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JNK inhibition and inflammation after cerebral ischemia ☆

Corinne Benakis [a], Christophe Bonny [b], Lorenz Hirt [a,*]

[a] Department of Neurology, University Hospital (CHUV), Lausanne, Switzerland
[b] Xigen Pharmaceuticals, Lausanne, Switzerland

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ABSTRACT

The c-Jun-N-terminal kinase signaling pathway (JNK) is highly activated during ischemia and plays an important role in apoptosis and inflammation. We have previously demonstrated that D-JNKI1, a specific JNK inhibitor, is strongly neuroprotective in animal models of stroke. We presently evaluated if D-JNKI1 modulates post-ischemic inflammation such as the activation and accumulation of microglial cells.

Outbred CD1 mice were subjected to 45 min middle cerebral artery occlusion (MCAo). D-JNKI1 (0.1 mg/kg) or vehicle (saline) was administered intravenously 3 h after MCAo onset. Lesion size at 48 h was significantly reduced, from 28.2 ± 8.5 mm^3 ($n = 7$) to 13.9 ± 6.2 mm^3 in the treated group ($n = 6$). Activation of the JNK pathway (phosphorylation of c-Jun) was observed in neurons as well as in Isolectin B4 positive microglia. We quantified activated microglia (CD11b) by measuring the average intensity of CD11b labelling (infra-red emission) within the ischemic tissue. No significant difference was found between groups. Cerebral ischemia was modelled *in vitro* by subjecting rat organotypic hippocampal slice cultures to oxygen (5%) and glucose deprivation for 30 min. *In vitro*, D-JNKI1 was found predominantly in NeuN positive neurons of the CA1 region and in few Isolectin B4 positive microglia. Furthermore, 48 h after OGD, microglia were activated whereas resting microglia were found in controls and in D-JNKI1-treated slices.

Our study shows that D-JNKI1 reduces the infarct volume 48 h after transient MCAo and does not act on the activation and accumulation of microglia at this time point. In contrast, *in vitro* data show an indirect effect of D-JNKI1 on the modulation of microglial activation.

1. Introduction

Cerebral ischemia induces neuronal damage in the territory of the occluded artery within minutes of the onset of ischemia. The injured neurons undergo cellular degeneration through excitotoxic mechanisms such as the expression of apoptotic proteins and cytotoxic enzymes as well as the up-regulation of stress-signaling pathways (Dirnagl et al., 1999; Mehta et al., 2007). This emergency state strongly activates nearby glial cells leading to the release of inflammatory mediators within the ischemic tissue and to the expression of adhesion molecules in vascular endothelial cells. The inflammatory response leads to the accumulation and activation of microglial cells as well as the recruitment of circulating T lymphocytes to the infarct area (Petty and Wettstein, 2001; Ishikawa et al., 2004; Wang et al., 2007; Benakis et al., 2009). It is postulated that this local inflammatory response contributes to the secondary injury to potentially viable tissue which could lead to a larger cerebral infarct and impaired clinical outcome in patients with ischemic stroke (Barone and Feuerstein, 1999; del Zoppo et al., 2001).

Despite promising data in experimental research, the inhibition of the delayed inflammatory response has failed to improve the outcome after stroke in clinical trials (Enlimomab Acute Stroke Trial Investigators, 2001; Sughrue et al., 2004). There is a great need to further characterize the cellular and molecular mechanisms underlying inflammation to improve chances of identifying promising therapeutic targets to prevent secondary injury. Furthermore, it is now clear that the efficacy of neuroprotective agents is greater if they target not only one single aspect of the ischemic cascade, but multiple mechanisms such as combined inhibition of neuronal cell death and of the detrimental effects of inflammation after stroke (Kaminska, 2005). Experimental protocols therefore need to explore such combined approaches.

D-JNKI1 is a selective non-ATP-competitive inhibitor that prevents the interaction of JNK with its targets including c-Jun (Bonny et al., 2001). This peptide prevents NMDA-induced neuronal death in primary cultures (Borsello et al., 2003; Centeno et al., 2007) as well as rescues the evoked potential response recorded in the CA1 region after oxygen and glucose deprivation (OGD) in organotypic hippocampal slice cultures (Hirt et al., 2004). Moreover, D-JNKI1 remarkably attenuates the lesion size in transient and permanent models of cerebral ischemia in both rats and mice (Borsello

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* Corresponding author. Address: Laboratory of Neurology, CHUV BH 19-208, 1011 Lausanne, Switzerland. Fax: +41 21 3141244.
E-mail address: Lorenz.Hirt@chuv.ch (L. Hirt).

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et al., 2003; Hirt et al., 2004; Repici et al., 2007; Esneault et al., 2008), even with delayed intravenous administration (Wiegler et al., 2008). These experimental studies have also shown that D-JNKI1 improves functional outcome at later time-points after ischemia. All together, these data strongly suggest that inhibition of the JNK pathway is a very promising target for stroke patients.

The JNK pathway is also activated in microglial cell cultures after stimulation by LPS or thrombin (Hidding et al., 2002; Waetzig et al., 2005), which leads to the expression of inflammatory molecules such as TNFα. It is well described that microglia undergo morphological changes after cerebral ischemia with accumulation and proliferation at the site of injury and secretion of inflammatory modulators, such as cytokines, chemokines and toxic factors (Feuerstein et al., 1998; del Zoppo et al., 2000).

Our aim was to test whether D-JNKI1 was also inhibiting the JNK pathway in microglia and if this contributed to the strong neuroprotective effect of delayed D-JNKI1 administration in our models. We examined whether the activation of microglia was reduced in D-JNKI1-treated animals, subjected to middle cerebral artery occlusion (MCAo), by investigating morphological changes and accumulation within the infarct area. Our data show that D-JNKI1 does not influence global morphology and number of microglia *in vivo*. We showed that D-JNKI1 is present mainly in neurons and in few microglia when administered in an *in vitro* ischemia model by subjecting hippocampal slice cultures to OGD. Interestingly, we identified changes in microglia *in vitro*, such as a modification of microglial morphology and localization after OGD or OGD + D-JNKI1. While D-JNKI1 has a strong neuroprotective effect in our stroke model, it does not appear to attenuate the activation by morphological criteria and accumulation of microglia in ischemic tissue at 48 h.

2. Materials and methods

2.1. Animals

Male Crl:CD1/ICR mice (20–30 g, 4- to 5-week-old) and gestant Sprague–Dawley (OFA) rats were purchased from Charles River Laboratories Inc. (Charles River France, L'Abresle, France). All procedures were in accordance with the Swiss Federal Law on Animal Welfare and were approved by the Swiss Federal Veterinary Office.

2.2. Transient middle cerebral artery occlusion (MCAo)

Focal cerebral ischemia was induced as previously described (Wiegler et al., 2008), but with a different anaesthesia protocol and a longer ischemia duration. CD1 mice were anesthetized and maintained with 1–3% isoflurane in 70% N_2O and 30% O_2 using a face mask. Body temperature was maintained at 37 ± 0.5 °C throughout surgery (FHC Inc., Bowdoinham, ME, US). Regional cerebral blood flow (CBF) was continuously recorded by laser Doppler flowmetry (LDF, Periflux 5000, Perimed, Sweden) during a period covering the induction of ischemia until 10 min after the end of ischemia. Transient left MCAo was induced as previously described (Lei et al., 2009) with a silicone-coated nylon monofilament (diameter: 0.17 mm, Doccol Co., Redlands, CA, USA) inserted through the common carotid artery and withdrawn after 45 min to allow reperfusion. Mice were given 0.025 mg/kg of buprenorphine subcutaneously for post-surgery analgesia and were housed in an incubator at 31 °C for 24 h for recovery.

2.3. Groups, treatments and neurological deficits

Randomly, saline solution (NaCl 0.85% Medium, bioMérieux) or D-JNKI1 (0.1 mg/kg, Xigen Pharmaceuticals) was injected intravenously (i.v.) into the tail vein of mice using a 1 ml syringe (Omnifix-1 ml, Terumo needle 25G) and a mouse restrainer (Braintree Scientific Inc.) 3 h after ischemia onset. The lesion volume was evaluated 48 h after cerebral ischemia and immunostainings were performed at 9 h, 24 h, 48 h and 7 days after surgery. The neurological deficit was assessed at 48 h. Neuroscore was graded for severity using the following scale: 0 = no observable neurological deficit, 1 = failure to extend the right forepaw, 1.5 = intermittent circling behavior, 2 = circling behavior, 3 = loss of walking or uncontrolled movement (Bederson et al., 1986; Hirt et al., 2004).

2.4. Tissue preparation and immunohistochemistry

Mice were anesthetized with intra-peritoneal injection of 8 mg/kg xylazine (Rompun® 2%, Bayer) + 100 mg/kg ketamine (Ketanarkon 100, Streuli Pharma AG) and transcardially perfused with PBS followed by 4% paraformaldehyde. Serial coronal 20-μm thick, 720-μm distant cryostat sections were used for lesion size measurements and immunolabelling.

2.4.1. Histological evaluation of neuronal damage

Digitalized images of the Nissl stained tissue were acquired under a light stereomicroscope (Leica MZ16FA) and the ischemic area was measured by a blinded observer with ImageJ 1.38x software (NIH, http://rsb.info.nih.gov/ij/). The direct infarct volume was calculated by multiplying the sum of the infarct areas on each section by the distance between the sections.

2.4.2. Staining of degenerating neurons: FluoroJade B

FluoroJade B staining (0.0004% FluoroJade® B, Millipore, UK) was performed on frozen sections according to the Chemicon International Company protocol. Sections were then mounted in Eukitt (O. Kindler GmbH & Co., Freiburg, Germany).

2.4.3. Immunohistochemistry staining for glial and inflammatory cells

Sections were blocked in 0.3% H_2O_2 for 20 min at room temperature (RT) followed by 10% normal goat serum (NGS, Invitrogen, Paisley, UK) in PBS with 1% bovine serum albumin (BSA) for 1 h at RT. Sections were incubated overnight at 4 °C with the following primary antibodies: rat anti-CD11b (Mac-1, 1:100, AbD SEROTEC) or biotinylated Isolectin B4 (1:500, *Griffonia simplicifolia* lectin I, Vector Laboratories) to label microglia, mouse anti-GFAP (1:500, Millipore) for astrocytes, hamster anti-TCRbeta (1:200, BioLegend) for T lymphocytes, hamster anti-CD11c (1:200, BioLegend) for dendritic cells. Using the immunoperoxidase method, biotinylated anti-rat (1:500, BA-9400, Vector Laboratories, Burlingame, CA), anti-mouse (1:500, Vector Laboratories, Burlingame, CA) or anti-hamster (1:500, Biolegend) IgG antibodies were applied for 1 h followed by avidin–biotin–horseradish peroxidase complex (Vectastain kit, Vector Laboratories, Burlingame, CA). Incubation with biotinylated secondary antibody was omitted for Isolectin B4. The immunocomplex was visualized by 3,3′-diaminobenzidine (DAB kit, Vector Laboratories, Burlingame, CA) and mounted with Eukitt.

2.4.4. Double immunofluorescence

After blocking in 10% NGS (±0.1% Triton X-100) sections were incubated overnight at 4 °C in various combinations of the following primary antibodies: mouse anti-NeuN (1:500, Millipore), mouse anti-GFAP (1:500), biotinylated Isolectin-B4 (1:500) and rabbit anti-phospho-c-Jun(Ser63) or phospho-c-Jun(Ser73) (1:100, Cell Signaling). Antigens were visualized with the appropriate fluorochrome-conjugated secondary antibodies: alexa fluor 488 anti-rabbit (1:500, Invitrogen), alexa fluor 594 anti-mouse (1:500, Invitrogen), Cy3-conjugated Streptavidin (1:500, Jackson ImmunoResearch Laboratories) for 1 h at RT. Sections were counterstained with DAPI (1:10,000, 1 mg/ml, SIGMA) and mounted in