Neuroscience 220 (2012) 1–10

COMBINED TISSUE PLASMINOGEN ACTIVATOR AND AN NK1 TACHYKININ RECEPTOR ANTAGONIST: AN EFFECTIVE TREATMENT FOR REPERFUSION INJURY FOLLOWING ACUTE ISCHEMIC STROKE IN RATS

R. J. TURNER * **AND R. VINK**

Adelaide Centre for Neuroscience Research, School of Medical Sciences, University of Adelaide, Adelaide, SA, Australia

Abstract—We have recently reported on the efficacy of an NK1 tachykinin receptor antagonist in improving outcome following stroke, including reduced blood–brain barrier (BBB) disruption, reduced cerebral edema and improved functional outcome. The clinically approved stroke treatment, tissue plasminogen activator (tPA), has been associated with an increased risk of hemorrhage and death, if given at later time points. Accordingly, adjunctive therapies have been investigated to reduce the adverse effects of tPA and improve outcome. The aim of the present study was to characterize the effects of a combination of an NK1 tachykinin receptor antagonist with tPA, on BBB permeability and functional outcome following transient ischemic stroke in rats. Stroke was induced in male Sprague–Dawley rats using a reversible thread model of middle cerebral artery occlusion where occlusion was maintained for 2 h, followed by reperfusion. Animals received either 25 mg/kg of N-acetyl-L-tryptophan or 1 mg/kg of tPA, either alone or in combination, or equal volume saline vehicle, intravenously at the time of reperfusion. Functional outcome was assessed by the rotarod, bilateral asymmetry test, modified neuroscore and open field tests. BBB permeability was assessed by Evans Blue extravasation. Combination therapy of an NK1 tachykinin receptor antagonist with tPA significantly reduced BBB permeability, functional deficits and the incidence of intracerebral hemorrhage and death. As such, combined tPA–NK1 tachykinin receptor antagonist treatment may represent a novel therapeutic intervention for the treatment of reperfusion injury in acute ischemic stroke.

Key words: ischemic stroke, NK1 tachykinin receptor antagonist, tissue plasminogen activator, blood–brain barrier, neuropeptides, neurogenic inflammation.

INTRODUCTION

Currently, thrombolysis with tissue plasminogen activator (tPA) within 4.5 h is the only clinically approved therapy for the treatment of ischemic stroke (Zhang et al., 2010). Reperfusion of the ischemic territory is desirable to salvage tissue (Yang and Betz, 1994; Aronowski et al., 1997) but it may also be associated with reperfusion injury and increased tissue damage (Przyklenk and Kloner, 1989). Indeed, a number of clinical studies have documented an increased risk of hemorrhagic complications and death associated with tPA therapy, which has prompted investigation into the safety profile of tPA. In addition, clinical application of tPA therapy is limited with as little as 5% of ischemic stroke patients receiving tPA treatment (Marler and Goldstein, 2003).

In the blood, tPA functions as a fibrinolytic agent, however when it gains access to the brain parenchyma it may mediate events associated with blood–brain barrier (BBB) damage, cerebral edema and cell death (Zhang et al., 2002; Polavarapu et al., 2007). Exogenous tPA may cross both the intact and damaged BBB with potentially neurotoxic effects (Tsirka et al., 1997; Wang et al., 1998; Goto et al., 2007). There is a need for an intervention that may be combined with tPA to reduce neurotoxicity, decrease the risk of hemorrhage and death, reduce reperfusion injury, amplify the neuroprotective effect and potentially increase the therapeutic window. Such adjunctive therapies may provide a means of safely administering tPA, enabling the beneficial thrombolytic effects while disabling the damaging extravascular effects. In this study we sought to examine the effects of tPA beyond fibrinolysis, such as those effects at the BBB.

Our laboratory has recently demonstrated the involvement of the neuropeptide substance P (SP) and neurogenic inflammation in BBB breakdown and resultant vasogenic edema following traumatic brain injury and stroke (Vink et al., 2004; Turner et al., 2006, 2011; Donkin et al., 2007, 2009, 2011; Turner and Vink, 2007; Donkin and Vink, 2011; Turner et al., 2011). We have established that SP release is a feature of ischemic stroke and that it is associated with profound BBB dysfunction, cerebral edema and persistent functional deficits (Turner et al., 2006, 2011; Turner and Vink, 2007). Furthermore, blocking the action of SP with an NK1 tachykinin receptor antagonist significantly reduces this BBB disruption, cerebral edema and functional deficits (Turner et al., 2011). An NK1 tachykinin receptor antagonist may therefore

*Corresponding author. Address: School of Medical Sciences, University of Adelaide, SA 5005, Australia. Tel: +61-8-8313-3114; fax: +61-8-8303-5384.
E-mail address: Renee.Turner@adelaide.edu.au (R. J. Turner).

Abbreviations: EB, Evans Blue; ECA, external carotid artery; ECASS, European Cooperative Acute Stroke Study; ICA, internal carotid artery; ICH, intracerebral hemorrhage; LDL, low-density lipoprotein; LRP, LDL-related receptor protein; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; *MMP*, matrix metalloproteinase; NAT, N-acetyl-L-tryptophan; SP, substance P; tPA, tissue plasminogen activator; TTC, 2,3,5-triphenyltetrazolium chloride.

0306-4522/12 $36.00 © 2012 IBRO. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neuroscience.2012.06.047

represent a novel adjunctive therapy for combination with tPA, to reduce reperfusion injury and treat ischemic stroke. Accordingly, the aim of the present study was to assess the efficacy of the combination therapy comprising tPA and an NK1 tachykinin receptor antagonist on outcome, particularly BBB permeability and functional outcome, following acute ischemic stroke in rats.

EXPERIMENTAL PROCEDURES

All experimental protocols were approved by the Animal Ethics Committees of the University of Adelaide and the Institute of Medical and Veterinary Science, and conducted according to guidelines established for the use of animals in experimental research as outlined by the Australian National Health and Medical Research Council.

Adult male Sprague–Dawley rats (n = 107; 365–395 g) were used in the study. Animals were group housed in a conventional rodent room on a 12-h day–night cycle and provided with a standard diet of rodent pellets and water *ad libitum*. After transport, animals were rested for several days before inclusion in any experiment. At the time of the experiment, they were randomly assigned to an experiment and then to naïve, sham surgery, stroke surgery and treatment groups.

Middle cerebral artery occlusion (MCAO)

Animals were fasted overnight before surgery and then anesthetized with isoflurane (1.5–3%; Abbot Australasia), intubated and MCAO was performed, as described in detail elsewhere (Longa et al., 1989), and occlusion maintained for 2 h. Briefly, a 4-0-monofilament nylon suture with a tip rounded by heating near a flame and coated with 0.1% poly-L-lysine (Sigma Castle Hill, NSW, Australia) was introduced into the lumen of the external carotid artery (ECA), and was subsequently advanced into the internal carotid artery (ICA). The suture was then advanced 17 mm beyond the ECA/ICA bifurcation to occlude the origin of the middle cerebral artery (MCA). Lignocaine (0.5 ml) was applied to the surgical area and the wound closed with wound clips (9 mm Autoclip, Becton Dickinson). Anesthesia was discontinued, and when animals were able to breathe spontaneously they were extubated and allowed to recover. Reperfusion of the ischemic territory was achieved at 2 h after the onset of ischemia via withdrawal of the suture into the ECA, under isoflurane anesthesia.

Study design

Animals (n = 107) were randomly assigned to naïve, sham and treatment groups. Following stroke, animals received either equal volume of sterile saline (Baxter Healthcare Regency Park, SA, Australia) vehicle or the drug treatments N-acetyl-L-tryptophan (NAT) (Sigma) (25μmole/kg) (Turner et al., 2011) and tPA (Actilyse, Boehringer Ingelheim) (1 mg/kg), either alone or in combination, administered via the tail vein at the onset of reperfusion. Drugs were prepared and stored as per the manufacturer's instructions and then administered in a blinded fashion. Dose of tPA was determined from previous experimental stroke studies (Wang et al., 1998).

Assessment of blood–brain-barrier permeability

Evans Blue (EB; FW 960.8; Sigma; 2 ml/kg of 4% solution) extravasation was used to assess BBB integrity as previously described in detail elsewhere (Kaya et al., 2001). The assessment of BBB permeability study was divided into two parts: the effects of tPA and NAT, given immediately after one another, on barrier integrity in naïve animals (n = 5–8/group); and the effects of combined tPA + NAT treatment on barrier integrity in stroke animals (n = 3–7/group). In the naïve group, tPA was administered first, followed by NAT, BBB permeability was then assessed 4 h after drug administration. Briefly, 2 ml/kg of 4% EB solution was injected intravenously at 23.5 h post-reperfusion or sham surgery. At 24 h post-reperfusion or sham surgery the chest cavity was opened under isoflurane anesthesia and the animal transcardially perfused with saline. Perfusion was discontinued when the perfusate from the right atrium was colorless, this was consistent among animals. The brain was removed and the left and right hemispheres dissected. Tissue samples of the left and right hemispheres were then weighed and homogenized in phosphate-buffered saline (2.5 ml). Undiluted trichloroacetic acid (2.5 ml; Sigma T-0699) was then added to the homogenate and samples vortexed for 2 min before being stored at 4 °C overnight. Following centrifugation at 1000g for 30 min, the absorbance of the supernatant was measured at 610 nm using a UV/Vis spectrophotometer. The level of extravasated EB was determined using a previously obtained EB standard curve.7

Assessment of functional outcome

Ischemic stroke produces short-term and long-term motor, sensory and neurological dysfunction (Modo et al., 2000; Ding et al., 2002) and accordingly, a battery of tests are required to evaluate post-stroke impairments. Commencing at 24 h post-reperfusion or sham surgery, a subset of animals (n = 6–9/group) were assessed using the rotarod, bilateral asymmetry test and the modified neuroseverity score on days 1–7 post-stroke and on the open field on days 1, 3, 5 and 7. Functional outcome testing was carried out by an observer blinded to the experimental grouping of the animals.

Assessment of motor outcome. Motor deficits were assessed using a rotarod device (Hamm et al., 1994), which comprises a metal frame with a rotating assembly of eighteen 1-mm rods. Animals were placed on the device and remained stationary for 10 s. The rotation speed was then increased to a maximum of 30 revolutions per min, with each speed being maintained for 10 s. Animals were required to grip the rods in order to walk on the rotarod. The score recorded was when the animal completed the 2-min trial, fell off completely or gripped the rungs for two revolutions without walking.

Assessment of sensory outcome. The bilateral asymmetry test was used to assess tactile extinction probing sensory neglect following stroke as previously described (Modo et al., 2000). Briefly, two strips of tape (2 cm × 3.5 cm) were applied to the saphenous part of the forepaws. Time to removal for the left and right forepaws was recorded. Each trial lasted 120 s and animals were given two consecutive trials. The mean of the two trials was taken as the bilateral asymmetry test latency.

Assessment of neurological outcome. A modified neuroscore was used to assess general neurological function (Li et al., 2000). One point was awarded for the inability to perform the task or the lack of a tested reflex. A score of 10–15 indicated severe injury, 5–9: moderate injury, 1–4: mild injury and 0: no observable injury.

Assessment of spontaneous exploratory behavior. The open field test (Giulian and Silverman, 1975) was used to assess spontaneous exploratory behavior, considered to reflect stress and anxiety. The open field comprises a white paneled 1 m × 1 m enclosure with 100 equal 10-cm squares marked on the base. Animals were placed in the center of the enclosure and allowed to explore for 5 min. The number of squares traveled through by the animals was taken as the spontaneous exploratory behavior. Naïve animals explore the entire open field and transverse > 150 squares, whereas stroke animals remain in the perimeter and exhibit large amounts of freezing behavior.