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Stachybotrys microspora triprenyl phenol-7, a novel fibrinolytic agent, suppresses superoxide production, matrix metalloproteinase-9 expression, and thereby attenuates ischemia/reperfusion injury in rat brain

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ABSTRACT

Stachybotrys microspora triprenyl phenol-7 (SMTP-7) is a novel fibrinolytic agent with anti-inflammatory effect. Previous study demonstrated that SMTP-7 further ameliorated infarction volume in a mouse embolic stroke model compared with tissue type plasminogen activator (tPA), but the reason SMTP-7 has more beneficial effect than tPA has not yet been determined. In the present study, we investigated whether SMTP-7 has an intrinsic neuroprotective effect against transient focal cerebral ischemia (tFCI). Sprague–Dawley rats were subjected to tFCI by intraluminal middle cerebral artery occlusion for 2 h. Following induction of tFCI, rats were randomized into two groups based on the agent administered: SMTP-7 group and vehicle group. We examined cerebral infarction volume 24 h after reperfusion, and evaluated superoxide production, the expressions of nitrotyrosine and matrix metalloproteinase-9 (MMP-9), which play major roles in secondary brain injury and hemorrhagic transformation. The findings showed that SMTP-7 significantly suppressed superoxide production, the expression of nitrotyrosine and MMP-9 after tFCI, and consequently attenuated ischemic neuronal damage. These results suggest that SMTP-7 has an intrinsic neuroprotective effect on ischemia/reperfusion injury through the suppression of oxidative stress and MMP-9 activation.

Fibrinolytic therapy using tissue-type plasminogen activator (tPA) is one of the most effective treatments for acute ischemic stroke. However, tPA may cause hemorrhagic transformation when administered after a significant amount of brain tissue has been damaged [25]. The indication for this treatment is therefore limited to a narrow therapeutic time window, which is only possible in 3–5% of acute ischemic stroke patients [27]. Furthermore, tPA is known to have an intrinsic neurotoxic effect due to protease and other mechanisms mediated by excitotoxicity and activation of the matrix metalloproteinase (MMP) family [10,26]. Therefore, an alternative fibrinolytic agent with a neuroprotective effect, allowing lengthy therapeutic time window, is needed to increase the opportunity for fibrinolytic therapy and improve patient outcomes in clinical settings.

Stachybotrys microspora triprenyl phenol (SMTP)-7 (orniplabin; CAS registry no. 273379-50-9), a novel fibrinolytic agent, is an analog of triprenyl phenol, designated staplabins which was isolated from a fungal culture of *S. microspora* (IFO 30018) [8]. Staplabin is a low-molecular-weight compound that stimulates plasminogen–fibrin binding [21], and causes the susceptible activation of plasminogen by inducing a conformational change [24]. A previous study demonstrated that SMTP-7 further ameliorated infarction volume in a mouse embolic stroke model compared with tPA, probably due to its anti-inflammatory effect [14,20]. However, the exact mechanism by which SMTP-7 provided a more beneficial effect than tPA has not been determined, and the detailed mechanisms of its anti-inflammatory effect are unclear. In the present study, we investigated whether SMTP-7 has an intrinsic neuroprotective effect against transient focal cerebral (tFCI) ischemia in

Abbreviations: SMTP, *Stachybotrys microspora* triprenyl phenol; tFCI, transient focal cerebral ischemia; MMP-9, matrix metalloproteinase-9; ROS, reactive oxygen species; tPA, tissue-type plasminogen activator; MCA, middle cerebral artery; ICA, internal carotid artery; ECA, external carotid artery; CCA, common carotid artery; TTC, triphenyltetrazolium chloride; Het, hydroethidine; DAPI, 46-diamidino-2-phenylindole; Ex, excitation; Em, emission; BBB, brain–blood barrier.

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rats by intraluminal blockade of the middle cerebral artery (MCA). To further elucidate the biochemical effect of SMTP-7, we examined the involvement of reactive oxygen species (ROS) and matrix metalloproteinase (MMP)-9, both of which play pivotal roles in ischemia/reperfusion injury in the central nervous system.

The present experiments were conducted in accordance with Japanese standards for the care and use of laboratory animals and the protocol was approved by the Animal Care Committee, Tohoku University Graduate School of Medicine. All surgical procedures were performed after the induction of anesthesia.

Male Sprague–Dawley rats weighing between 250 and 280 g (Charles River Japan Inc., Tsukuba, Japan) were initially anesthetized with 4% halothane and maintained with 1.5–2.0% halothane in a 30% O_2/70% N_2O gas mixture delivered via a facemask. tFCI was induced by occluding the middle cerebral artery using the intraluminal technique with silicon-coated 4–0 nylon suture [11]. Briefly, under an operating microscope, the left common carotid artery (CCA) was exposed through a midline neck incision and was carefully dissected free from surrounding nerves and fascia from its bifurcation to the base of the skull. The occipital artery branches of the external carotid artery (ECA) were then isolated, and these branches were coagulated and cut. The internal carotid artery (ICA) was isolated and carefully separated from the adjacent vagus nerve, and the pterygopalatine artery was ligated. Next, a silicon-coated 4–0 nylon suture was inserted via the proximal ECA into the ICA by 20 mm from the CCA bifurcation. After 2 h of tFCI, the suture was gently withdrawn to restore blood flow in the MCA territory.

Following induction of tFCI, rats were randomized into two groups based on agent administered: vehicle group and SMTP-7 group. Animals were infused intravenously with SMTP-7 solution (10 mg/kg in 1 mg/ml mannitol and sterile water at a ratio of 1–2) (TMS. Co., Ltd., Tokyo, Japan) or vehicle (10 ml/kg mannitol and sterile water at a ratio of 1–2) by investigators who were blind to the solution. For administration of SMTP-7 or vehicle, 10% of the agent was intravenously administered to the rats 5 min before reperfusion, followed by an intravenous infusion of the remaining agent for 30 min using a microinfusion pump (BeeHive; Bioanalytical Systems, West Lafayette, IN, USA) based on a previous study [20]. In present study, because it was technically difficult to dissolve SMTP-7 in normal saline, we used similar composition of mannitol (0.5 mg/ml) as a solvent of SMTP-7 as well as a vehicle.

In 10 rats for each group, the infarction volume was investigated in terms of the deficit shown by 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma Chemical Co., Tokyo, Japan) staining. Twenty-four hours after reperfusion, the rats were anesthetized again and sacrificed. Brains were sectioned in coronal slices 2 mm thick. The brain slices were incubated in 2% (w/v) TTC for 15 min at 37 °C. All slices were photographed and images were stored on a personal computer. The area of infarction was measured double blind using Image J software (version 1.31; NIH, Bethesda, MD, USA) and the infarction volume of the slice was calculated. The infarction volume is described using the following correction. Infarction area of each section = (contralateral hemisphere area/ipsilateral hemisphere area) × ipsilateral lesion area. The infarction volume was calculated by summation of the lesion areas of all sections and multiplying these by slice thickness [13]. Edema volume was estimated using image analysis by subtracting the volume of the contralateral hemisphere from that of the ipsilateral hemisphere [9]. In addition, we assessed the hemorrhagic transformation, identified as intracerebral hemorrhage by microscope within infarction area of each coronal slice used for TTC staining.

In 4 rats for each group, the early production of superoxide anions, which is one of the ROS in cerebral ischemia, was investigated using hydroethidine (Het) according to a previously described method [2,12]. Het solution (200 μl; 1 mg/ml in PBS) was administered intravenously 1 h after reperfusion. One hour later (2 h after reperfusion), the rats were sacrificed. Each cerebral hemisphere was homogenized. The concentration of superoxide anions in the supernatant was measured using a Hitachi F-2500 Fluorescence Spectrophotometer (Hitachi High Technologies America Inc., IL, USA). The concentration was evaluated using a Xanthine/Xanthine oxidase system as previously described [18,30].

In 4 rats for each group, to evaluate the spatial distribution of the expression of superoxide, Het solution (200 μl; 1 mg/ml in PBS) was administered intravenously 1 h after reperfusion. One hour later (2 h after reperfusion), all rats were sacrificed by transcardial perfusion with 200 ml of normal saline followed by 200 ml of 4% paraformaldehyde. After fixation with 4% paraformaldehyde for 24 h, the brains were sectioned at 50 μm on a vibratome. The sections were mounted with 46-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Fluorescence was assessed microscopically at excitation (Ex) = 355 nm and emission (Em) = 415 nm for Het detection or at Ex = 510–550 nm and Em = 580 nm for ethidium detection.

The MMP-9 protein level was investigated by Western blotting at 2, 12 and 24 h after reperfusion in 4 rats at each time point in vehicle group. Whole hemisphere of the ischemic side was rapidly sampled and frozen. Protein extraction of the whole cell fraction was performed and the sample proteins in Tris–glycine buffer were loaded as described previously [4]. The primary antibodies were rabbit polyclonal anti-MMP-9 antibody (Santa Cruz Biolaboratory, Santa Cruz, CA, USA) at a dilution of 1:200. The densitometry was scanned with a GS-700 imaging densitometer (Bio-Rad Laboratories, Hercules, CA, USA) and the results were quantified using Multi-Analyst software (Bio-Rad). The ratio of optical density to that of the vehicle was statistically evaluated. Immunoreactivities of nitrotyrosine were examined 2 h after reperfusion and those of MMP-9 were examined at 24 h after reperfusion in 4 rats in each group. All rats were sacrificed by transcardial perfusion and fixed as described above. Thereafter, the brains were sectioned at 50 μm on a vibratome. The brain sections were incubated with blocking solution and reacted with rabbit polyclonal anti-nitrotyrosine antibody (Santa Cruz Biolaboratory) and rabbit polyclonal anti-matrix metalloproteinase-9 antibody (Santa Cruz Biolaboratory) at a dilution of 1:200. Immunohistochemistry was performed using the avidin–biotin technique and then the nuclei were counterstained with methyl green solution for 2 min.

All data were expressed as mean ± standard deviation (SD). For comparison of mean infarct volume, production of superoxide anions and the expression of MMP-9 between the two groups, unpaired *t*-test was performed. Values of $P < 0.05$ were considered significant. All statistical analyses were performed on a personal computer with GraphPad Prism ver. 5.03 for Microsoft Windows (GraphPad Software, Inc., La Jolla, CA).

Infarction volume 24 h after reperfusion showed a significant reduction in the SMTP-7 group compared to that in the vehicle group ($P = 0.03$) (Fig. 1). Edema volume tended to be larger in the vehicle group ($100.3 \pm 36.8\ mm^3$) than in the SMTP-7 group ($78.7 \pm 44.7\ mm^3$). However, there were no significant differences the two groups ($P = 0.22$). Hemorrhagic lesions were found in three of ten in the vehicle group and one of ten in the SMTP-7 group (30% and 10%, respectively). However, these lesions were only small petechiae without mass effect.

Prominent oxidized Het and nitrotyrosine expression were observed in the peri-ischemic core lesion of rats 2 h after reperfusion. Compared with those in the vehicle group, oxidized Het signals were not increased in the SMTP-7 group (Fig. 2A). The concentration of superoxide anions was significantly greater in the SMTP-7 group that in vehicle group ($P = 0.0009$) (Fig. 2B). The SMTP-7 group showed weak immunoreactivity for nitrotyrosine in the