Title

Recombinant relaxin protects liver transplants from ischemia damage by hepatocyte glucocorticoid receptor: From bench-to-bedside.

Journal

Hepatology (Baltimore, Md.), 68(1)

ISSN

0270-9139

Authors

Kageyama, Shoichi
Nakamura, Kojiro
Fujii, Takehiro
et al.

Publication Date

2018-07-01

DOI

10.1002/hep.29787

Peer reviewed

HEPATOLOGY

HEPATOLOGY, VOL. 68, NO. 1, 2018

Recombinant Relaxin Protects Liver Transplants From Ischemia Damage by Hepatocyte Glucocorticoid Receptor: From Bench-to-Bedside

Shoichi Kageyama,[1*] Kojiro Nakamura,[1*] Takehiro Fujii,[1] Bibo Ke,[1] Rebecca A. Sosa,[2] Elaine F. Reed,[2] Nakul Datta,[1] Ali Zarrinpar,[1] Ronald W. Busuttil,[1] and Jerzy W. Kupiec-Weglinski[1]

Hepatic ischemia-reperfusion injury (IRI) represents a major risk factor of early graft dysfunction and acute/chronic rejection as well as a key obstacle to expanding the donor pool in orthotopic liver transplantation (OLT). Although glucocorticoid receptor (GR) signaling may enhance cytoprotective programs, clinical use of glucocorticoid is limited because of adverse effects, whereas clinical relevance of GR-facilitated cytoprotection in OLT remains unknown. We aimed to evaluate the significance of hepatic GR in clinical OLT and verify the impact of recombinant human relaxin (rhRLX), which may function as a GR agonist in a tissue/disease-specific manner. Fifty-one OLT patients were recruited under an institutional research board (IRB) protocol. Liver biopsies were collected after cold storage (presurgery) and 2 hours postreperfusion (before abdominal closure), followed by western blotting–assisted hepatic analyses. Forty-three percent of OLTs failed to increase GR perioperatively under surgical stress. Post-/pre-GR ratios at postoperative day 1 correlated negatively with serum aspartate aminotransferase (AST)/cleaved caspase-3 and positively with B-cell lymphoma-extra large (Bcl-xL)/B-cell lymphoma 2 (Bcl-2) levels. In a murine OLT model with extended (18-hour) cold storage, treatment with rhRLX ameliorated ischemia-reperfusion (IR) damage and improved survival while up-regulating hepatocyte GR and Bcl-xL/Bcl-2 expression in OLT. rhRLX-induced GR suppressed hepatocyte high-mobility group box 1 (HMGB1) translocation/release, accompanied by decreased Toll-like receptor 4 (TLR4)/receptor for advanced glycation end products (RAGE), suppressed interleukin 1 beta (IL1β), chemokine (C-C motif) ligand 2 (CCL2), C-X-C motif chemokine (CXCL)10, tumor necrosis factor alpha (TNFα), CXCL1, and CXCL2 levels, and attenuated neutrophil/macrophage accumulation in OLT. Inhibition of GR in hepatocyte culture and in OLT diminished rhRLX-mediated cytoprotection. *Conclusion:* This translational study underscores the role of rhRLX-GR signaling as a regulator of hepatocellular protection against IR stress in OLT. In the context of a recent phase III clinical trial demonstrating positive outcomes of rhRLX in patients with acute heart failure, studies on rhRLX for the management of IRI in OLT recipients are warranted. (HEPATOLOGY 2018;68:258-273).

Although orthotopic liver transplantation (OLT) has become the standard of care for patients with end-stage liver disease and those with hepatic malignancies, shortage of available organs remains important clinical challenge.[1] ischemia-reperfusion injury (IRI), a leading cause of early graft dysfunction and failure, represents a major risk factor in the development of acute and chronic rejection.[2] However, despite obvious clinical importance, the mechanisms that account for liver ischemia-reperfusion injury (IRI) are not well understood, and novel strategies to improve outcomes and expand donor pool are warranted.

Abbreviations: Ab, antibody; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma-extra large; Bx, biopsy; CCL2, chemokine (C-C motif) ligand 2; CXCL, C-X-C motif chemokine; DAMPs, damage-associated molecular patterns; ELISA, enzyme-linked immunosorbent assay; GR, glucocorticoid receptor; H&E, hematoxylin and eosin; HMGB1, high-mobility group box 1; HPFs, high power fields; IHC, immunohistochemistry; IL1β, interleukin 1 beta; IR, ischemia-reperfusion; IRB, Institutional Research Board; IRI, ischemia-reperfusion injury; LDH, lactate dehydrogenase; OLT, orthotopic liver transplantation; POD, postoperative day; RAGE, receptor for advanced glycation end products; rhRLX, recombinant human RLX-2; RLX-2, relaxin-2; ROS, reactive oxygen species; RXFP1, relaxin family peptide receptor-1; sALT, serum alanine aminotransferase; sAST, serum aspartate aminotransferase; siRNA, small interfering RNA; TLR4, Toll-like receptor 4; TNFα, tumor necrosis factor alpha; TUNEL, dT-mediated dUTP nick end labeling; UCLA, University of California, Los Angeles.

Received August 16, 2017; accepted January 12, 2018.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.29787/suppinfo.

Liver IRI is characterized by innate immune-driven sterile inflammation in which reoxygenation of hypoxic cells promotes reactive oxygen species (ROS) formation, including hydrogen peroxide and superoxide, followed by hepatocyte death.[2] The cellular insult leads to secretion of danger-associated molecular patterns (DAMPs), including hepatocyte high-mobility group box 1 (HMGB1), ATP, and histone/DNA,[3] which then trigger a cascade of inflammatory cytokine/chemokine programs, further contributing to target cell death. Given that hepatocyte damage is the initiating event in innate immune activation and ultimate graft dysfunction, preventing hepatocyte death is one of the obvious therapeutic strategies against organ IRI. Indeed, adenovirus-mediated gene transfer of antiapoptotic Bcl-2 attenuated liver IRI in mice,[4] whereas by up-regulating antiapoptotic B-cell lymphoma-extra large (Bcl-xL), recombinant erythropoietin improved hepatocellular function in rat livers exposed to ischemia-reperfusion (IR) insult.[5]

glucocorticoid receptor (GR), a ubiquitously expressed ligand-dependent nuclear hormone receptor, functions as a transcription factor regulating the expression of glucocorticoid-responsive genes.[6] Whereas GR signaling induces apoptosis in a wide range of cells, other cell types may exert antiapoptotic response.[7] Ligand-stimulated GR binds and activates Bcl-xL promoter sequences *in vitro* and *in vivo* and suppresses apoptosis in fibrosarcoma cells,[8] whereas dexamethasone was shown to increase Bcl-2/Bcl-xL and inhibit apoptosis in primary hepatocyte cultures.[9] In line with these findings, the efficacy of glucocorticoid to mitigate hepatic IRI was recorded in a cholestatic rat liver model[10] and in patients undergoing liver resection.[11] However, steroid-related metabolic disorders and well-known adverse effects, such as recurrence of hepatitis C, delayed wound healing, or infections, severely limit their use, whereas clinical relevance of GR hepatoprotection has not been examined to date.

Relaxin, a group of low-molecular-weight peptides of the insulin-growth factor family, consists of seven members, with relaxin-2 (RLX-2) accounting for most of the abundant physiological actions.[12] Isolated mainly from ovaries, RLX-2 is essential in hemodynamic adaptation by decreasing systemic vascular resistance, increasing cardiac output and improving global arterial compliance.[13] Besides its function at the maternal-fetal interface in pregnancy, studies on cytoprotective,[14] anti-inflammatory,[15] and antifibrotic[16] effects underpin the increasing interest in RLX-2 as a therapeutic agent. Indeed, a recent phase III randomized, clinical trial demonstrated the efficacy, safety, and tolerability of recombinant human RLX-2 (rhRLX) in patients with acute heart failure.[17] RLX-2 binds with high affinity to cognate relaxin family peptide receptor-1 (RXFP1)[18] expressed in reproductive tissues, heart, kidney, lung, and brain.[19] Others reported on protective effects of RLX-2 in cardiac,[12] renal,[20] and lung[21] IRI models. Although RLX-2 seemed to reduce cell damage in an isolated rat liver perfusion system,[22] the mechanism and putative clinical relevance of the finding remain unknown.

Supported by Novartis International AG; NIH grants PO1 AI120944, RO1 DK062357, DK107533, and DK102110 (to J.W.K.W.); and The Dumont Research Foundation.

Current address for Ali Zarrinpar: Department of Surgery, Division of Transplantation & Hepatobiliary Surgery, University of Florida College of Medicine.

**These authors contributed equally.*

View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep.29787

Potential conflict of interest: Nothing to report.

ARTICLE INFORMATION:

From the [1]The Dumont-UCLA Transplant Center, Department of Surgery, Division of Liver and Pancreas Transplantation; [2]Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at University of California, Los Angeles, CA.

ADDRESS FOR CORRESPONDENCE AND REPRINT REQUESTS TO:

Jerzy W. Kupiec-Weglinski, M.D., Ph.D.
Dumont-UCLA Transplant Center
77-120 CHS
10833 Le Conte Avenue
Los Angeles, CA 90095
E-mail: jkupiec@mednet.ucla.edu
Tel: +1-310-825-4196

Interestingly, recent studies have identified RLX-2 as a potential GR agonist that may function independently of RXFP1 in a tissue- and disease-specific manner.[23]

Here, we have identified a rhRLX-GR cytoprotection pathway in mouse and human OLT settings. We demonstrated that perioperative enhancement of hepatocyte GR expression triggers local antiapoptotic programs and hepatocellular protection in liver graft recipients. In the context of a recent clinical trial in acute heart failure patients,[17] our translational study validates the use of rhRLX in the management of IRI in OLT recipients.

Materials and Methods

CLINICAL OLT STUDY

Fifty-one adult OLT recipients recruited between May 2013 and August 2015 received routine standard of care and immunosuppressive therapy, as specified by University of California, Los Angeles (UCLA) OLT protocols. Study data were managed using REDCap electronic data capture tools. Recipient blood was collected before the transplant and at postoperative day (POD) 1-14. Liver function was evaluated by serum alanine aminotransferase (sALT) and aspartate aminotransferase (sAST). Protocol Tru-Cut needle biopsies (Bx) were obtained from the left liver lobe and snap-frozen. Pretransplant Bx were obtained after liver cold storage on the back table (presurgery), whereas posttransplant Bx were collected 2 hours after portal reperfusion (before the abdominal closure). Early allograft dysfunction was defined by the presence of one or more of the following: total bilirubin $\geq$10 mg/dL (171 μmol/L) at POD 7; international normalized ratio $\geq$1.6 at POD 7; and ALT/AST >2,000 IU/L within the first 7 PODs.

ANIMALS

C57BL/6 mice at 6-8 weeks of age were used (Jackson Laboratory, Bar Harbor, ME). Animals were housed in the UCLA animal facility under specific pathogen-free conditions and received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" (NIH publication 86-23 revised 1985).

REAGENTS

The recombinant form of human relaxin-2 (rhRLX; RLX030) was provided by Novartis International AG (East Hanover, NJ). Gene-specific small interfering RNA (siRNA) against GR (or scrambled siRNA) was purchased from Santa Cruz Biochemistry (Santa Cruz, CA); GR antagonist (RU-486) was obtained from Sigma-Aldrich (St. Louis, MO).

MOUSE OLT

We used a well-established mouse model of *ex vivo* hepatic cold storage followed by OLT, as described by our group.[24] To mimic a "marginal" human OLT setting, donor livers were stored in University of Wisconsin (UW) solution at 4°C for 18 hours before transplantation into syngeneic mice. Animals were treated with rhRLX (5 μg/kg intravenously) or lactate ringer solution (control) at the time of reperfusion. In some experiments, donor livers were preincubated with GR antagonist (RU-486, 500 nM) for 18 hours during the cold storage. OLT and serum samples were collected at 6 hours of reperfusion, the peak of hepatocellular damage in this model. Separate groups of OLT recipients were monitored for survival. The sham group underwent the same procedures except for OLT.

HEPATOCELLULAR FUNCTION ASSAY

sALT and sAST levels were measured with Infinity alanine aminotransferase (ALT) and aspartate aminotransferase (AST) Liquid Stable Reagent (Thermo Scientific, Rockford, IL) and validated with Validate GC3 (LGC Maine Standards, Cumberland Foreside, ME).

LIVER HISTOLOGY AND IRI GRADING

Formalin-fixed, paraffin-embedded liver sections (5 μm) were stained with hematoxylin and eosin (H&E). The severity of IRI was graded using Suzuki's criteria.[25]

IMMUNOHISTOCHEMISTRY

Expression of RXFP1 and GR (liver, heart, kidney, lung, and esophagus) was examined using rabbit anti-RXFP1 antibody (Ab; Santa Cruz) and rabbit anti-GR monoclonal antibody (Cell Signaling Technology, Danvers, MA). Immunostaining signals were visualized with a labeled polymer in the EnVision+ system horseradish peroxidase (HRP) kit (Dako, Carpinteria, CA). Liver-infiltrating macrophages and neutrophils

were detected with rat anti-CD11b Ab (BD Biosciences, San Jose, CA) and rat anti-Ly6G Ab (Bio-Rad, Hercules, CA), respectively. Signals were visualized with secondary Alexa Fluor 488 antirat immunoglobulin G. Results were scored semiquantitatively by blindly counting the number of positive cells in 10 high power fields (HPFs)/section (400×).

CELL ISOLATION AND CULTURES

Primary mouse hepatocytes were isolated by a two-stage collagenase perfusion method.[26] To trigger oxidative stress, H_2O_2; (Sigma-Aldrich) was added (2 mM for 5 hours) into culture with or without rhRLX pretreatment (1 μg/mL for 24 hours). In some experiments, hepatocytes were transfected with GR-siRNA (or scrambled siRNA) using Lipofectamine reagent (Invitrogen, Waltham, MA) in advance of rhRLX treatment. Culture medium was analyzed for lactate dehydrogenase (LDH) cytotoxicity and ALT/AST levels.

TdT-MEDIATED dUTP NICK END LABELING ASSAY

Cell death in formalin-fixed, paraffin-embedded liver sections (5 μm) was detected by the Apop Tag Plus Peroxidase in Situ Apoptosis Kit (Millipore, Temecula, CA). Results were scored semiquantitatively by blindly counting the number of positive cells in 10 HPFs/section. Cell death in cultured hepatocytes was assessed by a TdT-mediated dUTP nick end labeling (TUNEL) Kit (Sigma-Aldrich), and TUNEL-positive cells were counted in 4 HPFs/group (400×).

WESTERN BLOTTING ASSAY

Proteins were extracted from liver tissues/hepatocyte cultures and their concentration was measured (BCA Protein Assay Kit, Thermo Scientific). An equal amount of protein was electrophoresed, blotted, and incubated with primary Ab, secondary HRP-conjugated Ab, and developed. Primary Ab detecting GR, Bcl-xL, Bcl-2, cleaved caspase 3, β-actin (Cell Signaling), HMGB1 (Abcam, Cambridge, MA), and RXFP1 (R&D Systems, Minneapolis, MN) were used. To compare protein expression in human OLT samples, densitometry quantification was conducted, as follows. In a preliminary study, one of the Bx samples expressing all target proteins was assigned as a "reference" sample. Equal amount of protein lysate from each sample was applied to each well/gel, and target band intensity was normalized by reference sample, followed by normalization with β-actin.

QUANTITATIVE RT-PCR ANALYSIS

RNA was extracted from liver tissue samples using the RNAse Mini Kit (Qiagen, Germantown, MD). A total of 5.0 μg of RNA was reverse transcribed into complementary DNA. Quantitative PCR was performed using DNA Engine with Chromo 4 Detector (MJ Research, Waltham, MA). Primer sequences are listed (Supporting Table S1). Expression of the target gene was normalized to the housekeeping gene, hypoxanthine guanine phosphoribosyltransferase (HPRT).

ENZYME-LINKED IMMUNOSORBENT ASSAY

Serum HMGB1 concentration was measured by enzyme-linked immunosorbent assay (ELISA; MyBioSource, Inc., San Diego, CA), according to the manufacturer's protocol.

STATISTICAL ANALYSIS

In mouse studies, group comparisons were performed using a Student *t* test. For human data, continuous values were analyzed by Mann-Whitney U test and categorical variables by Fisher's exact test. Spearman's correlation coefficient (r) was used to evaluate the strength of linear relationship between variables. The cumulative survival rate was analyzed by the Kaplan-Meier method, and differences were compared using a log-rank test. EZR (Saitama Medical Center, Jichi Medical University), a graphical user interface for R (version 3.2.2; The R Foundation for Statistical Computing), and GraphPad Prism (5 for Mac; GraphPad Software, Inc., La Jolla, CA) were used for Fisher's exact test and for the other analyses, respectively. A P value of <0.05 was considered statistically significant.

STUDY APPROVAL

All studies were approved by the UCLA institutional research board (IRB) (IRB #13-000143) and Animal Research Committee.

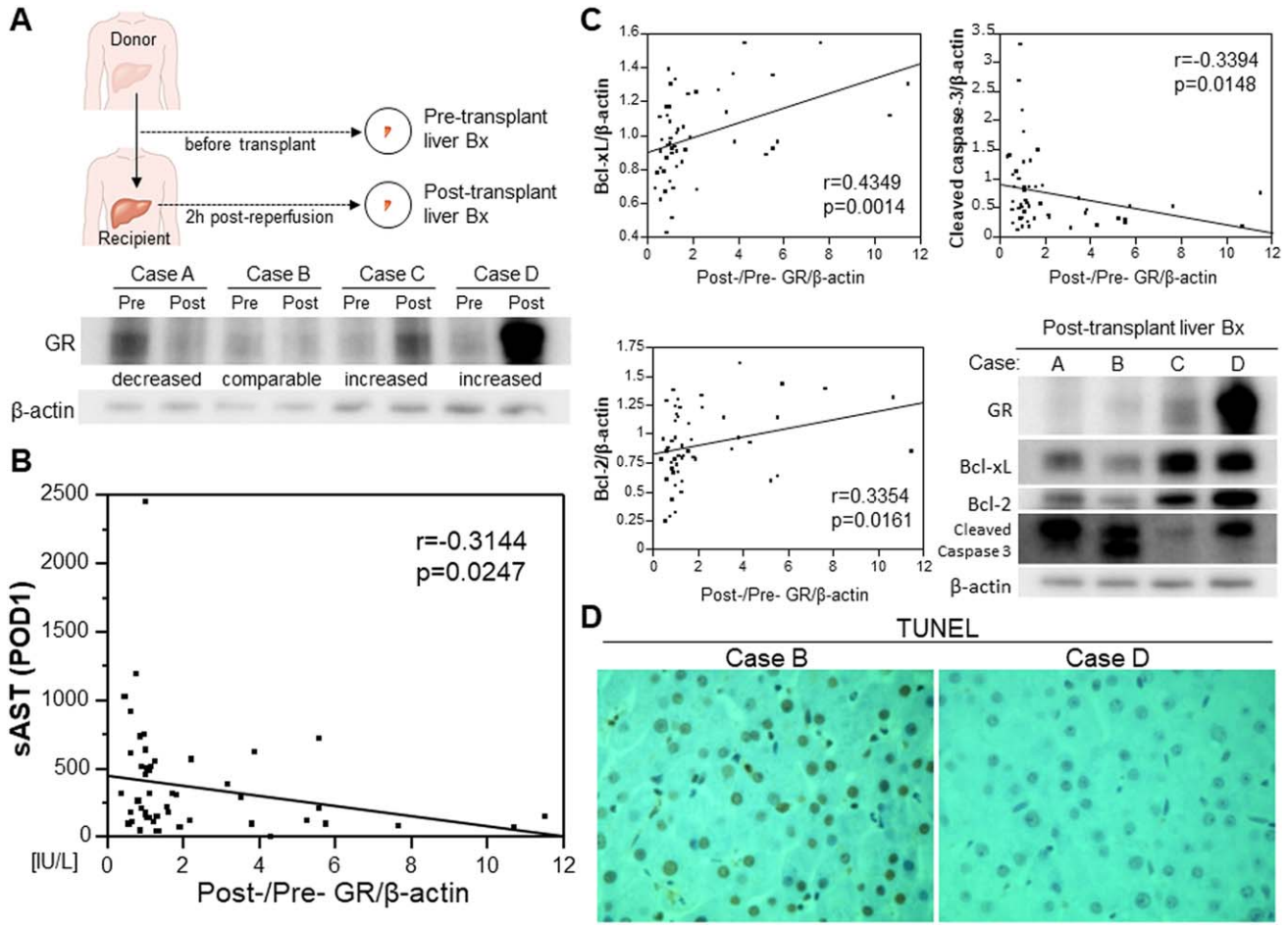

FIG. 1. Increased peritransplant GR levels correlate with improved hepatocellular function and cytoprotection in human OLT. (A) Pretransplant (post–cold storage) and posttransplant (2 hours after reperfusion) liver biopsies (Bx) were collected from 51 OLTs. Bx samples were analyzed by western blottings with β-actin normalization for posttransplant/pretransplant GR ratios. Four representative perioperative GR profiles are shown (Case A, decreased; B, comparable; C/D, increased). (B) Relationship between post-GR/pre-GR ratio and sAST level at POD1. (C) Relationship between post-GR/pre-GR ratio and posttransplant Bcl-xL, Bcl-2, and cleaved caspase 3 levels. Representative western blottings (lower right). (D) Representative TUNEL staining (original magnification, ×400). r, Spearman's correlation coefficient.

Results

HEPATIC GR EXPRESSION CORRELATES WITH HEPATOCELLULAR FUNCTION AND ANTIAPOPTOTIC PHENOTYPE IN HUMAN OLT

We aimed to evaluate retrospectively the perioperative graft GR enhancement and its correlation with liver function in our human OLT cohort (n = 51). Donor liver biopsies (Bx) were collected after cold storage at the back table (before the transplant surgery) whereas posttransplant liver Bx were obtained 2 hours after portal reperfusion (before the abdominal closure; Fig. 1A). Bx samples were analyzed by western blottings, and posttransplant/pretransplant expression levels were calculated to determine perioperative GR profile (representative 4 cases are shown in Fig. 1A). Interestingly, 43% of OLT recipients failed to increase graft GR perioperatively (22 of 51; post-GR/pre-GR: median, 1.09; range, 0.29-11.5; Supporting Fig. S1). There was no significant correlation between GR increase and cold ischemia time or intraoperative blood loss (factors of surgical stress; Supporting Fig. S2). Post-GR/pre-GR ratios negatively correlated with sAST levels at POD 1 (r = –0.3144; P = 0.0247; Fig. 1B), indicating that perioperative GR increase was accompanied by improved OLT function. Moreover,

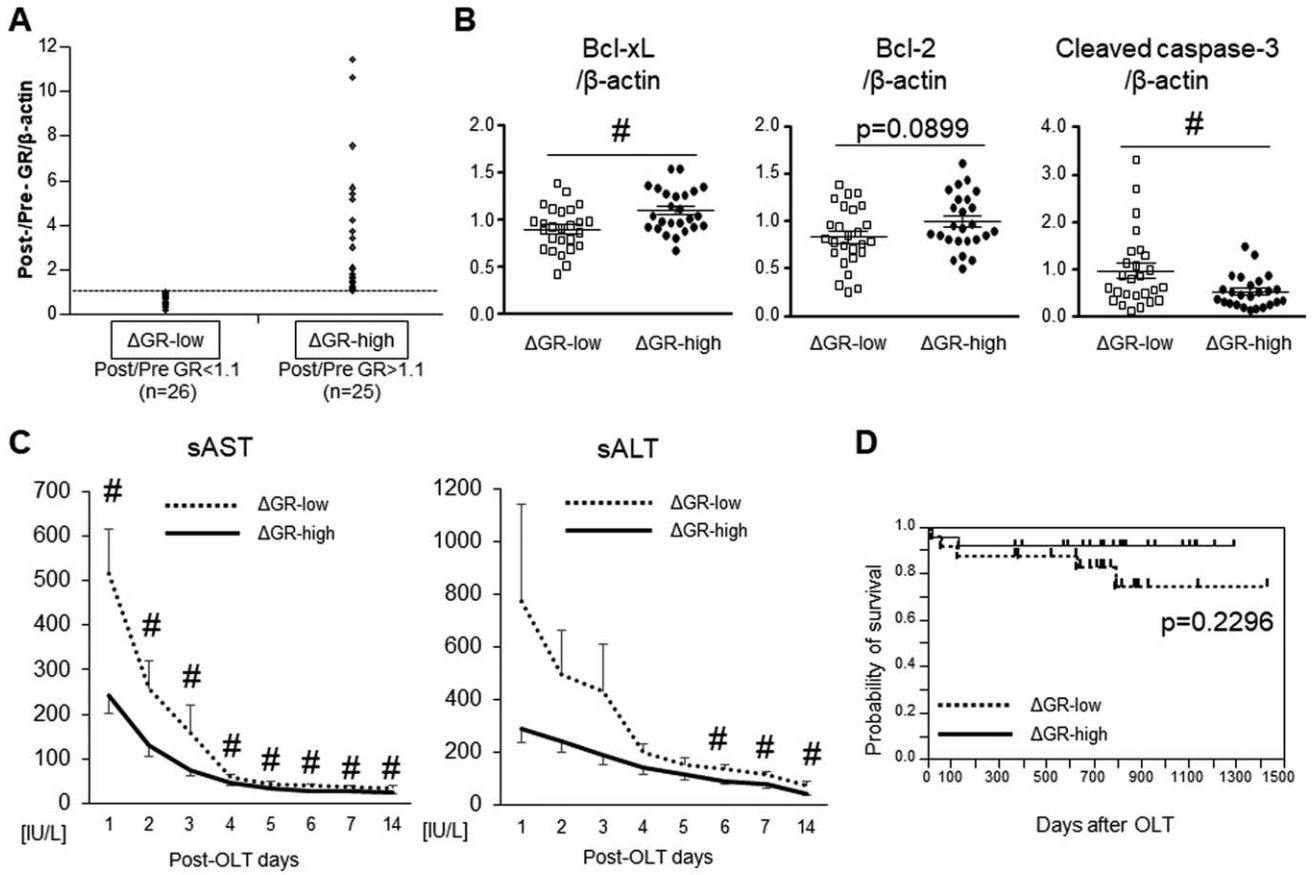

FIG. 2. Peritransplant GR levels are associated with clinical OLT outcomes. (A) Bx samples collected from 51 OLTs were divided into ΔGR-low (post-GR/pre-GR <1.1; n = 26) and ΔGR-high (post-GR/pre-GR >1.1; n = 25) groups. (B) Western blotting–assisted expression of Bcl-xL, Bcl-2, and cleaved caspase 3. Data shown in dot plots and bars indicate mean ± SEM. $^{\#}P < 0.05$ (Mann-Whitney U test). (C) Serum AST and ALT levels at POD 1-14. Dotted line indicates ΔGR-low, whereas solid line indicates ΔGR-high. $^{\#}P < 0.05$ (Mann-Whitney U test). (D) Cumulative probability of OLT survival (Kaplan-Meier method). Dotted line indicates ΔGR-low, whereas solid line ΔGR-high (log-rank test).

post-GR/pre-GR ratios correlated positively with Bcl-xL/Bcl-2 profile and negatively with cleaved caspase 3 levels (Fig. 1C). Western blottings (Fig. 1C) and TUNEL staining (Fig. 1D) from representative Bx samples (Fig. 1A) are shown.

To evaluate the impact of intragraft GR levels for clinical outcomes, 51 human OLTs were classified into ΔGR-low (post-GR/pre-GR <1.1; n = 26) and ΔGR-high (post-GR/pre-GR >1.1; n = 25) expression groups (Fig. 2A). Patients' demographic data and clinical parameters are shown (Supporting Table S2). There was no correlation between ΔGR classification and donor/recipient background, including cold ischemia time, Model for End-Stage Liver Disease (MELD) score, age, sex, or body mass index. Consistent with Fig. 1C data, ΔGR-high cases showed suppressed cleaved caspase 3 ($P < 0.05$) and enhanced Bcl-xL ($P < 0.05$) expression. Despite similar trends, Bcl-2 variables did not reach statistically significant difference ($P = 0.0899$) between ΔGR-low versus ΔGR-high groups (Fig. 2B). OLT recipients characterized by ΔGR-high exhibited lower levels of sAST at POD 1-14 ($P < 0.05$) and of sALT at POD 6-14 ($P < 0.05$) as compared to the ΔGR-low group (Fig. 2C). ΔGR-high recipients experienced shorter posttransplant intensive care unit (ICU) stay (14.4 ± 1.8 vs. 20.0 ± 5.5 days; $P = 0.1930$) and lower incidence of early allograft dysfunction (0.0% vs. 11.5%; $P = 0.1248$). To examine whether GR levels may predict long-term clinical outcomes, we analyzed cumulative posttransplant survival, with the median follow-up of 740 days (range, 4-1,432). None of the patients underwent secondary transplantation. Despite obvious trends, the improved survival in the ΔGR-high group

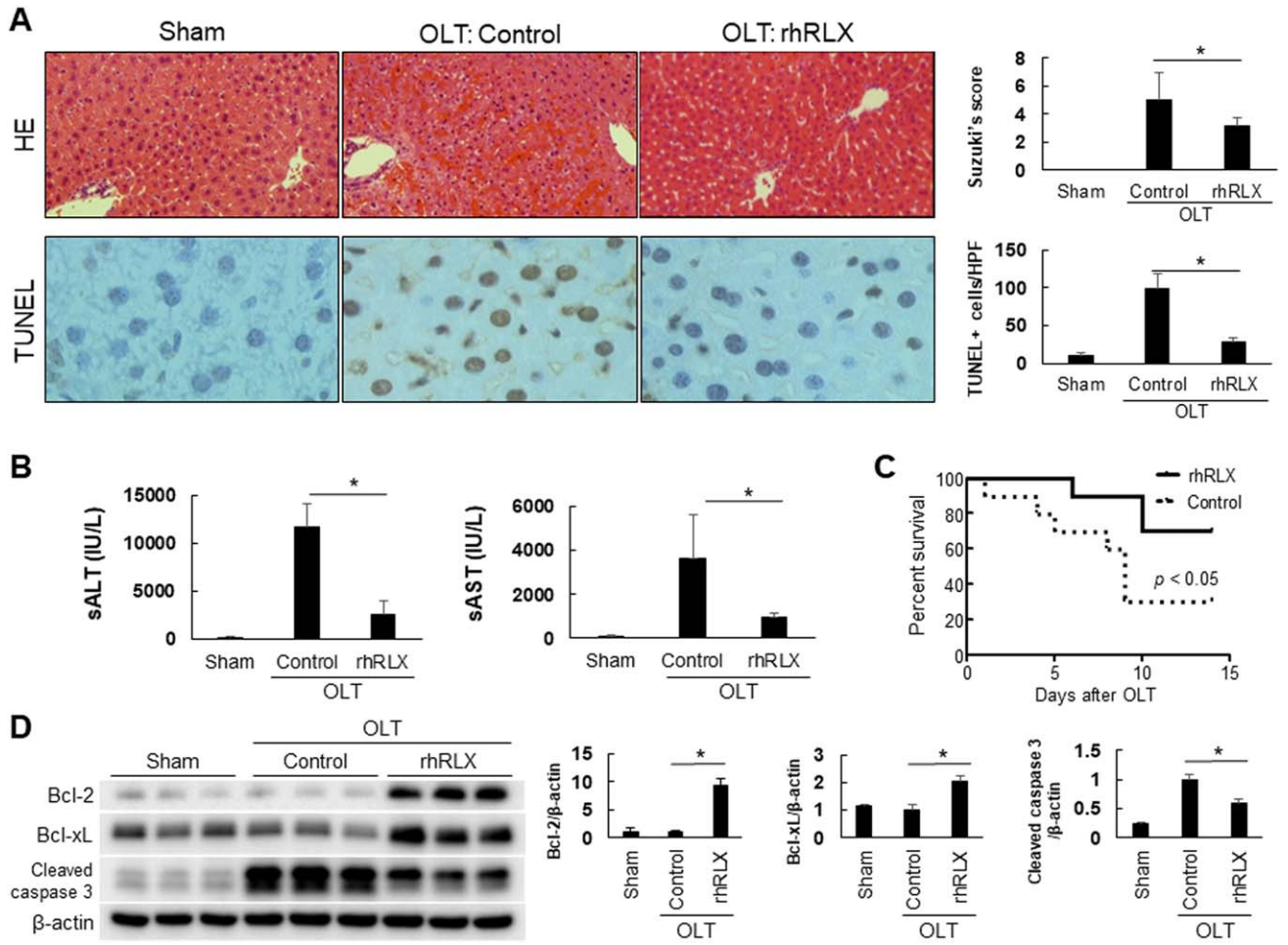

FIG. 3. rhRLX alleviates hepatocellular damage and improves posttransplant survival in IR-stressed mouse OLT. Mouse (C57/Bl6) livers subjected to 18 hours of cold storage were transplanted orthotopically to syngeneic mice. OLT recipients were treated with rhRLX (5 μg/kg intravenously) or equivalent amount of lactate ringer solution (control) at reperfusion. Liver grafts and serum samples were analyzed at 6 hours post-OLT. (A) Representative H&E staining (upper left, original magnification, ×100) and Suzuki's histological grading of liver IRI (upper right, n = 5-6/group). Representative TUNEL staining (lower left, original magnification, ×400) and quantification of TUNEL-positive cells/HPF (n = 5/group). (B) Serum ALT and AST levels (IU/L, n = 5-6/group). (C) rhRLX-treated or control recipients were monitored for 14 days and cumultive survival was analyzed by the Kaplan-Meier method. Solid line indicates rhRLX-treated whereas dotted line indicates control mice (n = 10/group; $P < 0.05$, log-rank test). (D) Western blottings–asisted detection and relative intensity ratio of Bcl-2, Bcl-xL, and cleaved caspase 3. β-actin expression served as an internal control and used for normalization (n = 3/group). Data are shown as mean ± SD (*$P < 0.05$, Student t test).

failed to reach statistical significance when compared to the ΔGR-low group ($P = 0.2296$; Fig. 2D).

rhRLX AMELIORATES HEPATOCELLULAR IR DAMAGE AND IMPROVES SURVIVAL IN MOUSE OLT

To determine the impact of pharmacological GR enhancement in the liver, we used rhRLX, a putative GR agonist, in a mouse model of extended (18 hours) *ex vivo* hepatic cold storage followed by OLT, which mimics a marginal human OLT setting.[24] After the optimal rhRLX dose and route of administration were determined (Supporting Fig. S3A,B), recipient mice were infused with a single dose of rhRLX (5 μg/kg intravenously) or lactate ringer (control) immediately before reperfusion at the completion of surgery. At 6 hours of reperfusion, rhRLX-treated OLTs showed attenuated sinusoidal congestion, vacuolization, and hepatocellular necrosis as compared to controls (Fig.

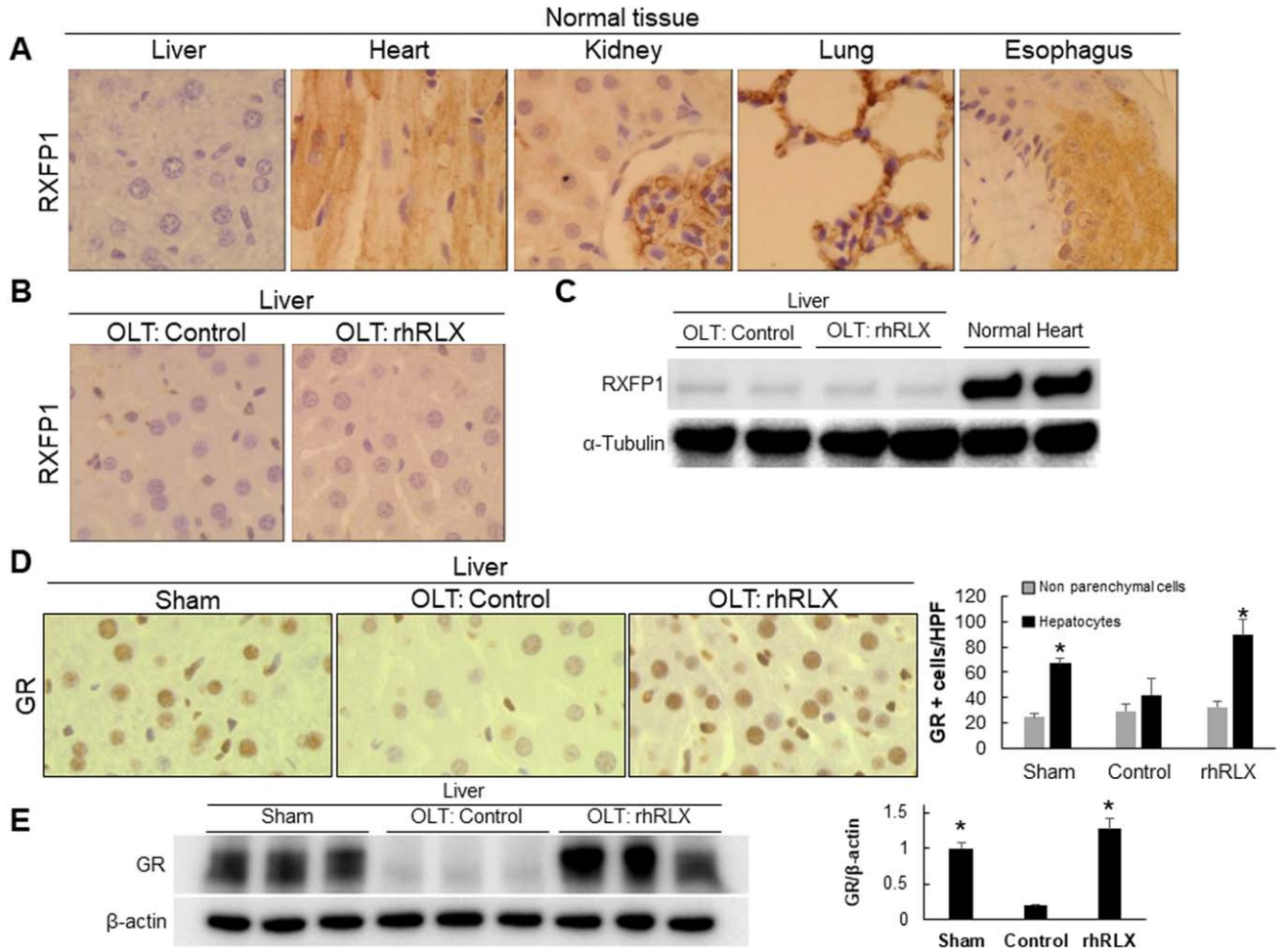

FIG. 4. rhRLX triggers GR, but not RXFP1 expression, in IR-stressed mouse OLT. (A) Representative RXFP1 staining in mouse liver, heart, kidney, lung, and esophagus under basal condition (original magnification, ×400; n = 3). (B) Representative RXFP1 staining in control and rhRLX-treated OLT (original magnification, ×400; n = 3). (C) Representative western blotting–asisted detection of RXFP1 in control OLT, rhRLX-treated OLT, and normal heart (n = 4/group). (D) Representative GR staining in sham-operated liver, control OLT, and rhRLX-treated OLT and quantification of GR-positive cells in nonparenchymal cells (gray) versus hepatocytes (black; n = 5/group). (E) Western blotting–assisted detection and relative intensity ratio of GR. *β*-actin expression served as an internal control and used for normalization (n = 3/group). Data shown as mean ± SD. $^*P < 0.05$ versus OLT: Control (Student *t* test).

3A). These correlated with diminished Suzuki's histological grading of hepatocellular damage ($P < 0.05$; Fig. 3A); decreased frequency of TUNEL + cells ($P < 0.0005$; Fig. 3A); depressed sALT ($P < 0.01$)/sAST ($P < 0.05$) levels (Fig. 3B); and improved OLT survival as compared to controls (70% vs. 30%; $P < 0.05$; n = 10/group; Fig. 3C). In addition, rhRLX markedly increased western blotting–assisted expression of antiapoptotic Bcl-2/Bcl-xL, while depressing proapoptotic cleaved caspase 3 (Fig. 3D). Thus, by enhancing antiapoptotic programs, rhRLX attenuated hepatocellular damage and markedly improved survival of OLTs subjected to prolonged cold storage.

rhRLX INTERACTS WITH HEPATIC GR RATHER THAN RXFP1 COGNATE RECEPTOR

We used immunohistochemistry (IHC) and western blottings to analyze hepatic levels of two major rhRLX binding partners, that is, RXFP1 (cognate receptor) and GR. In contrast to murine heart, kidney, lung, or

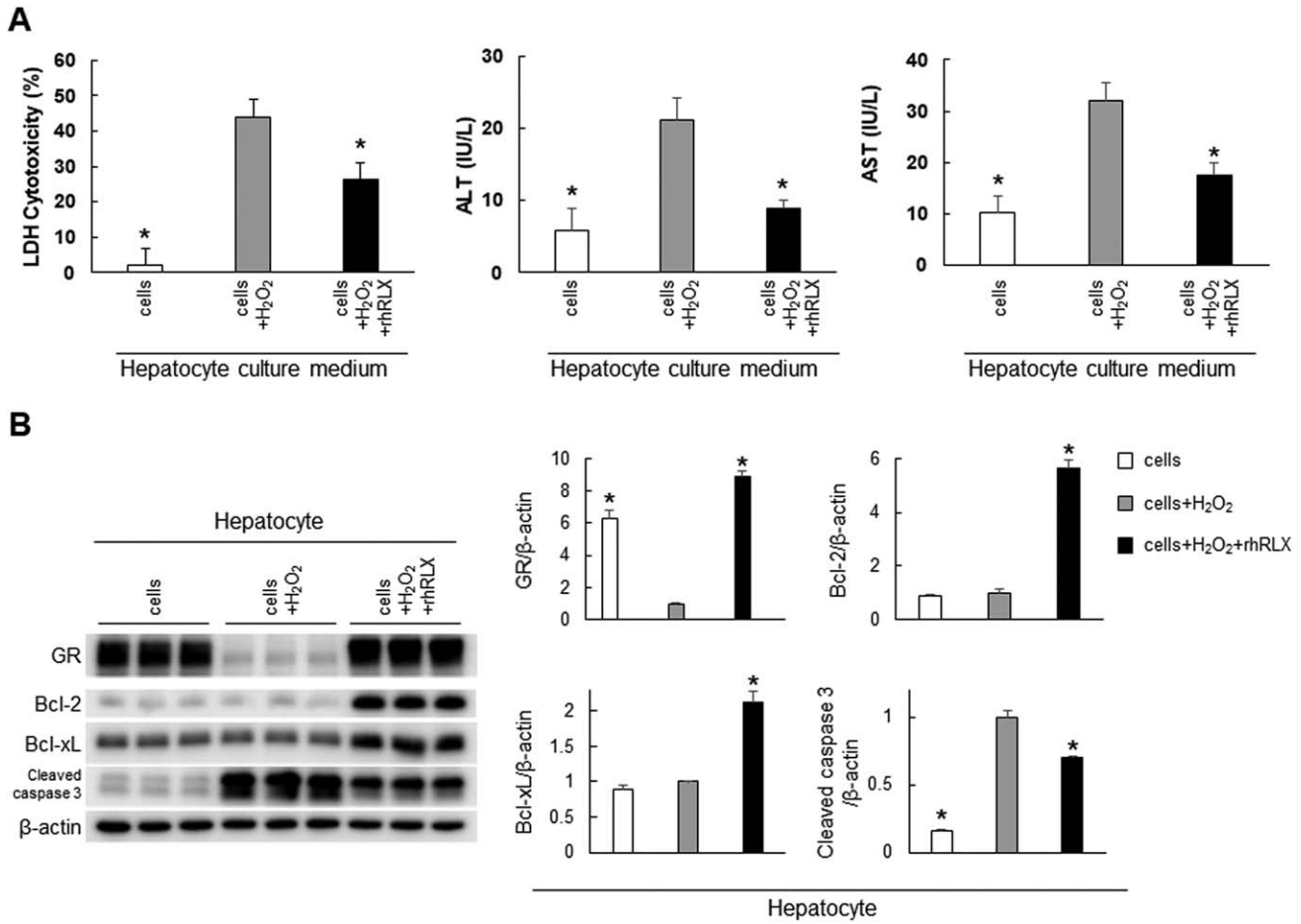

FIG. 5. rhRLX protects hepatocytes from H_2O_2-induced cell death while up-regulating GR/Bcl-2/Bcl-xL *in vitro*. Primary mouse hepatocytes exposed to H_2O_2-induced oxidative stress (2 mM; 5 hours) were pretreated with or without rhRLX (1 μg/mL; 24 hours). (A) Cell damage was assessed by LDH, ALT, and AST levels in the culture medium (n = 4/group). (B) Western blotting–assisted detection and relative intensity ratio of GR, Bcl-2, Bcl-xL, and cleaved caspase 3. β-actin expression served as an internal control and was used for normalization (n = 3/group). Data shown as mean ± SD. $^*P < 0.05$ versus cells + H_2O_2 (Student *t* test).

esophagus, RXFP1 expression was barely detectable in normal mouse liver (Fig. 4A), and neither affected by IR-stress nor rhRLX treatment (Fig. 4B). Moreover, marginal induction of RXFP1 was found in IR-stressed OLTs with or without adjunctive rhRLX (Fig. 4C), suggesting that hepatic RXFP1 signaling is not essential in rhRLX-mediated *in vivo* effects.

IHC confirmed predominantly hepatocyte GR expression (Fig. 4D). Although rhRLX treatment recreated otherwise blunted GR levels in mouse liver grafts (Fig. 4D,E), relatively low GR expression in OLT-infiltrating nonparenchymal cells was only marginally affected by rhRLX (Fig. 4D). Thus, rhRLX-hepatocyte GR cross-talk is essential for OLT protection against IR stress.

rhRLX ATTENUATES ROS-INDUCED HEPATOCYTE DEATH AND INCREASES GR/Bcl-2/Bcl-xL EXPRESSION *IN VITRO*

To focus on hepatocyte rhRLX-GR function, we investigated primary mouse hepatocyte cultures exposed to H_2O_2-induced oxidative stress. Pretreatment with rhRLX (1 μg/mL) mitigated hepatocellular death, evidenced by depressed LDH cytotoxicity as well as ALT/AST release into the culture supernatants (Fig. 5A). Consistent with our *in vivo* findings (Fig. 4D,E), adjunctive rhRLX suppressed otherwise elevated cleaved caspase 3 while increasing GR/Bcl-2/Bcl-xL expression profile (Fig. 5B).

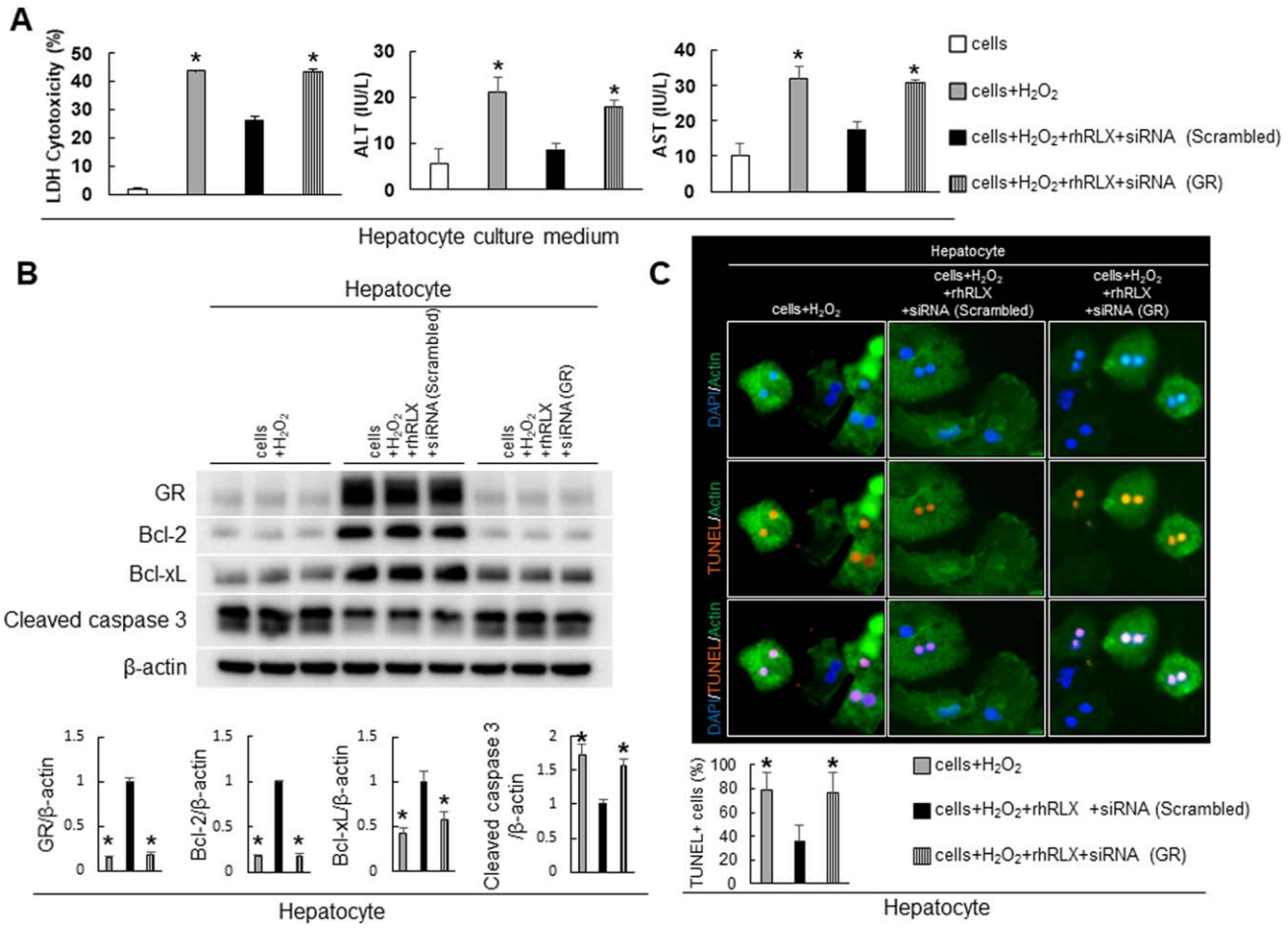

FIG. 6. Hepatocyte protection by rhRLX against ROS, reactive oxygen species; stress is GR-dependent. H_2O_2-stressed (2 mM; 5 hours) primary mouse hepatocytes were pretreated with or without rhRLX (1 μg/mL; 24 hours) and siRNA against GR. (A) Culture medium was screened for LDH, ALT, and AST levels (n = 4/group). (B) Western blotting–assisted detection and relative intensity ratio of GR, Bcl-2, Bcl-xL, and cleaved caspase 3. β-actin expression served as an internal control and used for normalization (n = 3/group). (C) Representative TUNEL-assisted detection of hepatocyte death (Actin, green; DAPI, blue; TUNEL, red) and quantification of TUNEL-positive cells (four randomly chosen HPF/group). Data shown as mean ± SD. $^*P < 0.05$ versus cells + H_2O_2 + rhRLX + siRNA (scrambled; Student t test). Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

rhRLX ATTENUATES H_2O_2-STRESSED HEPATOCYTE DEATH IN GR-DEPENDENT MANNER

Having shown GR induction and cytoprotection by exogenous rhRLX *in vivo* and *in vitro*, we focused on the functional significance of GR signaling in rhRLX-conditioned primary hepatocyte cultures. Unlike in the scrambled siRNA-treated group, adjunctive GR-siRNA knockdown recreated cellular damage in rhRLX-treated hepatocytes, evidenced by increased LDH and ALT/AST levels in otherwise H_2O_2-stress–resistant, rhRLX-treated cells (Fig. 6A). Neither rhRLX nor adjunctive GR knockdown by siRNA affected antioxidant heme oxygenase-1 (hsp 32) expression in hepatocyte cultures (Supporting Fig. S4). In addition, GR silencing suppressed Bcl-2/Bcl-xL while augmenting cleaved caspase 3 (Fig. 6B) and restoring TUNEL + (Fig. 6C) hepatocyte expression. These data indicate that rhRLX mitigates hepatocyte death by the GR/Bcl-2/Bcl-xL signaling axis.

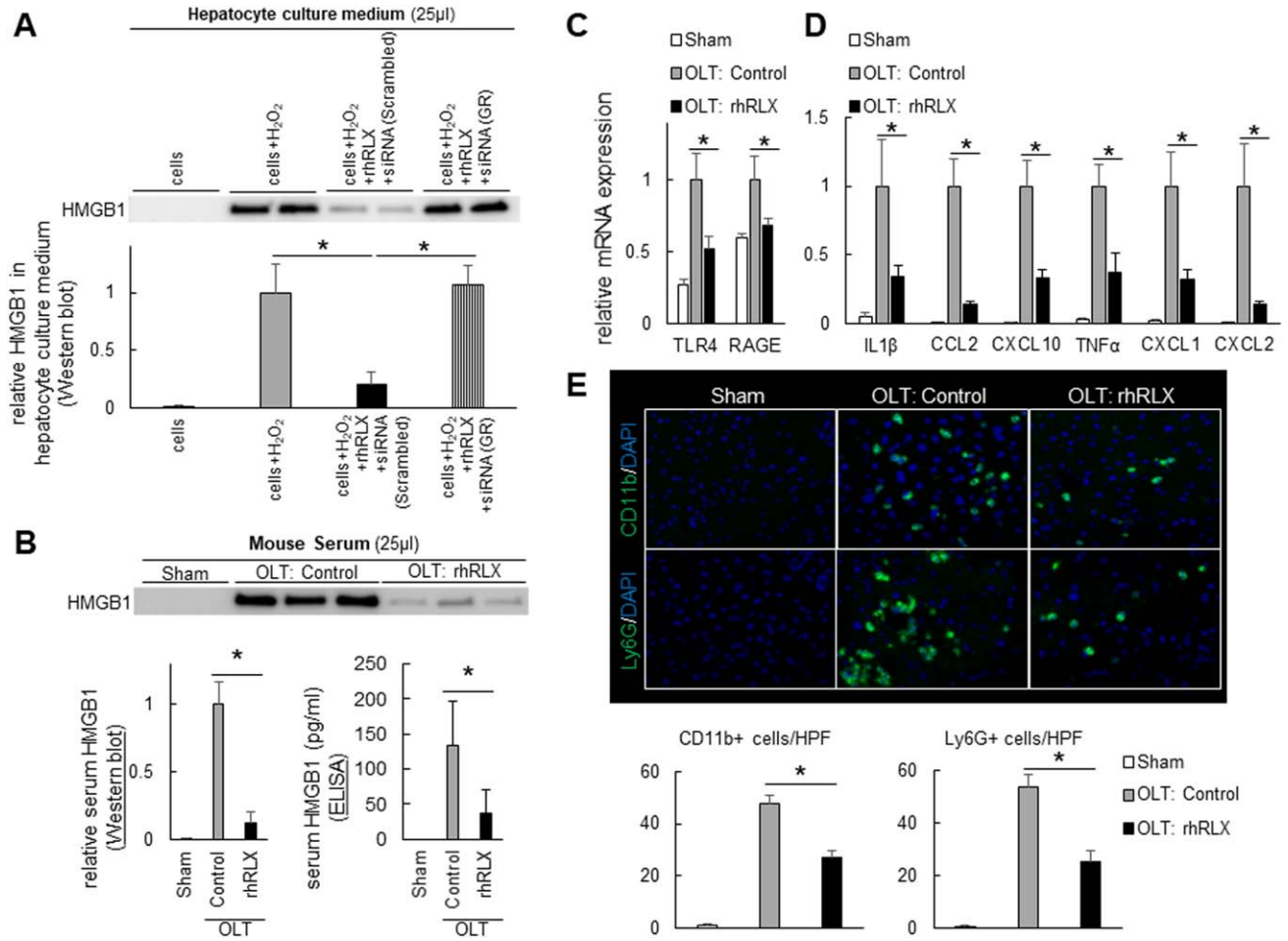

FIG. 7. rhRLX mitigates HMGB1 translocation and suppresses TLR4/RAGE as well as proinflammatory phenotype in IR-stressed OLT. (A) H_2O_2-stressed (2 mM; 5 hours) primary mouse hepatocytes were pretreated with or without rhRLX (1 μg/mL; 24 hours) and siRNA against GR. Representative western blotting–assisted detection of HMGB1 in culture medium and quantitation of relative expression values (n = 3-4/group; $^*P < 0.05$). (B-E) Mouse livers stored for 18 hours in Fig. 4C were transplanted orthotopically followed by 6 hours of reperfusion. rhRLX (5 μg/kg intravenously) or lactate ringer (control) was administered at the time of reperfusion. (B) Representative western blotting–assisted HMGB1 detection in serum and quantitation of relative expression values (n = 3-4/group; $^*P < 0.05$), and serum HMGB1 levels measured by ELISA (pg/mL; n = 4-5/each; $^*P < 0.05$). (C,D) qRT-PCR-assisted detection of mRNA coding for TLR4, RAGE, IL1β, CCL2, CXCL10, TNFα, CXCL1, and CXCL2. Data were normalized to HPRT gene expression (n = 4/group; $^*P < 0.05$) (E) Representative IHC staining and quantification of OLT-infiltrating $CD11b^+$ and $Ly6G^+$ cells (positive cells/HPF; original magnification, ×400; n = 5/group; $^*P < 0.05$). Data shown as mean ± SD (Student *t* test). Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

rhRLX PREVENTS HMGB1 TRANSLOCATION AND RELEASE FROM H_2O_2-STRESSED HEPATOCYTES

Because the release of DAMPs from injured cells is the key event in the early phase of innate immune activation, we next examined HMGB1 levels in H_2O_2-stressed mouse hepatocyte cultures. The efficacy of rhRLX to inhibit translocation of cellular HMGB1 into culture medium was blocked after adjunctive GR silencing (siRNA) as compared with scrambled siRNA-treated cells (Fig. 7A). Thus, rhRLX-GR signaling prevented cell death (Fig. 6A-C) and suppressed HMGB1 release from stressed hepatocytes (Fig. 7A).

rhRLX SUPPRESSES CELLULAR HMGB1 RELEASE TO MITIGATE IRI IN MOUSE OLT

To confirm the impact of rhRLX on HMGB1 translocation and release *in vivo*, we profiled HMGB1

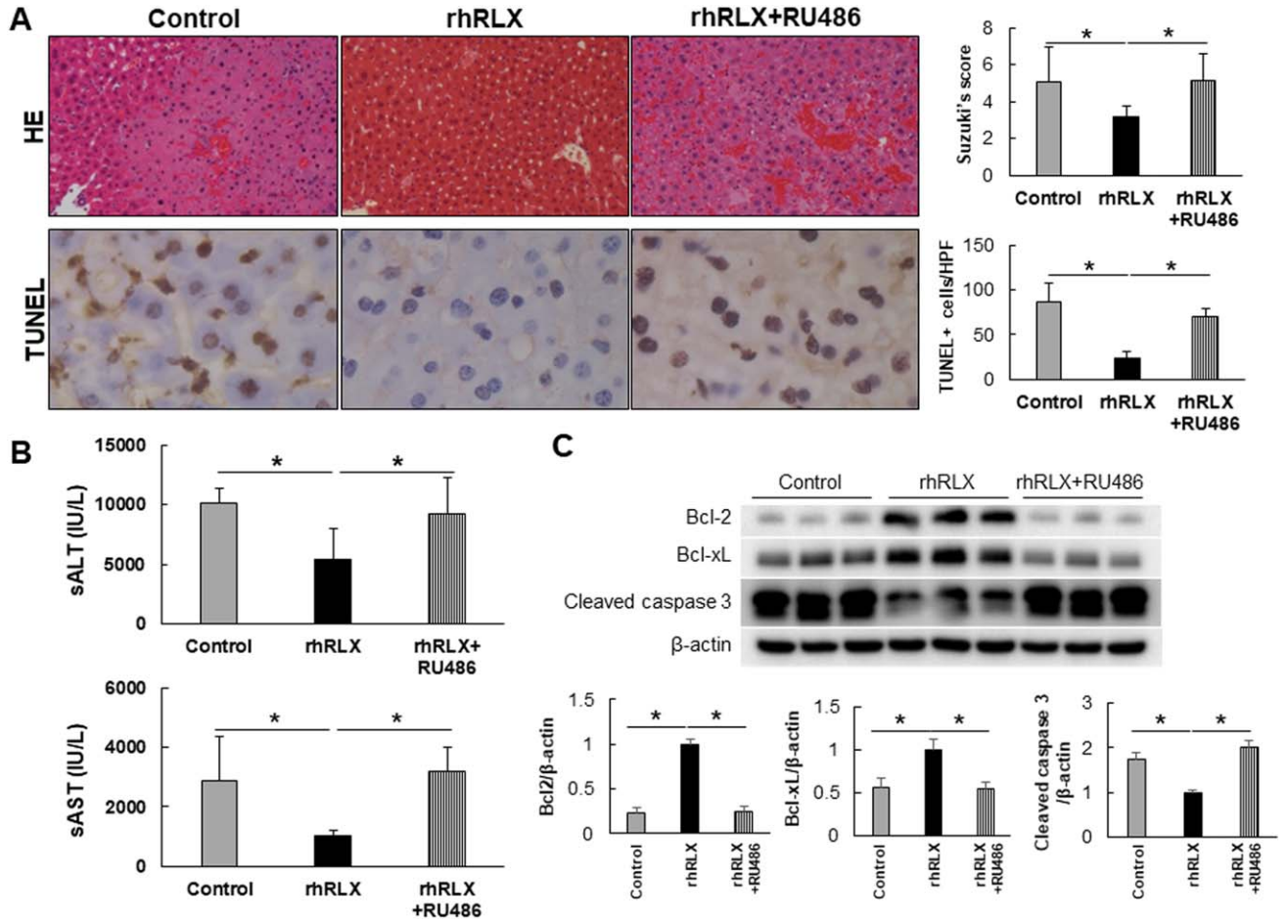

FIG. 8. GR antagonist (RU-486) abrogates rhRLX-induced hepatoprotection in IR-stressed mouse OLT. Mouse livers subjected to 18 hours of cold storage were transplanted to syngeneic recipients treated with rhRLX or lactate ringer (control) at the time of reperfusion. Separate donor livers were preincubated with UW containing GR antagonist (RU-486, 500 nM) for 18 hours during cold storage. (A) Representative H&E staining (upper left; original magnification, ×100) and Suzuki's histological IRI grading (upper right, n = 3-5/group). Representative TUNEL staining (lower left; original magnification, ×400) and quantification of TUNEL-positive cells/HPF (n = 3-4/group) (B) Serum ALT and AST levels (IU/L, n = 3-5/group). (C) Western blotting–assisted detection and relative intensity ratio of Bcl-2, Bcl-xL, and cleaved caspase 3. *β*-actin expression served as an internal control and was used for normalization (n = 3/group). Data shown as mean ± SD (Student *t* test).

and inflammatory markers in IR-stressed mouse OLTs. Consistently, rhRLX treatment reduced serum HMGB1 levels (western blottings and ELISA; Fig. 7B) as well as hepatic Toll-like receptor 4 (TLR4) and receptor for advanced glycation end products (RAGE), that is, major extracellular HMGB1-activated immune receptors (Fig. 7C). Consistent with proinflammatory pathway blockade, rhRLX mitigated mRNA levels coding for interleukin 1 beta (IL1*β*), chemokine (C-C motif) ligand 2 (CCL2), C-X-C motif chemokine (CXCL10), tumor necrosis factor alpha (TNF*α*), CXCL1, and CXCL2 (Fig. 7D) while decreasing macrophage (CD11b) and neutrophil (Ly6G) infiltration (Fig. 7E). Thus, rhRLX attenuated local inflammation *in vivo*, at least in part, by suppressing translocation of cellular HMGB1 in IR-stressed OLTs.

rhRLX HEPATOPROTECTION IN IR-STRESSED MOUSE OLT IS GR DEPENDENT

Having shown rhRLX facilitated GR-dependent hepatoprotection *in vitro* (Fig. 6), accompanied by

HMGB1-mediated immune regulation (Fig. 7), we aimed to determine the significance of a GR signaling *in vivo* model. Indeed, rhRLX-induced hepatoprotection was abolished after adjunctive treatment with RU486 (GR antagonist), as evidenced by H&E staining, Suzuki's scores, TUNEL staining (Fig. 8A), and sAST/sALT levels (Fig. 8B). In parallel, RU486 adjunct diminished Bcl-2/Bcl-xL while enhancing cleaved caspase 3 expression in rhRLX-treated OLT (Fig. 8C). RU486 treatment alone did not increase liver damage (sAST/sALT level and Suzuki's score) as compared to control OLT (Supporting Fig. S5). Thus, hepatocyte GR functions as an essential regulator in rhRLX-mediated protection against IRI.

Discussion

This study demonstrates the key regulatory role of hepatic GR signaling in inflammatory injury in a human liver. Indeed, increased perioperative GR expression correlated with enhanced antiapoptotic programs and preservation of hepatocellular function in OLT recipients (Figs. 1 and 2). Our parallel studies in a clinically relevant mouse model of hepatic cold storage and OLT have revealed cytoprotective function and putative mechanisms of hepatocyte rhRLX-GR cross-talk *in vivo* and *in vitro*. A single intravenous infusion of rhRLX, a GR agonist, conferred OLT protection against IR insult, evidenced by decreased release of liver enzymes, suppression of histopathological tissue injury scores, and modulation of apoptosis. These findings were confirmed in primary mouse hepatocyte cultures, where addition of rhRLX attenuated cell death by increasing GR/Bcl-2/Bcl-xL expression and preventing HMGB1 translocation from H_2O_2-stressed cells. GR-dependent cytoprotection after rhRLX treatment was confirmed in TNFα-stressed hepatocyte cultures (data not shown). Hence, this translational study highlights a function of hepatocyte GR and rhRLX therapy in IR-stressed livers.

A recent phase III randomized, clinical trial in a cohort of 1,161 patients with acute heart failure demonstrated positive outcomes after rhRLX treatment, only with controllable hypotension.[19] Of note, up to 48-hour continuous intravenous rhRLX administration did not increase the incidence of adverse infections. In addition, a single infusion of rhRLX at reperfusion in our murine IRI-OLT model was well tolerated and markedly improved posttransplant liver function and survival (Fig. 3C). Thus, by documenting hepatocyte-protective rhRLX-GR signaling, our findings complement published data in patients with acute heart failure and validate future rhRLX clinical trials. Failure to find significant differences in post-OLT survival between ΔGR-high versus ΔGR-low groups, despite obvious trends, may be attributed to limited patient cohort. Further studies are required given that the correlation between IRI severity and post-OLT graft/patient survival remains controversial.[27,28]

In the clinical arm of our study, 43% of human OLTs failed to increase GR expression (Supporting Fig. S1), despite a routine hydrocortisone bolus given at reperfusion. High ΔGR was associated with a trend toward more severe recipient status (higher MELD, longer pretransplant hospital/ICU stay) while favoring lower donor pretransplant transaminase levels (Supporting Table S2). However, these differences failed to reach statistical significance, and studies in larger patient cohorts are needed to further explore these clinical factors. Unable to elucidate the causality of such a GR decrease, we may envision the following scenarios. First, cellular stress may alter GR expression profile given that hypoxia did reduce GR function and its ligand-binding ability in a fetal heart.[29] Moreover, NLR family pyrin domain containing 3/caspase-1 axis, which is essential in liver IRI pathogenesis, cleaves cellular GR and diminishes cell sensitivity to glucocorticoid.[30] In our study, cold storage (18 hours) alone did not decrease GR expression in the liver graft (data not shown). However, 6 hours of reperfusion depressed hepatic GR *in vivo* (Fig. 4D,E); and H_2O_2-induced stress depressed hepatocyte GR *in vitro* (Fig. 5B) while increasing cleaved caspase-1 (data not shown). Second, despite hypothalamic-pituitary-adrenal axis secreting glucocorticoid in stress response to a trauma,[31] major surgery may increase glucocorticoid demand throughout the body, with actual demand sometimes exceeding physiological/pharmacological supply, and resulting in adrenal failure. In this case, GR suppression may result in insufficient ligand binding, implying that the 43% of GR-depressed OLTs might potentially be rescued by the adequate ligand supply. We are aware of putative differences between human and rodent glucocorticoid biological functions. Whereas corticosterone is the major circulating glucocorticoid in rodents attributed to the absence of a cortisol synthesis enzyme, in humans the corticosterone level is 10- to 20-fold lower than of cortisol, with the latter exhibiting far more powerful activity than corticosterone.[32] Thus, although rhRLX treatment markedly increased GR expression in murine OLT (Fig. 4D,E), it remains

unknown whether rhRLX can equally enhance hepatic GR in coritisol-dominated human OLT. Despite these shortcomings, however, our findings validate future studies on rhRLX-GR cross-talk in human liver graft GR expression/function.

RXFP1 expression was barely detectable in a mouse liver, and neither OLT nor rhRLX treatment made any difference (Fig. 4A-C). Indeed, although Fallowfield et al. reported undetectable RXPF1 protein levels in normal human or rat livers, hepatic stellate cells, myofibroblasts, and sinusoidal endothelial cells became $RXFP1^{+}$ in fibrotic livers.[33] However, despite marginal RXFP1 expression in OLTs, we cannot exclude a possibility of RXFP1-signaling functioning as a part of a complex rhRLX cytoprotection axis. Indeed, one of the distinct relaxin actions is mediated by endothelial RXFP1/phosphoinositide 3-kinase/protein kinase B/endothelial nitric oxide (NO) synthase signaling,[34] whereas relaxin dilated sinusoid in unstressed rat liver by a NO-dependent manner.[35] Further studies using RXFP1 knockout mice or "RXFP1-inactive relaxin (chemically modified porcine relaxin biologically inactive at RXFP1)"[36] are warranted to verify the involvement of RXFP1 in rhRLX cytoprotection. On the other hand, GR knockdown by siRNA abolished hepatocyte protection by rhRLX *in vitro* (Fig. 6), whereas adjunctive GR antagonist (RU486) diminished graft protection observed otherwise following rhRLX monotherapy *in vivo*. However, given that GR is expressed ubiquitously, we cannot exclude the possibility that RU486 might influence other cell types in IRI pathogenesis (e.g., macrophage, lymphocyte, and endothelial cells). Hepatocyte-specific GR knockout mice[37] are indispensable to confirm the significance of hepatocyte-specific GR signaling.

HMGB1, a multifunctional danger molecule (alarmin) orchestrating the inflammatory and immune cascades in organ IRI,[3] is a ubiquitously expressed protein that binds to the minor groove of nuclear DNA to control the activity of transcription factors by structurally modifying the DNA double helix. Once translocated and released passively or secreted actively from injured or dying cells into extracellular space, it acts as a key DAMP that alerts innate immune system to activate macrophages by downstream TLR4 and neutrophils by RAGE signaling.[38] Indeed, Ilmakunnas et al. reported that HMGB1 may represent a useful biomarker of hepatocellular damage in human LT.[39] The efficacy of pharmacological HMGB1 deactivation in warm hepatic rat and mouse[40] IRI models has been recently reported on. Thus, HMGB1 secretion from injured hepatocytes is not only an indicator of liver function, but it may also serve as a regulator of sterile inflammatory tissue injury. Consistent with the latter, suppression of HMGB1 mobilization and translocation from hepatocytes was accompanied by decreased TLR4/RAGE expression and IR inflammation, suggesting that an anti-inflammatory phenotype in mouse OLT after treatment with rhRLX could be attributed, at least in part, to suppressed HMGB1 secretion. We are aware that rhRLX-GR signaling may directly regulate macrophage activation. Indeed, porcine relaxin or RXFP1-inactive relaxin suppressed TNFα/IL6 production from THP-1 cells whereas adjunctive RU486 reversed that effect.[36] We found that rhRLX suppressed proinflammatory, while enhancing anti-inflammatory, gene expression programs in mouse bone-marrow–derived macrophage (BMDM) cultures (data not shown). On the other hand, RLX-2 stimulated THP-1 cell adhesion and migration through a RXFP1-dependent mechanism.[41] Although relaxin may promote inflammation through RXFP1 in decidual macrophage,[42] addition of rhRLX failed to alter total GR or RXFP1 protein expression in BMDM cultures (Nakamura, unpublished). The precise rhRLX macrophage-regulatory mechanism requires further in-depth studies.

Gujral et al. reported that the majority of cell death in rat livers subjected to warm ischemia occurred by necrosis and only a minority of damaged hepatocytes displaying features of apoptosis.[43] Indeed, pan-caspase inhibitors Z-Asp-cmk[43] and Z-VD-fmk[44] failed to protect livers against IRI. However, necrosis and apoptosis represent interdependent phenomena resulting from activation of shared pathways, and apoptosis precedes necrosis in the pathogenesis of liver IRI.[43,44] In addition, although some pan-caspase inhibitors paradoxically reported increased necrosis,[45] necroptosis,[46] or autophagic cell death,[47] it is noteworthy that a caspase inhibitor, IDN-6556, which reduced liver IRI in a murine model, was also tested in a phase II clinical OLT trial.[48] We recently reported that hepatoprotection after treatment with necrostatin-1 stable (Nec-1s; a necrosis inhibitor) was abolished in Kupffer cell–deficient mice, indicating that Nec-1s's direct hepatocyte effect failed to mitigate liver cell death in the mechanism of IRI.[49] Consistent with the antiapoptotic program critically important in liver IR resistance, in our clinical arm screen of 51 OLT patients, posttransplant Bcl-xL expression negatively correlated with AST levels at POD 1 ($r = -0.3148$; $P = 0.0245$); and rhRLX treatment attenuated

hepatocellular damage while increasing Bcl-xL/Bcl-2 and depressing cleaved caspase-3 in IR-stressed mouse OLT.

In conclusion, our results underscore the role of GR signaling as a regulator of hepatocellular protection in IR-stressed OLT. As a promising candidate to mitigate innate activation and sterile inflammatory tissue injury, future studies on rhRLX, a GR ligand and agonist, in the management of OLT recipients are warranted.

Acknowledgments: We thank Ko Takanashi (UCLA-TPCL) and Damla Oncel (undergrad UCLA student) for immunohistochemical assistance and Dr. Takahiro Ito and Antony Aziz for helping to collect clinical data.

REFERENCES

1) Dutkowski P, Linecker M, DeOliveira ML, Mullhaupt B, Clavien PA. Challenges to liver transplantation and strategies to improve outcomes. Gastroenterology 2015;148:307-323.
2) Zhai Y, Petrowsky H, Hong JC, Busuttil RW, Kupiec-Weglinski JW. Ischaemia-reperfusion injury in liver transplantation—from bench to bedside. Nat Rev Gastroenterol Hepatol 2013;10:79-89.
3) Tsung A, Tohme S, Billiar TR. High-mobility group box-1 in sterile inflammation. J Intern Med 2014;276:425-443.
4) Bilbao G, Contreras JL, Eckhoff DE, Mikheeva G, Krasnykh V, Douglas JT, et al. Reduction of ischemia-reperfusion injury of the liver by in vivo adenovirus-mediated gene transfer of the anti-apoptotic Bcl-2 gene. Ann Surg 1999;230:185-193.
5) Shawky HM, Younan SM, Rashed LA, Shoukry H. Effect of recombinant erythropoietin on ischemia-reperfusion-induced apoptosis in rat liver. J Physiol Biochem 2012;68:19-28.
6) Nicolaides NC, Galata Z, Kino T, Chrousos GP, Charmandari E. The human glucocorticoid receptor: molecular basis of biologic function. Steroids 2010;75:1-12.
7) Gruver-Yates AL, Cidlowski JA. Tissue-specific actions of glucocorticoids on apoptosis: a double-edged sword. Cells 2013;2:202-223.
8) Gascoyne DM, Kypta RM, Vivanco M. Glucocorticoids inhibit apoptosis during fibrosarcoma development by transcriptionally activating Bcl-xL. J Biol Chem 2003;278:18022-18029.
9) Bailly-Maitre B, de Sousa G, Zucchini N, Gugenheim J, Boulukos KE, Rahmani R. Spontaneous apoptosis in primary cultures of human and rat hepatocytes: molecular mechanisms and regulation by dexamethasone. Cell Death Differ 2002;9:945-955.
10) Subhas G, Gupta A, Bakston D, Silberberg B, Lobocki C, Andrus L, et al. Protective effect of methylprednisolone on warm ischemia-reperfusion injury in a cholestatic rat liver. Am J Surg 2010;199:377-380; discussion, 380-371.
11) Orci LA, Toso C, Mentha G, Morel P, Majno PE. Systematic review and meta-analysis of the effect of perioperative steroids on ischaemia-reperfusion injury and surgical stress response in patients undergoing liver resection. Br J Surg 2013;100:600-609.
12) Di Lascio G, Harmelin G, Targetti M, Nanni C, Bianchi G, Gasbarri T, et al. Cellular retrograde cardiomyoplasty and relaxin therapy for postischemic myocardial repair in a rat model. Tex Heart Inst J 2012;39:488-499.
13) Teichman SL, Unemori E, Teerlink JR, Cotter G, Metra M. Relaxin: review of biology and potential role in treating heart failure. Curr Heart Fail Rep 2010;7:75-82.
14) Lodhi RS, Nakabayashi K, Suzuki K, Yamada AY, Hazama R, Ebina Y, Yamada H. Relaxin has anti-apoptotic effects on human trophoblast-derived HTR-8/SV neo cells. Gynecol Endocrinol 2013;29:1051-1054.
15) Nistri S, Chiappini L, Sassoli C, Bani D. Relaxin inhibits lipopolysaccharide-induced adhesion of neutrophils to coronary endothelial cells by a nitric oxide-mediated mechanism. FASEB J 2003;17:2109-2111.
16) Lekgabe ED, Kiriazis H, Zhao C, Xu Q, Moore XL, Su Y, et al. Relaxin reverses cardiac and renal fibrosis in spontaneously hypertensive rats. Hypertension 2005;46:412-418.
17) Teerlink JR, Cotter G, Davison BA, Felker GM, Filippatos G, Greenberg BH, et al. Serelaxin, recombinant human relaxin-2, for treatment of acute heart failure (RELAX-AHF): a randomised, placebo-controlled trial. Lancet 2013;381:29-39.
18) Halls ML, Bathgate RA, Summers RJ. Relaxin family peptide receptors RXFP1 and RXFP2 modulate cAMP signaling by distinct mechanisms. Mol Pharmacol 2006;70:214-226.
19) **Bathgate RA**, **Halls ML**, van der Westhuizen ET, Callander GE, Kocan M, Summers RJ. Relaxin family peptides and their receptors. Physiol Rev 2013;93:405-480.
20) Collino M, Rogazzo M, Pini A, Benetti E, Rosa AC, Chiazza F, et al. Acute treatment with relaxin protects the kidney against ischaemia/reperfusion injury. J Cell Mol Med 2013;17:1494-1505.
21) Alexiou K, Matschke K, Westphal A, Stangl K, Dschietzig T. Relaxin is a candidate drug for lung preservation: relaxin-induced protection of rat lungs from ischemia-reperfusion injury. J Heart Lung Transplant 2010;29:454-460.
22) Boehnert MU, Hilbig H, Armbruster FP. Relaxin as an additional protective substance in preserving and reperfusion solution for liver transplantation, shown in a model of isolated perfused rat liver. Ann N Y Acad Sci 2005;1041:434-440.
23) Dschietzig T, Bartsch C, Stangl V, Baumann G, Stangl K. Identification of the pregnancy hormone relaxin as glucocorticoid receptor agonist. FASEB J 2004;18:1536-1538.
24) Shen XD, Gao F, Ke B, Zhai Y, Lassman CR, Tsuchihashi S, et al. Inflammatory responses in a new mouse model of prolonged hepatic cold ischemia followed by arterialized orthotopic liver transplantation. Liver Transpl 2005;11:1273-1281.
25) Suzuki S, Toledo-Pereyra LH, Rodriguez FJ, Cejalvo D. Neutrophil infiltration as an important factor in liver ischemia and reperfusion injury. Modulating effects of FK506 and cyclosporine. Transplantation 1993;55:1265-1272.
26) Tamaki N, Hatano E, Taura K, Tada M, Kodama Y, Nitta T, et al. CHOP deficiency attenuates cholestasis-induced liver fibrosis by reduction of hepatocyte injury. Am J Physiol Gastrointest Liver Physiol 2008;294:G498-G505.
27) Berberat PO, Friess H, Schmied B, Kremer M, Gragert S, Flechtenmacher C, et al. Differentially expressed genes in post-perfusion biopsies predict early graft dysfunction after liver transplantation. Transplantation 2006;82:699-704.
28) Ali JM, Davies SE, Brais RJ, Randle LV, Klinck JR, Allison ME, et al. Analysis of ischemia/reperfusion injury in time-zero

biopsies predicts liver allograft outcomes. Liver Transpl 2015;21: 487-499.
29) **Xue Q**, **Dasgupta C**, Chen M, Zhang L. Foetal hypoxia increases cardiac AT(2)R expression and subsequent vulnerability to adult ischaemic injury. Cardiovasc Res 2011;89:300-308.
30) **Yue S**, **Zhu J**, Zhang M, Li C, Zhou X, Zhou M, et al. The myeloid heat shock transcription factor 1/beta-catenin axis regulates NLR family, pyrin domain-containing 3 inflammasome activation in mouse liver ischemia/reperfusion injury. HEPATOLOGY 2016;64:1683-1698.
31) Desborough JP. The stress response to trauma and surgery. Br J Anaesth 2000;85:109-117.
32) Raubenheimer PJ, Young EA, Andrew R, Seckl JR. The role of corticosterone in human hypothalamic-pituitary-adrenal axis feedback. Clin Endocrinol (Oxf) 2006;65:22-26.
33) Fallowfield JA, Hayden AL, Snowdon VK, Aucott RL, Stutchfield BM, Mole DJ, et al. Relaxin modulates human and rat hepatic myofibroblast function and ameliorates portal hypertension in vivo. HEPATOLOGY 2014;59:1492-1504.
34) McGuane JT, Debrah JE, Sautina L, Jarajapu YP, Novak J, Rubin JP, et al. Relaxin induces rapid dilation of rodent small renal and human subcutaneous arteries via PI3 kinase and nitric oxide. Endocrinology 2011;152:2786-2796.
35) Bani D, Nistri S, Quattrone S, Bigazzi M, Bani Sacchi T. The vasorelaxant hormone relaxin induces changes in liver sinusoid microcirculation: a morphologic study in the rat. J Endocrinol 2001;171:541-549.
36) Dschietzig T, Bartsch C, Baumann G, Stangl K. RXFP1-inactive relaxin activates human glucocorticoid receptor: further investigations into the relaxin-GR pathway. Regul Pept 2009; 154:77-84.
37) **Mueller KM**, **Kornfeld JW**, Friedbichler K, Blaas L, Egger G, Esterbauer H, et al. Impairment of hepatic growth hormone and glucocorticoid receptor signaling causes steatosis and hepatocellular carcinoma in mice. HEPATOLOGY 2011;54:1398-1409.
38) Tsung A, Klune JR, Zhang X, Jeyabalan G, Cao Z, Peng X, et al. HMGB1 release induced by liver ischemia involves Toll-like receptor 4 dependent reactive oxygen species production and calcium-mediated signaling. J Exp Med 2007;204:2913-2923.
39) **Ilmakunnas M**, **Tukiainen EM**, Rouhiainen A, Rauvala H, Arola J, Nordin A, et al. High mobility group box 1 protein as a marker of hepatocellular injury in human liver transplantation. Liver Transpl 2008;14:1517-1525.
40) Kadono K, Uchida Y, Hirao H, Miyauchi T, Watanabe T, Iida T, et al. Thrombomodulin attenuates inflammatory damage due to liver ischemia and reperfusion injury in mice in Toll-like receptor 4-dependent manner. Am J Transplant 2017;17:69-80.
41) Figueiredo KA, Mui AL, Nelson CC, Cox ME. Relaxin stimulates leukocyte adhesion and migration through a relaxin receptor LGR7-dependent mechanism. J Biol Chem 2006;281:3030-3039.
42) Horton JS, Yamamoto SY, Bryant-Greenwood GD. Relaxin modulates proinflammatory cytokine secretion from human decidual macrophages. Biol Reprod 2011;85:788-797.
43) Gujral JS, Bucci TJ, Farhood A, Jaeschke H. Mechanism of cell death during warm hepatic ischemia-reperfusion in rats: apoptosis or necrosis? HEPATOLOGY 2001;33:397-405.
44) Yang M, Antoine DJ, Weemhoff JL, Jenkins RE, Farhood A, Park BK, Jaeschke H. Biomarkers distinguish apoptotic and necrotic cell death during hepatic ischemia/reperfusion injury in mice. Liver Transpl 2014;20:1372-1382.
45) Lemaire C, Andreau K, Souvannavong V, Adam A. Inhibition of caspase activity induces a switch from apoptosis to necrosis. FEBS Lett 1998;425:266-270.
46) Galluzzi L, Kroemer G. Necroptosis: a specialized pathway of programmed necrosis. Cell 2008;135:1161-1163.
47) Vandenabeele P, Vanden Berghe T, Festjens N. Caspase inhibitors promote alternative cell death pathways. Sci STKE 2006; 2006:pe44.
48) Baskin-Bey ES, Washburn K, Feng S, Oltersdorf T, Shapiro D, Huyghe M, et al. Clinical trial of the pan-caspase inhibitor, IDN-6556, in human liver preservation injury. Am J Transplant 2007;7:218-225.
49) **Yue S**, **Zhou H**, Wang X, Busuttil RW, Kupiec-Weglinski JW, Zhai Y. Prolonged ischemia triggers necrotic depletion of tissue-resident macrophages to facilitate inflammatory immune activation in liver ischemia reperfusion injury. J Immunol 2017;198: 3588-3595.

Author names in bold designate shared co-first authorship.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.29787/suppinfo.