Propofol Affects EGF’s Activity in Intestinal Cell by Down-Regulating EGFR-Mediated Intracellular Signaling

Hua Chai,*a Wenyong Peng,a Zhongquan Zhu,a Duojia Xu,a Yuanliang Chen,a Zhijian Lan,a and Xiayun Jinb

*a Anesthesiology, Jinhua Central Hospital; Jin Hua 321000, China; and b Oncology, Jinhua Central Hospital; Jin Hua 321000, China

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Propofol is a commonly used anesthetic drug in clinic. In recent years, a series of non-anesthetic effects of propofol have been discovered. Studies have shown that propofol has many effects on the intestine. Epidermal growth factor (EGF) is one of the most important growth factors that could regulate intestinal growth and development. In the current study, we studied the effect of protocol on the biological activity of EGF on intestinal tissue and cell models. Through flow cytometry, indirect immunofluorescence and Western-blot and other technologies, it was found that propofol reduced the activity of EGF on intestinal cells, which inhibited EGF-induced intestinal cell proliferation and changed the cell behavior of EGF. To further explore the potential mechanism by which propofol down-regulated epidermal growth factor receptor (EGFR)-induced signaling, we carried out a series of related experiments, and found that propofol may inhibit the proliferation of intestinal cells by inhibiting the EGFR-mediated intracellular signaling pathway. The current research will lay the theoretical and experimental basis for further study of the effect of propofol on the intestine.

Key words propofol; cell proliferation; epidermal growth factor receptor (EGFR); EGF; intestinal cell

INTRODUCTION

Propofol is a commonly used anesthetic drug in clinic. Since its introduction in the 1980s, propofol (2,6-diisopropylphenol) has been widely used in clinical anesthesia and intensive care unit for sedation. A large number of clinical studies have confirmed that propofol is a safe and effective anesthetic with fewer toxic and side effects.1 As a short-acting intravenous anesthetic, propofol has many advantages. However, in addition to the anesthetic effect, researches have shown that propofol has many potential biological effects. It has been reported that propofol can promote the development of rat nerve cells.2 In addition, many studies have focused on the effects of propofol on the intestine. It has been reported that propofol can prevent intestinal cell apoptosis in an animal model of burn injury.3 Furthermore, Propofol has a protective effect on the intestine in traumatic brain injury model of rats.4 In addition, Propofol relieves intestinal ischemia–reperfusion injury by inhibiting nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-mediated mast cell activation.5

Epidermal growth factor (EGF, which consists of 53 amino acids and a molecular weight of 6000kD) has important biological functions for many tissues, such as gastrointestinal tract.6 It binds to specific receptors (EGFR) and functions to promote DNA, RNA, protein and Polyamine synthesis, which results in the growth of intestinal cells.7 EGFR is a transmembrane glycoprotein (170kD) with an extracellular epidermal growth factor binding domain and an intracellular tyrosine kinase domain, which is composed of erbB1, erbB2, erbB3, and erbB4. After EGF binding, EGFR was activated, the divergent downstream signaling pathways (such as signal transducer and activator of transcription (STATs), extracellular signal-regulated kinase (ERK) 1/2, and phosphatidylinositol 3-kinase (PI3K)-AKT) were then triggered, which in turn regulates multiple biological processes such as cell survival and proliferation.8 EGF can also increase the nutritional absorption on the small intestinal mucosa.9 In addition, EGF can promote the maturation, regeneration and repair of gastrointestinal tissue.9

In the current study, we explored the effect of propofol on the biological activity of EGF on the intestinal cell model. For this, we carried out a series of experiments, and the results showed that propofol inhibited the EGF-induced proliferation of intestinal epithelial cells. We further explored the underlying mechanism by which propofol inhibits EGF-induced intestinal cell proliferation, and results showed that protocol inhibited EGFR-mediated intracellular signaling pathway. Further study showed that propofol accelerates EGF-induced EGFR down-regulation, this may be a potential mechanism by which EGFR-mediated signaling was down-regulated. In short, this study will lay an experimental and theoretical basis for further research on the effects of propofol on intestinal cells and tissues.

MATERIALS AND METHODS

Materials Anti-phospho-STAT3 (#9145S), Anti-phospho-AKT (#4060S), Anti-phospho-ERK1/2 (#9101S), and total STAT3 (#9139S), total AKT (# 4691S), total ERK1/2 (#9102S) antibodies were purchased from Cell Signaling Technology (U.S.A.). Cell culture medium and fetal bovine serum (FBS) were purchased from Thermo Fisher (U.S.A.). The polyvinylidene difluoride (PVDF) membrane was purchased from Millipore (Bedford, MA, U.S.A.). Cell lysis buffer, bicinchoninic acid (BCA) kit

* To whom correspondence should be addressed. e-mail: chaihua2021@126.com
and skim milk were purchased from Beyotime Biotechnology (China). EGF was purchased from Sigma-Aldrich (U.S.A.). Anti-EGFR antibody was purchased from Abcam (U.K.). Alexa Fluor 488-labeled secondary antibody was purchased from Sigma-Aldrich. Unless otherwise stated, other chemicals were obtained from Sigma-Aldrich.

**Cell Culture**  
Human IEC6 (hIEC6) and FHs-74 INT cells were purchased from ATCC and stored in our laboratory. The cells were removed from liquid nitrogen, the cell suspension was diluted with fresh culture medium, and the cells were then transferred into a 15 mL centrifuge tube. The cells were centrifuged at 1000 rpm for 5 min. The supernatant was discarded, and 6 mL of fresh culture medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS) were added and resuspended in a new culture dish, and cultured in a 37 °C, 5% CO₂ cell incubator.

**Western-Blot**  
After treating the cells with propofol alone or propofol/EGF mixture, the cells were rinsed 3 times with phosphate buffered saline (PBS) to remove the remaining medium. Sodium dodecyl sulfate (SDS) sample buffer (500–1000 µL) was added, and then cells were slightly scraped with a cell scraper. The cell samples were centrifuged at 12000 × g for 15 min. The supernatant was discarded, and the 6 mL of fresh culture medium (modified Eagle's medium (DMEM) supplemented with 10% FBS) were added and resuspended in a new culture dish, and cultured in a 37 °C, 5% CO₂ cell incubator.

**Indirect Immunofluorescence Assay (IFA)**  
One day before the start of the experiment, a single cell suspension containing 5 × 10⁴ cells were placed in a cell culture plate with a cover slip. When cells reached 30–50% confluence, propofol and/or EGF was added and incubate at 37 °C for different time points. Then the cells were washed three times with PBS, 4% PFA (1mL/well) was added at room temperature for 10 min. After washing, 1 mL of 0.1% Triton X-100 was added and incubated for 10 min. The cell samples were washed 3 times with PBS (3 min/time). Then the cells were blocked by adding 1 mL 3% BSA at room temperature for 2 h. The primary antibody was added and incubated at 4 °C overnight. After washing 4 times with PBS (10 min/time). Fluorescently labeled secondary antibody was added and incubated at room temperature for 1 h. After washing for times with PBS (10 min/time), 4-6-diamidino-2-phenylindole (DAPI) was used to stain the cell nuclei. Cell samples were observed and imaged using a laser confocal microscope (Leica, Germany). The images were analyzed with image J software.

**Enzyme-Linked Immunosorbent Assay (ELISA)**  
The intracellular signaling pathway was detected using the ELISA kit from CST (U.S.A.), and the operation process was carried out according to the manufacturer’s instructions.

**Detection of Cell Proliferation by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT)**  
A cell suspension was prepared with a culture solution containing 10% FBS, and was seeded into a 96-well plate (10000 cells per well), and the volume of each well was 200 µL. After treating with EGF/Propofol for different time points, 20 µL of MTT solution (5 mg/mL in PBS) was added to each well, and incubated for 4 h. Dimethyl sulfoxide (DMSO), 150 µL, was added to each well and shake for 10 min to fully dissolve the blue crystals. The samples absorbance at 450 nm was measured using a microplate reader.

**Analysis of Detection Proliferation by CCK8**  
The cells were cultured in a 96-well plate in 100 µL medium at a density of 10000 cells/well. The cells were cultured in a CO₂ incubator at 37 °C for 24 h. The cells were then treated with EGF, propofol, or mixture of Propofol and EGF at different time points. Ten microliter CCK8 solutions were added to each well in the culture plate, and cultured for 2 h, and the absorbance at
450 nm was measured using a microplate reader.

**Analysis of Apoptosis by Flow Cytometry** After the cells were treated with propofol, the experiment was carried out as follows. The cell samples were washed with PBS twice and then $5 \times 10^5$ cells were collected. Five hundred microliters of binding buffer were added to resuspend cell samples. After washing the cells, $5 \mu$L Annexin V-enhanced green fluorescent protein (EGFP) and $5 \mu$L Propidium Iodide were added and then mixed for 15 minutes. Flow cytometry was then used to detect the intracellular signaling molecules activated by EGF. The experimental process has been described in detail in Materials and Methods. Results are expressed as the mean plus or minus the standard error of the mean (S.E.M.).
mixed with cell samples. After incubation at room temperature in the dark for 10 min, Flow cytometer was used to analyze cell samples.

**Analysis of Cell Cycle** Cells were seeded on culture plate at a density of $1.5 \times 10^6$. After 24 h of culture, propofol, EGF or mixture of propofol/EGF were added, and cultured for 30–90 min. The cells were washed and collected. The cells were then centrifuged at 1000 rpm for 5 min to remove the cell debris. After the cells were fixed, 400 µL PI was added for staining for 30 min, and then analyzed by flow cytometry.

**Statistical Analysis** Statistical analysis was performed using Prism version 6 software (Graph Pad software). The data are expressed as mean ± standard error of the mean (S.E.M.). One-way ANOVA was used for comparison. $p < 0.05$ was defined as statistically significant.

**RESULTS**

**EGF Binds to EGFR Expressed on the hIEC6 and FHs-74 Int Cells** We first tested the EGFR expression on IEC6 cells and FHs-74 int cells, and the results showed that EGFR was mainly expressed on the cell membrane of IEC6 and FHs-74 int cells by confocal laser scanning microscope (CLSM) (please see Supplementary Fig. 1). In addition, Co-localization analysis showed that EGF could interact with EGFR on the cell surface (Fig. 1).

**EGF-Induced Signaling Profiles on hIEC6 Cells** As far as we know, there were no relevant literatures that have reported the EGFR’s signaling properties on human intestinal cells, therefore we first analyzed EGFR’s signaling properties on human IEC6 cells and FHs-74 int cells. IEC6 cells were stimulated with EGF at different time points, and then the phosphorylation level of EGFR-mediated signaling molecules (including EGFR, AKT, ERK1/2, STAT3) were evaluated by Flow cytometry. As shown in Figs. 2A–D, the results showed that EGF activated EGFR in a dose-dependent manner. When EGF was used at 5 nM, EGFR activation (phosphorylation) could be detected. With the increasing of EGF concentration, the degree of EGFR tyrosine phosphorylation gradually increased, when the concentration of EGF was at 20 nM, the tyrosine phosphorylation level of EGFR was peaked and then began to decline. In addition, EGF also induced the phosphorylation of AKT, ERK1/2 and STAT3 in a dose-dependent manner. Under the stimulation of 5 nM EGF, tyrosine phosphorylation of AKT, ERK1/2 and STAT3 can also be detected. The tyrosine phosphorylation level of these signaling molecules increased significantly with the increase of EGF concentration, and reached the peak under the stimulation of 10–20 nM EGF stimulation, and then decreased with the increase of EGF concentration. In addition, we further studied the signal properties of EGF in another human small intestinal epithelial cell line (FHs-74 int). We found that EGF can activate EGFR-mediated signaling pathways in a dose-dependent manner (Supplementary Fig. 2).

In addition, we further explored the EGF-mediated intracellular signaling in time course experiments by ELISA kit. We analyzed the phosphorylation levels of EGFR, AKT, ERK1/2, and STAT3 phosphorylation. As shown in Figs. 3A–D, the results indicated that EGF treatment activates intracellular signaling pathways in a time-dependent manner. EGFR phosphorylation can be detected after 5 min of EGF stimulation. The tyrosine phosphorylation level of EGFR gradually increased with time increasing, and peaked at about 30 min. Subsequently, the phosphorylation level of EGFR stated to
decrease. In addition, AKT and ERK1/2 tyrosine phosphorylation were also detected after 5 min of stimulation with EGF, and reached the maximum in 15–60 min, and then the phosphorylation levels of AKT and ERK1/2 began to decrease. At the same time, we also found that phosphorylation level of STAT3 was detected at 5 min after EGF treatment, and reached a peak during 30–60 min. In addition, we further studied the signal properties of EGF in FHs-74 int cells. We found that EGF could activate EGFR-mediated signaling pathways in a time-dependent manner (Supplementary Fig. 3).

Propofol Inhibits EGF-Induced Cell Proliferation

We further analyzed the effect of propofol on EGF-induced cell proliferation. As shown in Fig. 4A, EGF (20 nM) alone could significantly stimulate cell proliferation. However, the cell proliferation induced by EGF was significantly inhibited by propofol (10 \( \mu \)M), indicating that propofol can inhibit the biological activity of EGF. Cell cycle experiments showed that propofol regulate the cell cycle induced by EGF, and the proportion of S phase cells was significantly reduced (Fig. 4B).

Propofol Inhibits EGF-Mediated Intracellular Signal- ing Pathway

Here, we analyzed the effect of propofol on EGF-mediated signaling by Flow Cytometry, and the results showed that propofol significantly inhibited EGF-mediated signaling (Figs. 5A–D). It can be seen that propofol significantly inhibited EGFR-mediated signaling. In addition, Western blot analysis showed that propofol also significantly inhibited EGF/EGFR-mediated signaling at the indicated time points (Fig. 5E). However, propofol had no significant effect on the basic expression levels of EGFR, AKT, ERK1/2 and STAT3. In addition, we further analyzed the effect of propofol on EGFR-mediated signaling pathway in another human small intestinal epithelial cell line (FHs-74 int), and the results also showed that propofol significantly inhibited EGFR-mediated signaling (Supplementary Fig. 4). In summary, we found that propofol inhibited EGFR-mediated signaling pathways in two human intestinal epithelial cell lines.

Potential Molecular Mechanism by Which Propofol Inhibits EGF/EGFR Signaling

We first tested whether propofol affected the binding of EGF to EGFR, and the results showed that propofol did not affect EGF binding to EGFR, and propofol also did not affect EGFR’s internalization (Fig. 6A). Next, we further analyzed the molecular mechanism by which propofol inhibits EGF-mediated signaling. The effect of propofol on EGFR stability was evaluated, and the results showed that the EGFR is down-regulated by EGF stimulation with a dose/time-dependent manner (Fig. 6B), and the expression of membrane-localized EGFR was also significantly down-regulated (Fig. 6B). However, propofol accelerated EGF-induced EGFR down-regulation (Fig. 6C). Interestingly, propofol alone had no effect on EGFR expression (Fig. 6D).

To further explore why propofol can accelerate the EGF-induced EGFR degradation, we analyzed the effect of propofol on the intracellular sorting of internalized EGFR. As shown in Fig. 6E, we detected that propofol did not affect the co-localization of EGFR and Rab5/4-positive endosomes. But interestingly, propofol increased the co-localization of EGFR and Rab-9 (which can transport cargo molecules to lysosomes) (Fig. 6F). Furthermore, Propofol also enhanced the co-localization between internalized EGFR and Lysosomal markers (Fig. 6G). Based on the above findings, we speculate that propofol may inhibit EGF-mediated signaling by accelerating the degradation of the internalized EGFR.

DISCUSSION

Propofol is a commonly used anesthetic drug in clinic. Since its use in 1980s, it has shown good anesthetic effects with low toxicity and low side effects. However, many studies have reported that propofol is not only a clinically useful anesthetic drug, but also exhibits many other biological
activities (non-anesthetic effects). For example, it has been reported that propofol has anti-inflammatory effects. Inflammatory reactions are accompanied by the generation of oxygen free radicals. Propofol reacts with oxygen free radicals to form stable phenoxy groups and scavenge oxygen free radicals, which in turn exhibits an anti-inflammatory role. In addition, some studies have shown that propofol has an anti-anxiety effect. Some experimental studies have shown that

Fig. 5. Propofol Inhibited EGF-Mediated Intracellular Signaling Transduction
Intestinal cells are stimulated with the EGF and/or propofol. Then the cellular protein was extracted and subjected to Flow cytometry (A–D) and Western-blotting (E) using the indicated antibodies. The grayscale values of the bands were analyzed with Image J software. Picture is representative of at least three independent experiments. Results are expressed as the mean ± S.E.M. An asterisk indicates a statistically significant difference (p < 0.05). NS, not statistically significant.
Fig. 6. A. Propofol Has No Effect on EGF’s Internalization and the Interaction between EGF and EGFR

B. Detection of total EGFR and membrane-localized EGFR expression. C. Propofol accelerates EGF-induced EGFR down-regulation. D. Propofol alone has no effect on EGFR expression. E. Propofol did not affect the co-localization of EGFR and Rab5/4. F. Propofol increased the co-localization of EGFR and Rab-9. G. Propofol enhanced the co-localization of EGFR and Lysosomal markers. An asterisk indicates a statistically significant difference ($p < 0.05$). NS, not statistically significant. (Color figure can be accessed in the online version.)
propofol exhibits better anxiolytic effects in animal models.\textsuperscript{2)} Propofol (60 mg/kg) exerts a good anti-anxiety effect, and it has no effect on the exercise ability of experimental animals.\textsuperscript{2)}

In addition, propofol also exhibits a series of anti-tumor effects.\textsuperscript{16,17)} In addition, it has been reported that propofol also can enhance cisplatin-induced cervical cancer cell apoptosis through JAK2/STAT3 signaling pathway.\textsuperscript{18)} Additionally, a series of studies have shown that propofol also has anti-cancer effects against other types of tumor cells.\textsuperscript{19)}

The EGF/EGFR system has important biological activities.\textsuperscript{20–22)} In the current study, we mainly analyzed the effects of propofol on EGF/EGFR's activity in two intestinal cell lines. As far as we know, until now, there is no relevant research reporting the signaling profile of EGF on intestinal cells. Therefore, we first analyzed the EGFR-mediated intracellular signaling pathway. The results showed that EGF can activate multiple signaling molecules (including EGFR/STAT3/ERK1/2) in a time- and dose-dependent manner. However, when propofol was used, the EGF-induced the proliferation of intestinal cells was significantly inhibited by propofol treatment. But propofol alone has no effect on the cell proliferation. Further study showed that propofol could block EGF-mediated intracellular signaling, although EGF could still activate EGFR-mediated signaling in a dose and time-dependent manner, the phosphorylation level of EGF-mediated intracellular signaling was significantly down-regulated.

Next, we further analyzed why propofol can inhibit EGF-mediated intracellular signaling pathways, and the results showed that propofol resulted in a significant down-regulation of EGF expression in the presence of EGF, suggesting that propofol can accelerate the degradation of EGF-induced EGFR. To further explore why propofol can accelerate the degradation of EGF-induced EGFR, we analyzed the effect of propofol on the intracellular sorting of internalized EGFR. We found that propofol did not affect the co-localization of EGF and Rab5/4-positive endosomes. But it is interesting that we found that propofol could increase the co-localization of EGFR and Rab-9. Furthermore, Propofol also increased the co-localization of EGFR and Lysosomal markers. These findings suggest that propofol may promote lysosomal transport of internalized EGFR. These findings suggested that propofol inhibited EGF-mediated signaling possible via accelerating the degradation of the internalized EGFR. Of course, this is only our speculation, the potential mechanism remains speculative and needs further experimental confirmation.

In summary, in the current study, we first analyzed the signaling properties of EGF on two intestinal cell lines. Additionally, we further analyzed the effect of propofol on EGF’s bioactivities, and found that propofol can inhibit the biological activity of EGF by down-regulating the EGFR expression. Current research laid a foundation for further research on the relationship between propofol and growth factors.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES


