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Evidence for neuroprotection by the fenamate NSAID, mefenamic acid

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ABSTRACT

Fenamate NSAIDs are inhibitors of cyclooxygenases, antagonists of non-selective cation channels, subtype-selective modulators of $GABA_A$ receptors, weak inhibitors of glutamate receptors and activators of some potassium channels. These pharmacological actions are all implicated in the pathogenesis of ischemic stroke. The aim of this study was to investigate the hypothesis that the fenamate, mefenamic acid, is neuroprotective in an *in vitro* and *in vivo* model of stroke.

Embryonic rat hippocampal neurons were cultured and maintained for up to 14 days *in vitro*. At 9 or 14 days, cells were exposed to glutamate (5 μM) or glutamate (5 μM) plus mefenamic acid (10–100 μM) or the control agent, MK-801 (10 μM) for 10 min. 24 h later, cell death was determined by measuring lactate dehydrogenase (LDH) levels in the culture media.

In vivo, male Wistar rats (300–350 g) were subjected to 2 h middle cerebral artery occlusion (MCAO) followed by 24 h reperfusion. Animals received either a single i.v. dose of MFA (10 mg/kg or 30 mg/kg), or MK-801 (2 mg/kg) or saline prior to MCAO or, four equal doses of MFA (20 mg/kg) at 1 h intervals beginning 1 h prior to MCAO. Ischemic damage was then assessed 24 h after MCAO.

In vitro, mefenamic acid (10–100 μM) and MK-801 (10 μM) significantly reduced glutamate-evoked cell death compared with control cultures. *In vivo*, MFA (20 mg/kg × 4) significantly reduced infarct volume, total ischemic brain damage and edema by 53% ($p \leq 0.02$), 41% ($p \leq 0.002$) and 45% ($p \leq 0.002$) respectively. Furthermore, mefenamic acid reduced cerebral edema when measured as a function of brain water content. MK-801 was also neuroprotective against MCAO brain injury.

This study demonstrates a significant neuroprotective effect by a fenamate NSAID against glutamate-induced cell toxicity, *in vitro* and against ischemic stroke *in vivo*. Further experiments are currently addressing the mechanism(s) of this neuroprotection.

1. Introduction

Ischemic stroke is characterized by activation of multiple cellular pathways that can lead to extensive cell death (Sweeney et al., 1995; Traystman, 2003). The ischemic damage initiated by disruption of blood flow results in the loss of energy supply to the brain (Maiese, 1998; Walz, 1999), depletion of neuronal ATP and initiation of a cascade of events that includes over-stimulation of neuronal glutamate receptors (*excitotoxicity*), ion imbalances, intra-neuronal calcium overload, mitochondrial injury, generation of reactive oxygen species (ROS), inflammation, apoptosis and ultimately cell death (Santos et al., 1996; Small et al., 1999; Lyden and Wahlgren, 2000; Lo et al., 2003). Despite extensive research in the field of ischemic stroke, no standard, safe and effective treatment has yet been identified to combat these pathophysiological events (Hammerman and Kaplan, 1998; Muir, 2002; Cheng et al., 2004; Zivin, 2007).

More recently, it has been proposed that combination drug therapy, or agents with multiple pharmacological actions might better attenuate ischemic damage and offer significant advantages over single drug treatments (Gladstone et al., 2002; Fisher et al., 2006). Fenamate non-steroidal anti-inflammatory drugs (NSAIDs) are inhibitors of cyclooxygenases (COX) with some selectivity towards the COX-2 isoform (Lee and Wang, 1999; Asanuma et al., 2001). These agents are commonly used for their anti-inflammatory, analgesic and antipyretic actions (Flower et al., 1972). More recently, several additional pharmacological properties of the fenamates have been identified (Teramoto et al., 2003; Coyne et al., 2007). Asanuma et al. (2001), for example, demonstrated that the fenamate, mefenamic acid (*Ponstel*®) dose-dependently scavenged nitric oxide radicals, *in vitro* and reduced NO donor-induced death in the B65 neuroblastoma cell line. Similarly, in a study using stimulated human neutrophils, Ramos et al. (1995) reported that another fenamate, meclofenamic acid, inhibited myeloperoxidase-dependent hydroxyl radical generation through scavenging of hydrochlorous acid (Ramos et al., 1995). These actions are additional to COX inhibition and may contribute to the anti-inflammatory effects of fenamates.

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Several studies have now also demonstrated that fenamates modulate a variety of neuronal ion channels. Halliwell et al. (1999), for example, reported that mefenamic acid can potentiate or inhibit human recombinant $GABA_A$ receptors depending on the subunits forming the GABA receptor complex (Halliwell et al., 1999); more recently, Coyne et al. (2007) showed that fenamates not only potentiate but also directly activate native $GABA_A$ receptors expressed in rodent hippocampal neurons. The fenamates, niflumic and flufenamic acid, also inhibit NMDA-activated currents recorded from spinal neurons (Lerma and Martin del Rio, 1992) and flufenamic acid has been shown to block calcium-activated non-selective cation (CAN) channels in rat hippocampal CA1 neurons (Partridge and Valenzuela, 2000). Since CAN channels are activated in response to cytoplasmic calcium, they might play a key role in neuronal depolarization and in excitotoxicity (Partridge et al., 1994). In cultured cortical neurons, meclofenamic acid enhanced the M-type (potassium) currents and reduced evoked and spontaneous action potentials (Peretz et al., 2005). Together these pharmacological actions suggest that fenamates might reduce neuronal cell damage resulting from cerebral ischemia.

The present study was therefore undertaken to investigate the hypothesis that mefenamic acid has neuroprotective properties against glutamate-induced cell death *in vitro* and ischemic stroke damage *in vivo*.

2. Materials and methods

2.1. Primary hippocampal neuron cell cultures

Rat hippocampal neurons were obtained from 19-day embryonic brain and maintained in cell culture for our *in vitro* studies. Dissociated hippocampal neurons were plated on poly-D-lysine-coated 24-well plates in Neurobasal medium, supplemented with B27 (1%, v/v, Invitrogen, CA), Penicillin/Streptomycin (1%, v/v), and 0.5 mM L-glutamine. Cells were placed in an incubator (Isotemp, Fisher Scientific Inc., CA) at 37 °C, 100% relative humidity and 5% CO_2. After 48 h *in vitro*, non-neuronal cell division was halted by the addition of 10^{-5} M cytosine arabinoside into the culture medium. Thereafter 50% of the culture dish media was changed every 3 days. Under these conditions, and as described previously by others (e.g. Brewer et al., 1993) culture dishes contained neurons and were essentially devoid of non-neural elements at the time of our experiments (see Fig. 1 (top)).

2.2. Glutamate-induced neurotoxicity

Neurotoxicity was induced by exposure of cell cultures to mono-sodium glutamate (Na-glutamate) at 9 or 14 days *in vitro*. A stock solution of Na-glutamate (1 mM) was freshly prepared in HBSS and the cells were exposed to Na-glutamate (0.3–100 μM) in HBSS for 10 min; during this time the cells were kept in the incubator at 37 °C, 5% CO_2 100% relative humidity atmosphere. Following glutamate exposure, the cells were washed three times in fresh culture media to terminate the reaction and then returned to the incubator and maintained in normal culture media for a further 24 h.

2.3. Drug treatment

Mefenamic acid or MK-801 was applied to the cells at the same time as glutamate exposure. After 10 min, glutamate-containing HBSS was removed by three fresh media washes and then replaced with pre-warmed media containing the test drug (at the concentration applied during glutamate exposure) for a further 24 h until the neurotoxicity assay.

2.4. Cell death assessment

Cell death was quantified by measuring the release of lactate dehydrogenase (LDH) in the culture media using a CytoTox-96™ non-radioactive assay kit (Promega, WI). The extent of cell death was determined with reference to that induced by Triton X-100 (0.9%) which induced 100% neurotoxicity in the present experiments. The optical density (OD) was determined at 490 nm using an ELISA Microplate reader according to the manufacturer's protocol. The measurement of optical density was corrected for background absorbance, which includes phenol red and animal sera (both present in the culture media) and for naturally occurring cell loss (determined in control cultures) over the 24 h of the experiment.

2.5. Animal preparation

Animal studies conformed to the guidelines outlined in the *Guide for the Care and Use of Laboratory Animals* from the National Institutes of Health and were approved by the local Animal Use Committee. Rats were allowed free access to water and commercial rodent diet chow under standard laboratory conditions. The room temperature was maintained at 20–23 °C and the room illumination was on a 12/12-h light/dark cycle. Animals were acclimatized to the laboratory environment for at least 3–5 days prior to the start of the study.

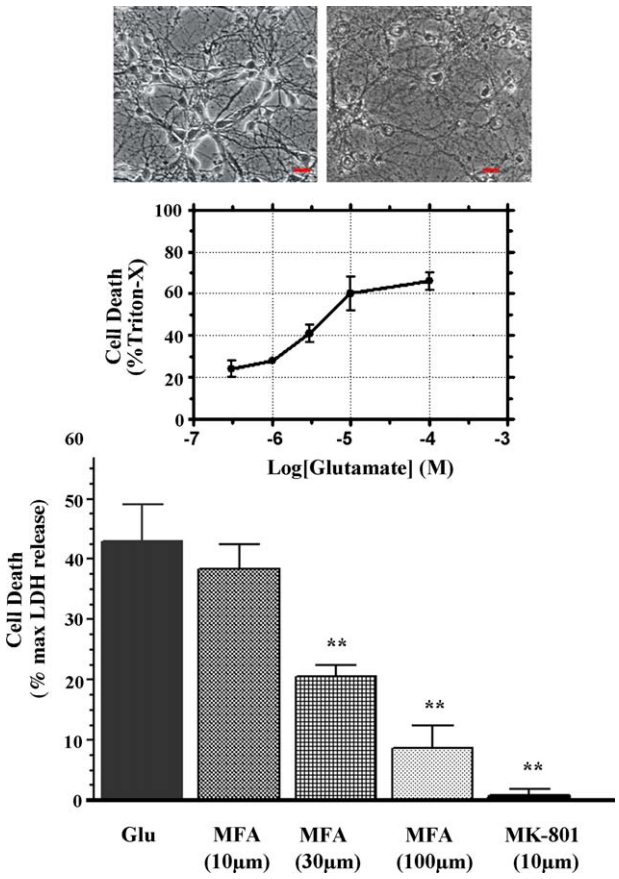

Fig. 1. Mefenamic acid reduces glutamate-evoked cell death in hippocampal cultures. (Top) Photomicrographs (×300) of primary hippocampal cells, 9 days *in vitro*, before (left) and 24 h after (right) exposure to glutamate (5 μM, for 10 min). The scale bars are 20 μm. The graph below shows the increase in cell death (on the *y*-axis) plotted against glutamate concentration (on the *x*-axis) applied to the cultured hippocampal cells. Each data point represents three independent experiments conducted in triplicate. The level of cell loss in control cultures over 24 h was 18% of that induced by triton, a value subtracted from glutamate and glutamate + drug exposed cultures to determine glutamate-evoked cell toxicity. The lower histogram depicts the reduction in glutamate (5 μM) induced cell death by mefenamic acid (MFA, 10 μM, 30 μM, or 100 μM) or MK-801 (10 μM). Each data point represents three independent experiments conducted in triplicate ($^{**}p < 0.01$).

2.6. Focal transient MCAO

Male Wistar rats (Harlan, IN) weighing 300–350 g were first anesthetized using 3% isoflurane (Aerrane, Front Dodge, IA) in 0.8% oxygen and then subjected to transient focal cerebral ischemic stroke by occluding the middle cerebral artery using an intraluminal filament for 2 h followed by 24 h of reperfusion. In brief, a midline incision on the ventral part of the neck was made to expose the external, internal and common carotid arteries. The arteries were then dissected free from the surrounding nerves and muscles. The right external and common carotid arteries were ligated by a suture (silk 5/0, Carlisle Laboratories, Farmers Branch, TX) and the right internal arteries were temporarily ligated using a microvascular clip (Fine Science Tool Inc., Foster City, CA). A small incision was made in the common carotid artery. A nylon filament, prepared from a fishing line (Stren Fishing Lines for 6 lb fish, Wilmington, DE) with its tip rounded by heating, was then inserted into the right common carotid artery and advanced into the internal carotid artery, approximately 18–20 mm from the point of the bifurcation of the internal and external arteries. A suture was tightly ligated around the filament to secure it in place. Anesthesia was then turned off and the animals were allowed to awaken. Two hours post-occlusion, the animals were re-anesthetized to allow removal of the filament and to allow reperfusion for 24 h.