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Propofol protects against hydrogen peroxide-induced injury in cardiac H9c2 cells via Akt activation and Bcl-2 up-regulation

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ABSTRACT

Propofol is a widely used intravenous anesthetic agent with antioxidant properties secondary to its phenol based chemical structure. Treatment with propofol has been found to attenuate oxidative stress and prevent ischemia/reperfusion injury in rat heart. Here, we report that propofol protects cardiac H9c2 cells from hydrogen peroxide (H_2O_2)-induced injury by triggering the activation of Akt and a parallel up-regulation of Bcl-2. We show that pretreatment with propofol significantly protects against H_2O_2-induced injury. We further demonstrate that propofol activates the PI3K–Akt signaling pathway. The protective effect of propofol on H_2O_2-induced injury is reversed by PI3K inhibitor wortmannin, which effectively suppresses propofol-induced activation of Akt, up-regulation of Bcl-2, and protection from apoptosis. Collectively, our results reveal a new mechanism by which propofol inhibits H_2O_2-induced injury in cardiac H9c2 cells, supporting a potential application of propofol as a preemptive cardioprotectant in clinical settings such as coronary bypass surgery.

Introduction

Propofol (2,6-diisopropylphenol), an intravenous anesthetic, has been found to attenuate oxidative stress induced mechanical and metabolic derangements in the isolated rat heart and prevent ischemia/reperfusion injury [1–3]. The antioxidant mechanism mainly relates to its phenolic chemical structure. Given its relatively weak antioxidant effect in vitro [4], we postulate a previously unappreciated mechanism of action to prevent myocardial ischemia/reperfusion injury. We believe the presence of the drug during the first few minutes of tissue reperfusion might serve to offset the cellular response to the ischemic insult, initiating the cell signaling events whereby cardiomyocytes opt for cell survival.

Phosphoinositide 3-kinase (PI3K)–Akt signaling pathway is one of the important signal transduction pathways controlling cardiomyocytes survival and function [5]. Various growth factors and cellular stresses activate Akt through phosphorylation of threonine 308 or serine 473 residues. Once activated, Akt proceeds to phosphorylate its downstream targets, in various subcellular locations including in the nucleus, which contribute to its anti-apoptotic effects [6,7]. A wide variety of putative downstream effectors have been identified that could contribute to the anti-apoptotic effects of Akt, including transcription factor NF (nuclear factor)-κB, B-cell lymphoma-2 (Bcl-2) family pro-apoptotic protein Bad, caspase family protein caspase 9, and endothelial nitric oxide synthase (eNOS) [7].

We previously reported that propofol attenuated tumor necrosis factor-α and hydrogen peroxide (H_2O_2)-induced apoptosis in human umbilical vein endothelial cells, and that this attenuation was associated with up-regulation of eNOS expression, increase in intracellular nitric oxide (NO) release and preservation of Bcl-2 expression [8,9]. Endothelial cell apoptosis is known to precede myocyte apoptosis in the setting of myocardial ischemia/reperfusion injury [10]. However, the effects of propofol on cardiomyocyte apoptosis have yet to be fully elucidated.

Recently, propofol was reported to protect cardiomyocytes against H_2O_2-induced apoptosis and the anti-apoptotic effect of propofol was attributed to the increased expression of heme oxygenase-1 [11] in the study. Of interest, it is believed that the cytoprotective effects of heme oxygenase-1 are mediated via interaction with and activation of the PI3K–Akt pathway [12]. However, whether propofol activates cell survival signaling through a PI3K–Akt signaling pathway is currently unclear and remains

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speculative. Therefore, the present study was designed to determine propofol's effect on PI3K–Akt signaling pathway as a potential alternative mechanism of propofol-mediated cytoprotection.

Materials and methods

Reagents. Propofol, wortmannin, dimethyl sulfoxide (DMSO) and H_2O_2 were from Sigma Chemical. The Fluorometric CaspACE™ Assay System and Cell Titer 96® Aqueous Non-radioactive Cell Proliferation Assay were from Promega Corporation. All culture reagent, Hoechst 33258 and Trizol reagent were from Invitrogen. All SDS–PAGE reagents and RT-PCR reagents were from Bio-Rad Laboratories, Inc. Rabbit antibodies against Akt, phospho-Akt, Bcl-2 and horseradish peroxidase (HRP) conjugated goat anti-rabbit IgGs were from Santa Cruz Biotechnology, Inc.

Cardiac H9c2 cells culture and treatment protocol. Cardiac H9c2 cells are a clonal heart muscle cell line derived from embryonic rat hearts that retains many cardiomyocyte phenotypes [13]. All the cells used were derived from the same initial CRL-1446 cell culture obtained from the American Type Culture Collection (ATCC). The cell culture growth medium was comprised of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown in a humidified incubator containing 95% air and 5% CO_2 at 37 °C with media replenishment every 3 days.

Before experimental intervention, confluent cultured cells were serum starved for 48 h in DMEM supplemented with 1% fetal bovine serum. The starved cells were then treated with H_2O_2 and propofol alone, or in combination for the times indicated in the figure legends. For inhibitor experiments, cells were pre-incubated with selective PI3K inhibitor (wortmannin, 500 nM) or vehicle (DMSO) for 30 min, and then treated with H_2O_2 and/or propofol. The concentration of H_2O_2 (400 μM) applied was determined from pilot studies by our group and based on similar methods described in the scientific literature [14]. The concentration of propofol (50 μM) applied was determined based on similar methods described in the scientific literature [8] and the average blood propofol concentration during human cardiac surgery in our clinical project [15].

Assessment of cell viability by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2- (4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. Cells were cultured in 24-well plates and treated according to the protocol. After 24 h, Cell Titer 96® Aqueous One Solution Reagent was added to each well according to the manufacturer's instructions. After 2 h incubation the cell viability was determined by measuring the absorbance at 490 nm using a plate reader. Four independent survival experiments were performed for each experimental condition.

Evaluation of cell apoptosis by Hoechst 333258 staining. Following 24 h treatment, cells from each group were washed with phosphate buffered saline and fixed with 4% formalin for 10 min. Hoechst 33258 (10 μg/ml) was applied for 30 min under dark condition to stain the nuclei of fixed cells. Cells were examined and photographed using a fluorescence microscope. Apoptotic cells were identified as those with a nucleus exhibiting brightly stained condensed chromatin or nuclear fragments, and normal nuclei have blue chromatin with organized structure. For each experimental condition, four separate cell populations were prepared. Apoptotic indices were determined by direct visualization and counting of a minimum of 500 cells per population (at least 100 cells from five randomly selected fields). The apoptotic index was calculated as the ratio of number of apoptotic cells to total cells counted × 100.

Assay for caspase-3 activity. Following treatment, cells from each group were lysed and caspase-3 activity was measured by the Fluorometric CaspACE™ Assay System according to the manufacturer's recommendation. The fluorogenic CPP32/caspase-3 (Ac-DEVD-AMC) substrate is labeled with the fluorochrome 7-amino-4-methyl coumarin (AMC). The uncleaved substrates produce a blue fluorescence that can be detected by exposure to UV light at 360 nm. AMC is released from these substrates upon cleavage by CPP32-like enzymes. Free AMC produces a yellow-green fluorescence that is monitored by a fluorometer at 460 nm. The amount of yellow-green fluorescence produced upon cleavage is proportional to the amount of caspase-3 activity present in the sample. Assays were performed in duplicate and three independent experiments were performed.

Western blot analysis of Akt, phospho-Akt, and Bcl-2 expression. Cells from each group were lysed using RIPA buffer containing 20 mm Tris–HCl (pH 7.4), 150 mm NaCl, 1% Nonider P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, 1% PMSF, 1% sodium orthovanadate and 1% protease inhibitor cocktail at 4 °C. The lysate was centrifuged at 10,000*g* at 4 °C for 30 min to remove the insoluble material. Supernatants were collected. The protein concentration was measured by Bradford protein assay. Then equal amounts of protein (50 μg) from each sample was separated by 12% SDS–PAGE and transferred to nitrocellulose membrane. The membranes were blocked for 1 h in 5% skim milk and then incubated overnight at 4 °C with primary antibody against Akt, or phospho-Akt (Ser 473), or phospho-Akt (Thr 308), or Bcl-2. Following a 30-min wash, the membranes were incubated with secondary antibody conjugated to HRP for 1 h at room temperature. The membranes were then washed for 30 min and visualized by enhanced chemiluminescence. The protein expression levels were determined by analyzing the signals captured on the nitrocellulose membranes using a Chemi-doc image analyzer (Bio-Rad).

Quantitative real-time RT-PCR (reverse transcription-polymerase chain reaction) analysis of Bcl-2 gene expression. Total cellular RNA was isolated using the Trizol reagent according to the manufacture's instruction. The RNA concentration and purity were determined spectrophotometrically. Total RNA (5 μg) extracted from each sample was reverse transcribed to cDNA in a 20 μl reaction mixture using iScript™ cDNA Synthesis Kit according to the manufacture's directions. Quantification of gene expression was measured using the iQ™ SYBR® Green Supermix in an iCycler Real-Time PCR System. Amplification was performed in a final 25 μl containing 1 μg of cDNA, optimized specific primers and iQ SYBR Green Supermix. The specific sense and anti-sense primers used were shown below. Bcl-2, sense: 5′-GACGCGAAGTGCTATTGGT-3′, anti-sense: 5′-TCAGGCTGGAAGGAGAAGAT-3′; GAPDH, sense: 5′-CACAGTCTTCTGAGTGGCAGTGAT-3′, anti-sense: 5′-GTGAAGGTCGG TGTGAACGG ATTT-3′. The amount of the PCR product was measured as fluorescent signal intensity after standardizing to GAPDH internal control. The comparative threshold cycle method was used for the calculation of amplification fold according to the mathematical model reported by Pfaffl [16]. All experiments were performed in triplicate.

Statistical analysis. All data represent the mean of samples from 3 to 4 separately performed experiments. Results were expressed as mean and standard deviation (mean ± SD). Difference was analyzed for significance by one-way ANOVA followed by Tukey's test. A value of $p < 0.05$ was considered statistically significant.

Results

Effects of H_2O_2 and propofol alone on H9c2 cells viability

We first determined the dose at which cytotoxicity develops in a period of 24 h upon H_2O_2 exposure in H9c2 cells through the MTS assay. Cells were treated with increasing doses of H_2O_2 for 24 h. As shown in Fig. 1A, H_2O_2 impaired cell viability in a concentration-

dependent manner over the tested concentration range (50–500 μm). A maximum reduction of 51 ± 8% was observed with 500 μm of H_2O_2. We chose the level of H_2O_2 400 μm for use in our subsequent experiments based on these results.

In order to evaluate whether propofol was cytotoxic to H9c2 cells, we determined the viability of cells treated with propofol (25–100 μm) for 24 h using the MTS assay. Cell viability was not significantly affected by treatment with any tested propofol concentrations (Fig. 1B). We chose the level of 50 μm for subsequent experiments based on similar methods described in scientific literature [8].

Effects of propofol on H_2O_2-induced cell death and apoptosis in H9c2 cells

In order to determine the effects of propofol on H_2O_2-stimulated cell death, the H9c2 cells were pretreated with propofol (50 μm) for 30 min, and then co-incubated with 400 μm of H_2O_2 for additional 24 h. As shown in Fig. 1C, viability of H_2O_2-treated cells was restored to control levels in the presence of propofol.

As a further test for apoptosis we counted representative Hoechst-staining images from control (DMSO) and propofol alone groups as shown in Fig. 1D. Most nuclei in the control or the propofol group displayed uniform blue chromatin with organized structure. Intense Hoechst stained nuclei, which indicate a cell undergoing apoptosis, were not frequently observed in these groups. However apoptotic nuclei were observed with increased frequency in H_2O_2-stimulated cells compared to control cells. Propofol pretreatment restored H_2O_2-induced apoptosis to control levels. The apoptotic index increased significantly in cells stimulated with H_2O_2, a condition that was prevented by prior incubation with propofol.

We also examined the effect of propofol on H_2O_2-stimulated caspase-3 activation. As shown in Fig. 1E, H_2O_2 (400 μm) treatment

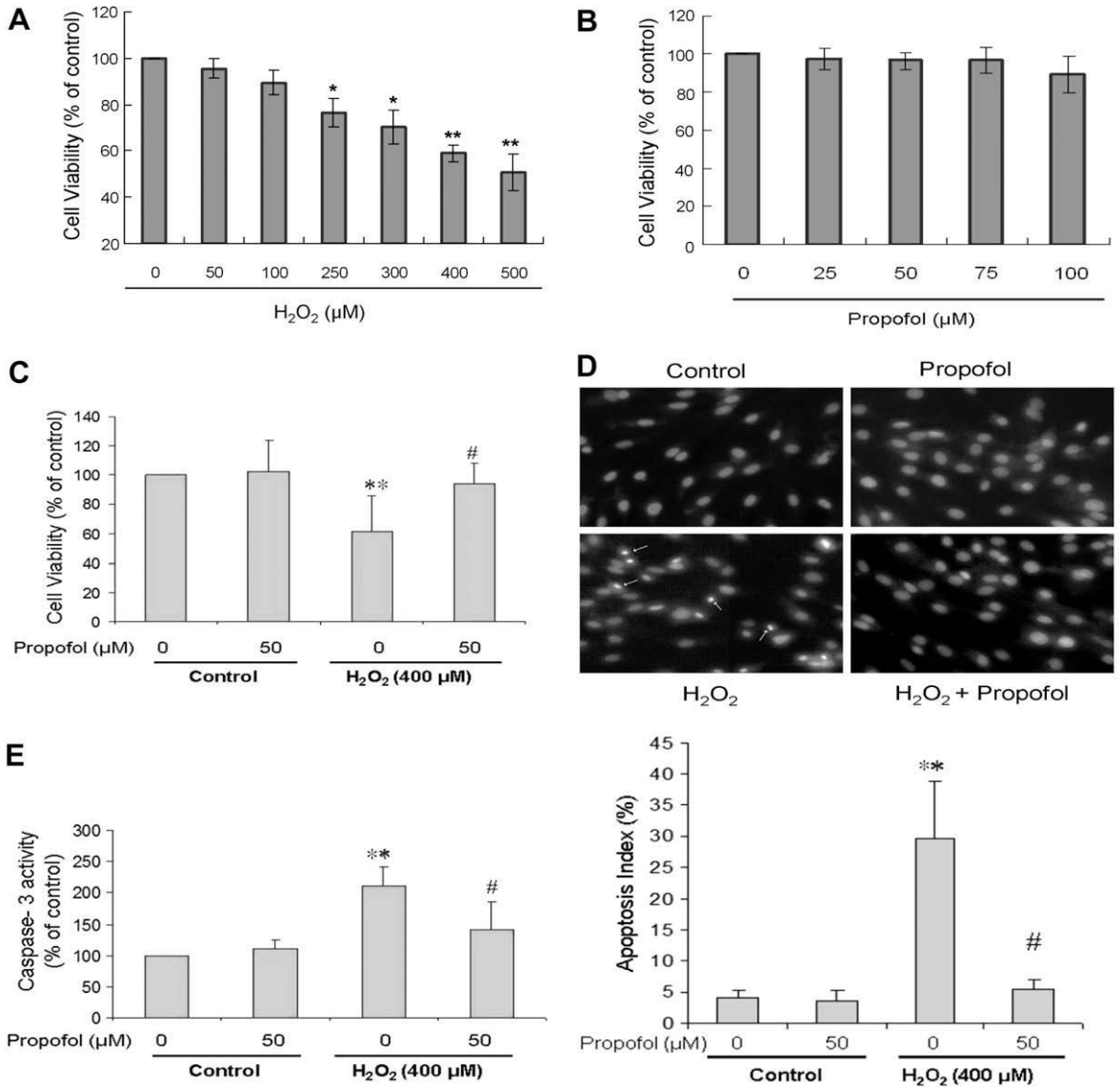

Fig. 1. Effects of H_2O_2 and/or propofol on H9c2 cells viability and apoptosis. (A, B) Cells were incubated with increasing concentrations of H_2O_2 (0–500 μM) or propofol (0–100 μM) for 24 h. Cell viability was measured by MTS assay. (A) H_2O_2 decreased H9c2 cells viability in a concentration-dependent manner. (B) Cell viability was not significantly affected by any tested propofol concentrations. (C–E) Cells were treated with propofol (50 μM) or vehicle (DMSO) in the presence or absence of H_2O_2 (400 μM) for 24 h. (C) Cell viability was measured by MTS assay. (D) Cell apoptosis was evaluated by Hoechst 33258 staining. Representative immunocytochemistry photomicrographs and corresponding histogram were displayed. (E) Caspase-3 activity was measured by the Fluorometric CaspACE™ Assay. $^{*}p < 0.05$ *vs* control, $^{**}p < 0.01$ *vs* control. $^{\#}p < 0.01$ *vs* H_2O_2 only group.

was associated with significantly increased caspase-3 activity as compared to control. Propofol (50 μm) pretreatment significantly decreased this H_2O_2-induced caspase-3 activation.

Effects of propofol on Akt phosphorylation

To explore the potential signaling pathways contributing to the anti-apoptotic function of propofol, we examined the kinetics of Akt activation. As shown in Fig. 2A, propofol stimulated Akt phosphorylation at serine 473 beginning at 10 min after treatment. Phosphorylation was still elevated 1 h after treatment. Increased phosphorylation of Akt at threonine 308 was also observed starting at approximately 1 h and remained high up to 24 h after treatment. DMSO control had no effects on Akt phosphorylation at either serine 473 or threonine 308.

Effects of PI3K inhibition on the protection of propofol against H_2O_2-stimulated cell death and apoptosis

We next determined whether the PI3K–Akt signaling pathway was involved in the pro-survival function of propofol. H9c2 cells were pre-incubated with wortmannin and then treated with H_2O_2 alone or in combination with propofol for 24 h. We showed that wortmannin reversed the protective effects of propofol on H_2O_2-stimulated cells and led to decreased cell viability, increased apoptosis and deceased Akt activation, as measured by MTS assay (Fig. 2B), by caspase-3 activity assay (Fig. 2C), and by Western blot (Fig. 2D), respectively.

Modulation of propofol on Bcl-2

In order to determine if the activation of the PI3K–Akt pathway by propofol is instrumental to the survival of H9c2 cells by modulating Bcl-2 levels, we first assessed its effects on Bcl-2 gene and protein expression. As shown in Fig. 3A, a decrease in Bcl-2 expression was observed in response to H_2O_2 treatment. However, cells treated with propofol showed an increase in Bcl-2 mRNA and protein expression as measured by real-time RT-PCR (Fig. 3B) and Western blot (Fig. 3A), respectively. Although gene expression was elevated for up to 6 h post-treatment, Bcl-2 protein levels were consistently increased over the period of 24 h.

We proceeded to investigate whether the inhibition of PI3K by wortmannin, could suppress the propofol effects on Bcl-2. As shown in Fig. 3C, wortmannin decreased propofol-induced Bcl-2

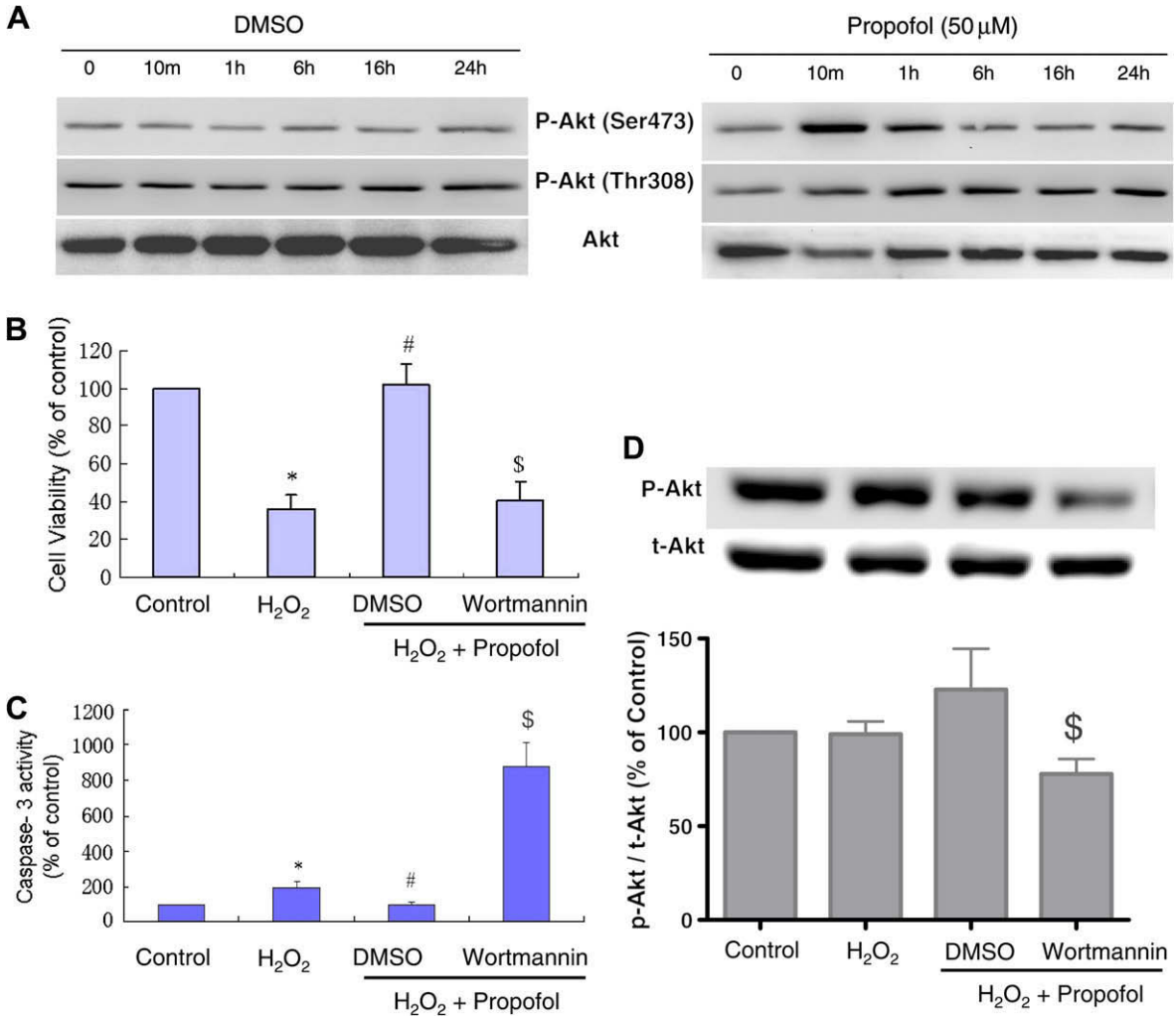

Fig. 2. Effects of propofol on Akt phosphorylation and PI3K inhibition on the protection of propofol against H_2O_2-stimulated cell death and apoptosis. (A) H9c2 cells were treated with vehicle (DMSO) or propofol (50 μM) for various times as indicated. Cell lystates were harvested and Western blot was performed to examine Akt phosphorylation. Propofol increased Akt phosphorylation at both serine 473 and threonine 308 sites while DMSO has no effects on Akt phosphorylation. (B–D) H9c2 cells were pre-incubated with 500 nM wortmannin for 30 min and then treated with H_2O_2 (400 μM) and propofol (50 μM) for 24 h. Wortmannin reversed the increases in cell viability (B) and the decreases in caspase-3 activity (C) induced by propofol in H_2O_2-stimulated cells. Western blotting results further showed that wortmannin treatment decreased Akt phosphorylation induced by propofol (D). $^{*}p < 0.01$ *vs* control, $^{\#}p < 0.01$ *vs* H_2O_2 only group, $^{\$}p<0.01$ *vs* H_2O_2 + propofol + DMSO group.

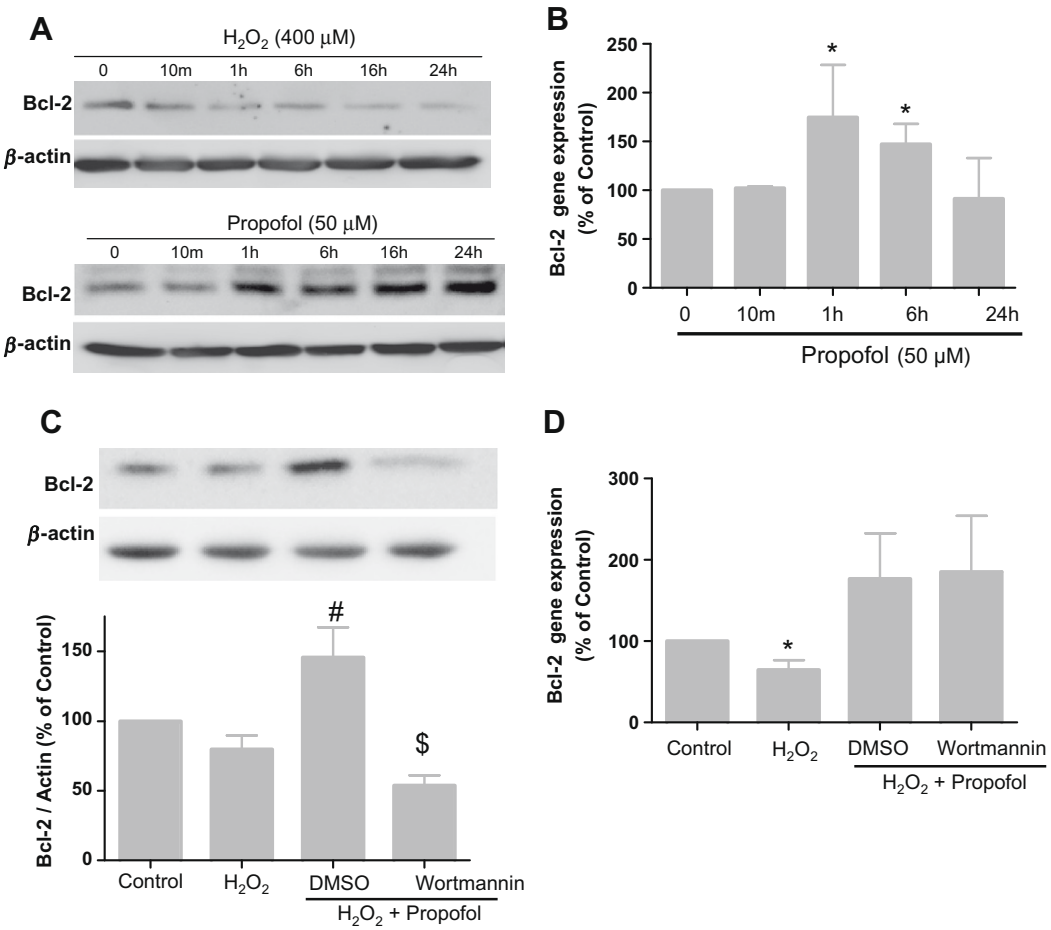

Fig. 3. Modulation of propofol on BCl-2. (A, B) H9c2 cells were treated with propofol (50 μM) or H_2O_2 (400 μM) for various times as indicated. (A) Cell lystates were harvested and Western blot was performed to examine Bcl-2 protein expression. Expression of β-actin was also examined for protein loading control. A decrease in Bcl-2 expression was observed in response to H_2O_2, while propofol increased Bcl-2 expression time-dependently. (B) Total cellular RNA were extracted and real-time RT-PCR was performed to examine Bcl-2 mRNA expression. Expression of GAPDH was also examined for internal control. Propofol increased Bcl-2 mRNA expression beginning at 1 h after treatment and was still elevated after 6 h treatment. (C, D) H9c2 cells were pre-incubated with 500 nM wortmannin for 30 min and then treated with H_2O_2 (400 μM) and propofol (50 μM) for 24 h. Wortmannin treatment decreased the Bcl-2 protein expression induced by propofol (C). Of interest, wortmannin treatment had no effects on propofol-induced Bcl-2 gene expression (D). $^{*}p < 0.01$ *vs* control, $^{\#}p < 0.01$ *vs* H_2O_2 only group, $^{\$}p < 0.01$ *vs* H_2O_2 + propofol + DMSO group.

protein expression. Of interest, wortmannin had no effects on propofol-induced Bcl-2 mRNA expression, as shown in Fig. 3D, which remained comparatively at the basal level.

Discussion

Consistent with the observed anti-apoptotic effect of propofol reported previously, we showed that propofol significantly protects cardiac H9c2 cells from cell death and apoptosis induced by H_2O_2 as determined by the MTS cell viability assay, caspase-3 activity assay and apoptotic nuclear morphological Hoechst staining. In addition, we found for the first time that propofol activates the PI3K–Akt signaling pathway as demonstrated by the suppression of the effect of propofol on cardiac H9c2 survival by wortmannin, which effectively suppresses the propofol-induced activation of Akt, up-regulation of Bcl-2 and protection from apoptosis. More importantly, we demonstrated that propofol may modulate anti-apoptotic Bcl-2 activation and expression through both Akt-dependent and Akt-independent pathways.

Several studies, including our own initial studies, have attributed propofol's cardioprotective effects largely to its antioxidant properties. However, propofol possesses diverse actions including anti-inflammatory properties, activation of atypical PKC isoforms, and inhibition of mitochondrial permeability transition that could confer protection against ischemia/reperfusion [8,17–20]. The results of the present study are consistent with our previous work, suggesting that propofol's effects cannot be explained strictly on the basis of an antioxidant mechanism. This provides a new direction of research examining alternative mechanisms of propofol-mediated cytoprotection in our laboratory.

Akt is a critical modulator of cell survival that antagonizes mitochondrial directed apoptosis. Acute activation of Akt can inhibit cardiomyocytes apoptosis induced by multiple stimuli, including H_2O_2 [7]. In order to determine if PI3K–Akt was the anti-apoptotic signaling pathway exploited by propofol in cardiac cells, we first evaluated its ability to induce Akt phosphorylation and activation. We found that propofol stimulated Akt phosphorylation at serine 473 beginning 10 min after treatment. Phosphorylation was still elevated after 1 h treatment. Increased phosphorylation of Akt at threonine 308 was also observed starting at approximately 1 h and remaining high up to 24 h after treatment (Fig. 2A).

In light of these results, we wanted to determine whether propofol activation of Akt is PI3K dependent in H9c2 cells. We verified whether the inhibition of PI3K by wortmannin, could suppress the effects of propofol on survival. As shown in Fig. 2B–D, wortmannin effectively suppressed the propofol-induced activation of Akt and protection from cell death and apoptosis.

Anti-apoptotic Bcl-2 is down-regulated during ischemia/reperfusion, but up-regulated in myocardium adapted to stress [21]. The activation of PI3K–Akt pathway is instrumental to the survival of cells by modulating Bcl-2 activation and expression. Akt phosphorylation induces the phosphorylation of Bad, a Bcl-2 family protein that promotes cell death by binding to and blocking the activity of Bcl-2. Phosphorylated Bad leads to the release and activation of Bcl-2 [22]. Moreover, Akt signaling also activates transcription factors that up-regulate anti-apoptotic genes. Akt can interact with and activate IκB (inhibitor of kappa B) kinases (IKKs) and lead to the activation of NF-κB [6,7], which then translocates to the nucleus and transcribes anti-apoptotic genes, such as Bcl-2 [23,24].

In order to determine if the activation of the PI3K–Akt pathway by propofol is instrumental to the survival of H9c2 cells by modulating Bcl-2 levels, we first assessed its effects on anti-apoptotic Bcl-2 gene and protein expression. We found that propofol treatment increased both Bcl-2 protein and gene expression. Although gene expression was elevated for up to 6 h post-treatment, Bcl-2 protein levels were consistently increased over the period of 24 h. We then investigated whether the inhibition of PI3K by wortmannin could suppress the propofol effects on Bcl-2 levels. We found that inhibition of Akt signaling by wortmannin in propofol-treated cells decreased Bcl-2 expression and was associated with increased cell death. Of interest, wortmannin treatment had no effects on propofol-induced Bcl-2 gene expression (Fig. 3). This important finding suggests that propofol may modulate Bcl-2 gene expression through another Akt-independent pathway as well.

Interestingly, in situations in which cardioprotection was obtained independently of Akt, the activation of another transcription factor transcription factor Signal Transducer and Activator of Transcription 3 (STAT-3) was observed [25]. STAT-3 is activated during ischemia and ischemia/reperfusion by the Janus Kinases (JAKs). STAT-3 is phosphorylated by activated JAK at the tyrosine level, which enables STAT-3 to form homodimers or heterodimers that translocates to the nucleus, resulting in gene transcription [26]. Preliminary data from our group demonstrated that propofol appeared to be able to modulate STAT-3 phosphorylation and activation in H9c2 cells (data not shown). However, further studies must be carried out to determine the effects of propofol on this Akt-independent modulation on Bcl-2. The postulated Akt-dependent and -independent modulation on Bcl-2 levels by propofol is summarized in Fig. 4 below.

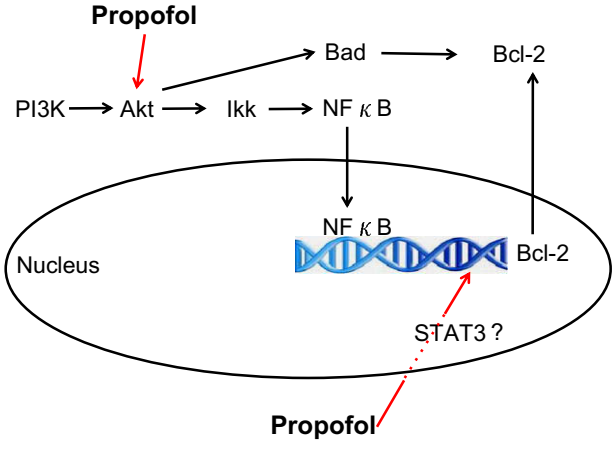

Fig. 4. Postulated Akt-dependent and -independent modulation on Bcl-2 by propofol. Akt phosphorylation induces the phosphorylation of Bad, which leads to the release and activation of Bcl-2. Moreover, Akt interact with and activate inhibitor of kappa B kinases (IKKs) and lead to the activation of nuclear factor (NF)-κB, which then translocates to the nucleus and transcribes anti-apoptotic Bcl-2 gene. We postulated that propofol might modulate Bcl-2 activation and expression through both Akt-dependent and -independent (STAT-3, signal transducer and activator of transcription 3) pathways.

Altogether, these findings suggest for the first time that propofol may provide an anti-apoptotic stimulus to cardiac H9c2 cells through the activation of PI3K–Akt signaling pathway and transcriptional modulation of Bcl-2 levels. This observation provides a potential support of propofol as a preemptive cardioprotectant in clinical settings such as during coronary bypass surgery.

Acknowledgments

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