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RIG-1 receptor expression in the pathology of Alzheimer's disease

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Abstract

Background: Neuroinflammation plays a critical role in the pathogenesis of Alzheimer's disease (AD) and involves activation of the innate immune response via recognition of diverse stimuli by pattern recognition receptors (PRRs). The inflammatory inducers and precise innate signaling pathway contributing to AD pathology remain largely undefined.

Results: In the present study we analyzed expression levels of innate immune proteins in temporal and occipital cortices from preclinical (no cognitive impairment, NCI, N = 22) to mild cognitive impairment (MCI, N = 20) associated with AD pathology (N = 20) and AD patients (N = 23). We found that retinoic acid-inducible gene-1 (RIG-1) is significantly elevated in the temporal cortex and plasma in patients with MCI. In addition, primary human astrocytes stimulated with the RIG-1 ligand 5'ppp RNA showed increased expression of amyloid precursor protein (APP) and amyloid- β (A β), supporting the idea that RIG-1 is involved in the pathology of MCI associated with early progression to AD.

Conclusion: These findings suggest that RIG-1 may play a critical role in incipient AD.

Keywords: Innate immunity, Rig signaling, RLR, Inflammation, Alzheimer's disease, Mild cognitive impairment

Background

Alzheimer's disease (AD) pathogenesis is associated with central nervous system (CNS) inflammatory responses [1-4]. Amyloid- β (A β) fibrils trigger inflammatory responses mediated by Toll-like receptors (TLR)4/TLR6 in the presence of CD36 [1-4]. Moreover, a polymorphism in the TLR4 extracellular domain has been reported to be associated with protection against late-onset AD in an Italian population [5], suggesting that a sterile inflammatory response could influence AD pathology through TLR4 signaling. In addition, TLR2 has been shown to act as a receptor for A β , and to trigger an inflammatory response [6]. Activation of innate immunity in the CNS appears to be a universal component of neuroinflammation. AD may be distinguished by a disease-specific mechanism for induction of inflammatory responses. In

addition, distinct pathways for production of inflammation inducers in vulnerable brain regions where these processes occur are potential biomarkers of AD pathophysiology.

Infection of cells by viruses and microorganisms activates innate immune inflammatory responses. The initial sensing of infection is mediated by pattern recognition receptors, which include TLRs, RIG-I-like receptors (RLR), NOD-like receptors (NLR), and C-type lectin receptors (CLR). The RLR family is a RNA sensing system that is comprised of retinoic acid inducible gene-like-1 (RIG-1), melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). RIG-1 recognizes relatively short dsRNA (up to 1 kb) whereas MDA5 detects long dsRNA (more than 2 kb) to activate synthesis of type I IFNs, including IFN- α and IFN- β [7]. RLRs are localized in the cytoplasm and recognize the genomic RNA of dsRNA viruses and dsRNA generated as the replication intermediate of ssRNA viruses and also act as sensors of cellular damage [8]. RLRs activate downstream signaling proteins evoking type I IFN production. Type I IFNs play central roles in antiviral responses by inducing apoptotic cell death in

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virally infected cells, rendering cells resistant to virus infection, activating acquired immunity, and stimulating hematopoietic stem cell turnover and proliferation. In addition, type I IFNs have been implicated in the inflammatory response in AD [9].

We have shown recently that RLR signaling proteins are present in CNS neurons and glial cells, and RLR signaling stimulation resulted in astrocyte activation [10]. In addition, activation of the inflammasome, an NLR innate immune complex, contributes to age-related cognitive decline in elderly animals [11]. However, limited information is available about the role of RLRs in AD pathology or early disease progression. Since MCI is considered a transitional phase between normal aging (or cognition) and AD [12-14], it is important to identify the molecular events that characterize MCI associated with AD pathology.

Methods

Patient consents and subjects demographics

The study was approved by the University of Miami Miller School of Medicine institutional review board. Written informed consent for research and brain autopsy was obtained for all subjects in this study.

Neuropathologic specimens (3 millimeters) of fresh-frozen human temporal (BA38) and occipital cortex (BA17) were obtained from the University of Miami Brain Endowment Bank™. The temporopolar cortex (BA38) was sampled from frozen tissue blocks at the level of the fundus of the temporopolar sulcus. The occipital cortex was sampled from the primary visual cortex (BA17). Postmortem specimens were selected from age-matched subjects with no cognitive impairment (NCI), MCI, and from AD patients. The diagnosis of AD

Table 1 Characteristics of subjects used in the study (brain cortex)

Characteristic	NCI	MCI	AD
Number of subjects	22	20	23
Male (%)	15 (67)	4 (19)	9 (36)
Female (%)	7 (33)	16 (81)	14 (64)
Age at death			
Median (IQR)	68 (61 to 79)	86 (70 to 91)	80 (70 to 85)
Range	59 to 95	61 to 105	60 to 88
Race	20C 1H	20C 1H	24C 1H
Brain weight			
Median (IQR)	1,352 (1,298 to 1,505)	1,210 (1,043 to 1,398)	1,115 (950 to 1,215)
Range	1,054 to 1,570	880 to 1,840	825 to 1,250
CDR score			
0 (%)	22 (100)	12 (60)	0 (0)
1 (%)	0 (0)	8 (40)	0 (0)
2 (%)	0 (0)	0 (0)	2 (9)
3 (%)	0 (0)	0 (0)	21 (91)
Braak score			
0 (%)	22 (100)	0 (0)	0 (0)
I (%)	0 (0)	6 (30)	0 (0)
II (%)	0 (0)	5 (25)	0 (0)
III (%)	0 (0)	9 (45)	0 (0)
IV (%)	0 (0)	0 (0)	1 (4)
V (%)	0 (0)	0 (0)	13 (56)
VI (%)	0 (0)	0 (0)	9 (40)
AD CERAD			
Not present (%)	22 (100)	1 (5)	0 (0)
Possible (%)	0 (0)	4 (20)	0 (0)
Probable (%)	0 (0)	5 (25)	0 (0)
Definite (%)	0 (0)	10 (50)	23 (100)

AD: Alzheimer's disease, CDR: clinical dementia rating, CERAD: Consortium to Establish a Registry for Alzheimer's Disease, IQR: interquartile range, MCI: mild cognitive impairment, NCI: no cognitive impairment. Race: C = Caucasian and H = Hispanic.

was made using standard diagnostic criteria [15]. Subjects with NCI, MCI, and AD were selected based on their antemortem clinical dementia rating (CDR) score one year prior to death and postmortem pathologic evaluation for AD pathology and Braak stage. Neuropathologic diagnosis was based on NIA-Regan criteria recommendations of the Consortium to Establish a Registry for AD (CERAD) [16] and Braak staging of neurofibrillary tangles [17]. The diagnosis of MCI included assessment of normal activities of daily living, normal general cognitive function, abnormal memory for age, and no dementia [17]. MCI patients met neuropathologic criteria for possible to probable AD and Braak stages I to IV [17]. AD cases selected for this study included patients with a diagnosis of clinical dementia and definite AD on postmortem examination (Braak stages V or VI; Table 1).

Plasma and serum samples

All plasma and serum samples were obtained from the University of Kentucky Alzheimer's Disease Center Brain Bank. The samples were obtained from patients diagnosed

postmortem as either age-matched controls with no cognitive impairment (NCI; Braak stage (0 to I), MCI (Braak stages II to IV), or AD (Braak stages V to VI). The section of the study included six age-matched controls (NCI; Braak stages 0 to I), seven MCI patients with possible AD, determined by pathological evidence of neurofibrillary tangles and senile plaques (Braak stages II to IV), and ten patients who met clinical diagnostic criteria for definite AD (Braak stages V to VI; Table 2).

Plasma and serum immunoglobulin isolation

To prevent interference of immunoglobulin G (IgG) during immunoblot analysis of plasma and serum, IgG was isolated using a Pierce Albumin/IgG Removal kit (Thermo Scientific Waltham, MA, USA) according to manufacturer's instructions.

Immunoblotting

Occipital and temporal cortices were homogenized in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM pyrophosphate, 1 mM β -glycerophosphate) with protease

Table 2 Characteristics of subjects used in the study (plasma and serum)

Group	Braak stage	AD (CERAD)	Age at death	Gender	ApoE	PMI (hours)
NCI	0	B = CERAD Probable	92	M	3/5	3.33
NCI	0	No	85	F	3/3	2.50
NCI	1	No	90	F	2/3	4.00
NCI	1	No	100	F	2/3	2.25
NCI	1	No	84	F	3/4	3.00
NCI	1	No	79	F	3/4	1.75
MCI	2	B = CERAD Probable	91	F	3/4	1.75
MCI	2	B = CERAD Probable	93	F	3/4	2.75
MCI	2	B = CERAD Probable	80	F	3/4	2.00
MCI	2	A = CERAD Probable	81	M	3/5	2.83
MCI	2	C = Definite AD	79	M	3/3	1.75
MCI	4	B = CERAD Probable	77	M	3/4	2.75
MCI	3	B = CERAD Probable	92	F	2/3	3.25
AD	6	C = Definite AD	78	M	3/4	3.50
AD	6	C = Definite AD	84	M	3/4	2.75
AD	6	C = Definite AD	83	F	3/3	3.50
AD	6	B = CERAD Probable	85	M	3/3	2.75
AD	6	B = CERAD Probable	80	M	3/3	2.75
AD	6	C = Definite AD	87	M	3/4	3.25
AD	6	C = Definite AD	73	M	3/3	2.00
AD	6	C = Definite AD	80	F	3/3	4.00
AD	6	C = Definite AD	83	F	3/4	2.25
AD	5	C = Definite AD	91	F	3/3	3.00

AD: Alzheimer's disease, ApoE, apolipoprotein e; CERAD: Consortium to Establish a Registry for AD; MCI: mild cognitive impairment, NCI: no cognitive impairment, PMI: postmortem interval.

inhibitor cocktail (Sigma). Twenty five micrograms of protein per sample were resolved in 10 to 20% Tris-HCl Criterion precasted gels (Bio-Rad, Hercules, CA, USA), transferred to polyvinylidene difluoride membranes (Applied Biosystems Waltham, MA, USA) and placed in blocking buffer (PBS, 0.1% Tween-20, 0.4% I-Block (Applied Biosystems Waltham, MA, USA) and then incubated for one hour with an antibody against RIG-1 (Anaspec Fremont, CA, USA) at a dilution of 1:1,000. To authenticate the presumptive bands shown in Figures 1 and 2, a RIG-1 positive control sample (Novus Biologicals Littleton, CO, USA) was used. Immunoblotting is more appropriate to demonstrate the authenticity of the bands. Membranes were incubated for one hour with primary antibodies followed by appropriate secondary horseradish peroxidase (HRP)-linked antibodies (Cell Signaling Danvers, MA, USA). Visualization of signal was enhanced by chemiluminescence using a Phototope-HRP detection kit (Cell Signaling Danvers, MA, USA). To control for protein loading, immunoblots were stripped with Restore, Western blot stripping buffer (Pierce Rockford, IL, USA) and blotted for β -actin using monoclonal anti- β -actin antibody (1:8,000, Sigma St. Louis, MO, USA). Quantification of band density was performed using the UN-SCAN-IT gel software, and data were normalized to β -actin. For immunoblotting of serum and plasma 5 μ g of protein were loaded equally across all samples used to keep data normalized.

Astrocyte culture preparation and RIG-1 stimulation

Human astrocytes were grown in culture as described in de Rivero Vaccari *et al.* in 2012 [10]. Primary human astrocytes (Lonza Basel, Switzerland) were grown in culture in complete Astrocyte Growth Medium (Lonza Basel, Switzerland) for seven days. RIG-1 signaling was stimulated with 5' triphosphate double-stranded RNA (5' ppp dsRNA, Invivogen San Diego, CA, USA) as a specific ligand to stimulate RIG-1 signaling at different concentrations (2 and 4 μ g/ml) for 18 hours. After stimulation, cells were harvested and immunoblotted for RIG-1 (Anaspec Fremont, CA, USA), phosphorylated IRF3 (Novus Biologicals Littleton, CO, USA), amyloid precursor protein (Abcam Cambridge, MA, USA) and amyloid- β (Epitomics Burlingame, CA, USA) expression as described.

Stimulation of human astrocytes with 3-42 amyloid- β

Human astrocytes were grown in culture for seven days and stimulated with 3-42 amyloid- β (Anaspec Fremont, CA USA) at a concentration of 0.5, 1 and 3 μ M for 18 hours. Then cells were harvested and immunoblotted for expression of caspase-1 (Imgenex San Diego, CA, USA) and RIG-1 (Anaspec Fremont, CA USA) as described.

Statistical analysis

The primary outcome measures were levels of immune proteins in two brain regions. The demographic, clinical

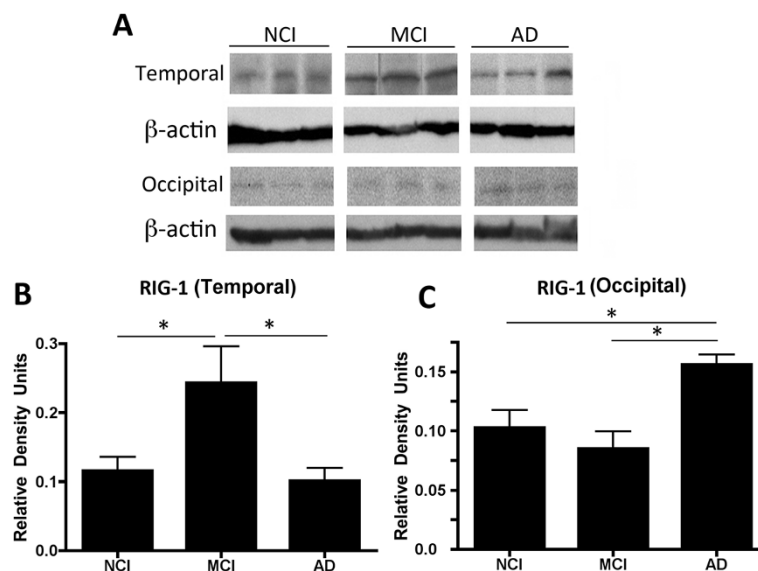


Figure 1 RIG-1 is elevated in the temporal cortex of mild cognitive impairment (MCI) patients. Representative immunoblots (A) of the temporal cortex (B) and occipital cortex (C) from age-matched controls (NCI), MCI and Alzheimer Disease (AD) patients analyzed for RIG-1 expression. β -actin was used as a protein loading control and internal standard. Data are presented as mean \pm SEM. * P < 0.05. N = NCI: 22, MCI: 20 and AD: 23.

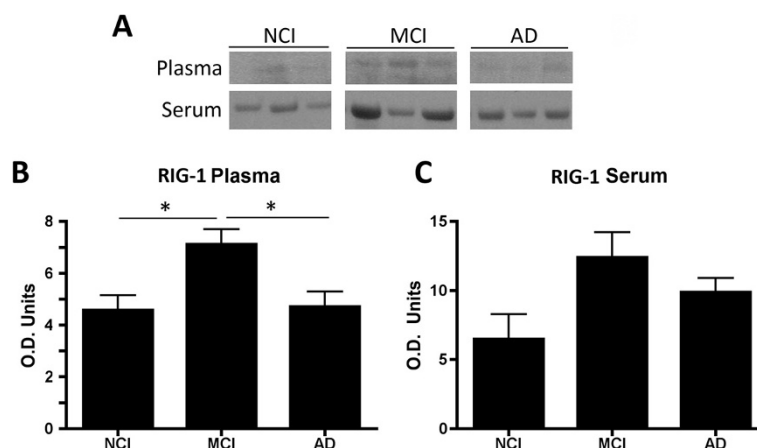


Figure 2 RIG-1 is elevated in the plasma of mild cognitive impairment (MCI) patients. Representative immunoblots (A) of plasma (B) and serum (C) from age-matched controls (NCI), MCI and Alzheimer Disease (AD) patients analyzed for RIG-1 expression. 5 μ g of protein were loaded for the plasma and serum samples after removal of IgG. Data presented as mean \pm SEM. * $P < 0.05$. N = NCI: 6, MCI: 7 and AD: 10 patients.

and neuropathological characteristics were used to group assignment. Association between individual protein measures and age, gender or postmortem interval were explored in multivariate analyses to ensure that the results were unchanged. Statistical comparisons between groups were made using one-way ANOVA and one-tailed Student's *t*-test. The level of statistical significance was set at * $P < 0.05$.

Results

RIG-1 is elevated in the temporal cortex of MCI patients

The demographic and neuropathology characteristics of the cohort used in this section of the study are summarized in Table 1. The study included 22 age-matched controls (NCI), 20 MCI patients with pathologic evidence of senile plaques and neurofibrillary tangles consistent with possible or probable AD (Braak stages I to IV), and 23 patients who met clinical diagnostic criteria for AD and definite pathologic evidence (Braak V to VI). Immunoblot analysis of temporal cortical samples revealed an increase in RIG-1 expression in the MCI group when compared to the NCI and AD groups (Figure 1B). In contrast, the levels of RIG-1 in the occipital cortex were higher in the AD group than in the NCI and MCI groups (Figure 1C). Thus, these results show for the first time that RIG-1 is increased in the temporopolar cortex of MCI patients.

RIG-1 is elevated in the plasma of MCI patients

To determine the levels of RIG-1 in the plasma and serum of patients with MCI associated with AD, immunoglobulin G was isolated from serum and plasma obtained from patients corresponding to the NCI, MCI and AD groups, as described above. Figure 2 shows that RIG-1 was significantly increased in the plasma

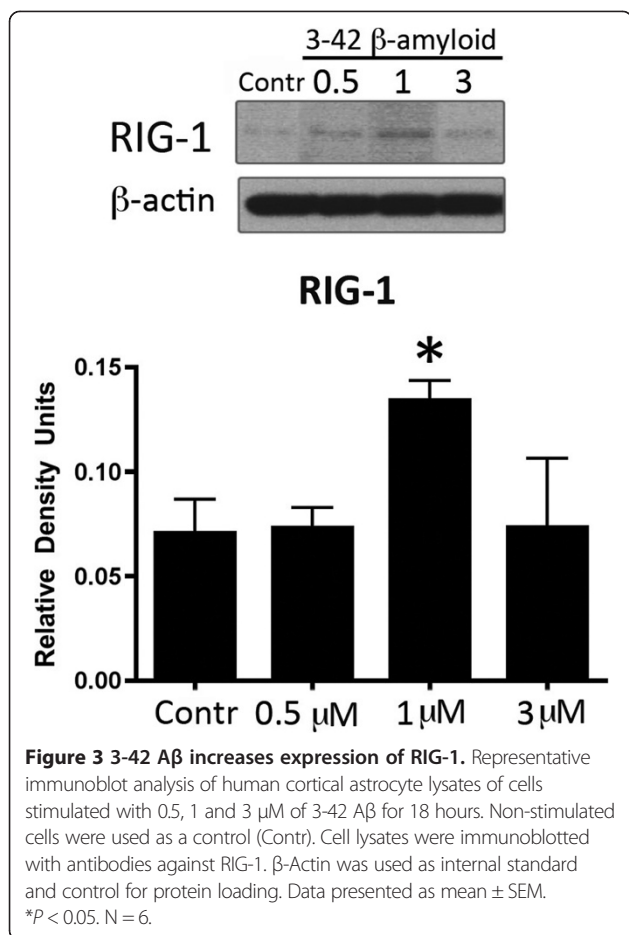
(Figure 2B) from MCI patients compared to the NCI and AD groups, whereas the levels of RIG-1 in serum (Figure 2C) did not differ among the three groups. Thus, these results show for the first time that RIG-1 is increased in the plasma of MCI patients.

3-42 A β increases expression of RIG-1

3-42 A β species have been shown to be the most prevalent form of A β peptides present in early and later stages of human AD amyloid pathology [18]. Since we found that levels of RIG-1 expression are elevated in the temporal cortex from MCI patients when compared to end-stage AD pathology (AD, Figures 1 and 2), we stimulated human cortical astrocytes with 3-42 A β for 18 hours at different concentrations (C, 0.5, 1 and 3 μ M) to determine if A β peptide levels regulate the protein expression levels of RIG-1. Interestingly, there was a concentration dependent effect of 3-42 A β on the expression of RIG-1. At 0.5 μ M treatment, the RIG-1 levels did not change when compared to the control/untreated group, whereas at 1 μ M, the levels of RIG-1 increased, and at 3 μ M, the protein levels of RIG-1 returned to basal/control levels (Figure 3). Importantly, no morphological or toxic changes were noticed in the cultured astrocytes at the concentrations of 3-42 A β used for 18 hours (data not shown). Thus, it appears that A β may be involved in regulating the levels of the RIG-1 protein.

5'ppp dsRNA activates RIG-1 signaling in primary human cortical astrocytes

5'ppp dsRNA has been shown to be a specific ligand of RIG-1 signaling activation [19]. To determine whether 5'ppp dsRNA is responsible for the activation of RIG-1 in primary human cortical astrocytes, 5'ppp dsRNA was administered to primary astrocytes in culture for



18 hours at two different concentrations (2 and 4 μ g/ml). As shown in Figure 4B and 4C, RIG-1 and phospho-interferon regulatory factor 3 (P-IRF3), respectively, were significantly elevated after the administration of 4 μ g/ml of 5'ppp dsRNA, thus indicating RIG-1 signaling activation.

5'ppp dsRNA increases expression of APP and A β in primary human cortical astrocytes

To identify if RIG-1 signaling stimulation is involved in the pathogenesis of AD, astrocytes were stimulated with the RIG-1 signaling agonist 5'ppp dsRNA (4 μ g/ml) for 18 hours. Samples were then resolved by immunoblotting using antibodies against two hallmark proteins of AD, APP (Figure 4E) and A β (Figure 4F). Stimulation of RIG-1 with 4 μ g/ml 5'ppp dsRNA, which activates RIG-1 signaling in astrocytes, resulted in a significant elevation in the expression of APP and A β when compared to the control group, suggesting an involvement of RIG-1 signaling in the expression of two hallmark proteins in AD pathology.

Discussion

The results of the present study demonstrate that RIG-1 is significantly elevated in the plasma and temporal

cortex of MCI patients with AD pathology whereas RIG-1 is elevated in the occipital cortex of AD patients. Stimulation of RIG-1 with 5'ppp dsRNA in human cortical astrocytes resulted in increased expression of APP and A β . Thus, these findings suggest a potential role of the RIG-1 signaling system in incipient AD.

AD is a progressive neurodegenerative disorder characterized by impaired judgment, confusion, changes in behavior, disorientation [20], impairment of daily living, and loss of the ability to function independently [21]. AD is expected to become more prevalent as life expectancy continues to rise. It has been estimated that by 2050, the number of AD cases could double or triple to between 11 to 16 million [22]. A major limitation in finding therapeutic solutions for AD has been the lack of reliable methods for early diagnosis of this devastating disease. AD is a neurodegenerative disorder characterized by a progressive cognitive impairment as a consequence of neuronal dysfunction and ultimately the death of neurons. MCI is considered a transitional phase between normal aging and AD [12-14]. The amyloid hypothesis of AD proposes that neuronal damage results from the accumulation of insoluble, hydrophobic, fibrillar peptides such as amyloid- β_{1-42} [23-26]. These peptides activate enzymes resulting in a cascade of second messengers including prostaglandins and platelet-activating factor. Apoptosis of neurons is thought to follow as a consequence of the uncontrolled release of second messengers. It is possible that RIG-1 signaling in the temporal cortex is involved in the early events leading to AD pathology such as the accumulation of APP. On the other hand, the presence of RIG-1 in the occipital cortex of AD patients may be associated with exacerbated production of cytokines in AD patients [27] as a result of disease progression in later stages of AD when the pathology spreads throughout the cortex from the limbic to koniocortical areas.

Neuroinflammation has been considered to play a critical role in the pathogenesis of AD [28-33], but the role of the innate immune response has not been thoroughly examined [34,35]. Human neurons, in the absence of glia, have the intrinsic machinery to trigger robust inflammatory, chemoattractive, and antiviral responses [36]. The innate immune system senses microbial and viral pathogen and danger signals released from damaged or stressed cells to trigger conserved intracellular signaling pathways that drive proinflammatory responses that are critical for productive innate and adaptive immunity. Excessive inflammatory responses become deleterious adding to tissue destruction. Here we have provided evidence demonstrating that the RIG-1 is elevated in the innate immune response in disease-affected brain areas of MCI patients.

RIG-1 signaling may be activated by small self-RNA cleavage products generated by RNase L that stimulate

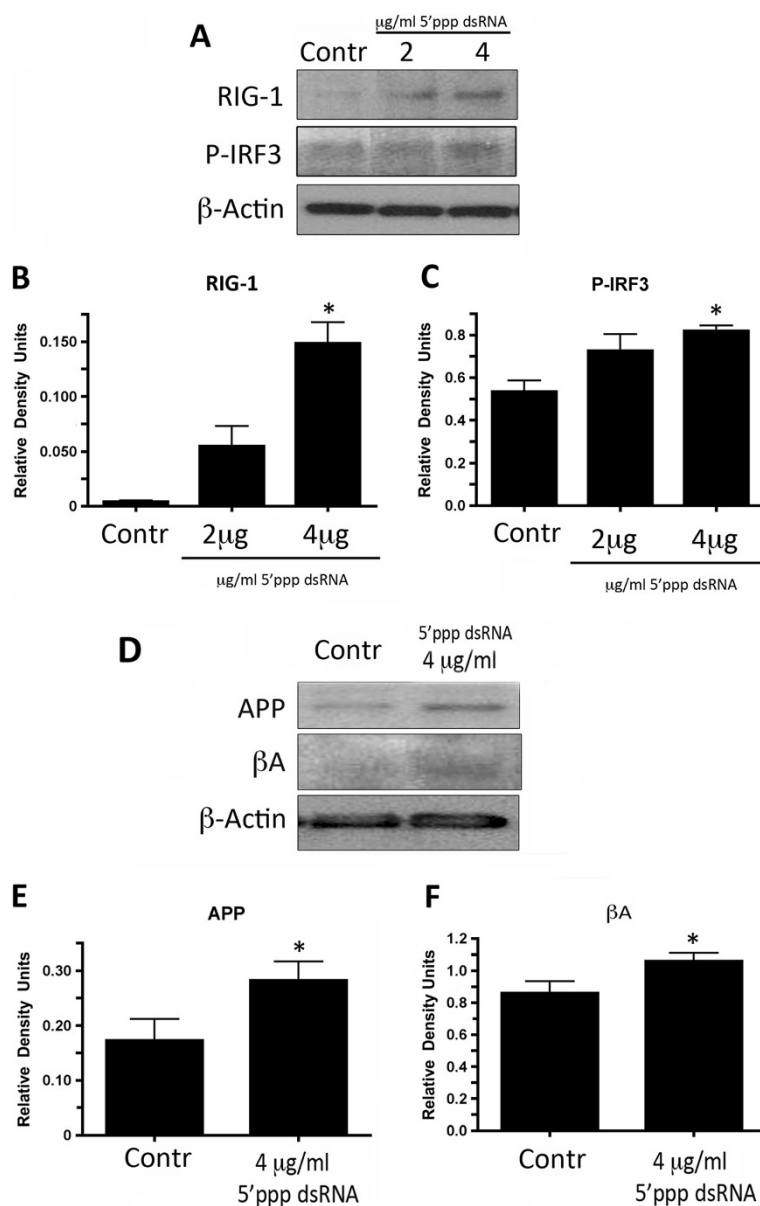


Figure 4 5'ppp dsRNA activates RIG-1 signaling and increases expression of APP and A β . Representative immunoblot analysis of human cortical astrocyte lysates (A) of cells stimulated with 2 or 4 μ g/ml of 5'ppp dsRNA for 18 hours. Non-stimulated cells were used as a control (Contr). Cell lysates were immunoblotted with antibodies against (B) RIG-1 and (C) P-IRF3. β -Actin was used as internal standard and control for protein loading. Data presented as mean \pm SEM. * P < 0.05. N = 6. Representative immunoblot analysis of human cortical astrocyte lysates (D) of cells stimulated with 4 μ g/ml of 5'ppp dsRNA for 18 hours. Non-stimulated cells were used as a control (Contr). Cell lysates were immunoblotted with antibodies against (E) APP and (F) A β . β -Actin was used as internal standard and control for protein loading. Data presented as mean \pm SEM. * P < 0.05. N = 6.

signaling of RIG-1 [37] or by reactive oxygen species (ROS) [38]. Since damaged CNS cells release small self-nucleic acids and ROS, these molecules may play an important role in the initiation of the innate immune response in MCI [39]. Alternatively, foreign nucleic acids, the signature of invading viruses and certain bacteria, are sensed intracellularly and then stimulate RIG-1 signaling [7]. Other, yet to be identified ligands may be involved in the

activation of RIG-1 signaling in MCI. Moreover, our data suggest that RIG-1 signaling activation results in increased expression of APP and A β , and that in addition A β contributes to the expression of RIG-1. It is important to consider that this study used samples from individuals in the MCI group that had a slightly greater number of females and a wider age range; thus, when interpreting these results one must take into account the effects of gender and age [40].

Conclusions

In this study, we used immunoblot analysis to determine whether RIG-1 signaling stimulation results in increased expression of A β and APP. In order to determine whether human cortical astrocytes respond to RIG-1 stimulation, we treated primary cortical astrocytes in culture with the specific RIG-1 ligand 5' ppp dsRNA and assayed for the expression of the RIG-1 signaling proteins RIG-1 and P-IRF3.

as well as APP and A β . The levels of these proteins were increased upon stimulation with the RIG-1 ligand, consistent with the hypothesis that RIG-1 signaling is involved in the pathogenesis of AD. Astrocytes have been previously implicated in the pathogenesis of AD [41-44]. In addition, we have previously shown that RIG-1 signaling is involved in the activation of astrocytes [10]. Thus, our findings further support an involvement of astrocytes in AD pathology.

Abbreviations

AD: Alzheimer's disease; PRRs: pattern recognition receptors; NCI: no cognitive impairment; MCI: mild cognitive impairment; RIG-1: retinoic acid-inducible gene-1; APP: amyloid precursor protein; A β : amyloid- β ; CNS: central nervous system; RLR: RIG-I-like receptors; NLR: NOD-like receptors; CLR: C-type lectin receptors; MDA5: melanoma differentiation-associated gene 5; LGP2: laboratory of genetics and physiology 2; CDR: clinical dementia rating; CERAD: Consortium to Establish a Registry for AD; Apoe: apolipoprotein e; 5' ppp dsRNA: 5' triphosphate double-stranded RNA.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JPdRV designed the study, performed experiments, analyzed the data, interpreted the results and prepared the manuscript. FJB and CS performed experiments and analyzed the data. DCM, WDD and RWK reviewed and discussed the manuscript. All authors have read and approved the final version of the manuscript.

Acknowledgments

These studies were supported by grants from the NIH to RWK (NS059836), to WDD (NS042133) and gifts to DCM (McGowan Endowment Fund). We thank David Sequeira for help with immunohistochemistry and Dennis Dixon, MD and Jocelyn Bruce, MD for expert neuropathological consultation. Postmortem neuropathological specimens were obtained from the University of Miami Brain Endowment BankTM. Plasma and serum samples were obtained from the University of Kentucky Alzheimer's Disease Center Brain Bank.

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Received: 9 December 2013 Accepted: 25 March 2014

Published: 2 April 2014

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doi:10.1186/1742-2094-11-67

Cite this article as: de Rivero Vaccari *et al.*: **RIG-1 receptor expression in the pathology of Alzheimer's disease.** *Journal of Neuroinflammation* 2014 **11**:67.

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