J Anesth (2013) 27:366–373
DOI 10.1007/s00540-012-1539-7

ORIGINAL ARTICLE

Propofol attenuates lipopolysaccharide-induced monocyte chemoattractant protein-1 production through p38 MAPK and SAPK/JNK in alveolar epithelial cells

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Received: 26 August 2012 / Accepted: 29 November 2012 / Published online: 21 December 2012

Abstract
Purpose Propofol is widely used in sedation and surgical procedures involving patients with acute lung injury (ALI), a common complication in critically ill patients. Monocyte chemoattractant protein-1 (MCP-1) plays an important role in pathological changes in ALI. The present study investigated the anti-inflammatory effect and mechanism of propofol on MCP-1 production and mitogen-activated protein kinase (MAPK) phosphorylation induced by lipopolysaccharide (LPS) in alveolar epithelial cells (AECs).
Methods AECs were treated with 1 μg/ml LPS for 30 min, 1 h, 6 h, or 24 h following pretreatment with 12.5–100 μM propofol for 30 min. Cytokines and chemokines secretion were profiled using cytokine array, and mRNA and protein levels of MCP-1 were measured by RT-PCR and ELISA. The phosphorylation of p38 MAPK, p44/42 MAPK, SAPK/JNK, ATF-2, and c-Jun were measured by Western blot analysis.
Results Propofol at 50 and 100 μM dose-dependently inhibited MCP-1 mRNA expression ($P < 0.05$), and also propofol at 50 μM decreased extracellular MCP-1 protein levels ($P < 0.05$) compared to the LPS group. Propofol at 12.5–50 μM inhibited LPS-induced phosphorylation of p38 MAPK, p44/42 MAPK, SAPK/JNK, ATF-2, and c-Jun in AECs.
Conclusions Propofol at clinically relevant concentrations attenuated LPS-induced MCP-1 mRNA expression and secretion by inhibiting the phosphorylation of p38 MAPK, SAPK/JNK, ATF-2, and c-Jun exerting its anti-inflammatory effects in AECs. These results suggest that propofol may modulate inflammatory response at clinically achievable concentrations in ALI.

Keywords Propofol · MCP-1 · P38 MAPK · ATF-2 · Alveolar epithelial cell

Introduction

Propofol is an intravenous anesthetic agent widely used to introduce and maintain anesthesia during surgical procedures. Several studies have reported that propofol has anti-inflammatory effects, such as suppression of biosynthesis of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS)-activated macrophages [1], and reduction of cluster of differentiation 14 (CD14) and Toll-like receptor 4 (TLR4) expression in endotoxin-induced lung injury [2]. Propofol also inhibits the activation of p38 mitogen-activated protein kinase (MAPK) upregulating the expression of Annexin A1 in human monocytic THP-1 cells [3], and attenuates granulocyte–macrophage colony-stimulating factor (GM-CSF) production by suppressing MAPK/extracellular signal-regulated kinase (ERK) activity and NF-κB translocation in hepatocytes [4]. It offers antioxidating potential to attenuate hydrogen peroxide-induced PC12 cell death through p38 MAPK, and protects against

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oxidative stress in human umbilical vein endothelial cells and human hepatic cells [5–8]. In addition, it is also known to inhibit cancer cell invasion, depress angiotensin II-induced cardiomyocyte hypertrophy, and inhibit human neutrophil chemotaxis [9–11].

Propofol has often been used in recent years as a sedative in patients with acute lung injury (ALI), which is a common and critical pulmonary complication with high mortality rate [12–16]. ALI is an inflammatory disorder characterized by an excessive infiltration of neutrophils, the release of inflammatory mediators, and destruction of the alveolar–capillary membrane with severe consequences for pulmonary gas exchange [17–19]. The pathological changes associated with ALI have multiple aspects, including activation of inflammatory cells and damage to epithelial cells and endothelial cells. Alveolar epithelial cells (AECs) have several important functions, including maintaining a tight barrier, regulating surfactant production, and removing excess alveolar fluid by vectorial ion transport. These functions have an important role in prevention of alveolar edema, and their impairment generally results in a poor prognosis for patients with ALI [20]. In such cases, the anti-inflammatory effects of propofol in AECs may improve the prognosis for ALI.

To clarify the anti-inflammatory effects of propofol in ALI, it is quite important to know the production of cytokines and chemokines inhibited by propofol in AECs. The present study investigated the effects of propofol on inflammatory response, in particular monocyte chemoattractant protein-1 (MCP-1), induced by LPS, a bacterial endotoxin capable of inducing inflammatory reactions with pathological events resembling ALI in AECs.

Materials and methods

Cell culture

L2 cells (JCRB9053, Japanese Collection of Research Bioresources, Japan) and A549 cells (DS Pharma Biomedical Co., Ltd, Japan) as AECs were derived from adult female rat lung and human lung carcinoma. AECs were incubated in an atmosphere of 5 % CO_2–95 % air at 37 °C, in F-12K Nutrient Mixture Kaighn' s Modification Medium for L2 cells and in Dulbecco's modified Eagle medium for A549 cells, supplemented with 10 % fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were grown in 75 cm^2 culture flask to >95 % confluence and routinely passaged with 0.25 % trypsin and 1 mM EDTA·4Na in Hanks solution. All the chemicals and reagents for tissue culture were purchased from Invitrogen Corporation (Carlsbad, CA, USA).

Experimental design

Preliminary experiments were tested with LPS (*Escherichia coli* 055, Wako Pure Chemical Industries, Ltd, Osaka, Japan) at 0.01, 0.1, 1, and 10 μg/ml in AECs. LPS at 1 μg/ml was the appropriate concentration in this study (data not shown). For intracellular and extracellular MCP-1 protein level assays, AECs were treated with 1 μg/ml LPS for 24 h following pretreatment with 12.5, 25, or 50 μM propofol (2,6-diisopropylphenol, Tokyo Chemical Industry Co., Ltd, Japan) for 30 min in medium with 0.5 % FBS. For RNA extraction, AECs were treated with 1 μg/ml LPS for 6 h following pretreatment with 25, 50, or 100 μM propofol, or 1 μM MAPK inhibitors (SB203580, p38 MAPK inhibitor, from Sigma-Aldrich Co., MO, USA; U0126, MEK1/2 inhibitor, from Cell Signaling Technology Inc., MA, USA; and/or SP600125, SAPK/JNK inhibitor, from Calbiochem, CA, USA) for 30 min or 1 h in medium with 0.5 % FBS. For phosphorylated MAPK determination, AECs were treated with 1 μg/ml LPS for 30 min or 1 h following pretreatment with 12.5, 25, or 50 μM propofol for 30 min in medium with 0.5 % FBS. No treatment with propofol, MAPK inhibitors, and/or LPS was used as a control. The resulting cell samples were kept at −80 °C until use.

Profiling cytokines and chemokines

Profiling in the cell culture supernatant was performed according to manufacturer's protocol, using the Proteome Profiler™ Array, Human Cytokine Array Panel A (R&D Systems Inc., MN, USA). Immunocomplexes were visualized by ECL Plus Western Blotting Detection Reagents (GE Healthcare UK Ltd, Buckinghamshire, UK), captured images with a digital camera, and analyzed by computerized densitometric scanning of the images using Lane Analyzer (Rise Co., Ltd and ATTO Corp., Japan).

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from AECs using the RNeasy Mini kit (QIAGEN, Japan). RNA purity and quantification were determined by spectrophotometry (Ultrospec 4000 Spectrophotometer, Pharmacia Biotech Ltd, UK). The RT-PCR reaction was carried out using the OneStep RT-PCR Kit (QIAGEN, Japan) according to the manufacturer's instructions. The PCR primers were 5′-TATGCA GGTCTCTGTCACGC-3′ for forward MCP-1, 5′-TTCCTT ATTGGGGTCAGCAC-3′ for reverse MCP-1, 5′-CGGAG TCAACGGATTTGGTCGTAT-3′ for forward GAPDH, and 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′ for reverse GAPDH. RT-PCR products were analyzed by electrophoresis

in a 2 % agarose gel (Invitrogen Corp., Carlsbad, CA, USA). mRNA was illuminated with UV light and photographed using a digital camera, and analyzed by computerized densitometric scanning of the images using Lane Analyzer (Rise Co., Ltd and ATTO Corp., Japan).

Enzyme-linked immunosorbent assay (ELISA)

To measure the amount of MCP-1 in cell culture supernatant and cell lysate, ELISA was performed according to the manufacturer's protocol using the RayBio® Rat MCP-1 ELISA Kit for serum, plasma and cell culture supernatants, and cell lysate and tissue lysate (RayBiotech Inc., GA, USA). The optical density was determined at 450 nm using a Microplate Reader MTP-450 (Corona Electric Co., Ltd, Japan).

Western blot analysis

AECs were lysed in a 200 μl Tris–SDS–β-Me sample buffer (Daiichi Pure Chemicals Co., Ltd, Tokyo, Japan) with 0.1 % pepstatin, aprotinin and leupeptin as a protease inhibitor (Sigma-Aldrich Co., MO, USA). Protein concentration was determined by the Bradford methods using the Bio-Rad Protein Assay. Equal amounts of proteins were separated electrophoretically on mini-protean precast gels and transferred to supported nitrocellulose membrane. All chemicals and reagents used to measure volumes of protein and electrophoresis were purchased from Bio-Rad Laboratories Inc. (CA, USA). Membranes were probed with the respective primary antibodies to phospho-p38 MAPK (Thr180/Thr182), p38 MAPK, phospho-p44/42 MARK (Thr202/Thr204), p44/42 MAPK, phospho-SAPK/JNK (Thr183/Thr185), SAPK/JNK, Phospho-ATF-2 (Thr71), Phospho-c-Jun (Ser73), and β-actin, and anti-rabbit IgG HRP-linked secondary antibody (Cell Signaling Technology Inc., MA, USA). Immunocomplexes were visualized by ECL Plus Western Blotting Detection Reagents (GE Healthcare UK Ltd, Buckinghamshire, UK).

Statistic analysis

Data were expressed as the mean ± SD. Differences among multiple groups were expressed by one-way ANOVA, followed by the Bonferroni post hoc test. *P* values of <0.05 were considered as statistically significant.

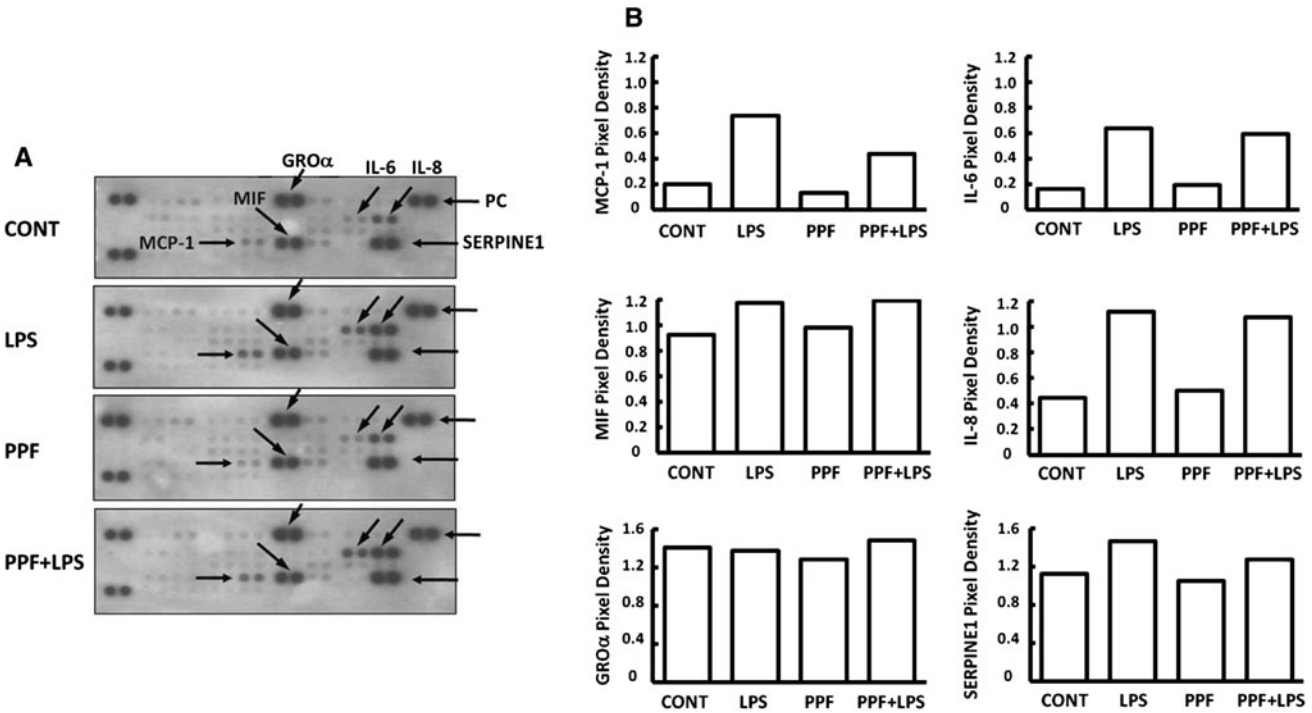

Fig. 1 Profiling cytokines and chemokines inhibited by propofol. A549 cells were treated with 1 μg/ml LPS for 24 h following pretreatment with 50 μM propofol for 30 min. **a** A representative image of cytokine array on profiling cytokines and chemokines by propofol in A549 cell culture supernatant. **b** Protein levels were analyzed by computerized densitometric scanning of images using Lane Analyzer. Values were adjusted for assay variation by dividing the integrated optical density of each protein level by that of the positive control protein level. Positive control (PC) was already prepared by the manufacturer. Results are mean of duplicate. LPS 1 μg/ml, propofol (PPF) 50 μM

Results

Profiling cytokines and chemokines inhibited by propofol

As a first step, we profiled cytokines and chemokines inhibited by propofol in LPS-treated AECs. A representative image of cytokines and chemokines protein levels are shown in Fig. 1a and the results of density analysis are summarized in Fig. 1b. LPS at 1 μg/ml increased MCP-1, macrophage migration inhibitory factor (MIF), IL-6, interleukin-8 (IL-8), and SERPINE1 protein levels in the cell culture supernatant, while 50 μM propofol slightly inhibited IL-6, IL-8, and SERPINE1 secretions in AECs. In particular, propofol led to marked inhibition of MCP-1 secretion. The inhibition of propofol for MCP-1 IL-6, IL-8, and SERPINE1 were 40.79, 6.51, 3.80, and 13.33 %, respectively.

MCP-1 mRNA expression with varying concentrations of propofol in AECs

The effect of MCP-1 mRNA expression by different concentrations of propofol is shown in Fig. 2. LPS at 1 μg/ml significantly increased MCP-1 mRNA expression in AECs ($P < 0.05$). Varying concentrations of propofol (25, 50, and 100 μM) were used to determine whether propofol inhibited LPS-induced MCP-1 mRNA expression in AECs. Propofol at 50 μM and 100 μM dose-dependently inhibited MCP-1 mRNA expression in LPS-treated AECs ($P < 0.05$).

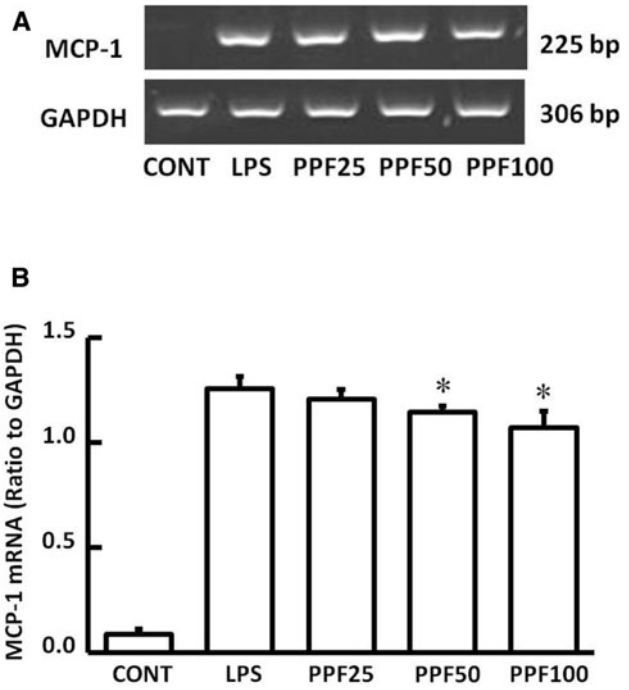

Fig. 2 MCP-1 mRNA expression by different concentrations of propofol in L2 cells. L2 cells were treated with 1 μg/ml LPS for 6 h following pretreatment with 25, 50, or 100 μM propofol for 30 min. **a** A representative image of RT-PCR products of MCP-1 and GAPDH separated on a 2 % agarose gel by electrophoresis. **b** Relative MCP-1 mRNA expression. Values were adjusted for assay variation by dividing the integrated optical density of MCP-1 mRNA by that of GAPDH mRNA. Results are mean ± SD of four trials. LPS 1 μg/ml, propofol 25 μM (PPF25), 50 μM (PPF50), and 100 μM (PPF100). *$P < 0.05$, compared to LPS alone

MCP-1 protein levels with varying concentrations of propofol in AECs

MCP-1 protein levels of the culture supernatant and lysate in AECs are shown in Fig. 3. LPS at 1 μg/ml significantly increased MCP-1 protein levels ($P < 0.05$) of the culture supernatant and lysate in AECs. Propofol at 50 μM significantly decreased MCP-1 protein levels ($P < 0.05$) of the culture supernatant compared to LPS alone in AECs

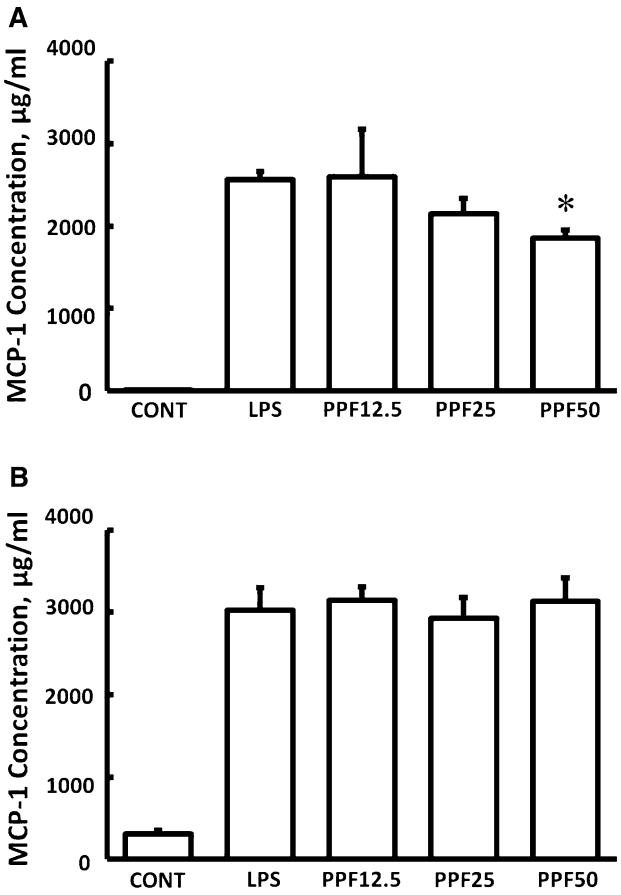

Fig. 3 Extracellular and intracellular MCP-1 protein level by different concentrations of propofol in L2 cell. L2 cells were treated with 1 μg/ml LPS for 24 h following pretreatment with 12.5, 25, or 50 μM propofol for 30 min. MCP-1 protein level in L2 cell was measured by ELISA. **a** Extracellular MCP-1 protein level. **b** intracellular MCP-1 protein level. Results are mean ± SD of four trials. LPS 1 μg/ml, propofol 12.5 μM (PPF12.5), 25 μM (PPF25), and 50 μM (PPF50). *$P < 0.05$, compared to LPS alone

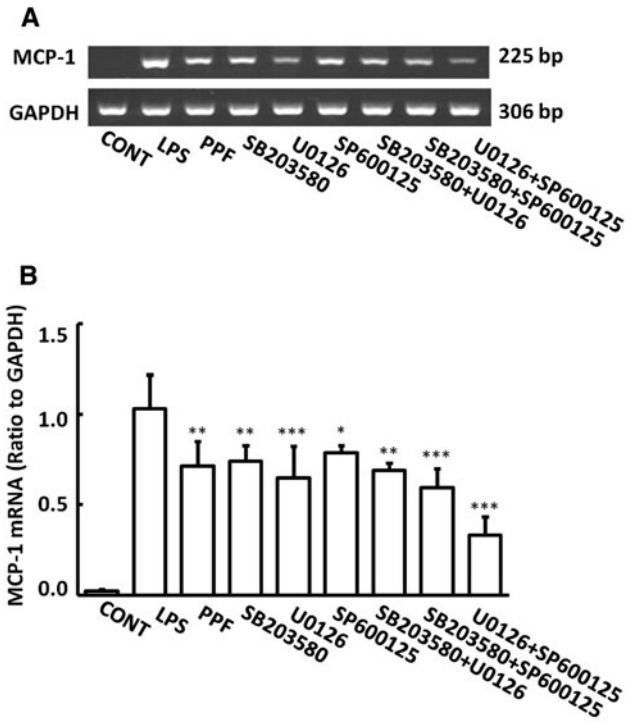

Fig. 4 MCP-1 mRNA expression by MAPK inhibitors in L2 cells. L2 cells were treated with 1 μg/ml LPS for 6 h following pretreatment with 50 μM propofol, 1 μM SB203580, 1 μM U0126, and/or 1 μM SP600125 for 1 h. **a** A representative image of RT-PCR products of MCP-1 and GAPDH separated on a 2 % agarose gel by electrophoresis. **b** Relative MCP-1 mRNA expression. Values were adjusted for assay variation by dividing the integrated optical density of MCP-1 mRNA by that of GAPDH mRNA. Results are mean ± SD of four trials. LPS 1 μg/ml, propofol 50 μM, SB203580; 1 μM, U0126; 1 μM, and SP600125; 1 μM. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$, compared to LPS alone

(Fig. 3a). However, propofol did not reduced MCP-1 protein levels of lysate compared to LPS alone in AECs (Fig. 3b).

Effects of MAPK inhibitors on LPS-induced MCP-1 mRNA expression in AECs

We examined the effects of MAPK inhibitors on LPS-induced MCP-1 mRNA expression, to confirm whether MCP-1 expression is mediated by MAPK in AECs. The effect of MCP-1 mRNA expression by MAPK inhibitors is shown in Fig. 4. SB203580 (p38 MAPK inhibitor 1 μM), U0126 (MEK1/2 inhibitor 1 μM), and SP600125 (SAPK/JNK inhibitor 1 μM) significantly decreased LPS-induced MCP-1 mRNA expression in AECs ($P < 0.01$, 0.001, and 0.5, respectively). Combination of MAPK inhibitors (SB203580 + U0126, SB203580 + SP600125, and U0126 + SP600125) significantly decreased LPS-induced MCP-1 mRNA expression in AECs ($P < 0.01$, 0.001, and 0.001, respectively).

Effects of propofol on LPS-induced MAPK activation in AECs

We hypothesized that propofol attenuates the LPS-induced production of MCP-1 by inhibiting activation of MAPK family in AECs. To assess this hypothesis, AECs were treated with 1 μg/ml LPS for 30 min or 1 h following pretreatment with 12.5, 25, or 50 μM propofol for 30 min, and then the protein levels of phospho-p38 MAPK (Thr180/Thr182), p38 MAPK, phospho-p44/42 MARK (Thr202/Thr204), p44/42 MAPK, phospho-SAPK/JNK (Thr183/Thr185), SAPK/JNK, phospho-ATF-2 (Thr71), phospho-c-Jun (Ser73) and β-actin were determined by Western blot analysis (Fig. 5). Propofol indisputably inhibited LPS-induced phosphorylation of p38 MAPK, p44/42 MAPK and SAPK/JNK ($P < 0.05$). In addition, 1 μg/ml LPS phosphorylated ATF-2 and c-Jun, well-known as a p38 MAPK and SAPK/JNK target, while propofol significantly inhibited LPS-induced ATF-2 (12.5 μM, $P < 0.05$; 25 μM, $P < 0.001$; and 50 μM, $P < 0.01$) and c-Jun (25 μM, $P < 0.05$ and 50 μM, $P < 0.01$) phosphorylation.

Discussion

The anti-inflammatory effects of propofol, an intravenous general anesthetic agent, have attracted growing attention. As a first step in this study, we profiled the cytokines and chemokines inhibited by propofol in LPS-treated AECs. Our results showed that 50 μM propofol inhibited IL-6, IL-8, and MCP-1 secretion; in particular, it had the strongest inhibitory effects on MCP-1 secretion in AECs. Several studies have reported that propofol inhibits the release of inflammatory mediators, including IL-1β, IL-6, and TNF-α in macrophages [1]. However, there is only limited information about the effects of propofol on MCP-1 production in AECs. Thus, we focused on the effects of propofol on MCP-1 expression and production in AECs.

As is widely known, LPS upregulates MCP-1 expression and production in human bronchial epithelial cell and rat type II AECs [21, 22]. MCP-1 has been shown to influence inflammatory processes in rat type II AECs [23] and has been implicated in an accumulation of neutrophil on a model of LPS-induced lung injury [24]. Therefore, MCP-1 plays a key role in acute inflammatory response in ALI [25, 26]. In the present study, propofol significantly inhibited MCP-1 mRNA expression and extracellular MCP-1 protein levels induced by LPS. Thus, propofol may exert its anti-inflammatory effects by inhibiting MCP-1 mRNA expression in AECs.

MAPK is an important intracellular signal transduction system of mediating extracellular signal response in cells. The MAPK family consists of three different subgroups of

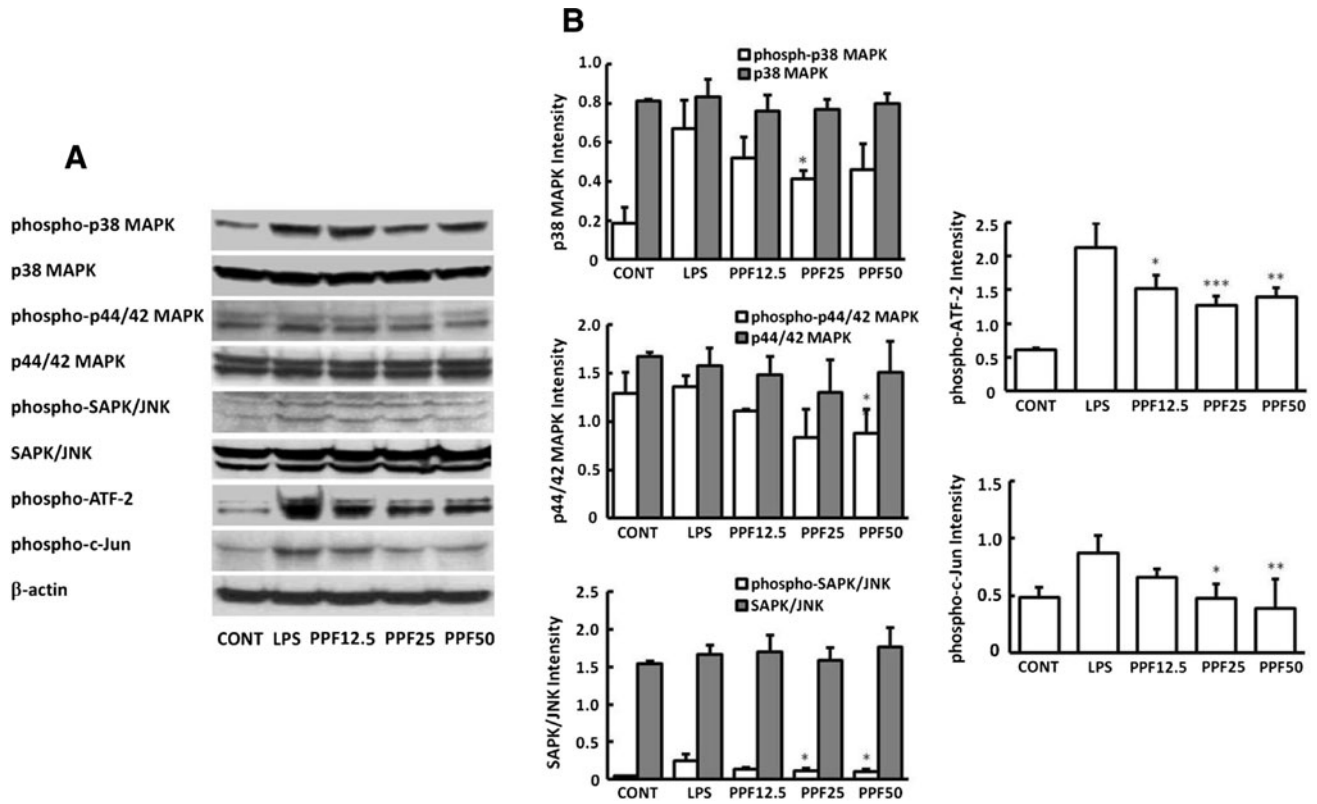

Fig. 5 Inhibitory effect of propofol on LPS-induced MAPK activation in L2 cell. L2 cell were treated with 1 μg/ml LPS for 30 min or 1 h following pretreatment with 12.5, 25, or 50 μM propofol for 30 min. The phosphorylation of p38 MAPK, p44/42MAPK, SAPK/JNK, ATF-2, and c-Jun were measured by Western blot analysis. **a** A representative image of visualized immunocomplexes. **b** Relative density of immunocomplex. Values were adjusted for assay variation by dividing the integrated optical density of immunocomplex by that of β-actin. Results are mean ± SD of four trials. LPS 1 μg/ml, propofol 12.5 μM (PPF12.5), 25 μM (PPF25), and 50 μM (PPF50). $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$, compared to LPS alone

molecules: ERK-1/2, JNK, and p38 MAPK [27]. In particular, the p38 MAPK signal pathway is the primary MAPK signal pathway involved in inflammation. Pro-inflammatory factors, bacterial components, and UV irradiation all activate the p38 MAPK signal pathway [28, 29]. The p38 MAPK signal pathway is activated by the phosphorylation of upstream MAPK kinases, such as mitogen-activated protein kinase kinase 3 (MKK3)/MKK6, and phosphorylate cellular transcription factors including Elk-1 and ATF-2 [29, 30]. SAPK/JNK is also activated by inflammatory cytokines and a wide variety of cellular stresses, and regulates the transcriptional activity of ATF-2 and c-Jun [31].

In the present study, the data showed that LPS induced the phosphorylation of p38 MAPK (Thr180/Thr182), SAPK/JNK (Thr183/Thr185), ATF-2 (Thr71), and c-Jun (Ser73), while propofol inhibited their LPS-induced phosphorylation. Several studies have indicated that propofol inhibits the phosphorylation of p38 MAPK in human monocytic cells, rat pheochromocytoma cells, and human umbilical vein endothelial cells [3, 6, 7], but not JNK or ERK-1/2 activation in rat pheochromocytoma cells [6]. More importantly, Marin et al. [32] reported that the phosphorylation of p38 MAPK and its substrate, ATF-2, participated in thrombin-induced endothelial IL-8 and MCP-1 production in human umbilical vein endothelial cells. Sung et al. [33] reported that the phosphorylation of MKK3/MKK6, p38 MAPK, ATF-2, and Elk-1 were involved in homocysteine-induced MCP-1 expression in endothelial cells. The present results clearly demonstrated that p38 MAPK inhibitor, MEK1/2 inhibitor, and SAPK/JNK inhibitor inhibited LPS-induced MCP-1 mRNA expression in AECs. Our data suggests that propofol attenuates the LPS-induced production of MCP-1 by inhibiting the phosphorylation of p38 MAPK and ATF-2 in AECs.

The clinically targeted blood concentrations of propofol are 3–6 μg/ml for induction and 2–5 μg/ml for maintenance in human [34]. In addition, it was reported that propofol concentrations in the lungs were similar or greater than in blood (the ratio of propofol concentrations in the lungs and blood being in the range of 0.8–2.6) and that lung concentrations declined in parallel with blood concentrations in rats [35]. Thus, the propofol concentrations at 12.5–100 μM (2.23–17.83 μg/ml) in the present

experiments are the range of clinically relevant concentrations during anesthesia. Our results showed that propofol significantly inhibited MCP-1 production at 50 μM and the phosphorylation of p38 MAPK, SAPK/JNK, ATF-2, and c-Jun at 12.5–50 μM in AECs. These results suggest that propofol may modulate inflammatory response at clinically achievable concentrations.

In conclusion, we found that propofol inhibited MCP-1 mRNA expression and secretion, as well as the phosphorylation of p38 MAPK, SAPK/JNK, ATF-2, as the transcription factor known as a p38 MAPK, and c-Jun, as a SAPK/JNK target, induced by LPS in AECs. These results suggest that propofol has the potential to attenuate injury by inhibiting the phosphorylation of p38 MAPK and ATF-2 and thereby inhibiting MCP-1 production and secretion in ALI.

Acknowledgments This study was supported by a Grant (B-03) from Research Institute of Oral Science, Nihon University School of Dentistry at Matsudo.

Conflict of interest None of the authors have any conflicts of interest associated with this study.

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