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Title:
SIKE1 deficiency accelerates hepatic ischemia/reperfusion (IR) injury through enhancing TLR-3-regulated inflammation

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Abstract. Although the pathogenesis of liver ischemia-reperfusion (IR) has been well reported, its molecular mechanism is not fully understood. Suppressor of IKKε (SIKE1) is an interaction partner for Inhibitor of κB Kinase ε (IKKε), a negative modulator of the interferon pathway. In the study, we explored the effects of SIKE1 on liver IR injury in the wild type (WT) and SIKE1-knockout (KO) mice. The findings indicated that after IR operation, reduced SIKE1 expressions were observed in liver of mice. And lipopolysaccharide (LPS) time-dependently decreased SIKE1 mRNA and protein levels in liver cells. Notably, SIKE1-deletion accelerated liver IR injury, as evidenced by the enhanced histological changes. Additionally, IR-induced inflammation was augmented by SIKE1-deficiency through potentiating the secretion of pro-inflammatory cytokines (interleukin (IL)-1β, IL-18, IL-6 and tumor necrosis factor-α (TNF-α)), which was associated with the stronger activation of IKKε, IκBα and nuclear factor κB (NF-κB) in liver of mice. Importantly, after IR operation, SIKE1-knockout aggravated Toll-like receptor (TLR)-3 and its down-streaming signal pathways, including TANK-binding kinase 1 (TBK-1)/IFN regulatory factor (IRF)-3, glycogen synthase kinase-3β (GSK-3β)/AKT/mammalian target of rapamycin (mTOR). The in vitro study using primary hepatocytes with LPS exposure demonstrated that SIKE1-deletion-accelerated inflammation was diminished by TLR-3 knockdown. Together, the data revealed that SIKE1-deficiency intensified hepatic IR injury, and that SIKE1 might be a potential therapeutic target against acute liver injury.

Keywords: hepatic IR injury, SIKE1, inflammation, TLR-3
1. Introduction

Liver ischemia-reperfusion (IR) injury remains the major cause of liver dysfunction and failure after hepatic trauma, resection and transplantation [1,2]. Accordingly, the tissue inflammatory immune response induced by innate immune receptor activation, including TLRs, plays an important role in the pathogenesis of hepatic IR [3,4]. Initially, the tissue damage and stress prompt the secretion or release of danger-related molecular pattern, activating pattern recognition receptors, such as TLR-3/4, to induce secretion of cytokines and chemokine [5]. In the reperfusion stage, inflammatory cytokines drive further hepatocellular injury [6]. Therefore, suppression of inflammation is one of the most effective approaches to attenuate hepatic IR injury.

Suppressor of IKKε (SIKE1), a small coiled-coil domain-containing protein, is comprised of 207 amino-acid residues, and is expressed in most tissues, such as the heart [7]. SIKE1 has been identified as an interaction partner for IKKε. The profound involvement of IKKε in inflammation suggests a potential value against many diseases [8]. In addition, SIKE1 is a negative modulator of interferon pathway through regulating TBK-1-involved signaling, which is closely associated with TLR-3 expression [9]. TLR-3 could induce the production of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, as well as associated chemokine in various cell types [10,11]. However, the functional relevance of SIKE1 expression levels in acute hepatic injury was little to be known.
In the present study, the down-regulated SIKE1 was observed in the liver of mice after IR operation. And also, in vitro, LPS exposure decreased SIKE1 expression from mRNA and protein levels in Kupffer cells and primary hepatocytes. In particular, we found that SIKE1-deletion accelerated hepatic IR injury, accompanied with severer histological alterations, elevated alanine transaminase (ALT), aspartate transaminase (AST) and lactate dehydrogenase (LDH) in serum of mice. And inflammatory response was significantly induced by IR operation in WT mice, whereas the process was worsen by SIKE1-deletion. Importantly, knockout of SIKE1-elevated activity of IKKε/IκBα/NF-κB, TBK-1/IRF-3, and GSK-3β/AKT/mTOR was dependent on TLR-3 expressions. Collectively, our findings indicated that SIKE1 could be considered as a potential therapeutic target against acute liver injury.

2. Materials and methods

2.1. Animals and treatment

30 male wild type (WT) and 30 male SIKE1-knockout (KO) mice on a C57Bl/6 background were obtained from Jacksons Laboratory and housed in a pathogen-free environment. Hepatic IR injury was induced as following described. Briefly, mice were anesthetized using intraperitoneal injection of a 30 mg/kg tiletamine/zolazepam solution containing 10 mg/kg of xylazine. Following a midline laparotomy, the hepatic hilum was cautiously dissected and a micro-vascular clamp was applied to the first branch of the liver artery and portal vein supplying the left lateral and median lobes of the liver. Circulation in the caudal lobes was retained intact to prevent
intestinal venous congestion. The peritoneum was closed with saline soaked sterile gauze to prevent dehydration, and the mice were placed on a heating pad. After hepatic ischemia for 90 min, the micro-vascular clamp was removed, initiating reperfusion, and the abdominal wall was closed using 6-10 nylon sutures. After 1 or 6 h of reperfusion, the mice were anesthetized, whole blood was harvested through retro-orbital puncture, and the liver samples were collected for molecular analyses. Sham-operated controls underwent the same procedure, but without vascular occlusion. All animal procedures were performed in line with the Guide for the Care and Use of Laboratory Animals, issued by the National Institutes of Health in 1996 and approved by the Animal Care and Use Committee of the Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China).

2.2. Cells and culture

Liver kupffer cells in mice were isolated by the improved perfusion digestion method [12]. The primary hepatocytes were isolated from mice following previous study [13]. All cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Hyclone, USA) containing 10% fetal bovine serum (FBS, Hyclone, USA) and 100 U/ml penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO2. The cells were incubated with 100 ng/ml LPS (Sigma Aldrich, USA) for 24 h. The following sequence was targeted for the TLR-3 mRNA to knockdown TLR-3 expression: 5’-TGGACCACGATCAGCTGCCT-3’. The cells were pre-transfected with TLR-3 siRNA using lipofectamine 2000 (Invitrogen, USA) following the manufacturer’s instructions for 24 h, followed by LPS administration for another 24
2.3. Real time-quantitative (RT-q) PCR analysis

Total RNA (2.5 µg) was reverse-transcribed into cDNA using SuperScriptTM III System (Invitrogen, USA). Total 1µg mRNA was reverse transcribed using cDNA RT Kits (Thermo, USA) according to the manufacturer’s protocols. Real-time PCR was carried out with SYBR Green Master Mix (Applied Biosystems, USA) on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems). The cycle threshold (Ct) values were analyzed by using the comparative Ct (ΔΔCt) method. The amount of target was obtained by normalizing to the endogenous reference (GAPDH) and exhibited as the relative to the control. The primers for RT-qPCR test were displayed in Supplementary Table 1.

2.4. Western blot test

Tissue or cellular proteins were extracted using ice-cold lysis buffer (pH 7.4, 1% Triton X-100, 10% glycerol, 0.5% sodium deoxycholate, 137 mM sodium chloride, 0.1% SDS, 20 mM Tris). Proteins (20 µg) were separated by 10-12% SDS–PAGE electrophoresis and transferred to PVDF nitrocellulose membrane. Western blot antibodies include SIKE1, p-AKT, AKT, p-IκBα, p-IKKε, p-NF-κB (p65), NF-κB (p65), TBK-1, p-TBK-1 (Abcam, USA), IRF-3, p-GSK-3β, GSK-3β, p-mTOR, mTOR, TLR-3 (Cell Signaling Technology, USA) and GAPDH (Santa Cruz, USA) and HRP-conjugated secondary antibody (KeyGen Biotech, Nanjing, China). Then, the membranes were developed in enhanced chemiluminescence (ECL, Bio-Rad Corporation, USA) solution. The intensity of the selected bands was captured, and
also exposed to Kodak X-ray film (Fujian, China), and finally analyzed with ImageJ software (version 1.42q, National Institutes of Health, MD).

2.5. ELISA measurement

Cytokine (IL-1β, TNF-α, IL-6 and IL-18) levels in serum were measured according to the manufacturer’s protocols (R&D System, Minneapolis, USA).

2.6. AST, ALT and LDH measurement

Serum ALT, AST, and LDH levels were measured using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions.

2.7. Histochemical analysis

The liver tissues were isolated, and fixed in 10% zinc formalin, embedded in paraffin, sectioned at 4 µm, and then subjected to H&E staining. After removing the paraffin, sections were treated with 0.3% H₂O₂ for 30 min, blocked with 3% bovine serum albumin (BSA) for 30 min at room temperature, and then incubated with p-IKKε, p-NF-κB (p65) and TLR-3 (1:300) at 4°C overnight. After washing three times in PBS, slides were incubated with secondary antibody (1:200; KeyGen Biotech) conjugated with horseradish peroxidase for 30 min at room temperature, and then visualized with diaminobenzidine (DAB) kit (Zsbio, Beijing, China) and nucleuses were counterstained with hematoxylin.

2.8. Statistical analysis

Data are presented as the mean±SEM. from at least three independent experiments. Group comparisons were analyzed using one or two-way ANOVA and Turkey’s post
A value of P<0.05 was considered statistically significant.

3. Results

3.1. The expression of SIKE1 in liver of mice with hepatic IR injury.

To calculate the effects of SIKE1 on acute liver injury, we first measured SIKE1 expression in liver of mice with hepatic IR injury. The WT mice showed significantly decreased SIKE1 expression from gene and protein levels immediately after IR operation for 1 and especially 6 h, which were comparable to the Sham group (Fig. 1A and B). And in vitro, LPS incubation time-dependently reduced SIKE1 expression in Kupffer cells and primary hepatocytes, which was comparable to the Con group (Fig. C-F). Next, the WT and SIKE1-knockout (KO) mice were subjected to hepatic IR injury. After IR operation for 1 and 6 h, H&E staining showed that SIKE1-deletion mice exhibited severer histological alterations along with higher histopathologic liver score compared to the WT/Sham group (Fig. 1G). Additionally, SIKE1 knockout markedly up-regulated serum ALT, AST and LDH levels in mice with hepatic IR injury (Fig. 1H-J). The results above indicated that SIKE1-deficiency accelerated liver IR injury.

3.2. SIKE1-deletion aggregates inflammation in the liver of mice with hepatic IR injury.

Inflammatory response has been well reported to be vital in inducing hepatic IR injury [3-5]. Therefore, we attempted to explore if SIKE1 was participated in the modulation of inflammatory response in mice with liver IR injury. Fig. 2A suggested
that compared to the WT/Sham group, the systematic levels of pro-inflammatory cytokines (IL-1β, IL-18, IL-6 and TNF-α) were higher in mice after IR operation. Significantly, SIKE1-deletion augmented the serum levels of pro-inflammatory cytokines, which was comparable to the WT/Sham group. Consistently, over-expression of IL-1β, IL-18, IL-6 and TNF-α was observed in liver of WT/Sham mice, which was aggregated by SIKE1-knockout through RT-qPCR analysis (Fig. 2B). As expected, protein levels of p-IKKε, p-IκBα and p-NF-κB in liver of WT mice were highly up-regulated after IR operation, and this was markedly accelerated by SIKE1-deficiency (Fig. 2C). A similar trend of p-IKKε, and p-NF-κB was observed by using immunohistochemical analysis (Fig. 2D and E). Thus, SIKE1-knockout enhanced inflammatory response in mice with hepatic IR injury.

3.3. The effects of SIKE1-knockout on TLR-3 signaling pathway.

In this regard, we documented that WT mice after IR injury showed significantly high expression of TLR-3 mRNA and protein levels in comparison to the WT/Sham group, and that SIKE1-deletion further increased this up-regulation (Fig. 3A). Also, the immunohistochemical analysis confirmed that SIKE1-deficiency accelerated liver TLR-3 expression after hepatic IR injury (Fig. 3B). In line with TLR-3 changes, its down-streaming signals of p-TBK-1 and IRF-3 were also expressed highly in liver of mice suffering from liver IR injury, which was augmented by SIKE1-knockout (Fig. 3C). Next, we tested the effects of SIKE1 on GSK-3β/AKT/mTOR pathway. As shown in Fig. 3D and E, IR operation markedly increased the levels of p-GSK-3β, p-AKT and p-mTOR, whereas SIKE1-deletion enhanced this increasing.
3.4. SIKE1-deficiency potentiated inflammation by dependence of TLR-3 expression.

To further verify the effects of SIKE1 on acute liver injury, and to reveal the underlying molecular mechanism, the in vitro study was performed using primary hepatocytes isolated from WT and KO mice. Fig. 4A indicated that compared to the WT/Con group, LPS-exposure stimulated the expression of pro-inflammatory cytokines (IL-1β, IL-18, IL-6 and TNF-α), which was elevated by SIKE1-knockout. Following, high expressions of inflammatory modulators, including (C–X–C motif) ligand (CXCL) 10 (CXCL10), CCL-5, IFN-γ and E-Selectin (ELAM-1), induced by LPS were further elevated in hepatocytes in the absence of SIKE1 (Fig. 4B). The increased p-IKKε, p-IκBα and p-NF-κB triggered by LPS were also potentiated when SIKE1 was deleted (Fig. 4C). In addition, the in vitro study supported that SIKE1-knockout significantly enhanced LPS-induced TLR-3 expression (Fig. 4D). Furthermore, RNA interference of TLR-3 was utilized. Western blot analysis indicated that TLR-3 was successfully knockdown (Fig. 4E). And both LPS stimulation and SIKE1-deletion showed no effects on TLR-3 expression in TLR-3-silenced hepatocytes (Fig. 4F). We also detected that in LPS-incubated cells, TLR-3 knockdown reversed SIKE1-elevated p-TBK-1 and IRF-3 expressions (Fig. 4G and Supplementary Fig. 1A). Also, after TLR-3 knockdown, no significant difference of p-GSK-3β, p-AKT and p-mTOR was observed in WT/LPS and KO/LPS groups (Fig. 4H and Supplementary Fig. 1B). And we found the similar protein levels of p-IKKε, p-IκBα and p-NF-κB stimulated by LPS in TLR-3 knockdown hepatocytes.
with or without SIKE1 expression (Fig. 4I and Supplementary Fig. 1C). Finally, RT-qPCR analysis indicated that TLR-3 silence significantly inhibited SIKE1 knockout-induced elevation of pro-inflammatory cytokines triggered by LPS (Fig. 4J). Together, the findings in vitro demonstrated that SIKE1-regulated inflammation was dependent on TLR-3 pathway.

4. Discussion

Ischemia-reperfusion (IR) injury is the primary driver of acute hepatic dysfunction after elective liver resection and prolonged hemorrhagic shock [1,14]. Reperfusion initiates a complex chain of cytokine-regulated events [15]. SIKE1 is the suppressor of IKKe and TBK1, and acts to inhibit TLR3-regulated IRF3 activation and interferon-stimulated response elements [8,9,16]. TLR-3-regulated inflammatory response has been well reported previously through various signaling pathways [17,18]. In our present study, the effects of SIKE1 on hepatic IR injury using the wild type mice or SIKE1-knockout mice were investigated, as well as the underlying molecular mechanism. Of note, we found that SIKE1 expression was suppressed liver of mice after IR operation. Employing SIKE1-knockout mice, SIKE1-deletion accelerated pathological liver injury, evidenced by the enhanced serum levels of AST, ALT and LDH, essential markers of hepatic injury [19], in compared to the WT mice with IR treatment. Importantly, our mechanistic exploration revealed the importance of inflammation during SIKE1-modulated hepatic IR injury, which was dependent on TLR-3 expressions.

IR activates pro-inflammatory cytokine/chemokine release. As previously
reported, TLRs-dependence of cytokine/chemokine programs are required for inflammation and ultimate tissue damage in liver IR injury immune cascade [20]. Neutrophils accumulate in the liver vasculature responding to the exposure to inflammatory mediators such as TNF-α, IL-1α or IL-1β, CXC chemokines [21,22]. Consistently, the WT mice after IR operation exhibited over-expression of pro-inflammatory cytokines, including IL-1β, IL-18, IL-6 and TNF-α. Additionally, the in vitro analysis indicated that inflammation-associated chemokines (CXCL-10, CCL-5, IFN-γ and ELAM-1) were highly induced by LPS-exposure. CXCL-10 is a down-streaming molecule in inflammation-associated signaling pathway [23]. The significance of activation of pro-inflammatory chemokines, such as CCL-5, has been highlighted in conditions of acute injury [24]. IFN-γ is vital in accelerating the secretion of inflammation-related signals, including CXCL-10 [25]. And ELAM-1 binds to macrophages, playing an important role in regulating inflammatory response [26]. Intriguingly, we detected that SIKE1-deficiency could significantly elevated the generation of these pro-inflammatory cytokines and chemokine.

Under un-stimulated conditions, NF-κB is sequestered in the cytoplasm as an inactive form by interaction with a family of inhibitor proteins known as IκB proteins [27]. While once being activated, IκB-α results in NF-κB translocation to the nuclear and leads to the expression of various pro-inflammatory cytokines [28]. Furthermore, IKKε over-expression could activate NF-κB [29]. In line with the results above, p-IKKε, p-IκBα and p-NF-κB were expressed highly in the liver of mice after IR, supporting the inflammatory response that participated in hepatic IR injury. Of note,
IR-induced phosphorylation of IKKe, IkBa and NF-kB was more prominent in SIKE1-knockout mice. Accordingly, NF-kB could be activated by the TRIF-dependent pathway. TRIF is used by TLR3, leading to NF-kB activation and induction of inflammatory cytokines [30,31]. Our in vitro study indicated that the extremely high expression of p-IKKε, p-IkBα and p-NF-kB induced by SIKE1-deletion was reversed by TLR-3-knockdown. TBK-1 and IRF-3 are important down-streaming signals of TLR-3. Under various disease conditions, IRF-3 could be stimulated by TBK-1 [32,33]. Consistent with previous study, suppressing SIKE1 expression resulted in the increase of p-TBK-1. However, SIKE1-knockout showed no effects on p-TBK-1 and IRF-3 in LPS-stimulated cells with TLR-3-knockdown. Therefore, we supposed that SIKE1-regulated hepatic IR injury might be, at least partly, dependent on TLR-3 expression.

GSK-3β is involved in TLR3-mediated pro-inflammatory cytokine production, including IL-6, TNF-α, and IL-1β [34]. We demonstrated that the activation of GSK-3β was further exacerbated by SIKE1-dificiency in mice after hepatic IR injury. Moreover, GSK-3β has been shown to mediate AKT in different diseases, such as cardiac injury [35]. And given that mTOR is a downstream target of AKT, we also explored if SIKE1 expression showed effects on AKT/mTOR pathway [36,37]. Consistent with GSK-3β changes, here SIKE1-deletion aggravated the enhanced AKT and mTOR activity after hepatic IR challenge, which was in line with previous study. Also, in TLR-3 knockdown cells, SIKE1-knockout exhibited no influences on AKT/mTOR, further indicated that SIKE1-regulated liver IR injury was tightly linked
to TLR-3 expression.

In conclusion, our study verified that SIKE played an essential role in regulating hepatic IR injury in mice. SIKE1-deletion elevated inflammatory response through aggravating IKKε/IκBα/NF-κB and GSK3β/AKT/mTOR pathways, which was dependent on TLR-3 expression. The results illustrated that SIKE1 could be a therapeutic target in acute liver injury. However, further study in future is still necessary to comprehensively reveal the connection among the signals mentioned above, and to verify if there are any other underlying mechanisms that were not found here.

References


Figure legends

Figure 1. The expression of SIKE1 in liver of mice with hepatic IR injury. (A,B) The WT mice were subjected to hepatic IR injury, followed by RT-qPCR and western blot analysis of SIKE1, respectively. (C,D) Kupffer cells were incubated with LPS (100 ng/ml) for the indicated time, followed by RT-qPCR and western blot assays of SIKE1, respectively. (E,F) The primary hepatocytes isolated from WT mice were incubated with 100 ng/ml LPS for different time, and then RT-qPCR and western blot analysis were used to measure SIKE1 mRNA and protein levels. WT and SIKE1-knockout (KO) mice were subjected to IR injury. And after IR operation for 1 or 6 h, further researches as described below were performed. (G) H&E staining of liver isolated form each group. (H) ALT, (I) AST and (J) LDH in serum were measured. Results are represented as mean ± SEM. *P<0.05, **P<0.01 and ***p<0.001 versus the Con group or WT/Sham group; +P<0.05 and ++P<0.01. n.s.: no significant difference.

Figure 2. SIKE1-deletion aggregates inflammation in the liver of mice with hepatic IR injury. (A) Serum IL-1β, IL-18, IL-6 and TNF-α levels were measured using ELISA methods. (B) RT-qPCR analysis of IL-1β, IL-18, IL-6 and TNF-α in
liver of mice from each group. (C) Western blot analysis was used to determine p-IKKε, p-IκBα and p-NF-κB levels in hepatic of mice. The immunohistochemical analysis of (D) p-IKKε, and (E) p-NF-κB in the liver tissue sections from each group of mice. Results are represented as mean ± SEM. * P<0.05, ** P<0.01 and *** p<0.001 versus the WT/Sham group; +P<0.05, ++P<0.01 and +++P<0.01.

Figure 3. The effects of SIKE1-knockout on TLR-3 signaling pathway. (A) RT-qPCR and western blot analysis were performed to evaluate liver TLR-3 mRNA and protein levels. (B) Immunohistochemical analysis of TLR-3 expression levels in hepatic section of mice. (C) The expressions of liver p-TBK-1 and IRF-3 from the indicated groups of mice were measured using western blot analysis. (D) Western blot analysis of p-GSK-3β. (E) The immunoblotting analysis of p-AKT, and p-mTOR. Results are represented as mean ± SEM. * P<0.05, ** P<0.01 and *** p<0.001 versus the WT/Sham group; +P<0.05, ++P<0.01 and +++P<0.01.

Figure 4. SIKE1-deficiency-intensified inflammation is dependent on TLR-3 expression. Primary hepatocytes were isolated from the WT or SIKE1-KO mice, and then were incubated with 100 ng/ml LPS for 24 h for the following study. (A) RT-qPCR analysis of IL-1β, IL-18, IL-6 and TNF-α. (B) RT-qPCR analysis of CXCL-10, CCL-5, IFN-γ and ELAM-1. (C) p-IKKε, p-IκBα and p-NF-κB protein levels were calculated using western blot analysis. (D) Western blot analysis of TLR-3. (E) TLR-3 expression was knockdown by transfecting with its specific siRNA sequence for 24 h, followed by TLR-3 examination using western blot analysis. Next, the primary hepatocytes were pre-treated with TLR-3 siRNA for 24 h, followed by
LPS exposure for another 24 h. And the studies below were carried out. (F,G) Western blot analysis of TLR-3, p-TBK-1 and IRF-3. (H) p-GSK-3β, p-AKT and p-mTOR protein expression levels were calculated using immunoblotting assay. (I) Western blot analysis of p-IKKε, p-IκBα and p-NF-κB. (J) RT-qPCR analysis of IL-1β, IL-18, IL-6 and TNF-α. Results are represented as mean ± SEM. * P<0.05, ** P<0.01 and *** p<0.001 versus the WT/Con group; + P<0.05 and ++ P<0.01.
Figure 1

Figure 2
Figure 3
Figure 4
Highlights

- SIKE1-deficiency accelerated liver ischemia reperfusion injury of mice.
- SIKE1-deficiency potentiated inflammation by dependence of TLR-3 expression.
- SIKE1-deletion aggregates inflammation in the liver of mice with hepatic IR injury.
- The crosstalk of SIKE1 and TLR3 synergistically influence hepatic IR injury related signaling pathway.