Inhibition of Tumor Necrosis Factor-α–Converting Enzyme by a Selective Antagonist Protects Brain from Focal Ischemic Injury in Rats

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Received October 1, 2003; accepted January 12, 2004 This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Tumor necrosis factor α (TNFα) is an immunomodulatory and proinflammatory cytokine implicated in neuroinflammation and neuronal damage in response to cerebral ischemia. Tumor necrosis factor-α converting enzyme (TACE or ADAM17) is a key sheddase that releases TNFα from its inactive cell-bound precursor. Using a selective small molecule inhibitor of TACE, DPH-067517, we tested the hypothesis that inhibition of TNFα formation might have a salutary effect in ischemic stroke induced by embolic occlusion of the middle cerebral artery (MCAO). DPH-067517 selectively inhibited TACE enzyme activity in vitro (Ki = 2.8 nM), and effectively suppressed ischemia-induced increase in soluble TNFα in brain tissue after systemic administration. DPH-067517 (3 and 30 mg/kg, i.p. administered 15 min before MCAO) produced 43% (n = 8, p = 0.16) and 58% (n = 8, p < 0.05) reduction in infarct size and 36% (p < 0.05) and 23% (p < 0.05) reduction in neurological deficits, respectively. The salutary effect of DPH-067517 in ischemic brain injury was also observed when the first dose was administered 60 min after the onset of ischemia. Inhibition of TACE had no effect on apoptosis measured by levels of active caspase-3 expression and DNA fragmentation. Our data suggest that inhibition of TACE might be a potential therapeutic strategy for neuroprotection after focal ischemic stroke.

Tumor necrosis factor-converting enzyme (TACE, or ADAM17) is a member of the ADAM (A Disintegrin and Metalloproteinase) family of proteases containing both a disintegrin and a metalloproteinase domain (Doedens and Black, 2000). ADAMs form a large group of cell surface proteins that combine features of both cell surface adhesion molecules and proteases. So far, 31 ADAMs have been identified in the public database, 17 of which contain the sequence motifs that were originally identified for its ability to release soluble TNFα from transmembrane pro-TNFα (Black et al., 1997; Reddy et al., 2000).

Focal stroke is a pathophysiological condition caused by decreased blood supply to the brain. The deprivation of oxygen and glucose in the ischemic brain eventually leads to cell death (necrosis and apoptosis), inflammation, and tissue repair (del Zoppo et al., 2000; Wang and Feuerstein, 2000). TNFα is a key inflammatory mediator that has been demonstrated to be upregulated in brain ischemia (Liu et al., 1994; Wang et al., 1994) and to play a detrimental role in neuronal survival. Administration of TNFα during an ischemic brain insult has been shown to augment the injury, as evidenced by increased tissue damage and neurological deficits (Barone et al., 1997). Correspondingly, experiments with neutralizing anti-TNFα antibodies or sTNF-R1, administered directly into the cerebroventricular system, reduced ischemic damage and improved functional outcome (Dawson et al., 1996; Barone et al., 1997; Nawashiro et al., 1997a). However, several other studies suggested a protective role of the TNFα signaling pathway in tolerance to ischemic or traumatic brain injuries (Bruce et al., 1996; Nawashiro et al., 1997b; Liu et al., 2000). TNFα null mutations in mice result in exacerbation of lesions and functional deficits after cerebral ischemia (Bruce et al., 1996). Studies conducted with animals exposed to TNFα before ischemic injuries showed remarkable tolerance to the ischemic insult, as

ABBREVIATIONS: TACE, tumor necrosis factor-converting enzyme; ADAM, a disintegrin and metalloproteinase; ADAMTS, ADAM family proteins with thrombospondin type I motifs; TNFα, tumor necrosis factor α; DPH-067517, N-(4-[2-(hydroxylamino)-2-oxoethyl]-2,6-[dimethyl-4-piperidinyl]-4-[2-methyl-4-quinolinyl][methoxy][benzamide]; MMP, matrix metalloproteinase; ELISA, enzyme-linked immunosorbent assay; MCAO, occlusion of the middle cerebral artery; MCA, middle cerebral artery; CBF, cerebral blood flow; TTC, 2,3,5-triphenyltetrazolium chloride; IL, interleukin; BB-1101, 2S-allyl-N-hydroxy-3R-isobutyl-N-(1S-methylcarbamoyl-2-phenylethyl)-succinamide.
Reactions were quenched by the addition of 20% H9262 mixtures were incubated for 1 hour on a norbital shaker at 27°C. The enzyme reaction contained 25 mM CaCl₂, and 1 mM ZnCl₂. The enzyme reaction was obtained from porcine spleen and partially purified TACE plus the diluted peptide in 200-nM partially purified TACE enzyme was obtained from porcine spleen and a synthetic fluorogenic substrate, (7-methoxy coumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys (2,4-dinitrophenyl)-NH₂. Procedures using lab animals were approved by our Institutional Animal Care and Use Committee. Male Sprague-Dawley rats at 20 weeks of age weighing 340 to 450 g were used for these experiments. Rats were anesthetized with gas inhalation composed of a 30% oxygen (0.3 liter/min) and 70% nitrous oxide (0.7 liter/min) mixture. The gas was passed through an isoflurane vaporizer set to deliver 3 to 4% isoflurane during initial induction and 1.5 to 2% during surgery. Details for animal care and monitoring during operation have been described previously (Wang et al., 2003b).

To induce thromboembolism in the cerebral circulation, an embolus was prepared using a procedure described previously (Zhang et al., 1997; Wang et al., 2003b). Briefly, a femoral artery was dissected from a donor rat under anesthesia and a small volume of blood (1–2 ml) was withdrawn into PE-50 tubing. The blood clot was retained from the middle cerebral artery. No clot was injected in sham-operated animals.

Drug Administration. To ensure the effect of TACE inhibitor in ischemic injury, our initial study started with a higher dose, i.e., 30 mg/kg DPH-067517, or vehicle (saline), administered to rats i.p., 15 min before and 6 h after MCAO. The same dose regimen was used to assess the therapeutic window by administering the first dose at 60 min or 180 min after MCAO and the second dose at 6 h (n > 8). A concentration-dependent study was then performed by dosing DPH-067517 (0.3, 3 or 30 mg/kg) or vehicle (saline) at 15 min before and 6 h after MCAO (n > 8). Because similar effects were observed on infarct volume and neurological deficits between the 3 and 30 mg/kg DPH-067517 groups administered 15 min before MCAO, the lower dose was also confirmed by administering at 60 min after MCAO. A dose of 3 mg/kg DPH-067517 was used to study physiological parameters, drug effects on cytokine and caspase-3 expression as well as apoptosis.

Physiological Measurements. Regional cerebral blood flow (CBF) was measured with a Laser Doppler Perfusion Monitor (Moor Instruments Inc., Wilmington, DE). Under anesthesia, a small incision was made at the midpoint between the right orbit and the external auditory canal. The temporalis muscle was retracted and
the underlying fascia cleared. A small area of skull about 1 mm posterior and 5 mm lateral to the Bregma in the ipsilateral hemisphere was thinned to allow placement of the laser Doppler probe. CBF was carefully monitored 15 min before and 15 min and 6 h after MCAO in 3 mg/kg DP-067517- or vehicle-treated animals.

Arterial blood pressure and heart rate were measured using an MP100 Workstation and analyzed using an AcqKnowledge software (BIOPAC Systems, Inc., Santa Barbara, CA) according to the manufacturer’s specifications. Femoral arterial blood samples were analyzed for pH, oxygen (pO2) and carbon dioxide (pCO2) by direct collection through PE-50 tubing into an i-STAT G3+ cartridge and processed with a portable clinical analyzer (Abbott Laboratories, Abbott Park, IL).

Measurement of Infarct Volume. To measure the infarct volume, brains were removed under deep anesthesia as described above 24 h after MCAO and evaluated using 2,3,5-triphenyltetrazolium chloride (TTC) staining of 2-mm thick brain slices. The stained brain tissue was fixed in 10% formalin in phosphate-buffered saline. The image was captured using a Microtek ScanMaker 4 DUO Scanner (Microtek, Carson, CA) and quantitated using Image Pro Plus 4.1 software (Media Cybernetics, Silver Spring, MD).

Neurological Deficits. Neurological deficits were examined at 24 h after MCAO or sham-operation using a 5-point scale as described previously (Wang et al., 2003b). Specifically, no neurological deficit, 0 point; right Horner’s syndrome, 1 point; failure to extend the left forelimb and hindlimb, each 1 point; turning to the left and circling to the left, each 1 point. The same groups of animals were subjected to rota-test using an Accelerating Speed Treadmill for rats (Stoelting, Wood Dale, IL). Four trials were allowed at each session, and the mean values collected for group data analysis.

Enzyme-Linked Immunosorbent Assay. Tissue lysate from ipsilateral and contralateral brain samples (24 h after MCAO, n = 8) was pulverized using a porcelain mortar and pestle under liquid nitrogen. The pulverized brain tissues were incubated in a lysis buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100) and 5 μl/ml of protease inhibitor cocktail (Sigma, St, Louis, MO) for 1 h at 4°C. After a 10-min centrifugation at 10,000g, the supernatant of tissue lysate was collected and aliquoted for ELISA and protein concentration measurement using a Bio-Rad detergent-compatible protein assay kit (Hercules, CA). The levels of TNFα and IL-1β protein in the brain tissue were measured using ELISA kits for rat TNFα and IL-1β (R&D Systems, Inc., Minneapolis, MN) following the manufacturer’s specifications. Tissue extracts (50 μl) were applied to each well for the ELISA, and the final measure was read out using a plate reader at 450 nm. The concentrations of TNFα and IL-1β protein in each sample were determined according to the standards (recombinant proteins) provided with the kits. TNFα and IL-1β levels in the brain samples were within the linear range of the standard curves. Each sample was normalized using its total protein concentration in milligrams and expressed as the ratio of ipsilateral versus contralateral tissues.

Western Blot Analysis. Western blot analysis was used to evaluate the levels of the active form of caspase-3 in rats (n = 8) 24 h after transient MCAO. The pulverized brain tissues were lysed and processed as described in the previous section. The soluble component of the tissue lysate was used for Western blot (100 μg protein/lane) using a mouse monoclonal IgG against caspase-3 as previously described in detail (Wang et al., 2003a). The blot was stripped and re-probed with a goat polyclonal anti-actin antibody (Santa Cruz Biotechnology, Inc.) for loading controls in each lane.

Apoptosis Analysis. Apoptosis was measured by quantitating DNA fragments in the ipsilateral and contralateral hemispheric brain tissue 24 h after MCAO in rats treated with 3 mg/kg DP-067517 or saline (n = 8) using a cell death detection ELISA kit (Roche Molecular Biochemicals; Wang et al., 2003a). This sandwich-enzyme immunoassay provides a quantitative determination of histone-associated DNA fragments (mono- and oligonucleosomes) based on a photometric reaction using monoclonal antibodies directed against both DNA and histones. Frozen, pulverized brain tissue was lysed using the lysing buffer provided in the kit (30 min at room temperature) and pelleted (200g). Aliquots of the supernatant were used in the assay according to the manufacturer’s protocol.

Statistical Analysis. All data are presented as mean ± S.E., and statistical comparisons were made by analysis of variance (analysis of variance; Fisher’s protected least-squares difference). Differences were considered significant when p < 0.05.

Results

DP-067517 Selectively Inhibited TACE Activity. Table 1 shows the selective inhibition by DP-067517 of TACE compared with other closely related metalloproteinases. DP-067517, with K of 2.8 nM for TACE, is selective for TACE compared with other MMPs and ADAMTSs tested, showing more than 100-fold selectivity with the exception of MMP-14 (i.e., 58-fold). The selective inhibition of TACE activity by DP-067517 in brain tissues was also observed by measuring the selective suppression of soluble TNFα release after brain ischemia (Fig. 2A). As a control, DP-067517 had no effect on the expression of soluble IL-1β after ischemic brain injury (Fig. 2B).

Effects of DP-067517 on Physiological Parameters. The effects of DP-067517 on regional cerebral blood flow, heart rate, arterial blood pressure, pH, blood oxygen (pO2), and blood carbon dioxide (pCO2) were evaluated in rats after

<table>
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<tr>
<th>Enzyme</th>
<th>Name</th>
<th>Ki (nM)</th>
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<td>MMP-1</td>
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<td>1786</td>
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<td>Gelatinase A</td>
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<td>1071</td>
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<td>Collagenase 2 (Neutrophil collagenase)</td>
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<td>732</td>
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<tr>
<td>MMP-9</td>
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<td>1071</td>
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Methods. The levels of soluble TNFα and IL-1β in the brain after ischemic injury. Rats (n = 8) were i.p. administered 3 mg/kg DPH-067517 or vehicle at 15 min before and 6 h after MCAO. Brain tissues were collected at 24 h after MCAO and processed for "sandwich" ELISA analysis as described in detail under Materials and Methods. The levels of soluble TNFα (A) and IL1β (B) in the brain were determined and the data are illustrated as ratios of ipsilateral/contralateral (IL/CL) with mean ± S.E. after normalizing for total protein concentration. * p < 0.05 relative to the vehicle-treated group.

sham-operation or MCAO (15 min before, 15 min and 6 h after MCAO). No significant difference was observed between DPH-067517- and vehicle-treated groups in any of the respiratory and cardiovascular parameters tested (Table 2).

Neuroprotective Effect of DPH-067517 on Rat Brain after MCAO. Figs. 3 and 4 illustrate the dose-dependent effect of DPH-067517 on ischemic lesion and neurological deficits. Similar levels of ischemic damage were observed comparing the vehicle group and rats treated with a low dose of DPH-067517 (0.3 mg/kg). Both infarct volume and neurological deficits were significantly reduced after treatment with either 3 or 30 mg/kg DPH-067517, with 43% (n = 8, p = 0.16) and 58% (n = 8, p < 0.05) reduction in ischemic lesion (Fig. 3A) and 36% (p < 0.05) and 23% (p < 0.05) reduction in neurological deficits, respectively, compared with controls (Fig. 4B). The reduction in infarct size was observed in both cortical and subcortical regions (Fig. 3; quantitative data not shown).

Therapeutic window was studied by providing the first dosing of 30 mg/kg DPH-067517, at 15 min before (n = 8), or 60 min (n = 10) or 180 min (n = 9) after MCAO. As shown in Fig. 5, significant neuroprotection was observed when the first dose (30 mg/kg) was given at 60 min (57% reduction in infarct size and 35% reduction in neurological deficits, p < 0.05) but not at 180 min after MCAO. Similarly, 3 mg/kg DPH-067517 administered at 60 min after MCAO (n = 7) significantly decreased in infarct size (54%, p < 0.05) and neurological deficits (37%, p < 0.05).

DPH-067517 Had No Effect on Expression of Caspase-3 and DNA Fragmentation (Apoptosis) in the Brain after MCAO. Because TNFα has been previously shown to be involved in apoptosis (Gupta 2001) and apoptosis represents one of the pathophysiological phenomena after focal brain ischemia (Moskowitz and Lo, 2003), the effects of DPH-067517 on caspase-3 expression and DNA fragmentation (an indicator of apoptosis) were examined. Western analysis was used to detect the expression of active caspase-3 (p20) in the brain after MCAO. As expected, the levels of p20 caspase-3 were markedly increased in the ischemic brain tissues compared with the contralateral tissues at 24 h after MCAO (Fig. 6), with a mean ipsilateral/contralateral ratio of 4.0 in vehicle-treated rats (Fig. 7A). A similar ratio of caspase-3 expression (ratio = 4.6, not statistically different from vehicle treatment) was observed in DPH-067517-treated animals.

The effect of DPH-067517 on apoptosis after cerebral ischemia was evaluated by monitoring DNA fragmentation using an ELISA method. Although apoptosis was induced in the ischemic brain tissues after MCAO, no significant difference was observed between groups after DPH-067517 and vehicle treatment (Fig. 7B). Sham operation did not induce caspase-3 expression and apoptosis in the brain.

Discussion

TNFα is one of the key immunomodulatory and pro-inflammatory cytokines up-regulated after focal stroke. However, the precise function of TNFα in ischemic brain has been the subject of much speculation, with data supporting both det-
It should be pointed out, however, that TACE has also been shown to be involved in the release of L-selectin, transforming growth factor-α, the p75 TNF receptor, type II interleukin-1 receptor, and amyloid precursor protein (Buxbaum et al., 1998; Peschon et al., 1998; Lammich et al., 1999), all of which are known to be associated with the pathology of various neurological disorders, including focal stroke and Alzheimer’s disease (Fassbender et al., 1995; Bruce et al., 1996; Wang et al., 1997; Ruocco et al., 1999; Citron, 2002). Therefore, although DPH-067517 is selective for inhibition of TACE relative to other MMPs and ADAMs, additional mechanisms associated TACE function cannot be excluded for the neuroprotective effect of DPH-067517 treatment in ischemic brain injury.

In addition to inflammation, apoptosis represents one of the pathophysiological events after ischemic brain injury (Moskowitz and Lo, 2003). Because TNFα has been shown previously to be involved in apoptosis (Gupta 2001), our present study explored the potential effect of DPH-067517 on apoptosis by examining the levels of caspase-3 expression and DNA fragmentation. Our data suggest that inhibition of apoptosis might not be the neuroprotective mechanism for DPH-067517 in ischemic brain injury. In addition, because the degree of apoptosis does not correlate with infarct volume after DPH-067517 treatment, these data suggest that the reduction in infarct volume might be more closely related to necrosis than to apoptosis. It also remains to be explored...
whether the levels of soluble TNFα expression and apoptosis might be affected when the TACE inhibitor is given after ischemic insult.

In contrast to our present result, one previous study concluded that TACE activity was neuroprotective because inhibition of TACE by a "selective" inhibitor, BB-1101, abolished the protective effect of ischemic preconditioning in rats (Cardenas et al., 2002). The evidence from this previous report and our present study suggests that TACE, like TNFα, might play either a detrimental or protective role in ischemic brain injury depending on the particular condition. On the other hand, these differences might reflect the use of different models (ischemic preconditioning versus ischemic injury) and different TACE inhibitors, so the key underlying differences remain to be investigated.

In summary, our present study demonstrates that the inhibition of TACE by a selective inhibitor is neuroprotective in ischemic brain injury, and this protection is probably associated with reduced expression of soluble TNFα in the brain. These data suggest that pharmacological manipulation of TACE/TNFα activity might be of therapeutic value for the treatment of ischemic stroke.

References


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