Selective Antegrade Cerebral Perfusion Attenuating the TLR4/NF-κB Pathway during Deep Hypothermia Circulatory Arrest in a Pig Model

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Key Words
Deep hypothermia circulatory arrest \cdot Cerebral injury \cdot Neuroinflammation \cdot Endogenous neuroprotection \cdot Toll-like receptor \cdot Nuclear factor-kappa B \cdot Interleukin-6

Abstract
Objectives: The alteration of the Toll-like receptor/nuclear factor-kappa B (TLR4/NF-κB) signaling pathway during deep hypothermia circulatory arrest (DHCA) has not yet been defined. The aim of this study was to explore the expression of the TLR4/NF-κB pathway cytokine in cerebral injury resulting from DHCA as well as the effect of selective antegrade cerebral perfusion (SACP) on TLR4/NF-κB pathway expression.

Methods: Twelve pigs were randomly assigned to DHCA alone (n = 6) or DHCA with SACP (n = 6) at 18°C for 80 min. Serum interleukin (IL)-6 was assayed by ELISA. Apoptosis and NF-κB proteins were detected by fluorescence TUNEL and Western blot, respectively. The level of TLR4 mRNA and protein were determined through qRT-PCR and Western blot.

Results: The serum IL-6 level of the SACP group was significantly lower than that of the DHCA group at the end of circulation arrest and experimentation. Apoptotic index and NF-κB protein were apparently lower in SACP animals (p < 0.05). Compared to the DHCA group, the levels of TLR4 protein and mRNA in the SACP group were lower with significance (p < 0.05).

Conclusions: The TLR4/NF-κB signaling pathway plays a critical role in the pathogenesis of DHCA cerebral injury. Attenuation of the TLR4/NF-κB inflammatory cytokines probably contributes to the neuroprotective effect of SACP. The TLR4/NF-κB inflammatory signaling pathway may be a novel therapeutic target for developing a new strategy for neuroprotection in DHCA.

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ated with an increased risk of adverse neurological outcomes. For this reason, it is necessary to determine, clinically, which cerebral protection strategy should be adopted preoperatively (especially for prolonged DHCA), such as complex aortic arch reconstruction and congenital heart disease. Other ancillary measures of cerebral protection should be combined with DHCA to minimize its detrimental effects and maximize its benefits. Selective antegrade cerebral perfusion (SACP) is used as a neuroprotection perfusion method during DHCA [5, 6].

Although the mechanism of cerebral injury resulting from DHCA has not been fully elucidated, the neuroinflammatory theory is a novel mechanism involved in this process [7, 8]. It is well documented that Toll-like receptors (TLRs) are the first-line molecules activating innate immune and neuroinflammatory responses. TLR4 is able to activate nuclear factor-kappa B (NF-κB) via mechanisms that are both dependent and independent of the adaptor MyD88 [9]. This pathway leads to the expression of inflammatory cytokine genes, including tumor necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6 [10]. There is increasing evidence demonstrating that TLR4 plays a key role in cerebral ischemia [11]. The infarction area, cerebral edema and neurological deficits were improved in TLR4 mutant mice compared with wild-type controls [12]. Nevertheless, there are no data concerning alterations in TLR4/NF-κB signaling pathway inflammatory cytokines in brain damage following DHCA. In addition, the effect of SACP on the expression of these cytokines remains unknown.

The aim of this study was to explore the expression and the putative role of neuroinflammatory changes induced by TLR4/NF-κB in cerebral injury following DHCA. We also examined the effect of SACP on this signaling pathway.

Materials and Methods

Animal Care

Twelve Wuzhishan miniature pigs (6–8 weeks old and weighing 9.7–13 kg, average 11.66 kg) were used in this study. The study was approved by the Institutional Animal Care Committee and was conducted in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996) and the Guidelines for Animal Experimentation issued by the 1st Hospital which is affiliated to the Sun Yat-sen University.

Study Design

The animals were randomly assigned to either the DHCA group or the SACP group. The groups were treated with 80 min of DHCA alone or in conjunction with 80 min of SACP at 18°C and followed by 180 min of reperfusion including 60 min of rewarming.

The experimental protocol is described in figure 1. The tag numbers were defined as follows:

1. Baseline (T1), at a cerebral temperature of 36°C, immediately before cooling.
2. After 45 min of cooling (T2), at a nasopharynx/esophagus temperature of 18°C, before DHCA/SACP.
3. After 80 min of DHCA/SACP (T3), before the initiation of cardiopulmonary bypass (CPB).
4. After 60 min of rewarming (T4).
5. After 120 min of reperfusion (T5).
6. End point (T6), after 180 min of reperfusion.

Blood was drawn from the animals at every time point. At the final time point, all of the animals were sacrificed in order to harvest the cerebral cortex.
Anesthesia and Preoperative Management

The animals were anesthetized using an intramuscular administration of 40 mg/kg ketamine (JiuXu Pharmaceutical Co., Ltd., Zhejiang, China) and 2 μg/kg fentanyl citrate (RenFu Pharmaceutical Co., Ltd., Yichang, China). The animals were incubated and mechanically ventilated at a tidal volume of 15 ml/kg and a respiratory rate of 16 breaths/min with 40% oxygen using a volume-cycled respirator (BIRD VELA, Model 606A). Anesthesia was maintained via an intravenous infusion of fentanyl, vecuronium bromide (0.1 mg/kg, Sida Pharmaceutical Co., Ltd., Hainan, China) and midazolam (0.03 mg/kg, Huá’en Pharmaceutical Co., Ltd., Jiangsu, China).

After intubation, an arterial pressure catheter was inserted into the right femoral artery for pressure monitoring and blood sampling. An 8-Fr central venous catheter was inserted into the left femoral vein for fluid administration and central venous pressure monitoring. Urine output was measured through a suprapubic catheter. Two temperature probes were applied: one inserted into the skull via a drilled hole and one located in the rectum.

Neuromonitoring

After intubation and the establishment of vascular catheters, the animals were equipped with intracerebral probes. Three small burr holes (0.8 mm in diameter) were drilled in three quadrants centered in the bregma of the skull with access to the superficial cerebral cortex (fig. 2).

Cerebral Temperature

Hole D (fig. 2) was used for taking the cerebral temperature through a microprobe (Licox Integra, Plainsboro, N.J., USA) connected to the transducer of a multichannel physiologic recorder (MP150, BIOPAC Systems, Inc., Calif., USA).

Surgical Procedure and CPB

A median sternotomy was performed, and the strings were sutured. After system heparinization (400 IU/kg intravenously), a 10-Fr aortic cannula (Edwards Life Sciences LLC, Irvine, Calif., USA) was inserted into the ascending aorta for systemic perfusion. In addition, 12-Fr venous cannulas (Edwards Life Sciences LLC) were placed into both the superior and inferior vena cava.

CPB was performed using two roller pumps for extracorporeal circulation and suction, respectively. The extracorporeal circuit and membrane oxygenator were primed (Minimax Plus Oxygenator SK3301, Medtronic Inc., USA) with multiple electrolyte injections, and heparin (3 mg/100 ml blood; 3 mg/100 ml crystal liquid) was added. CPB flow was maintained at 75–80 ml/kg/min with a mean systemic perfusion pressure of 50–80 mm Hg.

Deep Hypothermia Circulatory Arrest

The animals were cooled systemically for 45 min with cardiopulmonary bypass by means of a heat exchanger (Sarns Heater Cooler, Ann Arbor, Mich., USA). After the body temperature was reduced to 30°C, the ascending aorta was cross-clamped and the cardioplegic solution was administered. A saline ice-slush was topically applied to cool the surface of the heart. The heat exchanger continued to run until the body temperature was cooled to 18°C. The roller pumps were then turned off, and DHCA was performed for 80 min. After completing the DHCA, the aorta was unclamped, and the animal was rewarmed to normothermia over a 60-min period using a temperature gradient of 8°C. For the hearts that resumed deleterious arrhythmia after removing the clamp, direct-current defibrillation was used to restore the sinus rhythm. The animals were weaned from CPB when the hemodynamic data became steady after partial perfusion. The reperfusion lasted for 3 h, and the animals were then sacrificed for tissue harvesting.

Selective Antegrade Cerebral Perfusion

After reaching the target temperature, the aortic cross was clamped and CPB was stopped. Passive venous drainage into the blood reservoir was allowed after undergoing DHCA. SACP is dependent on temperature; therefore, blood was allowed to circulate in the heart-lung machine to reach the correct temperature. After 5 min, SACP was initiated through a 6-Fr catheter inserted into the innominate artery and was maintained at the same temperature for 80 min. The innominate artery was snared to minimize blood loss. The perfusion pressure was controlled at 40–50 mm Hg at a rate of 10 ml/kg/min. At the end of the 80-min DHCA period, the snare on the innominate artery was released and complete CPB was gradually restored. The animals were warmed until reaching a cerebral temperature of 36°C.

Tissue Harvesting

After 3 h of reperfusion, the skull was carefully opened and the frontal cortex was quickly removed. The tissue was fixed for 24 h either in 10% buffer paraformaldehyde solution or 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) at 4°C for 24 h for subsequent fluorescence TUNEL or else immediately snap frozen in liquid nitrogen. The samples were stored at −80°C for Western blot and qRT-PCR.

Biochemical Analysis

The serum IL-6 (P600B, Quantikine, R&D Systems, Inc.) concentration was determined with an immunoassay analyzer (Cobas e 601, Roche) using the ELISA method.
was examined by ELISA analysis. The serum IL-6 level of the DHCA group and SACP group was compared using a Student’s unpaired t test. The differences were considered statistically significant when p < 0.05.

Hematoxylin and Eosin Staining
Each section was deparaffinized in xylene, and the sections were dehydrated with a graded alcohol series. After being counterstained with hematoxylin for 2 min, the sections were incubated in the eosin staining solution for 1 min. They were washed in distilled water for 3 min after each staining, and then dehydrated with absolute alcohol followed by xylene. They were air-dried and coverslipped with Permount. All specimens were observed and photographed using a microscope by an experienced pathologist who was blind to the study design.

Fluorescence TUNEL
After deparaffinizing the sections in xylene for 5 min, they were hydrated with 100% ethanol for 3 min and 95% ethanol for 1 min. They were then incubated with proteinase K (20 μg/ml in 10 mM Tris/HCl, pH 7.4) for 30 min. The sections were treated with TdT Reaction Mixture for 60 min and then stained with Hoechst for 10 min at 37 °C in a humidified chamber. The samples were rinsed with PBS for 2 min after each staining step. A positive result was confirmed when the nuclei stained brown. To quantify apoptotic cells, sections were examined at a magnification of ×10–40, using a Zeiss eyepiece graticule with a 100-square grid. Apoptotic neurons were counted in 10 different fields in each region at ×40 magnification using defined areas in the cortex. The investigator who analyzed the TUNEL sections was blind to the group identities. Apoptotic neurons were expressed as an apoptotic index (AI) that was calculated using the equation: AI = apoptotic neurons/total neurons × 100%.

Quantitative Real-Time RT-PCR
Total RNA was isolated from tissue samples with Trizol (Invitrogen) according to the manufacturer’s instructions. RNA was reverse-transcribed using a ReverTra Ace qPCR RT kit (Toyobo Biochemicals, Osaka, Japan) according to the manufacturer’s protocol. Sequence-specific primers for *Sus scrofa* TLR4 and β-actin are as follows: TLR4-F: 5′-CTTCAGAGAGAGACGAGATCC-3′, TLR4-R: 5′-ACCACACGACAATACCTT-3′, actin-F: 5′-TGTTGACAATTGTCCGTA-3′ and actin-R: 5′-CTGTCAGAGGTGGAGAG-3′.

Real-time PCR was performed with a SoFast EvaGreen Supermix Kit (Bio-Rad, Hercules, Calif., USA) on a Bio-Rad Q5 instrument (Bio-Rad) using the SyBr Green detection protocol described by the manufacturer. Briefly, the amplification mixture consisted of 0.4 μM primers, 10 μl of SoFast EvaGreen supermix (Bio-Rad) and 2 μl of template DNA in a total volume of 20 μl. The samples were amplified with the following program: initial denaturation at 98°C for 2 min, followed by 40 cycles of denaturation for 5 s at 98°C and annealing/elongation for 30 s at 57°C. The fluorescence signals were continuously collected. All PCR reactions were performed in triplicate, and control reactions without a template were included. TLR4 mRNA expression levels were normalized and calculated with a standard curve using actin as the reference gene.

Western Blot
Total proteins were extracted by homogenizing samples in RIPA buffer (Sangon, Shanghai, China). The protein concentration was determined with a BCA protein assay kit (Sangon). The proteins were boiled with 4x SDS loading buffer and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (Pall, New York, N.Y., USA) using the Mini Trans-Blot electrophoretic transfer cell system (Bio-Rad). The membranes were blocked for 2 h at room temperature with 5% nonfat milk in Tris-buffered saline Tween 20 (NaCl, 0.1% Tween-20, Tris). NF-κB/P65 (Abcam, ab31481, 1:1,000) and TLR4 (Abcam, ab8376, 1:1,000) were incubated overnight at 4°C. The primary antibody was detected with a 1:4,000 dilution of peroxide-conjugated goat anti-piglet IgG (Zymed) for 1 h at room temperature. The protein bands were visualized with an ECL plus Western blot detection system (Forverogen) on X-ray film (Kodak, Rochester, N.Y., USA). The same membranes were also blotted with GAPDH (1:1,000) and H3 (Bioss, bs-0349R, 1:500) antibodies. Densitometry was performed using Kodak Molecular Imaging software.

Statistical Analysis
All statistical analyses were performed using SPSS 20.0 for Windows software (IBM SPSS, USA). Continuous variables are expressed as the mean ± standard deviation. ANOVA and Student’s unpaired t test were used to evaluate significance for continuous variables at different time points in the same group or at the same time point between different groups. Student’s unpaired t test was also applied to assess the differences in the qRT-PCR and Western blot results from both groups. p < 0.05 was defined as statistically significant.

Results
Biochemical Analysis
ELISA analysis demonstrated that the serum IL-6 levels of the SACP group were significantly lower than those of the DHCA group at T3 and T6 (p < 0.05; fig. 3).
Hematoxylin and Eosin Staining
At a high magnification (×400), most of the vasculature was dilated and congested and was surrounded by inflammatory cells. Liquefaction necrosis was observed in DHCA animals (fig. 4a). A milder vascular dilatation and congestion were observed, and the inflammatory cells were not obvious in the SACP-treated animals (fig. 4b).

Fluorescence TUNEL
There were more TUNEL-positive nuclei in the DHCA-treated cerebral cortex neurons (fig. 5a) than in the SACP group (fig. 5b). The AI of the DHCA group was significantly higher than that of the SACP group (17.89 ± 5.35 vs. 9.66 ± 1.97, t = 3.319). * p = 0.02.

Quantitative Real-Time RT-PCR
The TLR4 mRNA levels of the SACP animals were significantly lower than those of the DHCA animals (t = 3.09, p = 0.023; fig. 6).

Western Blot
The protein levels of NF-κB/P65 and TLR4 were significantly lower in the SACP group than in the DHCA group (t = 3.89, p = 0.037; fig. 7).

Discussion
Among all the molecular signaling pathways participating in the neuroinflammatory cascade, TLR4 was the first to be discovered and it is the TLR that has been studied the...
most. TLR4 plays a key role in innate immunity in the central nervous system [13] and cardiovascular system [14, 15]. Increased TLR4 expression levels of mRNA and proteins have been observed in heart tissue sampled from patients with dilated cardiomyopathy and atherosclerosis as well as in murine hearts exposed to ischemic injury [14, 15].

Substantial evidence indicates that TLR4 is a crucial factor for the central nervous system, not only in neurodegenerative diseases (e.g. Alzheimer’s disease) and brain trauma but also in hypoxia-associated diseases and cerebrovascular diseases (e.g. stroke) [11, 16, 17]. The pathogenic role of TLR4 in cerebral ischemia was established in TLR4 mutant mice with transient middle cerebral artery occlusion (MCAO). The infarction area, cerebral edema and neurological behavioral scores were improved in TLR4 mutant mice compared to wild-type animals [12]. Another study utilizing TLR4 mutant mice examined global cerebral ischemia reperfusion. The study showed ameliorated neuronal injury and apoptosis and reduced secretion of proinflammatory cytokines [18]. Recently, Yao et al. [19] reported that the microglia protein level of TLR4 was markedly enhanced in the corpus callosum and cerebellum from rats subjected to hypoxia. In our study, histology examinations show that there was evidence of cerebral injury and neuroinflammation. We found that both the mRNA and protein expression levels of TLR4 simultaneously increased in the DHCA group. This result suggests that TLR4 is involved in the mechanism of brain damage caused by DHCA. Attenuating the activation of TLR4 by SACP is one mechanism for inducing a neuroprotective effect. To our knowledge, this is the first report showing a change in the TLR4 signaling pathway during cerebral injury. We demonstrated that SACP is able to attenuate the TLR4/NF-κB inflammation axis and cytokine expression in a DHCA pig model.

NF-κB is a central mediator of inflammatory cascades. Abundant evidence demonstrates that NF-κB is activated in brain ischemia [9, 20]. Increased activity of NF-κB was examined in a transient MACO model [21]. A decreased infarction volume induced by transient and permanent ischemia was reported in mice deficient in the P50 subunit of NF-κB [21, 22]. Furthermore, reduced NF-κB levels were detected in TLR4 knockout mice receiving transient MACO treatment [18]. It is still unclear whether the function of NF-κB is proapoptotic or antiapoptotic [20, 21].

Our results indicate a proapoptotic function of NF-κB in cerebral injury induced by DHCA based on the high AI. Furthermore, SACP might protect the brain from injury by reducing apoptosis and inflammatory changes. The mechanism may be via suppressing the activity of NF-κB, which is a key factor for neuroinflammation.

A recent clinical trial reported that there was a correlation between serum IL-6 and cardiovascular pathology [23]. In the brain, the upregulation of IL-6 through NF-κB plays a key role in activating microglia, initiating the infiltration of immune cells and triggering a series of events that ultimately lead to neuronal damage [9, 20]. The level of proinflammatory cytokines such as TNF-α and IL-6 in TLR4-null mice was lower than that in wild-type controls [24]. Our experimental data showed that the serum IL-6 levels clearly increased in the DHCA group and were decreased in the SACP group. This result fur-
ther supports the severe neuroinflammatory evidence in cerebral injury caused by DHCA.

We are aware of the limitations of this study. First and foremost, there were no postoperative data relevant to cerebral function recovery, such as neurological behavioral scores. This study aimed to explore brain injury associated with acute ischemia rather than chronic cases, and postoperative variables were beyond its scope. In addition, inhibitor or enhancer agents of TLR4/NF-κB were not applied in this experiment to elucidate the mechanism of the TLR4/NF-κB signaling axis. This is our first study based on a large animal model; these limitations will be taken into consideration in a further study.

**Conclusion**

These findings suggest that the TLR4/NF-κB signaling axis plays an important role in the neuroinflammation mechanism of cerebral injury resulting from DHCA. SACP exhibits a neuroprotective effect by improving the histology and lowering the AI. These changes contribute to the attenuation of the TLR4/NF-κB signaling pathway mediators, including upstream factors (TLR4/NF-κB) and downstream cytokines (IL-6). The neuroprotective effect of SACP may be regulated in part by the TLR4/NF-κB signaling pathway. Therefore, the TLR4/NF-κB signaling pathway represents a novel endogenous therapeutic target.

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