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Cinnabar protects serum-nutrient starvation induced apoptosis by improving intracellular oxidative stress and inhibiting the expression of CHOP and PERK

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ABSTRACT

Cinnabar has been used for treatment of various disorders for thousands of years. The medical use of cinnabar, however, has been controversial because of its heavy metal mercury content. A large quantity of studies indicate that the toxicity of cinnabar is far below other inorganic or organic mercury-containing compounds. Yet, the underlying molecular basis has remained unresolved. Here, we investigated the beneficial effects of cinnabar on serum-nutrient starvation-elicited cell injury. Our findings showed that treatment of human renal proximal tubular cells (HK-2) with 4 nM cinnabar effectively inhibited nutrient deprivation induced apoptosis, reduced intracellular reactive oxygen species generation and increased GSH content, which was contrary to the exacerbated apoptotic cell death and oxidative stress in cells treated with $HgCl_2$ at equal mercury concentration. In addition, cinnabar exerted robust antioxidative and antiapoptotic effects in cells under dual challenges of nutrient deprivation and treatment of H_2O_2. The protein expression levels of both CHOP and PERK were remarkably down-regulated in the cells treated with cinnabar compared to the control cells or cells treated with $HgCl_2$. Overall, our data indicates that cinnabar at low concentration exerts anti-oxidative stress and anti-apoptosis effects by inhibiting the expression of the endoplasmic reticulum stress pathway proteins CHOP and PERK.

1. Introduction

Cinnabar has been widely used as an important ingredient in traditional Chinese medicines (TCM) such as Angong Niuhuang Wan (AGNHW), the most representative cinnabar-containing TCM, to treat various diseases [1,2]. Because of its high mercury content, the medical utilization of cinnabar has long been questioned due to concerns of mercury toxicities. Studies have demonstrated that mercury easily accumulates in kidney, liver and brain tissues. While no obvious damage was observed in kidney and liver of rats administrated with cinnabar at 10-fold clinically equivalent dose, certain brain histopathological changes occurred [3]. In addition, animal studies have shown that long-term exposure to low dosages of cinnabar also causes a wide range of neurotoxicological effects [4,5]. Indeed, the toxicity on the central nervous system has been one of the main concerns regarding the clinical application of cinnabar-containing TCM. However, compared to other inorganic mercury and organic mercury compounds which caused damage of various organ systems, cinnabar is much less toxic [6]. Cinnabar-containing TCM differs significantly from other common mercurial compounds in mercury toxicity, indicating that total mercury content appears to be insufficient as the sole evaluation of the safety of cinnabar [7].

A large number of studies show that cinnabar or cinnabar-containing TCM is much less toxic than other mercury containing compounds. Moreover, its mercury absorption and accumulation in the kidney, the toxic target organ of inorganic mercury, is also less than other mercury containing compounds such as $HgCl_2$ and methylmercury (MeHg) [8–13]. Therefore, the evaluation of the safety of cinnabar on the basis of total mercury contents alone is unreasonable. On the other hand, despite widespread applications, the therapeutic basis of cinnabar remains unclear [7].

Cinnabar contains 96% mercury sulfide (HgS) composed of only

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inorganic mercury showing very low solubility [14,15]. In vivo and in vitro studies have demonstrated that cinnabar absorption and bioaccumulation were much lower compared with $HgCl_2$ or other mercury-containing compounds [16–18]. In addition, it has been established that mercury will first bind to thiol groups of proteins or endogenous small molecules (e.g., GSH and cysteine) due to a high affinity for them after being absorbed into blood [19], and these mercuric S-conjugates of small endogenous thiols might be a transportable form for mercury entering target cells [11,14,19,20]. However, due to the extremely high molecular weight of the protein, the part of the mercury bound to the protein has difficulty entering the cell [21]. Thus, the final concentration of mercury absorbed into target cells by oral cinnabar may be very low. Studies have shown that HgS inhibits hypoxia induced oxidative stress [22]. These triggered us to investigate the antioxidant effect of cinnabar at low nanomolar concentration and compare this with the effect of $HgCl_2$ at equal mercury concentration.

The antioxidant effects of cinnabar were evaluated in HK-2 cells induced by serum-nutrient starvation. It has been shown that nutrient deprivation causes apoptosis and induces endoplasmic-reticulum (ER) stress [23]. Accumulating evidence implicates ER stress-induced cellular dysfunction and cell death as major contributors to many diseases. Studies have shown that excessive or adverse stress to the ER induced by mercury triggers apoptosis and ultimately leads to cell death [24,25]. However, the effects of cinnabar at low doses on ER stress remain less studied.

Therefore, the present study was designed to determine the antioxidant and antiapoptotic effects of cinnabar. Furthermore, we investigated the effect of cinnabar on the expression of ER stress gene stress pathway genes CHOP and PERK.

2. Materials and methods

2.1. Reagents and drugs

Mercuric chloride ($HgCl_2$) was purchased from Sigma-Aldrich, USA, and cinnabar (96% HgS) was purchased from Beijing Tong-Ren-Tang Co. (Beijing, China), respectively. Dulbecco's modified Eagle's medium (DMEM)/F12, trypsin-EDTA, fetal bovine serum (FBS) and antibiotic-antimycotic solution were purchased from GIBCO (Grand Island, NY, USA). ROS (Catalog No. S0033) and GSH detection kits (Catalog No. S0053) were purchased from Beyotime company (Jiangshu, China). LDH assay kit (Catalog No. A020-1-2) was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Apoptosis kit (Catalog No. A005-3) was purchased from Shanghai 7sea Pharmatech Co., Ltd (Shanghai, China). H_2O_2 (Catalog No. A7250) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture and preparation of cinnabar solution and $HgCl_2$

HK-2 cells were purchased from Chinese Academy of Sciences Kunming Cell Bank. Cells were cultured in (DMEM)/F12 medium supplemented with 10% fetal bovine serum, at 37 °C and 5% CO_2 in a humidified atmosphere. Cinnabar was prepared in DMSO, and then supplied with serum free medium and sonicated for 1 h. The prepared solution was subjected to centrifugation at 12,000 rpm for 10 min, and then filtered with 0.22 μm microporous filter membrane to obtain medium containing saturated cinnabar. $HgCl_2$ was prepared into 4 mM storage solution with sterile PBS, and then diluted into 4 nM with basic medium. A medium without serum served as control for serum deprivation, while 10% FBS indicated a medium containing 10% FBS.

2.3. Detection of mercury content of cinnabar

Taking 500 μL of the above cinnabar solution, 500 μL of extractant [0.12% (w/v) L-cysteine, 5% (v/v) methanol, 10 mM (w/v) ammonium acetate, 0.1% (v/v) HCl] was added to it, vortexed for 30 s, centrifugated at 12000 rpm for 10 min, and the supernatant was the saturated cinnabar solution without serum. C18 column (4.6 mm × 250 mm, 5 μm, ANPEL laboratory technologies, Shanghai, China) was used for separation. The mobile phase was 0.12% (w/v) L-cysteine, 5% (v/v) methanol, 10 mM (w/v) ammonium acetate, 0.1% (v/v) HCl, the flow rate was 1.2 ml/min, and the injection volume was 100 μL. Then mercury concentration of saturated cinnabar was evaluated by UPLC-ICP-MS (the detection limit of Hg was 0.039 ng/L).

2.4. Determination of cytotoxicity and cytosolic lactate dehydrogenase

The cytotoxic effect of the tested agents was determined by MTT assay. The cells were seeded in a 96-well plate with 0.5×10^5 cells/well and treated with $HgCl_2$ and cinnabar at low concentration (1 nM $HgCl_2$ and 1 nM cinnabar) and high concentration (4 nM $HgCl_2$ and 4 nM cinnabar), respectively, for 4 days. Upon the completion of the treatment, cells were exposed to MTT (5 mg/mL) for 4 h at 37 °C. The culture medium was then removed, and the cells were solubilized into dimethyl sulfoxide. Finally, absorbance was measured at 570 nm using a microplate reader. Cell survival rate (%) = OD treatment group/OD blank × 100%.

The release of cytosolic lactate dehydrogenase (LDH) was determined by the LDH assay. HK-2 cells (1.5×10^5 cells/well) were seeded in 6-well plates and treated with 4 nM cinnabar or $HgCl_2$ in serum-free medium. After 4 days incubation, the supernatant culture medium was collected and LDH activity was detected by an assay kit at 450 nm (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.5. Apoptosis assays

Cellular apoptosis was detected by flow cytometry. Cells were seeded in 6-well plate and then treated with 4 nM $HgCl_2$ and cinnabar with or without the present of 100 μM H_2O_2 in serum-free medium. After 4 days, cells were collected for apoptosis analysis, using Annexin V-FITC propidium iodide staining solution.

2.6. Measurement of cellular reactive oxygen species (ROS) production

Intracellular ROS production was estimated by a kit (Beyotime Institute of Biotechnology, Haimen, China). HK-2 cells (1.5×10^5 cells/well) were seeded in 6-well microplates for 24 h, and then were incubated with 4 nM $HgCl_2$ and cinnabar with or without 100 μM H_2O_2 in serum deprivation for 4 days. After treatment, the cells were washed with PBS three times, and then stained with DCFH-DA for 30 min at 37 °C in the dark. ROS generation was assessed utilizing a Gallios™ Flow Cytometer (Beckman) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

2.7. Measurement of cellular glutathione (GSH)

The intracellular content of GSH and GSSG was detected using the GSH/GSSG ratio detection assay kit from Beyotime. Upon the completion of treatment, cells were collected and processed per manufacturer's instruction. The total GSH and oxidized GSSG were measured by kinetic determination method. The reduced GSH was obtained by deducting the oxidized GSSG from the total GSH.

2.8. Measurement of real-time reverse transcription-polymerase chain reaction (RT-PCR)

Upon the completion of treatments, cells were collected and total RNA was isolated using Trizol reagent (Takara, Dalian, China). Total RNA was quantified by ND-2000 spectrophotometer. cDNA was synthesized using the PrimeScript™II first-strand cDNA synthesis kit (Takara, Dalian, China) with the temperature program: 37 °C for 15 min, 85 °C for 5 s. The sequences of the specific sets of primers were shown

below: GAPDH Fwd, 5′-CGACCACTTTGTCAAGCTCA-3′and GAPDH Rv, 5′-AGGGGTC

TACATGGCAACTG-3′; CHOP Fwd, 5′-GCGCATGAAGGAGAAA-GAAC-3′ and CHOP Rv,5′-CCAXTTGTTCATGCTTGGTG-3′; PERK Fwd, 5′-CTCACAGGCAAA

GGAAGGAG-3′ and PERK Rv, 5′-AACAACTCCAAAGCCACCAC-3'. The RT-PCR analysis was performed on Bio-Rad CFX96 Real-Time system (Bio-Rad Laboratories Inc., USA) and the conditions of RT-PCR were as follows: initial denaturating step at 95 °C for 3 min, 95 °C for 10 s, and annealing temperature for 45 s. Each sample was analyzed with three duplicates.

2.9. Western blot analysis

Upon the completion, cells were collected and lysed in RIPA lysis buffer. The whole cell lysates were centrifuged at 14,000×*g* for 15 min at 4 °C. The protein concentrations of the supernatants were detected using Pierce BCA Protein Assay Kit (Thermo Scientific). Samples containing 15 μg of total protein were separated on 10% SDS-PAGE gel, then transferred onto a polyvinylidene difluoride (PVDF) membrane (Cat# IPVH00010). The membranes were blocked with 5% non-fat milk at room temperature for 2 h and immunoblotted with indicated primary antibodies anti-β-actin (1:10,000; Proteintech), anti-CHOP (1:1000; Bioss) and anti-PERK (1:1,000, Bioss), respectively, overnight at 4 °C. Horseradish peroxidase-conjugated secondary antibodies were added and reacted with the membrane at room temperature for 1 h. The membrane-bound secondary antibody was detected with ECL Western blot detection kit.

2.10. Statistical analysis

Data are expressed as mean ± standard deviation. SPSS 24.0 software was used to analyze the data. One-way analysis of variance and *t*-test were used for analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Determination of mercury concentration in cinnabar solution

Saturated concentration of cinnabar was adopted in this study in order to clarify the pharmacological effect of cinnabar more rigorously and scientifically. UPLC-ICP-MS was employed for qualification of mercury content in saturated cinnabar solution. The mass spectrum is shown in Fig. 1, and the detection indicated a saturated concentration of 4 nM cinnabar.

3.2. Effect of cinnabar and $HgCl_2$ on the viability of HK-2 cells

To observe the effect of different concentrations of cinnabar and $HgCl_2$ on HK-2 cell survival, cells were treated with $HgCl_2$ and cinnabar at different concentrations (1 and 4 nM) for 4 days. As shown in Fig. 2, cinnabar at 1 and 4 nM did not cause significant cytotoxicity, as determined by MTT assay. While 1 nM $HgCl_2$ did not exert significant cytotoxic effect, 4 nM $HgCl_2$ induced dramatic cell loss as compared to the control. Based on these results, in the following experiments, we only used a high concentration (4 nM) to investigate the toxicity difference between cinnabar and $HgCl_2$ and related mechanism.

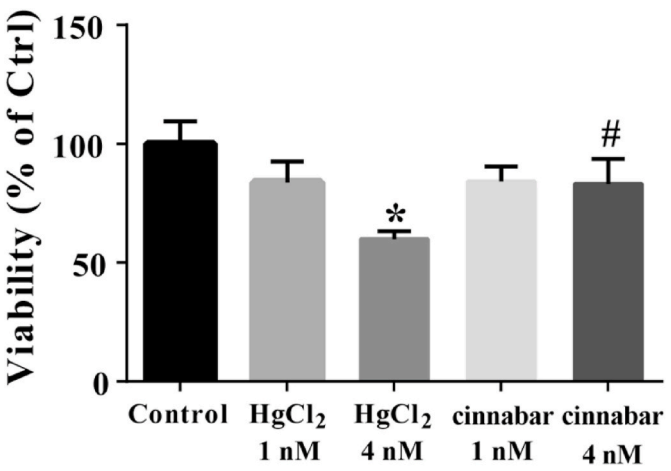

Fig. 2. Comparison of cytotoxicity of cinnabar and $HgCl_2$ on HK-2 cells. Cells were treated with $HgCl_2$ and cinnabar for 4 days at low concentration (1 nM $HgCl_2$ and 1 nM cinnabar) and high concentration (4 nM $HgCl_2$ and 4 nM cinnabar), respectively, for 4 days. Cytotoxicity was assessed by MTT assay. Values were calculated from three independent experiments and presented as mean ± SD. *, $P < 0.05$ *vs.* control cells; #, $P < 0.05$ *vs.* 4 nM $HgCl_2$ treated cells.

3.3. Differential effects of cinnabar and $HgCl_2$ on serum withdrawal induced cell injury and apoptosis

To investigate the effects of cinnabar and $HgCl_2$ on cell damage, HK-2 cells were treated with 4 nM cinnabar and $HgCl_2$, respectively, for 4 days. As illustrated in Fig. 3A, $HgCl_2$ treatment caused noticeable cytotoxicity as indicated by increased LDH leakage as compared to the control cells, whereas cinnabar exerted significant less cytotoxicity as compared to $HgCl_2$. Morphological examination also showed that suspended dead cells were more numerous in $HgCl_2$ treated cells than in cells treated with cinnabar, with the $HgCl_2$ cell bodies appearing longer and more shrunken, while in the cinnabar group, the cell morphology was closer to normal. Nutrient deprivation is well known to induce apoptosis [23]. As shown in Fig. 3B, serum withdrawal caused significant increase in cellular apoptosis relative to cells with nutrient support (10% FBS). Treatment of cells with 4 nM $HgCl_2$ further exacerbated apoptotic cell death. However, a dramatic opposite effect on nutrient deprivation-induced apoptosis was observed in HK-2 cells treated with cinnabar. At the same concentration of 4 nM, cinnabar significantly inhibited nutrient deprivation induced apoptosis (Fig. 3B).

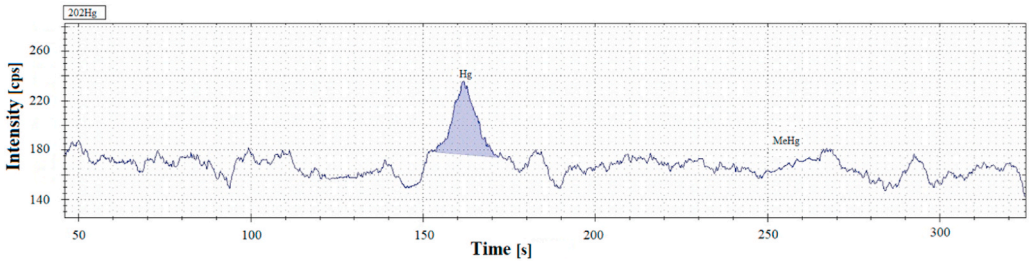

Fig. 1. Chromatogram of detection of mercury content in cell culture medium containing cinnabar. The cinnabar solution and cinnabar containing medium were prepared as described in the Materials and methods. The mercury content of cinnabar containing medium was detected by UPLC-ICP-MS.

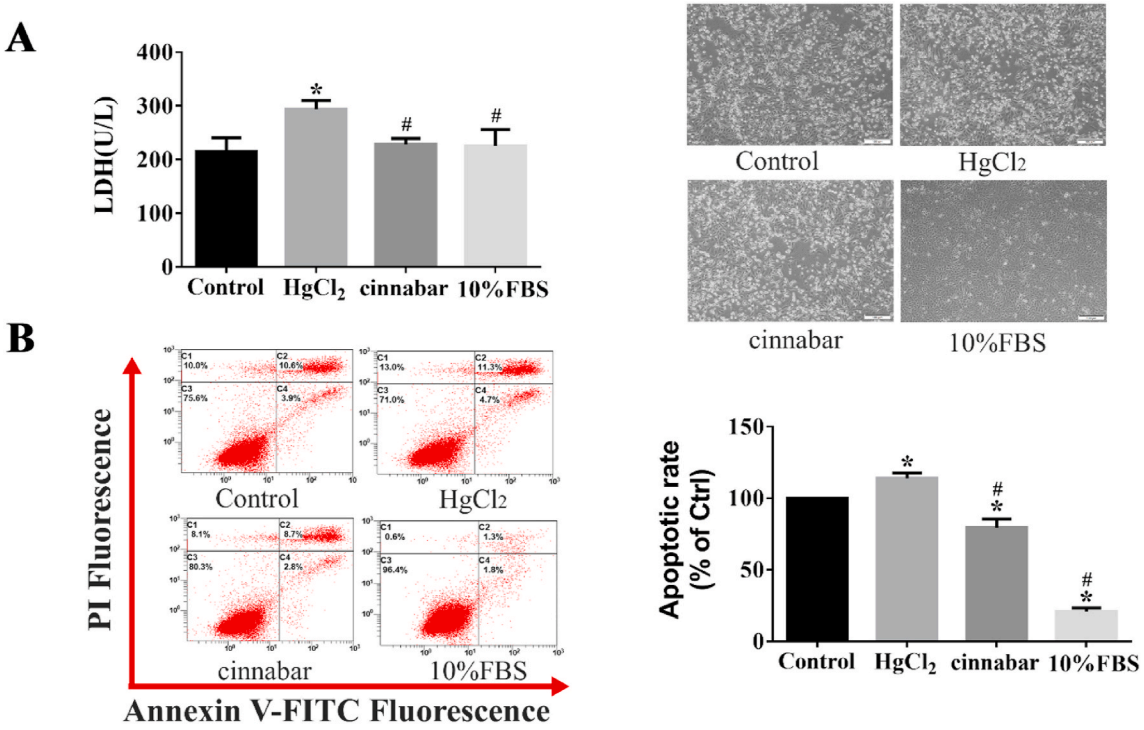

Fig. 3. Effects of the cinnabar and $HgCl_2$ on the injury and apoptosis of cells. Cells were exposed with cinnabar and $HgCl_2$ for 4 days. (A) Release of cytosolic LDH and the morphological changes in cultured HK-2 cells (scale bar = 100 μm). (B) Apoptotic cells were detected by flow cytometry using Annexin/PI double staining, and apoptotic rate was expressed as percent of control. Values were calculated from three independent experiments and presented as mean ± SD. *, $P < 0.05$ *vs.* control cells; #, $P < 0.05$ *vs.* $HgCl_2$-treated cells alone.

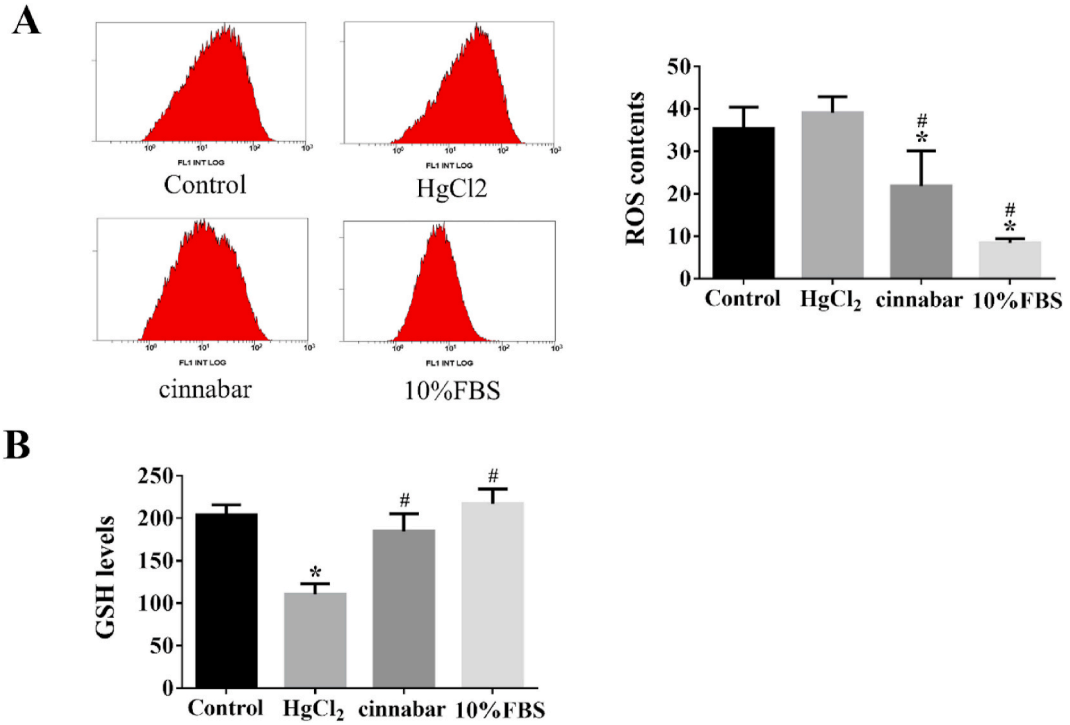

Fig. 4. Effect of cinnabar and $HgCl_2$ on intracellular ROS contents and GSH levels in HK-2 cells. Cells were treated with 4 nM cinnabar and $HgCl_2$ for 4 d. The determination of ROS and GSH levels was carried out as described in the Materials and methods section. (A) Intracellular ROS was detected by flow cytometry using DCFH-DA staining and quantification of ROS contents. (B) Intracellular GSH levels (μM). Values were calculated from three independent experiments and presented as mean ± SD. *, $P < 0.05$ *vs.* control group; #, $P < 0.05$ *vs.* $HgCl_2$-treated cells alone.

3.4. Effects of cinnabar and $HgCl_2$ on ROS production and intracellular oxidative status of HK-2 cells

To further understand the differential effects on nutrient deprivation induced apoptosis in HK-2 cells between cinnabar and $HgCl_2$, we determined the intracellular ROS contents by flow cytometry. The intracellular accumulation of ROS in cinnabar-treated cells was dramatically lower than those cells treated with $HgCl_2$ and the control cells (Fig. 4A). To illustrate the precise role of cinnabar in inhibiting apoptosis and accumulation of ROS, we next determined cellular oxidative status. GSH is one of the most important endogenous ROS scavengers, and GSH or the ratio of it with oxidized glutathione has been regarded as an index of oxidative stress implicated into various pathological conditions [26]. We therefore determined cellular GSH and found that intracellular GSH (μM) in cinnabar-treated cells was significantly higher than that in $HgCl_2$-treated cells (Fig. 4B). These results evidenced that low-concentration cinnabar (4 nM) may possess the ability to inhibit oxidative stress and protect intracellular antioxidants when cells are under stress state.

3.5. Cinnabar inhibits the expression of CHOP and PERK in the ER stress pathway

Endoplasmic reticulum (ER) stress induced apoptosis has been increasingly recognized in various pathological progress [27]. Here we determined the expression of CHOP and PERK, two major mediators of ER stress pathway in apoptosis. While the treatment of cells with $HgCl_2$ elevated the expression of CHOP and PERK levels, the treatment of cells with the low-concentration cinnabar significantly decreased protein levels of both CHOP and PERK (Fig. 5A and B). Next, the real-time RT-PCR analysis revealed that mRNA expression levels of CHOP and PERK were dramatically decreased following treatment with cinnabar, suggesting a transcriptional regulation of cinnabar on the expression of CHOP and PERK (Fig. 5C). Taken together, our data indicated that the antioxidative and antiapoptotic functions of low-concentration cinnabar under stress state in HK-2 cells may be mediated through down-regulating the expression of ER stress pathway mediators CHOP and PERK.

3.6. Antioxidative and antiapoptotic effects of cinnabar in cells challenged with H_2O_2

In order to further confirm the anti-apoptotic and anti-oxidative stress effects of cinnabar, we treated HK-2 cells with 100 μM of H_2O_2 in addition to cinnabar and $HgCl_2$. The results demonstrated that cinnabar was able to significantly decrease the apoptotic rate and intracellular ROS production as compared with control and $HgCl_2$-treated cells (Fig. 6A and B). Owing to the concentration of cinnabar and $HgCl_2$ was rather low, higher concentrations of H_2O_2 were not adopted in our experiments as the damage of high concentration H_2O_2 may mask the toxicity of $HgCl_2$.

3.7. Cinnabar-mediated inhibition of the expression of CHOP and PERK in cells treated with H_2O_2

We next explored the effect of cinnabar on the expression of both CHOP and PERK in cells subjected to dual challenges of nutrient deprivation and treatment of 100 μM H_2O_2. We examined the relative expression of CHOP and PERK in the mRNA and protein levels. As shown in Fig. 7A, cinnabar significantly decreased CHOP and PERK mRNA levels as compared with the control and $HgCl_2$ group. Furthermore, the down regulation of CHOP and PERK protein levels in HK-2 cells was

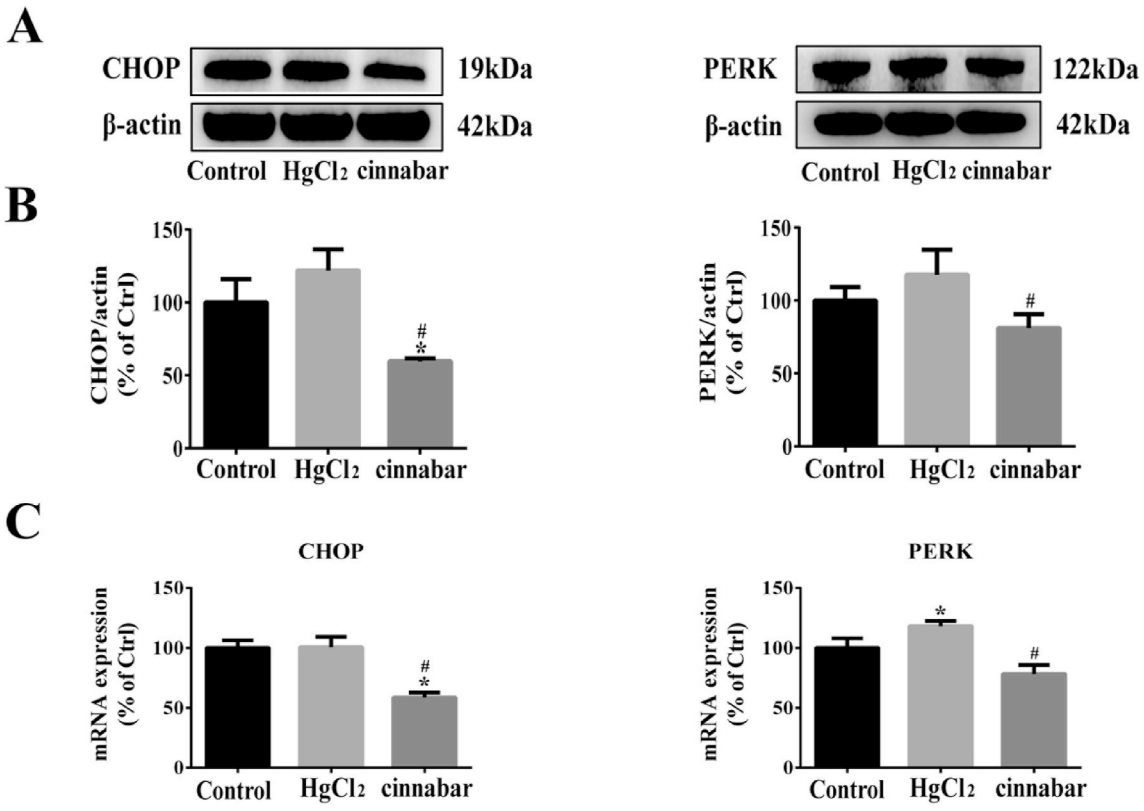

Fig. 5. Cinnabar inhibits the expression of CHOP and PERK in the ER stress pathway. Cells were incubated with cinnabar and $HgCl_2$ for 4 d. (A, B) Western blot analysis and quantified protein levels of CHOP and PERK, respectively. β-actin was used as loading control. (C) Relative transcript levels of CHOP and PERK. The mRNA levels of each target gene were normalized to the expression of the GAPDH gene. Values were calculated from three independent experiments and presented as mean ± SD. *, $P < 0.05$ *vs*. control cells; #, $P < 0.05$ *vs*. $HgCl_2$-treated cells.

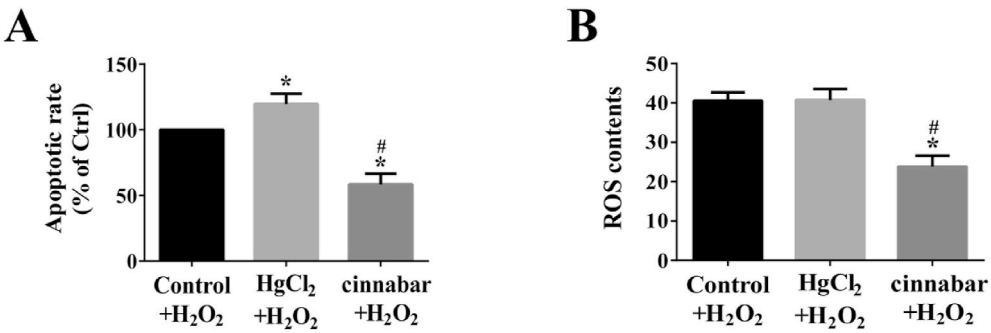

Fig. 6. Effect of cinnabar and $HgCl_2$ on apoptosis rate and intracellular ROS level in HK-2 cells in the presence of H_2O_2. Cells were treated simultaneously with 4 nM cinnabar or $HgCl_2$ and 100 μM H_2O_2 for 4 d. (A) Apoptotic rate (%) of control. (B) Intracellular ROS content. Values were calculated from three independent experiments and presented as mean ± SD. *, $P < 0.05$ *vs.* control cells; #, $P < 0.05$ *vs.* $HgCl_2$-treated cells.

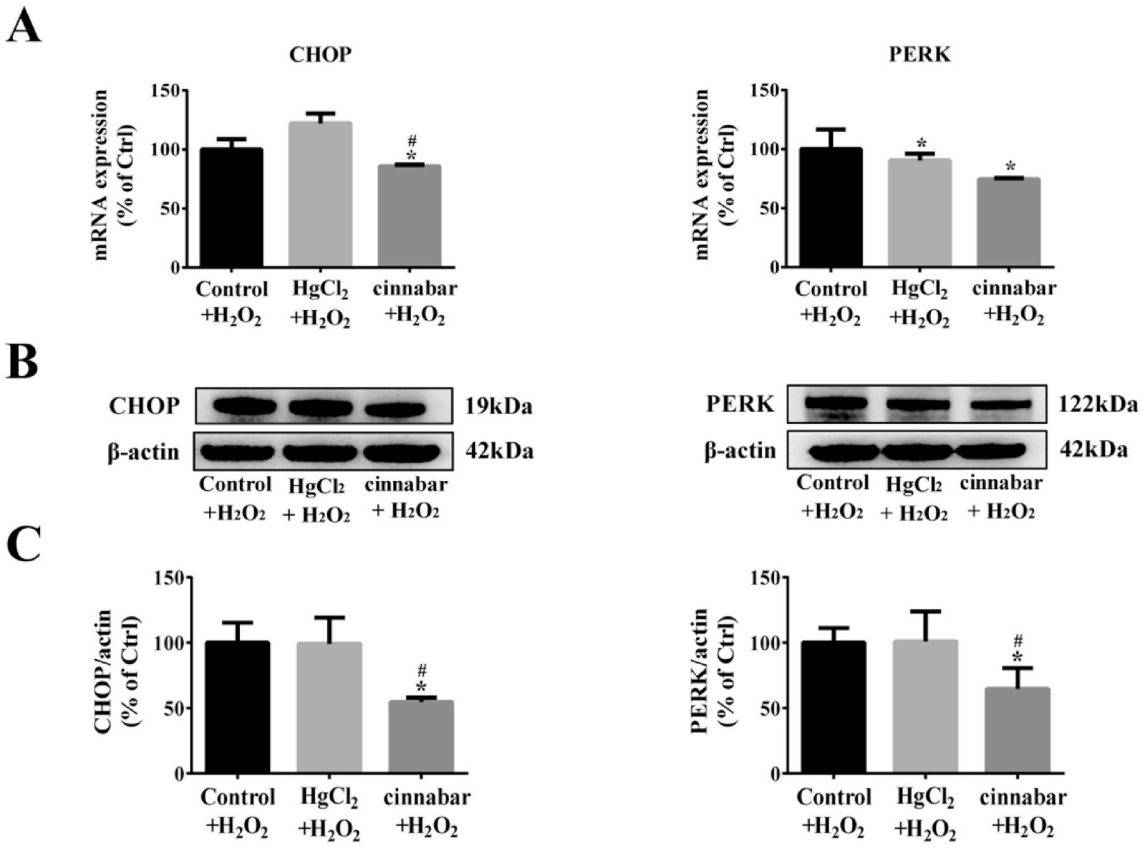

Fig. 7. Cinnabar-mediated inhibition of the expression of CHOP and PERK in HK-2 cells treated with H_2O_2. Cells were treated simultaneously with 4 nM cinnabar or $HgCl_2$ and 100 μM H_2O_2 for 4 d. (A) Relative transcript levels of CHOP and PERK. The mRNA levels of each target gene were normalized to the expression of the GAPDH gene. (B, C) Western blot analysis and quantified protein levels of CHOP and PERK, respectively. β-actin was used as loading control. Values were calculated from three independent experiments and presented as mean ± SD. *, $P < 0.05$ *vs.* control cells; #, $P < 0.05$ *vs.* $HgCl_2$-treated cells.

found in the cinnabar-treated cells as compared to control and $HgCl_2$ cells (Fig. 7B and 7C). Collectively, our data suggested that the anti-oxidation and anti-apoptosis effects of low-concentration cinnabar were mediated by the suppression of CHOP and PERK.

4. Discussion

Cinnabar has been used as an essential component in traditional Chinese medicines and in Indian Ayurvedic medicines for thousand years [7]. The medical use of cinnabar has been a big controversial issue over the last few decades because of toxicological concerns of high content of metal mercury. Due to this, many cinnabar containing traditional medicines have been banned, but some are still in active use [1,28,29]. Accumulating in vivo and in vitro studies have demonstrated that cinnabar and cinnabar-containing traditional medicines exhibit distinct toxicological profiles and are much less nephrotoxic than other mercury compounds. However, the exact mechanism behind this has not been clearly elucidated. On the other hand, though an important ingredient deliberately added in many traditional remedies, the rational for the inclusion of cinnabar remains to be fully described. Although it contains up to 96% HgS, cinnabar still possesses some other non-mercury compounds, such as arsenic compounds [30], and cannot be replaced with HgS clinically, partly due to these non-mercury compounds that may have a certain degree of influence on the pharmacological effect of cinnabar. It is worthy to note that cinnabar, as an insoluble mineral drug, has a very low soluble inorganic mercury

content. To accurately quantify the mercury content in cinnabar, inorganic mercury was used as the standard for accurate quantification, which is consistent with our previous studies [31]. Importantly, at the saturation concentration (4 nM) cinnabar did not cause significant cytotoxicity, while a dramatic toxic effect was observed for $HgCl_2$ at a concentration of 4 nM. We further reported that cinnabar exhibited a distinct effect on nutrient deprivation induced oxidative stress and apoptosis in the renal tubular epithelial cells HK-2. While the treatment of cells with $HgCl_2$ exacerbated oxidative stress and elevated cellular apoptosis induced by nutrient withdrawal, cells treated with cinnabar appeared to be well tolerant of the nutrient deprivation-induced oxidative stress and were less apoptotic, clearly demonstrating that cinnabar differs from $HgCl_2$ and is protective against oxidative stress at a low nanomolar concentration. Following exposure to inorganic mercury, the majority of mercury ions accumulate in the kidney and in short-term exposure, about 40% of inorganic mercury without nephrotoxic effect was absorbed and accumulated in the kidney [32]. Kidney is the main target organ of inorganic mercury, and renal tubules and proximal convoluted tubules are more sensitive to inorganic mercury and other toxic substances than other parts due to their involvement in the secretion and reabsorption of substances. Therefore, proximal convoluted tubules are the main toxic target cells of inorganic mercury. The HK-2 cells, which are immortalized human renal cortex proximal convoluted tubule epithelial cells, have various characteristics of normal cortical juxta convoluted tubule epithelial cells, and are often used as an effective model for the study of mercury nephrotoxicity in vitro [33,34]. Therefore, the Hk-2 cells were employed in the present study as well. Cinnabar is insoluble with low bioavailability, and thus gives rise to a very low concentration of inorganic mercury in blood once absorbed from the gastrointestinal tract. In addition, in the blood or in cell culture medium (with FBS), mercury combines with the protein containing sulfhydryl group in serum to form mercuric S-conjugates which may have difficulty crossing the cell membrane and exerting a toxic effect due to the excessive molecular weight of the protein [35]. With these considerations, a cell culture system without serum was employed in the present study, which allowed us to dissect the molecular basis behind the differences without complications from proteins binding to mercury. On the other hand, serum withdrawal induces cellular oxidative stress, a condition suitable for investigating the antioxidant properties of related agents. In serum-deprivation medium, cells are in a state of stress due to a lack of nutrients and are more sensitive to toxic agents. Indeed, it appeared clearly that, although at a very low concentration (4 nM), $HgCl_2$ obviously exacerbated cell damage and caused increased apoptotic cell death as compared to cells with serum withdrawal only. It distinctly showed that serum deprivation does increase the contribution of $HgCl_2$ to cytotoxicity. Interestingly, the treatment of 4 nM cinnabar exerted a completely different effect. Rather than enhancing toxicity cinnabar treatment significantly reduced cellular apoptosis induced by serum deprivation, which might be attributed to its antioxidant capabilities as evidenced by the suppressed ROS generation and elevated antioxidant GSH level.

Oxidative stress plays an imperative role in apoptosis of renal injury under physiological conditions from exposure to mercury, and studies demonstrated that intracellular ROS potently impacts the apoptotic extent of renal cells [36]. In agreement with the previous studies our results showed that intracellular ROS level increased in $HgCl_2$-treated cells accompanied by an increase in apoptosis. The depletion of antioxidants was also a significant sign in the development of oxidative stress. Glutathione, an endogenous free radical scavenger containing sulfhydryl group, possesses two functions: one is to combine with mercury ion, and the other is to eliminate mercury-induced ROS [37,38]. As expected, $HgCl_2$ treatment significantly depleted intracellular GSH level. However, a significant amount of intracellular GSH level was preserved in the cells treated with cinnabar as compared to the cells treated with $HgCl_2$. These results clearly demonstrated a differential effect between cinnabar and $HgCl_2$. To further confirm the antioxidative and antiapoptotic effects of cinnabar, the HK-2 cells were treated with H_2O_2 while challenged with nutrient deprivation. H_2O_2 is often used as a toxicant in vitro model to mimic oxidative stress-induced renal injury [39]. The results revealed that cinnabar was still able to decrease the apoptotic rate and reduce intracellular ROS production as compared with control and $HgCl_2$-treated cells. It has been reported previously that Angong Niuhuang Wan, a Chinese patent medicine containing cinnabar, can exert its neuroprotective effect by inhibiting oxidative/nitrative stress [28]. In the present study, we ascertained the protective effect of cinnabar on HK-2 cells after serum deprivation by detecting intracellular ROS which is the source of oxidative stress, and the GSH content which is a vital antioxidant in oxidative stress system. From our experimental results, we indicated that $HgCl_2$ caused more significant apoptosis in the absence or presence of H_2O_2, but there was no significant difference in ROS content, this may be due to the existence of other apoptosis mechanisms besides ROS. Another major finding in the present study relates to the ER stress mediated apoptosis. We identified two mediators CHOP and PERK that might be involved in the cinnabar-mediated modulation of apoptosis. Under ER stress, CHOP is activated by PERK, causing transcriptional activation of multiple genes and inducing ER stress-mediated apoptosis [40]. In endoplasmic reticulum stress, the expression of CHOP can be induced by ERN1, ATF6 and PERK, and the activation of CHOP is also the direct result of endoplasmic reticulum stress. However, compared with ERN1 and ATF6 signaling pathways, PERK-CHOP signaling pathway can remain activated for a longer time in the late stage of apoptosis [41–43]. Our findings clearly demonstrated that the low concentration cinnabar decreased the expression of PERK and concomitantly down-regulated CHOP expression, suggesting an ability for cinnabar to inhibit ER stress. This inhibitory effect of cinnabar on ER stress still existed when cells were challenged with additional stress from H_2O_2, suggesting that the modulation of the ER stress pathway might be a key event in cinnabar-mediated antiapoptotic action. However, the regulation of $HgCl_2$ on HK-2 cells was not obvious at gene or protein level relatived endoplasmic reticulum stress, so we speculated that CHOP-PERK signal pathway may not the main signal pathways of $HgCl_2$ mediated apoptosis.

It is worthy to note that cinnabar induces renal injury in chronically treated rats [44]. However, this does not conflict with our experimental results because the cinnabar concentration used in our experiment is extremely low. In traditional Chinese medication, cinnabar is an essential component of a plethora of medicines, but its mercury content in the body is extremely low. In particular, we detected the mercury concentration in medium, however, we did not measure the intracellular mercury content after HK-2 cells were treated with cinnabar or $HgCl_2$ because the detection of intracellular low concentration mercury was a severe restriction, and the toxicity of mercury may have a great relationship with the target transporter [17,45], which is worthy of a further investigation to yield more mechanisms of mercury toxicity as well as therapeutic effect of cinnabar. In this regard, our findings are of great significance, and provide new clues to further exploring not only the differential toxicities between cinnabar and other common mercury containing compounds, but also the therapeutic molecular basis of cinnabar. Moreover, studies show that the dissolved components of cinnabar is mercury polysulfide, which apparent permeability coefficient (P_{app}) is lower than $HgCl_2$. In addition, the combination compounds of mercury polysulfide with albumin have less effects on cell viability. Those may be the material basis of cinnabar as a therapeutic medicine exhibiting pharmacological. In conclusion, we demonstrate that low concentration cinnabar exerts antioxidative stress and antiapoptotic effects by inhibiting the expression of the endoplasmic reticulum apoptosis pathway genes CHOP and PERK.

5. Conclusion

In summary, the present study clearly illustrated that compared with $HgCl_2$, cinnabar effectively alleviated serum-nutrient starvation induced

apoptosis, reduced intracellular ROS generation and increased GSH content. This cinnabar conferred protective effect might occur through the down-regulation of CHOP and PERK expression, thus leading to increased antioxidative stress and anti-apoptosis effects. Furthermore, the antioxidative effect of cinnabar was further confirmed in cells under dual challenges of nutrient deprivation and treatment of H_2O_2. The findings in the present study provide a theoretical basis for further exploration of medication safety of cinnabar in the field of traditional Chinese Medicine.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.101055.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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