Silencing of soluble epoxide hydrolase 2 gene reduces H₂O₂-induced oxidative damage in rat intestinal epithelial IEC-6 cells via activating PI3K/Akt/GSK3 β signaling pathway

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Abstract Oxidative stress plays a vital role in the occurrence and development of intestinal injury. Soluble epoxide hydrolase 2 gene (EPHX2) is a class of hydrolytic enzymes. We aim to explore the effects and molecular mechanism of siEPHX2 on H₂O₂-induced oxidative damage in rat intestinal epithelial IEC-6 cells. IEC-6 cells were transfected with EPHX2-siRNA and control si RNA plasmids by lipofectamineTM 2000 transfection reagent. The transfected samples were treated with H₂O₂ (50, 100, 200, 300, 400, and 500 μ mol/L) for 12, 24, and 48 h, respectively. Cell viability was determined by cell counting kit-8 (CCK-8). Lactate dehydrogenase (LDH), malondialdehyde (MDA), and superoxide

dismutase (SOD) were assessed by respective detection kits. Mitochondrial membrane potential (MMP), cell apoptosis and reactive oxygen species (ROS) and the levels of factors were determined by flow cytometer, quantitative real-time PCR (qRT-PCR) and western blot assays, respectively. We found that the IC₅₀ of H₂O₂ was 200 μ mol/L at 24 h, and the transfection of siEPHX2 in H₂O₂-induced IEC-6 cells significantly promoted the cell viability, SOD activity and MMP rate, and reduced the rates of ROS and apoptosis as well as LDH and MDA contents. siEPHX2 up-regulated the B-cell lymphoma-2 (Bcl-2) level and down-regulated the levels of fibroblast-associated (Fas), Fas ligand (FasL), Bcl-2 associated X protein (Bax), and Caspase-3. Moreover, the phosphorylation levels of phosphoinositide 3 kinase (PI3K), protein kinase B (Akt), and glycogen synthase kinase3 β (GSK3 β) were up-regulated. We proved that siEPHX2 had a protective effect on H₂O₂-induced oxidative damage in IEC-6 cells through activating PI3K/Akt/GSK3 β signaling pathway.

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Introduction

Intestinal epithelium is a natural barrier in the body, and the integrity of its structure and function plays a vital role in maintaining the normal activities of life (Elliott and Kaestner 2015). It is generally accepted that oxidative stress plays a vital role in the occurrence and development of intestinal injury, and it is a leading cause of many serious diseases such as various cancers (Monti et al. 2017; Turan et al. 2017). Oxidative stress is a potential pathological damage process caused by excessive production of free radicals or decreased antioxidant capacity, as well as imbalances between antioxidant and antioxidant systems (Pal and Gupta 2017). In the state of intestinal oxidative stress, a large number of free radicals are generated to destroy the biofilm, affecting the normal function of cells and the DNA, RNA thermostability, and enzyme activity, and finally accelerating the decomposition of proteins (Rashid et al. 2017). Studies have shown that oxidative stress causes intestinal cells to produce large amounts of toxic reactive oxygen species (ROS) metabolites, and inhibits the active oxygen radical scavenging system, causing intestinal mucosal cellular damage, and eventually leading to inflammatory bowel disease, such as ulcerative colitis and Crohn disease (Bhat-tacharyya et al. 2014; Diaz de Barboza et al. 2017).

H₂O₂ is one of the main components of ROS and can diffuse into the cells through the membrane. When the H₂O₂ level exceeds the antioxidant capacity of the cells, the balance of intracellular redox changes and oxidative stress occurs (Ma et al. 2016). Moreover, H₂O₂ is a common inducer to cellular oxidative damage and widely used in inducing apoptosis model (Martin-Guerrero et al. 2017). In order to reduce the toxic effects of ROS, antioxidant system will produce antioxidant enzymes such as superoxide dismutase (SOD). Study found that SOD can catalyze the single electron reduction of H₂O₂ (Chen et al. 2015).

Soluble epoxide hydrolase (sEH) was first discovered in 1973 by researchers through investigating the metabolism of terpenoid epoxides of insect-like. Human sEH is encoded by the soluble epoxide hydrolase 2 gene (EPHX2) and located in the 8p21-12 region of the human chromosome 8 (Arand et al. 1991). It has been reported that high glucose-induced oxidative stress suppresses sEH levels in Hep3B cells in vivo (Oguro et al. 2015). EPHX2, a class of hydrolytic enzymes, is widely found in plants, insects,

mammals and microorganisms (Larsson et al. 1995). It can reduce the content of internal oxide in organism by chemical catalysis, and also metabolize endogenous aliphatic and aromatic epoxide to produce the corresponding diol (Fretland and Omiecinski 2000). In recent years, study suggests that epoxyeicosatrienoic acids (EETs), the substrate of EPHX2, have a variety of important biological functions, including dilatation of blood vessel, lowering blood pressure, anti-inflammation, promoting fibrinolysis and cell proliferation, etc. (Spector et al. 2004). Pedro et al. confirmed that in the oral squamous cell carcinoma (OSCC) cases, 21 genes encoding enzymes involved in antioxidant metabolism are differentially expressed, specifically, the level of EPHX2 was down-regulated (Pedro et al. 2018). Previous studies have reported that EPHX2 level in ISIAH (inherited stress-induced arterial hypertension) rats was significantly higher than that in normal WAG rats under stress condition (Abramova et al. 2013). In addition, study showed that inhibition of sEH reduces acute kidney injury (Oguro et al. 2015). Klocke et al. found that prophylactic inhibition of EPHX2 delays onset of nephritis and ameliorates kidney damage in NZB/W F1 mice (Klocke et al. 2019). A study found that EPHX2 inhibition could be used as a novel method in the treatment of inflammatory bowel disease (Reisdorf et al. 2019). These studies suggest that EPHX2 possibly contributes to the diseases caused by oxidative stress and inflammation. However, the relationship between EPHX2 and oxidative damage of intestinal epithelial cells remains unknown.

In the current work, we speculated that transfection of siEPHX2 in H₂O₂-induced IEC-6 cells might have a protective effect on oxidative damage of intestinal epithelial cells. We explored the effect of H₂O₂ on the viability of rat intestinal epithelial IEC-6 cells. Subsequently, H₂O₂ was selected as an inducer to induce oxidative damage in IEC-6 cells. Moreover, to analyze the effects of siEPHX2 on oxidative damage of intestinal epithelial cells, IEC-6 cells transfected with EPHX2-siRNA plasmid, and H₂O₂-induced cells were determined. Then cell apoptosis and oxidative stress were assessed by the commercial kits. Moreover, we further determined the signaling pathway, via which the effect of siEPHX2 on H₂O₂-induced oxidative damage in IEC-6 cells was realized.

Materials and methods

Cell culture

Rat intestinal epithelial IEC-6 cell line, which was derived from the small intestinal crypt of sterile rats, was a normal and untransformed cell line (Quaroni et al. 1979). IEC-6 cells were obtained from Procell Life Technology Co., Ltd (Wuhan, China). The cells were cultured in complete Dulbecco's Modified Eagle Medium (DMEM; Huayueyang, Beijing, China) containing 10% Fetal Bovine Serum (FBS; Hyclone, Shanghai, China), 10 µg/mL insulin (Sigma, San Francisco, USA) and 50 µg/mL gentamicin (Solarbio, Beijing, China) in a 95% humidified incubator (HWS-2000; Safu, Ningbo, China) with 5% CO₂ at 37 °C.

Cell transfection and treatment

The EPHX2-siRNA (targeting sequence: 5'-TGAGC-GAGCAGAATTACTA-3') and unspecific scrambled siRNA (targeting sequence: 5'-UUCUCCCCGAAC-GUGUCGUCACGCCUTT-3') plasmids were obtained from Genewiz Biotechnology Co., Ltd (Suzhou, China). IEC-6 cells were transfected with plasmids at 50–60% confluency by lipofectamineTM 2000 transfection reagent (Thermo, Beijing, China).

The cells were divided into five groups, namely, control group (0.1% PBS treatment), negative control (NC) group (control siRNA plasmid treatment), H₂O₂ group (200 µmol/L H₂O₂ treatment), NC + H₂O₂ group (control siRNA plasmid and 200 µmol/L H₂O₂ treatment), and siEPHX2 + H₂O₂ group (EPHX2-siRNA plasmid and 200 µmol/L H₂O₂ treatment).

Cell morphology analysis

The IEC-6 cells were cultured for 24 h and photographed under a fluorescent microscope (DYF-880; Dianying, Shanghai, China).

Cell counting kit-8 (CCK-8) analysis

Cell counting kit-8 (CCK-8) (Beyotime, Shanghai, China) was used to detect the cell viability according to the instructions. Briefly, the cells were cultured in 96-well plates (3.5×10^3 cell/well) in a 95% humidified incubator with 5% CO₂ at 37 °C. Next day, the level of H₂O₂ at 50, 100, 200, 300, 400, and 500 µmol/

L were added into part of the cells, while rest of the cells were respectively treated with 0.1% PBS, control siRNA plasmid, 200 µmol/L H₂O₂, control siRNA plasmid and 200 µmol/L H₂O₂, and EPHX2-siRNA plasmid and 200 µmol/L H₂O₂. After the treatment, the cells were transferred to the incubator, and incubated for 12, 24, and 48 h. After the incubation, CCK-8 reagent was dripped into the cells. The cells were incubated in a 95% humidified incubator with 5% CO₂ at 37 °C for 2 h. Finally, absorbance was measured at 450 nm using a microplate reader (SpectraMax iD3; Molecular devices, California, USA).

Reactive oxygen species (ROS) analysis

The rate of ROS was determined using reactive oxygen species (ROS) detection kit (Beyotime, Shanghai, China) following the protocols. The cells were cultured in 6-well plates (5×10^4 cell/well) in an incubator with 95% humidity and 5% CO₂ at 37 °C. Next day, the cells were respectively treated with 0.1% PBS, control siRNA plasmid, 200 µmol/L H₂O₂, control siRNA plasmid and 200 µmol/L H₂O₂, and EPHX2-siRNA plasmid and 200 µmol/L H₂O₂. After the treatment, the cells were incubated with 2',7'-dichlorofluorescein diacetate (DCFH-DA) at 37 °C for 25 min and washed by PBS for three times. Finally, a flow cytometer (BD Biosciences) was applied to analyze the ROS levels, at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Lactate dehydrogenase (LDH) analysis

The content of LDH was detected by LDH detection kit (Genmed, Shanghai, China) following the protocols of the manufacturer. In brief, the cells were treated by drugs and accordingly grouped. After the treatment, the cells were separated by reagent A, added to reagent B and transferred into an incubator and held for 1 h. After the culturing, the cells were centrifuged in MPC-P25 microplate (Allsheng, Hangzhou, China) at 250×g for 10 min, and the supernatant was then added into 96-well plates. Then, reagent C and reagent D were dripped into the supernatant, mixed to react for 30 min in the dark. After the reaction, reagent E was added into the mixture. Finally, the absorbance was measured at 490 nm using a microplate reader.

Malondialdehyde (MDA) analysis

MDA detection kit (Leagene, Beijing, China) was used to determine MDA content following the instructions. The cells were treated by drugs and accordingly grouped. After the treatment, the cells were crushed on ice using ATPIO-400SD ultrasonic cell breaker (ATPIO, Nanjing, China). Then, 200 μ L TAB solution was added into 100 μ L cell liquid, thoroughly mixed and heated in boiling water for 20 min, and centrifuged at $1000\times g$ at room temperature for 10 min. After the centrifugation, deposit was resuspended by DMEM, while the suspension was added into 96-well plates. Finally, the absorbance was read at 532 nm by microplate reader. The total proteins were detected by a BCA protein quantification kit (Yeasen, Shanghai, China).

Superoxide dismutase (SOD) analysis

SOD detection kit (Beyotime, Shanghai, China) was performed to determine the activity of SOD following the instructions. The cells were treated by drugs and accordingly grouped. After the treatment, the cells were crushed on ice using an ATPIO-400SD ultrasonic cell breaker. Then, hydroxylamine working fluid was dripped into cell liquid. After mixing, the mixture was reacted at 37 °C for 40 min and chromogenic reagent was added into the mixture to mix for 15 min. Next, suspension was dripped into 96-well plates. Finally, the absorbance was detected at 550 nm by microplate reader and the total proteins were determined by BCA protein quantification kit.

Mitochondrial membrane potential (MMP) analysis

MMP content was examined using JC-1 detection kit (Beyotime, Shanghai, China) according to instructions. The cells were treated by drugs and accordingly grouped. After the treatment, the cells were digested by 0.25% EDTA-trypsin (Solarbio, Beijing, China) and resuspended using DMEM. The cells were stained in JC-1 solution, incubated at 37 °C for 20 min and centrifuged at $600\times g$ at 4 °C for 3 min. The supernatant was discarded, and the cells were washed by PBS twice. MMP was detected using flow cytometer at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Cell apoptosis analysis

The Annexin V-FITC/PI apoptosis detection kit was used to assess cell apoptosis according to the manufacturer's instructions. Briefly, cells were treated by drugs and accordingly grouped. After the treatment, the cells were washed by cold PBS twice and then centrifuged at $300\times g$ at 4 °C for 5 min, and resuspended by $1\times$ Binding Buffer. Subsequently, the cells were stained with Annexin V-FITC and PI staining solution in the dark at room temperature for 10–15 min. $1\times$ Binding Buffer was added into cells on the ice. Finally, the sample was assessed using flow cytometer.

Quantitative real-time PCR (qRT-PCR) analysis

The cells were treated by drugs and accordingly grouped. After the treatment, total RNAs were collected by Trizol reagent (Takara, Beijing, China). 1 μ g RNA was used to synthesize cDNA by RT Master Mix kit (Takara, Beijing, China). Reverse transcriptional reaction conditions were set at 85 °C for 15 min, at 4 °C for 10 min. SYBR Premix TaqTM II kit (Takara, Beijing, China) was used to amplify cDNA and the protocol consisted of 25 μ L SYBR Green Mix, 1 μ L forward/reverse primers, 19 μ L ddH₂O, and 4 μ L cDNA. PCR reaction conditions were set at 95 °C for 10 min, 35 cycles at 95 °C for 15 s, at 60 °C for 45 s, at 95 °C for 15 s, at 60 °C for 1 min, at 95 °C for 15 s and at 60 °C for 15 s. The primer sequences used were as follows: EPHX2, 5'-TGACAGTGAAGCCAGG-GATC-3' (forward), 5'-ACCATCTCCTCACACAG-CAA-3' (reverse) (product: 229); fibroblast-associated (Fas), 5'-CCCGGAAAGCAAGATCGATG-3' (forward), 5'-TGGCTTGAATTTCTCTGCG-3' (reverse) (product: 176); Fas ligand (FasL), 5'-GCACAC CCTCTGAAACCAA-3' (forward), 5'-ACCCAGC CTCATTGATCACA-3' (reverse) (product: 159); Caspase-3, 5'-AAGCCATGGTGAAGAAGGAA-3' (forward), 5'-TCTCAATGCCACAGTCCAGT-3' (reverse) (product: 164); B-cell lymphoma-2 (Bcl-2), 5'-TGGGGATGACTTCTCTCGTC-3' (forward), 5'-CCGAAC TCAAAGAAGGCCAC-3' (reverse) (product: 162); Bcl-2 associated X protein (Bax), 5'-CAT CCAGGATCGAGCAGAGA-3' (forward), 5'-AAT-CATCCTCTGCAGCTCCA-3' (reverse) (product: 151); GAPDH, 5'-GTATGACTCTACCCACGGCA-3' (forward), 5'-AAGACGCCAGTAGACTCCAC-3' (reverse).

(reverse) (product: 162). GAPDH served as a sample control. The formula $2^{-\Delta\Delta CT}$ was used to calculate the gene expression.

Western blot analysis

After the transfection and treatment, the cells were washed by PBS for twice and lysed with RIPA buffer (high) (Solarbio, Beijing, China) to obtain protein extracts. The contents of protein extracts were detected by a BCA protein quantification kit. The proteins were separated on sodium dodecyl sulfate-polyacrylamide gelelectrophoresis (SDS-PAGE) and then transferred to PVDF membranes (Reno, Hangzhou, China). Subsequently, the membranes were blocked by 5% non-fat milk for 1.5 h and incubated with anti-EPHX2 (Abcam, ab155280, 1:1000), anti-Fas (Invitrogen, 12-9919-41, 1:800), anti-FasL (Invitrogen, 12-5911-81, 1:1200), anti-Caspase-3 (Invitrogen, 700182, 1:700), anti-Bax (Invitrogen, MA5-14006, 1:1000), anti-Bcl-2 (Invitrogen, MA5-11757, 1:1000), anti-phosphorylated-glycogen synthase kinase 3 β (p-GSK3 β) (Abcam, ab75745, 1:800), anti-GSK3 β (Abcam, ab32391, 1:1000), anti-phosphorylated-protein kinase B (p-Akt) (Invitrogen, 44-623G, 1:1200), anti-Akt (Invitrogen, 44-623G, 1:1600), anti-phosphorylated-phosphatidylinositol 3 kinase (p-PI3K) (Invitrogen, PA5-12799, 1:1600), anti-PI3K (Invitrogen, MA5-17149, 1:1000), and anti-GAPDH (Invitrogen, 39-8600, 1:1000) at 4 °C overnight. The membranes were washed by PBST 3 times for 6 min and then soaked in corresponding secondary antibodies (HRP mouse anti-goat IgG, Invitrogen, BA1074, 1:7000; HRP mouse anti-rabbit, Invitrogen, BA1034, 1:7000) at 37 °C for 60 min. Finally, secondary antibodies were recycled at 4 °C refrigerator. The membranes were washed by PBST 3 times for 6 min, and the proteins were exposed to ECL detection reagent (Taixin, Beijing, China). GAPDH served as a sample control.

Statistical analysis

The experimental data were analyzed with SPSS 13.0 software. The results were expressed as mean $\pm$ SD. The differences among groups were analyzed using One-way analysis of variance (ANOVA). Each experiment was conducted in triplicate for three times.

Statistically significant difference was considered as $P < 0.05$.

Results

Identification of IEC-6 cells

IEC-6 cells had flat monolayer with flat irregular polygons and a round nucleus, moreover, the cells were tightly connected into a monolayer. Furthermore, the proliferation of cells promoted the movement of the whole epithelial cellular membrane, and the cells at the edge of the epithelium were connected more closely to the membrane and were rarely isolated from the cell population. We observed that cells were in the shape of paving stones and the intercellular connection was closely (Fig. 1a). In addition, some cells, which showed epithelial growth, were often more separated from each other, resulting in a mesh shaped hole, and this was one of the characteristics of epithelial cells. Figure 1b showed that the structures of the nucleus and organelle were clearly visible.

The expression level of EPHX2 and viability of IEC-6 cells

QRT-PCR and western blot assays were performed to determine the mRNA and protein levels of EPHX2 in IEC-6 cells. The data showed that the expression level of EPHX2 in control group was similar to NC group, and a significant down-regulation of EPHX2 was observed in case of cells were transfected with EPHX2-siRNA plasmid ($P < 0.05$, Fig. 2a–c).

CCK-8 was used to determine the viability of IEC-6 cells, and we found that H₂O₂ could inhibit the viability of cells in time- and dose-dependent manners. The IC₅₀ of H₂O₂ was 200 μ mol/L at 24 h, moreover, the viability of cells in H₂O₂ group was similar to the NC + H₂O₂ group. SiEPHX2 increased the viability of IEC-6 cells treated by H₂O₂, compared with NC + H₂O₂ group ($P < 0.05$, Fig. 2d, e).

Fig. 1 Identification of IEC-6 cells. **a, b** After culturing IEC-6 cells for 24 h, the cells were photographed under a fluorescent microscope

Fig. 2 The expression level of EPHX2 and viability of IEC-6 cells. **a–c** IEC-6 cells were transfected with EPHX2-siRNA and unspecific scrambled siRNA plasmids by lipofectamineTM 2000 transfection reagent. The mRNA and protein levels of EPHX2 were determined by qRT-PCR and western bolt assays, respectively. GAPDH served as an internal control. The gray value was measured and counted by quality one. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared with control group. ^ $P < 0.05$; ^^ $P < 0.01$; ^^ $P < 0.001$, compared with NC group. **d, e** IEC-6 cells were treated with H₂O₂ (50, 100, 200, 300, 400,

and 500 µmol/L) for 12, 24, and 48 h, respectively. The experiments were divided into five groups, namely, control group (0.1% PBS treatment), negative control (NC) group (control siRNA plasmid treatment), H₂O₂ group (200 µmol/L H₂O₂ treatment), NC + H₂O₂ group (control siRNA plasmid and 200 µmol/L H₂O₂ treatment), and siEPHX2 + H₂O₂ group (EPHX2-siRNA plasmid and 200 µmol/L H₂O₂ treatment). Cell viability was analyzed by cell counting kit-8 (CCK-8). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared with control group. ^ $P < 0.05$, compared with NC + H₂O₂ group.

Fig. 3 siEPHX2 suppresses the oxidative stress in IEC-6 cells treated by H_2O_2 . **a**, **b** ROS was measured by ROS detection kit. **c–e** The contents of LDH, and MDA and SOD activity were assessed by LDH, MDA, and SOD detection kits, respectively.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared with NC group. ^ $P < 0.05$; ^^ $P < 0.01$; ^^ $P < 0.001$, compared with NC + H_2O_2 group

siEPHX2 suppresses the oxidative stress in IEC-6 cells treated by H_2O_2

ROS rate, LDH and MDA contents, and SOD activity were measured using respective kits to investigate the effect of siEPHX2 on oxidative stress in IEC-6 cells treated by H_2O_2 . Our data revealed that the rate of ROS in H_2O_2 group was significantly higher than that

in NC group, while siEPHX2 inhibited the increase of ROS rate induced by H_2O_2 . Moreover, H_2O_2 increased the contents of LDH and MDA but decreased the activity of SOD. We also found that siEPHX2 reduced LDH and MDA contents and promoted the activity of SOD compared with NC + H_2O_2 group ($P < 0.05$, Fig. 3).

Fig. 4 siEPHX2 inhibits the rate of apoptosis and increases MMP rate in IEC-6 cells treated by H₂O₂. **a, b** Cell apoptosis was determined by the Annexin V-FITC/PI apoptosis detection kit. (C, D) MMP was measured using JC-1 kit. **P* < 0.05; ***P* < 0.01; ****P* < 0.001, compared with NC group. ^*P* < 0.05; ^^*P* < 0.01; ^^*P* < 0.001, compared with NC + H₂O₂ group

siEPHX2 inhibits the rate of apoptosis and increases MMP rate in IEC-6 cells treated by H₂O₂

The Annexin V-FITC/PI apoptosis detection and JC-1 kits were performed to determine the rates of apoptosis and MMP in H₂O₂-induced IEC-6 cells, respectively. Our results showed that the rate of apoptosis of H₂O₂ group increased, compared with NC groups. The rate of apoptosis of siEPHX2 + H₂O₂ group was

obviously lower than that in NC + H₂O₂ group (*P* < 0.05, Fig. 4a, b). In addition, our results revealed that the rate of MMP in H₂O₂ group was obviously lower than that in NC group; whereas the rate of MMP in siEPHX2 + H₂O₂ group was significantly higher than that in NC + H₂O₂ group (*P* < 0.05, Fig. 4c, d).

siEPHX2 regulates apoptosis-related factors in IEC-6 cells treated with H₂O₂

To further determine the inhibitory effect of siEPHX2 on apoptosis of IEC-6 cells treated by H₂O₂, qRT-PCR and western blot assays were conducted to determine the mRNA and protein levels of apoptosis-related factors, respectively. We found that H₂O₂ markedly promoted Fas, FasL, Caspase-3, and Bax expressions but suppressed Bcl-2 expression. However, the levels of Fas, FasL, Caspase-3, and Bax were down-regulated,

Fig. 5 siEPHX2 regulates apoptosis-related factors in IEC-6 cells treated by H₂O₂. **a** The mRNA levels of Fas, FasL, Bax, Bcl-2, and caspase-3 were determined by qRT-PCR assay. **b**, **c** The protein levels of Fas, FasL, Bax, Bcl-2, and caspase-3 were determined by western bolt assay, and normalized to the levels

of GAPDH. The gray value was measured and counted by quality one. **P* < 0.05; ***P* < 0.01; ****P* < 0.001, compared with NC group. ^*P* < 0.05; ^^*P* < 0.01; ^^*P* < 0.001, compared with NC + H₂O₂ group

Fig. 6 siEPHX2 reduces H_2O_2 -induced oxidative damage in IEC-6 cells via activating PI3K/Akt/GSK3 β signaling pathway. **a–c** The protein levels of PI3K, p-PI3K, Akt, p-Akt, GSK3 β , and p-GSK3 β were determined by western bolt assay. GAPDH served as an internal control. The gray value was measured and counted by quality one. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared with NC group. $^{\wedge}P < 0.05$; $^{\wedge\wedge}P < 0.01$; $^{\wedge\wedge\wedge}P < 0.001$, compared with NC + H_2O_2 group

while Bcl-2 level was up-regulated, when the cells were transfected with EPHX2 siRNA plasmid, compared with NC + H_2O_2 groups ($P < 0.05$, Fig. 5).

siEPHX2 reduces H_2O_2 -induced oxidative damage in IEC-6 cells via activating PI3K/Akt/GSK3 β signaling pathway

Western blot assay was performed to determine the protein levels of PI3K, p-PI3K, Akt, p-Akt, GSK3 β , and p-GSK3 β , and the data revealed that H_2O_2 significantly down-regulated levels of p-PI3K,

p-Akt, and p-GSK3 β . Furthermore, siEPHX2 promoted p-PI3K, p-Akt, and p-GSK3 β expressions, compared with H_2O_2 and NC + H_2O_2 groups. However, the levels of PI3K, Akt, and GSK3 β did not change in different groups ($P < 0.05$, Fig. 6). The schematic representation of the findings of this study is presented in Fig. 7.

Fig. 7 The schematic representation of the findings of this study

Discussion

The IEC-6 cell line has epithelial morphology and single cellular growth. The epithelial cell line is characterized as undifferentiated and small intestinal epithelial crypt, which are the same as normal cells (Quaroni et al. 1979). Similar to previous studies, our results showed that IEC-6 cells had epithelial morphology, single cellular growth, and were undifferentiated, confirming that IEC-6 cells grew normally and could be used for later experiments.

H₂O₂ is an oxidant and often used to induce oxidative damage in cells such as PC12 (Wang et al. 2017a), IEC-6 (Bettaib et al. 2017), and human hepatic cells (Martin-Guerrero et al. 2017). It has shown that H₂O₂ treatment could inhibit the IEC-6 cell viability, and up-regulate the apoptotic and ROS levels (Zhuang et al. 2019). In the current study, we assessed the inhibitory effects of H₂O₂ on IEC-6 cells, and found that H₂O₂ inhibit the viability of IEC-6 cells, and the IC₅₀ of H₂O₂ was 200 μmol/L at 24 h. Therefore, 200 μmol/L H₂O₂ and 24 h were selected to induce oxidative damage in IEC-6 cells.

Human sEH is encoded by the EPHX2, and reports showed that inhibiting sEH attenuated MPTP-induced neurotoxicity in the nigrostriatal dopaminergic system by attenuating oxidative stress, ER stress and apoptosis (Huang et al. 2018). Previous research demonstrated that EPHX2 silencing can decrease the ability of oxidative stress in prostate cancer cells (Vainio et al. 2011), moreover, one study have reported that n – 3 polyunsaturated fatty acids decreased the protein expression of sEH through oxidative stress and p38 kinase (Okada et al. 2017). Thus, we speculated that EPHX2 is related to oxidative stress of intestinal epithelial cells. The IEC-6 cells were transfected with EPHX2 siRNA plasmid, then H₂O₂ was further used to induce oxidative damage, and we found that the transfection of siEHPX2 in H₂O₂-

induced IEC-6 cells down-regulated the level of EPHX2, and promoted the viability of cells. Meanwhile, ROS rate and the contents of LDH and MDA were decreased, while SOD activity was promoted, indicating that siEPHX2 could suppress the ability of oxidative stress in H₂O₂-induced IEC-6 cells. However, it will be more convincing if ROS was traced by fluorescence microscopy or images of cells exposed to ROS fluorescence probes were provided.

According to the report, oxidative stress-induced apoptosis is one of the main pathways of intestinal cellular damage (Ogura and Shimosawa 2014). It has been reported that ROS could mediate apoptosis and played an important role in cellular apoptosis, and the destruction of mitochondrial transmembrane potential is often accompanied by apoptosis (Cui et al. 2018; Xiao et al. 2016). After hypoxia in cells, ROS rapidly increases, which can damage DNA to activate multiple signal transduction pathways such as Bcl-2/Bax and Fas/FasL pathways and activate caspase enzymes, resulting in cell apoptosis (Gu et al. 2013; Ogura and Shimosawa 2014; Wang et al. 2017b). As expected, H₂O₂ promoted apoptosis of IEC-6 cells and reduced the rate of MMP, which up-regulated the levels of Fas, FasL, Bax, and Caspase-3 and down-regulated Bcl-2 level. Nevertheless, siEPHX2 reduced the rate of apoptosis and increased MMP rate. Meanwhile, the levels of Fas, FasL, Bax, and Caspase-3 were down-regulated and Bcl-2 level was up-regulated when H₂O₂-induced IEC-6 cells were transfected with EPHX2-siRNA plasmid. Thus, we proved that siEPHX2 had the potential protective effect on H₂O₂-induced oxidative damage in IEC-6 cells. In this study, we found no significant difference in the late apoptosis rate between the siEPHX2 + H₂O₂ group and the H₂O₂ group, however, the exact mechanism still remains to be further explored.

PI3K/Akt signal transduction pathway is an important pathway for membrane receptor signal

transduction into cells (Parcellier et al. 2008) and GSK3 β is a key target protein for PI3K/Akt signaling pathway. PI3K-activated Akt phosphorylates the N-terminal Ser9 site of GSK3 β , leading to GSK3 β inactivation (Chen et al. 2018; Chowdhury et al. 2017). PI3K/Akt signaling pathway is critical in mediating cell survival and apoptosis and could phosphorylate the downstream signalings (Chen et al. 2018), for example, GSK3 β inhibits mitochondria-mediated apoptosis (Liao et al. 2018). It has been reported that regulation of GSK3 β activity could inhibit the decrease of MMP to attenuate early mitochondria-dependent apoptosis (Lv et al. 2016; Zhao et al. 2019). It has been reported that mesencephalic astrocyte-derived neurotrophic factor-mediated protection against 6-OHDA-induced cytotoxicity by potentiating the Nrf2-related survival mechanism via PI3K/Akt/GSK3 β pathway (Zhang et al. 2017). Previous evidences have shown that quercetin reduces oxidative injury via activating PI3K/Akt/GSK3 β signaling pathway (Jiang et al. 2016). Moreover, researchers found that chicoric acid promoted apoptosis by ROS-mediated PI3K/Akt signaling pathway (Xiao et al. 2013). Thus, we suspected that siEPHX2 might reduce oxidative damage of H₂O₂-induced IEC-6 cells via regulating PI3K/Akt/GSK3 β signaling pathway. As expected, siEPHX2 activated the phosphorylation levels of PI3K, Akt, and GSK3 β in IEC-6 cells treated by H₂O₂. The result indicated that siEPHX2 protected against H₂O₂-induced oxidative damage in IEC-6 cells via activating PI3K/Akt/GSK3 β signaling pathway.

Conclusions

In conclusion, our study explored the potential effect and molecular mechanism of siEPHX2 on H₂O₂-induced oxidative damage in IEC-6 cells. 200 μ mol/L H₂O₂ and 24 h were used to induce oxidative damage in IEC-6 cells. Subsequently, we found that siEPHX2 suppressed the ability of oxidative stress and the rate of apoptosis by up-regulating phosphorylation levels of PI3K, Akt, and GSK3 β in H₂O₂-induced IEC-6 cells. Hence, it was proved that siEPHX2 had potential effect on H₂O₂-induced oxidative damage in IEC-6 cells via activating PI3K/Akt/GSK3 β signaling pathway.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Abramova TO, Redina OE, Smolenskaya SE, Markel AL (2013) Elevated expression of the Ephx2 mRNA in the kidney of hypertensive. ISIAH Rats Mol Biol 47:821–826
- Arand M, Knehr M, Thomas H, Zeller HD, Oesch F (1991) An impaired peroxisomal targeting sequence leading to an unusual bicompartamental distribution of cytosolic epoxide hydrolase. FEBS Lett 294:19–22
- Bettaib J, Talarmin H, Droguet M, Magne C, Boulaaba M, Giroux-Metges MA, Ksouri R (2017) Biomedicine *Tamarix gallica* phenolics protect IEC-6 cells against H₂O₂ induced stress by restricting oxidative injuries and MAPKs signaling pathways. Pharmacotherapy 89:490–498. <https://doi.org/10.1016/j.biopha.2017.02.047>
- Bhattacharyya A, Chattopadhyay R, Mitra S, Crowe SE (2014) Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. Physiol Rev 94:329–354. <https://doi.org/10.1152/physrev.00040.2012>
- Chen J et al (2015) Epigallocatechin-3-gallate attenuates lipopolysaccharide-induced mastitis in rats via suppressing MAPK mediated inflammatory responses and oxidative stress. Int Immunopharmacol 26:147–152. <https://doi.org/10.1016/j.intimp.2015.03.025>
- Chen X, Yan X, Guo L (2018) Inhibitory effect of Patrnia on BRL-3A cell apoptosis through the TLR4/PI3K/AKT/GSK3beta and TLR4/P38/JNK signaling pathways. Mol Med Rep 17:5344–5349. <https://doi.org/10.3892/mmr.2018.8466>
- Chowdhury D, Kumar D, Bhadra U, Devi TA, Bhadra MP (2017) Prohibitin confers cytoprotection against ISO-induced hypertrophy in H9c2 cells via attenuation of oxidative stress and modulation of Akt/Gsk-3beta signaling. Mol Cell Biochem 425:155–168. <https://doi.org/10.1007/s11010-016-2870-3>
- Cui L et al (2018) Apoptosis induction by alantolactone in breast cancer MDA-MB-231 cells through reactive oxygen species-mediated mitochondrion-dependent pathway. Arch Pharm Res 41:299–313. <https://doi.org/10.1007/s12272-017-0990-2>
- Diaz de Barboza G, Guizzardi S, Moine L, Tolosa de Talamoni N (2017) Oxidative stress, antioxidants and intestinal calcium absorption. World J Gastroenterol 23:2841–2853. <https://doi.org/10.3748/wjg.v23.i16.2841>
- Elliott EN, Kaestner KH (2015) Epigenetic regulation of the intestinal epithelium. Cell Mol Life Sci 72:4139–4156. <https://doi.org/10.1007/s00018-015-1997-9>

- Fretland AJ, Omiecinski CJ (2000) Epoxide hydrolases: biochemistry and molecular biology. *Chemico-Biol Interact* 129:41–59
- Gu D, Huang J, Shan Z, Yin Y, Zheng S, Wu P (2013) Effects of long-term ketamine administration on rat bladder protein levels: a proteomic investigation using two-dimensional difference gel electrophoresis system. *Int J Urol* 20:1024–1031. <https://doi.org/10.1111/iju.12100>
- Huang HJ, Wang YT, Lin HC, Lee YH, Lin AM (2018) Soluble epoxide hydrolase inhibition attenuates MPTP-induced neurotoxicity in the nigrostriatal dopaminergic system: involvement of alpha-synuclein aggregation and ER. *Stress Mol Neurobiol* 55:138–144. <https://doi.org/10.1007/s12035-017-0726-9>
- Jiang W et al (2016) Quercetin protects against Okadaic acid-induced injury via MAPK and PI3K/Akt/GSK3beta signaling pathways in HT22 hippocampal neurons. *PLoS ONE* 11:e0152371. <https://doi.org/10.1371/journal.pone.0152371>
- Klocke J et al (2019) Prophylactic inhibition of soluble epoxide hydrolase delays onset of nephritis and ameliorates kidney damage in NZB/W F1 mice. *Sci Rep* 9:8993. <https://doi.org/10.1038/s41598-019-45299-5>
- Larsson C, White I, Johansson C, Stark A, Meijer J (1995) Localization of the human soluble epoxide hydrolase gene (EPHX2) to chromosomal region 8p21-p12. *Hum Genet* 95:356–358
- Liao YX, Zhang ZP, Zhao J, Liu JP (2018) Effects of fibronectin 1 on cell proliferation, senescence and apoptosis of human glioma cells through the PI3K/AKT signaling pathway cellular physiology and biochemistry. *Int J Exp Cell Physiol Biochem Pharmacol* 48:1382–1396. <https://doi.org/10.1159/000492096>
- Lv D, Bai Z, Yang L, Li X, Chen X (2016) Lipid emulsion reverses bupivacaine-induced apoptosis of h9c2 cardiomyocytes: PI3K/Akt/GSK-3beta signaling pathway. *Environ Toxicol Pharmacol* 42:85–91. <https://doi.org/10.1016/j.etap.2016.01.004>
- Ma Z et al (2016) Safflower yellow B suppresses HepG2 cell injury induced by oxidative stress through the AKT/Nrf2 pathway. *Int J Mol Med* 37:603–612. <https://doi.org/10.3892/ijmm.2016.2462>
- Martin-Guerrero SM et al (2017) Poly(ADP-ribose)polymerases inhibitors prevent early mitochondrial fragmentation and hepatocyte cell death induced by H₂O₂. *PLoS ONE* 12:e0187130. <https://doi.org/10.1371/journal.pone.0187130>
- Monti DM et al (2017) Ferritin nanocages loaded with gold ions induce oxidative stress and apoptosis in MCF-7 human breast cancer cells. *Dalton Trans*. <https://doi.org/10.1039/c7dt02370g>
- Ogura S, Shimosawa T (2014) Oxidative stress and organ damages. *Curr Hypertens Rep* 16:452. <https://doi.org/10.1007/s11906-014-0452-x>
- Oguro A, Oida S, Imaoka S (2015) Down-regulation of EPHX2 gene transcription by Sp1 under high-glucose conditions. *Biochem J* 470:281–291. <https://doi.org/10.1042/bj20150397>
- Okada T et al (2017) N-3 polyunsaturated fatty acids decrease the protein expression of soluble epoxide hydrolase via oxidative stress-induced P38 kinase in rat endothelial cells. *Nutrients*. <https://doi.org/10.3390/nu9070654>
- Pal R, Gupta N (2017) Yogic practices on oxidative stress and of antioxidant level: a systematic review of randomized controlled trials. *J Complement Integr Med*. <https://doi.org/10.1515/jcim-2017-0079>
- Parcellier A, Tintignac LA, Zhuravleva E, Hemmings BA (2008) PKB and the mitochondria: AKTing on apoptosis. *Cell Signal* 20:21–30. <https://doi.org/10.1016/j.cellsig.2007.07.010>
- Pedro NF, Biselli JM, Maniglia JV, Santi-Neto D, Pavarino EC, Goloni-Bertollo EM, Biselli-Chicote PM (2018) Candidate biomarkers for oral squamous cell carcinoma: differential expression of oxidative stress-related genes. *Asian Pac J Cancer Prevent* 19:1343–1349. <https://doi.org/10.22034/apjcp.2018.19.5.1343>
- Quaroni A, Wands J, Trelstad RL, Isselbacher KJ (1979) Epithelioid cell cultures from rat small intestine. Characterization by morphologic immunologic criteria. *J Cell Biol* 80:248–265
- Rashid S et al (2017) Partial protection by 18beta Glycrrhetic acid against Cisplatin induced oxidative intestinal damage in wistar rats: possible role of NFkB and caspases. *Pharmacol Rep*. <https://doi.org/10.1016/j.pharep.2017.02.013>
- Reisdorf WC et al (2019) Preclinical evaluation of EPHX2 inhibition as a novel treatment for inflammatory bowel disease. *PLoS ONE* 14:e0215033. <https://doi.org/10.1371/journal.pone.0215033>
- Spector AA, Fang X, Snyder GD, Weintraub NL (2004) Epoxyeicosatrienoic acids (EETs): metabolism and biochemical function. *Prog Lipid Res* 43:55–90
- Turan I, Ozacmak HS, Ozacmak VH, Barut F, Arasli M (2017) Agmatine attenuates intestinal ischemia and reperfusion injury by reducing oxidative stress and inflammatory reaction in rats. *Life Sci* 189:23–28. <https://doi.org/10.1016/j.lfs.2017.08.032>
- Vainio P et al (2011) Arachidonic acid pathway members PLA2G7, HPGD, EPHX2, and CYP4F8 identified as putative novel therapeutic targets in prostate cancer. *Am J Pathol* 178:525–536. <https://doi.org/10.1016/j.ajpath.2010.10.002>
- Wang S, Zhao X, Yang S, Chen B, Shi J (2017a) Salidroside alleviates high glucose-induced oxidative stress and extracellular matrix accumulation in rat glomerular mesangial cells by the TXNIP-NLRP3 inflammasome pathway. *Chem Biol Interact* 278:48–53. <https://doi.org/10.1016/j.cbi.2017.10.012>
- Wang YL et al (2017b) Protective effect of curcumin against oxidative stress-induced injury in rats with Parkinson's disease through the Wnt/ beta-catenin signaling pathway cellular physiology and biochemistry. *Int J Exp Cell Physiol Biochem Pharmacol* 43:2226–2241. <https://doi.org/10.1159/000484302>
- Xiao H, Wang J, Yuan L, Xiao C, Wang Y, Liu X (2013) Chioric acid induces apoptosis in 3T3-L1 preadipocytes through ROS-mediated PI3K/Akt and MAPK signaling pathways. *J Agric Food Chem* 61:1509–1520. <https://doi.org/10.1021/jf3050268>
- Xiao H, Wu M, Shao F, Guan G, Huang B, Tan B, Yin Y (2016) N-Acetyl-L-cysteine protects the enterocyte against oxidative damage by modulation of mitochondrial

- function. *Mediators Inflamm* <https://doi.org/10.1155/2016/8364279>
- Zhang J et al (2017) Nrf2-mediated neuroprotection by MANF against 6-OHDA-induced cell damage via PI3K/AKT/GSK3beta pathway. *Exp Gerontol* 100:77–86. <https://doi.org/10.1016/j.exger.2017.10.021>
- Zhao Q et al (2019) Qiliqiangxin attenuates oxidative stress-induced mitochondrion-dependent apoptosis in cardiomyocytes via PI3K/AKT/GSK3beta signaling. *Pathway Biol Pharm Bull* 42:1310–1321. <https://doi.org/10.1248/bpb.b19-00050>
- Zhuang S, Yu R, Zhong J, Liu P, Liu Z (2019) Rhein from rheum rhabarbarum inhibits hydrogen-peroxide-induced oxidative stress in intestinal epithelial cells partly through PI3K/Akt-mediated Nrf2/HO-1. *Pathways J Agric Food Chem* 67:2519–2529. <https://doi.org/10.1021/acs.jafc.9b00037>

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