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The ameliorative effects of a hypnotic bromvalerylurea in sepsis

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ABSTRACT

Sepsis is a severe pathologic event, frequently causing death in critically ill patients. However, there are no approved drugs to treat sepsis, despite clinical trials of many agents that have distinct targets. Therefore, a novel effective treatment should be developed based on the pathogenesis of sepsis. We recently observed that an old hypnotic drug, bromvalerylurea (BU) suppressed expression of many kinds of pro- and anti-inflammatory mediators in LPS- or interferon-γ activated alveolar and peritoneal macrophages (AMs and PMs). Taken the anti-inflammatory effects of BU on macrophages, we challenged it to septic rats that had been subjected to cecum-ligation and puncture (CLP). BU was subcutaneously administered to septic rats twice per day. Seven days after CLP treatment, 85% of septic rats administrated vehicle had died, whereas administration of BU reduce the rate to 50%. Septic rats showed symptoms of multi-organ failure; respiratory, circulatory and renal system failures as revealed by histopathological analyses, blood gas test and others. BU ameliorated these symptoms. BU also prevented elevated serum-IL-6 level as well as IL-6 mRNA expression in septic rats. Collectively, BU might be a novel agent to ameliorate sepsis by preventing the onset of MOF.

1. Introduction

Sepsis and septic shock is a severe pathologic event, frequently causing death in critically ill patients. In the US alone, more than 200 thousand people die every year due to sepsis. The mortality rate is approximately 25%, which is increased by its severity. Sepsis has long been a critical issue in intensive care and emergency medicine, and great efforts have been made to develop improved or novel treatment modalities for sepsis and related disorders.

The severe systemic inflammation, which is often referred to as systemic inflammatory response syndrome (SIRS) [1], leads to multi-organ failure (MOF) [2]. Severe sepsis most frequently (18%) accompanies respiratory system failure. Renal (15%) and circulatory (7%) system failures are also frequent [3]. Excessive immune responses play a pivotal role in the aggravation of sepsis, causing increased release of proinflammatory cytokines into circulation, known as a cytokine storm [4], which results in MOF. To control sepsis, achieving appropriate control of the immune system may be as important as pathogen removal. Accordingly, many agents with diverse activities have been used clinically to reduce inflammatory responses [5,6]. However, most trials in sepsis have been unsuccessful. Consequently, there are currently no approved drugs to appropriately control the severe systemic inflammation.

In the present study, we used hypnotic bromvalerylurea (BU), which was originally discovered at the beginning of the 20th century [7] and is still sold as an over-the-counter sedative/hypnotic mainly in Asian countries including Japan. We found for the first time that BU has an anti-inflammatory action on

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lipopolysaccharide (LPS)-treated macrophages. Taken the anti-inflmmatory action of BU, we challenged BU to the rat model of sepsis.

2. Materials & methods

2.1. Isolation of peritoneal and alveolar macrophages

All experiments were conducted in accordance with the Guidelines for Animal Experimentation of Ehime University Graduate School of Medicine that were established in accordance with the guidelines published in the National Institute of Health *Guide for the Care and Use of Laboratory Animals*. Peritoneal macrophages (PMs) and alveolar macrophages (AMs) were harvested as described elsewhere [8]. Isolated macrophages were cultured in serum-free E2-LPS (from Escherichia coli serotype 055:B5; Sigma Chemical Co., St. Louis, MO) medium [9]. In some experiments, macrophages were incubated with BU and/or rat recombinant interferon-γ (IFN-γ; 1 pg/ml - 10 ng/ml; PeproTech, Rocky Hill, NJ).

2.2. Measurement of nitrite, cytokine and serum creatinine concentration

Released NO levels were determined by the Griess reaction [9]. Blood samples, macrophage conditioned media and fluid samples obtained by bronchoalveolar lavage were used for measurements of cytokines by ELISA kits (R&D Systems; Minneapolis, MN, USA). Serum creatinine levels were assayed using FUJI DRI-CHEM SLIDE CRE-PIII (Tokyo, Japan).

Table 1
Oligonucleotide primers for quantitative real-time RT-PCR.

Gene	Sense/Anti-sense
Gapdh	5′-CTG AGA ATG GGA AGC TGG TC-3′
	5′-TCA GAT GCC TGC TTC ATC AC-3′
Ccl2	5′-TTG TCA CCA AGC TCA AGA GA-3′
	5′-CAC ATT CAA AGG TGC TGA AG-3′
Ccl3	5′-TTG AGA CCA GCA GCC TTT GC-3′
	5′-TCA AGC CCC TGC TCT ACA CG-3′
Ccl4	5′-TTC TGC GAT TCA GTG CTG TC-3′
	5′-GAT TTG CCT GCC TTT TTT GG-3′
Cxcl3	5′-ACT GCT TCT GCT GCT TCT GC-3′
	5′-TGA CTT CTG TCT GGG TGC AG-3′
Il1β	5′-CAC CTT CTT TTC CTT CAT CTT TG-3′
	5′-GTC GTT GCT TGT CTC TCC TTG TA-3′
Il6	5′-CTT CCA GCC AGT TGC CTT CT-3′
	5′-GAG ACG ATT GGA AGT TGG GG-3′
Il10	5′-ATA ACT GCA CCC ACT TCC CA-3′
	5′-TTT CTG GGC CAT GGT TCT CT-3′
Il23	5′-ATC TTC ACA GGG GAG CCT TC-3′
	5′-CTG CGA AGG ATC TTG GAA CG-3′
Ifnγ	5′-AGG AAA GAG CCT CCT CTT GG-3′
	5′-GGA TCT GTG GGT TGT TCA CC-3′
iNos	5′-AGG GAG TGT TGT TCC AGG TG-3′
	5′-TCC TCA ACC TGC TCC TCA CT-3′
Irf1	5′-GCA AAA CCA AGA GGA AGC TG-3′
	5′-CAG AGA GAC TGC TGC TGA CG-3′
Stat1	5′-AGA GCG ACC AGA AAC AGG AA-3′
	5′-GCT CTC TGC AAC AAT GGT GA-3′
Stat3	5′-TCA CTT GGG TGG AAA AGG AC-3′
	5′-TGG GAA TGT CAG GGT AGA GG-3′
Tnfα	5′-CCC AGA CCC TCA CAC TCA GAT-3′
	5′-TTG TCC CTT GAA GAG AAC CTG-3′

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

Macrophages and ileum tissues were homogenized in ISOGEN (Nippon Gene, Tokyo, Japan) and total RNA was prepared. Then, cDNA was synthesized as previously described [10]. All gene-specific mRNA expression values were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA levels. The primer sequences for each gene are listed in Table 1.

2.4. Immunoblotting

Macrophages and ileum tissue were homogenized and lysed in Laemmli's sample solution. Lysates were immunoblotted with antibodies listed in Table 2 [11].

2.5. Human monocytes/macrophages

Human blood was collected from healthy volunteers, who gave informed consent under approval of internal review board of Ehime University Hospital. Mononuclear cell fraction was obtained with the use of Mono-Poly Resolving medium (DS Pharma Biochemical, Osaka Japan). Only the cells attached to suspension culture dishes were used for experiments.

2.6. Animals, CLP, and BU administration

Adult male Wistar rats were maintained on a 12-h light–dark cycle and were provided free access to food and water. CLP operation was done as described elsewhere [12]. Rats were randomly divided into sham, sepsis/control, and sepsis/BU groups. Sham and sepsis/control rats were subcutaneously administered a maintenance solution, SOLDEM 3A (Terumo Corp., Tokyo, Japan) and Sepsis/BU rats BU solution dissolved in SOLDEM 3A. As a control, the drug midazolam (MDZ; Astellas, Tokyo, Japan) was subcutaneously administered at 2 mg/kg body weight. Body temperature was measured using a rectal thermometer (DT 300, Inter Medical, Nagoya, Japan).

2.7. Immunohistochemistry

Immunohistochemical studies were done as described elsewhere [13]. For the lungs, rats were transcardially perfused with 20 mL PBS to remove blood, then, the lungs were dissected and immersed in 4% paraformaldehyde in PBS containing 2 mM $MgCl_2$ for 4 h for fixation. The fixed tissue sections were subjected to H&E staining and/or immunohistochemical staining. The primary antibodies are listed in Table 2. Hoechst 33258 (Sigma) was used for nuclear staining. Morphometrical analyses were done as has been described [11].

2.8. Assessments of lung edema

The upper lobe of the right lung was removed 24 h after CLP, weighed, and then dried in an oven at 80 °C for 48 h. The dry tissue weight was measured, and the wet-to-dry weight (w/d) ratio was calculated to evaluate the degree of lung edema [14].

2.9. Statistics

Statistical analyses were conducted using one-way analysis of variance with Bonferroni's post-hoc test, or the log-rank test for evaluating the survival rate.

3. Results

3.1. Suppressive effects of BU on LPS-treated macrophages in vitro

LPS increased the expression of PM mRNA transcripts encoding interferon-γ (Fig. 1Aa, IFN-γ) [15,16], interferon regulatory factor 1 (Fig. 1Ab, IRF1) [17], interleukin (IL)-6 (Fig. 1Ac) [18], and IL-23 (Fig. 1Ad) [19], which have all been implicated in sepsis. BU suppressed the mRNA expression. BU also suppressed LPS-induced increased expression of IL-10 (Fig. 1Ba) and STAT3 (Fig. 1Bb), which are mediators of anti-inflammatory signaling [20]. BU dose-dependently inhibited LPS-induced expression or release of various proinflammatory mediators by AMs (Fig. 1C–F). Thus, BU suppressed LPS-induced expression of a wide variety of both pro- and anti-inflammatory mediators by macrophages.

3.2. Suppressive effects of BU on IFN-γ or LPS-induced STAT1 phosphorylation in macrophages

BU suppressed the IFN-γ (10 ng/mL)-induced JAK1/2-mediated rapid phosphorylation of STAT1 [21] (Fig. 1G) and the expression of STAT1, IRF1, and iNOS mRNA (Fig. 1H). By contrast, IFN-β did not induce STAT1 phosphorylation that may be dependent on JAK1 and Tyk2 [21] (Fig. 1I). Additionally, BU did not suppress granulocyte macrophage-colony stimulating factor (GM-CSF)-induced JAK2-mediated STAT5 phosphorylation [22] (Fig. 1J). PMs expressed nearly two orders of magnitude more JAKs 1 and 2 mRNA transcripts than JAK3 and Tyk2 (Fig. 1K). BU suppressed LPS-induced JAKs 1 and 2 mRNA expression, whereas BU did no-t display significant effects on JAK3 and Tyk2 expression. BU was also effective in human macrophages (Fig. 1L).

3.3. BU did not prevent LPS-induced IκB degradation

BU did not inhibit LPS-induced IκB degradation, as indicated by immunoblotting of PM cell lysates collected 2 h after LPS treatment (Fig. 2A). Kinetic immunoblotting study revealed that LPS-induced IκB degradation (Fig. 2Ba) and TANK-binding kinase 1 (TBK1) phosphorylation (Fig. 2Bb) occurred 2 or 3 h earlier than STAT1 phosphorylation (Fig. 2Bc), increase of IRF1 (Fig. 2Bd) and iNOS expression (Fig. 2Be). Although BU did not affect LPS-induced phosphorylation of TBK1 [23], it did suppress STAT1 phosphorylation and IRF1 and iNOS expression. Furthermore, LPS-induced NF-κB nuclear translocation could be clearly observed in rat primary cultured microglial cells that were not prevented by BU (data not shown). Thus, BU did not inhibit LPS-induced nuclear translocation of NFκB.

3.4. BU improved the survival of septic rats through amelioration of MOF

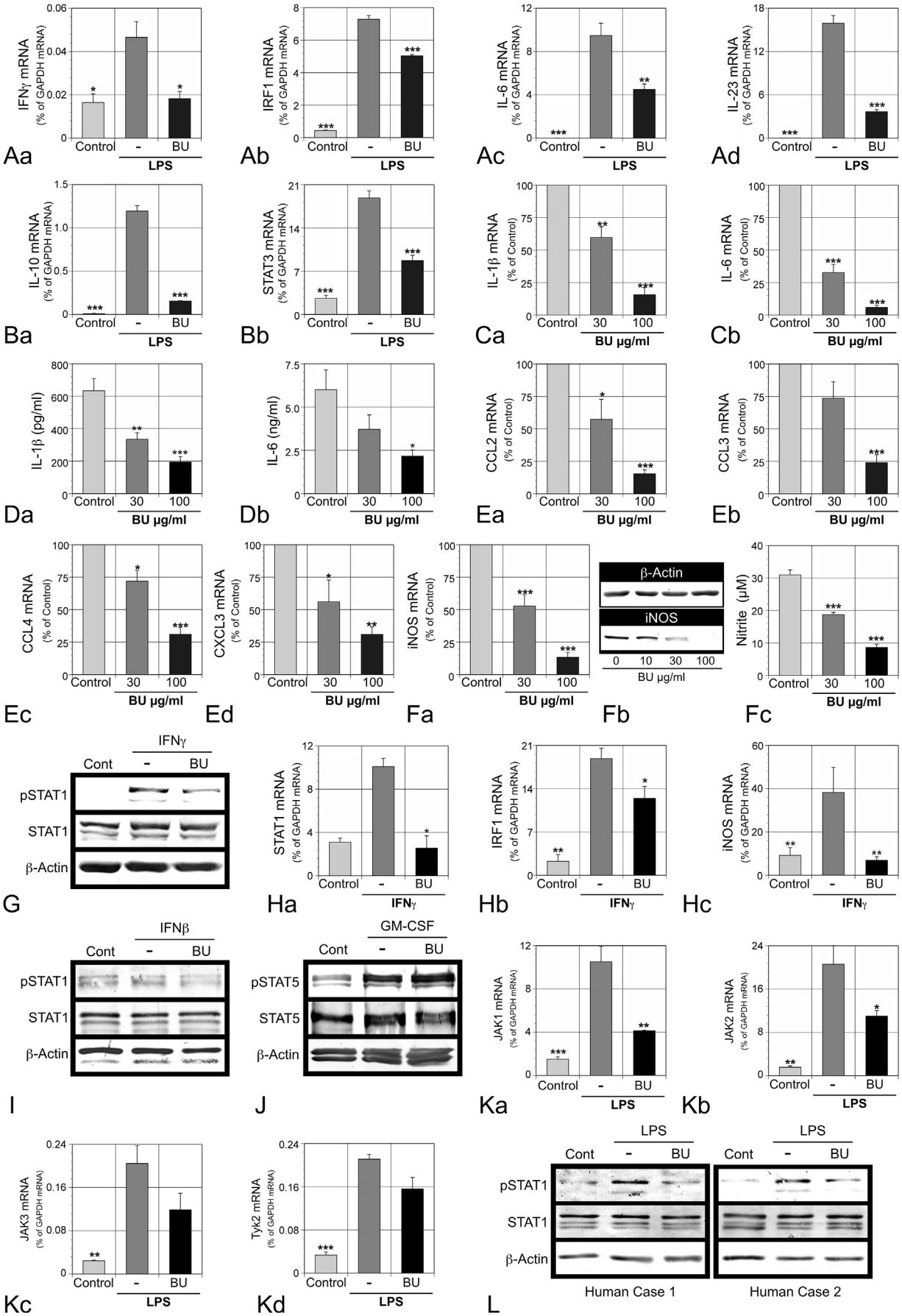
We administered BU to the CLP-induced septic rats. BU was dissolved in vehicle at a concentration of 500 mg/L and 10 mL of the solution was subcutaneously injected into male Wistar rats (mean bodyweight, 300 g) twice per day. The BU dose administered was 33 mg/kg bodyweight/day, which is within the normal applicable dose range for humans (clinical maximum dosage of BU, 3 g/day). BU administration improved the survival of septic rats (Fig. 3A). Seven days after CLP treatment, 85% of septic rats administrated vehicle (sepsis/control) had died, whereas the mortality rate of rats administered BU solution was 50% (sepsis/BU). For comparison, a benzodiazepine midazolam (MDZ) with sedative/hypnotic actions was similarly administered to septic rats (sepsis/MDZ; Fig. 3A). MDZ was ineffective despite its reported immunosuppressive and ameliorative effects on macrophages and septic rats [24]. When BU administration was started 3 h after CLP, the survival rate also improved, similarly to the rapid administration of BU (Fig. 3B).

The high mortality rate in sepsis might be attributable to MOF [2]. Therefore, we analyzed the morphology and functions of the lungs, kidney, and abdominal organs 24 h after CLP (Figs. 3 and 4). Lungs of septic rats (sepsis/control) showed septal thickening, reduced alveolar space, and many inflammatory cells in edematous interstitial tissue (Fig. 3C). As a consequence of sepsis-induced vascular dysfunction, extravasated IgG accumulated (Fig. 3D). Accumulation of leukocytes or inflammatory cells in the lung could be observed by immunofluorescence staining using antibodies to CD11b (Fig. 3E) and IL-1β (Fig. 3F). Lung edema was evaluated by determining the wet/dry weight ratio of the lung (Fig. 3G) [25]. The number of $CD11b^+$ myeloid and $IL\text{-}1\beta^+$ inflammatory cells was greatest in the lungs of sepsis/control rats (Fig. 3H). BU administration (sepsis/BU) largely overcame these sepsis-induced pathological changes in the lung. As determined by ELISA, IL-1β in the bronchoalveolar lavage fluid much increased in septic rats (Fig. 3I), and BU overcame the increase in IL-1β concentration.

CLP elicited many symptoms of bowel inflammation such as swelling and reddening in the sepsis/control rats, which were suppressed by BU (Fig. 4A). The ileum of sepsis/control rats showed dense leukocyte accumulation (Fig. 4B). A significant increase in serum creatinine levels (Fig. 4C) and the reduction of body temperature (Fig. 4D) were observed in sepsis/control rats. Physiological parameters and blood gas test data from the three groups of rats 24 h after CLP are shown in Table 3. BU largely normalized a variety of the CLP-induced pathologic changes and parameters. To determine whether BU could suppress STAT1 phosphorylation in septic rats, distal ileum tissues from three rat groups were subjected to immunohistochemical staining (Fig. 4E). Enhanced pSTAT1 immunoreactivity was generally distributed in and under the peritoneum in sepsis/control rats 8 h after CLP (Fig. 4Eb), where $CD11b^+$ myeloid leukocytes accumulated (arrowheads, Fig. 4Eb). Such diffuse pSTAT1 immunoreactivity was significantly reduced in sepsis/BU rats (Fig. 4Ec). Suppressive effects of BU on STAT1 phosphorylation were confirmed by immunoblotting (Fig. 4F and G). IL-6 mRNA and protein was elevated in the ileum 5 h after CLP, which was abolished by BU administration (Fig. 4H and I). BU administration much reduced elevated IL-6 protein level in circulation 24 h after CLP.

Table 2
Primary antibodies used in this study.

Antigen	Antibody	Source
β-Actin	Mouse monoclonal	Sigma–Aldrich (St. Louis, MO, USA)
CD11b	Mouse monoclonal	AbD serotec (Oxford, UK)
CD45	Mouse monoclonal	AbD serotec
CD68	Mouse monoclonal	AbD serotec
Iba1	Rabbit polyclonal	Wako (Osaka, Japan)
IL-1β	Rabbit polyclonal	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
iNOS	Mouse monoclonal	BD Biosciences (Franklin Lakes, NJ, USA)
IRF-1	Rabbit monoclonal	Cell Signaling Technology (Boston, MA, USA)
JAK1	Rabbit polyclonal	Cell Signaling Technology
NF-κB p65	Rabbit monoclonal	Cell Signaling Technology
Phospho-STAT1	Rabbit monoclonal	Cell Signaling Technology
STAT1	Rabbit polyclonal	Cell Signaling Technology


Aa
IFNγ mRNA (% of GAPDH mRNA)
Control
LPS
BU
Ab
IRF1 mRNA (% of GAPDH mRNA)
Ac
IL-6 mRNA (% of GAPDH mRNA)
Ad
IL-23 mRNA (% of GAPDH mRNA)
Ba
IL-10 mRNA (% of GAPDH mRNA)
Bb
STAT3 mRNA (% of GAPDH mRNA)
Ca
IL-1β mRNA (% of Control)
BU μg/ml
Cb
IL-6 mRNA (% of Control)
Da
IL-1β (pg/ml)
Db
IL-6 (ng/ml)
Ea
CCL2 mRNA (% of Control)
Eb
CCL3 mRNA (% of Control)
Ec
CCL4 mRNA (% of Control)
Ed
CXCL3 mRNA (% of Control)
Fa
iNOS mRNA (% of Control)
Fb
β-Actin
iNOS
Fc
Nitrite (μM)
G
IFNγ
Cont
pSTAT1
STAT1
β-Actin
Ha
STAT1 mRNA (% of GAPDH mRNA)
Hb
IRF1 mRNA (% of GAPDH mRNA)
Hc
iNOS mRNA (% of GAPDH mRNA)
I
IFNβ
J
GM-CSF
pSTAT5
STAT5
Ka
JAK1 mRNA (% of GAPDH mRNA)
Kb
JAK2 mRNA (% of GAPDH mRNA)
Kc
JAK3 mRNA (% of GAPDH mRNA)
Kd
Tyk2 mRNA (% of GAPDH mRNA)
L
Human Case 1
Human Case 2

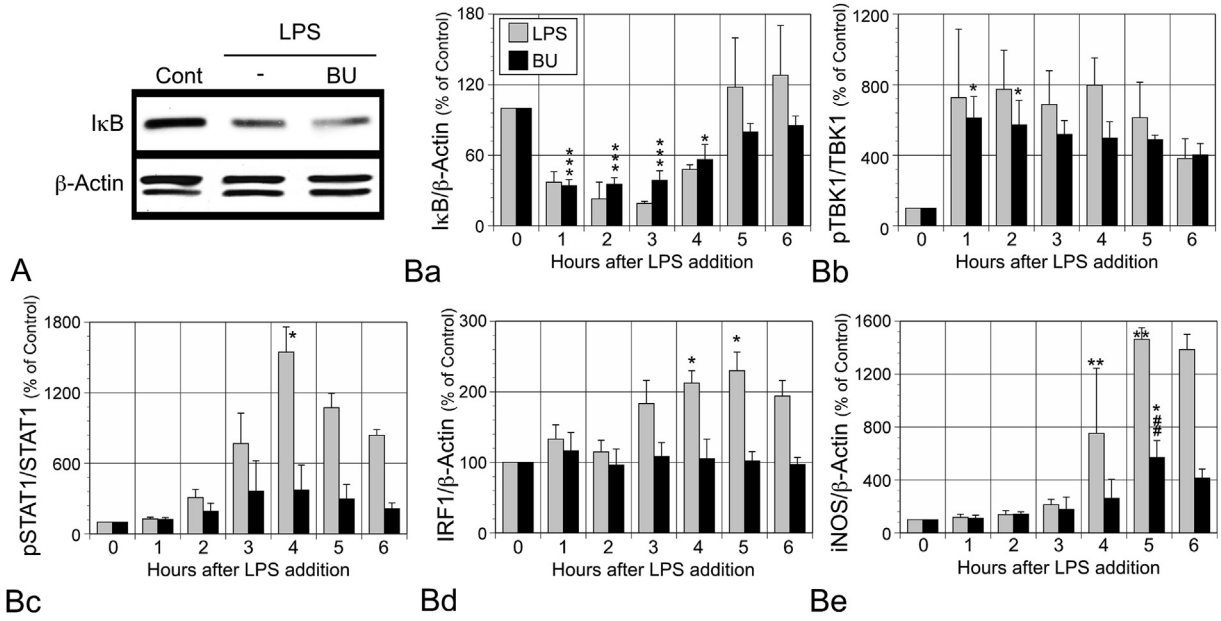


Fig. 2. BU suppressed JAK/STAT signaling, but not NF-κB activation. (A) A representative immunoblot showing that BU did not inhibit LPS-induced (2 h incubation) IκB degradation in PMs. (B) A kinetic immunoblotting study (n = 3) of proinflammatory signaling pathway components in LPS-treated PMs. IκB degradation (Ba) and TBK1 phosphorylation (Bb) occurred 1 h after LPS was added, whereas STAT1 phosphorylation (Bc) and IRF1 (Bd) expression occurred several hours later. Significant iNOS (Be) expression was observed at 4 h after addition of LPS. Graph data are expressed as means ± SEM. *, **, and *** indicate P < 0.05, 0.01, or 0.001, respectively, vs. the controls or 0 h time point; ## indicates P < 0.01 vs. LPS alone.

4. Discussion

This is the first report demonstrating that an old hypnotic/sedative agent BU has strong ameliorating effects on the CLP-induced sepsis rat model. BU suppressed the LPS-induced production of proinflammatory mediators by macrophages in vitro. BU increased the survival of septic rats. CLP-induced sepsis caused MOF accompanying histopathological changes characteristic for acute lung injury, such as pulmonary edema, accumulation of inflammatory cells, and enhanced vascular permeability. Such pathological changes of the lung result in the respiratory failure, the most critical problem causing death of the patients [2]. The sepsis model also accompanied renal and circulatory system failures as well as systemic inflammatory symptoms. These changes were all suppressed by BU administration. Thus, BU prevented the onset of MOF, presumably through the suppression of proinflammatory reactions by myeloid leukocytes, particularly macrophages.

In sepsis cases, anti-inflammatory reactions often follows the primary extreme proinflammatory reactions that might be responsible for the high mortality rate of sepsis [26]. In this anti-inflammatory response, the anti-inflammatory cytokine IL-10 may cause immunodeficiency allowing bacterial growth [6,26,27]. BU suppressed LPS-induced IL-10 and STAT3 mRNA expression by PMs, suggesting that BU functions as an immunomodulatory drug that inhibits both pro- and anti-inflammatory responses. The immunomodulatory actions of BU might be at least partly attributable to its suppressive effects on STAT1 phosphorylation and IRF1 expression. Phosphorylated STAT1 and IRF1 may strengthen LPS-induced upregulated expression of proinflammatory mediators including NO production [28,29].

It is currently unknown whether BU suppresses LPS- or IFNγ-induced STAT1 phosphorylation through a direct inhibitory effect on JAK1 activity. However, BU suppressed IFNγ-induced STAT1 phosphorylation but not GM-CSF-induced STAT5 phosphorylation in PMs, suggesting that BU inhibits JAK1 but not JAK2 activity. Although the IFN-γ receptor associates with JAK1 and JAK2 [30], JAK2 inhibition can cause side effects, such as anemia and thrombocytopenia [31] because receptors for erythropoietin and thrombopoietin employ JAK2 as a critical signal transduction molecule as GM-CSF receptor does. Collectively, JAK1-specific inhibition might be a leading therapeutic target.

BU has been used clinically for one century, mainly in East Asia, and there are no reports describing BU-associated immune deficiency. BU effectively suppressed IFN-γ-induced phosphorylation of STAT1 in human monocytes. In conclusion, BU may be a strong candidate agent to ameliorate the prognosis of sepsis because of its established safety. It is necessary to clarify the molecular mechanisms underling the immunomodulatory effects of BU.

Fig. 1. Suppressive effects of BU on activated macrophages in vitro. (A) Incubation with LPS for 3 h increased mRNA expression of IFN-γ (Aa), IRF1 (Ab), IL-6 (Ac), and IL-23 (Ad) by PMs, and the addition of BU with LPS suppressed this effect (n = 4). (B) BU suppressed mRNA encoding the anti-inflammatory cytokine IL-10 (Ba) and downstream signaling molecule STAT3 (Bb). (C) BU inhibited LPS-induced expression of mRNA encoding IL-1β (Ca) and IL-6 (Cb) by AMs as shown by qPCR. n = 4. (D) BU inhibited release of IL-1β (Da) and IL-6 (Db) by AMs as shown by ELISA. n = 11. (E) BU suppressed mRNA expression encoding CCL2 (Ea), CCL3 (Eb), CCL4 (Ec) and CXCL3 (Ed) chemokines as shown by qPCR. n = 4. (F) BU suppressed the expression of iNOS mRNA (Fa) and protein (Fb), as well as NO release (Fc) by AMs. (G) BU suppressed STAT1 phosphorylation caused by incubating PMs with IFN-γ for 30 min (pSTAT1; phosphorylated STAT1). (H) At the same time point, BU suppressed the expression of mRNA encoding STAT1 (Ha), IRF1 (Hb), and iNOS (Hc). n = 4. (I) IFN-β did not induce the phosphorylation of STAT1. (J) GM-CSF induced phosphorylation of STAT5 that was not suppressed by BU. (K) Expression levels of mRNA encoding JAK1 (Ka), JAK2 (Kb), JAK3 (Kc) and Tyk2 (Kd) by PMs. PMs expressed JAK1 and JAK2 at high levels. BU suppressed mRNA for the JAKs. n = 4. (L) BU suppressed LPS-induced STAT1 phosphorylation in human monocytes. Graph data are presented as means ± SEM; *, **, and *** indicate P < 0.05, 0.01, or 0.001, respectively, vs. controls.

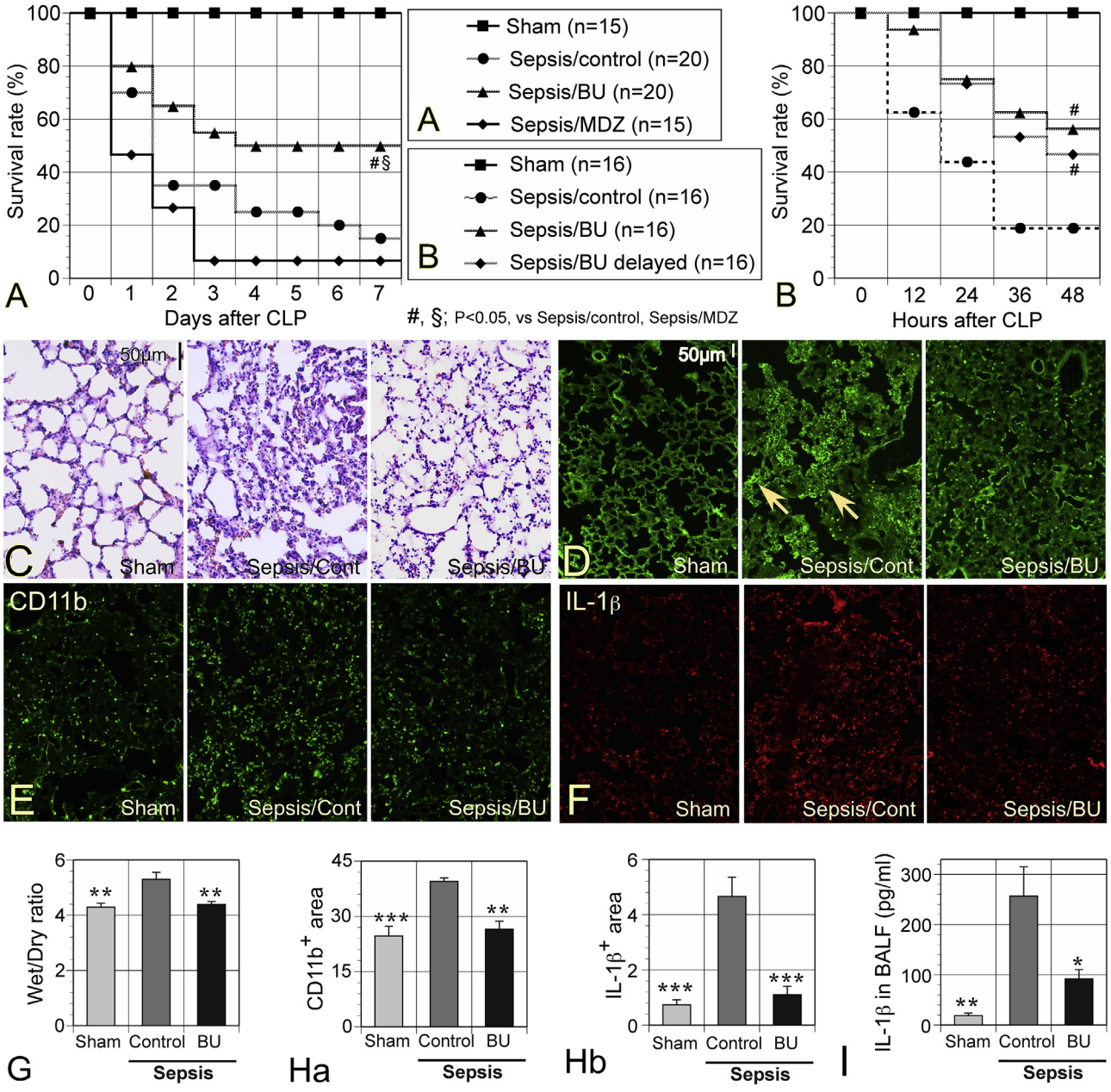


Fig. 3. BU improved the viability of septic rats and ameliorated CLP-induced pathological changes in septic rat lungs. (A) Kaplan–Meier survival plots of sham, sepsis/control, sepsis/BU, and sepsis/midazolam (MDZ) rats for 7 days after CLP. No sham-operated rats died within this period, while 85.0% of the sepsis/control rats and 93.3% of the sepsis/MDZ rats died. The mortality rate of sepsis/BU rats was 50.0%, which was significantly lower than for sepsis/control rats ($P < 0.05$; #, § vs. Sepsis/control and MDZ, respectively). (B) The graph shows the viability of CLP-rats when BU administration was started shortly and 3 h (delayed) after CLP. The delayed administration of BU was effective. Statistical significance was determined by a log-rank test. (C) H&E staining of lungs. Septal thickening of lungs of sepsis/control rats was apparent, whereas sepsis/BU rat lungs displayed thinner alveolar walls. (D) Immunofluorescence staining of extravasated IgG in rat lungs. IgG was deposited in edematous lung tissue from a sepsis control rat (arrows). In the lungs of sepsis/BU rats, extravasated IgG was not observed. (E and F) The number of $CD11b^+$ (E) or $IL\text{-}1\beta^+$ (F) cells in rat lungs was reduced when septic rats were treated with BU. (G) To evaluate the effects of BU on lung edema, freshly isolated and dried lungs were weighed, and the wet-to-dry ratio was calculated. BU suppressed lung edema ($n = 5$). (H) Morphometrical analyses of the number of inflammatory cells in the lungs ($n = 4$). BU reduced the number of myeloid cells ($CD11b^+$ cells; Ha) and inflammatory cells that expressed IL-1β (Hb). (I) Concentrations of IL-1β in bronchoalveolar lavage fluids were determined by ELISA ($n = 4$). IL-1 β concentration was much increased in the septic rat lungs and BU decreased the concentration. *, **, and *** indicate $P < 0.05$, 0.01, or 0.001, respectively, vs. sepsis/control.

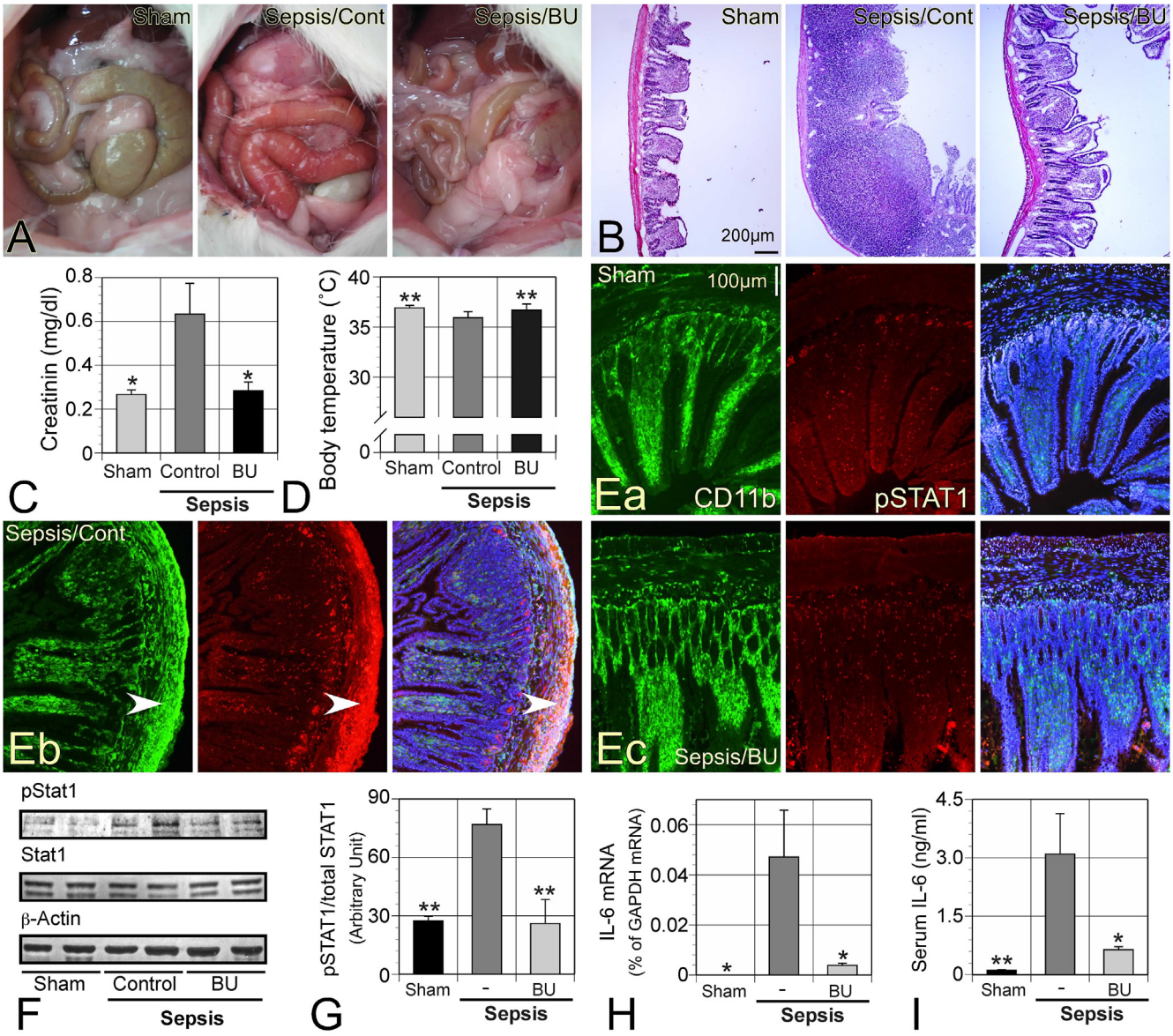


Fig. 4. The suppressive effects of BU on systemic inflammation after CLP. (A) Representative pictures of abdominal cavities of sham, sepsis/control, and sepsis/BU rats. (B) H&E staining shows the ileum walls. The thickened wall of the ileum of sepsis/control rats was mainly occupied by leukocytes. BU prevented the accumulation of cells in the ileum wall. (C) BU suppressed the sepsis-induced increase of serum creatinine concentrations (n = 6). (D) BU prevented the reduction of body temperature (sham, n = 7; sepsis/control, n = 17; sepsis/BU, n = 15). (E) The distal ileum was subjected to immunohistochemical staining for CD11b and pSTAT1 5 h after CLP (green, CD11b; red, pSTAT1; blue, nuclear staining with Hoechst 33432). Although thickening of the ileum wall was not apparent at this time point, strong pSTAT1-immunoreactivity could be observed in and under the peritoneum (arrowhead) of sepsis/control rats (Eb), where $CD11b^{+}$ myeloid cells also accumulated. The BU-treated rat ileum did not show strong pSTAT1 immunoreactivity (Ec). (F and G) Immunoblotting for pSTAT1 5 h after CLP. Representative immunoblots (F; n = 2) and graphs of densitometric data (G; n = 4). BU suppressed STAT1 phosphorylation nearly similarly to the sham group level. (H) BU suppressed the elevation of IL-6 mRNA expression in the distal ileum 5 h after CLP (n = 4). (I) BU prevented the elevation of serum IL-6 protein levels 24 h after CLP (n = 10). Data are expressed as means ± SEM. *, and ** indicate $P < 0.05$, and 0.01, respectively, vs. sepsis/control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Blood gas test data.

	Sham	Sepsis-cont	Sepsis-BU
Body weight (g)	302 ± 17.3	300 ± 18.3	298 ± 17.1
Body temperature before CLP (°C)	36.9 ± 0.24	36.9 ± 0.30	36.8 ± 0.24
Body temperature 24 h after CLP (°C)	36.9 ± 0.25	36.0 ± 0.63**	36.7 ± 0.60##
pH	7.47 ± 0.02	7.49 ± 0.04	7.47 ± 0.03
$PaCO_2$ (mmHg)	39.3 ± 2.49	32.3 ± 6.17	35.4 ± 2.56
PaO_2 (mmHg)	237.0 ± 3.16	201.8 ± 19.97**	240 ± 7.75##
Systolic ABP (mmHg)	148.4 ± 4.67	109.8 ± 12.19***	131.6 ± 6.35*,##
Diastolic ABP (mmHg)	107.8 ± 3.63	88.4 ± 8.62	97.8 ± 7.63
Mean ABP (mmHg)	121.3 ± 3.25	95.5 ± 9.48***	109.0 ± 6.39*,#
Glucose (mg/dL)	200.8 ± 10.18	170.6 ± 31.26	208.8 ± 19.43
Lactate (mg/dL)	13.0 ± 3.32	32.2 ± 6.87***	22.4 ± 3.21*,#
Na^+ (mmol/L)	134.6 ± 0.55	118.0 ± 4.74***	127.2 ± 3.96*,##
K^+ (mmol/L)	3.44 ± 0.22	5.02 ± 0.27***	4 ± 0.64##

* vs sham # vs sepsis, *,#P < 0.05, **,##P < 0.01, ***,###P < 0.001

Conflict of interest

The authors disclose no potential conflicts of interest.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.111.

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