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Toll-like receptor 4 is involved in myocardial damage following paraquat poisoning in mice

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ABSTRACT

The ingestion of the herbicide paraquat (PQ) can cause multiple organ injury including cardiac lesions. However, the underlying mechanism of myocardial damage is not known. Toll-like receptor 4 (TRL4) is a pattern-recognition receptor in the innate immune response to microbial pathogens. TLR4 is involved in heart dysfunction such as septic shock or myocardial ischemia. We investigated whether TLR4 would be linked to the pathogenesis of heart disease due to PQ exposure. Wild type mice (WT) and TLR4-deficient mice were injected intraperitoneally with 75 mg/kg of PQ to induce myocardial damage and tested for echocardiographic assessment, histopathology, pro-inflammatory cytokine and TLR4 expression. WT mice after PQ exposure displayed deteriorate cardiac function, pathological damages, increased TLR4 mRNA and protein levels as well as myocardial TNF-α and IL-1β levels. Compared with WT mice, TLR4-deficient mice were significantly resistant to the PQ-induced injury. We concluded that the TLR4 was required as a mediator and played an important role in myocardial damage due to PQ.

1. Introduction

Paraquat dichloride (1,1′-dimethyl-4,4′-bipyridinium dichloride) (PQ) has been an effective and widely used herbicide in more than 120 countries since 1962. However, PQ is highly toxic when ingested (Gunnell et al., 2007). Although some progress has been made regarding the treatment of PQ intoxication using animal models (Dinis-Oliveira et al., 2009; Dong et al., 2012; Hagiwara et al., 2007), there is still no vital antidote or method that can be used for detoxification. The deliberate and accidental ingestion of PQ has caused many human fatalities. In many countries, PQ is a suicide agent because of ease of access, extremely low cost, rapid mode of morbidity and high mortality rates (Hwang et al., 2002).

PQ ingestion is characterized by rapid systemic uptake that leads to multiple organ injury (Onyon and Volans, 1987). The effects of PQ on the lungs, including acute respiratory distress syndrome (ARDS) and pulmonary fibrosis, have been evaluated in many studies (Takahashi et al., 1994; Fukuda et al., 1985; Tomita et al., 2007). Cardiac injury due to PQ poisoning is also frequent. Cardiogenic events, including arrhythmias, heart failure, and cardiac arrest, occasionally lead to sudden death in patients (Povoa et al., 1992; Noguchi et al., 1990; Fukushima et al., 2010). Myocardial injury is an important effect secondary to extensive PQ poisoning, usually suggesting poor prognosis (Fang et al., 2004). However, the mechanism of myocardial injury due to PQ poisoning is not fully understood.

The Toll-like receptors (TLRs), transmembrane proteins, serve as pattern-recognition receptors in the innate immune response to microbial pathogens. Among these receptors, TLR4 is activated by bacterial lipopolysaccharide (LPS) and is therefore known as the LPS receptor (Baumgarten et al., 2001; Oyama et al., 2004a). TLR4 signaling occurs in a manner similar to that mediated by interleukin-1 (IL-1). TLR4 is also activated by several endogenous ligands associated with tissue injury (Ohashi et al., 2000). After recruiting the adapter protein MyD88 and interleukin receptor-associated kinase (IRAK), the protein kinase NF-κB-inducing kinase (NIK) is activated through TNF coronary receptor-activated factor-6 (TRAF6). NIK subsequently activates an I-κB kinase that results in phosphorylation of I-κB, thereby promoting NF-κB, translocation to the nucleus, and gene transcription, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). TLR4 is expressed in leukocytes and cardiomyocytes and has been linked to the pathogenesis of heart disease. TLR4 is involved in heart dysfunction, such as septic shock and myocardial ischemia (Baumgarten et al., 2006; Boyd et al., 2006; Tavener et al., 2004). However, the relationship between expression of TLR4 and heart dysfunction due to PQ poisoning is not known.

In this study, we analyzed the role of TLR4 in PQ-induced myocardial injury using wild type mice and TLR4 knockout mice.

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Our data showed the TLR4 was involved in the acute phase of cardiac dysfunction following PQ poisoning.

2. Materials and methods

2.1. Animals

Male wild type C57BL/6J mice (8–10 weeks old, weighing 18–22 g) and male TLR4 deficient mice (TLR4-ko, C57BL/10ScN) were purchased from the animal facility at the China Medical University. All animals were housed with a daily 12 h illumination cycle and free access to standard feed and water. All procedures involving animals were approved and performed in accordance with the guidelines provided by the institutional ethics commission.

2.2. Experimental protocols

There were four groups in the experiment: (1) Control group (WT mice treated with saline, $n = 6$); (2) TLR4-ko group (TLR4-ko mice treated with saline, $n = 6$); (3) WT + PQ group (WT mice, $n = 30$) and (4) TLR4-ko + PQ group (TLR4-ko mice, $n = 30$). Group 3 and Group 4 were injected intraperitoneally with 75 mg/kg of PQ (Sigma, St. Louis, MO, USA; 98% purity). Mice were randomly divided into the corresponding groups. PQ dosing was based on studies by Ge et al. (2010).

At 2, 4, 8, 16, and 24 h after PQ administration, 6 mice from the WT + PQ group and TLR4-ko + PQ group were euthanized by exsanguination of the abdominal aorta under deep anesthesia. All the specimens were analyzed by histology, while only the WT + PQ mice were evaluated for TLR4 mRNA. Samples from mice receiving PQ at 2, 8, and 24 h were used for cytokine detection, whereas only mice from the WT + PQ group were evaluated by Western blot analysis. At 8 h after treatment, control mice and TLR4-ko mice were euthanized by the same method. Heart tissue specimens were quickly removed and prepared as indicated below.

2.3. Echocardiographic assessment

Mice were anesthetized (with a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine) for cardiac geometry and functional assessment using a 2-D guide M-mode echocardiography (Agilent Sonos 5500) at 8 h following injection of either PQ or saline. Heart rate (BMP), diastolic and systolic left ventricular (LV) dimensions were recorded and fractional shortening was calculated using the following equation: (EDD − ESD)/EDD, where EDD represents end-diastolic diameter, and ESD represents end-systolic diameter.

2.4. Histopathology

The isolated apex of the heart was fixed in 4% paraformaldehyde, embedded in paraffin wax, sectioned and stained with hematoxylin–eosin (HE). For transmission electron microscopy, heart tissues were fixed by perfusion with 4% paraformaldehyde and 1% glutaraldehyde in phosphate buffer at pH 7.2, cut into 1 mm cubes, and washed with 0.1 M phosphate buffer. The tissues were post-fixed with 1% OsO_4 in Millonig's phosphate buffer, dehydrated, and embedded in Epon 812 resin (TAAB, UK). Ultrathin sections were examined with a JEM-1200EX electron microscope (JEOL, Japan).

2.5. Cytokine detection

The isolated hearts were snap-frozen and TNF-α and IL-1β were assessed using a TNF-α and IL-1β ELISA KIT (Wuhan Boster Bio-Engineering Co, Ltd, Wuhan, China). Cytokines were measured using 100 μg of protein. Standard reference cytokines were provided by the manufacturer. Assays were conducted with a microtiter plate reader (Dynex MRX, USA) (450-nm wavelength). Values are expressed as pictograms per milligram of protein.

2.6. Total RNA preparation and qualitative (q) reverse transcription (RT)-PCR

The heart tissue (30 mg) was snap-frozen in liquid nitrogen and stored at −70 °C for future RNA extraction. Total heart RNA was extracted using the RNAiso Reagent (Baoxin Biotechnology Co. Ltd., China), according to the manufacturer's instructions. The quantity of total RNA was determined by OD260 measurements. cDNA was synthesized from total RNA using the SYBR PrimeScript RT-PCR kit (Baoxin Biotechnology Co. Ltd., China), and qPCR was carried out on a Prism 7500 Fast PCR System (Applied Biosystems, Inc., USA). mRNA expression was assayed using the following primers:

TLR4, (sense) 5′-CAGCAAAGTCCCTGATGACA-3′
and (antisense) 5′-CCTGGGGAAAAACTCTGGAT-3′;
GAPDH, (sense) 5′-TGTGTCCGTCGTGGATCTGA-3′
and (antisense) 5′-TTGCTGTTGAAGTCGCAGGAG-3′.

Amplification of TLR4 mRNA required an initial denaturation step at 94 °C for 30 s. Temperature cycling consisted of 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 60 s, and elongation at 72 °C for 90 s. Transcript levels were normalized by comparison with GAPDH using the $2^{-\Delta\Delta CT}$ method.

2.7. Western blot analysis

Heart tissue (30 mg) was homogenized with RIPA buffer. The supernatant was collected for measuring protein concentration using a protein assay kit (Beyotime Institute of Biotechnology, China). Samples were then diluted to appropriate concentrations, and 30 μg of each were separated on 12% sodium dodecyl sulphate–polyacrylamide gels, followed by electrophoretic transfer to a nitrocellulose membrane. Membranes were blocked with 5% skimmed milk in Tris-buffered saline solution containing 0.1% Tween20, and then probed with rabbit anti-mouse TLR4 antibody (1:200; Santa Cruz Biotechnology, USA) for 60 min at 4 °C. After washing, horseradish peroxidase-coupled sheep anti-rabbit IgG monoclonal antibody (1:500, Wuhan Boster Bio-Engineering Co., Ltd., China) was applied. The proteins were detected by an enhanced chemiluminescence kit (Pierce, USA) according to the manufacturer's instructions and quantified by densitometry analysis using Image-Pro Plus software (Media Cybernetics, Inc., USA). We determined the density ratio of each band compared to its corresponding GAPDH band.

2.8. Statistical analysis

All statistical analyses were carried out using SPSS 16.0 software (SPSS, Inc., USA). Results are expressed as means ± standard deviation (SD). Analysis of variance (ANOVA) followed by Student–Neuman–Keuls post hoc test was performed to determine the statistical significance of differences between groups. A P-value less than 0.05 indicated significance.

3. Results

3.1. Echocardiographic changes in mice

Knockout TRL4 did not affect tested geometric or functional parameters, including heart rate, EDD, ESD and fractional shortening. Compared to the untreated mice, heart rate was significantly affected in WT + PQ mice, and less affected in TLR4-ko mice at 8 h after PQ treatment. Upon PQ treatment in both WT and TLR4-ko mice, EDD and ESD increased, and fractional shortening decreased. Compared to the WT + PQ group, changes in ESD and fractional shortening in TLR4-ko + PQ mice were significantly attenuated following PQ exposure (Fig. 1).

3.2. Histopathological changes in heart tissue in mice

The appearance of heart tissue in the control group is shown in Fig. 2A. Obvious pathological changes were observed in WT mice after PQ treatment. At 2 h, cardiac muscle fibers showed swelling, distension and disorderly alignment. Some cell nuclei appeared to swell, and the staining was lighter. There was edema in the interstitial tissues. Cross-striations of the cardiac muscle fibers and intercalated discs became vague and undistinguishable (Fig. 2B). At 4 h and 8 h, more of the cardiac muscle cell nuclei showed morphologic alterations (Fig. 2C and D). At 16 h, several cell nuclei dissolved and disappeared, and vacuoles appeared in the cell endoplasm (Fig. 2E). At 24 h, nuclei were not present in the majority of the cardiac muscle cells. The cardiac cells were disrupted, the cell membranes were no longer visible, and there were multiple vacuoles in the endoplasm of the cell (Fig. 2F).

The morphology was similar in the control and TRL4-ko groups without PQ treatment (Fig. 2G). Pathological changes were significantly attenuated in PQ-treated TLR4-ko mice. At 2 h, some cell nuclei and cardiac muscle fibers appeared to swell, but the level of edema was less severe (Fig. 2H). At 4 h and 8 h, cardiac muscle cell nuclei showed fewer morphologic alterations compared with WT mice (Fig. 2I and J). At 16 h and 24 h, the cell membranes and nuclei in most cardiac cells were still visible. There were fewer vacuoles in the cellular endoplasm. Few cardiac cells were disrupted in PQ-treated TLR4-ko mice (Fig. 2K–M).