Toll-Like Receptor 4–Dependent Microglial Activation Mediates Spinal Cord Ischemia–Reperfusion Injury

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Background—Paraplegia continues to complicate thoracoabdominal aortic interventions. The elusive mechanism of spinal cord ischemia–reperfusion injury has delayed the development of pharmacological adjuncts. Microglia, the resident macrophages of the central nervous system, can have pathological responses after a variety of insults. This can occur through toll-like receptor 4 (TLR-4) in stroke models. We hypothesize that spinal cord ischemia–reperfusion injury after aortic occlusion results from TLR-4–mediated microglial activation in mice.

Methods and Results—TLR-4 mutant and wild-type mice underwent aortic occlusion for 5 minutes, followed by 60 hours of reperfusion when spinal cords were removed for analysis. Spinal cord cytokine production and microglial activation were assessed at 6 and 36 hours after surgery. Isolated microglia from mutant and wild-type mice were subjected to oxygen and glucose deprivation for 24 hours, after which the expression of TLR-4 and proinflammatory cytokines was analyzed. Mice without functional TLR-4 demonstrated decreased microglial activation and cytokine production and had preserved functional outcomes and neuronal viability after thoracic aortic occlusion. After oxygen and glucose deprivation, wild-type microglia had increased TLR-4 expression and production of proinflammatory cytokines.

Conclusions—The absence of functional TLR-4 attenuated neuronal injury and microglial activation after thoracic aortic occlusion in mice. Furthermore, microglial upregulation of TLR-4 occurred after oxygen and glucose deprivation, and the absence of functional TLR-4 significantly attenuated the production of proinflammatory cytokines. In conclusion, TLR-4–mediated microglial activation in the spinal cord after aortic occlusion is critical in the mechanism of paraplegia after aortic cross-clamping and may provide targets for pharmacological intervention. (Circulation. 2013;128[suppl 1]:S152-S156.)

Key Words: aortic surgery ■ aorta ■ inflammation ■ ischemia ■ macrophages ■ reperfusion ■ spinal cord
Spinal cords were removed from wild-type (n=9) and mutant (n=9) mice with functional TLR-4 were obtained from Charles River Laboratories.

Histological Analysis and Neuronal Quantification
After 60 hours of reperfusion, the animals were euthanized. The vertebral column was removed en bloc from T8-L3. The spinal cord was removed from the vertebral column by injection of PBS (pH 7.4) into the spinal column. Spinal cords were transferred to 4% formalin where they remained for ≥24 hours before paraffin embedding, sectioning, and hematoxylin and eosin staining. Sections were evaluated blindly for neuronal viability quantified as neurons per microscopic field. Neurons within the anterior horn that contained prominent nucleoli and loose chromatin were considered normal.

Microglial Isolation
Adult male C3H/HeJ and C3H/HeNCrlBR mice were euthanized. The vertebral columns were removed en bloc as above. After dissection of spinal cords, the tissue slices were digested in a solution of Hibernate A (Invitrogen, Grand Island, NY) and papain (Worthington Biochemical Corporation, Lakewood, NJ). The digested tissues were triturated several times and applied to OptiPrep gradient (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum, glutamax, and pen/strep. Isolated cells cultures were matured for ≤10 days to obtain a confluent cell culture.

Oxygen and Glucose Deprivation
DMEM without glucose (Gibco, Grand Island, NY) was pretreated in a humidified hypoxic chamber (1% O2, 5% CO2, 94% N) at 37°C for 2 hours. Confluent cell cultures were transferred to hypoxic chamber and placed in pretreated media where they remained for 24 hours. After OGD, cell cultures were returned to DMEM containing 10% fetal bovine serum, glutamax, and pen/strep media (Gibco, Grand Island, NY) and returned to a 37°C incubator with atmospheric oxygen. Media and cell cultures were removed at 1- to 36-hour time points for protein analysis.

Immunoblotting
Spinal cords were removed from wild-type (n=9) and mutant (n=9) mice that had no manipulation or at 6 and 36 hours after IR surgery. Galectin-3/mic-2 (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a marker of microglial activation at 1:500 dilutions. Microglial cells were lysed with M-PER mammalian protein extraction reagent (Fisher Scientific, Pittsburg, PA). Microglial lysates were assessed for TLR-4 expression with TLR-4 polyclonal antibody (1:1000; Cell Signaling, Danvers, MA) at 1 to 24 hours after OGD. Protein extracts were placed in 4× Laemmli sample buffer with β-mercaptoethanol and boiled for 10 minutes at 100°C, loaded into a 15-wellgradient ready gels (Bio-Rad, Hercules, CA), and run at 160 V for 450 minutes. The gels were transferred to nitrocellulose membranes at 100 V for 60 minutes and then cross-linked using an ultraviolet Stratalinker (Stratagene, La Jolla, CA). The membranes were blocked in 5% dry milk in 0.1% Tween in PBS (T-PBS) and rinsed 3× in 0.1% T-PBS. The blocked membranes were incubated with primary antibody overnight at 4°C (5% BSA in 0.1% T-PBS). The membranes were washed in 0.1% T-PBS 3× and incubated in appropriate horseradish peroxidase-conjugated secondary antibodies diluted to 1:5000 in 5% BSA in 0.1% T-PBS for 1 hour at room temperature. SuperSignal West Dura Chemiluminescent Substrate for horseradish peroxidase was used for 5 minutes, and membranes were visualized by Chemidoc Hi Sensitivity (BioRad, Hercules, CA) scanner. Mean density values of bands were quantified by QuantityOne software (BioRad, Hercules, CA).

Cytokine Quantification
Expression of tumor necrosis factor (TNF)-α and interleukin-6 (IL-6) was chosen as likely outputs of activated microglia. Based on previous studies from our laboratory, time points of 6 and 36 hours were chosen. Spinal cords were removed from wild-type and mutant mice that had no manipulation or at 6 and 36 hours after IR surgery as above. A comparison of 3 mice in each genotype was made at each time point, with a total of 18 mice included in the analysis. Microglial cultures from 3 separate cell lines had media removed at 6, 24, and 36 hours after OGD, and cytokines were quantified using ELISA (R & D systems, Minneapolis, MN) performed according to the manufacturer’s instructions. Absorbance of standards and samples was determined spectrophotometrically at 595 nm using a microplate reader (Bio-Rad, Hercules, CA). Results were plotted against the linear portion of the standard curve, and the protein concentration of each sample was expressed as pg/mL of sample.

Results
Sham mice (n=4), 2 of each strain, showed no functional deficits at any point after surgery. Wild-type mice with functional TLR-4 (n=9) had a mild initial function deficit at 12 hours after IR, which progressed to moderate paralysis at 60 hours. Mutant mice (n=9) had mild functional deficit at 12 hours after aortic occlusion, which was not significantly different from wild-type mice. Mutant mice, however, did not exhibit the same progressive functional decline that was observed in mice with functional TLR-4. Statistical significance was observed at 24 hours until the duration of the experiment (Figure 1).

Histological Changes
Wild-type mice that underwent IR had an increased amount of pyknotic nuclei compared with mutant and sham mice, suggesting a greater degree of cellular injury. In addition, these...
mice had impressive inflammatory infiltrates in the gray matter surrounding the anterior horn motor neuron, with distortion of the gray–white junction (Figure 2).

**Neuronal Viability**

All sham mice had significantly more anterior horn neurons compared with both the mutant and wild-type mice undergoing IR. Mice with mutant TLR-4 receptor had significantly more viable anterior horn neurons compared with wild-type mice (Figure 3).

**Microglial Activation**

Spinal cord microglial activation was assessed by galectin-3/mac-2 at 6 (Figure 4A) and 36 hours (Figure 4B) postoperatively and compared with baseline values. At both postoperative time points, wild-type mice had increased microglial activation compared with mutant mice and baseline values.

**Cytokine Expression**

Spinal cord microglial activation paralleled the production of proinflammatory cytokines. The absence of functional TLR-4 on microglial cells inferred a significant reduction in the expression of proinflammatory cytokines TNF-α and IL-6 both in vivo and in vitro (Figures 5 and 6). Statistical significance (*P*<0.05) was observed for both IL-6 and TNF-α at 6 hours postoperatively. A reduction to values near baseline was observed at 36 hours, at which point statistical significance was lost (Figure 5). Similarly, wild-type microglia had a significant increase in the expression of proinflammatory cytokines. This was seen in a time-dependent manner and was significant with IL-6 at 24 and 36 hours. Although the concentrations of TNF-α were much lower than expected, there was a significant reduction in TNF-α expression by mutant microglia at 36 hours (Figure 6).

**Expression of TLR-4 on Microglia After OGD**

Isolated microglial cultures demonstrated the presence of TLR-4 with increased expression after 24 hours of OGD. This expression continued to increase after OGD and was significant at 24 hours when there was nearly a 30% increase in the expression of TLR-4 when normalized to GAPDH (Figure 7).

**Discussion**

These data demonstrate the pathological role of TLR-4-dependent activation of microglia in the development of delayed paraplegia seen clinically after aortic surgery. The in vivo data suggest that, after thoracic aortic occlusion in mice, the absence of a functional TLR-4 is associated with preserved hind-limb function and neuronal viability and an attenuation of microglial activation and proinflammatory cytokine production. Furthermore, the in vitro data demonstrate that TLR-4 is upregulated in microglia after OGD and microglia with intact TLR-4 express a significantly higher amount of proinflammatory cytokines.

The insult sustained by the spinal cord during thoracoabdominal aortic intervention has classically been defined as ischemia–reperfusion. The injury, however, should be separated into 2 distinctly different insults. Clinically, this is seen in the bimodal distribution of paraplegia after thoracoabdominal interventions. Patients experience either immediate paralysis after interventions, which is rarely reversible, or subsequently develop paraplegia in the hours to days after surgery, which can commonly be reversible, with the delayed injury more prominent in contemporary series. To further clarify these clinical entities, we have developed a murine model of immediate and delayed paraplegia.

Ischemia causes metabolic derangement, with wide variations in the degree of injury to motor neurons. If this ischemic injury is severe enough, the patient will experience immediate paraplegia secondary to neuronal apoptosis or necrosis in...
the motor neurons. Dense paraplegia from these irreversibly injured neurons will not recover. With reperfusion, the restoration of blood flow results in an amplified inflammatory response from both resident and systemic leukocytes, leading to further neuronal injury from production of reactive oxygen species and cytokines.13 The secondary injury attributed to reperfusion provides a window of intervention for the potential reversal or prevention of delayed injury.

To investigate the contribution of TLR-4 signaling to the development of delayed paraplegia, mice with TLR-4 loss-of-function mutation were subjected to lower body ischemia. The duration of ischemia was selected by titrating the ischemic duration until wild-type mice exhibited a delayed progressive function deficit. Mice with TLR-4 mutation had significantly preserved neurological function and neuronal viability compared with wild-type controls. Both groups subjected to IR had significantly worse functional outcomes compared with sham mice. The difference is likely related to the metabolic effects of initial ischemic insult. Ischemic spinal cord also demonstrated a significant increase in microglial activation with intact TLR-4 receptors. These data suggest the contribution of TLR-4 signaling to reperfusion injury in the spinal cord.

Microglia, the resident immune cells in central nervous system, serve a complicated role. Their presence is associated with a host of beneficial and detrimental effects. Although microglia are necessary for neuronal development and viability,14 dysregulation of these cells after physiological insults can result in neurotoxicity in a process known as reactive microgliosis.15 On activation, microglia undergo a dramatic phenotypic transformation and can produce a host of neurotoxic factors, such as TNF-\(\alpha\) and nitric oxide.17 Others have shown that microglial inhibition significantly attenuates IR injury in the brain and spinal cord.5 The role of the microglia seems to be significant with regard to the reperfusion arm of this injury.

Microglia have a plethora of receptors that can be activated by a variety of stimuli.7 Exogenous stimuli, such as bacterial and viral pathogens, can cause activations of microglia.18 Recently, TLRs, in particular TLR-4, have gained attention for their role in various other models of IR. These receptors represent a family of proteins that regulate the innate immune response. Although multiple cell lines, such as astrocytes19 and neurons, have TLR-4 receptors,20 the TLR-4 concentration is highest on microglial cells.21 In stroke models, TLR-4 has been associated with larger infarct volumes and worse functional deficit.22 In addition, TLR-4–mediated microglia activation has been implicated in the pathogenesis of Alzheimer

![Neuronal Quantification](image)

**Figure 3.** All mice that underwent ischemia–reperfusion (IR) surgery had significantly less viable anterior horn neurons compared with sham mice (*\(P<0.05\)). Mice with intact toll-like receptor-4 (wild type [wt]) that underwent IR surgery had the lowest number of normal-appearing anterior horn neuron, which was significantly different from mutant mice (mut; \(\#P<0.05\)).

![Microglial Cytokine Expression](image)

**Figure 5.** Compared with baseline values, the expression of proinflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor (TNF)-\(\alpha\) was significantly (*\(P<0.05\)) elevated at 6 hours after ischemia–reperfusion (IR) surgery in wild-type (wt) mice compared with mutant mice (mut). At 36 hours after IR surgery, values decrease and return to near baseline.

![Spinal Cord Cytokine Expression](image)

**Figure 4.** Immunoblotting of galectin-3/mac-2 in spinal cord after ischemia–reperfusion (IR) surgery, with a comparison to baseline values. **A.** Galectin-3/mac-2 immunoblotting 6 hours after IR surgery with increased expression in wild-type (wt) mice after surgery compared with mutant mice or baseline values. **B.** Representation of galectin-3/mac-2 immunoblotting 36 hours after IR surgery. wt mice had an increased expression of microglial activation marker compared with mutant mice (mut) or baseline values.

**Figure 6.** Expression of tumor necrosis factor (TNF)-\(\alpha\) and interleukin-6 (IL-6) after 24 hours of oxygen and glucose deprivation (OGD). Mice with intact toll-like receptor-4 (wild type [wt]) had a significant increased expression of IL-6 at 24 and 36 hours and TNF-\(\alpha\) at 36 hours (*\(P<0.05\)) compared with mutant mice (mut).
The in vitro data presented demonstrate that TLR-4 upregulation occurs in microglia after OGD. This upregulation of receptors, when it occurs, continues to increase for a period of time and likely sensitizes microglia to the progressive functional decline seen in wild-type mice after OGD, and significant increases were noted at 12 and 24 hours (*P<0.05).

To summarize, mice lacking functional TLR-4 did not exhibit the progressive functional decline seen in wild-type mice after thoracic aortic occlusion. In addition, a reduction of microglial activation and proinflammatory cytokine production was observed in mutant mice compared with wild-type controls. The in vitro data presented demonstrate that TLR-4 upregulation occurs in microglia after OGD and is associated with the expression of proinflammatory cytokine production. In conclusion, TLR-4 activation of microglia in the spinal cord after IR injury is critical in the mechanism of injury progression, which could be important in future prevention of the clinical entity of delayed paraplegia after thoracoabdominal aortic intervention.

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**Disclosures**

None.

**References**


