Glyoxalase I overexpression ameliorates renal ischemia-reperfusion injury in rats

Glyoxalase I 高発現はラットの腎虚血再灌流障害を改善する

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Abstract

Methylglyoxal (MG), a highly reactive carbonyl compound generated by carbohydrate oxidation and glycolysis, is the major precursor of protein glycation and induces cytotoxicity leading to apoptosis. Although recent studies have emphasized that MG accumulates in not only chronic oxidative stress-related diseases but also acute hypoxic conditions, the pathogenic contribution of MG in acute diseases is unclear. MG is efficiently metabolized by the glyoxalase system, namely glyoxalase I. Ⅰ investigated the pathophysiological role of glyoxalase I as an MG detoxifier in rat renal ischemia-reperfusion (I/R) injury. I/R-induced tubulointerstitial injury was associated with a deterioration in renal glyoxalase I activity independent of its cofactor, GSH, as well as an increase in renal MG level. In *in vitro* studies, knockdown of glyoxalase I by siRNA transfection in rat tubular cells exacerbated cell death by hypoxia-reoxygenation compared to control cells. I also examined whether glyoxalase I overexpression prevented renal I/R damage in rats overexpressing human glyoxalase I with enzyme activity in the kidney 17-fold higher than in wild-type. The histological and functional manifestations of I/R in these rats were significantly ameliorated in association with a decrease in intracellular MG accumulation, oxidative stress and tubular cell apoptosis. In conclusion, glyoxalase I exerts renoprotective effects in renal I/R injury via a reduction in MG accumulation in tubular cells.

Introduction

Methylglyoxal, a precursor of advanced glycation end products

Chemical modification of proteins by reactive aldehydes and ketones is a well-recognized phenomenon in diabetes. Such modifications occur through a reaction known as Maillard reaction (1-3). Maillard reaction in the early stage is well established (Figure 1) but the reaction in the advanced stage remains to be elucidated to date. Glucose reacts with the free amino groups of amino acids, proteins, phospholipids, and nucleic acids. This process begins with the conversion of reversible Schiff base adducts to more stable, covalently-bound Amadori rearrangement products (4). Widely-known Amadori pruducts are glycosylated hemoglobin (HbA1c). HbA1c is the Amadori product formation on the HbA1 amino terminal valine of the β chain and one of the most useful index of glycemic control (5). These Amadori products then undergo further rearrangements to form irreversible adducts on proteins that are known as advanced glycation end products (AGEs) (6). AGEs are thought to be responsible, in part, for the diabetic complications such as retinopathy, nephropathy and vascular injury (7-10). Alpha-dicarbonyl compounds are thought to be the major precursors of AGEs (Figure 2). Among the α-dicarbonyls, methylglyoxal (MG) has recently received considerable attention as a mediator to form AGEs. A recent study on the formation of AGEs in endothelial cells cultured under hyperglycemic conditions indicated that MG was the major precursor of AGEs (11). It has been reported that MG primarily reacts with arginine residues to form N^{δ}-(5-hydroxy-4,6-dimethylpyrimidine-2-yl)-L-ornithine (argpyrimidine) (12). MG also reacts with lysine residues to generate N^{ε}-carboxy-ethyllysine (CEL) (13). Representative AGEs formed by methylglyoxal is shown below (Figure 3). These AGE interact with the receptor for AGE (RAGE) and thereby lead to oxidative stress followed by cellular injury (14, 15). In addition, MG itself is thought to be a strong cytotoxic substance. MG selectively inhibits mitochondrial

respiration and glycolysis in the cells (16). In relation to this, MG has been shown to inactivate membrane ATPases and glyceraldehyde-3-phosphate dehydrogenase, a key enzyme of the glycolytic pathway (17). In addition, MG induces apoptosis through various mechanisms. Kim et al. reported that MG-induced apoptosis in retinal pericytes may occur via an oxidative stress mechanism involving NF-κB (18). MG enhances cisplatin-induced apoptosis through activation of protein kinase Cδ (19).

Glyoxalase system for detoxification of methylglyoxal

The glyoxalase system detoxifies MG. The glyoxalase system is composed of two enzymes; glyoxalase I, GLO I, which metabolizes MG to S-D-lactoylglutathione, and GLO II, which converts S-D-lactoylglutathione to D-lactate (20) (Figure 4). The glyoxalase system was originally found in 1913. The complete function remains to be elucidated. Previous reports demonstrated that overexpression of GLO I in bovine endothelial cells prevented an increase in MG and intracellular AGE accumulation when the cells were cultured in the presence of high glucose, indicating that GLO I has a critical role for suppression of AGE formation (11). Moreover, a series of studies indicated that certain tumor primary cultures and cell lines overexpress GLO I, suggesting that increased amounts of this enzyme prevent tumor cell apoptosis, possibly by limiting MG production. GLO I is a GSH-dependent enzyme. Under physiological conditions *in situ*, the rate of fragmentation of hemithioacetal to GSH and MG is of the order of 10^3 times faster than the rate of isomerization by GLO I. Therefore, there is a rapid pre-equilibrium of GSH and MG with hemithioacetal, and the activity of GLO I *in situ* is proportional to the cellular concentration of GSH (21). GLO I is expressed ubiquitously in the integral part of the cells. In human kidney, GLO I is more abundant in the cortex than in the medulla (22).

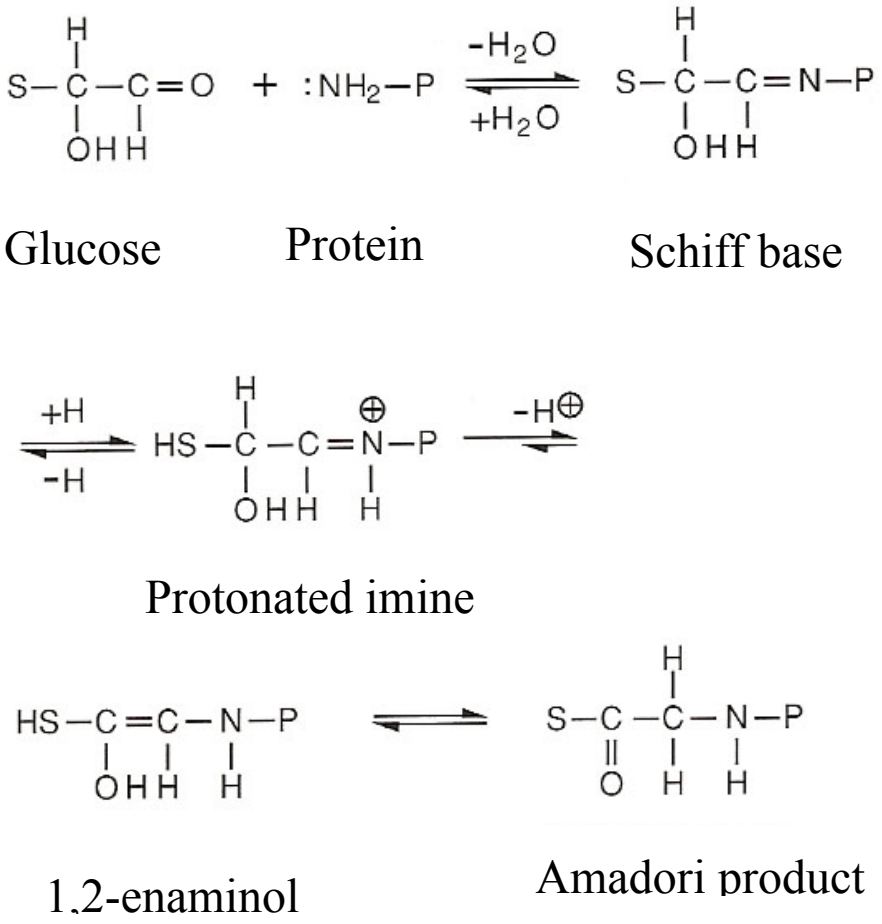

Figure 1. Schematic representation of Maillard reaction in the early stage. Glucose reacts with proteins and reversible Schiff base products are formed. Schiff base adducts are converted to more stable, covalently-bound Amadori rearrangement products.

Takeuchi M et al. Mol Med 2001 (Reference 4)

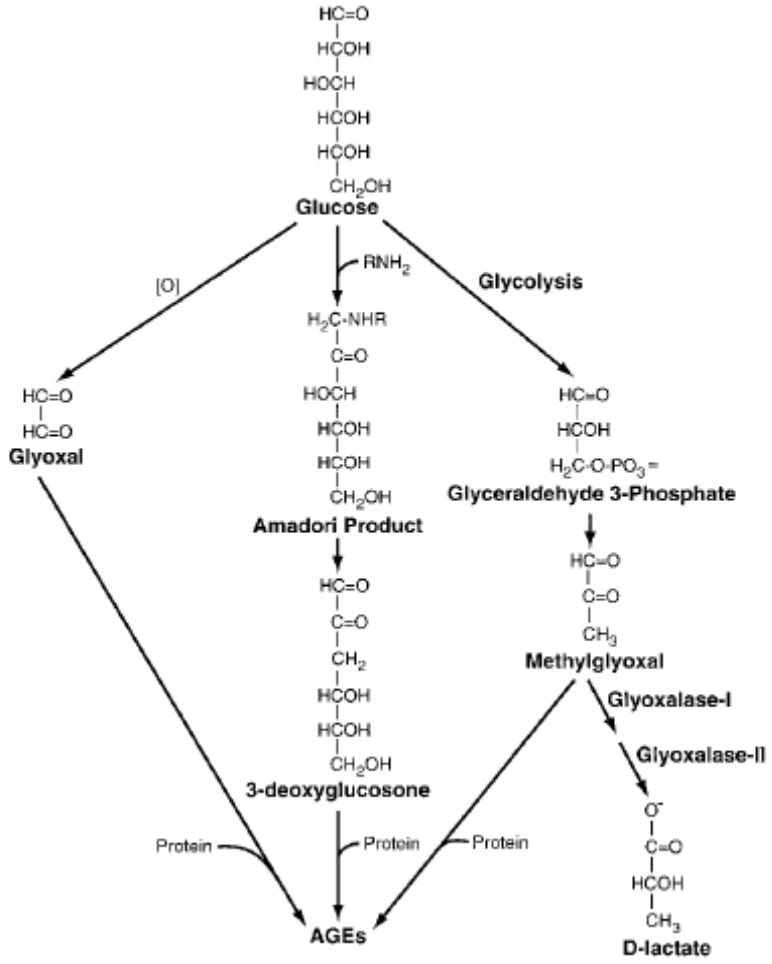

Figure 2. Schematic representation of potential pathway leading to AGE formation. AGEs arise from autoxidation of glucose to glyoxal, decomposition of the Amadori product to 3-DG, and the glyceraldehyde fragmentation product MG, which all react with amino groups of proteins. MG is metabolized to the unreactive D-lactate by the glyoxalase system.

Shinohara M et al. J Clin Invest 1998 (Reference 11)

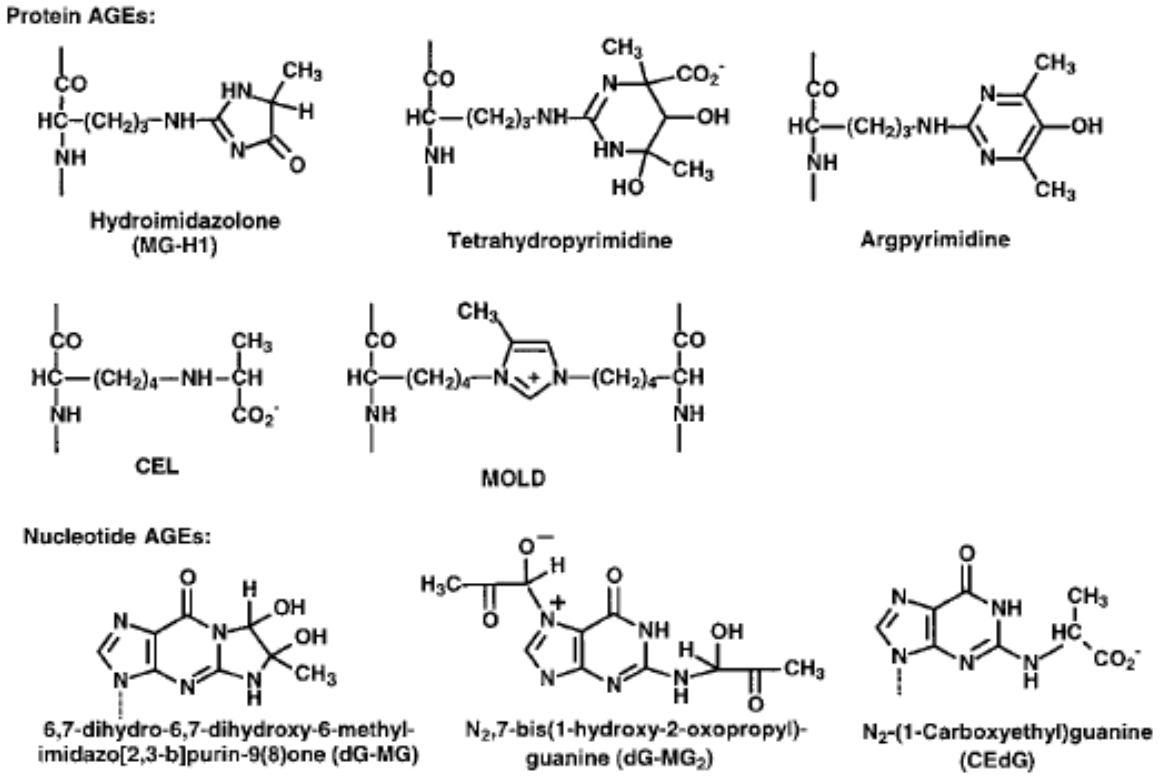

Figure 3. Protein and nucleotide AGEs formed by methylglyoxal.

Thornallry PJ et al. Biochem Soc Trans 2003 (Reference 21)

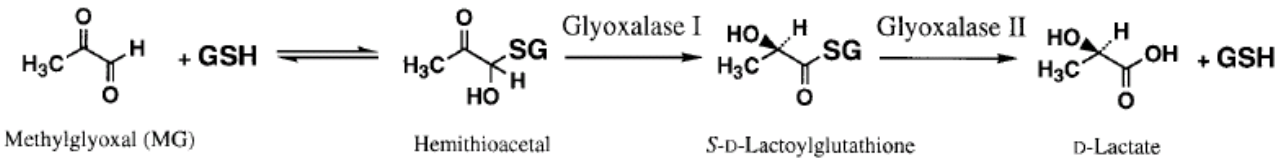

Figure 4. The glyoxalase system.

Thornallry PJ et al. Biochem Soc Trans 2003 (Reference 21)

Sequence of human GLO I

The human cDNA clones encoding GLO I was isolated by Kim N.S. et al. and the sequence of human GLO I was reported (23). The human cDNA encodes a protein of 184 amino acids with a calculated Mt of 20719. Nucleotides sequence of human GLO I cDNA and the deduced amino acid sequence are indicated below (Figure 5).

Crystal structure of human GLO I

The structure of human GLO I was determined by multiple isomorphous replacement (24). The monomer is built up from two domains (residues 31-104 and 124-183), linked by a 20 residue connection and preceded by a long N-terminal arm. This arm does not interact with either domain (Figure 6A). The active enzyme is a dimer of identical subunits. In the dimer, the first strand of the first domain of one monomer interacts in an anti-parallel fashion with the first strand of the second domain of the opposing monomer so that an eight-stranded β sheet is formed. This sheet combines with the extra helix of domain 1 and the C-terminal helix of domain 2 to form a barrel containing the active site at the dimer interface (Figure 6B).

Pathophysiological functions of the glyoxalase system in the diseases

MG level is increased in various oxidative stress-related diseases. The glyoxalase system-related pathological conditions include diabetic nephropathy (25, 26), diabetic retinopathy (27, 28), Alzheimer disease (29-31), anxiety (32-34), resistance to anti-cancer drugs (35, 36), and aging (37-40). In addition, GLO I deficiency was associated with unusually high plasma levels of AGEs in a hemodialysis patient (41). A list of the glyoxalase system-related diseases is expanding at the present time.

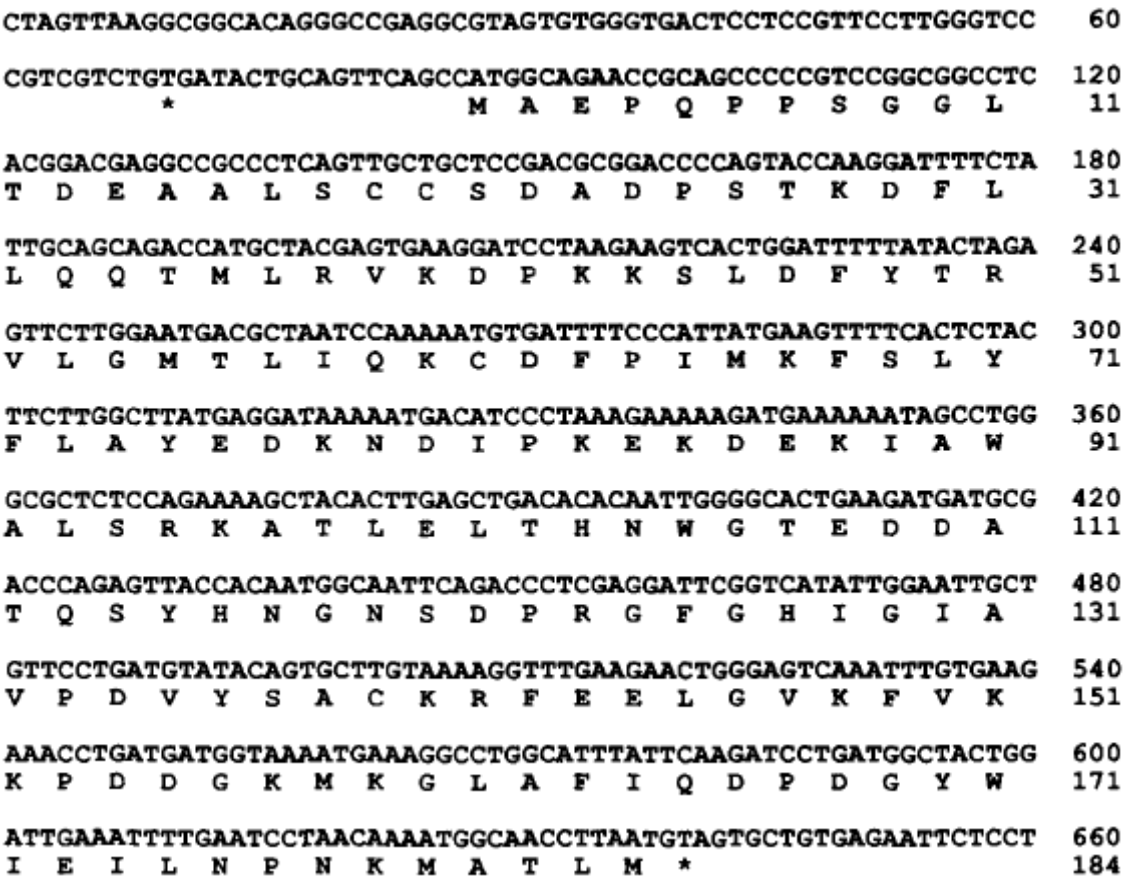

Figure 5. Nucleotide sequence of human GLO I cDNA and the deduced amino acid sequence. The asterisks indicate a termination codon. The open reading frame is preceded by an inframe termination codon.

Kim NS et al. J Biol Chem 1993 (Reference 23)

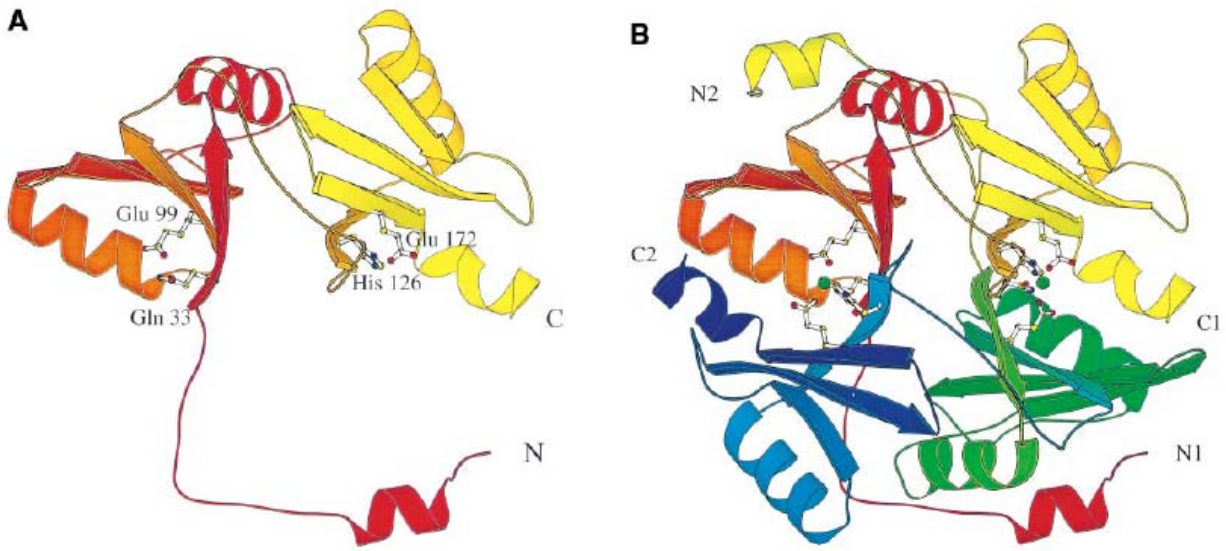

Figure 6. Schematic representation of GLO I. (A) Monomer, (B) dimer. The dimer has been colour ramped according to residue number, starting with red at the N-terminus of one molecule, passing through yellow at the C-terminus of that molecule and finishing with blue at the C-terminus of the other monomer. The zinc and its coordinating residues are shown in a ball and stick representation with the zinc colored green. The active site is situated in a barrel which is formed only on dimerization.

Cameron AD et al. EMBO J 1997 (Reference 24)

Glyoxalase system and kidney diseases

Regarding the relationship between the glyoxalase system and kidney diseases, diabetic nephropathy has been the focus of intensive researches. For example, Beisswenger et al. demonstrated that MG levels are elevated in diabetic subjects with more rapid progression of nephropathy (26). On the other hand, relationship between acute kidney injury and the glyoxalase system remains to be elucidated, although acute kidney injury has remained a significant health care concern with high mortality rates.

Glyoxalase system and ischemia-reperfusion injury

Ischemia due to hypotension or sepsis is the most common cause of human acute renal failure. Also in kidney transplantation, ischemia-reperfusion (I/R) injury is the significant problem. These backgrounds emphasize clinical importance of renal I/R injury. The mechanisms underlying renal I/R injury are most likely multifactorial and interdependent, involving hypoxia, inflammatory responses, and cellular damage by free radical.

Recently several reports demonstrated that methylglyoxal (MG), advanced glycation end-products (AGEs) precursor produced by the fragmentation of triose phosphates, is increased in myocardiac or cerebral I/R. In cardiac I/R injury, MG content is increased in the I/R heart associated with the accumulation of N^{ε}-carboxymethyl lysine (CML) (42) (Figure 7). In cerebral I/R injury, MG-arginine adducts, argpyrimidine, is detected in arterial walls within the infarcted zone after 24 hr reoxygenation (43) (Figure 8). However, the relationship between MG and renal I/R injury has not been reported yet.

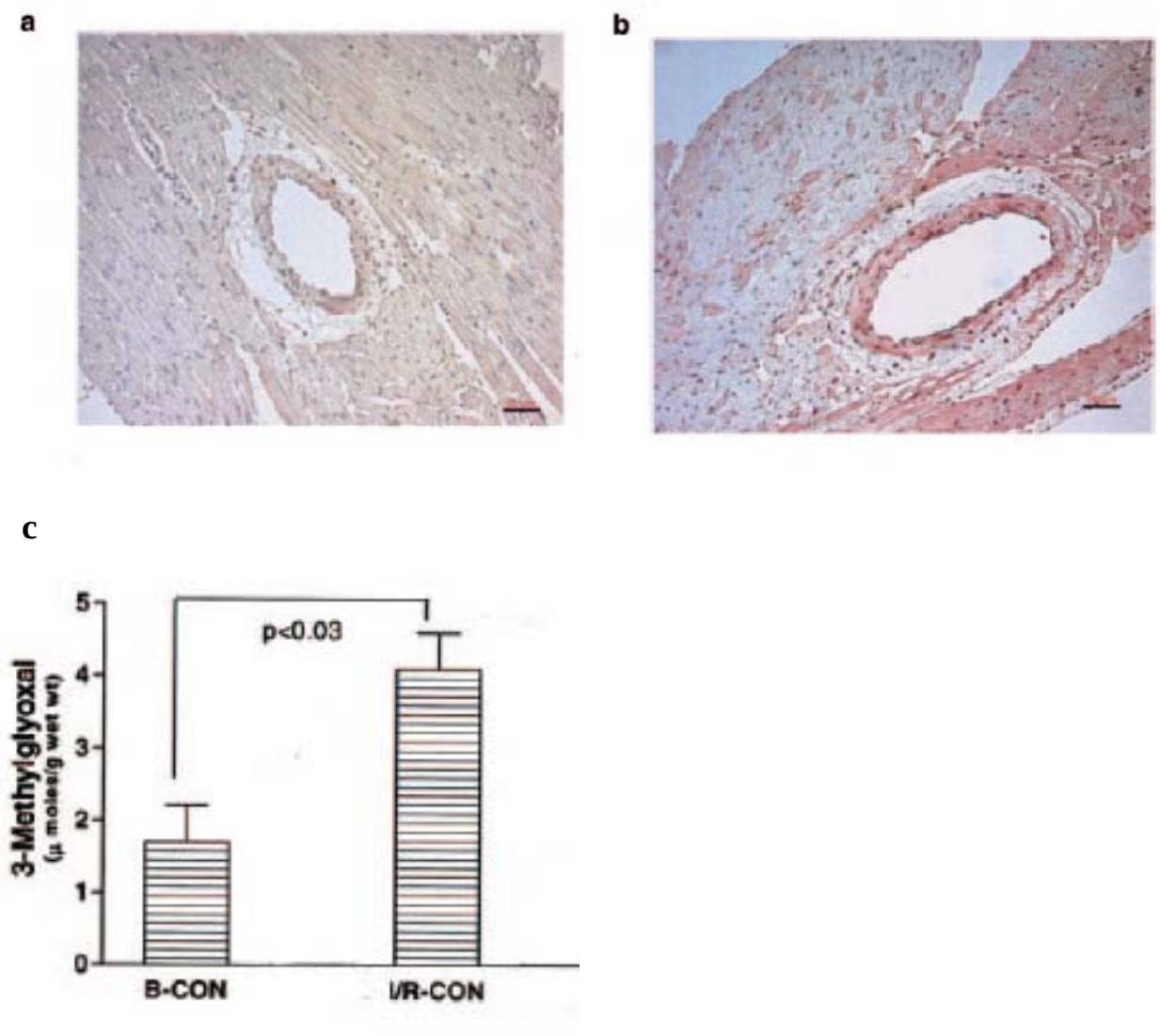

Figure 7. Immunostaining of CML/AGEs in baseline and after I/R hearts is shown. (a) In hearts subjected to normoxic perfusion, CML epitopes were not detected. (b) In contrast, heart tissue retrieved from ischemic hearts displayed immunoreactive epitopes for CML-AGE. (c) The concentration of MG in heart tissue is shown under baseline and after I/R. The concentration of MG was increased after I/R.

Bucciarelli LG et al. Circulation 2006 (Reference 42)

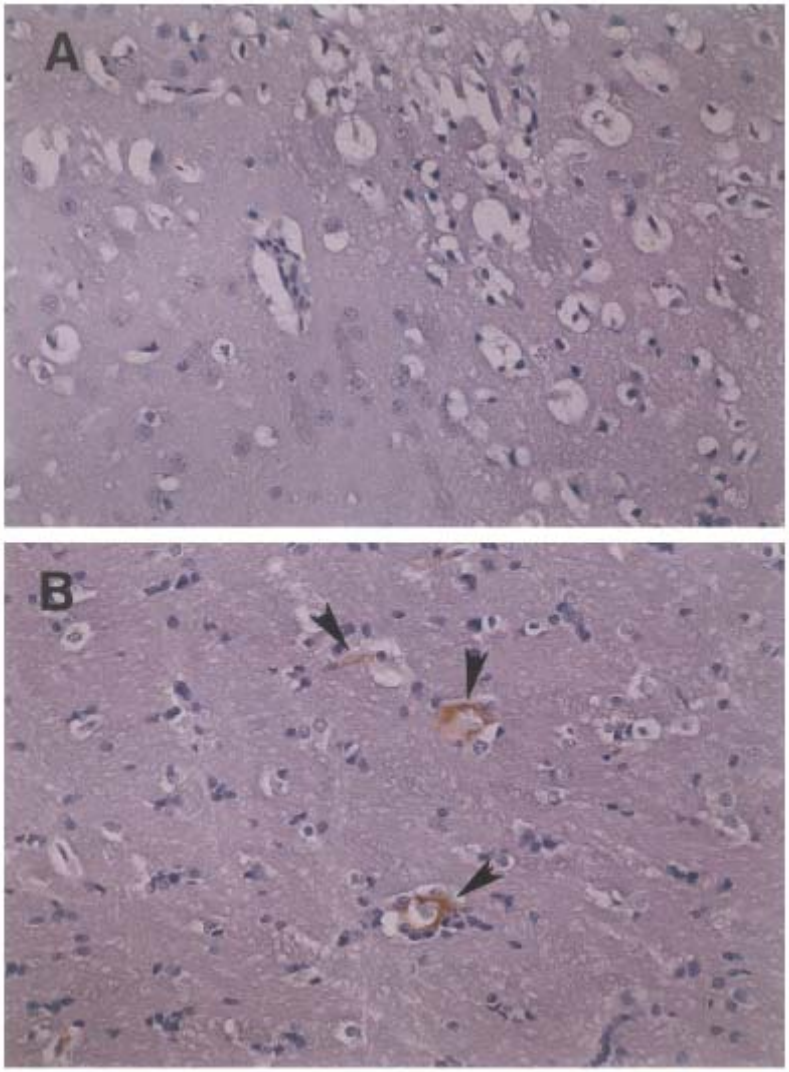

Figure 8. Immunohistochemical detection of argpyrimidine in rat brains after reperfusion following 3 h of middle cerebral artery occlusion. (A) Immunoreactivity was not seen at 6 h after reperfusion. (B) Antigenic materials were detected in some arterial walls (arrowheads) within the infracted zone at 24 h after reperfusion.

Oya T et al. J Biol Chem 1999 (Reference 43)

Inhibition of GLO I in the presence of S-nitrosoglutathione (GSNO)

Nitric oxide (NO), a small lipophilic molecule, has diverse physiological and pathological roles in biological system. Nitric oxide is produced by a family of enzymes known as nitric oxide synthases by the conversion of L-arginine into L-citruline. NO reacts with GSH and forms S-nitrosoglutathione (GSNO). Sahoo R. et al. reported that GLO I activity is inhibited at very low GSNO concentration, which is physiologically possible (44). GSNO and GSH may compete for the same binding site in the GLO I due to their similar molecular structure. Once GSNO binds to that site it permanently alters the substrate-binding pocket, which could not be reversed in the presence of excess GSH.

Phosphorylation of GLO I

Post-translational protein modification of GLO I may be the other conceivable mechanism of lowering activity of GLO I. Past reports indicated that amino sequences of GLO I contains several potential phosphorylation sites (45). For example, serine residues (8, 21, 26 residues) and threonine residue (107 residue) are potential phosphorylation sites. Van Herreweghe F. reported that tumor necrosis factor (TNF) induces an increased phosphorylation of GLO I that is mediated by protein kinase A (46, 47). They suggested the possibility that the TNF-induced phosphorylation of GLO I does not lead to inhibition of its MG-detoxifying activity, which implies that the phosphorylated form of GLO I is not involved in MG catabolism. However this suggestion is yet to be proven. In addition, there may be phosphorylation of tyrosine residues of GLO I.

In this study, I aimed to elucidate a role of the glyoxalase system in renal I/R injury and demonstrated that GLO I has renoprotective effects in renal I/R injury by reducing MG in tubular cells.

Materials and Methods

GLO I transgenic rats

To generate the human GLO I transgenic construct, entire coding sequence of human GLO I cDNA was cloned into the EcoRI site of the pBsCAG-2. The GLO I transgene isolated by digestion of pBsCAG-2 containing GLO I cDNA with Kpn I and Sac I was microinjected into one pronucleus of fertilized Wistar rats eggs, followed by transfer into the oviducts of pseudopregnant rats as described previously (48) (Figure 9). Rat genomic DNA extracted from tail tissue was used to detect the transgene by polymerase chain reaction (PCR) using specific primers for pBsCAG-2 vector. Primers for cytomegalovirus enhancer contained in pBsCAG-2, sense (5'-GTC GAC ATT GAT TAT TGA CTA G-3') and antisense (5'-CCA TAA GGT CAT GTA CTG-3'), amplified a 320 bp fragment.

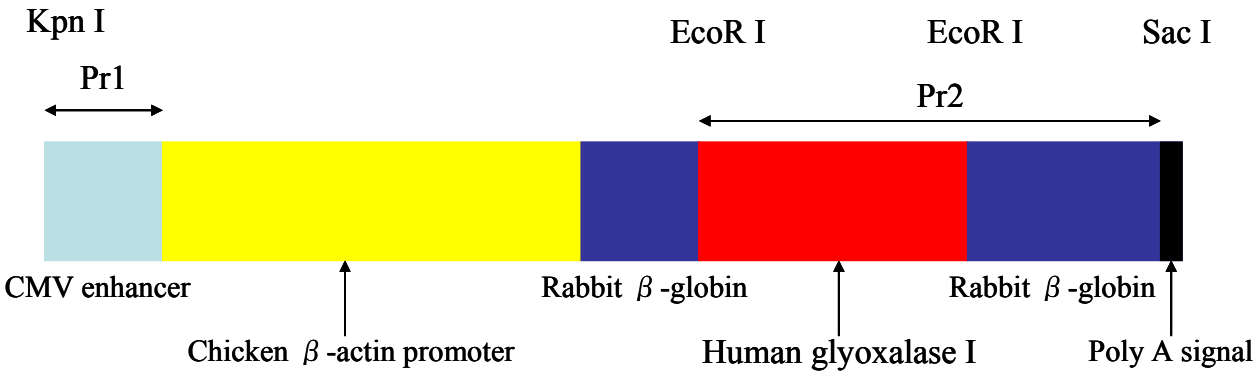

Figure 9. GLO I transgene construct. Full-length human GLO I cDNA was cloned in the rabbit β-globin gene including a part of the second intron, the third exon, and the 3' untranslated region. The positions of primers (Pr1 and Pr2) for PCR analysis are indicated above the construct.

Inagi R et al. Kidney Int 2002 (Reference 48)

Animal experimental protocol

All experiments were conducted in accordance with the Guide for Animal Experimentation at the University of Tokyo. Male Wistar rats were purchased from Nippon Seibutsu Zairyo Center Co. (Saitama, Japan). Male Wistar rats and GLO I transgenic rats at the age of 6 to 8 weeks were used. All rats were housed in individual cages in a temperature- and light-controlled environment in an accredited animal care. Rats were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg, Nembutal; Dainippon Sumitomo Pharma, Osaka, Japan). One week before induction of ischemic injury, the right renal pedicle and the right ureter were ligated, and right nephrectomy was performed. Ischemic injury was induced by clamping of left renal artery and vein for 45 min. Core body temperature was maintained at 37℃ using a homeothermic table during surgery. Twenty-four hours after induction of injury, blood samples were obtained via cardiac puncture and rats were euthanized and the kidneys were harvested for histologic analysis. Blood urea nitrogen (BUN) levels were measured using a commercial kit (Wako, Osaka, Japan).

GLO I activity and GSH concentration

Whole kidney tissues (25 mg) from wild or heterozygous GLO I transgenic rats were homogenized in 0.5 ml of NaPB, pH 7.0, containing 0.02 % TritonX-100 and centrifuged at 20,000$\times g$ for 20 minutes at 4℃. The supernatant was used as a lysate to assess GLO I activity. GLO I activity was assayed by spectrophotometry according to the method by McLellan and Thornalley (49), monitoring the increase in absorbance at 240nm due to the formation of S-D-lactoylglutathione for three minutes at 25℃. GSH concentrations in the kidney homogenates were measured by Bioxytech GSH-400 Assay reagent (Oxis Internationl, Inc., Portland, OR).

Histological analysis

Tissue fixed in methyl Carnoy's solution was processed and paraffin-embedded. Four-micrometer sections were stained with the periodic acid-Schiff (PAS) reagent and counterstained with hematoxylin. Vimentin was stained with murine monoclonal IgG antibody V9 (Dako, Carpinteria, CA) and monocyte/macrophages were identified with murine monoclonal IgG1 antibody ED-1 (Chemicon, Temecula, CA) by an indirect immunoperoxidase method. Tubular injury in terms of tubular dilatation, tubular epithelial injury, debris accumulation, and cast formation was graded with an arbitrary score of 0 to 3: 0, normal; 1, mild; 2, moderate; 3, severe (50). Tubules that are vimentin-positive or are surrounded by vimentin-positive cells were counted in 5 randomly selected cortical fields (×200). ED-1 positive cells were counted in 5 randomly selected fields (×200) including both cortex and outer medulla. All quantification was performed in a blinded manner.

Oxidative stress marker staining

For nitrotyrosine staining, four-micrometer sections were stained with anti-nitrotyrosine (1:1000; Sigma-Aldrich, St. Louis, MO), biotinylated anti-rat IgG (1:400; Vector), and horseradish peroxidase (HRP)-avidin (1:1000; Vector). For 4-hydroxynonenal (4-HNE) staining, sections were stained with monoclonal anti-4-HNE antibody, NA59 (1:100) using Histofine mouse stain kit (Nichirei Bioscience, Tokyo, Japan).

Carboxyethyl lysine (CEL) staining

Renal tissues were embedded in OCT compound (Sakura Fine Technical, Tokyo, Japan), frozen in liquid nitrogen, and stored at -80℃ until use. Sections were cut 4 μm thick with a cryostat MICROM and were mounted on poly-L-lysine-coated slides. After inhibition of endogenous peroxidase activity, the sections were incubated with the monoclonal anti-CEL antibodies (10

μg/ml; Trans Genic, Inc., Kumamoto, Japan), washed, and reacted with a biotinylated anti-mouse IgG (1:400; Vector Laboratories, Burlingame, CA) and horseradish peroxidase (HRP)-avidin (1:1000; Vector). The specificity of these monoclonal anti-CEL antibodies was indicated in the past reports (Figure 10).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) assay

Formalin-fixed section of renal tissues (5 μm) were deparaffinized in Histoclear and were hydrated in graded ethanol. The TUNEL-positive cells were identified using TACS^{TM} 2 TdT-Blue Label *In Situ* Apoptosis Detection Kit (Trevigen, Inc., Gaithersburg, MD). For each renal section, 5 randomly selected fields (×200) were examined and the numbers of positive cells were counted.

Cell culture and induction of hypoxic environment

IRPTC is a cultured cell line derived from proximal tubular cells of male Wistar rats, immortalized by infection with the temperature-sensitive (ts) SV40 mutant viruses (51). General characteristics of ts SV40 immortalized cell lines have been described elsewhere (52, 53). Cells were cultured in Dulbecco's modified Eagle's medium (Nissui Seiyaku, Tokyo, Japan) buffered with 25mM Hepes (Sigma, St. Louis, MO) at pH 7.4 supplemented with 5% fetal bovine serum (JRH Biosciences, Lenexa, KS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.01mM nonessential amino acid, at 37℃ under a humidified atmosphere of 5%CO_2/95%air. Hypoxic conditions were provided by exposure to 1%O_2/5%CO_2 with the balance as nitrogen in a multi-gas incubator, APM-30D (ASTEC, Fukuoka, Japan). Hypoxic stimulation was initiated at 70% confluence and IRPTC were cultured in the culture medium containing 1% FBS during the treatment.

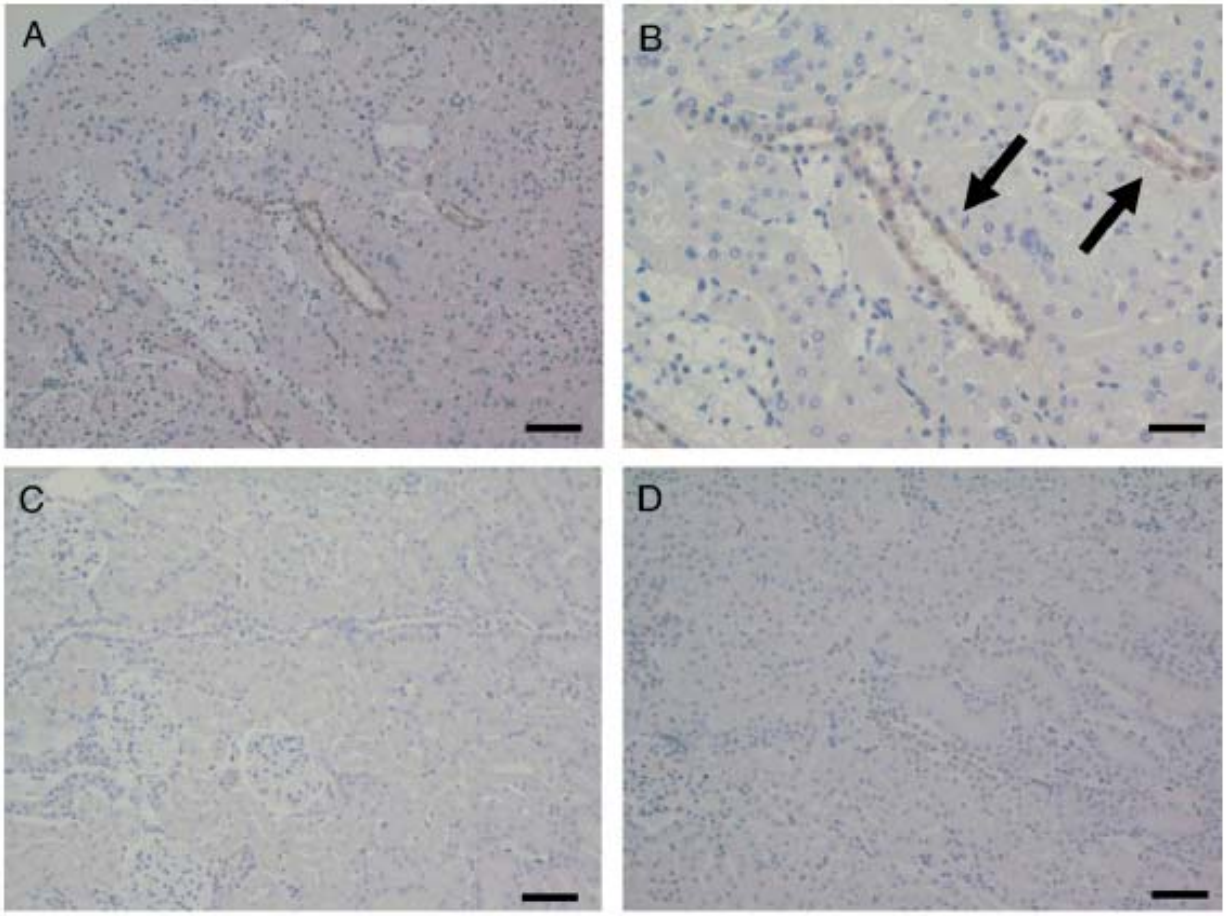

Figure 10. Immunohistochemical localization of CEL in diabetic rat kidney. (A, B) Paraffin-embedded diabetic rat renal tissues were stained with the monoclonal anti-CEL antibodies. Distal tubular epithelial cells (arrows) were positively stained. (C) No positive signal was observed in healthy rat. (D) No positive signal was observed in diabetic section stained with pretreated monoclonal anti-CEL antibodies with excess amount of CEL-BSA.

Nagai R et al. J Immunol Methods 2008 (Reference 55)

siRNA transfection procedures

The siRNA oligonucleotides were synthesized by TaKaRa Bio (Shiga, Japan). IRPTC were transfected with siRNA at 30% confluence by the use of Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were incubated for an additional 24 h before starting hypoxic stimulation. To control for off-target effects of siRNA, a separate well of IRPTCs was transfected with a control GC content-matched siRNA. The sequence of the GLO I siRNA duplex and the scramble control duplex were as follows: GLO I: forward 5'-GUU CUC GCU CUA UUU CUU AGC-3', reverse 5'-UAA GAA AUA GAG CGA GAA CUU-3'; control: forward 5'-GUC UGG CGU UCG UUC AAG AUC-3', reverse 5'-UCU UGA ACG AAC GCC AGA CCU-3'.

Total RNA isolation and real-time quantification PCR

Total RNA of cultured IRPTC were isolated with ISOGEN (Nippon Gene, Tokyo, Japan) and reverse-transcribed with Im-Prom II (Promega, Madison, WI). cDNA was subjected to real-time quantitative PCR using iQ SYBR Green PCR supermix (Bio Rad) and iCycler PCR system (Bio Rad). The relative amounts of GLO I were calculated with calibration by β-actin. The primers used here were as follows: GLO I: forward 5'-ATT TGG CCA CAT TGG GAT TGC-3', reverse 5'-TTC AAT CCA GTA GCC ATC AGG-3'; and β-actin: forward 5'-CTT TCT ACA ATG AGC TGC GTG-3', reverse 5'-TCA TGA GGT AGT CTG TCA GG-3'. The PCR was performed using the following conditions: 95℃ for 15 min, followed by 40 cycles of denaturation at 94℃ for 30s, annealing at 60℃ for 30s, and extension at 72℃ for 60s.

Cell viability test

Cell damage was evaluated using the trypan blue exclusion test and LDH release. Unstained (viable) and stained (nonviable) cells suspended in 0.4% trypan blue were counted by hemacytometer, and death rate was expressed as the percentage of nonviable cells. LDH release from cells was calculated by determining the ratio of LDH in the culture medium compared to that in the lysed cell plus the culture medium (Kainos Laboratories, Tokyo, Japan) and expressed as a percentage.

Western blot analysis

The kidney tissue of the experimental rats or IRPTC (20μg) was homogenized in a sample buffer (0.35M Tris-HCl, pH6.8, 10% SDS, 36% glycerol, 5% β-mercaptoethanol, and 0.012% bromophenol blue), electrophoresed on 10-15% SDS-PAGE, and transferred to a polyvinylidene fluoride membrane (Amersham Biosciences, Piscataway, NJ).

For detection of GLO I and CEL, rabbit polyclonal antibody to rat GLO I peptide; GIAVPDVYEA (5μg/ml), and mouse anti-CEL monoclonal antibody (54, 55) (CEL-SP; 4F5, 2.5μg/ml; donated by Dr. Ryoji Nagai) was used as the first antibody. Rabbit anti-actin polyclonal antibody (1:1000; Sigma Chemical Co., St. Louis, MO, USA) was also used as control. The bands were detected by the enhanced chemiluminescence system (Amersham Biosciences), and quantitative densitometry using NIH ImageJ was performed.

Immunocytochemistry

IRPTC was seeded on 4-well Lab-Tek chamber slides (Nalge Nunc International, Rochester, IL, USA) and cultured in normoxic or hypoxic conditions. Samples were fixed with Aceton/Methanol (1:1) at -20 ^{o}C for 10 minutes and stained with anti-CEL monoclonal antibody (10 μg/ml; Trans Genic, Inc., Kumamoto, Japan), washed, and reacted with a biotinylated anti-mouse IgG (1:400) and horseradish peroxidase (HRP)-avidin (1:1000).

Statistical analysis

Statistical analysis was performed using SAS software version 5.1.2 (SAS Institute, Cary, NC, U.S.A.). Data were expressed as mean ± SEM. Student paired *t* test was used to assess the statistical differences. All reported *p* values are two-sided. Nonparametric data were analyzed with the Mann-Whitney test when appropriate. Values of $p<0.05$ were considered statistically significant.

Results

Decreased renal GLO I activity and increased MG content in rat renal I/R injury

First, I evaluated the change of GLO I activity and the level of GSH, a cofactor of GLO I, in the kidney of I/R injury. I/R induced tubulointerstitial injury, such as loss of tubular epithelial cells, dilation of tubules, macrophage infiltration, and cast formation (Figure 11A: PAS staining). Interestingly, these tubulointerstitial damages were associated with a significant lowering of renal GLO I activity (I/R; 13.8±1.1 U/g versus Sham; 21.3±3.6 U/g, $P<0.01$; n=6 per group) but not its mRNA or protein expression level (Figure 11B). GSH level, which regulates GLO I activity, did not statistically change by renal I/R injury (I/R; 3.41±0.65μmol/g protein versus Sham; 4.39±0.55μmol/g protein, $P=0.28$; n=6 per group, Figure 12A). Immunohistochemistry revealed that MG content estimated by MG-lysine adducts, carboxyethyl lysine (CEL), was also markedly augmented in the tubulointerstitial lesion. These results were confirmed by western blot analysis for detection of CEL (Figure 12B).

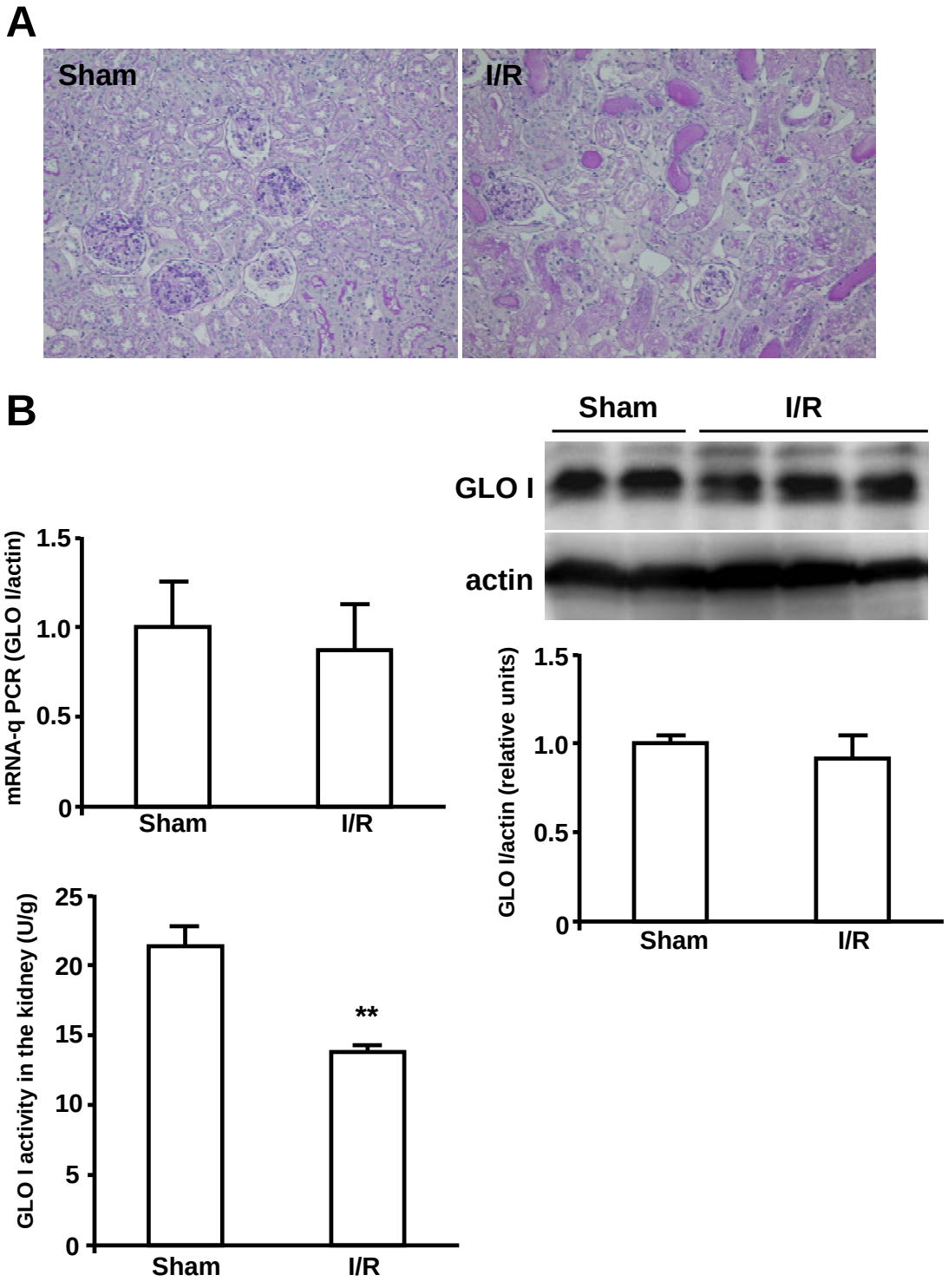

Figure 11. Renal GLO I activity, the level of GSH and the accumulation of CEL in wild-type rats with I/R. (A) Representative light microscopy of the kidney from sham operated rats and rats with I/R. Tubular injury, tubular dilatation, tubular epithelial injury, debris accumulation, and cast formation were apparent in wild-type rats with I/R. Periodic acid-Schiff staining. Original magnification, x200. (B) GLO I activity in kidney homogenates was decreased by I/R. On the other hand, mRNA or protein expression level did not change. **P<0.01 versus sham operated rats.

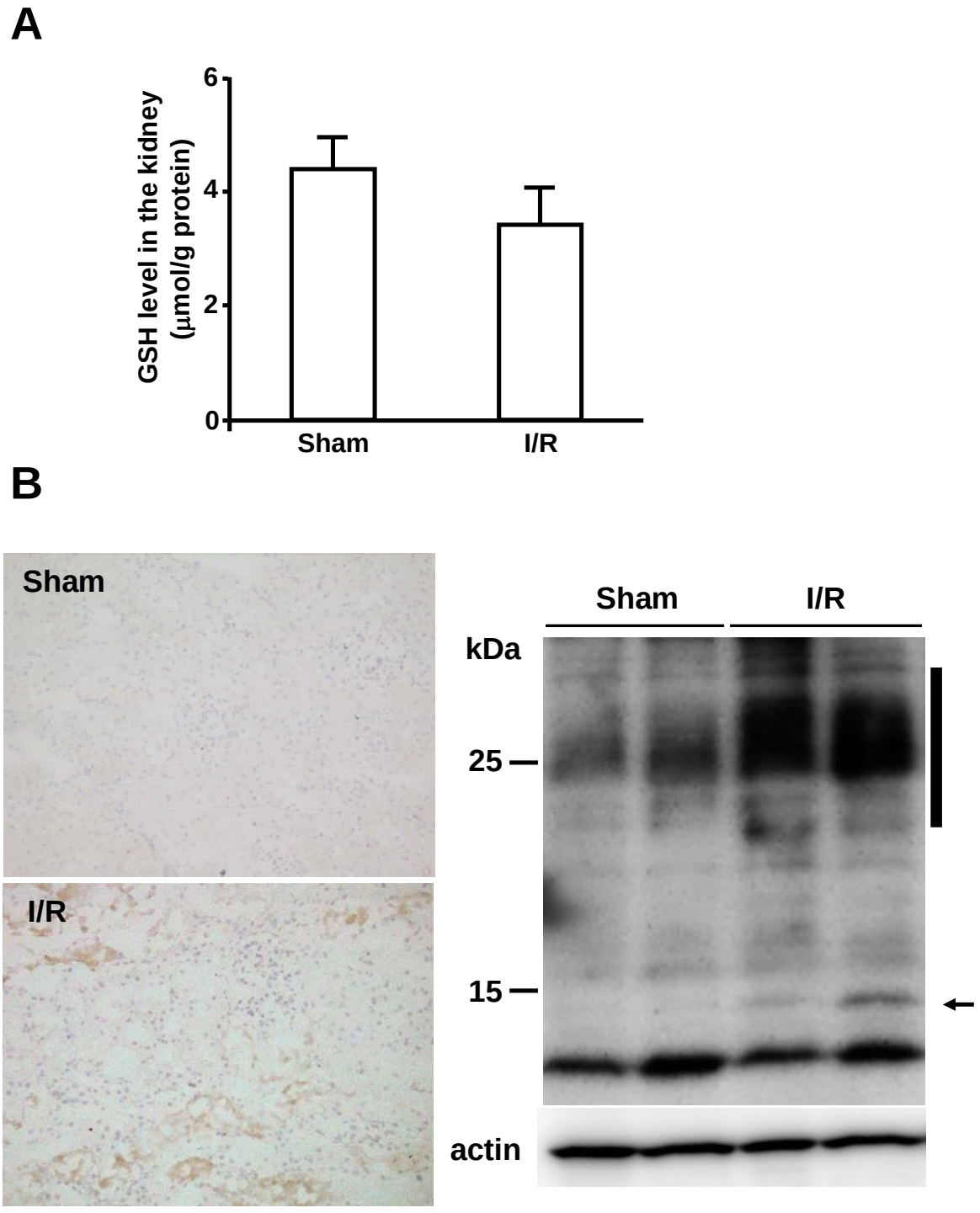

Figure 12. (A) The level of GSH in kidney homogenates did not change. (B) Immunohistochemistry of MG adducts, CEL in the kidney and immnoblots for CEL in kidney homogenates. Immunostaining of CEL in the tubulointerstitium and the protein modified by CEL was increased by I/R. (n=6, each) The intensity of the bands, the molecular weight of which corresponded to 24-30 kDa (indicated by a black bar) and 14 kDa (indicated by an arrow), was increased in kidney from rats with I/R as compared to control rats.

Decreased GLO I activity in association with intracellular MG accumulation in cultured tubular epithelial cells undergoing hypoxia/reoxygenation

To confirm my results in *in vivo* studies, I investigated the change of GLO I activity in cultured tubular epithelial cells, immortalized rat proximal tubular cells (IRPTC), by hypoxia/reoxygenation (H/R). GLO I activity was significantly decreased in IRPTC exposed to hypoxia (0.2 % O_2) 24h/reoxygenation 2h (Normoxia (n=6): 0.437±0.017, H/R (n=6): 0.243±0.013 U/mg protein; $P<0.01$) without affecting glyoxalase expression at mRNA and protein level (Figure 13). Immunocytochemistry and western blot analysis showed that lowered GLO I activity enhanced CEL accumulation in IRPTC with H/R, suggesting an increase in intracellular MG content (Figure 14). These data were consistent with my findings observed in *in vivo* I/R study.

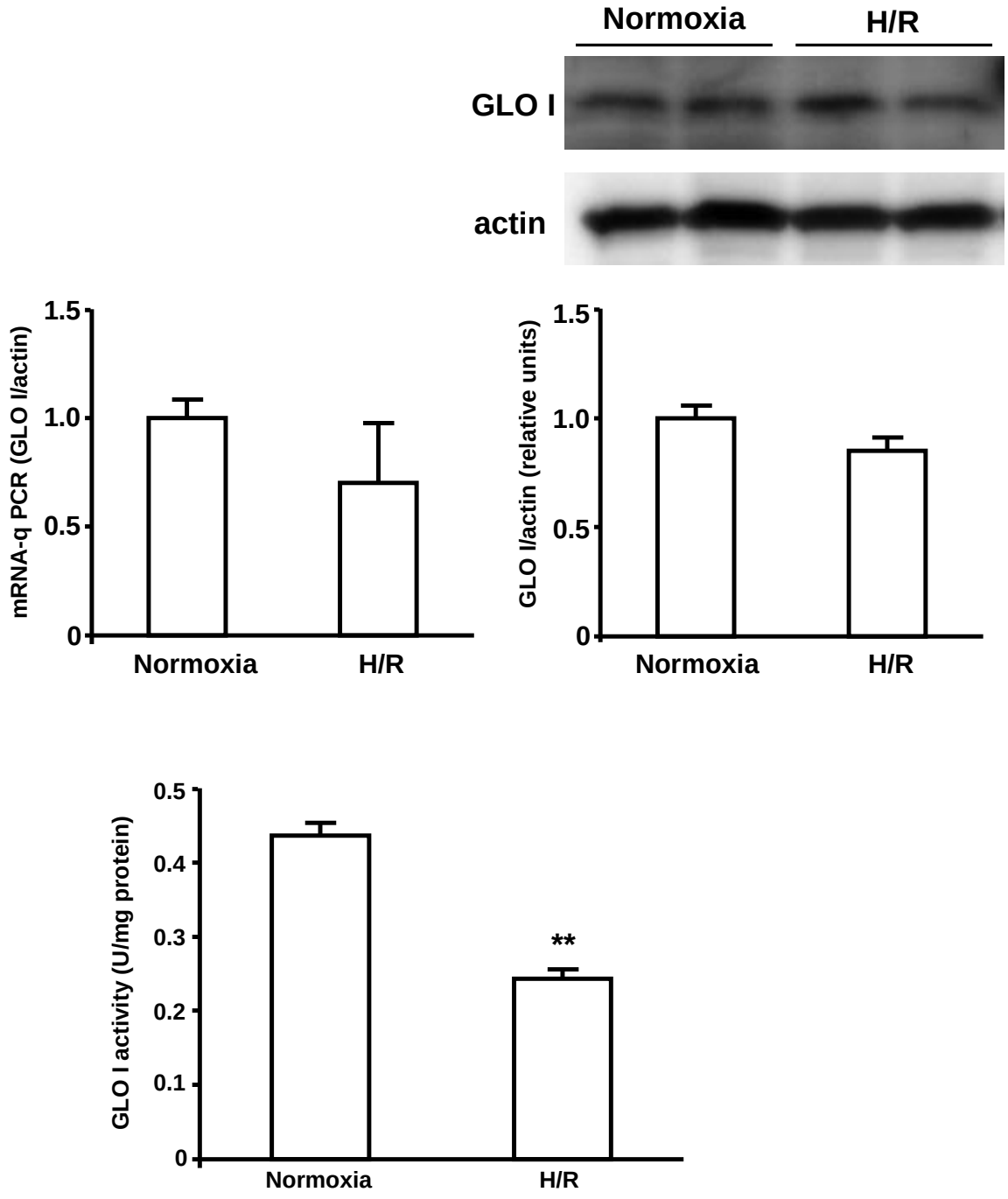

Figure 13. In vitro study using IRPTCs showed that GLO I activity was also decreased by Hypoxia/reoxygenation (H/R) without the change of mRNA or protein expression level (n=6, each).

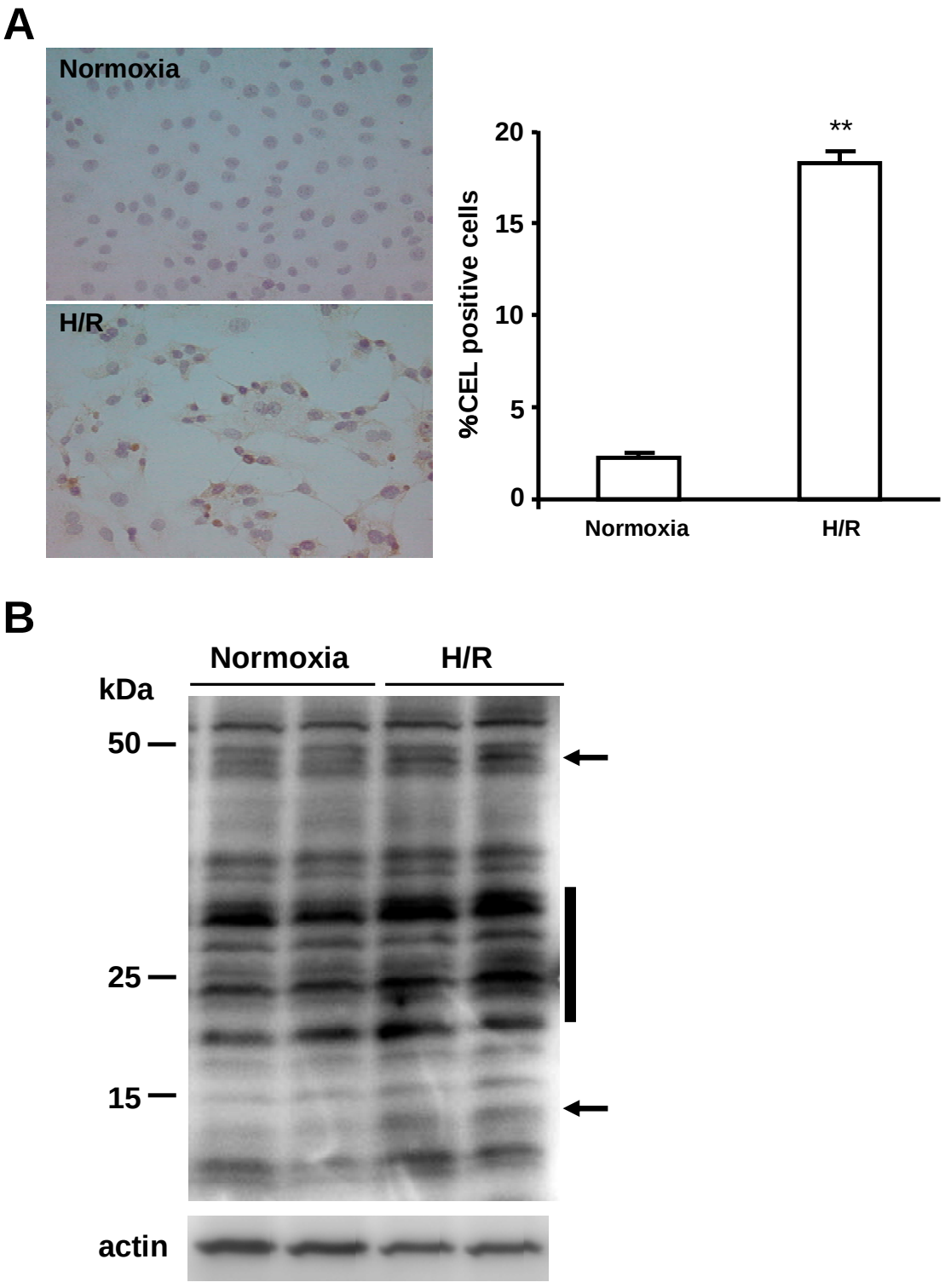

Figure 14. (A) Immunocytochemistry of CEL in IRPTCs. CEL positive cells were counted in 15 randomly selected fields each. Immunostaining of CEL was increased by H/R. **P<0.01 versus normoxia. (B) Immunoblot analysis for CEL in IRPTCs. Representative bands were indicated. The protein modified by CEL was increased by H/R.

Exacerbation of H/R-induced cellular injury in GLO I-knockdown cultured tubular cells

To confirm a functional contribution of GLO I in H/R-induced cellular injury, I performed siRNA transfection specific for knockdown of GLO I gene into IRPTC. GLO I specific siRNA transfection to IRPTC knocked down its gene expression by approximately 60 % compared to control siRNA transfection. This enzymatic activity was also decreased by 38% in IRPTC transfected with GLO I siRNA (Figure 15A). I then used trypan blue exclusion and LDH release assays to assess whether knockdown of GLO I aggravates H/R-induced cell damage. To enhance the difference in cell death by H/R, this experiment employed relatively mild H/R conditions, namely hypoxia by 1% O_2 for 40 h followed by reoxygenation for 2 h. Survival of GLO I-knocked down IRPTC was similar to that of IRPTC transfected with control siRNA under normoxia during the experimental period. Under H/R conditions, in contrast, knockdown of GLO I in IRPTC significantly exacerbated cellular injury estimated by trypan blue exclusion assay and LDH assay as compared to IRPTC transfected with control siRNA (death rate; 13.2±1.0 % in control siRNA versus 22.3±2.0 % in GLO I siRNA, $P<0.05$; %LDH released, 14.5±0.2 % in control siRNA versus 20.0±0.8 % in GLO I siRNA, $P<0.01$, Figure 15B). Augmented H/R-induced cell death by knockdown of GLO I was associated with increased intracellular CEL accumulation (Figure 16).

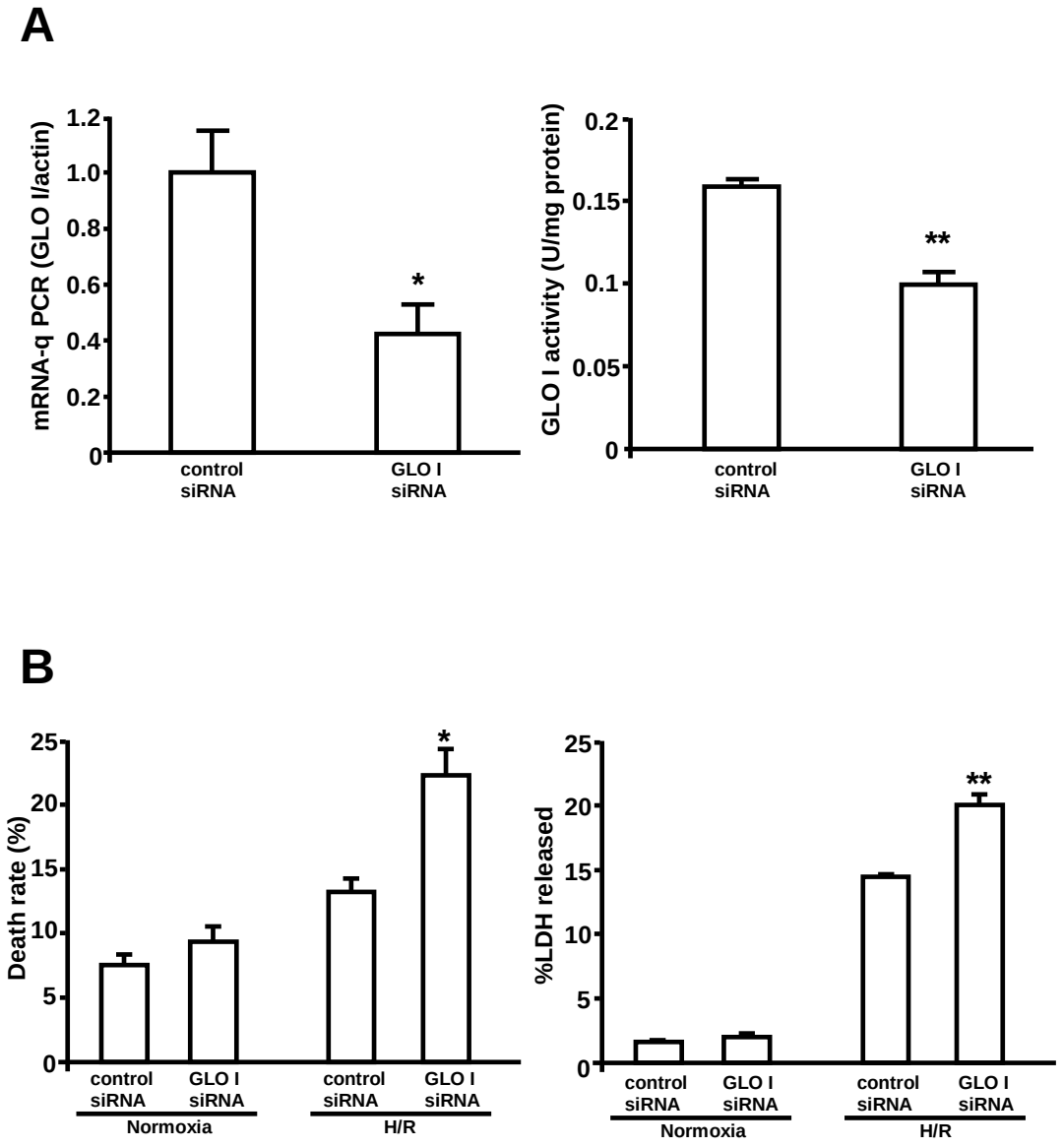

Figure 15. In vitro studies using small interference RNA (siRNA) specific for GLO I showed that GLO I has a critical role in alleviating H/R injury. (A) Expression of GLO I mRNA expression and GLO I activity in IRPTCs was suppressed by siRNA targeted against GLO I (n=4 per group for mRNA, n=3 per group for GLO I activity). These studies were performed two times independently, and the representative data is shown. *P<0.05 versus control-siRNA. **P<0.01 versus control-siRNA. (B) Trypan blue staining and LDH assay showed that knockdown of GLO I induced severer cellular injury in hypoxia (1 %) 40hr/reoxygenation 2hr (n=6, each). *P<0.05 versus control-siRNA in H/R. **P<0.01 versus control-siRNA in H/R.

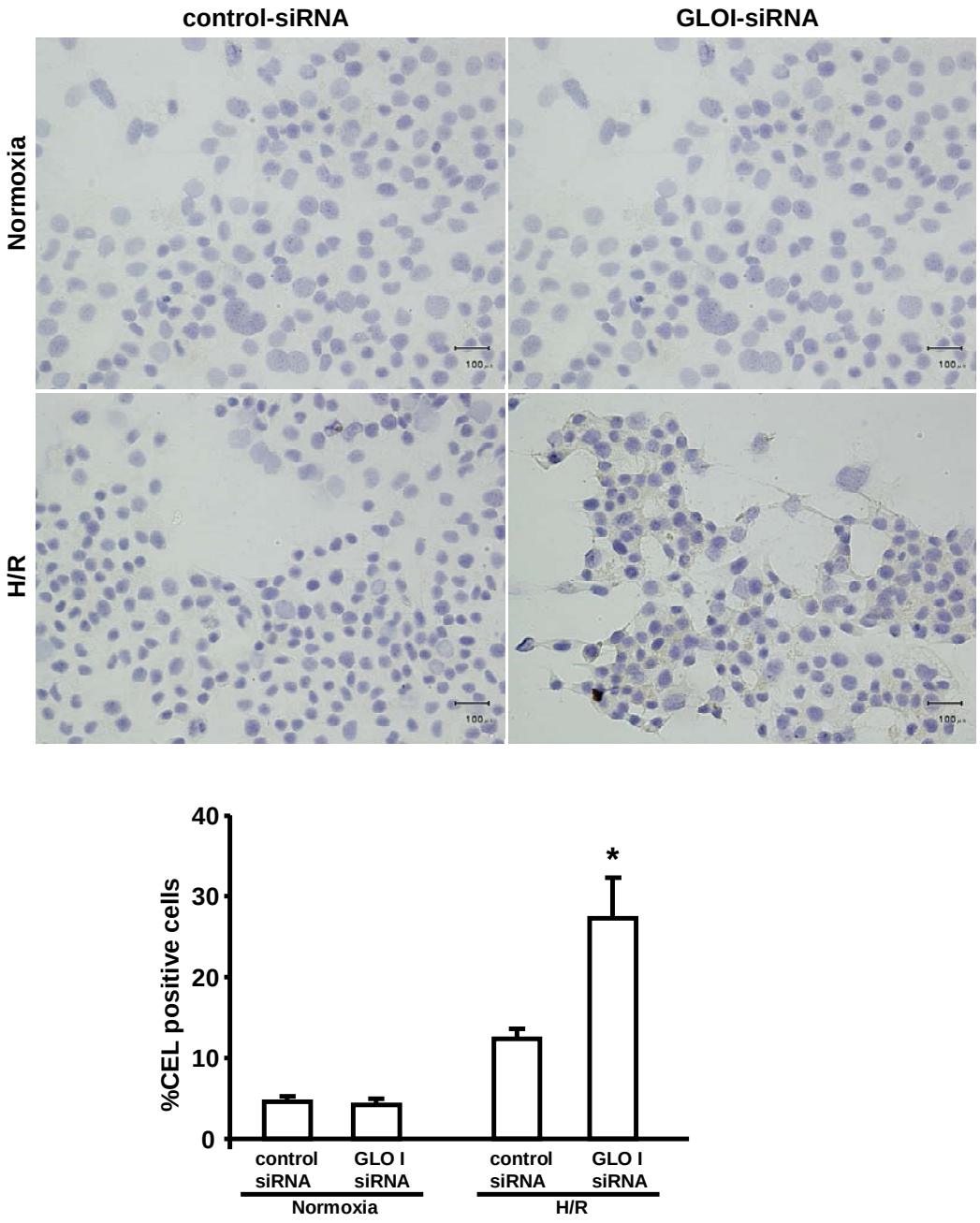

Figure 16. Immunocytochemical analysis of CEL showed that knockdown of GLO I increased accumulation of CEL in H/R. CEL positive cells were counted in 5 randomly selected fields each. *P<0.05 versus control-siRNA in H/R.

Amelioration of I/R-induced renal disease manifestations by GLO I overexpression

My *in vitro* study demonstrated that GLO I is important for cell survival in H/R. Therefore I hypothesized that overexpression of GLO I in the kidney can ameliorate the severity of renal I/R injury. I generated the rats overexpressing GLO I, GLO I Tg rats, as described previously(48) and assessed renoprotective effects of GLO I against I/R injury in these rats. GLO I Tg rats showed significantly high level of GLO I activity in various organs including the kidney: renal GLO I activity in GLO I Tg rats was approximately 17-fold higher than that in wild-type rats (GLO I Tg rats (n=4), 268.6±22.4 U/g versus wild-type rats (n=3), 18.7±0.3 U/g).

Importantly, when I/R injury was induced in GLO I Tg rats, the severity of disease manifestations such as tubular dilatation and tubular epithelial loss was markedly ameliorated as compared in wild-type rats (score for TI damage, GLO I Tg rats with I/R (n=11), 1.32±0.21 vs. wild-type rats with I/R (n=8), 2.36±0.18; $P<0.01$; Figure 17A). Renal function, estimated by BUN levels, was also ameliorated in the GLO I Tg rats compared to wild-type rats (GLO I Tg rats with I/R (n=11), 87.1±6.7 mg/dl versus wild type rats with I/R (n=8), 108.3±3.1 mg/dl; $P<0.05$; Figure 17B). The amelioration of TI damage observed in GLO I Tg rats with I/R injury was positively correlated with improvement of renal function estimated by BUN level (Figure 17C). Renal GLO I activity in GLO I Tg rats did not change by I/R injury with a statistical difference, while it was significantly decreased in wild-type rats (Figure 17D).

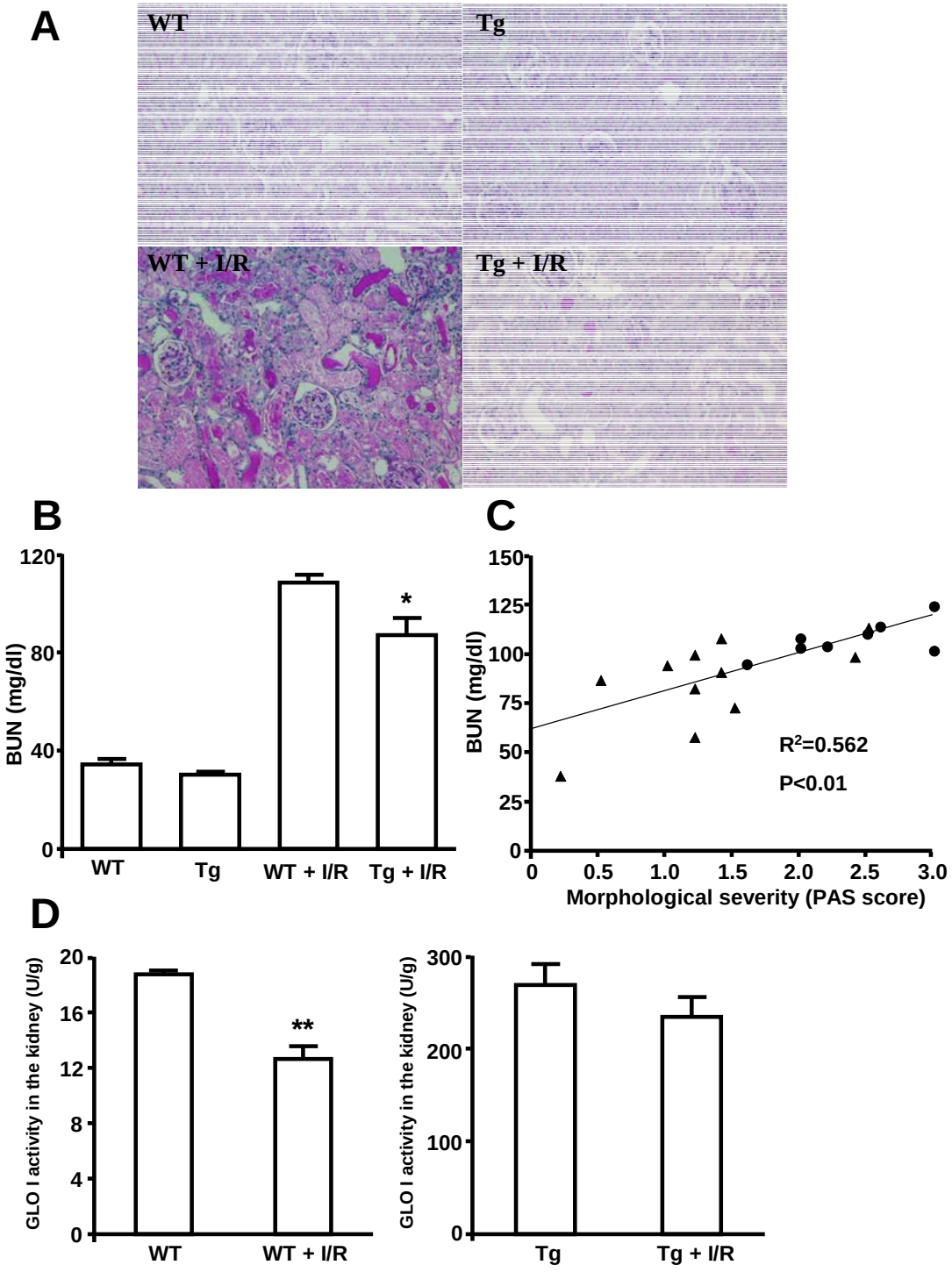

Figure 17. Overexpression of GLO I ameliorated renal injury induced by I/R *in vivo*. (A) Representative light microscopy of the kidney from wild-type and GLO I Tg rats with or without I/R. The pathologic features of renal I/R, including loss of tubular epithelial cells, dilation of tubules and interstitium and macrophage infiltration, were remarkably improved in GLO I Tg rats compared to wild-type rats. Periodic acid-Schiff staining. Original magnification, x200. (B) Renal function was also improved in GLO I Tg rats with I/R compared to wild-type rats with I/R. *P<0.05 versus wild-type rats with I/R. (C) There was a significant positive correlation between the morphological severity (PAS score) and BUN levels. ●: wild type rats with I/R, ▲: GLO I Tg rats with I/R (D) GLO I activity in kidney homogenates. GLO I activity in the kidney was decreased by renal I/R in wild-type but not GLO I Tg rats. **P<0.01 versus wild-type rats without I/R. (WT: n=3, Tg: n=4, WT + I/R: n=8, Tg + I/R: n=11).

To confirm the improvements of the renal histology in GLO I Tg rats with I/R, I performed immunohistochemical studies for detection of vimentin, a marker of TI injury, and ED-1, a marker of macrophage. Vimentin-positive staining in the tubulointerstitium was slightly detected in wild-type rats or GLO I Tg rats without I/R. While expression of vimentin in the injured tubular cells or interstitium was remarkably increased in wild-type rats with I/R, it was significantly reduced in GLO I Tg rats with I/R (Wild + I/R (n=8), 42.3±2.6 vimentin-positive tubules per field versus Tg + I/R (n=11), 19.5±2.5; $P<0.01$; Figure 18). I/R injury was associated with an increase in macrophage infiltration, estimated by ED-1-positive cell number, in the interstitium in wild-type rats. The number of infiltrating macrophages induced by I/R was significantly reduced in GLO I Tg rats as compared to wild-type rats (WT + I/R (n=8), 115.0±9.4 ED-1 positive cells per field versus Tg + I/R (n=11), 73.9±4.3; $P<0.01$, Figures 18).

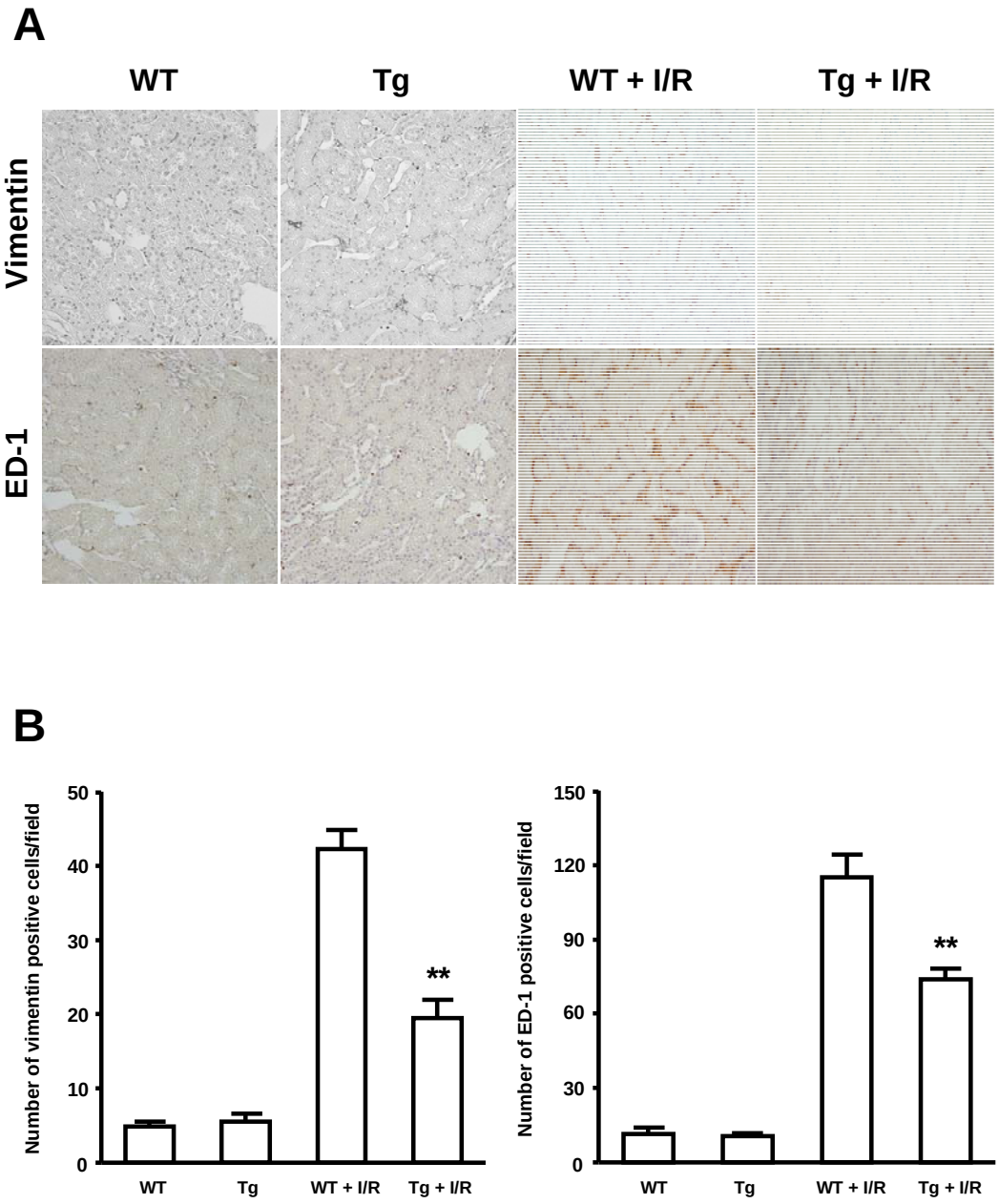

Figure 18. Amelioration of renal injury by overexpressing GLO I was confirmed by immunohistochemical analysis of markers of tubulointerstitial injury, vimentin and markers of monocytes/macrophages, ED-1. (A) While expression of vimentin intermediate filaments was recognized in interstitial cells in wild-type rats with I/R, the number of vimentin-positive cells was reduced in GLO I Tg rats with I/R. In wild-type rats, ischemic injury caused massive macrophage infiltration. In contrast, fewer ED-1 positive cells were recruited in GLO I Tg rats. Original magnification, x200. (B) Computer-assisted morphometry was performed for evaluation of vimentin positive cells and ED-1 positive cells. The number of vimentin positive cells and ED-1 positive cells was decreased in GLO I Tg rats with I/R compared to wild-type rats with I/R. **P<0.01 versus wild-type rats with I/R. (WT: n=3, Tg: n=4, WT + I/R: n=8, Tg + I/R: n=11).

Improvement in I/R injury by GLO I overexpression associated with a decrease in MG accumulation, oxidative stress, and apoptosis in tubulointerstitium

I then investigated whether the improvement of renal I/R injury in GLO I Tg rats was associated with a decrease in MG level and oxidative stress in the kidney. Immunohistochemistry for detection of MG-lysine adducts, CEL, revealed that the CEL was slightly detected in non-disease wild-type and GLO Tg rats and its accumulation was increased in I/R-injured tubulointerstitium of wild-type rats. In contrast, the degree of CEL accumulation was markedly decreased in GLO I Tg rats with I/R as compared to wild-type rats with I/R (Figure 19). To estimate oxidative stress status in the kidney of I/R-injured GLO I Tg rats, I performed immunohistochemical analysis for detection of 4-HNE, which is an oxidative stress marker derived from lipid peroxidation. While positive-stained area for this oxidative marker was remarkably increased in tubulointerstitium of wild-type rats with I/R, it was ameliorated in GLO I Tg rats with I/R (Figure 19). Furthermore, to evaluate apoptosis of tubular epithelial cells, I analyzed the kidney tissues by TUNEL staining. Increased numbers of TUNEL-positive nuclei were detected in wild-type rats with I/R compared to sham controls. In GLO I Tg rats with I/R, TUNEL-positive nuclei were reduced compared to wild type rats with I/R (Figure 20A). The results were supported by computer-assisted morphometry (Figure 20B).

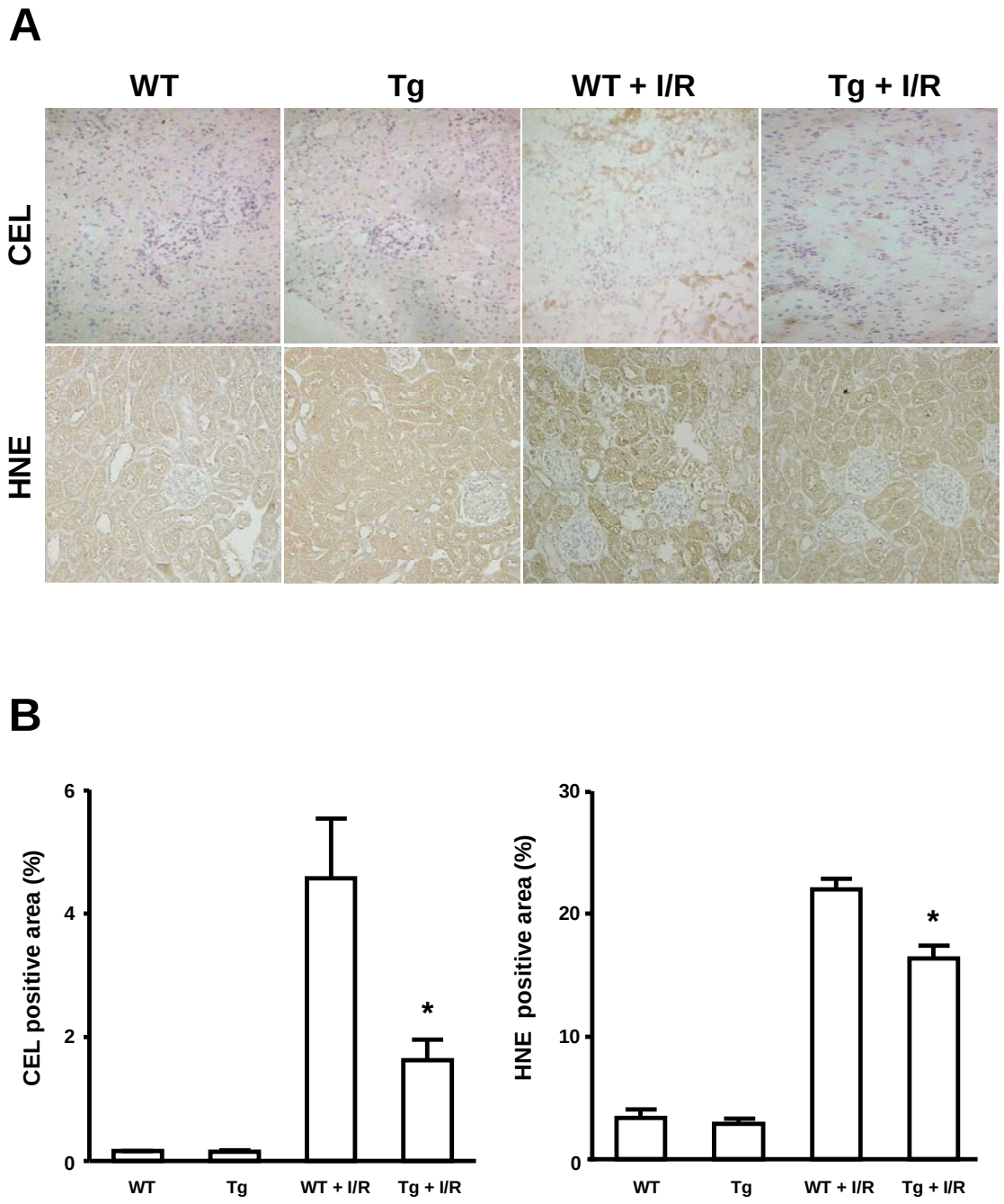

Figure 19. Amelioration of renal injury by overexpressing GLO I was accompanied by reduction of AGEs, oxidative stress. (A) Immunohistochemical analysis of CEL and 4-HNE which is oxidative stress marker in kidney tissues. While CEL and 4-HNE staining was obviously increased in TI region in wild-type rats by renal I/R, staining in GLO I Tg rats was scarce and faint. Original magnification, x200. (B) Computer-assisted morphometry was performed for evaluation of CEL and 4-HNE-positive area. CEL and 4-HNE-positive area was decreased in GLO I Tg rats with I/R compared with wild-type rats with I/R. *P<0.05 versus wild-type rats with I/R.

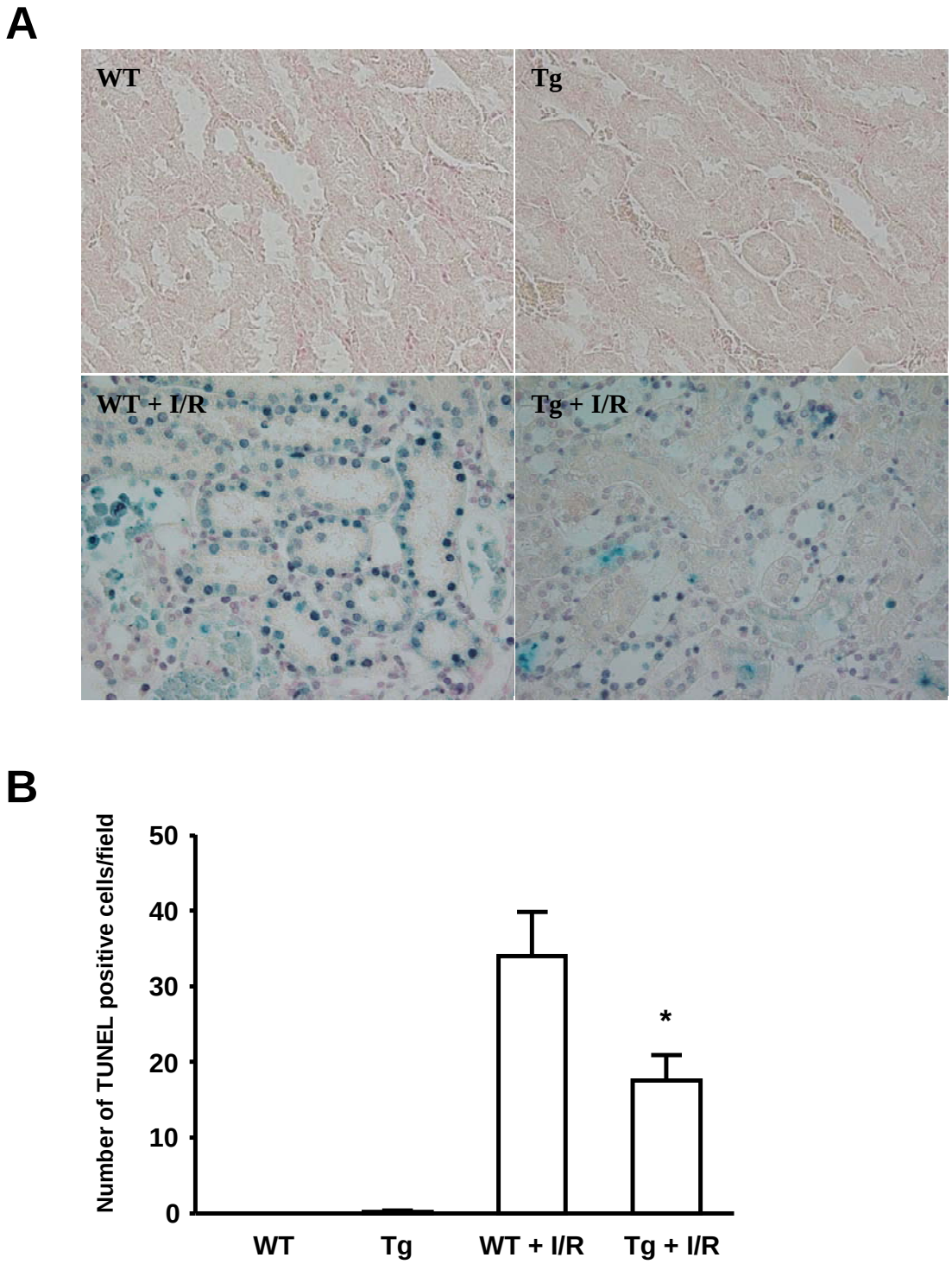

Figure 20. Amelioration of renal injury by overexpressing GLO I was accompanied by reduction of apoptotic cells. (A) TUNEL staining in kidney tissues. Almost no staining was observed before ischemia in wild-type rats and GLO I Tg rats. While apoptosis of tubular cells were obvious in wild type rats with I/R, the number of apoptotic cells was reduced in GLO I Tg rats with I/R. Original magnification, x200. (B) TUNEL positive cells were counted in 5 randomly selected fields. The number of TUNEL positive cells was decreased in GLO I Tg rats with I/R compared to wild-type rats with I/R. *P<0.05 versus wild-type rats with I/R. (WT: n=3, Tg: n=4, WT + I/R: n=8, Tg + I/R: n=11).

Discussion

I for the first time demonstrated that GLO I activity was lowered by renal I/R in *in vivo* and by hypoxia/reoxygenation in *in vitro* study utilizing cultured tubular epithelial cells. Previous reports demonstrated MG is increased in cardiac or cerebral I/R but the mechanism leading to an increase in MG has been almost no more than speculation. My results indicated that lowering *in situ* GLO I activity accelerates an increase in MG accumulation in renal I/R at an independent manner with GSH level, a co-factor of GLO I. Previous reports demonstrated that glyoxalase activity was decreased in some states such as diabetes and aging (56). The formation of the substances and post-translational protein modification which inhibit GLO I activity may be the conceivable mechanisms of lowering GLO I activity in renal I/R lesion. For example, S-nitrosoglutathione, which was formed from nitric oxide and GSH, interact with GLO I and converts GLO I into inactive form (44, 57). Tumor necrosis factor (TNF) induced phosphorylation of GLO I, which is required of TNF induced cell death in murine fibrosarcoma L929 cells (46). My preliminary study indicated that phosphorylation of GLO I was increased *in vivo* renal I/R injury and *in vitro* H/R. To analyze the activity of phosphorylated GLO I, the activity of GLO I was measured in the presence or absence of the kinase inhibitors in the H/R-exposed IRPTC. The three kinds of kinase inhibitors (protein tyrosine kinase inhibitor, Genistein and Herbimycin and protein kinase A inhibitor, H89) were used. These kinase inhibitors did not affect the activity of GLO I in the H/R-exposed IRPTC. However the possibility that the activity of phosphorylated GLO I is decreased was not completely ruled out because of the cytotoxicity and other biological effects of these kinase inhibitors. Clarification of a mechanism of lowering GLO I activity in I/R is a subject of future studies.

GSH is a cofactor of GLO I and the activity of GLO I is proportional to intracellular GSH level (21). My results in this study demonstrated that renal I/R injury did not change GSH levels in the kidney statistically. Therefore, overexpression of GLO I can lead to sufficient elevation of *in situ* GLO I activity in renal I/R.

It is the most interesting finding that overexpression of GLO I ameliorates renal injury induced by I/R *in vivo*. My histological analysis followed by computer-assisted morphology showed improvement of I/R-induced TI injury and renal dysfunction by overexpression of GLO I. These findings were confirmed by decreased number of macrophage infiltration and tubulointerstitium positive for vimentin, a marker for TI injury. To my knowledge, this is the first report demonstrating the effects of GLO I against acute renal failure such as renal I/R injury.

Although the precise mechanism by which overexpression of GLO I ameliorated renal I/R injury is unclear, I could propose two possibilities. One is the reduction of MG and the other is the suppression of oxidative stress.

Renal I/R leads to elevation of MG at least in part from the decrease in GLO I activity. Past reports indicated that MG itself is cytotoxic and induces apoptosis in various cells. In my study GLO I overexpression prevented an increase in renal MG level estimated by MG adducts (CEL) accumulation and tubular cell apoptosis. Therefore, the reduction of renal MG may contribute to amelioration of renal I/R injury via reducing apoptosis.

MG reacts with proteins to form advanced glycation endproducts (AGEs). The interaction of AGEs with the receptor for AGE (RAGE) leads to oxidative stress. Oxidative stress may further increase the production of MG in part from the decrease in the activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (58). That is to say, the elevation of MG forms the vicious cycle. In my study immunohistochemistry of oxidative stress marker (4-HNE) showed that *in situ* oxidative stress was reduced by overexpressing GLO I. Therefore, cutting off

the vicious cycle via reducing oxidative stress may contribute to amelioration of renal I/R injury. The schematic mechanism for improvement of renal I/R injury by overexpressing GLO I was indicated in Figure 21 (59).

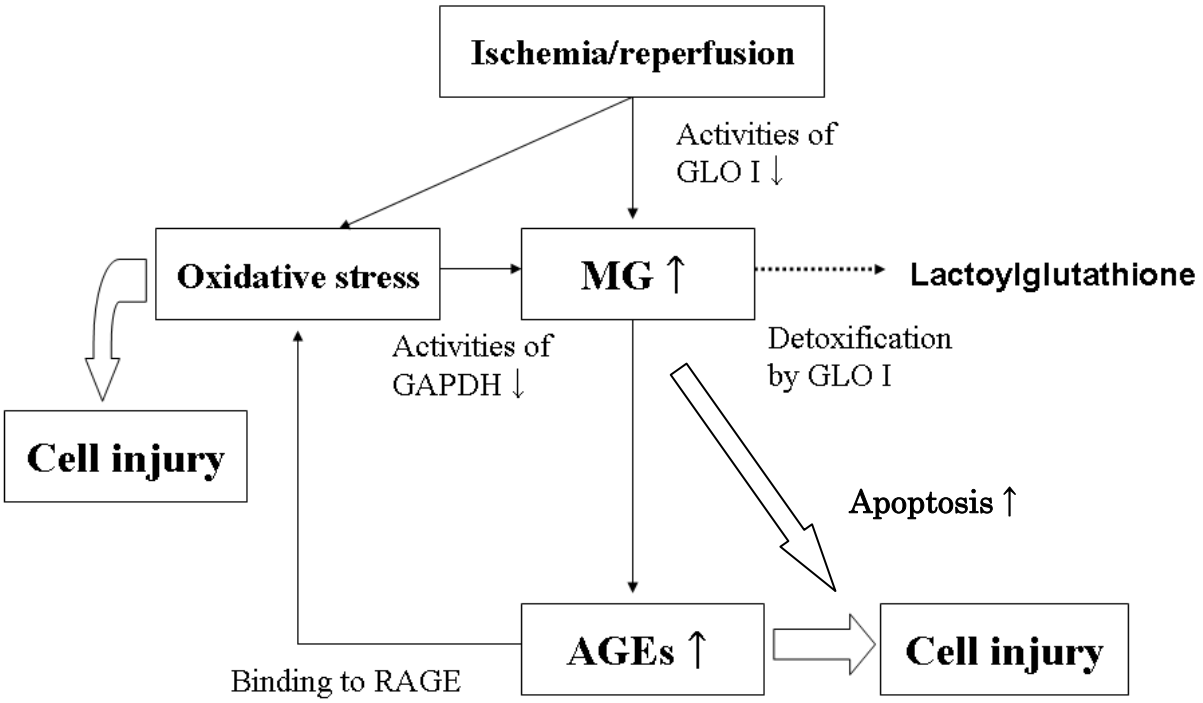

Figure 21. Proposed mechanism for improvement of renal ischemia-reperfusion injury by overexpressing GLO I. Ischemia/reperfusion leads to elevation of methylglyoxal (MG) from the decrease in GLO I activity. MG reacts with proteins to form advanced glycation end products (AGEs). The interaction of AGEs with the receptor for AGE (RAGE) leads to oxidative stress. Oxidative stress may further increase MG. Overexpression of GLO I detoxifies MG and cut off the vicious cycle. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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In *in vitro* study knockdown of GLO I in tubular epithelial cells exacerbated the cellular injury and increased apoptosis under hypoxia/reoxygenation. This results indicated the essential role of GLO I in renal tubular cells in alleviating the cellular injury under hypoxia/reoxygenation, suggesting a direct renoprotection in *in vivo* renal I/R by the increase of *in situ* GLO I activity. According to the past reports, inhibitors of GLO I, S-p-bromobenzylglutathione diesters had antitumour activity *in vitro* and *in vivo* through leading to the cellular accumulation of MG and induction of apoptosis (60, 61). My *in vitro* study also indicated that knockdown of GLO I induced cellular injury although types of cells and conditions in which cells were incubated were different. In addition, it was suggested that overexpression of GLO I is associated with multidrug resistance in various classes of refractory tumor (carcinomas of the lung and prostate, and some leukemias). Therefore, it is thought that my *in vivo* study applied the strategy that refractory tumors develop as resistance to antitumor agent-induced apoptosis.

Several past reports indicated that blockade of RAGE attenuates cardiac or hepatic I/R injury (42, 62). Initially, RAGE was described as a receptor for AGEs that accumulate in hyperglycemic conditions. However, subsequent studies have shown that S100/calgranulins, amphoterin (or high-mobility group box 1), and amyloid-β-peptide also can bind to RAGE, resulting in activation of signal transduction and modulation of gene expression (63-66). Therefore, blockade of RAGE includes the influence of several pathways besides AGEs-RAGE axis. In my present study, I could focus on the effect of the substrate of GLO I, MG in renal I/R injury.

GLO I TG rats showed the histological and functional improvement of I/R-induced renal damage with a statistical significance. However, the degree of the functional improvement was milder than that of the histological improvement. One possible explanation of this phenomenon is that dehydration during the I/R operation may cause an increase in BUN concentration, thereby

overestimated renal dysfunction resulted in the apparent mild functional improvement.

There are several limitations to this study. I did not evaluate the time course of renal GLO I activity after I/R. So whether deterioration of GLO I activity occurs before renal injury was not evident. GLO I Tg rats were generated by introducing humsan GLO I under cytomegalovirus enhancer and GLO I was overexpressed ubiquitously. Therefore, the possibility that infiltrating cells (for example, macrophages) which overexpress GLO I have good influence on renal pathophysiology is not completely ruled out. In addition, renal GLO I activity in GLO I Tg rats were far beyond physiological range. So application of this strategy to human requires further prudent evaluation about adverse effects.

In conclusion, GLO I has renoprotective effects in renal I/R injury by reducing MG in the tubular cells. My findings give an important insight in understanding the pathophysiology of renal I/R injury and suggest a possibility of therapeutic approach targeting the glyoxylase system.

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