AS for 1 h, fixed, and permeabilized. Cells were incubated with an anti-Nrf2 antibody, followed by incubation with a FITC-labeled secondary antibody. The subcellular and nuclear localization of Nrf2 was visualized using a confocal microscope user 40× magnification. (C) ARE-luciferase activity was measured after transient transfected RAW264.7 cells incubated with various concentrations of AS (25-100 μg/mL) for 6 h. The results are the mean ± SD of three assays. * indicates significant difference in comparison to LPS-activated group ($p < 0.05$).

Fig. 4 AS induces the expression of the antioxidant genes HO-1 and NQO-1 in macrophages. (A) RAW264.7 cells were incubated with or without 50 μg/mL AS for 0.5-12 h, and total RNA was subjected to RT-PCR using one-step RT-PCR master mix. The RT-PCR products were separated by 1% agarose gel electrophoresis. GAPDH served as an internal control. Relative changes in mRNA bands were measured by densitometry. (B) Cells were incubated with or without 50 μg/mL AS for 1-12 h, and total cell lysate was subjected to Western blotting to monitor the HO-1 and NQO-1 protein expression using specific antibodies. Equal amounts (50 μg) of total lysate from each sample were resolved by 8-15% SDS-PAGE, with β-actin as a control. The relative changes in the intensities of the protein bands were measured by densitometry. The results are the mean ± SD of three assays. * indicates significant difference from the LPS-activated group ($p < 0.05$).

Fig. 5 AS induces GSH formation and γ-GCLC protein expression in macrophages. (A) Intracellular GSH was measured using a commercially available ELISA kit, as described in the Materials and Methods. RAW264.7 cells were incubated with 50 μg/mL AS for 4-12 h. (B) Total cell lysate was subjected to Western blotting using specific antibodies to monitor γ-GCLC protein expression. Cells were incubated with or without AS (50 μg/mL) for 1-12 h. Equal

amounts (50 μg) of total lysate from each sample were resolved by 8-15% SDS-PAGE with β-actin as a control. The relative changes in the intensities of the protein bands were measured by densitometry. The results are the mean ± SD of three assays. * indicates significant difference from the LPS-activated group ($p < 0.05$).

Fig. 6 Nrf2 knockdown attenuates the protective effects of AS in LPS-stimulated macrophages. (A) RAW264.7 cells were transfected with siRNA targeting Nrf2 or a non-silencing control. Following transfection for 24 h, the cells were incubated with or without AS (50 or 100 μg/mL) for 18 h. The Nrf2 knockdown was confirmed by Western blotting. (B-E) The control and Nrf2 siRNA-treated macrophages were incubated with or without AS (50 or 100 μg/mL) for 18 h in the presence or absence of LPS (1 μg/mL). (B) NO production was determined by measuring the formation of nitrite, the stable end-metabolite of NO in the culture media using the Griess assay. The levels of PGE_2 (C), TNF-α (D), and IL-1β (E) in the culture media were measured using a commercial ELISA kit, as described in the Materials and methods. The results are the mean ± SD of three assays. * indicates a significant difference from the LPS-activated group ($p < 0.05$).

Fig. 7 AS activates the PI3K and MAPK signaling pathways in macrophages. RAW264.7 cells were incubated with 50 μg/mL AS for 5-60 min, and the levels of phosphorylated PI3K, JNK1/2, ERK1/2, and p38 MAPK were determined using western blot analysis. Equal amounts of total cell lysate (50 μg) were resolved by 8-12% SDS-PAGE, and Western blotting was performed. β-actin as used as an internal control. The relative changes in the intensities of the protein bands were determined by densitometry.

Fig. 8 AS inhibits LPS-induced ICAM-1 expression in macrophage cells. RAW264.7 cells were treated with AS (12.5-100 □g/mL) in the absence or presence of LPS (1 □g/mL) for 24 h.

Equal amounts (50 μg) of total lysate from each sample were resolved by 8-15% SDS-PAGE with β-actin as a control. The relative changes in the intensities of the protein bands were measured by densitometry. The results are the mean ± SD of three assays. * indicates significant difference from the LPS-activated group ($p < 0.05$).

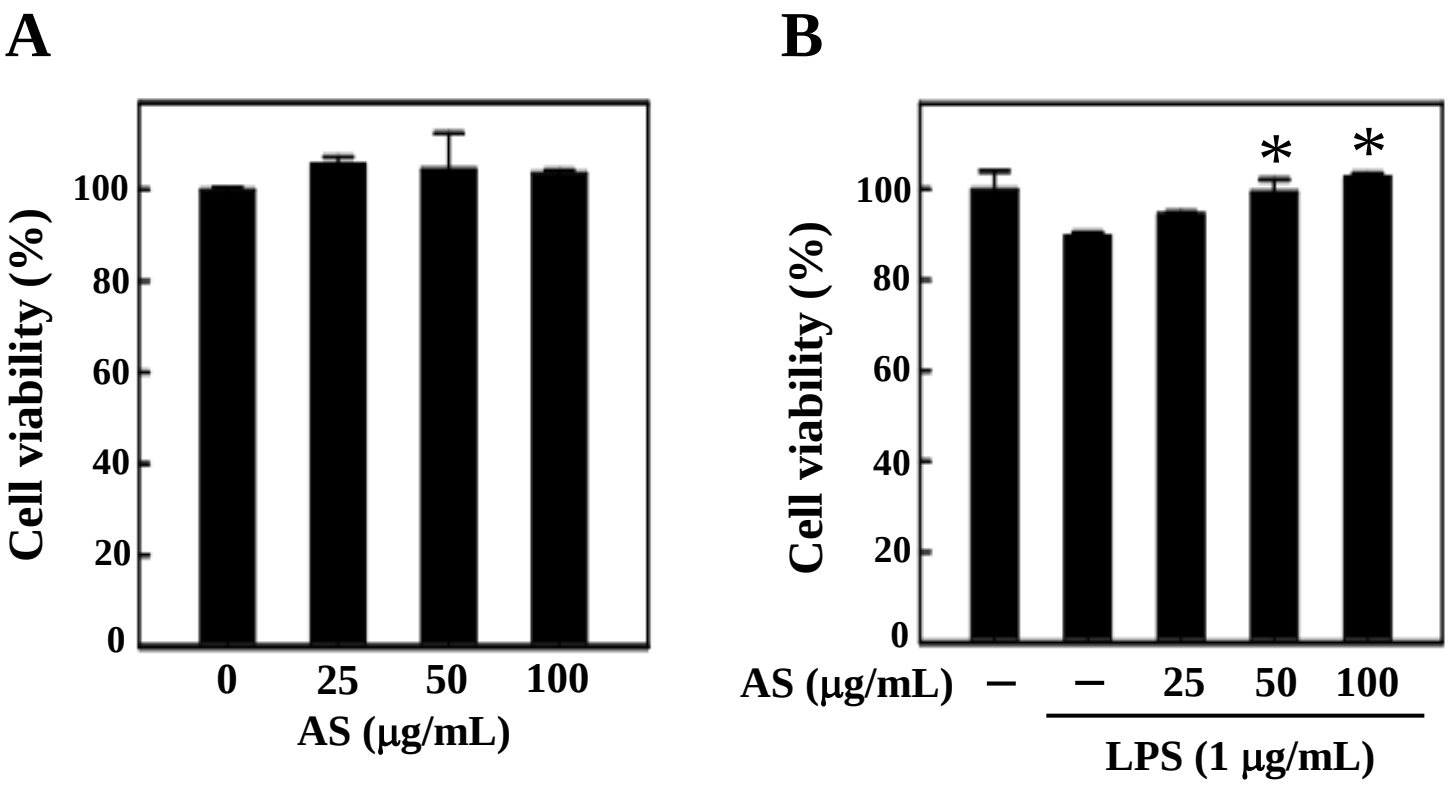
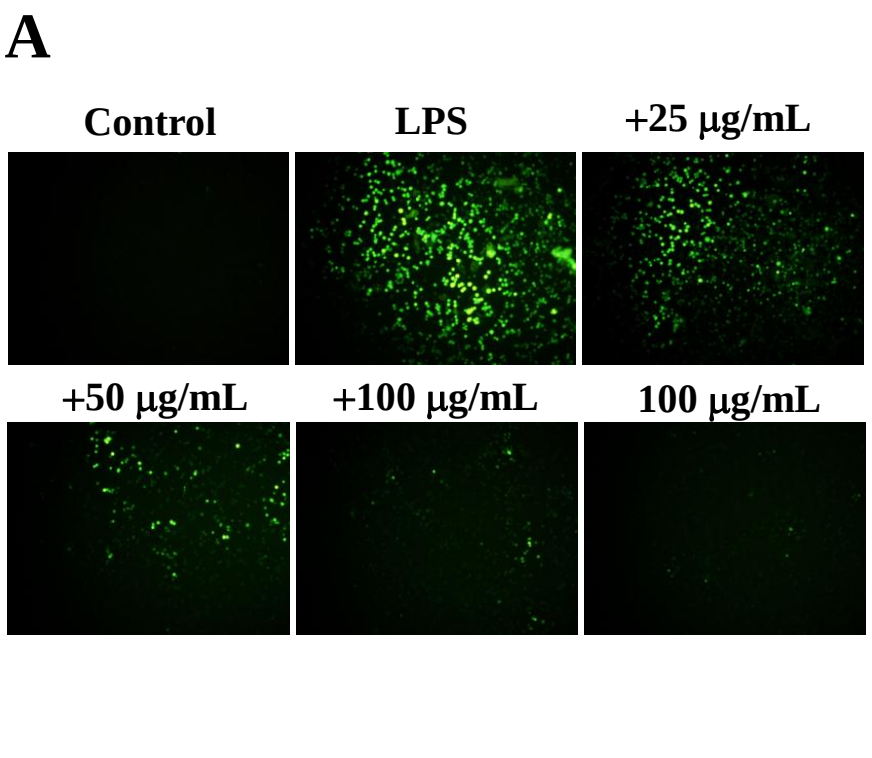


Fig 1



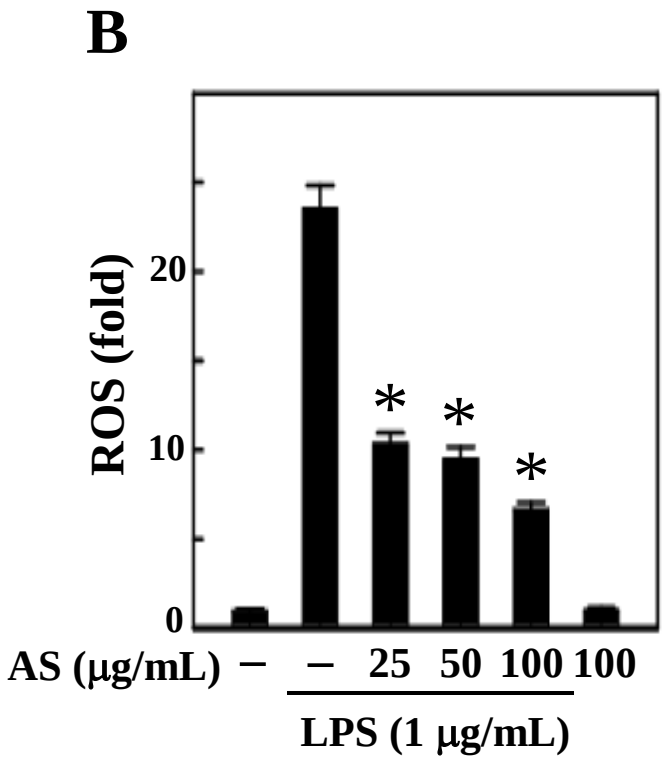


Fig 2

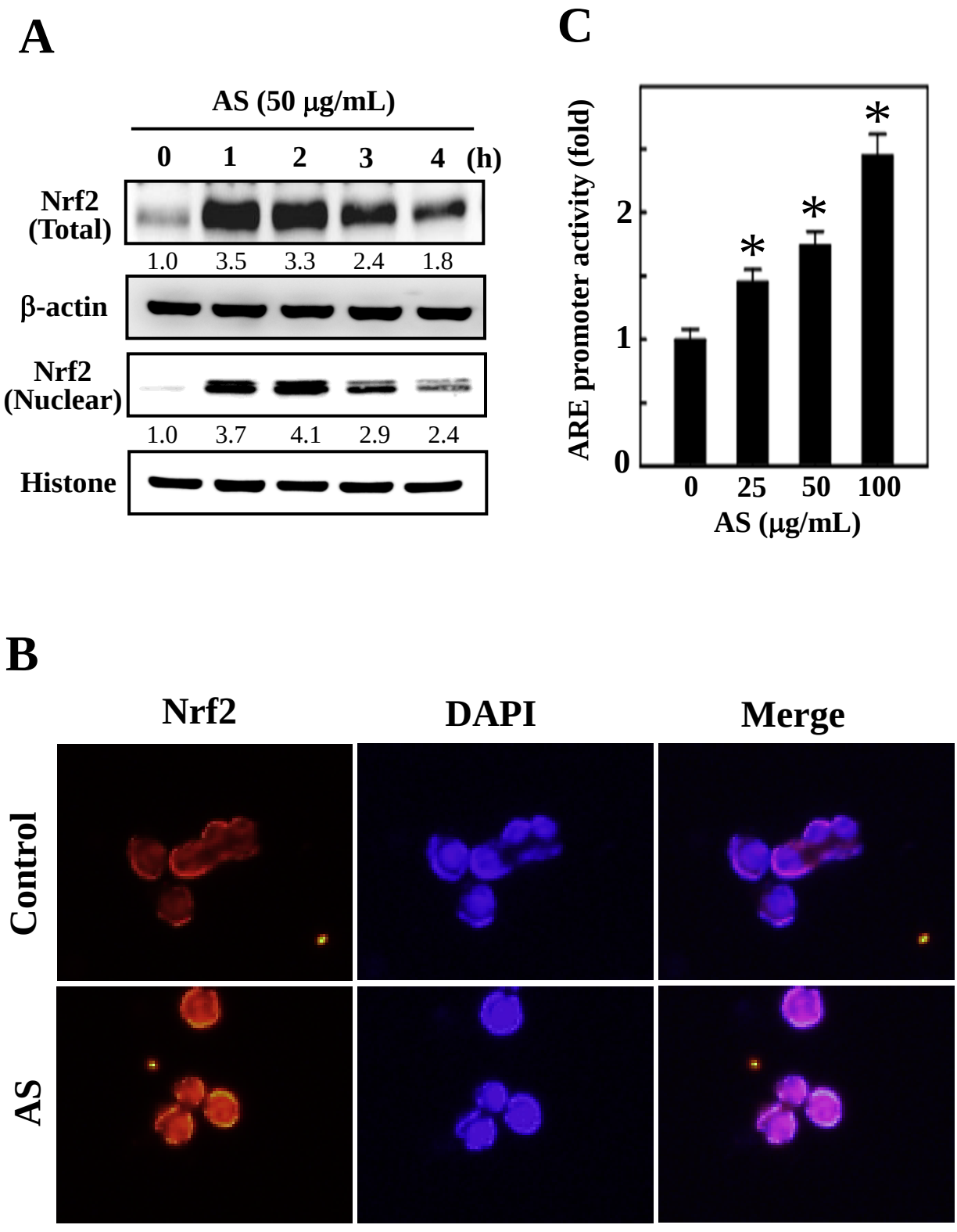


Fig 3

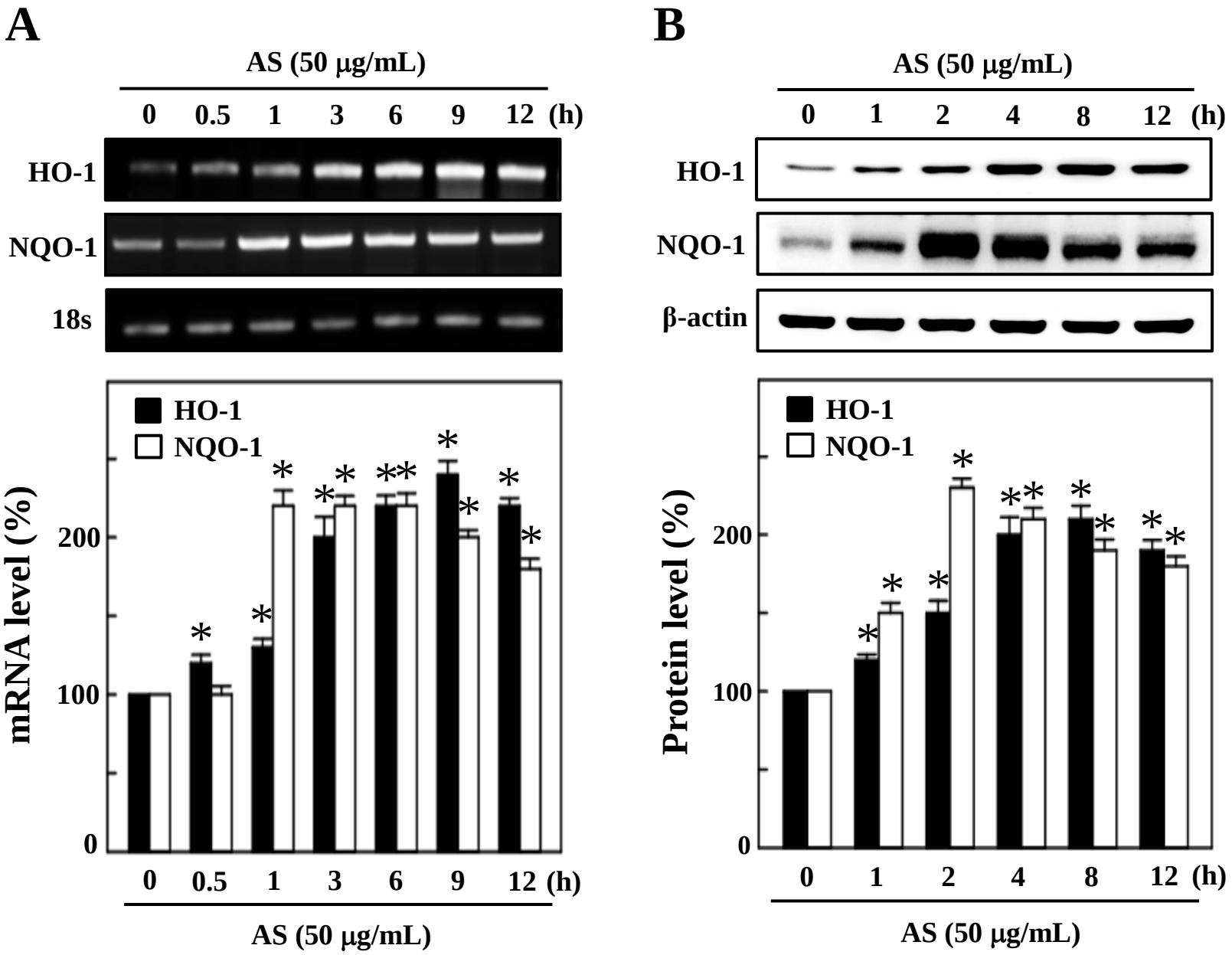


Fig 4

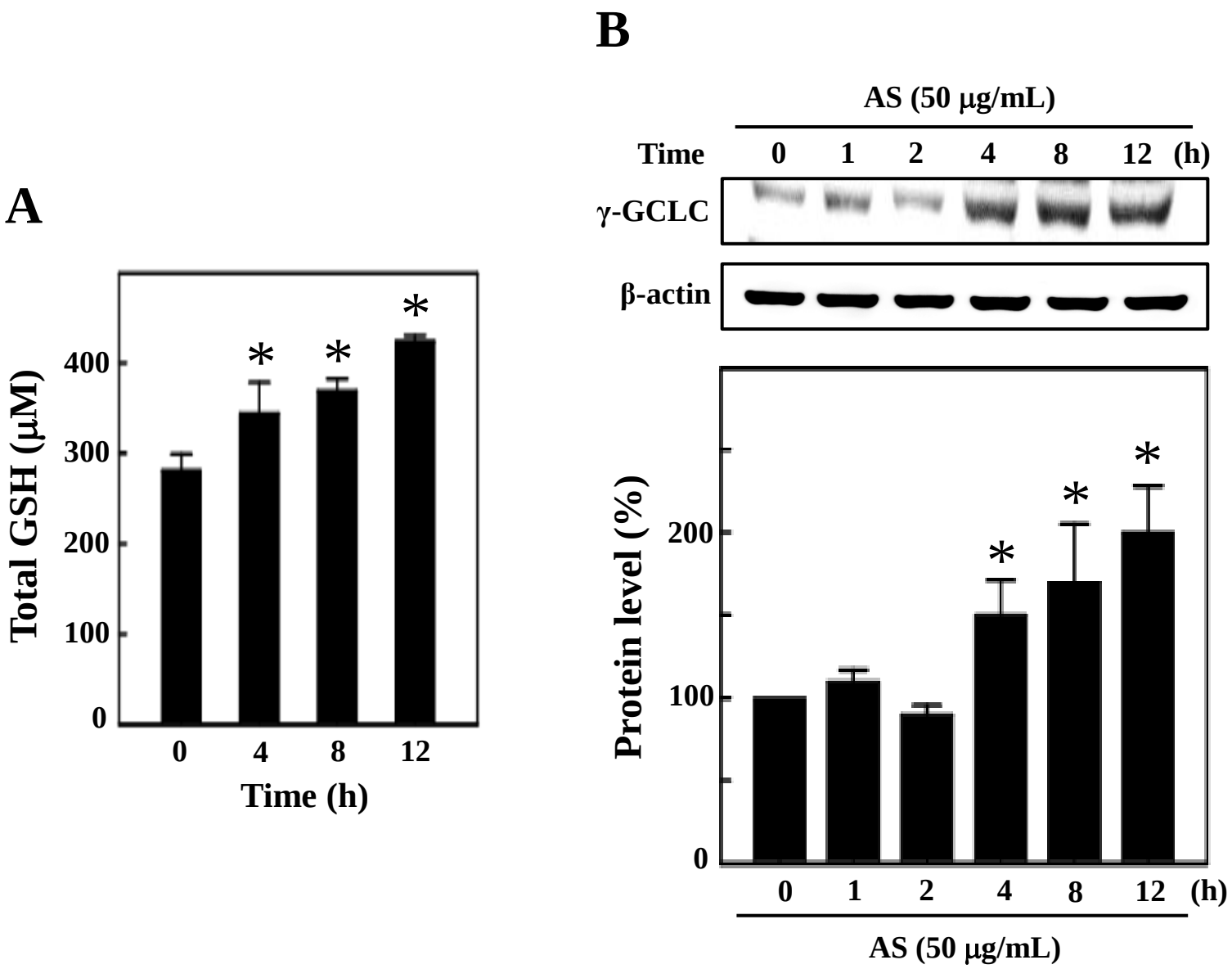


Fig 5

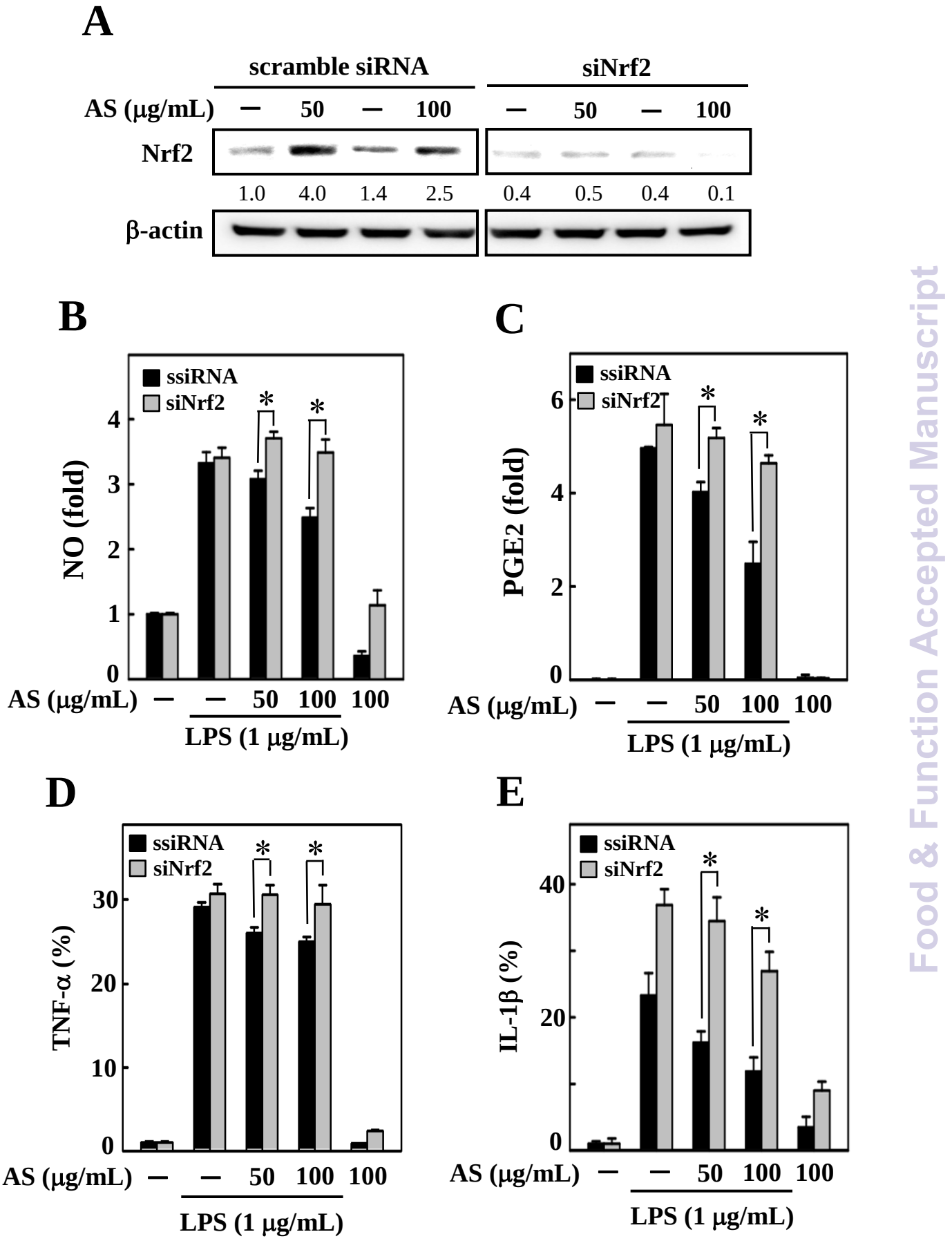


Fig 6

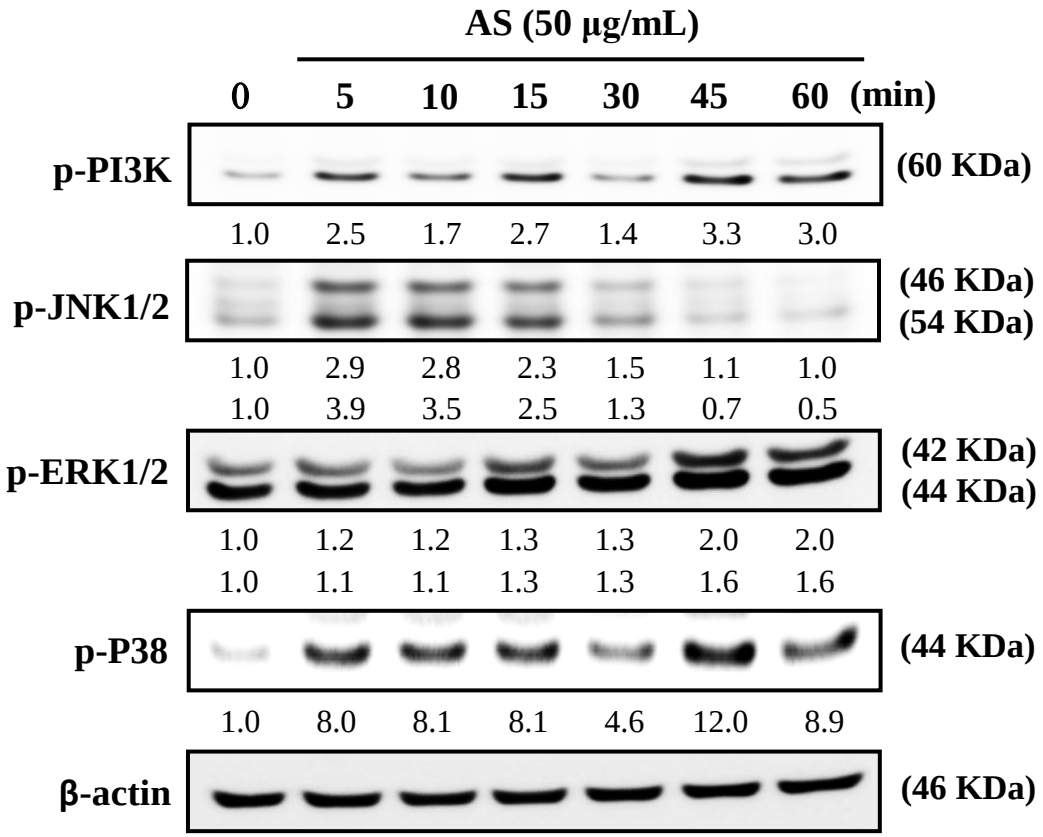


Fig 7

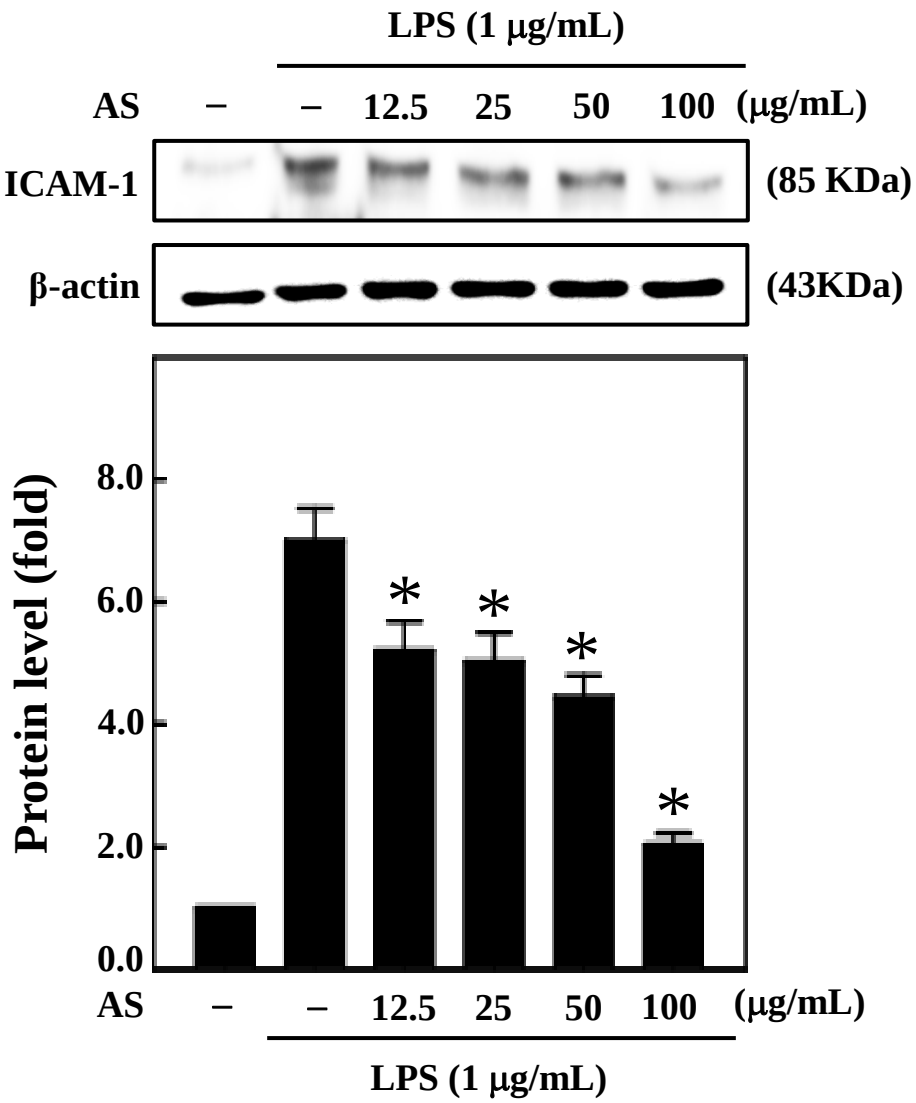


Fig 8