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Lauroylethanolamide and linoleoylethanolamide improve functional outcome in a rodent model for stroke

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Abstract

Ischemic stroke is a significant health problem affecting over 6 million people in the United States alone. In addition to surgical and thrombolytic therapeutic strategies for stroke, neuroprotective therapies may offer additional benefit. *N*-acylethanolamines (NAEs) are signaling lipids whose synthesis is upregulated in response to ischemia, suggesting that they may be neuroprotective. To date only three NAEs, arachidonylethanolamide (NAE 20:4), palmitoylethanolamide (NAE 16:0) and oleoylethanolamide (NAE 18:1) have shown to exert neuroprotective effect in animal models for stroke. Here, we describe neuroprotective effects of the hitherto uncharacterized NAEs, lauroylethanolamide (NAE 12:0) and linoleoylethanolamide (NAE 18:2) in a middle cerebral artery occlusion model of stroke. Pretreatment with NAE 18:2 prior to ischemia/reperfusion (I/R) injury resulted in both significantly reduced cortical infarct volume and improved functional outcome as determined using the neurological deficit score. NAE 12:0 improved neurological deficits without a significant reduction lesion size. Our results suggest that NAEs, as a whole, provide neuroprotection during I/R injury and may have therapeutic benefit when used as complementary treatment with other therapies to improve stroke outcome.

Keywords

N-acylethanolamine; neuroprotection; middle cerebral artery occlusion; ischemia

Introduction

Stroke is a significant health problem and the third leading cause of death in the United States [31]. Ischemia-reperfusion (I/R) injury resulting from stroke leads to metabolic distress, oxidative stress and neuroinflammation, making it likely that multiple therapeutic intervention strategies may be needed for successful treatment [17]. Current therapeutic strategies for stroke, including thrombolytic drugs, such as tissue plasminogen activator (TPA) [45], offer great promise for treatment, but complimentary neuroprotective treatments are likely to provide a better outcome [18,41]. Animal models of stroke used in pre-clinical studies have led to the identification of a large number of neuroprotective compounds including anti-epileptic drugs, COX-2 inhibitors, inducible nitric oxide synthase (iNOS)

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inhibitors, minocycline, antioxidants and polyphenols [1,2,6-9,14]. Some of these putative neuroprotectants have been tested in human clinical trials, but they have yielded little positive outcome [47]. Nevertheless, neuroprotection studies utilizing animal models still provide new strategies for limiting stroke severity as they continue to offer translational potential for improving stroke outcome in the future [24].

N-acylethanolamines (NAEs) are endogenous bioactive lipids involved in numerous physiological functions in mammals, including neurotransmission, reproduction, inflammation, analgesia, appetite and cytoprotection and widely expressed in mammals [as reviewed in, 42]. The physiological functions of NAEs are largely unknown [42]. The classical known molecular targets of NAEs include the cannabinoid receptors, CB1R and CB2R, and the vanilloid receptor 1 (VR1) [12,13,29,30,33]. However, there is ample evidence for non-cannabinoid receptor and non-vanilloid receptor mediated action of NAEs [16,29,30,36,39,42]. Importantly, non-cannabinoid NAEs are synthesized in many tissues including the brain and they are elevated in response to a variety of stimuli such as excessive glutamate and ischemia [3,4,11,20,21,35,43,44].

Some NAEs exhibit neuroprotective properties in models of Alzheimer's disease, Parkinson's disease and ischemic stroke [15,16,25,28,44,48]. The NAE, arachidonylethanolamide NAE 20:4, is an endogenous cannabinoid (CB1) receptor ligand and is neuroprotective in experimental models of stroke [46]. Similar results have been obtained for the saturated NAE 16:0 [16,28,44]. However, we demonstrated that NAE 16:0 reduces infarct volume, functional neurological deficit and neuroinflammation in rats following I/R injury by a mechanism independent of CB1 nor vanilloid (VR1) receptors [16].

Given that all NAE species are synthesized and degraded by the same enzymatic pathway, we hypothesized that the NAEs lauroylethanolamide (NAE12:0) and linoleoylethanolamide (NAE18:2) may exhibit similar neuroprotective properties in an *in vivo* surgical model of ischemic stroke response to middle cerebral artery occlusion (MCAO).

NAE 18:2 does activate neither CB1R nor CB2R; however, it has been postulated to activate VR1 [37]. In contrast, NAE 12:0, a plant-derived compound [5,10], is not found in mammalian tissues and has no known function in neurons, but due to its structural similarity to NAE 16:0, we hypothesized it may play a cytoprotective role. Of particular relevance, NAE 12:0 is highly expressed in plant seeds, potently inhibits lipoxygenase activity [5,10,26], and has been shown to be an endogenous lipid mediator of protection against oxidative stress in cut-flower senescence [55].

We here show that both NAE12:0 and NAE18:2 reduced neurological deficits following I/R injury. In addition, NAE 18:2 also significantly reduced lesion size following MCAO. Our data support the hypothesis that NAEs may have potential pharmaceutical benefit as complementary neuroprotective therapy in stroke.

Materials and methods

Animals

Male Sprague Dawley rats (300-325 g) were obtained from Harlan Laboratories (Indianapolis, IN) and housed in the animal care facility for one week prior to experiments for acclimatization. Rats were kept in a temperature-controlled vivarium (22-25° C) with a 12-hour light dark cycle, and *ad libitum* access to food and water. All animal experiments had been reviewed and approved by the Institutional Animal Care and Use Committee.

Middle cerebral artery occlusion (MCAO) to induce focal cerebral ischemia

MCA occlusion and reperfusion was performed as described by us previously, using an intraluminal filament model [16,53]. Briefly, anesthesia was with Ketamine (60 mg/kg) and Xylazine (10 mg/kg). A 3-0 monofilament Ethilon nylon suture (Ethicon Inc., Sommerville, N.J., USA) was introduced through a puncture into the lumen of the left internal carotid artery, for 90 minutes. Reperfusion period was 24 hr post MCAO. After recovery from anesthesia, animals were returned to their cages with *ad libitum* access to food and water.

Experimental groups

Animals were randomly divided into four experimental groups (n=3 animals per group) for the present study: (1) control ischemic-reperfusion group (I/R, 90 minutes of MCAO followed by 24 hours of reperfusion) with vehicle treatment, (2) I/R with NAE 12:0 (10 mg/kg, i.p.) pretreatment, 6 hours and 30 minutes before MCAO, (3) I/R with NAE 18:2 (10 mg/kg, i.p.) pretreatment, 6 hours and 30 minutes before MCAO, (4) I/R with NAE 18:2 (20 mg/kg, i.p.) pretreatment, 6 hours and 30 minutes before MCAO. All parameters were measured at 24 hours after 90 minutes of MCAO. NAE12:0 was synthesized from ethanolamine and lauroylchloride (Nu-Check Prep, Elysian, MN), and purity determined by GC/MS [19]. NAE 18:2 was purchased from Cayman Chemical (Ann Arbor, MI, USA) at a purity of ≤98% and administered intraperitoneally at indicated times and dosed with ethyl alcohol as the vehicle control. Behavioral analyses and measurements of cerebral infarct volume were performed on the same animals.

Measurement of cerebral infarct volume

Animals were euthanized with an overdose of pentobarbital, decapitated and brains removed. Following 5 min. incubation in ice-cold saline, seven coronal slices (2 mm thickness) were cut from each brain and incubated in 2% 2,3,5- triphenyltetrazolium chloride (TTC) for 15 min at 37° C. Pale colored region indicated areas of infarction infarct whereas colored region indicated viable areas. Infarction volume was calculated with a previously described method to compensate for brain swelling in the ischemic hemisphere [16,49], using ImageJ software (National Institutes of Health, U.S.A.).

Neurological evaluation

Neurological evaluation was performed following 24 hours of reperfusion after MCAO. The method of neurological scoring was essentially as described previously [40]. We here used six criteria of neuromuscular function assigning a score based on the severity of the phenotype (the maximum score is given in parentheses, scores are in incremental steps of 0.5): 1. forelimb flexion (1.0); 2. torso twisting (1.0); 3. Lateral push (1.0); 4. hindlimb placement (1.0); 5. forelimb placement (1.0); 6. mobility (2.0). Hence, the maximum score using our modified version of the test is 7.0. The behavior assigned to each scoring criterion is described in detail in the original publication [40].

Statistical data analysis

Data are expressed as the mean ± standard error of mean. Statistical significance was determined by analysis of variance (ANOVA) with post-hoc Student-Newman-Keuls multiple comparison test, using SigmaStat 3.5 statistical software (Systat Software Inc., San Jose, CA) and a p value of less than 0.05 was considered significant.

Results

NAE 18:2 treatment reduces infarct volume following I/R injury

The neuroprotective properties of two NAEs were measured in rats subjected to MCAO followed by 24 h reperfusion. In vehicle treated rats, MCAO-induced infarct volume as determined by TTC staining in coronal brain slices (Fig. 1A) was 38.1 ± 3.2 % (Fig. 1B), was contributed by lesions in the cortical (23.3 ± 3.5 %) and subcortical (14.6 ± 2.5 %) brain areas (Fig. 1C). The extent of the lesion is consistent with previous reports by us and others [16,50,54]. Administration of NAE 12:0 (10 mg/kg) at 6 hr and 30 min prior to MCAO had no statistically significant effect on lesion size compared with vehicle (39.6 ± 2.4 %; n=3; P=0.86; Fig. 1A-C). In contrast, NAE 18:2 reduced infarct volume by 80.9 ± 17.7 % compared with vehicle control (to 7.3 ± 6.8 %; n=3; P<0.05; Fig. 1A-B), when administered at 20 mg/kg. A lower dose of 10 mg/kg reduced infarct volume by 52.5 ± 21.3 % (n=3, P<0.05; Fig. 1A-B).

A differential analysis of the cortical versus subcortical lesion size revealed an even more substantial reduction in the cortical areas by 94.2 ± 13.6 % (to 1.3 ± 3.2% compared with 23.3 ± 3.5 % for vehicle control; n=3, P<0.01; Fig. 1A,C). Despite a 90% and 69% reduction in stroke damage volume for the 10 mg/kg and 20 mg/kg dose, respectively, we did not identify statistically significant effects on subcortical lesion size upon administration with NAE 18:2 (Fig. 1A,C).

NAE 12:0 and NAE 18:2 reduce neurological deficits following MCAO

In order to assess whether the cytoprotective effects of NAEs on infarct volume are concomitant with improved functional outcome after I/R injury, we scored neurological deficits using a six-test scale [modified from 40].

Functional outcome after NAE 12:0 (10 mg/kg) administration improved significantly, reflected by improvement in all parameters tested (Fig. 2A). The overall neurological deficit score was reduced from 5.8 ± 0.2 for the vehicle-treated group to 3.8 ± 0.3 for the NAE 12:0 treated group (n=3; P<0.05; Fig. 2B).

Similarly, administration of NAE 18:2 reduced the functional deficits after I/R injury in a dose-dependent fashion. Score following 10 mg/kg administration was 3.7 ± 0.7 (n=3, P<0.05; Fig. 2B), whereas a dose of 20 mg/kg reduced neurological deficit score to 2.8 ± 0.4 (n=3, P<0.01, Fig. 2B).

Discussion

In the present study, we determined that the previously uncharacterized NAEs, NAE 12:0 and NAE 18:2, significantly improve functional outcome after I/R injury in our rodent model for stroke. NAE 18:2 furthermore significantly reduced cortical lesion volume when administered prior to MCAO. We previously showed that NAE 16:0 reduced cortical and subcortical lesion size and improved neurological deficit in the same MCAO stroke model [16,28]. Together, these data provide strong evidence that exogenously-applied NAEs are neuroprotective against ischemic injury.

NAEs 12:0 and 18:2 were selected for this study due to their structural similarity to other neuroprotective NAE species (such as NAE 16:0 and NAE 20:4), as well as their unknown function in neuronal injury. Furthermore, the differing acyl chain lengths and degree of saturation between NAE 12:0 and NAE 18:2 shed some light on whether these structural requirements are critical for neuroprotection.

Overall, the reduction in lesion size by NAE 18:2 was similar to that observed by us previously for NAE 16:0 [16] and significantly greater than reported effects on lesion size by NAE 18:1 [48] and NAE 20:4 [44]. Of note, only NAE 16:0 improved functional outcome after I/R injury, as reported by us and others [16,44], while NAE 20:4 showed no behavioral improvement [44]. The study investigating NAE 18:1 did not include a detailed behavioral assessment of neurological deficits [48].

Our previous work demonstrated that NAE 16:0 acts through an intracellular mechanism that is independent of CB1/CB2 receptors and VR1 [16]. Whilst we did not experimentally determine the mechanism of action for the NAE 12:0- and NAE 18:2-mediated neuroprotection in the present study, it is likely that the NAEs tested here also act through a cannabinoid-independent mechanism: NAE12:0 is not present in mammalian tissue and has no known molecular target [34]. Its abundance and protective effects in plants in response to oxidative stress suggest an intracellular mechanism other than the cannabinoid system, which is absent in plants [28,34]. NAE18:2 does not activate CB1/CB2 receptors, however, it has been reported to activate VR1 [37]. In neuroblastoma cells, activation of VR1 is linked to the initiation of apoptotic pathways [32]; similarly, VR1 down-regulation is neuroprotective in an *in vivo* excitotoxicity model [52], thus making it unlikely that VR1 activation is mediating the neuroprotective response of NAE18:2 in this study. Interestingly, the known cannabinoid NAE 20:4 did not improve functional outcome after MCAO [44], in contrast, NAE 16:0 significantly improved behavioral deficits while acting on targets other than cannabinoid receptors [16,44], further lending support to a cannabinoid receptor-independent mechanism of action for NAE 12:0 and NAE 18:2. Taken together, these data suggest that the mechanism of action of NAE neuroprotection in ischemic injury is through a yet unidentified mechanism not involving CB1 or VR1 receptors.

In this study, NAE12:0 did not significantly reduce overall lesion size as determined by estimating the infarct volume using TTC staining, while we observed a trend towards reduced lesion size in subcortical areas. The shorter acyl chain length of NAE 12:0, compared with those of NAE 16:0 and 18:2, may be subject to faster degradation and metabolism and thus be less efficient in reducing the lesion size following MCAO [51]. However, NAE 12:0 did statistically significantly reduce the neurological deficits associated with MCAO. It is known that lesion size may only exhibit a weak correlation with behavioral deficit measurements in animals experiencing MCAO [47]. Importantly, enhancement of functional recovery is considered a more biologically-relevant endpoint measure in pre-clinical studies, as it directly relates to the potential efficacy of neuroprotective compounds for clinical trials [41,47].

The perceived discrepancy between lesion size and functional outcome may be explained by neuroprotective effects in the stroke penumbra [22,38]. A variety of pathophysiological events take place in and around the stroke core (measured by our histochemical assay) and spread outward over time leading to delayed neuronal death [27]. Neurons in the stroke penumbra also exhibit cellular markers of apoptotic death several hours or even days after the initial ischemic event, unlike in the stroke core [38]. It is likely that NAE12:0 as well as NAE 18:2 administered at the lower dose of 10 mg/kg offer some protection in the stroke penumbra, underlying the improved neurological outcome. Unfortunately, the identification and measurement of the stroke penumbra can only be reliably determined using positron emission tomography (PET) scanning [22,23], and is beyond the scope of this study. Overall, neuroprotective therapies may be more useful for protecting against delayed neuronal death in the stroke penumbra some distance away from the core ischemic area [22,38]. This suggests that the stroke penumbra is a suitable target for neuroprotection in stroke, and that treatment with NAEs may provide therapeutic benefit for this aspect of stroke outcome.

We here show that the two NAE species, lauroylethanolamide (NAE 12:0) and linoleoylethanolamide (NAE 18:2), are neuroprotective in a rat model for stroke. The data presented here supports our hypothesis that NAEs act through an intracellular mechanism independent of the classic endocannabinoid pathways [16]. As such, NAEs prove useful candidates for neuroprotection in stroke that could complement existing thrombolytic and surgical approaches in stroke therapy.

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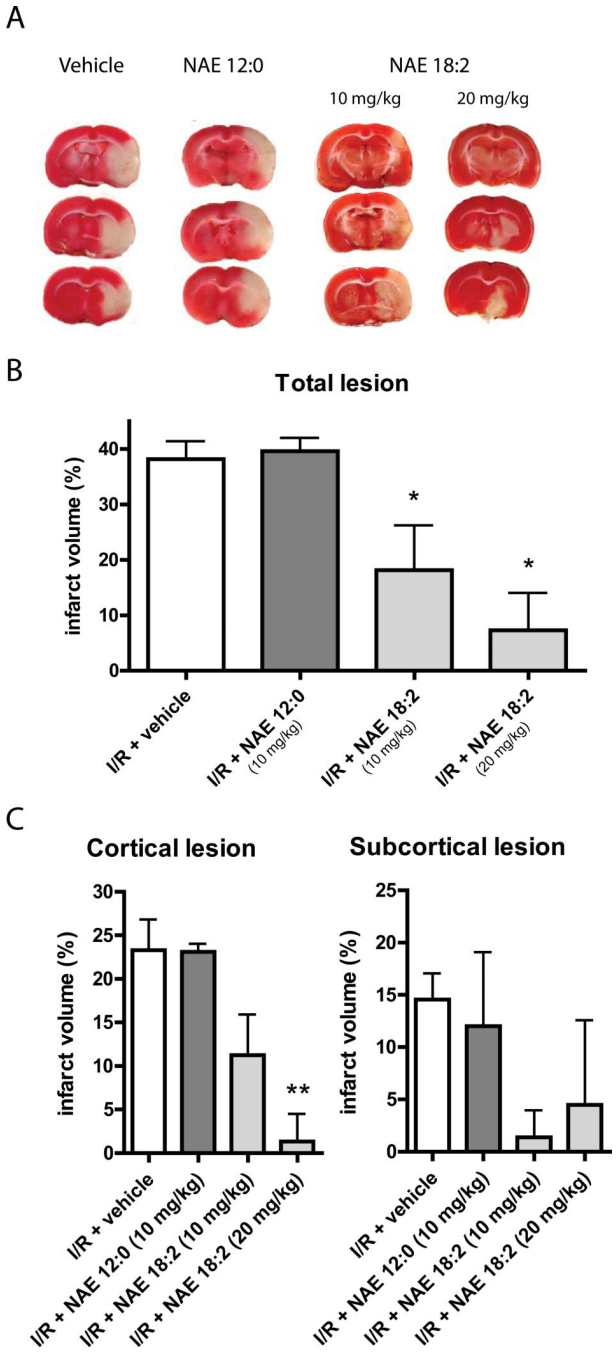

Figure 1. NAE 18:2 is neuroprotective against ischemic tissue damage and reduces MCAO-induced infarct volume

(**A**) Representative TTC-stained sections of rat brain following 90 min. MCAO/24 hr reperfusion (ischemia/reperfusion; I/R), treated with vehicle or NAEs (as indicated). Viable tissue stains red, whereas damaged ischemic brain tissue appears unstained/white. (**B**) Quantification of the volume of the ischemic lesion (infarct volume) revealed that NAE 12:0 had no significant effect on lesion size, whereas NAE 18:2 was neuroprotective when administered at both 10 mg/kg and 20 mg/kg. (**C**) The overall reduction in infarct volume by NAE 18:2 can be attributed to a significant reduction of cortical lesion size, whereas there

was no statistically significant subcortical effect. Data are shown as mean ± s.e.m. * $p<0.05$, ** $p<0.01$, compared with vehicle treated control group.

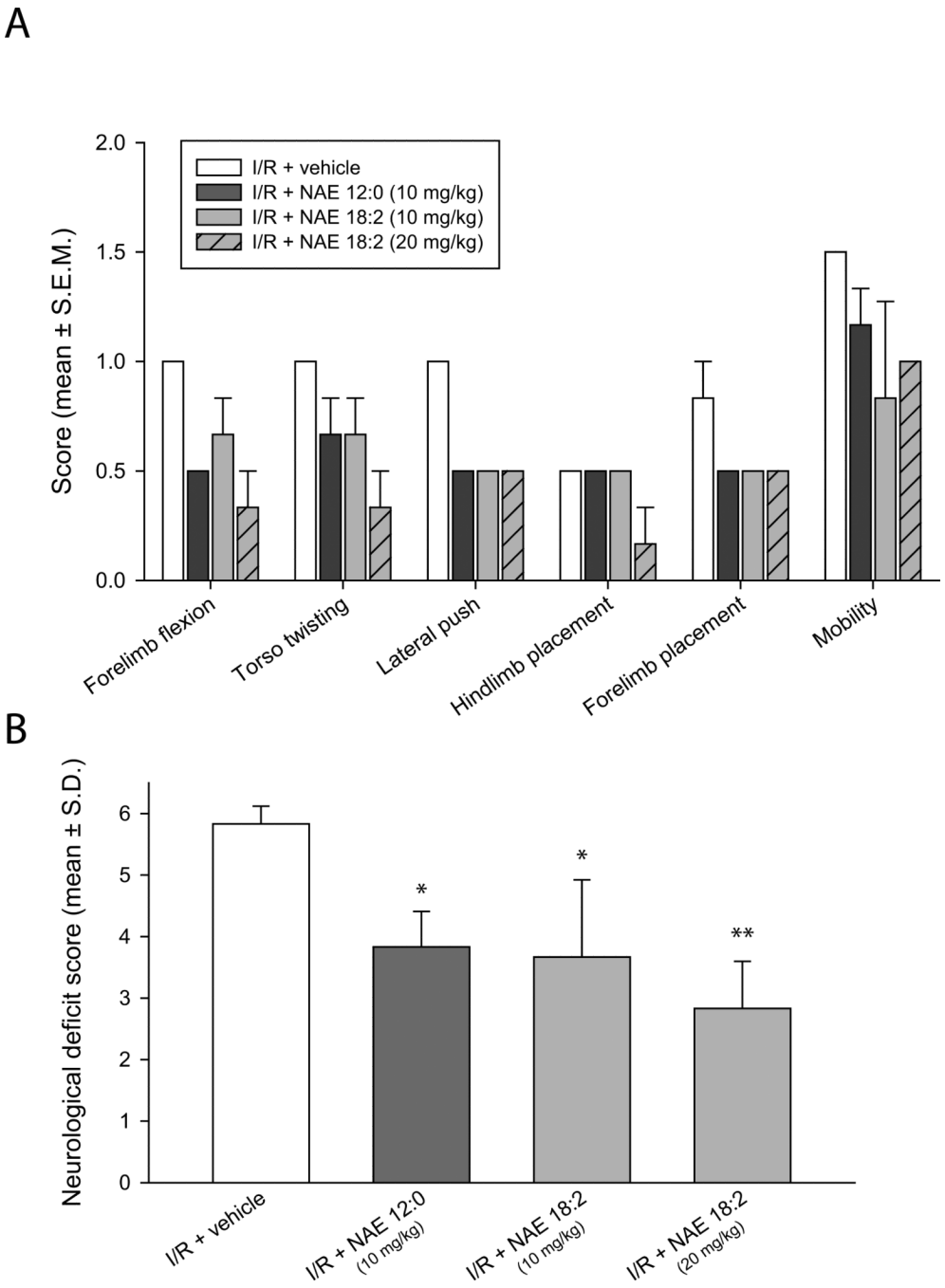

Figure 2. NAE 12:0 and NAE 18:2 improve neurological outcome after I/R injury

(**A**) I/R causes a moderate to severe neurological phenotype, as determined by analysis of the neurological deficit using the modified scale of Petullo et al. (1999). Both NAE 12:0 and NAE 18:2 reduced I/R-induced neurological deficits in all parameters tested. (**B**) Combined neurological deficit score was significantly reduced by both NAE 12:0 and NAE 18:2 prior to MCAO. Data are shown as mean ± s.e.m. * $p<0.05$, ** $p<0.01$, compared with vehicle treated control.