Propofol protects against erastin-induced ferroptosis in HT-22 cells.

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Research Article

Keywords: Propofol, Ferroptosis, ALOX5, ROS, Lipid, Mitochondria

Posted Date: February 15th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1332526/v1

Abstract

Propofol is a short-acting intravenous anesthetic, which is widely used in clinical treatment. Previous articles indicated that propofol is a therapeutic target for anti-apoptosis, anti-inflammation, anti-lipid peroxidation, and anti-Reactive oxygen species (ROS). Moreover, cell ferroptosis has strong correlations with cellular ROS, inflammatory responses, and lipid peroxidation. However, the mechanisms of propofol attenuating neuron injury by reducing ferroptosis remain unknown. Hence, we hypothesized that propofol could protect neuron cells via reducing cell ferroptosis. To test this hypothesis, HT-22 cells were treated with a specific ferroptosis activator (erastin) in the presence of propofol (50 μM). In such a study, we found propofol reduced erastin induced high Fe 2+ concentration, lipid peroxides, and excess ROS. The western blot also helps us understand that propofol could rescue erastin-induced low expression of GXP4 and system Xc - . Further experiments indicated that propofol attenuated p-ALOX5 expression at Ser663 independent of ERK. In addition, we built two transient transfection cell lines, ALOX5 OE, and Ser663Ala-ALOX5 OE to confirm the target of propofol. We found that Ser663 point is the critical role of propofol in rescuing erastin induced cell injury/lipid peroxidation. In conclusion, propofol may help attenuates ferroptosis, which may provide a new therapeutic method to treat neuron injury or brain inflammatory response.

Introduction

Neurodegenerative diseases are neuronal dysfunction and death in age-related diseases, which are becoming common diseases over past decades (Kaur, Flores Gutiérrez, Nistri, & Bolam, 2016). Recent reports indicated that ferroptosis has a high correlation with neurodegenerative diseases (Do Van et al., 2016). Ferroptosis differs from classic cell death, such as autophagy, apoptosis, and necrosis. It is characterized by the overwhelming iron-dependent accumulation of lethal lipid ROS, which could significantly cause inflammatory responses in cell (Scott J. Dixon & Stockwell, 2012). In ferroptosis cells, the heaps of reactive oxygen occur in cytoplasm and lipid, and shrunken, electron-dense mitochondria have a strong correlation with ferroptosis, which may induce cell death. Ferroptosis is an iron-dependent cell death process, that would be induced once glutathione-dependent antioxidant enzyme glutathione peroxidase 4 (GPX4) is inhibited (Xie et al., 2016). Meanwhile, ferroptosis could be triggered by many small molecules, including erastin and RAS selective lethal (RSL), which directly inhibit the cell surface cysteine or GPX4 expression level. Besides, someone indicates that suppressing wild type p53 expression reduces ferroptosis through up-regulating cysteine expression, which could be widely used in tumor therapy (Webers, Heneka, & Gleeson, 2019).

Propofol is an important drug in clinical application. It is considered a rapid-acting intravenous anesthetic agent, and it is commonly used in induction or maintenance of general anesthesia or as a sedation agent for critically ill patients. Its off-label uses include the treatments of postoperative nausea and vomiting and refractory status epilepticus. In previous studies, propofol was shown to bind gamma-aminobutyric acid (GABA) receptors for sedative and anticonvulsant effects (Giuliani, 2019). In addition, Kevin et al. (Kevin, Novalija, & Stowe, 2005; Liu Jia, 2011; Wu Xingjun, 2007) indicated that propofol in clinically

used concentration could reduce H_2O_2-induced excess lipid oxidation, and low concentration propofol can protect cell membranes. The decent fat-soluble of propofol can assemble in the cell membrane and enhance anti-ROS ability. Some papers also indicated that propofol could inhibit H_2O_2-induced superoxide radical to inactivate NF-κB, which may indirectly protect cells (Pinho Ricardo A, 2019; Shintoku et al., 2017; Solaroglu et al., 2004; Z. Yang, Cheng, Yan, Xiong, & Liu, 2017).

Interestingly, cells with ferroptosis may actively produce ROS, which may involve multiple sources. In addition, nicotinamide adenine dinucleotide phosphate (NADPH)-dependent lipid peroxidation and GSH depletion are also crucial for the induction of ferroptosis. Previous studies indicated that GSH depletion inactivated GPX4, which triggers ferroptosis. Mitochondrial fatty-acid metabolism provides the specific lipid precursor required for ferroptosis(Cao Jennifer Yinuo, 2016; Grieco Maddalena, 2019). However, no article that declares the protective effects of propofol in anti-ferroptosis, despite the decent protective effects of propofol in reducing ROS and lipid oxidant. Hence, we hypothesized that propofol reduces ferroptosis. To test this hypothesis, we utilized HT-22 cells treated with erastin as a cell model.

The organisms with membranes having polyunsaturated lipids that contain bis-allylic carbons can be easily destroyed to lipid peroxidation by oxygen. And Fe^{2+} can significantly accelerate this procession(Xie et al., 2016). Consequently, reducing lipid peroxidation could attenuate Fe^{2+} dependent cell injury. In addition to being formed through non-specific propagation of radicals, oxidized lipids can also be synthesized in a controlled manner by cyclooxygenases (COXs), cytochrome p450s (CYPs), and lipoxygenases (LOXs). LOX enzymes are the most significant contributor to the synthesis of lipid hydroperoxides. The 5-lipoxygenase is an enzyme encoded by the ALOX5 gene responsible for oxidizing arachidonic acid at carbon 5, forming 5- hydroperoxy eicosatetraenoic acid (5-HPETE)(Bruno et al., 2018). A recent PNAS article indicated that a high ALOX5 level may drive ferroptosis through peroxidation in polyunsaturated fatty acids (W. S. Yang et al., 2016). Pharmacological inhibition of ALOX5 by zileuton exhibited a neuroprotective role in glutamate-induced HT22 cells by blunting ferroptosis (Y. Liu et al., 2015). Consistently, it was also previously confirmed that the inhibition of ALOX5 protected neurons from ferroptosis death in mice with hemorrhagic stroke via neutralizing lipid peroxides (Karuppagounder et al., 2018).

Here, we found that erastin may cause alxo5 phosphorylation at ser 663 site, and propofol can significantly reduce erastin-induced cell injury, lipid peroxidation, Fe^{2+} accumulation, and high p-ALOX5 (ser 663) level. Consequently, we hypothesized that propofol could attenuate ferroptosis to protect neuron cells.

Materials And Methods

Cell culture

In this study, Hippocampal neuronal cell line (HT-22 cell line) was kindly gifted from Weilin Jin (Shanghai Jiao Tong University). HT-22 cells were cultured in DMEM/F12 medium containing with 10% fetal bovine

serum (all from Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (#C0222, Beyotime, China). All these cells were passaged by trypsin (TrypLE™, 12563-01, Thermofisher, US) when cells grew over 90%. The cells were re-seeded into a culture dish with 10% density. The cells should be put into a cell incubator with 5% CO2 and 37°C humid air after treatments.

Erastin treatment and transient transfection:

In this study, HT-22 cells were added with neuron injury induced drug (erastin, 0.5 μM, #S7242, Selleck, US) for 8 hours to build a neuron injury model. Propofol (50 μM, P-076, Sigma, Germany)(Jan et al., 2009) as a protective drug was added into medium two hours before neuron injury induced drug adding. Ferroptosis specific inhibitor (Ferrostain-1, 1 μM, S7243, Selleck, US) were used in different experiments.

Cells were seeded onto a culture dish and transfected with mouse ALOX5 OE or ALOX5 mutation plasmids (Ser663Ala, Ser at 663th amino acid site change to Ala) at 50 nM using Lipofectamine 2000 (Invitrogen, US) for 6 hours according to the instructions.

Cell viability and cell necrosis rate:

The CCK-8 kit only detects indirect cell viability. Almost 5000 WT-ALOX5 or Ser663Ala cells/100 μl were seeded into 96-well-plate per well, cells were treated with erastin for 8 hours in the presence of propofol. Then, 10 μl CCK-8 reagent was added into per well. After 2 hours incubated with CCK-8 reagent, the indirect viability could be measured by a microplate reader (Molecular Devices, US) at 450 nm.

PI/Hoechst 33342 staining ((PI, P4170, sigma, Germany), (Hoechst, #b2261, sigma, Germany)) and CCK-8 (#C0037, Beyotime, China) was used to detect cell necrosis rate in such study. In the PI/HO staining experiment, HT22 cells were seeded in the 24-wells-plates at a density of 1×10^5/ mL. After overnight incubated, pretreated with the indicated concentration of PPF for 2 h before erastin adding. After 8 h treatments of erastin, cells were incubated with 5 μg/mL PI and 10 μg/mL Hoechst 33342 for 15 min. The results were captured by Olympus fluorescence microscope. Cell necrosis rate was calculated by PI (+)/Hoechst (+).

ROS and lipid peroxides measurements:

Dihydroethidium (DHE) staining method was used to measure erastin induced intracellular ROS formation in this study. At first, HT-22 cells were treated with erastin, propofol, and Ferrostain-1. Then, DHE was added into the medium at a final concentration of 10 μM. After incubated for 30 mins, cells were washed by PBS one time. Different color pictures could be observed and taken by a fluorescence microscope. And these pictures were transferred to data by ImageJ software. To detect lipid peroxidation level, cells were treated as previously described and then incubated with 1 μM BODIPY 581/591 C11 (Invitrogen, US) for 30 min. After incubation, cells were washed by PBS one time. Then cells were

incubated by trypsin and were resuspended by 400 μL PBS. At least 10000 cells were detected and analyzed by a fluorescent microplate reader (Molecular Devices, ID3) for lipid peroxides at 530 nm and 585 nm.

Immunofluorescences

4-HNE staining method was used to determine lipid oxidant levels, p-ALOX5 staining was used to identify the nucleated location in HT-22 cells. After previous cell treatment, cells were fixed in 4% paraformaldehyde for 15 min and then were permeabilized by Triton X-100 for 5 min. Next, cells were blocked by 10% donkey serum for 1 h. After blocking, cells were incubated with primary antibody ((4-HNE, #ab46545, Abcam, UK), (ALOX5 Ser663, #bs-3252R, Bioss)) overnight at 4°C. After incubating, cells were washed at 3 times by PBS and then incubated with Alexa Fluor 488–conjugated secondary antibody (#A11001, Invitrogen, US) at room temperature 1 hour. After DAPI staining, the samples were observed in a fluorescence microscope. All fluorescent intensity of pictures were transferred into data by Image J software.

Iron ion concentration measurement:

According to product instruction, intracellular iron ion (Fe^{2+}) concentration in HT22 cells was stained with FeRhoNox-1 (Goryo, Japan). FeRhoNox-1 was dissolved in DMSO to make 1 mM stock solution, and then FeRhoNox-1 was diluted in HBSS (Gibco, US) to 5 μM working solution. After cells were incubated at 37°C for 30 min in the darkness, the pictures which contain color red and DAPI were taken by Olympus fluorescence microscope. In this study, microplate reader (Molecular Devices, ID3, US) was also used to get data, and fluorescence signal was observed at 570 nm.

Electron microscopy

For the measurement of mitochondrial structural changes, the electron microscopy method was used in HT-22 cells. After paraformaldehyde fixation, cells were processed and sectioned with a diamond knife on copper grids. Grids were examined with a Hitachi (Tokyo, Japan) electron microscope, and pictures were captured using a MegaView III digital camera.

qRT-PCR (Quantitative-real-time PCR)

Total RNA from HT22 cells was isolated by TRIzol (Invitrogen, US) according to product instructions. After RNA isolation, the PrimeScript RT kit (#RR037, Takara, Japan) was used to reverse-transcribe RNA into cDNA according to the product specification. Quantitative real-time PCR was performed using the two-step RT-PCR method by CFX connect (Bio-Rad, US). The sequences of Primers for RT-PCR of PTGS2 are TGCACTATGGTTACAAAAGCTGG (Forward Primer), TCAGGAAGCTCCTTATTTC CCTT (Reverse Primer).

Western blot

Total protein was lysed in ice-cold RIPA (#P0013B, Beyotime Biotechnology, China) containing protease and phosphatase inhibitor cocktail. The lysate was quantified by BCA protein assay kit (#23227, Thermo fisher, US). 35 μg protein was loaded into per well and was separated by SDS-PAGE. After electrophoresis (100 volt, 80 minutes), PVDF membranes (Millipore, Germany) were used to block all protein at 200 mA for 0.5-1 hour. Then, membranes were incubated with blocking buffer for 2 hours at room temperature. Then membranes were incubated with primary antibodies overnight at 4°C. After primary antibodies incubated, membranes were incubated with horseradish peroxidase–linked secondary antibodies for 1 hour. The protein membranes were taken by ChemiDoc Imaging System with the help of an ECL detection reagent (#34095, Thermo Fisher, US). The primary antibodies mentioned above contained p-PKA (ab75991, Abcam, UK), PKA (ab38949, Abcam, UK), p-MK2 (ab63378, Abcam, UK), MK2 (ab32567, Abcam, UK), p-ERK (#9101, CST, US), ERK (#9102, CST, US), ALOX5 (Ser663) (#bs-3252R, Bioss), ALOX5 (Ser271) (#3748, CST, US), ALOX5 (Ser523) (PA5-99150, Invitrogen), ALOX5(#ab169755, abcam), p53 (#ab32389, abcam, UK), xCT (ab175186, Abcam, UK), GPX4(ab125066, Abcam, UK), β-actin (#sc-47778, Santa Cruz, US)(Lu et al., 2019).

Statistical analysis:

In this study, we used PRISM 7.0 to analyze the data. And all the results were presented as means ± SD. ANOVA method or Student's t-test method was respectively used in statistical significances' analysis.

Results

Propofol prevents erastin-induced cell injury:

Firstly, we used CCK-8 kits to identify the suitable concentration of erastin in this study. The results suggest that 0.5 μM erastin may significantly inhibit cell viability (only 0.5 v.s. control) (Figure 1 (A)). Previous study indicated that 25-100 μM propofol were acceptable *in vitro* experiments (Jan et al., 2009; D. Liu, Sun, Du, & Kong, 2018; Wang, Zhang, Zhang, & Yan, 2018; Zhang et al., 2019). Here, we used CCK-8 kits, and it helped us understand that 50 μM propofol may protect erastin-induced cell injury (Figure 1 (B)). We also added 25-75 μM propofol into normal HT-22 cells, and Cell viability results suggest that 50 μM propofol has no cell toxicity for neuron cells (Figure 1 (C)). To investigate the effects of erastin in neuronal HT-22 cells and the role of propofol in cells, we set four cell groups, control, vehicle (erastin), vehicle + propofol, and vehicle + Ferrostatin-1 (fer-1, specific ferroptosis inhibitor). According to microscope pictures, we found normal HT-22 cells morphology in the control group, whereas we found that erastin caused cell injury, which cells changed to point like morphology. The propofol could rescue erastin-induced abnormal cell morphology, and fer-1 also attenuate erastin-induced cell injury. In addition, we found fer-1 can significantly inhibit erastin-induced cell injury (Figure 1 (D)). We further used PI/Hoechst 33342 staining to detect the function of propofol in cells. Here, we found erastin significantly

caused high necrosis rates compared with control cells, whereas propofol and fer-1 could reduce erastin-induced cell necrosis (Figure 1 (E)). These results demonstrate that propofol attenuates erastin-induced cell injury in HT-22 cells.

Propofol prevents erastin-induced ROS, lipid peroxidation, Fe^{2+} accumulation, and mitochondrial injury:

ROS may cause inflammatory responses, cellular organelles injury, and apoptosis. Lipid peroxidation is a metabolic disorder problem that induces the accumulation of lipid peroxides and cellular organelle injury. It also been reported that excess ROS and lipid peroxides involve deeply with ferroptosis (Neitemeier et al., 2017). In this study, we used Dihydroethidium (DHE) staining method to identify the function of propofol. Here, we found that erastin caused a high total ROS level in HT-22 cells compared with control cells, whereas propofol and fer-1 can significantly reduce erastin-induced high positive DHE levels (Figure 2 (A)). Next, we used IF to identify the 4-HNE level in cells. We found that erastin cause a high 4-HNE level, whereas propofol and Fer-1 can significantly inhibit it expression (Figure 2 (B)). In addition, we used a multi-function microplate reader to identify the lipid oxidation level. In this study, BODIPY C11 was chosen because it could specifically monitor the ROS level in membrane and lipid(Cheloni & Slaveykova, 2013). The BODIPY C11 results show us that erastin causes lipid peroxidation compared with control cells, whereas propofol and fer-1 significantly inhibited lipid peroxidation (Figure 2 (C)). Furthermore, we stained cells with FeRhoNox™-1 to monitor Fe^{2+} levels in cells. The fluorescence pictures and analysis suggest that erastin caused high fluorescence light density in HT-22 cells compared with control cells, whereas propofol and Fer-1 could significantly inhibit cell storing Fe^{2+} (Figure 2 (D)). Moreover, the electron microscope (EM) pictures show that erastin treatment caused injured mitochondrial, which edema mitochondrial, mitochondrial fragments were seen in erastin-treated cells. In contrast, propofol can significantly eliminate erastin-induced damaged mitochondrial (Figure 2 (E)).

Propofol prevents erastin-induced cell ferroptosis:

Our previous results suggest that propofol may attenuate erastin-induced ferroptosis in HT-22 cells. To identify our hypothesis, we detected ferroptosis markers in this study. Firstly, we used qRT-PCR to detect the *PTGS2* gene folds. The results show us that erastin cause a high level of *PTGS2*, whereas propofol reduces its high expression (Figure 3 (A)). The western blots also suggest that erastin caused a high expression of COX2, whereas propofol could inhibit it expression (Figure 3 (B)). Many pieces of research indicated that GPX4 and xCT are the target proteins of ferroptosis (Xie et al., 2016; W. S. Yang et al., 2016). Hence, we used western blot to detect these two protein levels. As expected, low expression of xCT and GPX4 protein levels were seen in erastin-treated cells compared with control, whereas propofol could significantly rescue them (Figure 3 (C)). These results help us confirm that propofol could attenuate erastin-induced ferroptosis in neuron cells.

Propofol reduces p-ALOX5 to attenuate erastin-induced ferroptosis.

Recent articles highlight the function of ALOX5 protein, and they indicated that phosphorylation of ALOX5 might involve in ferroptosis(Karuppagounder et al., 2018; Proneth & Conrad, 2019). In this study, we used western blot to identify the role of ALOX5 in erastin-induced ferroptosis. According to the results, we found that erastin caused high phosphorylation of ALOX5 at ser 663, whereas propofol can significantly reduce erastin-induced high level. However, we found that erastin and propofol did not influence the phosphorylation of ALOX5 at ser 523 and ser 271 (Figure 4 (A)). Some articles indicated that only nuclear-located ALOX5 has activity(Rådmark, Werz, Steinhilber, & Samuelsson, 2007). Here, we used immunofluorescence to identify the phospho-ALOX5 ser 663 nuclear translocation level. The pictures show that high nuclear translocation was seen in erastin-treated cells, whereas propofol inhibits its nuclear translocation (Figure 4 (B)). ALOX5 has been reported to be phosphorylated by different kinases at several sites, including Ser271 (p38 MAPK-activated protein kinase 2 (MK2))(Werz, Szellas, Steinhilber, & Radmark, 2002), Ser523 (protein kinase A (PKA))(O, J, B, & O, 2000), Ser663 (extracellular signal-regulated kinase (ERK)) (Werz et al., 2002). Further, we used western blot to identify the upstream of phosphorylation of ALOX5. The results suggest that erastin did not influence p-MK2 and p-PKA protein levels in HT-22 cells (Figure 4 (C)). Interestingly, the high p-ERK level was seen in erastin-treated cells, whereas propofol could not reduce erastin-induced high p-ERK level (Figure 4 (D)). These suggest that erastin may activate ferroptosis through ERK, whereas propofol could directly bind ALOX5 to reduce phosphorylation level.

The protection effect of propofol from erastin-induced ferroptosis is suppressed in Ser663Ala overexpressed HT-22 cells:

In this study, because we focused on function of ALOX5 phosphorylation, total knockdown or overexpression technology may not work well. Then we transfected cells with overexpression plasmids encoding ALOX5 or ALOX5 with mutation (Ser663Ala, Ser at 663th amino acid site change to Ala) to further determined the effect between propofol and p-ALOX5 (S663). The western blot results show that these two plasmids could enhance total ALOX5 protein level, whereas they cannot regulate p-ALOX5 (S663) level (Figure 5 (A)). Interestingly, erastin could enhance p-ALOX5 (S663) level in ALOX5 OE group, whereas no change was seen in Ser663A OE group (Figure 5 (B)). We further investigated cell viability. According to Cell viability data, propofol enhanced cell viability in Ser663Ala cells (about 2 folds), whereas propofol could significantly enhance cell viability in ALOX5 OE cells (20 folds) (Figure 5 (C)). To further investigate the ALOX5 Ser663 phosphorylation functions in ferroptosis, we also detected iron ion (Fe^{2+}) concentration by FeRhoNox™-1 staining (Figure 5 (D)) and lipid peroxides by BODIPY C11 staining (Figure 5 (E)) according to fluorescent microplate reader. As expected, propofol attenuate erastin-induced high Fe^{2+} concentration and lipid peroxidation level in Ser663Ala cells, whereas propofol could

significantly attenuate Fe^{2+} concentration and lipid peroxidation level in ALOX5 cells. In summary, we confirmed that the phosphorylation of Ser663 is a crucial point in the propofol inhibitory effect of ferroptosis.

Discussion

Propofol is a commonly used intravenous anesthetic that works mainly via the GABAA receptor in the central nervous system and is widely used in various clinical settings(Bowery & Smart, 2009; Lewerenz & Maher, 2015; Trapani, Altomare, Sanna, Biggio, & Liso, 2000). It is used as anesthesia induction and maintenance drugs in operating rooms and is also administered as a sedative in the intensive care unit, commonly infused continuously(Chiara Adembri, 2007; Hutchens, Memtsoudis, & Sadovnikoff, 2006). Propofol had been used as a sedative especially to those ventilated traumatic patients. Moreover, propofol had a neuroprotective effect by reducing cerebral metabolism and decreasing intracranial pressure or increasing blood supply(Martin, Hicks, Holbrook, & Cox, 2020; Okuno et al., 2017; Pinho Ricardo A, 2019). Propofol was also proved to have direct neuroprotective effects *in vitro*. In our study, we determined to investigate whether propofol serves as a ferroptosis inhibitor to reduce neuron cell death. Articles have previously shown that propofol can substantially rescue neuron injury in cerebral ischemia and cerebral injury-induced neuron inflammation(Pinho Ricardo A, 2019). However, the role of propofol in the development of ferroptosis related neuron injury remains elusive. Therefore, we studied the role of propofol in the pathogenesis of erastin-induced neuron cell ferroptosis by various genetic and pharmacological approaches. Our findings indicated that propofol protected against erastin-induced low cell viability, morphology damages, and cell necrosis rate.

Ferroptosis is closely associated with the intracellular iron, and the process of ferroptosis required iron-dependent production of ROS and lipid peroxidation. Previous studies indicated that ferroptosis-sensitive cells with Ras mutation have increased TFR1 expression compared with ferroptosis-resistant cells(Cao Jennifer Yinuo, 2016). Interestingly, TFR1 protein is responsible for reducing Fe^{3+} to Fe^{2+}(W. S. Yang & Stockwell, 2008). This evidence supports that Fe^{2+} may contribute to iron overload during ferroptosis rather than increasing iron uptake or reducing iron storage in cells because only Fe^{2+} could catalyze Fenton reaction. Indeed, Fenton reaction is the final step that may accelerate lipid to lipid peroxidation in cells(Xie et al., 2016), which may cause ferroptosis. In such study, our data showed that propofol treatment significantly inhibited iron (Fe^{2+}) accumulation in cells. Here, our results help us confirm that propofol significantly reduce Fe^{2+} accumulation in cells, rather than inhibiting iron releasing from HT-22 cells, or absorbing iron from the culture medium.

It has been reported that ferrostatin-1 (Fer-1) is the most potent inhibitor of erastin-induced ferroptosis in HT-1080 cells. It specifically inhibited RSL-induced death, but not cell death induced by other oxidative lethal compounds and apoptosis-inducing agents, because fer-1 could scavenge lipid ROS. In addition, glutamate-induced death in OHSCs and erastin-induced death in cancer cells share a core lethal mechanism that can be inhibited by iron chelation or Fer-1(W. S. Yang et al., 2016). In this study, we used

fer-1 as positive control (specific inhibitor) in erastin-induced ferroptosis, in which we can identify the protective efficiency of propofol in attenuating ferroptosis compared with fer-1.

In this study, propofol significantly reduced erastin-induced high fold change numbers of PTGS2 gene and high expression of COX2 protein. Wan Yang indicated that the PTGS2 gene encodes COX2 protein, which is used as a pharmacodynamic marker associated with ferroptosis cell death(Wan S. Yang et al., 2014). Further, some articles indicated that erastin-induced ferroptosis might involve various mechanisms, including inhibition of the xCT, GSH depletion, and subsequent lipid peroxidation. GPX4 is considered as an essential negative regulator of lipid peroxidation(Neitemeier et al., 2017). Erastin is supposed to mediate directly detrimental effects at mitochondrial in neuronal HT-22 cells. It was reported that erastin altered the permeability of the outer mitochondrial membrane via interaction with particular isoforms of voltage-dependent anion channel (VDAC), thereby inducing mitochondrial-triggered oxidative cell death in tumor cells(Wan S. Yang et al., 2014). Our data suggest that propofol could rescue erastin-induced low expression of GPX4 and xCT. Consequently, we confirmed that propofol is a decent ferroptosis inhibitor, which may inhibit neuron injury.

Although, we confirmed that propofol could up-regulate GPX4 and xCT in erastin-treated cells. However, no evidence supports that propofol could directly act on GPX4 or xCT. Interestingly, the previous article suggested that ALOX5 could be a target of propofol(Y. Liu et al., 2015). In addition, some articles also indicated that ALOX5 plays critical role in ferroptosis because lipid peroxidation is the main factor in ferroptosis, whereas activated ALOX5 caused high lipid peroxidation level(Rådmark et al., 2007). They expressed GFP-ALOX5 in HT-1080 cells and observed the ferroptosis(W. S. Yang et al., 2016). A paper indicated that edaravone's neuroprotective effect in ischemia was dependent on 5-lipoxygenase (ALOX5) signalling pathway, because edaravone could inactivate ALOX5 to protect mitochondrial function and structure(Song et al., 2018). We acknowledged that mitochondrial dysfunction and injured mitochondrial might cause mtROS(Weinberg, Sena, & Chandel, 2015), which could accelerate cell death or another kind of cell injury. Moreover, the significance of ALOX5 in ferroptosis also arises from the fact that ALOX5 is an iron-containing enzyme and iron accumulation often occurs in ferroptosis(Sun, Zhou, & Mao, 2019). Given that protein phosphorylation is vital for ALOX5 activity, ALOX5 protein phosphorylation may be involved in the ferroptosis process.

ALOX5, one of six human lipoxygenases, primarily expressed in leukocytes. Some studies indicated that propofol could bind ALOX5 to influence its activity(Shintoku et al., 2017). We hypothesized that ALOX5 spatial conformation was transformed to attenuate ALOX5 activity after propofol binding ALOX5. Also, some indicated that there are some docks in which propofol can bind ALOX5, which propofol can inhibit ALOX5 activity in HEK 293 cells according to transiently transfected with WT ALOX5 or point mutants(Okuno et al., 2017). Consequently, we wanted to investigate the function of propofol in erastin-induced neuron injury. We found that propofol reduced erastin-induced high p-ALOX5 (Ser663), whereas no significant changes were seen in p-ALOX5 (Ser271 and Ser523)(Rådmark et al., 2007). These results suggested that ALOX5 (Ser663) phosphorylation site could target propofol reduction neuron injury induced by ferroptosis. High p-ALOX5 (ser663) fluorescence intensity was seen in nuclear in erastin-

treated cells in IF pictures. ERK pathway, which was identified that RAS–RAF–MEK signaling renders other tumor cells sensitive to erastin(Yagoda et al., 2007). Here we wanted to determine whether propofol activates ERK to phosphorylate ALOX5 protein. We used the western blot method to detect the ERK signaling pathway proteins, and the results show us that erastin could up-regulate p-ERK, whereas propofol did not reduce erastin-induced high p-ERK level. This evidence suggests that erastin may activate ALOX5 to induce ferroptosis through ERK, whereas propofol could attenuate ALOX5 activity. In addition, the p-MK2 (the up-stream of p-ALOX5 ser 523) and p-PKA (the up-stream of p-ALOX5 ser 271) level were not influenced by erastin. Consequently, these results help us understand why p-ALOX5 (Ser 523) or p-ALOX5 (Ser 271) was not activated in erastin-treated cells. Further, we built two transient transfection cell lines that were transfected with plasmids encoding ALOX5 or ALOX5 with mutation (Ser663Ala, Ser at 663th amino acid site change to Ala), to verify whether propofol directly inhibits phosphorylating ALOX5 at ser663. Considering these results, we confirmed that propofol might directly inhibit phosphorylation ALOX5, which can inhibit ferroptosis.

In conclusion, treatment with propofol attenuates erastin-induced ROS and lipid peroxides and attenuates excess Fe^{2+}. Furthermore, propofol just works in binding ALOX5, which avoids Ser663 phosphorylation, to inhibit ferroptosis, and it cannot influence ERK protein. These findings highlight the mechanism of ferroptosis in the pathogenesis of neuron injury. It also provides the potential therapeutic target reducing neuron cell death via pharmacological treatment with propofol in ameliorating the neuron injury. Hence, propofol may help prove a promising therapeutic target to intervene with the progression of neuron injury by reducing ferroptosis.

Abbreviations

4-HNE: 4-Hydroxy-2E-Nonenal; CCK: Cell counting kit; COX: cyclooxygenases; DHE: Dihydroethidium; Era: Erastin; EM: Electronic microscopy; ERK: extracellular regulated protein kinases; Fer-1: Ferrostain-1; GPX4: Glutathione Peroxidase 4; IF: Immunofluorescences; MK2: MAPK-activated protein kinase 2; PPF: Propofol; PKA: protein kinase A; qRT-PCR: Quantitative-real-time PCR; PI: Propidium iodide; PTGS2: Prostaglandin-endoperoxide synthase 2; RSL: RAS selective lethal; ROS: Reactive oxygen species; mtROS: mitochondrial ROS; xCT: system Xc^-;

Declarations

Ethics statement

This study does not include human or animal samples. Nanjing University Medical School Research Ethics Committee has confirmed that no ethical approval is required.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Data availability statement

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Conflict of interests

The authors declare no conflict of interest in commercial use.

Funding

YL received Anhui Province Research and Development Program (NO. 17041f0804021).

Authors' contribution

WX, ZY, designed, and performed the experiments. XL analyzed the data. XL participated in manuscript writing/editing. LY., and WJ contributed significantly to the experimental design and funds.

Acknowledgements

Over the course of my researching and writing this paper, I would like to express my thanks to all those who have helped me.

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Figures

Figure 1

Propofol prevents erastin-induced cell injury: CCK-8 results: (A) HT-22 cells were treated with 0, 0.1, 0.25, 0.5, or 1 μM erastin for 8 h, (B) HT-22cells were treated with 0, 2.5, 5, 10, 25, 50 μM propofol in the presence of 0.5 erastin, or (C) HT-22 cells were treated with 25, 50, 75 μM propofol. The cell vitality was measured by microplate reader after CCK-8 treatment. (DE) Analysis of HT-22 cells on control, erastin (Era, 0.5 μM), Era + propofol (PPF, 50 μM), or Era+ Ferrostain-1 (Fer-1, 1 μM). (D) Microscopic examination. After cells were treated with erastin, propofol, or ferrostain-1, the cells were observed by microscopic. Scare bar = 100 μm. (E) PI/Hochest33342 staining analysis. After erastin, propofol, or ferrostain-1 treatments, cells were stained with PI and hochest 33342 to identify the necrosis rate. *$p < 0.05$, **$p < 0.01$.

Figure 2

Propofol prevents erastin-induced ROS, lipid peroxidation and damaged mitochondrial: Analysis of HT-22 cells on control, erastin (Era, 0.5 μM), Era + propofol (PPF, 50 μM), or Era+ Ferrostain-1 (Fer-1, 1 μM). (A) DHE staining images and analysis. Cells were stained with DHE for 15 min and were observed by a fluorescence microscope. Scare bar = 100 μm. (B) IF staining images and analysis. Cells were fixed and immunostained by anti-4-hydroxy-2- nonenal (4-HNE) antibody. Fluorescence signals were visualized by microscopy with constant fluorescence parameters. Scare bar = 100 μm. (C) Cells were stained with BODIPY C11 for 30 min to identify the lipid ROS level. The data were measured by a multi-function microplate reader. (D) FeRhoNox staining images and analysis. Living cells were stained with FeRhoNox to identify the Fe^{2+} level, and cells were observed by fluorescence microscopy. Scare bar = 100 μm. (E) Representative EM image of HT-22 cells and quantification of injured mitochondria counts (red arrow indicate the injured mitochondria). Scare bar = 3 μm. Each experiment was repeated 3 times. Data are expressed as the mean ± SEM. *$p < 0.05$, **$p < 0.01$.

Figure 3

Propofol prevents erastin-induced cell ferroptosis: Analysis of HT-22 cells on control, erstin (Era, 0.5 μM), or Era + propofol (PPF, 50 μM). (A) qRT-PCR was used to identify the *PTGS2* gene fold in HT-22 cells. (BC) Western blot results showing the protein (COX2, GPX4, xCT) level in HT-22 cells. β-actin was used as an internal reference in whole-cell lysates. Each experiment was repeated 3 times. Data are expressed as the mean ± SEM. *$p < 0.05$, **$p < 0.01$.

Figure 4

Propofol reduces p-ALOX5 to attenuate erastin-induced ferroptosis: Analysis of HT-22 cells on control, erastin (Era, 0.5 μM), or Era + propofol (PPF, 50 μM). (A) Western blot results showing the protein (ALOX5, p-ALOX5 (Ser 271, Ser 523, Ser 663)) level in HT-22 cells. β-actin was used as an internal reference in whole-cell lysates. (B) IF staining images and analysis. Cells were fixed and immunostained by anti-p-ALOX5 (Ser 663) antibody. Fluorescence signals were visualized by microscopy with constant fluorescence parameters. Scare bar = 20 μm. (CD) Western blot results showing the protein (MK2, p-MK2, PKA, p-PKA, ERK, p-ERK) level in HT-22 cells. β-actin was used as an internal reference in whole-cell lysates. Each experiment was repeated 3 times. Data are expressed as the mean ± SEM. *$p < 0.05$, **$p < 0.01$.

Figure 5

Propofol protecting erastin-induced neuron injury is suppressed in Ser663Ala overexpressed HT-22 cells: Analysis of HT-22 cells on Ser663A OE+erastin (0.5 μM), Ser663A OE+ erastin + propofol (50 μM), ALOX5 OE +erastin, or ALOX5 OE +erastin + propofol. (AB) Western blot results showing the protein (ALOX5, p-ALOX5 (Ser 663)) level in HT-22 cells. β-actin was used as an internal reference in whole-cell lysates. (C) CCK-8 results: The cell vitality was measured by microplate reader after CCK-8 treatment. (D) FeRhoNox staining and analysis. Living cells were stained with FeRhoNox to identify the Fe^{2+} level, and cells were measured by a multi-function microplate reader. (E) Cells were stained with BODIPY C11 for 30 min to identify the lipid ROS level. The data were measured by a multi-function microplate reader. Each experiment was repeated 3 times. Data are expressed as the mean ± SEM. *$p < 0.05$, **$p < 0.01$.