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ORIGINAL PAPER

Silibinin ameliorates cisplatin-induced acute kidney injury via activating Nfe2l1-mediated antioxidative response to suppress the ROS/MAPK signaling pathway

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Abstract

Cisplatin, a first-line chemotherapeutic agent commonly used to treat various solid tumors, induce severe adverse effects, especially nephrotoxicity, which largely limits its clinical application. However, the currently used measures to prevent nephrotoxicity are not ideal owing to the mechanisms underlying cisplatin-induced nephrotoxicity are not comprehensively understood. Herein, we examined the effects of silibinin on cisplatin-induced nephrotoxicity and found that silibinin exerted cytoprotection effects during cisplatin treatment in HEK293 cells and in a cisplatin-induced acute kidney injury (AKI) model. Mechanistically, silibinin ameliorated cisplatin-induced AKI via decreasing ROS-mediated MAPK signaling pathway activation, which was confirmed using the inhibitor N-acetylcysteine. Moreover, the protective effect of silibinin against cisplatin-induced ROS generation through the antioxidant transcription factor nuclear factor-erythroid 2-related factor 1 (Nfe2l1), rather than Nfe2l2, mediates HO1 expression. Furthermore, interference with the abundance of Nfe2l1 using siRNA or an overexpression plasmid enhanced or decreased the effect of cisplatin-induced apoptosis, respectively, in HEK293 cells. Interestingly, Nfe2l1 protein stability was more sensitive to cisplatin than that of Nfe2l2. More importantly, the mechanism that silibinin activates Nfe2l1-mediated antioxidant responses was confirmed in a cisplatin-induced AKI model. Silibinin rescued cisplatin-induced Nfe2l1 inhibition by regulating its transcription and post-translational modifications. Taken together, our results reveal a novel mechanism by which silibinin ameliorates cisplatin-induced AKI via activating Nfe2l1-mediated antioxidative response, which provides a new insights to protect patients receiving cisplatin-based cancer treatment against AKI.

Keywords Cisplatin · Silibinin · Acute kidney injury · Nfe2l1 · Antioxidant reaction · MAPK

Introduction

In the past four decades, cisplatin has been used a first-line chemotherapy agent for the treatment of solid tumors, including testicular, breast, and other cancers (Volarevic et al. 2019). However, several clinical trials have demonstrated that severe adverse effects, such as nephrotoxicity, neurotoxicity, and ototoxicity accompanied the use of cisplatin (Cersosimo 1989; Miller et al. 2010; Karasawa and Steyger 2015). The kidney is the main organ responsible for the metabolic processing of cisplatin, thus nephrotoxicity is a prevalent side effect (30–40%) and clinical symptoms caused by cisplatin administration are severe (Volarevic et al. 2019), which markedly limits its clinical application.

In addition to affecting the DNA synthesis, cisplatin can also cause cancer cell death through triggering apoptotic pathways, provoking cytoplasmic organelle dysfunction,

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inflammation, and oxidative stress (Karasawa and Steyger 2015; Manohar and Leung 2018). Among these processes, oxidative stress is a crucial factor contributing to cisplatin-induced cell death in normal cells with low proliferation cells during the initial period, because substantial evidence suggests that cisplatin can directly bind to the antioxidant glutathione upon entry into cells (Ishikawa and Ali-Osman 1993; Fuertes et al. 2003); and it can inhibit antioxidant enzymes such as glutathione peroxidase, catalase and superoxide dismutase (Khynriam and Prasad 2002) to disrupt the redox steady state, leading to reactive oxygen species (ROS) generation (Karasawa and Steyger 2015). Furthermore, a large amount of cisplatin accumulating in the cytoplasm causes mitochondrial DNA damage, disrupts the mitochondrial respiratory chain, and decreases fatty acid oxidation. All these processes may lead to elevated ROS production and induce oxidative stress.

The cap'n'collar-basic region leucine zipper subfamily members nuclear factor-erythroid 2-related factor 1 (Nfe2l1) and Nfe2l2 are two most important transcription factors involved in oxidative stress response. They can directly bind to specific cis-regulatory elements within the promoter regions of target genes by forming homo- or heterodimers with their cognate partners to regulate a series of antioxidant enzymes, including heme oxygenase 1 (HO1), NAD(P):quinone oxidoreductase 1, and glutamate-cysteine ligase modifier (Zhang and Xiang 2016). Although they share many target antioxidant genes, Nfe2l1 and Nfe2l2 performe distinct roles in response to oxidative stress. In vivo experiments demonstrated that global Nfe2l2 knockout mice and cells derived from them are more susceptible to carcinogen-induced oxidative stress, however, such mice do not spontaneously develop any clinical symptoms (Chan 1996, Xu et al. 2006). In contrast, global knockout of Nfe2l1 in mouse results in severe oxidative stress and causes embryonic lethality (Farmer 1997, Chan et al. 1998, Kwong et al. 1999; Leung et al. 2003), and conditional knockout of Nfe2l1 in different mouse organs leads to distinct pathological phenotypes, such as non-alcoholic steatohepatitis-based spontaneous hepatoma (Xu et al. 2005; Ohtsuji et al. 2008) and neurodegenerative diseases (Kobayashi et al. 2011; Lee et al. 2011). Moreover, knockout of Nfe2l1 and/or Nfe2l2 in mouse liver and human hepatoma carcinoma cells revealed that Nfe2l2 mainly maintains cellular redox homeostasis under severe stress conditions, and Nfe2l1 is responsible for defense responses against endogenous stressors to maintain the basal steady state under normal conditions (Ohtsuji et al. 2008; Zhang and Xiang 2016, Xiang et al. 2018a). Therefore, activating Nfe2l1 and Nfe2l2 signaling to elevate their antioxidant ability in maintaining cellular basal and adaptive response homeostasis is a promising approach to prevent kidney injury arising due to cisplatin-induced oxidative stress. In fact, a large number of naturally extracted compounds were confirmed to defend against cisplatin insult by enhancing Nfe2l2 signaling, but it also seems to be applicable to cancer cells, which markedly decreases the effects of chemotherapy drugs (Choi 2016). Thus, improving the Nfe2l1-dependent cellular basal protective response of normal kidney cells may be an effective method to coping with cisplatin-induced acute kidney injury (AKI). However, the role of Nfe2l1 in this process remains unclear.

Silibinin is a traditional hepatoprotective flavonoid derived from the seeds of milk thistle *Silybum marianum*, and it exerts protective effects through its ant-fibrotic, anti-inflammatory and antioxidant properties (Jahanafrooz et al. 2018). Of note, the antioxidative mechanism of silibinin has rarely been elucidated during cisplatin-induced AKI, except in a recent study on mice, which demonstrated that silibinin can defend against this injury by improving mitochondrial functioning through activation of sirtuin 3 (SIRT3) (Li et al. 2017). Based on the established functions of silibinin and Nfe2l1/2, we investigated the effects of silibinin on Nfe2l1- and Nfe2l2- mediated antioxidative responses during cisplatin-induced AKI.

Materials and methods

Chemicals and antibodies

Cisplatin injection was purchased from Haosoh Pharma (Jiangsu, China). Silibinin and N-acetylcysteine (NAC) were purchased from MedChemExpress (Shanghai, China). Dimethyl sulfoxide (DMSO) was obtained from Solarbio Life Sciences (Beijing, China). The specific antibody targeting Nfe2l1 was kindly gifted from Prof. Yiguo Zhang (Chongqing University, China). The primary antibodies of Nfe2l2 (ab62352), HO1 (ab68477), and p97 (ab109240) were obtained from Abcam (Shanghai, China). Other primary antibodies against PARP (#9542), γH_2AX (#9718), H_2AX (#7631), p-JNK (Thr183/Tyr185) (#4668), JNK (#9252), p-ERK (#4370), ERK (#9102), p-p38 (#9211), p38 (#9212), and Sirt3 (#5490) were purchased from Cell Signaling Technology (Shanghai, China). In addition, the primary antibody of DDI-1 (13968-1-AP), β-actin (TA-09), and all the corresponding secondary antibodies (IRDye 800CW Goat anti-Mouse or Rabbit, #926-32210 or 926-32211) were acquired from Proteintech (Wuhan, China), ZSGB-BIO (Beijing, China), and LI-COR Biosciences (Lincoln, NE, USA), respectively.

Cell culture

Human embryonic kidney HEK293 cells were obtained from ATCC (Zhong Yuan Ltd., Beijing, China) and were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco,

USA) containing 10% fetal bovine serum (Biological Industries, Israel), penicillin (100 units/ml) and streptomycin (100 units/ml) in a humidified atmosphere of 5% CO_2 and 95% ambient air at 37 °C.

Drug treatment

Silibinin was dissolved in DMSO at 200 mM, which was hold as stock solution at -80 °C. When the confluence of HEK293 cells reached to 80% in six-well plates, different concentrations of cisplatin injection solution (0, 2.5, 5, and 10 μg/mL), silibinin (0, 12.5, 25, 50, and 100 μM), or a conbination of cisplatin injection (5 μg/mL) and silibinin (100 μM) were employed for indicated time treatmint (12 h or 24 h). During these settings, normal saline (NS) and DMSO were used as vehicle control of cisplatin injection and silibinin, respectively.

ROS detection

The intracellular ROS of HEK293 cells were detected using Reactive Oxygen Species Assay Kit (Beyotime Biotechnology, Shanghai, China). In brief, HEK293 cells (2×10^5) were seeded into six-well plates, and treated with indicated chemicals when the confluence reached to 80%. Subsequently, these cells were cultured with DCFH-DA probe for 1 h, and then, the signals of ROS were obtained using fluorescence microscope (IX-73P2F, Olympus) after washing with serum-free culture medium. The intensity of ROS was measured by ImageJ software.

Small interference RNA (siRNA) or plasmid transfection

The cellular protein level of Nfe2l1 was intervene with specific targeted siRNA or plasmid (Zhang et al. 2014), which was gifted from Prof. Yiguo Zhang (Chongqing University, China), for knockdown and overexpression using Lipofectamine 3000 reagent (Invitrogen, USA) according to the manufacturer's instructions. Once the transfection was finished, these experimental cells were stimulated with cisplatin and/or silibinin for indicated time.

Quantitative real-time PCR analysis

Experimental cells were lysed with lysis buffer from the RNAsimple Total RNA Kit (Tiangen Biotech CO., LTD, Beijin, China) and total RNAs were isolated according to the instructions. Then, 500 ng samples were used for reverse transcription by using the PrimeScript RT Master Mix (Perfect Real Time) Kit (Taraka, Beijing, China). Subsequently, specific primers against Nfe2l1 (F: GAGCTTCCCTGCACA GTTTCCA, R: GACATGAGATCTTGCCACTGCTG), β-actin (F: CATGTACGTTGCTATCCAGGC, R: CTCCTT AATGTCACGCACGAT) were employed to detect their content according to the instructions of TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) Kit (Taraka, Beijing, China).

Western blotting

Experimental cells or tissue samples were lysed with RIPA buffer (Beyotime Biotechnology, Shanghai, China). Whereafter, the concentration of each sample was calculated with BCA kit (Beyotime Biotechnology, Shanghai, China). Equal protein of each sample was loaded and separated by 10% SDS-PAGE, and then electrolytically transferred to nitrocellulose (NC) membranes, followed by blocking nonspecific binding sites with 5% non-fat milk. Subsequently, specific primary antibodies were hybridized with NC membranes over night at 4℃. Then the membrane was incubated with secondary antibodies labeled with IRDye700 (Rockland Immunochemicals, Gilbertsville, PA, USA), and visualized by the Odyssey system (LiCor, Lincoln, NE, USA).

Animal experiments

The mice experimental procedures were conducted strictly in accordance with the operating procedures of the Specific Pathogen Free (SPF) in Experimental Animal Center of Southwest Medical University. Briefly, 6–8 weeks-old C57BL/6J male mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China) and housed in SPF grade room with free access to water and standard chow. All the mice were randomly divided into four groups: vehicle group (n = 4), silibinin group (n = 4), cisplatin group (n = 5), and silibinin plus cisplatin group (n = 4). The mice were intragastrically administered with 500 mg/kg silibinin per day for 14 days before intraperitoneal injection with cisplatin (20 mg/kg) to establish AKI model. Three days later, these mice were anesthetized with pentobarbital (50 mg/kg) to collect blood samples, whereafter sacrificed for obtaining kidney samples. All the samples were prepared for blood urea nitrogen (BUN) assay, histologic staing, and western blot analysis. Among them, BUN assay was performed according to the instructions of Urea Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Hematoxylin–eosin (HE) stain

The HE stain of mice kidney tissue slides were performed as previously discribed (Chen 2020). Briefly, mice kidney tissues from different groups were fixed with 4% paraformaldehyde and embedded within paraffin. Then, these paraffins were sectioned to slices and transfered to slides, followed by dewaxing, washing, and rehydration, respectively. Finally,

the prepared slides were stained with hematoxylin and eosin solution (Beyotime Biotechnology, Shanghai, China), and visualized by microscopy.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8. Results were presented as mean ± SD Comparisons between two groups or multiple groups of means were performed with *t* -tests or one-way analysis of variance (ANOVAs), respectively. $P < 0.05$ was considered statistically significant.

Results

Apoptosis in HEK293 cells elicited by Cisplatin induced DNA damage

To confirm the role of cisplatin-induced cytotoxicity in HEK293 cells, different concentrations of cisplatin were employed and the protein levels of cleaved PARP (c-PARP), a marker of apoptosis, and γH_2AX, a marker of DNA damage, were evaluated by western blotting. The results showed that the abundance of c-PARP and γH_2AX gradually increased with cisplatin treatment in a dose-dependent manner (Fig. 1a–c). Similar results were observed in the treatment with cisplatin for 12 and 24 h (Fig. 1d–f), especially regarding γH_2AX, which showed a significant difference after cisplatin treatment for 12 h (Fig. 1f). These results implied that cisplatin can induce DNA damage-triggered apoptosis in HEK293 cells within a short period of time.

Silibinin prevents cisplatin-induced DNA damage and apoptosis

To investigate the effect of silibinin on cisplatin-induced DNA damage and apoptosis in HEK293 cells, we first examined changes in c-PARP and γH_2AX protein levels after treatment with silibinin at different concentrations. As shown in Fig. 2a, abundances of c-PARP and γH_2AX showed no marked changes. Interestingly, the concentrations of these proteins gradually decreased with increasing silibinin concentration in cisplatin-treated HEK293 cells (Fig. 2b). To further confirm the protective effects of silibinin on cells, HEK293 cells were pre-treated with 100 μM silibinin for 12 h before cisplatin treatment and were examined using

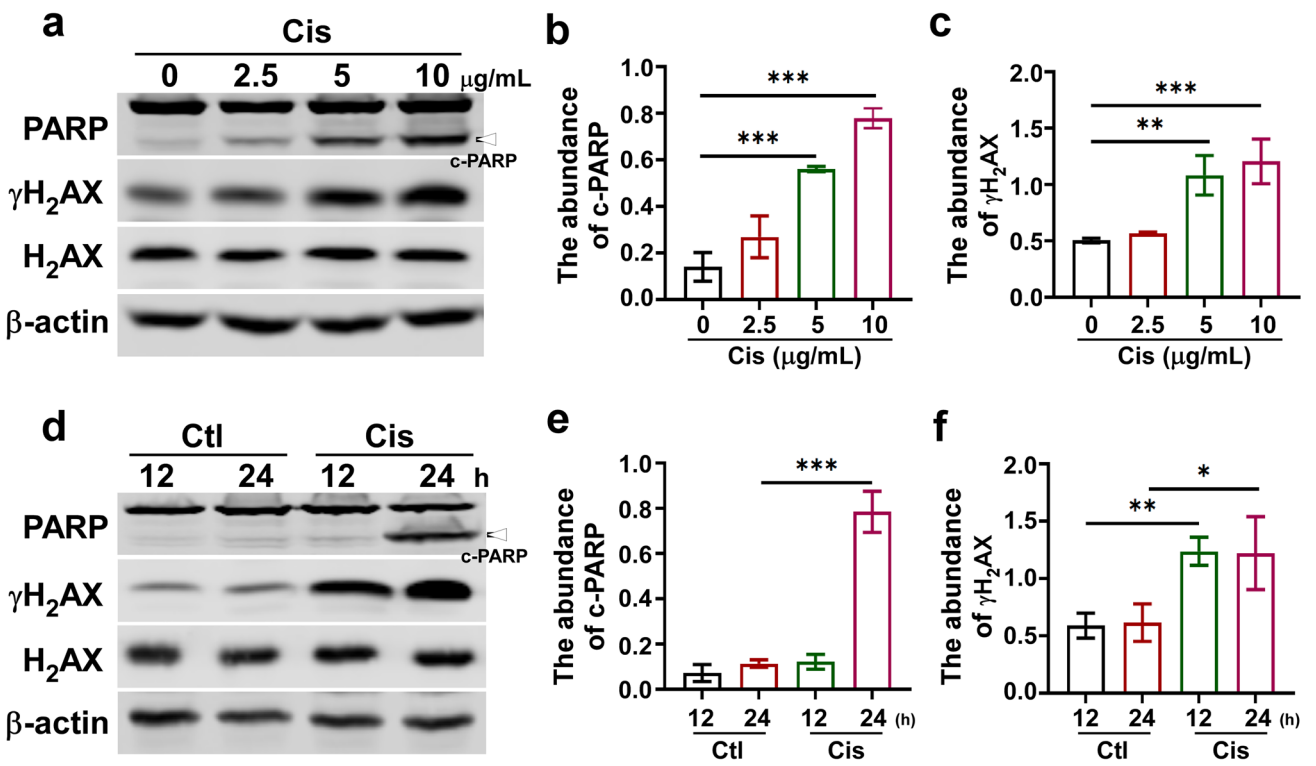

Fig. 1 The effect of cisplatin on DNA damage and apoptosis of HEK293 cells. **a**–**c** HEK293 cells were treated with different concentrations of cisplatin (Cis) for 24 h, and the protein levels of PARP and γH_2AX were detected by western blot. **d**–**f** Cis (5 μg/mL) and its vehicle control (normal saline, Ctl) were employed to treat HEK293 cells for indicated time periods, and the protein levels of PARP and γH_2AX were detected by western blot. β-actin was used as a loading control. The arrows indicated cleaved PARP (c-PARP). The intensity of target bands were calculated with ImageJ. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

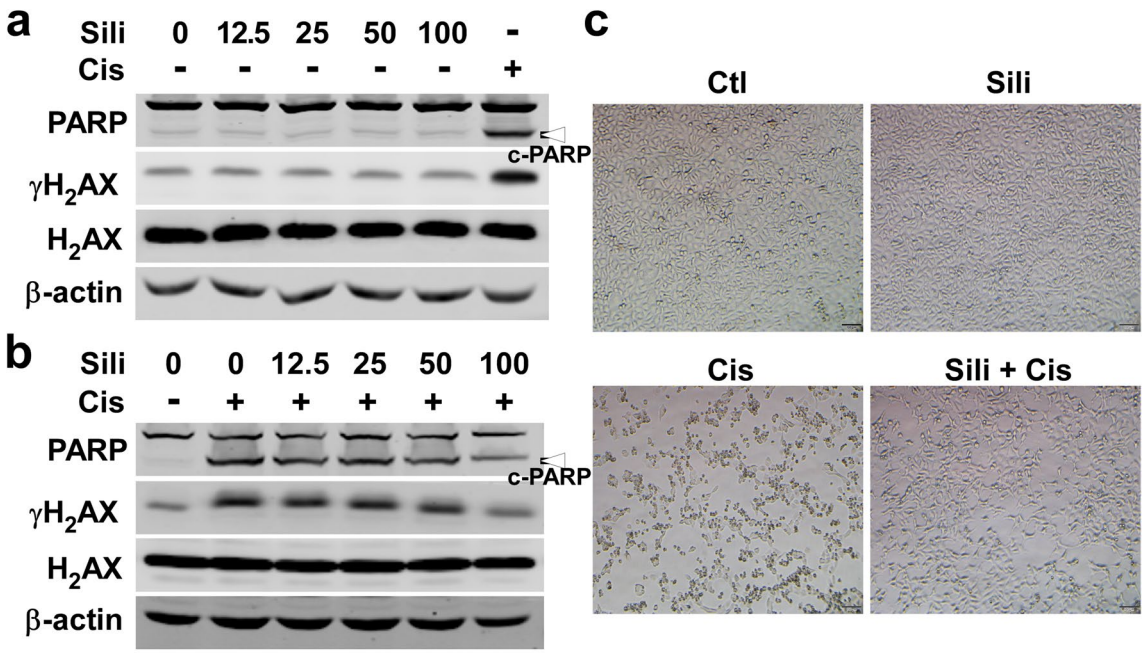

Fig. 2 The effect of silibinin on HEK293 cells apoptosis. **a**, **b** HEK293 cells were treated with different concentrations of silibinin (Sili) alone (**a**) or pre-treated 12 h followed by adding 5 μg/mL cisplatin (**b**) for 24 h, and then the protein levels of PARP and γH_2AX were evaluated by western blot. (**c**) The morphological changes of HEK293 cells were observed by microscope after experimental cells were pre-treated with DMSO (Ctl) or Sili (100 μM) for 12 h befor treating cells with Cis (5 μg/mL) or its vehicle control normal saline for 24 h. The arrows indicated cleaved PARP (c-PARP). β-actin was used as a loading control

light microscope. Silibinin did not affect the morphology of HEK293 cells and alleviated cisplatin-induced cell death (Fig. 2c), which was consistent with the changes in c-PARP and γH_2AX. Taken together, these results demonstrated that silibinin can exert cytoprotective effects against cisplatin damage by attenuating cisplatin-induced apoptosis.

Silibinin prevents cisplatin-induced apoptosis through inhibiting the ROS-mediated MAPK signaling pathway

Multiple signaling pathways are activated during cisplatin-induced cell apoptosis and death, especially the mitogen-activated protein kinase (MAPK) pathway, including JNK, p38, and ERK. Thus, we detected the abundance of these MAPK pathway members in cisplatin-treated HEK293 cells and found that phosphorylation of three members of MAPK (i.e. JNK, ERK, and p38) were markedly increased after treatment with cisplatin for 12 and 24 h (Fig. S1). Intriguingly, the activation of these phosphorylated proteins was significantly suppressed by combined treatment with silibinin and cisplatin, compared to treatment with cisplatin only (Fig. 3a, b). Our previous work and other studies indicated that ROS are vital mediators in the process of cell apoptosis and MAPK siganling activation (Yu et al. 2018; Checa and Aran 2020). Therefore, to confirm whether the inhibition of cisplatin-induced MAPK activation contribute to the elimination of ROS by silibinin, we detected the intracellular ROS levels using a DCFH-DA probe and observed that ROS levels of HEK293 cells were notably elevated after stimulation with cisplatin, whereas in the presence of silibinin, this increase was not observed (Fig. 3c, d). This result was consistent with the effect of N-acetylcysteine (NAC), an ROS scavenging agent, on cisplatin-induced MAPK signaling pathway activation (Fig. S2a). In addition, the increase in DNA damage and apoptosis markers induced by cisplatin was also attenuated with the removal of ROS (Fig. S2b). These results suggested that silibinin can relieve cisplatin-induced apoptosis via decreasing ROS-mediated MAPK signaling pathway.

Silibinin antagonizes the inhibitory effect of cisplatin on Nfe2l1, rather than Nfe2l2, mediated HO1 expression to suppress the ROS-trigered MAPK siganling pathway in HEK293 cells

Nfe2l1 and Nfe2l2 are the major transcription factors that mediate cellular antioxidant responses in eukaryotes. To confirm the role of these two antioxidant transcription factors in cisplatin-treated HEK293 cells, we first measured changes in Nfe2l1 and Nfe2l2 protein levels after treatment with different concentrations of cisplatin. Western blotting

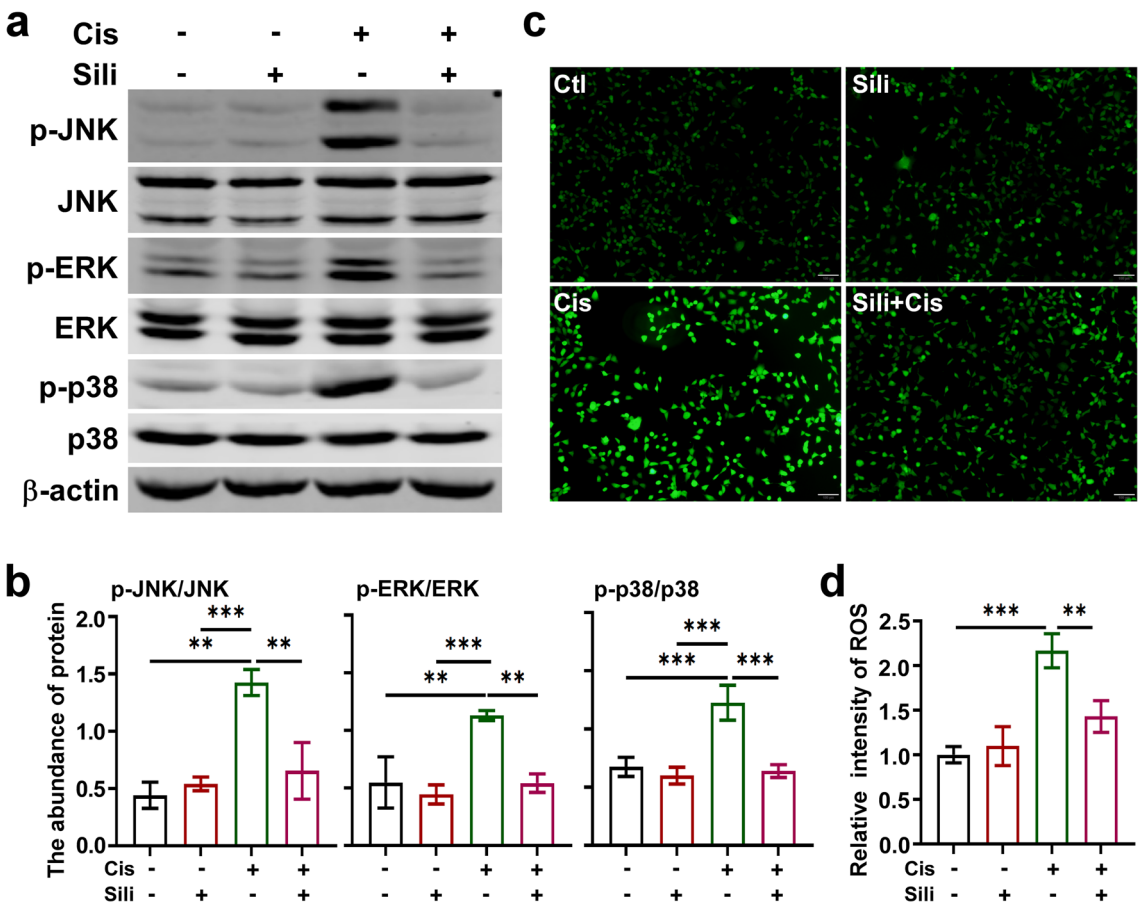

Fig. 3 The role of silibinin in cisplatin-induced MAPK signaling pathway in HEK293 cells. **a**–**d** HEK293 cells were pre-treated with DMSO (Ctl) or silibinin (Sili, 100 μM) for 12 h before adding cisplatin (Cis, 5 μg/mL) or its vehicle control normal saline for 24 h incubation, and the abundance of MAPK signaling pathway members, p-JNK, p-ERK, and p-p38, were evaluated with western blot (**a**, **b**). β-actin was used as a loading control. Meanwhile, the ROS level was detected by specific probe, and imaged with fluorescence microscope (**c**, **d**). $^{**}P<0.01$, $^{***}P<0.001$

demonstrated that Nfe2l2 was significantly suppressed in the treatment with cisplatin at 10 μg/mL for 24 h, and the effect on Nfe2l1 was observed at 2.5 μg/mL cisplatin (Fig. S3a-c), implying that Nfe2l1 was more sensitive to cisplatin than Nfe2l2. Moreover, the downstream antioxidant enzyme HO1 also gradually decreased with increasing cisplatin concentrations, which was pronounced at 2.5 μg/mL cisplatin (Fig. S3a, b), indicating that cisplatin increased cellular ROS levels by inhibiting the Nfe2l1/HO1 axis. To further determine which transcription factor is regulated by silibinin in the process of mitigating cisplatin-induced ROS, Nfe2l1 and Nfe2l2 concentrations were examined after pre-treatment with silibinin only or in combination with cisplatin. Silibinin elevated the basic level of Nfe2l1, but not that of Nfe2l2, which was, however, not significant, and it markedly suppressed cisplatin-induced Nfe2l1 reduction (Fig. 4a, b). Furthermore, silencing Nfe2l1 with specific target siRNA displayed that downregulation of the Nfe2l1/HO1 axis augmented the phosphorylation of ERK, p38, JNK, and c-PARP (Fig. 4c–h). In contrast, overexpression of Nfe2l1 decreased, to some extent, the phosphorylation of ERK, p38, JNK, and c-PARP (Fig. 4k–n). These results indicated that silibinin exerted cytoprotective effects against cisplatin by upregulating the Nfe2l1/HO1 axis-mediated antioxidant responses to clear intracellular ROS.

Silibinin ameliorates cisplatin-induced AKI via Nfe2l1 mediated antioxidant responses in vivo

To explore whether silibinin also protects the kidney and whether silibinin triggers the Nfe2l1/HO1 axis-mediated antioxidant response in cisplatin-induced AKI in vivo, C57BL/6J male mice were subjected to intragastric silibinin administrated (500 mg/kg) for 14 days before intraperitoneal injection with 20 mg/kg cisplatin to induce AKI. Compared with the vehicle group, the silibinin group showed normal kidney tubules as assessed after HE staining. However, the cisplatin group exhibited severe kidney abnormalities

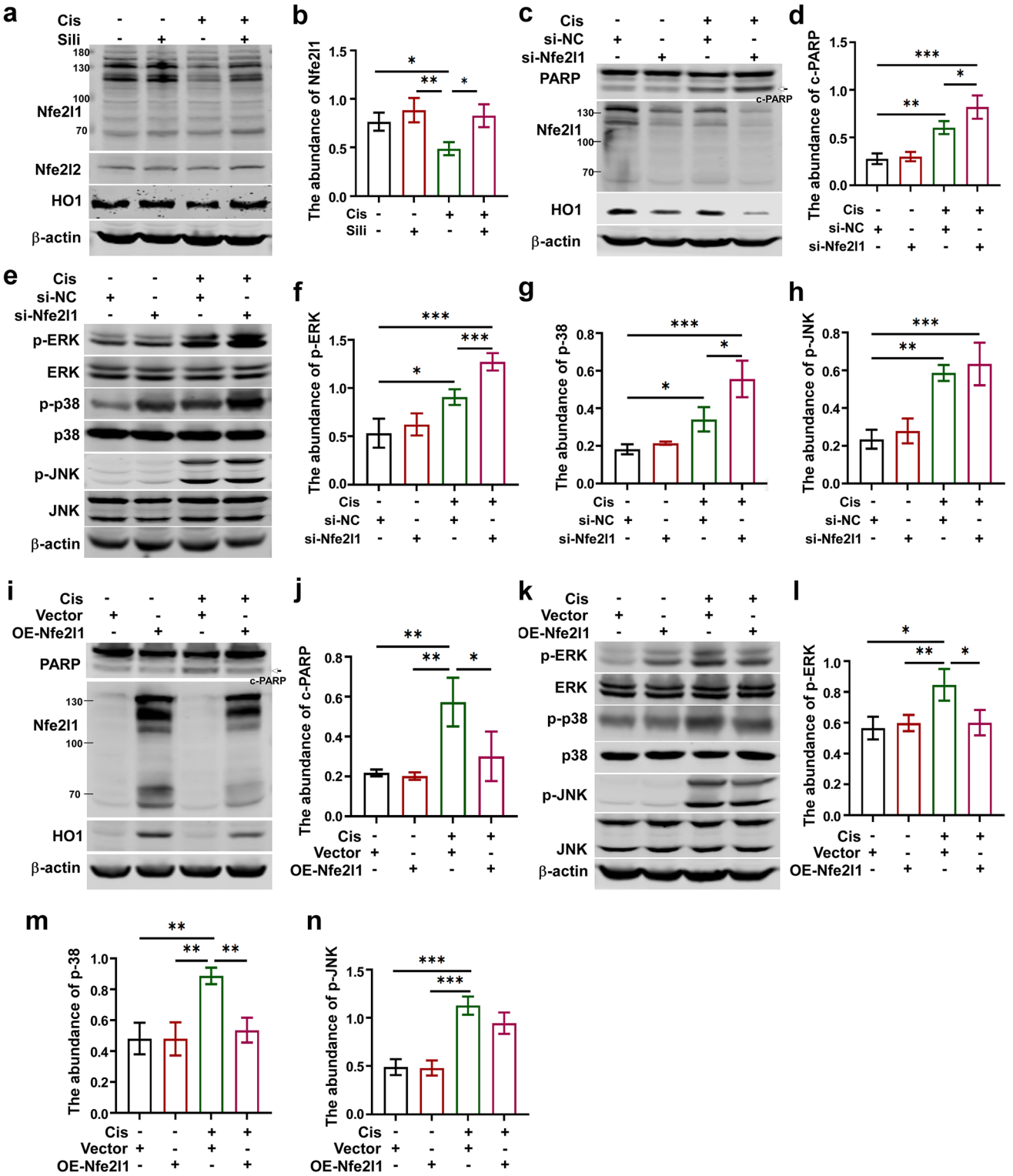

Fig. 4 The role of Nfe2l1 in cisplatin-induced MAPK signaling pathway activation and apoptosis. **a**, **b** HEK293 cells were pre-treated with DMSO or silibinin (Sili, 100 μM) for 12 h before adding cisplatin (Cis, 5 μg/mL) for 24 h incubation, the protein levels of Nfe2l1, Nfe2l2, and HO1 were detected with western blot. **c**–**n** HEK293 cells were transfected with siRNA (si-Nfe2l1) or overexpression plasmid (OE-Nfe2l1) to interfere the expression of Nfe2l1 before subjected to indicated treatment for 24 h, then the abundance of c-PARP, HO1, p-ERK, p-p38, and p-JNK were measured by western blot. The arrows indicated cleaved PARP (c-PARP). β-actin was used as a loading control. *P < 0.05, **P < 0.01, ***P < 0.001

regarding histology, especially necrosis of the renal tubular epithelial cells (Fig. S4a). Notably, cisplatin-induced injury was attenuated by silibinin treatment, accompanied by a significant decrease in blood urea nitrogen (Fig. S4a, b). These observations indicated that silibinin improved kidney functioning during cisplatin-induced AKI. The tissue samples were further analyzed by western blotting which showed that the abundance of Nfe2l1 (especially in isoforms at 105 kDa, 85 kDa, and 65 kDa), and HO1 was markedly lower in the cisplatin group than in vehicle group, whereas Nfe2l2 demonstrated the opposite results (Fig. 5a, b). Consistent with the results in HEK293 cells, silibinin restored the effect of cisplatin on Nfe2l1/HO1, accompanied by the restoration of Nfe2l2 to normal levels (Fig. 5c, d). Importantly, the augment of phosphorylation of p38 and JNK, rather than ERK, was also suppressed by silibinin in the cisplatin-induced

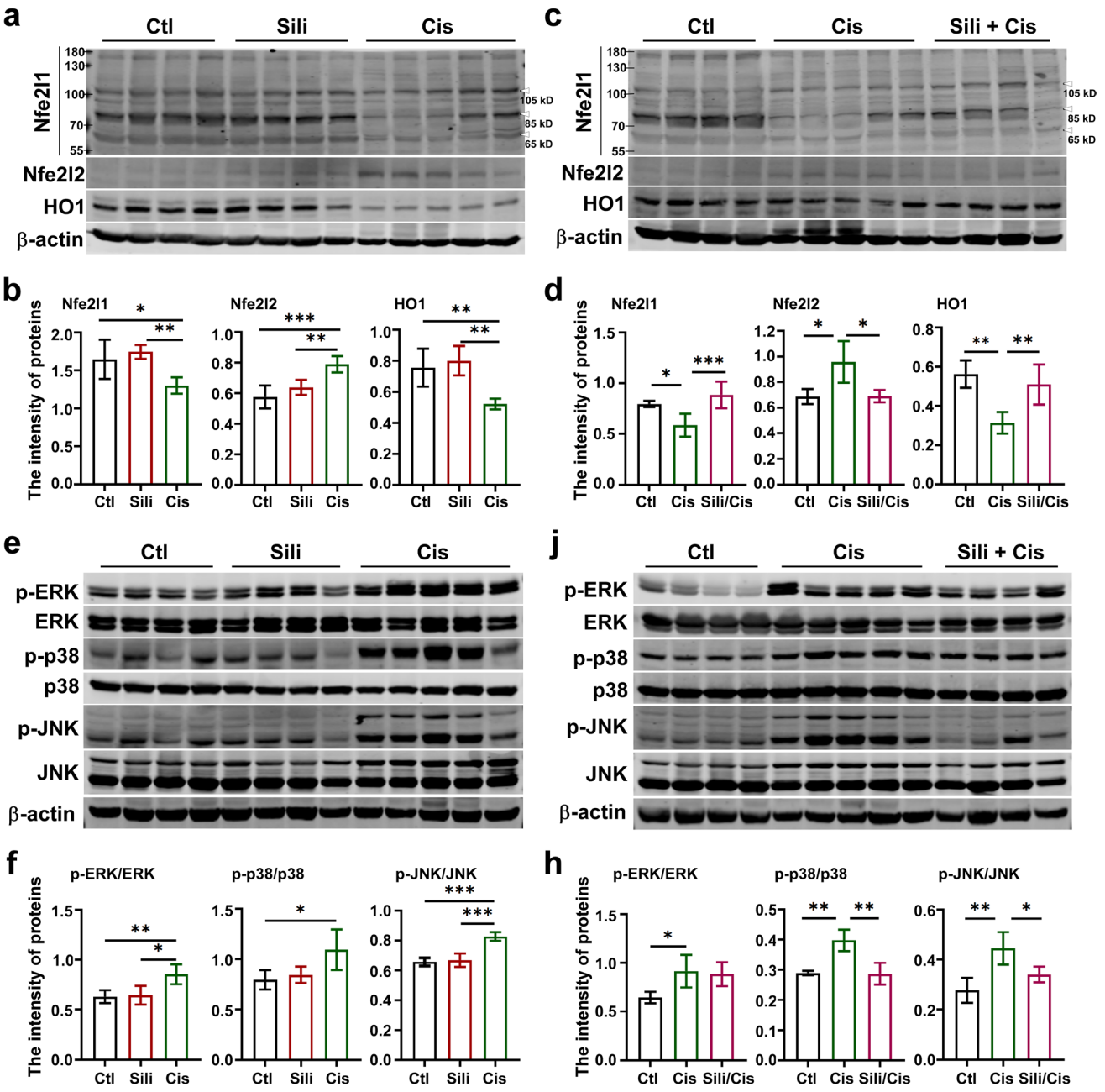

Fig. 5 The effect of silibinin on Nfe2l1/HO1 mediated MAPK signaling pathway in cisplain-induced AKI model. **a**–**h** The kindey tissues of experiment mice were collected and subjected to analyze the expression of Nfe2l1, Nfe2l2, HO1, p-ERK, p-p38, and p-JNK by using western blot. β-actin was used as a loading control. Ctl, Sili, and Cis are the abbreviation of vehicle control (normal saline), silibinin, and cisplatin theatment, respectively. *P < 0.05, **P < 0.01, ***P < 0.001

AKI model (Fig. 5e–h). Taken together, these results suggest that silibinin ameliorates cisplatin-induced AKI via the Nfe2l1/HO1 axis-mediated antioxidant response, thereby suppressing the activation of the MAPK signaling pathway in vivo.

Silibinin can rescue cisplatin-induced Nfe2l1 inhibition by regulating its transcription and post-translational modification

As described above, silibinin ameliorated cisplatin-induced kidney cell apoptosis by inhibiting the Nfe2l1/HO1 axis in vivo and in vitro, and almost all isoforms of Nfe2l1 were decreased following treatment with cisplatin (Figs. 4a, 5a). To reveal the underlying mechanism, we first recorded two key post-translational modification proteins involved in the process of Nfe2l1 protein maturity. The protein levels of p97, an AAA-ATPase that drives Nfe2l1 retro-translocation from the endoplasmic reticulum (ER) lumen into the cytoplasm, were decreased by cisplatin treatment and were significantly restored by silibinin treatment (Fig. 6a, b). However, protease DDI-1, which contributes to the release of Nfe2l1 from the ER membrane did not display marked changes when treated with cisplatin or silibinin (Fig. 6a, c). We further evaluated the mRNA level of Nfe2l1 in the same settings and found that silibinin also elevated Nfe2l1 mRNA levels and reduced cisplatin-induced inhibition (Fig. 6d). These results indicated that silibinin rescued cisplatin-induced Nfe2l1 inhibition by regulating its transcription and post-translational modification.

Discussion

Cisplatin-induced AKI is the most common side effect that limits the clinical application of cisplatin in solid tumor treatment. Although many respective mechanisms have been revealed, they are still not comprehensively understood. In the present study, we explored the effects of silibinin on cisplatin-induced nephrotoxicity and found that silibinin exerted cytoprotective effects against cisplatin treatment in HEK293 cells and in cisplatin-induced AKI model. We demonstrated, for the first time, that cisplatin can downregulate the protein concentrations of the antioxidant transcription factor Nfe2l1 to decrease the expression of the antioxidant enzyme HO1, which increases ROS-induced oxidative stress, thereby triggering DNA damage and inducing apoptosis via the MAPK signaling pathway in kidney cells. Moreover, cisplatin-induced Nfe2l1 reduction, and its downstream cascade reactions were mitigated by silibinin, in vitro and in vivo.

Cisplatin-induced oxidative stress is a vital factor that exerts cytotoxicity mainly mediated by ROS. Accumulating evidence suggests that ROS is an important physiological

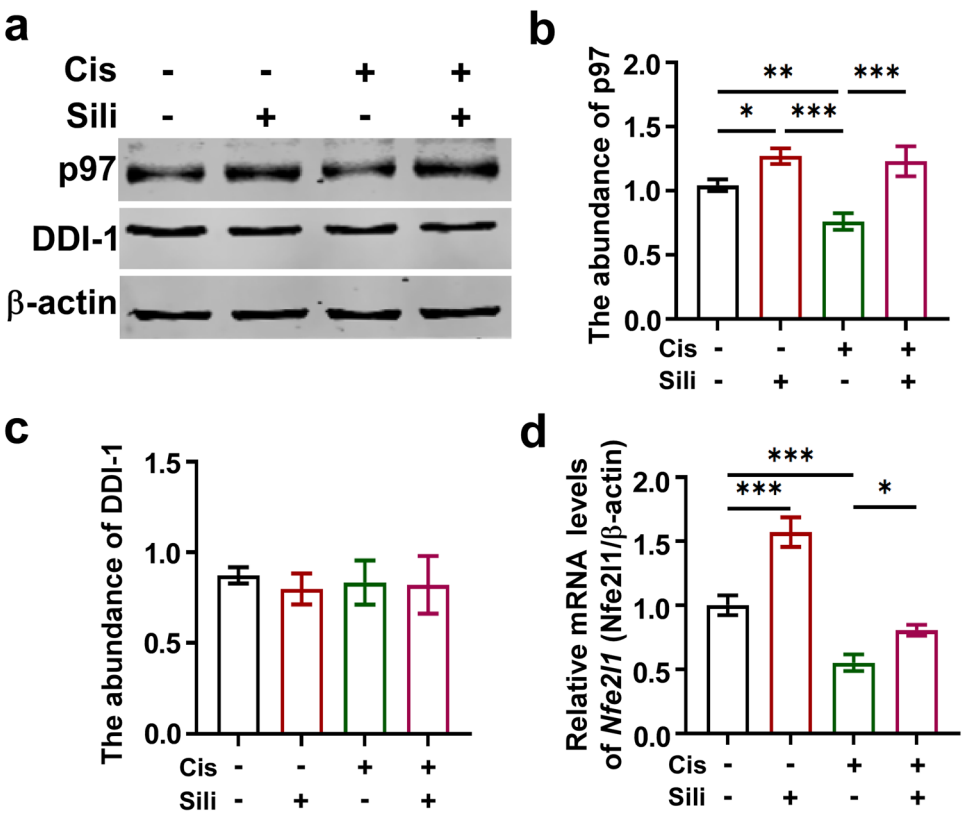

Fig. 6 Silibinin rescued cisplatin-induced Nfe2l1 inhibition by regulating its transcription and posttranslational modification. **a**–**d** The protien levels of p97 and DDI-1 (**a**–**c**) and mRNA levels of Nfe2l1 (**d**) in HEK293 cells were analyzed after pre-treated with DMSO or silibinin (Sili, 100 μM) for 12 h before adding cisplatin (Cis, 5 μg/mL) or its vehicle control normal saline for 24 h incubation. β-actin was used as a loading control. *P < 0.05, **P < 0.01, ***P < 0.001

modulator that regulates several intracellular signaling pathways, particularly the MAPK pathway (Checa and Aran 2020). Following continuous activation of this signaling pathway by ROS overproduction, cell death is elicited by multiple negative effects including cell cycle arrest, senescence, and apoptosis (Yue and Lopez 2020). In the present study, we found that the ROS-mediated MAPK signaling pathway was significantly activated in HEK293 cells, resulting in DNA damage-triggered apoptosis. This process was significantly mitigated by ROS scavenger NAC. These results confirmed that ROS plays a crucial role in cisplatin-induced kidney injury.

The mechanisms of cisplatin-induced ROS generation are mainly involved in three aspects after cisplatin is passively transferred into renal tubular cells via channel proteins, including organic cation transporter 2, solute carrier family 22 member 2, and copper ion transporter 1 (Fang et al. 2021). First, once it enters into the cells, cisplatin undergoes hydrolysis and forms a positively charged electrophile complex, thereby binding to DNA, which results in DNA cross-links and prevents its replication in dividing cells (Fujikawa et al. 2014). As proximal tubular cells cannot divide (Tanase et al. 2019), cisplatin mainly interferes with mitochondrial DNA to disrupt mitochondrial function and generate ROS in the target cells (Fang et al. 2021). Second, with the accumulation of cisplatin in the cytoplasm, several enzymatic sources of ROS production, including NADPH oxidase family members, are upregulated and accelerate intracellular ROS production (Cao et al. 2018; Zhang et al. 2021). Third, intracellular cisplatin can disrupt the redox balance system by directly binding to endogenous antioxidant enzymes, such as glutathione (Karasawa and Steyger 2015) and CYP2E1 (Zhang et al. 2021), to decrease their activity or indirectly decrease the expression of antioxidant enzymes such as superoxide dismutase, glutathione, and catalase by transcriptional regulation (Holditch et al. 2019). In fact, the transcription factor Nfe2l2, a master molecular switch regulating intracellular adaptive redox balance, has been shown to play protective role during cisplatin-induced kidney cell damage (Mirzaei et al. 2021), which was also observed in the current study. Surprisingly, however, the decrease in Nfe2l1 was more sensitive to cisplatin than that of Nfe2l2, implying that Nfe2l1 is the primary factor in maintaining the redox system balance for coping with cisplatin-induced oxidative stress.

Considering the importance of ROS in determining the fate of kidney cells, numerous natural antioxidants have been explored to reduce cisplatin-induced oxidative stress (Fang et al. 2021). Silibinin, a flavonoid compound derived from the seeds of milk thistle *Silybum marianum*, can exert a hepatoprotective role by activating Nfe2l2 related signaling pathways (Liu et al. 2019). However, the abundance of Nfe2l2 did not change markedly in HEK293 cells after silibinin treatment in the present study. By contrast, we found that silibinin specifically activated the expression of Nfe2l1-mediated antioxidant reactions, implying that silibinin may be an effective compound to elevate the antioxidant capacity of cells to defend against cisplatin-induced kidney injury. Interestingly, this protective role was observed in a cisplatin-induced AKI model, which is consistent with the observations from Li et al. (Li et al. 2017). In addition, the mechanism by which silibinin alleviated the downregulation of sirt3 in vivo as revealed by Li et al. (Li et al. 2017) was also reproduced in the current study (Fig. S4c–f), together with changes in BUN and histological changes, which indicated that the established cisplatin-induced AKI model of present study was more reliable, although only few mice were used. However, no change in sirt3 expression was observed in HEK293 cells (Fig. S4g, h), suggesting that sirt3 is not a general target protein of silibinin. Therefore, the role of sirt3 in cisplatin-induced AKI in human cells needs to be further elucidated in future studies. Of note, compared to HEK293 cells, a consistent result of Nfe2l1 in protein level was showed in cisplatin-induced AKI model, accompanied by the same changes in HO1, whereas Nfe2l2 demonstrated an opposite trend compared to that of Nfe2l1. The discrepancy in Nfe2l2 protein levels between animal tissue and human cells may be a result of the different doses of cisplatin in these two cell types (i.e. kidney tissue cells in vivo were subjected to a lower concentration of cisplatin than cells in vitro) because a low concentration of cisplatin can act as a stimulus to activate the intracellular adaptive antioxidant response. This hypothesis is partly supported by our observation that the average protein level of Nfe2l2 was increased in the presence of cisplatin at a concentration of 0.5 μg/mL, compared to that in the control group (Fig. S3c). Importantly, even though a relatively low concentration of cisplatin occurred in kidney tissue cells in this setting, the concentration of Nfe2l1 was decreased and recovered by silibinin, which strongly indicated that Nfe2l1 is a potential target for improving cisplatin-induced AKI.

It is should be noted that Nfe2l1 is an ER membrane-anchored protein and progresses to mature isoforms through multiple modifications with the aid of p97 and DDI-1 (Xiang et al. 2018a, b). When the post-translational modification process is disrupted, Nfe2l1 can be degraded into different isoforms through proteasomes and calpains (Zhang et al. 2015; Yang 2020). Therefore, we examed abundances of these proteins and found that p97 was marked affected by cisplatin treatment. This observation is also supported by the work of Karasawa et al. who reported that cisplatin can directly bind to p97 and calreticulin (Karasawa et al. 2013). Interestingly, the mRNA level of Nfe2l1 was also decreased in the presence of cisplatin and was rescued by silibinin. These results suggest that silibinin relieves cisplatin-induced Nfe2l1 down regulation via transcriptional and

post-translational regulation, although the detailed mechanism warrants further research.

In addition, since Nfe2l1 is mainly responsible for maintaining the basal steady state of intracellular oxidative reactions and silibinin has been used for liver protection during clinical treatments, as a priority target of cisplatin, activating the function of Nfe2l1 to elevate intracellular antioxidant ability is an attractive way to prevent cisplatin-induced AKI.

Conclusion

In summary, we revealed a novel mechanism by which silibinin ameliorates cisplatin-induced nephrotoxicity by activating Nfe2l1-mediated antioxidant response to reduce ROS-raised MAPK signaling pathway activation, which provides a new clue for AKI protection of patients receiving cisplatin-based cancer treatment.

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Declarations

Conflict of interest The authors have no potential conflicts of interest to disclose.

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