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Apolipoprotein E Isotype-dependent Modulation of microRNA-146a in plasma and brain

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Abstract

The apolipoprotein E gene allele e4 (apoE4) is a prevalent genetic risk factor for Alzheimer's disease (AD) that can modulate systemic and central inflammation, independent of amyloid accumulation. Although disruption of innate immune toll receptor (TLR) signaling is modulated by apoE and observed in AD, ApoE isotype specific effects remain poorly understood. Therefore, we examined the effect of apoE isotype on brain levels of major regulators of TLR signaling including miR146a, a microRNA enriched in the brain. We used 6 month-old apoE3 or apoE4 targeted replacement mice with and without mutant familial AD transgenes. ApoE4 reduced levels of miR146a compared to apoE3, both in the brain (29%; $p < 0.0001$) and plasma (47%; $p < 0.05$), which correlated with each other ($r^2=0.74$; $p < 0.05$). The presence of 5xFAD transgenes increased brain miR146a in both apoE3 (E3FAD) and apoE4 (E4FAD) mice; however, miR146a levels in E4FAD mice remained lower than in E3FAD mice (62%; $p < 0.05$), despite increased amyloid and inflammation. Supporting these observations, apoE4 brains showed increased expression of interleukin receptor associated kinase-1 (160%; $p < 0.05$) (normally downregulated by miR146) that inversely correlated with miR146a levels ($r^2=0.637$; $p < 0.0001$). Reduced negative feedback of toll-like receptor signaling (by miRNA146a) can explain early-life hypersensitivity to innate immune stimuli (including Aβ) in apoE4 carriers. Thus, apoE4 causes early dysregulation of a central controller of the innate immune system both centrally and systemically. This defect persists with FAD pathology and may be relevant to ApoE4 AD risk.

Keywords

microRNA-146a; inflammation; transgenic mice; gene regulation; ApoE3; ApoE4; Toll-Like Receptor Signaling; interleukin receptor associated kinase-1 (IRAK-1); Alzheimer's disease

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Conflicts of Interest: None

Introduction

The apolipoprotein E gene isotype E4 (apoE4) is the most prevalent major genetic risk factor for developing Alzheimer's disease (AD), accelerating the age of onset of AD, but also in young adults causing prodromal AD-related phenotypes. The mechanisms remain elusive, but include inflammation-related pathways such as dysregulation of the innate immune system, as several new AD genes are specific to innate immunity. Although apoE is generally anti-inflammatory [2], apoE4 is deficient in this activity compared to the apoE3 and E2 isotypes and can exacerbate neurodegeneration associated with microglial innate immune response [1]. Relative to ApoE3, ApoE4 increases inflammatory responses to, while impeding recovery from inflammatory stimuli (e.g. LPS and Aβ) in both peripheral innate immune cells and in the brain and cultured glia; this was mediated by via hyperstimulation of toll-like receptor (TLR)-4 signaling, which activates NFκB [1,3–7].

Similar pro-inflammatory effects of ApoE4 have been reported in humans *in vivo* and this may be relevant to AD risk because non-steroidal anti-inflammatory drug (NSAID) use, repeatedly reported to reduce AD risk (when used for at least two year and prior to onset), was predominantly in apoE4 carriers when examined for genotype effects [8].

ApoE4's mechanisms for increasing inflammatory responses could involve defective inhibitory feedback that prevents resolution. MicroRNAs play important role in responding to and turning off the inflammatory response. MicroRNAs are ~22 nucleotide RNAs that post-transcriptionally repress gene mRNAs by base pairing leading to mRNA degradation [9]. Among the thousands of identified miRNAs, a few are identified as regulating inflammatory responses in brain, particularly miR146a (reviewed in [10,11]). Specifically, the inflammation associated activation and translocation of the transcription factor NFκB require increased miR146a expression to resolve the inflammatory response. With TLR signaling and other inflammatory responses, NFκB normally increases miR146a expression, and miR146a downregulates NFκB p65 and Interleukin-1 Receptor-Associated Kinase 1 (IRAK1), an essential TLR4 and IL1R signal transduction pathway mediator, as a normal feedback mechanism to control the inflammatory response, to prevent uncontrolled inflammation and keep the immune response "in check" (reviewed in [10–13]). The proinflammatory stimuli LPS or Aβ will normally activate this inhibitory feedback loop by downregulating downstream IRAK1 [13], but feedback inhibition becomes dysfunctional with age, for example in peripheral macrophages [14]. MiR146a is also a target of the inflammation hub regulator, transcription factor PU.1, identified as a hub of AD-modified gene expression [15]. AD patients show increased miR-146a in brain [16] and similarly, several mouse models of AD, including the 5xFAD, also show increased brain miR146a, associated with pathology [17]. Mouse apoE increases transcription of miR146a gene in peripheral macrophages, at least in part, by increasing PU.1 mRNA and protein levels to suppress pro-inflammatory responses [2]. However, ApoE isotype differences and early changes in young adult life are unknown. To fill these knowledge gaps, we sought to identify apoE4 isotype-specific effects on miR146a levels and activity on its target IRAK1 in the brain and plasma of 6 month-old apoE transgenic mice, and whether effects were dependent or independent of amyloid.

Methods

Animals

This study used apoE3 and apoE4 targeted replacement mice [18] in the presence or absence of the 5xFAD transgenes [19]. Mice were 6–8 months old with equal male and female sexes. Group sizes (n) were apoE3 (10), apoE4 (10), E3FAD (7) and E4FAD (6). Mice were euthanized with a lethal dose (100 mg/kg) of pentobarbitol, and during intracardiac perfusion with non-fixative buffer, atrial blood was collected and plasma prepared. After intracardiac perfusion, the mouse was decapitated and the brain removed and dissected into regions including cortex, which was snap frozen and powderized for RNA preparation.

RNA preparation and gene expression measurement

Total RNA from brain cortex and plasma was prepared using the MirVana miRNA Isolation Kit (Ambion). Brain RNA was quantified using a Nanodrop spectrophotometer. For measures of mRNAs, 1 μg of total brain RNA was reverse-transcribed, then PCR amplified using gene-specific Taqman primers and probes (Life Technologies); levels of mRNA for PU.1 and IRAK1 were measured and were normalized to levels of GAPDH mRNA in the same RNA samples. For measures of miRNA, 10ng total RNA was reverse-transcribed using gene specific primers then PCR amplified with gene specific primers and probes (Taqman miRNA gene specific reagents); levels of miR146a were measured and were normalized to the levels of U6snRNA in the same samples.

For measures of miRNA in plasma, plasma was pooled from pairs of mice that had similar brain levels of miR146a, for a total volume of 130 μL plasma, and total RNA was isolated using the MirVana Kit. For downstream normalization of RNA recovery and yield, pure Cel-miR39 (Qiagen) was spiked into the Lysis Buffer to a final concentration of 6 pM, and 1300 μL of this Lysis Buffer was used for each 130 μL plasma sample; total RNA was eluted into 100 μL H_2O; 9 μL of this total RNA was used for each gene RNA measure. RNA recovery and yield was corrected by normalization to the level of Cel-miR39 in each sample; after this normalization, levels of miR146a or miR124 were normalized to U6snRNA in each sample. Normalizations were by the delta-delta Ct method [20], and levels in apoE3 samples were set to 1.0.

Statistics

SPSS software (V 23, IBM) was use for statistical analysis. 2×2 ANOVA was performed to evaluate apoE isotype and FAD effects and interactions on miRNAa146a, and Fishers LSD post hoc analysis was used to assess differences between pre-planned comparisons. T test was used to determine apoE isotype effects on brain IRAK1, PU.1 and plasma miRNA146a levels.

Results

Brain miR146a levels

2×2 Anova demonstrated significant effects of ApoE ($P < 0.001$) and FAD ($P = 0.002$) but no effect of sex and no interaction between ApoE and FAD. However there was an

interaction between sex and FAD (P =0.024), with female ApoE3 mice showing the largest FAD-associated increase in miRNA146a.

Data showed that ApoE4 mice expressed only 29% of the level of miR146a expressed in E3 mice ($p < 0.0001$). Similarly, E4FAD mice expressed less (62% of the level of miR146a) than E3FAD mice ($p < 0.05$; Fig. 1). Increased miR146a in both these strains with 5xFAD is consistent with the reported large increase in miR146a in the parent strain 5xFAD with wildtype mouse apoE, seen at 3 months of age when the FAD pathology is developed but not at 1 month of age in the absence of pathology [17].

Pu.1 and IRAK1 mRNA levels and correlation with miR146a levels in brain

PU.1 and IRAK1 mRNA levels normalized to levels of GAPDH mRNA in the same samples are shown in Fig. 2a.

Although mean PU.1 levels in apoE4 were 15% less than that of apoE3 mice, this was not statistically significant. This suggests reduced PU.1 (by itself) cannot account for the robust 71% drop in miR146a in apoE4. Since, as reviewed above, IRAK1 mRNA is a well-established target for miR146a regulation, we measured IRAK1 mRNA and found that it was elevated in E4 by 60% ($p < 0.05$; Fig 2a). IRAK1 mRNA levels were correlated with miR146a levels in the same samples ($r^2=0.637$; $p < 0.0001$; Fig. 2b).

Levels of miR146a in mouse plasma and correlation with levels in brain

Plasma was pooled from pairs of mice with similar brain levels of miR146a, n=4 or 5 pools.

Plasma miR146a levels in E4 mice were 47% that of E3 ($p < 0.05$) (Fig. 3a). Plasma miR146a correlated with brain miR146a (the average brain level for each pair of mice used to make the plasma pools); Pearson correlation $r^2=0.74$; $p < 0.05$ (Fig. 3b). Important controls for measuring plasma miRNA included normalization to an exogenous miRNA spike in control for normalizing total RNA recovery (using Cel-miR39 spike) and normalization to an endogenous U6snRNA to control for genotype-dependent variation in total plasma RNA; further, potential biological variation in total miRNA from plasma (apoE3 versus apoE4) was ruled out by showing that another miRNA, miR124, did not differ between apoE3 and apoE4 plasma (miR124 levels: E3=2.4+/−0.62SEM; E4=2.6+/−1.57SEM; $p>0.05$).

Discussion

There is increased expression of miR146a in brains of AD patients [16] and AD models [17] but the role of miR146a in AD pathogenesis and its modulation by apoE isotype were unknown. This study supports evidence that FAD increases miR146a expression in the mouse brain, emulating increases seen in patients, and for the first time identifies an apoE isotype specific response that can be independent of the stimulatory effects of FAD. Compared to young apoE3 mice, age-matched apoE4 mice show markedly lower expression of miR146a in the brain, corresponding to lower expression in plasma. This E4-specific deficit in miR146a, one of the main CNS regulators of inflammation, suggests that apoE4 perturbs a major inhibitory feedback loop. Insufficient miR146a obstructs negative feedback

regulation of inflammation, resulting in chronic inflammation, generally observed in brains of apoE4 carriers. This would be consistent with the hypothesis that miR146a defects may be central to the known increased sensitivity of E4-carriers to TLR and other pro-inflammatory NFκB-driven pathways.

Our data support the idea that E4-specific defects in miR146a responses will be present in the prodromal stage where it contributes to disease risk. In patients, it is unclear if the defects may eventually be masked after onset of the disease, because there is a disproportionally higher risk of amyloid deposition (which increases miR146a). Although a lower sensitivity to Aβ induction of miR146a may be difficult to detect after disease onset in humans, in the well-controlled mouse models, we show that even though miR146a levels are increased by FAD, levels in E4FAD are still lower than E3FAD. This suggests that E4FAD mice manifest a striking defect in miR146a regulation, since this occurs despite increased amyloid deposition [19] and aberrant neuroinflammation, driven by amyloid deposits that stimulate IL-1β and TLR signaling, which is already high in young E4FAD mice [6]. Our data support the hypothesis that increased miR146a in AD and AD model mice brain is a beneficial response that is blunted in apoE4 carriers, consistent with increased TLR signaling. Further, apoE knockout mice, despite increased brain NFκB activation, have unexpectedly reduced levels of miR146a, in part because mouse apoE stimulates miR146a transcription [2]. Although the presence of E4 results in amplification of amyloid deposition relative to E3, recent findings raise questions about amyloid-independent roles of E4 that may contribute directly to innate immune dysregulation. Our data on dramatically reduced miR146a in apoE4 mouse brain and plasma in the absence of the 5xFAD transgenes and amyloid provide an amyloid-independent mechanism of apoE4 on both central and systemic inflammation.

The miR146a gene expression is under complex transcriptional control, including by PU.1, which can be upregulated by ApoE in peripheral innate immune cells [2]. However, since neither the levels of PU.1 mRNA, nor its upstream regulator miR124, showed apoE-isotype dependent differences (data not shown), reduced PU.1 is unlikely to explain the reduced central miR146a in apoE4.

miR146a regulates the inflammatory response not only by regulating NFκB p65 mRNA, but by regulating the mediators of inflammatory signaling through TLR pathways, most compellingly the TLR4 pathway mediators, IRAK-1 and TFAF6. We show that the severely reduced miR146a in apoE4 mice is correlated with increased IRAK1 mRNA. This differential regulation of TLR4 pathway IRAK-1 by apoE4 may explain the well-established increased sensitivity of apoE4 to inflammatory stimuli signaling through TLR4 (see Introduction). The miR146a transcription promoter has been extensively studied, and is a model system for NFκB regulation of gene expression. Thus, even though baseline NFκB activation and miR146a are both elevated with the chronic inflammation observed in peripheral tissues and brain with aging and AD, our results demonstrate that in young apoE4 mice (similar to in apoE-knockout mice [2]), miR146a responses are markedly blunted. This baseline deficit in negative or anti-inflammatory feedback may accelerate chronic inflammation, microglial priming and functional alterations contributing to aging and AD. ApoE2 remains one of the few known longevity genes, with ApoE4 showing an adverse

effect [21] and the other major AD risk factor, aging, also involving a loss of this negative miR146a feedback brake on innate immune hyperactivation [14]. Consistent with a possible causal role in ApoE4 as an AD risk factor, a rare polymorphism in the coding region of miR146a results in reduced miR146a levels and has been recently associated with increased AD risk [22]. Reduced miR146a negative feedback and increased pro-inflammatory drive with ApoE4 may contribute to ApoE4 effects accelerating AD and aging.

We also found that miR146a was reduced in young E4 mouse plasma similar to and correlated with levels in brain. This may help explain increased peripheral pro-inflammatory mediators with ApoE4 [5]. We previously reported that, at relatively young ages in human FAD mutation carriers and non-carriers, apoE4 carriers showed increased inflammatory plasma markers, including several interleukins and Complement factor H (CFH) [23]. CFH is an innate immune protein with AD risk-related polymorphisms that is upregulated in AD plasma [24]. Since CFH mRNA is downregulated by miR146a, miR146a reductions in young ApoE4-carriers can explain upregulation of CFH with ApoE4. Further studies are needed to determine whether monitoring apoE4-related inflammatory effects in blood by miR146a could provide a peripheral biomarker relevant to apoE4-dependent CNS pathology [25]. A peripheral biomarker central to ApoE4's pro-inflammatory activity could aid in the development of preventive therapeutic interventions that mitigate ApoE4's pro-inflammatory impact during the more easily modifiable and prolonged prodromal stages of AD.

Acknowledgments

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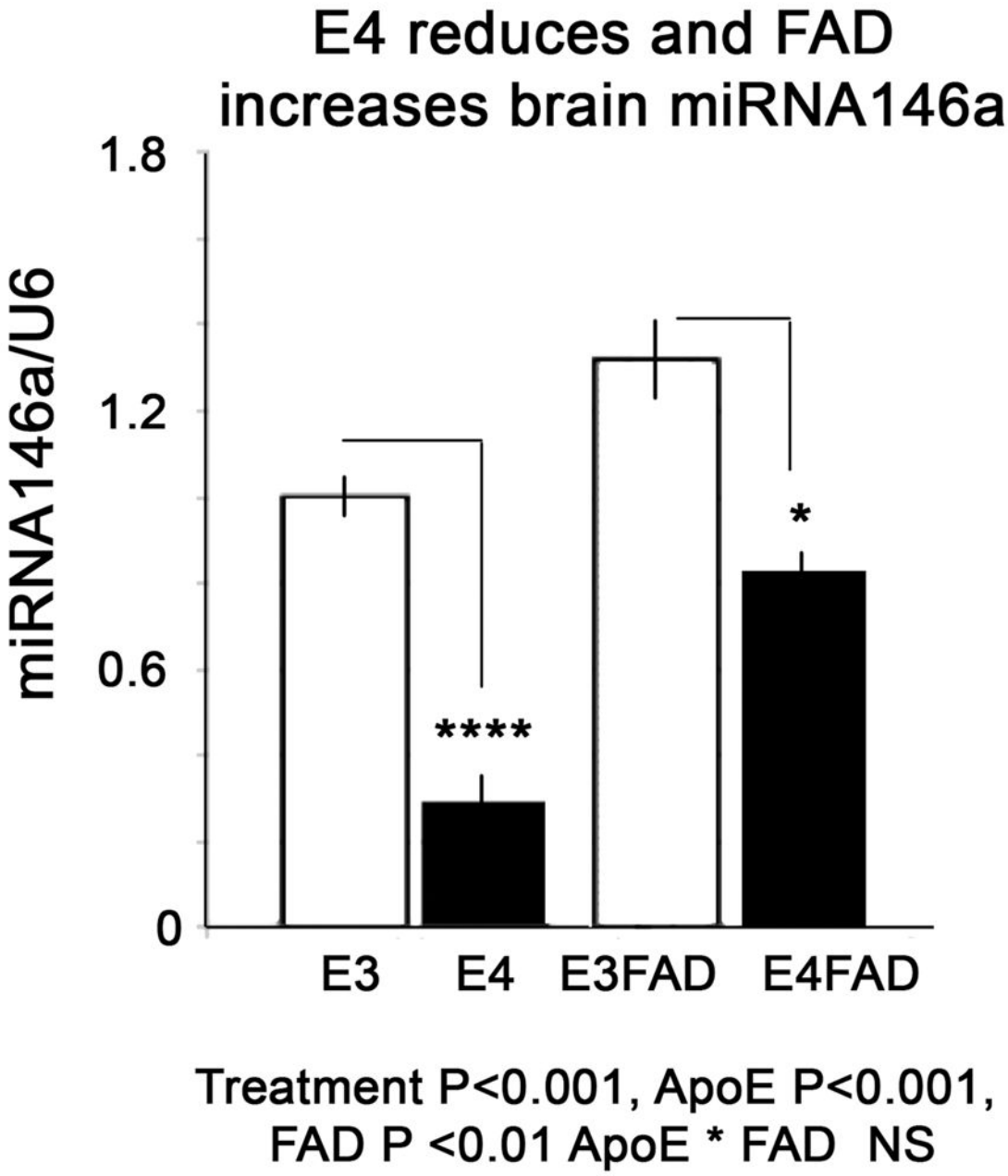

Fig. 1. Brain miR146a levels

Levels of miR146a were measured in young adult apoE3 and apoE4 mice, with and without the 5x-FAD transgenes, and normalized to U6snRNA. Relative levels were calculated by the delta-delta Ct method with E3 set to 1.0 (SEM are shown). The level of miR146a in apoE4 mice was 29% of that in E3 mice ($p < 0.0001$), and the level of miR146a in E4FAD mice was 62% of that in E3FAD mice ($p < 0.05$). Group sizes are n=6 to10 per group.

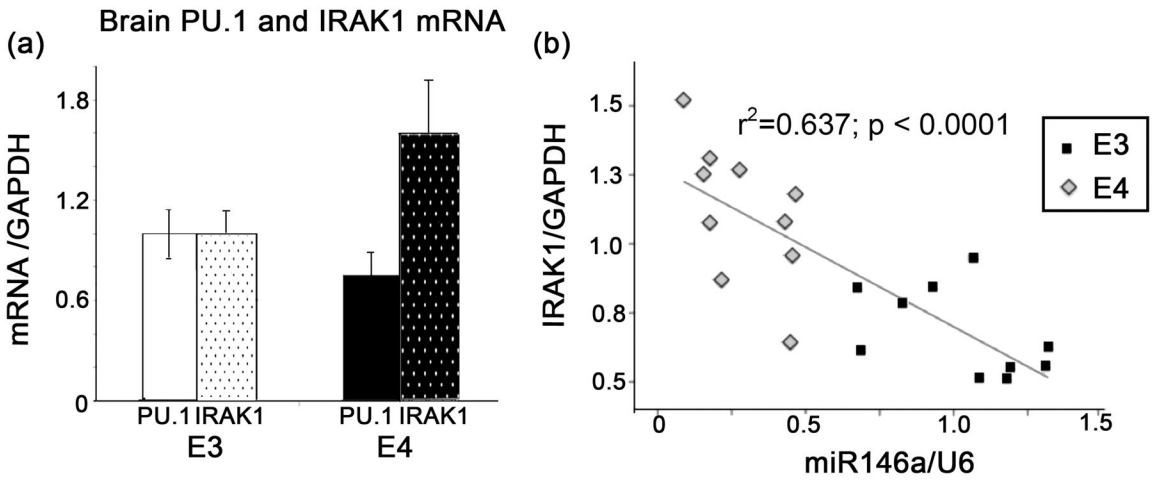

Fig. 2. Pu.1 and IRAK1 mRNA levels and correlation with miR146a levels in brain

Pu.1 and IRAK1 mRNA levels are normalized to levels of GAPDH mRNA in the same samples by the delta-delta Ct method; E3 is set to 1.0; SEM are shown (2a). The 15% decrease in mean PU.1 levels in apoE4 is not statistically significant. IRAK1 mRNA is elevated in E4 by 60% ($p < 0.05$). IRAK1 mRNA levels were correlated with miR146a levels in the same samples; $r^2=0.637$ ($p < 0.0001$); data points for individual animals are shown for apoE4 (diamonds) and apoE3 (squares) (2b).

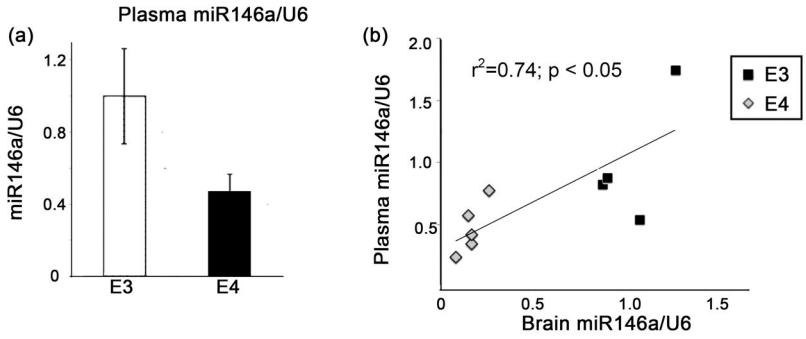

Fig. 3. Levels of miR146a in mouse plasma and correlation with levels in brain

Each point represents values from two mice to ensure detection with low blood volumes collected; that is, plasma was pooled from pairs of mice with similar brain levels of miR146a. Results show that plasma miR146a level in E4 mice was 47% that of E3 ($p < 0.05$) (3a). Plasma miR146a correlated with brain miR146a (the average brain level for each pair of mice used to make the plasma pools) (E3: black square; E4: grey diamonds). Pearson correlation $r^2=0.74$; $p < 0.05$ (3b).