Basic Research

5-aminolevulinic acid exerts renoprotective effect via Nrf2 activation in murine rhabdomyolysis-induced acute kidney injury

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ABSTRACT

Aim: Acute kidney injury (AKI) is associated with chronic kidney disease (CKD), as well as high mortality, but effective treatments for AKI are still lacking. A recent study reported the prevention of renal injury, such as ischemia-reperfusion injury (IRI), by 5-aminolevulinic acid (ALA), which induces an antioxidant effect. The current study aimed to investigate the effect of ALA in a rhabdomyolysis-induced mouse model of AKI created by intramuscular injection of 50% glycerol.

Methods: Rhabdomyolysis-induced AKI was induced by an intramuscular injection of glycerol (5 ml/kg body weight) into mice. Administration of ALA (30 mg/kg, by gavage) was started from 48 hours before or 24 hours after glycerol injection. The mice were sacrificed at 72 hours after glycerol injection. The roles of nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1), which is one of the Nrf2-related antioxidants, were further investigated through in vivo and in vitro methods.

Results: ALA markedly reduced renal dysfunction and tubular damage in mice with rhabdomyolysis-induced AKI. ALA administration decreased oxidative stress, macrophage infiltration, and inflammatory cytokines and apoptosis. The expression of Nrf2 was upregulated by ALA administration. However, administration of Zinc protoporphyrin-9 (ZnPPIX) to inhibit HO-1 activity did not abolish these improvements by ALA. The expression of Nrf2-associated antioxidant factors other than HO-1 was also increased.

Conclusions: These findings indicate that ALA exerts its antioxidant activity via Nrf2-associated antioxidant factors to provide a renoprotective effect against rhabdomyolysis-induced AKI.

Keywords: aminolevulinic acid, antioxidant effect, HO-1, Nrf2, rhabdomyolysis-induced AKI

INTRODUCTION

As the general population ages, the prevalence of diseases such as diabetes, hypertension, and cardiovascular disease is also increasing. One consequence has been an increased incidence of acute kidney injury (AKI), which multiple cohort studies have identified as an independent risk factor for mortality.[1-3] Reduction of kidney function by AKI was previously thought to be temporary and reversible; however, epidemiological studies and meta-analysis have shown that AKI can lead to chronic kidney disease (CKD). One common injury target during AKI is tubulointerstitial disorder, which is also involved in the progression from AKI to CKD.[4] Therefore, amelioration of this disorder is important for the treatment of AKI, as well as for the prevention of progression from AKI to CKD. However, no effective treatment is presently available, other than hydration.

AKI is one of the most severe complications of rhabdomyolysis, a muscle degeneration and myoglobin leakage syndrome that can develop from many causes such as crush syndrome, drugs, infections, and other diseases. The global prevalence of rhabdomyolysis is not fully known and appears to be underestimated, but no large epidemiologic studies or updated registers on rhabdomyolysis are available.[5] Recent publications indicate that rhabdomyolysis causes between 7% and 10% of the AKI cases each year in the United States,[6] so that an estimated 4% to 33% of patients with rhabdomyolysis will develop AKI.[7]

The detailed mechanism by which rhabdomyolysis induces AKI has not been fully elucidated, but it appears to involve oxidative stress, inflammation, myoglobin casts, myoglobin toxicity, and apoptosis of tubular cells.[8] These pathological processes activate and/or upregulate multiple signaling pathways and numerous genes involved in cell death and inflammatory responses.[8, 9] As an endogenous natural amino acid and mediator of heme

synthesis, 5-aminolevulinic acid (ALA) shows renoprotective effects by inducing heme oxygenase-1 (HO-1) expression against renal ischemia-reperfusion injury (IRI) and cisplatin nephropathy in the mouse kidney,[10, 11] whereas ALA ameliorates cardiomyocyte hypertrophy via nuclear factor erythroid 2-related factor 2 (Nrf2) activation.[12] Transcription factor Nrf2 upregulates antioxidant genes such as NAD(P)H: quinone oxidoreductase 1 (NQO1), HO-1, and glutamate-cysteine ligase modifier (GCLM), thereby decreasing oxidative stress.[13, 14] However, the effects of ALA have not yet been investigated in rhabdomyolysis-induced AKI. Focusing on Nrf2, especially HO-1, we examined the potential renoprotective effects of ALA in rhabdomyolysis-induced AKI.

METHODS

Animal and models of rhabdomyolysis-induced AKI

We induced rhabdomyolysis in age-matched, 10-week-old C57BL/6J male mice by a single intramuscular injection of 50% glycerol in saline to the femoral muscle. The animals were fed standard laboratory animal chow and had free access to tap water, but were deprived of drinking water for 24 hours before and after the injection. Body weight was recorded, and blood pressure was measured by the tail-cuff method with an automatic sphygmomanometer (BP98A; Softron, Tokyo, Japan). The mice were placed in metabolic cages for 24 hours to collect urine and were finally sacrificed 72 hours after the glycerol injection under sevoflurane inhalation anesthesia for collection of kidney tissue and blood samples.

The mice were randomly divided into the following four groups (n=8):

- a control group (Cont) given saline (5 ml/kg body weight, intramuscular [i.m.]);
- a rhabdomyolysis group (Rhab) given 50% glycerol (5 ml/kg body weight, [i.m.]);
- an ALA (Sigma-Aldrich, St. Louis, MO, USA) premedication treatment group (Rhab/Pre-ALA) treated with ALA (ALA: 30 ml/kg/day and ferrous citrate

[Eisai, Tokyo, Japan] 15 mg/kg/day, by gavage) each day from 48 hours before the glycerol injection; and

- an ALA treatment group (Rhab/ALA) treated with ALA each day from 24 hours after the glycerol injection.

The same amount of phosphate-buffered saline (PBS) as administration of ALA was administered for gavage to ALA non-administration groups.

In an additional experiment, mice were randomly assigned into the following four groups (n=8):

- a control group (Cont);
- a rhabdomyolysis group (Rhab);
- a Rhab/Pre-ALA group; and
- an ALA premedication + Zinc protoporphyrin-9 (ZnPPIX) (Enzo Life Sciences, Farmingdale, NY, USA) group (Rhab/Pre-ALA+HO-1i) treated with ALA and ZnPPIX (50 mg/kg body weight was injected [intraperitoneally] 30 minutes before each ALA administration).

The same amount of PBS as administration of ALA was administered by gavage to ALA non-administration groups, and the same amount of PBS as administration of ZnPPIX was administered intraperitoneally in non-ZnPPIX administration groups.

Cell Culture

We used human proximal tubule epithelial cells (hPTECs) at passage 8–10 (Lonza, USA). The cells were cultured in 12-well plates and propagated in REGM™ Renal Epithelial Cell Growth Medium BulletKit™ (Lonza) medium supplemented with 5% heat-inactivated newborn bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37℃ in 5% carbon dioxide.

Histopathological examination

Kidney sections (4 μm thick) were prepared from paraffin-embedded tissues. The severity of the tubulointerstitial injury was evaluated by examining 10 fields of randomly selected tissue samples (Hematoxylin-Eosin (H-E) staining). Blue-stained scarred areas were quantified using a color image analyzer (WinROOF; Mitani, Fukui, Japan).

Immunohistochemical analysis

Serial cryostat sections (4 μm thick) of paraffin-embedded specimens were rehydrated in PBS and subjected to antigen retrieval in a microwave or treated with proteinase K (Dako Japan, Kyoto, Japan). An antibody against F4/80 (MCA497GA; AbD Serotec, Raleigh, NC, USA) was used, and detection was carried out using the Histofine Simple Stain Max-Po kit (Nichirei, Tokyo, Japan) and 3,3-diaminobenzidine (Sigma-Aldrich). The number of F4/80-postive cells in 20 fields of the tubular interstitium at high magnification (×400) was counted, and the average for each mouse was expressed as the number of positive cells per field.

Physiologic and biochemical analyses

Blood samples obtained at the time of killing were assessed by enzymatic methods for serum creatinine and blood urea nitrogen. Urinary neutrophil gelatinase-associated lipocalin (NGAL) levels and urinary creatinine (UCr) were measured using commercially available ELISA kits (BioPorto, Gentofte, Denmark) and LabAssay™ Creatinine (Wako Pure Chemical Industries, Osaka, Japan), respectively. Urine samples were centrifuged at 5,000 × g for 10 min, and, after proper dilution, the supernatant was used for the determination of 8-hydroxy-2'-deoxyguanosine (8-OHdG) by a competitive ELISA kit (Highly sensitive 8-OHdG Check ELISA; Japan Institute for the Control of Aging, Fukuroi, Shizuoka). The determination range was 0.125–10 ng/ml. Urinary 8-OHdG was expressed as the total amount excreted in 24 hours. The urinary 8-OHdG/creatinine ratio was alternatively used in analysis, with consistent results.

Western blot analysis

Kidney and cell lysates were prepared as described previously.[15, 16] Protein samples (60–80 μg per lane) were subjected to immunoblotting analysis with antibodies against α-actin (Cell Signaling Technology, Danvers, MA, USA), β-actin (Sigma-Aldrich), GAPDH (Merck Millipore, Danvers, MA, USA), cleaved caspase-3 (Cell Signaling), Nrf2 (Cell Signaling), HO-1 (Abcam, Cambridge, UK), NQO1 (Abcam), and GCLM (Abcam). Signals were detected using an enhanced chemiluminescence system (GE Healthcare Japan, Tokyo, Japan).

Real-time reverse transcription–quantitative PCR

Total RNA was extracted from the kidney using TRIzol (Life Technologies, CA, USA) and complementary DNA (cDNA) was synthesized from 1 μg of total RNA using a ReverTra Ace® qPCR RT Master Mix (Toyobo Life Science, Osaka, Japan) according to the manufacturer's protocol. Subsequently, a quantitative real-time polymerase chain reaction (PCR) was performed with a THUNDERBIRD® Probe qPCR Mix (TaqMan) (Toyobo Life Science). The primers and probes for TaqMan analysis were designed using sequence information from GenBank using Primer 3 online software. RT-qPCR was performed using an AriaMx real-time PCR system (Agilent Technologies, Tokyo, Japan). Targeted gene expressions were calculated with absolute quantitation using calibration curves. Expression levels of the target gene were normalized to 18S rRNA and expressed with fold changes expression compared with the control group.

Detection of LDH release

Culture supernatants were collected, centrifuged at 200 × g for 5 minutes, and transferred to new tubes. Lactate dehydrogenase (LDH) activity was measured using an LDH cytotoxicity detection kit (TaKaRa BIO, Shiga, Japan).

WST-1 assay

The percentage of surviving cells was measured with a premixed water-soluble tetrazolium salt-1 (WST-1) Cell Proliferation Assay System (TaKaRa BIO). The percentage of surviving cells was calculated using the following formula: percentage of surviving cells (experimental optical density cell-free optical density) / (untreated optical density cell-free optical density) x 100.

Transient transfection with siRNA

Cells were cultured to 70% to 80% confluence on a 6-well plate. SiRNA was transfected by using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies) with Control-siRNA (Santa Cruz Biotechnology, Dallas, TX, USA) and Nrf2-siRNA (Santa Cruz Biotechnology) at a concentration of 12.5 nmol/well. After 24 hours from the transfection, the medium was changed to fresh Renal Epithelial Basal Medium (REBM), and various stimuli were performed. The extent of knockdown (KD) was assessed by Western blotting. Evaluation of antioxidant genes were assessed 6 hours after hemin stimulation.

Statistical analysis

The values are expressed as the mean ± standard error of the mean (SEM). Parameters were evaluated using a two-tailed, unpaired Student's *t*-test or one-way analysis

of variance for comparison of multiple means. A value of $p < 0.05$ was considered statistically significant.

RESULTS

ALA ameliorated renal dysfunction and tubular injury in rhabdomyolysis-induced AKI

Body weight was decreased in glycerol-injected groups compared to before glycerol injection. However, there were no significant differences in the rate of body weight change. There were also no significant differences in blood pressure, pulse rate and urine volume in glycerol-injected groups. Blood urea nitrogen and serum creatinine levels were significantly higher in the rhabdomyolysis group than in the control group. ALA ameliorated those parameters, especially in the ALA premedication group (Table 1). The H-E staining revealed a number of casts, extension of the tubular lumen, shedding of the renal tubular epithelial cells, and infiltration of inflammatory cells in the rhabdomyolysis group. These changes were attenuated by ALA treatment, especially in the ALA premedication group (Fig. 1a, b). Urinary NGAL levels were significantly increased in the rhabdomyolysis group compared with the control group. This change was attenuated by ALA treatment (Fig. 1c).

ALA reduced oxidative stress and upregulated HO-1

Urinary 8-OHdG excretion was significantly higher in the rhabdomyolysis group than in the control group, and it was attenuated by ALA treatment (Fig. 1d). Expression of HO-1 protein was higher in the rhabdomyolysis group than in the control group, and it was more elevated in the ALA treatment groups (Fig. 1e, f).

ALA reduced infiltration of macrophages, expression of inflammatory cytokines, and apoptosis

The rhabdomyolysis group showed significantly increased infiltration of macrophages, as evaluated by F4/80 staining. This change was suppressed by ALA treatment, especially in the ALA premedication group (Fig. 2a, b). The expression of mouse MCP-1, IL-1β, and TNFα mRNA was significantly higher in the rhabdomyolysis group than in the control group. These changes were ameliorated by ALA treatment (Fig. 2c–e). The apoptosis of tubules, evaluated by cleaved caspase-3 expression, was higher in the rhabdomyolysis group than in the control group. This change was significantly ameliorated by ALA treatment (Fig. 2f, g).

ALA protected hPTECs from hemin-induced cell injury

Cell death was measured by lactate dehydrogenase (LDH) assay. Stimulation of hemin increased LDH release to the medium, but this change was attenuated by ALA (Fig. 3a). Cell proliferation, measured by the WST-1 assay, was suppressed by stimulation of hemin, and this change was ameliorated by ALA (Fig. 3b). Cleaved caspase-3 expression was higher in the hemin group than in the control group, and this change was significantly ameliorated by ALA treatment. HO-1 protein expression was higher in the hemin group than in the control group, and it was more elevated in the ALA treatment groups (Fig. 3c–e).

ALA exerted a renoprotective effect even when HO-1 was inhibited

Renal dysfunction induced by rhabdomyolysis was ameliorated by ALA treatment, even when HO-1 was inhibited (Table 2). H-E staining revealed that HO-1 inhibition did not abolish the amelioration of rhabdomyolysis-induced renal tubular injury by ALA (Fig. 4a, b). Urinary 8-OHdG excretion and the expression of mRNA associated with inflammatory

cytokines were significantly higher in the rhabdomyolysis group than in the control group. These changes were ameliorated by ALA treatment despite the HO-1 inhibition (Fig. 4c–e). Expression of Nrf2, a transcription factor upstream of HO-1, was elevated by administration of ALA in rhabdomyolysis-induced AKI mice (Fig. 4f, g). We identified that Nrf2 expression was also elevated by ALA treatment in control mice (Supplemental Fig. 1). Other Nrf2-related factors, NQO1 and GCLM, were also elevated by ALA treatment (Fig. 4f, h–i). Cleaved caspase-3 expression was higher in the rhabdomyolysis group than in the control group. This change was significantly improved by ALA treatment, and this improvement was maintained despite the HO-1 inhibition (Fig. 4j, k).

Knockdown (KD) of Nrf2 exacerbated cell death in hemin-induced cell injury of hPTECs

The expression of Nrf2 and Nrf2-related genes were decreased by transfection of Nrf2 siRNA in hPTECs (Fig. 5a–e). The expression of NQO1 mRNA was not increased in the Nrf2-KD group even by hemin stimulation (Fig. 5c). However, the expression of HO-1 and GCLM mRNA were significantly increased in the Nrf2-KD + hemin group compared to the Nrf2-KD group (Fig. 5d–e). The extent of cytotoxicity due to hemin stimulation was exacerbated in the Nrf2 KD + hemin group compared to the control + hemin group (Fig. 5f).

DISCUSSION

This study confirmed that ALA attenuated the oxidative stress, inflammatory cytokine release, and macrophage infiltration into the tubulointerstitium associated with rhabdomyolysis-induced AKI, while preventing apoptosis, to exert a renoprotective effect. This effect was more significant in the group given ALA as a premedication. ALA

administration upregulated the expression of Nrf2 and Nrf2-related antioxidant genes. The specific HO-1 inhibitor, ZnPPIX, did not overcome the renoprotective effect of ALA against rhabdomyolysis-induced AKI. In vitro experiment, it was suggested that Nrf2 has a protective function through expression of antioxidant genes, especially NQO1, in hPTECs. These results suggested that ALA had a protective role against rhabdomyolysis-induced AKI via Nrf2 regulation.

HO-1 is an important enzyme that regulates the inflammatory response and biological defense reaction in tissue injury.[17, 18] The renoprotective effect of HO-1 has been demonstrated in various renal injury animal models. Salom et al. reported that HO-1 had a renoprotective effect via suppressing the excessive production of nitric oxide (NO) and peroxynitrite in IRI.[19] Shiraishi et al. reported that HO-1 ameliorated cisplatin nephropathy by preventing apoptosis.[20] Those mechanisms include deletion of endogenous heme; induction of cytoprotective substances such as bile pigment, carbon monoxide, and ferritin; and maintenance of iron homeostasis. These processes lead to vasorelaxant, antioxidant, anti-inflammatory, and anti-apoptotic effects and exert a renoprotective effect against AKI.[17, 21–24] Furthermore, ALA, an amino acid synthesized in mitochondria, is involved in heme metabolism and is known as an HO-1 inducer. Hou et al. reported that ALA had a renoprotective effect in an IRI model via upregulation of HO-1 and suppressing expression of TNFα and macrophage infiltration.[11] Terada et al. reported that ALA upregulated HO-1 and suppressed tubule cell apoptosis in cisplatin nephropathy.[10] However, in our experiment, the specific HO-1 inhibitor, ZnPPIX, did not overcome the renoprotective effect of ALA against rhabdomyolysis-induced AKI. This result indicates that the protective effect of HO-1 is limited, and other Nrf2-related antioxidants are strongly involved in the renoprotective effect of ALA in rhabdomyolysis-induced AKI. A relationship between ALA and Nrf2 has not been

previously established in AKI. The findings presented here indicate only a limited role for HO-1 in the antioxidant activity of ALA, whereas NQO1 and GCLM may be part of the main mechanism of the antioxidant-mediated renoprotective effect of ALA against rhabdomyolysis-induced AKI. This difference from previous reports on renoprotection in conventional AKI models indicates a need for further investigation of other AKI models using HO-1 inhibitors.

Previous reports have demonstrated that the mechanisms of rhabdomyolysis-induced AKI are caused by myoglobin flowing out of the destroyed muscle tissue and binding with Tamm-Horsfall protein in the renal tubule to form a cast that increases consumption of NO, vasoconstriction, and reactive oxygen species production, resulting in apoptosis and tubular cell damage.[5, 25, 26] Although it is considered that the main mechanism of renoprotective effect of ALA is a reduction of oxidative stress and inhibition of apoptosis, it is also notable that ALA reduces cast formation in tubule in our experiment. A recent report suggested that the apoptosis inhibitor of macrophage (AIM) accumulates on cell debris within the kidney proximal tubules during AKI and binds to the kidney injury molecule (KIM)-1, which is expressed in injured tubular epithelial cells. These processes enhance phagocytic removal of debris, thereby facilitating renal cell repair.[27] We did not investigate the detailed mechanism by which ALA decreased casts, but ALA could influence the debris clearance mechanism, which will be the subject of future investigation. Another possibility of renoprotective effect by ALA in rhabdomyolysis-induced AKI was amelioration of rhabdomyolysis. We checked levels of blood myoglobin after the glycerol injection in each group. However, no significant change occurred between treated and nontreated groups by ALA (data not shown). Therefore, the suppression of muscle damage by ALA is unlikely to be a preventive factor of renal injury in rhabdomyolysis-induced AKI.

In conclusion, ALA ameliorated renal dysfunction and inflammatory change in rhabdomyolysis-induced AKI. ALA increased the expression of Nrf2 and its downstream antioxidant factors, including NQO1, HO-1, and GCLM. Therefore, the renoprotective effect of ALA is attributable to an antioxidant effect exerted through induction of Nrf2. ALA could have therapeutic benefits as a remedy for rhabdomyolysis-induced AKI, for which a cure other than rehydration has not been found.

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CONFLICT OF INTEREST STATEMENT

This investigation was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No. 15H04838 to N.K.), the Research Project Grant from Kawasaki Medical School (No. 28D-002 to A.U.), and scholarship donations from Kyowa Hakko Kirin and Chugai Pharmaceutical. A portion of the results from this study was presented as an abstract at the Annual Meeting of the American Society of Nephrology, November 15–20, 2016, in Chicago, Illinois.

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Figure legends

Figure 1. Evaluation of tubular injury, oxidative stress, and HO-1 expression in mouse kidney after rhabdomyolysis.

(a) H-E staining of tubules. Bar = 100 μm. (b) Tubular injury score (semiquantitative interstitial injury in the renal cortico-medullary junction). (c) Urinary NGAL excretion. (d) Urinary 8-OHdG excretion. (e) Representative Western blot for HO-1. (f) Western blot analysis of HO-1.

n=8 in each group. Data are expressed as mean ± SEM. $^{*}P < 0.05$ versus control mice; $^{\dagger}P < 0.05$ versus rhabdomyolysis-induced AKI mice; $^{\ddagger}P < 0.05$ versus rhabdomyolysis-induced AKI mice pretreated with 5-aminolevulinic acid (pre-ALA).

Figure 2. In vivo evaluation of infiltration of macrophages, inflammation-associated mRNA expression, and apoptosis in mouse kidney after rhabdomyolysis.

(a) F4/80 staining of tubules. Bar = 20 μm. (b) F4/80 positive cell area. (c) Expression of MCP-1 mRNA. (d) Expression of IL-1β mRNA. (e) Expression of TNFα mRNA. (f) Representative Western blot of cleaved caspase-3. (g) Western blot analysis of cleaved caspase-3.

n=8 in each group. Data are expressed as mean ± SEM. $^{*}P < 0.05$ versus control mice; $^{\dagger}P < 0.05$ versus rhabdomyolysis-induced AKI mice; $^{\ddagger}P < 0.05$ versus rhabdomyolysis-induced AKI mice pretreated with 5-aminolevulinic acid (pre-ALA).

Figure 3. Evaluation of the protective effect of 5-aminolevulinic acid (ALA) against hemin in human proximal tubule epithelial cells (hPTECs).

(a) LDH assay. (b) WST-1 assay. (c) Representative Western blot of cleaved caspase-3 and HO-1. (d) Western blot analysis of cleaved caspase-3. (e) Western blot analysis of HO-1.

Data are expressed as mean ± SEM. $^{*}P < 0.05$ versus control group; $^{\dagger}P < 0.05$ versus hemin group.

Figure 4. In vivo evaluation of the renoprotective effect of ALA under the condition of HO-1

inhibition in mouse kidney after rhabdomyolysis.

(a) H-E staining of tubules. Bar = 100 μm. (b) Tubular injury score (semiquantitative interstitial injury in the renal cortico-medullary junction). (c) Urinary 8-OHdG excretion. (d) Expression of F4/80 mRNA. (e) Expression of TNFα mRNA. (f) Representative Western blot of Nrf2, NQO1, and GCLM. (g) Western blot analysis of Nrf2. (h) Western blot analysis of NQO1. (i) Western blot analysis of GCLM. (j) Representative Western blot of cleaved caspase-3. (k) Western blot analysis of cleaved caspase-3.

n=8 in each group. Data are expressed as mean ± SEM. $^{*}P < 0.05$ versus control mice; $^{\dagger}P < 0.05$ versus rhabdomyolysis-induced AKI mice; $^{\ddagger}P < 0.05$ versus pre-ALA treated rhabdomyolysis-induced AKI mice.

Figure 5. Evaluation of the protective effect of Nrf2 against hemin in hPTECs.

(a) Representative Western blot of Nrf2. (b) Western blot analysis of Nrf2. (c) Expression of NQO1 mRNA. (d) Expression of HO-1XZ mRNA. (e) Expression of GCLM mRNA. (f) LDH assay. Data are expressed as mean ± SEM. $^{*}P < 0.05$ versus control group; $^{\dagger}P < 0.05$ versus control + hemin group; $^{\ddagger}P < 0.05$ versus siRNA group.

Supplemental Figure 1. In vivo evaluation of Nrf2 expression by ALA.

(a) Representative Western blot of Nrf2. (b) Western blot analysis of Nrf2.

Data are expressed as mean ± SEM. $^{*}P < 0.05$ versus control mice

Table 1. Physiological and biochemical characteristics of mice in the experiment using ALA

Group	Cont	Rhab	Rhab/Pre-ALA	Rhab/ALA
Blood pressure (mmHg)	102 ± 2	101 ± 2	106 ± 1	103 ± 2
Pulse rate (/min)	541 ± 34	497 ± 30	510 ± 27	521 ± 21
Baseline body weight (g)	26.0 ± 0.6	26.4 ± 0.9	24.8 ± 0.5	24.3 ± 0.4†
Body weight (g)	26.3 ± 0.3	24.4 ± 0.9	23.7 ± 0.7	22.3 ± 0.4†
Change rate of body weight (%)	101.4 ± 1.4	92.4 ± 2.5†	95.7 ± 1.9	92.6 ± 1.0†
Kidney/Body weight (%)	1.21 ± 0.04	1.67 ± 0.09†	1.25 ± 0.06‡	1.49 ± 0.05
Urine volume (ml)	1.7 ± 0.1	2.4 ± 0.4	1.4 ± 0.2	1.7 ± 0.3
UN (mg/dl)	28.1 ± 1.8	149.7 ± 24.1†	24.8 ± 1.3‡	110.7 ± 21.5†,§
Serum Cre (mg/dl)	0.07 ± 0.02	0.72 ± 0.21†	0.11 ± 0.01‡	0.41 ± 0.07

n=8 in each group. UN, urea nitrogen; Cre, creatinine. Values are expressed as mean ± SEM. † $P < 0.05$ vs. Cont, ‡ $P < 0.05$ vs. Rhab, § $P < 0.05$ vs. Rhab/Pre-ALA.

Table 2. Physiological and biochemical characteristics of mice in the experiment using ALA and ZnPPIX

Group	Cont	Rhab	Rhab /Pre-ALA	Rhab /Pre-ALA+HO-1i
Baseline body weight (g)	22.8 ± 0.2	23.5 ± 0.3	22.1 ± 0.4‡	24.5 ± 0.3†,§
Body weight (g)	23.4 ± 0.3	21.6 ± 0.5	21.1 ± 0.4	23.0 ± 0.6
Change rate of body weight (%)	102.9 ± 0.9	91.8 ± 1.3†	95.6 ± 0.5	95.6 ± 2.7
Kidney/Body weight (%)	0.91 ± 0.02	1.12 ± 0.05†	0.90 ± 0.03‡	0.81 ± 0.06‡
Urine volume (mL)	1.0 ± 0.1	1.2 ± 0.4	1.4 ± 0.2	1.2 ± 0.2
UN (mg/dL)	19.5 ± 1.2	181.7 ± 46.0†	37.5 ± 10.9‡	48.2 ± 20.5‡
Serum Cre (mg/dL)	0.09 ± 0.01	1.28 ± 0.42†	0.18 ± 0.04‡	0.33 ± 0.19‡

n=8 in each group. UN, urea nitrogen; Cre, creatinine. Values are expressed as mean ± SEM. † $P < 0.05$ vs. Cont, ‡ $P < 0.05$ vs. Rhab, § $P < 0.05$ vs. Rhab/Pre-ALA.

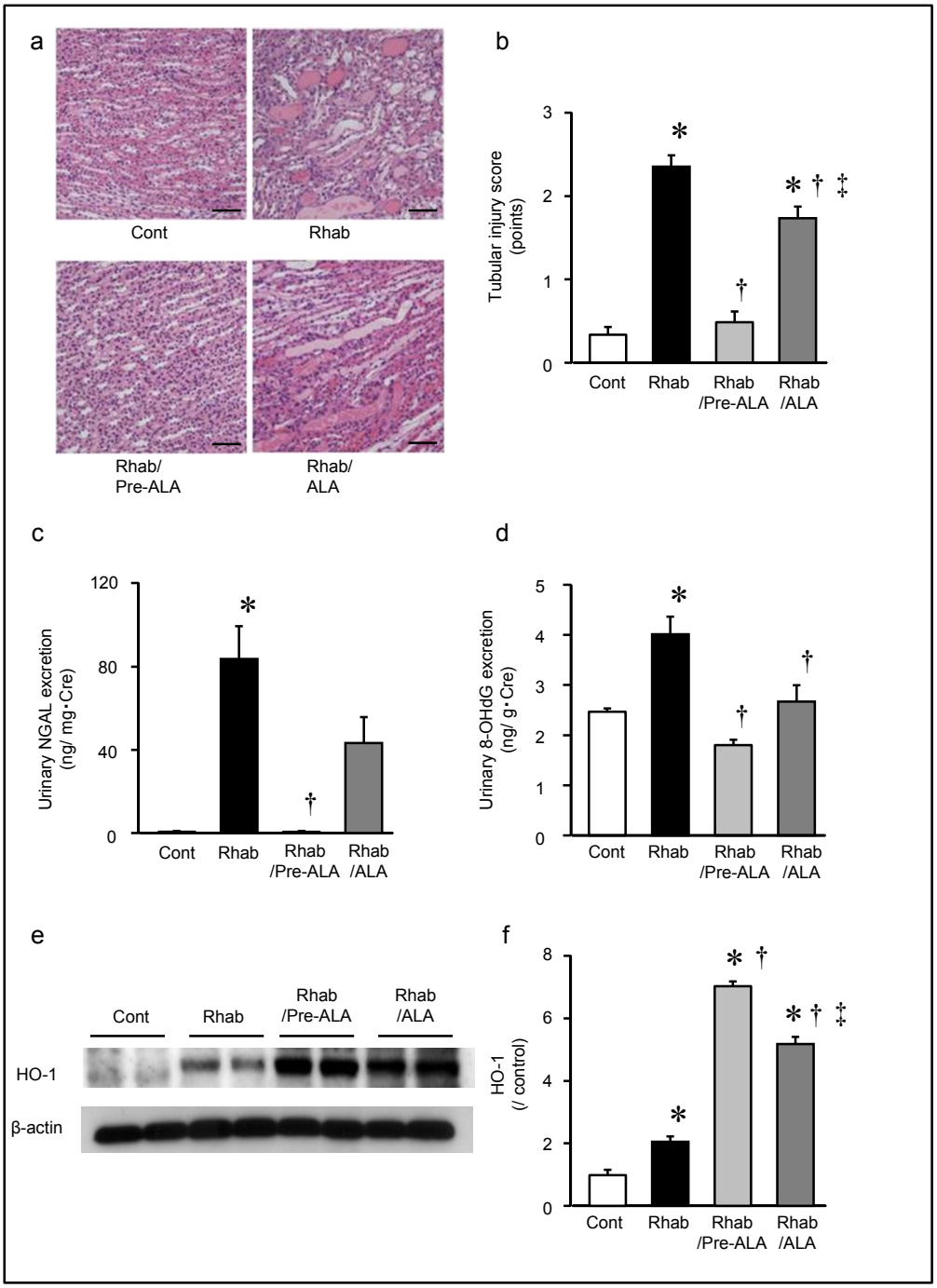

Figure 1. Evaluation of tubular injury, oxidative stress, and HO-1 expression in mouse kidney after rhabdomyolysis.

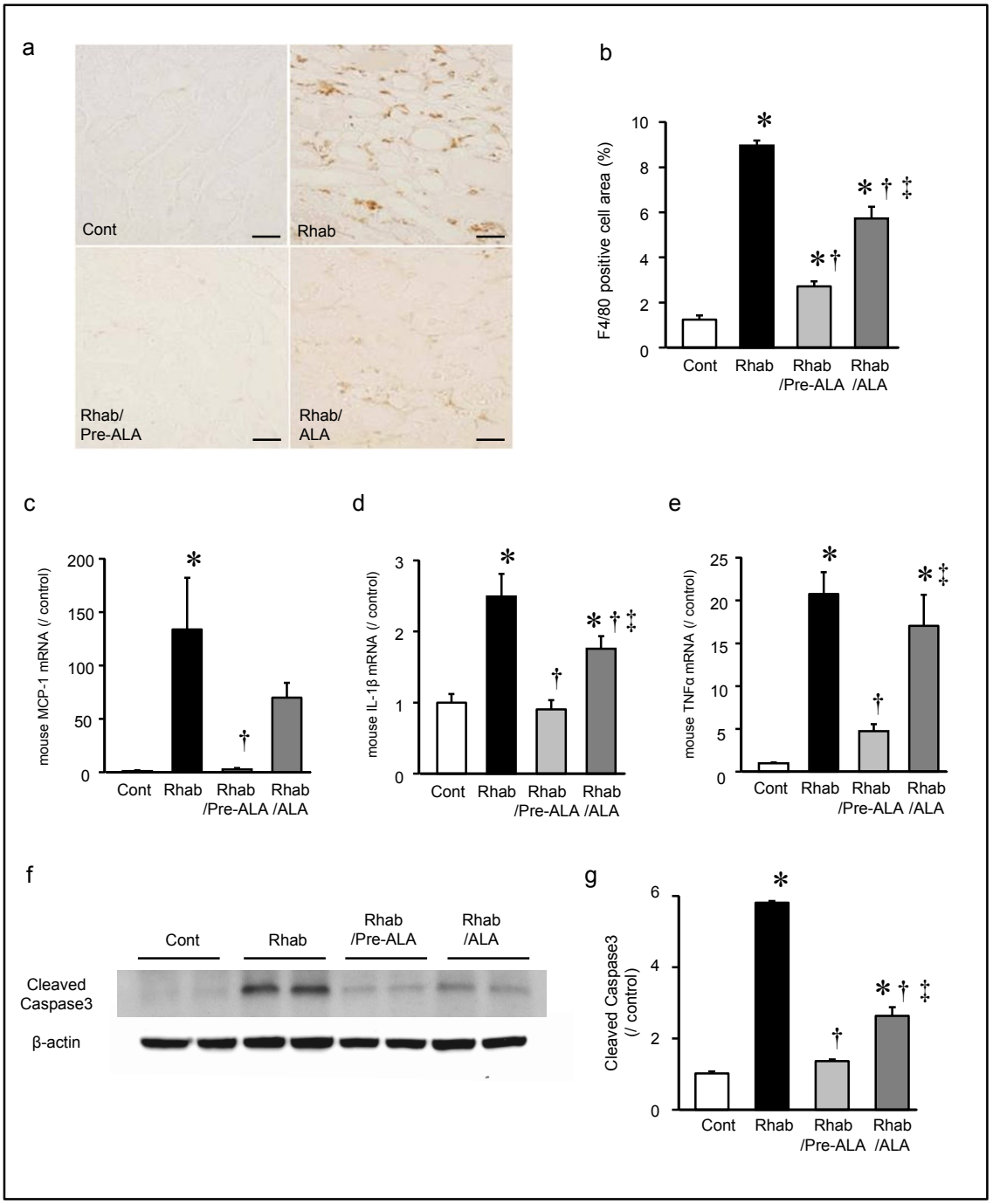

Figure 2. In vivo evaluation of infiltration of macrophages, inflammation-associated mRNA expression, and apoptosis in mouse kidney after rhabdomyolysis.

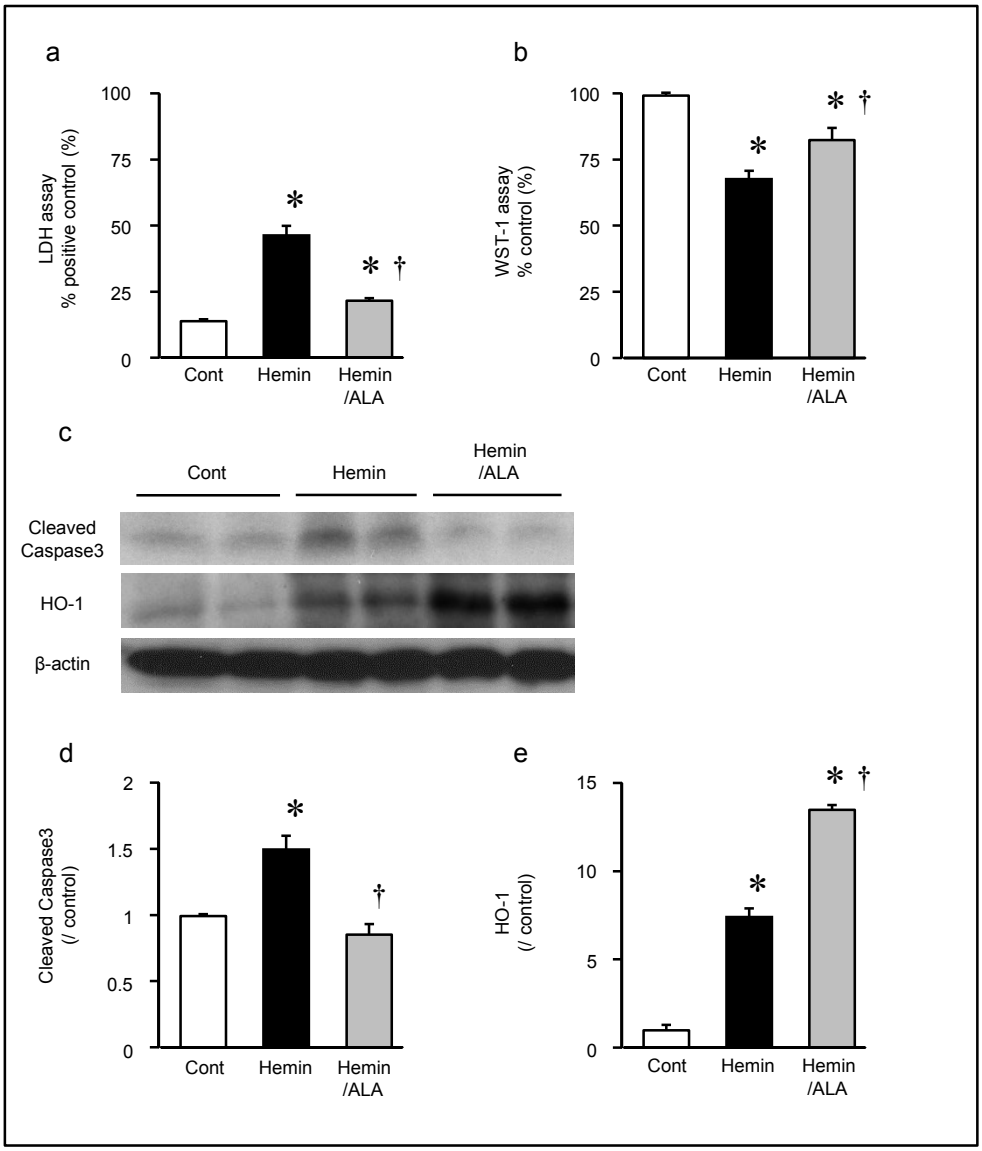

Figure 3. Evaluation of the protective effect of 5-aminolevulinic acid (ALA) against hemin in human proximal tubule epithelial cells (hPTECs).

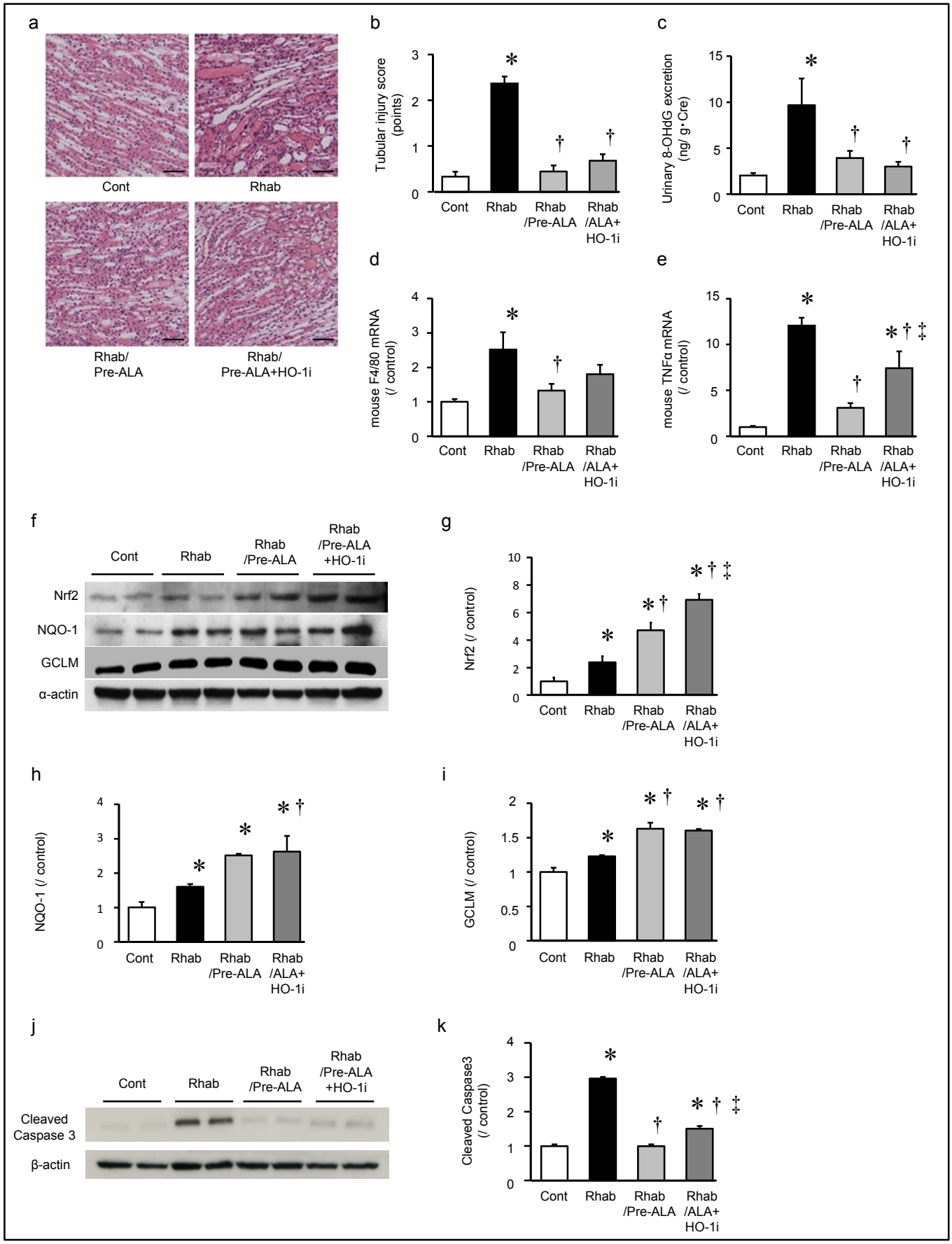

Figure 4. In vivo evaluation of the reno-protective effect of 5-aminolevulinic acid (ALA) under the condition of HO-1 inhibition in mouse kidney after rhabdomyolysis.

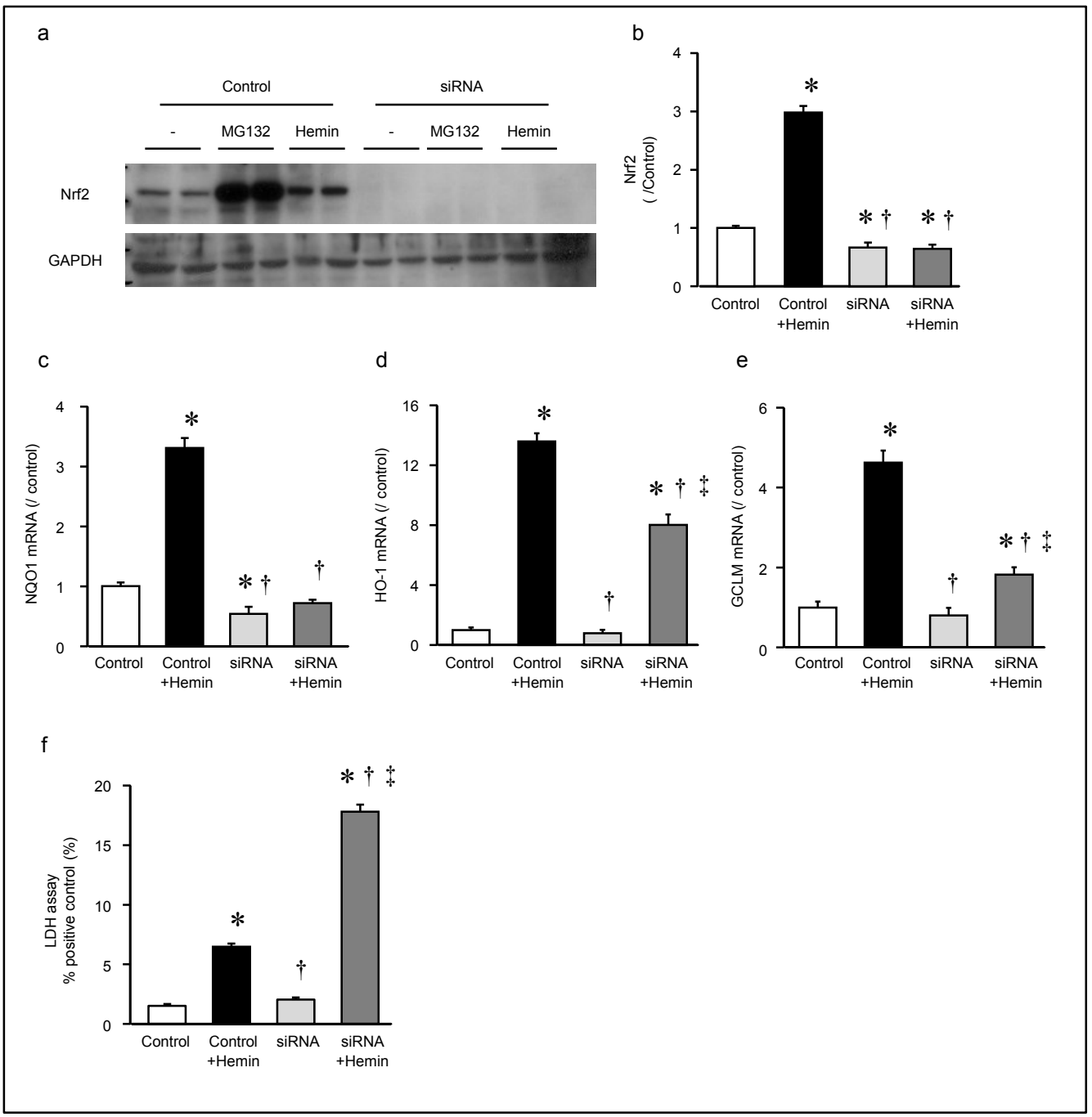

Figure 5. Evaluation of the protective effect of Nrf2 against hemin in hPTECs.

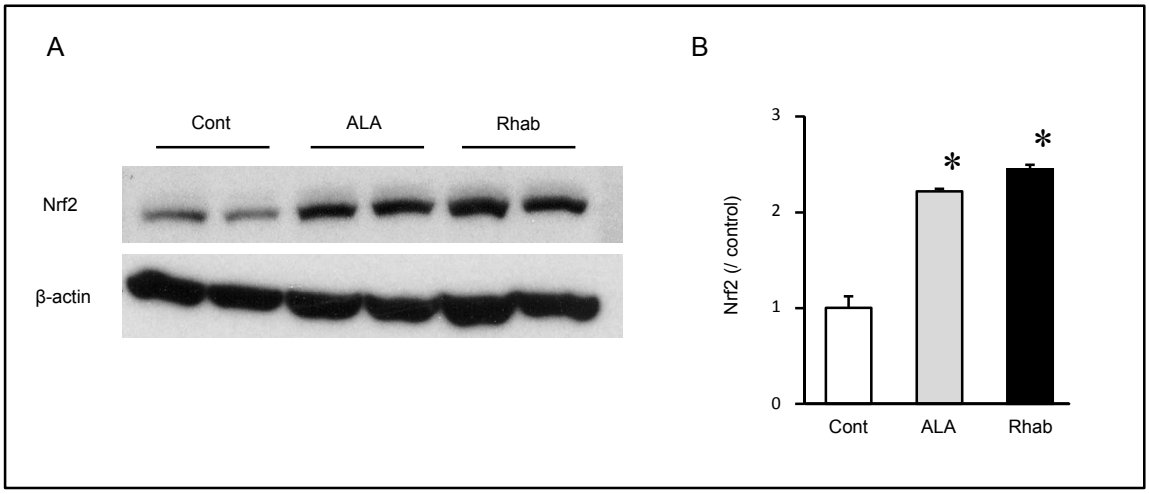

Fig. S1. In vivo evaluation of Nrf2 expression by ALA.