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Original Article

Peptidomic Analysis Reveals that Novel Peptide LDP2 Protects Against Hepatic Ischemia/Reperfusion Injury

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Abstract

Background and Aims: Hepatic ischemia/reperfusion (I/R) injury has become an inevitable issue during liver transplantation, with no effective treatments available. However, peptide drugs provide promising regimens for the treatment of this injury and peptidomics has gradually attracted increasing attention. This study was designed to analyze the spectrum of peptides in injured livers and explore the potential beneficial peptides involved in I/R injury. ***Methods:*** C57BL/6 mice were used to establish a liver I/R injury animal model. Changes in peptide profiles in I/R-injured livers were analyzed by mass spectrometry, and the functions of the identified peptides were predicted by bioinformatics. AML12 cells were used to simulate hepatic I/R injury *in vitro*. After treatment with candidate liver-derived peptides (LDPs) 1–10, the cells were collected at various reperfusion times for further study. ***Results:*** Our preliminary study demonstrated that 6 h of reperfusion caused the most liver I/R injury. Peptidomic results suggested that 10 down-regulated peptides were most likely to alleviate I/R injury by supporting mitochondrial function. Most importantly, a novel peptide, LDP2, was identified that alleviated I/R injury of AML12 cells. It increased cell viability and reduced the expression of inflammation- and apoptosis-related proteins. In addition, LDP2 inhibited the expression of proteins related to autophagy. ***Conclusions:*** Investigation of changes in the profiles of peptides in I/R-injured livers led to identification of a novel peptide, LDP2 with potential function in liver protection by inhibiting inflammation, apoptosis, and autophagy.

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Keywords: Hepatic ischemia/reperfusion (I/R) injury; Peptidomics; Peptide LDP2; Apoptosis; Autophagy.

Abbreviations: AML12, alpha mouse liver 12; CCK-8, Cell Counting Kit-8; GO, Gene Ontology; I/R, ischemia/reperfusion; KEGG, Kyoto Encyclopedia of Genes and Genomes; LAP3, leucine aminopeptidase 3; LDP, liver-derived peptide; PPI, protein-protein interaction; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

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Introduction

Liver transplantation is the ultimate treatment for end-stage liver failure.[1,2] However, the limited number of liver donors and hepatic ischemia/reperfusion (I/R) injury accompanying the operation limit the availability and clinical outcomes of liver transplantation. Alleviating hepatic I/R injury not only improves clinical outcomes, but also allows for the use of marginal liver transplants, thereby expanding the pool of donors available for liver transplantation.[3] Despite its clinical importance, there is no effective drug to preclude hepatic I/R injury after liver transplantation. Effective preventive and therapeutic strategies are urgently needed.

Peptidomics, is a high-throughput sequencing technology that paves the way for large-scale exploration of novel valuable peptides.[4,5] Low molecular weight, high bioactivity, easy entry into cells, and easy synthesis are the distinctive advantages of peptides that are candidates for drug development.[6–8] Previous studies have found that a number of synthetic or endogenous peptides, such as octreotide[9] and humanin,[10] have anti-hepatic I/R injury activity, indicating the great potential of peptides as therapeutic candidates for hepatic I/R injury. During hepatic I/R injury, numerous liver-derived active substances are released, such as the anti-inflammatory cytokine interleukin (IL)-13[11] and secretory leukocyte protease inhibitor (SLPI),[12] which have profound effects in limiting liver inflammation and damage, maintaining homeostasis, and eventually resolving liver in-

jury after I/R. Thus far, no liver-derived peptides (LDPs) have been investigated for the treatment of hepatic I/R injury. Therefore, it has been speculated whether liver-derived active peptides released in response to I/R injury can protect livers after transplantation by counteracting hepatic I/R injury.

We constructed a hepatic I/R injury mouse model and used liquid chromatography tandem-mass spectrometry (LC-MS/MS) as a tool[13,14] for analyzing the liver peptidome to detect differentially expressed endogenous peptides in sham-operated controls and in mice with hepatic I/R injury. The physical and chemical properties and biological functions of identified peptides were analyzed by bioinformatics. Low-expression peptides extracted from sham-operated livers were identified and subsequently subjected to anti-hepatic I/R injury assays. We assessed the effectiveness and mechanism of the candidate liver-derived peptides (LDPs) for protecting mouse hepatocytes from hypoxia/reoxygenation (H/R), a standard treatment that mimics the occurrence of hepatic I/R injury. Screening identified a novel peptide LDP2, whose precursor protein is leucine aminopeptidase 3 (Lap3), that protected against mouse hepatocyte injury. A mechanistic investigation highlighted inhibitory effects of LDP2 on inflammation, apoptosis, and autophagy. This study validated the potential of a new peptide to alleviate hepatic I/R injury and provides a new perspective for exploring effective therapeutic approaches.

Methods

Animals

Six- to eight-week-old male C57BL/6 mice were used to construct the experimental model. Animals were purchased from SPF Biotechnology Co., Ltd. (Beijing, China) and temporarily raised at our facility. All experimental protocols were performed in keeping with the National Institutes of Health Guidelines for the Use of Laboratory Animals (NIH publication 86–23, revised 1985) and were approved by the Animal Care and Use Committee of Shanghai Tongren Hospital (approval number: 2021-089-01).

Mouse hepatic I/R injury model

A partial 70% warm hepatic I/R injury mouse model was used in this trial.[15] Briefly, overnight-fasted mice were anesthetized with isoflurane and placed on a thermostatic (37°C) heating pad with their abdomen upward. A microvascular clamp was used to block the blood supply of the left and middle hepatic lobe. The ischemic state lasted 60 m, and then the clamps were removed. The mice were then sacrificed, and tissue and blood samples were collected for further study. A sham operation was performed in the control group mice; blood and tissue samples were collected as described above.

Serum assays

Mouse blood samples were centrifuged at 3,000 rpm for 15–20 m at 4°C to obtain serum. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed with a Rayto Chemray 800 Chemistry System analyzer (Shengzhen, China) to assess liver function. A Luminex assay (Wayen Biotechnologies, Shanghai, China) was used to measure the serum levels of inflammatory cytokines.

Histology and immunofluorescence staining

The injury caused by ischemia-reperfusion was assessed in liver tissue stained with hematoxylin and eosin (HE) as previously reported.[16] Three fields were randomly selected in each slide, and images were captured by light microscopy. Suzuki scores of the stained slides were recorded independently by three experienced pathologists to quantify the damage to the liver.[17] Immunofluorescence staining was performed as previously described.[18] The antibodies that were used are listed in Supplementary Table 1. Images were acquired by fluorescence microscopy.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

TUNEL staining was performed in paraffin sections with a staining kit (G1501; Servicebio, Wuhan, China) following to the manufacturer's protocol to indicate apoptotic cells.

Quantitative real-time PCR (qPCR)

The total mRNA was extracted from liver with TRIzol reagent (15596018; Invitrogen, Carlsbad, CA, USA). cDNA was obtained with reverse transcription kit (FSO-301; TOYOBO, Osaka, Japan), amplified with SYBR Premix Ex Taq (RR420A; Takara, Tokyo, Japan), and the signal was detected with QuantStudio™ Design and Analysis Software (Invitrogen; Woodlands; Singapore). Primers were searched in the PrimerBank website (https://pga.mgh.harvard.edu/primerbank/), and synthesized by the GENWIZ company (Suzhou, China).

Peptide extraction and peptide identification

Three mice from each of the hepatic I/R-injured and sham-operated groups were used for peptidomic analysis. The experimental protocol has been previously reported.[19] The peptides were identified with a Q-Extractive HF (ThermoFisher Scientific, Waltham, MA, USA) mass spectrometer with a 3000 UPLC system (ThermoFisher Scientific).

Bioinformatics analysis

Detailed information of the possible properties and functions of peptides was acquired as previously described.[20] Peptide precursor proteins were matched with those in the UniProt database (http://www.uniport.org/). The molecular weight (MW) and isoelectric point (pI) of each peptide were searched online (https://web.expasy.org/protparam/). Gene Ontology (GO) or Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis were performed to predict the potential function of different peptides on DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/). The interactions between precursor proteins of different peptides were analyzed with the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database, and the results were modified in Cytoscape 3.5.1 software.

Peptide synthesis

The peptide sequences are shown in Supplementary Table 2. All the peptides were synthesized by GL Biochem (Shanghai, China). Lyophilized peptides were dissolved into sterile

ultrapure water to make 10 mM stock solutions.

Hypoxia/reoxygenation (H/R) in vitro

To construct a hypoxia condition, complete DMEM/F12 medium was replaced with glucose- and serum-free DMEM/F12, and then the cells were moved to a hypoxic incubator with a 94% N_2, 5% CO_2, and 1% O_2 atmosphere for 6 h at 37°C. For the reoxygenation phase, the medium was replaced with complete DMEM/F12 medium, and the cells were moved back to a normal incubator. When the time of reperfusion reached 6 h, the cells, and supernatants were collected for further study.

Assay of cell viability

Cell viability was determined with a Cell Counting Kit-8 (CCK-8) assay following the manufacturer's instructions. Alpha mouse liver 12 (AML12) cells were seeded in 96-well plates at a density of 1×10^4 cells/well, and upon reaching 70-80% confluency, they were treated with candidate peptides at 100, 30, 10, 3, or 1 μM for 2 h at 37°C before hypoxia induction. After 2 h of treatment, the peptides were replaced with glucose- and serum-free DMEM/F12 medium, followed by H/R treatment. Two hours before the end of reoxygenation, 10 μL of CCK-8 reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well, and the cells were cultured away from light in 37°C incubators for 2 h. The intensity of the light absorption was measured at 450 nm with a microplate reader.

Western blotting

The expression levels of proteins related to apoptosis (Bax, Bcl-2) and autophagy (ATG7, p62, LC3B) in mice livers or AML12 cells were detected as previously reported.[21] The antibodies used are listed in Supplementary Table 1.

Statistical analysis

The data are from three or more independent assays. The percentage (%) of positive cells in the fluorescent staining assay and the expression of different proteins in western blotting assays were quantified by ImageJ software 1.26. The analysis of experimental data and the production of graphs were carried out with GraphPad Prism 8.0.1 software. The results were reported as means±standard deviations. Differences in data were analyzed by unpaired two-sided Student's *t*-tests or one-way analysis of variance (nonparametric or mixed) for comparisons. Differences with *p*-values <0.05 were considered to be statistically significant.

Results

Ischemic liver at 6 h post-reperfusion had the most severe damage

We first aimed to construct a liver I/R model by subjecting mice to partial hepatic ischemia followed by a period of reperfusion. Serum biochemical and pathological parameters were used to assess liver damage. Compared with those in the sham-operated group, the serum ALT and AST levels significantly increased at 3, 6 and 12 h post-injury and peaked at 6 h reperfusion in the trial groups (Fig. 1A, B). Consistent with the serological indices, the Sukizi score of HE-stained liver tissue based on the visual inspection of liver necrotic areas further confirmed the dynamic profiles of liver injury during I/R (Fig. 1C). As liver injury was also most evident in the trial group at 6 h after reperfusion, we used 6 h as the standard reperfusion time for the ischemic model group in further procedures.

Exacerbated inflammation and hepatocyte death at 6 h post-reperfusion

Proinflammatory cytokine expression levels were assayed by qPCR and a Luminex liquid suspension chip. As shown in Figure 2A, the expression of proinflammatory cytokines (IL1β, IL6, and TNF-α) in post-reperfusion livers in the model groups was significantly increased compared with that in the sham-operated group. Similarly, model mice had a stronger pattern of serum proinflammatory cytokine expression than sham-operated mice (Fig. 2B). Monocyte/macrophage infiltration and elevated inflammation have been previously reported to drive liver I/R injury.[22] We thus investigated whether macrophage/neutrophil infiltration was increased after hepatic I/R. Immunofluorescence staining for macrophage and neutrophil antigens showed a significant increase in infiltration of these two monocyte types in the postreperfusion livers at 6 h (Fig. 2C).

Hepatic necrosis/apoptosis has been shown to positively correlate with liver damage.[23] TUNEL staining of liver biopsies demonstrated that post-reperfusion livers had a higher percentage of apoptotic cells than the livers in the sham-operated group (Fig. 2D). Taken together, the serum biochemical and histopathological analyses verified that severe liver damage was present at 6 h postreperfusion, confirming that we constructed a reliable hepatic I/R injury mouse model.

Expression profiles of peptides in livers with ischemia-reperfusion injury

Endogenous peptides from IR-injured and sham-operated livers were analyzed by using LC-MS/MS. A total of 20,045 peptides were identified, 682 of which had differential expression in injured livers (fold change ≥2 and *p*-values <0.05), with 584 down-regulated peptides and 98 up-regulated peptides (Fig. 3A). The heatmap indicated the markedly different distribution of those peptides between the hepatic I/R-injured and sham-operated groups (Fig. 3B). The MW, peptide length and pI of 684 peptides were determined (Fig. 3C–E). The MWs of the peptides ranged from 0.8–2.2 kDa. Most down-regulated peptides were 1.2–1.4 kDa and most up-regulated peptides were 1.6–1.8 kDa. Peptide length ranged from 9–17 amino acids, and the pI values of peptides were enriched in the 8–9 range. Investigation of the association between the distribution of the MW and pI of peptides found that the peptides were mainly clustered into four groups, near pI 4, pI 6, pI 9 and pI 10 (Fig. 3F).

GO and KEGG pathway analysis of the precursor proteins of differentially expressed peptides

To acquire an overview of the potential functions of the 682 differentially expressed peptides, GO and pathway analyses were performed on their precursor proteins. In both biological process and molecular function analyses, the up-regulated peptides were mainly involved in body metabolism, especially in ATP biosynthetic and metabolic processes (Fig.

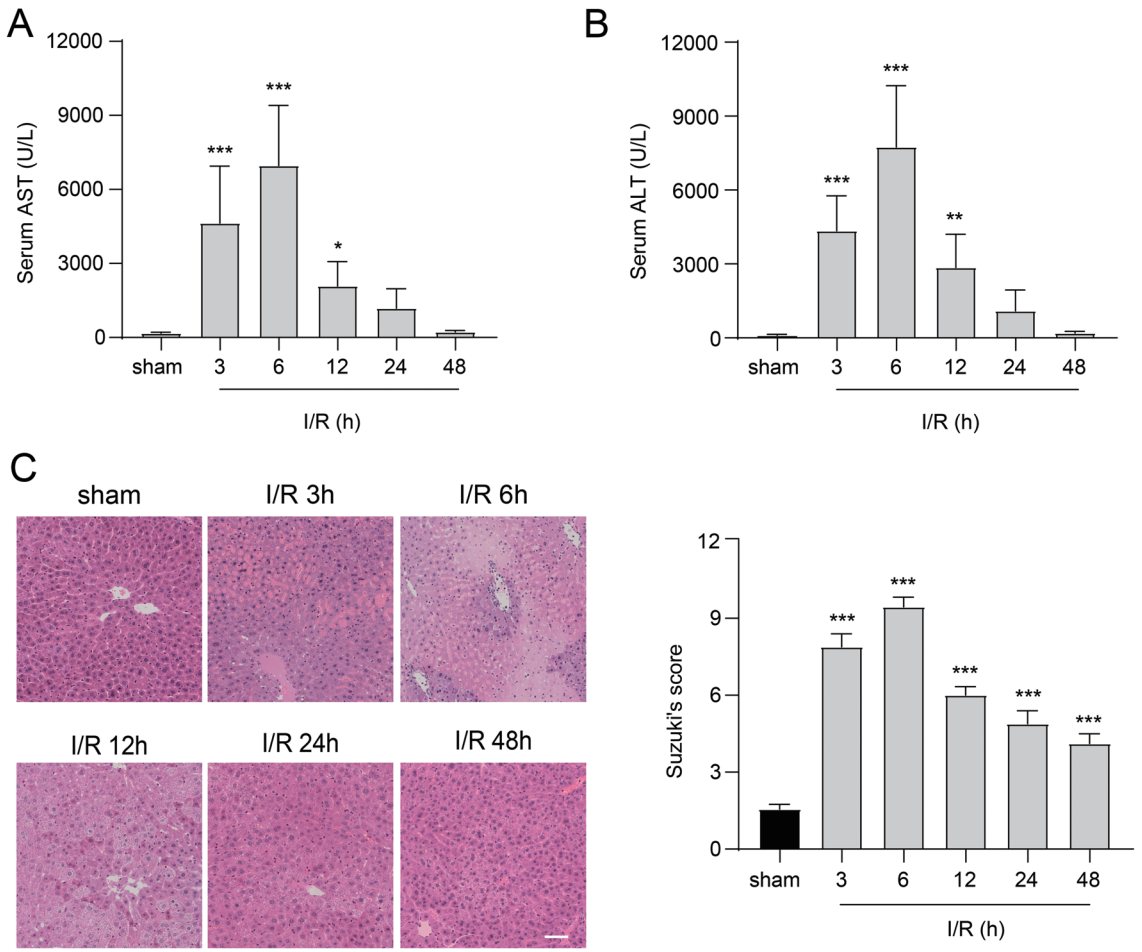

Fig. 1. Serum biochemistry and pathology parameters indicate the most severe liver damage. (A, B) Serum ALT and AST levels in the study groups. (C) Representative images of HE-stained liver sections (left) and the Sukizi scores of the positive areas (right), in the mice following treatment. The data are means ± SDs. *p<0.05, **p<0.01, ***p<0.001 vs. the sham-operated group; scale bar: 50 μm, n=6–8. ALT, alanine aminotransferase; AST, aspartate aminotransferase; I/R, hepatic ischemia/reperfusion injury group; sham, sham-operated group.

4A, B). For down-regulated peptides, the top 10 enriched biological process were oxidation-reduction process, metabolic process, tricarboxylic acid cycle, fatty acid beta-oxidation, lipid metabolic process, fatty acid metabolic process, protein homotetramerization, protein folding, fatty acid metabolism, and ATP metabolism (Fig. 4C, D). The most enriched molecular functions were oxidoreductase activity, catalytic activity, lyase activity, poly(A) RNA binding, NAD binding, 3-hydroxyacyl-CoA dehydrogenase activity, fatty-acyl-CoA binding, isomerase activity, amino acid binding and aldehyde dehydrogenase (NAD) activity (Fig. 4E, F). Interestingly, cellular component analysis found that most precursor proteins of both up-regulated and down-regulated peptides were associated with mitochondrial construction.

To analyze which biological pathway the differentially expressed peptides might participate in, we performed a comprehensive analysis of their precursor proteins using pathway analysis software. For up-regulated peptides, the precursor proteins were involved in metabolic pathways, carbon metabolism, oxidative phosphorylation, nitrogen metabolism, propanoate metabolism, butanoate metabolism, and alanine, aspartate, and glutamate metabolism. For down-regulated peptides, the top 20 pathways were associated with fatty acid, carbon, branched chain amino acids, oxidative phosphorylation and antibiotics biosynthesis (Supplementary Fig. 1).

Protein-protein interaction (PPI) network of the precursor proteins of differentially expressed peptides

The top 100 up- and down-regulated peptide precursor proteins with the highest fold change were used to construct a PPI network (Fig. 5A, B). Analysis of this PPI network with Cytoscape identified 10 hub molecules in up- and down-regulated peptide precursor protein PPI network. They were Hmgcs2, Acat1, Acaa2, Hadh, Abat, Sucla2, Atp5a1, Acox1, Gapdh, and Etfb in the up-regulated PPI network and Aco2, Got2, Hspa9, Hspd1, Glud1, Uqcrc1, Eno1, Aldob, Mdh1, and Sucla2 in the down-regulated PPI network (Fig. 5C, D). The hub molecules are key enzymes and molecules active in ATP binding, glycolysis, TCA, and mitochondrial beta-oxidation. The result clearly demonstrated that mitochondrial metabolism pathways were altered in I/R-injured livers.

Verification of the hepatoprotective function of differentially expressed peptides in hepatocytes

The relatively down-regulated peptides in I/R-injured livers may protect against hepatic I/R injury. To validate their functional role against injury, we synthesized 10 of the most

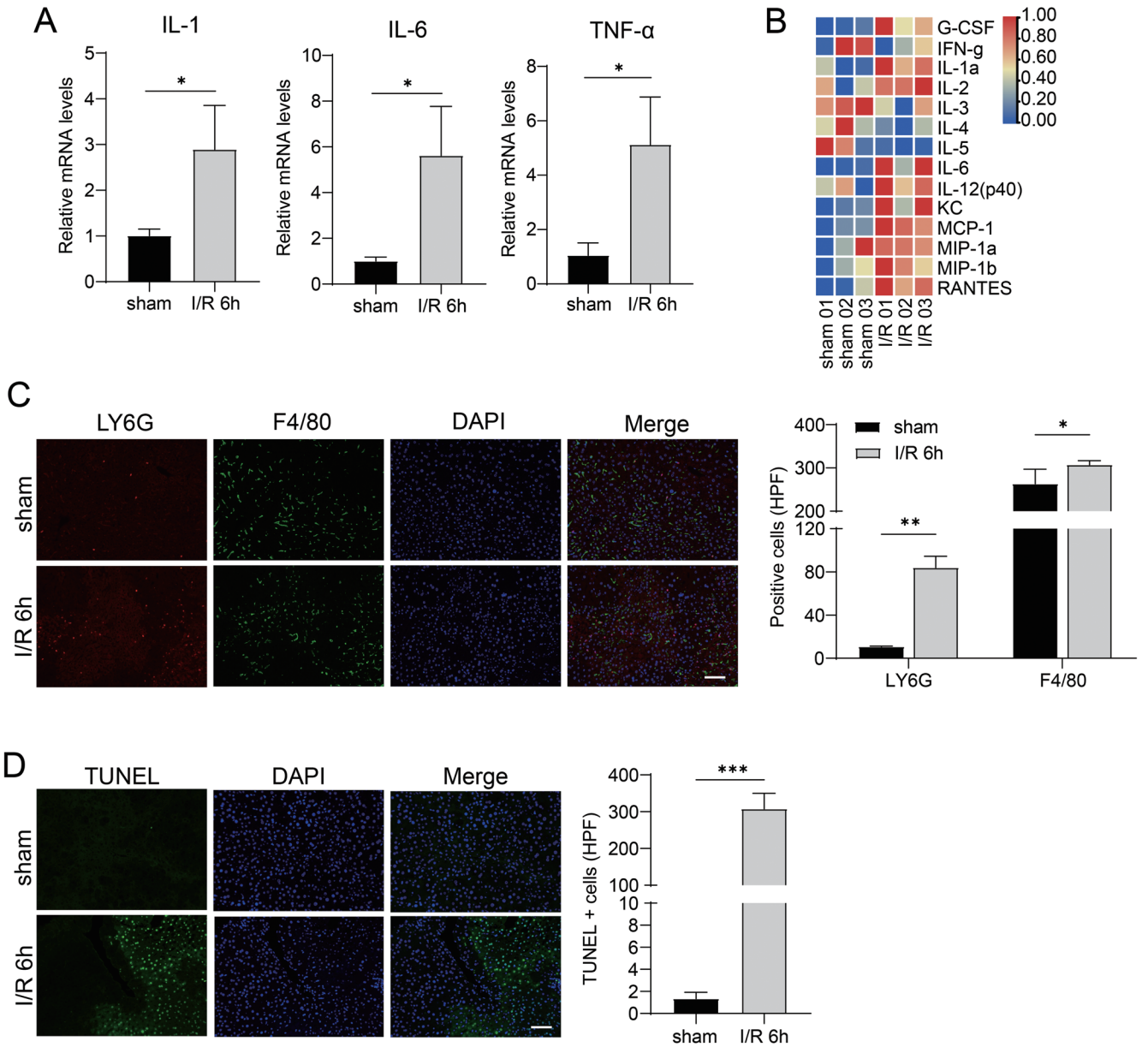

Fig. 2. Increased inflammation and hepatocyte death at 6 h post-perfusion. (A) Levels of mRNA related to pro-inflammatory factors were tested by qPCR 6 h after injury or sham operation. (B) The heat map shows serum cytokines and chemokines assayed by Luminex liquid suspension chips. (C) Representative immunofluorescence images of the LY6G- or F4/80-positive inflammatory cells in livers and the quantification of positive cells (right). (D) Representative images of TUNEL staining 6 h after hepatic I/R injury to assess apoptosis in the study groups. Data are means ± SDs. *p<0.05, **p<0.01, ***p<0.001 compared with the sham-operated group, scale bar: 50 μm, n=3–5. TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

differentially expressed LDPs for easy presentation (Supplementary Table 2), and tested their effects on cell viability in mouse liver cells. After H/R, the CCK-8 assay results showed that LDP2, one of the 10 candidate peptides, increased the survival of cells at concentrations of 30 μM and 100 μM, and the optimum effect was at 30 μM (Fig. 6A, B). Detailed results are shown in Table 1. We also explored whether the LDP2 itself damaged hepatocytes, and found that LDP2 had no adverse effect on AML12 cells (Fig. 6C). Consistent with the increased survival of H/R-induced hepatocytes, increased expression of cell death negative regulators, such as Bcl-2, and lower levels of pro-apoptotic and pro-necrotic factors, such as Bax, were found in the LDP2 treatment group than in the control (Fig. 6D). Moreover, the study demonstrated that LDP2 alleviated the inflammation of hepatocytes induced by reperfusion injury, as indicated by a decreased level of pro-inflammatory cytokines CXCL-1 and MCP-1 (Fig. 6E). Considering that increased production of reactive oxygen species (ROS) and autophagy are the main participants during hepatocyte injury, we investigated ROS production and hepatocyte autophagy LDP2 treatment. As shown in Figure 6F and Supplementary Figure 2, LDP2 significantly reduced the levels of proteins associated with autophagy, but had no effect on ROS production. The results demonstrated that LDP2 had a protective role in H/R-induced injury in cells through of inhibition of apoptosis, inflammation, and autophagy.

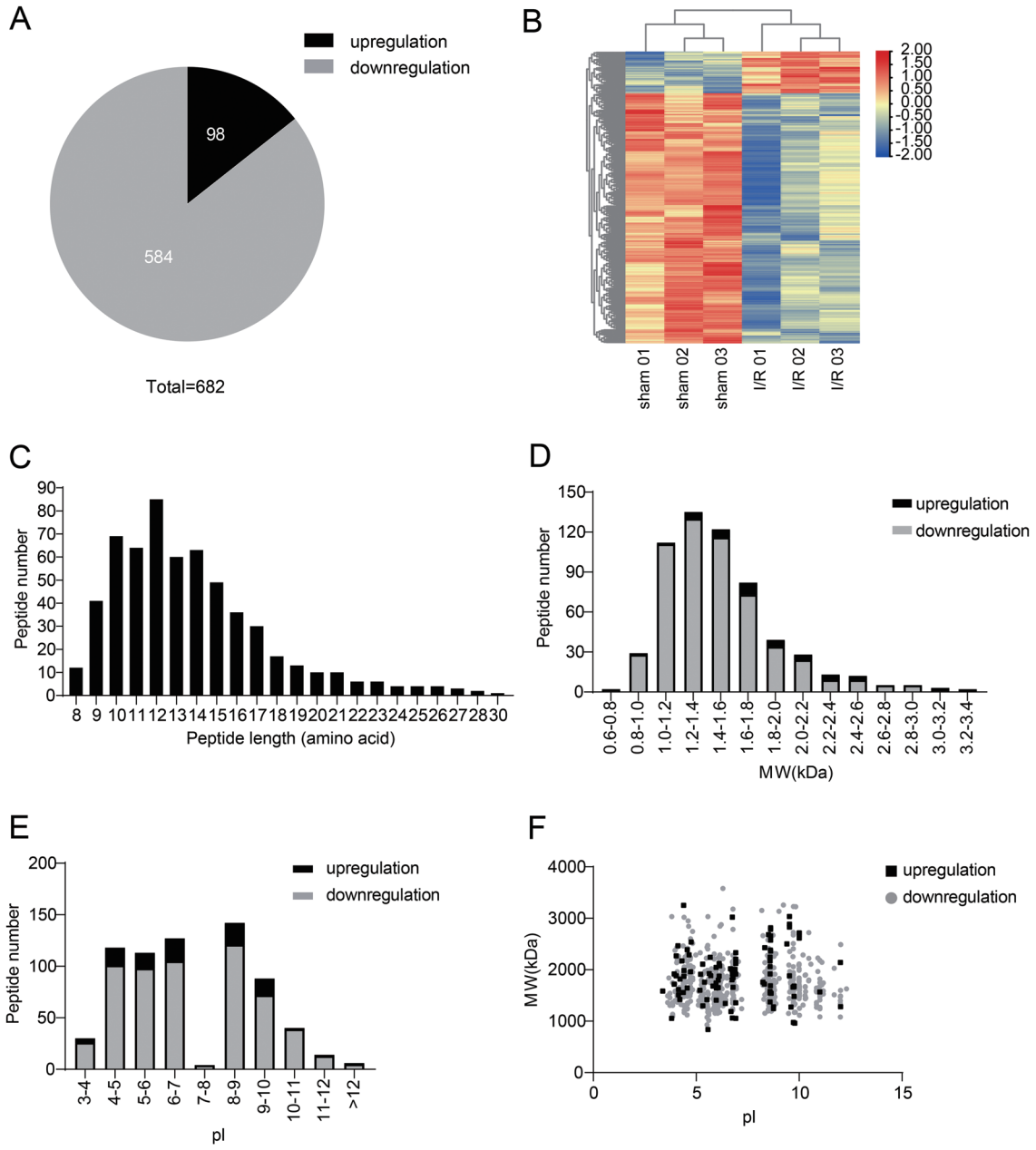

Fig. 3. Identification of the expression spectrum of the peptide. (A) The number of up- and down-regulated differentially expressed endogenous peptides from I/R-injured livers compared with the sham-operated group. (B) The heat map indicates the peptide profiles with significant differences between the indicated groups. (C–E) Distribution of the length, MW and pI of peptides. (F) Correlation between MW and pI distribution of peptides. MW, molecular weight; pI, isoelectric point.

Discussion

Hepatic I/R injury has long been considered a problem in the prognosis of liver surgery, particularly liver transplantation.[24] Several methods have shown the potential function of liver protection against I/R injury, but very few basic and translational studies have been successfully performed at the bedside.[25,26] In this study, we performed peptidomic studies to analyze changes in peptides in I/R-injured livers and discovered their differential expression patterns. We provided evidence, such as cell viability and the expression of proteins associated with apoptosis or autophagy, showing that a novel peptide LDP2 was a pivotal regulator protecting against hepatic I/R injury. Our research demonstrates that LDP2 may be a promising clinical therapeutic molecule for the treatment and prevention of hepatic I/R injury.

In this study, the construction of an animal model of hepatic I/R injury was the cornerstone of the whole trial and the timing of reperfusion is the key to the success of this model. In previous studies, an ischemia time of 1 h has usually been chosen as the ideal ischemia time, but the time of reperfusion is very different in different studies.[27–29] To determine the appropriate duration of reperfusion that could induce 70% liver injury, a criterion for assessing the success of the injury model,[27] we selected 3, 6, 12, and

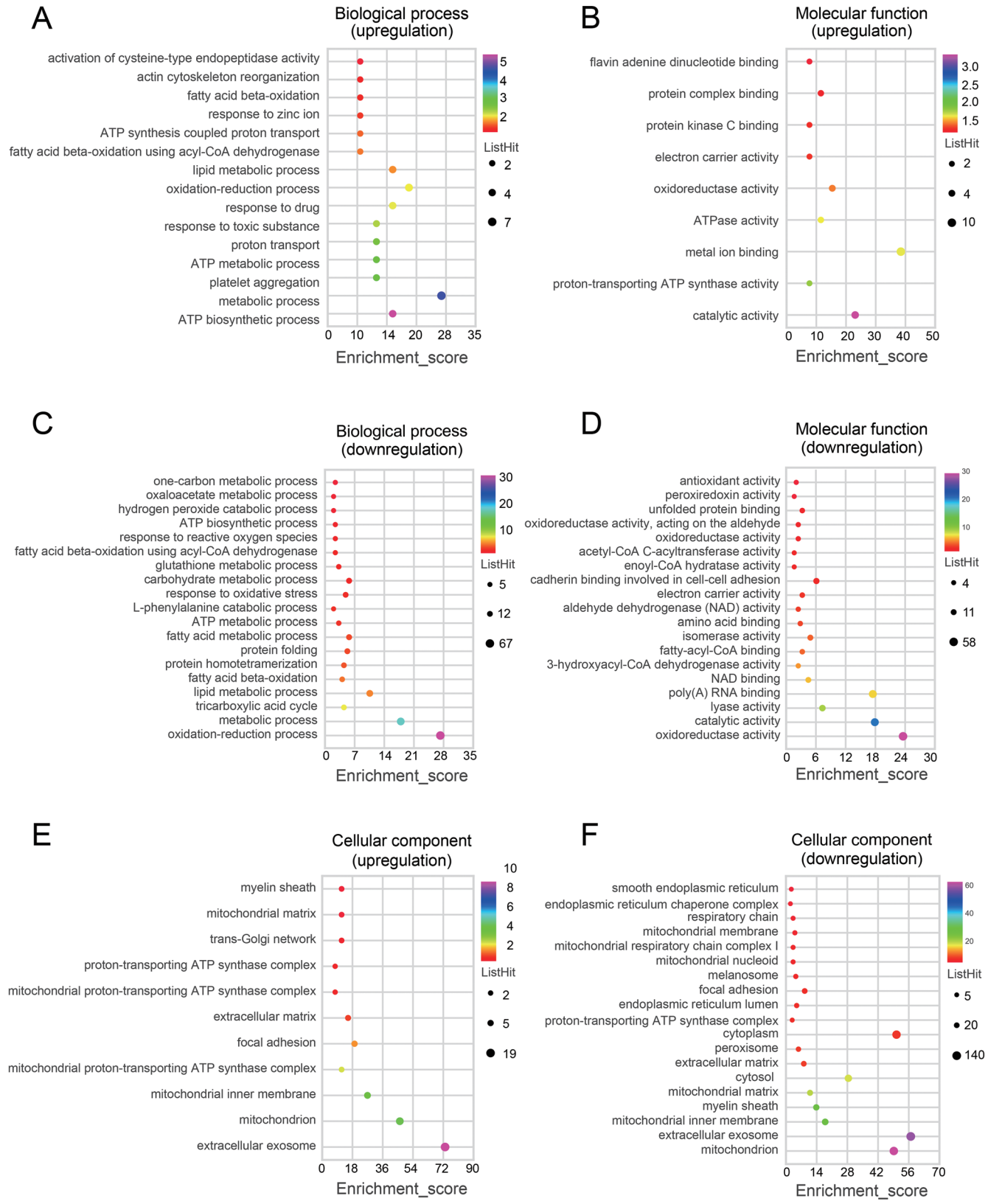

Fig. 4. Bioinformatics analysis of differentially expressed peptide precursor proteins. GO analysis of (A, C) Molecular function, (B, D) Cellular component, and (E, F) Biological process of up- and down-regulated differentially expressed endogenous peptides. GO, Gene Ontology.

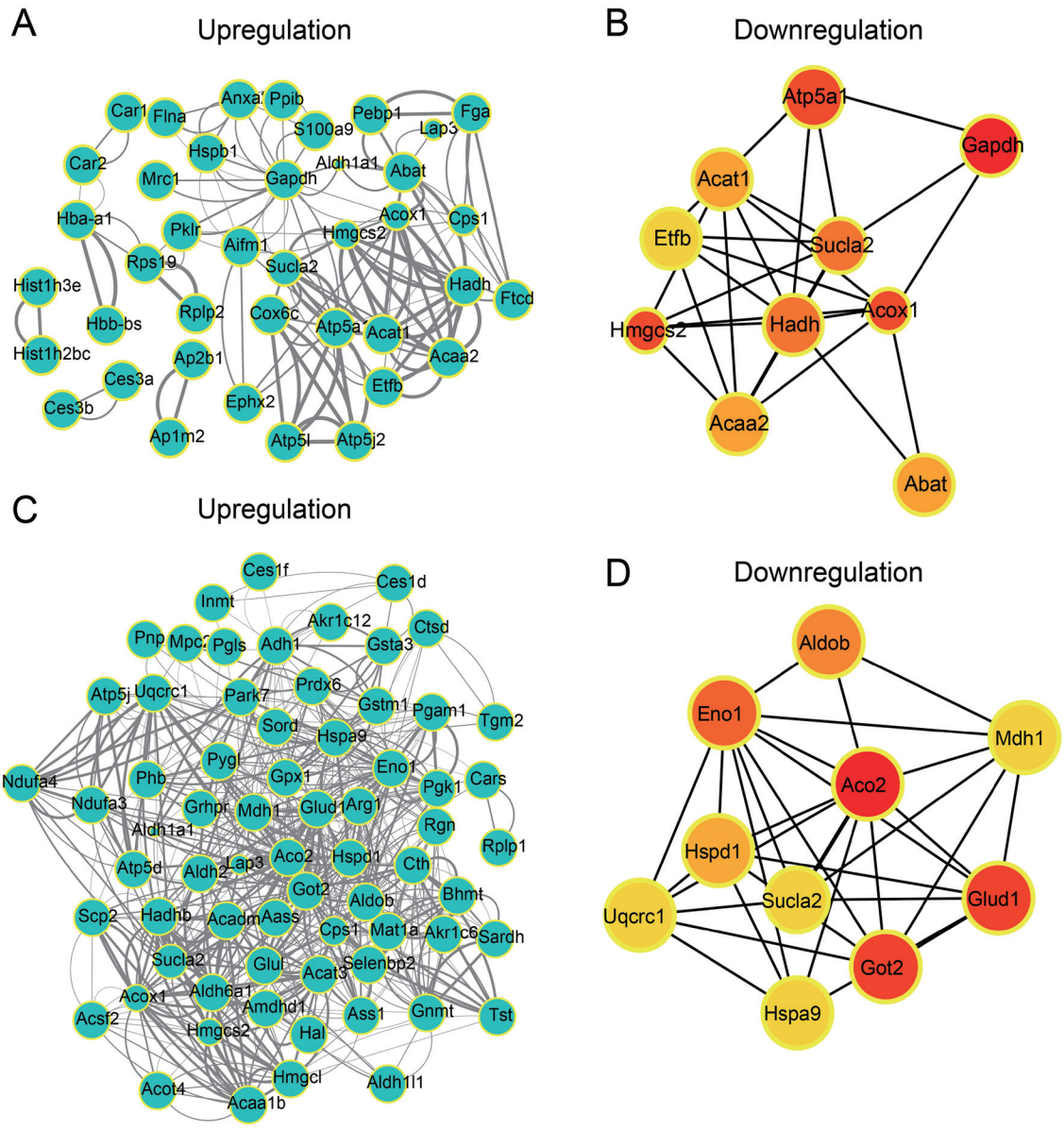

Fig. 5. Protein-protein interaction (PPI) network analysis of peptide precursor proteins. (A, B) PPI constructed with the top 100 up- and down-regulated differentially expressed peptides; (C, D) Hub molecules screened by Cytoscape.

24 h as candidate times of reperfusion. The inflammation response and cell death, including the recruitment of inflammatory cells, secretion of circulating proinflammatory factors by various cells and the consequent enhancement of hepatocyte death, have been used in many studies as two indicators of the extent of liver damage.[30,31] As shown in Figure 2, this study showed that by comparing these different reperfusion candidate time points, 6 h of reperfusion induced higher levels of pro-inflammatory chemokines, more LY6G and F4/80-positive inflammatory cell infiltration, and more severe hepatocyte death, indicating that 6 h of reperfusion triggered the most severe liver injury. Therefore, 1 h of ischemia followed by 6 h of reperfusion was an appropriate operation to build a stable hepatic I/R injury mouse model in our study.

Based on our successful establishment of the hepatic I/R injury model, we performed an injured liver peptide omics analysis that identified 682 differentially expressed peptides in the injured liver compared with those in the livers in the sham-operated group. Most peptides consisted of 9–17 amino acids, and the MW distribution was between 0.8 and 2.2 kDa, indicating that the peptides were valid. In our study, all the peptides had precursor proteins, and some were derived from the same protein. Several studies have shown that peptides often have biological effects similar to those of their precursor proteins.[32] Therefore, analysis of the biological functions of these precursor proteins will help us understand the role of the differentially expressed peptides in the process of hepatic I/R injury. Through analyzing these precursor proteins by bioinformatics, the cellular components of these peptides were found to be enriched in mitochondria, and the biological functions were mainly involved in metabolic processes. Maintaining the function and integrity of mitochondria is essential for normal cellular

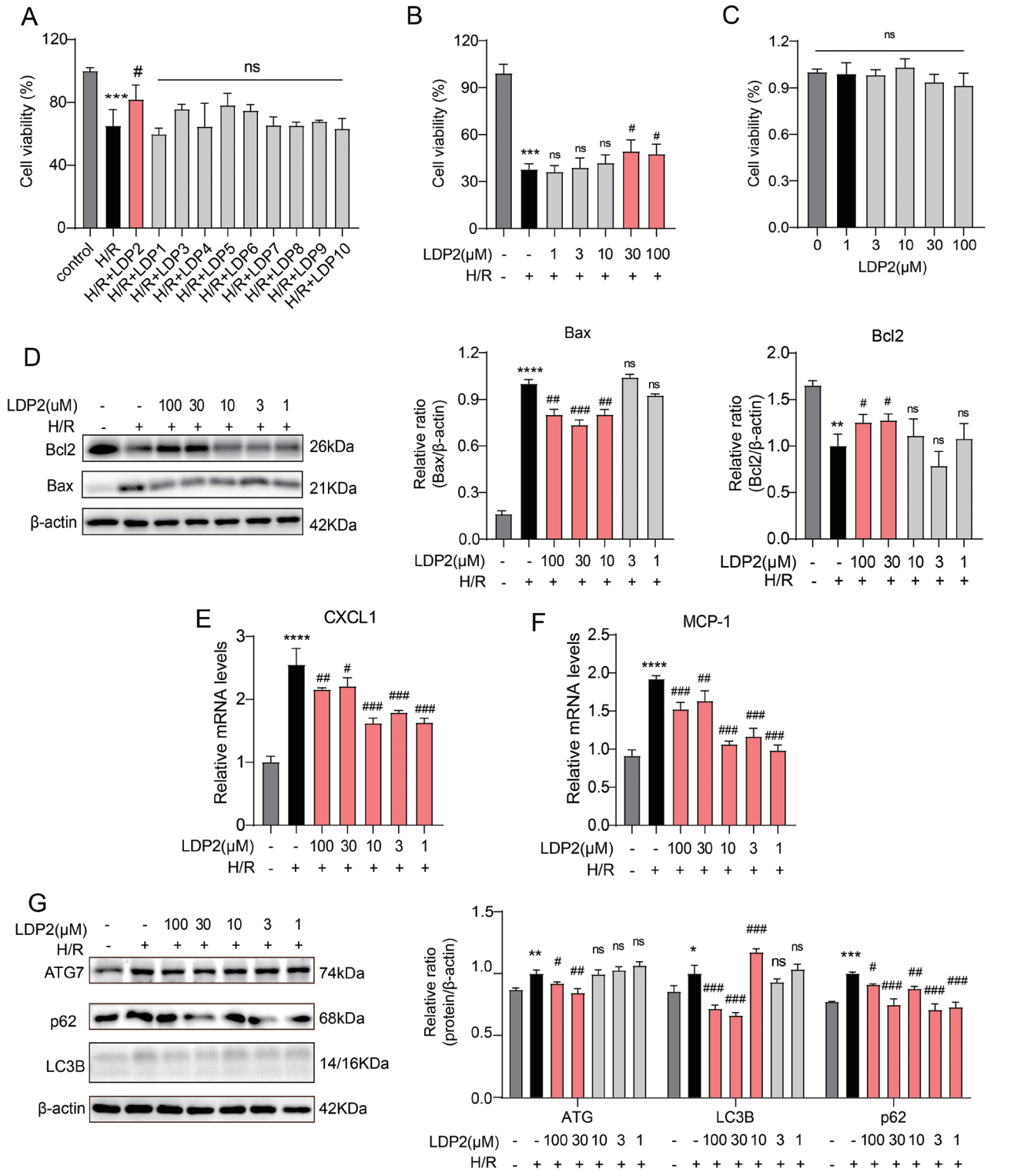

Fig. 6. Functional LDP2 peptide attenuates apoptosis, inflammation, and autophagy of hepatocytes induced by hypoxia/reoxygenation (H/R) treatment. (A) Function screening of identified potential peptides by assessing hepatocyte viability. (B) LDP2 peptide had no adverse effects at high concentrations in hepatocytes. (C) LDP2 significantly increased hepatocyte viability at concentrations of 30 and 100 μM. The peak effect was at 30 μM. (D) CXCL-1 or MCP-1 mRNA expression in hepatocytes with different concentrations of LDP2 treatment. (E) Western blot analysis of proteins related to apoptosis (Bcl-2 and Bax) in hepatocytes treated with or without H/R for 12 h. (F) ATG7, p62 and LC3B protein expression in hepatocytes treated with or without H/R for 12 h were assayed by western blotting. Data are means±SDs. *p<0.05, **p<0.01, and ***p<0.001 compared with the control group, $^{\#}p$<0.05, $^{\#\#}p$<0.01, and $^{\#\#\#}p$<0.001 compared with the H/R-treated group, n=3. LDP, liver-derived peptide; H/R, hypoxia/reoxygenation.

Table 1. Properties of LDP2

Name	Protein	Uniprot Accession	Site	Sequence	Molecular weight in kDa	Isoelectric point
LDP2	Q9CPY7	Lap3	319–330	AAKLNLPINIIG	1,237	9

LDP, liver-derived peptide.

homeostasis, particularly in the liver and heart.[33] Studies have revealed that mitochondrial dysfunction has a strong correlation with I/R injury.[34] Mitochondrial dynamics, including mitochondrial fusion,[35] fission,[36] biogenesis[37] and mitophagy[38] have been implicated in renal and cardiovascular diseases associated with I/R injury. The peptide functional analysis suggests that an imbalance in mitochondrial dynamics may have occurred in the injured liver, which promotes mitochondria as a therapeutic goal for hepatic I/R injury and in which the differentially abundant peptides may have an important role.

The underlying mechanisms of hepatic I/R injury are mainly related to hepatocyte viability, inflammation, apoptosis, and oxidative stress injury.[39,40] Our studies first focused on the role of candidate peptides in hepatocyte viability, and identified a peptide named LDP2 that has a hepatocyte-protective function. The results demonstrated that LDP2 inhibited the apoptosis and inflammation of hepatocytes induced by H/R. Interestingly, the bioinformatics analysis identified differentially expressed peptides that may be involved in the regulation of mitochondrial function in I/R-injured livers, reactive ROS levels, a major indicator of abnormal mitochondrial function,[41] were not affected by LDP2, suggesting that there may be alternative mechanisms for LDP2 regulation of mitochondrial dynamics. Autophagy, as the other vital event of mitochondrial dysfunction, is also participated in hepatic I/R injury. Zhu *et al*.[42] reported that rapamycin alleviated hepatic I/R injury by enhancing autophagy, but others have debated whether inactivation of autophagy ameliorates hepatic I/R injury.[43] Although the evidence is inconclusive, recent studies highlight the therapeutic potential of controlled autophagy in the suppression of hepatic I/R injury. In our study, LDP2 significantly inhibited autophagy in hepatocytes, as evidenced by the reduced expression of autophagy marker proteins, indicating that autophagy contributed to hepatocyte injury and that LDP2 had a protective effect against hepatocyte damage via autophagy inhibition. LDP2 is derived from cytosol aminopeptidase (also called leucine aminopeptidase, LAP3), located at amino acids 319–330. Previous work revealed that LAP3 regulates apoptosis,[44] inflammation,[45] and autophagy.[46] Based on those results and the findings of this study, we speculate that the LAP3 protein may be cleaved into a shorter peptide to protect against hepatocyte injury from I/R-induced apoptosis and inflammation through an underlying mechanism of autophagy inhibition. The protective effectiveness of LDP2 on I/R needs to be more specifically identified, and how LDP2 functions has yet to be investigated.

In conclusion, to the best of our knowledge, this is the first study to use peptidomics to analyze the peptide spectrum of I/R-injured livers. We identified a novel liver-derived active peptide, LDP2, that protected H/R-treated hepatocytes from apoptosis, inflammation and autophagy induced by I/R injury. The study results provide novel insights into the treatment of hepatic I/R injury.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (PS, BH), acquisition of data (QB, SC), analysis and interpretation of data (QB, LL, JZ), drafting of the manuscript (QB, BH, PS), critical revision of the manuscript for important intellectual content (XW, XG), administrative, technical, or material support (ZW, DC, YZ), and study supervision (PS, XG).

Ethical statement

All experimental protocols were performed in keeping with the National Institutes of Health Guidelines for the Use of Laboratory Animals (NIH publication 86–23, revised 1985) and were approved by the Animal Care and Use Committee of Shanghai Tongren Hospital (approval number: 2021-089-01).

Data sharing statement

Data and materials are available upon reasonable request to the corresponding author.

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