Research Article

Methanol Extract of *Myelophycus caespitosus* Inhibits the Inflammatory Response in Lipopolysaccharide-stimulated BV2 Microglial Cells by Downregulating NF-κB via Inhibition of the Akt Signaling Pathway

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Abstract

Purpose: To determine whether the methanol extract of *Myelophycus caespitosus* (MEMC) downregulates the expression of pro-inflammatory mediators in lipopolysaccharide (LPS)-stimulated BV2 microglial cells.
Methods: Reverse transcription-polymerase chain reaction (RT-PCR) together with Western blot analysis was used to evaluate the expression of pro-inflammatory mediators such as nitric oxide (NO) and prostaglandin E_2 (PGE_2) as well as their regulatory genes such as inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2), in LPS-stimulated BV2 microglial cells. The level of NO production was analyzed using Griess reaction. The release of PGE_2 was determined using sandwich enzyme-linked immunosorbent assay. The DNA-binding activity of nuclear factor-κB (NF-κB) was measured by electrophoretic mobility shift assay.
Results: MEMC inhibited LPS-induced pro-inflammatory mediators, NO and PGE_2, as well as their respective genes, iNOS and COX-2, at both protein and mRNA levels, without any significant cytotoxicity. Treatment with MEMC also substantially reduced the LPS-induced DNA-binding activity of NF-κB and nuclear translocation of NF-κB subunits p65 and p50 via the inhibition of IκBα phosphorylation and degradation. MEMC promoted dephosphorylation of Akt that subsequently suppressed the DNA-binding activity of NF-κB in LPS-stimulated BV2 microglial cells.
Conclusion: Collectively, these data suggest that MEMC attenuates expression of pro-inflammatory mediators such as NO and PGE_2 by suppression of their regulatory genes through the inhibition of Akt-mediated NF-κB activity.

Keywords: *Myelophycus caespitosus, Nitric oxide, Prostaglandin E_2, Nuclear factor-κB.*

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INTRODUCTION

Inflammatory response functions in normal cells to protect against tissue wounds and microbial infections induced by biological, chemical or physical stimuli. However, an aberrant inflammatory response can lead to overexpression of pro-inflammatory mediators such as nitric oxide (NO) and prostaglandin E_2 (PGE_2), which can result in tissue destruction and organ damage [1,2]. Specifically, over-activated microglial cells exert self-cytotoxicity by secreting these pro-inflammatory mediators, and this plays an important role in the pathogenesis of central nervous system diseases such as Parkinson's and Alzheimer's diseases [3]. Therefore, agents that attenuate pro-inflammatory mediators in microglial cells may represent promising strategies for reducing brain injury and neurodegenerative disease. According to a recent study, inhibition of nuclear factor-κB (NF-κB) results in an anti-inflammatory response by downregulation of the expression of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) [4]. Targeting specific cell signaling pathways in the inflammatory process is expected to be a critical aspect in the treatment against inflammation-mediated diseases [5].

Marine algae have been extensively studied as they are a rich source of novel structural compounds that possess biological activity against malignant neoplasms, heart disease, high blood pressure, cerebrovascular disease, senility, diabetes and liver disease [5,6]. In recent reports, we also noted that many seaweed extracts significantly attenuate the expression of lipopoly-saccharide (LPS)-stimulated pro-inflammatory mediators in microglial or macrophage cells and may therefore have the potential for modulating the LPS-induced inflammatory response [7−9]. *Myelophycus caespitosus* has been used as a traditional medicine in Northeastern Asia for treating inflammatory diseases, skin cancer, colon cancer, hematologic malignancy and skin whitening. Nevertheless, no scientific reports have yet investigated the anti-inflammatory properties of *M. caespitosus*.

This study focused on evaluating the effects of the methanol extract of *M. caespitosus* (MEMC) on the expression of NO and PGE_2 and their respective regulatory genes iNOS and COX-2, in LPS-stimulated BV2 microglial cells.

EXPERIMENTAL

Reagents and chemicals

MEMC was purchased from Jeju HI-Tech Industry Development Institute (Jeju, Republic of Korea). LPS and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rabbit anti-human antibodies against iNOS, COX-2, p65, p50, phospho (p)-IκBα, and IκBα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-human antibodies against β-actin, c23, (p)-Akt and Akt were obtained from Cell Signaling (Beverly, MA, USA). Peroxidase-labeled anti-rabbit antibody was purchased from KOMA Biotechnology (Seoul, Republic of Korea) and LY294002 was purchased from Calbiochem (San Diego, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from WelGENE Inc. (Daegu, Republic of Korea). Other chemicals used were purchased from Sigma.

Cell culture and viability assay

BV2 microglial cells were cultured at 37 °C in 5 % CO_2 in DMEM supplemented with 5 % FBS and antibiotics. In all experiments, cells were pre-treated with the indicated concentrations of MEMC 1 h before the addition of LPS (1 µg/ml) in serum-free DMEM. MTT assays were used to determine cell viability.

NO assay

BV2 microglial cells (1 × 10^5 cells/ml) were plated onto 24-well plates and pretreated with

the indicated concentrations of MEMC for 1 h prior to stimulation with 1 µg/ml of LPS for 24 h. The cell supernatants were collected and assayed for NO production using Griess reagent. Briefly, the samples were mixed with equal volume of Griess reagent (1 % sulfanilamide in 5 % phosphoric acid and 0.1 % naphthylethylenediamine dihydrochloride) and then incubated at room temperature for 5 min. The absorbance was measured at 540 nm on a microplate reader (MULTISKAN EX, Thermo Electron Corporation, Marietta, OH, USA). Nitrite concentration was determined from a sodium nitrite standard curve.

Measurement of PGE$_2$

The expression levels of PGE$_2$ were measured by enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, BV2 microglial cells (2×10^5 cells/ml) were plated in 24-well plates and pretreated with the indicated concentrations of MEMC for 1 h prior to stimulation with 1 µg/ml of LPS for 24 h. One hundred microliters of culture media were collected for the determination of PGE$_2$ concentrations by ELISA.

Reverse transcriptase polymerase chain reactions (RT-PCR)

Total RNA was extracted from BV2 microglial cells using Easy-blue reagent (iNtRON Biotechnology, Sungnam, Republic of Korea) according to the manufacturer's instruction. One microgram of RNA was reverse-transcribed using MMLV reverse transcriptase (Bioneer, Daejeon, Republic of Korea). Then, cDNA was amplified by PCR using specific primer iNOS (forward 5'-cct cct cca ccc tac caa gt-3' and reverse 5'-cac cca aag tgcttc agt ca-3'), COX- 2 (forward 5'-aag act tgc cag gct gaa ct-3' and reverse 5'-ctt ctg cag tcc agg ttc aa-3') and β-actin (forward 5'-tgt gat ggt ggg aat ggg tca g-3' and reverse 5'-ttt gat gtc acg cac gat ttc c-3'). The following PCR conditions were applied: COX-

2 and iNOS, 25 cycles of denaturation at 94 ºC for 30 s, annealing at 59 ºC for 30 s and extended at 72 ºC for 30 s; β-actin, 23 cycles of denaturation at 94ºC for 30 s, annealing at 57 ºC for 30 s and extended at 72 ºC for 30 s. β-actin was used as an internal control to evaluate relative expression of COX-2 and iNOS.

Western blot analysis

Total cell extracts were prepared using PRO-PREP protein extraction solution (iNtRON Biotechnology). The preparation of cytosolic and nuclear extracts was conducted using NE-PER nuclear and cytosolic extraction reagents (Pierce, Rockford, IL, USA). Cell extracts were separated on polyacrylamide gels and then standard procedures were used to transfer them to the nitrocellulose membranes. The membranes were developed using an ECL reagent (Amersham, Arlington Heights, IL, USA).

Electrophoretic mobility shift assay (EMSA)

DNA-protein binding assays were carried out with nuclear extract. Synthetic complement-tary NF-κB binding oligonucleotides (5'-AGT TGA GGG GAC TTT CCC AGG C-3') were 3'-biotinylated using the biotin 3'-end DNA labeling kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Assays were performed using a Lightshift EMSA Optimization kit (Pierce) according to the manufacturer's protocol.

Statistical analysis

All data were derived from at least three independent experiments. Statistical analyses were conducted using SigmaPlot software (version 11.0) Values were presented as mean ± SE. Significant differences between the groups were determined using one-way ANOVA, with statistical significance set at $p < 0.05$.

RESULTS

MEMC is not cytotoxic to BV2 microglial cells

To evaluate the effects of MEMC on the viability of BV2 microglial cells, we incubated the cells with various concentrations of MEMC (0, 2.5, 5.0, 7.5 and 10.0 μg/ml) for 24 h with or without incubation with LPS. Cell viability was assessed by the MTT assay and showed that MEMC did not exert significant cytotoxicity at all concentrations tested (Fig 1). Solvent control (0.1 % DMSO) or LPS alone did not exert significant cytotoxicity, but a high concentration of MEMC of 20.0 μg/ml substantially induced cell death (data not shown). Therefore, concentrations of MEMC ranging from 0.0 to 10.0 μg/ml were selected for further studies.
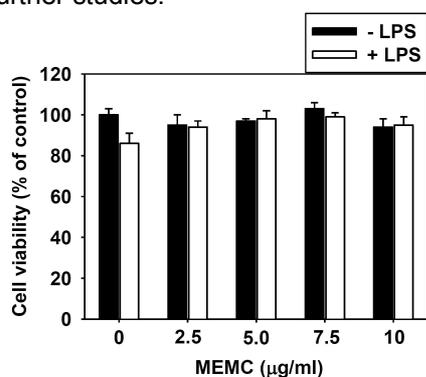
Fig 1: Effect of MEMC on BV2 microglial cell viability. Cells (1 × 10^5 cells/ml) were incubated with the indicated concentrations of MEMC 1 h before LPS (1.0 μg/ml) treatment for 24 h. Data are presented as mean ± SE, n = 3; *$p < 0.05$.

MEMC suppresses NO and PGE$_2$ production

To confirm the anti-inflammatory effects of MEMC in LPS-stimulated BV2 microglial cells, we investigated the production of NO using the Griess reagent assay. Fig 2A shows that LPS significantly increases NO production to 14.1 ± 1.2 μM; however, pretreatment with MEMC decreased LPS-induced NO production to 10.4 ± 0.8 μM, 7.8 ± 0.7 μM, 6.2 ± 0.8 μM and 4.1 ± 0.3 μM in a dose-dependent manner. In particular, the

highest concentration of MEMC reduced the NO levels comparable to that of the control group (3.2 ± 0.3 μM). In parallel, ELISA was performed to assess the effect of MEMC on LPS-stimulated secretion of PGE$_2$ in BV2 microglial cells. Similar to NO, MEMC significantly attenuated LPS-mediated PGE$_2$ production in a dose-dependent manner (Fig 2B). Following LPS stimulation, PGE$_2$ levels reached 1324 ± 35 pg/ml. However, a high concentration of MEMC (10 μg/ml) completely reduced the PGE$_2$ levels to 421 ± 12 pg/ml, comparable to the levels of the control group (311 ± 21 pg/ml). These data suggest that MEMC suppresses pro-inflammatory mediators such as NO and PGE$_2$ in LPS-stimulated BV2 microglial cells.
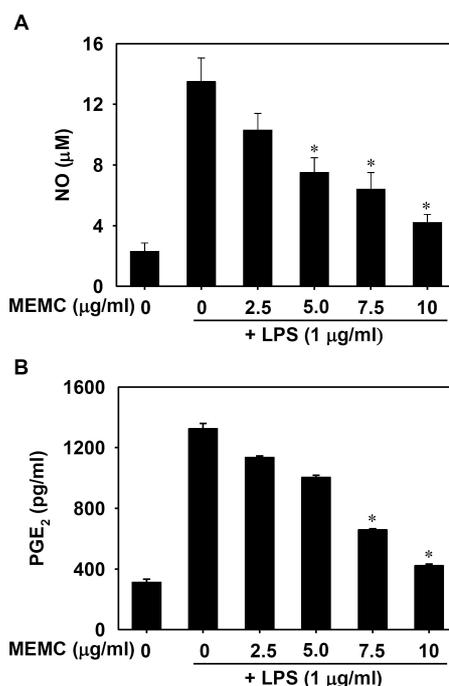
Fig 2: Effect of MEMC on LPS-induced NO (A) and PGE$_2$ (B) production in BV2 microglial cells; Cells (1 × 10^5 cells/ml) were treated with the indicated concentrations of MEMC 1 h before LPS (1.0 μg/ml) treatment for 24 h. (A) The levels of NO were determined using Griess reagent and a standard curve was constructed using NaNO$_2$ in culture medium. (B) The levels of PGE$_2$ in the media were detected using a specific enzyme-linked immunosorbent assay. Data are presented as mean ± SE, n = 3; *$p < 0.05$.

MEMC attenuates iNOS and COX-2 expression

To investigate the expressional mechanism of pro-inflammatory mediators such as NO and PGE_2 in LPS-induced BV2 microglial cells, we measured the expression of their respective regulatory genes, iNOS and COX-2, both the protein and mRNA level using western blot analysis and RT-PCR. LPS treatment increased the expression of iNOS and COX-2 (Fig 3A) as well as their mRNA (Fig 3B). However, MEMC treatment significantly inhibited LPS-induced iNOS and COX-2 expression at both the mRNA and protein level. MEMC alone had no effect on the expression of these proteins and genes. These data indicate that MEMC regulates the transcription of iNOS and COX-2 in a LPS-dependent manner.

A

| – | + | – | + | MEMC (10 μg/ml) |
| – | – | + | + | LPS (1 μg/ml) |

◄ iNOS
◄ COX-2
◄ β-actin

B

| – | + | – | + | MEMC (10 μg/ml) |
| – | – | + | + | LPS (1 μg/ml) |
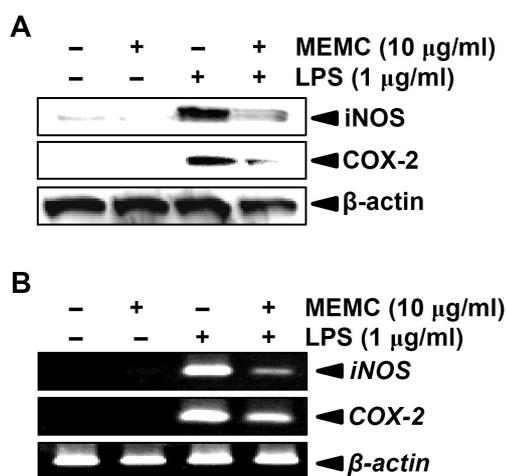
◄ *iNOS*
◄ *COX-2*
◄ *β-actin*

Fig 3: Effect of MEMC on LPS-induced iNOS and COX-2 protein (A) and mRNA (B) expression in BV2 microglial cells. Cells (1×10^5 cells/ml) were incubated with MEMC (10 μg/ml) 1 h before LPS (1.0 μg/ml) treatment for 6 h. (A) Cell lysates were resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with antibodies against iNOS or COX-2. (B) Total RNA was isolated and RT-PCR analyses of *iNOS* and *COX-2* were performed. *Note:* β-Actin was used as an internal control for the RT-PCR and Western blot analysis.

MEMC modulates LPS-stimulated NF-κB activity

NF-κB activation is essential to transactivate LPS-induced iNOS and COX-2 gene expression [10]. We measured the DNA-binding activity of NF-κB and the nuclear translocation of NF-κB subunits p65 and p50 as well as IκB degradation and phosphorylation. Treatment with LPS significantly increased DNA-binding activity of NF-κB as measured by the EMSA (Fig 4A). However, pretreatment with MEMC completely reduced LPS-induced DNA-binding activity of NF-κB. In a parallel experiment, we found that LPS reduces the expression of p65 and p50 in the cytosolic fraction, and this correlated with an increase in phosphorylated IκB as measured by western blot analysis (Fig 4B). However, prior treatment with MEMC maintained stable p65 and p50 levels in the cytosolic fractions and decreased the amount of phosphorylated IκB. In addition, we found that LPS treatment induced the nuclear translocation of p65 and p50; however, pretreatment with MEMC resulted in a decrease of these NF-κB subunits in the nuclear fraction (Fig 4C). These data indicate that MEMC inhibits NF-κB activity by suppressing IκB-mediated nuclear translocation of NF-κB.

MEMC inhibits LPS-stimulated NF-κB activity through suppression of Akt phosphorylation

The Akt signaling pathway has been shown to regulate pro-inflammatory genes such as *iNOS* and *COX-2* through activation of NF-κB [11]. Therefore, we examined the effect of MEMC on Akt phosphorylation in LPS-stimulated BV2 microglial cells using western blot analysis. Phosphorylated Akt was significantly increased in LPS-stimulated BV2 microglia cells; however, MEMC treatment produced an inhibition of Akt phosphorylation (Fig 5A).
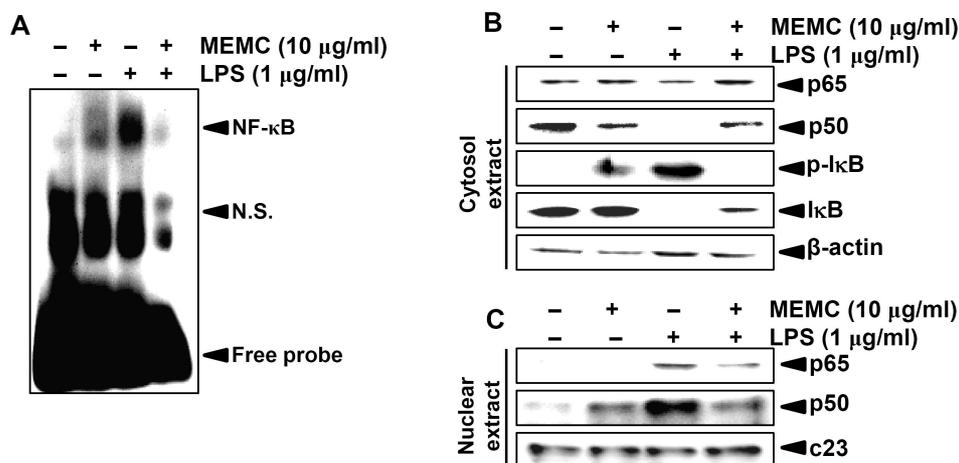
Fig 4: Effect of MEMC on NF-κB activity in LPS-stimulated BV2 microglial cells. Cells were pretreated with MEMC (10 µg/ml) 1 h before stimulation with LPS (1.0 µg/ml) for 30 min. (A) The nuclear extracts were assayed for NF-κB activity by EMSA. The cytosolic (B) and nuclear (C) extracts were prepared to determine the levels of p65, p50, phosphor-IκBα and IκBα by western blot analysis. ***Note:*** β-Actin and c23 were used as an internal control for Western blot analysis. N.S. = non-specific.
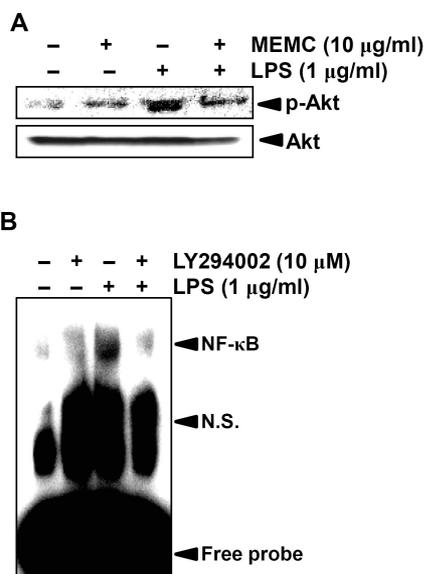
Fig 5: Effect of MEMC on Akt phosphorylation in LPS-stimulated BV2 microglial cells. (A) Cells were pretreated with MEMC (10 µg/ml) 1 h before stimulation with LPS (1.0 µg/ml) for 30 min. Western blot analysis was performed to determine Akt phosphorylation. (B) Cells were treated with LY294002 (20 µM) 1 h before LPS (1.0 µg/ml) treatment for 30 min. EMSA measured NF-κB binding to its DNA promoter region in the nuclear protein extract. N.S. = non-specific.

To verify that Akt activity is directly involved in NF-κB activation, we investigated the effect of the Akt inhibitor LY294002 on LPS-stimulated NF-κB DNA-binding activity. We found that treatment with LY294002 substantially reduced NF-κB-DNA binding in nuclear extracts (Fig 5B). These data indicate that MEMC inhibits LPS-induced NF-κB activation by inhibiting Akt phosphorylation

DISCUSSION

Several studies have reported that MEMC possesses anti-allergic and anti-cancer activities [12,13]. Nevertheless, there have been no reports on the effect of MEMC in LPS-stimulated inflammatory response. Therefore, we studied the anti-inflammatory properties of MEMC in LPS-stimulated BV2 microglial cells. We first found that MEMC attenuates NO and PGE$_2$ production and the expression of their respective regulatory genes, *iNOS* and *COX-2*, at both the mRNA and protein levels. In addition, we presented data that suggest that MEMC inhibits iNOS and COX-2 expression by suppressing NF-κB activity through the inhibition of Akt phosphorylation.

Microglias are glial cells that function in the central nervous system's immune response against bacterial infection [14]. Microglias represent 20% of the total glial cell population in the mature brain [15]. When stimulated with LPS, microglias release NO and PGE$_2$, which mediate traumatic brain injury following cerebral stroke [16]. Moreover, there is extensive evidence indicating that neural damage is absent in iNOS-deficient mice [17] and that inhibition of COX-2 expression after stroke *in vivo* exerts neuroprotective effects [18]. Therefore, the inhibition of pro-inflammatory mediators such as NO and PGE$_2$ is a potential therapeutic approach against neurodegenerative diseases. Our data demonstrated that MEMC significantly attenuates the expression of iNOS and COX-2 as well as their respective gene products in LPS-stimulated BV2 microglial cells. We hypothesize that MEMC exerts neuro-protective effects by inhibiting LPS-induced inflammatory responses.

NF-κB is known to play an important role in the regulation of pro-inflammatory genes such as *iNOS* and *COX-2* [4]. Therefore, targeting the NF-κB pathway represents a potential strategy to treat and prevent inflammatory diseases such as asthma and nervous disorders [19]. In the present study, we found that MEMC inhibits NF-κB activity by suppressing nuclear translocation of NF-κB subunits p65 and p50 via the inhibition of IκB phosphorylation and degradation. To further explore NF-κB regulation, we investigated the relationship between Akt dephosphorylation and regulation of NF-κB activity, as it has been reported that many anti-inflammatory mediators are modulated by the Akt-dependent NF-κB signaling pathway [11]. In this study, MEMC significantly inhibited Akt phosphorylation, which suppresses NF-κB activation. These data demonstrate the potential of MEMC as a pharmaceutical agent for LPS-induced inflammatory disease. Nevertheless, recent studies showed that mitogen-activated protein kinases (MAPKs) and reactive oxygen species (ROS) are effector molecules that

regulate NF-κB-dependent inflammatory reactions [20,21], although the role of ROS in NF-κB regulation remains controversial [22]. In addition, the transcription factor, AP-1, is also involved in the regulation of inflammation-associated gene expression in LPS-stimulated alveolar macrophages [23]. Therefore, further studies are required to elucidate the role of AP-1 and MAPKs in LPS-induced inflammatory responses.

CONCLUSION

Our data demonstrate that MEMC inhibits anti-inflammatory mediators such as NO and PGE$_2$, and their respective regulatory genes *iNOS* and *COX-2* in LPS-stimulated BV2 microglial cells. This anti-inflammatory effect results from the inhibition of Akt phosphorylation and the subsequent attenuation of NF-κB activation.

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COMPETING INTERESTS

The authors report no conflict of interest and they alone are responsible for the contents of this work.

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