Hepatoprotective effects of sevoflurane against hepatic ischemia-reperfusion injury by regulating microRNA-124-3p-mediated TRAF3/CREB axis

Yi-Liang Wang¹, Ying Zhang² and Da-Sheng Cai¹✉

© The Author(s) 2022

The purpose of the present study is to define the role of sevoflurane (SEV) in hepatic ischemia-reperfusion (I/R) injury as well as its underlying mechanism. Initially, hepatic I/R animal models and I/R hepatocyte models were established in C57BL/6 mice and normal mouse hepatocytes (BNL CL.2) after SEV preconditioning, respectively, followed by detection of microRNA-124-3p (miR-124-3p), TRAF3, and CRED expression by RT-qPCR and Western blot analysis. In addition, miR-124-3p, TRAF3 and CRED expression in hepatocytes was altered to identify their roles in modulating the levels of glutathione transferase (GST), aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and inflammation-related factors and in hepatic apoptosis by ELISA and flow cytometry respectively. The effects of SEV on the miR-124-3p/TRA3/CRED axis were also verified in vitro and in vivo. IP assay was performed to verify the effect of TRAF3 on CRED ubiquitination in BNL CL.2 cells, and the cycloheximide (CHX) intervention experiment to detect the stability of CRED protein. SEV augmented the miR-124-3p expression in I/R animal and cell models. Moreover, SEV was observed to suppress I/R-induced liver damage, GST, ALT, and AST levels, hepatic apoptosis and inflammation. Overexpression of miR-124-3p resulted in alleviation of hepatic I/R injury, which was countered by TRAF3 overexpression. miR-124-3p targeted TRAF3, while TRAF3 promoted CRED ubiquitination and reduced protein stability of CRED. SEV could impede I/R-induced liver damage, GST, ALT, and AST levels, hepatic apoptosis and inflammation via mediation of the miR-124-3p/TRA3/CRED axis in vitro and in vivo. Collectively, SEV may upregulate miR-124-3p to inhibit TRAF3 expression, thereby reducing the ubiquitination and degradation of CRED, alleviating hepatic I/R injury.

Cell Death Discovery (2022) 8:105; https://doi.org/10.1038/s41420-021-00784-7

INTRODUCTION

Hepatic ischemia-reperfusion (I/R) injury is a common occurrence following blood supply restoration after blood vessel occlusion during the course of countless liver surgeries [1]. Furthermore, hepatic I/R injury is recognized as the leading cause of abnormal liver function and hepatic failure, whereas there are only a limited number of options to effectively tackle this major complication of liver surgery [2]. In addition, hepatic I/R injury has long been a major cause of liver dysfunction and failure after surgical procedures [3, 4]. Besides, hepatic I/R injury implicates a persistent pro-oxidant and pro-inflammatory mechanisms that result in hepatocyte dysfunction and inflammatory or apoptotic responses [5, 6]. Notably, pretreatment with sevoflurane (SEV) has been reported to protect liver tissues against I/R injury, as well as confer protection to hepatocytes against I/R-triggered necrosis [7]. However, the molecular mechanisms of SEV underlying the highly-beneficial hepatoprotection remain largely unknown.

Recently, studies have uncovered the association between microRNAs (miRNAs) and liver protection conferred by anesthetic preconditioning in hepatic I/R injury [8]. miRNAs are small non-coding RNAs endogenously expressed to serve the function of posttranscriptional gene expression mediation [9]. For example, miR-182-5p has been proposed to suppress liver damage and inflammation by targeting TLR4 in an animal model of hepatic IR injury and in a cell model of lipopolysaccharide-induced inflammation [10]. Interestingly, prior evidence has also suggested that miR-124-3p was downregulated in hepatic I/R injury, whereas its overexpression was closely-related to arrested inflammation in vivo and in vitro [11]. Furthermore, another study has indicated that the mechanism underlying the SEV hepatoprotection function may be associated with the upregulation of said miR-124-3p [12]. Initial bioinformatics analysis in the current study further identified the necrosis factor (TNF) receptor-associated factor 3 (TRA3) gene as a putative target of miR-124-3p. TRAF3 has been known to be mediated by and contributes to multiple ubiquitination actions, and plays a wide range of regulatory roles in inflammatory reactions [13]. The evidence presented by Wang et al. demonstrated that TRAF3 was highly-expressed in human liver samples and animal liver with hepatic steatosis, and its interaction with TAK1 contributed to the modulation of ubiquitination in hepatocytes [14]. Furthermore, TRAF3 has also been noted to repress the stability of CAMP-responsive element-binding protein 3 (CREB3), as well as to augment the process of CREB ubiquitination [15]. Moreover, CREB upregulation has also been
previously demonstrated to protect the brain from I/R injury in vivo [16]. Therefore, we hypothesized that miR-124-3p/TRA3F3-CREB axis participates in the protective effects of SEV against hepatic IR injury.

RESULTS

SEV promotes miR-124-3p expression to alleviate hepatic I/R injury

It has been documented that SEV pretreatment could repress I/R injury by protecting live tissues and conferring protection to hepatocytes against I/R-triggered necrosis [7]. Moreover, the mechanism underlying the SEV hepatoprotection function may be associated with the upregulation of miR-124-3p [11]. In addition, miR-124-3p was downregulated in hepatic I/R injury [11]. Therefore, C57BL/6 mice were employed to establish hepatic I/R models after SEV intervention. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) demonstrated that compared with sham-operated mice, I/R mice exhibited decreased miR-124-3p expression, whereas I/R mice treated with SEV presented with elevated miR-124-3p expression relative to I/R mice (Fig. 1A). Enzyme-linked immunosorbent assay (ELISA) showed that serum levels of glutathione transferase (GST), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were higher in I/R mice than in sham-operated mice, while further SEV treatment reduced GST, ALT, and AST levels significantly in the I/R mice, which suggested alleviated liver injury (Fig. 1B). As shown by hematoxylin and eosin (HE) staining and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining, I/R in mice displayed more severe liver tissue injury and increased hepatocyte apoptosis compared to sham-operated mice, which was abrogated by SEV treatment (Fig. 1C, D).

Subsequently, ELISA and RT-qPCR results further demonstrated that necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 levels in serum and liver tissues of I/R mice were notably higher than that in sham-operated mice, whereas a significant reduction in the aforementioned indicators was observed in I/R mice treated with SEV compared to I/R mice (Fig. 1E, F). Moreover, Western blot analysis determined that relative to sham-operated mice, I/R mice displayed an increase in cleaved-caspase-3 and Bcl-2-associated X protein (Bax) expression as well as a decrease in B-cell lymphoma 2 (Bcl-2) expression, whereas all the said changes were reversed by SEV treatment (Fig. 1G and Supplementary Fig. 1A).

SEV inhibits I/R Injury of hepatocytes via upregulation of miR-124-3p

To determine whether SEV could inhibit I/R injury of hepatocytes by promoting expression of miR-124-3p, I/R cell models were induced on BNL CL.2 (mouse fetal hepatocytes) using H2O2, miR-124-3p expression was increased with the increasing concentration of H2O2, and stabilized at the concentration of 200 μM (Supplementary Fig. 2A).

RT-qPCR revealed that H2O2 treatment led to decreased miR-124-3p expression, which could be elevated by further treatment with SEV or miR-124-3p mimic. However, SEV + miR-124-3p inhibitor brought about a marked decline in miR-124-3p expression compared with SEV + NC inhibitor (Fig. 2A). In addition, ELISA showed that serum levels of GST, ALT, and AST were increased after H2O2 treatment, and could be reversed by further treatment with SEV or miR-124-3p mimic. Moreover, SEV + miR-124-3p inhibitor reversed the decreased serum levels of GST, ALT, and AST caused by treatment with SEV in H2O2-treated hepatocytes (Fig. 2B). Flow cytometry further demonstrated elevated apoptosis rate in hepatocytes solely subjected to H2O2 treatment, which was negated following additional treatment with SEV or miR-124-3p mimic. In contrast to treatment of SEV + NC inhibitor, H2O2-treated hepatocyte apoptosis was elevated in response to treatment with SEV + miR-124-3p inhibitor (Fig. 2C).

Moreover, ELISA and RT-qPCR results displayed that TNF-α, IL-1β, and IL-6 levels were all elevated in the supernatant of H2O2-treated hepatocytes and in H2O2-treated hepatocytes, which was counteracted by further treatment with SEV or miR-124-3p mimic. Meanwhile, in the presence of SEV, miR-124-3p inhibitor treatment enhanced TNF-α, IL-1β, and IL-6 levels in the supernatant of H2O2-treated hepatocytes and in H2O2-treated hepatocytes (Fig. 2D, E). Meanwhile, H2O2 treatment resulted in an increase in cleaved-caspase-3 and Bax expression, while decreasing that of Bcl-2, which was neutralized by further treatment with SEV or miR-124-3p mimic. SEV + miR-124-3p inhibitor further reversed changes caused by treatment with SEV in H2O2-treated hepatocytes (Fig. 2F).

miR-124-3p targets and downregulates TRAF3, thus inhibiting H2O2-induced hepatocyte apoptosis and release of inflammatory factors

The downstream target genes of miR-124-3p were predicted using online bioinformatics databases, microT, starBase, mirDB, and TargetScan. The prediction results reared a total of 372, 3281, 261, and 750 genes respectively, while 111 intersecting genes were obtained from these four prediction results (Fig. 3A). The co-expression network of the 111 candidate target genes was then obtained using the Coexpedia website (Fig. 3B), among which 30 genes exhibited a high score in this network (score >22). The differential expression of the aforementioned 30 genes in the GSE50884 dataset was then determined, and highly-expressed TRAF3 was found in I/R, with the most significant p value (Fig. 3C). The starBase website further predicted the presence of binding sites between miR-124-3p and TRAF3 (Fig. 3D), and dual-luciferase reporter gene assay demonstrated that miR-124-3p mimic failed to bring about significant change in the luciferase activity of mutant type (MUT) TRAF3, but notably diminished the luciferase activity of wild type (WT) TRAF3 (Fig. 3E). miR-124-3p mimic contributed to increased miR-124-3p expression, but decreased TRAF3 expression, indicating that miR-124-3p could target and inhibit the TRAF3 gene (Fig. 3F and Supplementary Fig. 1B).

Next, miR-124-3p mimic increased miR-124-3p expression but reduced that of TRAF3, whereas oe-TRAF3 brought about an increase in TRAF3 expression. However, treatment with miR-124-3p mimic + oe-TRAF3 resulted in a marked increase in TRAF3 expression in BNL CL.2 cells relative to the treatment of miR-124-3p mimic + oe-NC (Fig. 3G and Supplementary Fig. 1C). ELISA results illustrated decreases in GST, ALT, and AST serum levels in the supernatant of hepatocytes following treatment with miR-124-3p mimic, whereas increases in GST, ALT, and AST serum levels were observed after treatment with oe-TRAF3. Relative to treatment with miR-124-3p mimic + oe-NC, GST, ALT, and AST serum levels were found to be increased in treatment with miR-124-3p mimic + oe-TRAF3 (Fig. 3H). Meanwhile, flow cytometric data demonstrated that miR-124-3p mimic led to a decrease in hepatocyte apoptosis, but oe-TRAF3 showed an opposite trend, whereas miR-124-3p mimic + oe-TRAF3 led to increased hepatocyte apoptosis relative to miR-124-3p mimic + oe-NC (Fig. 3I). ELISA and RT-qPCR results demonstrated that TNF-α, IL-1β, and IL-6 levels were all declined in hepatocyte supernatant and hepatocytes upon miR-124-3p mimic, which was normalized by TRAF3 overexpression. Meanwhile, miR-124-3p mimic + oe-TRAF3 brought about increased TNF-α, IL-1β, and IL-6 levels in hepatocyte supernatant and hepatocytes relative to miR-124-3p mimic + oe-NC (Fig. 3J, K). Furthermore, Western blot analysis demonstrated that miR-124-3p mimic resulted in decreases in cleaved-caspase-3 and Bax in expression, as well as an increase in expression of Bcl-2, which could all be abolished by TRAF3 overexpression. Elevated cleaved-caspase-3 and Bax expression, as well as reduced expression of Bcl-2 was detected upon miR-124-3p mimic + oe-TRAF3 relative to miR-124-3p mimic + oe-NC (Fig. 3L and Supplementary Fig. 1D).
Fig. 1  SEV promotes the expression of miR-124-3p and alleviates hepatic I/R injury in mice. A The expression of miR-124-3p in liver tissues of sham-operated mice, I/R mice or I/R mice treated with SEV detected by RT-qPCR. B Serum levels of GST, ALT, and AST in sham-operated mice, I/R mice or I/R mice treated with SEV detected by ELISA. C Liver tissue injury in sham-operated mice, I/R mice or I/R mice treated with SEV determined by HE staining (200×). D Hepatocyte apoptosis in sham-operated mice, I/R mice or I/R mice treated with SEV detected by TUNEL staining (200×). E Serum levels of TNF-α, IL-1β, and IL-6 in sham-operated mice, I/R mice or I/R mice treated with SEV detected by ELISA. F The expression of TNF-α, IL-1β, and IL-6 in liver tissues of sham-operated mice, I/R mice or I/R mice treated with SEV detected by RT-qPCR. G The protein expression of Bax, Bcl-2, and cleaved-caspase-3 in liver tissues of sham-operated mice, I/R mice or I/R mice treated with SEV detected by Western blot analysis. *p < 0.05 (n = 8).
TRAF3 negatively regulates CREB expression through ubiquitination, thus promoting H₂O₂-induced hepatocyte apoptosis and release of inflammatory factors

In order to further explore the downstream regulatory factors of TRAF3, the GPS-Prot interaction network of TRAF3 (Fig. 4A) was retrieved using the GeneCards database. The results showed that there was a total of 279 hepatic I/R injury-related genes. Using the Jvenn tool, 12 intersecting genes were found interacting with TRAF and correlated with hepatic I/R injury (Fig. 4B).

TRAF3 has been exhibited to accelerate the process of CREB ubiquitination [15]. A prior study manifested the participation of CREB upregulation in repression of hepatic I/R injury [17]. Results showed that TRAF3 expression was notably increased, while the CREB protein expression was markedly decreased by oe-TRAF3, along with insignificantly increased CREB mRNA expression (Fig. 4C, D and Supplementary Fig. 1E). Myc-CREB, Myc-CREB + hemagglutinin (HA)-ubiquitin (Ub), or Myc-CREB + HA-Ub + Flag-TRAF3 were subsequently transfected into BNL CL.2 hepatocytes, and Myc-CREB + HA-Ub + Flag-TRAF3 was used in immunoprecipitation (IP) assay to detect the ubiquitination modification of CREB. Relative to Myc-CREB, Myc-CREB + HA-Ub resulted in upregulated ubiquitination level of CREB, which were further increased upon Myc-CREB + HA-Ub + Flag-TRAF3. Meanwhile, Myc-CREB or Myc-CREB + Flag-TRAF3 was transfected into BNL CL.2 hepatocytes, and the interaction between CREB and TRAF3 was detected with IP assay using Myc. TRAF3 bands were then detected in the presence of Myc-CREB + Flag-TRAF3, indicating that CREB could interact with TRAF3 (Fig. 4E). The results displayed that the degradation rate of CREB was increased following oe-TRAF3 treatment (Fig. 4F and Supplementary Fig. 1F). Then, oe-TRAF3 led to increased expression of TRAF3, but reduced CREB protein expression in H₂O₂-treated BNL CL.2 cells, along with unchanged CREB mRNA expression. However, a combination of oe-TRAF3 and oe-CREB resulted in a marked elevation of CREB protein expression with no significant changes in TRAF3 expression and CREB mRNA expression when compared to the treatment with oe-TRAF3 (Fig. 4G and Supplementary Fig. 1G). In addition, ELISA demonstrated that GST, ALT, and AST serum levels were elevated by oe-TRAF3, and could be reversed by additional transfection with oe-CREB (Fig. 4H). Additionally, flow cytometry showed that oe-TRAF3 led to augmented hepatocyte apoptosis, which was rescued by additional treatment oe-CREB (Fig. 4I). ELISA and RT-qPCR results revealed that oe-TRAF3 resulted in elevated TNF-α, IL-1β, and IL-6 levels in hepatocyte supernatant and hepatocytes, which was neutralized by further CREB overexpression (Fig. 4J). Western blot analysis results displayed that oe-TRAF3 brought about an increase in cleaved-caspase-3 and Bax expression and decreased that of Bcl-2. However, these changes were reversed in entirety following oe-TRAF3 + oe-CREB treatment (Fig. 4K and Supplementary Fig. 1H).
SEV orchestrates miR-124-3p/TRAF3/CREB axis to inhibit H2O2-induced hepatocyte apoptosis and release of inflammatory factors.

Results illustrated reduced miR-124-3p expression and CREB expression, while increased TRAF3 expression in H2O2-treated hepatocytes, which was normalized by further SEV treatment. Moreover, in the presence of SEV, miR-124-3p inhibitor led to decreased miR-124-3p and CREB expression, whereas increased TRAF3 expression in H2O2-treated hepatocytes (Fig. 5A). In addition, in the presence of SEV, miR-124-3p mimic treatment elevated the CREB expression in H2O2-treated hepatocytes (Supplementary Fig. 2B). Treatment of SEV + miR-124-3p inhibitor + oe-CREB led to elevated CREB expression in H2O2-treated hepatocytes compared with treatment with SEV + miR-124-3p inhibitor + oe-NC (Fig. 5A).

In addition, ELISA depicted that ALT, AST, and GST levels were increased by SEV + miR-124-3p inhibitor in H2O2-treated hepatocytes, whereas oe-CREB declined the said levels in H2O2-treated hepatocytes in the presence of SEV + miR-124-3p inhibitor (Fig. 5B). Flow cytometric data demonstrated that hepatocyte apoptosis was notably increased upon SEV + miR-124-3p inhibitor in H2O2-treated hepatocyte, which was reversed by SEV + miR-124-3p inhibitor + oe-CREB (Fig. 5C). In addition, ELISA and RT-qPCR documented that TNF-α, IL-1β, and IL-6 levels were all markedly
CREB protein stability after CHX intervention. TRAF3 + presence of oe-TRAF3 or oe-TRAF3 detected by RT-qPCR and Western blot analysis. TRAF3 or oe-TRAF3 treatment, SEV treated hepatocytes, which was then abrogated in the presence of H2O2. miR-124-3p inhibitor contributed to increased expression of cleaved-caspase-3 and Bax and decreased expression of Bcl-2, whereas SEV brought about the decreased expression of Bcl-2. Meanwhile, ELISA and RT-qPCR showed that TNF-α, IL-1β, and IL-6 levels in the serum and liver tissues of mice were notably diminished in liver tissues of I/R mice than in sham-operated mice, which was reduced upon SEV treatment. However, SEV alleviated the said liver tissue injury and apoptosis caused by I/R modeling. However, SEV + sh-CREB counteracted the changes brought about by SEV (Fig. 6C, D).

Fig. 4 TRAF3 attenuates the expression of CREB through ubiquitination, thus promoting the H2O2-induced hepatocyte apoptosis and release of inflammatory factors. A The GPS-Prot interaction network of TRAF3 in the GeneCards database. B The Venn map of the 12 intersecting genes interacted with TRAF and related to hepatic I/R injury. C The mRNA expression of TRAF3 and CREB in the presence of oe-TRAF3 or oe-TRAF3 + oe-CREB detected by RT-qPCR. D The protein expression of TRAF3 and CREB in the presence of oe-TRAF3 or oe-TRAF3 + oe-CREB detected by Western blot analysis. E The effects of TRAF3 on CREB ubiquitination detected by IP assay. F The effects of TRAF3 on CREB protein stability after CHX intervention. G The expression of CREB and TRAF3 in hepatocytes in the presence of oe-TRAF3 or oe-TRAF3 + oe-CREB detected by RT-qPCR and Western blot analysis. H Serum levels of GST, ALT, and AST in hepatocyte supernatant in the presence of oe-TRAF3 or oe-TRAF3 + oe-CREB detected by ELISA. I Hepatocyte apoptosis in the presence of oe-TRAF3 or oe-TRAF3 + oe-CREB detected by flow cytometry. J The expression of TNF-α, IL-1β, and IL-6 in hepatocyte supernatant and hepatocytes in the presence of oe-TRAF3 or oe-TRAF3 + oe-CREB detected by ELISA and RT-qPCR. K The protein expression of Bax, Bcl-2, and cleaved-caspase-3 in hepatocytes in the presence of oe-TRAF3 or oe-TRAF3 + oe-CREB detected by Western blot analysis. *p < 0.05. The cell experiment was repeated three times.

Table 1

| Condition         | ALT Activity (IU/L) | AST Activity (IU/L) | GST Activity (IU/L) |
|-------------------|---------------------|---------------------|---------------------|
| oe-NC             | 1000                | 2000                | 3000                |
| oe-TRAF3          | 1500                | 2500                | 3500                |
| oe-TRAF3 + oe-CREB| 2000                | 3000                | 4000                |

Table 2

| Condition         | Relative Expression of TRAF3 | Relative Expression of CREB |
|-------------------|-------------------------------|-----------------------------|
| oe-NC             | 0.5                           | 1.0                         |
| oe-TRAF3          | 2.0                           | 1.5                         |
| oe-TRAF3 + oe-CREB| 2.5                           | 2.0                         |

Table 3

| Condition         | Relative Protein Expression of TRAF3 | Relative Protein Expression of CREB |
|-------------------|--------------------------------------|-------------------------------------|
| oe-NC             | 0.5                                  | 1.0                                 |
| oe-TRAF3          | 2.0                                  | 1.5                                 |
| oe-TRAF3 + oe-CREB| 2.5                                  | 2.0                                 |

SEV manipulates miR-124-3p/TRAF3/CREB axis to alleviate the hepatic I/R injury in mice

It was found that compared with sham-operated mice, miR-124-3p and CREB expressions were significantly diminished in liver tissues of I/R mice in addition to increased expression of TRAF3, which was reversed by SEV treatment. SEV + sh-CREB enhanced CREB expression in contrast to SEV + sh-control (Fig. 6A). ELISA data displayed that ALT, AST, and GST levels were dramatically higher in I/R mice than in sham-operated mice, which was reduced upon SEV. Relative to SEV + sh-control treatment, the ALT, AST, and GST levels in the serum and liver tissues of mice were notably diminished in liver tissues of I/R mice than in sham-operated mice, which was reduced upon SEV.
opposite findings in I/R mice, which was neutralized by SEV + sh-CREB (Fig. 6G and Supplementary Fig. 1J).

DISCUSSION

I/R injury is one of the leading causes of liver damage occurring during hepatic resection and transplantation, significantly contributing to liver dysfunction and post-transplantation failure [18]. Prior evidence has indicated that the prevention of SEV preconditioning against hepatic I/R injury was closely associated with miRNAs along with their downstream signaling axis [8]. Therefore, in this study, our data clarified that miR-124-3p confers hepatoprotective effects against hepatic I/R injury via the TRAF3/CREB axis.

Firstly, our findings revealed that SEV preconditioning rescued the down-regulation of I/R-induced miR-124-3p in mouse and cell models. Furthermore, the I/R-induced liver damage, in addition to...
serum levels of GST, ALT and AST, hepatocyte apoptosis and inflammation were all relieved by SEV. Hepatic I/R is also accompanied by acute self-sustaining inflammation resulting in necrosis [19]. Similarly, various studies have documented the critical importance of aberrantly expressed miRNAs in hepatic I/R injury, with evidence even dictating alteration of miRNA expression as a promising therapeutic strategy to prevent or alleviate hepatic I/R injury [20]. For instance, upregulation of several miRNAs, such as miR-27a [21] and miR-191 [22] in response to hepatic I/R has been reported. Meanwhile, other miRNAs, such as miR-20a [23] and miR-125b [24] are known to exhibit downregulated levels in hepatic I/R, contributing to diminished inflammation and liver damage by inhibiting the transcription of their target genes. Notably, Li et al. also uncovered that miR-124 was downregulated in I/R animal and cell models, and further suggested that miR-124 could alleviate I/R-triggered liver injury by targeting the Rab38 gene [25]. However, the aforementioned study solely evaluated cell apoptosis levels in response to miR-124 expression alteration, whereas in the current study, several factors such as the levels of liver damage, ALT, AST and GST contents, hepatocyte apoptosis and inflammation were extensively studied using in vivo models. As a result, we hypothesized that both SEV and miR-124 restoration may result in amelioration of hepatic I/R injury.

Subsequently, we explored the role of miR-124 in the alleviation of SEV in hepatic I/R injury in vitro, and came across results indicating that SEV inhibited hepatic I/R injury by elevating the miR-124-3p expression, as evidenced by repressed ALT, AST and GST contents, and diminished hepatocyte apoptosis and inflammation. SEV has been revealed to prevent hepatic I/R injury by reducing ALT/AST contents and histological changes in liver tissues [26]. Moreover, SEV is known to exert its hepatoprotective role on the liver against I/R via different mechanisms, including inflammatory signaling and immunological mechanisms [27]. Additionally, a prior research revealed that SEV possesses the ability to regulate macrophages to confer a protective role during the course of cerebral ischemic injury [28], whereas the effects of SEV on macrophages in hepatic I/R injury remain to be unclear and will be addressed in our future studies. Nevertheless, the miRNA-mediated translational repression of mRNAs has been extensively studied in inflammatory regulation and organ injury following I/R. One particular study highlighted that SEV preconditioning has the potential to suppress oxidative stress and hepatocyte apoptosis caused by I/R modeling through the modulation of miR-200c [29]. Prior evidence has also indicated that miR-124-3p expression was enhanced in SEV treated cell lines, while the restoration of miR-124-3p has been shown to augment the beneficial pharmacological effects of inhaled SEV [12]. Accordingly, we speculated that the promotive effect of miR-124-3p on SEV impact could be achieved through the mediation of target genes.

Consequently, we employed miR target detection program for predicting the potential target genes of miR-124-3p, wherein the
TRAF3 gene was predicted as a core candidate target of miR-124-3p, and further verified using a dual-luciferase reporter assay. TRAF3 is a ubiquitously expressed adapter protein, which could accelerate the apoptotic potential of mature B cells. TRAF3 also plays a critical role in inflammation regulated by T cells [30]. In addition, studies have found that ablation of TRAF3 could abrogate hepatocyte death, inflammation and pro-inflammatory cytokine secretion in animal and cell models of hepatic I/R [31]. On the contrary, the adverse role of TRAF3 in liver steatosis has also been unraveled via stimulation of TAK1 ubiquitination and degradation of CREB. Upregulation of Bax, cleaved-caspase-3, TNF-α, IL-1β and IL-6 expression, as well as promoted hepatocyte apoptosis and inflammatory response further contributes to hepatic I/R injury. SEV promotes the expression of miR-124-3p and resulted in ameliorated hepatic I/R injury.

In conclusion, the current study provides new insight into the hepatoprotective mechanisms of SEV in hepatic IR injury via miR-124-3p. The augmented expression of miR-124-3p regulated by SEV resulted in attenuated hepatic IR injury by impairing TRAF3-mediated CREB ubiquitination. Directly targeting inflammation and hepatocyte apoptosis by manipulating miR-124-3p should be considered as a novel means to combat IR-induced injury in liver surgeries (Fig. 7). However, how does the outcome of this study contributed to therapy/treatment of liver injury needs to be further explored in future studies.

**MATERIALS AND METHODS**

**Ethics statement**

The current study was conducted under the approval of the Medical Ethics Committee of The First Hospital of China Medical University. Animal experiments were performed following the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, and all efforts were made to minimize the suffering of the included animals.

**Establishment of hepatic I/R injury mouse models**

In total, 45 male C57BL/6 mice (aged 8–10 weeks, weighing 18–21 g) purchased from Animal Experiment Center of The First Hospital of China Medical University were enrolled to establish the animal models of hepatic I/R injury. The portal vein and hepatic artery of the middle lobe and left lobe of the liver were clamped for 90 min using a special clamp after the mice were anesthetized with 2% pentobarbital sodium. After 24 h of reperfusion, the mice were subsequently euthanized, and serum and liver specimens were collected for subsequent experimentation. Sham-operated mice (n = 8) were only subjected to laparotomy without vascular occlusion or reperfusion, while the I/R mice (n = 37) were injected with 2.0% SEV (0426, Fusopharmaceutical Industries, Ltd., Osaka, Japan) three times every 15 min. Following the instructions of AAV construction, the mouse livers post-anesthesia were administrated with AAV sh-control and sh-CREB. Twenty-four hours after injection, hepatic I/R was performed on the mice. The animal model of hepatic I/R was successfully established in 32 mice, with a calculated success rate of 86.49%. The 32 mice were subsequently assigned into four groups, with eight mice in each group.

Inflammation and metabolic disease progression in liver injury. The expression of miR-124-3p was downregulated when hepatic I/R occurs, which resulted in increased expression of TRAF3 targeted by miR-124-3p, promoting the ubiquitination and degradation of CREB. Upregulation of Bax, cleaved-caspase-3, TNF-α, IL-1β and IL-6 expression, as well as promoted hepatocyte apoptosis and inflammatory response further contributes to hepatic I/R injury. SEV promotes the expression of miR-124-3p and resulted in ameliorated hepatic I/R injury.

**Fig. 7** The proposed regulatory mechanism illustrating the role of miR-124-3p, TRAF3, and CREB in hepatic I/R injury. The expression of miR-124-3p was downregulated when hepatic I/R occurs, which resulted in increased expression of TRAF3 targeted by miR-124-3p, promoting the ubiquitination and degradation of CREB. Upregulation of Bax, cleaved-caspase-3, TNF-α, IL-1β and IL-6 expression, as well as promoted hepatocyte apoptosis and inflammatory response further contributes to hepatic I/R injury. SEV promotes the expression of miR-124-3p and resulted in ameliorated hepatic I/R injury.
RT-qPCR
mRNA or miRNA was collected from tissues or cells, followed by extraction of tissue RNA using the TRIzol reagent (15069020 or AM1561, Thermo Fisher Scientific Inc., Waltham, MA, USA). For miR-124-3p detection, the total RNA was reversely transcribed using miRNA First Strand cDNA Synthesis (Tailing Reaction) Kit (BS32451-0010, Shanghai Sangon Biotechnology Co., Ltd., Shanghai, China). For mRNA detection, 5 μg of tissue RNA was transformed to complementary DNA (cDNA) via reverse transcription according to the manufacturer’s instructions of the RT-qPCR kit (ABI Company, Oyster Bay, NY, USA). U6 was used as the internal reference for miR-124-3p, while glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the reference for the other genes. The fold changes in gene expression were calculated using means of relative quantification (the 2–ΔΔCT method). The primers are depicted in Supplementary Table 1.

Western blot analysis
Cells were rinsed with phosphate buffer saline (PBS), and then lysed with cell lysis buffer (P0013, Beyotime Biotechnology Co., Shanghai, China). The tissues were ground in liquid nitrogen and lysed with cell lysis buffer (P0013, Beyotime), followed by incubation at 4°C for 30 min. After incubation, the lystate was collected into a 1.5 ml Eppendorf tube, and then centrifuged at 10,000 rpm, at 4°C and 12,000 x g for 15 min, and the supernatant was collected. Sample protein concentration was determined using a bicinchoninic acid (BCA) protein detection kit (Beyotime). The protein loading buffer was added to the supernatant and boiled for 5 min. Subsequently, 20 μg of protein was resolved in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and placed onto a polyvinylidenefluoride membrane (Millipore, Billerica, MA, USA). Following blockade with 5% skim milk, the membrane was incubated for one night at 4°C along with Tris-buffered saline containing Tween-20 (TBST)-diluted primary antibodies (Abcam Inc., Cambridge, UK) to TRAF3 (ab36988, dilution ratio of 1–2 μg/ml), CREB (ab31387, dilution ratio of 1:1000), cleaved-caspase-3 (ab2302, dilution ratio of 1:500), Bax (ab23503, dilution ratio of 1:1000), Bcl-2 (ab182858, dilution ratio of 1:2000), ubiquitin (Ub; ab7780, dilution ratio of 1:2000), and β-actin (used as internal reference; ab8267, dilution ratio of 1:10,000). Following three rinses with TBST, the membrane was then immunoblotted with horseradish peroxidase-labeled secondary antibodies (ab205718, goat anti-rabbit, dilution ratio of 1:10000; ab205719, goat anti-mouse, dilution ratio of 1:10,000), followed by 1-hour incubation at room temperature. The blots were developed utilizing a chemiluminescence reagent (Baoman Biotechnology, Shanghai, China) and bands areas were quantified using the gel image analysis software, Image J Software.

ELISA
An ADVIA 2400 biochemical analysis instrument (Siemens, Taunya, NY, USA) was employed to detect the serum levels of ALT, AST, and GST. The levels of ALT, AST, GST, TNF-α, and β-actin in the supernatant were similar as performed in the treatment group. For the treatment group, the sections were incubated with 50 μl TUNEL reaction mixture in a dark wet box with humidity at room temperature for 1 h, while 50 μl of the fluorescein-labeled dUTP solution was supplemented to the NC group. Apoptotic cells were counted under a fluorescence microscope (excitation light wavelength: 450–490 nm, emission: 515–565 nm). The sections were subsequently treated with 50 μl DIG-POD at room temperature for 30 min, and with 50–100 μl dianaminobenzidine substrate for 10 min. Next, hematoxylin or methyl green was employed to counterstain the sections once photographed. The sections underwent dehydration with gradient alcohol, clearing with xylene and sealing with neutral gum. Finally, the apoptotic cells (200–500 cells in total) were observed under a light microscope and the morphology was photographed. Apoptotic cells were then comprehensively judged based upon morphological characteristics. The unstained cells presented with a smaller size, and complete but foaming cell membrane; whereas the apoptotic bodies appeared in the late stage, and the adherent cells were shrunk and became round, and then fall off; while the stained cells showed concentrated and marginalized chromatin into massive/apoptotic bodies, along with cracked nuclear membrane.

Flow cytometry
Cells in the culture dish were collected after being detached with ethylenediaminetetraacetic acid-free trypsin. The cells in the supernatant were then centrifuged at 2000 rpm for 5 min and collected. The collected cells were stained with 5 μl fluorescein isothiocyanate (FITC)-labeled Annexin V (Annexin V-FITC) and 5 μl propidium iodide (RA016, Nanjing Keygen Biotech CO., LTD, Nanjing, China) for 15 min. Cell apoptosis was detected using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Cell treatment
Normal BNL CL2 mouse hepatocytes (Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin (Gibco, Carlsbad, California, USA) at 37°C with 5% CO2 and 95% O2. The hepatocytes were subcultured for 3–5 days, detached with 0.25% trypsin and sub-cultivated in medium containing 10% fetal bovine serum, 5% glucose, and 1% penicillin and streptomycin. The cell density was adjusted to 2.5 × 10⁴ cells/ml and cultured in tips of 96-well plates. After 24 h, the cells were treated with 50 μl of H₂O₂ solution (5 mM) at room temperature for 5–30 min, and then reacted with PBS containing 2% hydrogen peroxide at room temperature for 5 min. After preparation of the TUNEL reaction mixture, the treatment group was mixed with 50 μl DTT and 450 μl fluorescein-labeled dUTP solution, while the NC group was added with 50 μl fluorescein-labeled dUTP solution. The control group was firstly added with 100 μl D-Nase 1, and then incubated for 10–30 min at room temperature, while the subsequent steps were similar as performed in the treatment group. For the treatment group, the sections were incubated with 50 μl TUNEL reaction mixture in a dark wet box with humidity at room temperature for 1 h, while 50 μl of the fluorescein-labeled dUTP solution was supplemented to the NC group. Apoptotic cells were counted under a fluorescence microscope (excitation light wavelength: 450–490 nm, emission: 515–565 nm). The sections were subsequently treated with 50 μl DIG-POD at room temperature for 30 min, and with 50–100 μl diaminobenzidine substrate for 10 min. Next, hematoxylin or methyl green was employed to counterstain the sections once photographed. The sections underwent dehydration with gradient alcohol, clearing with xylene and sealing with neutral gum. Finally, the apoptotic cells (200–500 cells in total) were observed under a light microscope and the morphology was photographed. Apoptotic cells were then comprehensively judged based upon morphological characteristics. The unstained cells presented with a smaller size, and complete but foaming cell membrane; whereas the apoptotic bodies appeared in the late stage, and the adherent cells were shrunk and became round, and then fall off; while the stained cells showed concentrated and marginalized chromatin into massive/apoptotic bodies, along with cracked nuclear membrane.
5 h, whereas for SEV treatment, 2% SEV was used to intervene the hepatocytes for 2 h.

**IP assay**
In order to detect the ubiquitination levels of CREB, Flag-TRAF3, Myc-CREB, and HA-Ub plasmids were co-transfected into BNL CL.2 cells. Following 48 h of transfection, the total protein was extracted from the cells and quantified using the BCA method. Afterwards, 1 mg total protein was incubated with 30 μl Protein G Agarose and 1 μg normal rabbit immunoglobulin G primary antibody. Centrifugation was then carried out to remove the supernatant, followed by denaturation of precipitates. The protein was subsequently transferred onto a polyvinylidene fluoride membrane after agarose-gel electrophoresis separation. Blots were then probed with primary antibodies to Flag (#14793, dilution ratio of 1:5000, rabbit antibody, Abcam), and HA (ab1424, dilution ratio of 10 μg/ml, mouse antibody, Abcam), followed by 1 h incubation at 4 °C. Afterwards, the bands were detected using an enhanced chemiluminescence reagent (Baoman Biotechnology, Shanghai, China), and the gray value for each band was analyzed using the gel image analysis software, Image J Software [35–38].

**Protein stability test**
In order to test the effect of ubiquitination on CREB protein stability, 48 h post-transfection with TRAF3 and empty plasmids, cells were incubated with CHX (10 μg/ml, HY-12320, MCE) at 37 °C. The cells were harvested at the 0-, 1-, 2-, 4-, and 8-h time intervals. The protein expression patterns of CREB were quantified using Western blot analysis. The degradation curve of CREB was drawn subsequently.

**Dual-luciferase reporter gene assay**
The target gene of miR-124-3p was predicted using a miRNA bioinformatics software. The TRAF3 3' untranslated region gene fragment was artificially synthesized and introduced into the reporter plasmid pmiR-reporter (Huayueyang Biotechnology Co., Ltd., Beijing, China) with the help of endonuclease sites, Spe I and Hind III. The complementary sequence mutation site of seed sequence was designed based on the TRAF3 WT. T4 DNA ligase was then used to insert the target fragment into the pmiR-reporter plasmid following restriction endonuclease digestion. The luciferase reporter plasmids WT and MUT were co-transfected with miR-124-3p into BNL CL.2 cells. The cells were collected 48 h after transfection, and the protein was extracted. Luciferase activity test was used as a luciferase detection kit (K801-200, BioVision, Palo Alto, USA) with a lomax20/20 luminometer fluorescence detector (Promega Corporation, Madison, WI, USA).

**Statistical analyses**
Data analyses were performed using SPSS 21.0 software (IBM Corp., Armonk, NY, USA). Measurement data were presented as mean ± standard deviation. Paired data between two groups obeying normal distribution and homogeneous variance were compared utilizing paired t-test. Data among multiple groups were compared using one-way analysis of variance (ANOVA) and a Tukey’s test. Data among groups at different time points were compared by repeated-measures ANOVA and Bonferroni post hoc test. A value of p < 0.05 reflected a statistically significant difference.

**DATA AVAILABILITY**
The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

**REFERENCES**
1. Li S, Fujino M, Takahara T, Li XK. Protective role of heme oxygenase-1 in fatty liver ischemia-reperfusion injury. Med Mol Morphol. 2019;52:61–72.
2. Yan ZZ, Huang YP, Wang X, Wang HP, Ren F, Tian RF, et al. Integrated omics reveals Tollip as a regulator and therapeutic target for hepatic ischemia-reperfusion injury in mice. Hepatology. 2019;70:1750–69.
3. Anger F, Camara M, Ellinger E, Germer CT, Schlegel N, Otto C, et al. Human mesenchymal stromal cell-derived extracellular vesicles improve liver regeneration after ischemia reperfusion injury in mice. Stem Cells Dev. 2019;28:1451–62.
4. Mohammed SG, Ibrahim I, Mahmoud MF, Mahmoud AA. Carvedilol protects against hepatic ischemia/reperfusion injury in high-fructose/high-fat diet-fed mice: Role of G protein-coupled receptor kinase 2 and 5. Toxicol Appl Pharm. 2019;382:114750.
5. Xing Y, Li J, Li SP, Xi J, Ma N, Liu L, et al. MiR-27a-5p regulates apoptosis of liver ischemia-reperfusion injury in mice by targeting Bcl1. J Cell Biochem. 2018;119:10357–63.
6. Dossi CG, Gonzalez-Manan D, Romero N, Silva D, Videla LA, Tapia GS. Anti-oxidative and anti-inflammatory effects of Rosa Mosqueta oil supplementation in rat liver ischemia-reperfusion. Food Funct. 2018;9:4857–471.
7. Li J, Yuan T, Zhao X, Lv GY, Liu HQ. Protective effects of sevoflurane in hepatic ischemia-reperfusion injury. Int J Immunophathol Pharm. 2016;29:300–7.
8. Morita T, Ishikawa M, Sakamoto A. MicroRNA regulates liver protection during anesthetic and ischemic preconditioning in rats: an animal study. PLoS ONE. 2015;10:e0125866.
9. Desvignes T, Loher P, Elbeek K, Ma J, Urgese G, Fromm B, et al. Unification of miRNA and isomiR research: the mirGFF3 format and the mirtop API. Bioinformatics. 2020;36:698–703.
10. Jiang W, Liu G, Tang W. MicroRNA-182-5p ameliorates liver ischemia-reperfusion injury by suppressing toll-like receptor 4. Transl Proc. 2016;4:2809–14.
11. Zhang M, Ying Y, Ye Q, Niu Y. Comprehensive circRNA expression profile during ischemic postconditioning attenuating hepatic ischemia/reperfusion injury. Sci Rep. 2019;9:264.
12. Gao C, Shen J, Meng ZX, He XF. Sevoflurane inhibits glioma cells proliferation and metastasis through mirRNA-124-3p/ROCK1 axis. Pathol Oncol Res. 2020;26:947–54.
13. Lin WW, Hostager BS, Bishop GA. TRAF3 ubiquitination, and B-lymphocyte receptor signalling enhances liver ischemia-reperfusion injury in mice. Liver Transpl. 2012;18:659–70.
14. Jimenez-Castro MB, Cornide-Petronio ME, Gracia-Sancho J, Peralta C. Inflammassome-mediated inflammation in liver ischemia-reperfusion injury. Cells. 2019;8:1131.
15. Liu D, Jin X, Zhang C, Shang Y. Sevoflurane relieves hepatic ischemia-reperfusion injury by inhibiting the expression of Grp78. Biosci Rep. 2018;38:BSR20180549.
16. Xu CF, Yu CH, Li YM. Regulation of hepatic microRNA expression in response to ischemic preconditioning following ischemia reperfusion injury in mice. OMICS. 2009;13:513–20.
17. Christou CD, Toulas G. The role of microRNA in hepatic ischemia/reperfusion injury. Micros. 2020;248–54.
18. Tomhe S, Yazedzi HO, Sud V, Leighman P, Huang H, Zamora R, et al. Computational analysis supports IL-17A as a central driver of neutrophil extracellular trap-mediated injury in liver ischemia reperfusion. J Immunol. 2019;202:268–77.
19. Dar WA, Sullivan E, Bynon JS, Eltzschig H, Ju C. Ischaemia reperfusion injury in liver transplantation: cellular and molecular mechanisms. Liver Int. 2019;39:788–801.
20. Chi X, Jiang Y, Chen Y, Yang F, Cai Q, Fan P, et al. Suppression of microRNA27a protects against liver ischemia/reperfusion injury by inhibiting PPARgamma and inhibiting endoplasmic reticulum stress. Mol Med Rep. 2019;20:4003–8.
21. Pan W, Wang L, Zhang XF, Zhang H, Zhang J, Wang G, et al. Hypoxia-induced microRNA-191 contributes to hepatic ischemia-reperfusion injury through the ZONAB/Cyclin D1 axis. Cell Death Differ. 2019;26:391–401.
22. Zhang L, Song Y, Chen L, Li D, Feng H, Lu Z, et al. MiR-20a-containing exosomes from umbilical cord mesenchymal stem cells alleviates liver ischemia/reperfusion injury. J Cell Physiol. 2020;235:3698–710.
23. Huang Z, Zheng D, Pu J, Dai J, Zhang Y, Zhang W, et al. MicroRNA-125b protects liver from ischemia/reperfusion injury via inhibiting TRAF6 and NF-kappaB pathway. Biosci Biotechnol Biochem. 2019;83:829–35.
preconditioning in the liver and lung in a rat model of liver ischemia/reperfusion injury. Mol Immunol. 2016;72:1–8.

31. Dang DD, Saiyin H, Yu Q, Liang WM. Effects of sevoflurane preconditioning on microglia/macrophage dynamics and phagocytosis profile against cerebral ischemia in rats. CNS Neurosci Ther. 2018;24:364–71.

32. Wu Y, Gu C, Huang X. Sevoflurane protects against hepatic ischemia/reperfusion injury by modulating microRNA-200c regulation in mice. Biomed Pharmacother. 2016;84:1126–36.

33. Xie P, Kraus ZJ, Stunz LL, Liu Y, Bishop GA. TNF receptor-associated factor 3 is required for T cell-mediated immunity and TCR/CD28 signaling. J Immunol. 2011;186:143–55.

34. Hu J, Zhu XH, Zhang XJ, Wang PX, Zhang R, Zhang P, et al. Targeting TRAF3 signaling protects against hepatic ischemia/reperfusion injury. J Hepatol. 2016;64:146–59.

35. Chen Z, Shen H, Sun C, Yin L, Tang F, Zheng P, et al. Myeloid cell TRAF3 promotes metabolic inflammation, insulin resistance, and hepatic steatosis in obesity. Am J Physiol Endocrinol Metab. 2015;308:E460–9.

36. Rajasethupathy P, Fiumara F, Sheridan R, Betel D, Puthanveettil SV, Russo JJ, et al. Characterization of small RNAs in Aplysia reveals a role for miR-124 in constraining synaptic plasticity through CREB. Neuron. 2009;63:803–17.

37. Suzuki S, Toledo-Pereyra LH, Rodriguez FJ, Cejalvo D. Neutrophil infiltration as an important factor in liver ischemia and reperfusion injury. Modulating effects of FK506 and cyclosporine. Transplantation. 1993;55:1265–72.

38. Kim JH, Kang JW, Lee YS, Lee JW, Chi XZ, Li YH, et al. RUNX family members are covalently modified and regulated by Pias1-mediated sumoylation. Oncogenesis. 2014;3:e101.

ACKNOWLEDGEMENTS

We would like to acknowledge the technical support provided by the First Hospital of China Medical University.

AUTHOR CONTRIBUTIONS

Y.-L. Wang conceived and designed research. YZ performed experiments and interpreted results of experiments. D-SC analyzed data and prepared figures. Y.-L. Wang drafted paper, edited and revised manuscript together with D-SC. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41420-021-00784-7.

Correspondence and requests for materials should be addressed to Da-Sheng Cai.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022