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Albumin fusion renders thioredoxin an effective anti-oxidative and anti-inflammatory agent for preventing cisplatin-induced nephrotoxicity

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ABSTRACT

Background: A strategy for preventing cisplatin nephrotoxicity due to enhanced oxidative stress and inflammatory response is highly desirable. Thioredoxin-1 (Trx), an endogenous redox-active protein, has a short retention time in the blood. A long acting form of Trx, human serum albumin-Trx (HSA-Trx), was produced by recombinant HSA fusion and its effectiveness in preventing cisplatin nephrotoxicity was examined.
Methods: HSA-Trx was prepared in *Pichia* expression system. Cisplatin-induced nephropathy mouse model was established by a single administration of cisplatin.
Results: Compared to saline, Trx or N-acetylcysteine, an intravenous administration of HSA-Trx attenuated the cisplatin-induced elevation in serum creatinine, blood urea nitrogen and urinary N-acetyl-β-D-glucosaminidase along with the decrease in creatinine clearance. HSA-Trx caused a substantial reduction in the histological features of renal tubular injuries and the apoptosis-positive tubular cells. Changes in superoxide, 8-OHdG, glutathione and nitrotyrosine levels indicated that HSA-Trx significantly suppressed renal oxidative stress. HSA-Trx also suppressed the elevation of TNF-α, IL-1β and IL-6. Administered fluorescein isothiocyanate-labeled HSA-Trx was found partially localized in the proximal tubular cells whereas majority remained in the blood circulation. Specific cellular uptake and the scavenging of intracellular reactive oxygen species by HSA-Trx were observed in HK-2 cells.
Conclusion: HSA-Trx could be a novel and effective approach for preventing cisplatin nephrotoxicity due to its prolonged anti-oxidative and anti-inflammatory action not only in extracellular compartment but also inside the proximal tubular cell.
General significance: We report the renoprotective effect of HSA-Trx against cisplatin nephrotoxicity. This work would enhance developing therapeutics against acute kidney injuries including cisplatin nephrotoxicity.

1. Introduction

Cisplatin, dichloro-diamino platinum, is a widely used and potent chemotherapy agent that is used in the treatment of a variety of malignant solid tumors of the lung, bladder, colon, testis and brain [1]. However, its clinical use is limited due to dose-dependent nephrotoxicity in about 30% of patients which prevents the use of high doses for maximum therapeutic efficacy [2]. Unfortunately, there is no specific treatment for cisplatin-induced renal dysfunction or injury.

Experiments with rodents revealed that renal damage from cisplatin occurs primarily in proximal tubules, especially the S3 segment of the outer medullary stripe [3]. Cisplatin accumulates in the kidney to a greater degree than other organs because the major route for its elimination from the body is by renal excretion. The cisplatin concentration in the proximal tubular epithelial cells is about 5 times higher than the serum concentration [4]. This disproportionate accumulation of cisplatin in kidney tissue contributes to cisplatin-induced nephrotoxicity [5]. The highest concentration of cisplatin accumulates in the S3 segment of the proximal tubule, where organic cation transporter 2 (OCT2) mediated cisplatin uptake is likely to be the major pathway for its uptake [6].

The *in vivo* mechanisms of cisplatin nephrotoxicity are complex. Findings reported in recent studies suggest that numerous interrelated processes appear to be involved in the development of cisplatin-induced nephrotoxicity, including the formation of reactive oxygen species (ROS) [7,8] and nitrogen species [9,10], apoptosis [11] and inflammation [12,13]. In fact, ROS have been found to be produced

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via the xanthin–xanthine oxidase system, the mitochondria and the NADPH oxidase system in the cisplatin-treated animals [14]. In addition, the renal content of reactive nitrogen species (RNS) such as peroxynitrite and nitric oxide was also increased [9]. These oxidative stress factors initiate the mitochondrial pathway, resulting in the activation of caspase-9 which is the initiator caspase that activates caspase-3, the principal executioner caspase in renal tubule apoptosis [15]. Cisplatin also induces the production of a series of inflammatory cytokines, such as TNF-α, IL-1β and IL-6 that mediate renal injuries [15]. Moreover, cisplatin-induced ROS have been reported to further activate p38 mitogen-activated protein kinase (MAPK) pathway that enhances the production of these inflammatory cytokines, and consequently increases the extent of renal tubular damage [16]. Taken into consideration of the accumulation of cisplatin in renal proximal tubular cells, to prevent cisplatin-induced nephrotoxicity, it would be desirable to develop a therapeutics that possesses to exert both anti-oxidative and anti-inflammatory activities at kidney, especially inside the proximal tubular cells. Recently, it was reported that resveratrol, a phytoalexin, attenuated the cisplatin-induced structural and functional renal changes by reducing ROS and inhibiting inflammatory cell infiltrate [17].

Trx is a ubiquitous, redox-active, low-molecular-weight protein that has anti-oxidative, anti-inflammatory and anti-apoptotic properties [18]. Although Trx has great potential for use as a therapeutic agent against several types of oxidative stress diseases [18], its short half-life (1 h in mouse, 2 h in rat) limits its clinical application. We recently engineered a genetic fusion protein that is comprised of human serum albumin (HSA) and thioredoxin-1 (Trx) (HSA-Trx) and is expressed by a *Pichia* expression system [19–22]. The pharmacokinetic properties of the HSA-Trx fusion protein are similar to that of HSA itself, but have a 10 times longer plasma half-life than Trx in normal mice. Interestingly, HSA-Trx showed a higher distribution to the lung and kidney than to other organs [19]. HSA-Trx has proven to be therapeutically effective in a septic shock mouse model [19], as well as in alleviating oxidative stress related pulmonary disease [20,22]. Furthermore, our recent study demonstrated that HSA-Trx prevents experimental contrast-induced nephropathy *via* inhibition of oxidative stress in the general circulation [21]. These findings prompted us to investigate the issue of whether HSA-Trx might be effective for the treatment of other types of acute kidney injuries (AKI), such as cisplatin-induced nephropathy if HSA-Trx could exert it's biological activity not only in extracellular compartment, but also inside of the proximal tubular cells.

The objective of this study was to investigate the therapeutic effect of HSA-Trx on a cisplatin-induced nephropathy mouse model. The model was produced by intraperitoneal administration of 15 mg/mL cisplatin to mice. The renoprotective effect, anti-oxidative, and anti-inflammatory action of HSA-Trx on the model were evaluated *in vivo*. To clarify whether HSA-Trx acts in the kidneys or in the general circulation, or both, the renal localization and renal tubular uptake of HSA-Trx were evaluated using FITC-labeled HSA-Trx.

2. Materials and methods

2.1. Materials

Cisplatin (Randa™) was purchased from Nippon Kayaku Co., Ltd. (Tokyo, Japan). The *Pichia* Expression Kit was purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals were of reagent grade or of the highest purity available commercially.

2.2. Production of HSA-Trx fusion protein

The genetic fusion of Trx and HSA was performed and the fusion protein was produced following a previously reported method [19]. Briefly, transformed *Pichia pastoris* was incubated in 5 L of BMGY liquid media (1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base with ammonium sulfate without amino acids, 4×10^{-5}% biotin, 1% glycerol) (growth phase) for 2 days ($OD_{600} = 2$), and then cultured in 800 mL of BMMY media that contained a protein expression inducer as well as a carbon source, methanol (1% yeast extract, 2% pepton, 100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base with ammonium sulfate without amino acids, 4×10^{-5}% biotin, 1% methanol) (protein induction phase) for 3 days at 30 °C. Methanol was added daily, to permit the concentration of methanol to be maintained at 1% in order to sustain the protein expression induction effect. Purification of the fusion protein was initially carried out by chromatography on a Blue Sepharose 6 Fast Flow column (GE Healthcare, Tokyo, Japan) equilibrated with 200 mM sodium acetate buffer (pH 5.5) after dialysis against the same buffer. Next, using AKTA prime, a 5 mL HiTrap Phenyl HP column (GE Healthcare, Tokyo, Japan) was used for hydrophobic chromatography, under the following conditions: Buffer A, 50 mM Tris–HCl + 1.5 M ammonium sulfate, pH 7.0; Buffer B, 50 mM Tris–HCl, pH 7.0; Gradient, 0–100% (Buffer B) 100 mL; Flow rate, 3 mL/min. The desired fusion protein was treated with activated carbon for lipid removal and analyzed by SDS-PAGE using a 15% polyacrylamide gel, with Coomassie blue R250 staining. The fusion protein was estimated to be more than 95% pure.

2.3. Cisplatin-induced nephrotoxicity in mice

All animal experiments were conducted in accordance with the guidelines of Kumamoto University for the care and use of laboratory animals. Mature male ICR mice, weighing approximately 20 to 22 g at the start of the experiment, were housed in individual cages under controlled conditions of light (12 h/12 h light/dark cycle) and temperature (21–23 °C) and allowed free access to standard laboratory diet and tap water. After a 7-day period of acclimation to the experimental area, the mice were randomized to receive the Trx alone (100 nmol/kg), NAC (300 μmol/kg), HSA-Trx (100 nmol/kg) or saline 30 min before the intraperitoneal administration of cisplatin (15 mg/kg). Controls were administered saline injections only. All drugs were injected *via* the tail vein. The mice were allowed to recover in metabolic cages for an additional 96 h, and urine samples were collected and used for the determination of N-acetyl-β-D-glucosaminidase (NAG) activity and urinary creatinine excretion. After the blood samples were collected for the determination of SCr and BUN, the mice were sacrificed under diethyl ether anesthesia at 96 h after the administration of cisplatin. The kidneys were removed and bisected in the equatorial plane, the right kidney was divided for the ratio of reduced and oxidized glutathione (GSH/GSSG) and inflammatory cytokine assay, and the left kidney was fixed in phosphate buffered 4% formalin and prepared for routine histological examination.

2.4. Biochemical evaluation of blood and urine samples

Mean SCr and urinary creatinine concentrations were measured by enzymatic methods, using the respective assay kits (Wako Pure Chemical, Osaka, Japan). The mean BUN concentration was determined by the diacetylmonoxime method using an assay kit (Wako Pure Chemical). The activity of NAG in the urine was determined by the enzymatic degradation of sodium cresol sulfonephthaleinyl N-acetyl-β-D-glucosaminide (as the substrate) using a commercial assay kit (Shionogi Pharmaceutical, Osaka, Japan). NAG activity was expressed as units per g of urinary creatinine. Creatinine clearance during 24 h (from 72 h to 96 h) after the injection of cisplatin was calculated as ml/min.

2.5. Histologic examination of renal tissues

The left kidney fixed in 4% formalin was dehydrated in a graded series of ethyl alcohol concentrations and embedded in paraffin. Kidney blocks cut into 2-μm sections were subjected to periodic acid-Schiff (PAS) staining for morphologic analysis and TUNEL staining for cell

apoptosis and immunohistochemistry (8-hydroxy-2′-deoxygenase (8-OHdG) and nitrotyrosine (Nitro-Tyr)). PAS-stained tissue sections were viewed by light microscopy at a ×200 magnification. For the semi-quantitative analysis of morphological changes, 20 high-magnification (×400) fields of the cortex and the outer stripe of the outer medulla in mice were randomly selected. The injury ratio (extent of degeneration, detachment, tubulitis and dilatation of tubular cells/total tubular cells × 100) was then measured. All quantifications were performed in a blind manner. For TUNEL staining, sections were stained using an *In situ* cell death detection kit, Fluorescein (Roche, Basel, Switzerland) according to the manufacturer's protocol for paraffin-embedded sections. For the immunohistochemistry of 8-OHdG and Nitro-Tyr, first, antigen retrieval was conducted by means of immunosaver (Nisshin EM Corporation, Tokyo, Japan). A solution containing 50 mM Tris–HCl + 0.1% Tween-20 (T-TB) was then used to solubilize the kidney slices, followed by blocking with Block Ace (Dainippon Pharmaceutics, Osaka, Japan) at room temperature for 15 min. The primary antibody reaction was then conducted overnight at a temperature below 4 °C. In addition, the primary antibody containing inducible 8-OHdG [15A3] (Santa Cruz, California, USA, cat#: sc-651) or Nitro-Tyr (Santa Cruz California, USA, cat#: sc-66036) was diluted 50 times prior to use. The kidney slices were then washed with T-TB, followed by reaction with the secondary antibody at room temperature for 1.5 h. For 8-OHdG immunostaining, Alexa Fluor 488 goat anti-mouse IgG (H + L) (Invitrogen, Tokyo, Japan), for Nitro-Tyr immunostaining, Alexa Fluor 546 goat anti-mouse IgG (H + L) (Invitrogen, Tokyo, Japan) was diluted 100 times before use. After the reaction, the slide was observed using a Microscope (Keyence, BZ-8000, Osaka, Japan).

2.6. Measurement of renal glutathione levels

Kidney homogenates were prepared by homogenizing the tissues with 5% (w/v) sulfosalicylic acid (SSA). Reduced and oxidized glutathione levels in the kidney were determined using a GSSH/GSH quantification kit (Dojinkagaku, Kumamoto, Japan).

2.7. Quantification of TNF-α, IL-1β, and IL-6

At 96 h after cisplatin administration, the kidneys were removed and homogenized in RIPA/PI buffer (150 mM NaCl, 1% nonidet-P40, 10 mM Tris–HCl, pH 7.4 including 1% protease inhibitor (Nacalai tesque, Tokyo, Japan)). After centrifugation at 1600 *g* for 10 min at 4 °C (two times), the supernatants were recovered. The amounts of TNF-α, IL-β, and IL-6, in the supernatant were measured using ELISA, according to the manufacturer's protocol.

2.8. Preparation of fluorescein isothiocianate-labeled HSA-Trx (FITC-labeled HSA-Trx)

HSA-Trx was labeled with fluorescein isothiocianate (FITC). HSA-Trx (final concentration 4 mg/mL) and FITC (final concentration 1 mg/mL) were dissolved in 0.15 M K_2HPO_4 (pH 9.5), followed by mixing for 4 h at room temperature. That solution was desalted by passage through a PD-10 desalting column (GE Healthcare UK Ltd.) and eluent was concentrated by Vivapore (Sartorius Stedim Biotech S.A., France).

2.9. Evaluation of renal localization and plasma levels of fluorescein isothiocyanate (FITC)-labeled HSA-Trx after intravenous administration to mice

ICR mice were injected FITC-labeled HSA-Trx (400 nmol/kg) into a tail vein. Blood samples were collected for the determination of plasma levels of FITC-labeled HSA-Trx at 1 h after the administration of FITC-labeled HSA-Trx. The kidneys were also removed and fixed in phosphate buffered 4% formalin, after the mice were killed under diethyl ether anesthesia at 1 h after the administration of FITC-labeled HSA-Trx. The plasma levels of FITC-labeled HSA-Trx were quantified by using a spectral photometer (JASCO Corporation, MODEL: PTL-3965, Tokyo, Japan). Kidney sections were prepared by the same methods as described above (Histological examination). FITC-derived fluorescence was observed using a Microscope (Keyence, BZ-8000, Osaka, Japan). Immunostainings of HSA, human Trx, and megalin were also performed by the same method as described above. The primary antibody against HSA (BETHYL, Montgomery, cat#: A80-229A), human Trx (R&D system, Minneapolis, cat#: AF1970), or megalin (Santa Cruz Biotechnology, USA, cat#: sc-16478) was diluted 100, 13, or 50 times prior to use. For the secondary antibody for HSA, Trx, and megalin immunostaining, Alexa Fluor 647 donkey anti-goat IgG (H + L) (Invitrogen, Tokyo, Japan) was diluted 100 times before use. After the reaction, the slide was observed using a microscope (Keyence, BZ-8000, Osaka, Japan).

2.10. Cell cultures

HK-2 cells were used as a model of human proximal tubular cells. HK-2 cells (American Type Culture Center, Manassas, VA) was cultured at 37 °C in 5% CO_2 in K-SFM, supplemented with 5 ng/mL human recombinant EGF and 0.05 μg/mL bovine pituitary extract.

2.11. Cellular uptake experiments using FITC-labeled HSA-Trx

To quantify the uptake of FITC-labeled HSA-Trx, HK-2 cells were incubated with FITC-labeled HSA-Trx in an incubator at 37 °C (total uptake) or 4 °C (non-specific uptake). All uptake assays were performed in the absence of serum in the cell culture medium. After incubation with FITC-labeled HSA-Trx, the cells were trypsinized and washed twice with PBS. FITC-labeled HSA-Trx-incorporated cells were quantified using a FACSCalibur® flow cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with a 488-nm argon laser. In inhibition experiments of each endocytic pathway, the inhibitor [excess amount of unlabeled HSA (50-fold HSA)] was incubated with concurrent addition of FITC-labeled HSA-Trx.

2.12. Measurement of intracellular ROS levels

To measure ROS levels (mostly peroxide), CM-H_2DCFDA, a ROS-sensitive fluorescent dye, was used as a ROS probe. To evaluate the effect of HSA-Trx on cisplatin induced ROS production, HK-2 cells were incubated in 96-well plates (1×10^4 cells/well) in K-SFM medium containing 5 ng/mL human recombinant EGF and 0.05 μg/mL bovine pituitary extract at 37 °C for 24 h, and then incubated with 5 μM CM-H_2DCFDA for 30 min in D-PBS. After removing the D-PBS from the wells, the HK-2 cells were incubated with different concentrations of HSA-Trx (0.1, 0.5, 1, 5, 10 μM) for 1 h, and then incubated with 5 μg/mL cisplatin for 3 h. Fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a fluorescence microplate reader (SPECTRA FLUOR, TECAN, Mannedorf, Switzerland).

2.13. Statistical analyses

All data are expressed as the mean ± SD. The means for groups were compared by analysis of variance followed by Tukey's multiple comparison. In fig. 8G, the groups were compared by student's *t*-test. A probability value of $P < 0.05$ was considered to be significant.

3. Results

3.1. Effect of HSA-Trx on cisplatin-induced renal dysfunction

The levels of serum creatinine (SCr), blood urea nitrogen (BUN) and creatinine clearance were measured at 96 h after the intraperitoneal administration of cisplatin (15 mg/kg). As shown in Fig. 1, cisplatin administration resulted in elevations in SCr, BUN, and a decrease in

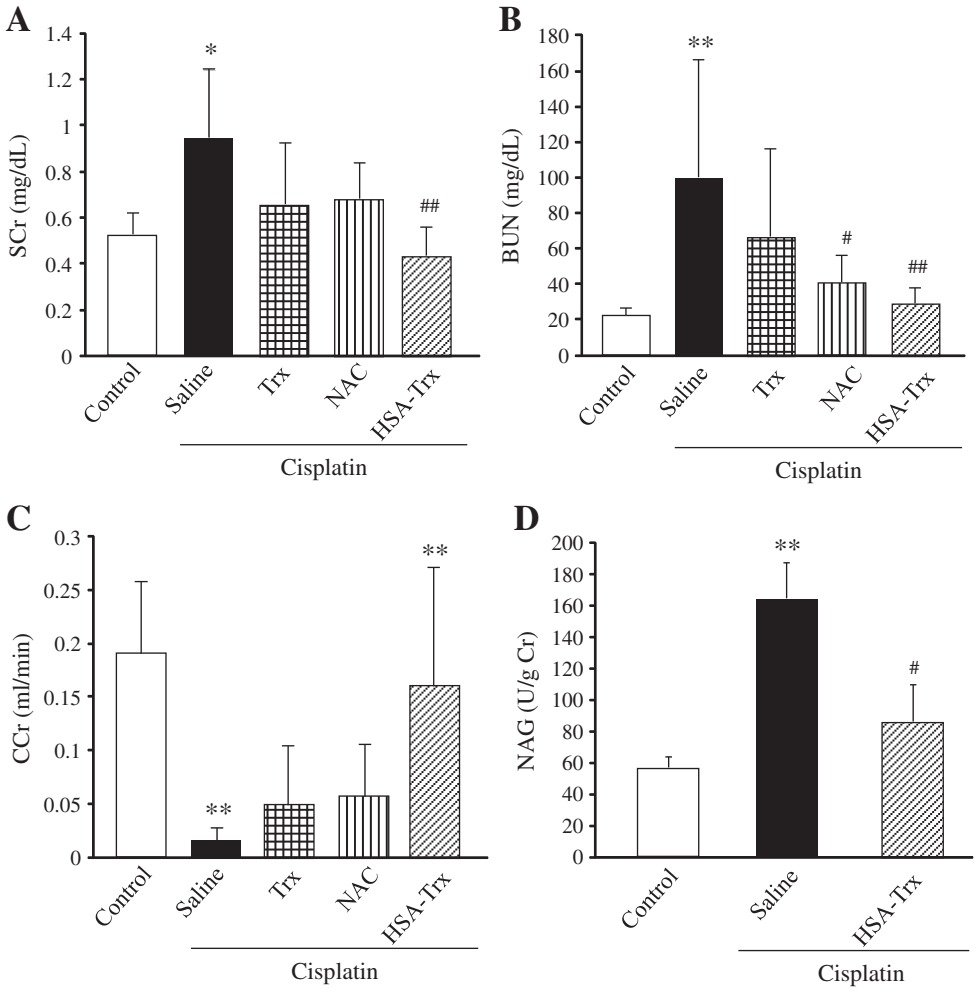
creatinine clearance compared to the control group. The administration of a single intravenous dose of HSA-Trx (100 nmol/kg) at 30 min prior to the cisplatin injection significantly attenuated cisplain-induced renal dysfunction compared with the saline-administered control group (Fig. 1A–C). On the other hand, the administration of an equal molar amount of Trx (100 nmol/kg) or N-acetylcysteine (NAC), a radical scavenger, tended to improve cisplain-induced renal dysfunction. However, these effects were inferior to that for the HSA-Trx group. Interestingly, the levels of urinary N-acetyl-β-D-glucosaminidase (NAG) activity, an indicator of renal tubular dysfunction peaked at 3 to 12 h, and the administration of HSA-Trx significantly attenuated the increased urinary NAG at 12 h after cisplatin administration.

3.2. Effects of HSA-Trx on histologic alterations of renal tissue caused by cisplatin

Fig. 2 shows histological alterations, as evidenced by Periodic acid-Sciff (PAS) staining and a semiquantitative scoring analysis of the kidneys of the control and cisplatin groups with or without HSA-Trx, Trx alone or NAC. It is well known that, because cisplatin accumulates in proximal tubular epithelial cells, the most severe and pronounced alterations occur in the renal tubule cortex and outer stripe of the outer medulla rather than the inner stripe of the outer medulla or inner medulla [23]. As shown in Fig. 2A, the most severe and pronounced alterations were observed in the renal cortico-medullary boundary zone (cortex and outer stripe of the outer medulla) rather than inner stripe of the outer medulla or inner medulla. The cisplatin group (Fig. 2B, F and G) showed evidence of tubular damage, detachment and foamy degeneration of the tubular cells, in addition to subtle tubular dilation compared with the control group. The histological features of the cisplatin group that had been pretreated with HSA-Trx included reduced renal tubular injuries (Fig. 2E). Pretreatment with Trx alone or NAC tended to decrease renal tubular damage but the effects were less than the decrease caused by HSA-Trx (Fig. 2C, D, E and G). These morphological changes are entirely consistent with the alteration in renal functions shown in Fig. 1.

3.3. Effects of HSA-Trx on renal tubular apoptosis caused by cisplatin

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) immunostaining was performed to evaluate renal tubular cell apoptosis (Fig. 3). Kidneys from the cisplatin group treated with saline showed a marked increase in the number of TUNEL-positive tubular cells, mainly in the outer medulla (Fig. 3B). In the cisplatin group that had been pretreated with HSA-Trx, the number of TUNEL-positive cells was markedly decreased



**P<0.01, *P<0.05 vs control , ##P<0.01,#P<0.05 vs cisplatin treatment with saline.

Fig. 1. Changes in the levels of (A) serum creatinine (SCr), (B) blood urea nitrogen (BUN), (C) creatinine clearance (CCr) and (D) urinary N-acetyl-β-D-glucosaminidase (NAG) activity after an intraperitoneal injection of cisplatin (15 mg/kg). Saline, HSA-Trx, Trx alone or NAC was injected 30 min before the injection of cisplatin. Each column represents the mean ± SD (n = 4–7).

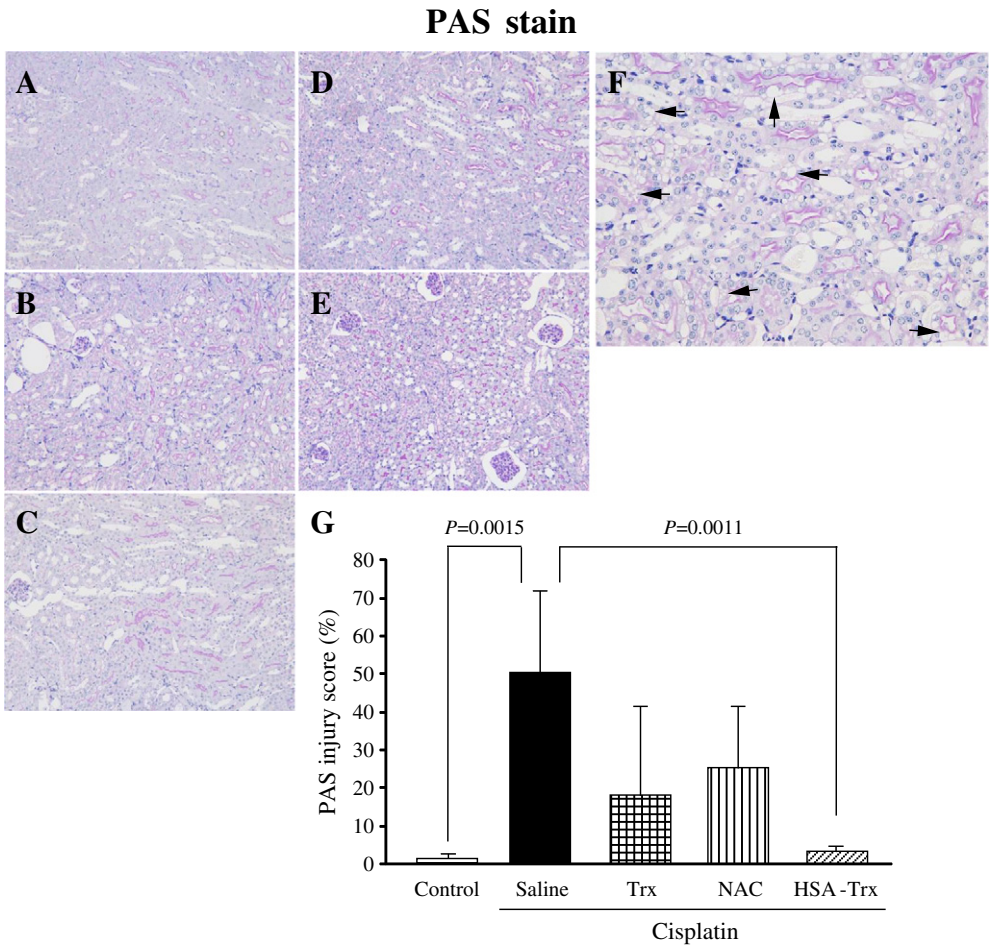


Fig. 2. Histological assessment of the kidney of control or cisplatin-induced nephropathy mice with or without HSA-Trx pretreatment. Representative photomicrographs of PAS-stained kidney sections (A–F) and semiquantitative scoring analysis of tubular degeneration (G) are presented. (A) Control mice, (B) mice with cisplatin after a saline injection, (C) mice with cisplatin after a Trx alone injection, (D) mice with cisplatin after a NAC injection and (E) mice with cisplatin after a HSA-Trx injection. (F) Degeneration (arrow) of tubular cells was observed in the case of the mice with cisplatin after a saline injection. Saline, HSA-Trx, Trx alone or NAC was injected 30 min before the cisplatin (15 mg/mL) injection. The marked tubular injuries caused by cisplatin (B) were diminished as a result of the HSA-Trx treatment (E). Tubular injury (detachment and foamy degeneration of tubular cells and subtle tubular dilation) induced by cisplatin was significantly suppressed by the HSA-Trx treatment (G). For the semi-quantitative analysis of morphological changes, 20 high-magnification (×400) fields of the cortex and the outer stripe of the outer medulla in the mice were randomly selected. The injury ratio (extent of degeneration, detachment, tubulitis and dilatation of tubular cells/total tubular cells × 100) was measured. Magnifications: ×200 in A through E. Data are presented as mean ± SD (n = 4–5). Statistical analyses were performed using the Tukey multiple comparison.

compared to the saline treatment group (Fig. 3E and F). On the other hand, the number of positive cells tended to decrease but could be partially observed in mice that had been pretreated with Trx alone or with NAC (Fig. 3C, D and F).

3.4. Effects of HSA-Trx on ROS induced by cisplatin

Previous findings suggested that ROS released from renal tubules that contain accumulated cisplatin played a critical role in the development of cisplatin-induced nephropathy [23]. Therefore, to investigate whether HSA-Trx would cause a decrease in the ROS induced by cisplatin, kidney sections at 96 h after cisplatin administration were examined for superoxide production (dihydroethidium (DHE) staining) and immunostained to detect the presence of an oxidized derivative of deoxyguanosine (8-OHdG) (Fig. 4A). In addition, cisplatin has been reported to increase renal iNOS protein expression followed by NO release [24]. NO could react with cisplatin-induced superoxide to produce RNS such as peroxynitrite. Hence, kidney sections collected at 96 h after cisplatin administration were also immunostained for nitrotyrosine (Nitro-Tyr) as a reactant of RNS and protein (Fig. 4A). In the cisplatin group that had been pretreated with saline, the number of superoxide, 8-OHdG and Nitro-Tyr-positive cells in the renal tubules (specially boundary zone) was markedly increased compared to the control group. In contrast, the HSA-Trx pretreatment clearly resulted in a decrease in the number of superoxide, 8-OHdG and Nitro-Tyr-positive cells compared with the saline treatment (Fig. 4A).

We next determined the ratio of reduced and oxidized glutathione in the kidney (GSH/GSSG) and the levels of hydroperoxide in plasma at the same experimental conditions. Cisplatin administration decreased the renal GSH/GSSG ratio and increased plasma hydroperoxide levels. However, the HSA-Trx treatment restored the renal GSH/GSSG ratio and decreased plasma hydroperoxide levels, suggesting that HSA-Trx suppresses both renal and plasma ROS levels in cisplatin treated mice.

3.5. Effects of HSA-Trx on inflammatory cytokines induced by cisplatin

Cisplatin-induced ROS cause an increase in the levels of a variety of inflammatory cytokines *via* the activation of the p38 MAPK intracellular signaling pathways [16]. Therefore, we examined the effect of HSA-Trx

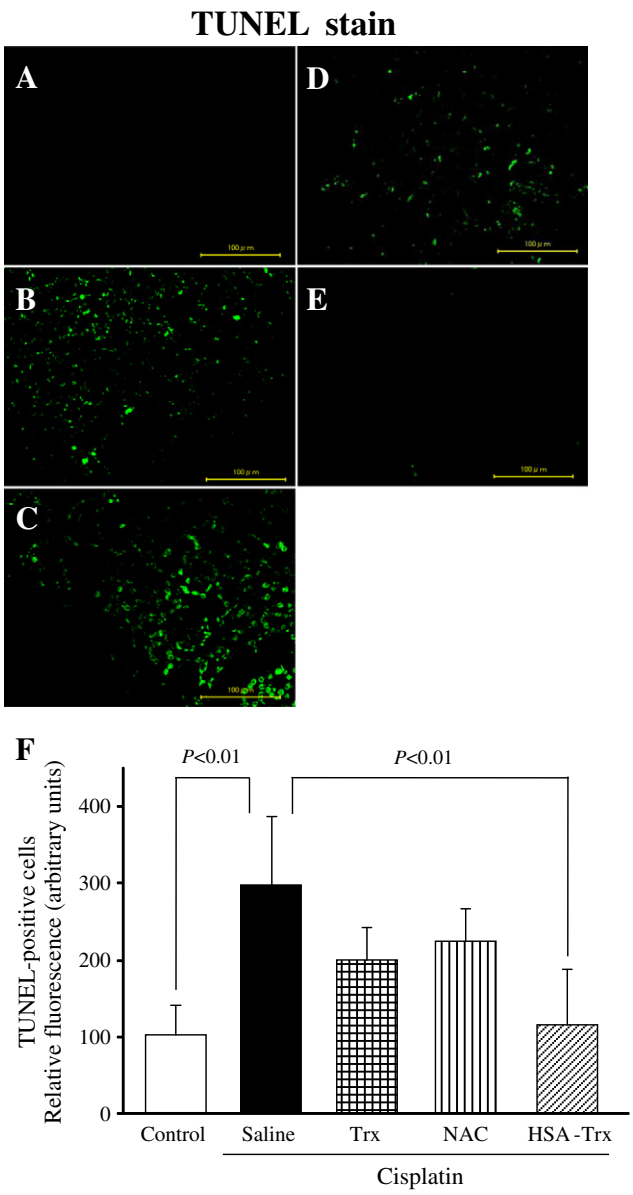


Fig. 3. TUNEL staining of kidneys of control or cisplatin-induced nephropathy mice with or without HSA-Trx. Representative photomicrographs of TUNEL-stained kidney sections from (A) control mice, (B) mice treated with cisplatin after saline injection, (C) mice treated with cisplatin after treatment with Trx alone, (D) mice treated with cisplatin after NAC injection and (E) mice treated with cisplatin after HSA-Trx injection. (F) Image analysis of the extent and intensity of TUNEL-staining was performed. While the number of TUNEL-positive renal tubular cells was increased in the case of cisplatin-induced nephropathy, the numbers were markedly decreased by HSA-Trx treatment. Data are presented as the mean ± SD (n = 4–5).

on TNF-α, IL-1β and IL-6 levels in the kidney of cisplatin-induced nephropathy. As shown in Fig. 5, the levels of TNF-α (Fig. 5A), IL-1β (Fig. 5B) and IL-6 (Fig. 5C) in the kidney, that were increased by cisplatin injection, were significantly decreased as a result of the HSA-Trx treatment.

3.6. Renal localization of FITC-labeled HSA-Trx

To investigate the renal distribution of HSA-Trx, kidney sections were observed at 1 h after the administration of fluorescein isothiocyanate (FITC)-labeled HSA-Trx. As shown in Fig. 6A, FITC-derived fluorescence was observed inside tubular epithelial cells with the exception of the glomus and interstice in the kidney. In addition, the FITC-labeled HSA-Trx was extensively distributed in the cortex of the kidney, partly in the cortio-medullary boundary zone (the cortex and outer stripe of the outer medulla). Interestingly, the distribution of FITC-labeled HSA-Trx is entirely consistent with the renal morphological changes shown in Fig. 2.

To confirm whether the intracellular fluorescence observed in Fig. 6 was derived from the intact form of FITC-labeled HSA-Trx, kidney sections were subjected to immunostaining with an anti-HSA antibody (Fig. 6C), an anti-human Trx antibody (Fig. 6F) and an anti-megalin antibody (Fig. 6I). The anti-HSA and anti-human Trx antibodies do not crossreact with mouse species. The immunostaining by the anti-HSA antibody (Fig. 6C), the anti-human Trx antibody (Fig. 6F) and the FITC-derived fluorescence (Fig. 6D and G) was overlapped extensively with each other. These data strongly indicate that the observed FITC-derived fluorescence was derived from the intact form of FITC-labeled HSA-Trx. As expected, the expression of megalin was observed in the brush border membranes at the luminal side of the proximal tubules. Interestingly, the expression of megalin was partially overlapped with the localization of FITC-labeled HSA-Trx.

To determine whether HSA-Trx is taken up by renal tubular cells, we next examined the uptake of FITC-labeled HSA-Trx by human proximal tubular cells (HK-2 cells). Specific uptake was determined by subtracting the nonspecific uptake measured at 4 °C from the uptake at 37 °C. As shown in Fig. 6K, the FITC-labeled HSA-Trx was taken up in a time-dependent manner. Moreover, the uptake of FITC-labeled HSA-Trx was markedly suppressed by an excess amount of unlabeled HSA (50-fold HSA) (Fig. 6L). These data indicate that HSA-Trx is incorporated into HK-2 cells *via* a megalin/cublin mediated endocytic pathway, the same as for HSA.

3.7. Effect of HSA-Trx on intracellular ROS levels induced by cisplatin in HK-2 cells

The effect of HSA-Trx on intracellular ROS levels induced by cisplatin was examined using HK-2 cells. The presence of cisplatin caused a significant increase in ROS production in a time-dependent manner, reaching a maximum at 3 h (data not shown). Intriguingly, the extent of cisplatin-induced ROS levels was significantly suppressed by HSA-Trx in a concentration-dependent manner (Fig. 7).

3.8. Kidney and plasma distributions of FITC-labeled HSA-Trx with the co-administration of unlabeled HSA

The renal localization (Fig. 8A–F) and plasma levels (Fig. 8G) of FITC-HSA-Trx were evaluated at 1 h after the administration of FITC-labeled HSA-Trx with the co-administration of a 50-fold amount of unlabeled HSA to investigate the mechanism of distribution of HSA-Trx to the kidney. Kidney sections were immunostained by an anti-megalin antibody (Fig. 8B), an anti-human Trx antibody (Fig. 8D) and an anti-HSA antibody (Fig. 8F). As shown in Fig. 8D, the renal tubular localization of HSA-Trx was markedly decreased by 50-fold excess amount of unlabeled HSA. Immunostained HSA was observed at the basal side of the tubular cells (Fig. 8F). This stained HSA was considered to be concomitantly administered unlabeled HSA. Under the same experimental conditions, no significant difference was observed in plasma levels of FITC-labeled HSA-Trx with or without the co-administration of unlabeled HSA. These data suggest that the majority of HSA-Trx remained in the blood circulation and a small portion of the fusion protein was distributed to tubular cells at 1 h after the administration of HSA-Trx.

4. Discussion

Cisplatin is a widely used and potent chemotherapy agent that is used in the treatment of solid tumors. However, cisplatin-induced nephrotoxicity is a major dose-limiting factor and a serious clinical problem associated with this type of therapy. Therefore, a treatment strategy that could mitigate cisplatin nephrotoxicity would be highly

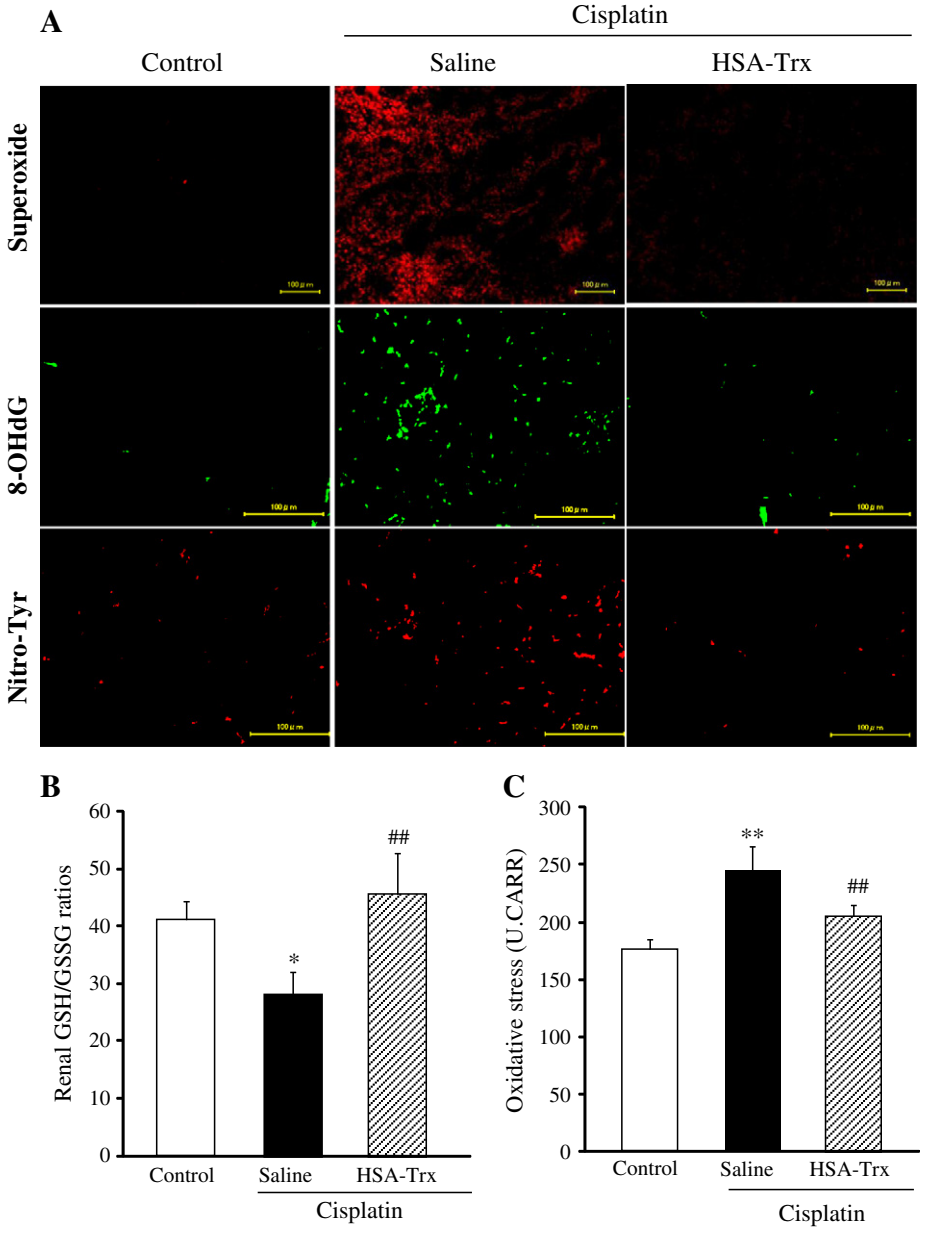


Fig. 4. The redox effect of HSA-Trx on the plasma and renal oxidative stress in cisplatin-induced nephropathy mice. (A) Representative photomicrographs of renal superoxide (DHE staining) (upper panels), immunostaining of renal 8-OHdG (8-hydroxy-2′-deoxygenase) (middle panels) and Nitro-Tyr (nitrotyrosine) (lower panels) in control mice, mice treated with cisplatin after a saline injection or after the injection of HSA-Trx, (B) renal GSH/GSSG ratios and (C) level of plasma hydroperoxide contents (dROMs test). HSA-Trx suppressed the cisplatin-induced oxidative stress in kidney and plasma. Data are presented as mean ± SD (n = 4–5).

desirable. Chemotherapies using cisplatin are seldom given on an outpatient basis, because aggressive hydration is required to reduce cisplatin-induced nephrotoxicity. This vigorous hydration decreases the quality of life in cancer patients. Cisplatin is usually given in 3 or more doses, often with several weeks between doses [25]. This implies that a therapeutic agent for preventing cisplatin-induced nephropathy will need to have a long duration of action. Using albumin fusion technology, we were able to successfully extend the plasma half-life of Trx to about 10 times longer in mice, with the plasma elimination profile of HSA-Trx being similar to that for HSA [19,20,22]. The half-life of HSA-Trx in humans is expected to be about 20 days, similar to HSA in a clinical setting. Albumin fusion also prolongs the anti-oxidative and anti-inflammatory modulating actions of Trx, rendering HSA-Trx the required desirable therapeutic properties for the treatment or prevention of cisplatin-induced nephrotoxicity. In fact, we have previously reported that HSA-Trx had the potential to effectively prevent contrast-induced nephropathy [21]. Pre- and post-treatment of HSA-Trx also had suppressive effect against bleomycin-induced pulmonary fibrosis by its long-acting antioxidative and anti-inflammatory modulation [22]. Therefore, HSA-Trx could have therapeutic properties for the treatment or prevention of cisplatin-induced nephrotoxicity. In addition, considering its half-life, HSA-Trx might also be expected to prevent the late emergence of cisplatin nephrotoxicity. The protocol for the culture, purification and formulation established for recombinant HSA could be easily modified to produce HSA-Trx by simply switching the cDNAs because the recombinant HSA product has already been approved for use.

The mechanisms of cisplatin nephrotoxicity involve oxidative stress, inflammation and apoptosis [23]. HSA-Trx markedly attenuated cisplatin-induced renal oxidative stress, as shown in the evaluation of renal superoxide production, the accumulation of 8-OHdG and

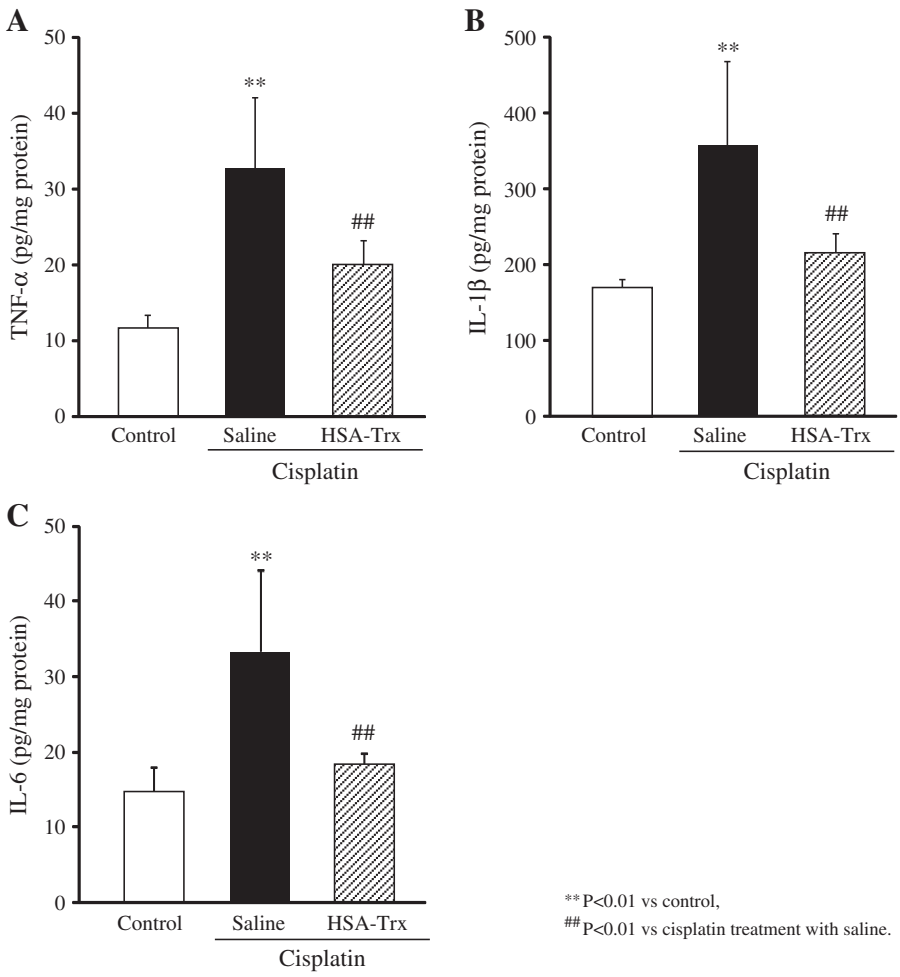


Fig. 5. Effect of HSA-Trx on the expression of renal inflammatory cytokines in cisplatin-induced nephropathy mice. The expression of (A) TNF-α (B) IL-1β, and (C) IL-6 in kidney was determined by ELISA kit. HSA-Trx suppressed the cisplatin-induced inflammatory cytokine expressions in kidney. Data are presented as the mean ± SD (n = 6).

intracellular reduced glutathione content (Fig. 4). Trx suppresses oxidative stress *via* a number of mechanisms. For instance, Trx directly scavenges ROS such as hydroxyl radicals and singlet oxygen *via* the reversible oxidation of its redox-active cysteine residues [26]. It also indirectly enhances the reduction of hydroperoxides *via* the activation of peroxiredoxine, a family of peroxidases, by serving as an electron source [27]. These mechanisms likely contribute to the anti-oxidative effect of HSA-Trx against cisplatin-induced renal injury. Like Trx, HSA also contains a reduced thiol in Cys34, which accounts for about 70–80% or more of the total thiol groups in the blood circulation [28,29], and thereby plays an important role in the anti-oxidant defense system in blood [30]. Therefore, it is highly possible that HSA in HSA-Trx also contributes to the anti-oxidative activity of the molecule. However, the administration of an equal molar amount of HSA had no renoprotective effect on cisplatin-treated mice in comparison with the HSA-Trx group (data not shown), and Trx alone had little effect in preventing cisplatin-induced renal injury, in comparison with the HSA-Trx group. Taking into account the fact that the plasma level of HSA-Trx used in this study was estimated to be below 1/200 of the endogenous mouse serum albumin level, these findings suggest that the ROS scavenging effect of HSA-Trx is mainly dependent on the long acting Trx.

Previous studies demonstrated that inducible nitric oxide synthetase (iNOS) expression in the kidney was increased in cisplatin-treated mice, and selective iNOS inhibition reduced cisplatin-induced renal injury [10]. A simultaneous increase in superoxide and iNOS-derived NO production may facilitate the formation of peroxynitrite, potent cytotoxin. A previous study reported that Trx scavenged NO and peroxynitrite with its Cys69 and Cys73 residues, which are located outside of the active site [31]. In the present study, HSA-Trx inhibited the production of nitrotyrosine as a reactant of RNS and protein, suggesting that HSA-Trx also suppresses the nitrosative stress induced by cisplatin.

Cisplatin induces a series of inflammatory changes that mediate renal injuries. Recent evidence indicates that inflammation plays an important role in the pathogenesis of cisplatin-induced renal injuries. Among the inflammatory cytokines, TNF-α is thought to play a central role in mediating renal injuries. It induces apoptosis and ROS production, and coordinates the activation of a large network of chemokines and cytokines in the kidney [23]. Ramesh G et al. reported that TNF-α inhibitors ameliorated cisplatin-induced renal dysfunction by 50% and also reduced renal structural damage [32]. They also showed that TNF-α-deficient mice were protected to a greater extent against cisplatin nephrotoxicity [33]. Trx has been reported to exert its anti-inflammatory properties by suppressing the migration and/or infiltration and extravascular leakage of leukocytes *via* modulating the expression of macrophage migration inhibitory factors [34,35], resulting in a reduced expression of inflammatory cytokines such as TNF-α, IL-1β and IL-6 [36]. HSA-Trx was shown in the present study to markedly suppress the cisplatin-induced elevation in inflammatory cytokines such as TNF-α, IL-1β and IL-6, clearly suggesting that HSA-Trx exerted an anti-inflammatory action in this mouse model.

Apoptosis is now recognized as an important mode of cell death in cisplatin nephrotoxicity. Fig. 3 clearly shows that HSA-Trx suppressed the production of TUNEL-positive cells. Fujino G et al. recently demonstrated the role of endogenous Trx on redox regulation associated

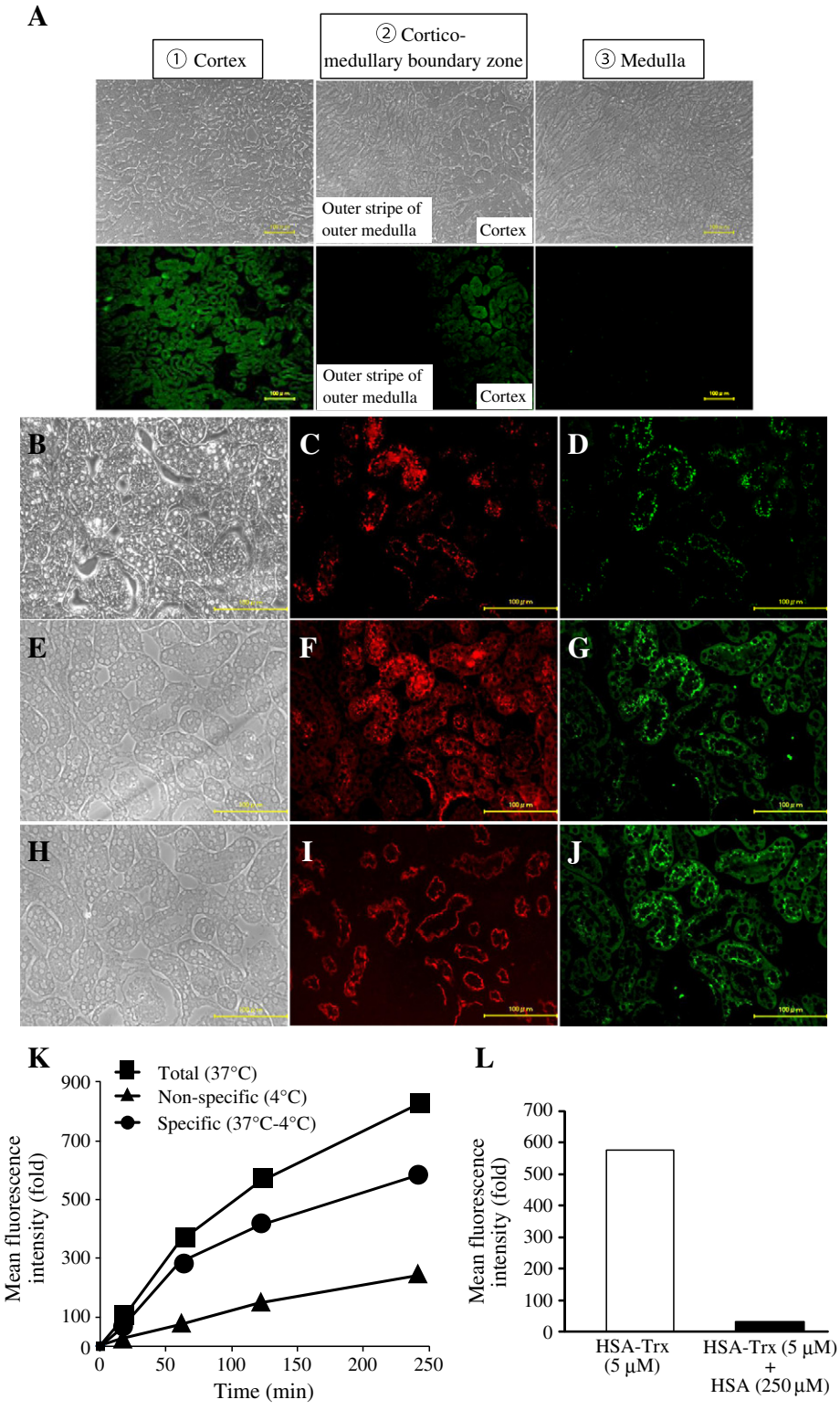
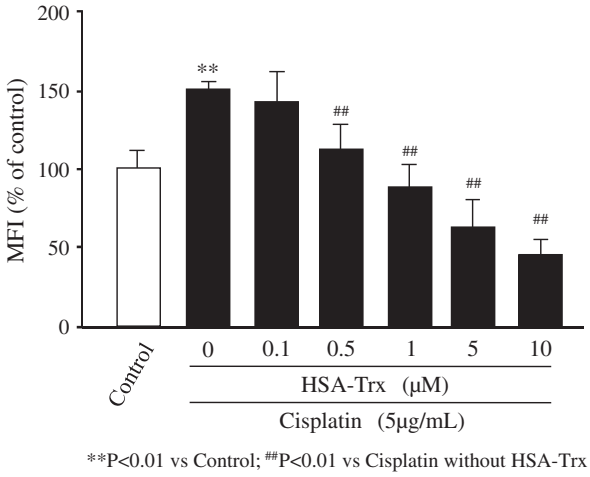
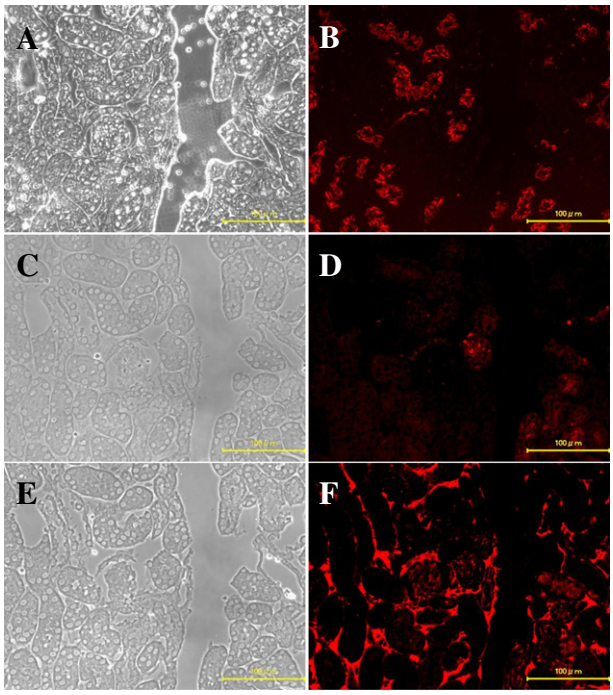


Fig. 6. Renal localization of FITC-labeled HSA-Trx in mouse and uptake of FITC-labeled HSA-Trx by HK-2 cells. (A) Kidney sections of mice at 1 h after FITC-labeled HSA-Trx injection. Each panel indicates (1) cortex, (2) cortico-medullary boundary zone, (3) medulla. FITC-derived fluorescence was observed inside tubular epithelial cell with the exception of glomus and interstice in kidney. Representative photomicrographs of immunostaining of (C) HSA, (F) human-Trx, and (I) megalin, (B, E, and H) bright-field, (D, G, and J) FITC-derived fluorescence. All sections (B–J) are serial sections of the kidney. Immunostaining by anti-HSA antibody, anti-human Trx antibody and FITC-derived fluorescence was well overlapped with each other. Magnifications: ×400 in B through J. HK-2 cells were incubated for each time point with FITC-labeled HSA-Trx (400 nmol/kg) at 37 °C or 4 °C (K), and in the absence or presence of excess amount of unlabeled HSA (50-fold HSA) (L). The uptake of FITC-labeled HSA-Trx was assessed by flow cytometry and expressed as the ratio of the mean fluorescence intensity estimated from untreated cells at 37 °C. The specific uptake was calculated by subtracting the uptake at 4 °C (nonspecific uptake) from the uptake at 37 °C. The uptake of FITC-labeled HSA-Trx was markedly suppressed in the presence of excess amount of unlabeled HSA (50-fold HSA).



**P<0.01 vs Control; ##P<0.01 vs Cisplatin without HSA-Trx

Fig. 7. Effect of HSA-Trx on intracellular ROS levels induced by cisplatin in HK-2 cells. HK-2 cells were incubated in 96-well plates (1×10^4 cells/well) in K-SFM medium containing 5 ng/mL of human recombinant EGF and 0.05 μg/mL bovine pituitary extract at 37 °C for 24 h, and then incubated with 5 μM CM-H_2DCFDA for 30 min in D-PBS. After removing the D-PBS from the wells, the HK-2 cells were incubated with different concentrations of HSA-Trx (0.1, 0.5, 1, 5, 10 μM) for 1 h, and then incubated with 5 μg/mL cisplatin for 3 h. Fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Each column represents the mean ± SD (n = 4). $^{**}P < 0.01$ vs control; $^{\#\#}P < 0.01$ vs cisplatin treatment without HSA-Trx.



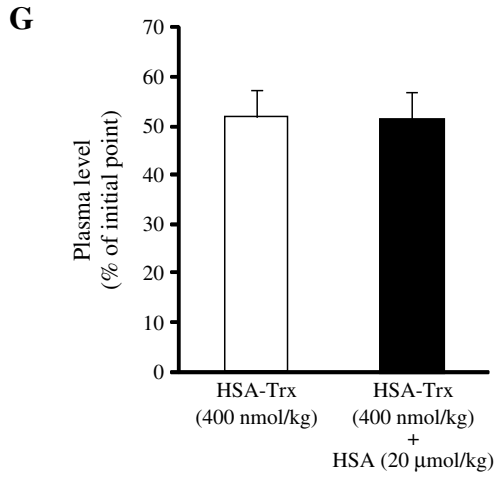


Fig. 8. Kidney and plasma distributions of FITC-labeled HSA-Trx. (A–F) Renal localization of FITC-HSA-Trx was evaluated at 1 h after administration of FITC-labeled HSA-Trx with co-administration of a 50-fold amount of unlabeled HSA. Representative photomicrographs of immunostaining of (B) megalin, (D) human-Trx, and (F) HSA, (A, C, and E) bright-field. All section (A–F) is serial section of kidney. No significant difference was observed in the plasma levels of FITC-labeled HSA-Trx in the absence or presence of unlabeled HSA. On the other hand, the renal tubular distribution of HSA-Trx was markedly decreased in the presence of excess amount of unlabeled HSA. Magnifications: ×400 in A through F. (G) Plasma level of FITC-HSA-Trx was evaluated at 1 h after the administration of FITC-labeled HSA-Trx with or without the co-administration of 50-fold amount of unlabeled HSA using a spectral photometer.

with apoptosis [37]. The apoptosis signal regulating factor 1 (ASK1) binds to Trx inside the cell, under normal conditions. When Trx is oxidized by ROS, ASK1 dissociates from Trx and interacts with TRAF2/6, inducing the phosphorylation of JNK and p38 MAPK to transduce the signal for apoptosis [37]. Therefore, one of the possible mechanism by which HSA-Trx suppresses apoptosis could be *via* the inhibition of ROS-induced endogenous Trx oxidation, or the complementation of intracellular Trx, which can bind to ASK1. Furthermore, suppression of the increased production of the inflammatory cytokine TNF-α could also contribute to the inhibition of apoptosis signaling, as mentioned above.

It would also be interesting to know whether HSA-Trx acts in the kidneys or in the general circulation, or both. We previously proposed that it is highly possible that HSA-Trx exerts its biological effect in the extracellular compartment [19–22]. That is, HSA-Trx scavenges ROS extracellularly thereby enhancing total anti-oxidant capacity, which would facilitate the regeneration, replenishment and cellular translocation of glutathione and other SH-containing molecules. In fact, as shown in Fig. 4B, HSA-Trx treatment restored the renal GSH/GSSG ratio. On the other hand, receptor-mediated endocytosis *via* megalin and cubilin reabsorbed the glomerularly filtered albumin from the luminal side of the proximal tubule [38]. Recently, Tenten V et al. provided evidence for the existence of a transcytosis mechanism for the reabsorption of albumin *via* the megalin/cubilin system across tubular cells in an FcRn-dependent manner [39]. Therefore, the possibility that HSA-Trx could exert its pharmacological effects in the proximal tubule after being taken up *via* the megalin/cublin system cannot be excluded. In fact, when kidney sections obtained at 1 h after the administration of FITC-labeled HSA-Trx were immunostained using anti-HSA, anti-human Trx and anti-megalin antibodies, HSA-Trx was found to be localized in a punctate pattern along the apical region of proximal tubular cells, whereas partial localization was observed in proximal tubular cells (Fig. 6A–J). In addition, since HSA-Trx distribution was suppressed by an excess amount of unlabeled HSA (Fig. 8), it is highly possible that a portion of the glomerularly filtered HSA-Trx could be taken up by proximal tubular cells *via* the same route as HSA. These data are supported by the observation that HSA-Trx is specifically taken up by HK-2 cells (Fig. 6K), and the uptake was suppressed in the presence of unlabeled HSA (Fig. 6L). Furthermore, HSA-Trx significantly suppressed cisplatin-induced intracellular ROS formation (Fig. 7). Therefore, HSA-Trx could potentially exert its effect, in part, inside of proximal tubules in addition to the extracellular space. However, since no significant difference in the plasma levels of FITC-labeled HSA-Trx with or without the coadministration of unlabeled HSA could be observed (Fig. 8), only a small portion of the administered HSA-Trx was distributed to the kidney. These findings indicate that HSA-Trx exerts its biological effect predominantly in the extracellular compartment, and partially in the intracellular compartment of proximal tubular cells. Further investigation using megalin/cubilin knockout mice will be necessary to confirm the present findings.

Several reports have demonstrated that NAC improved cisplatin-induced nephrotoxicity [40,41]. In this study, NAC is working in a similar manner as HSA-Trx, but its renoprotective effect was lower than

HSA-Trx. Our finding suggests that the difference in the preventive effect between HSA-Trx and NAC in cisplatin nephrotoxicity could be mainly due to the prolonged anti-oxidative and anti-inflammatory action of HSA-Trx in kidney, especially inside the proximal tubular cells as well as in the general circulation. Therefore, HSA-Trx could retain its activity in the kidney longer than NAC. It might be also possible to treat kidney impairment with HSA-Trx at a lower dose and a lesser dosing frequency than NAC.

In conclusion, a single dose of HSA-Trx administered before cisplatin treatment exerts significant renoprotective effects in a mouse model. The results indicate that HSA-Trx could be a novel and effective approach for preventing cisplatin nephrotoxicity.

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