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Tissue Inhibitor of Metalloproteinase 3 Deficiency Disrupts the Hepatocyte E-Cadherin/β-Catenin Complex and Induces Cell Death in Liver Ischemia/Reperfusion Injury

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Tissue inhibitor of metalloproteinase (TIMP) 3 is a naturally occurring inhibitor of a broad range of proteases, with key roles in extracellular matrix turnover and in the pathogenesis of various diseases. In this study, we investigated the response of mice lacking TIMP3 (TIMP3–/–) to hepatic ischemia/reperfusion injury (IRI). We report here that TIMP3–/– mice showed an enhanced inflammatory response, exacerbated organ damage, and further impaired liver function after IRI when compared with their wild-type littermates. Loss of TIMP3 led to the cleavage and shedding of E-cadherin during hepatic IRI; the full-length 120-kDa E-cadherin and the ratio of 38-kDa C-terminal fragment/120-kDa E-cadherin were decreased and increased, respectively, in TIMP3–/– livers after IRI. Moreover, GI254023X, a potent inhibitor of a disintegrin and metalloprotease (ADAM) 10, was capable of partially rescuing the expression of E-cadherin in the TIMP3-null hepatocytes. The proteolysis of E-cadherin in the TIMP3–/– livers was also linked to the loss of β-catenin from the hepatocyte membranes and to an increased susceptibility to apoptosis after liver IRI. In a similar fashion, depression of the E-cadherin/β-catenin complex mediated by TIMP3 deletion and knockdown of β-catenin by small interfering RNA were both capable of inducing caspase activation in isolated hepatocytes subjected to H_2O_2 oxidative stress. Hence, these results support a protective role for TIMP3 expression in sheltering the hepatocyte E-cadherin/β-catenin complex from proteolytic processing and inhibiting apoptosis after hepatic IRI.

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Hepatic ischemia/reperfusion injury (IRI) is a common feature in various clinical settings that span from surgical procedures to liver pathologies, where the blood supply to the liver is temporarily interrupted.[1,2] Despite improvements in surgical techniques and perioperative care, IRI remains a major clinical problem in orthotopic liver transplantation.[3,4] Indeed, hepatic IRI is largely responsible for the morbidity associated with liver transplantation and for the increased risk of acute and chronic organ dysfunctions.[5,6] Besides, the severe organ shortage for transplantation has led to the use of extended criteria donor (ECD) livers, which have a higher degree of susceptibility to hepatic IRI.[7,8] Hence, a better understanding of the molecular pathophysiology of liver ischemic damage and the subsequent development of improved protective strategies against hepatic IRI are of prime importance.

Extracellular matrix (ECM) turnover is essential in several biological processes, including cell migration, tissue repair, and remodeling.[2] ECM turnover is

Abbreviations: ADAM, a disintegrin and metalloprotease; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motif; ALT, alanine aminotransferase; AST, aspartate aminotransferase; B, bile duct; cDNA, complementary DNA; CTF, C-terminal fragment; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; E-cadherin, epithelial cadherin; ECM, extracellular matrix; H & E, hematoxylin-eosin; HPF, high-power field; IgG, immunoglobulin G; IL, interleukin; IRI, ischemia/reperfusion injury; Ly-6G, lantigen 6 complex locus G6D; Mac-1, macrophage antigen 1; MMP, matrix metalloproteinase; mRNA, messenger RNA; NS, nonsilencing; P, portal vein; SD, standard deviation; siRNA, small interfering RNA; TIMP, tissue inhibitor of metalloproteinase; TNF-α, tumor necrosis factor α; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling; WT, wild-type.

largely modulated by the interactions between matrix metalloproteinases (MMPs) and their endogenous tissue inhibitors of metalloproteinases (TIMPs).[9] MMPs are zinc-dependent endopeptidases that cleave ECM proteins and non-ECM substrates, such as cytokines and chemokines, and deregulation of their activity often results in major tissue damage and impaired organ functions.[2,10] There is growing evidence that disruptions in MMP-TIMP balances underlie the pathogenesis of various diseases.[11-14] There are 4 identified TIMPs (TIMP1 through TIMP4) that form tight 1:1 molar stoichiometric complexes with MMPs but that differ in tissue-specific expression and MMP inhibitory profiles.[12,15,16] Unique among TIMPs, TIMP3 has poor aqueous solubility, binds tightly to the ECM, and has the broadest inhibitory profile.[17,18] TIMP3 is not only a natural inhibitor of MMPs, but it is also capable of inhibiting members of the related adamalysins (a disintegrin and metalloprotease [ADAM]) and adamalysins with thrombospondin motifs (a disintegrin and metalloproteinase with thrombospondin motif [ADAMTS]), including the tumor necrosis factor α (TNF-α)–converting enzyme (also called ADAM17).[19] In humans, loss of TIMP3 has been related to various pathological conditions.[20] Several studies have suggested a protective role for TIMP3 expression in disorders like inflammatory vascular disease, osteoarthritis, obesity, and various cancers.[21-24] In general, the functional significance of MMPs and TIMPs is determined by the local environment, and consequently their functions can be cell type–specific and/or disease-specific.[10] In this study, we investigated the response of TIMP3 deficient mice (TIMP3–/–), which have normal hepatic structure and function, to hepatic IRI. Our data documents that the loss of TIMP3 is detrimental in hepatic IRI and that TIMP3 shelters the cadherin/β-catenin complex from proteolytic processing in hepatocytes.

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Correction statement: Correction added on December 20, 2019, after initial online publication: In the bar graph in Fig. 3C, "Act." was spelled out to read "Active."

Materials and Methods

MICE AND MODEL OF HEPATIC IRI

Male TIMP3–/– knockout (TIMP3–/–) mice in the C57BL/6 background and matched TIMP3 wild-type (WT; TIMP3+/+) littermates were obtained from the Jackson Laboratory (Sacramento, CA). Mice were housed in the University of California, Los Angeles animal facility under specific pathogen-free conditions. Hepatic IRI was performed as described.[25] Briefly, arterial and portal venous blood supplies were interrupted to the cephalad lobes of the liver for 60 minutes using an atraumatic clip. After partial hepatic warm ischemia, the clip was removed, initiating hepatic reperfusion. Mice were killed at 6 and 24 hours after reperfusion. Sham mice underwent laparotomy without vascular occlusion. All animals received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health.

ASSESSMENT OF LIVER DAMAGE

Serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) levels were measured using a commercially available kit (Teco Diagnostics, Anaheim, CA), following the manufacturer's instructions. Liver specimens were fixed with a 10% formalin-buffered solution, embedded in paraffin, and processed for hematoxylin-eosin (H & E) staining.

RNA EXTRACTION AND REVERSE-TRANSCRIPTASE POLYMERASE CHAIN REACTION

RNA was extracted from snap-frozen livers and isolated hepatocytes with Trizol (Life Technologies, Carlsbad, CA) as described.[26] Reverse transcription was performed using 5 μg of total RNA in a first-strand complementary DNA (cDNA) synthesis reaction with

SuperScript III RNaseH Reverse Transcriptase (Life Technologies) as recommended by the manufacturer. The cDNA product was amplified by polymerase chain reaction using primers specific for each target cDNA.

IMMUNOHISTOCHEMISTRY

Immunostaining was performed in cryostat sections as previously described.[27,28] Anti-macrophage antigen 1 (Mac-1) (M1/70), anti-lymphocyte antigen 6 complex locus G6D (Ly6G) (1A8; BD Biosciences, San Jose, CA), anti-E-cadherin, and anti-β-catenin (Vanderbilt University, Nashville, TN) primary antibodies were used at optimal dilutions. Bound primary antibodies were detected using biotinylated secondary antibodies and streptavidin peroxidase–conjugated complexes (Vector Laboratories, Burlingame, CA). Immunofluorescence staining was performed using rabbit polyclonal anti-TIMP3 (Abcam, Cambridge, MA) and anti-β-catenin (D13A1; Cell Signaling, Danvers, MA) primary antibodies and an anti-rabbit immunoglobulin G (IgG; Alexa Fluor 594; Molecular Probes, Eugene, OR) secondary antibody. AlexaFluor 488 phalloidin (Invitrogen, Carlsbad, CA) and Vecta-shield mounting media with 4´,6-diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA) were used for F-actin and nuclear staining, respectively. Sections were blindly evaluated by counting 10 high-power fields (HPFs)/section in triplicate for leukocyte counting. Immunofluorescence staining of β-catenin was performed using a rabbit and a secondary anti-rabbit IgG antibody (Alexa Fluor 594; Molecular Probes).

WESTERN BLOT ANALYSIS

Western blots were performed as described.[28] Proteins (30 μg/sample) in sodium dodecyl sulfate–loading buffer were electrophoresed through sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were incubated with specific antibodies against ADAM10 (B-3; Santa Cruz Biotechnology, Santa Cruz, CA), E-cadherin (4A2C6), and β-catenin (1A1; Vanderbilt University, Nashville, TN), E-cadherin (24E10), cleaved caspase 3 (Asp175), cleaved caspase 6 (Asp162), caspase 8 (which detects the active p10 subunit-8D35G2), and XIAP (Cell Signaling, Danvers, MA), and hypoxia inducible factor 1 alpha subunit (0737R; One World Lab, San Diego, CA). After development, membranes were stripped and reblotted with anti-actin antibody (Santa Cruz Biotechnology).

TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE–MEDIATED DEOXYURIDINE TRIPHOSPHATE NICK-END LABELING STAINING

Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining was conducted using an In Situ Cell Death Detection Kit (Sigma-Aldrich, St. Louis, MO) following the manufacturer's instructions. Nuclei were stained with DAPI (H-1200; Vector Laboratories).

ISOLATION AND CULTURE OF HEPATOCYTES

Primary hepatocytes were isolated from TIMP3+/+ and TIMP3−/− mice and cultured as described.[29] Briefly, anesthetized mice were subject to a midline laparotomy and cannulation of the inferior vena cava for liver perfusion with ethylene diamine tetraacetic acid chelating and collagenase perfusion buffers. Hepatocytes were separated from nonparenchymal cells by successive low-speed centrifugation steps. Isolated mouse hepatocytes were cultured overnight in Williams' E medium with 10% fetal bovine serum on 12-well collagen-coated plates at 37°C with 5% CO_2. Oxidative stress was achieved by treating hepatocytes (1×10^5/well) with 2 mM H_2O_2 in serum-free medium for 5 hours. When applicable, primary hepatocytes were preincubated with different doses of ADAM10 inhibitor (GI254023X; Sigma-Aldrich) or vehicle for 60 minutes prior to oxidative stress.

SMALL INTERFERING RNA PREPARATION AND TRANSFECTION

Small interfering RNAs (siRNAs) directed against murine β-catenin were designed as previously described[30] and supplied by Qiagen (Chatsworth, CA). The siRNA sequences targeting murine β-catenin were 5′-AGCUGAUAUUGAUGGACAG-3′ (sense) and 5′-CUGUCCAUCAAUAUCAGCU-3′ (antisense). The nonsilencing (NS) siRNA, 5′-UUCUCCGAACGUGUCACGU-3′ (sense) and 5′-ACGUGACACGUUCGGAGAA-3′ (antisense) served as negative controls. Primary hepatocytes were transfected for 24 hours with 100 nM of siRNA using lipofectamine 2000 reagent (Invitrogen) prior to oxidative stress.

DATA ANALYSIS

Results are expressed as mean ± standard deviation (SD). Statistical comparisons between groups of normally distributed data were performed with the Student *t* test using the statistical package SPSS (SPSS Inc., Chicago, IL). *P* values <0.05 were considered statistically significant.

Results

TOTAL LOSS OF TIMP3 EXPRESSION RESULTED IN EXACERBATED LIVER DAMAGE AFTER IRI

TIMP3 was abundantly detected in hepatocytes of naïve WT (TIMP-3+/+) livers, and its expression was found decreased in damaged WT livers after IRI (Supporting Fig. 1). To test the significance of TIMP3 expression in liver IRI, our experiments included TIMP3-deficient mice and WT control littermates. There were no detectable differences in histology and liver enzyme (AST and ALT) serum levels between naïve TIMP3−/− and TIMP3+/+ mice as well as sham-operated TIMP3−/− and TIMP3+/+ mice; naïve and sham TIMP3−/− and TIMP3+/+ mice all presented good liver histological preservation and serum aminotransferase levels within the normal range (Fig. 1). Nevertheless, TIMP3 deficiency was associated with exacerbated sinusoidal congestion, necrosis, and lobular architecture disruption at 6 and 24 hours after reperfusion, when compared with the already significantly damaged TIMP3+/+ control livers after IRI (Fig. 1A). Moreover, the serum levels of AST and ALT were both significantly ($P < 0.05$) increased in TIMP3−/− mice at 6 and 24 hours after IRI relative to TIMP3+/+ counterparts (Fig. 1A). Thus, these data suggest that the loss of TIMP3 is associated with aggravated hepatic IRI.

TIMP3 DEFICIENCY FAVORED LEUKOCYTE ACCUMULATION AND ACTIVATION AFTER HEPATIC IRI

Leukocyte infiltration was scarcely observed in naïve TIMP3−/− and TIMP3+/+ livers (Fig. 2A,B). Infiltration of Ly-6G neutrophils was significantly increased in the portal areas of TIMP3−/− livers at 6 ($P < 0.05$) and 24 ($P < 0.05$) hours after hepatic IRI compared with controls (Fig. 2A). In addition, Mac-1–positive leukocytes were also detected in higher numbers in the TIMP3−/− livers at 6 hours ($P < 0.05$) after reperfusion (Fig. 2B). The increase in leukocyte recruitment correlated with upregulated levels of proinflammatory interleukin (IL) 1β ($P < 0.05$), IL6 ($P < 0.05$), and TNF-α ($P < 0.05$) in TIMP3−/− livers at 6 hours after reperfusion (Fig. 2C).

TIMP3 DEFICIENCY RESULTED IN INCREASED CLEAVAGE AND SHEDDING OF E-CADHERIN DURING LIVER IRI

Epithelial cadherin (E-cadherin) is a key molecule involved in the maintenance of tissue integrity.[31] E-cadherin is normally expressed in periportal hepatocytes and virtually undetectable in the regions surrounding the central veins of naïve adult livers.[32] In our settings, there were no significant differences on E-cadherin expression between TIMP3+/+ and TIMP3−/− naïve livers; naïve TIMP3+/+ and TIMP3−/− stained similarly strongly for E-cadherin in the periportal areas and expressed comparable levels of the mature full-length E-cadherin (120 kDa; Fig. 3A,B). Conversely, although E-cadherin staining was still readily detectable in the periportal hepatocytes of TIMP3+/+ livers after IRI, particularly at 6 hours, it was largely absent from the periportal areas of TIMP3−/− livers after reperfusion (Fig. 3A). When compared with TIMP3+/+ controls, the full-length 120-kDa E-cadherin was significantly depressed in TIMP3−/− livers at both 6 ($P < 0.05$) and 24 ($P < 0.05$) hours after IRI (Fig. 3B). Proteolytic cleavage of the extracellular domain of the mature 120-kDa E-cadherin protein generates an intracellular 38-kDa C-terminal fragment (CTF).[33] Moreover, the ratio 38-kDa CTF/120-kDa E-cadherin was increased by approximately 2-fold ($P < 0.05$) in TIMP3−/− livers after IRI compared with TIMP3+/+ livers (Fig. 3B). A previous study has implicated ADAM10 in the ectodomain shedding of E-cadherin in fibroblasts and keratinocytes.[31] In our settings, loss of the mature 120-kDa E-cadherin protein in the TIMP3−/− livers

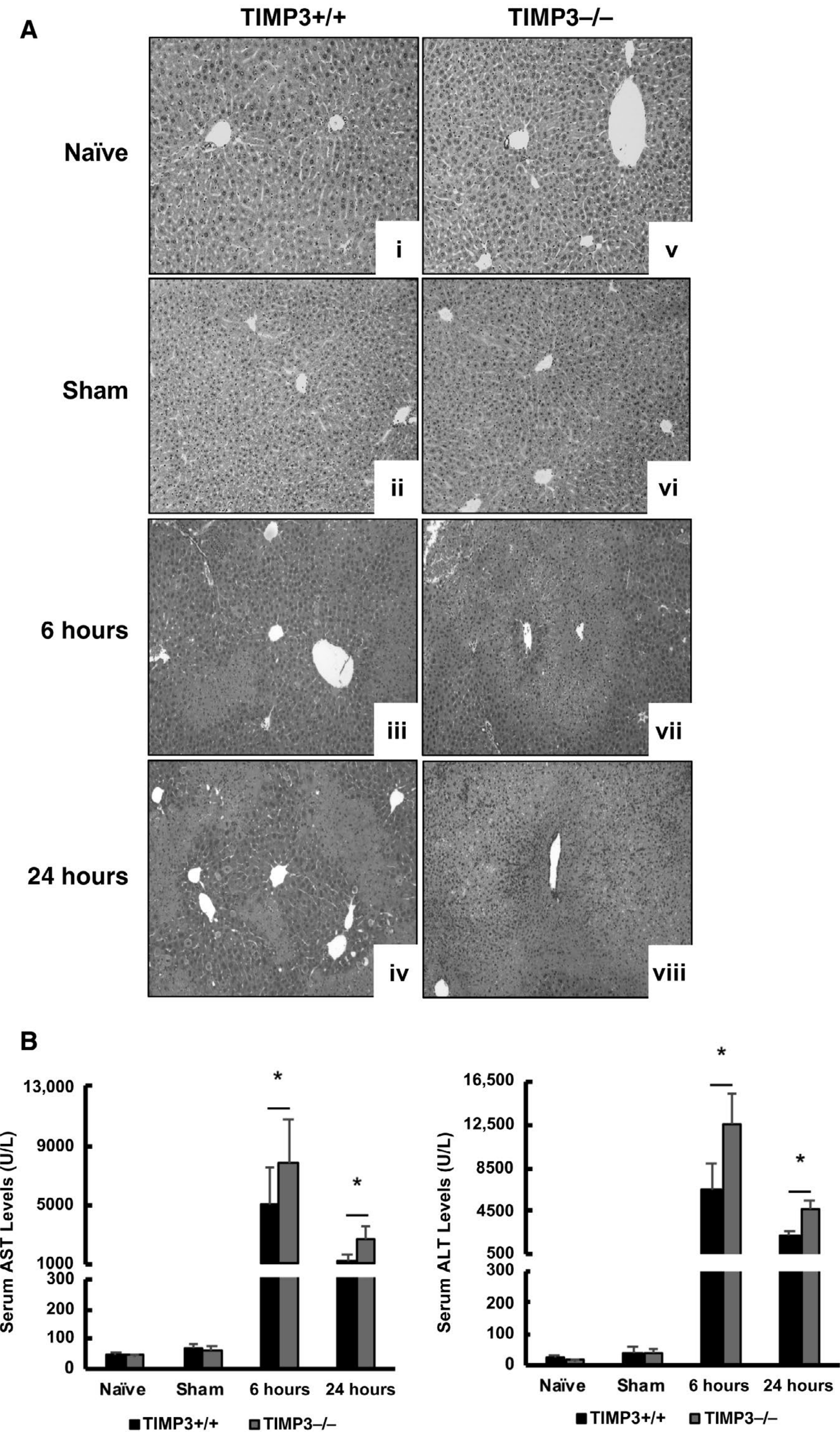

FIG. 1. Histological preservation and serum AST and ALT levels in TIMP3+/+ and TIMP3−/− mice. (A) Representative H & E staining of TIMP3+/+ (i-iv) and TIMP3−/− (v-viii) naïve (i and v) and sham (ii and vi) livers, and livers after 6 hours (iii and vii) and 24 hours (iv and viii) of hepatic IRI. TIMP3 deficiency was associated with increased cell death and lobular architecture disruption when compared with TIMP3 expressing livers. (B) Serum AST and ALT levels measured in naïve mice, sham mice, and in mice subjected to hepatic IRI. Serum AST and ALT levels measured in blood samples retrieved after 6 hours and 24 hours of IRI were significantly increased in TIMP3−/− mice (gray bars) relative to TIMP3+/+ control mice (black bars; n = 4-6/group; magnification ×10; *$P < 0.05$).

FIG. 2. Leukocyte infiltration and cytokine expression were increased in TIMP3−/− livers after IRI. (A) Ly-6G+ and (B) Mac-1+ leukocyte infiltration was scarcely noticeable in naïve livers (i and ii) and amplified in livers after 6 hours (iii and iv) and 24 hours (v and vi) after IRI in TIMP3−/− mice (ii, iv, and vi; gray bars) when compared with their respective WT TIMP3+/+ controls (i, iii, and v; black bars). (C) The expression of proinflammatory cytokines IL1β, IL6, and TNF-α was also significantly enhanced in TIMP3−/− livers (gray bars) at 6 hours after IRI (n = 4-6/group; magnification ×40; $^*P < 0.05$).

was associated with significantly increased levels of the ADAM10 active form after IRI (Fig. 3C). Taken together, these observations suggest that TIMP3 has a role in hampering the proteolytic processing of ADAM10 and in preserving E-cadherin expression in hepatocytes.

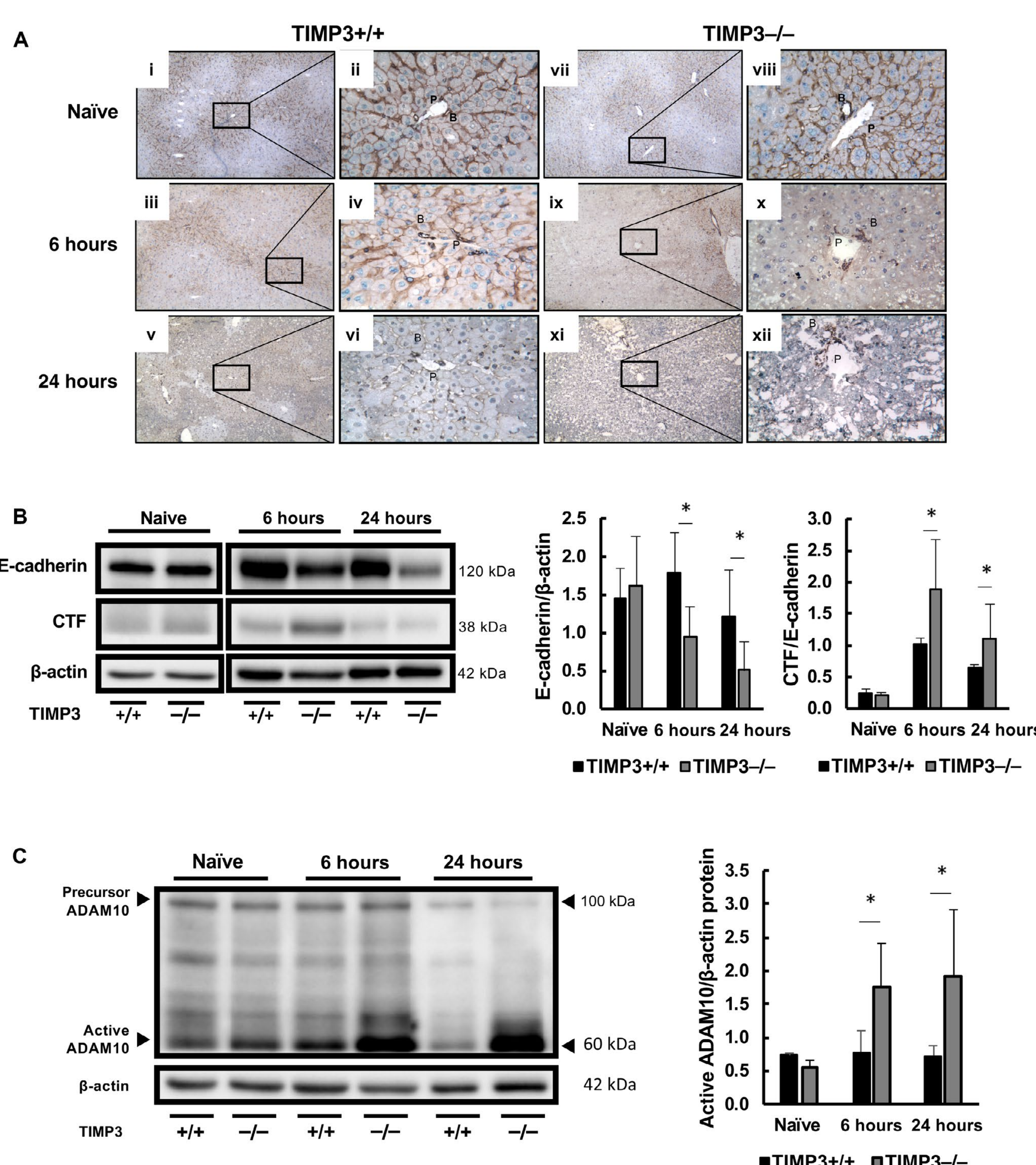

FIG. 3. Total loss of TIMP3 resulted in increased cleavage and shedding of E-cadherin and enhanced active ADAM10 levels in livers after IRI. (A) E-cadherin staining in TIMP3+/+ (i-vi) and TIMP3–/– (vii-xii) livers. TIMP3+/+ and TIMP3–/– naïve livers (i, ii, vii, and viii) stained similarly strongly for E-cadherin in the periportal areas. E-cadherin staining was largely absent from the TIMP3–/– periportal areas after 6 hours (iii, iv, ix, and x) and 24 hours (v, vi, xi, and xii) of liver IRI. (B) When compared with TIMP3+/+ controls (black bars), the full-length 120-kDa E-cadherin and the 38-kDa CTF/120-kDa E-cadherin ratios were decreased and increased, respectively, in TIMP3–/– livers (gray bars) after IRI. (C) The active form of ADAM10 (~60 kDa) was markedly amplified in TIMP3–/– livers (gray bars) compared with respective controls (black bars) after IRI (n = 4-6/group; i, iii, v, and vii: magnification ×10; ii, iv, vi, and viii: magnification ×40; *$P < 0.05$).

E-CADHERIN PROTEIN SHEDDING IS INCREASED IN CULTURED TIMP3–/– HEPATOCYTES AND PARTIALLY DISRUPTED BY ADAM10 INHIBITION

We observed a robust depression of the full-length 120-kDa E-cadherin protein expression in TIMP3–/– hepatocytes cultured overnight when compared with control WT hepatocytes. This led us to perform a timeline study and to evaluate the dynamics of E-cadherin messenger RNA (mRNA) and protein expressions in both TIMP3–/– and TIMP3+/+ hepatocytes during the first 24 hours after cell isolation. There were no significant differences in E-cadherin mRNA expression following hepatocyte isolation from TIMP3–/– and TIMP3+/+ livers; TIMP3–/– and TIMP3+/+ hepatocytes expressed comparably high levels of E-cadherin mRNA at various time points after isolation (Fig. 4A). On the other hand, E-cadherin protein was virtually depleted from both TIMP3–/– and TIMP3+/+ hepatocytes during the isolation process (due to enzymatic digestion) and gradually restored in these cells during the 24 hours of culture. However, when compared with TIMP3+/+ hepatocytes, the restoration of E-cadherin protein was profoundly impaired in TIMP3–/– hepatocytes at both 12 and 24 hours after cell isolation, suggesting protein degradation by proteases (Fig. 4B). We next tested whether GI254023X, a potent and selective inhibitor of ADAM10, was capable of enhancing E-cadherin membrane stability. As shown in Fig. 4C, GI254023X-mediated inhibition of ADAM10 significantly increased the levels of the mature 120-kDa E-cadherin protein in TIMP3–/– hepatocytes after 24 hours of culture. However, when compared with TIMP3+/+ hepatocytes, the restitution of the 120-kDa E-cadherin protein in TIMP3–/– hepatocytes was only partial by ADAM10 inhibition (Fig. 4C). A similar observation was also made in isolated hepatocytes subjected to H_2O_2 oxidative stress; compared with TIMP3+/+ hepatocytes, the TIMP3–/– hepatocytes showed an increased 38-kDa CTF/120-kDa E-cadherin ratio, which was partially reduced by the addition of the ADAM10 inhibitor (Fig. 4D). Taken together, these results suggest that in addition to regulating ADAM10 activity, TIMP3 inhibits other proteases that are also responsible for E-cadherin shedding in hepatocytes.

TIMP3–/– DEFICIENCY WAS ASSOCIATED WITH LOSS OF β-CATENIN EXPRESSION AND EXACERBATED APOPTOSIS AFTER LIVER IRI

Under normal conditions, E-cadherin is mostly present as an E-cadherin/β-catenin complex at cell–cell junctions,[34] and E-cadherin proteolysis can lead to loss of β-catenin from the cell membrane.[35] We therefore evaluated the expression of β-catenin in the presence and absence of TIMP3. β-catenin expression was comparably detected in hepatocytes of naïve TIMP3+/+ and TIMP3–/– livers (Fig. 5A,B). Like E-cadherin, β-catenin staining was virtually absent from the hepatocyte membranes of TIMP3–/– livers (particularly in portal areas) after reperfusion, and it was noticeably better preserved in the TIMP3+/+ controls (Fig. 5A). Loss of TIMP3 resulted in a significantly depressed β-catenin protein at both 6 and 24 hours after liver IRI (Fig. 5B), which correlated with the increased proteolytic cleavage of E-cadherin observed in these mice (Fig. 3B). In addition, the loss of TIMP3 was also associated with an increased susceptibility to apoptosis after hepatic IRI. The expressions of active caspase 3, 6, and 8 were overall increased in TIMP3–/– livers after IRI (Fig. 6A). Moreover, TUNEL-positive hepatocytes were also markedly increased in TIMP3–/– livers after 6 hours of reperfusion, particularly in the almost β-catenin–negative tissue areas (Fig. 6B).

TIMP3 DEFICIENCY AND siRNA-MEDIATED β-CATENIN KNOCKDOWN RESULTED IN INCREASED HEPATOCYTE APOPTOSIS

To support a potential link between loss of β-catenin in TIMP3–/– hepatocytes and their susceptibility to apoptosis, we subjected both isolated TIMP3+/+ and TIMP3–/– hepatocytes to H_2O_2-mediated oxidative stress. Compared with TIMP3+/+ hepatocytes, both E-cadherin and β-catenin protein levels were significantly depleted in the cultured TIMP3–/– hepatocytes (Fig. 7A,B). Moreover, loss of the E-cadherin/β-catenin complex in the TIMP3–/– hepatocytes correlated with an increase in caspase 3 activity in these cells (Fig. 7C). We next treated TIMP3+/+ hepatocytes

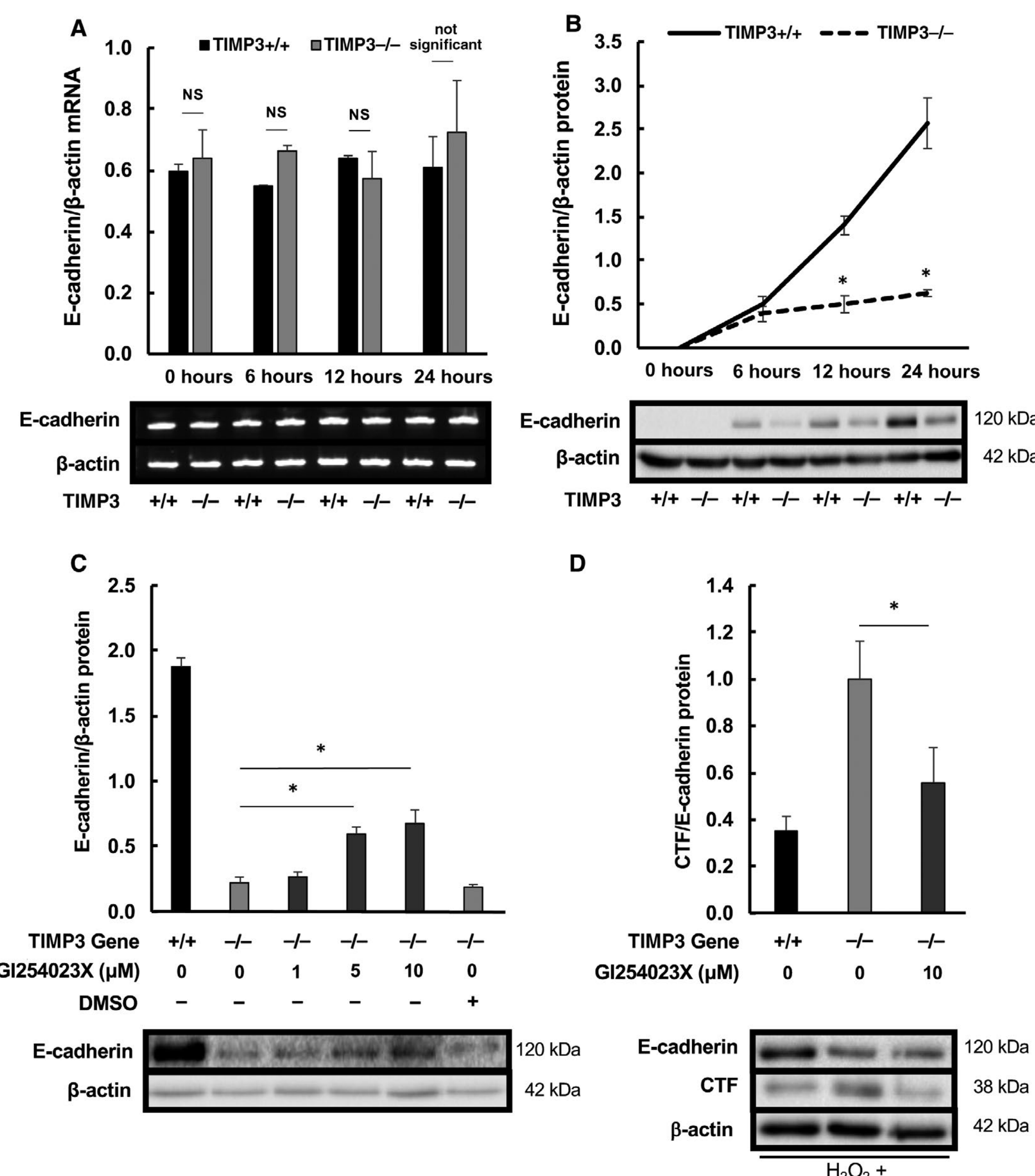

FIG. 4. E-cadherin protein expression was partially restored by GI254023X in isolated TIMP3–/– hepatocytes. (A) E-cadherin mRNA and (B) E-cadherin protein expressions in cultured TIMP3–/– and TIMP3+/+ hepatocytes during the first 24 hours after cell isolation. TIMP3–/– and TIMP3+/+ hepatocytes expressed comparably high levels of E-cadherin mRNA at various time points after isolation. Conversely, the restoration of E-cadherin protein was profoundly impaired in TIMP3–/– hepatocytes at both 12 hours and 24 hours after the isolation process compared with respective TIMP3+/+ controls. (C) The levels of the 120-kDa E-cadherin protein were partially restored by the presence of GI254023X, a selective ADAM10 inhibitor, in TIMP3–/– cultured hepatocytes. (D) Compared with TIMP3+/+ controls, TIMP3–/– hepatocytes subjected to H_2O_2-mediated oxidative stress showed a markedly increased 38-kDa CTF/120-kDa E-cadherin ratio; this ratio was significantly reduced by the addition of GI254023X (in vitro data are expressed as mean ± SD of 3 independent experiments; *$P < 0.05$).

with siRNA directed against β-catenin as a proof of concept for hepatocyte apoptosis induced by loss of β-catenin (Fig. 7D). Indeed, the knockdown of β-catenin in TIMP3+/+ hepatocytes resulted in a significant increase of both caspase 3 and caspase 6 activation (Fig. 7E-G), supporting the view that TIMP3 protects hepatocytes from undergoing apoptosis by sheltering the cadherin/β-catenin complex in these cells.

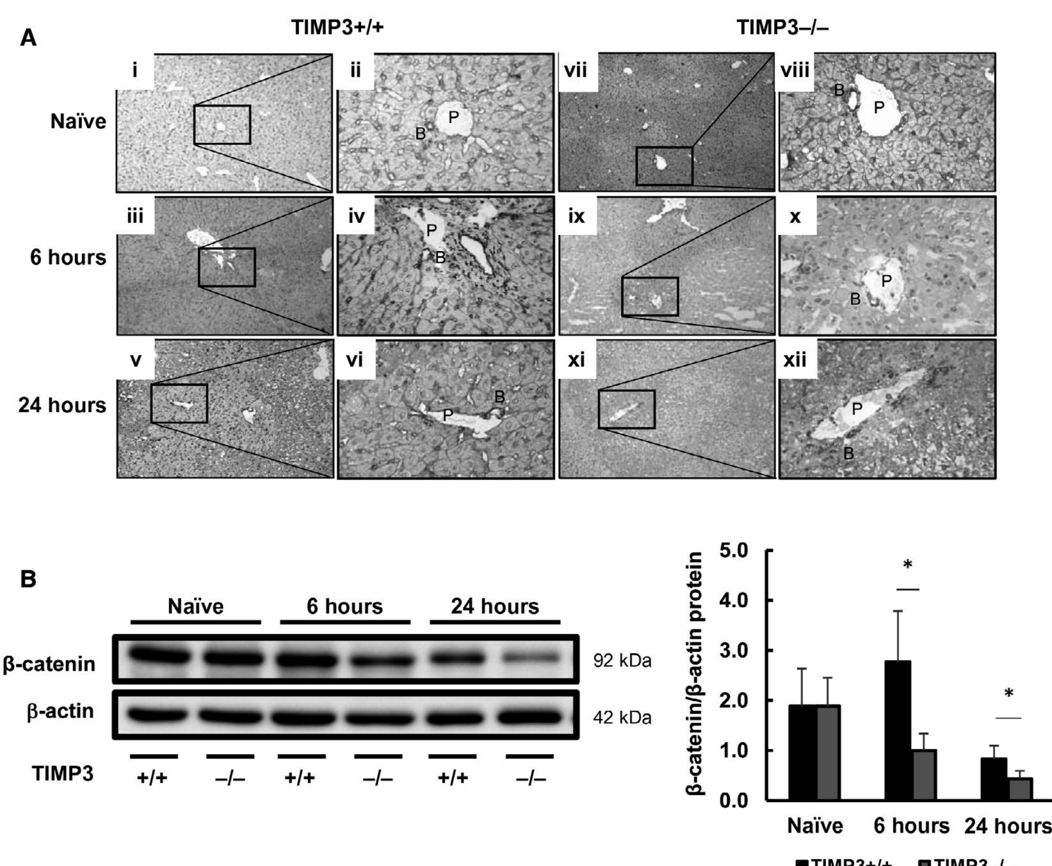

FIG. 5. Loss of β-catenin in the absence of TIMP3 in liver IRI. (A) Compared with TIMP3+/+ livers (i-vi), β-catenin staining was similarly detected in naïve livers (i, ii, vii, and viii) and profoundly reduced in the portal areas at 6 hours (i, iv, ix, and x) and 24 hours (v, vi, xi, and xii) after liver IRI in TIMP3–/– mice (vii-xii). (B) β-catenin protein expression was significantly depressed in TIMP3–/– livers (gray bars) compared with respective controls (black bars) after IRI (n = 4-6/group; i, iii, v, vii: magnification ×10; ii, iv, vi, viii: magnification ×40; *$P < 0.05$).

Discussion

A better understanding of the functions of TIMPs can aid in the development of novel therapeutic approaches against liver IRI and, therefore, improve the outcome of liver transplantation.[2] TIMP3 is a natural occurring inhibitor of a broad range of MMPs, ADAMs, and ADAMTSs, which are key mediators in the regulation of ECM integrity, cell migration, and inflammatory cytokine activation.[19] In this study, we examined the functional significance of TIMP3 expression in hepatic IRI. We found TIMP3 expression to be reduced in damaged WT livers after hepatic IRI, similarly to what has been previously reported for other pathologies, such as human and experimental heart failure and osteoarthritis.[36,37] Using TIMP3-deficient mice, we determined that total loss of TIMP3 expression leads to further exacerbated liver damage after IRI. In general, TIMP3–/– mice showed increased leukocyte accumulation and activation, more severe organ lesions, and further impairment of liver function after hepatic IRI. Our findings are in agreement with previous murine studies in which TIMP3 genetic ablation has been shown to induce dysregulated inflammation in a number of disease-like phenotypes, including lung injury, partial hepatectomy, kidney fibrosis, diabetes, atherosclerosis, and dilated cardiomyopathy.[21,37-41]

E-cadherin is one of the main components of adherens junctions with roles in maintaining liver homeostasis.[31,42] Indeed, it has been demonstrated that loss of E-cadherin induces sclerosing cholangitis and is the first step of tumor cell migration and metastasis.[42-44] Our analysis of mouse tissue samples showed

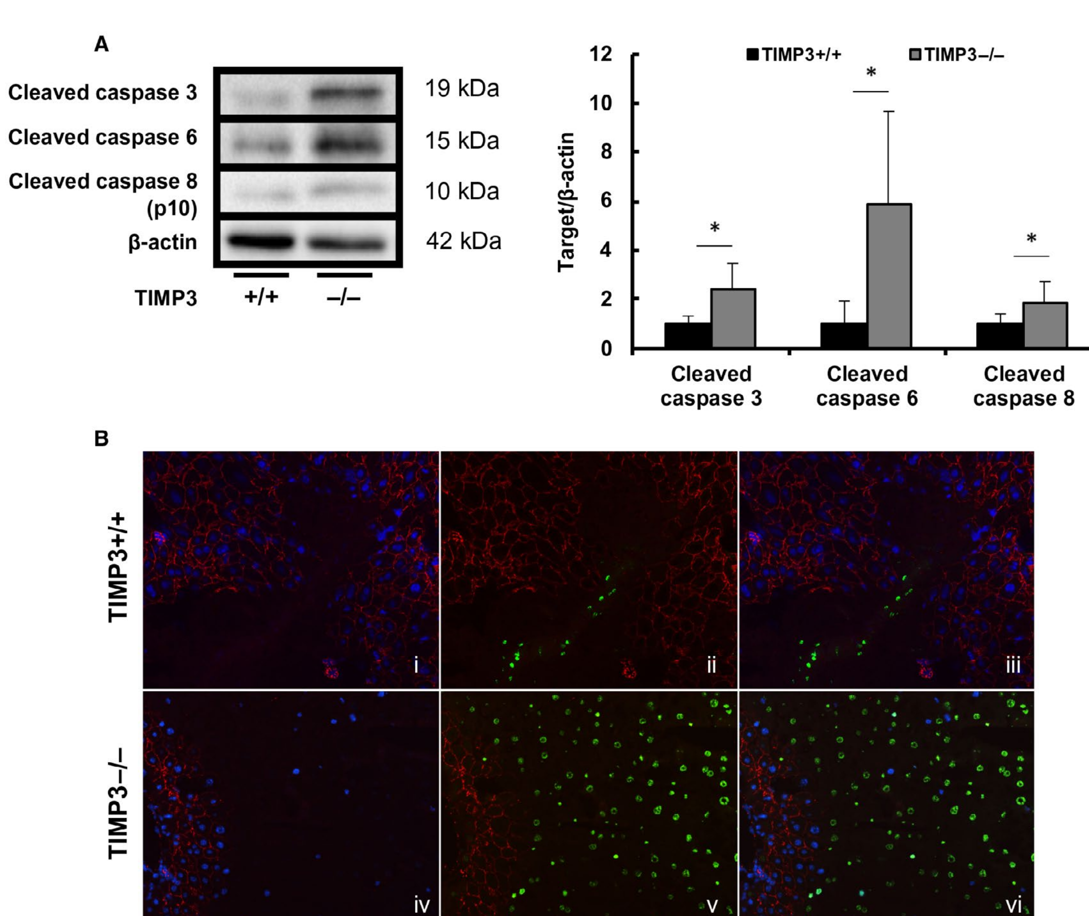

FIG. 6. Loss of TIMP3 resulted in exacerbated cell death after hepatic IRI. (A) Compared with TIMP3+/+ control livers (black bars), the expressions of active caspase 3, 6, and 8 were overall increased in TIMP3−/− livers (gray bars) at 6 hours after IRI. (B) Triple immunofluorescence of β-catenin in red (i-vi; Alexa Fluor 594), TUNEL in green (ii, iii, v, and vi), and nuclear stain in blue (i, iii, iv, and vi; DAPI) in TIMP3+/+ (i-iii) and TIMP3−/− (iv-vi) livers after reperfusion; areas of TIMP3−/− livers in which β-catenin staining was depressed exhibited a considerably more intense TUNEL-positive staining (n = 6/group; magnification ×20; $^*P < 0.05$).

that E-cadherin expression was largely absent from the periportal areas of TIMP3−/− livers after reperfusion. The proteolytic cleavage of the E-cadherin extracellular domain generates an intracellular 38-kDa CTF,[33] and the ratio CTF/E-cadherin was significantly increased in TIMP3−/− livers after IRI. ADAM10 has been implicated in the ectodomain shedding of E-cadherin in fibroblasts and keratinocytes[31]; however, the role of ADAM10 in hepatic IRI is virtually unknown. In our setting, the levels of the ADAM10 active form were particularly higher in the TIMP3−/− livers after IRI, suggesting a potential role for TIMP3 in hampering the proteolytic processing of ADAM10. GI254023X, a selective ADAM10 inhibitor,[45] was able to partially increase the E-cadherin hepatocyte membrane stability and reduce the CTF/E-cadherin ratio in isolated TIMP3−/− hepatocytes subjected to oxidative stress. The latter could perhaps be explained by the possibility of GI254023X being a partial antagonist; however, it is important to stress that TIMP3 has a broad inhibitory range, and there are several other proteases capable of cleaving E-cadherin,[33] which can potentially contribute to the observed results in hepatic IRI. In this regard, in addition to the known 38-kDa E-cadherin CTF, we have detected other E-cadherin fragments and enhanced MMP9 activity in TIMP3-null livers after reperfusion (data not shown). MMP9 has been associated with the loss of E-cadherin in ovarian carcinoma cells,[46] and it is expressed by infiltration leukocytes in damaged livers.[26] Thus, our data suggest that TIMP3 regulates E-cadherin shedding in hepatocytes by affecting the activity of proteolytic enzymes

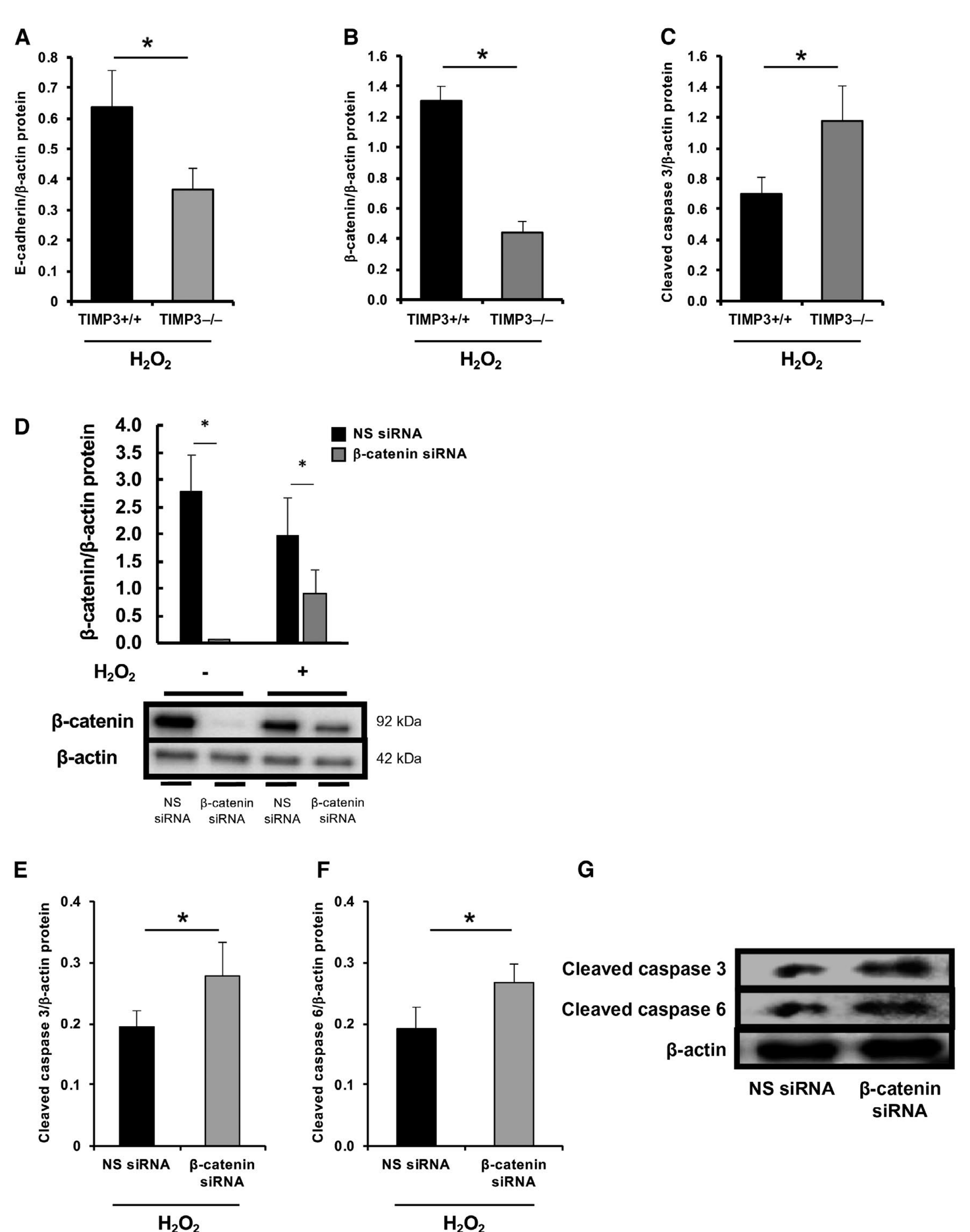

FIG. 7. Caspase activity was enhanced in both TIMP3–/– and β-catenin siRNA-treated TIMP3+/+ hepatocytes. Compared with control TIMP3+/+ hepatocytes (black bars), (A) E-cadherin and (B) β-catenin expressions were depressed, and (C) active caspase 3 expression was increased in TIMP3–/– hepatocytes (gray bars) subjected to H_2O_2-mediated oxidative stress. (D) siRNAs directed against β-catenin markedly depressed its expression in TIMP3+/+ hepatocytes before and after oxidative stress. (E and G) Active caspase 3 and (F and G) active caspase 6 were significantly increased in TIMP3+/+ hepatocytes treated with siRNAs against β-catenin (gray bars) and subjected to H_2O_2-mediated oxidative stress; the NS siRNA-treated TIMP3+/+ hepatocytes are shown in black bars (in vitro data are expressed as mean ± SD of 3 independent experiments; *$P < 0.05$).

capable of degrading E-cadherin, one of them being ADAM10.

Under normal conditions, E-cadherin is mostly present as an E-cadherin/β-catenin complex at the cell–cell junctions,[34] and the proteolysis of E-cadherin leads to the loss of β-catenin from the cell membrane.[35] In our setting, TIMP3 deficiency resulted also in the depression of β-catenin levels after liver IRI. Moreover, the loss or dissociation of the E-cadherin/β-catenin complex, in the absence of TIMP3, correlated with an increased susceptibility to apoptosis in isolated hepatocytes, and in livers after hepatic IRI. TIMP3 expression has been linked to both induction and reduction of apoptosis in distinct types of cells.[17,47,48] In our settings, TUNEL-positive hepatocytes were particularly increased in the almost E-cadherin/β-catenin–negative areas of TIMP3−/− livers after reperfusion, which correlated with elevated caspase activation detected in these livers. Moreover, when TIMP3+/+ hepatocytes subjected to oxidative stress were treated with siRNAs directed against β-catenin, as a proof of concept for hepatocyte apoptosis induced by loss of β-catenin, the knockdown of β-catenin resulted in a significantly increased caspase activation in these cells. These results are in line with previous reports showing that β-catenin–deficient hepatocytes have loss of viability due to apoptosis and that mice with β-catenin–deficient hepatocytes are significantly more susceptible to liver IRI.[49,50]

In this study, we assessed for the first time the response of TIMP3-null mice to hepatic IRI. Our results demonstrate that the loss of TIMP3 is detrimental in hepatic IRI and that TIMP3 shelters the cadherin/β-catenin complex from proteolytic processing in hepatocytes and, likely as a result, protects these cells from undergoing apoptosis. Moreover, they also provide evidence of a role for the disintegrin-like metalloproteinase ADAM10 in liver IRI. IRI is a common feature of several liver pathologies.[50] Hence, this study supports the view that therapeutic restoration of normal TIMP3 levels in hepatocytes (currently under development in our laboratory) may have a broad clinical applicability.

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