Propofol Suppresses LPS-Induced Inflammation in Amnion Cells via Inhibition of NF- κ B Activation

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Abstract

BACKGROUND: Preterm labor is a leading risk factor for neonatal death and long-term impairment and linked closely with inflammation. Non-obstetric surgery is occasionally needed during pregnancy and the anesthetic drugs or surgery itself can give rise to inflammation. Here, we examined the influence of propofol pretreatment on the expression of cyclooxygenase-2 (COX-2) and prostaglandin E₂ (PGE₂) after lipopolysaccharide (LPS) stimulation. In addition, we evaluated the expression of pro-inflammatory cytokines and nuclear factor kappa B (NF- κ B).

METHODS: Human amnion-derived WISH cells were used to investigate the effect of propofol on the LPS-induced expression of inflammatory substances involved in preterm labor. For the experiment, WISH cells were pretreated with various concentrations propofol (0.01–10 μ g/ml) for 1 h and then treated with LPS (1 μ g/ml) for 24 h. Cytotoxicity was evaluated using MTT assay. PGE₂ concentration was assessed by ELISA. Protein expressions of COX-2, PGE₂ and NF- κ B were analyzed by western blotting analysis. RT-PCR was used for analysis of mRNA expression of COX-2, PGE₂, interleukin (IL)-1 β and tumor necrosis factor (TNF)- α .

RESULTS: Propofol showed no cytotoxicity on the WISH cells. LPS-induced PGE₂ production and COX-2 and PGE₂ expression were decreased after propofol pretreatment. Propofol also attenuated the LPS-induced mRNA expression of IL-1 β and TNF- α . Moreover, the activation of NF- κ B was inhibited by propofol pretreatment on LPS-stimulated WISH cells.

CONCLUSION: We demonstrated that propofol suppresses the expression of inflammatory substances enhanced by LPS stimulation. Furthermore, this inhibitory effect of propofol on the inflammatory substance expression is mediated by suppression of NF- κ B activation.

Keywords Amnion · NF- κ B · Inflammation · Preterm labor · Propofol

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1 Introduction

An estimated 15 million babies are born preterm (delivered before 37 complete weeks of gestation) every year and this number is rising [1]. Preterm birth increases the morbidity and mortality of neonates and is the second most common cause of death in children under 5 years of age, responsible for approximately 1 million deaths in 2015 [2, 3]. Infection and/or inflammation are known as common causes of preterm labor; in addition, the use of anesthetic agents is associated with preterm labor [4]. Anesthesia during pregnancy is occasionally needed for non-obstetric surgery,

such as cervical cerclage, surgeries for appendicitis, ovarian disorders (torsion or neoplasm), and trauma [5]. The anesthetic agents used for pregnant patients during non-obstetric surgery may have an adverse effect on preterm labor or birth. Therefore, it is necessary to ensure the safety of anesthetic agents for use in pregnant patients.

Infection and/or inflammation are related to early preterm birth and, according to the literature, account for approximately 40% of spontaneous preterm birth [2, 6]. Microbial invasion to the amniotic membrane activates pro-inflammatory signaling cascades, which trigger the production of pro-inflammatory cytokines (e.g., TNF- α and IL-1 β) and cyclooxygenase-2 (COX-2) expression, and sequentially induce prostaglandin (PG) production and myometrial contraction and cervical ripening leading to preterm labor [7, 8]. Among prostaglandins (PGs), prostaglandin E₂ (PGE₂) has been reported as a major labor-inducing factor, and the concentration of PGE₂ increases in the amniotic tissue at the beginning of labor [9].

PGs are formed from arachidonic acid via the action of COX enzyme (COX-1 and COX-2). It is known that COX-2 has a major role in PG production in the amnion during labor. According to the literature, the mRNA concentration of COX-2, rather than COX-1, increases near to full term and with the onset of labor [10]. In the amnion, COX-2 expression is regulated by nuclear factor-kappa B (NF- κ B) [11]. NF- κ B is an inducible transcription factor, which regulates the expression of various genes involved in immune and inflammatory responses [12]. NF- κ B is retained in the cytoplasm in an inactivated form by binding to inhibitory proteins. The activation of NF- κ B is upregulated through the activation of various cytokines, TNF, or Toll-like receptors on the cell surface, which leads to the promotion of inflammatory gene expression, including pro-inflammatory cytokines and COX-2 [13, 14].

Propofol is a widely used as an intravenous anesthetic for general anesthesia and sedation. Despite concerns about the possibility of neonatal depression and teratogenicity, propofol has been used for the induction of general anesthesia during non-obstetrical surgery and cesarean section [15, 16]. In some studies, propofol suppressed PGE₂ synthesis in human mononuclear cell lines and reduced PGE₂ production and COX-2 expression in amniotic membrane cells [17, 18]. In addition, propofol is known to have antioxidant or anti-inflammatory effects [19, 20]. However, there are no studies on the effect of propofol on the expression of pro-inflammatory cytokines and the transcription factors involved in the stimulation of preterm labor.

In this study, we investigated the effect of propofol pretreatment on the expression of PGE₂ and COX-2 on the lipopolysaccharide (LPS)-induced inflammatory response using amnion-derived WISH cells, and analyzed nitric

oxide (NO) production, pro-inflammatory cytokines, and NF- κ B expression.

2 Materials and methods

2.1 Cell culture

The establishment of a line (WISH) of human amnion cells was purchased from the American Type Culture Collection (ATCC® CCL25™, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle medium (DMEM; ATCC® 30-2003™, Manassas, VA, USA), supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) in a 5% CO₂ atmosphere at 37 °C. After 3 days, adherent cells were removed, and the culture was continued, with the medium replaced twice per week.

2.2 Propofol treatment

This study used a commercially available propofol (Fresenius Kabi Austria GmbH, Hafnerstrabe, Austria), which was dissolved in dimethyl sulfoxide (DMSO; Biosesang, Seongnam, Korea) and further diluted in phosphate buffered saline (PBS). Propofol was added to cultured cells at concentrations between 0.01 and 10 μ g/mL for 1 h and then LPS treatment (1 μ g/mL) was applied for 24 h.

2.3 MTT assay

The cells (1×10^5 /well) were seeded in 24-well plates and cultured for 24 h at 37 °C in a 5% CO₂ incubator. Subsequently, the cells were exposed to propofol (0.01–10 μ g/mL) for 1 h and then incubated with LPS (1 μ g/mL) for 24 h. After treatment with propofol, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Affymetrix, Inc. Santa Clara, CA, USA] assay was performed by the addition of 100 μ L MTT solution (5 mg/mL in PBS at pH 7.4) into each well and incubation of the plate at 37 °C. After 1 h, the medium was removed, and 100 μ L DMSO was added into each well. The plate was gently rotated on an orbital shaker for 15 min to completely dissolve the precipitate. The absorbance at 540 nm was detected by using a microplate reader (Bio-Rad Model 680; Bio-Rad, Hercules, CA, USA). All experiments were repeated three times.

2.4 NO assay

WISH cells were seeded at 1×10^4 cells per well into 24-well plates. The concentration of NO in the culture supernatant was determined as nitrite (NO₂[−]) using Griess reagent (Cell Signaling Technologies, Denver, MA, USA).

Briefly, WISH cells were pretreated with propofol (0.01–10 $\mu\text{g/mL}$) for 1 h and then incubated with LPS (1 $\mu\text{g/mL}$) for 24 h. After incubation, the supernatant was collected and mixed with an equal amount of Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 2.5% orthophosphoric acid). The samples were incubated at room temperature for 10 min, and absorbance at 540 nm was detected by using a microplate reader (Bio-Rad Model 680). Each assay was performed in triplicate.

2.5 Measurement of PGE₂ concentration

WISH cells were seeded in 6-well plates at a density of 1×10^5 cells/1.5 mL and cultured at 37 °C in a 5% CO₂ incubator. Then, the cells were pretreated with propofol (0.01, 0.1, 1, and 10 $\mu\text{g/mL}$) for 1 h and incubated in the presence of LPS (1 $\mu\text{g/mL}$) for 24 h. Following the manufacturer's instructions, a volume of 100 μL of cell lysate was collected for the determination of PGE₂ concentration by using the Correlate-EIA high-sensitivity PGE₂ enzyme immunoassay (ELISA) (Enzo Life Sciences, Plymouth Meeting, PA, USA).

2.6 Western blotting

All cells were extracted with chilled RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% NP40, 5 mM DTT, 0.2 mM sodium orthovanadate, 100 mM NaF, and 1 mM PMSF) containing protease inhibitor/phosphatase Inhibitor Cocktail (Cell Signaling Technology, Danvers, MA, USA). Samples (25 μg protein/well) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA).

The membranes were blocked in TBS-0.1% Tween 20 (TBST) containing 3% skim milk for 1 h at room temperature. Subsequently, the membranes were then incubated with α -tubulin (1:1000; Santa Cruz, CA, USA), NF- κ B p65 (1:1000; Santa Cruz, CA, USA), phospho-NF- κ B p65 27.Ser 536 (1:500; Santa Cruz, CA, USA), PGE synthase 2 (A-2) antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA), or COX-2(D5H5) rabbit mAb (1:1000; Santa Cruz, CA, USA) overnight at 4 °C in TBST with 3% skim milk. After three washes with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit (1:1000; Enzo Life Sciences) and anti-mouse (1:1000; Santa Cruz, CA, USA) secondary antibodies for 1 h at room temperature. After three washes with TBST, the bands were visualized by use of the enhanced chemiluminescence detection reagents (Promega, Madison, WI, USA). α -Tubulin expression was used as the control; the target protein bands were normalized relative

to the control band by using the NIH image program (ImageJ Launcher, Bethesda, MD, USA).

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

Total RNA was extracted from WISH cells by using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total mRNA (1 μg) was synthesized to cDNA using oligo (dT) PrimeScriptTM first-strand cDNA Synthesis Kits (TaKaRa Clontech, BD Biosciences, Palo Alto, CA, USA) in accordance with the manufacturer's instructions. RT-PCR was performed on a SimpliAmp Thermal Cycler (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). Polymerase chain reaction (PCR) primers were as follows: COX-2, Forward: 5'-CCT TCC TCC TGT GCC TGA TG-3', Reverse: 5'-CTG GCC TCG CTT ATG ATC T-3'; PGE₂, Forward: 5'-TGA AGG CTG TGA ACG AGC AG-3', Reverse: 5'-CAT TGG GGG AGA TCA GGT GC-3'; IL-1 β , Forward: 5'-CTC GCC AGT GAA ATG ATG GCT-3', Reverse: 5'-GTC GGA GAT TCG TAG CTG GAT-3'; TNF- α , Forward: 5'-CCA GGC AGT CAG ATC ATC TTC-3', Reverse: 5'-GTT ATC TCT CAG CTC CAC GC-3'; β -actin, Forward: 5'-GAC CTG ACT GAC TAC CTC ATG-3', Reverse: 5'-CGC TCA TTG CCA ATG GTG ATG-3'. The amplification of COX-2, PGE₂, IL-1 β , and β -actin was performed for 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension period of 72 °C for 7 min. The amplification of TNF- α and β -actin was performed for 35 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s, with a final extension period of 72 °C for 10 min. The PCR-amplified products were separated on 1.5% agarose gels and stained. The Gel Doc ImageQuant LAS 500 System (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used to assay the PCR products; all target genes were normalized to β -actin. The data were analyzed by using the NIH image program (ImageJ Launcher, Bethesda, MD, USA).

2.8 Statistical analysis

Data values are presented as the mean $\pm$ standard deviation (SD). All experiments were repeated three times. Statistical significance was determined by using SigmaPlot v10 software. *P* value of < 0.05 was considered to indicate statistically significant.

3 Results

3.1 Propofol and LPS were not toxic to WISH cells

The effects of propofol and LPS on the viability of WISH cells were analyzed by MTT assay. WISH cells were

pretreated with various concentration of propofol (0.01–10 $\mu\text{g/mL}$) and then exposed to LPS (1 $\mu\text{g/mL}$). As shown in Fig. 1, there were no significant differences in cell viability at the propofol concentrations tested.

3.2 Propofol did not affect NO production

NO is known to be involved in the relaxation of the smooth muscle myometrial cells, which leads to uterine quiescence throughout gestation [21, 22]. To investigate the effect of propofol on NO production in WISH cells, the concentrations of the stable nitrite end products in all the culture supernatants were measured by the Griess reaction microassay. As shown in Fig. 2, there was no difference in NO production at the pretreated propofol concentrations.

3.3 Propofol pretreatment decreased PGE₂ production and COX-2 and PGE₂ expression in LPS-stimulated WISH cells

We used an ELISA to investigate the effect of propofol on the LPS-induced production of PGE₂. As shown in Fig. 3A, the level of PGE₂ was significantly elevated by LPS stimulation compared with that in the control cells. This elevation was significantly inhibited by propofol pretreatment at 0.1, 1 and 10 $\mu\text{g/mL}$ concentrations compared with that in the LPS group.

To examine the effect of propofol on the mRNA expression of COX-2 and PGE₂, we used RT-PCR analysis. As shown in Fig. 3B, propofol significantly restricted the LPS-induced mRNA expression of COX-2 and PGE₂ at all concentrations of propofol.

The protein expression of COX-2 and PGE₂ was evaluated by western blotting. After treatment with LPS (1 $\mu\text{g/mL}$), the expression of COX-2 and PGE₂ was significantly increased compared with that in the control group. Propofol pretreatment before LPS exposure led to a dose-dependent decrease

Fig. 1 Propofol and LPS did not affect the viability of WISH cells in MTT assay. WISH cells were cultured in media with various concentrations propofol (0.01–10 $\mu\text{g/mL}$) for 1 h and then exposed to LPS (1 $\mu\text{g/mL}$) for 24 h. Data are presented as mean $\pm$ SD. All experiments were repeated three times

Fig. 2 Nitric oxide (NO) production was not changed after LPS and propofol treatment in WISH cells. Stable nitrite end products were measured by the Griess reaction microassay. The results are presented as mean $\pm$ SD. All experiments were repeated three times

in COX-2 expression between 0.01 and 10 $\mu\text{g/mL}$ propofol, with significant differences compared with that in the LPS group. In addition, PGE₂ expression showed a significant decrease at all concentrations of propofol (0.01–10 $\mu\text{g/mL}$) compared with those in the LPS group (Fig. 3C).

3.4 Propofol pretreatment reduced the mRNA expression of IL-1 β and TNF- α in LPS-stimulated WISH cells

To evaluate the effect of propofol on the LPS-induced mRNA expression of pro-inflammatory cytokines (IL-1 β and TNF- α), we used RT-PCR analysis. As shown in Fig. 4, LPS increased the mRNA expression of IL-1 β and TNF- α significantly compared with the control group. Propofol pretreatment exerted an inhibitory effect on the mRNA expression of IL-1 β at concentrations of 0.01 and 10 $\mu\text{g/mL}$, which was statistically significant. However, mRNA expression of TNF- α was significantly reduced by propofol pretreatment at all concentrations (Fig. 4).

Fig. 3 LPS-induced PGE₂ production and COX-2 and PGE₂ expression were inhibited by propofol. WISH cells were cultured in media after treatment with propofol (0.01–10 μg/mL) for 1 h and/or LPS (1 μg/mL) for 24 h. **A** PGE₂ production was analyzed using ELISA. **B** The mRNA expression of COX-2 and PGE₂ was analyzed using RT-PCR analysis. Relative density of the mRNA expression of COX-

2 and PGE₂ was normalized by β-actin. **C** The protein expression of COX-2 and PGE₂ was evaluated by western blotting. Relative density analysis was performed using NIH Image program and normalized by α-tubulin. Values are mean ± SD of three independent experiments. #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 versus control group; **p* < 0.05, ***p* < 0.01, ****p* < 0.001 versus LPS group

3.5 LPS-induced NF-κB activation was inhibited by propofol pretreatment

To investigate the effect of propofol on the activation of NF-κB, which plays a key role in regulation of the expression of various inflammatory genes, western blotting was performed. To analyze the activation of NF-κB, the expression of phospho-NF-κB (phospho-p65, the activated

form of NF-κB) was examined by western blotting. In the LPS group, the expression of phospho-NF-κB was significantly increased compared with that in the control group. The expression of phospho-NF-κB was significantly decreased by propofol pretreatment at 0.01 and 1 μg/mL compared with that in the LPS group. Although there was no significant difference in the expression of phospho-NF-

Fig. 4 Propofol attenuated the LPS-induced mRNA expression of IL-1 β and TNF- α . RT-PCR was used to detect the mRNA expression of IL-1 β and TNF- α in WISH cells and assessed by band densitometry. Relative mRNA level was normalized by β -actin and presented as mean $\pm$ SD of three independent experiments. ### p < 0.01, ### p < 0.001 versus control group; * p < 0.05, ** p < 0.01 versus LPS group

κ B at 0.1 and 10 μ g/mL propofol, it was decreased slightly compared with that in the LPS group (Fig. 5).

4 Discussion

In this study, propofol decreased PGE₂ production and the expression of COX-2 and PGE₂ in LPS-stimulated WISH cells. This result is in agreement with other recent studies that have reported that propofol suppressed PGE₂ production through the inhibition of COX expression in human immune cells or amniotic membranes [17, 18, 23]. Here, we stimulated amnion-derived WISH cells with LPS to induce inflammatory responses that lead to the upregulation of pro-inflammatory cytokines as well as the synthesis of PGs. Bacterial LPS has been used widely in experiments on inflammation because LPS stimulates the release of many inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6 [24]. Intrauterine infection or infection-led activation

of inflammatory responses have been considered as a main risk factor related to spontaneous preterm birth [25, 26]. Therefore, we stimulated the inflammatory response in WISH cells with LPS after propofol pretreatment to verify whether propofol exerts a protective effect against the production of inflammatory substances in WISH cells.

We showed that NO production was not affected by propofol pretreatment in LPS-stimulated WISH cells. Luo et al. [27] reported that NO production was increased by propofol concentrations between 12.5 and 100 μ M in human umbilical vein endothelial cells. In our study, we evaluated the NO concentration in WISH cells using propofol concentrations of 0.01–10 μ g/mL (0.056–56 μ M). The difference in results between the study of Luo et al. [27] and our study may arise from the cell types in each experiment. Each cell type has a different sensitivity to drug and different effect on the activation of nitric oxide synthase (NOS). This is the first study to investigate the effect of propofol on NO production in amnion-derived

Fig. 5 LPS-induced NF- κ B activation was inhibited by propofol pretreatment in WISH cells. Protein expression of activated NF- κ B was examined using western blotting analysis by immunoblotting for phospho-NF- κ B (phospho-p65). Relative amounts of protein were analyzed by triplicate experiments and normalized by NF- κ B. ### $p < 0.001$ versus control group; ** $p < 0.01$ versus LPS group

WISH cells, but the results are inconclusive. Therefore, further study is required to demonstrate the influence of propofol on NO production in the amnion.

In previous studies, the molecule NO, a free radical generated from the amino acid L-arginine by NOS, is known to induce uterine quiescence during pregnancy [28, 29]. However, some literature showed that NOS activity and localization was not changed in fetal membranes and that NO metabolites were increased in amniotic fluid during labor [30–32]. Therefore, the role of NO produced by the fetal membrane and placenta is still under debate.

In this study, we used propofol concentrations between 0.01 and 10 μ g/mL with reference to previous study with a similar study design [18]. Our data demonstrate that propofol treatment at this range was not toxic to WISH cells. It was reported that the blood concentrations of propofol in clinical use were 0.8–1.0 μ g/mL to induce awakening from anesthesia, 1–2 μ g/mL for long-term intensive care unit sedation, and 3–11 μ g/mL to maintain general anesthesia [33]. Therefore, the propofol concentrations used in this study are appropriate for clinical practice.

It has been demonstrated that NF- κ B is important in the expression of COX-2 in amnion-derived WISH cells [34, 35]. Therefore, we hypothesized that the decrease in PGE₂ and COX-2 induced by propofol may be mediated

through blocking of NF- κ B activation. NF- κ B is composed of five subunits: NF- κ B1 (encoding p50 and its precursor p105), NF- κ B2 (encoding p52 and its precursor p100), RelA (p65), RelB, and c-Rel, mainly with p65 and p50 subunits [36]. NF- κ B is sequestered in the cytoplasm in an activated form by I κ B proteins (inhibitory protein) or by the p105 and p100 subunits, where they act as I κ B proteins [37]. The activation of NF- κ B occurs through two signaling pathways, the canonical and non-canonical pathways. Canonical NF- κ B activation involves the degradation of I κ B by I κ B kinase (IKK) complex and p50/Rel A and p50/c-Rel dimers were predominant; in contrast, noncanonical NF- κ B activation is mediated by the phosphorylation of p100, the NF- κ B2 precursor protein. Subsequently, mature NF- κ B2 p52 was generated and led to the nuclear translocation of p52/RelB [38, 39]. However, the mechanism of NF- κ B activation in the amnion prior to labor is not established. In this study, we examined NF- κ B activation through the detection of phosphorylated p65 expression by using western blotting and found that it was related to the canonical NF- κ B pathway. However, additional experiments with regard to the noncanonical pathway are needed to confirm the mechanism of NF- κ B activation in WISH cells.

In conclusion, we have shown that propofol attenuated LPS-induced PGE₂ production, as well as the expression of COX-2, PGE₂, IL-1 β , and TNF- α in amnion-derived WISH

cells. These results suggested that propofol exerts a protective effect on preterm labor induced by the inflammatory response. Moreover, this study showed that LPS-induced NF- κ B activation was inhibited by propofol pretreatment at 0.01 and 1 μ g/mL. On the basis of this result, we provided the evidence that a decrease in the expression of inflammatory substances induced by propofol may occurred through the inhibition of NF- κ B activation. Although additional studies are necessary to identify the effect of propofol on preterm labor, we propose that propofol can confer protection against preterm labor induced by PGE₂ and pro-inflammatory cytokines when used during pregnancy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Statement There are no animal or human experiments carried out for this article.

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