SUPPLEMENTAL MATERIAL

MATERIALS AND METHODS

A graphical abstract and experimental schedule are shown in supplementary figures 1 and 2.

Foot fault test

To evaluate hindlimb motor function, the foot fault test was performed with normal and experimental rats 56 days after ET-1 administration. Based on the previous report, a 30-rung horizontal ladder (6 cm across, 3-cm intervals between rungs) was created (Supplementary figure 3A, B).[1]

SC blood flow measurements before and after ET-1 administration

Referring to the literature, changes in SC blood flow before and after ET-1 administration were evaluated using a laser tissue blood flowmeter (FLO-N1; Omegawave, Inc., Tokyo, Japan). Th13 was removed under general anaesthesia according to the method described above. Blood flow at Th13 was measured from the dorsal side using an NS probe (Omegawave, Inc.) before, just after, and at 15 min, 30 min, and 60 min after ET-1 administration. The probe was connected to a blood flow meter.[2 3] The method relies on the measurement of Doppler frequency shifts in laser light reflected from moving red blood cells, and the results are displayed after conversion to unit millilitres per minute per 100g. The average value of 5 consecutive seconds was taken as the value at that time. Statistical analysis was performed on data obtained before ET-1 administration and the post-administration time points.

In vivo magnetic resonance imaging (MRI) study

MRI studies were performed as previously reported with minor modifications.[4] Rats were anesthetized with 1.5% isoflurane. All MRI measurements were performed using a 7.0-T Bruker PharmaScan system (Bruker Biospin, Ettlingen, Germany) with a transmit volume coil and a 30-mm-diameter receive surface coil. In brief, T2-weighted images were obtained using a 2D Turbo RARE sequence under the following parameters: TR=3000 ms, TE=30 ms (RARE factor=4, flip angle=90°), field of view=40 mm×40 mm, matrix size=200×200 (in-plane resolution=200 μm×200 μm), number of slices=16, slice thickness=1 mm, slice gap=0 mm, and number of averages=2. Positioning of the spine was set to centre the probe at Th13. SC areas were calculated from T2-weighted images using Fiji Image J imaging software (National Institutes of Health, Bethesda, MD). Thereafter, 11 interval moving averages were calculated and compared at each time point for T2-weighted images. MR images were obtained at 1, 3, 7, and 14 days after ET-1 administration.

Western Blotting Assay

Western blotting was performed to evaluate the effects of ET-1 administration on inflammation and apoptosis in SC tissue. A 6-mm section of the SC centred on the infarct site was removed from the SC infarction model at 6 h, 1 day, and 3 days after ET-1 administration. In normal rats, a 6-mm section of SC tissue centred at Th13 was removed.

The SC was homogenised with RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Waltham, MA, USA). After homogenisation, the samples were incubated on ice for 10 min. Protein concentrations were calculated by the Bradford assay using a spectrophotometer. Afterward, the samples were added to the same volume of sample buffer (30% glycerol, 4% sodium dodecyl sulphate, 125-mM Tris–HCl at pH 6.8, 100-mM dithiothreitol, 10-mM EDTA, 10% b-

mercaptoethanol, and 0.1% bromophenol blue) and incubated at 100℃ for 10 min. Extracted proteins were run on either a 10% or 15% SuperSep Ace agarose gel (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and then transferred to an Immobilon-P Transfer Membrane (Merck Millipore, Billerica, MA, USA). After blocking with 5% skim milk (Nacalai Tesque, Kyoto, Japan) for 30 min at room temperature, the transfer membranes were incubated with the following primary antibodies diluted in 5% skim milk at 4℃ overnight: rabbit anti-cleaved caspase-9 antibody (1:1000; 9507, Cell Signaling Technology, Danvers, MA), rabbit anti-cleaved caspase-3 antibody (1:1000; 9661, Cell Signaling Technology), rabbit anti-TNF-α antibody (1:1000; ab6671, Abcam), rabbit anti-IL-1β antibody (1:500; Cell Signaling Technology), and mouse anti-β-actin antibody (1:5000; ab6276, Abcam). After overnight incubation, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (1:5000; 115-035-071, Jackson ImmunoResearch) and HRP-conjugated goat anti-rabbit IgG antibody (1:2500; 111-035-144, Jackson ImmunoResearch), were used as secondary antibodies. The transfer membranes were incubated for 1 h at room temperature with secondary antibodies diluted in 5% skim milk. After washing, an enhanced chemiluminescence reaction was performed using Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific) for 5 min. Images were captured using the ImageQuant LAS 4000 IR digital imaging system (Fuji Film, Tokyo, Japan). For comparison among groups, relative protein expression was calculated as the ratio to b-actin expression in each sample.

RESULTS

Foot fault test

The foot fault score of the ET-1 group (day 56) was significantly lower than that of normal rats (12.0±7.6 vs 147.7±13.9, $p<0.01$) (Supplementary figure 3C).

In vivo MRI study

The signal intensity in T2-weighted magnetic resonance images at the site of SC infarction increased over time, and the difference in the signal intensity from that in the surrounding area became clearer (Supplementary figure 4). The grey line shows the actual values, and the black line shows the 11-section moving average.

Vasoconstriction process after ET-1 injection

Blood flow at the level of Th13 was 61.6±7.4 ml/min/100g before ET-1 administration, 15.9±4.0 ml/min/100g immediately after ET-1 administration, 15.3±4.3 ml/min/100g at 15 min, 31.9±1.4 ml/min/100g at 30 min, and 55.3±9.0 ml/min/100g at 60 min. The blood flow was significantly decreased immediately, 15 min, and 30 min after ET-1 administration compared with that before ET-1 administration (Fig 3B, Before vs Just after; $p<0.001$, Before vs 15 min; $p<0.001$, Before vs 30 min; $p<0.05$). After 60 min, blood flow improved to the same level as before ET-1 administration (Before vs 60 min; not significant).

Western blot analysis of SC tissues

The signal intensity of cleaved caspase-9 at 6 h was significantly higher than that before ET-1 administration ($n = 4$, $p<0.05$). The signal intensity of cleaved caspase-3 was significantly higher

on day 1 than before ET-1 administration ($n = 4$, $p<0.05$). Evaluation of the serial changes in inflammatory cytokine expression revealed the highest expression at 3 days after ET-1 administration (TNF-α; $n = 3$, $p<0.05$, IL-1β; $n = 4$, $p<0.05$) (Supplementary figure 6).

References

1. Martins LA, Schiavo A, Xavier LL, et al. The Foot Fault Scoring System to Assess Skilled Walking in Rodents: A Reliability Study. *Front Behav Neurosci* 2022;16:892010. doi: 10.3389/fnbeh.2022.892010 [published Online First: 20220429]
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