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5-Aminolevulinic acid combined with ferrous iron induces carbon monoxide generation in mouse kidneys and protects from renal ischemia-reperfusion injury

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Hou J, Cai S, Kitajima Y, Fujino M, Ito H, Takahashi K, Abe F, Tanaka T, Ding Q, Li XK. 5-Aminolevulinic acid combined with ferrous iron induces carbon monoxide generation in mouse kidneys and protects from renal ischemia-reperfusion injury. *Am J Physiol Renal Physiol* 305: F1149–F1157, 2013. First published July 31, 2013; doi:10.1152/ajprenal.00275.2013.—Renal ischemia reperfusion injury (IRI) is a major factor responsible for acute renal failure. An intermediate in heme synthesis, 5-aminolevulinic acid (5-ALA) is fundamental in aerobic energy metabolism. Heme oxygenase (HO)-1 cleaves heme to form biliverdin, carbon monoxide (CO), and iron (Fe^{2+}), which is used with 5-ALA. In the present study, we investigated the role of 5-ALA in the attenuation of acute renal IRI using a mouse model. Male Balb/c mice received 30 mg/kg 5-ALA with Fe^{2+} 48, 24, and 2 h before IRI and were subsequently subjected to bilateral renal pedicle occlusion for 45 min. The endogenous CO concentration of the kidneys from the mice administered 5-ALA/Fe^{2+} increased significantly, and the peak concentrations of serum creatinine and blood urea nitrogen decreased. 5-ALA/Fe^{2+} treatments significantly decreased the tubular damage and number of apoptotic cells. IRI-induced renal thiobarbituric acid-reactive substance levels were also significantly decreased in the 5-ALA/Fe^{2+} group. Furthermore, mRNA expression of HO-1, TNF-α, and interferon-γ was significantly increased after IRI. Levels of HO-1 were increased and levels of TNF-α and interferon-γ were decreased in the 5-ALA/Fe^{2+}-pretreated renal parenchyma after IRI. F4/80 staining showed reduced macrophage infiltration, and TUNEL staining revealed that there were fewer interstitial apoptotic cells. These findings suggest that 5-ALA/Fe^{2+} can protect the kidneys against IRI by reducing macrophage infiltration and decreasing renal cell apoptosis via the generation of CO.

5-aminolevulinic acid; carbon monoxide; hemeoxygenase-1; kidney; ischemia-reperfusion injury; oxidative stress

RENAL ISCHEMIA-REPERFUSION INJURY (IRI) is a complex pathophysiological process involving programmed cell death (PCD) and oxidant damage that leads to acute renal failure (AFR). The mortality rate of ARF remains between 50% and 70% among patients who receive intensive care who require dialysis and ranges between 25% and 100% in postoperative patients suffering from ARF. Renal IRI is unavoidable in renal transplantation and may lead to acute posttransplant tubular necrosis (21, 24, 49, 55) and delayed graft function (27, 47). Recent findings have indicated that carbon monoxide (CO), an endogenous byproduct of heme degradation through the heme oxygenase (HO) system, exerts cytoprotective effects by reducing the expression of proinflammatory mediators, preventing vascular constriction, decreasing platelet aggregation, and inhibiting apoptosis (41, 66). Subsequent studies have actively used exogenous CO to treat various experimental disease conditions. In the field of transplantation, CO has been shown to inhibit acute and chronic allograft rejection (37, 57) and the rejection of xenografts (53). Furthermore, studies in the area of renal disease have reported that CO-releasing molecules (CORMs) protect against the renal damage in ischemia-induced ARF in mice (63), cisplatin-induced nephrotoxicity in rats (60), and cold ischemia and reperfusion injury during kidney transplantation of both iso- and allografts in rats (5) and mice (55).

5-Aminolevulinic acid (5-ALA), an intermediate in heme synthesis, is fundamental for aerobic energy metabolism. It is used as a photo sensitizer precursor for photodynamic diagnosis and photodynamic therapy to identify and kill tumor cells (8, 23). 5-ALA showed very low toxicity because of the substance's short half-life. The half-Life of 5-ALA after oral administration (20 mg/kg) is 55.2 min in humans (unpublished data), 45 min in humans (100 mg) (10), and 40.7 min in dogs (7.29 mg/kg) (9). Therefore, there is almost no clinically relevant photosensitivity.

HO isoforms catalyze the conversion of heme to CO and biliverdin/bilirubin with the concurrent release of iron (Fe^{2+}). Many studies have demonstrated that exogenously added biliverdin/bilirubin and CO exert strong antioxidant and protective effects during renal IRI (29). For instance, Adin et al. (1) demonstrated that bilirubin treatment resulted in a significant improvement of renal vascular resistance, urine output, glomerular filtration rate, tubular function, and mitochondrial integrity after IRI. Neto et al. (39) showed that, in a rat model of transplant-induced IRI, exposure of CO to the recipients resulted in a significant improvement of graft renal functions. The authors also observed ultrastructural improvement using transmission electron microscopy evidencing viable podocytes, the preservation of foot processes, less frequent vacuolization, and the maintenance of internal cellular architecture. Furthermore, CO inhalation resulted in a significant reduction of PCD of tubular epithelial cells and, similar to biliverdin administration, showed a significant decrease in IL-6, IL-1β, ICAM-1, indicible nitric oxide synthase, and nitrite/nitrate formation (39). Nakao et al. (36) showed that in renal transplant recipients, treatment with CO gas or biliverdin alone failed to

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recover creatinine clearance decrease as well as proteinuria; in contrast, all these parameters were normalized by the concomitant administration of CO gas and biliverdin. Furthermore, IRI-induced upregulation of proinflammatory mediators and the extravasation of inflammatory infiltrates were significantly less with dual treatment than untreated controls (36). These previous reports have suggested that biliverdin/bilirubin and CO probably act through different mechanisms, because, in addition to the results of the above-mentioned report (36), additive protection was observed when the two compounds were administered simultaneously (33).

Renal ischemia is a consequence of arterial occlusion, shock, and organ transplantation and is a common cause of renal cell death, delayed graft function, renal graft rejection, and ARF, which has a complex pathophysiology with a number of contributing factors, such as local neutrophil accumulation, macrophage activation, and the release of proinflammatory cytokines, all of which lead to cell injury (3). In the field of transplantation, CO has been shown to inhibit acute and chronic allograft rejection as well as xenograft rejection (6, 37, 40, 43, 53). Kidney IRI represents an important problem affecting the outcome of renal transplantation (20). The biological actions of CO are corroborated by the pharmacological effects of CO itself, observed at concentrations ranging from 10 to 500 ppm, which exhibited protective effects against ischemic injury (43). Evidence indicates that CO can provide beneficial anticell death and anti-inflammatory effects in the context of IRI (34). Low concentration CO inhalation provided protection against cold IRI in a rat kidney transplantation model (12). However, exogenous CO administration has been shown to increase carboxyhemoglobin, with a theoretical risk of impaired O_2 delivery to organs and tissues (26, 35). During IRI, endogenous CO is generated from heme degradation through the activity of HO-1. The effects of HO-1 upregulation could be mimicked by CO administration in a mouse-to-rat heart transplant model (53).

The purpose of the present study was to test the hypothesis that the administration of 5-ALA and Fe^{2+} has a salutary effect in ameliorating renal IRI in mice via the generation of CO during renal IRI.

MATERIALS AND METHODS

Animals. Male Balb/c mice (8~12 wk of age and weighing 20~25 g) were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). All mice were maintained under standard conditions and fed rodent food and water, in accordance with the guidelines of the Animal Use and Care Committee of the National Research Institute for Child Health and Development (Tokyo, Japan). All animal experiments were performed according to the recommendations of the Committee of Care and Use of Laboratory Animals in National Research Institute for Child Health and Development.

Reagents. 5-ALA/HCl (COSMO ALA, Tokyo, Japan) and Fe^{2+} (sodium ferrous citrate, Eisai Food & Chemical, Tokyo, Japan) were dissolved in distilled water (DW), and the molar ratio of 5-ALA to Fe^{2+} was 1:0.5. Fe^{2+} was diluted in DW immediately before administration. Light exposure was limited as much as possible. Zinc protoporphyrin and cobalt protoporphyrin (ZnPPIX and CoPPIX, respectively; Porphyrin Products, Logan, UT) were diluted in 100 mM NaOH as a stock solution of 50 mM and were kept at −80°C until use. 5-ALA/Fe^{2+} was administrated orally in 0.3 ml DW, and the volume of 0.3 ml of ZnPPIX and CoPPIX was administered intraperitoneally.

Measurement of tissue CO concentrations. Kidneys were obtained from naïve Balb/c mice and DW-, 5-ALA-, Fe^{2+}-, and 5-ALA/Fe^{2+}-treated groups on *day 3* ($n = 3$ for all). ZnPPIX and CoPPIX were administered (5 mg/kg ip) simultaneously with 5-ALA/Fe^{2+} (30 mg/kg). The kidneys were dissociated into thorough homogenates using a gentleMACS dissociator (MiltenyiBiotec, Bergisch Gladbach, Germany), tissue CO concentrations were then measured by a sensor gas chromatograph (FIS, Kobe, Japan) according to the operations manual.

Mouse model of renal IRI. Renal ischemia-reperfusion was induced by bilateral clamping of the renal pedicle for 45 min followed by reperfusion for 24 h. Briefly, the renal pedicles were bluntly dissected with animals on a temperature-controlled operating table (Marukan, Osaka, Japan) heated to body temperature, and mice were anesthetized intraperitoneally with 0.5% pentobarbital sodium (50 mg/kg). For mice subjected to ischemia-reperfusion, bilateral renal pedicle occlusion for 45 min was performed using microvascular clamps (Natume Seisakusho, Tokyo, Japan). Reperfusion commenced once the artery clamps were removed. Occlusion was verified visually by a change in the color of the kidneys to a paler shade, and reperfusion was confirmed by a blush. Other mice were subjected to a sham operation (sham mice): they underwent identical surgical procedures as renal ischemia-reperfusion mice but did not undergo bilarterial renal pedicle clamping and were maintained under anesthesia for the duration of the experiment. Except for the mice retained for observations of the survival rate, animals were euthanized 24 h after reperfusion, and the kidneys were isolated, quick frozen in liquid nitrogen, and stored at −80°C until further analysis.

Study design. Mice were randomly divided into the following four groups: sham (*group 1*; $n = 20$), DW treatment control (*group 2*; $n = 25$), 5-ALA/Fe^{2+} (30 mg/kg) treatment (*group 3*; $n = 25$), and ZnPPIX (5 mg/kg) plus 5-ALA/Fe^{2+}(30 mg/kg) treatment (*group 4*; $n = 10$). Mice in the control and 5-ALA/Fe^{2+} groups were given 0.5 ml of DW or 30 mg/kg of 5-ALA/Fe^{2+} at 48, 24, and 2 h before ischemia, respectively.

Assessment of renal function. Blood samples were obtained from an orbital angular vein at 24 h after reperfusion. The blood was centrifuged (4,500 rpm for 10 min) to separate serum. To monitor renal function, levels of serum creatinine and blood urea nitrogen (BUN) were measured with a FUJIFILM DRI-CHEM3500i analyzer (FUJIFILM, Tokyo, Japan).

Thiobarbituric acid-reactive substances. Level of renal thiobarbituric acid-reactive substances (TBARS), considered to be an indicator of oxidative stress, were measured using a Lab Assay TBARS kit (Cay Chemical, Greensboro, NC) according to the manufacturer's instructions.

Table 1. *The primers and probes used in this study*

Genes	Forward Primer	Reverse Primer	Probe
Heme oxygenase-1	5′-CAGGGTGACAGAAGAGGCTAAGAC-3′	5′-TTGTGTTCCTCTGTCAGCATCAC-3′	5′-TCCTGCTCAACATTGAGCTGTTTGAGGA-3′
TNF-α	5′-TGTCTACTGAACTTCGGGGTGAT-3′	5′-AACTGATGAGAGGGAGGCCAT-3′	5′-TCCCCAAAGGGATGAGAAGTTCCCAA-3′
Interferon-γ	5′-CAAGGCGAAAAAGGATGCA-3′	5′-CGGATGAGCTCATTGAATGCT-3′	5′-TGCCAAGTTTGAGGTCAACAACCCACA-3′
18S rRNA	5′-ATGAGTCCACTTTAAATCCTTTAACgA-3′	5′-CTTTAATATACGCTATTGGAGCTGGAA-3′	5′-ATCCATTGGAGGGCAAGTCTGGTGC-3′

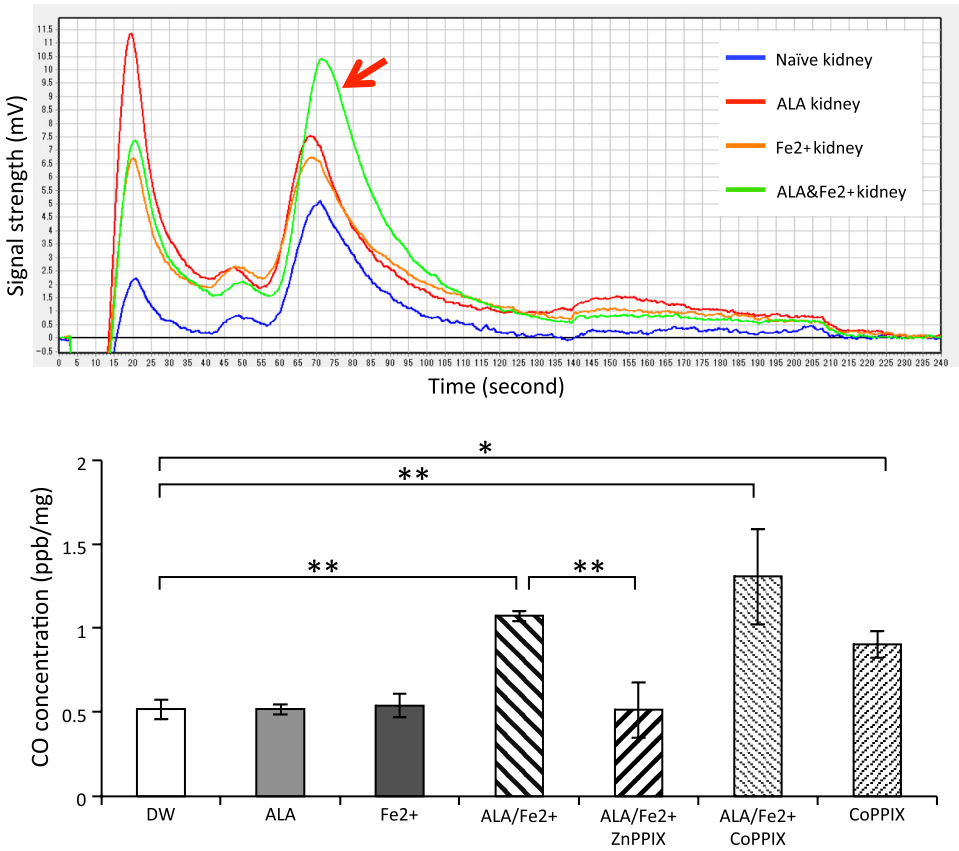

Fig. 1. Changes in the carbon monoxide (CO) concentration in mouse kidneys. Mice were treated with distilled water (DW), 5-aminolevulinic acid (5-ALA), Fe^{2+}, 5-ALA/Fe^{2+}, 5-ALA/Fe^{2+} + zinc protoporphyrin (ZnPPIX), 5-ALA/Fe^{2+} + cobalt protoporphyrin (CoPPIX), or CoPPIX. 5-ALA/Fe^{2+} (30 mg/kg) was administrated orally in 0.3 ml DW, and the volume of 0.3 ml ZnPPIX and CoPPIX (5 mg/kg, respectively) was administered intraperitoneally. Data shown are means ± SD; $n = 6$ mice/group. *$P < 0.05$ and **$P < 0.01$ compared with the corresponding value of the DW-treated control group.

Histopathological evaluation of the kidneys. For the histopathological examination, kidneys were collected, cut coronally, fixed in 10% formaldehyde, and embedded in paraffin. Five-micrometer sections were prepared and stained with hematoxylin and eosin. Sections were scored with a semiquantitative scale designed to evaluate changes in the kidney 24 h after IRI. Histological changes were mainly evaluated by quantitative measurement of the tubular injury by assessing specific variables in 10 individual high-power fields (magnification:

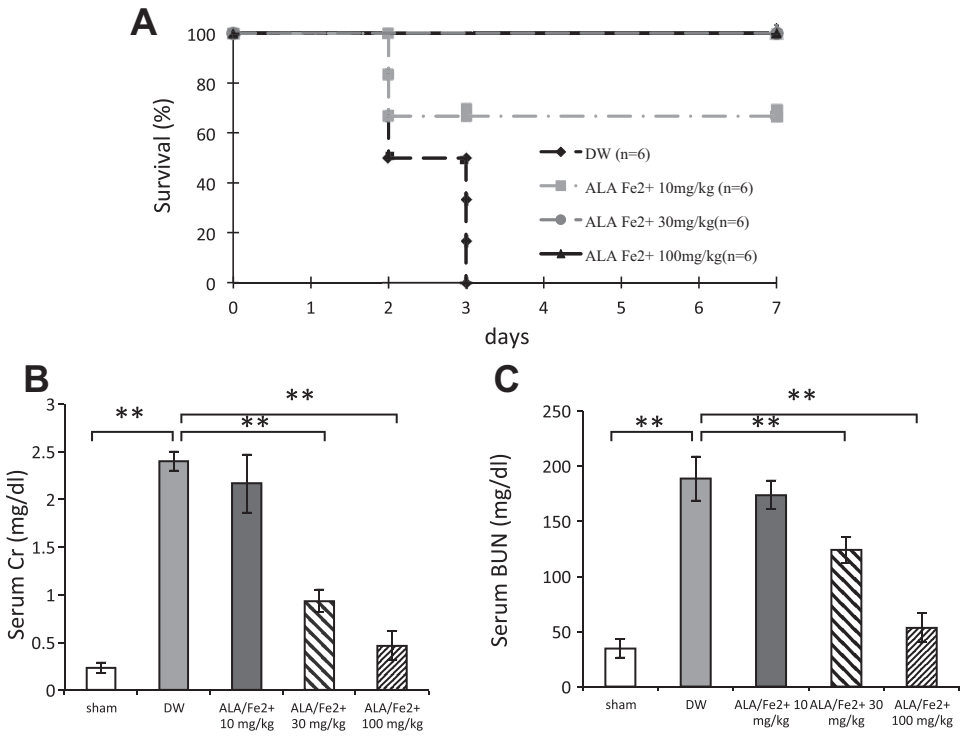

Fig. 2. 5-ALA/Fe^{2+} treatment protects against lethal renal ischemia-reperfusion (I/R) injury (IRI). *A*: Balb/c mice were pretreated with 5-ALA/Fe^{2+} (10, 30, or 100 mg/kg) or DW after clamping of the renal pedicles for 45 min. The survival of mice was observed for >7 days. The difference in the survival curves was significant (**$P < 0.01$). DW-treated animals rapidly developed worsened renal function after I/R, as indicated by the lower survival rate and increased serum levels of creatinine (Cr; *B*) and blood urea nitrogen (BUN; *C*) by 24 h after I/R. Data shown are means ± SD; $n = 6$ mice/group. *$P <$ 0.05 and **$P < 0.01$ compared with the corresponding value of the DW-treated control group.

×400). The percentage of the area affected was estimated for the number of necrotic cells, loss of the brush border, cast formation, and tubule dilation, and damages were scored as follows: *0* = 0~5%, *1* = 5~10%, *2* = 11~25%, *3* = 26~45%, 4 = 46~75%, and *5* = >76%. The 10 fields analyzed in each section were selected at random (22).

Immunohistochemical analysis. Macrophage infiltration was assessed with a monoclonal anti-mouse F4/80 antibody (BioLegend, San Diego, CA). Briefly, after being dewaxed, kidney sections were blocked with 10% heat-inactivated rat serum in PBS for 1 h. Primary antibodies were used at a 1:100 dilution, and samples were incubated overnight in antibodies at 4°C. Secondary biotin-conjugated antibodies (Zymed Laboratories, San Francisco, CA) were used at 1:200 dilution and were incubated with samples for 1 h. Labeled proteins were visualized with horseradish peroxidase (VECTORSTAIN Elite ABC Reagent, VECTOR Laboratories, Burlingame, CA) and diaminobenzidine (DAB) peroxidase substrate solution (DAB substrate kit for peroxidase, VECTOR Laboratories). Sections were rinsed and mounted in aqueous mounting medium. For each mouse, average values from both kidneys were included in the analysis. Ten random visual fields were analyzed per kidney section, and the percentage of the Sirius red-positive area or number of F4/80-positive cells was determined using the ImageJ software program.

TUNEL assay. To detect PCD, a TUNEL assay was performed on paraffin-embedded sections using the ApopTag Plus peroxidase in situ apoptosis detection kit (CHEMICON, Billerica, MA) according to the manufacturer's instructions.

RNA preparation and quantitative RT-PCR. Total RNA was extracted from frozen renal tissue samples using ISOGEN (NipponGene, Tokyo, Japan). Each 0.8-μg aliquot of RNA was reverse transcribed to cDNA using oligo(dT) primers and Super Script reverse transcriptase (Invitrogen). The sequences used in our study are shown in Table 1. Quantitative RT-PCR was performed using a TaqMan system on the Applied Biosystem PRISM7700 instrument (ABI Japan, Tokyo, Japan), and experiments were conducted using 0.9 mM of each primer in a final reaction volume of 25 μl of Premix Ex Taq (Takara Bio, Shiga, Japan). The PCR cycling conditions were as follows: 50°C for 2 min, 95°C for 15 min, and 50 cycles of 95°C for 30 s, 60°C for 1 min, and 25°C for 2 min. The normalized threshold cycle (C_t) value of each gene was obtained by subtracting the C_t value of 18S rRNA.

Statistical analysis. Data are presented as means ± SD and were analyzed statistically using one-way ANOVA followed by a Fisher's protected least-significant difference test or Mann-Whitney *U*-test. *P* values of <0.05 were considered to be statistically significant.

RESULTS

Generation of a high concentration of endogenous CO in the kidneys of mice administered 5-ALA/Fe^{2+}. We first examined whether the administration of 5-ALA/Fe^{2+}would lead to the generation of endogenous CO in mice. To test this, mice were administered DW, 5-ALA, Fe^{2+}, or 5-ALA/Fe^{2+}, respectively. As shown in Fig. 1, the endogenous CO concentrations of the kidneys from the mice administered 5-ALA/Fe^{2+} were significantly increased compared with those of mice administered DW, 5-ALA, and Fe^{2+} alone ($P < 0.01$). To study the mechanism responsible for this increase, mice were administered 5-ALA/Fe^{2+} and were treated with CoPPIX, an inducer

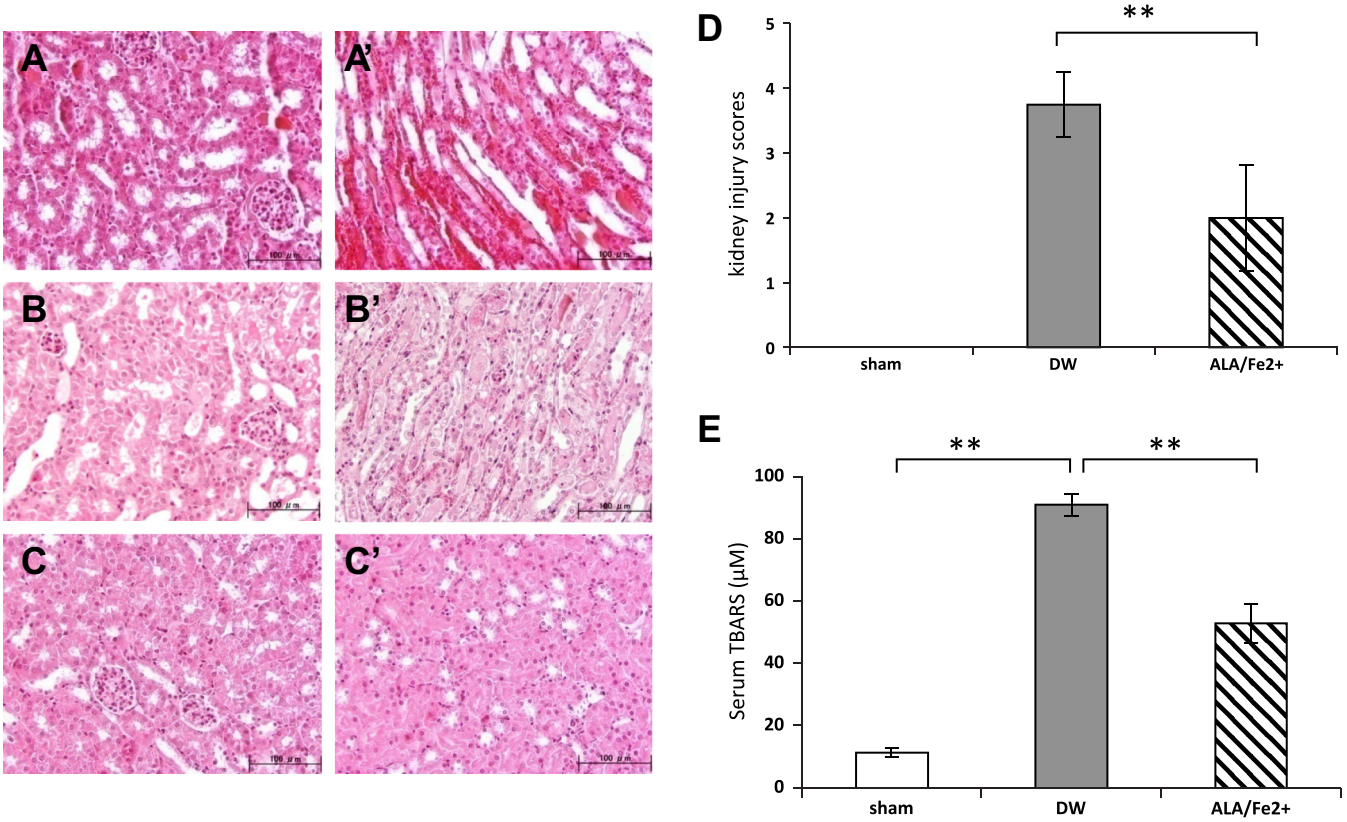

Fig. 3. 5-ALA/Fe^{2+} attenuated the renal injury and oxidative stress during I/R. *A–C* and *A′–C′*: effects of pretreatment with 5-ALA/Fe^{2+} on the renal histology 24 h after reperfusion. Scale bars = 100 μm. Severe tubular dilation and necrosis were observed in DW-treated control IRI mice [cortex (*B*) and medulla (*B′*)] but not in sham-operated (sham) mice [cortex (*A*) and medulla (*A′*)], whereas 5-ALA/Fe^{2+} pretreatment was associated with less histological damage [cortex (*C*) and medulla (*C′*)]. Representative photographs are shown (n = 3–5). *D*: results of the quantification of the corresponding histological scores. *E*: blood was collected 24 h after reperfusion to determine the levels of serum thiobarbituric acid-reactive substances (TBARS). Data shown are means ± SD; n = 3–5. *$P <$ 0.05 and **$P < 0.01$ compared with the corresponding value of the DW-treated control group.

of HO-1, or ZnPPIX, an inhibitor of HO-1. The results showed that the enzymatic activity of HO-1 was significantly enhanced by CoPPIX administration, whereas this did not occur in the presence of ZnPPIX. Inhibition of HO-1 activity by ZnPPIX administration in mice administered 5-ALA/Fe^{2+} led to a significant decrease in the endogenous CO concentration in the kidneys. In contrast, the induction of HO-1 activity by CoPPIX administration alone or in the presence of 5-ALA/Fe^{2+} led to an increase in the endogenous CO concentration in the kidneys ($P < 0.01$).

Effects of 5-ALA/Fe^{2+} treatment on IRI and renal dysfunction. We evaluated the effects of 5-ALA/Fe^{2+} treatment on renal IRI in male Balb/c mice. A dose range from 10 to 100 mg/kg body wt was administered. As expected, DW-treated control animals exhibited rapidly worsened renal function after IRI, as indicated by their decreased survival rate and increased serum levels of creatinine and BUN 24 h after IRI (Fig. 2). 5-ALA/Fe^{2+} pretreatment protected renal function in a dose-dependent manner, as shown by the increasing serum creatinine levels at doses varying from 100 to 10 mg/kg (Fig. 2, *B* and *C*). The protective effect continued, and mice pretreated at the dose of 100 mg/kg 5-ALA/Fe^{2+} survived for >1 wk after reperfusion, and the same result was observed at a dose of 30 mg/kg (Fig. 2*A*). Therefore, the 30 mg/kg dose was used for subsequent experiments.

5-ALA/Fe^{2+} pretreatment reduced the extent of acute tubular damage and suppressed oxidative stress during IRI. The preventive effect of 5-ALA/Fe^{2+} was associated with histological modifications. In fact, 5-ALA/Fe^{2+} was able to prevent tubular dilation, swelling, necrosis, congestion, and vacuolization, which were observed 24 h after reperfusion after ischemia. Scoring the histopathological damage of the tubules confirmed the protective effect of 5-ALA/Fe^{2+} (Fig. 3, *A–C*, *A′–C′*, and *D*). Sham mice displayed no tubular injury. The histopathological scores clearly showed that 5-ALA/Fe^{2+} pretreatment decreased the renal histological damage after IRI.

To assess the renal oxidative stress during IRI, we examined the expression of TBARS, a marker of the lipid peroxidation levels, which is commonly used to evaluate oxidative stress and cellular injury. Low expression of TBARS was detected in the normal kidneys, and the level was significantly higher in DW-treated control kidneys subjected to IRI, whereas the administration of 5-ALA/Fe^{2+} dramatically decreased the levels of TBARS in the kidneys subjected to IRI (Fig. 3*E*).

Kidneys exhibited increased PCD and macrophage infiltration during IRI, which were both decreased by 5-ALA/Fe^{2+} treatment. To examine the kidneys for PCD, we performed a TUNEL assay. After IRI, DW-treated control kidneys showed a significant increase in the number of TUNEL-positive cells compared with sham kidneys. The administration of 5-ALA/Fe^{2+} significantly decreased the number of TUNEL-positive cells

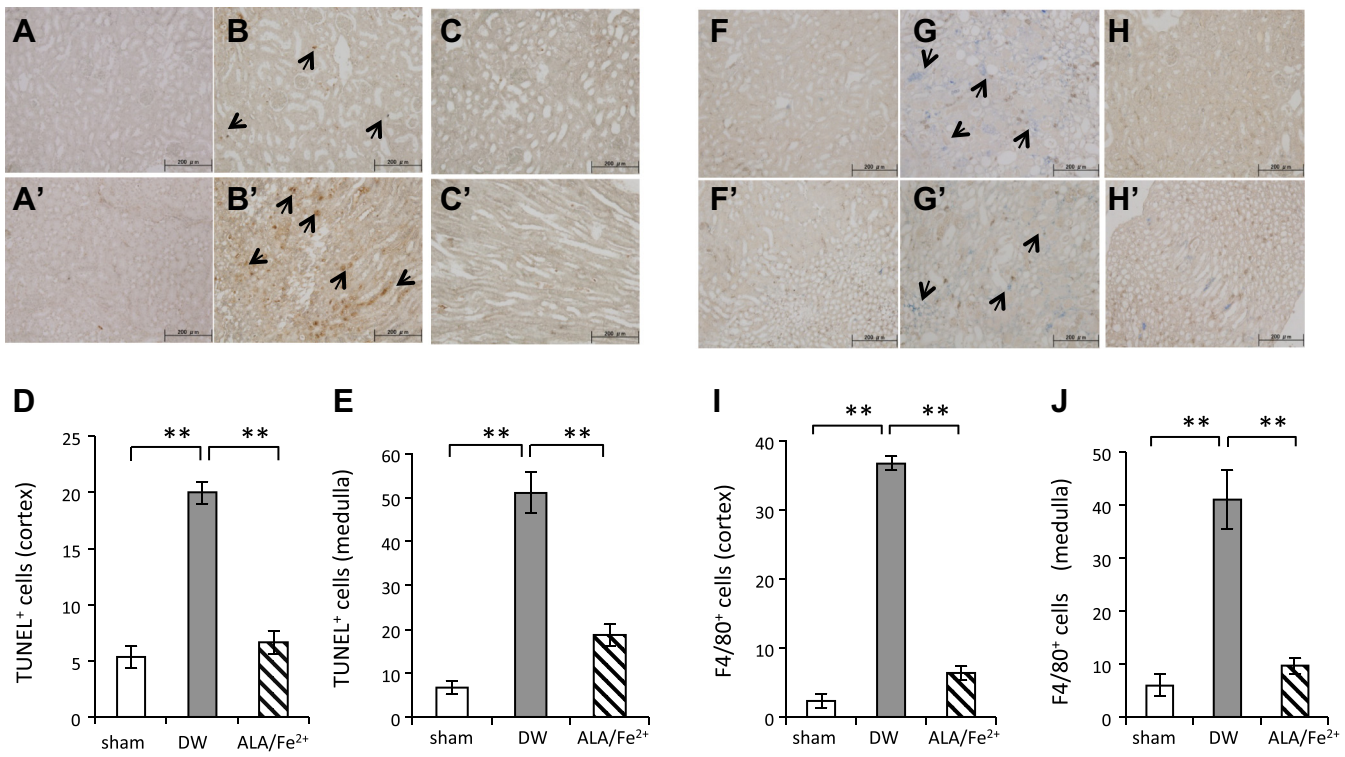

Fig. 4. Effects of 5-ALA/Fe^{2+} treatment on the programmed cell death and macrophage infiltration induced by IRI. *A–C* and *A′–C′*: light photomicrographs of kidney sections from the sham [cortex (*A*, *F*) and medulla (*A′*, *F′*)], DW-treated control [cortex (*B*) and medulla (*B′*)], and 5-ALA/Fe^{2+}-treated [cortex (*C*) and medulla (*C′*)] group 24 h after reperfusion. The dark arrows correspond to representative TUNEL-positive cells. *D* and *E*: results of the quantification of TUNEL-positive cells for the kidney cortex (*D*) and medulla (*E*). Scale bars = 200 μm. *F–H* and *F′–H′*: F4/80-positive cells (arrows) were evident in the kidneys after IRI [cortex (*G*) and medulla (*G′*)], and the numbers were reduced by the pretreatment of mice with 5-ALA/Fe^{2+} [cortex (*H*) and medulla (*H′*)]. Scale bars = 200 μm. *I* and *J*: results of the quantification of F4/80-positive cells in the kidney cortex (*I*) and medulla (*J*). Data are means ± SD and are representative of three independent experiments; n = 3–5 mice/condition and 5 observations/representative section. $^{**}P < 0.01$ compared with the corresponding value of the DW-treated control group.

not only in the cortex but also in the medulla (Fig. 4, *A–C*, *A′-C′*, *D*, and *E*). Furthermore, we also performed an immunohistochemical analysis to identify the inflammatory cell types that were present. We found that F4/80-positive cells were more prevalent in DW-treated control kidneys after IRI than in sham kidneys. Treatment with 5-ALA/Fe^{2+} effectively reduced the number of F4/80-positive cells in both the cortex and medulla (Fig. 4, *F–H*, *F′–H′*, *I*, and *J*).

5-ALA/Fe^{2+} pretreatment induced HO-1 and suppressed the expression of inflammatory cytokine mRNA expression in kidneys subjected to IRI. Our experiments suggested that 5-ALA/Fe^{2+} might ameliorate ischemic acute kidney injury by its effect on HO-1. In many studies, increasing the HO-1 expression level ameliorated ischemic acute kidney injury. Similarly, as shown in Fig. 5, we found that mRNA expression of HO-1 was significantly increased in kidneys subjected to IRI compared with sham kidneys and that 5-ALA/Fe^{2+} pretreatment exhibited the tendency of further enhancement of HO-1 mRNA expression.

On the other hand, the injury occurring as a result of renal IRI has also been demonstrated to be associated with various inflammatory cytokines. We therefore compared the levels of TNF-α and interferon (IFN)-γ in kidney homogenates from either 5-ALA/Fe^{2+}-treated mice or DW-treated control mice with those from sham mice 24 h after reperfusion. We found that mRNA expression levels of TNF-α and IFN-γ were significantly increased in the kidneys after IRI. Furthermore, the increase of IFN-γ mRNA expression was significantly reduced by 5-ALA/Fe^{2+} treatment along with the tendency of reduction of TNF-α mRNA expression.

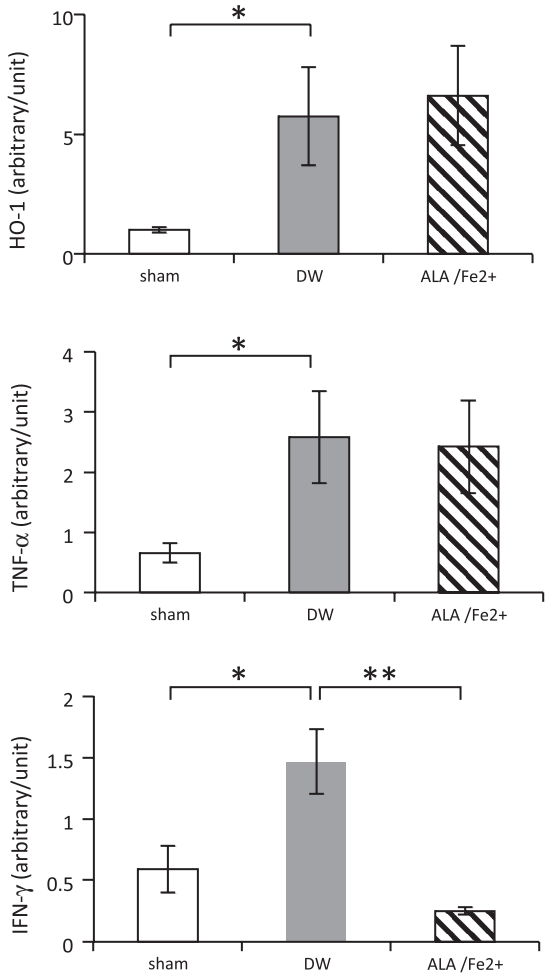

Fig. 5. 5-ALA/Fe^{2+} treatment induced heme oxygenase (HO)-1 and suppressed inflammatory cytokine mRNA expression in kidneys after I/R. RT-PCR analysis revealed that treatment with 5-ALA/Fe^{2+} slightly increased HO-1 and decreased TNF-α and interferon (IFN)-γ mRNA expression markedly. The relative quantity is presented as the ratio of the comparative threshold cycle (C_t) of the target genes against those of the housekeeping gene 18S rRNA. Data are representative of 3 independent experiments and indicate the mean ratio of triplicate results from each experiment. Data are means ± SE; $n = 3–7$. *$P < 0.05$ and **$P < 0.01$ compared with the corresponding value of the DW-treated control group.

DISCUSSION

In this study, we provided the first evidence showing that the administration of 5-ALA/Fe^{2+} can ameliorate renal IRI in mice with the generation of CO. HO-1 is a stress-responsive enzyme that acts during inflammatory reactions as the rate-limiting step in the catabolism of heme, yielding equimolar amounts of Fe^{2+}, biliverdin, and CO gas (11, 61). Our study has shown that the simultaneous administration of 5-ALA and Fe^{2+} generated high concentrations of endogenous CO in mice (Fig. 1). Several recent studies (17, 28) have shown that CO mediates cytoprotection through the induction of HO-1. HO-1 was thought to act as one of the key enzymes required to generate CO and has previously been proposed to have protective effects during ischemic acute kidney injury. We found that 5-ALA/Fe^{2+} ameliorates ischemic acute kidney injury by increasing HO-1; it may do so by satisfying these requirements. We found that both 5-ALA/Fe^{2+} and IRI increased the mRNA abundance of HO-1 (Fig. 5). The benefit of increased HO-1 was that it generated a high concentration of endogenous CO in the kidneys (Fig. 1). Using the HO-1 inhibitor ZnPPIX, we also demonstrated that the concentration of endogenous CO in the mouse kidney was reduced when HO-1 was inhibited (Fig. 1).

The biological and physiological functions of endogenous CO have been experimentally demonstrated (64). Previous studies have shown that CO could regulate nonadrenergic/noncholinergic intestinal relaxation (50, 68), intrahepatic vascular resistance (58), pulmonary vascular resistance (59), and relaxation of tail artery tissues (66). Exogenously delivered CO has exerted potent protective functions in numerous experimental models of inflammation, sepsis/endotoxemia, hemorrhagic shock, autoimmune diseases, and fibrosis (7, 18, 52, 57, 65, 70).

In our study, we found another way to increase the concentration of endogenous CO in the kidneys and other organs by simultaneously administering 5-ALA and Fe^{2+} to mice. The present study showed that pretreatment of mice with 5-ALA/Fe^{2+} resulted in better kidney perfusion than was observed in DW-treated control mice. Animals pretreated with 5-ALA and Fe^{2+} had lower plasma levels of BUN and creatinine caused by IRI (Fig. 2) and lower histopathological injury scores (Fig. 3).

Our observations prompted us to investigate the possible mechanism(s) underlying these effects. Renal tissue damage has been well documented to be due to cell death. The cellular mechanisms underlying cell death have been extensively discussed in a number of studies, and it has been suggested that PCD, which incorporates both apoptosis and regulated necro-

sis, is likely responsible for the renal tubular atrophy induced by ischemia-reperfusion (30, 54). PCD is increasingly recognized as a major form of cell death during IRI and can even impact the functional outcome independent of inflammation. It has been shown that abrogation of early renal IRI-induced PCD prevents the development of subsequent inflammation and organ dysfunction (30). We therefore examined PCD in the kidneys of DW-treated control mice and 5-ALA/Fe^{2+}-treated mice. As shown in Fig. 3, TUNEL staining revealed that there was considerable PCD in both the cortical and medullar regions of the kidneys in control mice 24 h after IRI. However, 5-ALA/Fe^{2+} effectively inhibited PCD of tubular cells after renal ischemia. CO is known to have anti-PCD effects in both in vivo and in vitro models (2, 72). The direct inhibition of PCD afforded by CO may be mediated by several different mechanisms, probably depending on the stimulus inducing the PCD and the types of cells involved (4, 31, 46, 71).

In renal IRI, reperfusion triggers the inflammatory process by the activation of chemical mediators and enzymes (e.g., ROS, phospholipase A_2, lysozymes, leukotrienes, prostaglandins, etc.). Significant cellular damage also activates macrophages and other parenchymal cells and results in the release of numerous inflammatory mediators, including TNF-α, followed by an extravasation of macrophages, neutrophils, and T cells to the interstitial space (13, 45, 56, 69). In addition, it has been found that activated macrophages play an important role in IRI. The early infiltration of macrophages plays an important pathogenic role in renal IRI, presumably by their release of proinflammatory cytokines and chemokines (25). In the present study, 5-ALA/Fe^{2+} inhibited the accumulation of F4/80-expressing cells in the mouse kidneys after IRI compared with the control group.

CO has been known to exert anti-inflammatory actions in various injury models. Typically, LPS-induced inflammatory tissue injury was inhibited by CO by the downregulation of proinflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6) (32, 42). In the present study, the increased levels of inflammatory cytokines, such as IFN-γ, were coincident with macrophage recruitment. IFN-γ, which is an important cytokine typically elevated in the control group, showed more prominent and specific upregulation, and this might be related to the fact that inflammatory cytokines can be directly expressed by activated macrophages (16, 62). Of interest, the increase in the expression of these inflammatory cytokines was suppressed by the pretreatment of mice with 5-ALA/Fe^{2+} (Fig. 5). In the present study, the increase of mRNA expression of HO-1 in ALA/Fe^{2+}-treated animals was modest, even though 5-ALA treatment is known to induce HO-1 (14, 15, 48). There are several possibilities for this phenomenon. As a previous report (19) has indicated, HO-1 expression is induced strongly after IRI. So, the induced expression of HO-1 by 5-ALA/Fe^{2+} might be hidden. Furthermore, a previous in vitro study (15) has demonstrated that the peak of HO-1 expression by 5-ALA is 12 h after treatment. Although we did not perform quantitative RT-PCR just after 5-ALA/Fe^{2+} treatment before IRI and the evaluation of time-dependent HO-1 expression, the expression of HO-1 by 5-ALA might already have started to decline from the peak level. Additionally, there might be the feasibility of a predominant role of CO and bilirubin compared with HO-1 by 5-ALA/Fe^{2+} treatment toward the protection of cellular injury after IRI. Since in the present study we did not depict the importance of biliverdin/bilirubin generated by 5-ALA/Fe^{2+} in the kidney in that renal intracellular concentrations of these metabolites were not measured, we cannot rule out the possible contributions of biliverdin/bilirubin as well as CO generation in the response to 5-ALA/Fe^{2+}; as previous research has demonstrated, CO and bilirubin themselves have cytoprotective ability (29, 44, 51, 67). Further studies with biliverdin reductase, small interfering RNA against biliverdin/bilirubin, or another inhibitor elucidating the involvement of biliverdin/bilirubin in the protective effect of 5-ALA/Fe^{2+} in renal IRI will be done.

In summary, in the present study, we provide the first evidence showing that pretreatment with 5-ALA/Fe^{2+} noticeably decreased the level of injury in kidneys after IRI. We also demonstrated that the protective effects of 5-ALA/Fe^{2+} were associated with its antioxidant, anti-inflammatory, and anti-PCD mechanisms of action with the generation of CO. Thus, 5-ALA/Fe^{2+} may be a promising candidate for the pretreatment of patients before kidney transplantation.

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