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INTRACELLULAR MECHANISMS OF N-ACYLETHANOLAMINE-MEDIATED NEUROPROTECTION IN A RAT MODEL OF STROKE

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Abstract—N-acyl ethanolamines (NAEs) are endogenous lipids that are synthesized in response to tissue injury, including ischemia and stroke, suggesting they may exhibit neuroprotective properties. We hypothesized that NAE 16:0 (palmitoylethanolamine) is neuroprotective against ischemia-reperfusion injury in rats, a widely employed model of stroke, and that neuroprotection is mediated through an intracellular mechanism independent of known NAE receptors. Administration of NAE 16:0 from 30 min before to 2 h after stroke significantly reduced cortical and subcortical infarct volume, and correlated with an improvement of the neurological phenotype, as assessed by the neurological deficit score. We here show that NAE 16:0-mediated neuroprotection was independent of cannabinoid (CB1) and vanilloid (VR1) receptor activation, known NAE receptors on the plasma membrane, as determined by inclusion of specific inhibitors. The inclusion of an NAE uptake inhibitor (AM404), however, completely reversed NAE 16:0-mediated neuroprotection, suggesting that NAE 16:0s effects are through an intracellular mechanism. NAE 16:0 produced a significant reduction in the number of cells undergoing apoptosis and reversed ischemia-induced upregulation of several proteins, including inducible nitric oxide synthase and transcription factor NFκB. Our findings suggest that NAE 16:0-mediated neuroprotection is due to the reduction of neuronal apoptosis and inflammation in the brain.

Key words: neurodegeneration, neuroprotection, receptors, ischemia.

Stroke is the third leading cause of death in the USA with approximately 5.8 million current cases and approximately 780,000 new cases every year, making it a significant health problem (Lloyd-Jones et al., 2009). Potential neuroprotective treatment and intervention strategies have been tested over the last several years, but with limited success (White et al., 2000; Lo et al., 2003). Studies utilizing animal surgical models of stroke, such as ischemia-reperfusion injury induced by middle cerebral artery occlusion (MCAO), have been utilized to identify potential neuroprotective agents (Wen et al., 2004a,b; Hu et al., 2004; Green and Ashwood, 2005). Assessment of the efficacy of neuroprotective compounds against ischemia-reperfusion injury is an important *in vivo* experimental strategy relevant to ischemic stroke. Pharmacological agents used in these studies include anti-epileptic drugs (Calabresi et al., 2003), cyclooxygenase-2 (COX-2) inhibitors (Iadecola and Gorelick, 2005), estrogens (Wen et al., 2004a,b, 2007; Gibson et al., 2006), free radical scavengers (Green and Ashwood, 2005) and tissue plasminogen activator (Sheehan and Tsirka, 2005).

N-acyl ethanolamines (NAEs) are lipids present in the central nervous system (CNS) and involved in cellular signaling and a variety of physiological funtions (for review, see Fride, 2002). One particular NAE, NAE 20:4 (arachidonylethanolamine; AEA), is an endogenous ligand for cannabinoid (CB1) receptors (Fride, 2002) and activation of CB1 receptors protects cultured cortical neurons from excitotoxicity and oxidative stress (Marsicano et al., 2003; Kim et al., 2005, 2006; Shouman et al., 2006). AEA has since been demonstrated to have significant neuroprotective effects in experimental models for stroke (Sinor et al., 2000). Other targets of NAE action include vanilloid receptor 1 (VR1), protein kinases, nitric oxide synthase (NOS) and possibly ion channels (Di Marzo et al., 2002; Fride, 2002).

Of particular relevance, some NAE species and NAE precursor molecules, N-acyl phosphatidylethanolamines (NAPEs), are increased in response to multiple chemical and traumatic insults (Epps et al., 1979, 1980; Natarajan et al., 1986; Moesgaard et al., 1999; Schabitz et al., 2002; Berger et al., 2004), suggesting a role in cytoprotection. In addition, NAEs and NAPEs occur at higher levels in aged rat cortical neuron cultures and aged rats lose the ability to accumulate NAPE in the brain in response to ischemia (Moesgaard et al., 2000).

We previously hypothesized based on the structural and biochemical similarities to AEA that other NAEs that do not activate cannabinoid receptors are neuroprotective as well (Koulen and Chapman, 2006). Recently, the lesser characterized non-cannabinoid NAE 16:0 has been shown to reduce infarct volume in rats following ischemia/reperfusion (I/R) injury (Schomacher et al., 2008). However, the therapeutic window for NAE 16:0 and the mechanisms underlying this NAE 16:0-mediated protection remain unclear.

We here determined the therapeutic window of NAE 16:0-mediated neuroprotection against I/R injury in the MCAO model of ischemic stroke, and show that neuropro-

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Abbreviations: AEA, arachidonylethanolamine; ANOVA, analysis of variance; CB1/2, cannabinoid receptor type 1/2; CNS, central nervous system; COX-2, cyclooxygenase-2; CPZ, capsazepine; HE, hematoxylin and eosin; ICA, internal carotid artery; I/R, ischemia/reperfusion; iNOS, inducible nitric oxide synthase; MCAO, middle cerebral artery occlusion; NAE, N-acyl ethanolamine; NAPE, N-acyl ethanolamine-hydrolyzing phospholipase D; NFκB, nuclear factor kappa B; nNOS, neuronal nitric oxide synthase; PB, phosphate buffer; PBS, phosphate-buffered saline; tPA, tissue plasminogen activator; TdT, terminal deoxynucleotidyl transferase; TTC, 2,3,5-triphenyltetrazolium chloride; TUNEL, TdT-mediated dUTP Nick-end labeling; VR1, vanilloid receptor 1.

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tection occurs by an intracellular mechanism independent of CB1 and vanilloid receptor 1 (VR1) activation, by inducing a reduction in activity of apoptotic and neuroinflammatory pathways.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague Dawley rats weighing 300–325 g were purchased from Harlan Sprague Dawley (Indianapolis, IN, USA). The animals were acclimatized for 1 week in the animal care facility prior to use in present studies. Animals were maintained in a temperature-controlled room (22–25 °C) with 12 h light/dark cycles. Rats had free access to food and water. All animal experiments had been reviewed and approved by the Institutional Animal Care and Use Committee and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The authors attest that all efforts were made to minimize the number of animals and their suffering.

MCAO to induce focal cerebral ischemia

For MCA occlusion and reperfusion, an intraluminal filament model was used as described by us previously (Wen et al., 2004a,b, 2007). Briefly, animals were anesthetized with ketamine (60 mg/kg) and xylazine (10 mg/kg). For MCAO the left common carotid artery, left internal carotid artery (ICA) and the left external carotid artery were exposed, and a 3–0 monofilament nylon suture (Ethilon; Ethicon Inc., Sommerville, NJ, USA) was introduced into the ICA lumen through a puncture and was gently advanced until proper resistance was felt. After 90 min, the suture was gently withdrawn from the ICA and 24 h of reperfusion followed the MCAO. Rectal temperature of the animals was maintained at 37.0±0.5 °C throughout the surgery procedure using heating lamps. After recovery from anesthesia, animals were returned to their cages with free access to food and water.

Experimental groups

Animals were randomly divided into eight experimental groups for the present study: (1) control group of sham-operated rats with vehicle (ethyl alcohol) treatment, (2) control ischemic-reperfusion group (I/R, 90 min of MCAO followed by 24 h of reperfusion) with vehicle treatment, (3) I/R with NAE 16:0 (10 mg/kg i.p.) pretreatment, 6 h and 30 min before MCAO, (4) I/R+NAE 16:0 concomitant with MCAO, (5) I/R+NAE 16:0 at 2 h after MCAO, (6) I/R+NAE 16:0 at 3 h after MCAO, (7) AM251 (10 mg/kg i.p., 15 min before MCAO) + Capsazepine (CPZ) (10 mg/kg i.p., 10 min before MCAO) + I/R+NAE 16:0 at 0 h after MCAO, (8) AM404 (10 mg/kg i.p., 15 min before MCAO) + I/R+NAE 16:0 at 0 h after MCAO. All parameters were measured at 24 h after 90 min of MCAO. All compounds used in this study were administered i.p. at indicated times and dosed with ethyl alcohol as the vehicle control.

Measurement of cerebral infarct volume

For evaluation of infarct volume and other parameters an overdose of pentobarbital was given to rats prior to decapitation. Brains were quickly removed and placed in ice cold saline for 5 min. Seven coronal slices of 2 mm thickness were cut from each brain and incubated in 2% 2,3,5-triphenyltetrazolium chloride (TTC) for 15 min at 37 °C. In the TTC stained sections, pale-colored region indicated infarct area and colored region indicated viable areas. Stained brain sections were stored in 10% formalin and refrigerated at 4 °C for further processing and storage. Analysis for infarct volume in each brain slice was done using Simple PCI version 5.3.1 High Performance Imaging Software (Compix Inc., Cranberry Township, PA, USA).

Infarction volume was calculated with a previously described method to compensate for brain swelling in the ischemic hemisphere (Swanson et al., 1990). Briefly, the infarction area in each section was calculated by subtracting the non-infarct area of the ipsilateral side from the area of the contralateral side. Infarction areas on each section were summed and multiplied by section thickness to give the total infarction volume, which is expressed as a percentage of total volume.

Neurological evaluation

Neurological evaluation was performed before euthanasia at 24 h of reperfusion after MCAO for neurological deficits and scored as described elsewhere as follows: 0, no observable neurological deficit (normal); 1, failure to extend left forepaw on lifting the whole body by tail (mild); 2, circling to the contralateral side (moderate); 3, leaning to the contralateral side at rest or no spontaneous motor activity (severe) (Huang et al., 1994).

Hematoxylin and eosin stain

Hematoxylin and Eosin (HE) staining was conducted on animals sacrificed 24 h after sham surgery or I/R with and without NAE 16:0 treatment at the time of occlusion. Brains were flash frozen in liquid nitrogen, cryosectioned at 16 μm onto StarFrost adhesive slides (Mercedes Medical, Sarasota, FL, USA) and stored at −80 °C until staining. Sections were fixed in 4% (w/v) paraformaldehyde in phosphate puffer (pH 7.4) for 1 h at room temperature and washed two times for 7 min each with phosphate-buffered saline (PBS) containing 0.5% (w/v) glycine. HE staining was performed on these sections (Hu et al., 2004). Five microscopic fields in each section (from areas marked in Fig. 3C) were analyzed. Images were acquired using a Zeiss Axiovert 200 M inverted microscope equipped with an AxioCam MRC5 camera (Carl Zeiss, Thornwood, NY, USA).

TUNEL stain

DNA fragmentation was detected using terminal deoxynucleotidyl-transferase, recombinant enzyme mediated dUTP Nick-End Labeling (TUNEL) method with the Dead-End Fluorometric TUNEL assay according to the manufacturer's protocol (Promega, Madison, WI, USA). Animals were sacrificed at 24 h after post MCAO reperfusion, brains were removed, flash frozen on liquid nitrogen, cryosectioned at 16 μm onto adhesive slides and stored at −70 °C until further processing. Sections were fixed in 4% (w/v) paraformaldehyde in PB (pH 7.4) for 30 min at room temperature and washed two times for 7 min each with PBS containing 0.5% (w/v) glycine. Sections were then permeabilized for 10 min in 0.2% v/v Triton X-100 and then further processed for staining with Dead-End Fluorometric TUNEL. As a negative control, sections of ischemic brain were used after the standard procedures, but recombinant terminal deoxynucleotidyl-transferase (rTdT) was omitted. To counterstain with DAPI for detecting the total number of cells the sections were mounted in Prolong Gold containing DAPI (Invitrogen, Carlsbad, CA, USA). Total of five sections from each rat brain were taken and five microscopic fields (see Fig. 3C) in each section were acquired and analyzed. Optically sectioned images were acquired by using a Zeiss LSM 510 Duo META confocal laser-scanning microscope (Carl Zeiss, Thornwood, NY, USA). For counting cells stereologically in each field, the maximum projection of optically sectioned images was used. From each image, the total number of cells (nuclei stained with DAPI) and the number of TUNEL positive cells is counted and percentage of cells (TUNEL positive) in each image was calculated using Simple PCI version 5.3.1 software (Compix Inc., Cranberry Township, PA, USA).