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Propofol induces apoptosis of non-small cell lung cancer cells via ERK1/2-dependent upregulation of PUMA

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Abstract. – OBJECTIVE: Propofol is one of the most commonly used intravenous anesthetic agents used in cancer resections, but the effect of propofol on non-small cell lung cancer (NSCLC) remains unclear. Previous researches have reported that propofol can inhibit extracellular signal-regulated kinase (ERK) 1/2 phosphorylation or activate p53-upregulated modulator of apoptosis (PUMA) signaling, resulting in apoptosis. In addition, PUMA is negatively regulated by ERK1/2 activation. In the present work, we determined the effect of propofol on NSCLC A549 cells and explored its signaling pathway.

MATERIALS AND METHODS: A549 cells were treated with different concentrations of propofol (1-10 μg/mL) for 6 h. After washing, cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and antibiotics for another 72 h. Cell survival and apoptosis were determined by MTT, flow cytometry, and TUNEL analyses. To assess whether propofol functions via ERK1/2 and PUMA signaling pathways, A549 cells were transfected with small interfering RNA (siRNA) to target PUMA, or treated with human recombinant ERK1/2 (hrERK1/2) to activate ERK1/2.

RESULTS: Propofol treatment inhibited viability and induced apoptosis of A549 cells in a dose-dependent manner *in vitro*. Propofol inhibited phosphorylation of ERK1/2 (pERK1/2) and increased ERK1/2-dependent PUMA expression. Knockdown of PUMA by siRNA or treatment with hrERK1/2 to activate ERK1/2 blocked propofol-induced apoptosis and cell viability. Upregulation of PUMA expression by propofol requires pERK1/2 inactivation.

CONCLUSIONS: Propofol inhibits viability and induces apoptosis of A549 cells via an ERK1/2-dependent PUMA signaling.

Key Words:
Non-small cell lung cancer, Propofol, Apoptosis, ERK1/2, PUMA.

Introduction

The extracellular signal-regulated kinase (ERK) 1/2 is a member of the mitogen-activated protein kinase family, which regulates essential cellular functions including proliferation, differentiation, cell survival, and cell death[1]. ERK1/2 can be activated by numerous extracellular agents such as growth factors, cytokines, hormones, and tumor promoters[2]. ERK1/2 kinases positively regulate the cell cycle by increasing the availability of building blocks for cell growth[3], stimulating the cyclin-dependent kinase-cyclin complexes required for cell cycle progression[4], and preventing cell death[5]. Recent results have indicated that the inactivation of ERKs may activate tumor suppressor pathways and induce cell apoptosis[6]. These conflicting results of pro- and anti-apoptotic functions may reflect differences in the cellular context. The p53-upregulated modulator of apoptosis (PUMA) is a downstream target of p53 and a BH3-only family member. PUMA is activated by p53 following exposure to DNA damaging agents, such as γ-irradiation and commonly used chemotherapeutic drugs[7-9]. It is also activated by a variety of nongenotoxic stimuli independent of p53, such as serum starvation, kinase inhibitors, glucocorticoids, endoplasmic reticulum stress, and ischemia/reperfusion[10,11]. The pro-apoptotic function of PUMA is mediated by its interactions with anti-apoptotic Bcl-2 family members[12], which lead to mitochondrial dysfunction and caspase activation[13].

Propofol (2,6-diisopropylphenol) is an intravenous sedative-hypnotic agent administered to induce and maintain anesthesia. It has recently been revealed that propofol exhibits anticancer properties in LoVo colon cancer cells[14], gastric cancer cells[15], and gallbladder cancer cells[16]. In a

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prostate cancer cell line (PC3), propofol prevented isoflurane-induced hypoxia-inducible factor (HIF)-1α activation, and partially reduced cancer cell malignant activity[17]. Therefore, propofol might be a better agent for cancer surgery compared with other anesthetics. Propofol regulates apoptosis both *in vitro* and *in vivo* by different molecular mechanisms. In myocardial cells, combination therapy of propofol and sevoflurane may protect myocardial cells from damage during IR by decreasing levels of MAP2K3 and reducing apoptosis via the Bcl-2/Bax pathway[18]. In cervical cancer cells, propofol inhibits tumor size, cell viability, and promoted apoptosis via inhibition of the mTOR/p70S6K pathway[19]. Propofol can effectively inhibit proliferation and induce apoptosis of epithelial ovarian carcinoma cells and modulation of expression of the microRNA let-7i, possibly contributing to the antitumor action of propofol[20]. Propofol has also been shown to activate caspase-3, and trigger apoptosis and inhibit proliferation of neuronal cells[21]. However, some studies have found that propofol can inhibit apoptosis. Propofol exerts a cardioprotective effect when administered in the early phase of reperfusion. The effect is mediated by reducing cardiomyocyte apoptosis and activating nuclear translocation of ERK1/2-dependent nuclear factor (NF)-κB[22]. Wang et al[23] showed that propofol protected hepatic L02 cells from H_2O_2-induced apoptosis, partly via activation of the MEK/ERK pathway and further suppressing the expression of Bad and Bax. These conflicting findings of pro- and anti-apoptotic functions of propofol may be related to cell specificity.

Several researches[24-27] have demonstrated that inhibition of the MEK/ERK pathway could effectively induce upregulation of PUMA expression and trigger apoptosis. The aim of this study was to assess the roles of propofol on apoptosis and the growth of A549 cells and to explore its molecular mechanisms.

Materials and Methods

Cell Line and Culture

Human lung cancer A549 cells were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (Jinan, China), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

Agents

Primary antibodies against pERK1/2 (T202/Y204) (9101S), activated caspase-3 (Asp-175) (9661), ERK1/2 (9102S), and PUMA (4976T) were purchased from Cell Signaling Technology (Shanghai, China). PUMA small interfering RNA (siRNA) (sc-37153) and anti-β-actin (A19789) antibody were purchased from Santa Cruz Biotechnology (Guangzhou, China). Human recombinant ERK2 (hrERK2) (LS-G18540) was obtained from Ziqi.com (Shanghai, China). Cells incubated with culture medium and culture medium with dimethyl sulfoxide (DMSO) served as controls.

Transient siRNA Transfection

A549 cells were seeded in six-well plates and grown to 50-60% confluence. A549 cells were transfected with PUMA siRNA (2 μg) in OptiMEM (Gibco, BRL, Grand Island, NY, USA) for 24 h using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Stimulation of A549 Cells With Propofol

The dose of propofol used clinically typically ranges from 1-10 μg/mL (blood concentration) with higher doses used for induction of anesthesia and lower doses for maintenance[28,29]. In addition, cell culture and whole animal studies have shown that propofol can induce toxicity at high doses or prolonged exposure times after a single exposure[30]. Thus, we selected 1-10 μg/mL for further study. A549 cells were cultured in 96-well plates (3×10^4 per well) and treated with 1-10 μg/mL propofol for 6 h. After washing, cells were then cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and antibiotics for another 72 h. To determine the signaling pathways involved in the production of ERK1/2, A549 cells were preincubated with hrERK2 (10 ng/mL) or DMSO (control) 6 h prior to the addition of propofol. To determine the signaling pathways involved in the production of PUMA, A549 cells were transfected with PUMA or control siRNA 24 h before propofol exposure.

Western Blot Assay

For total protein extraction, cells were washed once with phosphate-buffered saline (PBS) and lysed with radioimmunoprecipitation buffer for 30 min on ice. Cytoplasmic extracts were prepared using the Extract Kit (Active Motif, Inc., Shanghai, China) according to the manufacturer's instructions. Protein concentration was quan-

tified with Coomassie Plus (Bradford) Protein Assay Reagent according to the manufacturer's instructions. Extracts (40 μg) were resolved with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to Hybond-C Extra nitrocellulose membranes (GE Healthcare; Germany). Membranes were probed with primary antibodies against pERK1/2 (1:100), ERK1/2 (1:100), PUMA (1:100), and activated caspase-3 (1:200), followed by incubation for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit or anti-goat IgG, respectively. Immunoblotting for β-actin (1:500) served as a protein loading control. All experiments were performed at least three independent times.

Cell Viability Assay

A549 cells were seeded in 96-well plates at a density of 5,000 cells/well in 100 mL of medium and incubated for 24 h. To determine the effect of ERK1/2, A549 cells were treated with hrERK1/2 for 6 h prior to propofol exposure. To determine the effect of PUMA, A549 cells were transfected with PUMA or control siRNA for 24 h prior to propofol exposure. Cells incubated in culture medium with or without DMSO served as controls. Determination of viable cells was performed by the addition of 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H- tetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. MTT measurements were performed after 3 days.

Flow Cytometry

The induction of apoptosis was quantified by annexin V/propidium iodide (PI) double staining followed by flow cytometry. Annexin V/PI double staining was performed using an apoptosis detection kit (Biovision, Mountain View, CA, USA) according to the manufacturer's instructions. Briefly, following exposure to various treatments, cells were gently detached by brief trypsinization and washed with ice cold PBS. After another wash with binding buffer, cells were suspended in 300 μL binding buffer containing annexin V and PI, and incubated for 5 min at room temperature. Early apoptotic cells were identified as annexin V-positive and PI-negative, while late apoptotic/necrotic cells were identified as annexin V-positive and PI-positive using a BD LSR II cell analyzer.

TUNEL Assay

DNA fission associated with apoptosis was analyzed using a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate *in situ* nick end labeling (TUNEL) detection kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. The cells cultured on coverslips were rinsed with PBS and fixed with ice-cold 1% paraformaldehyde. Terminal deoxynucleotidyl transferase, a template-independent polymerase, was used to incorporate nucleotides at the sites of DNA breaks. Nuclei were stained with TO-PRO-3. Fluorescent images were taken from three different fields on each coverslip with a confocal microscope. Apoptotic index was calculated as a percentage according to the following formula: number of TUNEL-positive nuclei/number of total cell nuclei.

Statistical Analysis

Student's *t*-test (two-tailed) was performed to analyze the data. p-value of < 0.05 was considered to be statistically significant.

Results

Effect of Propofol on pERK1/2, ERK1/2, PUMA, and Cleaved Caspase-3 Expression in A549 Cells

A549 cells were treated with 1-10 μg/mL propofol for 6 h. After washing, cells were cultured in DMEM supplemented with 10% FBS and antibiotics for another 72 h. Western blot analysis was used to assess the protein levels of pERK1/2, ERK1/2, PUMA, and cleaved caspase-3 following propofol treatment in A549 cells. As shown in Figure 1A, C, and D, treatment with propofol resulted in a dose-dependent increase in the expression of PUMA and cleaved caspase-3. However, pERK1/2 displayed a dose-dependent decrease (Figure 1A, B). No change was evident in the expression of total ERK1/2 (T-ERK1/2) following propofol treatment (Figure 1A).

Effect of Propofol on Cell Survival and Apoptosis

A549 cells were treated with 1-10 μg/mL propofol as above, and the effects of propofol on both cell survival and apoptosis were determined *in vitro*. Analysis of cell survival using the MTT assay showed a dose-dependent decrease following propofol treatment (Figure 2A). Treatment with 1-10 μg/mL propofol exhibited a dose-dependent increase in apoptosis using annexin V/PI double staining followed by flow cytometry (Figure 2B).

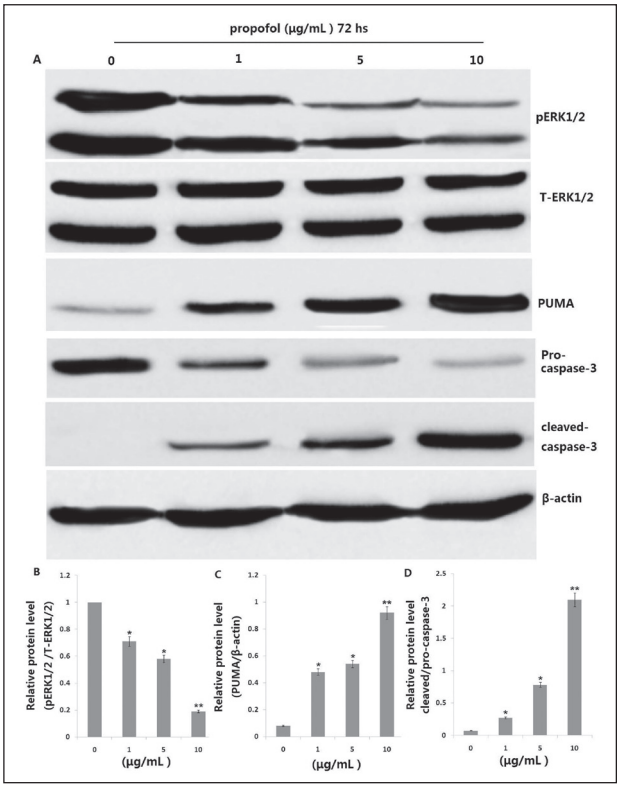

Figure 1. Effect of propofol on protein expression in A549 cells. A549 cells were treated with 1-10 μg/mL propofol for 6 h. **A,** Representative images showing expression of pERK1/2, ERK1/2, PUMA, and cleaved caspase-3 as analyzed by western blotting. β-actin was used as a loading control. **B,** Densitometry analysis of p-ERK1/2; **C,** Densitometry analysis of PUMA; **D,** Caspase-3 is activated as shown by the conversion of pro-caspase-3 to activated cleaved caspase-3. All values represent the mean ± standard error.

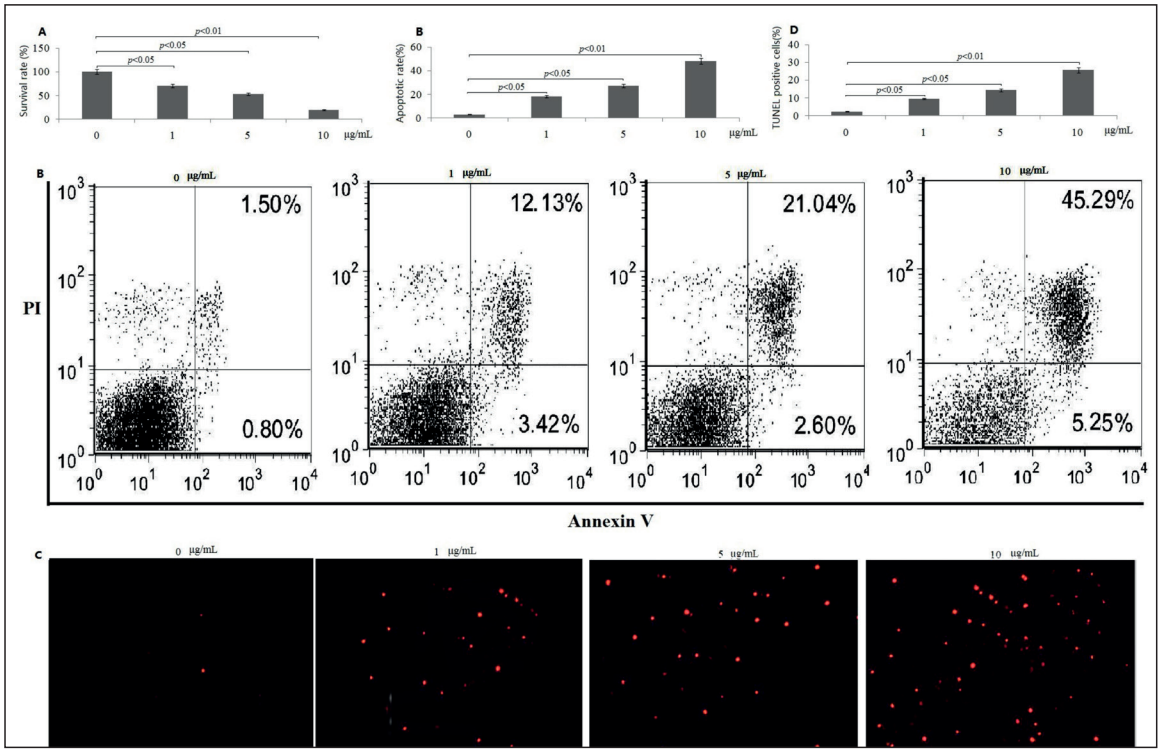

Figure 2. Effect of propofol on cell survival and apoptosis. A549 cells were treated with 1-10 μg/mL propofol for 72 h. **A,** Cell survival was detected by the MTT assay. **B,** Cell apoptotic rate was detected by flow cytometry of cells stained with annexin V and counterstained with propidium iodide after 6 h. Percentage of cells is shown in the corner of each quadrant. The results are representative of two independent experiments. **C,** Cell apoptotic rate was detected by TUNEL assay. **D,** Images show A549 cells incubated with propofol (×200; scale bar: 100 mm).

We then examined DNA damage by TUNEL staining. More TUNEL-positive cells were observed in propofol-treated A549 cells (Figure 2C). Most of the TUNEL-positive cells were located in condensed nuclei, a hallmark of apoptosis. As shown in Figure 2C, treatment of A549 cells with 1.5 and 10 μg/mL propofol for 72 h resulted in 8.4 ± 0.8%, 14 ± 1.3%, and 28 ± 1.4% apoptotic cells, respectively.

Upregulation of PUMA by Propofol Correlated With the Induction of Apoptosis in A549 Cells

To determine if propofol induced cell apoptosis and inhibited proliferation by upregulating PUMA, A549 cells were transfected with PUMA siRNA for 24 h, followed by treatment with 10 μg/mL propofol for 72 h. As shown in Figure 3A, 80% of the PUMA protein in A549 cells was inhibited by PUMA siRNA transfection and propofol treatment. In PUMA siRNA-transfected A549 cells, the rate of apoptosis was only 8.3%, which was significantly lower than propofol treatment alone ($p < 0.01$) (Figure 3B). In addition, the cell survival rate was significantly decreased in propofol-treated A549 cells (17.3 ± 26.2%) than PUMA siRNA transfected A549 cells (78.4 ± 90%) ($p < 0.01$) (Figure 3C). The results of the TUNEL staining were consistent with the flow cytometry (Figure 3D, E).

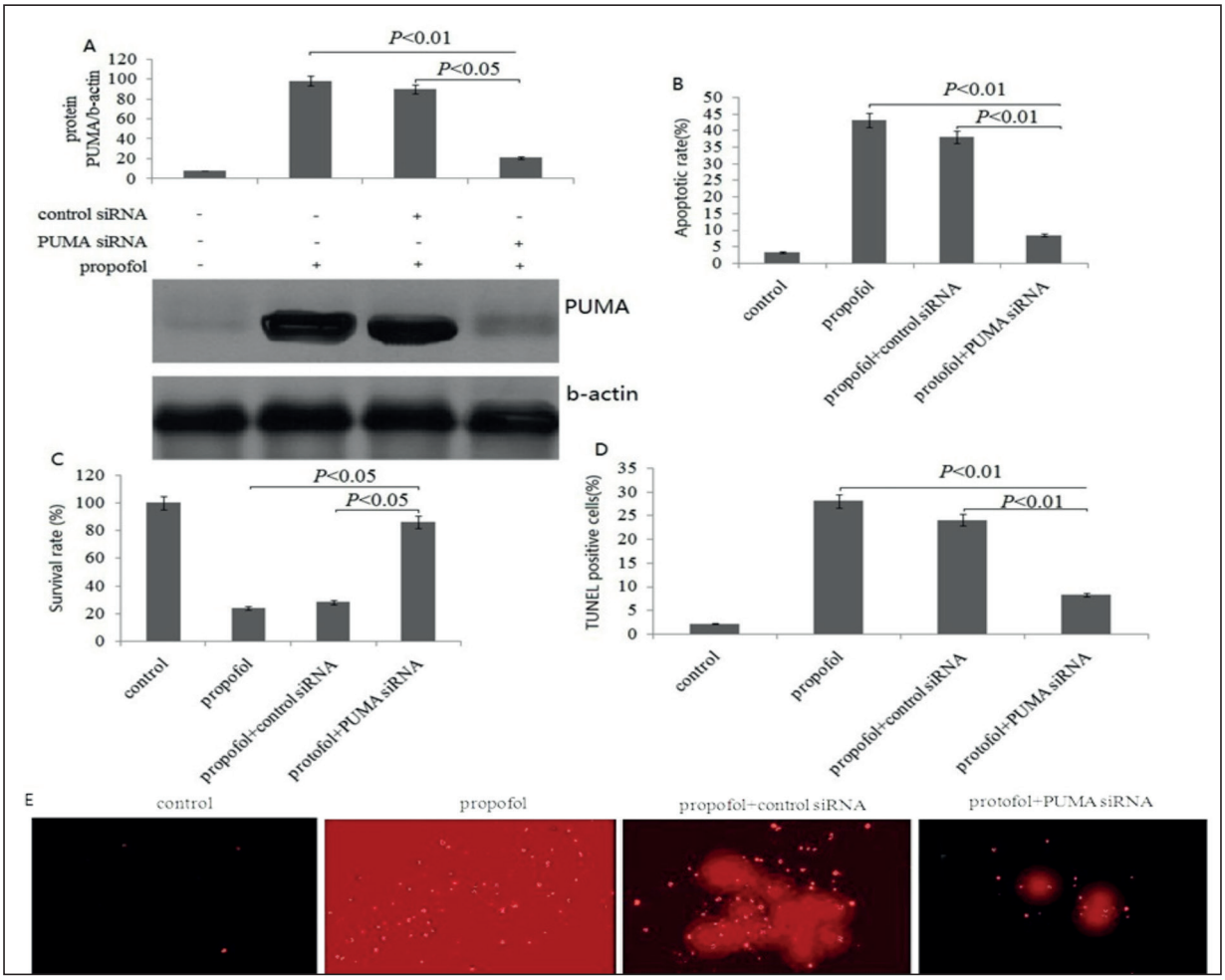

Figure 3. Effect of PUMA on propofol-induced apoptosis in A549 cells. A549 cells were transfected with PUMA small interfering RNA (siRNA) for 24 h, followed by treatment with 10 μg/mL propofol for 72 h. **A,** Representative images showing expression of PUMA as analyzed by western blotting. β-actin was used as a loading control. **B,** Cell apoptotic rate was determined by flow cytometry. **C,** Cell survival was determined by the MTT assay. **D,** Cell apoptotic rate was determined by the TUNEL assay. **E,** Images show apoptotic A549 cells incubated with propofol and/or siRNA (×200; scale bar: 100 mm).

Propofol Induced ERK1/2-dependent PUMA Upregulation

Propofol inhibited proliferation and induced apoptosis of A549 cells by PUMA upregulation. To determine the effect of ERK1/2 on propofol-induced apoptosis and growth inhibition, A549 cells were treated with hrERK1/2 for 6 h prior to propofol treatment. In the presence of hrERK1/2, PUMA protein was inhibited in propofol-treated A549 cells (Figure 4A). It has been suggested that propofol treatment abrogates ERK1/2-dependent induction of PUMA. Flow cytometry and the TUNEL assay revealed that apoptosis was also significantly decreased (Figure 4B, C). The cell survival rate was significantly increased with hrERK1/2 exposure (Figure 4D).

Discussion

The ability of a malignant tumor to become metastatic begins with the hallmarks of motility and invasiveness. It is estimated that 90% of cancer-associated mortality contributes to metastasis. Circulating tumor cells (CTCs) are shed into the vasculature from primary and/or metastatic tumor deposits[31]. The presence of CTCs in the peripheral blood appears to be an early indicator of cancer metastasis and may demonstrate tumor spread before clinical symptoms or detection by imaging[31,32]. Circulating cancer cells could be present in almost all patients prior to surgery[33]. The presence of CTCs in the blood represents a poor prognosis in a variety of carcinomas. In addition, surgery has long been suspected to enhance the growth and metastases of malignant tumors, which results in surgeons avoiding the tumor unless absolutely necessary. Moreover, during the perioperative period, surgery might enhance the release of CTCs into the bloodstream[34-38]. Once in the circulation, CTCs persist for a short time, and there is evidence of such cells at 24 h following surgical resection[39]. Tumor cells shed in the circulation arrest in the vasculature of a secondary organ during surgery within a few minutes. During this period, platelets form or neutrophils aggregate around CTCs or arrested tumor cells[40,41]. It has been demonstrated that extravasation typically occurs within the first 24-72 h after the initial arrest. After this period, most tumor cells have exited the bloodstream and have seeded into the stroma of the secondary site[42]. Therefore, killing the "seeded" cells within 72 h of extravasation is essential.

Propofol is widely used in clinical practice, largely due to its advantageous pharmacokinetic

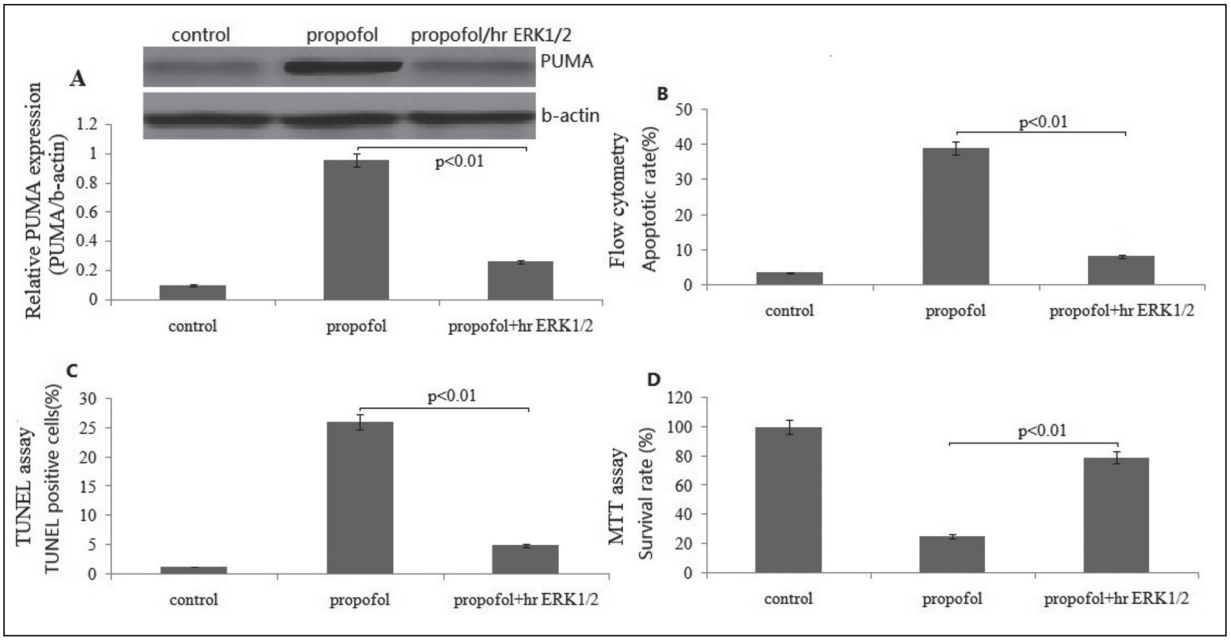

Figure 4. Effect of ERK1/2/PUMA signal on propofol-induced apoptosis in A549 cells. A549 cells were treated with hrERK1/2 for 6 h prior to propofol treatment. **A,** Representative images showing expression of PUMA following treatment with hrERK1/2 as analyzed by Western blot. β-actin was used as a loading control. **B,** Cell apoptotic rate was determined by flow cytometry. **C,** Cell apoptotic rate was determined by TUNEL assay. **D,** Cell survival rate was determined by MTT assay..

profile. It has been reported that propofol can influence the invasion and growth of human cancer cells, such as lung cancer cells[43], osteosarcoma[44], esophageal squamous cell carcinomas[45,46] and ovarian cancer[47]. A recent retrospective analysis[48] reported that cancer patients that receive total intravenous anesthesia have a better prognosis than those that receive volatile inhalational, suggesting that propofol might be able to kill the cancer cells released into the circulation in the perioperative period. In this study, we assessed the effect of propofol on the proliferation and apoptosis of A549 cells *in vitro*. Our results demonstrated that propofol can effectively inhibit growth and induce apoptosis of A549 cells *in vitro*. Although propofol clearly affects cell growth and apoptosis, the central signaling pathway in propofol-mediated cell death is not clear. ERK1/2 is an important mitogen-activated protein kinase that controls several cellular activities and physiological processes. In general, activation of ERK1/2 promotes cell survival[48], and inactivation of ERK1/2 effectively controls cell growth and induces apoptosis[49]. Miao et al[50] reported that propofol stimulation inhibits cancer cell invasion and that the effect is partly due to ERK1/2-dependent downregulation of matrix metalloproteinases. Another investigation[51] showed that propofol inhibits the ERK1/2 pathway, resulting in caspase-3 activation and inducing cell apoptosis. We found that propofol inhibited activation of ERK1/2, followed by enhanced apoptosis and decreased growth of A549 cells. However, restoration of ERK1/2 activity by treatment with hrERK1/2 inhibited propofol-induced apoptosis and growth inhibition of A549 cells, suggesting that propofol induced cell apoptosis by inhibiting the ERK1/2 signal.

A study showed that targeting Ras/Raf/MEK/ERK signaling promoted PUMA-dependent apoptosis of tumor cells[52,53], suggesting that PUMA was negatively regulated by ERK signaling. We found that propofol promoted the expression of PUMA. Propofol-induced apoptosis and growth inhibition in A549 cells was inhibited by transfection with PUMA siRNA. PUMA is necessary for propofol-induced apoptosis as shown by pro-caspase-3 cleavage, resulting in caspase-3 activation. In addition, restoration of ERK1/2 activity by hrERK1/2 reduced propofol-induced PUMA upregulation. From the observations above, we concluded that propofol induced apoptosis and inhibited growth of A549 cells via inhibition of ERK1/2 activity and induction of PUMA expression. In this study, propofol activated ERK1/2-PUMA signaling, resulting in apoptosis and growth inhibition of A549 cells. We, therefore, suggest that propofol killed lung cancer cells released into the circulation in the perioperative period.

Conclusions

We showed that propofol inhibited proliferation and induced apoptosis of A549 cells *in vitro* by modulation of ERK1/2-PUMA signaling. Therefore, propofol might be an appropriate anesthetic in NSCLC surgery. Further studies are needed to validate the clinical relevance of propofol.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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