Journal of Pharmacological Sciences 134 (2017) 116–123

Contents lists available at ScienceDirect

Journal of Pharmacological Sciences

journal homepage: www.elsevier.com/locate/jphs

Full paper

Effects of hypnotic bromovalerylurea on microglial BV2 cells

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ARTICLE INFO

Article history:
Received 24 February 2017
Received in revised form
25 May 2017
Accepted 29 May 2017
Available online 8 June 2017

Keywords:
NO
ATP
Microglia
Macrophage
Rotenone

ABSTRACT

An old sedative and hypnotic bromovalerylurea (BU) has anti-inflammatory effects. BU suppressed nitric oxide (NO) release and proinflammatory cytokine expression by lipopolysaccharide (LPS)-treated BV2 cells, a murine microglial cell line. However, BU did not inhibit LPS-induced nuclear translocation of nuclear factor-κB and subsequent transcription. BU suppressed LPS-induced phosphorylation of signal transducer and activator of transcription 1 (STAT1) and expression of interferon regulatory factor 1 (IRF1). The Janus kinase 1 (JAK1) inhibitor filgotinib suppressed the NO release much more weakly than that of BU, although filgotinib almost completely prevented LPS-induced STAT1 phosphorylation. Knockdown of JAK1, STAT1, or IRF1 did not affect the suppressive effects of BU on LPS-induced NO release by BV2 cells. A combination of BU and filgotinib synergistically suppressed the NO release. The mitochondrial complex I inhibitor rotenone, which did not prevent STAT1 phosphorylation or IRF1 expression, suppressed proinflammatory mediator expression less significantly than BU. BU and rotenone reduced intracellular ATP (iATP) levels to a similar extent. A combination of rotenone and filgotinib suppressed NO release by LPS-treated BV2 cells as strongly as BU. These results suggest that anti-inflammatory actions of BU may be attributable to the synergism of inhibition of JAK1/STAT1-dependent pathways and reduction in iATP level.

1. Introduction

Macrophages are the main player in a wide range of inflammatory diseases through various mechanisms. Microglial cells in the brain,[1] which have characteristic ramified morphology and similar natures to macrophages, may also play a pivotal role in health and disease. Once inflammatory reactions occur in tissues or organs, macrophages and microglial cells become activated and profoundly involved in disease pathogenesis, mainly through their proinflammatory functions. Appropriate controls for proinflammatory reactions by activated macrophages and related cells are necessary for the better outcome of inflammatory diseases.

Bromovalerylurea (BU; $C_6H_{11}BrN_2O_2$, CAS: 496-67-3) was developed as a sedative and hypnotic more than 100 years ago.[2] We have recently found that BU markedly suppresses lipopolysaccharide (LPS)-induced proinflammatory reactions of macrophages[3] and microglial cells.[4] BU ameliorates rat sepsis models improving survival of the animals, and suppresses the expression of proinflammatory mediators by macrophages. BU inhibits neuronal death in LPS-treated neuron-microglia coculture by suppressing nitric oxide (NO) release by microglial cells.[4] BU

Abbreviations: ALI, acute lung injury; AMs, alveolar macrophages; BU, bromovalerylurea; JNK, c-Jun N-terminal kinases; cDNA, complementary DNA; Dex, dexamethasone; DMEM, Dulbecco's Modified Eagle Medium; *E. coli*, *Escherichia coli*; ERK, extracellular signal-regulated kinase; LPS, lipopolysaccharide; iNOS, inducible NO synthase; IκB, inhibitor κB; IRF1, interferon regulatory factor 1; IL-1β, interleukin-1β; iATP, intracellular ATP; JAK1, Janus kinase 1; MSK1, mitogen- and stress-activated protein kinase 1; NO, nitric oxide; NRE, NFκB-responsive element; NFκB, nuclear factor-κB; p38, p38 mitogen activated protein kinase; PBS, phosphate buffered saline; PMs, peritoneal macrophages; qPCR, quantitative real-time RT-PCR; STAT1, signal transducer and activator of transcription 1; siRNA, small interfering RNA.

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Peer review under responsibility of Japanese Pharmacological Society.

http://dx.doi.org/10.1016/j.jphs.2017.05.007

prevents dopaminergic neuronal loss in the substantia nigra pars compacta in a rat Parkinson's disease model, resulting in amelioration of motor deficits.

The mechanisms underlying the anti-inflammatory effects of BU have yet to be elucidated. In our previous studies, we have shown that BU suppresses LPS- or interferon-γ-induced phosphorylation of signal transducer and activator of transcription 1 (STAT1) and interferon regulatory factors (IRFs) 1 and 8.[3,4] Furthermore, we have observed that BU suppresses LPS-induced phosphorylation of Janus kinase 1 (JAK1). However, it is not yet clarified whether the JAK1/STAT1/IRFs-dependent signaling pathway is the sole target of BU. To elucidate the mechanisms for the anti-inflammatory effects of BU in this study, we used the murine microglial cell line BV2.

2. Materials and methods

2.1. Agents

BU was purchased from Wako (Osaka, Japan). Inhibitors for p38 mitogen activated protein kinase (p38) (SB203580), mitogen- and stress-activated protein kinase 1 (MSK1) (SB747651A), and JAK1 (GLPG0634 or filgotinib) were from Cell Signaling (Danvers, MA), R&D Systems (Minneapolis, MN), and Selleckchem (Houston, TX), respectively. Dexamethasone (Dex) and rotenone were from Sigma–Aldrich (St. Louis, MO). Meropenem was from Sumitomo Dainippon Pharma (Osaka, Japan). LPS (from *Escherichia coli* (*E. coli*) serotype 055:B5) was from Sigma–Aldrich.

2.2. BV2 cell culture

Murine microglial cell line BV2 was kindly provided by Prof. S. Yoshida (Asahikawa Medical University). BV2 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. BV2 cells were seeded onto wells in 4-well culture plates and incubated with LPS for 30 min to 24 h. When the effects of BU or other agents were investigated, BV2 cells were incubated with the appropriate agent for 30 min before the addition of LPS.

2.3. Quantitative real-time RT-PCR (qPCR)

BV2 cells were incubated either alone or with LPS (1 μg/ml) for 60 or 180 min in the presence or absence of BU or rotenone. After incubation, complementary DNA (cDNA) was prepared and qPCR analysis was performed as described elsewhere.[5] Primer sequences for each gene are listed in Supplementary Table 1.

2.4. Assay for NO release

Conditioned media were obtained from BV2 cell cultures that had been incubated for 24 h in E2 medium [serum-free DMEM containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.3; Gibco, Grand Island, NY, USA), 4.5 mg/mL glucose, 5 μg/mL insulin, 5 nM sodium selenite, 5 μg/mL transferrin (Gibco) and 0.2 mg/mL bovine serum albumin (Sigma–Aldrich)] containing 1 μg/ml LPS, with or without BU (1–100 μg/ml) or other agents, and subjected to NO determination based on the Griess reaction.[6] To normalize the released NO level by the cellular protein contents, cells were solubilized with RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM sodium chloride, 0.5% w/v sodium deoxycholate, 0.1% w/v sodium dodecyl sulfate, 1.0% w/v NP-40 substitute) and the protein contents were determined by Pierce BCA protein assay reagents (Thermo Scientific, Rockford, IL).

2.5. Immunoblotting

BV2 cells were preincubated with BU, enzyme inhibitors or rotenone for 30 min, followed by the addition of LPS for 30 or 180 min. After incubation, cell lysates were prepared using Laemmli's sample buffer containing phosphatase inhibitor cocktail solution II (Wako). The lysates were then subjected to immunoblotting as described elsewhere.[7] Immunoreactive bands were analyzed by densitometry using ImageJ 1.43u (Wayne Rasband, National Institutes of Health, Bethesda, MD). The densitometry data were standardized to the internal standard, β-actin. Antibodies are listed in Supplementary Table 2.

2.6. Preparation of nuclear fraction and determination of nuclear factor-κB (NFκB) translocation into nuclei

Nuclear fractions of BV2 cells were prepared using Nuclear Extract kit (Active Motif, Tokyo, Japan), after incubation with LPS with or without BU for 150 min. The fractions were subjected to immunoblotting for detection of NFκB (p65) and histone H2A protein to determine whether NFκB was translocated into nuclei.

2.7. Luciferase reporter assay

NFκB-responsive element (NRE) (5′-GGG AAT TTC CGG GGA CTT TCC GGG AAT TTC CGG GGA CTT TCC GGG AAT TTC C-3′) was subcloned into the MluI–Bgl II site of a PGV-P2 vector (PGV-P2-NRE; TOYO INK, Tokyo, Japan), and transfected into BV2 cells together with a pCX4pur vector, which carried a puromycin-resistant gene in one-tenth the amount of PGV-P2-NRE. Stable cell lines of BV2-PGV-P2-NRE and negative control BV2-PGV-P2 transfected cells were established by puromycin selection. Cells were stimulated with LPS with or without BU for 24 h, and harvested in luciferase lysis buffer. Luciferase activities were measured according to the manufacturer's instructions (Pikka-gene, TOYO INK) using FlexStation (Molecular Devices, Sunnyvale, CA).

2.8. Small interfering RNA (siRNA)-mediated gene knockdown

siRNA-mediated knockdown was done in BV2 cells and peritoneal macrophages (PMs). The siRNA sequences targeting JAK1, STAT1 and IRF1 were as follows: JAK1, 5′- CCA UCA UGA GGG ACA UUA A - 3′; STAT1, 5′- CUA AGA GCC CGA CCC UAU U -3′; IRF1, 5′- GGC AUA UGC AGA UGG ACA U -3′ (Sigma–Aldrich Japan, Tokyo, Japan). BV2 cells were transfected with 20 nM of the siRNA duplexes using Viromer Blue (lipocalyx, Halle, Germany) as manufacture's instruction. Cells were incubated for 24 h in the growth medium, and then subjected to analyses of LPS-induced inflammatory responses. As a control, a siRNA duplex with an irrelevant sequence (Dharmacon Research, Pittsburgh, PA, USA) was used. PMs were isolated from male Wistar rats (8–9 weeks old; mean body weight, 300 g) by peritoneal lavage with 20 ml phosphate buffered saline (PBS) as described previously.[3] Protein knockdown in the PMs was performed as described elsewhere.[5]

2.9. WST1 assay

The effects of BU and rotenone on cellular metabolic rates were evaluated using WST1 (TAKARA BIO, Kusatsu, Japan), a stable tetrazolium salt, which is reduced by nicotinamide adenine dinucleotide forming a soluble formazan. BV2 cells were seeded into 96-well plates at 10,000 cells/well. The cells were pre-incubated for 30 min alone, or with BU or rotenone, followed by the addition of LPS and WST-1. The absorbance of the formazan

product was measured at 450 nm, with the reference wavelength at 600 nm. The metabolic activity of cells was expressed by $\Delta OD = OD_{450} - OD_{600}$.

2.10. ATP assay

Intracellular ATP (iATP) contents were measured using a kit (AMERIC-ATP kit, Wako) based on luciferase activity. BV2 cells were seeded on 6 cm dishes and were preincubated for 30 min alone, or with BU or rotenone, followed by incubation with LPS for 2 h in the presence of each agent. The cells were lysed with ATP-extraction solution provided in the kit, and ATP contents were measured according to the manufacturer's instructions.

2.11. Acute lung injury (ALI) model

All animal experiments were carried out in accordance with the Guidelines for Animal Experimentation of Ehime University Graduate School of Medicine and were approved by Ehime University animal experiment committee. The ALI model was prepared as described previously.[8] *E. coli* (*E. coli*; XL1-Blue MR) were cultured in 250 ml brain–heart broth (Thermo Fisher Scientific, Yokohama, Japan). The turbidity of the *E. coli* suspension in saline was adjusted to 1.0 optical density. Wistar rats (8–9 weeks old) were anesthetized, a 16-gauge venous catheter inserted into the trachea, and 0.2 ml of the *E. coli* suspension instilled. Rats were divided into four groups; control (no treatment; n = 12), the antibiotic meropenem alone (n = 10; 30 mg/kg meropenem in saline), meropenem + BU (n = 10; 30 mg/kg; 1% solution in propylene glycol; Wako, Osaka, Japan) and meropenem + Dex (n = 10; 0.5 mg/kg; 0.05% solution in propylene glycol). Shortly after instilling bacteria, the drugs were subcutaneously injected once. The survival of animals was monitored every 12 h for 48 h after the instilling of bacteria.

2.12. Preparation of alveolar macrophages (AMs)

AMs were harvested by bronchoalveolar lavage with 10 ml ethylenediaminetetraacetic acid (0.05% w/v) in PBS as described previously.[3] AMs were activated by stimulation with 1 μg/ml LPS (from *E. coli* serotype 055:B5; Sigma–Aldrich, St Louis, MO, USA) in E2 medium.

2.13. Evaluation of the binding of NFκB to NRE by enzyme-linked immuno-sorbent assay (ELISA)

Nuclear extracts were prepared using the Nuclear Extract kit (Active Motif) from BV2 cells that had been incubated with LPS, with or without BU or Dex, for 150 min. Subsequently NFκB DNA binding activity of the extracts was determined using the ELISA-based TransAM NFκB p65 Activation Assay (Active Motif) according to manufacturer's protocol and as described elsewhere.[9]

2.14. Statistical analysis

Numerical data were statistically analyzed using InStat3 software (GraphPad Software, La Jolla, CA). Statistical significance was assessed with one-way ANOVA and Tukey's *post hoc* test. The log-rank test was employed for evaluating the survival rate of ALI model rats. Differences between groups with *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. BU does not affect LPS-induced NFκB translocation into nuclei and activation

Subcutaneously administered BU prevented animal death from the acute lung injury (ALI) model in rats (Supplementary Fig. 1A). A

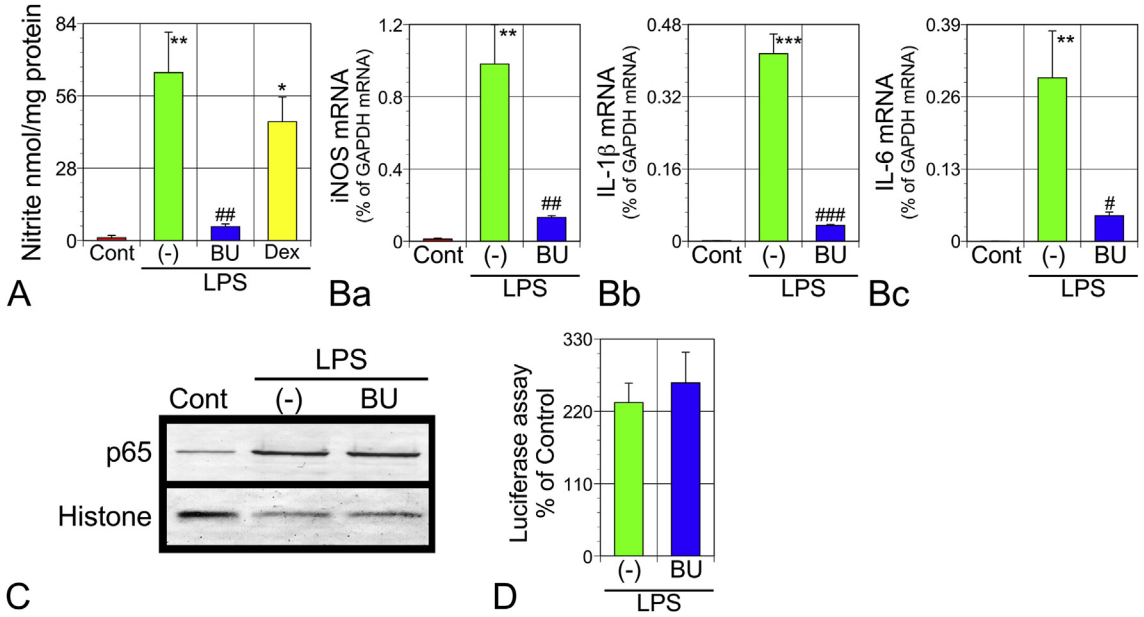

Fig. 1. Antiinflammatory effects of BU on BV2 cells are not attributable to inhibition of NFκB. (A) BV2 cells were incubated with LPS for 24 h in the presence of none, BU (100 μg/ml), or Dex (100 nM), then the supernatants were subjected to nitrite determination. BU but not Dex markedly suppressed LPS-induced NO release. (B) BV2 cells were incubated with LPS for 180 min and iNOS (Ba), IL-1β (Bb) and IL-6 (Bc) mRNA levels were evaluated by qPCR. (C) Nuclear fractions of LPS-treated BV2 cells in the presence or absence of BU were subjected to immunoblotting. Histone H2A protein was detected to show that the nuclear fraction was properly obtained. p65 was densely found in the nuclear fractions after LPS treatment regardless of the addition of BU. A representative result is shown. (D) Luciferase reporter assay shows that LPS treatment increased the NFκB-mediated transcription by more than twice compared with the basal condition. BU did not affect the LPS-stimulated transcription. Data (n = 4) in A, B and D are expressed as means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 vs Control (Cont); #P < 0.05, ##P < 0.01, ###P < 0.001 vs LPS alone (LPS (−)).

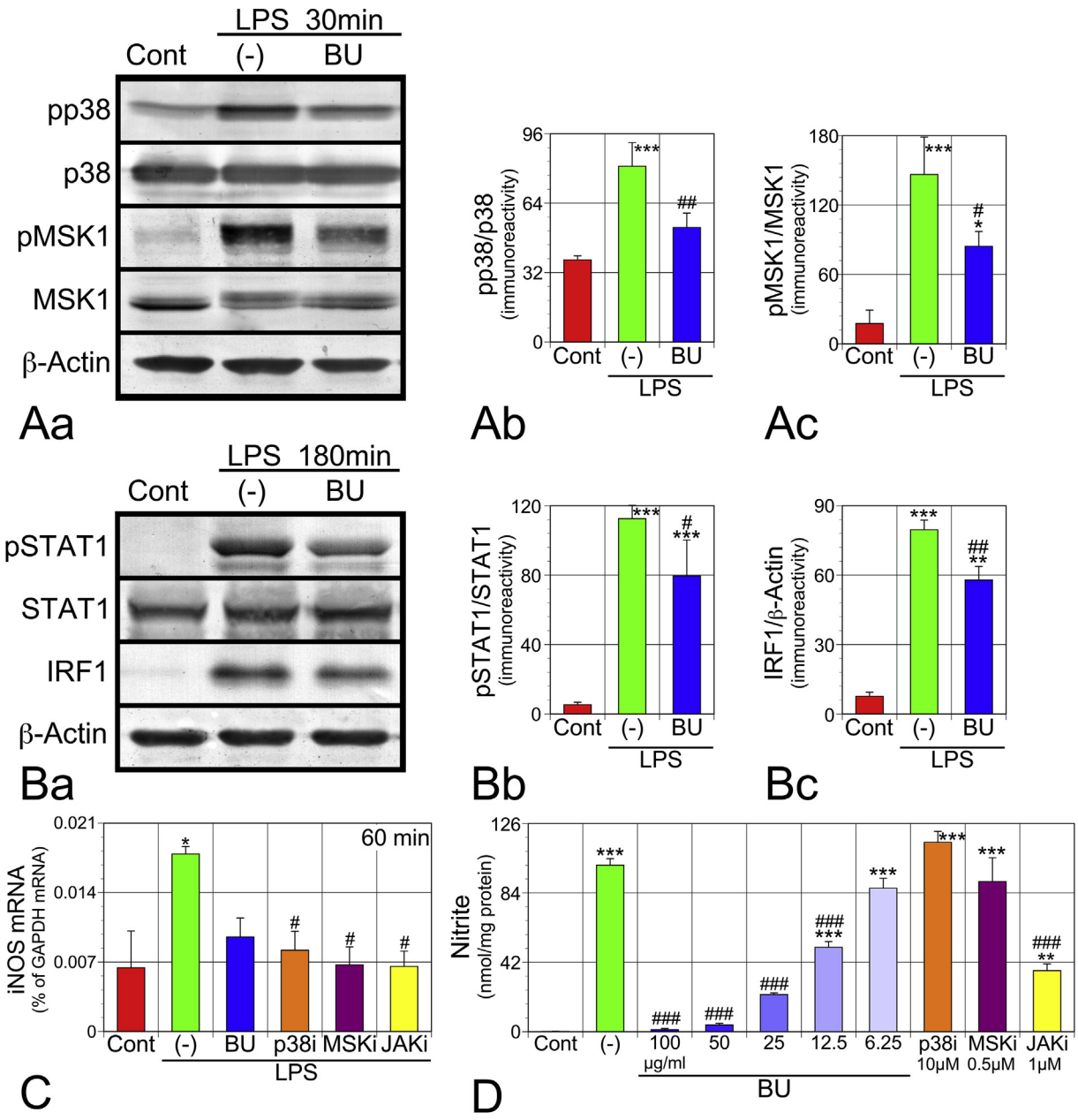

Fig. 2. Suppressive effects of BU on p38 and JAK1-mediated signaling pathway and LPS-induced NO release. (A) BV2 cells were preincubated with BU or vehicle for 30 min, then incubated with LPS with BU or vehicle for 30 min, and phosphorylation of p38 (Ab) and MSK1 (Ac) was examined by immunoblotting. BU inhibited phosphorylation of both enzymes. (B) STAT1 phosphorylation (Bb) and upregulated expression of IRF1 (Bc) were marked 180 min after addition of LPS. BU weakly but significantly suppressed the changes. Densitometric data (n = 4) in A and B are shown as mean ± SEM. (C) A significant increase in iNOS mRNA expression was observed 60 min after LPS addition. Inhibitors for p38 (10 μM), MSK1 (0.5 μM) and filgotinib (1 μM), but not BU (100 μg/ml), significantly inhibited the increase. (D) BU suppressed NO release in a dose-dependent manner. Filgotinib at 1 μM but not the inhibitors for p38 and MSK1 significantly suppressed the release. Data (n = 4) in C and D are expressed as mean ± SEM. **P < 0.01, ***P < 0.001 vs Control; #P < 0.05, ##P < 0.01, ###P < 0.001 vs LPS alone (LPS(−)).

synthetic glucocorticoid Dex in conjunction with the ultra-broad-spectrum antibiotic meropenem slightly prevented death. However, when BU was administered with the antibiotic, death was completely prevented. Isolated rat AMs were treated with LPS for 24 h and the released NO measured as nitrite (Supplementary Fig. 1B). BU and Dex suppressed NO release to a similar extent. BU did not inhibit LPS-induced NFκB binding to NRE, as revealed by an ELISA-based assay with nuclear extracts of AMs[9] (Supplementary Fig. 1C).

Distinct from the effects on AMs, BU (100 μg/ml, ~450 μM) but not Dex (100 nM) suppressed LPS-induced NO release by BV2 cells (Fig. 1A). The concentrations ~25–130 μg/ml of BU has been reported in blood of BU-intoxicated humans, who were unconscious as well as conscious.[10] Furthermore, 100 μg/ml of BU does not affect the viability of primary cultured rat neurons and microglial cells.[4] BU suppressed mRNA expression for inducible NO synthase (iNOS), interleukin-1β (IL-1β) and IL-6, 180 min after LPS was added to BV2 cell cultures (Fig. 1B), suggesting that BU suppresses the LPS-induced proinflammatory activation of BV2 cells at the transcription level. To determine whether BU suppresses translocation of NFκB into nuclei, a nuclear fraction was prepared after addition of LPS, with or without BU, to BV2 cell cultures (Fig. 1C). BU did not

inhibit LPS-induced translocation of p65, a component of NFκB, consistent with a previous report.[4] The luciferase reporter assay showed that BU did not affect the NFκB-mediated transcription (Fig. 1D).

3.2. BU suppressed LPS-induced phosphorylation of p38, MSK1, and STAT1

LPS treatment increased phosphorylation of p38 (SB203580), MSK1 in BV2 cells within 30 min of LPS addition; BU administration partially suppressed the increased phosphorylation (Fig. 2A). LPS increased STAT1 phosphorylation and IRF1 expression 180 min after LPS addition; BU weakly inhibited the LPS-induced changes (Fig. 2B). Inducible NO synthase (iNOS) mRNA expression was slightly increased as early as 60 min after addition of LPS (Fig. 2C). Inhibitors for p38 (SB203580; 10 μM), MSK1 (SB747651A; 0.5 μM) and JAK1 (GLPG0634 or filgotinib; 1 μM) weakly suppressed iNOS mRNA expression. BU at 12.5–100 μg/ml inhibited the LPS-induced NO release in a concentration-dependent manner, whereas inhibitors for p38 and MSK1 did not inhibit the NO release (Fig. 2D). Filgotinib (1 μM) suppressed NO release, consistent with its reported anti-inflammatory effects.[11] Additionally, 100 μM of p38, 5 μM of MSK1 inhibitors and 10 μM of filgotinib caused marked BV2 cell degeneration (data not shown).

3.3. STAT1 phosphorylation and the inhibitory effects of BU

BU at 50 μg/ml and lower concentrations did not significantly suppress STAT1 phosphorylation (Fig. 3A). The combination of BU (12.5 μg/ml) and filgotinib (1 μM) showed an additive inhibitory effect on NO release by BV2 cells (Fig. 3B), whereas filgotinib almost completely inhibited STAT1 phosphorylation and the addition of BU did not show any additive effect on the phosphorylation (Fig. 3C). The findings suggest that BU may exert its anti-inflammatory effects on BV2 cells mainly through mechanisms other than inhibition of STAT1 phosphorylation.

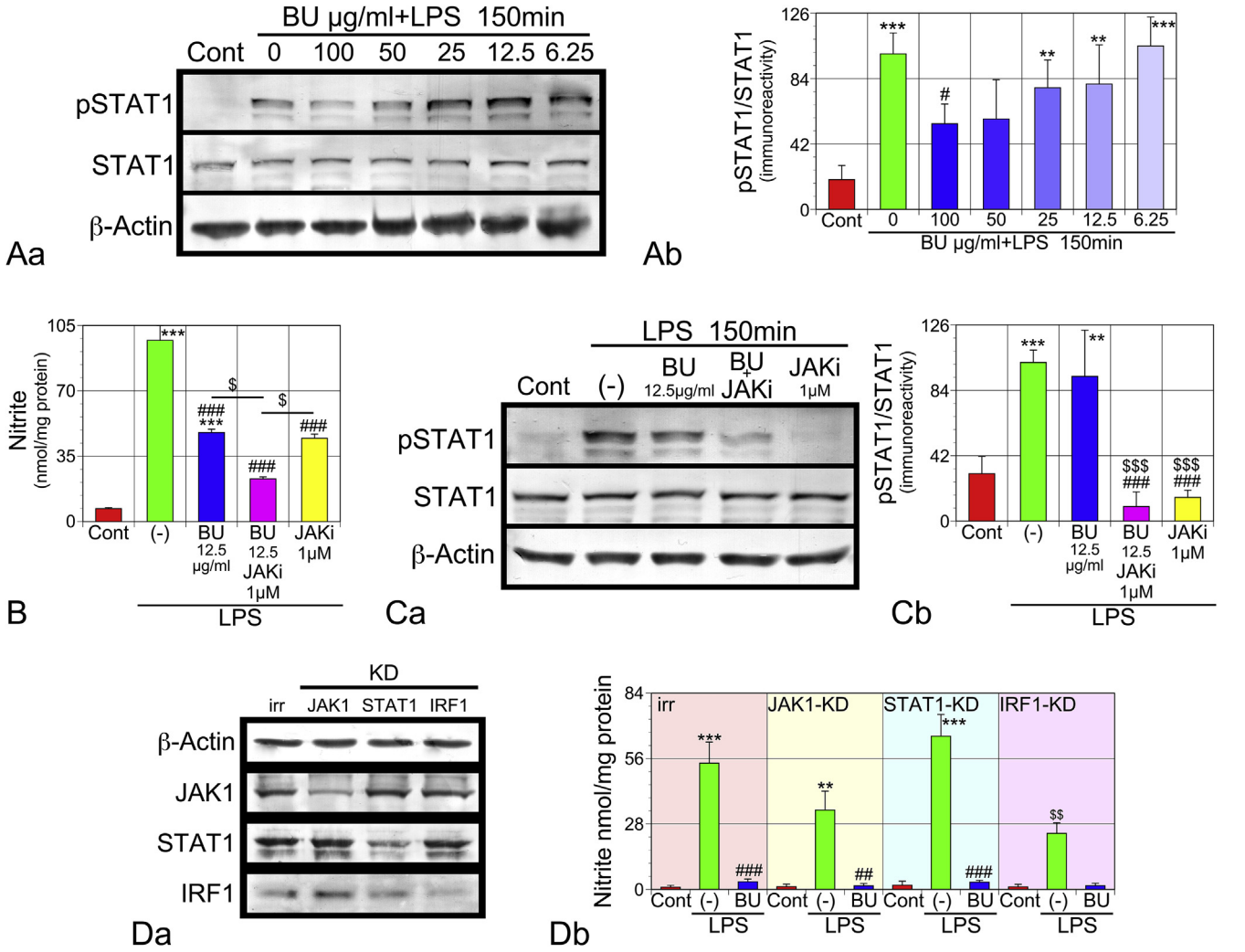

Fig. 3. The inhibitory BU effects are not solely attributable to the inhibition of JAK1/STAT1/IRF1-dependent pathway. (A) Concentration-dependent inhibitory effects of BU on LPS-induced phosphorylation of STAT1 in BV2 cells. Representative immunoblots (Aa) and densitometric data (Ab; n = 4) expressed as mean ± SEM are shown. BU only at 100 μg/ml significantly suppressed STAT1 phosphorylation. (B) BU (12.5 μg/ml) and filgotinib (JAKi, 1 μM) suppressed NO release by LPS-treated BV2 cells. The combination of BU and filgotinib displayed significant additive effects. (C) Representative immunoblots (Ca) showing inhibition of STAT1 phosphorylation by BU (12.5 μg/ml), filgotinib (1 μM) or their combination. Filgotinib alone almost completely inhibited STAT1 phosphorylation and the addition of BU did not cause any significant changes (Cb). (D) Transfection of siRNA to knockdown (KD) mRNA encoding JAK1, STAT1, and IRF1 suppressed the expression of these proteins by BV2 cells as revealed by immunoblotting (Da). irr denotes the results obtained with cells treated with siRNA of irrelevant sequence. (Db) Effects of KD on LPS-induced NO release and the suppression by BU. Only IRF1-KD caused significant inhibition of NO release. BU showed significant inhibitory effects on NO release in the JAK1- and STAT1-KD cells. Data (n = 4) are expressed as mean ± SEM. **P < 0.01, ***P < 0.001 vs Control; #P < 0.05, ##P < 0.01, ###P < 0.001 vs LPS (−); $ P < 0.05, $$ P < 0.01, $$$ P < 0.001 vs BU or JAKi alone in B and C, or irr-treated LPS (−) cells.

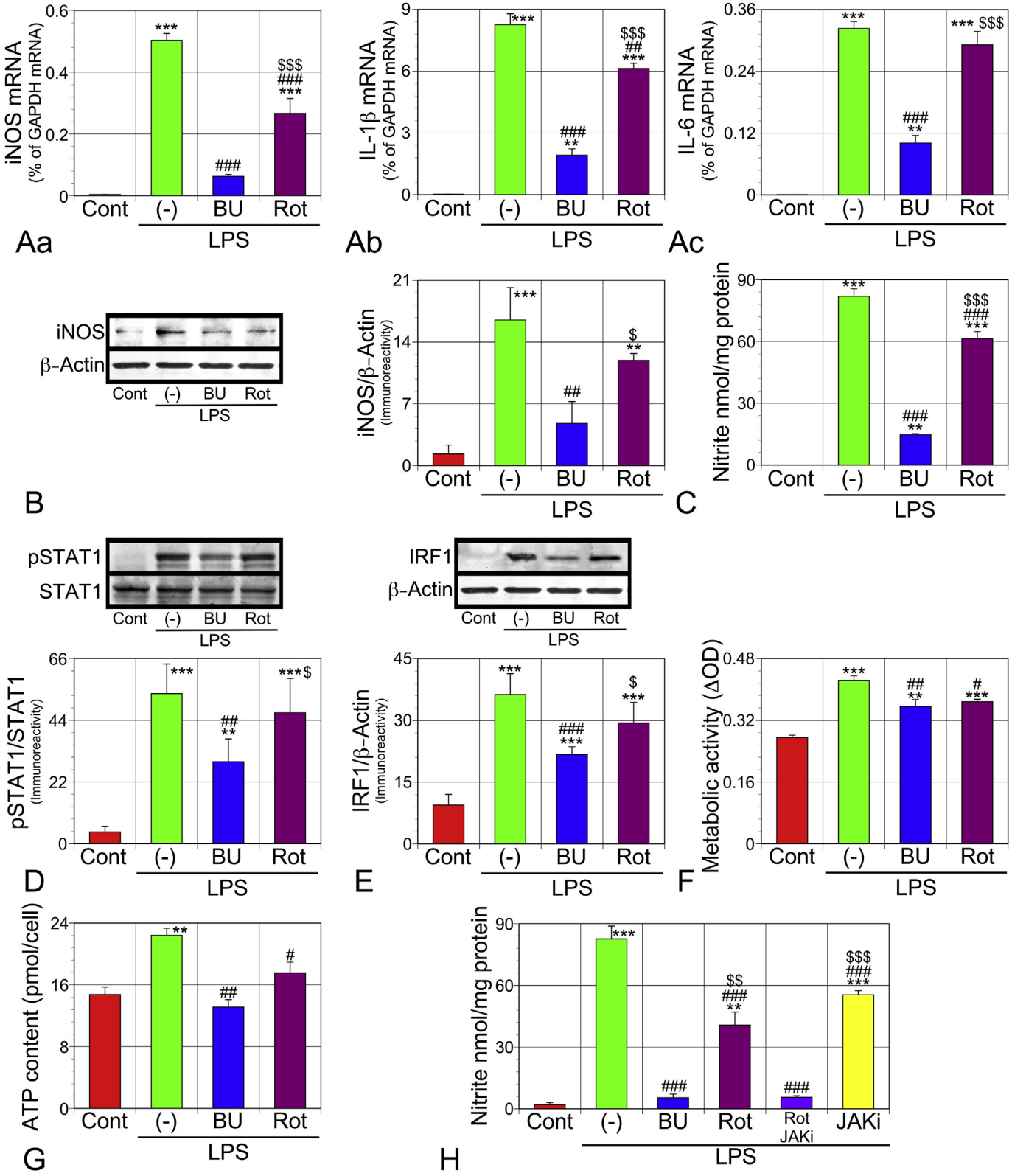

Fig. 4. Effects of BU and rotenone on proinflammatory reactions, metabolic activities and ATP contents. (A) Effects of BU (100 μg/ml) and rotenone (10 nM) on iNOS (Aa), IL-1β (Ab), and IL-6 (Ac) mRNA expression by BV2 cells incubated with LPS for 3 h, as revealed by qPCR. Data (n = 4) are expressed as mean ± SEM. (B) Effects of BU and rotenone on iNOS protein expression by BV2 cells incubated with LPS for 3 h, as revealed by immunoblotting. Representative immunoblots and densitometric data (n = 4) are shown as mean ± SEM. (C) BU and rotenone inhibited LPS-induced NO release by BV2 cells that had been incubated with LPS for 24 h. The inhibitory effect of rotenone was less significant than that of BU. (D and E) BV2 cells were incubated with LPS for 3 h and subjected to immunoblotting analyses. BU, but not rotenone, suppressed STAT1 phosphorylation (D) and IRF1 expression (E). (F) LPS treatment increased metabolic activity of BV2 cells as revealed by WST1 assay after 45-min-incubation with LPS. BU and rotenone partly reversed the LPS-induced increase in metabolic activity. (G) iATP content in BV2 cells was increased after 2-h-incubation with LPS. BU and rotenone abolished the increase. Data (n = 4) in B–G are expressed as mean ± SEM. (H) LPS-induced NO release by BV2 cells was inhibited by a combination of rotenone and filgotinib (1 μM) as strongly as BU. Data (n = 7) are shown as mean ± SEM. **P < 0.01, ***P < 0.001 vs Control; ###P < 0.001 vs LPS alone (LPS (−)); $ P < 0.05, $$$ P < 0.001 vs BU.

To further confirm the notion, JAK1, STAT1, and IRF1 expression was silenced in BV2 cells using specific siRNAs (Fig. 3D). When JAK1 or IRF1 expression was knocked down, the LPS-induced NO release was weakly suppressed (Fig. 3Db). STAT1-knockdown did not affect NO release. BU strongly suppressed NO release regardless of knockdown. By contrast, knockdown of JAK1, STAT1, and IRF1 expression in PMs caused their unresponsiveness to LPS and/or BU (Supplementary Fig. 2). The results suggest that the main mechanism for the anti-inflammatory effects of BU on BV2 cells is not inhibition of LPS-induced JAK1/STAT1/IRF1-dependent pathway.

3.4. BU suppressed cellular ATP synthesis to a similar extent as rotenone

Rotenone, a mitochondrial complex I inhibitor, has been reported to affect the activity of primary murine microglial cells[12] and BV2 cells.[13] Low concentrations of rotenone (lower than 0.2 μM) are nontoxic for primary mouse microglial cells.[12] In this study, a low concentration (10 nM) of rotenone suppressed LPS-induced mRNA expression of iNOS (Fig. 4Aa) and IL-1β (Fig. 4Ab) by BV2 cells, and had little effect on expression of IL-6 mRNA (Fig. 4Ac), consistent with a previous report.[12] Rotenone inhibited LPS-induced iNOS protein expression (Fig. 4B) and NO release (Fig. 4C) less significantly than BU. In contrast to BU, rotenone did not affect LPS-induced STAT1 phosphorylation (Fig. 4D) or IRF1 protein expression (Fig. 4E). BU and rotenone weakly suppressed LPS-enhanced cellular metabolic activity. BV2 cells increased intracellular ATP (iATP) content in response to incubation with LPS for 2 h (Fig. 4G), which was abolished by BU and rotenone. Finally, BV2 cells were incubated with a combination of rotenone and JAK1 inhibitor in the presence of LPS (Fig. 4H). The combination exerted significant stronger suppressive effect than did rotenone or filgotinib alone, and its effect was as strong as that of BU.

4. Discussion

Strong anti-inflammatory actions of BU on LPS-treated macrophages and microglial cells lead to curative effects on animal disease models such as ALI (Supplementary Fig. 1), sepsis[3] or Parkinson's disease.[4] Although nuclear translocation of NFκB is one of the critical steps for proinflammatory activation,[14] BU did not prevent the translocation. Furthermore, BU did not affect LPS-induced upregulated transcription of a luciferase reporter gene, which was under the control of the NRE. p38[15,16] and its substrate MSK1[17,18] have been implicated in proinflammatory activation of macrophages and microglial cells. However, inhibitors for p38 and MSK1 did not inhibit NO release in BV2 cells. Therefore, the weak inhibitory effects of BU on p38 or MSK1 are not critical mechanisms for the anti-inflammatory effects of BU.

Knockdown of JAK1, STAT1, or IRF1 in PMs abolished the inhibitory effects of BU on LPS-induced NO release (Supplementary Fig. 2). In primary microglial cells, knockdown of IRF1 and IRF8 reduces LPS-induced NO release.[4] Thus, the inhibitory effects of BU may be partly attributable to the inhibition of the JAK/STAT/IRF-dependent pathway. However, the maximum inhibitory effect of the JAK1 inhibitor filgotinib on NO release by BV2 cells was much weaker than that of BU, despite that 1 μM of filgotinib completely prevented STAT1 phosphorylation (Fig. 3D). Furthermore, knockdown of JAK1, STAT1, or IRF1 in BV2 cells did not significantly affect the antiinflammatory effects of BU. Inhibition of STAT1 phosphorylation by BU was much weaker than that by filgotinib. BU at 12.5 μg/ml, which did not inhibit STAT1 phosphorylation, suppressed NO release to a similar extent as 1 μM of filgotinib. A combination of BU (12.5 μg/ml) and filgotinib (1 μM) displayed stronger suppressive effects on NO release than each agent alone. These results suggest that the anti-inflammatory effects of BU are not attributable to the weak inhibition of STAT1 phosphorylation alone.

Rotenone at 1 or 10 μM has been reported to prevent LPS-induced NFκB translocation into nuclei while inhibiting IκB degradation.[19] We found that rotenone at 10 nM weakly suppressed LPS-induced activation, while it did not affect the cellular viability. BU suppressed mitochondrial metabolic activity to a similar extent as rotenone, presumably leading to reduced iATP. It has been reported that a decrease in iATP level leads to suppression of proinflammatory cytokine release.[20] Reduction in iATP level in endothelial cells caused by incubation with 2-deoxy-D-glucose, an inhibitor for glycolysis, prevents phorbol 12-myristate 13-acetate-induced translocation of NFκB into the nuclei.[21] iATP may be related to suppression of some inflammatory kinases, such as c-Jun N-terminal kinases (JNK) or extracellular signal-regulated kinase (ERK), and inhibitor κB (IκB) kinase.[22] Therefore, it would be of value to elucidate whether BU affects JNK or ERK activities, taken the observation that BU did not affect the NFκB activation.

5. Conclusion

BU has strong anti-inflammatory effects, which are not correlated with NFκB activation. BU has two distinct actions in association with anti-inflammatory effects: weak inhibition of LPS-induced STAT1 phosphorylation and IRF1 expression, and suppression of mitochondrial activities, leading to reduced iATP levels. Because the combination of rotenone and filgotinib suppressed NO release to a similar extent as BU, the marked anti-inflammatory effects of BU could be attributable to the synergistic effects of JAK1 inhibition and reduction in iATP. The mechanism behind BU's effects on iATP contents and JAK1 activity remains to be clarified with future research.

Conflict of interest statement

The authors have no conflicts of interest.

Acknowledgements

We are grateful to the staff of the Animal Center of INCS, Ehime University, for their careful and gentle handling of the animals. This study was partly supported by grants from Ehime University (#0503003000) and the Ministry of Education, Culture, Sports, Science, and Technology, Japan (#815K143510, 22390037 to JT).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jphs.2017.05.007.

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