

Propofol protects against hydrogen peroxide-induced apoptosis in cardiac H9c2 cells is associated with the NF- κ B activation and PUMA expression

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Abstract. – BACKGROUND AND AIMS:

Treatment with propofol has been found to attenuate oxidative stress injury in rat cardiac cells through the inhibition of programmed cell death. However, the underlying mechanism of this beneficial effect is not clear. Previous studies showed NF- κ B (NF- κ B) could be activated after oxidative stress in cardiac cells, and p53 up-regulated modulator of apoptosis (PUMA) is a direct target of NF- κ B and mediates oxidative stress - induced apoptosis. Here, we report that propofol protects cardiac H9c2 cells from hydrogen peroxide (H₂O₂)-induced apoptosis by preventing the activation of NF- κ B and PUMA upregulation.

MATERIALS AND METHODS: We first determined NF- κ B activity, PUMA expression, proliferation and apoptosis after hydrogen peroxide treatment of the H9c2 cell line using electrophoretic mobility shift assay (EMSA), western blot assay, MTT and annexin V/PI assay. We, then, examined the effect of inhibition of NF- κ B activity on the PUMA expression and hydrogen peroxide (H₂O₂)-induced apoptosis. We next examined the effect of inhibition of PUMA on hydrogen peroxide (H₂O₂)-induced apoptosis. In the end, we examined the effect of propofol pretreatment on NF- κ B activity, PUMA expression and hydrogen peroxide (H₂O₂)-induced apoptosis.

RESULTS: We show that H₂O₂ treatment led to increases in NF- κ B activity, PUMA protein levels in cardiac H9c2 cells. The induction of PUMA was mediated by the p65 component of NF- κ B. The apoptotic effect of PUMA induction by H₂O₂ was in an NF- κ B-dependent manner. We, further, demonstrate that pretreatment with propofol significantly decreased H₂O₂-induced apoptosis. Furthermore, propofol inhibits H₂O₂ induced NF- κ B activity and subsequent PUMA expression.

CONCLUSIONS: our results reveal a new mechanism by which propofol inhibits H₂O₂-induced injury in cardiac H9c2 cells via decreasing NF- κ B activation and PUMA expression. This inhibition improves cell survival, which provides a novel explanation for the pleiotropic effects of propofol that benefit the cardiac cells.

Key Words:

Propofol, Hydrogen peroxide, Apoptosis, PUMA, NF- κ B.

Introduction

NF- κ B is a group of dimeric transcription factors comprising members of the NF- κ B/Rel family, including p50, p52, p65 (Rel-A), Rel-B, and c-Rel. Although p50 and p65 regulate the canonical NF- κ B pathway, p52 and Rel-B are components of the noncanonical NF- κ B pathway. The activity of NF- κ B is normally kept in check by the I κ B family of inhibitors, which bind to and sequester NF- κ B in the cytoplasm¹. Activation of NF- κ B is triggered by I κ B phosphorylation by I κ k kinases and subsequent proteasomal degradation, which allows NF- κ B to translocate to the nucleus, where it binds to the κ B consensus sequences and modulates numerous target genes².

Previous study³ demonstrated exposure of H9c2 cells to 50 μ M H₂O₂ for 6h caused a significant increase in cell death, followed by increased NF- κ B activity. In intestinal epithelial cells, early activation of NF- κ B was one of the mechanisms of apoptosis by reactive oxygen species⁴. Wang et al⁵ also found hydrogen peroxide promotes apoptosis in endothelial cells and myocytes via activation of nuclear factor- κ B⁵. Marangolo et al⁶ has found H₂O₂ induced a transient activation of nuclear factor κ B. Pre-treatment of cells with the antioxidant N-acetylcysteine, (NAC), prevented both the activation of NF(Kappa)B and the induction of apoptosis by H₂O₂, suggesting a possible role for this transcription factor in oxidant-induced apoptosis in glial cells. The pro-survival activity of NF- κ B

in response to a variety of stimuli has been extensively characterized. Although there have been a few reports addressing the pro-cell death role of NF- κ B, the precise mechanism of NF- κ B's pro-cell death function still remains elusive.

PUMA (p53 upregulated modulator of apoptosis) is a Bcl-2 homology 3 (BH3)-only Bcl-2 family member and a critical mediator of p53-dependent and -independent apoptosis induced by a wide variety of stimuli, including genotoxic stress, deregulated oncogene expression, toxins, altered redox status, growth factor/cytokine withdrawal and infection⁷⁻⁸. PUMA is also involved in ischemia/reperfusion-induced intestinal injury⁹, which can induce an inflammatory response.

Gurzov et al¹⁰ has found that transcriptional activation of PUMA is regulated by NF- κ B and endoplasmic reticulum stress but is independent of p53. Qiu et al¹¹ has found the induction of PUMA was p53-independent but required NF- κ B. Absence of PUMA, but neither absence of p53 nor that of another BH3-only protein (Bid), relieved DSS- and TNBS-induced colitis and inhibited IEC apoptosis. Sun et al¹² has found PUMA was directly activated by p65 through the canonical NF- κ B pathway following AKT inhibition. Furthermore, PUMA was necessary for the chemosensitization and *in vivo* antitumor effects of aurora kinase inhibitors in colon cancer cells. We have recently found¹³ PUMA is a direct target of NF- κ B and mediates doxorubicin-induced apoptosis in cardiac H9c2 cells *in vitro*. Datta et al¹⁴ has recently reported that p53, activated by NF- κ B, is essential for H₂O₂ induced apoptosis in glioma cells, and p53 is the upstream of PUMA. We, therefore, suggested H₂O₂ induced apoptosis in glioma cells by PUMA activation.

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 injury¹⁵⁻¹⁷. It has been proposed that this action of propofol may be mediated by its ability to act as a free radical scavenger¹⁸. Oxidative stress is known to be associated with reperfusion injury and is probably responsible, either directly or indirectly, for damaging the myocyte¹⁹⁻²⁰. However, the underlying mechanism of this beneficial effect is not clear.

Xu et al²¹ has reported that propofol inhibited proliferation, invasion and angiogenesis of human Eca-109 cells *in vitro* through modulation of ERK-VEGF /MMP-9 signaling. Chen et al²² has

found propofol, by inhibiting p38 MAPK and NF- κ B activity, decreasing NOS expression, reducing NO production, could protect HUVECs which are exposed to oxidative stress and becoming dysfunctional. Cui et al²³ has found propofol prevents cerebral ischemia-triggered autophagy activation and cell death in the rat hippocampus through the NF- κ B/p53/PUMA signaling pathway.

In the present study, we first sought to define NF- κ B's role in cardiac H9c2 cells induced by H₂O₂. Here we present evidence that exposure to H₂O₂ induces a NF- κ B dependent cell death and that NF- κ B potentiates cell death through the activity of PUMA. We then investigated that propofol inhibited hydrogen peroxide-induced injury in cardiac H9c2 cells via the NF- κ B activation and PUMA expression.

Materials and Methods

Cells and Culture Conditions

Cardiac H9c2 cells were 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 (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were grown in a humidified incubator containing 95% air and 5% CO₂ at 37°C with media replenishment every 3 days.

Propofol and H₂O₂ Treatment

H9c2 cells (2×10^6) were incubated with 50 μ M propofol for 30 minutes and then incubated for a further 4 hours after the addition of H₂O₂ (St. Louis, MO, USA) to a final concentration of 400 μ M H₂O₂. Propofol (Diprivan, AstraZeneca Pharmaceutical, Harbin, China) was dissolved in dimethyl sulfoxide (DMSO; Sigma Chemical, St. Louis, MO, USA), yielding a final dimethyl sulfoxide (DMSO) concentration in media of 0.0025-0.0125%. Tests showed that DMSO over this concentration range had no effect on oxidative stress or cell viability (data not shown). The concentration of H₂O₂ (400 μ M) applied was based on similar methods described in the scientific literature²⁴. The concentration of propofol (50 μ M) applied was determined based on similar methods described in the scientific literature²⁵ and the average blood propofol concentration during human cardiac surgery in our clinical project²⁶.

NF- κ B Inhibition and PUMA siRNA Transfection

For inhibitor experiments (1) H9c2 cells (2×10^6) were pre-incubated with NF- κ B inhibitor (PDAC 25 μ M) or vehicle (DMSO) for 30 min and, then, treated with 400 μ M H₂O₂; (2) H9c2 cells (2×10^6) were transfected with PUMA siRNA or control siRNA for 24 hs according to the manufacture's instruction and, then, treated with 400 μ M H₂O₂.

Electrophoretic Mobility Shift Assays (EMSAs)

EMSA experiments were carried out as described in (27) using a ³²P-labeled oligonucleotide probe (5'-GCTACAAGGGACTTTC-CGCTGGGGACTTTCAGGGAGG-3').

Western Blotting Assays

Centrifuged cells were suspended in lysis buffer composed of 20 mM Tris, pH 7.5, 1% SDS, 2 mM EDTA, 2 mM EGTA and 6 mM β -mercaptoethanol. After a 10 min incubation on ice, cell suspensions were sonicated. Fifty micrograms of protein were subjected to 12% SDS-PAGE. Membranes were exposed to rabbit anti-PUMA polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:200 dilution followed by goat anti-rabbit secondary antibody at a 1:20,000 dilution.

MTT Assay

The amount of cell death was determined by examining cell number with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacture's instruction. The percent cell survival was calculated by taking the optical density (OD) reading of cells given a particular treatment, dividing that number by the OD reading for the untreated control cells, and then multiplying by 100.

Quantification of Apoptosis With Annexin V/PI Staining

Simultaneous flow cytometric quantification of apoptotic and viable cells was performed with an annexin V/propidium iodide kit (Annexin V-FITC/PI) (Assay Designs, Ann Arbor, MI, USA). H9c2 cells was exposure to up to 400 μ M H₂O₂ with or without propofol were harvested, resuspended in binding buffer, incubated with annexin-FITC and propidium iodide and analyzed by flow cytometry (CyFlow ML, Partec, Germany).

Statistical Analysis

All numerical results are reported as means \pm SE and represent data from a minimum of three independent experiments. Significance was evaluated using analysis of variance followed by paired Student's t test or paired two-way ANOVA. $p < 0.05$ was considered statistically significant.

Results

Propofol Inhibits H₂O₂-induced H9c2 Cell Death

We first determined the dose at which cytotoxicity develops in a period of 24 hours upon H₂O₂ exposure in H9c2 cells through the MTT assay. Cells were treated with increasing doses of H₂O₂ for 24 hours. As shown in Figure 1A, H₂O₂ impaired cell viability in a concentration-dependent manner over the tested concentration range (50 to 400 μ M). A maximum reduction of $53.7 \pm 8.6\%$ was observed with 400 μ M of H₂O₂.

In order to evaluate whether propofol was cytotoxic to H9c2 cells, we determined the viability of cells treated with propofol (1 to 100 μ M) for 24 hours using the MTT assay. Cell viability was not significantly affected by treatment with any tested propofol concentrations (Figure 1B). We chose the level of 50 μ M for subsequent experiments based on similar methods described in scientific literature²⁵.

We, next, investigates whether propofol inhibits H₂O₂-induced H9c2 cell death. H9c2 cells were treated with propofol 1-100 μ M or vehicle for 8 h, after that, the cells was exposed to 400 μ M H₂O₂ or vehicle in fresh medium for 24 h. As shown in Figure 1C, the survival rates of H9c2 cells treated with 1 μ M, 10 μ M, 25 μ M and 100 μ M propofol were 54.3%, 67.8%, 79.5% and 91.6%, respectively. The results indicate that a dose of propofol does not contribute significantly to cytotoxicity in H9c2 cells, and affords cytoprotection to the cell cultures in a concentration-dependent manner. Taken together, these data show that propofol inhibits H₂O₂-induced growth in H9c2 cells.

Propofol Inhibits H₂O₂-Induced H9c2 Cell Apoptosis

We first determined the effect of propofol or H₂O₂ exposure in H9c2 cells alone in a period of 24 hs on apoptosis through the Annexin V/PI assay. As shown in Figure 2A, H₂O₂ increases cell

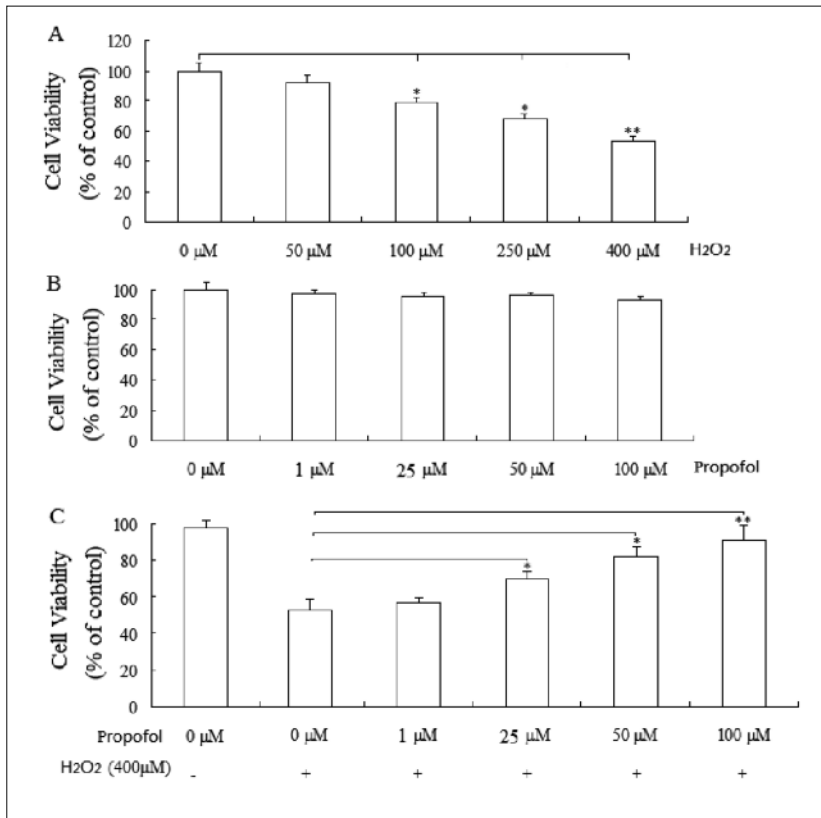


Figure 1. Propofol prevents H₂O₂-induced cell death. **A**, Cells were exposed to various concentrations of H₂O₂ for 24 h before being subjected to the MTT assay. **B**, Cells were treated with various concentrations propofol for 24 h before being subjected to the MTT assay. **C**, Cells were treated with 1-100 μM propofol for 8 h then incubated with H₂O₂ (400 μM) for a further 24 h, Cell viability was measured by MTT assays. The results (presented as mean ± SD) are from three independent tests. *and **significant differences (**p* < 0.05 and ***p* < 0.01 respectively).

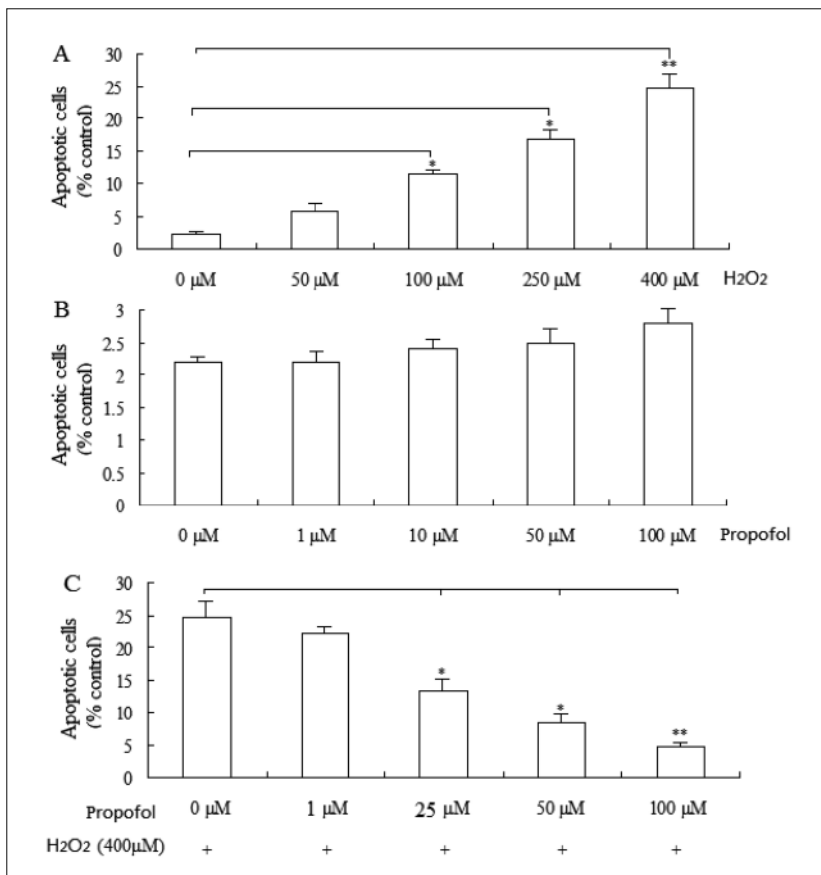


Figure 2. Effect of propofol on H₂O₂-stimulated cell apoptosis in H9c2 cells. **A**, Cells were exposed to various concentrations of H₂O₂ for 24 h before being subjected to the flow cytometry assay. **B**, Cells were treated with various concentrations propofol for 24 h before being subjected to the flow cytometry assay. **C**, Cells were treated with 1-100 μM propofol for 8 h then incubated with H₂O₂ (400 μM) for a further 24 hs, apoptotic cells was measured by the flow cytometry assays. Results (presented as mean ± SD) are from three independent tests. *and **significant differences (**p* < 0.05 and ***p* < 0.01, respectively).

apoptosis in a concentration-dependent manner over the tested concentration range (50 to 400 μM). A maximum reduction of $24.6 \pm 3.8\%$ was observed with 400 μM of H_2O_2 . We also found cell apoptosis was not significantly affected by treatment with any tested propofol concentrations (1-100 μM) (Figure 2B).

In order to determine the effects of propofol on H_2O_2 -stimulated cell apoptosis, the H9c2 cells were pretreated with propofol (50 μM) for 30 min and, then, co-incubated with 50-400 μM of H_2O_2 for additional 24 hs. As shown in Figure 2C, apoptotic cells of H_2O_2 -treated cells was restored to control levels in the presence of propofol.

H₂O₂-Induced NF- κ B Dependent PUMA Activation in H9c2 Cells

H9c2 cells were exposed to H_2O_2 (400 μM) for 24 h. NF- κ B activity was detected by EMSA assay, and PUMA expression was detected by western blot assay. As shown in Figure 3, H_2O_2 significantly increases cell NF- κ B activity (Figure 3A) and PUMA levels (Figure 3B).

We next investigated whether NF- κ B activity by H_2O_2 is PUMA dependent. To accomplish this, we first silenced PUMA with siRNA using the Lipofectamine 2000 according to the manufacturer's protocol. A panel of three different siRNA was screened for the ability to suppress PUMA expression in H9c2 cells. The expressing siRNA clone 3 reduced PUMA protein levels by more than 90% (Figure 3B) without affecting the NF- κ B activity of H_2O_2 -induced (Figure 3A).

We, then, investigated whether PUMA activity by H_2O_2 is NF- κ B dependent. H9c2 cells (2×10^6) were pre-incubated with NF- κ B inhibitor (PDAC 25 μM) for 30 min, and then treated with 400 μM

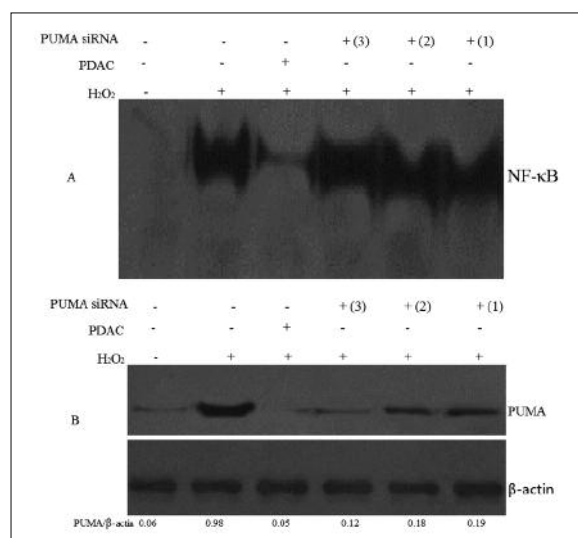


Figure 3. Effects of H_2O_2 on NF- κ B activity and PUMA expression. H9c2 cells (2×10^6) were pre-incubated with NF- κ B inhibitor (PDAC 25 μM) for 30 min, and then treated with 400 μM H_2O_2 ; H9c2 cells (2×10^6) were transfected with PUMA siRNA1, 2 and 3 for 24 hs, respectively according to the manufacturer's instruction and, then, treated with 400 μM H_2O_2 . A, EMSA for NF- κ B activity. B, Western blot assay for PUMA expression.

H_2O_2 for 24 h. As shown in Figure 3, NF- κ B activity (Figure 3A) and PUMA levels (Figure 3B) was significantly inhibited.

PUMA is Indispensable for H₂O₂-Induced Apoptosis and NF- κ B Activation in H9c2 Cells

H9c2 cells (2×10^6) were pre-incubated with PDAC 25 μM for 30 min and, then, treated with H_2O_2 (400 μM) for 24 hours, H_2O_2 -induced apoptosis was significantly inhibited (Figure 4). These data show H_2O_2 -induced apoptosis in H9c2 cells by NF- κ B activity.

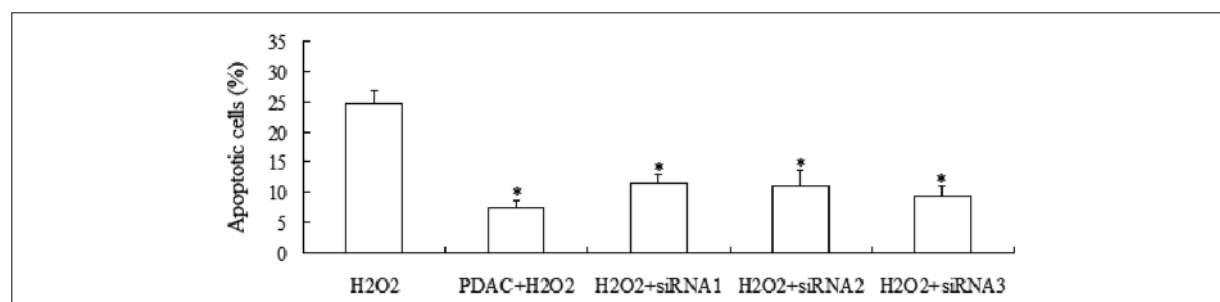


Figure 4. H_2O_2 -induced apoptosis depends on NF- κ B activation and PUMA expression in H9c2 cells. H9c2 cells (2×10^6) were pre-incubated with PDAC 25 μM for 30 min or transfected with PUMA siRNA1, 2 and 3 for 24 hs, and then treated with H_2O_2 (400 μM) for 24 hours, H_2O_2 -induced apoptosis was detected by flow cytometry assay. Results (presented as mean \pm SD) are from three independent tests. Compared to control, * $p < 0.05$.

H9c2 cells (2×10^6) were transfected with PUMA siRNA1, 2 and 3 for 24 hs to inhibit PUMA expression, respectively (Figure 3B) according to the manufacture's instruction and, then, treated with $400 \mu\text{M}$ H_2O_2 for 24 hs, H_2O_2 -induced apoptosis was also significantly inhibited (Figure 4), however, NF- κB activity was not significantly changed (Figure 3A). These results demonstrate that NF- κB -dependent PUMA upregulation is indispensable for H_2O_2 -induced cell apoptosis.

Propofol inhibits H_2O_2 -Induced NF- κB Activation and PUMA Expression in H9c2 Cells

H9c2 cells (2×10^6) were incubated with $50 \mu\text{M}$ propofol for 30 minutes and then incubated for a further 4 hs after the addition of H_2O_2 to a final concentration of $400 \mu\text{M}$. NF- κB activity was detected by EMSA assay, and PUMA expression was detected by western blot assay. As shown in Figure 5, propofol ($50 \mu\text{M}$) pretreatment significantly decreased this H_2O_2 -induced NF- κB activity (Figure 5A) and PUMA expression (Figure 5B).

Discussion

The main finding of the present study is that propofol significantly protects cardiac H9c2 cells from cell death and apoptosis induced by H_2O_2 as determined by the MTT cell viability assay and flow cytometry assay. In addition, we found for

the first time that H_2O_2 activates the NF- κB dependent PUMA expression, which results in H_2O_2 -induced H9c2 cell apoptosis. More importantly, we demonstrated that propofol protects H9c2 against H_2O_2 -induced apoptosis by inhibiting NF- κB activation and PUMA expression. Propofol may modulate PUMA expression through NF- κB -dependent pathways.

Oxidative stress induces DNA damage leading to either growth arrest and senescence or apoptosis, depending on the nature and intensity of the stimulus and cell type (i.e., normal vs. tumor cells)²⁸⁻²⁹. Here we show that H_2O_2 induced oxidative stress caused the apoptosis of cardiac H9c2 cells.

A large body of evidence has demonstrated a protective role of NF- κB activity in most tissues and cell types. For example, targeted deletion of *p65* in mice leads to increased apoptosis in several tissues³⁰. The protection by NF- κB is due to transcriptional activation of a number of anti-apoptotic proteins, such as c-FLIP, Bcl-2, Bcl-XL, cIAP2, and A1/Bfl-2.6 Cells without appropriate NF- κB activation can be eliminated by death-inducing signaling complex-mediated apoptosis through the extrinsic pathway³¹. Conversely, NF- κB has been found to promote apoptosis under certain conditions by activating the expression of proapoptotic proteins, such as PUMA^{13,32}. Here we show that H_2O_2 induced oxidative stress caused the apoptosis of cardiac H9c2 cells through NF- κB activation and subsequent PUMA upregulation.

Several studies have attributed propofol's cardioprotective effects largely to its antioxidant properties. However, propofol possesses diverse actions including anti-inflammatory properties, activation of a typical PKC isoforms, and inhibition of mitochondrial permeability transition that could confer protection against ischemia/reperfusion³³⁻³⁷. Propofol functions depending on the stimulus and cell type. In pancreatic cancer, inactivation of the NF- κB signaling pathway by propofol might abrogate gemcitabine-induced activation of NF- κB , resulting in chemosensitization of pancreatic tumors to gemcitabine³⁵. In ovarian cancer cell, propofol inhibits invasion and metastasis, enhances paclitaxel-induced ovarian cancer cell apoptosis through suppression of Slug³⁹. In esophageal squamous cell carcinoma cells, propofol inhibited proliferation, invasion and angiogenesis of human Eca-109 cells *in vitro* through modulation of ERK-VEGF/MMP-9 signaling⁴⁰. The studies above in-

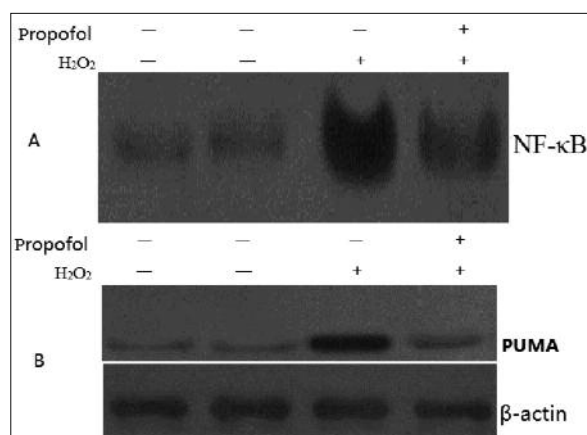


Figure 5. Modulation of propofol on H_2O_2 -induced NF- κB activation and PUMA expression in H9c2 cells. H9c2 cells were treated with $50 \mu\text{M}$ propofol for 30 minutes and, then, incubated for a further 4 hs after the addition of H_2O_2 to a final concentration of $400 \mu\text{M}$. **A**, EMSA for NF- κB activity. **B**, Western blot assay for PUMA expression.

licated that propofol played an important role in promoting apoptosis and inhibiting proliferation in tumor cells. However, Zhang et al⁴¹ has found propofol may protect PC12 cells from A β 25-35-induced apoptosis. Wang et al⁴² found propofol inhibits H₂O₂-induced apoptosis in cardiac H9c2 cells via Akt activation and Bcl-2 up-regulation. Wang et al⁴³ found propofol protects hepatic L02 cells from H₂O₂-induced apoptosis, partly through activating the MEK-ERK pathway and further suppressing Bad and Bax expression. The studies above indicated that propofol played an important role in inhibiting apoptosis and promoting proliferation in normal cells. We, therefore, suggested propofol in normal vs. tumor cells can have radically different outcomes. The results of the present study showed that propofol inhibited H₂O₂ induced oxidative stress via inhibiting of H₂O₂ induced NF- κ B activation and PUMA expression. This provides a new direction of research examining mechanisms of propofol-mediated cytoprotection in our laboratory.

Conclusions

Altogether, these findings suggest for the first time that propofol may provide an antiapoptotic stimulus to cardiac H9c2 cells through inhibiting NF- κ B activation and subsequent PUMA expression. This observation provides a potential support of propofol as a preemptive cardioprotectant in clinical settings such as during coronary bypass surgery.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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