Bradykinin contributes to immune liver injury via B2R receptor-mediated pathways in trichloroethylene sensitized mice: a role in Kupffer cell activation

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Declaration of interest

The authors report no conflicts of interest.

Bradykinin contributes to immune liver injury via B2R receptor-mediated pathways in trichloroethylene sensitized mice: a role in Kupffer cell activation

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Abstract: We have previously shown trichloroethylene (TCE) induced occupational medicamentosa-like dermatitis due to TCE (OMLDT) with immune liver injury, and kallikrein-kinin system (KKS) activation as a probably mechanism underlying the immune damage. Bradykinin (BK) is an important active component of KKS system function, but the specific role of BK in the immune liver injury has never been examined. The present study aimed to explore the important role of BK and mechanisms of action in immune liver injury induced by TCE. TCE sensitization significantly increased the expression of BK receptor (B2R) in the liver. Compared to blank and vehicle control group, TCE sensitization positive mice developed exacerbated liver injury evidenced by elevated AST, ALT levels and hepatocyte damage. TCE sensitization also stimulated MAPK and STAT3 activation in liver tissue. B2R antagonist HOE140 ameliorated these changes. Kupffer cells (KCs) of the liver were also activated following TCE sensitization; both CD68⁺ KCs and CD16/CD32⁺ M1 type KCs were increased in TCE positive group. Further experiments isolated the KCs from the liver in each group and showed that TCE sensitization resulted activation of MAPK signal pathway which in turn caused release of the pro-inflammatory cytokines, IL-1 β , IL-6, TNF- α , in KCs; the antagonist HOE140 again decreased these changes in KCs. These results uncover a novel role of BK and B2R cross-talk in KCs activation in TCE sensitized mice, mediated by pro-inflammatory cytokine release via MAPK and STAT3 activation, contributing to the immune liver injury.

Keywords: trichloroethylene; bradykinin; liver injury; Kupffer cell

1. Introduction

Trichloroethylene (TCE) is a volatile and non-flammable lipophilic liquid of the chlorinated aliphatic solvent family (Dumas et al., 2018). TCE is mainly used as a

solvent to remove grease from metal parts or lenses in industrial production (Xueqin et al., 2018). It is also widely used as a chemical intermediate and extractant in chemical, dry-cleaning and textile industries (ATSDR, 2006). TCE came into intensive commercial production in the United States and Germany in the 1920s, and the scale of the production reached hundreds and thousands of tons every year (NICNAS, 2000). Workers exposed to TCE develop occupational medicamentosa-like dermatitis due to TCE (OMLDT), also called TCE hypersensitivity syndrome (THS). THS is characterized by fever, generalized rash, liver dysfunction and superficial lymphadenopathy (Zhang et al., 2017). There is consistent data to indicate that TCE has also kidney and liver toxicity. However, the mechanism of the organ injury in THS is unknown.

Many clinical studies have found that OMLDT patients exhibit changes in complement system and a variety of cytokines, but the mechanism remains unclear (Zhang et al., 2017; Xueqin et al., 2018). A previous study shows that complement C3 can act as a substrate for kallikrein (KLK) -mediated inflammatory response. Through the activation of C3, KLKs promote complement activation in local tissues in the process of innate immunity. Conversely, inhibition of complement activation by C1-inh can also suppress the activity of KKS system (Oikonomopoulou et al, 2013).

In our previous studies, we have found that kallikrein-kinin system (KKS) plays an important role in organ injury in trichloroethylene-sensitized mice (Wang et al., 2016; Wang et al., 2015). The mechanism however is unclear. The level of BK reflects the strength of the KKS in immune activities. BK generated by plasma kallikrein proteolytic action on high-molecular-weight kininogen (HK), primarily mediates KKS actions. BK can be found in the blood, but can also be produced locally in many tissues. BK is a vasodilator peptide with pro-inflammatory actions via its membrane receptor, bradykinin B2 receptor (B2R), a G protein-coupled receptor (Ricciardolo et al., 2016). B2R has been shown to mediate the proinflammatory effect promoted by BK (Terzuoli et al., 2018). Many researchers show that BK and B2R activate the pathways of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) (Izumi et al., 2018). ERK and p38 MAPK activation are

known to increase gene expression of inflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6). The cytokines important in the inflammatory response, such as TNF- α and IL-6, on the other hand, have been reported to activate the pathways of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) (Izumi et al., 2018; Meng et al., 2005). Kupffer cells (KCs) are a specific type of resident macrophages in the liver and are the main cells of the innate immune system in this tissue. KCs are divided into two types, M1 (CD16/CD32⁺) and M2 (CD206⁺). M1-type KCs secrete proinflammatory cytokines (such as TNF- α , IL-1 β , IL-6, IL-12); M2-type KCs have a weak antigen presentation ability and counteract the immune response by secreting potent immunosuppressive factors, such as IL-10. (Zeng T, et al., 2016). The release of pro-inflammatory cytokine by KCs relies on the inflammatory signaling pathway molecules p38 MAPK, NF- κ B and STAT3 in liver (Liu et al., 2015; Seo et al., 2018). To date, whether TCE sensitization leads to BK activation in the liver and whether BK exerts an effect on Kupffer cells and causes a release of proinflammatory cytokines through MAPK related pathways have not been examined. Answers to these questions will provide molecular insight into KKS-mediated immune mechanisms in liver injury due to TCE sensitization.

To reveal the molecular mechanisms of KKS activation in immune liver injury due to TCE sensitization, we examined the KCs activation in BALB/c mice sensitized by TCE based on our previous experiments. In the present study, we used a specific B2R antagonist, HOE140, to dissect the BK/B2R signaling pathway mediating the immune liver injury in TCE sensitized mice. Furthermore, we isolated the KCs from the liver in these TCE sensitization mice and investigated MAPK and STAT3 pathway activation and its associated cytokine release.

2. Materials and Methods

2.1 Animals

A total of 110 female BALB/c mice at 6-8-week-old were purchased from Experimental Animal Center of Anhui Medical University, weighing between 17.3

and 25.4g. The animals were housed in certified barrier facilities in micro-isolation cages with five animals per cage at room temperature, and freely accessed a standard mouse food and water on a 12-h-light/12-h-dark cycle every day. The experimental protocol and the use of animals were approved by the Animal Care and Use Committee of Anhui Medical University. The mice were randomly divided into four groups: blank control group (n=5), vehicle (olive oil and acetone) control group (n=5), TCE-treated groups (TCE group) (n=48), TCE and B2R antagonist-treated groups (TCE + HOE140 group) (n=52).

2.2 Reagents

TCE and Freund's complete adjuvant (FCA) were purchased from Sigma (St. Louis, MO, USA); acetone and olive oil were purchased from the Shanghai Chemical Reagent Company. HOE140 was from TOCRIS bioscience (Avonmouth, Bristol, United Kingdom). Goat anti-mouse BK polyclonal IgG antibody was purchased from Santa Cruz (CA, USA), rabbit anti-mouse B2R antibody from Abcam (Cambridge Science Park, UK), the second antibody donkey anti-goat IgG H&L (Alexa Fluor® 594) and donkey Anti-Rabbit IgG H&L (Alexa Fluor® 488) were purchased from Abcam (Cambridge Science Park, UK), 4, 6-diamidino-2-phenylindole (DAPI) from Sigma (St. Louis, MO). Immunohistochemistry kits were purchased from ZSJQ-BIO (Beijing, China). Rabbit anti-mouse CD68, TNF- α , IL-1 β and IL-6 polyclonal IgG antibodies were purchased from Bioss (Beijing, China). Rat anti-mouse CD16/CD32 polyclonal IgG antibody was purchase from R&D systems (Minneapolis, USA). Other notable reagents/supplies purchased were: Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Pittsburgh, PA), Histostain Plus and DAB substrate kits (ZSJQ, Beijing, China), RIPA buffer (Beyotime Biotechnology, Beijing, China) containing PMSF (Beyotime Biotechnology, Beijing, China), 0.45um PVDF membrane (GE healthcare, Germany), collagenase (type IV, Sigma-Aldrich, St. Louis, USA), Percoll (Sigma-Aldrich, St. Louis, USA), The BK, IL-1 β , IL-6 and TNF- α ELISA kit (USCN, China), and Light Cycler 480 SYBR Green I Master kit (Roche, Basel, Switzerland).

2.3 Animal model and experiment grouping design

A BALB/c mouse model of TCE sensitization was generated as reported previously (Wang et al., 2015). On Day 1, the mice in the TCE-treat group were intradermally injected with 100 μ l of a mixture of 50% TCE (TCE : olive oil : acetone=5:2:3, 50 μ l) and FCA (50 μ l) for the first stimulation. A 100 μ l of 50% TCE was painted on the shaved area of dorsalis for continued sensitization on the 4th, 7th, and 10th day. A week later, 100 μ l of 30% TCE (TCE : olive oil : acetone =3:2:5) was painted twice to challenge the dorsal skin on 17th and 19th days. TCE + HOE140 group followed the same protocol as the TCE group except intraperitoneal injection of HOE140 (0.1 mg/kg) 1h before the TCE challenge on the 17th and 19th days (Gama et al., 2004). In the vehicle control group, mice were treated similarly with the same proportions of olive oil and acetone without TCE. The blank control group received no treatment. The shaved area of each mouse was covered with filter paper and sealed with nonirritating tape for 24 hours after painting. On the 20th day, 24 hours after the last challenge, the cutaneous reactions of the dorsal skin were scored on a 4-point scale: 0, no reaction; 1, scattered mild redness; 2, moderate and diffuse redness; and 3, intensive erythema and swelling. If the cutaneous reaction score of any animal was equal to 1 or greater, it was judged as a positive reaction and classified as sensitization positive or otherwise as sensitization negative. Blood, liver and other samples were collected according to experimental requirements after the mice were euthanized at 20th day (24h time point) and 22nd day (72 h time point). Additional blood samples were collected into Vacutainer tubes which contained EDTA and aprotinin (0.6 TIU/ml blood) to inhibit proteinase activity. The blood was centrifuged to separate plasma from blood cells and plasma was transferred into polypropylene tubes.

Thereafter all the specimens were grouped as follows, blank control group, vehicle control group, 24h TCE positive group, 24 TCE negative group, 72h TCE positive group, 72 TCE negative group, 24h TCE + HOE140 positive group, 24h TCE + HOE140 negative group, 72h TCE + HOE140 positive group, and 72h TCE + HOE140 negative group. The schematic protocol is shown in Figure 1.

2.4 Determination of liver enzyme activity

The blood was collected from eye vein plexus, and then centrifuged at 3000 rpm for

15 min at 4°C. Activities of the serum enzymes alanine aminotransferase (ALT) and aspartate transaminase (AST) were determined by automatic biochemistry analyzer.

2.5 Transmission electron microscopy (TEM) examination of liver tissue

Rice grain size fresh liver tissue from the same site of the liver in each mouse was fixed in 0.1mol/l glutaraldehyde-paraformaldehyde solution for 10h and made into specimens. The specimen sections were then examined by transmission electron microscope and pictures were taken.

2.6 Isolation of liver KCs

Liver KCs were isolated by in situ collagenase perfusion and differential centrifugation on Percoll density gradient as described previously (Hansen et al., 2002; Wu et al., 2016). Briefly, a 20-G catheter was put into the portal vein, and the inferior vena cava cut. The liver was perfused with PB, a mixture of digestion buffer: 1× PBC, collagenase, Pronase E, and 4.76 mM CaCl₂. After digestion, the liver was minced and triturated in BSA solution (1× PBC, supplemented with 0.5% FBS). The tissue suspension was passed through a 200-mesh cell strainer, and the single cells isolated. The cells were then fractionated by using 25% Percoll and 50% Percoll. The cell suspension was washed and allowed for adhesion to the culture dish. The KCs were able to adhere to the plastic, whereas the nonadherent fraction was washed away in DMEM with 10% FBS. The KCs from 2 mice (equal scores) were pooled, given the limited number of KCs available from each animal for RNA and protein isolation.

2.7 Double immunofluorescence detection for BK in combination with B2R

Fresh liver specimens were fixed in 10% formalin, dehydrated, embedded in paraffin and cut into 2µm thick slices. The sections were then deparaffinized in xylenes and rehydrated by a graded series of alcohols, followed by antigen retrieval and incubation in a blocking buffer containing 5% goat serum in 0.1 mmol/l PBS for 2 hours (pH, 7.4). Then sections were incubated with a mixture of goat anti-mouse BK polyclonal antibody (1:400) and rabbit anti-mouse B2R antibody (1:200) at room temperature for 1 h, and then left at 4 °C overnight. On the next day, the sections were warmed up in room temperature for 30min, washed with PBS and incubated with fluorescence-labeled secondary antibodies – a mixture of donkey anti-goat IgG H&L

(Alexa Fluor® 594, 1:1000) and donkey anti-Rabbit IgG H&L (Alexa Fluor® 488, 1:1000) for 2 h. The sections were then washed in PBS and the nucleus was counter-stained with DAPI for 15min. BK and B2R binding was examined using an inverted fluorescence microscope with appropriate excitation and emission filters.

2.8 Western blot for B2R, MAPK pathway molecules and STAT3 protein

Liver tissues and isolated KCs were homogenized with RIPA buffer (Beyotime Biotechnology, Beijing, China) containing PMSF (Beyotime Biotechnology, Beijing, China) and phosphatase inhibitors (10mM NaF, 1mM Na₃VO₄, 1mM Na₂P₂O₄). After determination of protein concentrations, a total of 50 µg protein was put for electrophoresis via 15% sodium dodecyl sulfate-polyacrylamide gel before being transferred to PVDF membranes. The membranes were blocked in 5% non-fat powdered milk in Dulbecco's PBS (DPBS) for 2 h at room temperature. Thereafter, the membranes were incubated with B2R antibody (1:500), P38, p-P38, ERK1/2, p-ERK1/2, JNK, p-JNK, STAT3, p-STAT3 (1:1000) antibodies and GAPDH (1:1000) antibody overnight. On the next day, the membranes were washed in DPBS containing 0.05% Tween-20 three times and in DPBS once, each for 10 min, and then incubated with goat anti-rabbit IgG antibody for 2 h. Thereafter, the membranes were washed three times in DPBS containing 0.05% Tween-20 and in DPBS once. Finally, the signal was developed using an enhanced chemiluminescence (ECL) detection kit from Pierce Biotechnology (Rockford, USA). The densitometry of the signal was analyzed by Image-Pro plus software 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

2.9 Total RNA extraction and quantitative real-time PCR (qRT-PCR) for gene expression

Total RNA was extracted from KCs using TRIzol reagents. The RevertAid First Strand cDNA Synthesis Kit was used to produce cDNA. The cDNA obtained was amplified by PCR in a Light Cycler 480 system using the primers shown in Table 1. The amplification protocol was: initial denaturation at 95 °C for 10min, followed by 45 cycles, including 95 °C for 15 sec, 60 °C for 15 sec and 72 °C for 20 sec. The relative level of gene expression was analyzed by the $2^{-\Delta\Delta Ct}$ method. The fold-change

in IL-1 β , IL-6 and TNF- α mRNA in the samples was normalized to GAPDH levels.

2.10 ELISA assay

The BK concentration in mouse serum was determined using USCN mouse ELISA kit. All reagents, samples and standards were prepared as instructed. 100 μ l standard or sample was added to each well and incubated 1.5 h at 37 °C. Next, 100 μ l of prepared biotin antibody was added to each well and samples were incubated for 1 h at 37 °C. 100 μ l of prepared streptavidin solution was added and the plate was incubated for 30 min at 37 °C. Thereafter, 100 μ l of TMB one-step substrate reagent was added to each well with 15 min incubation at 37 °C in dark. The reaction was finally stopped by adding 50 μ l stop solution to each well. The absorbance at 450 nm in each well was immediately measured using a Thermomax microplate reader. All experiments were performed in triplicate.

2.11 Immunohistochemistry (IHC) staining

Fresh liver specimens were fixed in 10% formalin, dehydrated, embedded in paraffin and cut into 5- μ m-thick slices. The sections were then deparaffinized in xylenes and rehydrated by a graded series of alcohols. Endogenous peroxidase was inhibited by incubation with 3% H₂O₂ for 15 min. Slides were immersed in 0.01M sodiumcitrate buffer for antigen retrieval in a microwave oven. The sections were incubated with anti-CD68, IL-1 β , IL-6 and TNF- α antibodies overnight at 4 °C after blocking non-specific binding with 5% normal goat serum in PBS for 15 min. On the morning of the next day, the sections were processed with Histostain Plus kit and DAB substrate kit for peroxidase reactions. Images were captured using an Olympus microscope. Image-Pro Plus version 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) was used to assess the area and density of the dyed region, and the integrated optical density (IOD) value of the IHC section. The mean densitometry of the digital image (magnification, $\times$ 400) was designated as representative DAB staining intensity. The signal density of the tissue areas from five randomly selected fields was counted in a blinded manner and subjected to statistical analysis.

2.12 Statistical Analysis

All data are expressed as the mean $\pm$ SD and the statistical analysis was performed

with SPSS 23.0, the professional statistical analysis software. A one-way analysis of variance (ANOVA) followed by a post-hoc least-significant test (LSD) and Dunnett test were used to examine the difference between the groups. A p value <0.05 was considered as statistically significant.

3. Results

3.1 Sensitization rates

Based on the cutaneous reaction scores, the mice were divided into sensitization-positive (positive) and sensitization-negative (negative) groups on the 20th day. Sensitization rates were calculated (as number of redness or swelling / number of mice) in both TCE group and TCE + HOE140 group. The sensitization rate was 39.58% (19/48 positive) in the TCE group and 32.69% (17/52 positive) in the TCE + HOE140 group. The difference between the two groups was not significant.

3.2 CD68 and CD16/CD32 detection in liver by IHC

CD68 is a highly glycosylated membrane protein. When KCs are activated, CD68 expression is increased and hence used as marker for KC activation. CD16/CD32 are M1 type KCs marker. The CD68⁺ and CD16/CD32⁺ cells in liver tissue were therefore assessed by IHC staining in the current TCE mouse model. Data showed increased CD68⁺ cells in liver tissue in each TCE positive group compared with blank control group and vehicle control group (Figure 2), suggesting KC activation in liver tissue by TCE sensitization.

3.3 Expression of BK in plasma

There was no significant difference in BK level between the blank control and vehicle control groups. However the plasma BK level was increased in 72h TCE and TCE + HOE104 groups compared to the blank control group, with statistical significance ($P<0.05$, Figure 3).

3.4 Expression of B2R protein in liver

The 72h time point was chosen to detect the protein expression of B2R as the liver injury was the most severe at this time point. The B2R level was increased by 6~8 folds in 72h TCE positive group compared with than blank control, vehicle control

group and 72h TCE negative group, showing marked B2R upregulation specific to TCE sensitization. The absolute mean value of B2R expression in 72h TCE + HOE positive group was slightly lower than that in 72h TCE positive group, but with no statistical significance (Figure 4).

3.5 HOE140 intervention demonstrating BK and B2R interaction

HOE140, a specific antagonist of B2R, was used to examine the binding of BK to B2R using fluorescence double-labeling of both molecules. Imaging data showed the BK and B2R binding in TCE sensitization positive liver (saffron yellow), and the binding was separated in TCE + HOE140 positive liver (red and green) (Figure 5). The results show that HOE140 prevented the interaction of BK with B2R.

3.6 Liver injuries in TCE sensitized mice

Serum ALT and AST activity was quantified as hepatic function. There was no significant difference between blank control group and vehicle control group. Compared to the blank control group, the serum AST was significantly higher in 24h and 72h TCE positive groups. The serum ALT in 72h TCE positive group was also significantly higher than that in blank control group (Figure 6).

The transmission electron microscopic examination showed no significant difference between the blank control and vehicle control group. In the liver tissue from 24h and 72h TCE positive group, there were a loss of intracellular organelles, appearance of a large number of vacuoles, loss of mitochondria and significant reduction of glycogen particles in hepatocytes. However, these histomorphological changes were alleviated in 24h and 72h TCE + HOE140 positive group and hepatocyte ribosome was only partially reduced. Vacuolar degeneration was seen in a small number of mitochondria, while most of the mitochondria clearly had normal morphology in 72h TCE + HOE140 positive group (Figure 7). These data clearly show that TCE sensitization caused significant cellular and sub organelle damages in hepatocytes and blocking B2R receptor by HOE140 antagonized these changes.

3.6 MAPK and STAT3 protein expression in liver

The expression MAPK and STAT3 signaling pathway proteins in the liver was determined. P38, ERK1/2, JNK and STAT3 expression showed no significant

difference in all groups. The expression of phosphorylated protein, p-P38, p-ERK1/2, p-JNK and p-STAT3, in liver showed no significant difference between the blank control group and the vehicle control group. However, these phosphorylated proteins were increased significantly in 72h TCE positive group except p-P38, showing phosphorylation and hence activation of these MAPK and STAT3 signaling proteins following TCE sensitization. Of interest, the expression of the phosphorylated proteins in 72h TCE + HOE140 group returned to the normal level which was close to that in 72h TCE negative group (Figure 8), suggesting that blocking BR2 receptor inhibited activation of these MAPK and STAT3 proteins. These parallel changes in both hepatocyte damages and MAPK and STAT3 signaling proteins following B2R antagonism support the involvement of MAPK and STAT3 pathways in hepatocellular damage in TCE sensitization.

3.7 MAPK protein expression in isolated KCs

To further explore the role of MAPK signal pathway activation in KC function in immune liver injury sensitized by TCE, the KCs were directly isolated from liver tissue and the protein expression was determined by western blot in blank control, vehicle control and 72h time point groups. In contrast to the measurement in liver tissue, p-P38 expression was significantly high in 72h TCE positive group. The p-ERK1/2 and p-JNK expressions in KCs also increased in 72h TCE positive group, similar to findings in liver tissue. However, HOE140 reduced the stimulated expression of p-P38, p-ERK1/2 and p-JNK significantly in TCE positive group (Figure 9), suggesting involvement of BR2 in activation of these signaling proteins in KCs.

3.8 Proinflammatory cytokine release from KCs and their deposition in liver tissue

To explore the downstream signaling pathways, the deposition of KC-related cytokines, IL-1 β , IL-6 and TNF- α , in the liver tissue was examined in each group by IHC. The results showed that the cytokine deposition was enhanced at 24h and 72h TCE positive groups. Compared to 72h TCE positive group, the expression of IL-1 β , IL-6 and TNF- α was reduced in 72h TCE + HOE140 positive group (Figure 10, 11, 12). Further experiments measured IL-1 β , IL-6 and TNF- α mRNA expressions

directly in KCs by qRT-PCR. The results showed that the mRNA expression of IL-1 β , IL-6 and TNF- α levels was markedly increased in KCs from 72h TCE positive group. Again, HOE140 alleviated the simulated mRNA expression of these cytokines in KCs from 72h TCE + HOE140 positive group mice (Figure 13). The cytokine expression in KCs was similar to the deposition in liver. These data together suggest a mechanism whereby increased expression of these key KC derived cytokines and their release into liver tissue promoting inflammatory reactions.

4. Discussion

TCE has long been recognized as an occupational and environmental pollutant and it induces OMLDT among exposed workers. OMLDT is an autoimmune disease, which has become one of the major occupational health problems in some countries (Liu et al., 2015; Zhang et al., 2017). In recent years, liver injury of OMLDT is receiving increased attention in the public health field. However, the mechanism of action is not fully understood (Zhang et al., 2018; Venkatratnam et al., 2018; Zhou et al., 2017). Many clinical data suggest involvement of type IV allergic reactions or delayed type hypersensitivity in OMLDT (Jia et al., 2012). However, increasing evidence suggest that type IV allergic reactions cannot fully explain the multi-organ injuries induced by TCE, especially in liver injury (Zhang et al., 2013). Several studies suggest that the liver injury induced by TCE is due to immune injury (Tang et al., 2008; Bradford et al., 2011). Macrophages activation was also observed in TCE treated MRL^{+/+} mice (Cai et al., 2007). Of significance, we found that KCs (CD68⁺) were activated in TCE sensitized mice in the present study. We further examined the increased expression of M1-type KCs, CD16/CD32, in the liver. An important finding is that CD16/CD32 expression was increased in TCE positive group. This supports TCE sensitization stimulating the activation of M1 type KCs as an immune response in liver tissue. The finding suggests that KCs and a release of KC-related cytokines are important intermediate steps in immune liver injury induced by TCE. This begs a further question how KCs and their secreted cytokines are regulated. In our previous study, we found KKS activation and plasma BK expression increased significantly in TCE sensitized mice (Wang et al., 2016). Many studies have revealed BK stimulated

macrophage activation and induced inflammatory cytokine expression in immune injury in other tissues (Feng et al., 2016; Vorlova et al., 2016; Desposito et al., 2015). In the present study, we examined the BK and B2R expression in the liver from TCE sensitized mice. We found BK was increased significantly in TCE positive group. Consistent with BK rise, B2R expression was also increased in 72h TCE positive group. These data suggest that BK release and its action on B2R may occur following TCE sensitization. The next question is how the BK and B2R interaction contributes to the immune liver injury following TCE sensitization.

To establish a causal relationship, we selected specific potent BK receptor (B2R) antagonist, HOE140, to investigate the pathophysiological role of BK and B2R in vivo. To ascertain the antagonist effect via the specific interaction between BK and its receptor B2R, we performed fluorescent double-labeling that showed co-localization of BK and B2R in the TCE positive liver tissue but separation of these two molecules after the antagonist treatment. To establish the relevance of BK and B2R interaction to liver damage, we examined changes to key liver function enzymes AST and ALT and direct histopathology. AST and ALT were increased significantly in TCE positive groups, compared to blank control and vehicle control group. Transmission electron microscopic examination showed loss of hepatocyte intracellular organelles, formation of a large number of vacuoles, loss of mitochondria and reduced glycogen particles in 24h and 72h TCE positive group. . All these results provide strong evidence that TCE sensitization can induce liver injury. Of great interest, the liver injury that manifested as above was attenuated by HOE140, evidenced by a recovery of histomorphological changes, and decreased levels of AST and ALT in mice pretreated with the antagonist, compared with its corresponding TCE positive mice. The results show a protective effect by HOE140 and supports a key role for B2R in TCE-sensitization induced liver injury.

We have also explored what pathway(s) were involved leading to the inflammatory response after the BK and B2R interaction. Previous studies have revealed that BK significantly increased p-ERK1/2 MAPK (Li et al., 2017), p-JNK MAPK (Frigolet et al., 2017) and STAT3 (Breit et al., 2015) in other tissues. Activation of the MAPKs,

including ERK1/2, JNK, leads to phosphorylation and activation of P38 transcription factors present in the cytoplasm or nucleus, which initiates the inflammatory response in other tissues (such as IL-1, TNF- α , and IL-6) (Pearson et al, 2001; Chen et al., 2017). The highly conserved JAK-STAT pathway involves regulation of the expression of diverse cytokines and activates the STAT proteins that are translocated into the nucleus and bind target gene promoter regions to regulate transcription of inflammatory genes (Boengler et al., 2008). We have, therefore, examined the expression of key MAPK and STAT3 pathway proteins following TCE sensitization in our experimental model, with or without B2R antagonist treatment. The results showed increase of p-ERK, p-JNK and p-STAT3 as result of activation of their inactive protein forms by TCE sensitization in liver tissue. Specifically, blocking the BK receptor with its antagonist reduced the stimulated expressions of these proteins, showing involvement of BK and B2R interaction in activating these pathways. Although we did not observe a difference in P38 and its phosphorylated protein – a major protein that regulates inflammation and cytokine of MAPK cascade, the difference was clearly shown in KCs as discussed later. KCs are the main cells which secrete the proinflammatory cytokines in liver, such as IL-1, TNF- α , TGF- β and IL-6. We have asked the next question about the down-stream mechanisms leading to inflammatory damage in liver tissue. Thus, we isolated the KCs from the liver tissue and measured the MAPK protein and cytokine mRNA expressions in each group. Indeed, p-P38 was increased significantly in 72h TCE positive group, and HOE140 alleviated the stimulated p-P38 protein increase, as well as the p-ERK and p-JNK expression. These data support a mechanism that BK binding to its receptor activates the MAPK pathways in KCs and liver tissue. The cytokine mRNA measurement showed that IL-1 β , IL-6 and TNF- α mRNA expressions were increased significantly in KCs from 72h TCE positive liver. These cytokine changes were also alleviated after the block of B2R. We also investigated the changes of the key pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α in liver tissue and obtained data similar to those in KCs. It is noteworthy that KCs' activation induces the secretion of IL-1 β , IL-6 and TNF- α (Figure 14). The results were corroborated by data from a previous

epidemiological study where TNF- α , interferon (IFN)- γ , IL-6 and IL-10 concentrations were remarkably higher in the OMLDT patients than in the healthy workers (Kamijima et al., 2013). These together suggest that MAPK pathway mediated cytokine release in TCE sensitization has relevance to human conditions of TCE sensitization and the levels of these proinflammatory cytokines may be potential biomarkers to reflect the immunological responses to TCE sensitization in OMLDT patients.

In conclusion, we sensitized BALB/c mice through skin challenge with TCE with or without pretreatment with the B2R antagonist (HOE140). Liver function and electron microscopic examinations proved that there was structural and functional liver damage after TCE sensitization. These changes were accompanied by KCs (CD68+) activation in the liver. B2R antagonist pretreatment attenuated TCE sensitization-induced increase of expressions of phosphorylated ERK, JNK, STAT3 proteins and inflammatory cytokines IL-1 β , IL-6, TNF- α in liver tissue, and alleviated the liver injury. These changes were accompanied by upregulated MAPK proteins and expressions of their associated pro-inflammatory cytokines in the KCs. The present study suggests that KCs activation is involved in the immune liver injury in BALB/c mice after TCE sensitization, and BK binding to its receptor B2R can activate MAPK signal pathway to mediate pro-inflammatory cytokine expressions in KCs and their release to liver tissue in these sensitized mice. The mechanism plays an important role in TCE-mediated immunological liver damage. These findings provide new insight into molecular mechanisms in TCE sensitization-induced immunological liver damage. It is the first such finding on molecular immunological mechanisms in organ damage following environmental chemical sensitization and may be fundamental to understanding the effect of environmental chemicals on the body with public health implications. The sensitization rate of HOE140 did not showed significant difference compared with TCE treatment group, and this implies that BK acts on post-sensitization pathways for inflammatory amplification through complement activation and secondary cytokine release rather than blocking the initial antigen presentation process. TCE sensitization may also involve both B2R-dependent and/or

independent pathways. The effect of directly blocking MAPK activation on the pro-inflammatory cytokine release in isolated KCs warrants further study.

Conflict of interest

The authors report no conflicts of interest.

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Figure legends

Figure 1 Experiment schedule for TCE sensitization in the present study. After 7 days of adaptive feeding, 100μl of a mixture of 50% TCE was intradermally injected in TCE-treat group; this was defined as the 1st day. On the 4th, 7th and 10th days, 50% TCE was painted on the back of the mice for sensitization. On the 17th day and 19th day, the mice were challenged twice with 30% TCE. The HOE140 was applied to the TCE + HOE140 groups 1 h before the challenge. Animals were euthanized, and tissues and samples were taken at 20th day and 22nd day.

Figure 2 CD68⁺ and CD16/CD32⁺ KCs in the liver in TCE mouse model. The expression of CD68 and CD16/CD32 in liver tissue was determined by immunohistochemical (IHC) staining analysis.

Figure 3 The expression of BK in plasma in TCE mouse model. ^a*P*<0.05, vs. the blank control group.

Figure 4 B2R expression in liver tissue in TCE mouse model. The expression of B2R protein in liver tissue was analyzed by Western-blot. ^a*P*<0.05, vs. the blank control group, ^b*P*<0.05, vs. corresponding TCE negative group.

Figure 5 Double immunofluorescence detection of BK and B2R and antagonistic effect of HOE140 in TCE positive group.

Figure 6 Serum levels of AST and ALT in different groups. ^a*P*<0.05, vs. blank control group, ^b*P*<0.05, vs. corresponding TCE negative group.

Figure 7 Liver cell ultra-structure changes in the mouse model by transmission electron microscope.

Figure 8 Total P38, ERK1/2, JNK and STAT3 and their phosphorylated proteins expressed in liver tissue from TCE sensitized mice. The results were normalized by arbitrarily setting the densitometry are expressed as mean ± SD, ^a*P*<0.05, vs. the blank control group.

Figure 9 P38, ERK1/2 and JNK and their phosphorylated proteins expressed in

isolated KCs from the liver in TCE sensitized mice. The results were normalized densitometry units and are expressed as mean $\pm$ SD, ^a*P*<0.05, vs. the blank control group.

Figure 10 Deposition of IL-1 β in liver tissue in TCE sensitization mouse model detected by IHC. ^a*P*<0.05, vs. the blank control group.

Figure 11 Deposition of IL-6 in liver tissue in TCE sensitization mouse model detected by IHC. ^a*P*<0.05, vs. the blank control group.

Figure 12 Deposition of TNF- α in liver tissue in TCE sensitization mouse model detected by IHC. ^a*P*<0.05, vs. the blank control group.

Figure 13 The relative mRNA expression of IL-1 β , IL-6 and TNF- α in isolated KCs. ^a*P*<0.05, vs. the blank control group, ^c*P*<0.05, vs. corresponding TCE positive group.

Figure 14 A schematic model of the molecular mechanisms whereby BK and B2R act on MAPK pathway to regulate Kupffer cell cytokine release in TCE sensitization mouse model.

Table 1. qRT-PCR primer pairs used.

Primer	Sequences
GAPDH forward	5'-ACCCCAGCAAGGACACTGAGCAAG-3'
GAPDH reverse	5'-GGCCCCTCCTGTTATTATGGGGGT-3'
IL-1 β forward	5'-GAAGAAGAGCCCATCCTC-3'
IL-1 β reverse	5'-GTTTCATCTCGGAGC-CTGTAG-3'
TNF- α forward	5'-CCCTCCTGGCCAACGGCATG-3'
TNF- α reverse	5'-TCGGGGCAGCCTTGTCCCTT-3'
IL-6 forward	5'-CCACTTCACAAGTCGGAGGCTTA-3'
IL-6 reverse	5'-GCAAGTGCATCATCGTTGTTTCATAC-3'

CD68

CD16/CD32

Blank control Vehicle control 72h TCE positive 72h TCE negative 72h TCE+HOE140 positive 72h TCE+HOE140 negative

B2R

GAPDH

72h TCE positive

72h TCE + HOE140
positive

BK

B2R

DAPI

Merged

Blank control

Vehicle control

24h TCE positive

72h TCE positive

24h TCE+ HOE140 positive

72h TCE+ HOE140 positive

p-P38

P38

p-ERK1/2

ERK1/2

p-JNK

JNK