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Full Length Research Paper

Asphyxial hypoxic preconditioning induces neuroprotective effect via activation of toll-like receptor 4 (TLR4) signal pathway in rats

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Hypoxic preconditioning (HP) induces neuroprotective effect against cerebral ischemia, but the mechanism still remains unclear. In this study, we investigated whether the toll-like receptor 4 (TLR4) signal pathway was involved in cerebral ischemic tolerance. According to the treatment of HP and asphyxial cardiac arrest (ACA), rats were assigned to ACA group, HP + ACA group, HP group and Sham group. Rat mortality was 5% in HP + ACA group and 30% in ACA group (P<0.01); neurofunctional scores in HP and HP + ACA group were better than in ACA group (P<0.05). Compared with Sham group, TLR4 mRNA expression, NF-κB activity and the production of TNF-α and IL-6 in HP or HP + ACA or ACA group were significantly increased. The increase was progressively significant in groups (AHP < AHP + ACA < ACA) (P<0.01). HP induced mild inflammation via activating the TLR4 signal pathway and then further inhibited inflammatory response induced by ACA.

Key words: Asphyxial cardiac arrest, hypoxic preconditioning, cerebral ischemic tolerance, toll-like receptor 4.

INTRODUCTION

Asphyxial cardiac arrest (ACA), a clinical correlate of airway loss, is a rare but important cause of death during anesthesia. Cardiac arrest (CA) leads to severe or even fatal injury, with only 37% survival rate of in-hospital CA and only 9% of out-hospital CA. Functional recovery of survivors can be highly variable, but as low as 3 to 10% can recover to their original state (Peters and Boyde, 2007). Cardiopulmonary resuscitation (CPR) has obtained suboptimal curative effects. Although current treatments can acquire restoration of spontaneous circulation (ROSC), about 40% cases will never wake up when they are sent to hospitals, and about 30% will suffer from permanent cerebral damage, including cognitive handicap and motor deficits. Poor neurological recovery after CA needs an improved pathophysiologic understanding on global cerebral ischemic injuries resulted from CA (Xiao et al., 2004; Gillum et al., 1989; National Center for Health Statistics, 1995; Peberdy et al., 2010). Pre-exposure on brain with sub-threshold level of pathologic stimulation, which is also termed as preconditioning, can significantly diminish neuronal vulnerability to ischemic injuries. The molecular mechanisms of preconditioning remain poorly understood. Stimuli used to precondition the brain and induce neuroprotection include transient global and focal ischemia, hypoxia, and administration of lipopolysaccharide (LPS) and so on. Interestingly, LPS treatments induce not only ischemic tolerance but also endotoxin tolerance.

Furthermore, ischemic preconditioning can induce tolerance for subsequent LPS attack. Between ischemic

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and endotoxin tolerance, an improved understanding on ischemic tolerance may be gained by further exploration on the mechanisms of endotoxin tolerance. It is well-known that endotoxin tolerance is closely related with toll-like receptor 4 (TLR4) signal pathway. According to classic theory of endotoxin tolerance, the first exposure to LPS can activate TLR4. Stimulation on TLR4 triggers signaling cascades and results in transcription activation of several inflammatory mediators including TNF and IL-6. The signal transduction initiated by LPS and cytokines can induce a hyporesponsive state for subsequent LPS attacks. TLR4, a member of the TLR family, participates in inherent immune response. Most research on TLR4 has focused on its roles on sepsis, however, an increasing number of studies suggest that TLR4 may also serve as a proinflammatory receptor (Davis and Patel, 2003; Gidday, 2006; Ran et al., 2005; Zemke et al., 2004; Kariko et al., 2004, 2004; Fan and Cook, 2004; Li and Wang, 2005, 2006). At present, it is controversial that whether the TLR4 signal pathway is also involved in brain ischemic tolerance. After cardiopulmonary resuscitation, cerebral secondary lesion seriously threatens survivors.

The inflammatory process plays a key role in the development of brain secondary injuries (Stanimirovic and Satoh, 2000; Iadecola and Alexander, 2001; del Zoppo et al., 2001; Bowen et al., 2006). To explore the mechanisms of cerebral ischemic tolerance benefits for the prevention and treatment of brain injury resulted from CA, we investigated the role of TLR4 signal pathway in cerebral ischemic tolerance induced by hypoxic preconditioning (HP).

MATERIALS AND METHODS

Experimental animals

Male adult Wistar rats, weighing 250 to 350 g (mean 300 ± 50 g), were provided by Experimental Animal Center of Capital University of Medical Sciences. These rats were bred with enough food and water in 12 h light/dark cycle cages. These experiments were approved by Animal Care and Use Committee, Capital University of Medical Sciences.

Surgical procedures

160 adult male Wistar rats were randomly divided into sham surgical group (without asphyxia, n = 20), HP group (4 times of HP, no ACA, n = 42), HP + ACA group (ACA 24 h after HP, n = 44) and ACA group (no HP, n = 54). HP and ACA were performed according to previous studies (Xiao et al., 2004; Jin et al., 2005; Fink et al., 2004; McCaul et al., 2006; Xu et al., 2004). The rats were anesthetized with chloral hydrate (30 mg/kg) and intubated with a 14-G plastic catheter under direct laryngoscope. After intubation, 2 mg/kg vecuronium was intraperitoneally administered. The rat was ventilated at 50 breaths/min by a rodent ventilator (Harvard Apparatus Model 683), with a tidal volume of 10 ml/kg and positive end-expiratory pressure (PEEP) of 3 cm H_2O. Femoral vein and artery were cannulated (Intermedic Non-Radioopaque PE-90 catheters, Becton Dickinson) for drug infusion, and then arterial blood gas analysis and mean arterial blood pressure (MAP) were determined. The rectal temperature of rats was maintained at 37.0 ± 0.5℃. Before asphyxia, the animals were ventilated with room air.

ACA

The animals were asphyxiated by disconnecting the respiratory tube of the ventilator for 4 min (resulting in cardiac arrest for 1 min). When asphyxia lasted for 3 min, CA was defined as MAP ≤10 mmHg and then CA was maintained for 1 min. After CA for 1 min, the rats were resuscitated by resumed ventilation (100% oxygen), cardiac compressions, intravenous administration with 0.005 mg/kg epinephrine and 1 mEq/kg sodium bicarbonate. Resuscitation was sustained to ROSC which was defined as MAP ≥ 50 mmHg. T_{CA} (time of cardiac arrest: time from asphyxia to MAP <10 mmHg) and T_{ROSC} (time to restore spontaneous circulation: from resuscitation to MAP □ 50 mmHg) were recorded. The mortality of rats 24 h after resuscitation was recorded.

Hypoxic preconditioning

Hypoxic preconditioning was performed by disconnecting the respiratory tube of the ventilator for 1 min followed by re-ventilation for 5 min. The preconditioning procedure was repeated 4 times.

Neurological deficit score (NDS)

The neurological functions and outcome assessment after graded injuries were performed as described previously (Geocadin et al., 2000, 2005). NDS was used to assess global neurological function after hypoxia cerebral injuries. Like human clinical neurological examination, NDS consists of consciousness level, cranial function, sensorimotor function, reflexes and simple behavioral assessments. Scoring ranges from 100 (completely normal) to 0 (brain death).

Tissue preservation

To analyze TLR4 mRNA expression, NF-κB activity and the production of TNF-α and IL-6 at 1, 3, 6, 12, 24, 48 and 72 h after injury, the rats were re-anesthetized with 30 mg/kg chloral hydrate and then sacrificed. The brain tissues were preserved in liquid nitrogen for further analysis.

Real-time reverse transcription-PCR (RT-PCR) analysis for TLR4 mRNA

Total RNA was extracted from brain tissues with a RNA extraction kit (Roche) according to manufacturer's instruction. The RNA samples were preserved at -80℃ for further use. Only RNA samples with 1.8 to 2.1 of A_{260}/A_{280} were used for further study. 100 g total RNA template was reversely transcribed at 42℃ for 15 min. The cDNA was preserved at -20℃ for amplification. The TLR4 mRNA expression was determined with TaqMan primers designed by Beacon designer 5.0. The primers and probes were listed in Table 1. PCR was carried out with ABI 7500 sequence detector (PE Applied Biosystems, Foster City, CA). 25 µl reaction systems contained 2 µl first-strand cDNA, 1 × PCR master mix, 300 nmol/l forward and reverse primer and 75 nmol/l taqman probe. All reactions were performed in the following reaction parameters: 50℃ for 2 min and 95℃ for 10 min, followed by 40 cycles of 95℃ for 15 s and 60℃ for 1 min. Simultaneously, positive control (plasmids) and negative control (no samples) were set. The standard curve for quantifying mRNA copy numbers was constructed with known numbers of TLR4 cDNA plasmid and GAPDH cDNA plasmid (provided by Han JQ, Basic Medical

Table 1. Real-time RT-PCR primer and probe sequences.

	Oligonucleotide sequence	Amplicon
TLR4 (Accession NM_019178)	Left: 5'- TGAGAAACGAGCTGGTAAAGAATT -3' Right: 5'- GTGGAAGCCTTCCTGGATGATG -3' Probe: 5'- AGTGCCCCGCTTTCAGCTTTGCCT -3'	122 bp
GAPDH (Accession NM_001090946)	Left: 5'- CATGACCTTCCGTGTTCCTACC -3' Right: 5'- TAGCCCAGGATGCCCTTCAG -3' Probe: 5'- CCTCAGACGCCTGCTTCACCACCT -3'	138 bp

Table 2. General physiologic parameters of each group.

Groups	Weight (g)	HR (bpm)	MAP (mmHg)	NDS
Sham (n = 20)	271.6 ±1 4.7	381.9 ± 40.2	98.4 ±10.5	100 ± 0
HP (n = 42)	275.8 ± 8.8	386.4 ± 34.8	99.8 ± 8.6	100 ± 0
HP + ACA (n = 44)	273.7 ± 12.2	382.2 ± 38.1	97.4 ± 9.7	100 ± 0
ACA (n = 54)	275.7 ± 9.5	386.7 ± 36.4	101.7 ± 9.5	100 ± 0

Sciences Academy of Military Medical Sciences of Chinese PLA). Standard curves for each internal control were generated with CT (threshold cycle) of templates with known number dilutions.
The absolute copy numbers of TLR4 and GAPDH mRNA in each sample were calculated based on its CT values in their plasmid DNA standard curve. The absolute copy number of TLR4 mRNA was then normalized according to the differences of RT efficiency and RNA integrity among test samples.

Determination of NF-kB activity

NF-kB activity was determined with TransAM kit (Active Motif, Carlsbad, CA), according to manufacturer's instruction. Nuclear extracts of brain tissues in each group were prepared with a Nuclear Extract kit (Active Motif, Carlsbad, CA). Briefly, diluted standard protein and nuclear extracts were added into plates and then were pre-coated with oligonucleotide containing NF-kB consensus sequence (5'-GGGACTTTCC-3'). Subsequently, the reaction system was incubated at room temperature for 1 h to facilitate conjugation. Primary antibody, which only conjugates with activated NF-kB/p50 was added into each well and then incubated at room temperature for 1 h. Then, horseradish peroxidase conjugated anti-IgG antibody was added and then incubated at room temperature for 1 h. Finally, developer and fixing bath were added. The absorbance was read at 450 nm by an automated micro plate reader (Synergy HT, Bio-Tec instrument, USA). This assay is specific for NF-kB/p50 activation and more sensitive than electrophoretic mobility shift assay.

Enzyme-linked immunosorbent assay (ELISA)

The TNF-α and IL-6 expressions in brain tissues in each group were determined according to the standard protocol of ELISA kits (Sigma, USA).

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical analyses were performed by SPSS 12.0 statistical software. The differences between groups were compared by analysis of variance (ANOVA). $P<0.05$ was considered statistically significant.

RESULTS

The general physiologic parameters were shown in Table 2 and there were no significant differences among the four groups.

Mortality of rats, T_{CA} and T_{ROSC}

The mortality of rats was 5% in the HP + ACA group and 30% in the ACA group ($P<0.01$). T_{CA} was 210.1 ± 18.4 s in the HP + ACA group and 190.6 ± 21.2 s in the ACA group ($P<0.01$). T_{ROSC} was 58.3 ± 11.1 s in the HP + ACA group and 99.6 ± 16.4 s in the ACA group ($P<0.01$). The detailed data were shown in Table 3.

Neurological deficit score (NDS)

The DNS in the HP + ACA group was better than the ACA group at 24, 48 h or 72 h. The detailed data were shown in Table 4.

TLR4 mRNA expression

Real time RT-PCR revealed that the expression of TLR4 mRNA was significantly higher in the HP or HP + ACA or ACA group compared with the sham group at each time point (except 72 h). The up-regulation started at 1 h, and peaked at 3 to 6 h and gradually down-regulated from 12

Table 3. The mortality of rats, T_{CA} and T_{ROSC}.

Groups	Mortality (%)	TCA (s)	TROSC (s)
Sham	0/20 (0)		
HP	0/20 (0)		
HP + ACA	1/20 (5)	210.1 ± 18.4	58.3 ± 11.1
ACA	6/20 (30) △	190.6 ± 21.2△	99.6 ± 16.4△

□*P*<0.01 *vs* HP + ACA, T_{CA} and T_{ROSC} refer to: time from asphyxia to cardiac arrest, time from resuscitation to restoration of spontaneous circulation, respectively.

Table 4. NDS after resuscitation (X ± SD, n = 6).

Groups	24 h	48 h	72 h
sham	100 ± 0	100 ± 0	100 ± 0
HP	100 ± 0	100 ± 0	100 ± 0
HP + ACA	68.45 ± 4.3	72.66 ± 4.88▲	80.34 ± 2.44*
ACA	55.40 ± 6.4△	62.12 ± 5.34△▲	69.32 ± 3.52△*

□*P*<0.01 *vs*. HP + ACA; ▲*P*<0.05 *vs*. 24 h, **P*<0.05 vs 48 h.

h. The increase intensity in all groups was progressive (HP < HP + ACA < ACA). The detailed data were shown in Figure 1.

Variation of NF-κB activation

TransAM NF-κB kit analyses revealed that activity of NF-κB was significantly higher in the HP or HP + ACA or ACA group compared with sham group at each time point (except 72 h). The kinetic variation of NF-κB activation was similar with TLR4 mRNA expression. The up-regulation started at 1 h, and peaked at 3 to 6 h and gradually down-regulated from 12 h. The increase intensity in all groups was progressive (HP < HP + ACA <ACA). The detailed data was shown in Figure 2

Variation of TNF-α and IL-6 production

ELISA analyses showed that the production of TNF-α and IL-6 was significantly higher in the HP or HP + ACA or ACA group compared with sham group at each time point. The production of TNF-α increased from 1 h, and peaked at 3 h and then decreased fast at 24 h. IL-6 production slowly increased from 1 h, and peaked at 12 h and then slowly decreased. The increase of TNF-α and IL-6 production in all groups was progressive (HP< HP + ACA < ACA). The detailed data were shown in Figures 3 and Figure 4.

DISCUSSION

Functional recovery of survivors from ACA are usually not satisfactory, thus their family is subject to severe economic and mental burden. Therefore, it is important to reduce secondary nerve injuries after cardiopulmonary resuscitation. Preconditioning induces the endogenous protective mechanisms of tissues, and then genetic manipulation and administration of drugs simulate the body's natural defense mechanisms to provide the possibility for prevention from neuronal damage and intervention after ACA (Zemke et al., 2004). Methods of preconditioning include hypoxia, short periods of ischemia, cortical spreading depression, oxygen glucose deprivation, hyperthermia and chemical preconditioning (Davis and Patel, 2003). Although hypoxic-preconditioning has shown promising neuro-protection in the subsequent ischemic brain injury, the underlying mechanisms and its influence on autonomic regulation have not yet well-understood. In this study, it was found that asphyxial hypoxic preconditioning could increase survival rate, relieve brain injury and improve nervous function after cardiopulmonary resuscitation in ACA rats, indicating that asphyxial hypoxic preconditioning was a good method of preconditioning. At present, it is recognized that ischemic tolerance involves the following putative components (Davis and Patel, 2003; Gidday, 2006; Ran et al., 2005; Zemke et al., 2004): adenosine, protein kinase C, ATP-sensitive potassium channels, NF-κB, vascular endothelial growth factor, erythropoietin, nitricoxide synthase, hypoxia-inducible factor, N-methyl-D-aspartate, superoxide dismutase, TNF, heat shock protein and free radicle. Unfortunately, different molecules have not yielded known mechanisms of ischemia tolerance. The inflammatory response plays a key role in brain ischemic injuries. Thus, we investigated

whether TLR4 inflammatory signal pathway participated in brain ischemic tolerance. Although the majority of

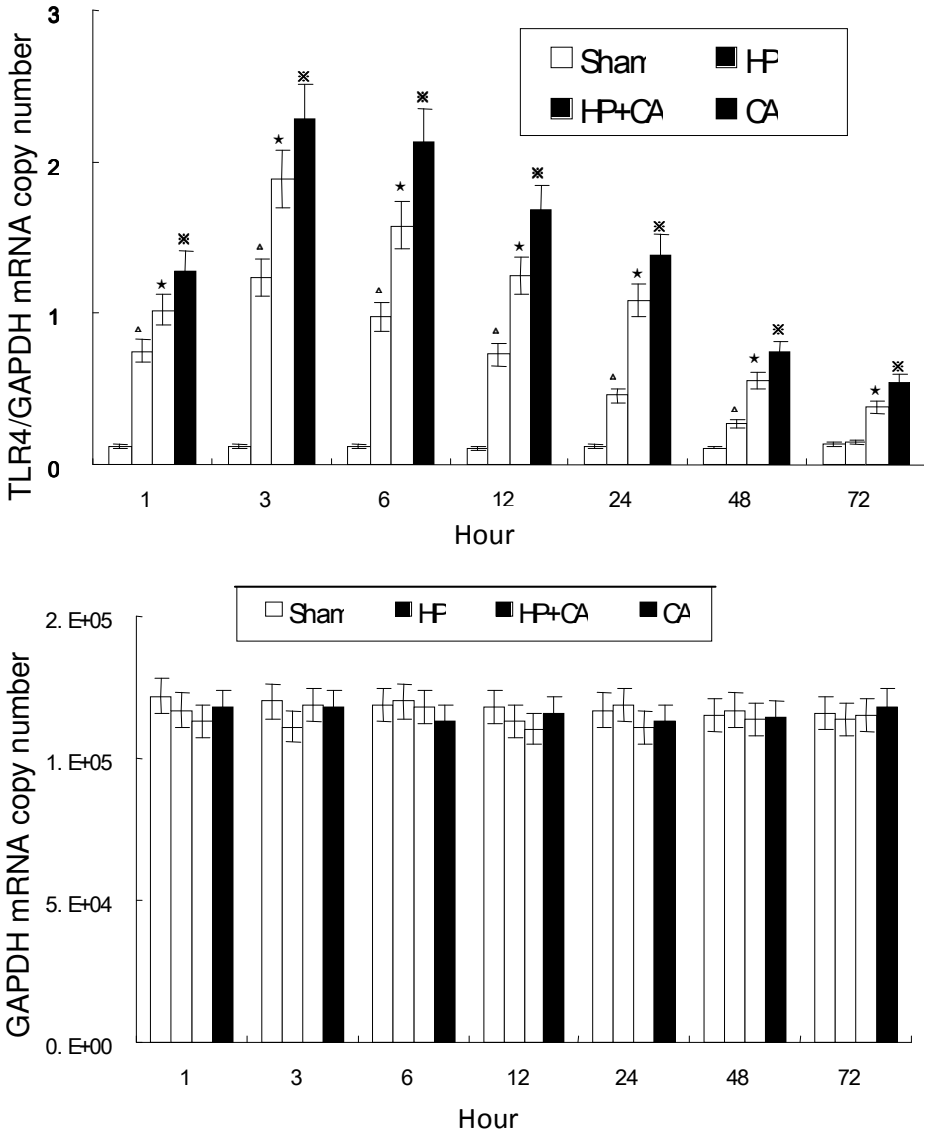

Figure 1. Real-time RT-PCR analysis of TLR4 mRNA expression. The absolute copy numbers of TLR4 mRNA were determined. TLR4/GAPDH mRNA copy numbers were displayed graphically (up). GAPDH mRNA copy numbers were displayed graphically (down). HP and ACA refer to hypoxic preconditioning and asphyxial cardiac arrest, respectively. There were 6 rats at each time point in the HP or HP + ACA or ACA group and 2 rats at each time point in the sham group (□*P*<0.01 *vs.* sham; □*P*<0.01 *vs.* HP; □*P*<0.01 *vs.* HP + ACA).

investigations on TLR4 focus on its roles as the receptor of LPS, it is also found that TLR4 can bind with multiple endogenous ligands including heat shock proteins, fibronectin, hyaluronan, fibrin/fibrinogen and elastase (Kariko et al., 2004). This study revealed hypoxic preconditioning could up-regulate TLR4 mRNA expression, but the increase of TLR4 mRNA in the HP + ACA or HP group was less than the ACA group.

Activation of TLR4 results in inflammatory responses. After TLR4 is activated, among intracellular signal transductions, the intracellular domain of TLR4 interacts with an intracellular adaptor protein MyD88. Then, phosphorylation cascades involving IL-1 receptor-associated kinase (IRAK) and TNF receptor-associated factor 6 (TRAF6) are triggered to activate transcription factor NF-κB. Activated NF-κB combines with specific κB gene sequence and then regulates the expression of many inflammation mediators (such as TNF-α, IL-1 and IL-6). These signals are cumulated in inflammatory reactions (Trudler et al., 2010). TNF-α and IL-6, two most

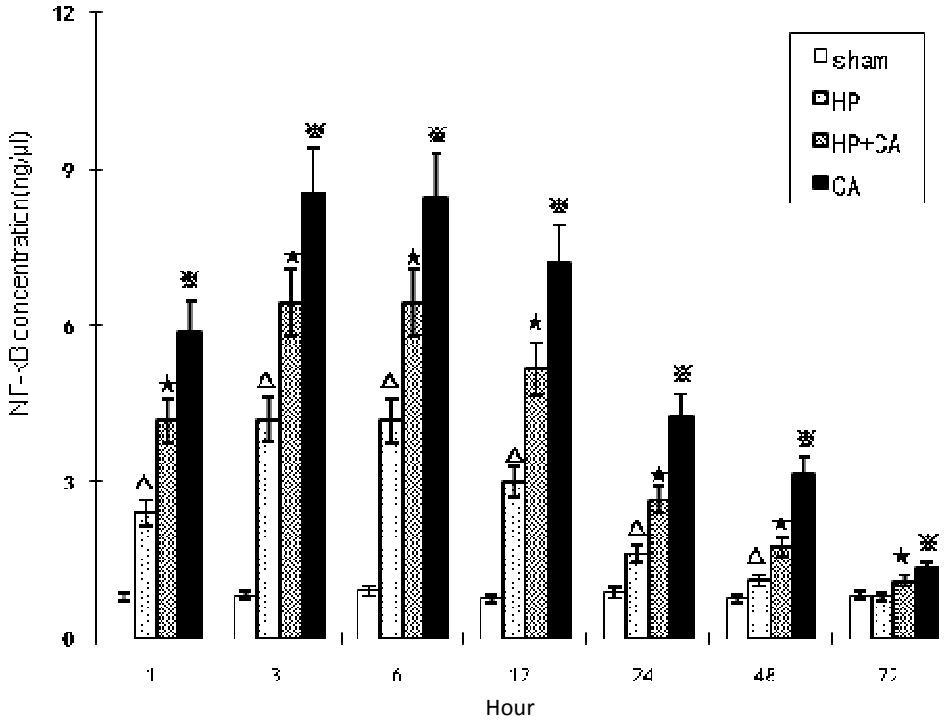

Figure 2. Change of NF-κB activation. HP and ACA refer to hypoxic preconditioning and asphyxial cardiac arrest, respectively. There were 6 rats at each time point in the HP or HP + ACA or ACA group and 2 rats at each time points in the sham group (▫*P*<0.01 *vs.* sham; ▫*P*<0.01 *vs.* HP; ▫*P*<0.01 *vs.* HP + ACA).

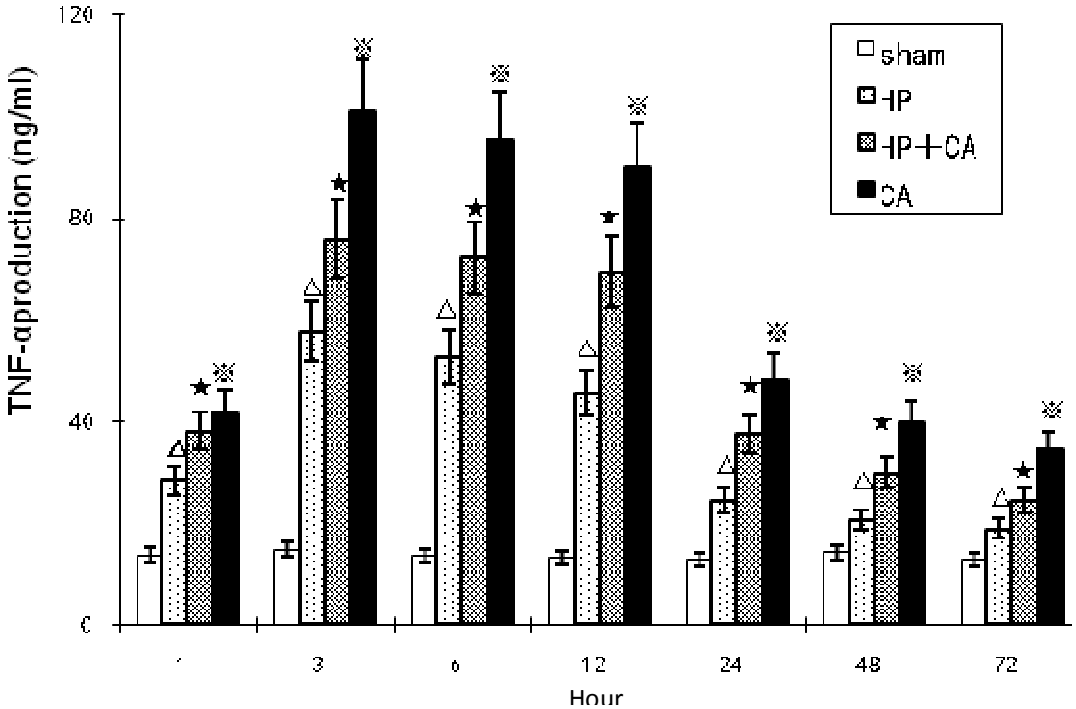

Figure 3. Changes of TNF-α production. HP and ACA refer to hypoxic preconditioning and asphyxial cardiac arrest, respectively. There were 6 rats at each time point in the HP or HP + ACA or ACA group

and 2 rats at each time point in the sham group (□*P*<0.01 *vs.* sham; □*P*<0.01 *vs.* HP; □*P*<0.01 *vs.* HP + ACA).

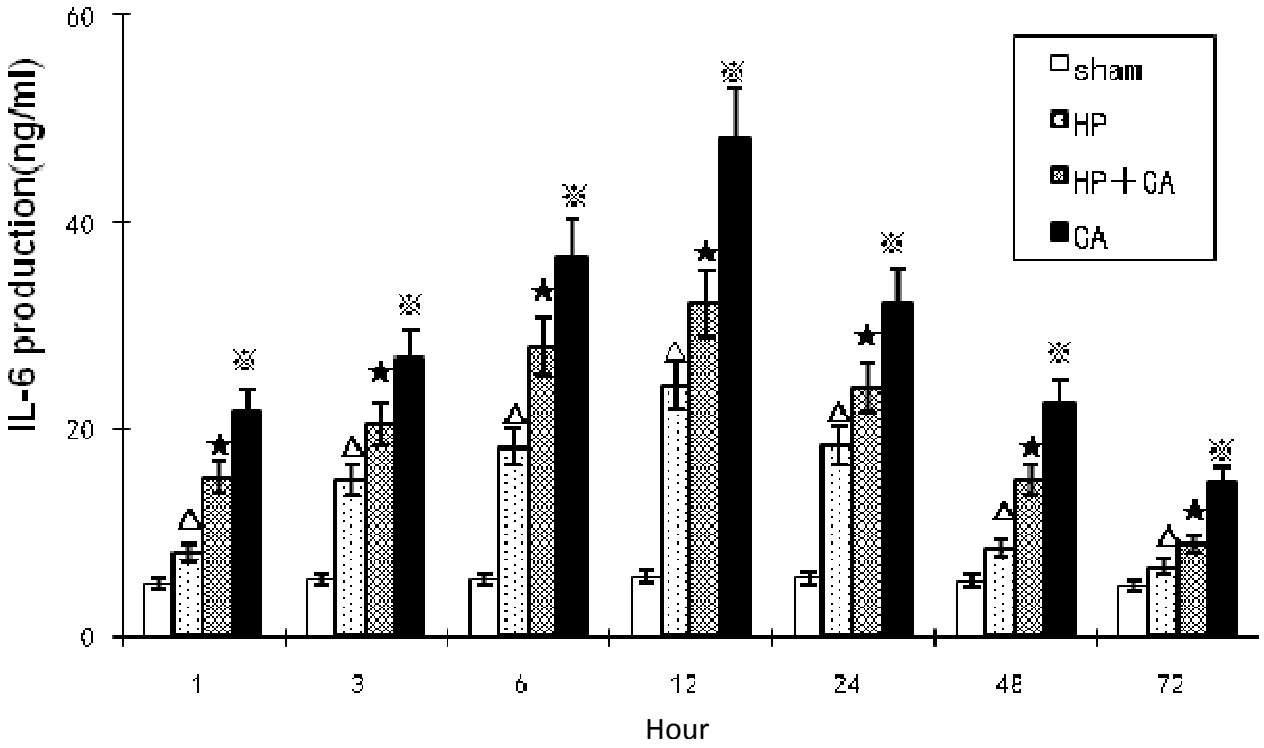

Figure 4. Changes of IL-6 production HP and ACA refer to hypoxic preconditioning and asphyxial cardiac arrest, respectively. There were 6 rats at each time point in the HP or HP + ACA or ACA group and 2 rats at each time point in the sham group (□*P*<0.01 *vs.* sham; □*P*<0.01 *vs.* HP; □*P*<0.01 *vs.* HP + ACA).

Increased systemic TNF-α concentration is closely related with mortality rates. TNF-α can induce hypotension, tissue injury and death of animals (Baeburn et al., 2002). A larger number of studies have shown close association between mean plasma IL-6 concentration and mortality rate (Song and Kellum, 2005). Thus, we chose NF-κB, TNF-α and IL-6 as research objects, and we found that hypoxic preconditioning could increase the activation of NF-κB, and induce the production of TNF-α and IL-6. It was also found that the kinetic variation of NF-κB activation, TNF-α and IL-6 production was similar to TLR4 mRNA expression. The mechanisms that asphyxial hypoxic preconditioning induces brain ischemic tolerance may result from the following reasons: hypoxic preconditioning induces mild damages of cells, blood vessels, or extracellular matrix and then small amounts of TLR4 ligands are released to activate TLR4 inflammatory pathway.

Subsequently, activated TLR4 inflammatory pathway not only results in a low level of cytokine release associated with mild inflammation but also simultaneously up-regulates feedback inhibitors of inflammation. Then, these inhibitors, including signal inhibitors, decoy receptors and anti-inflammatory cytokines, reduce the inflammatory responses of subsequent ischemic episodes (Fan and Cook, 2004; Kariko et al., 2004). However, these hypotheses should be further verified in the future.

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