Biochemical and Biophysical Research Communications 595 (2022) 47–53

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Blocking protease-activated receptor 4 alleviates liver injury induced by brain death

Hongbo Fang [a], Zibo Yuan [a], Yaohua Zhu [a], Hongwei Tang [b, c], Chun Pang [a], Jie Li [a], Jihua Shi [b, c], Wenzhi Guo [a, b, c], Shuijun Zhang [a, b, c, *]

[a] Department of Hepatobiliary and Pancreatic Surgery, the First Affiliated Hospital of Zhengzhou University, Zhengzhou City, Henan Province, China
[b] Henan Key Laboratory for Digestive Organ Transplantation, Zhengzhou City, Henan Province, China
[c] Zhengzhou Key Laboratory for Hepatobiliary & Pancreatic Diseases and Organ Transplantation, Zhengzhou City, Henan Province, China

ARTICLE INFO

Article history:
Received 25 November 2021
Accepted 19 January 2022
Available online 21 January 2022

Keywords:
Brain death
Liver injury
Protease-activated receptor 4
Inflammation
Apoptosis

ABSTRACT

Brain death (BD) induces a systemic inflammatory response that influences donor liver quality. Protease-activated receptor 4 (PAR4) is a thrombin receptor that mediates platelet activation and is involved in inflammatory and apoptotic processes. Therefore, we investigated the role of PAR4 blockade in liver injury induced by BD and its associated mechanisms. In this study, we constructed a BD rat model and treated rats with TcY-NH2, a selective PAR4 antagonist, to block PAR4 signaling at the onset of BD induction. Our results revealed that PAR4 protein expression increased in the livers of rats with BD. PAR4 blockade alleviated liver injury induced by BD, as indicated by lower serum ALT/AST levels and an improvement in histomorphology. Blood platelet activation and hepatic platelet accumulation in BD rats were reduced by PAR4 blockade. Additionally, PAR4 blockade attenuated the inflammatory response and apoptosis in the livers of BD rats. Moreover, the activation of NF-κB and MAPK pathways induced by BD was inhibited by PAR4 blockade. Thus, our results suggest that PAR4 contributes to liver injury induced by BD by regulating inflammation and apoptosis through the NF-κB and MAPK pathways. Thus, PAR4 blockade may provide a feasible approach to improve the quality of organs from BD donors.

1. Introduction

Organ donation after brain death (BD) rescues many patients with end-stage organ diseases and is a major source of organ transplants. However, BD affects organ quality and transplant outcomes [1]. Studies have demonstrated that liver grafts from BD donors are associated with worse ischemia-reperfusion injury (IRI) and inferior graft survival compared to living donors [2]. BD is defined as an irreversible cessation of all functions of the whole brain, including the brainstem, usually caused by traumatic brain injury, cerebral hemorrhage, and hypoxic-ischemic encephalopathy. A series of pathophysiological changes occur in BD donors, including hemodynamic disturbances, coagulation disorders, and systemic inflammatory responses [3]. Although therapeutic approaches are initiated to maintain organ function during BD, liver graft injury still arises before the transplantation procedure [4]. Therefore, there is a need to investigate the mechanisms of injury and the therapeutic approaches to prevent them.

Coagulation is activated during BD, and is characterized by significant prothrombin and platelet activation [5]. Hypercoagulation may lead to the formation of microthrombi in potential grafts, resulting in tissue injury. Meanwhile, thrombin and activated platelets can regulate the expression of inflammatory cytokines and leukocyte recruitment to participate in inflammatory response [6,7]. The proinflammatory genes interleukin-6 (IL-6), IL-1β, tumor necrosis factor-α (TNF-α), and P-selectin are significantly upregulated in DB donor livers compared with living donor livers [8]. These molecules recruit inflammatory cells, induce hepatocyte

Abbreviations: BD, Brain death; PAR4, Protease-activated receptor 4; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; NF-κB, Nuclear factor kappa B; MAPK, Mitogen-activated protein kinase; IL, Interleukin; TNF-α, Tumor necrosis factor-α; CD41, Cluster of differentiation 41; MPO, Myeloperoxidase; ICAM-1, Intercellular adhesion molecule-1; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; Bcl2, B-cell lymphoma 2; Bax, Bcl2-associated x; cl-Caspase3, cleaved-Caspase3; IκB, inhibitor of NF-κB; ERK, Extracellular signal regulated protein kinase; JNK, c-Jun N-terminal kinase.

* Corresponding author. Department of Hepatobiliary and Pancreatic Surgery, The First Affiliated Hospital of Zhengzhou University, Jianshe East Road No. 1, Zhengzhou City, Henan, 450052, China.
E-mail address: zhangshuijun@zzu.edu.cn (S. Zhang).

https://doi.org/10.1016/j.bbrc.2022.01.074
0006-291X/

death, and aggravate liver injury, which in turn promotes inflammation-associated coagulation activation [9,10]. The molecular pathways linking inflammation and hemostasis are complex. Thus, the identification of these key pathway regulators are essential to reduce liver injury during BD.

Protease-activated receptor 4 (PAR4) is a member of the seven transmembrane domain G protein-coupled receptor family, and is activated by thrombin, cathepsin G, factor Xa. Aside from its role in thrombin-induced platelet aggregation, PAR4 activation is involved in inflammatory lesions [11]. PAR4 knockout mice exhibit mild myocardial IRI [12]. Treatment with PAR4 antagonist attenuates hepatic IRI-induced platelet and T-cell recruitment, and alleviates apoptotic and necrotic injury [13]. Additionally, PAR4 antagonists have been examined in clinical trials [14]. However, the exact role of PAR4 in liver injury under BD conditions remains unknown. In this study, we used a rat BD model and selective PAR4 antagonist, TcY-NH2, to explore the effects of PAR4 blockade in liver injury induced by BD and its associated mechanism.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats weighing about 300 g were used. The rats were housed in a light and temperature-controlled environment and had free access to food and water. All rats received human care according to the local animal welfare guidelines. All rat experiments in this study conformed to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and were approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (No.2021-KY-0465).

2.2. BD model and experimental groups

Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (6 mg/100 g body weight). BD was induced by gradually increasing intracranial pressure by slow inflation of a No.3 Fogarty catheter balloon according to a previously described method [15]. BD was confirmed by cessation of brain electrical activities, apnea, dilated and fixed pupils, and absence of corneal reflexes. All rats were mechanically ventilated during BD. Hydroxyethyl starch was infused via the tail vein to maintain a mean arterial pressure above 80 mmHg.

Rats were randomly allocated to three groups (six rats per group): Sham-operated rats underwent the same surgical procedures without BD induction (Sham group); rats subjected to BD (BD group); rats subjected to BD plus TcY-NH2 (Tocris Bioscience, Ellisville, MO, USA) treatment (BD + TcY group). TcY-NH2 was injected (0.6 mg/kg bodyweight in 500 μL of saline) into the tail vein at the start of BD induction. Liver and blood samples were collected 6 h after BD and sham operations.

2.3. Measurement of blood components

Blood was collected by a direct puncture of the inferior vena cava. Serum alanine transaminase (ALT) and aspartate transaminase (AST) levels were measured using a standard clinical automatic analyzer, according to the manufacturer's protocols, to evaluate liver function. The platelet activation state was assessed by measuring plasma soluble P-selectin levels. The measurement was taken using an enzyme-linked immunosorbent assay kit (BYabscience, Nanjing, China), according to the manufacturer's protocol.

2.4. Histological and immunohistochemical staining

Liver samples were fixed in 10% formalin, embedded in paraffin, and sectioned (4 μm per section). To evaluate the histopathological changes, liver sections were stained with hematoxylin and eosin (H&E). Both cluster of differentiation 41 (CD41) and myeloperoxidase (MPO) were detected by immunohistochemistry staining described previously [16], to separately analyze platelet accumulation and neutrophil infiltration in the liver tissue. The primary antibodies anti-CD41 (1:200, Boster, Wuhan, China) and anti-MPO (1:200, Servicebio, Wuhan, China) were used. At least three fields per section were examined under a light microscope.

2.5. Terminal deoxynucleotidyl transferase 2′-deoxyuridine 5′-triphosphate nick-end labeling (TUNEL) assay

Apoptotic cells in paraffin-embedded liver tissue sections (4 μm per section) were identified via TUNEL assay using the In Situ Cell Death Detection Kit (Servicebio, Wuhan, China), according to its protocol. Cells with DNA fragments were stained nuclear positive by fluorescent labels, visualized directly by fluorescence microscopy, and thereafter counted. At least three fields per section were examined.

2.6. Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated from snap-frozen liver tissue using the RNAiso Plus kit (Takara, Dalian, China) and quantified using a Nanodrop 2000 spectrophotometer. cDNA was synthesized using the PrimeScript™ RT reagent Kit (Takara, Dalian, China) according to the manufacturer's protocol. Sample preparation for real-time PCR was performed according to the manufacturer's protocol using the SYBR® Premix Ex Taq™ kit (Takara, Dalian, China), and PCR was run on an Applied Biosystems 7500 Fast Real-Time PCR System.

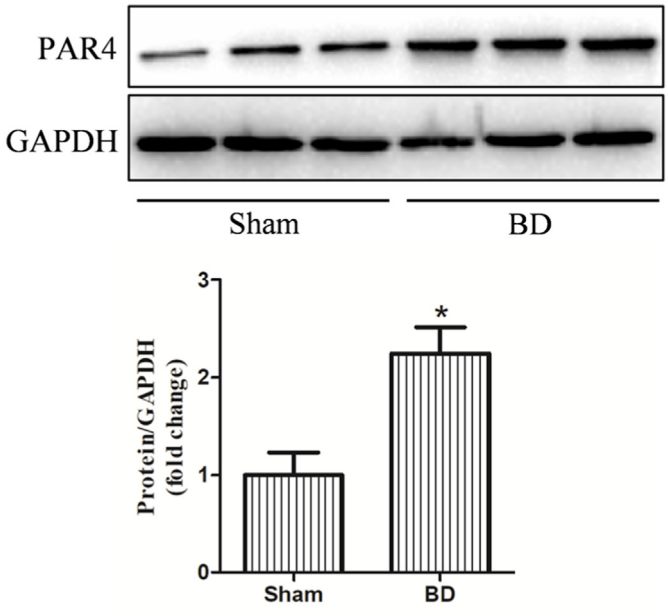

Fig. 1. Expression of hepatic PAR4 protein after BD. Hepatic PAR4 protein was detected by western blotting from rats 6 h after BD and sham operations ($n = 6$ per group), with GAPDH used as loading control. Representative immunoblots were presented. Quantified and normalized results of each group were presented as fold change compared to the sham group. Data are expressed as mean ± SD. *$P < 0.05$ versus Sham group.

All reactions were performed in duplicates. The results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels. The primer sequences are provided in Supplementary Material Table 1.

2.7. Western blotting

Total protein was extracted from liver tissue samples using a phosphorylated protein extraction kit (Solarbio, Beijing, China) according to the manufacturer's protocol. Western blotting were performed as described previously [17]. The primary antibodies were as follows: PAR4 (1:1000, Bioss, Beijing, China), p-IκBα (1:1000, CST, Danvers, MA, USA), p-p65(1:1000, CST, Danvers, MA, USA), p-ERK(1:2000, CST, Danvers, MA, USA), p-JNK(1:1000, CST, Danvers, MA, USA), and p-p38 (1:1000, CST, Danvers, MA, USA), IκBα(1:1000, Proteintech, Wuhan, China), Bcl2 (1:1000, Proteintech, Wuhan, China), Bax (1:5000, Proteintech, Wuhan, China), cl-Caspase-3 (1:1000, Proteintech, Wuhan, China), and GAPDH (1:20000, Proteintech, Wuhan, China). GAPDH was used as a loading control.

2.8. Statistical analysis

Statistical analysis was performed using SPSS for Windows (version 26.0). Data are expressed as mean ± standard deviation. The differences between two groups were assessed using the Student's t-test, while the differences among multiple groups were assessed using ANOVA. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Upregulated PAR4 expression in rat liver after BD

We investigated PAR4 expression in the livers of rats subjected to BD. Western blotting revealed that hepatic PAR4 protein expression in BD rats was significantly upregulated compared to that of sham group rats (Fig. 1).

3.2. PAR4 blockade alleviates liver injury after BD

To investigate the role of PAR4 blockade in liver injury during BD, we used TcY-NH2 (a selective PAR4 antagonist) to treat rats. H&E staining obviously confirmed that congestion and microthrombosis in hepatic sinusoids, inflammatory cell infiltration, hepatocyte vacuolar degeneration and edema, and spotty necrosis in liver sections of BD rats were inhibited with TcY-NH2 treatment (Fig. 2A). Serum ALT and AST levels significantly increased in rats subjected to BD compared to rats subjected to the sham operation.

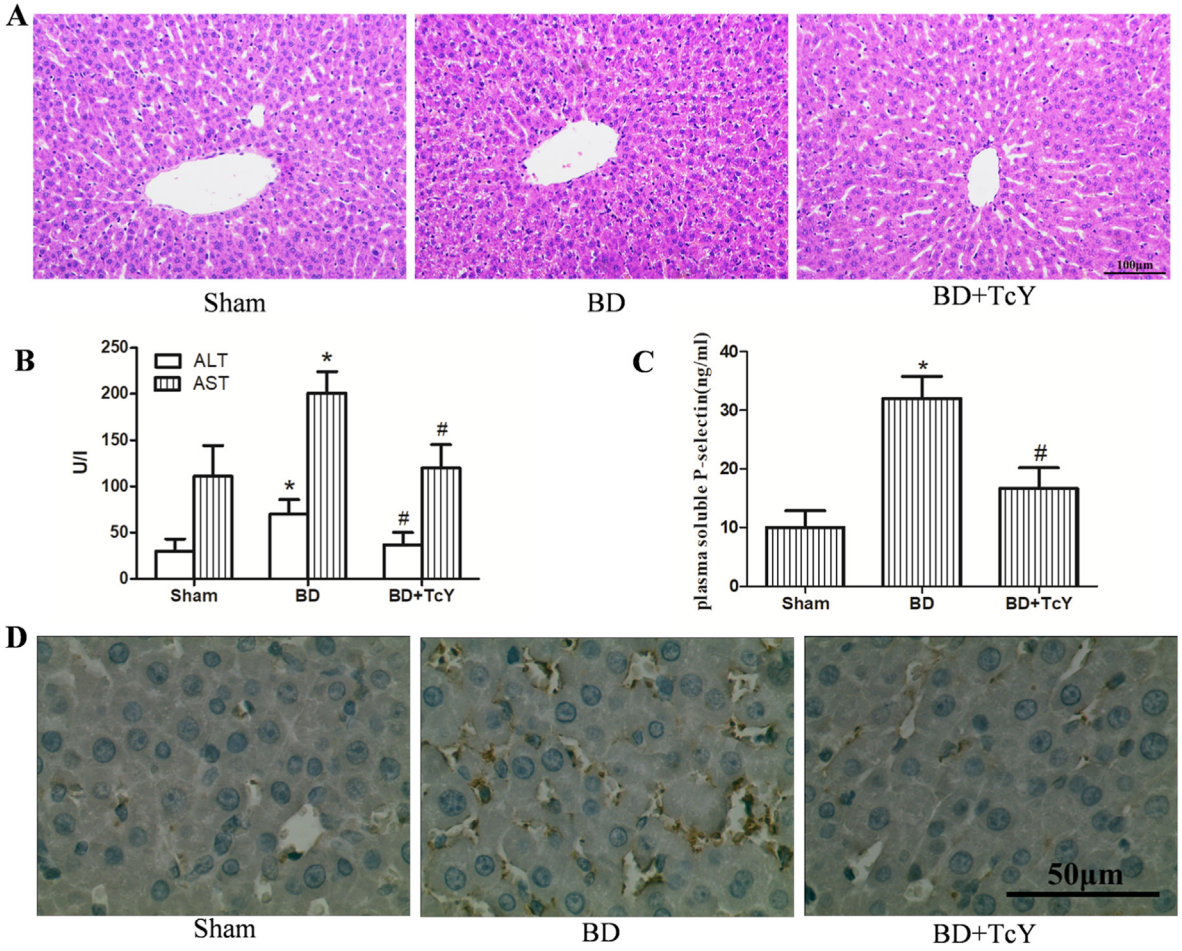

Fig. 2. PAR4 blockade alleviates liver injury and platelet accumulation after BD. (A) Representative H&E staining of paraffin-embedded liver sections of each group. Scale bar, 100 μm. (B) Levels of serum ALT/AST in each group. (C) Plasma levels of soluble P-selectin in each group. (D) Representative CD41 immunohistochemical staining of paraffin-embedded liver sections and counterstained with hematoxylin. Scale bar, 50 μm. Sham group: rats subjected to sham operation; BD group: rats subjected to BD; BD + TcY: rats treated with PAR4 antagonist, TcY-NH2, at the onset of BD induction. Samples were collected 6 h after BD and sham operations ($n = 6$ per group). Data are expressed as mean ± SD. *$P < 0.05$ versus Sham group. #$P < 0.05$ versus BD group.

However, the increased serum ALT and AST levels in rats subjected to BD were significantly decreased by TcY-NH2 treatment (Fig. 2B).

3.3. PAR4 blockade reduces blood platelet activation and hepatic platelet accumulation after BD

Plasma soluble P-selectin levels in TcY-NH2-treated rats were significantly higher in BD rats compared to sham group rats, but was significantly decreased by TcY-NH2 treatment (Fig. 2C). We evaluated platelet accumulation in the hepatic tissue by immunohistochemical staining for CD41 (Fig. 2D). There was a significant increase in platelet accumulation, mostly adjacent to sinusoidal endothelial cells, in the livers of BD rats. TcY-NH2 treatment decreased platelet accumulation in the livers of BD rats.

3.4. PAR4 blockade inhibits inflammatory response in liver after BD

We investigated the effect of PAR4 blockade on the inflammatory response in the liver during BD. Immunohistochemical staining demonstrated a significantly increased number of MPO-positive cells in the livers of BD rats compared to that of sham-operated rats, but was significantly decreased by TcY-NH2 treatment (Fig. 3A and B). The upregulated mRNA expression levels of IL-1β, TNF-α, intercellular adhesion molecule-1 (ICAM-1) and IL-6 were significantly inhibited by PAR4 blockade (Fig. 3C). NF-κB plays a critical role in regulating the expression of many genes involved in inflammatory liver injuries. The activation of NF-κB signaling in the liver of TcY-NH2-treated BD rats was significantly inhibited, accompanied by decreased protein levels of phospho-inhibitor α of NF-κB (p-IκBα) and p-p65, and increased protein levels of IκBα, compared to rats subjected to BD only (Fig. 3D).

3.5. PAR4 blockade suppresses MAPK signaling and apoptosis in liver after BD

We further explored the effect of PAR4 blockade on MAPK signaling and apoptosis in the liver during BD. The protein levels of phospho-extracellular signal-regulated protein kinase (p-ERK), phospho-c-Jun N-terminal kinase (p-JNK), and p-p38 were significantly upregulated in the livers of BD rats compared with sham-operated rats, which was significantly decreased by TcY-NH2 treatment (Fig. 4A). Western blotting demonstrated an upregulated level of anti-apoptotic protein B-cell lymphoma 2 (Bcl2), whereas the levels of proapoptotic proteins Bcl2-associated x (Bax) and cleaved-Caspase3 (cl-Caspase3) decreased in the livers of TcY-NH2-treated BD rats compared with rats subjected to BD only (Fig. 4B). TUNEL staining demonstrated that the number of apoptotic cells significantly increased in the livers of BD rats compared with sham-operated rats, which was significantly decreased by TcY-NH2 treatment (Fig. 4C).

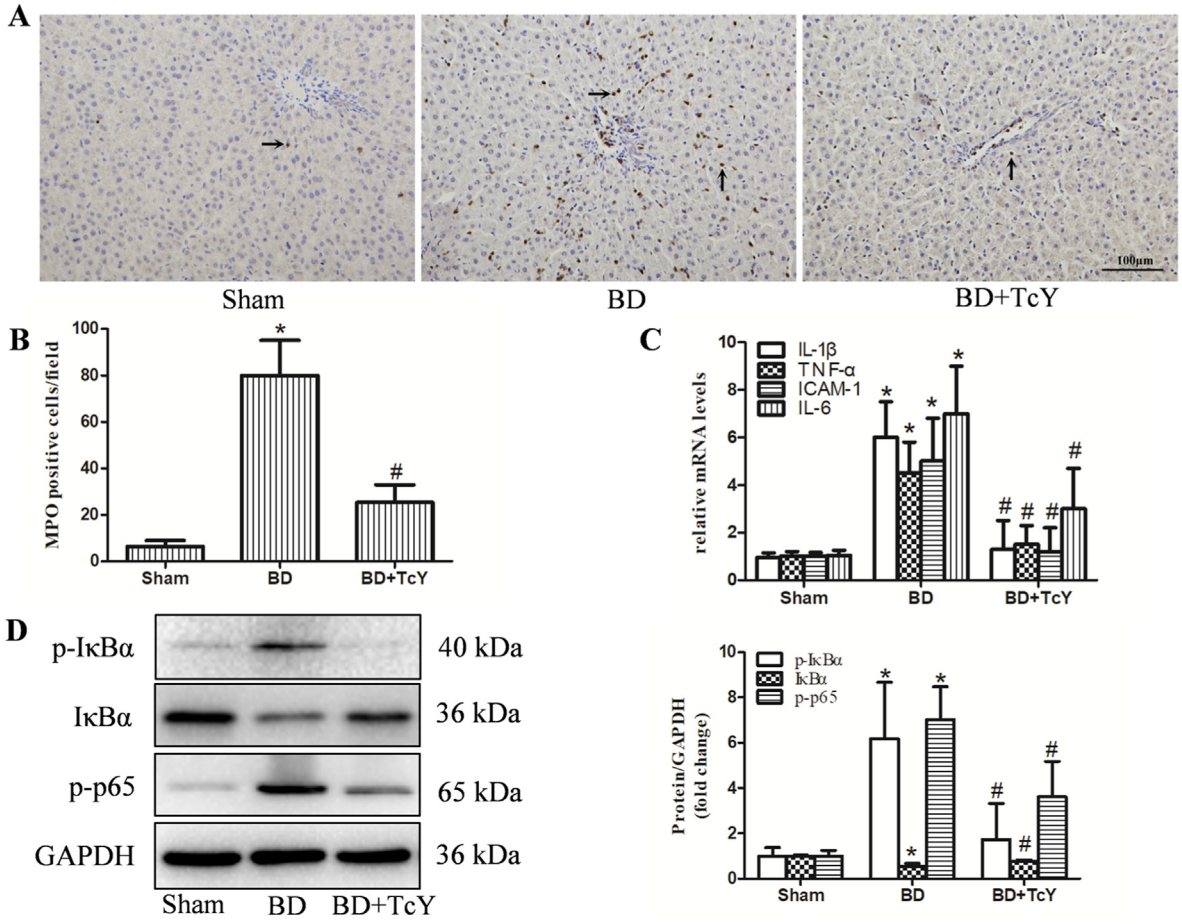

Fig. 3. PAR4 blockade inhibits inflammatory response in liver after BD. (A) Representative MPO immunohistochemical staining of paraffin-embedded liver sections to detect MPO-positive inflammatory cells (arrow) in each group and counterstained with hematoxylin. Scale bar, 100 μm. (B) MPO-positive inflammatory cells were counted. (C) mRNA levels of proinflammatory factors (IL-1β, TNF-α, ICAM-1 and IL-6) in livers of each group. (D) Protein levels of NF-κB signaling molecules (p-IκBα, IκBα and p-p65) in livers of each group. GAPDH was used as loading control. Representative immunoblots were presented. Quantified and normalized results of each group were presented as fold change compared to the sham group. Sham group: rats subjected to sham operations; BD group: rats subjected to BD; BD + TcY: rats treated with PAR4 antagonist, TcY-NH2, at the onset of BD induction. Samples were collected 6 h after BD and sham operation ($n = 6$ per group). Data are expressed as mean ± SD. *$P < 0.05$ versus Sham group. #$P < 0.05$ versus BD group.

4. Discussion

PAR4, expressed in platelets, endothelial cells, leukocytes, and several cell types, plays a vital role in hemostasis and inflammation [11,18,19]. In this study, we observed that PAR4 protein levels were upregulated in the livers of BD rats, which were consistent with previous reports on hearts [12,20]. Our study demonstrated that liver injury induced by BD was attenuated by blocking PAR4 with TcY-NH2, which was reflected by lower serum ALT/AST levels, less inflammatory response, fewer apoptotic cells, and improvement of histomorphology. We found that PAR4 blockade attenuated blood platelet activation and platelet accumulation in the livers of BD rats. Further research revealed that activation of NF-κB and MAPK signaling in the liver of BD rats was also inhibited by PAR4 blockade. Thus, our data demonstrated that PAR4 is a key mediator in the pathological process of liver injury after BD.

This study demonstrated that platelets were activated and accumulated in the liver, mostly adjacent to sinusoidal endothelial cells, during BD. These processes were attenuated by PAR4 blockade. PAR4 is the major thrombin receptor in human and murine platelets and is capable of transducing signals for thrombin-mediated platelet activation in mice [21]. TcY-NH2 treatment inhibited thrombin-induced platelet aggregation *in vitro* [22]. Platelet activation and accumulation may be partly attributed to the thrombin generated by prothrombin activation during BD. There is possible interaction of platelets with sinusoidal endothelial cells during BD due to their close proximity. PAR4 expressed in endothelial cells may also be activated by thrombin during BD. Dysfunction of liver sinusoidal endothelial cells induced by acetaminophen is relieved in PAR4-deficient mice [23]. Platelet activation plays an important role in the development of thrombosis. The procoagulant function of platelets is impaired by PAR4 inhibition during thrombus formation in human blood [24]. We found that congestion and microthrombosis in the hepatic sinusoid after BD could be inhibited by PAR4 blockade. Thus, our study demonstrated the role of PAR4 in platelet activation and accumulation in the liver after BD.

Cytokines and adhesion molecules are the primary outcome in many studies of inflammation after BD. NF-κB signaling in the liver is activated by BD and accompanied by elevated levels of serum IL-1β, TNF-α, and IL-6 [25]. NF-κB is an important nuclear transcription factor in the inflammatory response pathway, with p65 as a

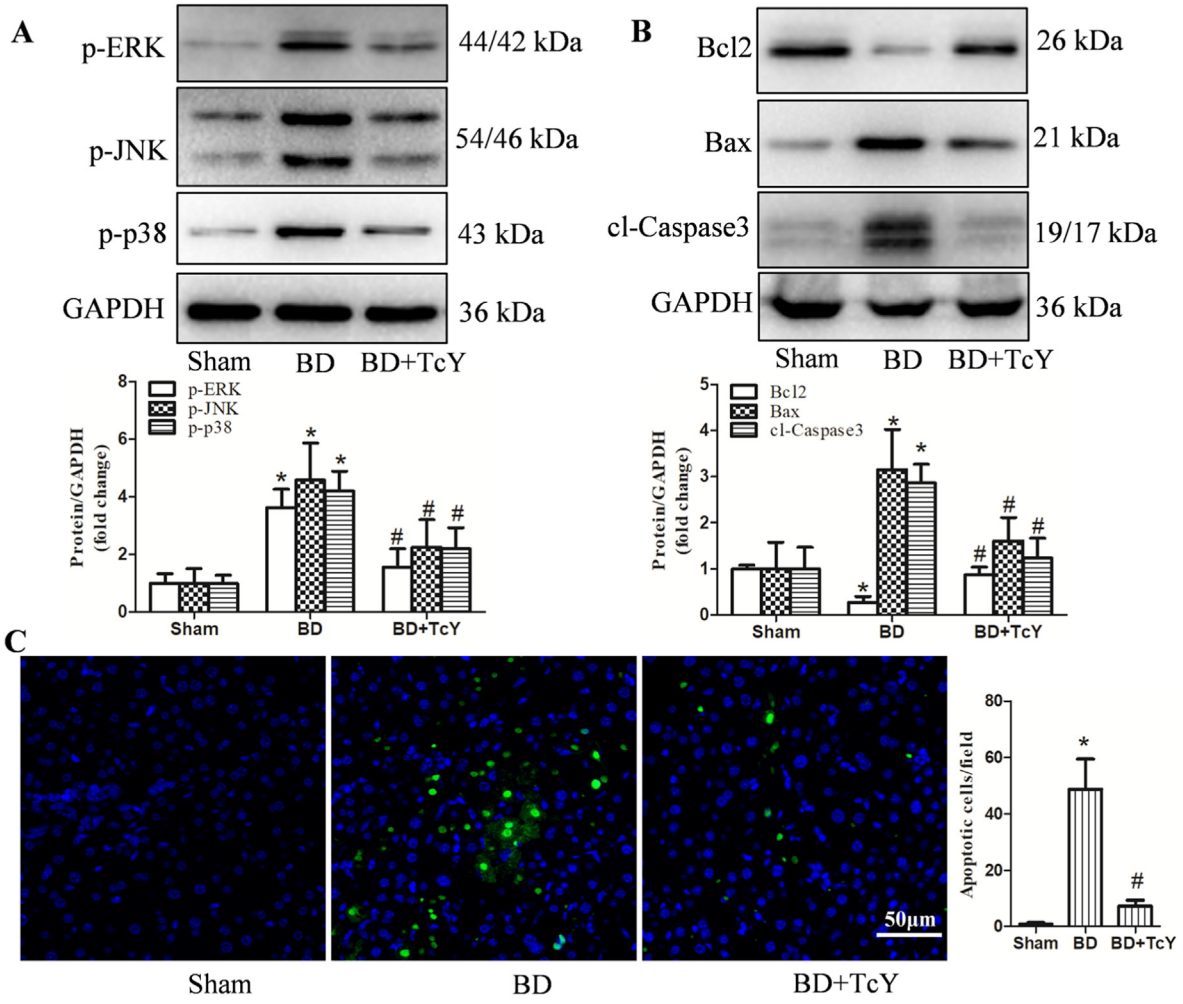

Fig. 4. PAR4 blockade suppresses MAPK signaling and apoptosis in liver after BD. (A–B) Protein levels of MAPK signaling molecules (p-ERK, p-JNK and p-p38) and apoptosis-related molecules (Bcl2, Bax and cl-Caspase3) in livers of each group. GAPDH was used as loading control. Representative immunoblots were presented. Quantified and normalized results of each group were presented as fold change compared to the sham group. (C) TUNEL staining in paraffin-embedded liver sections of each group and quantification of apoptotic cells (green) in each field. Scale bar, 50 μm. Sham group: rats subjected to sham operations; BD group: rats subjected to BD; BD + TcY: rats treated with PAR4 antagonist, TcY-NH2, at the onset of BD induction. Samples were collected 6 h after BD and sham operations ($n = 6$ per group). Data are expressed as mean ± SD. *$P < 0.05$ versus Sham group. #$P < 0.05$ versus BD group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

canonical component [26]. Once IκBα is phosphorylated and degraded, NF-κB is activated and translocated from the cytoplasm to the nucleus. Phosphorylation of p65 enhances its transcriptional activity and this regulates the expression of many inflammatory molecules. During the BD and liver ischemia-reperfusion process, proinflammatory mediators attract leukocytes to the tissue, resulting in aggravated liver injury [2]. Neutrophil infiltration is a key feature of hepatic IRI [27]. The inflammatory response caused by BD can worsen IRI in BD donor liver transplantation. Inhibition of inflammation can effectively attenuate liver injury during BD and improve outcomes after liver transplantation [28,29]. PAR4 inhibition attenuates traumatic brain injury by suppressing NF-κB-mediated neuroinflammation [30]. Similarly, we found that PAR4 blockade reduced the expression of IL-1β, TNF-α, ICAM-1, and IL-6 in the livers of BD rats. PAR4 blockade suppressed NF-κB activation and decreased MPO-positive inflammatory cell infiltration (mainly neutrophils) in the livers of BD rats. Therefore, we speculate that PAR4 activates the NF-κB pathway to promote the expression of inflammatory molecules and recruitment of neutrophils to the liver after BD.

Intriguingly, we observed that the phosphorylation of ERK, JNK, and p38 significantly increased in the livers of BD rats compared to sham-operated rats, but was suppressed by PAR4 blockade. The family of MAPKs includes ERK, JNK, and p38, and plays an important role in proliferation, inflammation, and apoptosis [31]. The JNK and p38 signaling pathways are activated by IL-1β, TNF-α, oxidative stress. p38 signaling is involved in neutrophil chemotaxis and NETosis in sterile liver inflammation [32] and can mediate p65 phosphorylation and TNF-α expression [33]. Our previous research showed that inhibition of JNK activation can attenuate liver injury induced by BD via regulation of Bcl2 and Bax expression [16]. ERK phosphorylation is activated during hepatic ischemia-reperfusion and is associated with neutrophil infiltration and cell death [34]. PAR4 triggers ERK phosphorylation, via $G\alpha_{q/11}$-mediated calcium signaling in HEK-293 cells, and platelet aggregation [35]. Additionally, PAR4 activates the p38 pathway and enhances the inflammatory response in endothelial cells [36]. PAR4 also induces myocyte apoptosis via JNK activation during myocardial IRI [12]. Meanwhile, this study is the first of its kind to demonstrate that PAR4 participates in the activation of MAPK signaling in the livers of BD rats. In general, these data suggest that MAPK signaling participate in inflammatory response and apoptosis induced by BD.

PAR4 is also involved in apoptosis. PAR4 activation induces myocyte apoptosis [12]. PAR4 blockade also reduces apoptosis during hepatic IRI [13]. Consistently, we found that PAR4 blockade inhibited apoptosis in the livers of BD rats. PAR4 blockade downregulated Bax and cl-Caspase3 protein expression and upregulated Bcl2 protein expression in the livers of BD rats. Bax, located in the mitochondrial outer membrane, mediates the release of cytochrome *c* to activate the caspase cascade reaction, which promotes apoptosis. However, upregulated Bcl2 inhibits the apoptotic processes [37]. The involvement of MAPK signaling in regulating the apoptotic pathway has been verified in published reports [26,32,35]. The activation of MAPK signaling in the livers of BD rats was inhibited by PAR4 blockade. Thus, our results suggest that PAR4 blockade suppresses apoptosis in the liver during BD, possibly via inhibiting the MAPK pathway.

In conclusion, this study suggests that PAR4 contributes to liver injury during BD. PAR4 blockade inhibits inflammation and apoptosis induced by BD, possibly by regulating NF-κB and MAPK signaling. The PAR4 blockade approach to prevent organ injury from BD donors seems feasible in the future. However, the mechanisms whereby PAR4 contribute to NF-κB and MAPK signaling activation, and the interactions of NF-κB and MAPK signaling are not entirely clear, needing to further study.

Funding

This study was supported by the Medical Science and Technology Research Project of the Health Commission of Henan Province (Grant No. SBGJ2018024, SB201901045), and the National Natural Science Foundation of China (Grant No.81971881).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.01.074.

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