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Anesthetic Propofol Causes Glycogen Synthase Kinase-3β-regulated Lysosomal/mitochondrial Apoptosis in Macrophages

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Summary

**Background:** Overdose of propofol treatment following a prolong treatment causes injury to multiple cell types including cardiomyocytes, skeletal muscle cells, neuron cells, and immune cells; however, its molecular mechanisms remain unclear. Activation of glycogen synthase kinase (GSK)-3β is pro-apoptotic under death stimuli. We therefore hypothesize that propofol overdose induces macrophage apoptosis through GSK-3β.

**Methods:** Phagocytic analysis by uptaking fluorescence-labelled *Staphylococcus aureus* showed the effects of propofol overdose-treated murine macrophages RAW264.7 and BV2 and primary human neutrophils *in vitro*. We further investigated cell apoptosis *in vitro* and *in vivo*, lysosomal membrane permeabilisation (LMP), and the loss of mitochondrial transmembrane potential (MTP) by propidium iodide, annexin V, acridine orange, rhodamine 123 staining, respectively. Protein analysis identified the expression and activation of apoptotic signals, and pharmacological inhibition and genetic knockdown using lentiviral-based short hairpin RNA were further used to clarify the roles of apoptotic signals.

**Results:** A high dose of propofol caused phagocytic inhibition and apoptosis *in vitro* for 24 h (25 μg/ml) and *in vivo* for 6 h (10 mg/kg/h). Propofol induced LMP and MTP loss while stabilising MTP and inhibiting caspase protected cells from mitochondrial apoptosis. Activation of lysosomal cathepsin B was required for propofol-induced LMP, MTP loss, and apoptosis. Propofol decreased anti-apoptotic Bcl-2 family proteins and then caused pro-apoptotic Bax activation. Propofol activated GSK-3β and inhibiting GSK-3β prevented Mcl-1 destabilization and lysosomal/mitochondrial apoptosis. Forced expression of Mcl-1 prevented the apoptotic effects of propofol. Decreased Akt was important for GSK-3β activation caused by propofol.

**Conclusions:** These results suggest an essential role of GSK-3β in propofol-induced lysosomal/mitochondrial apoptosis, and targeting GSK-3β is a new strategy against overdose propofol-induced apoptosis.
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Introduction

The anesthetic propofol (2,6-diisopropylphenol) is widely used for short-term sedation in surgical procedures.\(^1\) Under therapeutic conditions, the anesthetic concentrations of propofol used for clinical medication is less than 5 mg/kg/h to provide satisfactory sedation. In addition to its anesthetic properties, a safe range of doses of propofol is neuroprotective against ischemia-reperfusion\(^2-3\) and has cardiovascular benefits against oxidative stress.\(^4-7\) Propofol has immunomodulating actions by decreasing production of pro-inflammatory cytokines and inhibiting neutrophil functions.\(^8-13\) However, abuse of propofol treatment causes severe complications in patients with critical illness and is called propofol infusion syndrome (PRIS).\(^14,15\) Clinical manifestations and pathological observation showed metabolic imbalance and a variety of cellular injuries in PRIS patients, including lypemic plasma, fatty liver enlargement, metabolic acidosis, rhabdomyolysis, and myoglobinuria. In the immune system, propofol overdose impairs immune responses and causes increased susceptibility to severe infection.\(^14\) However, the molecular mechanisms for propofol overdose-induced immune suppression remain unclear, and its effects on cytotoxic injury need further investigation.

Chen et al.\(^16\) reported that propofol (3, 30, or 300 \(\mu\)M) suppresses macrophage function through the disruption of mitochondrial transmembrane potential (MTP) and cellular adenosine triphosphate synthesis. However, there is no significant cellular injury caused by propofol. Nevertheless, the pro-apoptotic effects of propofol have been previously demonstrated \textit{in vitro} and \textit{in vivo}.\(^17,18\) Tsuchiya et al.\(^19\) found that treatment of human promyelocytic leukaemia HL-60 cells with propofol (150 or 250 \(\mu\)M) resulted in growth inhibition accompanied by death receptor-associated activation of the caspase cascade followed by the mitochondrial pathway of apoptosis. Interestingly, Straiko et al.\(^18\) demonstrated that propofol (50 or 100 mg/kg) suppresses the phosphorylation of extracellular signal-regulated kinase (ERK), an important survival kinase, and causes caspase-3 activation to induce developmental neuroapoptosis in mouse brain. The molecular mechanisms for propofol-induced cell apoptosis remain unclear.

Dysregulation of intracellular organelles is generally processed for cell apoptosis involving either intrinsic or extrinsic pathways.\(^19,20\) The loss of MTP induces mitochondrial membrane permeabilisation (MMP) causing the formation of the apoptosome at the onset of mitochondrial apoptosis.\(^21,22\) In addition, apoptotic stimuli cause lysosomal membrane permeabilisation (LMP) through calcium, reactive oxygen species (ROS), ceramide, sphingosine, phospholipase, Bax, Bim, Bid, and caspases, and most of them are also regulators of MMP.\(^20,23,24\) After LMP, lysosomal cathepsin B and cathepsin D translocate to the cytoplasm and cause Bid truncation,
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caspase activation, and the loss of MTP before mitochondrial damage.\textsuperscript{25} It is speculated that lysosomes may function as death signal integrators to link the crosstalk in mitochondrial apoptosis.\textsuperscript{26}

Overexpression of glycogen synthase kinase (GSK)-3β or blockage of phosphatidylinositol 3-kinase (PI3K)/Akt, the negative regulator of GSK-3β, causes cells to undergo apoptosis.\textsuperscript{27,28} Generally, protein phosphatases (PPases) such as PP1 and PP2A dephosphorylate GSK-3β at its serine residue (Ser9) and activate it directly or indirectly by down-regulating PI3K/Akt, p70 S6 kinase (S6K), ERK, p38 mitogen-activated protein kinase (MAPK), and integrin-linked kinase (ILK), which are the negative regulators of GSK-3β.\textsuperscript{29-38} Furthermore, tyrosine phosphorylation of GSK-3β (Tyr216) by calcium-activated Pyk2 causes GSK-3β activation.\textsuperscript{39} A number of studies have demonstrated the apoptotic signalling cascades generally regulated by GSK-3β.\textsuperscript{39,40-42} For apoptosis, GSK-3β can phosphorylate Bax to promote mitochondrial injury.\textsuperscript{43} In addition, GSK-3β can phosphorylate Mcl-1 and cause its degradation via an ubiquitin-proteasome system.\textsuperscript{44} Mcl-1 has been demonstrated as being involved in maintaining either MMP or LMP.\textsuperscript{45,46} It is notable that lithium chloride, a GSK-3β inhibitor, reverses propofol-inactivated ERK as well as caspase-3 activation.\textsuperscript{18} However, the role of GSK-3β signalling has not been further characterised. In the present study, we found an essential role of GSK-3β in propofol overdose-induced lysosomal/mitochondrial apoptosis. GSK-3β regulated Mcl-1 stability upstream of LMP and then caused MTP loss through lysosomal cathepsin B. The activation of GSK-3β and its apoptotic effects in macrophages were also studied.
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Materials and Methods

Cell Cultures

Murine macrophages RAW264.7 and BV2 were provided by C-C Huang, MD, Department of Pediatrics, College of Medicine, National Cheng Kung University. Human HepG2 hepatoma cells were provided by H-Y Lei, PhD, Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University. Cells were routinely grown on plastic in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine and 15 mM HEPES supplemented with 10% foetal bovine serum (FBS), 100 units of penicillin, and 100 µg/ml of streptomycin and maintained at 37°C in 5% CO₂. Cells were used at a passage of 7 to 10 in this study.

Human peripheral whole blood, donated by healthy volunteer, was suspended in 4% dextran (Sigma-Aldrich, St Louis, MO, USA) at room-temperature for 30 min and collected supernatant. Then, human peripheral blood leukocytes suspension was gently overlaid onto Ficoll-plaque™ plus (GE Healthcare, Amersham Biosciences, Sweden), and centrifuged at 1,800 rpm for 20 min. Pallet containing neutrophils were collected, washed, and resuspended in RPMI 1640 medium (Invitrogen Life Technologies) with 10% FBS.

Animal treatment

The six to eight-week-old male progeny of BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, Me). They were fed standard laboratory chow and water ad libitum in the Laboratory Animal Center of National Cheng Kung University. The animals were raised and cared for according to the guidelines set up by the National Science Council, Taiwan. Experimental protocols adhered to the rules of the Animal Protection Act of Taiwan and were approved by the Laboratory Animal Care and Use Committee of National Cheng Kung University (IACUC Approval No.: 99013). Mice (n = 5 for each group) were intraperitoneally (IP) or intravenously (IV) injected with propofol, which was prepared from Diprivan (Zeneca Limited, Macclesfield, Cheshire, UK) dissolved in sterile PBS, for 6 h (10 mg/kg/h for IP and 5 mg/kg/h for IV). The vehicle control contains glycerol, soybean oil, purified egg phosphatide/egg lecithin, sodium hydroxide, and water.

Materials

The broad-spectrum caspase inhibitor, benzylxoycarbonyl-Val-Ala-Asp(O-Me)-fluoro methyl ketone (z-VAD-fmk), caspase-8 inhibitor benzylxoycarbonyl-Ile-Glu(O-Me)-Thr-Asp(O-Me)-fluoromethyl ketone (z-IETD-fmk), and caspase-3 inhibitor benzylxoycarbonyl-Asp(O-Me)-Glu(O-Me)-Val-Asp(O-Me)-fluoromethyl
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eketone (z-DEVD-fmk) were purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO). Cathepsin B inhibitor benzoyloxycarbonyl-Phe-Ala-fluoromethyl ketone (z-FA-fmk), cathepsin D inhibitor pepstatin A, GSK-3β inhibitors SB415286 and BIO, cyclosporin A (CSA), FK506, and proteasome inhibitor MG132 were purchased from Calbiochem (San Diego, CA). DAPI, propidium iodide (PI), and acridine orange were purchased from Sigma-Aldrich. PP2A inhibitor okadaic acid (OA), antioxidants diphenylene iodonium (DPI) and caffeic acid phenethyl ester (CAPE), intracellular calcium chelator BAPTA, Pyk2 inhibitor tyrphostin A9, PI3K inhibitor LY294002, mTOR inhibitor rapamycin, and MEK inhibitor PD98059 were obtained from Sigma-Aldrich and dissolved in DMSO prior to dilution with PBS and use in experiments. Rabbit anti-mouse Akt, Akt (Ser473), GSK-3α/β, GSK-3β (Ser9), GS, ERK, ERK (Thr202/Tyr204), p38 MAPK, p38 MAPK (Thr180/Tyr182), p70 S6K, p70 S6K (Thr389), Mcl-1, Bcl-2, Bcl-xL, active Bax, PARP, and PTEN were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). β-actin antibodies and horseradish peroxidase-conjugated anti-rabbit IgG were obtained from Chemicon. All drug treatments in cells were assessed for their cytotoxic effects using cytotoxicity assays before experiments. Non-cytotoxic dosages were used in this study.

**Phagocytic Analysis**

*S. aureus* was obtained from C-F Shen, MD, Department of Pediatrics, College of Medicine, National Cheng Kung University and fixed with 1% formaldehyde in phosphate-buffered saline (PBS) and then stained with PI (Sigma-Aldrich). Macrophages (2 x 10^5) were treated with or without propofol for 6 h at 37°C. Cells were then co-cultured with PI-stained *S. aureus* (2 x 10^7 cocci) for 1 h at 37°C. For flow cytometric analysis, the cells were washed twice with PBS and analyzed using a FACSCalibur (BD Biosciences, San Jose, CA) with excitation set at 488 nm and emission detected with FL-2 channel (565-610 nm).

**Viability Assay**

To evaluate cell viability, WST-8 assay (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), based on the extracellular reduction of WST-8 by NADH produced in the mitochondria via trans-plasma membrane electron transport and an electron mediator, was assayed using a colorimetric assay (WST-8 Detection kit; Dojindo Molecular Technologies, Gaithersburg, MD) according to the manufacturer’s instructions. The cells were cultured in 96-well tissue culture plates in DMEM medium with propofol treatment. WST-8 reagent (5
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µl/well) was added after 24 h of culture. A microplate reader (Spectra MAX 340PC; Molecular Devices, Sunnyvale, CA, USA) was used to measure the absorbance at 450 nm and data was analyzed with Softmax Pro software (Molecular Devices).

Cytotoxicity Assay

To evaluate cell damage, lactate dehydrogenase (LDH) activity was assayed using a colorimetric assay (Cytotoxicity Detection kit; Roche Diagnostics, Lewes, UK) according to the manufacturer’s instructions. Aliquots of the culture media were transferred to 96-well microplates. A microplate reader (Spectra MAX 340PC) was used to measure the absorbance at 620 nm with a reference wavelength of 450 nm and data was analyzed with Softmax Pro software.

Apoptosis Assay

Apoptosis was analyzed using PI staining as described previously \(^{47}\) and then analyzed using flow cytometry (FACSCalibur) with excitation set at 488 nm and emission detected with FL-2 channel (565-610 nm). The levels of apoptosis were reported and gated as percentages of sub-G1. \(^{29}\) In addition to PI staining, annexin V staining was also performed using a commercial kit (Sigma-Aldrich) according to the manufacturer’s instructions. To observe nuclear condensation, 4’,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich)-stained cells were observed using a fluorescence microscope (IX71; Olympus, Tokyo, Japan).

Complete blood counter (CBC) test

CBC test was conducted on peripheral whole blood collected in heparinized Pasteur pipettes by retro-orbital venipuncture. Analysis was conducted using a *scil Vet Focus* \(^{TM}\) 5 hematology analyzer (scil animal care company, Gurnee, IL).

LMP Assay

Lysosomal membrane stability was evaluated by determining the uptake of acridine orange (Sigma-Aldrich) as described previously. \(^{42}\) Briefly, cells were treated with 5 µg/ml of acridine orange in serum-free DMEM for 15 min at 37 °C and then washed with PBS. Using flow cytometer (FACSCalibur), emissions from fluorescent acridine orange were detected by FL-3 channel (>650 nm) with excitation wavelength at 488 nm.

Mitochondrial Functional Assay

The loss of MTP value was determined using rhodamine 123 (Sigma-Aldrich) as described previously. \(^{42}\) Cells were incubated with 50 µM of rhodamine 123 in cultured medium for 30 min
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at 37 °C. After being washed with PBS, cells were resuspended in cold PBS and immediately underwent flow cytometric analysis (FACSCalibur) with excitation wavelength at 488 nm and emission detected with FL-1 channel (515-545 nm).

Western Blotting

Harvested cells were lysed with a buffer containing 1% Triton X-100, 50 mM of Tris (pH 7.5), 10 mM of EDTA, 0.02% NaN3, and a protease inhibitor cocktail (Roche Boehringer Mannheim Diagnostics, Mannheim, Germany). Following one cycle of freeze-thaw, cell lysates were centrifuged at 10,000 × g at 4°C for 20 min. Lysates were boiled in sample buffer for 5 min.

The proteins were then subjected to SDS-PAGE and transferred to PVDF membrane (Millipore, Billerica, MA, USA) using a semi-dry electroblotting system. After blocking with 5% skim milk in PBS, the membranes were incubated with a 1/1000 dilution of primary antibodies at 4°C overnight. The membranes were then washed with 0.05% PBS-Tween 20 and incubated with a 1/5000 dilution of horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. After washing, the membranes were soaked in ECL solution (PerkinElmer Life Sciences Inc., Boston, MA, USA) for 1 min, and then exposed to film (BioMax; Eastman Kodak, Rochester, NY, USA). The relative signal intensity was quantified using ImageJ software (version 1.41o) from W. Rasband (National Institutes of Health, Bethesda, MD) (http://rsb.info.nih.gov/ij/).

Lentiviral-based Short Hairpin RNA (shRNA) Transfection

GSK-3β and PTEN knockdown in RAW264.7 cells were performed using lentiviral transduction to stably express shRNA that targeted GSK-3β and PTEN, respectively. shRNA clones were obtained from the National RNAi Core Facility located at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica, Taiwan. The mouse library should be referred to as TRC-Mm 1.0. The construct that was most effective in RAW264.7 cells (TRCN0000012615 containing the shRNA target sequence 5′-CATGAAAGTTAGCAGAGATAA-3′ for mouse GSK-3β and TRCN0000028992 containing the shRNA target sequence 5′-GCTAGAACCTTATCAAACCCCTT-3′ for mouse PTEN) and in HepG2 cells (TRCN0000040001 containing the shRNA target sequence 5′-GCTGAGCTGTACTAGGACAA-3′ for human GSK-3β) was used to generate recombinant lentiviral particles. Human TE671 cells were co-transfected with two helper plasmids pCMVdeltaR8.91 and pMD.G (gift from Dr. H. K. Sytwu, Graduate Institute of Life Sciences, National Defense Medical Center, Taiwan.), plus pLKO.1-puro-shRNA, using GeneJammer
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transfection reagent (Stratagene). The transfected cells are incubated at 37°C in an atmosphere of 5% CO₂ for 24 h, and then the medium is replaced with fresh medium. Cell supernatants containing the viral particles were harvested at 36, 48, 60 and 72 h after transfection. The supernatants were filtered using a 0.45-μm low-protein-binding filter and concentrated by centrifugation at 20,000 × g at 4°C for 3 h using a JA25.50 (Beckman) rotor. The virus pellets were re-suspended with fresh medium and stored at -80°C. Cells are transduced by lentivirus with appropriate multiplicity of infection in complete growth medium supplemented with 8 μg/ml polybrene. After transduction for 24 h, protein expression is monitored using Western blot analysis.

Mcl-1 Overexpression

For Mcl-1 overexpression, pcDNA3-HA and pcDNA3-HA-Mcl-1 were kindly provided by Dr. H-F Yang-Yen (Institute of Molecular Biology, Academia Sinica, Taiwan). These plasmids were prepared for transfection using a plasmid miniprep kit (Bio-Rad). For transfection, Lipofectamine 2000 (Invitrogen) reagent and 4 μg DNA plasmids were used.48

Statistical Analysis

Values are expressed as means ± standard deviation (SD). Groups were compared using Student’s two-tailed unpaired t test or one-way ANOVA analysis followed by Dunnet post-hoc test, as appropriate. Statistical significance was set at P < 0.05.
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Results

Propofol Overdose Induces Phagocytosis Inhibition and Apoptosis in Phagocytes and Causes Leukopenia and Neutropenia In Vivo

Propofol overdose causes cellular cytotoxicity results via the death of multiple cell types. In this study, we investigated the effects of propofol on cell survival and cytotoxicity in RAW264.7 murine macrophages. Viability and cytotoxicity analysis, using WST-8 and LDH, respectively, showed that propofol did not cause RAW264.7 cell death until the overdose of 12.5 μg/ml (84 μM). According to our results, the concentration at which 50% of cells were killed (LC50) by propofol in RAW264.7 cells was 25 μg/ml (140 μM; data not shown). These results indicate that propofol induces cell death depending on the dosage of the treatment.

To test the cytopathogenic effects of propofol, we used clinically relevant (10 μg/ml or 56 μM) or overdose (25 μg/ml) propofol to test its effects on phagocytosis in RAW264.7 (fig. 1A) and BV2 (fig. 1B) macrophages. The dose responses of propofol-induced phagocytic inhibition in these cells were shown by uptake of PI-stained, heat-killed S. aureus followed by flow cytometric analysis. We found that propofol overdose effectively reduced phagocytic activity in RAW264.7 (100% with vehicle and 84.5 ± 3.2% with propofol overdose) and BV2 (100% with vehicle and 54.4 ± 4.3% with propofol overdose) macrophages. In addition to macrophages, propofol also decreased the phagocytic activity in primary human neutrophils (fig. 1E, left).

We examined the cytotoxic effects of propofol, using PI staining followed by flow cytometric analysis. These analyses showed that propofol overdose caused RAW264.7 (fig. 1C, top) and BV2 (fig. 1D, top) to undergo apoptosis. DAPI staining confirmed this result by characterising the presence of chromatin condensation and fragmentation only in propofol overdose-treated macrophages (figs. 1C and D, bottom). Annexin V staining also showed that propofol overdose induced apoptosis of human neutrophils (fig. 1E, right). These results show that propofol overdose inhibits phagocytosis and induces apoptosis in murine macrophages as well as human neutrophils.

To examine the in vivo cytotoxic effects by propofol overdose, we did the in vivo experiment using BALB/c mice. The clinical dosage of propofol is less than 5 mg/kg/h. For propofol overdose used in this study, mice (n = 5) were intraperitoneally (10 mg/kg/h) or intravenously (5 mg/kg/h) injected with propofol for 6 h. Annexin V staining showed that propofol overdose significantly (P < 0.05) caused peritoneal macrophage apoptosis in vivo (fig. 1F). However, there was no increase apoptotic cells detected in peripheral blood cells, which might be caused...
Propofol causes macrophage apoptosis by rapid clearance in circulation \textit{in vivo} (data not shown). Meanwhile, the number of white blood cells and neutrophils in circulation was significantly ($P < 0.05$) decreased in propofol overdose-treated mice (fig. 1G). These results show the \textit{in vivo} cytotoxic effects of propofol overdose.

\textit{Propofol Overdose Induces LMP, MTP Loss, and Caspase-dependent Cell Apoptosis}

Dysregulation of intracellular organelles and activation of the caspase cascade are generally involved in cell apoptosis.\textsuperscript{19} To investigate the molecular mechanisms of propofol overdose (25 $\mu$g/ml)-induced apoptosis, the time kinetics (fig. 2A) of propofol-induced LMP in RAW264.7 cells were shown by a metachromatic fluorophore acridine orange staining followed by flow cytometric analysis. Using lipophilic cationic fluorochrome, rhodamine 123 staining, we found that propofol significantly ($P < 0.001$) and time-dependently induced MTP loss (fig. 2B) in RAW264.7 cells. In addition, PI staining followed by flow cytometric analysis showed that propofol caused significant ($P < 0.001$) cell apoptosis in RAW264.7 via a time-dependent manner (fig. 2C). The induction of LMP was earlier and more severe in propofol overdose-treated RAW264.7 cells than controls. These results indicate that propofol overdose induces lysosomal destabilization and mitochondrial damage followed by apoptosis.

To further investigate whether the mitochondrial pathway is involved in propofol overdose-induced RAW264.7 cell apoptosis, experimental approaches by stabilising MTP and inhibiting caspases were performed. CSA (1 $\mu$M), a mitochondrial permeability transition pore stabiliser, significantly ($P < 0.001$) inhibited propofol overdose-induced apoptosis (fig. 2D). FK506 (1 $\mu$M), a PP2B inhibitor similar to CSA, did not reverse propofol overdose-induced cell apoptosis. Using PI staining followed by flow cytometric analysis, we found that treatment with the pan-caspase inhibitor z-VAD-fmk (20 $\mu$M) and caspase-3 inhibitor z-DEVD-fmk (20 $\mu$M) effectively ($P < 0.001$) blocked propofol overdose-induced apoptosis in RAW264.7 cells (fig. 2E). Furthermore, inhibiting caspase-8 using z-IETD-fmk (20 $\mu$M) also effectively ($P < 0.001$) decreased propofol-induced apoptosis in macrophages. These results demonstrate that propofol overdose induces mitochondrial apoptosis as well as death receptor-regulated pathway followed by causing caspase-dependent apoptosis.

\textit{Propofol Overdose Induces Lysosomal/mitochondrial Apoptosis via a Lysosomal Protease Cathepsin B-regulated Manner}

Proteases cathepsin B/L and cathepsin D are mainly expressed in lysosomes and are activated and released following LMP.\textsuperscript{20,25} We thus examined the effects of lysosomal cathepsins on
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propofol overdose-induced lysosomal/mitochondrial apoptosis in RAW264.7 cells. PI staining followed by flow cytometric analysis showed that treatment of the cathepsin B inhibitor z-FA-fmk (20 μM) but not the cathepsin D inhibitor pepstatin A (10 μg/ml) significantly \( (P < 0.001) \) blocked propofol overdose-induced RAW264.7 cell apoptosis (fig. 3A). Using the cathepsin B activity assay, results showed that propofol overdose caused significant \( (P < 0.001) \) activation of lysosomal cathepsin B (fig. 3B). Using acridine orange and rhodamine 123 staining followed by flow cytometric analysis, respectively, we found that inhibiting cathepsin B with z-FA-fmk significantly \( (P < 0.001) \) blocked propofol overdose-induced LMP (fig. 3C) and MTP loss (fig. 3D). These results show that propofol overdose induces LMP followed by cathepsin B-regulated lysosomal/mitochondrial apoptosis.

**GSK-3β-mediated Mcl-1 Destabilization Is Involved in Propofol Overdose-induced Lysosomal/Mitochondrial Apoptosis**

Bcl-2 family proteins are important for controlling lysosomal/mitochondrial function.\(^{19,49}\) We next investigated the effects of propofol overdose on the expression of Bcl-2 family proteins, including anti-apoptotic Mcl-1, Bcl-2, and Bcl-xL and pro-apoptotic Bax in RAW264.7 cells. Western blot analysis showed that treatment of propofol effectively decreased the expression of Mcl-1 and Bcl-2, but not Bcl-xL, and induced the activation of Bax (fig. 4). These results indicate a dysregulation effect of propofol overdose on the expression of Bcl-2 family proteins followed by the induction of lysosomal/mitochondrial apoptosis.

GSK-3β down-regulates Mcl-1 through a mechanism involving phosphorylation followed by proteasome-mediated degradation.\(^{44}\) Upon propofol overdose treatment in RAW264.7 cells, Western blot analysis showed that GSK-3β was activated as shown by its dephosphorylation at serine 9 and by the down-regulation of its substrate GS (fig. 5A) as showed previously.\(^{50}\) Using a lentiviral-based shRNA approach, propofol overdose-induced Mcl-1 and GS destabilization and PARP cleavage was defective in GSK-3β-silencing cells (fig. 5B). These results demonstrate that GSK-3β mediates Mcl-1 destabilization in propofol overdose-treated cells. Using acridine orange, rhodamine 123, and PI staining, respectively, we found that propofol overdose significantly \( (P < 0.001) \) induced LMP (fig. 5C, top), MTP loss (fig. 5C, middle), and apoptosis (fig. 5C, bottom) via a GSK-3β-dependent manner. To further confirm the effects of GSK-3β activity, a pharmacological inhibition approach was used. Treating RAW264.7 cells with the GSK-3β inhibitor SB415286 showed a significant \( (P < 0.001) \) decrease in propofol overdose-induced LMP (fig. 5D, top), MTP loss (fig. 5D, middle), and apoptosis (fig. 5D,
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bottom). Furthermore, we found that the proteasome inhibitor MG132 significantly \( (P < 0.001) \) inhibited propofol-induced LMP (fig. 5E, top), MTP loss (fig. 5E, middle), and apoptosis (fig. 5E, bottom). Furthermore, annexin V staining showed that propofol overdose significantly \( (P < 0.05) \) induced GSK-3-dependent apoptosis in RAW264.7 and HepG2 cells, as demonstrated by GSK-3\(\beta\) knockdown approach, and primary human neutrophils, as demonstrated by treatment of GSK-3 inhibitor BIO (fig. 5F). These results demonstrate that propofol overdose causes GSK-3\(\beta\)-regulated Mcl-1 destabilization followed by lysosomal/mitochondrial apoptosis.

Overexpression of Mcl-1 Reduces Propofol Overdose-induced Lysosomal/mitochondrial Apoptosis

To test the hypothesis of whether deregulated Mcl-1 acts upstream of LMP in propofol overdose-induced lysosomal/mitochondrial apoptosis, we next examined whether Mcl-1 is important for lysosomal stabilisation as demonstrated previously.\(^{45}\) Using an overexpression approach, forced expression of Mcl-1 in RAW264.7 cells was resistant to propofol overdose-induced Mcl-1 destabilization as shown by Western blotting (fig. 6A). Using acridine orange, rhodamine 123, and PI staining followed by flow cytometric analysis, respectively, Mcl-1-overexpressing cells were significantly \( (P < 0.001) \) defective in propofol overdose-induced LMP (fig. 6B), MTP loss (fig. 6C), and apoptosis (fig. 6D). These results demonstrate that Mcl-1 is sufficient for lysosomal stabilisation, which protects cells from propofol overdose-induced apoptotic signalling of the lysosomal/mitochondrial axis pathway.

The Potential Mechanisms for Propofol Overdose-induced GSK-3\(\beta\)-regulated Lysosomal/mitochondrial Apoptosis

We next examined the regulation of GSK-3\(\beta\) in propofol overdose-treated RAW264.7 cells. For GSK-3\(\beta\) activation, both OA-sensitive PPases such as PP1 and PP2A\(^{30,31}\) and calcium-activated Pyk2\(^{39}\) are positive for GSK-3\(\beta\) activation. However, we found that inhibiting Pyk2 with the calcium chelator BAPTA (5 \( \mu \)M) or the specific inhibitor tyrphostin A9 (1.5 \( \mu \)M) and inhibiting PPases with OA did not reduce propofol overdose-induced cell apoptosis (fig. 7A). In propofol overdose-treated RAW264.7 cells, Western blot analysis showed that propofol overdose caused Akt dephosphorylation (Ser 473) and inactivation (fig. 7B). In addition to Akt, p70 S6K, ERK, p38 MAPK, and ILK are also involved in GSK-3\(\beta\) inactivation.\(^{30-38}\) Notably, results showed that propofol caused inactivation of p70 S6K and ERK but neither p38 MAPK nor ILK (data not shown) in propofol-treated RAW264.7 cells. Further studies using pharmacological approaches demonstrated that inhibiting Akt signalling considerably caused GSK-3\(\beta\) activation, indicating
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Akt acts upstream of GSK-3β in macrophages (fig. 7C). To investigate the possible mechanisms for Akt inactivation, PTEN, a phosphatase that negatively regulates Akt signalling, was silenced using the shRNA approach (fig. 7D). However, knockdown of PTEN expression did not inhibit propofol overdose-induced cell apoptosis (fig. 7E). These results indicate that Akt-regulated pathway contributes to propofol-activated GSK-3β and apoptosis.

Notably, propofol confers antioxidant activity. We hypothesize that propofol inactivates Akt through redox regulation while Akt is positively activated by ROS. In DPI- or CAPE-treated RAW264.7 cells with or without shGSK-3β, using acridine orange, rhodamine 123, and PI staining followed by flow cytometric analysis, respectively, we found that an overdose of antioxidants significantly \((P < 0.001)\) induced LMP (data not shown), MTP loss (data not shown), and apoptosis (fig. 7F) via a GSK-3β-independent manner. Furthermore, inhibiting either cathepsin B or cathepsin D did not reduce DPI-induced lysosomal/mitochondrial apoptosis (fig. 7G), indicating the different apoptotic mechanisms caused by propofol and other antioxidants. These results demonstrate that the antioxidant activity of propofol overdose does not contribute to GSK-3β-mediated lysosomal/mitochondrial apoptosis.
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Discussion

While cytotoxic effects caused by propofol are identified in PRIS patients, the molecular mechanisms are generally studied using the in vitro cell culture system. However, it is not documented how propofol induces cell injury and what are the targets for preventing cytotoxic propofol. Consistent with the previous findings, propofol overdose characteristically induces apoptotic cell death in neurons and monocytes/macrophages. In this study, we show that propofol overdose causes phagocyte apoptosis in vitro and in vivo using an intraperitoneally infusion model. However, using intravenously fusion model, even through the results show that propofol overdose significantly induced leukopenia and neutropenia in peripheral blood, the in vivo evidence by which propofol overdose causes cell apoptosis is still unidentified (data not shown). We hypothesize that a rapid clearance of apoptotic cells in circulation causes such effects. Furthermore, the cellular cytotoxicity caused by propofol overdose directly or indirectly through propofol-induced cellular stress responses is still unclear. To characterize the involvement of apoptosis and the molecular mechanism in PRIS, specific organ failure accompanied by cell apoptosis caused by intravenously infusion of propofol overdose is speculated to be an appropriate model for in vivo study.

In the present work, an in vitro model of propofol overdose-induced apoptosis in macrophages was used to investigate the molecular mechanisms. According to our findings, we provide a model, as summarised in fig. 8, to explore the potential mechanisms for propofol overdose-induced macrophage apoptosis through GSK-3β-regulated lysosomal/mitochondrial pathways. Propofol overdose activates GSK-3β by inhibiting Akt. However, the mechanisms for propofol-induced Akt inactivation need further investigation. After GSK-3β is activated, the anti-apoptotic Mcl-1 is degraded followed by dysregulation of LMP and MMP to induce caspase-3-mediated apoptosis. Meanwhile, lysosomal cathepsin B is activated to connect the lysosomal/mitochondrial axis apoptotic signalling. Based on our findings, we hypothesize that targeting GSK-3β may be a potential strategy for cellular protection from propofol overdose-associated cell death, particular in macrophages, neutrophils, and hepatocytes as demonstrated in the present work. However, the findings need to further investigate using an appropriate in vivo model of PRIS in the future. Limitations such as cell types and propofol responses may determine the signalling of GSK-3-regualted apoptotic pathway. For immunoregulation in PRIS, it needs further investigation particularly on the significance of propofol overdose-induced macrophage apoptosis.

The immunomodulation by propofol is currently purposed as a mechanism for its additional
Propofol causes macrophage apoptosis

pharmacological actions. Propofol has anti-inflammatory effects *in vivo* on inhibition of endotoxaemia-induced production of pro-inflammatory cytokines and chemokines, iNOS/NO biosynthesis, and generation of inflammatory mediators.\textsuperscript{8,11,53,54} Mechanistic studies showed that the molecular mechanism for propofol-conferring anti-inflammatory status is generally targeted on NF-κB activation.\textsuperscript{55-58} Additionally, propofol also decreases LPS- or lipoteichoic acid-activated MAPK/ERK, an upstream regulator of NF-κB nuclear translocation.\textsuperscript{57,58} In this study, we found that propofol overdose disrupts phagocytic activities, which is consistent with the previous studies that propofol represses the biological function of phagocytes.\textsuperscript{16} Notably, the mechanisms for the inhibitory effects are down-regulation of mitochondrial activities and the induction of lysosomal/mitochondrial apoptotic pathways. Dysfunction of phagocytosis and abnormal cell apoptosis will affect host phagocyte-mediated innate immunity. Propofol overdose reduces innate immunity against infection in PRIS patients.\textsuperscript{14} We further provide evidence that propofol overdose causes macrophages to undergo apoptosis *in vitro*. We therefore hypothesize that propofol overdose also induces apoptotic effects in circulating immune cells, including T cells and neutrophils. This speculation needs further investigation.

Maintenance of MMP and cellular adenosine triphosphate synthesis is critical for macrophage functions.\textsuperscript{59,60} Propofol reduces macrophage function by destabilising MMP and decreasing adenosine triphosphate synthesis.\textsuperscript{16} In addition, Tsuchiya *et al.*\textsuperscript{17} demonstrated that propofol causes apoptosis in HL-60 cells via a mechanism involving the activation of the death receptor pathway and the mitochondrial pathway. In this study, we first clarified that propofol overdose-induced apoptosis is sequentially caused by LMP, MTP loss, and caspase activation. Furthermore, inhibiting caspase-8 also decreased propofol-induced apoptosis, suggesting the involvement of a death receptor pathway caused by propofol. According to the results of our and others studies,\textsuperscript{17} the crosstalk between death receptor signaling and lysosomal/mitochondrial axis of apoptotic pathway is therefore speculated and needs further investigation. In contrast, regulation on caspase-8 and/or death receptor signaling by propofol-activated GSK-3β and lysosomal cathepsin B remains unclear. Under propofol overdose treatment such as in PRIS patients, we hypothesize that propofol may cause immunosuppression not only through inflammatory inactivation but also the induction of cell apoptosis. Notably, we further show that propofol overdose induces phagocytic inhibition via a mechanism involving GSK-3β-regulated apoptotic signalling (data not shown).

For the first time, we provide evidence that propofol overdose causes lysosomal/mitochondrial apoptosis in macrophages. Lysosomal protease cathepsin B is critical
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for cell death before mitochondrial damage. Previous studies showed that cathepsin B can cleave Bid to pro-apoptotic truncated Bid and then activates the mitochondrial pathway to cause caspase-9/caspase-3-mediated cell death.61,62 Our studies also showed that propofol causes caspase-3-dependent apoptosis following mitochondrial damage. In this study, we further showed crosstalk between lysosomal and mitochondrial injury through cathepsin B. For the lysosomal pathway, we found that Mcl-1 protects cells from propofol overdose-induced LMP while it is down-regulated by propofol-activated GSK-3β as similar to previous studies.44 Because Mcl-1 is important for maintaining either MMP or LMP,45,46 we hypothesize a GSK-3β-regulated lysosomal/mitochondrial apoptosis under propofol overdose treatment. In addition to Mcl-1, we also demonstrated that propofol overdose induces the activation of Bax while GSK-3β and Mcl-1 are important to control Bax activation.43 Interestingly, a current study showed that the GSK-3β inhibitor lithium chloride can protect mouse neuronal cells from propofol-induced caspase-3-mediated neuroapoptosis.18 Combined with our findings, the pro-apoptotic mechanisms of propofol overdose are therefore speculated to be regulated by pro-apoptotic GSK-3β.

The most challenging and important aspect of this study was exploring the mechanisms for propofol overdose-induced GSK-3β activation followed by Mcl-1 degradation, LMP, cathepsin B activation, MMP, and caspase-3 activation. Activation of GSK-3β is multifactorial depending on the kinds of stimuli, the periods of treatment, and the cell types. First, we showed that propofol overdose inactivates Akt, an important kinase for negatively regulating the pro-apoptotic GSK-3β through protein phosphorylation.27,28 However, inhibiting PPases such as PP1 and PP2A, a positive activator of GSK-3β, and PTEN, a negative regulator of Akt, did not rescue propofol overdose-induced apoptosis suggesting an independent role of PPase-mediated GSK-3β activation following Akt inactivation. Second, as demonstrated using pharmacological inhibition, we also excluded the involvement of calcium-modulated Pyk2, a positive kinase for GSK-3β activation.39 Third, the antioxidant property of propofol was also investigated in this study8 because ROS is important for maintaining Akt activity.52 However, our findings rule out the involvement of anti-ROS activities by propofol overdose because antioxidant overdose did not cause a GSK-3β- and cathepsin B-mediated apoptosis. Fourth, inhibiting HSP90, which has been demonstrated previously to regulate GSK-3β activation,63 using 17-AAG did not reverse apoptosis induced by propofol overdose (data not shown). Finally, further investigation showed that propofol overdose inactivates p70 S6K and ERK but not ILK and p38 MAPK, which are
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both negative regulators for GSK-3β. The possible mechanisms for propofol-induced down-regulation of Akt, p70 S6K, and ERK are currently under investigation while there are reports showing that propofol decreases ERK for anti-inflammation and for neuron cell death.

In conclusion, using an in vitro model of propofol overdose-induced apoptosis in macrophages, we define the pro-apoptotic signalling of propofol through inactivating GSK-3β-regulating kinases Akt. Activated GSK-3β causes Mcl-1 destabilization followed by LMP and induces lysosomal cathepsin B-mediated MTP loss followed by mitochondrial apoptosis. Taken together, GSK-3β acts an integrator in propofol overdose-induced lysosomal/mitochondrial apoptosis. Undoubtedly, these results provide evidence and implications for medicating propofol overdose-induced cellular cytotoxicity by targeting GSK-3β signalling.
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Acknowledgements

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**Legends for Figures**

Fig. 1. Propofol overdose causes phagocytic inhibition and apoptosis **in phagocytes and induces leukopenia and neutropenia in vivo**. (A) RAW264.7, (B) BV2 cells, and (E, left) **primary human neutrophils** (2 × 10^5 cells/well in 12-well culture plates) were treated with propofol (10 or 25 µg/ml) or vehicle with the same volume for 6 h. Formaldehyde-fixed, heat-killed *S. aureus* (2 × 10^7 cocci) were stained with PI then co-incubated with propofol-treated cells for 1 h. After washing with PBS to remove nonphagocytic cocci, flow cytometry was used to determine the phagocytic activity. A representative histogram obtained from three individual experiments is shown, and the mean fluorescence intensity and the percentages of relative phagocytic activity are means ± SD as compared with the normalised vehicle group. (C) RAW264.7, (D) BV2 cells, and (E, right) **primary human neutrophils** (1 × 10^6 cells/well in 6-well culture plates) were treated with propofol (10 or 25 µg/ml) or vehicle with the same volume for 24 h. PI or annexin V staining followed by flow cytometric analysis and DAPI staining followed by fluorescent microscopic observation were used to detect cell apoptosis. A representative histogram obtained from three individual experiments is shown, and the percentages of apoptotic cells are means ± SD. (F) BALB/c mice (n = 5 for each group) were intraperitoneally (IP) or intravenously (IV) injected with vehicle or propofol (10 mg/kg/h for IP and 5 mg/kg/h for IV) for 6 h. Peritoneal macrophages were isolated. Annexin V staining followed by flow cytometric analysis was used to detect cell apoptosis. The percentages of apoptotic cells are means ± SD. *P < 0.05 compared with vehicle. (G) Meanwhile, a CBC test (shown as total amount, × 1000 per µl) was used to quantify the number of white blood cells (WBC) and neutrophils in the whole blood of mice as indicated. The data are shown as means ± SD obtained from three mice. *P < 0.05 compared with vehicle.

Fig. 2. Propofol overdose induces LMP, the loss of MTP, and caspase-dependent cell apoptosis. RAW264.7 cells (1 × 10^6 cells/well in 6-well culture plates) were treated with propofol (25 µg/ml) or vehicle for the indicated time periods. Acridine orange (A), rhodamine 123 (B), and PI (C) staining followed by flow cytometric analysis were used to determine the induction of LMP, the loss of MTP, and cell apoptosis, respectively. A representative histogram obtained from three individual experiments is shown, and the percentages of LMP, MTP loss, and apoptotic cells are means ± SD. ***P < 0.001 compared with vehicle. RAW264.7 cells (2 × 10^5 cells/well in
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12-well culture plates) were pretreated with (D) the MTP stabiliser cyclosporin A (CSA, 1 μM) or a drug target control FK506 (1 μM) or (E) the pan-caspase inhibitor z-VAD-fmk (20 μM), the caspase-8 inhibitor z-IETD-fmk (20 μM), or the caspase-3 inhibitor z-DEVD-fmk (20 μM) for 0.5 h followed by propofol (25 μg/ml) or vehicle treatment for 24 h. PI staining followed by flow cytometric analysis was used to detect cell apoptosis. DMSO was used as a negative control. The percentages of apoptotic cells are means ± SD of three experiments. ***$P < 0.001$ compared with the propofol-treated group.

Fig. 3. Cathepsin B mediates propofol overdose-induced LMP, MTP loss, and cell apoptosis. RAW264.7 cells (2 × 10^5 cells/well in 12-well culture plates) were pretreated with the cathepsin D inhibitor pepstatin A (10 μg/ml) or the cathepsin B inhibitor z-FA-fmk (20 μM) for 0.5 h followed by propofol (25 μg/ml) or vehicle treatment for 24 h. PI (A), acridine orange (C), and rhodamine 123 (D) staining followed by flow cytometric analysis were used to determine the induction of cell apoptosis, LMP, and the loss of MTP, respectively. The percentages of apoptotic cells, LMP, and MTP loss are means ± SD of three individual experiments. ***$P < 0.001$ compared with the propofol-treated group. (B) RAW264.7 cells (1 × 10^6 cells/well in 6-well culture plates) were treated with propofol (25 μg/ml) or vehicle for 24 h. Cathepsin B activity was detected by using a cathepsin B activity assay kit. Data, obtained from three individual experiments, are means ± SD. ***$P < 0.001$ compared with the propofol-treated group.

Fig. 4. Propofol overdose deregulates the expression of Bcl-2 family proteins. RAW264.7 cells (1 × 10^6 cells/well in 6-well culture plates) were treated with propofol (25 μg/ml) or vehicle for the indicated time periods. (A) Western blot analysis was used to determine the expression of Mcl-1, Bcl-2, Bcl-xL, and active Bax. β-actin was the internal control. The ratios of these proteins to β-actin are shown as compared with the normalised vehicle group. Data are representative of three individual experiments. (B) A quantitative accumulated Western blot data has been shown. Data, obtained from three individual experiments, are means ± SD. *$P < 0.05$ compared with the control group.

Fig. 5. Propofol overdose induces GSK-3β-dependent Mcl-1 and glycogen synthase destabilization, LMP, MTP loss, and cell apoptosis. (A) RAW264.7 cells (1 × 10^6 cells/well in 6-well culture plates) were treated with propofol (25 μg/ml) or vehicle for the indicated time
Propofol causes macrophage apoptosis

periods. Western blot analysis was used to determine the expression of phospho-GSK-3β (Ser9), GSK-3β, glycogen synthase (GS), and PARP. (B) Expression of GSK-3β was silenced in RAW264.7 cells (1 × 10^6 cells/well in 6-well culture plates) using lentiviral-based shRNA (GSK-3β shRNA; shGSK-3β) constructs and a negative control construct (luciferase shRNA; shLuc). shLuc- or shGSK-3β-transfected cells were treated with propofol (25 µg/ml) or vehicle for 24 h. Western blot analysis was used to determine the expression of Mcl-1, GSK-3α/β, GS, and PARP. β-actin was the internal control. The ratios of these proteins to β-actin are shown as compared with the normalised vehicle group. Data are representative of three individual experiments. Meanwhile, RAW264.7 cells (2 × 10^5 cells/well in 12-well culture plates) were pretreated with shGSK-3β (C), the GSK-3β inhibitor SB415286 (25 µM) for 0.5 h (D), or the proteasome inhibitor MG132 (0.1 µM) for 0.5 h (E) followed by propofol (25 µg/ml) or vehicle treatment for 24 h. Acridine orange, rhodamine 123, and PI staining followed by flow cytometric analysis were used to determine the induction of LMP, the loss of MTP, and cell apoptosis, respectively. DMSO was used as a negative control. The percentages of LMP, MTP loss, and apoptotic cells are means ± SD of three individual experiments. ***P < 0.001 compared with the control or propofol-treated group. (F) RAW264.7 and HepG2 cells (2 × 10^5 cells/well in 12-well culture plates) were pretreated with shGSK-3β and primary human neutrophils (2 × 10^5 cells/well in 12-well culture plates) were pretreated with the GSK-3β inhibitor BIO (10 µM) for 0.5 h followed by propofol (25 µg/ml) or vehicle treatment for 24 h. Annexin V staining followed by flow cytometric analysis was used to determine the induction of cell apoptosis. DMSO was used as a negative control. The percentages of apoptotic cells are means ± SD of three individual experiments. *P < 0.05 compared with the control group.

Fig. 6. Overexpression of Mcl-1 resists propofol overdose-induced LMP, MTP loss, and cell apoptosis. RAW264.7 cells (1 × 10^6 cells/well in 6-well culture plates) were transfected with mouse Mcl-1 (pcDNA3-HA-mMcl-1) or control vector (pcDNA3-HA) for 24 h. Cells were then treated with propofol (25 µg/ml) or vehicle for 24 h. Western blot analysis was used to determine the expression of Mcl-1 (A). β-actin was the internal control. The ratios of Mcl-1 to β-actin are shown as compared with the normalised vehicle group. Data are representative of three individual experiments. Acridine orange (B), rhodamine 123 (C), and PI (D) staining followed by flow cytometric analysis were used to determine the induction of LMP, the loss of MTP, and cell
Propofol causes macrophage apoptosis apoptosis, respectively. The percentages of LMP, MTP loss, and apoptotic cells are means ± SD of three individual experiments. ***P < 0.001 compared with the control group.

Fig. 7. The possible mechanisms for propofol overdose-induced GSK-3β activation. (A) RAW264.7 cells (2 × 10⁵ cells/well in 12-well culture plates) were pretreated with the intracellular calcium chelator BAPTA (5 µM), the tyrosine Pyk2 inhibitor tyrphostin A9 (1.5 µM), or the PPase inhibitor OA (0.1 µM) for 0.5 h followed by propofol (25 µg/ml) or vehicle treatment for 24 h. PI staining followed by flow cytometric analysis was used to detect cell apoptosis. DMSO was used as a negative control. The percentages of apoptotic cells are means ± SD of three experiments. (B) RAW264.7 cells (1 × 10⁶ cells/well in 6-well culture plates) were treated with propofol (25 µg/ml) or vehicle for the indicated time periods. Western blot analysis was used to determine the expression of phospho-Akt (Ser473), Akt, phospho-p70 S6K (Thr389), p70 S6K, phospho-ERK (Thr202/Tyr204), ERK, phospho-p38 MAPK (Thr180/Tyr182), and p38 MAPK. β-actin was the internal control. The ratios of phosphorylated protein to total protein or β-actin are shown as compared with the normalised vehicle group. Data are representative of three individual experiments. (C) RAW264.7 cells (1 × 10⁶ cells/well in 6-well culture plates) were treated with LY294002, rapamycin, or PD98059 for 6 h. Western blot analysis was used to determine the expression of phospho-GSK-3β (Ser9), GSK-3β, phospho-Akt (Ser473), Akt, phospho-p70 S6K (Thr389), p70 S6K, phospho-ERK (Thr202/Tyr204), and ERK. β-actin was the internal control. The ratios of phosphorylated protein to total protein or β-actin are shown as compared with the normalised vehicle group. Data are representative of three individual experiments. (D) Expression of PTEN was silenced in RAW264.7 cells (1 × 10⁶ cells/well in 6-well culture plates) using lentiviral-based shRNA (PTEN shRNA; shPTEN) constructs and a negative control shLuc. Western blot analysis was used to determine the expression of PTEN. β-actin was the internal control. (E) Cells transfected with shLuc or shPTEN were treated with propofol (25 µg/ml) or vehicle for 24 h. PI staining followed by flow cytometric analysis was used to determine cell apoptosis. The percentages of apoptotic cells are means ± SD of three individual experiments. (F) shLuc- or shGSK-3β-transfected RAW264.7 cells (1 × 10⁶ cells/well in 6-well culture plates) were treated with DPI (20 µM) or CAPE (20 µM) for 24 h. (G) RAW264.7 cells (1 × 10⁶ cells/well in 6-well culture plates) were pretreated with z-FA-fmk (20 µM) or pepstatin A (10 µg/ml) for 0.5 h followed by DPI (20 µM) treatment for 24 h. DMSO
Propofol causes macrophage apoptosis

was used as a negative control. PI staining followed by flow cytometric analysis was used to determine the induction of cell apoptosis. The percentages of apoptotic cells are means ± SD of three individual experiments.

Fig. 8. Schematic model for propofol overdose-induced lysosomal/mitochondrial axis of apoptosis in macrophages. Propofol overdose causes Akt down-regulation through an unknown mechanism independent of PPases. It sequentially induces GSK-3β activation followed by Mcl-1 destabilization. This process induces LMP and cathepsin B activation followed by cathepsin B-mediated loss of MTP followed by MMP and cell apoptosis. Pro-apoptotic propofol overdose therefore causes a GSK-3β-regulated lysosomal/mitochondrial axis of apoptotic signalling pathway in macrophages.
Hsing et al. Figure 1

A

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G

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Hsing et al. Figure 2

A
Vehicle

Propofol (25 μg ml⁻¹)

9.7±2.2%  

75.7±4.6%

Acridine orange (FL-3)

B
Cell number

a  

8.5±1.3%  

b  

48.0±6.8%

Rhodamine 123 (FL-1)

C
Cell number

0.8±0.6%  

36.7±0.7%

PI (FL-2)

D
% of MTP

E
% of MTP loss

Apoptotic cells (%)

Apoptotic cells (%)

Time (h) - 1 3 6 12 24

Propofol (μg ml⁻¹) - - 25 25 25 25

CSA (μM) - - - 1 - -

FK506 (μM) - - - 1 - -

DMSO (μl) - - - 1 - -

Propofol (μg ml⁻¹) - - - 25 25 25 25

z-VAD-fmk (μM) - - - 20 - -

z-IETD-fmk (μM) - - - 20 - -

z-DEVD-fmk (μM) - - - 20 - -
Hsing et al. Figure 3

A

Apoptotic cells (%)

Propofol (μg ml⁻¹) 25 25 25 25
Pepstatin A (μg ml⁻¹) - 10 - -
z-FA-fmk (μM) - - 20 -
DMSO (μl) - - - 2

B

Cathepsin B activity (A.U./μg of protein)

Propofol (μg ml⁻¹) 25

C

% of LMP

D

% of MTP loss

Propofol (μg ml⁻¹) 25 25 25
z-FA-fmk (μM) - - 20 -
DMSO (μl) - - - 2
Figure 4

Hsing et al. Figure 4

A

Mcl-1

Bcl-2

Bcl-xL

Active Bax

β-actin

Time (h) 0 1 3 6 12
Propofol (μg ml⁻¹) - 25 25 25 25

B

Relative density (ration to untreated)

Time (h) 0 1 3 6 12
Propofol (μg ml⁻¹) - 25 25 25 25

*
Hsing et al. Figure 8

Propofol
↓?
Akt
↓
GSK-3β
↓
Mcl-1
LMP
Cathepsin B
↓?
MMP △ψm
Caspase
APOPTOSIS

SB415286
shRNA
Mcl-1
MG132
z-FA-fmk
CSA
z-VAD-fmk
z-DEVD-fmk