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

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ABSTRACT

Background: Overdose propofol treatment with a prolonged time causes injury to multiple cell types; however, its molecular mechanisms remain unclear. Activation of glycogen synthase kinase (GSK)-3β is proapoptotic under death stimuli.

Propofol infusion syndrome (PRIS) involves injury, by an unknown mechanism, of cardiomyocytes, skeletal muscle cells, neurons and immune cells.

What We Already Know about This Topic

• Propofol overdose induces macrophage apoptosis through GSK-3β.

What This Article Tells Us That Is New

• Propofol-induced lysosomal membrane permeabilization and MTP loss while stabilizing MTP and inhibiting caspase protected cells from mitochondrial apoptosis. Lysosomal cathepsin B was required for propofol-induced lysosomal membrane permeabilization, MTP loss, and apoptosis. Propofol decreased antiapoptotic Bcl-2 family proteins and then caused proapoptotic Bcl-2-associated X protein (Bax) activation. Propofol-activated GSK-3β and inhibiting GSK-3β prevented Mcl-1 destabilization, MTP loss, and apoptosis.

The authors therefore hypothesize that propofol overdose induces macrophage apoptosis through GSK-3β.

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

Results: A high dose of propofol caused phagocytic inhibition and apoptosis in vitro for 24 h (25 μg/ml, in triplicate) and in vivo for 6 h (10 mg/kg/h, n = 5 for each group). Propofol induced lysosomal membrane permeabilization and MTP loss while stabilizing MTP and inhibiting caspase protected cells from mitochondrial apoptosis. Lysosomal cathepsin B was required for propofol-induced lysosomal membrane permeabilization, MTP loss, and apoptosis.

The authors therefore hypothesize that propofol overdose induces macrophage apoptosis through GSK-3β.
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.

The anesthetic concentration of propofol (2,6-diisopropylphenol) 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 and has cardiovascular benefits against oxidative stress. Propofol has immunomodulating actions by decreasing production of proinflammatory cytokines and inhibiting neutrophil functions. However, abuse of propofol treatment causes severe complications in patients with critical illness and is called propofol infusion syndrome (PRIS).

Chen et al. reported that propofol (3, 30, or 300 μM) suppresses macrophage function through the disruption of mitochondrial transmembrane potential (MTP) and cellular adenosine triphosphate synthesis. The proapoptotic effects of propofol have been previously demonstrated in vitro and *in vivo*. Tsuchiya et al. found that treatment of human promyelocytic leukemia HL-60 cells with propofol (150 or 250 μM) resulted in growth inhibition accompanied by death receptor-associated activation of the caspase cascade followed by the mitochondrial pathway of apoptosis. Straiko et al. 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. The loss of MTP induces mitochondrial membrane permeabilization (MMP), causing the formation of the apoptosome at the onset of mitochondrial apoptosis. In addition, apoptotic stimuli cause lysosomal membrane permeabilization (LMP) through calcium, reactive oxygen species, ceramide, sphingosine, phospholipase, Bax, Bid, and caspasas, and most of them are also regulators of MMP. After LMP, lysosomal cathepsin B and cathepsin D translocate to the cytoplasm and cause Bid truncation, caspase activation, and the loss of MTP before mitochondrial damage. It is speculated that lysosomes may function as death signal integrators to link the crosstalk in mitochondrial apoptosis.

Overexpression of glycogen synthase kinase (GSK)-3β or blockade of phosphatidylinositol 3-kinase (PI3K)/Akt, the negative regulator of GSK-3β, causes cells to undergo apoptosis. 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. Furthermore, tyrosine phosphorylation of GSK-3β at its tyrosine residue (Tyr216) by calcium-activated proline-rich tyrosine kinase (Pyk2) causes GSK-3β activation. A number of studies have demonstrated the apoptotic signaling cascades generally regulated by GSK-3β. In addition, GSK-3β can phosphorylate Mcl-1 and cause its degradation via an ubiquitin-proteasome system. Mcl-1 has been demonstrated as being involved in maintaining either MMP or LMP. It is notable that lithium chloride, a GSK-3β inhibitor, reverses propofol-inactivated ERK as well as caspase-3 activation. However, the role of GSK-3β signaling has not been further characterized. In the current study, we found an essential role of GSK-3β in propofol overdose-induced lysosomal/mitochondrial pathways of apoptosis. The activation of GSK-3β and its apoptotic effects in macrophages were also studied.

**Materials and Methods**

**Cell Cultures**

Murine macrophages RAW264.7 and BV2 were provided by Professor Chao-Ching Huang, M.D., Department of Pediatrics, College of Medicine, National Cheng Kung University, Tainan, Taiwan. Human HepG2 hepatoma cells were provided by Professor Huan-Yao Lei, Ph.D., Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University, Tainan, Taiwan. Cells were routinely grown on plastic in Dulbecco modified Eagle medium with 1-glutamine and 15 mm HEPES supplemented with 10% fetal bovine serum, 100 units of penicillin, and 100 μg/ml streptomycin and maintained at 37°C in 5% CO2. Cells were used at a passage of 7–10 in this study. Human peripheral whole blood, donated by healthy volunteer, was suspended in 4% dextran (Sigma–Aldrich, St. Louis, MO) 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, Gaithersburg, MD) with 10% fetal bovine serum.

**Animal Treatment**

The 6- to 8-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, Tainan, Taiwan). Mice (n = 5 for each group) were intraperitoneally or intravenously injected with propofol (2,6-diisopropylphenol, Sigma–Aldrich) for 6 h (10 mg/kg/h for intraperitoneal injection and 5 mg/kg/h for intravenous injection).

Materials
Propofol (Zeneca Limited, Macclesfield, Cheshire, United Kingdom) was dissolved in sterile phosphate-buffered saline (PBS). The vehicle control contained glycerol, soybean oil, purified egg phosphatide/egg lecithin, sodium hydroxide, and water. The broad-spectrum caspase inhibitor, benzoxycarbonyl-Val-Ala-Asp(O-Me)-fluoro methyl ketone (z-VAD-fmk), caspase-8 inhibitor benzoxycarbonyl-Ile-Glu(O-Me)-Thr-Asp(O-Me)-fluoromethyl ketone (z-IETD-fmk), and caspase-3 inhibitor benzoxycarbonyl-Asp(O-Me)-Glu(O-Me)-Val-Asp(O-Me)-fluoromethyl ketone (z-DEVD-fmk) were purchased from Sigma–Aldrich and dissolved in dimethyl sulfoxide. Cathepsin B inhibitor benzoxycarbonyl-Phe-Ala-fluoromethyl ketone (z-FA-fmk), cathepsin D inhibitor pepstatin A, GSK-3β inhibitors SB415286 and BIO, cyclosporin A, FK506, and proteasome inhibitor MG132 were purchased from Calbiochem (San Diego, CA). 4′,6-diamidino-2-phenylindole, propidium iodide (PI), and acridine orange were purchased from Sigma–Aldrich. PP2A inhibitor okadaic acid, Pyk2 inhibitor tyrphostin A9, PI3K inhibitor LY294002, mTOR inhibitor rapamycin, and MAP kinase kinase inhibitor PD98059 were obtained from Calbiochem. All drug treatments in cells were assessed for noncytotoxic dosages were used in this study.

Phagocytic Analysis
Staphylococcus aureus was obtained from Ching-Fen Shen, M.D., Department of Pediatrics, College of Medicine, National Cheng Kung University, Tainan, Taiwan and fixed with 1% formaldehyde in PBS and then stained with PI (Sigma–Aldrich). Macrophages (2 × 10⁵) were treated with or without propofol for 6 h at 37°C. Cells were then cocultured with PI-stained S aureus (2 × 10⁵ 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 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphonyl)-2H-tetrazolium, monosodium salt) assay, based on the extracellular reduction of WST-8 by nicotinamide adenine dinucleotide hydrogenase produced in the mitochondria via transplasma 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 Dulbecco modified Eagle medium with propofol treatment. WST-8 reagent (5 μl/well) was added after 24 h of culture. A microplate reader (Spectra MAX 340PC; Molecular Devices, Sunnyvale, CA) was used to measure the absorbance at 450 nm and data were analyzed with Softmax Pro software (Molecular Devices).

Cytotoxicity Assay
To evaluate cell damage, lactate dehydrogenase activity was assayed using a colorimetric assay (Cytotoxicity Detection kit; Roche Diagnostics, Lewes, United Kingdom) 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 were analyzed with Softmax Pro software.

Apoptosis Assay
Apoptosis was analyzed using PI staining as described previously⁷ 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 phase of cells under cell cycle analysis.⁹ 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-stained cells were observed using a fluorescence microscope (IX71; Olympus, Tokyo, Japan).

Complete Blood Counter Test
The complete blood counter test was conducted on peripheral whole blood collected in heparinized Pasteur pipettes by retroorbital venipuncture. Analysis was conducted using a Scil Vet Focus® 5 hematology analyzer (Scil Animal Care Company, Gurnee, IL).

LMP Assay
Lyososomal 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 acridine orange in serum-free Dulbecco modified Eagle medium for 15 min at 37°C and then washed with PBS. Using flow cytometer (FACS Calibur), emissions from fluorescent acridine orange were detected by FL-3 channel (more than 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 rhodamine 123 in cultured medium for 30 min at 37°C. After being washed with PBS, cells were resuspended in cold PBS and immediately underwent flow cytometric analysis (FACS Calibur) 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(hydroxymethyl)aminomethane (pH 7.5), 10 mM EDTA, 0.02% NaN3, and a protease inhibitor cocktail (Roche Boehringer Mannheim Diagnostics, Mannheim, Germany). After 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 sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA) using a semi-dry electroblotting system. After blocking with 5% skim milk in PBS, the membranes were incubated with a 1/5,000 dilution of primary antibodies at 4°C overnight. The membranes were then washed with 0.05% PBS-Tween 20 and incubated with 1/5,000 dilution of horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. After washing, the membranes were soaked in enhanced chemiluminescence solution (PerkinElmer Life Sciences Inc., Boston, MA) for 1 min, and then exposed to film (BioMax; Eastman Kodak, Rochester, NY). The relative signal intensity was quantified using ImageJ software (version 1.41o) from W. Rasband (National Institutes of Health, Bethesda, MD).

Lentiviral-based Short Hairpin RNA Transfection
GSK-3β and PTEN knockdown in RAW264.7 cells were performed using lentiviral transduction to stably express short hairpin RNA (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 was cotransfected with two helper plasmids pCMV deltaR8.91 and pMD.G (gift from Professor Huey-Kang Sytwu, M.D., Ph.D., Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan), plus pLKO.1-puro-shRNA, using GeneJammer transfection reagent (Stratagene, La Jolla, CA). The transfected cells were 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 rotor (Beckman, Danvers, MA). The virus pellets were resuspended 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 Professor Hsin-Fang Yang-Yen, Ph.D., Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan. These plasmids were prepared for transfection using a plasmid miniprep kit (Bio-Rad, Hercules, CA). For transfection, Lipofectamine 2000 (Invitrogen, Frederick, MD) reagent and 4 μg DNA plasmids were used.48

Statistical Analysis
Values are expressed as means ± SD. Groups were compared using Student two-tailed unpaired t test or one-way ANOVA analysis followed by Dunnet, post hoc test, as appropriate with commercially available statistical software (SigmaPlot 8.0 for Windows; Systat Software, Inc., San Jose, CA). Statistical significance was set at P < 0.05.

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.14,15 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 lactate dehydrogenase, 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 Staphylococcus aureus (2 × 10^7 cocci) then coincubated with propofol-treated cells for 1 h. After washing with phosphate-buffered saline 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 normalized vehicle group. (C) RAW264.7, (D) BV2 cells, and (F) 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 4',6-diamidino-2-phenylindole (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. (G) 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. (H) Meanwhile, a complete blood counter test (shown as total amount, × 1,000 per μl) was used to quantify the number of leukocytes (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.
Propofol overdose induces lysosomal membrane permeabilization (LMP), the loss of mitochondrial transmembrane potential (MTP), and caspase-dependent cell apoptosis. RAW264.7 cells (1 x 10^5 cells/well in 6-well culture plates) were treated with propofol (25 μg/ml) or vehicle for the indicated time periods. (A) Acridine orange, (B) rhodamine 123, and (C) propidium iodide (PI) 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 x 10^5 cells/well in 12-well culture plates) were pretreated with (D) the MTP stabilizer cyclosporin A (CSA, 1 μM) or a drug target control FK506 (1 μM) or (E) the pan-caspase inhibitor benzylxoycarbonyl-Val-Ala-Asp(O-Me)-fluoromethyl ketone (z-VAD-fmk) (20 μM), the caspase-8 inhibitor benzylxoycarbonyl-Ile-Glu(O-Me)-Thr-Asp(O-Me)-fluoromethyl ketone (z-IETD-fmk) (20 μM), or the caspase-3 inhibitor benzylxoycarbonyl-Asp(O-Me)-Glu(O-Me)-Val-Asp(O-Me)-fluoromethyl ketone (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. Dimethyl sulfoxide (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.

**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. To investigate the molecular mechanisms of propofol overdose (25 μg/ml)-induced apoptosis, the time kinetics (fig. 2A) of propofol-induced LMP in RAW264.7 cells were shown by a metachromatic fluorochrome 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 occurred earlier and was more severe in propofol overdose-treated RAW264.7 cells than control samples. 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 stabilizing MTP and inhibiting caspases were performed. Cyclosporin A (1 μM), a mitochondrial permeability transition pore stabilizer, significantly (P < 0.001) inhibited propofol overdose-induced apoptosis (fig. 2D). FK506 (1 μM), a PP2B inhibitor similar to cyclosporin A, 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 μM) and caspase-3 inhibitor z-DEVD-fmk (20 μM) effectively (P < 0.001) blocked propofol overdose-induced apoptosis in RAW264.7 cells (fig. 2E). Furthermore, inhibiting caspase-8 using z-IETD-fmk (20 μM) 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.
Propofol Causes Macrophage 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.\textsuperscript{19,43,44} We next investigated the effects of propofol overdose on the expression of Bcl-2 family proteins, including antiapoptotic Mcl-1, Bcl-2, and Bcl-xL and proapoptotic Bax in RAW264.7 cells. Western blot analysis showed that treatment of propofol effectively decreased the expression of Mcl-1 (\( P = 0.02 \)) and Bcl-2 (\( P = 0.018 \)) but not Bcl-xL, and induced the activation of Bax (\( P = 0.032 \)) (fig. 4). These results indicate a dysregulation effect of propofol overdose on the induction of lysosomal/mitochondrial apoptosis.

GSK-3β down-regulates Mcl-1 through a mechanism involving phosphorylation followed by proteasome-mediated degradation.\textsuperscript{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 glycogen synthase (fig. 5A) as shown previously.\textsuperscript{50} Using a lentiviral-based shRNA approach, propofol overdose-induced Mcl-1 and glycogen synthase destabilization and poly (ADP-ribose) polymerase 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, MTP loss, and apoptosis (fig. 5D). Furthermore, we found that the proteasome inhibitor MG132 significantly (\( P < 0.001 \)) inhibited propofol-induced-oxidation, MTP loss, and apoptosis (\( \text{via a GSK-3β-dependent manner (fig. 5E).} \)) To further confirm the effects of GSK-3β activity, a pharmacologic 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, MTP loss, and apoptosis (fig. 5D). Furthermore, we found that the proteasome inhibitor MG132 significantly (\( P < 0.001 \)) inhibited propofol-induced-oxidation, MTP loss, and apoptosis (fig. 5E). Furthermore, annexin V staining showed that propofol overdose significantly induced GSK-3-dependent apoptosis in RAW264.7 (\( P < 0.001 \)) and HepG2 cells (\( P = 0.003 \)), as demonstrated by GSK-3 inhibitor BIO (fig. 5F). These results demonstrate that propofol overdose causes GSK-3β-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 stabilization as demonstrated previously.\textsuperscript{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

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We next examined the regulation of GSK-3. Apoptosis

PP2A30,31 and calcium-activated Pyk239 are positive for both okadaic acid-sensitive PPases such as PP1 and ethane-N,N,N
carbamoyl-ethane-N,N,N’-N’-tetraacetic acid (5 μM) or the specific inhibitor tyrphostin A9 (1.5 μM) and inhibiting PPases with okadaic acid 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. 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.8 We hypothesize that propofol inactivates Akt through redox regulation whereas Akt is positively activated by reactive oxygen species.52 In diphenylene iodonium- or caffeic acid phenethyl ester-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 diphenylene iodonium-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.

The Potential Mechanisms for Propofol Overdose-induced GSK-3β-regulated Lysosomal/Mitochondrial Apoptosis

We next examined the regulation of GSK-3β in propofol overdose-treated RAW264.7 cells. For GSK-3β activation, both okadaic acid-sensitive PPases such as PP1 and PP2A30,31 and calcium-activated Pyk239 are positive for GSK-3β activation. However, we found that inhibiting Pyk2 with the calcium chelator 1,2-bis(2-aminophenoxy)-ethane-N,N,N’,N’-tetraacetic acid (5 μM) or the specific inhibitor tyrphostin A9 (1.5 μM) and inhibiting PPases with okadaic acid 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 ILK17,18 are also involved in GSK-3β 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 pharmacologic approaches demonstrated that inhibiting Akt signaling considerably caused GSK-3β activation, indicating 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 signaling,51 was silenced using the shRNA approach (fig. 7D). However, knockdown of PTEN

Discussion

Although cytotoxic effects caused by propofol are identified in PRIS patients,14,15 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,17,18 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 an intravenous fusion model, even though the results show that propofol overdose significantly induced leukopenia and neutropenia in peripheral

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 stabilization, which protects cells from propofol overdose-induced apoptotic signaling of the lysosomal/mitochondrial axis pathway.

Fig. 4. Propofol overdose deregulates the expression of Bcl-2 family proteins. 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. (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 compared with the normalized 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 glycogen synthase kinase (GSK)-3β-dependent Mcl-1 and glycogen synthase destabilization, lysosomal membrane permeabilization (LMP), mitochondrial transmembrane potential (MTP) loss, and cell apoptosis. (A) RAW264.7 cells (1 x 10^6 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-GSK-3β (Ser9), GSK-3β, glycogen synthase (GS), and poly (adenosine diphosphate-ribose) polymerase (PARP). (B) Expression of GSK-3β was silenced in RAW264.7 cells (1 x 10^6 cells/well in 6-well culture plates) using lentiviral-based short hairpin RNA (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 compared with the normalized vehicle group. Data are representative of three individual experiments. Meanwhile, RAW264.7 cells (2 x 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 propidium iodide staining followed by flow cytometric analysis were used to determine the induction of LMP, the loss of MTP, and cell apoptosis, respectively. Dimethyl sulfoxide (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 x 10^5 cells/well in 12-well culture plates) were pretreated with
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 intravenous infusion of propofol overdose is speculated to be an appropriate model for in vivo study.

In the current study, 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 summarized in figure 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 antiapoptotic 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 signaling. Based on our findings, we hypothesize that targeting GSK-3β may be a potential strategy for cellular protection from propofol overdose-associated cell death, particularly in macrophages, neutrophils, and hepatocytes as demonstrated in the current study. However, the findings indicate a 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 signaling of GSK-3-regulated 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 pharmacologic actions. Propofol has antiinflammatory effects in vivo on inhibition of endotoxemia-induced production of proinflammatory cytokines and chemokines, inducible nitric oxide synthase/nitric oxide biosynthesis, and generation of inflammatory mediators.8,11,53,54 Mechanistic studies showed that the molecular mechanism for propofol-conferred antiinflammatory status is generally targeted on nuclear factor-kappa B (NF-κB) activation.55–58 In addition, propofol also decreases lipopolysaccharide- or lipoteichoic acid-activated MAPK/ERK, an upstream regulator of NF-κB nuclear translocation.57,58 In this study, we found that propofol overdose disrupts phagocytic activities, which is consistent with the previous studies that propofol represses the biologic function of phagocytes.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 patients with PRIS.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.59,60 Propofol reduces macrophage function by destabilising...
MMP and decreasing adenosine triphosphate synthesis. In addition, Tsuchiya et al. 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 our results and those of other studies, 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 signaling (data not shown).
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 proapoptotic GSK-3β through protein phosphorylation. 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 after Akt inactivation. Second, as demonstrated using pharmacologic inhibition, we also excluded the involvement of calcium-modulated Pyk2, a positive kinase for GSK-3β activation. Third, the antioxidant property of propofol was also investigated in this study because reactive oxygen species is important for maintaining Akt activity. However, our findings rule out the involvement of antioxidant species activities by propofol overdose because antioxidant overdose did not cause a GSK-3β- and cathepsin B-mediated apoptosis. Fourth, inhibiting heat shock protein 90, which has been demonstrated previously to regulate GSK-3β activation, using 17-N-Allylamino-17-demethoxygeldanamicin 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 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 proapoptotic signaling of propofol through inactivating GSK-3β-mediated kinase 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 signaling.

The authors thank the Immunobiology Core, Research Center of Clinical Medicine, National Cheng Kung University Hospital, Tainan, Taiwan, for providing services that include training, technical support, and assistance with experimental design and data analysis using Flow Cytometry Core facilities.

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