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

Chung-Hsi Hsing, M.D., Ph.D.,* Yu-Hong Chen, M.S.,† Chia-Ling Chen, Ph.D.,‡ Wei-Ching Huang, Ph.D.,§ Ming-Chung Lin, M.D.,‖ Po-Chun Tseng, M.S.,§ Chi-Yun Wang, M.S.,§ Cheng-Chieh Tsai, M.S.,# Pui-Ching Choi, B.S.,** Chiou-Feng Lin, Ph.D.††

ABSTRACT

Background: Overdose propofol treatment with a prolong 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. 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. 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

What We Already Know about This Topic

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

What This Article Tells Us That Is New

- 10 mg/kg/h of intraperitoneal propofol caused inhibition of phagocytosis and apoptosis of peritoneal macrophages obtained from BALB/c mice. Intravenous propofol reduced circulating leukocytes in the mice. These mechanisms may be involved in PRIS.

* Staff Anesthesiologist, Department of Anesthesiology, Chi Mei Medical Center, Tainan, Taiwan, and Assistant Professor of Anesthesiology, Department of Anesthesiology, College of Medicine, Taipei Medical University, Taipei, Taiwan. † Graduate Student, Department of Microbiology and Immunology, College of Medicine, National Cheng Kung University, Tainan, Taiwan. ‡ Postdoctoral Fellow, Center of Infectious Disease and Signaling Research, College of Medicine, National Cheng Kung University. § Graduate Student, Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University. ‖ Graduate Student, Institute of Clinical Medicine, College of Medicine, National Cheng Kung University. # Graduate Student, Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, and Lecturer, Department of Nursing, Chung Hwa University of Medical Technology, Tainan, Taiwan. ** Research Associate, Institute of Clinical Medicine, College of Medicine, National Cheng Kung University. †† Associate Professor, Center of Infectious Disease and Signaling Research, College of Medicine, National Cheng Kung University, and Associate Professor, Institute of Clinical Medicine, National Cheng Kung University.

Received from the Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan. Submitted for publication March 16, 2011. Accepted for publication January 10, 2012. This work was supported by the cooperation project CMNCKU9903 and CMNCKU10001 of National Cheng Kung University and Chi Mei Medical Center, Tainan, Taiwan, grant NSC 96–2320-B-006–018-MY3 from the National Science Council, Taipei, Taiwan, and grant D100–35B07 from the Ministry of Education, Taipei, Taiwan, R.O.C. under the National Cheng Kung University Project of Promoting Academic Excellence and Developing World Class Research Centers.

Address correspondence to Dr. Lin: Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan. cflin@mail.ncku.edu.tw. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

 Anesthesiology 2012; 116:868–81

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.[1] 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 proinflammatory 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]

Chen *et al.*[16] 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*.[17,18] Tsuchiya *et al.*[17] 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.*[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 permeabilization (MMP), causing the formation of the apoptosome at the onset of mitochondrial apoptosis.[21,22] In addition, apoptotic stimuli cause lysosomal membrane permeabilization (LMP) through calcium, reactive oxygen species, 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, caspase activation, and the loss of MTP before mitochondrial damage.[25] It is speculated that lysosomes may function as death signal integrators to link the crosstalk in mitochondrial apoptosis.[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.[27,28] 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.[29–38] Furthermore, tyrosine phosphorylation of GSK-3β at its tyrosine residue (Tyr216) by calcium-activated proline-rich tyrosine kinase (Pyk) 2 causes GSK-3β activation.[39] A number of studies have demonstrated the apoptotic signaling cascades generally regulated by GSK-3β.[29,40–42] For apoptosis, GSK-3β can phosphorylate Bax to promote mitochondrial injury.[43] In addition, GSK-3β can phosphorylate Mcl-1 and cause its degradation *via* an ubiquitin-proteasome system.[44] Mcl-1 has been demonstrated as being involved in maintaining either MMP or LMP.[45,46] It is notable that lithium chloride, a GSK-3β inhibitor, reverses propofol-inactivated ERK as well as caspase-3 activation.[18] 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 L-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% CO_2. 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, benzyloxycarbonyl-Val-Ala-Asp(O-Me)-fluoro methyl ketone (z-VAD-fmk), caspase-8 inhibitor benzyloxycarbonyl-Ile-Glu(O-Me)-Thr-Asp(O-Me)-fluoromethyl ketone (z-IETD-fmk), and caspase-3 inhibitor benzyloxycarbonyl-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 benzyloxycarbonyl-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, antioxidants diphenylene iodonium and caffeic acid phenthyl ester, intracellular calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, Pyk2 inhibitor tyrphostin A9, PI3K inhibitor LY294002, mTOR inhibitor rapamycin, and MAP kinase kinase inhibitor PD98059 were obtained from Sigma–Aldrich and dissolved in dimethyl sulfoxide before dilution with PBS and use in experiments. Rabbit antimouse 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, poly (ADP-ribose) polymerase, and phosphatase and tensin homolog (PTEN) were purchased from Cell Signaling Technology (Beverly, MA). β-actin antibodies and horseradish peroxidase-conjugated antirabbit IgG were obtained from Chemicon (Temecula, CA). All drug treatments in cells were assessed for their cytotoxic effects using cytotoxicity assays before experiments. 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 (2×10^5) were treated with or without propofol for 6 h at 37°C. Cells were then cocultured with PI-stained *S aureus* (2×10^7 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 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-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 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-G_1 phase of cells under cell cycle analysis.[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-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

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 acridine orange in serum-free Dulbecco modified Eagle medium 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 (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 (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(hydroxymethyl)aminomethane (pH 7.5), 10 mM EDTA, 0.02% NaN_3, 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 semidry electroblotting system. After blocking with 5% skim milk in PBS, the membranes were incubated with a 1/1,000 dilution of primary antibodies at 4°C overnight. The membranes were then washed with 0.05% PBS-Tween 20 and incubated with a 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 (TRCN0000012615 containing the shRNA target sequence 5′-CATGAAAGTTAGCAGAGATAA-3′ for mouse GSK-3β and TRCN0000028992 containing the shRNA target sequence 5′- GCTAGAACTTATCAA-ACCCTT −3′ for mouse PTEN) and in HepG2 cells (TRCN0000040001 containing the shRNA target sequence 5′-GCTGAGCTGTTACTAGGACAA-3′ for human GSK-3β) was used to generate recombinant lentiviral particles. Human TE671 cells are cotransfected with two helper plasmids pCMVdeltaR8.91 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 are incubated at 37°C in an atmosphere of 5% CO_2 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 (LC_{50}) by propofol in RAW264.7 cells was 25 μg/ml (140 μM; data not shown). These results indicate

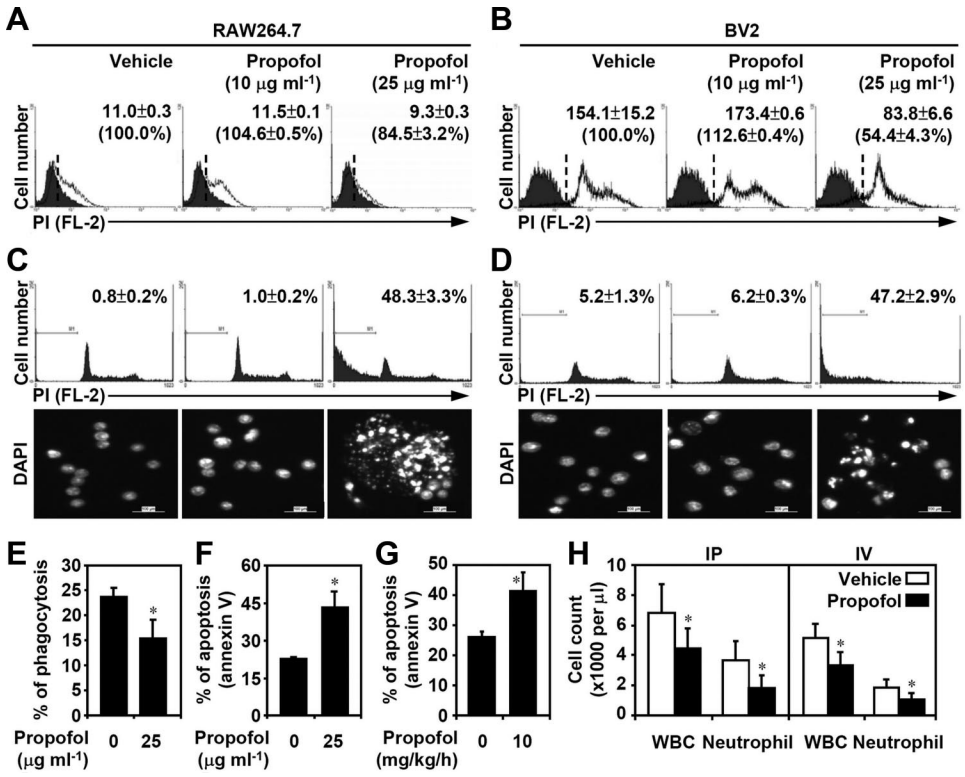

Fig. 1. Propofol overdose causes phagocytic inhibition and apoptosis in phagocytes and induces leukopenia and neutropenia *in vivo*. (*A*) RAW264.7, (*B*) BV2 cells, and (*E*) primary human neutrophils (2×10^5 cells/well in 12-well culture plates) were treated with propofol (10 or 25 μg/ml) or vehicle with the same volume for 6 h. Formaldehyde-fixed, heat-killed *Staphylococcus aureus* (2×10^7 cocci) were stained with propidium iodide (PI) 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.

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.

We examined the cytotoxic effects of propofol, using PI staining followed by flow cytometric analysis and 4′,6-diamidino-2-phenylindole staining by characterizing the presence of chromatin condensation and fragmentation. These analyses showed that propofol overdose caused RAW264.7 (fig. 1C) and BV2 (fig. 1D) to undergo apoptosis. In addition to macrophages, propofol also significantly ($P = 0.017$) decreased the phagocytic activity in primary human neutrophils (fig. 1E). Annexin V staining also showed that propofol overdose significantly ($P = 0.022$) induced apoptosis of human neutrophils (fig. 1F). 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 intraperi-

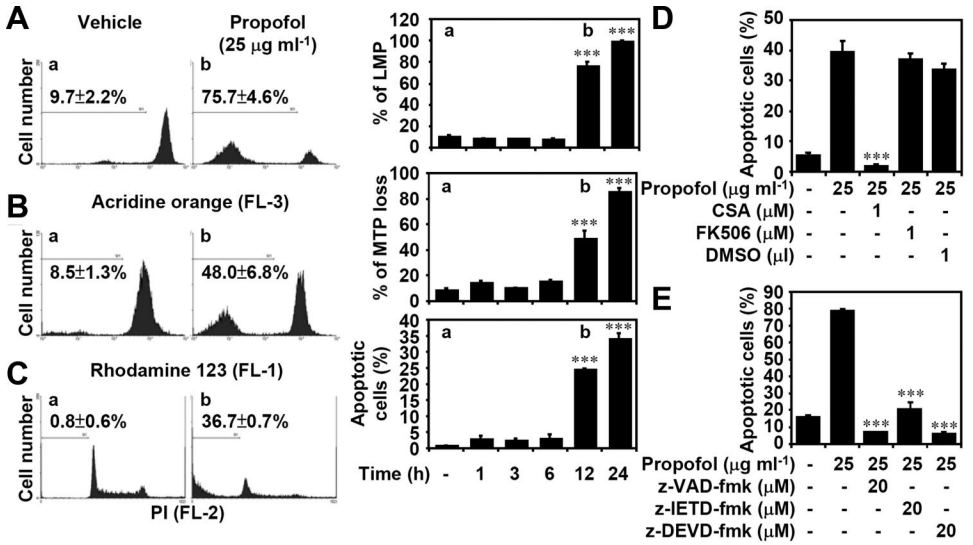

Fig. 2. Propofol overdose induces lysosomal membrane permeabilization (LMP), the loss of mitochondrial transmembrane potential (MTP), and caspase-dependent cell apoptosis. RAW264.7 cells (1×10^6 cells/well in 6-well culture plates) were treated with propofol (25 μg/ml) or vehicle for the indicated time periods. (*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 (2×10^5 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 benzyloxycarbonyl-Val-Ala-Asp(O-Me)-fluoro methyl ketone (z-VAD-fmk) (20 μM), the caspase-8 inhibitor benzyloxycarbonyl-Ile-Glu(O-Me)-Thr-Asp(O-Me)-fluoromethyl ketone (z-IETD-fmk) (20 μM), or the caspase-3 inhibitor benzyloxycarbonyl-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.

toneally (10 mg/kg/h) or intravenously (5 mg/kg/h) injected with propofol for 6 h. Annexin V staining showed that propofol overdose significantly ($P = 0.045$) caused peritoneal macrophage apoptosis *in vivo* (fig. 1G). However, there was no increase in apoptotic cells detected in peripheral blood cells, which might be caused by rapid clearance in circulation *in vivo* (data not shown). Meanwhile, the number of leukocytes and neutrophils in circulation was significantly decreased in propofol overdose-treated mice with intraperitoneal model (leukocytes, $P = 0.011$; neutrophil, $P = 0.013$) and with intravenous model (leukocytes, $P = 0.011$; neutrophil, $P = 0.027$) (fig. 1H). These results show the *in vivo* cytotoxic effects of propofol overdose.

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.[19] 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 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 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.

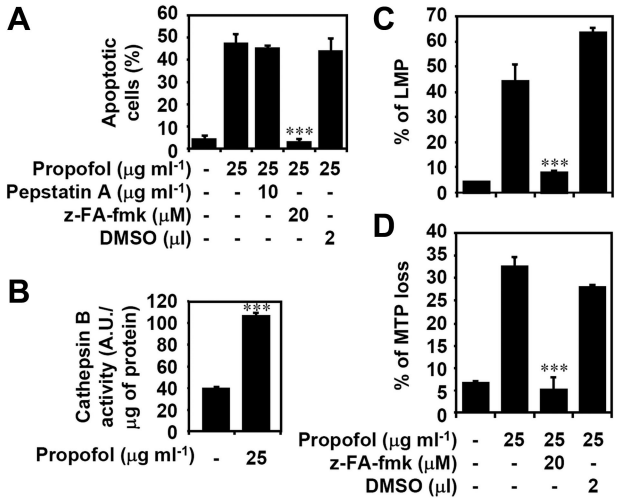

Fig. 3. Cathepsin B mediates propofol overdose-induced lysosomal membrane permeabilization (LMP), mitochondrial transmembrane potential (MTP) loss, and cell apoptosis. RAW264.7 cells (2×10^5 cells/well in 12-well culture plates) were pretreated with the cathepsin D inhibitor pepstatin A (10 μg/ml) or the cathepsin B inhibitor benzyloxycarbonyl-Phe-Ala-fluoromethyl ketone (z-FA-fmk) (20 μM) for 0.5 h followed by propofol (25 μg/ml) or vehicle treatment for 24 h. (*A*) Propidium iodide, (*C*) acridine orange, and (*D*) rhodamine 123 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.

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 after LMP.[20,25] We thus examined the effects of lysosomal cathepsins on 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 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 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 glycogen synthase (fig. 5A) as shown previously.[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 *via* a GSK-3β-dependent manner (fig. 5C). 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 LMP, 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-3β knockdown approach, and primary human neutrophils ($P = 0.007$), as demonstrated by treatment of 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.[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

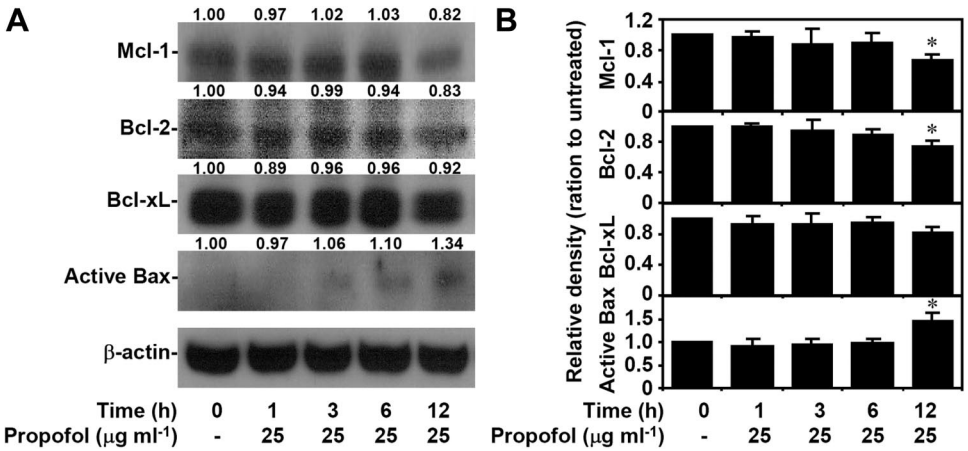

Fig. 4. Propofol overdose deregulates the expression of Bcl-2 family proteins. RAW264.7 cells (1×10^6 cells/well in 6-well culture plates) were treated with propofol (25 μg/ml) or vehicle for the indicated time periods. (*A*) Western blot analysis was used to determine the expression of Mcl-1, Bcl-2, Bcl-xL, and active Bax. β-actin was the internal control. The ratios of these proteins to β-actin are shown 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.

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.

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 PP2A[30,31] and calcium-activated Pyk2[39] 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 ILK 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 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 phenthyl 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.

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

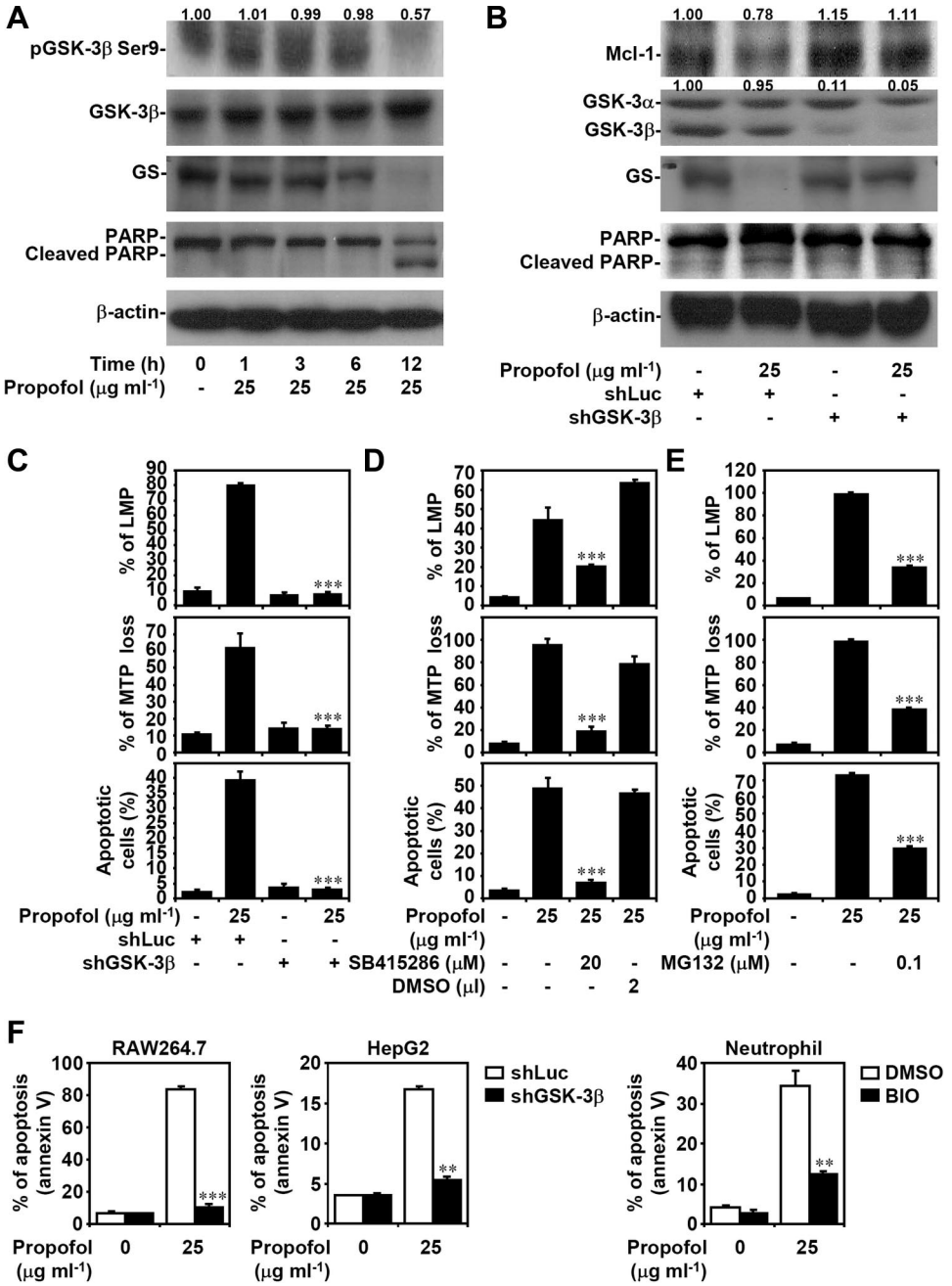

Fig. 5. Propofol overdose induces glycogen synthase kinase (GSK)-3β-dependent Mcl-1 and glycogen synthase destabilization, lysosomal membrane permeabilization (LMP), mitochondrial transmembrane potential (MTP) loss, and cell apoptosis. (*A*) RAW264.7 cells (1×10^6 cells/well in 6-well culture plates) 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 (1×10^6 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 (2×10^5 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 (2×10^5 cells/well in 12-well culture plates) were pretreated with

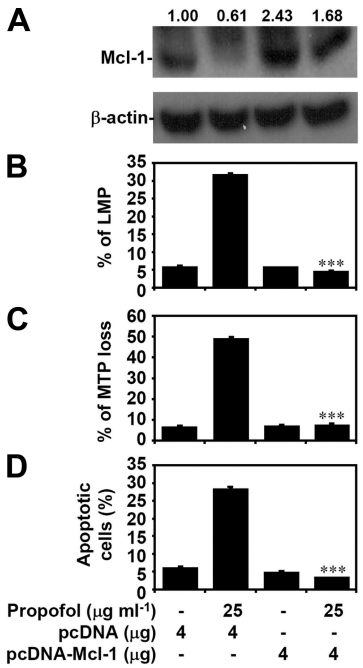

Fig. 6. Overexpression of Mcl-1 resists propofol overdose-induced lysosomal membrane permeabilization (LMP), mitochondrial transmembrane potential (MTP) loss, and cell apoptosis. RAW264.7 cells (1×10^6 cells/well in 6-well culture plates) were transfected with mouse Mcl-1 (pcDNA3-HA-mMcl-1) or control 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 normalized vehicle group. Data are representative of three individual experiments. (*B*) Acridine orange, (*C*) rhodamine 123, and (*D*) propidium iodide staining followed by flow cytometric analysis were used to determine the induction of LMP, the loss of MTP, and cell 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.

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-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 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

Fig. 5. (Continued) 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. **$P < 0.01$ and ***$P < 0.001$ compared with the control group.

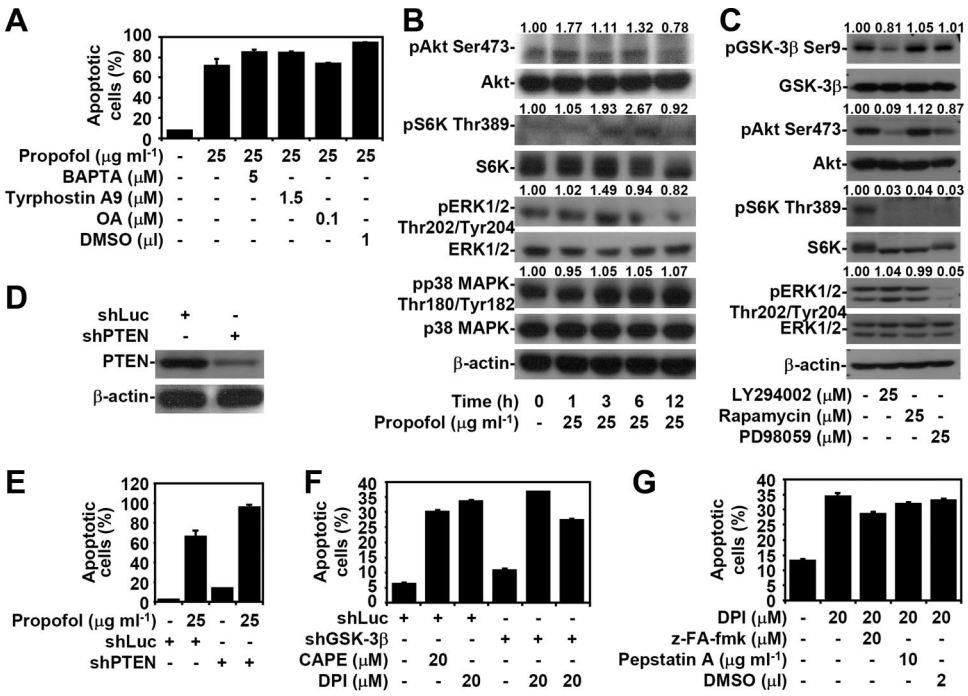

Fig. 7. The possible mechanisms for propofol overdose-induced glycogen synthase kinase (GSK)-3β activation. (*A*) RAW264.7 cells (2×10^5 cells/well in 12-well culture plates) were pretreated with the intracellular calcium chelator BAPTA (5 μM), the Pyk2 inhibitor tyrphostin A9 (1.5 μM), or the protein phosphatase inhibitor okadaic acid (OA) (0.1 μM) for 0.5 h followed by propofol (25 μg/ml) or vehicle treatment for 24 h. Propidium iodide (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. (*B*) 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. Western blot analysis was used to determine the expression of phospho-Akt (Ser473), Akt, phospho-p70 S6 kinase (S6K) (Thr389), p70 S6K, phospho-extracellular signal-regulated kinase (ERK) (Thr202/Tyr204), ERK, phospho-p38 mitogen-activated protein kinase (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 compared with the normalized vehicle group. Data are representative of three individual experiments. (*C*) RAW264.7 cells (1×10^6 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 compared with the normalized vehicle group. Data are representative of three individual experiments. (*D*) Expression of PTEN was silenced in RAW264.7 cells (1×10^6 cells/well in 6-well culture plates) using lentiviral-based short hairpin RNA (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 (1×10^6 cells/well in 6-well culture plates) were treated with diphenylene iodonium (DPI) (20 μM) or caffeic acid phenethyl ester (CAPE) (20 μM) for 24 h. (*G*) RAW264.7 cells (1×10^6 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 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.

MMP and decreasing adenosine triphosphate synthesis.[16] In addition, Tsuchiya *et al.*[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 our results and those of other studies,[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 signaling (data not shown).

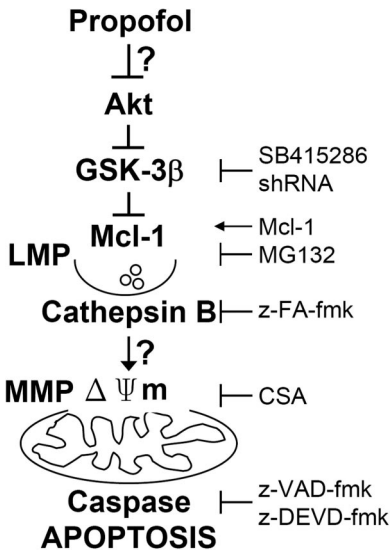

Fig. 8. Schematic model for propofol overdose-induced lysosomal/mitochondrial axis of apoptosis in macrophages. Propofol overdose causes Akt down-regulation through an unknown mechanism independent of protein phosphatases. It sequentially induces glycogen synthase kinase (GSK)-3β activation followed by Mcl-1 destabilization. This process induces lysosomal membrane permeabilization (LMP) and cathepsin B activation followed by cathepsin B-mediated loss of mitochondrial transmembrane potential followed by mitochondrial membrane permeabilization (MMP) and cell apoptosis. Proapoptotic propofol overdose therefore causes a GSK-3β-regulated lysosomal/mitochondrial axis of apoptotic signaling pathway in macrophages.

For the first time, we provide evidence that propofol overdose causes lysosomal/mitochondrial apoptosis in macrophages. Lysosomal protease cathepsin B is critical for cell death before mitochondrial damage. Previous studies showed that cathepsin B can cleave Bid to proapoptotic 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 after 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 whereas 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 proapoptotic mechanisms of propofol overdose are therefore speculated to be regulated by proapoptotic 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 proapoptotic 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 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.[39] Third, the antioxidant property of propofol was also investigated in this study[8] because reactive oxygen species is important for maintaining Akt activity.[52] However, our findings rule out the involvement of anti-reactive oxygen 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,[63] using 17-N-Allylamino-17-demethoxygeldanamycin 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.[29–38] 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 antiinflammation[57,58] and for neuron cell death.[18]

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-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 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.

References

1. Mackenzie N, Grant IS: Propofol for intravenous sedation. Anaesthesia 1987; 42:3–6
2. Shao H, Li J, Zhou Y, Ge Z, Fan J, Shao Z, Zeng Y: Dose-dependent protective effect of propofol against mitochondrial dysfunction in ischaemic/reperfused rat heart: Role of cardiolipin. Br J Pharmacol 2008; 153:1641–9
3. Li J, Han B, Ma X, Qi S: The effects of propofol on hippocam-

pal caspase-3 and Bcl-2 expression following forebrain ischemia-reperfusion in rats. Brain Res 2010; 1356:11–23
4. Karashima Y, Oike M, Takahashi S, Ito Y: Propofol prevents endothelial dysfunction induced by glucose overload. Br J Pharmacol 2002; 137:683–91
5. Wang B, Luo T, Chen D, Ansley DM: Propofol reduces apoptosis and up-regulates endothelial nitric oxide synthase protein expression in hydrogen peroxide-stimulated human umbilical vein endothelial cells. Anesth Analg 2007; 105: 1027–33
6. Chen J, Gu Y, Shao Z, Luo J, Tan Z: Propofol protects against hydrogen peroxide-induced oxidative stress and cell dysfunction in human umbilical vein endothelial cells. Mol Cell Biochem 2010; 339:43–54
7. Xia Z, Luo T, Liu HM, Wang F, Xia ZY, Irwin MG, Vanhoutte PM: L-arginine enhances nitrative stress and exacerbates tumor necrosis factor-alpha toxicity to human endothelial cells in culture: Prevention by propofol. J Cardiovasc Pharmacol 2010; 55:358–67
8. Marik PE: Propofol: An immunomodulating agent. Pharmacotherapy 2005; 25:28S–33S
9. Chen RM, Chen TG, Chen TL, Lin LL, Chang CC, Chang HC, Wu CH: Anti-inflammatory and antioxidative effects of propofol on lipopolysaccharide-activated macrophages. Ann N Y Acad Sci 2005; 1042:262–71
10. Takaono M, Yogosawa T, Okawa-Takatsuji M, Aotsuka S: Effects of intravenous anesthetics on interleukin (IL)-6 and IL-10 production by lipopolysaccharide-stimulated mononuclear cells from healthy volunteers. Acta Anaesthesiol Scand 2002; 46:176–9
11. Taniguchi T, Kanakura H, Takemoto Y, Kidani Y, Yamamoto K: Effects of ketamine and propofol on the ratio of interleukin-6 to interleukin-10 during endotoxemia in rats. Tohoku J Exp Med 2003; 200:85–92
12. Rodriguez-Lpez JM, Snchez-Conde P, Lozano FS, Nicols JL, Garcia-Criado FJ, Cascajo C, Muriel C: Laboratory investigation: Effects of propofol on the systemic inflammatory response during aortic surgery. Can J Anaesth 2006; 53:701–10
13. Heller A, Heller S, Blecken S, Urbaschek R, Koch T: Effects of intravenous anesthetics on bacterial elimination in human blood *in vitro*. Acta Anaesthesiol Scand 1998; 42:518–26
14. Vasile B, Rasulo F, Candiani A, Latronico N: The pathophysiology of propofol infusion syndrome: A simple name for a complex syndrome. Intensive Care Med 2003; 29:1417–25
15. Fudickar A, Bein B: Propofol infusion syndrome: Update of clinical manifestation and pathophysiology. Minerva Anestesiol 2009; 75:339–44
16. Chen RM, Wu CH, Chang HC, Wu GJ, Lin YL, Sheu JR, Chen TL: Propofol suppresses macrophage functions and modulates mitochondrial membrane potential and cellular adenosine triphosphate synthesis. ANESTHESIOLOGY 2003; 98: 1178–85
17. Tsuchiya M, Asada A, Arita K, Utsumi T, Yoshida T, Sato EF, Utsumi K, Inoue M: Induction and mechanism of apoptotic cell death by propofol in HL-60 cells. Acta Anaesthesiol Scand 2002; 46:1068–74
18. Straiko MM, Young C, Cattano D, Creeley CE, Wang H, Smith DJ, Johnson SA, Li ES, Olney JW: Lithium protects against anesthesia-induced developmental neuroapoptosis. ANESTHESIOLOGY 2009; 110:862–8
19. Ferri KF, Kroemer G: Organelle-specific initiation of cell death pathways. Nat Cell Biol 2001; 3:E255–63
20. Guicciardi ME, Leist M, Gores GJ: Lysosomes in cell death. Oncogene 2004; 23:2881–90
21. Garrido C, Galluzzi L, Brunet M, Puig PE, Didelot C, Kroemer G: Mechanisms of cytochrome c release from mitochondria. Cell Death Differ 2006; 13:1423–33
22. Kroemer G, Galluzzi L, Brenner C: Mitochondrial membrane permeabilization in cell death. Physiol Rev 2007; 87:99–163
23. Kroemer G, Jaattela M: Lysosomes and autophagy in cell death control. Nat Rev Cancer 2005; 5:886–97
24. Boya P, Kroemer G: Lysosomal membrane permeabilization in cell death. Oncogene 2008; 27:6434–51
25. Chwieralski CE, Welte T, Bhling F: Cathepsin-regulated apoptosis. Apoptosis 2006; 11:143–9
26. Jttela M, Cand C, Kroemer G: Lysosomes and mitochondria in the commitment to apoptosis: A potential role for cathepsin D and AIF. Cell Death Differ 2004; 11:135–6
27. Pap M, Cooper GM: Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. J Biol Chem 1998; 273:19929–32
28. Jope RS, Johnson GV: The glamour and gloom of glycogen synthase kinase-3. Trends Biochem Sci 2004; 29:95–102
29. Lin CF, Chen CL, Chiang CW, Jan MS, Huang WC, Lin YS: GSK-3beta acts downstream of PP2A and the PI 3-kinase-Akt pathway, and upstream of caspase-2 in ceramide-induced mitochondrial apoptosis. J Cell Sci 2007; 120:2935–43
30. Stambolic V, Woodgett JR: Mitogen inactivation of glycogen synthase kinase-3 β in intact cells *via* serine 9 phosphorylation. Biochem J 1994; 303(Pt 3):701–4
31. Rayasam GV, Tulasi VK, Sodhi R, Davis JA, Ray A: Glycogen synthase kinase 3: More than a namesake. Br J Pharmacol 2009; 156:885–98
32. Sutherland C, Leighton IA, Cohen P: Inactivation of glycogen synthase kinase-3 beta by phosphorylation: New kinase connections in insulin and growth-factor signalling. Biochem J 1993; 296:15–9
33. Eldar-Finkelman H, Seger R, Vandenheede JR, Krebs EG: Inactivation of glycogen synthase kinase-3 by epidermal growth factor is mediated by mitogen-activated protein kinase/p90 ribosomal protein S6 kinase signaling pathway in NIH/3T3 cells. J Biol Chem 1995; 270:987–90
34. Wang QM, Fiol CJ, DePaoli-Roach AA, Roach PJ: Glycogen synthase kinase-3 beta is a dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation. J Biol Chem 1994; 269:14566–74
35. Thornton TM, Pedraza-Alva G, Deng B, Wood CD, Aronshtam A, Clements JL, Sabio G, Davis RJ, Matthews DE, Doble B, Rincon M: Phosphorylation by p38 MAPK as an alternative pathway for GSK3beta inactivation. Science 2008; 320: 667–70
36. Bikkavilli RK, Feigin ME, Malbon CC: p38 mitogen-activated protein kinase regulates canonical Wnt-β-catenin signaling by inactivation of GSK3β. J Cell Sci 2008; 121:3598–607
37. Delcommenne M, Tan C, Gray V, Rue L, Woodgett J, Dedhar S: Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. Proc Natl Acad Sci U S A 1998; 95: 11211–6
38. Troussard AA, Mawji NM, Ong C, Mui A, St -Arnaud R, Dedhar S: Conditional knock-out of integrin-linked kinase demonstrates an essential role in protein kinase B/Akt activation. J Biol Chem 2003; 278:22374–8
39. Hartigan JA, Xiong WC, Johnson GV: Glycogen synthase kinase 3beta is tyrosine phosphorylated by PYK2. Biochem Biophys Res Commun 2001; 284:485–9
40. Beurel E, Jope RS: The paradoxical pro- and anti-apoptotic actions of GSK3 in the intrinsic and extrinsic apoptosis signaling pathways. Prog Neurobiol 2006; 79:173–89
41. Brewster JL, Linseman DA, Bouchard RJ, Loucks FA, Precht TA, Esch EA, Heidenreich KA: Endoplasmic reticulum stress and trophic factor withdrawal activate distinct signaling cascades that induce glycogen synthase kinase-3 beta and a caspase-9-dependent apoptosis in cerebellar granule neurons. Mol Cell Neurosci 2006; 32:242–53
42. Huang WC, Lin YS, Wang CY, Tsai CC, Tseng HC, Chen CL, Lu PJ, Chen PS, Qian L, Hong JS, Lin CF: Glycogen synthase kinase-3 negatively regulates anti-inflammatory interleu-

kin-10 for lipopolysaccharide-induced iNOS/NO biosynthesis and RANTES production in microglial cells. Immunology 2009; 128:e275–86

43. Linseman DA, Butts BD, Precht TA, Phelps RA, Le SS, Laessig TA, Bouchard RJ, Florez-McClure ML, Heidenreich KA: Glycogen synthase kinase-3beta phosphorylates Bax and promotes its mitochondrial localization during neuronal apoptosis. J Neurosci 2004; 24:9993–10002
44. Maurer U, Charvet C, Wagman AS, Dejardin E, Green DR: Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. Mol Cell 2006; 21:749–60
45. Werneburg NW, Guicciardi ME, Bronk SF, Kaufmann SH, Gores GJ: Tumor necrosis factor-related apoptosis-inducing ligand activates a lysosomal pathway of apoptosis that is regulated by Bcl-2 proteins. J Biol Chem 2007; 282:28960–70
46. Bivik C, Ollinger K: JNK mediates UVB-induced apoptosis upstream lysosomal membrane permeabilization and Bcl-2 family proteins. Apoptosis 2008; 13:1111–20
47. Wang Y, Huang WC, Wang CY, Tsai CC, Chen CL, Chang YT, Kai JI, Lin CF: Inhibiting glycogen synthase kinase-3 reduces endotoxaemic acute renal failure by down-regulating inflammation and renal cell apoptosis. Br J Pharmacol 2009; 157: 1004–13
48. Wang CY, Lin YS, Su WC, Chen CL, Lin CF: Glycogen synthase kinase-3 and Omi/HtrA2 induce annexin A2 cleavage followed by cell cycle inhibition and apoptosis. Mol Biol Cell 2009; 20:4153–61
49. Yang-Yen HF: Mcl-1: A highly regulated cell death and survival controller. J Biomed Sci 2006; 13:201–4
50. MacAulay K, Blair AS, Hajduch E, Terashima T, Baba O, Sutherland C, Hundal HS: Constitutive activation of GSK3 down-regulates glycogen synthase abundance and glycogen deposition in rat skeletal muscle cells. J Biol Chem 2005; 280:9509–18
51. Cantley LC, Neel BG: New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc Natl Acad Sci U S A 1999; 96:4240–5
52. Azar ZM, Mehdi MZ, Srivastava AK: Activation of insulin-like growth factor type-1 receptor is required for H2O2-induced PKB phosphorylation in vascular smooth muscle cells. Can J Physiol Pharmacol 2006; 84:777–86
53. Takemoto Y: Dose effects of propofol on hemodynamic and cytokine responses to endotoxemia in rats. J Anesth 2005; 19:40–4
54. Hsu BG, Yang FL, Lee RP, Peng TC, Chen HI: Effects of post-treatment with low-dose propofol on inflammatory responses to lipopolysaccharide-induced shock in conscious rats. Clin Exp Pharmacol Physiol 2005; 32:24–9
55. Brasil LJ, San-Miguel B, Kretzmann NA, Amaral JL, Zettler CG, Marroni N, Gonzlez-Gallego J, Tun MJ: Halothane induces oxidative stress and NF-kappaB activation in rat liver: Protective effect of propofol. Toxicology 2006; 227:53–61
56. Song XM, Wang YL, Li JG, Wang CY, Zhou Q, Zhang ZZ, Liang H: Effects of propofol on pro-inflammatory cytokines and nuclear factor kappaB during polymicrobial sepsis in rats. Mol Biol Rep 2009; 36:2345–51
57. Jawan B, Kao YH, Goto S, Pan MC, Lin YC, Hsu LW, Nakano T, Lai CY, Sun CK, Cheng YF, Tai MH, Eng HL, Wang CS, Huang CJ, Lin CR, Chen CL: Propofol pretreatment attenuates LPS-induced granulocyte-macrophage colony-stimulating factor production in cultured hepatocytes by suppressing MAPK/ERK activity and NF-kappaB translocation. Toxicol Appl Pharmacol 2008; 229:362–73
58. Chiu WT, Lin YL, Chou CW, Chen RM: Propofol inhibits lipoteichoic acid-induced iNOS gene expression in macrophages possibly through downregulation of toll-like receptor 2-mediated activation of Raf-MEK1/2-ERK1/2-IKK-NFkappaB. Chem Biol Interact 2009; 181:430–9
59. Diehl AM, Hoek JB: Mitochondrial uncoupling: Role of uncoupling protein anion carriers and relationship to thermogenesis and weight control "the benefits of losing control". J Bioenerg Biomembr 1999; 31:493–506
60. Ayala A, Chaudry IH: Immune dysfunction in murine polymicrobial sepsis: Mediators, macrophages, lymphocytes and apoptosis. Shock 1996; 6(Suppl 1):S27–38
61. Turk B, Turk V: Lysosomes as "suicide bags" in cell death: Myth or reality? J Biol Chem 2009; 284:21783–7
62. Blomgran R, Zheng L, Stendahl O: Cathepsin-cleaved Bid promotes apoptosis in human neutrophils *via* oxidative stress-induced lysosomal membrane permeabilization. J Leukoc Biol 2007; 81:1213–23
63. Lochhead PA, Kinstrie R, Sibbet G, Rawjee T, Morrice N, Cleghon V: A chaperone-dependent GSK3beta transitional intermediate mediates activation-loop autophosphorylation. Mol Cell 2006; 24:627–33