Transient receptor potential melastatin 2–mediated heme oxygenase-1 has a role for bacterial clearance by regulating autophagy in peritoneal macrophages during polymicrobial sepsis

XiaoWei Qian1, Hao Cheng1,2 and XinZhong Chen1

Abstract
Our previous study indicated an important protective role of transient receptor potential melastatin 2 (TRPM2) in controlling bacterial clearance in macrophages during polymicrobial sepsis by regulating heme oxygenase-1. Autophagy is necessary for macrophages to kill invasive bacteria. In the present study, TRPM2 knockout (KO) mice show decreased heme oxygenase-1 and autophagy in peritoneal macrophages after caecal ligation and puncture surgery. Caecal ligation and puncture-induced autophagy in peritoneal macrophages is dependent on heme oxygenase-1. TRPM2 KO mice treated with heme oxygenase-1 inducer before caecal ligation and puncture significantly increase autophagy of peritoneal macrophages, bacterial clearance rate and survival rate. In addition, TRPM2 KO mice treated with heme oxygenase-1 inducer before caecal ligation and puncture significantly attenuate organ injury and systemic inflammation. These improvements are reversed by autophagy inhibitor. Therefore, our findings suggest that TRPM2-mediated heme oxygenase-1 has a role for bacterial clearance possibly by regulating autophagy in peritoneal macrophages during polymicrobial sepsis.

Keywords
Transient receptor potential melastatin 2, heme oxygenase-1, autophagy, macrophage, sepsis

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Introduction
Sepsis is characterised as a life-threatening organ dysfunction caused by a dys-regulated host response to infection, which represents a major health-care problem worldwide and results in high mortality every yr.1,2 Macrophages serve as the first line of defence against microbial invasion. However, in sepsis, the bactericidal function of macrophages is severely reduced, leading to uncontrolled microbial growth.3

Transient receptor potential melastatin 2 (TRPM2) is a non-selective Ca2+–permeable cation channel which is highly expressed in macrophages.4 Accumulative studies have shown that TRPM2 is involved in the pathogenesis of sepsis,5–7 possibly by regulating the bacterial clearance of macrophages.6–8 Heme oxygenase-1 (HO-1) is an anti-inflammatory and anti-apoptotic protein which may be induced by inflammation, infection and hypoxia.9 HO-1 also plays a key role in bacterial clearance during polymicrobial sepsis.10 Our previous study indicated an important protective role of TRPM2 in controlling bacterial clearance during polymicrobial sepsis by regulating HO-1.6 However, the downstream signaling pathway of TRPM2-mediated HO-1 in bacterial clearance in peritoneal macrophages during polymicrobial sepsis remains unclear.
Autophagy is a catabolic process known for maintaining metabolic homeostasis by degraded misfolded proteins and damaged organelles. There is growing evidence that ligands of TLRs and other PRRs may trigger autophagy, which is necessary for innate clearance of invasive pathogens. Conventional autophagy is characterised by the formation of a double membrane–bound autophagosome embedded with LC3 II. Unlike conventional autophagy, in LC3-related phagocytosis, pathogens are engulfed into a single membrane–bound autophagosome embedded with LC3 II and degraded by rapid phagosome–lysosome fusion.

Numerous studies have shown that HO-1 can induce autophagy in a variety of cell models when treated with different stimulants. The purpose of the present study was to test the hypothesis that TRPM2-mediated HO-1 plays an important role in bacterial clearance by regulating autophagy in peritoneal macrophages during polymicrobial sepsis.

Materials and methods

Animals

Male mice aged 6–8 wk were used for all experiments. TRPM2 knockout (KO) mice were backcrossed onto the C57BL/6 background for 20 generations. Male C57BL/6 wild type (WT) mice were purchased from Zhejiang Province Experimental Animal Centre (Hangzhou, PR China). TRPM2 KO and WT mice were acclimated to a 12 h/12 h d/night cycle with free access to food and water under pathogen-free conditions in our laboratory. All animal experiments used in this study were approved by the Animal Care and Protection Committees of Zhejiang University (Hangzhou, PR China). The authors confirm that all animal experiments were performed according to the relative guidelines and regulations.

Caecal ligation and puncture model

The caecal ligation and puncture (CLP)-induced sepsis model was generated as previously described. Briefly, mice were anaesthetised with pentobarbital (80 mg/kg i.p.), the caecum was exposed by a midline abdominal incision and was ligated with a 4-0 silk ligature midway between the ileocaecal junction and the tip of the caecum. Using a 21 G needle, the caecum was punctured once through both surfaces at the middle of the ligation and the tip of the caecum, and a small amount of faeces was extruded. The caecum was replaced to the peritoneal cavity, and the abdomen was closed. All mice were administrated 1 ml 0.9% saline s.c. after surgery. Sham CLP mice were subjected to the same surgical procedure as described above without being ligated and punctured.

According to our previous study, HO-1 inducer (hemin, 10 mg/kg; Sigma–Aldrich, St Louis, MO) or vehicle (0.1% ammonium hydroxide containing 0.15 M NaCl) were injected i.p. every other d (three times) before CLP. HO-1 inhibitor (tin protoporphyrin (SnPP), 50 mg/kg; Sigma–Aldrich) or vehicle (0.9% NaCl) were injected i.p. 1 h before CLP. Chloroquine (60 mg/kg; Sigma–Aldrich) or 3-methyladenine (3-MA; 30 mg/kg; Sigma–Aldrich) was injected i.p. 1 h after CLP. Mice were randomly assigned to experimental groups. Mortality rate was monitored twice daily for 7 d.

Peritoneal macrophage isolation

Peritoneal macrophage isolation was performed as in our previous study. At 24 h after CLP or sham operation, the mice were anaesthetised, euthanized and damped with 70% EtOH for 1 min. A 25 G needle was inserted into the abdominal cavity after the posterior part of the abdominal wall was exposed. After fixing the needle with a vascular clamp, three separate injections of 2 ml PBS were administered into the abdominal cavity. After gently shaking the whole body for 10 s, the peritoneal lavage fluid (PLF) containing peritoneal cells was slowly extracted and centrifuged. RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA) containing 10% FBS (Moregate Biotech, Bulimba, Australia), 100 U/ml penicillin and 100 μg/ml streptomycin was used to suspend the cell pellets. The cells were then cultured in six-well plates to adhere for 2 h at 37°C in a humidified atmosphere with 5% CO₂ and 95% air. After removing the non-adherent cells by gentle washing twice with PBS, the adherent macrophages were cultured in RPMI 1640 medium.

Bacterial burden determination

Bacterial burden was determined as in our previous study. Briefly, PLF was harvested by washing the abdominal cavity with 5 ml sterile PBS. After serial dilutions with sterile PBS, 100 μl diluent was plated on tryptic soy agar plates and cultured at 37°C. CFU were counted 24 h after incubation and expressed as CFU/ml PLF.

Western blot assay

Western blot was performed as in our previous study. Briefly, equal amounts (30 μg) of protein were separated by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA). The membranes were then incubated with TBS with 0.05% TBST; Sigma–Aldrich) containing 5% non-fat dry milk before incubation with primary rabbit anti-HO-1 and LC3 Ab (Epitomics, Inc., Burlingame, CA) at 1:1000 dilution overnight on a
shaker on ice. α-Tubulin (Sigma–Aldrich) was concomitantly probed as a sample loading control. Thereafter, HRP-conjugated secondary goat anti-rabbit Ab (1:2000 dilution; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used to recognise the primary Ab. The bands were visualised by enhanced chemiluminescence solution (Thermo Fisher Scientific) and subsequently exposed to Kodak film (Carestream Health, Rochester, NY).

**Tissue histological analyses**

The left lung and left lobe of the liver were fixed in 4% paraformaldehyde and embedded in paraffin and sectioned serially. Sections were stained with hematoxylin and eosin and assessed by an observer blinded to the treatment groups. A scale was used to assess lung injury based on capillary congestion, alveolar congestion, leucocyte infiltration and alveolar wall thickness, where 0 = normal lungs, 1 = mild injury < 25% lung involvement, 2 = moderate injury 25–50% lung involvement, 3 = severe injury 50–75% lung involvement and 4 = very severe injury > 75% lung involvement. Leucocyte infiltration in the lung was evaluated by an image analysing system (automated image analysis software; Olympus, Tokyo, Japan). The sum of the above four indicators represents the lung injury score (range 0–16). Liver injury was evaluated based on liver cell diffuse vacuolar degeneration, loss of architecture and karyolysis. A scale was used to evaluate liver injury, where 0 = normal liver, 1 = mild injury, 2 = moderate injury, 3 = severe injury and 4 = complete necrosis of the liver.

**Lung wet/dry mass ratio**

At 24 h after CLP or sham operation, bilateral lungs were removed and weighed. The lungs were kept at 60°C for 48 h and then reweighed. The percentage of wet-to-dry mass represented the lung wet/dry mass ratio.

**Serum alanine aminotransferase activity assay**

Blood was harvested from the orbit at 24 h after CLP or sham operation. The serum level of alanine aminotransferase (ALT) was detected by enzymatic assay kit (Abcam, Cambridge, MA) according to the protocols recommended by the manufacturer.

**Cytokine measurement**

Blood was harvested from the orbit at 24 h after CLP or sham operation. The serum level of TNF-α was measured using an ELISA kit (R&D Systems, Minneapolis, MN) according to the protocols recommended by the manufacturer.

**Statistical analyses**

All data are presented as the mean ± SEM. Comparisons between two groups were analysed using Student’s t-tests. Comparisons among multiple groups were analysed using one-way ANOVA followed by a post hoc analysis using a Bonferroni test. The survival rate was calculated using the log-rank test. All data were analysed with SPSS Statistics for Windows v17.0 (SPSS, Inc., Chicago, IL). P < 0.05 was considered statistically significant.

**Results**

**TRPM2 KO mice show decreased HO-1 expression and autophagy in peritoneal macrophages after polymicrobial sepsis**

In our previous study, we clearly confirmed that septic TRPM2 KO mice had decreased bacterial clearance and impaired outcome. TRPM2 plays a protective role in controlling bacterial clearance by promoting HO-1 expression. In order to explore whether TRPM2-mediated HO-1 has a role in bacterial clearance by regulating autophagy in macrophages, first we investigated the effect of TRPM2 on autophagy induction. At 24 h after CLP, the peritoneal macrophages from septic WT and TRPM2 KO mice showed a significant increase in HO-1 expression compared to sham mice. HO-1 expression in peritoneal macrophages from septic TRPM2 KO mice were also markedly lower than that of septic WT mice (Figure 1a). As expected, compared to sham mice, the peritoneal macrophages from septic WT and TRPM2 KO mice also showed a significant increase in LC3 II/LC3 I expression. LC3 II/LC3 I expression in peritoneal macrophages from septic TRPM2 KO mice was also markedly lower than that of septic WT mice (Figure 1b).

**Polymicrobial sepsis–induced autophagy in peritoneal macrophages is dependent on HO-1**

To examine whether HO-1 is critical for autophagy in macrophages after sepsis, a known chemical HO-1 inhibitor (SnPP) was used to evaluate the role of HO-1 in regulating autophagy. Treatment of WT mice with SnPP before CLP resulted in decreased HO-1 and LC3 II/LC3 I expression in peritoneal macrophages (Figure 2a). Furthermore, treatment of WT mice with HO-1 inducer (Hemin) every other d (three times) prior to CLP increased HO-1 and LC3 II/LC3 I expression (Figure 2b). These results indicate that polymicrobial sepsis–induced autophagy is dependent on HO-1 in peritoneal macrophages.
Pre-treatment with HO-1 inducer increases autophagy in peritoneal macrophages in TRPM2 KO mice after polymicrobial sepsis

To confirm further whether TRPM2-mediated HO-1 is required for autophagy induction in macrophages, we next examined whether the HO-1 inducer increased autophagy in peritoneal macrophages from septic TRPM2 KO mice. At 24 h after CLP, treatment of TRPM2 KO mice with hemin prior to CLP increased HO-1 and LC3 II/LC3 I expression in peritoneal macrophages (Figure 3). These results suggest that TRPM2-mediated HO-1 plays a role in autophagy induction in peritoneal macrophages in CLP-induced septic mice.

Increased bacterial clearance of septic TRPM2 KO mice pre-treated with HO-1 inducer is reversed by autophagy inhibitor

To confirm further whether TRPM2-mediated HO-1 has a role for bacterial clearance by regulating macrophagic autophagy during polymicrobial sepsis, we investigated the role of autophagy inhibitors (chloroquine and 3-MA) in bacterial clearance in TRPM2 KO mice treated with hemin prior to CLP. At 24 h after CLP, pre-treatment of TRPM2 KO mice with hemin significantly increased bacterial clearance in the PLF (Figure 4). However, compared to TRPM2 KO mice pre-treated with hemin, chloroquine or 3-MA treatment significantly decreased bacterial clearance (Figure 4).

Improved outcome of septic TRPM2 KO mice pre-treated with HO-1 inducer is reversed by autophagy inhibitor

Pre-treating TRPM2 KO mice with hemin significantly improved their survival compared to TRPM2 KO mice pretreated with vehicle ($P = 0.02$; Figure 4). However, this improvement was reversed by chloroquine ($P = 0.02$; Figure 4) or 3-MA administration ($P = 0.005$; Figure 4). Compared to TRPM2 KO mice
pre-treated with vehicle control, pretreatment of TRPM2 KO mice with hemin significantly attenuated lung and liver injury, as well as TNF-α level in the serum (Figure 5a–d). As expected, these improvements

**Figure 2.** Effects of HO-1 inhibitor and HO-1 inducer on the HO-1 expression and autophagy in peritoneal macrophages after polymicrobial sepsis. (a) The WT mice were injected i.p. with HO-1 inhibitor (tin protoporphyrin, 50 mg/kg) or vehicle (0.9% NaCl) 1 h before CLP. CLP surgery was performed at 24 h after last hemin or vehicle injection. At 24 h after sham and CLP surgery, peritoneal macrophages were isolated. HO-1 activation was analysed by Western blot from two independent experiments (n = 4 per group). The HO-1 protein concentration was normalised by α-tubulin. LC3 I and LC3 II activation was analysed by Western blot from two independent experiments (n = 4 per group). The ratio of LC3 II/LC3 I was used to evaluate the intensity of autophagy. (b) WT mice were injected i.p. with 10 mg/kg hemin or vehicle every other d (three times) prior to CLP. CLP surgery was performed at 24 h after last hemin or vehicle injection. At 24 h after sham and CLP surgery, peritoneal macrophages were isolated. HO-1 activation was analysed by Western blot from two independent experiments (n = 4 per group). The HO-1 protein concentration was normalised by α-tubulin. LC3 I and LC3 II activation was analysed by Western blot from two independent experiments (n = 4 per group). The ratio of LC3 II/LC3 I was used to evaluate the intensity of autophagy. *P < 0.05; **P < 0.01, Student’s t-test. Error bars denote the mean ± SEM.

**Figure 3.** The effects of HO-1 inducer on the HO-1 expression and autophagy in peritoneal macrophages in TRPM2 KO mice after polymicrobial sepsis. (a) The TRPM2 KO mice were injected i.p. with 10 mg/kg hemin or vehicle every other d (three times) prior to CLP. CLP surgery was performed at 24 h after last hemin or vehicle injection. At 24 h after sham and CLP surgery, peritoneal macrophages were isolated. HO-1 activation was analysed by Western blot from three independent experiments (n = 3 per group). (b) The HO-1 protein concentration was normalised by α-tubulin. LC3 I and LC3 II activation was analysed by Western blot from three independent experiments (n = 3 per group). The ratio of LC3 II/LC3 I was used to evaluate the intensity of autophagy. *P < 0.05; **P < 0.01, compared to KO+vehicle group, one-way ANOVA. Error bars denote the mean ± SEM.
were reversed by chloroquine or 3-MA administration (Figure 5a–d). Taken together, these results indicate that TRPM2-mediated HO-1 has a role for bacterial clearance possibly by regulating autophagy in macrophages and contributes to the outcome of polymicrobial sepsis.

Discussion

The present study aimed to explore whether TRPM2-mediated HO-1 has a role in bacterial clearance by regulating autophagy in peritoneal macrophages during polymicrobial sepsis. Several findings are observed in this study. First, at 24 h after CLP, TRPM2 KO mice show decreased HO-1 and LC3 II/LC3 I expression in peritoneal macrophages. Second, CLP-induced LC3 II/LC3 I expression in peritoneal macrophages is dependent on HO-1. Third, treatment of TRPM2 KO mice with hemin (a HO-1 inducer) before CLP increased HO-1 and LC3 II/LC3 I expression in peritoneal macrophages. Fourth, treatment of TRPM2 KO mice with hemin prior to CLP significantly increased bacterial clearance and improved the survival rate, and these improvements were reversed by chloroquine or 3-MA (an autophagy inhibitor) administration. Finally, pre-treatment of septic TRPM2 KO mice with hemin significantly attenuated organ injury and the serum TNF-α level, and these improvements were also reversed by chloroquine or 3-MA treatment. Our findings suggest that TRPM2-mediated HO-1 has a role for bacterial clearance possibly by regulating autophagy in peritoneal macrophages during polymicrobial sepsis.

Macrophages are the first line of defence in innate immunity and play an important role in the elimination of bacteria. Following uptake in macrophages, autophagy targets intracellular bacteria in the cytosol-formed autophagosome, controlling their growth by degrading it with lysosome. Increasing evidence shows that autophagy is necessary for host defence against invasive bacteria. Disruption of certain autophagy genes severely reduces the host’s ability to remove invasive pathogens. Recent studies have demonstrated the role of TRPM2 in bacterial clearance in macrophages and the possible mechanism. Zhang et al. demonstrated that the macrophagic TRPM2 channel is critical for host resistance to bacterial invasion by enhancing phagosome maturation through promoting the recruitment of early endosomal Ag. Another study found that TRPM2-mediated cation influx is essential for acidification of phagosomes during phagosome maturation in macrophages undergoing phagocytosis. TRPM2 KO mice showed reduced bacterial clearance resulting from the decreased acidification in phagosomes in macrophages.

Recent studies have reported that TRPM2 promotes autophagy induction through different mechanisms. TRPM2 promotes autophagy which plays an important role in the formation of extracellular reticular traps of neutrophils stimulated by hydrogen peroxide. Jiang et al. found that the TRPM2 KO significantly inhibits zinc oxide-stimulated autophagy in human cerebrovascular pericytes. TRPM2 disruption significantly reduces mitochondrial autophagy and promotes cancer cell death. Herein, we observed that genetic disruption of TRPM2 indeed resulted in decreased the ratio of LC3 II/LC3 I, a surrogate marker for autophagy, in peritoneal macrophages after CLP. In our previous study, we clearly showed that the TRPM2 KO significantly reduces bacterial clearance of macrophages and increases bacterial burden in septic mice. These results indicate that TRPM2 may play a role in bacterial clearance by promoting autophagy in peritoneal macrophages during polymicrobial sepsis.

It has been reported that HO-1 plays a key role in LPS-stimulated autophagy. HO-1-mediated autophagy is also critical for preventing liver injury during sepsis. Using HO-1 inhibitor and HO-1 inducer, we further confirmed that CLP-induced autophagy is dependent on HO-1 in peritoneal macrophages. The decreased HO-1 and LC3 II/LC3 I expression were also observed in TRPM2 KO peritoneal macrophages after CLP, suggesting that TRPM2 plays a role in

![Figure 4](image-url)
Figure 5. Improved outcome of septic TRPM2 KO mice pre-treated with HO-1 inducer is reversed by autophagy inhibitor. TRPM2 KO mice were injected i.p. with 10 mg/kg hemin or vehicle every other d (three times) prior to CLP. CLP surgery was performed at 24 h after last hemin or vehicle injection. Chloroquine (60 mg/kg) or 3-MA (30 mg/kg) was injected i.p. 1 h after CLP. Mice were euthanized at 24 h after CLP. (a) Survival was monitored for 7 d. Data consist of two independent experiments (n = 15 per group). *P < 0.05, Kaplan–Meier log-rank test. (b) Lung and liver were collected and stained with hematoxylin and eosin (original magnifications, × 400). (c) Lung injury score, leukocyte infiltration and lung wet/dry mass ratio from three independent experiments (n = 6 per group) represent the severity of lung injury. (d) Liver injury score (n = 6 per group) and serum alanine aminotransferase (ALT) level from three independent experiments (n = 6 per group) represent the severity of liver injury. (e) Serum samples were collected, and TNF-α level was detected by ELISA from three independent experiments (n = 6 per group). *P < 0.05; **P < 0.01; ***P < 0.001, one-way ANOVA. Error bars denote the mean ± SEM.
autophagy induction in macrophages, possibly by regulating HO-1.

The decreased HO-1 expression likely underlies the decreased autophagy, which results in impaired bacterial clearance in macrophages observed in septic TRPM2 KO mice. To confirm this hypothesis further, TRPM2 KO mice were treated with HO-1 inducer (hemin) before CLP. TRPM2 KO mice pre-treated with hemin showed a significant enhancement of HO-1 and LC3 II/LC3 I expression in peritoneal macrophages, as well as decreased bacterial burden in the PLF after CLP. Enhanced autophagy promoted bacterial clearance which explained the associated improved survival rate, alleviated lung and liver injury and decreased systemic inflammation in TRPM2 KO mice with CLP. To validate this hypothesis further, we used chloroquine or 3-MA to see if it reversed this improved effect. As expected, chloroquine or 3-MA significantly decreased the bacterial clearance and reversed the improved outcome of TRPM2 KO mice pre-treated with hemin. These findings suggest that TRPM2-mediated HO-1, possibly by regulating autophagy, promotes bacterial clearance in peritoneal macrophages during CLP-induced polymicrobial sepsis.

There are some limitations to the present study. First, the data could not elucidate in detail how TRPM2-mediated HO-1 activates autophagy in macrophages. Second, the role of TRPM2-mediated HO-1 in regulating the acidification of phagosome or phagosome maturation was not investigated in the present study. Finally, we did not have a macrophage-specific TRPM2 KO mouse to examine the role of macrophage-specific TRPM2 in bacterial clearance in sepsis and the underlying mechanism. The deficiency of TRPM2 in multiple tissues may make it difficult to determine the function of TRPM2 in macrophages in vivo. Further studies are needed to elucidate these.

In summary, our study identifies a role for TRPM2-mediated HO-1 in bacterial clearance possibly by regulating autophagy in peritoneal macrophages during polymicrobial sepsis. Our data further reveal the mechanism of TRPM2 in the pathogenesis of sepsis, and immune intervention through TRPM2 may contribute to the treatment of sepsis.

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ORCID iD
XiaoWei Qian https://orcid.org/0000-0001-9553-1098

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