Toll-like receptor 4 contributes to acute kidney injury after cardiopulmonary resuscitation in mice

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Abstract. Toll-like receptor 4 (TLR4) activation mediates renal injury in regional ischemia and reperfusion (I/R) models generated by clamping renal pedicles. However, it remains unclear whether TLR4 is causal in the kidney injury following global I/R induced by cardiac arrest (CA) and cardiopulmonary resuscitation (CPR). The present study used wild-type (C3H/HeN) and TLR4-mutant (C3H/HeJ) mice to produce the CA/CPR model. CA was induced by injection of cold KCl and left untreated for different time periods. After resuscitation (72 h), the level of blood urea nitrogen (BUN) and serum creatinine (Scr), as well as histological changes in renal tissue were assessed to evaluate the severity of acute kidney injury (AKI). The expression of TLR4, intercellular adhesion molecule-1 (ICAM-1), myeloperoxidase (MPO) and growth-regulated oncogene-β (GRO-β) in kidney tissues was detected. The results demonstrated that the levels of Scr and BUN increased significantly in C3H/HeN and C3H/HeJ mice after CPR. CPR also resulted in increased expression of TLR4, ICAM-1, GRO-β and MPO in a CA-duration dependent manner. However, there was decreased expression of ICAM-1, GRO-β and MPO in C3H/HeJ mice compared with that in C3H/HeN mice. C3H/HeJ mice were resistant to AKI as demonstrated by the minor changes in renal histology and function following CPR. In conclusion, mice suffered from AKI after successful CPR and severe AKI occurred in mice with prolonged CA duration. TLR4 and its downstream signaling events that promote neutrophil infiltration via ICAM-1 and GRO-β may be important in mediating inflammatory responses to renal injury after CPR.

Introduction

Even if first-stage resuscitation is successful following cardiac arrest (CA) and cardiopulmonary resuscitation (CPR), the body is subjected to systemic ischemia/reperfusion (I/R) injury due to the return of spontaneous circulation (ROSC), thereby leading to multiple organ dysfunction syndrome (MODS) (i.e. post-resuscitation syndrome). Acute kidney injury (AKI) is one of the features of post-resuscitation syndrome. AKI is most commonly caused by systemic hypoperfusion and can lead to acute renal failure (1,2). AKI is common in the survivors of CA with a reported incidence of 23.2%. Incomplete recovery of renal function from AKI may cause excessive long-term morbidity and mortality and result in a higher risk of chronic kidney disease (3-5). Following ROSC, focus is placed on the condition of the brain and heart; however, prevention against AKI is also important and requires investigation.

Toll-like receptors (TLRs) are a family of signal transduction molecules that are implicated in the induction of innate and adaptive immunity. Recently, accumulating evidence has demonstrated that Toll-like receptor 4 (TLR4) is activated by endogenous proteins released from damaged tissues and participates in mediating renal injury following I/R (6,7). However, the majority of previous studies have employed regional I/R models generated by clamping renal pedicles, which are different from the global I/R models induced by CA/CPR. In addition, it remains unclear whether TLR4 is causal within the kidney after CA/CPR.

It is well known that larger animal models that allow for whole body hypoperfusion, lack access to the full toolset of genetic manipulation possible in the mouse. However, recently a mouse model of CA/CPR has emerged, which can be adapted to model AKI (8-10). This model is reliable to reproduce physiological, functional and histological outcomes observed in clinical AKI. In the present study, a mouse model of CA/CPR was used to test the hypothesis that TLR4 activation contributes to renal injury following resuscitation.

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Materials and methods

Animals. C57BL/6 male mice (n=40; age, 10-12 weeks) were obtained from the Animal Resource Center of Wuhan University (Wuhan, China). TLR4 mutant male mice (C3H/HeJ; n=20; age, 10-12 weeks) were purchased from Shanghai SLAC Animal Center (Shanghai, China). In these mice, the intracellular region of TLR4 amino acids had a mutation at the 712 site from proline to histidine, which resulted in no response of TLR4 to its ligand, lipopolysaccharide (11). TLR4 wild-type male mice (C3H/HeN; n=20; age, 10-12 weeks) were purchased from Beijing Vitalriver Experimental Animal Center (Beijing, China). All animals were housed in an animal facility with a specific-pathogen free environment. The mice were kept in a dry, ventilated and clean environment. The room temperature was maintained at ~25°C and humidity was maintained at 40-70%. The mice were allowed ad libitum access to food and water under automatic day/night control (12:12 h). Animal protocols were approved by the Laboratory Animal Ethics Committee of Huazhong University of Science and Technology (Huazhong, China).

Experimental protocols.

Experiment 1: Assessing the role of TLR4 in renal injury as the duration of CA increases. C57BL/6 mice were randomly assigned to 4 groups. In the sham group, the animals underwent surgical preparation without CPR. In the CA 3 min, CA 5 min and CA 8 min groups, resuscitation was started after 3, 5 and 8 min CA, respectively (n=10, per group).

Experiment 2: Assessing the inflammatory response to CA/CPR in TLR4-mutant mice. Animals were randomly assigned to 4 groups (n=10, per group) which were the C3H/HeN sham group (sham-ej), C3H/HeN CPR group (model-en) and C3H/HeJ CPR group (model-ej). Chest compressions and mechanical ventilation were started after 5 min of CA.

Surgical preparation. Mice were anesthetized with 40 mg/kg body weight of 1% pentobarbital sodium (Merck & Co., Inc., Whitehouse Station, NJ, USA) delivered by intraperitoneal injection. The mice were immobilized by taping their four legs in the supine position, and the rectal temperature was controlled at close to 37°C during surgery with a heating pad and lamp. The mice were immobilized 4 extremities by tape in a supine position on a heating pad. Rectal temperature was controlled at ~37°C during surgery with a heating pad and lamp. The mice were orally intubated with a 22-gauge catheter, connected to a mouse ventilator (ALC-V8S, Shanghai AlcBio Company, Shanghai, China) set to a respiratory rate of 130 breaths/min. Needle electrodes were placed subcutaneously on the chest for electrocardiogram monitoring throughout the experimental procedures. A middle incision was made in the neck of the mice and the external jugular vein was carefully separated and a catheter was inserted.

CA/CPR procedure. CA was induced by injection of 0.08 mg/g body weight KCl via the jugular catheter, and confirmed by the appearance of asystole on the electrocardiography monitor and no spontaneous breathing (12,13). At this time, mechanical ventilation was interrupted for the length of CA duration. After different CA durations, CPR was begun by injection of 0.4 µg/g epinephrine followed by chest compressions at a rate of ~300 beats/min and ventilation with 100% oxygen at a respiratory rate of 160 breaths/min. As soon as ROSC was achieved, defined as electrocardiographic activity with visible cardiac contractions, chest compressions were stopped (12,13). If ROSC could not be achieved within 10 min of CPR, resuscitation was stopped. Sham animals underwent anesthesia, oral intubation, mechanical ventilation, surgical preparation and insertion of vascular catheters. An equivalent volume of isotonic saline was given as a placebo control of KCl and epinephrine in the sham groups. All mice were alive in the sham groups after 3 days. However, in experiment 1, 9 and 8 mice survived in the CA 3 min and CA 5 min groups, respectively. While only 6 mice survived for 72 h in the CA 8 min group. In experiment 2, 7 and 8 mice survived in the C3H/HeN and C3H/HeJ CPR groups after ROSC 72 h, respectively.

Measurement of blood biochemical parameters. After ROSC (72 h), the surviving mice were sacrificed by cervical dislocation, and blood samples were collected from the heart. After centrifugation (4,000 x g/min, 20 min), the supernatant was stored at -80°C. Plasma serum creatinine (Scr) and blood urea nitrogen (BUN) levels were measured by an automatic biochemical analyzer (BECKMAN LX20, Beckman Coulter, Brea, CA, USA).

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The left kidneys were taken from the surviving mice and preserved at -80°C. Total RNA was extracted from 100 mg kidney tissue using TRIzol reagent (Invitrogen, Thermo Fisher, Waltham, MA, USA) in accordance with the manufacturer’s instructions. The concentration and purity of RNA were tested using an ultraviolet spectrophotometer (UV-2802, Unico Co., Dayton, NJ, USA). The reverse transcription of RNA to cDNA was performed using a reverse transcription kit (Takara Bio Inc., Shiga, Japan). The primers used were: TLR4 sense, 5’-TGAGGACCTTGGGGAAATGAGC-3’ and antisense, 5’-CTGCACTTGT TTGCAATCTCAT-3’; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sense, 5’-CTCTGATTTGGTGCGATT GG-3’ and antisense, 5’-CTTGAAGATGTGGAAT-3’. The PCR reaction (using an LY96G PCR instrument; Hangzhou LongYang Scientific Instruments Co., Ltd., Hangzhou, China) was conducted in a thermal cycler with initial 4 min denaturation at 95°C, followed by 30 (TLR4) or 28 cycles (GAPDH) of denaturation at 94°C for 50 sec, annealing at 58°C for 40 sec (TLR4) or 56°C for 30 sec (GAPDH), extension at 72°C for 60 sec (TLR4) or 50 sec (GAPDH), and a final extension at 72°C for 7 min (TLR4) or 10 min (GAPDH). The target bands were quantitatively determined by the relative intensity of TLR4 gene compared with that of GAPDH by using the GeneSnap gel image acquisition system (GeneGenius; Syngene UK, Cambridge, UK) and GeneTools gel image analysis software (version 2.0; Syngene UK) (14).

Protein preparation and western blotting. Proteins from the left kidney tissues were prepared using radioimmunoprecipitation assay buffer. All protein samples were subjected to concentration determination with the bicinchoninic
acid assay method. Protein samples were separated using 10% SDS/PAGE and transferred onto polyvinylidene fluoride membranes (Merck Millipore, Billerica, MA, USA). Membranes were blocked for 40 min with a 5% bovine serum albumin solution at room temperature, and subsequently incubated with the appropriate antibodies overnight at 4°C, and then the membranes were washed three times with Tris-buffered saline/Tween 20 (TBST), followed by incubation with a secondary antibody. Subsequently, the membranes were washed four times with TBST. The indicated antibodies included: Rabbit anti-mouse TLR4 polyclonal antibody (1:500, cat. no. ab13867, Abcam, Cambridge, UK), rabbit anti-mouse intercellular adhesion molecule-1 (ICAM-1) polyclonal antibody (1:500, cat. no. ab7815, Abcam) and rabbit anti-mouse growth-related gene product β (GRO-β) polyclonal antibody (1:500, cat. no. ab9950, Abcam, UK). Goat anti-mouse β-actin antibody (1:5,000, cat. no. ANTOO9, Antgene, Wuhan, China) was used as an internal control, and the secondary antibodies included goat anti-rabbit polyclonal antibody (1:5,000; cat. no. BA1003, Boster Biological Technology, Wuhan, China) and mouse anti-goat polyclonal antibody (1:5,000, cat. no. BA1006, Boster Biological Technology). The membranes were visualized using a Kodak Image Station 4000 MM imaging system (Kodak, Tokyo, UK) in accordance with the manufacturer's instructions. Semi-quantitative analysis was conducted on strips with Quantity one software (version 4.6.2; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Morphological studies. In experiment 1 and 2, the mice were sacrificed by cervical dislocation and the right kidneys were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (5 µm) were stained with hematoxylin and eosin according to standard protocols. The changes in renal histopathology were observed with electron microscopy. Jablonski grading was used to analyze the injury severity of renal tubules (0-4 levels) (15). In experiment 2, tissue samples for transmission electron microscopy were obtained from the left kidney. The cortex and outer section of the outer medulla were separated and cut into pieces of ~1 mm³. The tissue sections were prefixed with 4% glutaraldehyde, stored at 4°C until processed and then post-fixed with 1% osmium tetroxide. The specimens were immersed in propylene oxide after dehydration with a graded series of ethanol, embedded with epoxy resin and cut into ultrathin sections (0.1 µm). The sections were stained subsequently with lead-uranium and the changes in ultrastructural organization were then observed by a transmission electron microscope.

Myeloperoxidase (MPO) activity assay. Tissues from the left kidney were homogenized and centrifuged (12,000 x g, 20 min). Then, the supernatant was harvested and stored at -80°C. The MPO activity in the supernatant was detected using an enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (Nanjing JianCheng Bioengineering Institute, Nanjing, China).

Statistical analysis. Data are presented as the mean ± standard deviation for each experiment. The data were analyzed by SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA) as appropriate. Two-group comparisons were analyzed using Student’s t-test, whereas multiple-group comparisons were conducted using analysis of variance followed by Bonferroni’s multiple comparisons test. P<0.05 was considered to indicate a statistically significant difference.

Results

Indicators of kidney function. In experiment 1, the plasma concentration of Scr and BUN were markedly increased after CA/CPR. Moreover, the levels of Scr and BUN increased significantly with the increasing duration of CA, suggesting that the length of CA duration may be important in AKI following CPR (Fig. 1A and B). In experiment 2, there were also higher plasma concentrations of Scr and BUN following CPR in C3H/HeJ and C3H/HeN mice. However, the levels of Scr and BUN in the model-ej group were significantly lower than those in model-en group, indicating that TLR4-mutant mice exhibited minor renal dysfunction following CPR (Fig. 1C and D).

TLR4 mRNA and TLR4 protein expression. In experiment 1, to address whether CA/CPR stimulates TLR4 activation, the mRNA and protein expression of TLR4 in the kidney were measured on day 3 after ROSC. Normal renal tissue expressed TLR4 at a basal level. However, TLR4 mRNA and protein levels were significantly increased following CA/CPR, and increased in a CA-time dependent manner (Fig. 2A and B).

ICAM-1 and GRO-β protein expression. To determine the effect of CA/CPR on the expression of the downstream factors regulated by TLR4 signaling the protein expression of ICAM-1 and GRO-β, which are classically known for their role in neutrophil infiltration and aggregation were measured. In experiment 1, ICAM-1 and GRO-β levels were significantly increased with the extension of CA duration (Fig. 3A and B). In experiment 2 it was also demonstrated that CPR induced an increase in ICAM-1 and GRO-β expression. However, the expression of ICAM-1 and GRO-β in C3H/HeJ mice was significantly lower than that in the C3H/HeN mice, indicating that TLR4 mutation reduced neutrophil infiltration into renal tissues following CPR (Fig. 3C and D).

Activity of MPO. In the two experiments, CPR contributed to the elevation of MPO activity in the renal tissues, a biochemical marker of neutrophil infiltration. As the length of CA duration was prolonged, the activity of MPO gradually increased significantly with the extension of CA duration (Fig. 4A). However, in experiment 2 there was a reduction in MPO activity in the model-ej group compared with that in the model-en group, suggesting that TLR4 may be required for neutrophil infiltration into renal tissues after CPR (Fig. 4B).

Renal histopathology. Examination by light microscopy revealed that in experiment 1, the glomerular and tubular structures were clear and intact in the sham group. However, histological abnormalities that reflected renal tissue damage were observed after CPR, including atrophy of tubules, interstitial edema, denaturation, swelling, vacuolation of tubule epithelium and tubular necrosis. The renal abnormalities were most severe in the CA 8 min group as shown by the
highest renal damage scores (Fig. 5A and B). In experiment 2, the epithelium of the tubules showed signs of denaturation, swelling and vacuolation in the model-ej and model-en groups. However, the renal damage score was significantly lower in model-ej group than that in model-en group, suggesting that TLR4-mutant mice were more resistant to renal injury after CPR (Fig. 5C and D).

Examination by electron microscopy revealed that the membranes of the microvilli remained intact and the brush borders were closely arrayed in the sham-ej and sham-en groups. However, the loss of microvilli and increased cell vacuolization were shown in the model-ej and model-en groups. The ultrastructure abnormalities of the microvilli were more severe in the model-en group than that in the model-ej group (Fig. 6A). In addition, it was demonstrated that the glomerular epithelium in the model-en group presented more evident swollen endoplasmic reticulum and mitochondria with broken or absent cristae, some of which appeared to be vacuolized when compared with the model-ej group (Fig. 6B).

Discussion

The present study demonstrated that the mice suffered from AKI after successful CPR and severe AKI occurred in mice with prolonged CA. Notably, the results showed that CA/CPR increased the expression of TLR4, ICAM-1, GRO-β and MPO in kidney tissues in a CA-duration dependent manner. However, TLR4-mutant mice were protected from renal I/R injury as shown by the minor changes in renal function and histology, suggesting that TLR4-mediated inflammatory responses may be involved in AKI triggered by CA/CPR.

Post-resuscitation syndrome is one of the leading causes of mortality in patients following ROSC (16). Renal damage can cause metabolic acidosis and hyperkalemia, which are associated with cardiovascular events (such as ventricular fibrillation), threatening the life of critical patients. Incomplete recovery of renal function following AKI may cause excessive long-term morbidity and mortality and may be associated with a higher risk of chronic kidney disease. A number of survivors require renal replacement therapy during the advanced life-support phase. Thus, it is worth paying more attention to prevention of AKI following CPR.

Recently, it has been demonstrated that injection of KCl is a feasible method to induce immediate CA and allow successful resuscitation in a high fraction of animals (9). In the present study, the results demonstrated successful reproducibility of the AKI model after CA/CPR as judged by the changes in renal histology and function. In addition, AKI became more severe with prolonged CA duration. The results were consistent with clinical studies, which showed that transient impaired renal function is common in patients surviving CA (17). Duration of CA, pre-existing impaired renal function and blood pressure at admission were not independent risk factors associated with renal outcome (18). Moreover, a recent study demonstrated that AKI may not just be a consequence of CA but of the time without spontaneous circulation (19). In experiment 1, it was demonstrated that there was a relatively high survival rate with notable renal histopathological changes after CPR in the CA 5 min group. Therefore 5 min duration of CA was selected in experiment 2.

The mechanism of AKI after CPR is hypothesized to be associated with I/R injury, which is now shown to be involved in inflammation. TLR4, is a pattern recognition
A receptor that recognizes exogenous microbial or endogenous ligands resulting in the induction of natural immune and inflammatory responses (20). I/R rapidly activates innate immune responses. TLR4 has been shown to be upregulated in kidney I/R by clamping the renal pedicles (6,7). However, it is unclear whether TLR4 exhibits a causal role within
the kidney after the global I/R induced by CA/CPR. In the present study, it was demonstrated that TLR4 was activated by CA/CPR as shown by the significant increase in TLR4, which occurred in a CA-time dependent manner. Moreover, the present study aimed to demonstrate the critical role for TLR4 in the pathophysiology of AKI using TLR4 geneti-
cally mutant mice. Notably, C3H/HeJ mice were resistant to CPR-induced AKI as shown by the protection associated with a concomitant decrease in Scr and BUN levels and attenuation in histological changes, suggesting that TLR4 may contribute to AKI following CPR.

It has been suggested that endogenous TLR ligands, such as high-mobility group box 1 were released from damaged or necrotic cells in response to I/R (21). TLR4 engagement by its ligands triggers multiple downstream effects, including the activation and expression of chemokines responsible for neutrophil accumulation, which are also the features of pathological changes of AKI. MPO is one of the principal enzymes released from neutrophil azurophilic granules, and MPO activity was evaluated as an index of neutrophil accumulation (22). The results showed that MPO activity was increased in C3H/HeN and C3H/HeJ mice after resuscitation, indicating that neutrophils were recruited within the kidney following CPR. ICAM-1 is predominantly expressed on the surface of endothelial cells, involved in regulating a variety of effector cells to migrate to areas of inflammation. In addition, ICAM-1-deficient mice are protected against ischemic renal injury (23), indicating that ICAM-1 is a key mediator of acute ischemic renal failure likely acting via potentiation of neutrophil-endothelial interactions. Similar to ICAM-1, GRO-β can also induce inflammatory cells, such as neutrophils, mononuclear cells and lymphocytes, to migrate towards I/R injury regions and exacerbate inflammatory reactions. The inhibition of neutrophil migration by genetic deletion of GRO-β receptor or its ligand suppresses I/R-induced kidney injury (24,25). In the present study, the expression of ICAM-1 and GRO-β were elevated in kidney tissues after CPR, which are associated with the upregulation of TLR4 in C3H/HeN mice. This suggests that TLR4 and its downstream signaling events that promote neutrophil infiltration via ICAM-1 and GRO-β may be important in mediating inflammatory responses to renal injury following CPR.

It is noteworthy that expression of renal ICAM-1, GRO-β and MPO were also significantly increased following CPR in C3H/HeN mice, albeit less so than in C3H/HeJ mice. This implies that there is a response to CPR-induced I/R through TLR4-independent pathways. One of the possible explanations may be that renal TLR2, which is predominantly expressed by tubular cells, also mediates I/R injury in the kidney. Genetic absence or knockdown of TLR2 was previously shown to reduce cytokine and chemokine production, reduce leukocyte infiltration, and protect against kidney dysfunction and tubular damage (26,27).

The present study had certain limitations. It is better to use a mechanical compressor instead of manual chest compressions after CA. However, as far as we know, a mechanical compressor for small animals was not commercially available when the experiments were conducted. In addition, the immediate reperfusion period following ROSC is characterized by an abrupt increase in the plasma tumor necrosis factor-α concentration (28). In the present study, the levels of inflammatory cytokines were not measured following ROSC. In addition, it is better to observe the dynamic changes in inflammatory cytokines after resuscitation. Furthermore, patients treated with CPR generally have a clinical disease; however, the mice in the present study were healthy. Therefore, the outcome of this study in a mouse model of CPR remains to be demonstrated in large-animal and clinical studies.

In conclusion, the results documented that AKI after successful CPR is not rare and the duration of CA is associated with the renal outcome. In addition, renal TLR4 is crucial in mediating CPR-induced AKI, via systemic cytokine release and subsequent intrarenal events, such as renal neutrophil infiltration. Our study suggests that TLR4 has a potential

![Figure 6.](image-url)

Figure 6. Electron microscopy results. (A) The loss of microvilli and increased cell vacuolization in microvilli occurred in the model-ej and model-en groups. The abnormalities were more severe in the model-en group than that in model-ej group. (B) The glomerular epithelium presented more evident swollen endoplasmic reticulum and mitochondria with broken or absent cristae, some of which appeared to be vacuolized, in the model-en group than in the model-ej group.
therapeutic application for acute kidney injury after cardiopulmonary resuscitation.

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