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Hemin mitigates contrast-induced nephropathy by inhibiting ferroptosis via HO-1/Nrf2/GPX4 pathway

Running head: Hemin mitigates CIN by inhibiting ferroptosis

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Abstract

Contrast-induced nephropathy (CIN) is a common complication with adverse outcome after iodinated-contrast injection, yet still lacking effective medication. Heme oxygenase-1 (HO-1) has been reported to play an important role against renal injuries. Hemin, a HO-1 inducer and anti-porphyria medicine, may have a promising effect against CIN. In this study, we aim to investigate the effect of hemin on CIN model and the underlying molecular mechanisms in human proximal tubule epithelial cells (HK-2) cells. To mimic a common condition in PCI patients, CIN was induced by intravenous iopromide in high-fat fed diabetic rats. We found hemin, given right before iopromide, mitigated CIN with enhanced antioxidative capacity and reduced oxidative stress. HK-2 cells insulted by iopromide demonstrated decreased cell vitality and rising ROS, which could also be inhibited by hemin. The effects of hemin involved a key molecule in ferroptosis, glutathione peroxidase (GPX4), whose down-expression by siRNA reversed the effect of hemin on HK-2 cells. Furthermore, hemin's induction of GPX4 involved HO-1 and nuclear factor Nrf2. Either HO-1 or Nrf2 inhibitor prevented hemin's effect on GPX4 to a comparable extent, and over-expression of Nrf2 increased GPX4 expression. Besides, intervention of ferroptosis inhibitor liproxstatin-1 also alleviated CIN in vivo. Thus, we showed hemin mitigated CIN, inhibiting oxidative stress and ferroptosis, by upregulation of GPX4 via activation of HO-1/Nrf2. Hemin, as a clinical medicine, has a translational significance in treating CIN, and anti-ferroptosis is a potential therapeutic strategy for CIN.

Keywords: contrast-induced nephropathy; ferroptosis; GPX4; hemin;HO-1; Nrf2

Introduction

Contrast-induced nephropathy (CIN), also called contrast-inducedacute kidney injury(CI-AKI), is a common complication after exposure to iodinated contrast medium (CM) during diagnostic or interventional procedures, most commonly the percutaneous coronary intervention (PCI) [1]. CIN is the third cause of iatrogenic renal injury, besides that induced by drug and low circulating volume,and occurs in up to 30% of patients who receivedCM, leading to an abrupt landslide-like poor prognosis[2]. CMexposure is rather safe for normal kidney, but highly toxic for impaired kidney(typically from patients with diabetes mellitus, chronic heart failure,chronic kidney diseases and advanced age). Diabetes, with its unavoidable microvascular complication in kidney and macrovascular complication in coronary artery requiring PCI treatment, has been recognized as a major independent risk factor for CIN in clinic[3]. However, other than some prophylactic methods like hydration and hemofiltration, there still lacks an effective guideline-recommended medicine for CIN. Studies on oxidative stress in the pathogenesis of CIN has attracted wide attention, but the effect of various anti-oxidative medications so far is barely satisfactory, which warrants further research [4].

Heme oxygenase-1 (HO-1), as a new therapeutic anti-oxidative target, has demonstrated robust protective effect on acute kidney injury in a large number of experimental studies[5-7]. In clinical medication, there already exist a class of drug as HO-1 inducer in treating acute porphyria: hemin (brand name Panhematin, USA) and heme arginate (HA, brand name NORMOSANG, France)[8]. Hemin can increase plasma HO-1 in healthy volunteers and increase HO-1 expression in peripheral blood mononuclear cell (PBMC), with only mild side effects, without impact on liver and kidney function and blood coagulation function, laying a foundation for the safe use of hemin in indications other than porphyria[9,10]. In a clinical study of hemin intervention in 40 kidney transplant patients during the perioperative period,

the expression of HO-1 in PBMC and renal biopsy increased significantly without side effects [11]. The interventional study of hemin on CIN patients has not been reported yet. Thus, we speculated that the HO-1 inducer hemin may has a significant preventive and therapeutic effect on CIN and serve as a potential clinical candidate drug.

Kidney is such a bloody organ that whole-body circulating blood continuously flows through it. It's easy to understand why interstitial congestion and hemoglobin cast are typical pathological features of acute tubular necrosis in AKI. Heme (iron (II)-protoporphyrin IX), released from extravascular hemoglobin, induces reactive oxygen species (ROS) production, and possesses extraordinary pro-oxidative and pro-inflammatory properties [12]. HO-1 can metabolize cytotoxic heme to biliverdin, iron and carbon monoxide (CO) [8]. In light of the abnormal increase of iron in AKI, which would definitely disrupt the iron homeostasis and release free irons for free radical reactions, iron metabolism-related ferroptosis attracts our attention. Ferroptosis has also been reported to play an important role in acute kidney injury [13].We wondered if hemin could protect against CIN through regulating ferroptosis and the role of anti-ferroptosis intervention in coping CIN.

In the present study, we explored the protective effects of hemin against CIN in diabetic rats and investigated its possible molecular mechanism in antioxidative pathway and ferroptosis.

Results

1. Hemin protect against renal injury in CIN rats

Since acute kidney injury is hard to be induced by iodinated contrast in normal kidney with intact compensated function to cope with the CM insult and diabetic renal insufficiency is a common clinical comorbidity and predisposing risk factor for CIN in PCI patients, we established CIN model by intravenously injection of iopromide in high-fat fed diabetic rats...

Firstly, we ensured the success of the CIN model establishment in diabetic rats. We found that 24 h after the infusion of iopromide (1.8 g(I)/kg wt), both serum Cr and BUN levels were sharply increased relative to the DM control rats, even though diabetes background per se has impact on renal function at the baseline **(Fig. 1a, b)**. At the same time, the level of calculated CCr was remarkably decreased **(Fig. 1c)**, indicating that CIN was successfully induced by iopromide in diabetic rats.

Then, we tested the effect of hemin on renal function. Results showed that treatment with hemin (10 mg/kg wt) right before the infusion of iopromide significantly prevented the increase in serum Cr and BUN levels **(Fig. 1a, b)**, and the decrease in CCr level **(Fig. 1c)** in the CIN model. Next, we observed the H&E-stained histopathological changes under microscope. Consistent with our previous report [14], CIN was characterized by marked proximal tubule dropouts, extensive hemoglobin and erythrocyte casts, interstitial congestion and inflammation 24 h after CM injection, which were mainly located in the outer medulla. We found the above-mentioned pathological changes were significantly reduced by hemin treatment,as proved by the reduction in renal pathological scores**(Fig. 2a)**.Besides necrosis, regulated cell death (RCD), like apoptosis, ferroptosis, is another pathologicalmanifestation of injury.We found that CM increased cell death, determined by TUNEL staining,mostly in the boundary between the cortex and medulla, while hemin reducedthe TUNEL-positive cells to approximately one third of the CIN group**(Fig. 2b)**. The same effect of hemin on apoptosis was also verified by immunoblotting of Bax and Bcl-2 expression, where theBaxwas decreased and Bcl-2 elevated afteracute kidney injury compared to the DM control, which was attenuated by hemin treatment as shown in a decreased Bax/Bcl-2 ratio **(Fig. 2c)**.

2. Hemin reduced renal oxidative stress with influence on GPX4

Oxidative stress is an initiator and major component in pathogenesis of CIN [15].As anantioxidant, hemin exerts its protectiverole in many diseases mainly via down-regulation of

ROS formation. Our present study showed that CM induced an acute oxidative stress in kidney, as evaluated by DHE probe, MDA,T-AOC, SOD levels and the ratio of GSH/GSSG.DHE is an oxidant-sensitive probe for detection of ROS. MDA is a marker for lipid peroxidation. Heminsignificantly prevented the CM-induced increase in ROS and MDA production (**Fig. 3a, b**)and decrease inT-AOC, SOD and GSH/GSSG levels(**Fig. 3c-e**). GPX4 is a lipid repair enzyme by converting iron-dependent-ROS related lipid peroxides to non-toxic lipids, simultaneously converting the reduced GSH to oxidized GSSG [16]. GPX4 has been identified as a key regulatory factor in ferroptosis. Thus, we wondered whetherGPX4 was involved in the protective effect of hemin on CIN. Results demonstrated that GPX4 expression, determined by immunoblotting, was remarkably reduced in CIN group compared with DM control, which was reversed by hemin treatment **(Fig. 3f)**, indicating a role of GPX4 and ferroptosis in hemin's protective effect on CIN.

3. Hemin-induced GPX4 is essential in protecting CM-insulted HK-2 cells

To explore the molecular mechanism of hemin on CIN, we first selected a proper concentration of iopromide to consistently induce an in vitro model of CM-induced acute injury in HK-2 cells. We observed that iopromidedecreased the cell vitality of HK-2 cells in a concentration-dependent manner (10-100 mg/mL), determined by MTT uptake (**Supplementary Fig. S1a**). Iopromide with 30mg/mL was chosen to induce a moderate insult in the upcoming experiments. Although hemin, by itself, didn't have an impact on the cell vitality of normal HK-2 cells in the range of 1 to 100 μmol/L (**Supplementary Fig. S1b**), it preserved the CM-injured cell vitality in a concentration-dependent manner from 10-50 μmol/L **(Fig. 4a)**. The CM-induced ROS production in HK-2 cells was also obviously decreased by hemin (50 μmol/L) treatment **(Fig. 4b)**. To observe the effect of hemin on GPX4 expression in CM-injured HK-2 cells, we examined the changes of GPX4 expression first to find that within 24 hours iopromide time-dependently reduced the mRNA expression of

GPX4 **(Fig. 4c)**, which was reversed in the presence of hemin (50 mg/mL) **(Fig. 4d)**. The effect of hemin on GPX4 expression was also confirmed by immunoblotting, where hemin significantly reversed the down-regulated GPX4 protein expression in CM-injured HK-2 cells in a dose-dependent manner from 10-50 μmol/L, the protein levels were nearly normalized at the higher dosages **(Fig. 4e)**.

To further clarify whether GPX4 plays an essential role in hemin's down-regulation of ROS generation in HK-2 cells, GPX4 siRNA was employed to interfere with the expression of GPX4. The interference effects of candidate siRNAs were tested in **Fig. 4f**. siRNA with the best interference effect was selected for subsequent GPX4 interference test. Our results showed that the effects of hemin on ROS reduction and cell vitality promotion were completely eliminated when GPX4 expression was silenced **(Fig. 4g, h)**. These results indicate that GPX4 is a downstream signal of hemin and plays an important role in hemin's protective effect in CM-insulted HK-2 cells. In addition, exogenous administration of hemin could protect HK-2 cells against injuries induced respectively by two independent ferroptosis inducers: GPX4 inhibitor RSL-3 and non-GXP4-targeted inducer erastin, which further confirmed the inhibitory effect of hemin on ferroptosisis essentialin its protective effect in HK-2 cells.**(Fig. 4i, j)**.

4. Hemin promoted GPX4 expression through HO-1/Nrf2 pathway

To further explore the mechanism of the up-regulation effect of hemin on GPX4, firstly we tested that hemin could promote the protein expression of GPX4 in concentration-dependent (1-50 μmol/L) and time-dependent (3-24 hours) manners in normal HK-2 cell without CM insult **(Fig. 5a, b)**. Thus, 50 μmol/L of hemin incubated with HK-2 cells for 24 hours was selected for the following experimental condition. By literature reviewing, we found that Nrf2 can directly or indirectly up-regulate the expression of GPX4. However, whether hemin could regulate GPX4 expression through Nrf2 has not been reported. On the

one hand, Nrf2, as a robust transcription factor for numerous antioxidant genes with antioxidant response element (ARE), is a well-known regulator of HO-1 [17]; on the other hand, HO-1 in turn can also interact with Nrf2 to modulate its function through stabilization and subcellular distribution [18]. Thus, we wondered how the blocked effects of HO-1 inhibitor (ZnPP) and Nrf2 inhibitor (ML385) respectively on hemin would be, in terms of inducing GPX4 expression. Results revealed that either HO-1 inhibitor or Nrf2 inhibitor effectively blocked the up-regulation effect of hemin on GPX4 mRNA and protein expression to a comparable extent, indicating that hemin induced GPX4 expression via HO-1/Nrf2 pathway (**Fig. 5c, d**). To examine the efficacy of Nrf2 on GPX4 expression in HK-2 cells, we constructed a cell line over-expressing Nrf2 by plasmid transfection carrying Nrf2 gene (**Fig. 5e**) and found that Nrf2 over-expression efficiently up-regulated GPX4 gene transcription and translation (**Fig. 5e, f**).

5. Inhibitionofferroptosisprevented CIN in vivo

Studies have demonstrated that ferroptosis plays an important role in various type of acute kidney injuries[19] whichis regulated by GPX4, lipid synthesis, iron metabolism, and other factors like Nrf2 [16]. Inhibition of ferroptosis in treating AKI has become a new research hotspot recently, but the effect in CIN has not been reported yet. Besides the above-studied inhibition of ferroptosis by hemin via Nrf2/ GPX4 promotion, we tried with another commonly used ferroptosis inhibitor liproxstatin-1 (LIP-1) in vivo to see the effect. Results demonstrated that the renal function of CIN was significantly improved by the LIP-1 (10 mg/kg wt, ip) intervention, assessed by serum creatinine and BUN **(Fig. 6a, b)**, as well as the histopathological changes of CIN **(Fig. 6c)**.LIP-1 also reduced ROS production detected by DHE probing, MDA level and the ratio of GSH/GSSG **(Fig. 6d-f).** In addition, results of in vitro studies also showed that LIP-1, a well-known ferroptosis inhibitor, and DFO, an iron-chelator,couldprotect HK-2 cells against cell damages induced by iopromide**(Fig. 6g, h).**

These results demonstrated that inhibition of ferroptosis can decrease the renal damage caused by CM. Ferroptosis plays an important role in the pathogenesis of CIN and ferroptosis inhibition is also a promising therapeutic target and strategy in coping CIN.

Discussion

CM is highly nephrotoxic and mainly accumulates in kidney [20]. An acute decrease in renal blood flow leading to oxygen imbalance and direct cytotoxic effect are believed to be the main causes of CM in inducing renal tubular damage in CIN. However, CIN rarely occurs in normal kidneys. Homeostasis of the medullary oxygenation largely relies on the normal microvascular environment modulated by local prostaglandin and nitric oxide. It may already be disturbed with microvascular endothelial diastolic dysfunction at the very early stage of a renal disease, such as diabetic nephropathy and senile renal dysfunction, which easily induces the continuous contraction of blood vessels in the kidney under the stress of an acute CM insult [21]. Only those with an impaired renal function, even in the early compensatory stage, are vulnerable candidates for CIN. Diabetic patients with coronary heart disease in a condition requiring PCI treatment is a common scene happened in clinic, which CM would inevitably exacerbate the renal insufficiency after PCI with an accelerating poor prognosis. Since it is hard to establish CIN model in normal rat without predispositions, we used diabetic rats to establish it. By evaluation of renal function and histopathology, we confirmed a successful and typical histopathological changes in our CIN model, characterized by tubular necrosis, cast formation, interstitial congestion and infiltration of mononuclear cells mainly in the outer medullary region, where the ischemic-sensitive mTAL (medullary thick ascending limb of Henle's loop), rich in mitochondria, locates, which is consistent with previous reports of the CIN model [22].

Oxidative stress and necrosis related aseptic inflammation induced by CM injection are the main pathological mechanisms of CIN [23]; they can ignite each other reciprocally, forming a sustaining vicious circle with escalating self-damage in kidney after acute CM insult [24]. Antioxidative strategy has been widely investigated in treating CIN, such as N-acetylcysteine [15] and EGCG [14], but so far none of them has a satisfactory therapeutic effect in clinic, which warrants further research. Hemin, a HO-1 inducer, as an efficient clinical drug for porphyria characterized with an acute load of free iron and free radicals, attracted our attention. The transcriptomics study of hemin intervention in renal ischemia-reperfusion showed that genes regulated by hemin involve cell differentiation, metabolic signalling pathways, cell cycle, cell division, cytoskeletal actin and arachidonic acid metabolism, suggesting the multi-potency effect of hemin [25]. HO-1 has a close relationship with kidney showing a consistent protective effect in various kinds of AKI, like cisplatin-induced nephropathy, sepsis related AKI, ischemia-reperfusion injury of transplanted kidney, obstructive nephropathy, cardiorenal syndrome, hepatorenal syndrome and rhabdomyolysis-induced AKI [26]. Our results showed that hemin efficiently prevented CI-AKI with excellent properties in reducing the whole ROS level (detected by DHE) by inducing a robust antioxidative capability (evaluated by T-AOC), which reciprocally evidenced with a recent report from Brazil also demonstrating renoprotective effects of hemin on CIN in uninephrectomized diabetic rats [27]. Former study on the distribution of HO-1 expression in rat kidney showed that HO-1 is mainly expressed in the medulla part, more than three times that of the cortex [28]. It indicates that hemin might exert its effect partly by increasing HO-1 expression in the medulla area, consistent with the injured area in CIN kidney.

Ferroptosis is defined as anonapoptotic form of regulated cell death (RCD), mediated by iron-dependent free radical reactions, driven by the oxidative degradation of lipids, in a intracellular microenvironment controlled by GPX4 constitutively.Ferroptosis can be

inhibited by iron chelators and lipophilic antioxidants [16]. The regulation network of ferroptosis mainly consists of three parts: the increase of free iron providing free radicles, the lipid peroxidation damage, and the malfunction of GPX4 in repairing the lipid peroxidation damage. Apparently, ferroptosis，as a special form of RCD with regulation network distinct from apoptosis, is much more important for AKI than apoptosis. Many studies have found an important role of ferroptosis in thepathogenesis of AKI [13].Inhibition of ferroptosis can protect against AKI induced by variousinsults, such as ischemia-reperfusion, cisplatinand folic acid [29-31]. Since our results showed that hemin reduced the lipid peroxidation (measured by MDA) and the ratio of GSH/GSSG, we wondered if hemin also influenced the key modulator of ferroptosis, GPX4. We found hemin could induce the GPX4 protein expression of normal HK-2 cells in concentration and time- dependent manners. Treatment with hemin could reverse the impaired GPX4 expression, the increased ROS and decreased cell vitality in CM-insulted HK-2 cells, which could totally be offset by GPX4 siRNA indicating a pivotal role of GPX4 in hemin's protective effect as a downstream target. Besides the strategy of antioxidant, anti-ferroptosis may also be a promising method to treat CIN. Therefore, we tried this hypothesis with a common inhibitor of ferroptosis, LIP-1, by reducing lipid peroxidation in vivo, and found a similar protective effect in CIN as hemin.

Why the antioxidative effect of hemin is so powerful? Though the mechanism is not fully understood, a key reason should be its ability in activating Nrf2, a transcription factor of numerous antioxidant target genes with ARE (antioxidant response element) sequence. HO-1, induced by hemin, is not only a target gene of Nrf2, but also a modulator to activate Nrf2. HO-1 and Nrf2 constitute such a positive inter-promotive cycle that produces a self-enhancing and sustaining antioxidative capacity. Previous studies have demonstrated that GPX4 can be regulated by Nrf2 [16, 32]. In our study, we tested the blockage effects of HO-1 (ZnPP) and Nrf2 (ML385) respectively on hemin in inducing GPX4 and found both could efficiently

prevent hemin from inducing GPX4 mRNA and protein expression to a similar extent. Moreover, over-expression of Nrf2 by plasmid transfection could induce the mRNA and protein expression of GPX4.Notably, the current literature has reported the opposing role of HO-1 in the regulation of ferroptosis. Though many studies have demonstrated the protective effect of HO-1 against AKI using the gene knockout and transgene [33,34], showing a protective effect of HO-1 against ferroptosis [33],there areother reports of pro-ferroptotic effect of HO-1 in cancer cells [35,36]. We consider the different effect of HO-1 on ferroptosis may bedue to the different cell types.The abnormalpathophysiological state of cancer cells is quite different from normal cells, which may lead to the opposite effect of HO-1 on ferroptosis. HO-1 is known to metabolize heme into biliverdin/bilirubin, carbon monoxide and ferrous iron, and catalytic iron plays a key role in promoting AKI progression.HO-1 is a well-known key regulator of iron metabolism. Due to the strong oxidizing properties of iron and its ability to generate ROS via the Fenton reaction, certain antioxidants and ROS scavengers have demonstrated protective effects, that's where GPX4 exerts an important role in anti-ferroptosis.It has also been pointed out that the amount of cellular iron and ROS is the decisive driving force for HO-1 to exert different effects on ferroptosis, where excess cellular iron and ROS tend to switch HO-1 from anti-ferroptosis to a promoter of ferroptosis, suggestinga bidirectional effect of HO-1 on ferroptosis[36].Such bidirectional effect may be due to a dynamic imbalance between iron production by HO-1 and iron-dependent ROS depletion by GXP4 in cancer cells, which requires more in-depth study.

In conclusion, we demonstrated the protective effect of hemin on CIN rats and investigated the possible mechanism in HK-2 cells. Hemin treatment improved the morphological changes and dysfunction of the contrast-injured kidney with its antioxidative and anti-ferroptosis capacity, which is related to activation of HO-1-NRF2/GPX4 pathway. This study suggests that hemin, an anti-porphyria medicine, may has translational significance

in treating CIN, and anti-ferroptosis may be a potential therapeutic strategy for CIN. However, whether the treatment of hemin has an evidence-based effect on CIN in real world still needs further research.

Methods

1. Experimental animals

Adult male Sprague-Dawley (SD) rats, weighting 180-200 g, were purchased from Beijing Vital River Laboratory Animal Technology Co. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Hainan Medical University (Haikou, China). All procedures were performed under appropriate anesthesia, and all efforts were made to minimize animal suffering.

2. CIN model

Rats were fed with a high-fat diet, containing 20 g fat/100 g diet for 4 weeks before the induction of diabetes. The diabetic rats were established by intraperitoneal injection of Streptozotocin (40 mg/kg wt, STZ, Sigma-Aldrich) in 0.1M citrate buffer (pH 4.5). Non-fasting blood glucose levels from the tail were measured at 72 h after administration of STZ using a commercially available glucometer (Accu-Chek, Roche; measurement range: 0.6-33.3 mmol/L). A level higherthan 16.7 mmol/L in two consecutive days were considered hyperglycemia [19].

Experiments with contrast medium were performed, as previously reported with minor change [14], at 4 weeks following the establishment of diabetes. The rats were deprived of water 24 h before the acute CM insult and anesthetized with sodium pentobarbital (50mg/kg wt, ip), then placed on a heating table to maintain body temperature at 37°C. The left external jugular vein was cannulated with PE-10 tubing. CIN was introduced by intravenous injection of iopromide (Bayer HealthCare China, China) (1.8g(I)/kg wt). In the hemin group, hemin

(Sigma-Aldrich, USA), prepared for immediate use (dissolved in 0.1M NaOH, diluted with deionized water to 7.7mM, adjusted to pH 7.4 with 0.1M HCl), was injected (10mg/ kg wt) intravenously right before the CM injection. The vehicle group received equivalent amount of solvents. The intervention of ferroptosis inhibitor liproxstatin-1 (LIP-1, HY-12726, MedChemExpress China) was injected (10 mg/kg wt) intraperitoneally right after the successful operation of CIN model. Rats were placed in metabolic cages for 24 h urine collection. Blood and kidneys were collected 24 h after the injection of CM. Rats were sacrificed under anesthesia with sodium pentobarbital (100mg/kg wt).

3. Renal function

Blood sera was extracted by centrifugation. Creatinine (Cr) and blood urea nitrogen (BUN) were analysed with commercial kits (Sigma-Aldrich, USA) according to manufacturer's instructions. Creatinine clearance (CCr) was calculated according to the formula: CCr = UV/P: U is the urinary Cr concentration (μmol/L); V is the total urine volume collected in 24 h (ml/min); and P is serum Cr concentration (μmol/L).

4. Renal histopathology

The longitudinal half of the kidney samples were fixed in 4% paraformaldehyde, then dehydrated in increasing concentrations of ethanol, cleared in xylene and embedded in paraffin. Sections (4-μm thick) were transected, deparaffinized, and stained with haematoxylin and eosin. Morphological damages in kidney were scored to evaluate the degree of renal damage, including tubular epithelial cell vacuolization and necrosis, cast formation, interstitial congestion. The grading criteria was performed as previously described [14]. Ten fields per section from three different sections were examined.

5. Apoptosis in kidney

Apoptotic cell death of kidney was detected by TUNEL assay using a fluorescent in situ cell death detection kit (Roche, Switzerland) as previously described [14]. Nuclei were

identified with DAPI staining. TUNEL positive cells were counted in 10 non-overlapping fields of outer medulla in each slide for three different sections.

6. Measurement of oxidative markers

Oxidative stress of the injured kidney was evaluated by ROS, malondialdehyde (MDA), superoxide dismutase (SOD) levels, the ratio of reduced and oxidized glutathione (GSH/GSSG), and total antioxidant capacity (T-AOC) with commercial assay kits (Beyotime, China). For ROS detection by dihydroethidium (DHE) probing, kidney tissues were quicklyfrozen with liquid nitrogen, cut to a thickness of 8 μm at -20℃, then mounted on glass slides and incubated with DHE. The fluorescence intensityin renal outer medulla was viewed under an immunofluorescence microscope and analysed by Image J, according to the manufacturer's protocol.The renal tissues were homogenized in ice-cold sucrose buffer (pH 7.4) for the detection of MDA, SOD, T-AOC and GSH levels according to their respective manufacturer's protocol. The level of MDA was analysed using the thiobarbituric acid method, total SOD activity the nitroblue tetrazolium method, GSH levels the DTNB method, and T-AOC the rapid ABTS method.

7. Cell Culture

The human proximal tubular epithelial cell line HK-2 was purchased from ATCC (Hercules, CA). Cells were cultured in 10% FBS-DMEM containing100 U/mL penicillin, and 100 U/mL streptomycin and incubated in a CO_2 incubator with 5% CO2 at 37°C. Cells from subconfluent (70-80%) cultures were trypsin-dissociated and seeded onto collagen-coated 96-well plates at a density of 1×10^5 cells/well in a culture medium containing 2% FBS. They were allowedto attach overnight, and then treated with iopromide (30mg(I)/mL) for 24 hours. Other chemicals used to treat the cells were as follows: hemin (Sigma-Aldrich, 50 μM), ZnPP (282820, Sigma-Aldrich, 5 μM), ML385 (HY-100523, MedChemExpress, 5 μM), RSL-3 (HY-100218A, MedChemExpress, 5 μM), erastin (HY-15763, MedChemExpress, 30μM),

Liproxstatin-1 (LIP-1, HY-12726, MedChemExpress, 200nM) and Deferoxamine mesylate (DFO, HY-12726, MedChemExpress, 100μM). Cells were harvested and used for various morphological and biochemical studies. The cellular data were obtained from at least three independent experiments with three replicates performed in each trial.

8.Cell vitality assay

Cell vitality of HK-2 cells was determined by 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) (Beyotime, China)and LDH activity detection, as previously described [37]. HK-2 cells were seeded into 96-well culture plates at a density of 1×10^5cells/well and were allowed to grow to subconfluence (70-80%), then were serum starved for another 6 hours. Quiescent cells were treated with indicated reagents. After 24 hours, 10μl of MTT (5 mg/mL) were added to each well, and the incubation continued for an additional 4 hours at 37°C. Thereafter, 150μl of DMSO was added to each well, and the absorbance OD was read at 490 nm on a microplate reader (model 680, Bio-Rad). The activity of LDH released from injured cells was detected by LDH cytotoxicity assay kit (Beyotime, China). LDH detection working solution was added to the cell culture supernatant in a 96-well plate accordingly, and incubated in the dark at 37°C for 30 minutes, and then the absorbance was measured on the microplate reader at 490nm.

9.Plasmid transfection and siRNA interference

Plasmid transfection and siRNA interference were performed as previously described [37]. Nrf2 plasmid (Sangon, China) and GPX4 siRNAs (RIBOBIO, China) were synthesized for the transfection in HK-2 cells. When cells reached 80% confluence, plasmid (1μg/mL) or siRNA (50 nM) was transfected by using Lipofectamine 2000 reagent (Thermo Fisher Scientific) in Opti-MEM™ (ThermoFisher Scientific) according to the manufacturer's protocol. After 48 hours, the transfected cells were collected to determine the GPX4 protein

level and Nrf2 mRNA. The siRNA sequence with the best interfering effect was chosen and applied to our experiments.

10. Quantitative RT-PCR

Total RNA was isolated from cells using TRIzol Reagent (Invitrogen, USA) following the manufacturer's instruction. Spectroscopy was then used to detect the concentration and purity of the RNA samples. QuantiTect Reverse Transcription Kit (Qiagen, Netherlands) was used for reverse transcription of RNA to cDNA. Then quantitative real time PCR (RT-PCR) was performed with TB Green® Premix ExTaqII (Takara, Japan). The mRNA expression level of GPX4 gene and Nrf-2 gene was normalized by GAPDH. The primer sequences for GPX4 were 5'- CCGCCTTTGCCGCCTAC-3' (forward) and 5'- TTTACTTCGGTCTTGCCTCACT-3' (reverse). The primer sequences for Nrf2 were 5'- AGGTTGCCCACATTCCCAAA-3' (forward) and 5'- ACGTAGCCGAAGAAACCTCA-3' (reverse). The primer sequences for GAPDH were 5′- GAATACGGCTACAGCAACAGG-3′ (forward) and 5′- GGTCTGGGATGGAAATTGT-G-3′ (reverse) [37].

11. Western blot analysis

Kidney samples were grounded and homogenized in ice-cold lysis buffer. Protein extraction, electrophoresis, transfer, immunodetection and densitometric evaluation were performed as previously described [14]. Anti-Bcl-2 (Millipore, USA; dilution 1:500), anti-Bax (Millipore, USA; dilution 1:500), and anti-GPX4 (Gene Tex, USA; dilution 1:500) wereapplied. GAPDH (Santa Cruz, USA; dilution 1:1000) were used as housekeeping protein.

12. Statistical analysis

All data were analysed using SPSS19.0 (IBM) and expressed as mean ± SD from at least three repeated independent experiments. One-way analysis of variance (ANOVA) with Bonferroni post-hoc test was employed for comparisons among the experimental groups, after checking for normality (Kolmogorov-Smirnov) and homogeneity (Levene). Student’s t-test

was for comparison between only two groups. The histopathological scores among groups were compared by the nonparametric Kruskal-Wallis test. $P<0.05$ was considered statistically significant.

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Conflict of Interest Statement

The authors have no relevant financial or non-financial interests to disclose.

Data Availability

The research article data used to support the findings of this study are included within the article.

Author Contributions

Z.G. conceived and designed the study. ZY.Z, YQ.L., K.Z., XL.D. and JM.C. performed the experiments. DZ.W. gave experiment suggestions and contributed materials. DQ.G., LL.L. and JY.Z. analyzed the data. ZY.Z, and DQ.G. drafted the paper. Z.G. and M.Z. edited and revised the manuscript.

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Figure Legends

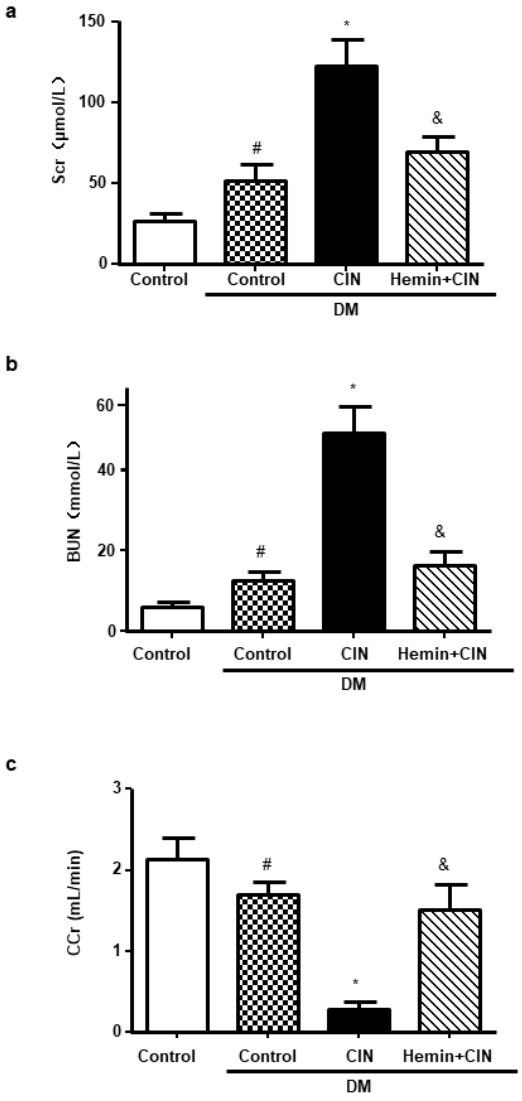

Fig. 1Hemin improved renal function in diabetic CIN rats

CIN models were established by intravenously injection of iopromide (1.8 g(I)/kg wt) in diabetic rats. Hemin (10mg/kg wt) or vehicle was given right before CM.Serum Cr (**a**) and BUN (**b**) were measured 24 h after CM injection. (**c**) CCr were calculated at 24 h after CM injection. Scr: serum creatinine; BUN: blood urea nitrogen; CCr: creatinine clearance; DM: diabetes mellitus; CM: contrast medium. Data are expressed as mean ± SD. (n=7; $^{\#}P<0.05$ vs. normal control; $^{*}P<0.05$ vs. DM control; $^{\&}P<0.05$ vs. CIN)

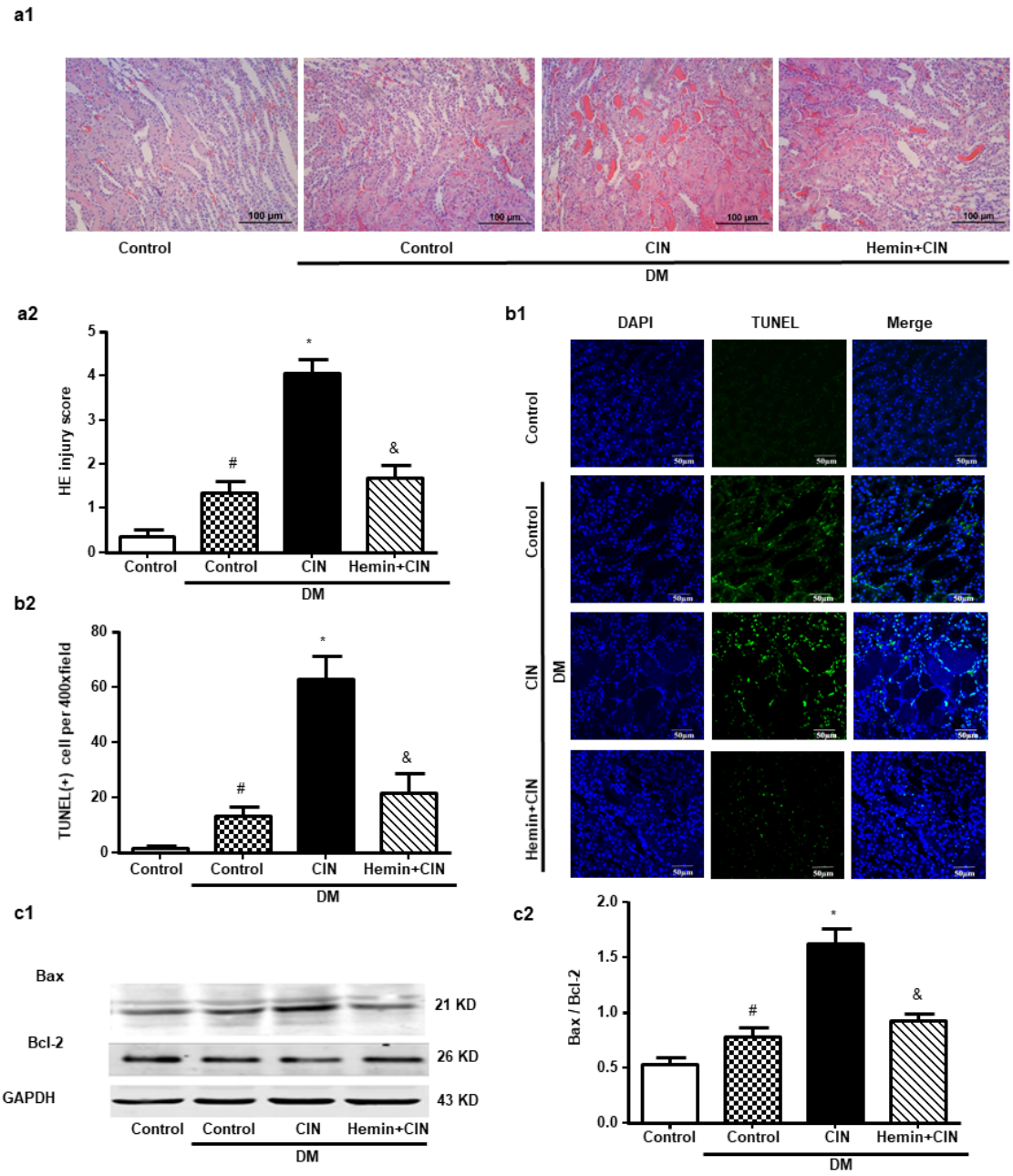

Fig. 2 Hemin alleviated pathological injury and apoptosis in diabetic CIN rats

Protocols of CIN model and hemin intervention was performed as Fig. 1. Kidney samples were collected 24 h after CM injection. (**a1**) Representative images of HE staining in renal outer medulla (scale bar = 100 μm) and (**a2**) Medullary pathological scores. (**b1**) Representative images of TUNEL staining in renal outer medulla (scale bar = 50 μm) and (**b2**) TUNEL-positive cells counted in 10 high-power (400×) fields. (**c1**) Western blot analysis of Bax (a marker of apoptosis) and Bcl-2(a marker of anti-apoptosis) and (**c2**) The

ratios of Baxand Bcl-2 in each group. Data are expressed as mean ± SD. (n=7; #P<0.05 vs. normal control; *P<0.05 vs. DM control; &P<0.05 vs. CIN)

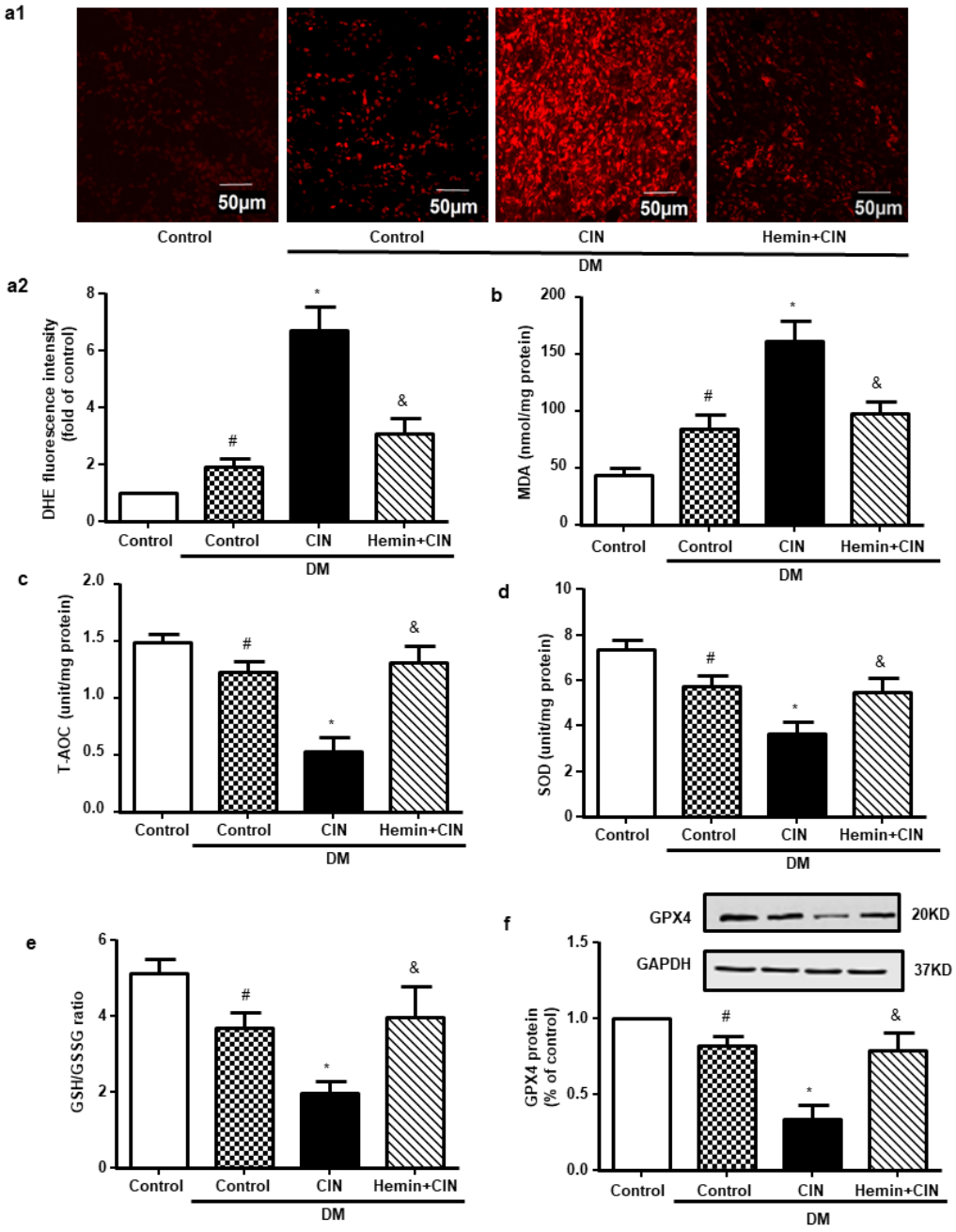

Fig. 3 Hemin reduced renal oxidative stress with increased GPX4 in diabetic CIN rats

Protocols of CIN model and hemin intervention was performed as Fig. 1. Kidney samples were collected at 24 h after CM injection. (**a1**) Tissue ROS level measured by DHE probing. Representative images were shown (scale bar = 50 μm). (**a2**) The fluorescence intensity of

DHE in each group. Values were presented with fold-change of control group. The renal levels of MDA (**b**), T-AOC (**c**), SOD (**d**) and GSH/GSSG ratio (**e**) were determined to evaluate oxidative stress. (**f**) Western blot of GPX4 (a regulator of ferroptosis). Values were presented as fold-change of control group. Data are expressed as mean ± SD. (n=7; #P<0.05 vs. normal control; *P<0.05 vs. DM control; &P<0.05 vs. CIN)

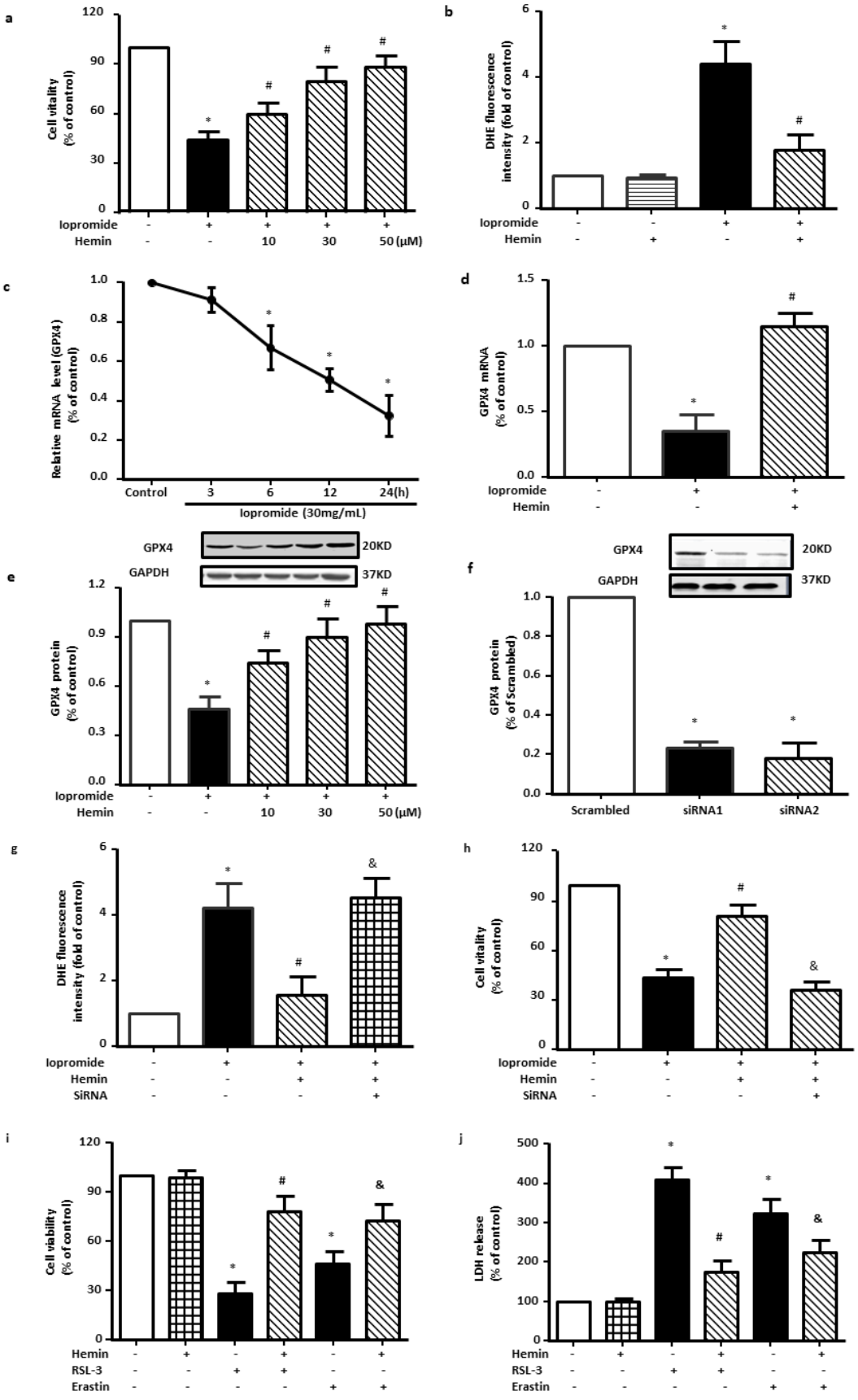

Fig. 4 Hemin-induced GPX4 is essential in protecting CM-insulted HK-2 cells

(**a**) HK-2 cells were incubated with CM iopromide (30mg(I)/mL) in the presence or absence of incremental concentrations of hemin (10-50 μM) for 24 hours. Cell vitality was determined by MTT assay. (**b**) Total ROS in CM-insulted cells incubated with or without hemin (50 μM) was determined by DHE fluorescence probing. Fluorescence intensity was analyzed by Image J. (**c**) GPX4 expression in cells incubated with iopromide (30mg(I)/mL) for incremental hours (3-24 h) was determined by q-PCR. (**d**) HK-2 cells were treated with iopromide (30mg(I)/mL)/ and hemin (50 μM) for 24 hours before GPX4 mRNA detection by q-PCR. (**e**) HK-2 cells were treated with iopromide (30mg(I)/mL) in the presence or absence of incremental concentrations of hemin (10-50 μM) for 24 hours. Protein expression of GPX4 was determined by immunoblotting. (**f**) HK-2 cells were transfected with different GPX4 siRNAs for 48 hours. GPX4 protein expression was determined by western blot. siRNA2 was selected for subsequent GPX4 interference test. (**g**) GPX4 siRNA transfected HK-2 cells were co-incubated with iopromide (30mg(I)/mL) or/and hemin (50 μM) for 24 hours. Total ROS in cells was detected by DHE fluorescence probing, and (**h**) cell vitality was determined by MTT assay. Data were presented as fold-change of control group.Data are expressed as mean ± SD. ($^{*}P<0.05$ vs. control; $^{\#}P<0.05$ vs. Iopromide alone; $^{\&}P<0.05$ vs. CM+Hemin).Further, HK-2 cells were incubated with RSL-3 (5 μM) or erastin (30 μM) in the presence or absence ofhemin (50 μM) for 24 hours. Cell vitality was determined by MTT assay(**i**) and LDH release assay (**j**).Values were presented as fold-change of control group.Data are expressed as mean ± SD.($^{*}P<0.05$ vs. control; $^{\#}P<0.05$ vs. RSL-3 alone; $^{\&}P<0.05$ vs. erastinalone)

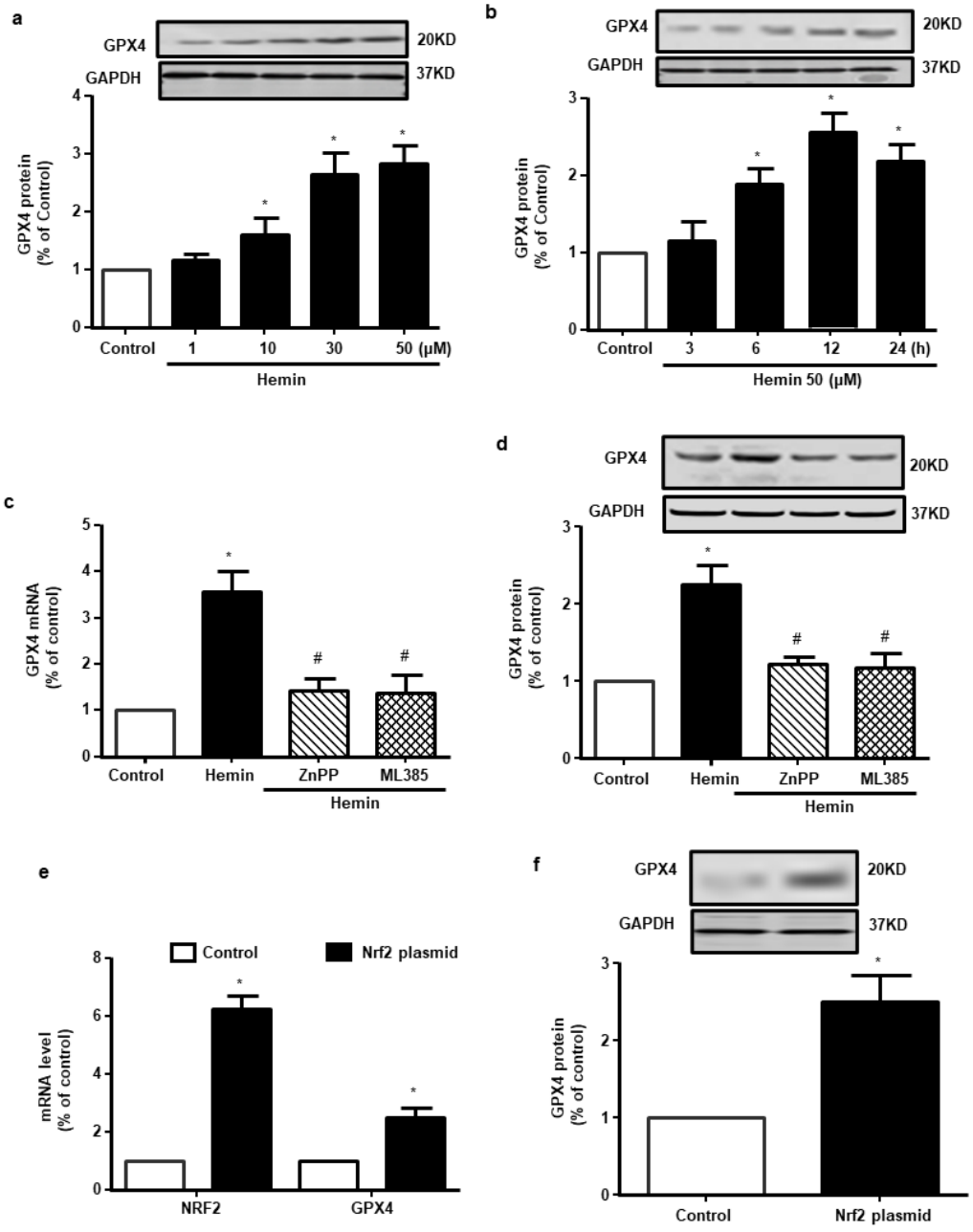

Fig. 5 Hemin promoted GPX4 expression through HO-1/NRF-2 pathway in HK-2 cells

(**a**) and (**b**) GPX4 protein level was determined by western blot after HK-2 cells were incubated with incremental concentrations (1-50μM) of hemin alone for 24h or with a fixed concentration of hemin (50μM) for incremental times (3-24h). (**c**) and (**d**) HK-2 cells were treated with 50 μM of hemin for 24 hours in the presence of either HO-1 inhibitor ZnPP (5 μM) or Nrf2 inhibitor ML385 (5 μM). The expression of GPX4 was detected by q-PCR and

western blot. (**e**) and (**f**) HK-2 cells were transfected with plasmid carrying Nrf2 over-expression genes. GPX4 and Nrf2 expressions in cells were determined by q-PCR. GPX4 protein expression was determined by western blot.Data were presented as fold-change of control group.Data are expressed as mean ± SD. ($^{*}P<0.05$ vs. control, $^{\#}P<0.05$ vs. hemin alone)

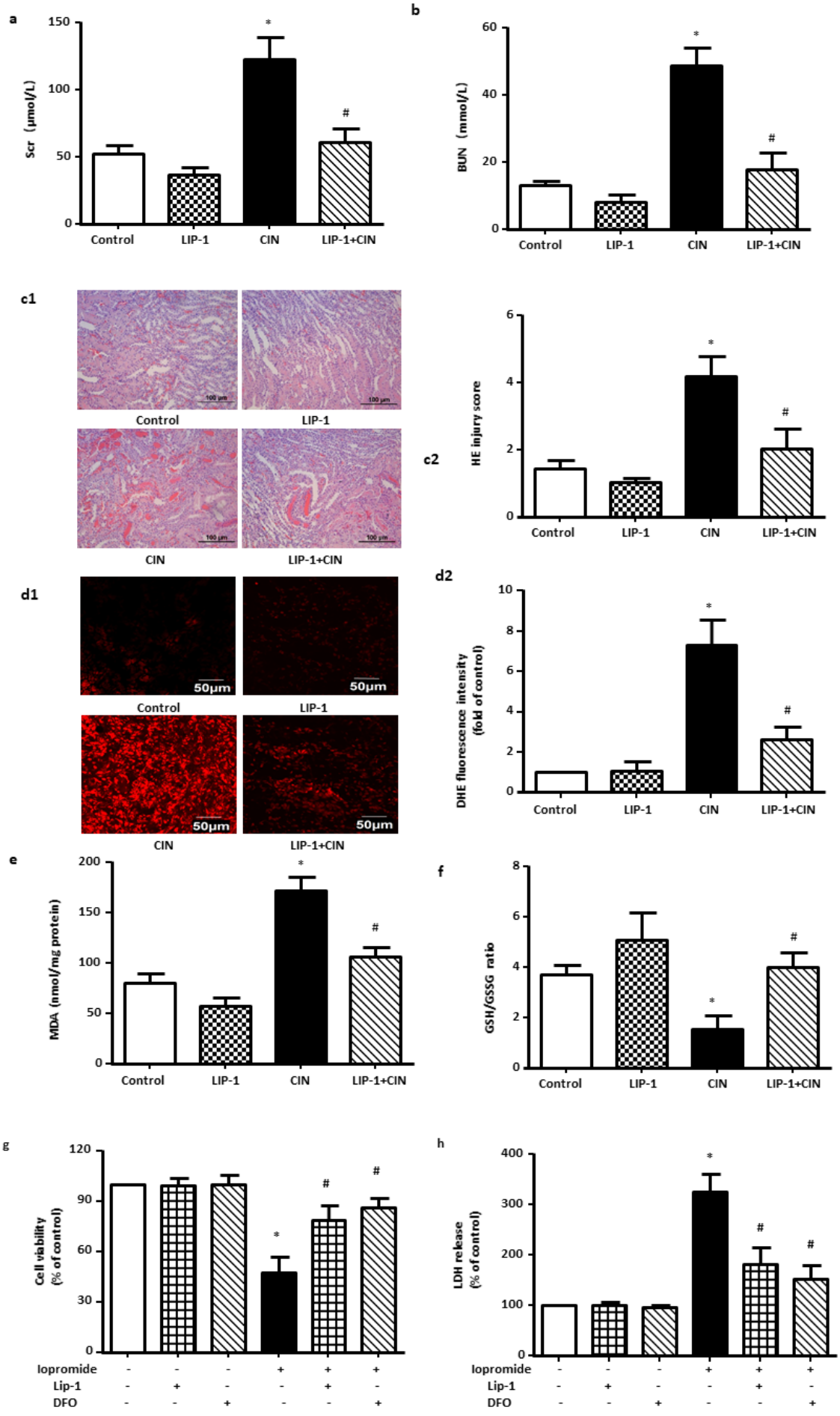

Fig. 6 LIP-1 prevented renal injury in diabetic CIN rats

LIP-1 (10 mg/kg wt) was intraperitoneally injected right after the successful operation of CIN in diabetic rats. Serum Cr (**a**) and BUN (**b**) levels were measured 24 h after CM injection. Kidney samples were collected 24 h after CM injection. (**c1**) Representative images of HE staining in renal outer medulla (scale bar = 100 μm). (**c2**) Medullary pathological scores were calculated. (**d1**) Tissue ROS level measured using DHE probing. Representative images were shown (scale bar = 50 μm). (**d2**) The fluorescence intensity of DHE in each group was calculated. Values were presented with fold-change of control group. The renal levels of MDA (**e**) and GSH/GSSG ratio (**f**) were measured.Data are expressed as mean ± SD. (n=7, $^{*}P<0.05$ vs. control, $^{\#}P<0.05$ vs. CIN).In addition, cell vitality in CM-insulted HK-2 cells incubated with or without LIP-1 (200nM) or DFO (10μM)was determined by MTT assay(**g**) and LDH release assay (**h**).Data were presented with fold-change of control group. Data are expressed as mean ± SD.($^{*}P<0.05$ vs. control; $^{\#}P<0.05$ vs. Iopromide alone)