ELSEVIER

Neurobiology of Aging 24 (2003) 187–190

NEUROBIOLOGY OF AGING

www.elsevier.com/locate/neuaging

Brief communication

Alteration of nitric oxide synthase activity in young and aged apolipoprotein E-deficient mice

A. Law [a,b], S. Gauthier [a,b,c], R. Quirion [a,b,c,*]

[a] *Douglas Hospital Research Centre, 6875 LaSalle Boulevard, Verdun, Que., Canada H4H 1R3*
[b] *Department of Neurology and Neurosurgery, McGill University, Montréal, Que., Canada H3B 2A1*
[c] *McGill Centre for Studies in Aging, Verdun, Que., Canada H4H 1R3*

Received 5 October 2001; received in revised form 11 February 2002; accepted 23 April 2002

Abstract

Impairments in cognitive performance have been observed in aged apolipoprotein E (apoE)-deficient mice, and apoE ε4 allele is a risk factor in Alzheimer's disease (AD). The absence of apoE correlates with diminished antioxidative capacity in animals, and elevated cerebral oxidative stress has been observed in AD individuals carrying the ε4 alleles. Nitric oxide (NO) is a neurosignaling molecule that has significant roles in cognition. NO has also been implicated in neurodegenerative diseases due to its oxidative properties. The current study examined the possible relationship between apoE and nitric oxide synthase (NOS) by comparing hippocampal and cortical NOS activities in wild-type and apoE-knockout mice. Our results showed that apoE deficiency had no effect on NOS activity in these animals; however, aged animals uniformly exhibited significantly higher NOS activity levels. These findings suggest that increased NOS activity may contribute to cognitive impairments in aged wild-type and apoE-knockout mice due to excess accumulation of oxidative damages in areas involved in learning and memory.

Keywords: Aging; Alzheimer's disease; Apolipoprotein E; Beta-amyloid; Cholinergic; Cognitive impairments; Cortex; Hippocampus; Inducible nitric oxide synthase; Microglia; Neurotoxicity; Neuronal nitric oxide synthase; Nitric oxide; Oxidative stress; Transgenic mice

1. Introduction

Apolipoprotein E (apoE) is a product of a polymorphic gene located on chromosome 19 [29]. ApoE has three genotypes, namely ε2, ε3, and ε4, with ε3 being the most common coding sequence variant [24]. ApoE is one of the primary apolipoproteins found in the brain, carrying important roles in cholesterol metabolism and neural plasticity [17]. Recent evidence shows hyperlipidemia, increased loss of synaptic terminals, and neuronal cytoskeleton abnormalities in the cortices and hippocampi of aged apoE-knockout animals [18,32,35]. Cholinergic deficits have also been observed in some, but not all, of these mutants [7,10]. Behaviorally, these animals showed learning deficits in the water maze [18,23]. The frequency of the ε4 allele has been shown to be higher in Alzheimer's disease (AD) patients, and is positively correlated with age of onset as well as amyloid plaque (Aβ) density, but negatively with cholinergic system integrity [19,24].

Nitric oxide (NO) is produced by three isoforms of nitric oxide synthase (NOS). The neuronal (nNOS, type I) and endothelial (eNOS, type III) are constitutive isoenzymes [37], whereas the inducible isoform (iNOS, type II) would only show significant expression upon stimulation [37]. NO is a signal transducing molecule which possess neurophysiological roles in the central nervous system [4]. It is involved in neurodevelopment, and is the apparent retrograde messenger in the formation and maintenance of long-term potentiation and depression [15]. The overproduction of NO by nNOS or iNOS has been implicated in neurotoxicity [3,8], and in a number of neurodegenerative diseases which oxidative stress is likely to be involved, such as AD [15]. Although, it has been reported that apoE modulates microglia-mediated NO production [11], and that apoE ε4 and NO have been implicated in AD, knowledge regarding the relationships between apoE and NOS is limited. The aim of this study was to examine the possible alteration in NOS activity secondary to apoE deficiency, thereby providing information on the putative links between apoE and the NOS system.

* Corresponding author. Tel.: +1-514-762-3048; fax: +1-514-762-3034.
E-mail address: quirem@douglas.mcgill.ca (R. Quirion).

0197-4580/02/$ – see front matter © 2002 Elsevier Science Inc. All rights reserved.
PII: S0197-4580(02)00068-4

2. Methods

2.1. Animals

ApoE-knockout and c57/blk6 mice were purchased from Charles River Canada, St. Constant, Que. All animals were obtained at 2 months of age. These animals were housed four per cage until the time of experiment, maintained on a 12-h light–dark schedule (lights on and off at 08:00 and 20:00 h, respectively) at 22 °C, and had free access to food (Purina Lab Chow) and water. The health of the animals was regularly monitored during housing; animals which developed signs of neoplasm were removed from the study. At the time of the experiment, the young adult animals were 6-month-old, and the aged animals were between 20- and 22-month-old. All procedures were conducted in accordance with approved animal care guidelines of McGill University and the Canadian Council on Animal Care.

2.2. Sample preparation

Six each of young adult and aged apoE-knockout and six each of young adult and aged c57/blk6 wild-type mice were sacrificed and their brains were removed. All animals used in this study were males. Frontal cortical and hippocampal tissues were dissected from the brains, and were stored at −80 °C until use. On the day of the experiment, tissue samples were placed in lysate buffer and were homogenized by sonication.

2.3. NOS assay

All reagents used for the assays were purchased from Calbiochem, San Diego, CA. The linearity of reaction—expressed in correlation coefficient (CC)—toward each reagent used have been tested to ensure assay accuracy. All supplied reagents showed CC > 0.99. Tissue samples (300 mg each) were centrifuged at 1400 × *g* for 10 min and the supernatants were extracted for NOS assaying. The protein concentrations of the homogenized tissue samples were standardized. As a control measure, a portion of the samples were placed in a water bath at 100 °C for 10 min. NOS activity was inferred by measuring total nitrite concentration and subsequent colorimetric assay [21]. Triplicates of tissue samples and duplicates of nitrate standards were placed in a 96-well microtiter plate with the addition of assay buffer for volume adjustment. Freshly prepared 1 mM NADPH solution and nitrate reductase were then added to each well. These reagents were required for enzyme action and the conversion of nitrate to nitrite, respectively. The samples were then incubated for 1 h at room temperature, followed by the additions of a cofactor preparation (flavin adenine dinucleotide and pyruvate) and lactate dehydrogenase solution to all sample wells for excess NADPH removal. The samples were incubated for an additional 20 min at room temperature, and Griess reagents were added to all sample wells to convert the nitrite in solution to an azo product with maximum absorbance at a wavelength of 540 nm. The absorbances of samples were quantified at 540 nm by using a micro-plate reader (Bio-Tek Instruments Inc., Ville St.-Laurent, Qué.). The resultant NOS activity of each sample was then calculated from the nitrate standard curve.

2.4. Statistical analysis

One-way analysis of variance with Bonferroni's multiple comparison tests and unpaired Student's *t*-tests were used in statistical analysis to compare the obtained data for the apoE-knockout animals and wild-type controls, with $P < 0.05$ being considered as statistically significant. All data are presented as mean ± S.E.M.

3. Results

Hippocampal and cortical NOS activities of young and aged apoE-knockout mice were compared to age-matched wild-type controls.

In the hippocampus, aged mice showed significantly higher NOS activity than the young animals (Table 1). Within either the young adult or aged groups, no difference

Table 1
Nitric oxide synthase (NOS) activity in the hippocampi of young adult (6 months) and aged (20–22 months) wild-type and apolipoprotein E (apoE)-knockout mice ($n = 6$ for all animal groups)

	NOS activity level (nmol/min ml)	
	Wild-type	ApoE-knockout
Young adults	0.07 ± 0.01	0.06 ± 0.01
Aged	0.17 ± 0.03*	0.19 ± 0.03*

Aged animals showed significantly higher NOS activity than young adult mice. No significant difference in NOS activity was seen between wild-type and apoE-knockout animals in either the young adult or aged animal group. All data are presented as mean ± S.E.M.

* $P < 0.01$ compared to young adults of the same animal type.

Table 2
Nitric oxide synthase (NOS) activity in the cortices of young adult (6 months) and aged (20–22 months) wild-type and apolipoprotein E (apoE)-knockout mice ($n = 6$ for all animal groups)

	NOS activity level (nmol/min ml)	
	Wild-type	ApoE-knockout
Young adults	0.09 ± 0.01	0.08 ± 0.01
Aged	0.25 ± 0.04**	0.22 ± 0.06*

Aged animals exhibited significantly higher NOS activity levels than young adult animals. No significant difference in NOS activity was observed between wild-type and apoE-knockout animal groups. All data are presented as mean ± S.E.M.

* $P < 0.05$.

** $P < 0.01$ compared to young adults of the same animal type.

was observed between apoE-knockout animals and wild-type controls.

In the cortex, NOS activity levels were significantly higher in the aged animals (Table 2). The apoE-knockout mice showed no significant difference in NOS activity when compared to the wild-type mice in both the young adult and aged groups.

Tissue samples that were subjected to boiling prior to incubation did not show NOS activity (data not shown).

4. Discussion

The present findings have demonstrated that aged mice showed higher hippocampal and cortical NOS activities than young adult animals, and apoE-knockout animals exhibited similar NOS activity levels to wild-type mice in both the young adult and aged groups.

The presence of apoE ε4 allele is considered as a risk factor for AD [24]. In recent years, the possible links between NO and AD pathogenesis has become a research focus [16,31]. Literature has documented significant modulatory effects of apoE on eNOS and iNOS in the vascular system [5,28]. Vitek et al. and other laboratories have demonstrated apoE genotypic-dependent enhancement or downregulation of macrophagic and microglial activation and subsequent iNOS-mediated NO release in human and animal models [11,34]. However, there is little documentation regarding possible interactions between apoE and nNOS. The findings of the present study indicate that apoE deficiency does not have a significant effect on NOS activity in brain areas associated with cognitive behaviors. The NOS assaying method employed in the current study does not distinguish between NOS isoforms, hence the demonstrated NOS activity could be attributed to nNOS or iNOS. However, under physiological conditions, NO production in the brain is mediated primarily by nNOS, with a minimal contribution of iNOS expression and activity [37]. Hence, the results obtained here using healthy young adult mice suggest that apoE deficiency does not significantly affect nNOS functioning. Nonetheless, the current findings do not preclude possible interactions between apoE and nNOS. Meda et al. have demonstrated the relationship between Aβ and NO release [20], and our laboratories as well as others have shown that Aβ can stimulate nNOS-mediated NO release [13,36]. Interestingly, mutual modulating effects between Aβ and apoE have been documented [22]. Whether apoE can directly or indirectly influence nNOS activity in a genotype-dependent manner is unclear. Moreover, since eNOS and nNOS share structural and functional similarities, and Kauser et al. have shown that apoE deficiency affects eNOS-mediated NO release in atherosclerosis [9], the possibility that apoE could also alter nNOS functioning should not be ignored. Further studies involving the use of transgenic animals expressing one specific apoE allelic type—ε3 or ε4 in particular—would likely to be useful in providing greater details on the association between apoE and nNOS.

We have previously shown that the number of nNOS transcripts was significantly elevated in the hippocampi and cortices of aged cognitively-impaired Long–Evans rats [12]. More recently, we demonstrated an age-associated nNOS expression decline and elevated iNOS expression in aged Long–Evans rats [14]. The findings in this study—with aged animals exhibiting increased hippocampal and cortical NOS activity regardless of their genetic backgrounds—may therefore, reflect enhanced iNOS activity in these aged mice, which is in accordance to previous studies which showed increased iNOS expression and activity in aging animals [30,33]. Elevated iNOS-mediated NO release would heighten oxidative stress, and cognitive impairments would result due to the accumulation of oxidative damages [6].

Interestingly, it has been reported that levels of antioxidants such as alpha-tocopherol and superoxide dismutase were decreased in apoE-deficient mice [27]. Moreover, apoE3, but not apoE4, has been shown to block iNOS-mediated NO release in microglia [2], and human data have also shown apoE genotype-dependent variations regarding oxidative defense in AD [25,26]. Aβ is an AD histopathological hallmark, and this peptide has been shown to induce iNOS-mediated NO release in the AD brain [1,8]. Hence, the relationships between apoE, NOS, and oxidative stress may carry significant implications in AD pathogenesis, and further investigation on these aspects is warranted.

Taken together, the present findings suggest that apoE likely does not have a major influence on nNOS functioning; however, future studies with animals expressing specific apoE genotype would be of interest.

Acknowledgments

This study was supported by research grants from Canadian Institutes for Health Research (CIHR) and the Alzheimer Society of Canada.

References

[1] Akama KT, Albanese C, Pestell RG, Van Eldik LJ. Amyloid beta-peptide stimulates nitric oxide production in astrocytes through an NFkappaB-dependent mechanism. Proc Natl Acad Sci USA 1998;95:5795–800.
[2] Barger SW, Harmon AD. Microglial activation by Alzheimer amyloid precursor protein and modulation by apolipoprotein E. Nature 1997;388:878–81.
[3] Dawson DA. Nitric oxide and focal cerebral ischemia: multiplicity of actions and diverse outcome. Cerebrovas Brain Meta Rev 1994;6:299–324.
[4] Dawson TM, Dawson VL, Snyder SH. A novel neuronal messenger in brain: the free radical, nitric oxide. Ann Neurol 1992;32:297–311.
[5] Detmers PA, Hernandez M, Mudgett J, Hassing H, Burton C, Mundt S, et al. Deficiency in inducible nitric oxide synthase results in reduced atherosclerosis in apolipoprotein E-deficient mice. J Immunol 2000;165:3430–5.

[6] Forster MJ, Dubey A, Dawson KM, Stutts WA, Lal H, Sohal RS. Age-related losses of cognitive function and motor skills in mice are associated with oxidative protein damage in the brain. Proc Natl Acad Sci USA 1996;93:4765–9.
[7] Gordon I, Grauer E, Genis I, Sehayek E, Michaelson DM. Memory deficits and cholinergic impairments in apolipoprotein E-deficient mice. Neurosci Lett 1995;199:1–4.
[8] Ii M, Sunamoto M, Ohnishi K, Ichimori Y. Beta-amyloid protein-dependent nitric oxide production from microglial cells and neurotoxicity. Brain Res 1996;720:93–100.
[9] Kauser K, da CV, Fitch R, Mallari C, Rubanyi GM. Role of endogenous nitric oxide in progression of atherosclerosis in apolipoprotein E-deficient mice. Am J Physiol Heart Circ Physiol 2000;278:H1679–85.
[10] Krzywkowski P, Ghribi O, Gagne J, Chabot C, Kar S, Rochford J, et al. Cholinergic systems and long-term potentiation in memory-impaired apolipoprotein E-deficient mice. Neuroscience 1999;92: 1273–86.
[11] Laskowitz DT, Matthew WD, Bennett ER, Schmechel D, Herbstreith MH, Goel S, et al. Endogenous apolipoprotein E suppresses LPS-stimulated microglial nitric oxide production. Neuroreport 1998;9:615–8.
[12] Law A, Dore S, Blackshaw S, Gauthier S, Quirion R. Alteration of expression levels of neuronal nitric oxide synthase and haem oxygenase-2 messenger RNA in the hippocampi and cortices of young adult and aged cognitively unimpaired and impaired Long–Evans rats. Neuroscience 2000;100:769–75.
[13] Law A, Gauthier S, Quirion R. Neuroprotective and neurorescuing effects of isoform-specific nitric oxide synthase inhibitors, nitric oxide scavenger, and antioxidant against beta-amyloid toxicity. Br J Pharmacol 2001;133:1114–24.
[14] Law A, O'Donnell J, Gauthier S, Quirion R. Neuronal and inducible nitric oxide synthase expressions and activities in the hippocampi and cortices of young adult, aged cognitively-unimpaired, and -impaired Long-Evans rats. Neuroscience 2002;112:267–75.
[15] Law A, Gauthier S, Quirion R. Say, NO to Alzheimer's disease: the putative links between nitric oxide and dementia of the Alzheimer's type. Brain Res Rev 2001;35:73–96.
[16] Lee SC, Zhao ML, Hirano A, Dickson DW. Inducible nitric oxide synthase immunoreactivity in the Alzheimer disease hippocampus: association with Hirano bodies, neurofibrillary tangles, and senile plaques. J Neuropathol Exp Neurol 1999;58:1163–9.
[17] Mahley RW, Nathan BP, Pitas RE. Apolipoprotein E—structure, function, and possible roles in Alzheimer's disease. Ann NY Acad Sci 1996;777:139–45.
[18] Masliah E, Samuel W, Veinbergs I, Mallory M, Mante M, Saitoh T. Neurodegeneration and cognitive impairment in apoE-deficient mice is ameliorated by infusion of recombinant apoE. Brain Res 1997;751:307–14.
[19] McGeer PL, Walker DG, Pitas RE, Mahley RW, McGeer EG. Apolipoprotein E4 (ApoE4) but not ApoE3 or ApoE2 potentiates beta-amyloid protein activation of complement in vitro. Brain Res 1997;749:135–8.
[20] Meda L, Cassatella MA, Szendrei GI, Otvos Jr L, Baron P, Villalba M, et al. Activation of microglial cells by beta-amyloid protein and interferon-gamma. Nature 1995;374:647–50.
[21] Nims RW, Cook JC, Krishna MC, Christodoulou D, Poore CM, Miles AM, et al. Colorimetric assays for nitric oxide and nitrogen oxide species formed from nitric oxide stock solutions and donor compounds. Methods Enzymol 1996;268:93–105.
[22] Ohm TG, Hamker U, Cedazo-Minguez A, Rockl W, Scharnagl H, Marz W, et al. Apolipoprotein E and beta A4-amyloid: signals and effects. Biochem Soc Symp 2001;121–9.
[23] Oitzl MS, Mulder M, Lucassen PJ, Havekes LM, Grootendorst J, de Kloet ER. Severe learning deficits in apolipoprotein E-knockout mice in a water maze task. Brain Res 1997;752:189–96.
[24] Poirier J, Davignon J, Bouthillier D, Kogan S, Bertrand P, Gauthier S. Apolipoprotein E polymorphism and Alzheimer's disease. Lancet 1993;342:697–9.
[25] Ramassamy C, Averill D, Beffert U, Bastianetto S, Theroux L, Lussier-Cacan S, et al. Oxidative damage and protection by antioxidants in the frontal cortex of Alzheimer's disease is related to the apolipoprotein E genotype. Free Radic Biol Med 1999;27:544–53.
[26] Ramassamy C, Averill D, Beffert U, Theroux L, Lussier-Cacan S, Cohn JS, et al. Oxidative insults are associated with apolipoprotein E genotype in Alzheimer's disease brain. Neurobiol Dis 2000;7:23–37.
[27] Ramassamy C, Krzywkowski P, Averill D, Lussier-Cacan S, Theroux L, Christen Y, et al. Impact of apoE deficiency on oxidative insults and antioxidant levels in the brain. Mol Brain Res 2001;86:76–83.
[28] Riddell DR, Vinogradov DV, Stannard AK, Chadwick N, Owen JS. Identification and characterization of LRP8 (apoER2) in human blood platelets. J Lipid Res 1999;40:1925–30.
[29] Roses AD, Pericak-Vance MA, Yamaoka L, Haynes C, Speer M, Gaskell P, et al. Linkage studies in familial Alzheimer's disease. Prog Clin Biol Res 1989;317:201–15.
[30] Sloane JA, Hollander W, Moss MB, Rosene DL, Abraham CR. Increased microglial activation and protein nitration in white matter of the aging monkey. Neurobiol Aging 1999;20:395–405.
[31] Thorns V, Hansen L, Masliah E. nNOS expressing neurons in the entorhinal cortex and hippocampus are affected in patients with Alzheimer's disease. Exp Neurol 1998;150:14–20.
[32] Veinbergs I, Mallory M, Mante M, Rockenstein E, Gilbert JR, Masliah E. Differential neurotrophic effects of apolipoprotein E in aged transgenic mice. Neurosci Lett 1999;265:218–22.
[33] Vernet D, Bonavera JJ, Swerdloff RS, Gonzalez-Cadavid NF, Wang C. Spontaneous expression of inducible nitric oxide synthase in the hypothalamus and other brain regions of aging rats. Endocrinology 1998;139:3254–61.
[34] Vitek MP, Snell J, Dawson H, Colton CA. Modulation of nitric oxide production in human macrophages by apolipoprotein E and amyloid beta-peptide. Biochem Biophys Res Commun 1997;240:391–4.
[35] Xu PT, Schmechel D, Rothrock-Christian T, Burkhart DS, Qiu HL, Popko B, et al. Human apolipoprotein E2, E3, and E4 isoform-specific transgenic mice: human-like pattern of glial and neuronal immunoreactivity in central nervous system not observed in wild-type mice. Neurobiol Dis 1996;3:229–45.
[36] Yang SN, Hsieh WY, Liu DD, Tsai LM, Tung CS, Wu JN. The involvement of nitric oxide in synergistic neuronal damage induced by beta-amyloid peptide and glutamate in primary rat cortical neurons. Chin J Physiol 1998;41:175–9.
[37] Zhang J, Snyder SH. Nitric oxide in the nervous system. Ann Rev Pharmacol Toxicol 1995;35:213–33.