SUPPLEMENTAL MATERIAL

Delayed paraplegia after spinal cord Ischemic injury requires caspase-3 activation in mice

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Supplemental Methods

Mouse model of spinal cord ischemia

The operative procedures to produce transient SCI in mice were performed according to a method described by Lang-Lazdunski and colleagues[1] with some modifications. After the animals were weighed, anesthesia was induced by 5 % isoflurane, and trachea was intubated by 20 G custom made catheter for mechanical ventilation. Mice were positioned in the spine position and anesthesia was maintained by 1.5% to 2% isoflurane with 100% oxygen. Paravertebral muscle temperature was measured by a thermocouple placed at the level of L1-L3 and maintained at 37.5 ± 0.2°C during surgery using a heating pad. Subsequently, PE-10 catheter was inserted into the left femoral artery to monitor distal arterial pressure. A ventral midline cervicothoracic incision was made, submaxillary glands were retracted, and the chest wall was incised from the apex of the manubrium caudal along the left sternal border, to the second rib. The thymus was retracted superiorly, and the aortic arch was gently isolated between the brachiocephalic artery and the left subclavian artery (LSA), avoiding the vagus nerve and the left recurrent laryngeal nerve. Heparin (1000 IU/kg) was injected via the left femoral arterial catheter. Then, under direct vision, the first clip was placed on the aortic arch between the left common carotid artery and the LSA, and then the second clip was placed on the origin of the LSA (within 30 seconds). The completeness of the occlusion was ascertained by an immediate and sustained loss of any detectable pulse pressure in the femoral artery pressure tracing. After ischemia, the clips were removed, and the chest was closed in layers. Protamine sulfate (1 mg) was then administered subcutaneously. At 10 minutes of reperfusion, the arterial catheter was removed, incisions were closed, and animals were allowed to recover from anesthesia. In sham-operated mice, all surgical procedures were performed as described, but no clips were placed. All mice were placed in a cage kept at 30 - 31°C for the following 2 hours.

These modifications enabled us to enhance the completeness of the surgical isolation and clamping of aortic arch that resulted in a highly reproducible reduction of rSCBF down to less than 10 % of baseline compared to ~30% in the Lang-Lazdunski study.[1] While 11 min of aortic occlusion resulted in immediate paraplegia in ~80% of

mice in the Lang-Lazdunski study,[1] 9 min of SCI was sufficient to produce immediate paraplegia in 100 % of mice at 72h after reperfusion in the current study with minimum operative mortality.

Measurements of physiological parameters

In addition to paravertebral muscle temperature, rectal temperature was also monitored and recorded periodically (before ischemia, at the end of ischemia, and 10 min of reperfusion) as core temperature during surgery. Blood gas analysis was performed before aortic occlusion and at 10 min of reperfusion. Arterial blood gases and pH were measured before ischemia and 10 minutes of reperfusion in 50μL samples obtained from the left femoral arterial catheter by a blood gas /pH analyzer (IRMAs TRUPOINT^{TM}, ITC. USA).

We used a qualitative real-time measure of regional spinal cord blood flow (rSCBF) by laser-Doppler flowmetry (PF2B, Perimed) with a 0.8-mm fiberoptic extension. As described by Lang-Lazdunski et al.,[1] the probe was affixed perpendicularly as much as possible on the intervertebral ligament surface between vertebra L1 and L2 through a limited skin incision.

Quantal bioassay for the relationship between the duration of SCI and neurological function

For the quantal bioassay of the relationship between the duration of SCI and neurologic function[2,3], the duration of SCI was selected to span all grades of neurologic function ranging from walking (BMS = 6 - 9) to paraplegia or paraparesis (BMS = 0 - 5). Based on our pilot experiments, the duration of SCI for individual animals was varied from 1 min up to 10 min. The P50i and P50d represent the duration of ischemia (in minutes) associated with 50% probability of immediate and delayed paraplegia, respectively. The onset of neurologic deficit (BMS = 0 - 5) was considered immediate if it was present at the initial examination (2 hrs after reperfusion) and delayed if the deficit occurred after a period during which mice exhibited the ability to walk (BMS = 6 - 9).

Histological studies

After SCI, lumbar enlargement of the spinal cord was perfusion fixed and embedded in paraffin. Spinal cord embedded in paraffin was sectioned to 5 μm thickness and 50 sections of each spinal cord were divided on ten slide glasses (five sections each on one slide glass). For each histopathological analysis described below, one slide glass of each spinal cord was chosen randomly by the investigator (KK) without the knowledge of the identity of the sample.

Detection and quantification of viable neurons with Nissl staining—Viability of neurons in paraffin-embedded spinal cord sections obtained from mice subjected to sham operation or 9 or 5 min of SCI were evaluated with Nissl staining.[4] Cells that contained Nissl substance in the cytoplasm, loose chromatin, and prominent nucleoli were considered to be viable. For quantitative analysis, the number of Nissle-positive neuron in the spinal ventral horn was counted in a randomly-chosen section under high-power magnification (200x) by an investigator (KK) blinded as to the identity of mice. N=4 for Sham, 8, 24, and 48h after 9 or 5 min SCI. N=6 for 72h after 5 min SCI. N=7 for 72h after 9 min SCI.

Immunohistochemical detection of glial activation and viable neurons—Sections were incubated for 1h in blocking solution and incubated overnight at 4°C with primary antibodies against markers of activated astrocytes (glial fibrillary acidic protein, GFAP, 1:500, Dako, Carpinteria, CA) or activated microglia (ionized calcium binding adaptor molecule 1, Iba-1, 1:500, Wako Chemicals USA, Richmond, VA). After rinsing, sections were incubated with secondary antibodies for 1h: Rhodamine RedTX-conjugated goat anti-rabbit antibody (for GFAP, 1:200, Jackson ImmunoReasearch, West Grove, PA) or Alexa Fluor 488 goat anti-rabbit antibody (for Iba-1, 1:200, Invitrogen, Carlsbad, CA). Viable neurons were detected by mouse anti-neuronal nuclei (NeuN) conjugated to Alexa Fluor 488 (1:100, Millipore, Billerica, MA). The fluorescence images were captured using appropriate filters with a fluorescence microscope (Nikon ECLIPSE TE-2000-S).

Detection of cleaved caspase 3—Activation of caspase-3 was assessed by immunohistochemistry in paraffin-embedded spinal cord sections using a rabbit polyclonal antibody against cleaved caspase-3 (1:50, Cell Signaling) according to the protocol recommended by the manufacturer.

Supplemental Tables

Table S1. Comparison of the Effects of Spinal Cord Ischemic Time on Neurological Outcome

Duration of SCI (min)	Total number of mice	Number of mice exhibiting		
		Normal motor function	Delayed paraplegia	Immediate paraplegia
1	3	3	0	0
2	4	4	0	0
3	5	5	0	0
3.5	5	2	3	0
4	5	1	4	0
4.5	5	0	5	0
5	7	0	7	0
5.5	7	0	7	0
6	8	0	6	2
6.5	10	0	6	4
7	8	0	2	6
7.5	6	0	1	5
8	6	0	0	6
9	6	0	0	6
10	5	0	0	5

Table S2. Physiological variables –Immediate paraplegia-

	Wild-type	Caspase-3$^{-/-}$
Ischemic Time (min)	9	9
Femoral artery pressure (mmHg)		
Pre-ischemia	79±5	82±6
Intra-ischemia	8±1	8±1
10 min of reperfusion	75±19	87±26
Paravertebral muscle temperature (ºC)		
Pre-ischemia	37.4±0.1	37.4±0.1
Intra-ischemia	37.4±0.1	37.4±0.1
10 min of reperfusion	37.4±0.1	37.5±0.2
Rectal temperature (ºC)		
Pre-ischemia	36.6±0.3	36.9±0.3
Intra-ischemia	36.8±0.4	36.3±0.5
10 min of reperfusion	36.9±0.2	36.8±0.2

Table S3. Physiological variables –Delayed paraplegia-

	Wild-type	Caspase-3$^{-/-}$
Ischemic Time (min)	5	5
Femoral artery pressure (mmHg)		
Pre-ischemia	83±7	79±6
Intra-ischemia	7±1	5±1
10 min of reperfusion	84±9	87±9
Paravertebral muscle temperature (ºC)		
Pre-ischemia	37.4±0.0	37.6±0.1
Intra-ischemia	37.4±0.1	37.5±0.0
10 min of reperfusion	37.4±0.1	37.3±0.1
Rectal temperature (ºC)		
Pre-ischemia	36.6±0.3	37.1±0.4
Intra-ischemia	37.4±0.4	37.5±0.3
10 min of reperfusion	36.9±0.3	36.9±0.2

Table S4. Regional spinal cord blood flow in Wild-type and Caspase-3$^{-/-}$ mice

	Wild-type	Caspase-3$^{-/-}$
rSCBP (%)		
Pre-ischemia	100	100
Intra-ischemia	8±4	7±3
10 min of reperfusion	91±14	101±13

rSCBF : regional spinal cord blood flow

Figure S1.

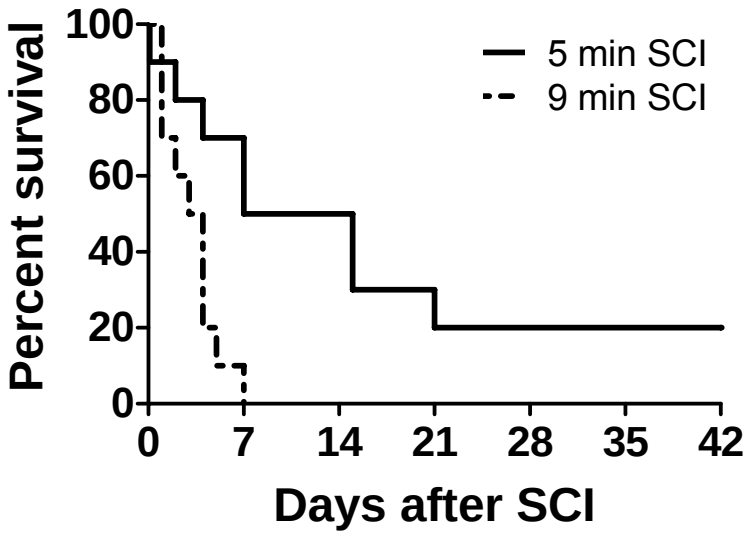

Figure S1. Long-term survival rates of mice subjected to 5 or 9 min of spinal cord ischemia (SCI). N=10 in each group.

Supplemental Reference List

1. Lang-Lazdunski L, Matsushita K, Hirt L, Waeber C, Vonsattel JP, Moskowitz MA, Dietrich WD. Spinal cord ischemia. Development of a model in the mouse. *Stroke*. 2000;31:208-213.

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