

Brief Report

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Brief Report

ABA Supplementation Rescues IRS2 and BDNF mRNA Expression in a Triple-Transgenic Mice Model of Alzheimer's Disease

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Abstract: Strong evidence indicates that insulin resistance contributes to the development of Alzheimer's disease by promoting inflammation, oxidative stress, and blood vessel damage. Conversely, neuroinflammation induces insulin resistance by targeting insulin signaling molecules: the insulin receptor substrates 1 and 2 (IRS1/2). In addition, neuroinflammation is strongly linked to reduced levels of brain-derived neurotrophic factor (BDNF) expression. BDNF is a key factor for neuronal survival, and its expression is compromised in neurodegenerative disorders. In this study, we show that, in a triple-transgenic (3xTg) mice model of Alzheimer's disease, the BDNF and IRS2 but not the IRS1 mRNA expression levels were reduced in the hippocampus, together with higher levels of TNF α , compared with wild-type mice. Subgroups of control and 3xTg mice received short and long treatments of Abscisic Acid (ABA), a phytohormone with anti-inflammatory and insulin-sensitizing capabilities. We found that the short ABA treatment can increase the IRS1 and IRS2 mRNA expression both in wild-type and 3xTg mice, concomitant with reducing pro-inflammatory cytokine TNF α in 3xTg mice. However, earlier, and thus longer treatments are required to rescue the BDNF mRNA levels. Our data strongly confirm that ABA administration is a potential treatment to prevent Alzheimer's disease, via lowering neuroinflammation, potentially rescuing insulin signaling molecules and BDNF mRNA expression.

Keywords: hippocampus; Alzheimer's disease; neuroinflammation; insulin signal; phytohormones; neurotrophic factor; neuroprotector

1. Introduction

Alzheimer's disease (AD) is the most common type of dementia, and it is characterized by progressive memory loss, cognitive impairment, and functional decline. The main histopathological hallmarks of AD are the accumulation of extracellular amyloid beta-peptide (A β) and hyperphosphorylated tau protein in intracellular neurofibrillary tangles that correlate with significant glucose hypometabolism. Scientific evidence has demonstrated that the onset of neurodegeneration occurs decades before the first clinical symptoms appear [1]. At these early stages, the neuroinflammatory status and insulin resistance are key players at the onset of the disease, as they can cause irreversible neuronal damage [2].

Late-onset AD has an idiopathic etiology; however, several risk factors have been identified, and metabolic syndrome leading to type II diabetes are considered major risks factors for AD [3]. One of the well-studied targets of insulin resistance is Tau phosphorylation, and the presence of hyperphosphorylated Tau in the patient blood serum is a significant predictive biomarker of AD [4]. Insulin signaling starts by the binding to the insulin receptor, and it is mediated by the insulin receptor substrates isoforms 1 and 2 (IRS1 and IRS2). We previously observed reduced levels of

hippocampal IRS2 mRNA in an animal model of metabolic syndrome that also displayed cognitive impairment [5]. Furthermore, human studies have shown that IRS2 mRNA and protein expression is significantly decreased in the hippocampus and temporal [6] and prefrontal cortices [7] of AD patients compared to control subjects. The administration of molecules with anti-inflammatory capability can rescue the IRS2 mRNA and protein levels, exemplified by ABA in rats with metabolic syndrome [5], and curcumin in a rat model of Alzheimer's disease [8]. IRS2 rescuing, in all cases, is concomitant with cognitive function improvement.

Interestingly, insulin signaling is strongly linked with BDNF, a key neurotrophic factor for neuronal survival and plasticity [9]. In fact, diabetic patients display distinctly reduced BDNF serum levels compared to non-diabetic controls [10]. Low BDNF expression is strongly correlated with high brain inflammatory status [11]. Not only is the BDNF function reduced in Alzheimer's disease, but it is also considered a fundamental target for disease management, given the fundamental role in neuroprotection [12].

In our previous studies, we evaluated two ABA treatments in a 3xTg model of AD: early ABA administration (duration of 5 months) and later (duration of 3 months). In this model, early treatment could effectively ameliorate memory impairment and prevent microglia switching to a reactive state [13]. According to the literature, and our previous findings, we hypothesized that the action of ABA would be mediated by enhancing the BDNF and IRS2 expression levels, thereby improving insulin signaling and preventing neurodegeneration.

2. Results

2.1. ABA Treatment Reduces Inflammatory Cytokine TNF α While Increasing BDNF mRNA Expression in Hippocampi of Triple-Transgenic Mice

We first evaluated the levels of cytokine tumor necrosis factor α (TNF α) to confirm the inflammatory status of the 3xTg mice and the effect of ABA (Figure 1A). We have previously shown that the microglia morphology in 3xTg mice is less ramified, corresponding to an M1/M2 status, compared to the age-matched controls. However, M1 and M2 differ in their cytokine profile, being M1 pro-inflammatory with higher levels of TNF α .

Here we show that, as expected, at the age of 8 months, untreated 3xTg mice have significantly higher levels of TNF α than wild-type animals ($*** p = 0.0004$; $t = 4.34$; $df = 17$). Confirming our previous effect on inflammatory microglia, ABA administration significantly prevented the increase in the TNF α mRNA levels in the 3xTg mice hippocampi, both at 3 months ($* p = 0.042$; $t = 2.298$; $df = 11$) and 5 months ($* p = 0.036$; $t = 2.33$; $df = 13$) of treatment.

Next, we measured the brain neurotrophic factor (BDNF) (Figure 1B), given its relevance in neuron survival and activity, and the evidence showing that BDNF activity is compromised in Alzheimer's models [14]. We confirmed the reduced BDNF mRNA levels in the hippocampi of the untreated 3xTg mice compared to the wild type ($** p = 0.009$; $t = 2.268$; $df = 16$). Next, we evaluated the potential benefit of ABA treatment and found that 3 months of ABA exposure did not increase the BDNF mRNA levels; however, with the earlier and longer ABA treatment, the hippocampal BDNF levels were significantly higher than in the untreated 3xTg mice ($**** p < 0.0001$; $t = 8.865$; $df = 12$). Interestingly, we found that 5 months of ABA exposure also increased the hippocampal BDNF in the wild-type mice ($** p = 0.002$; $t = 3.79$; $df = 13$)

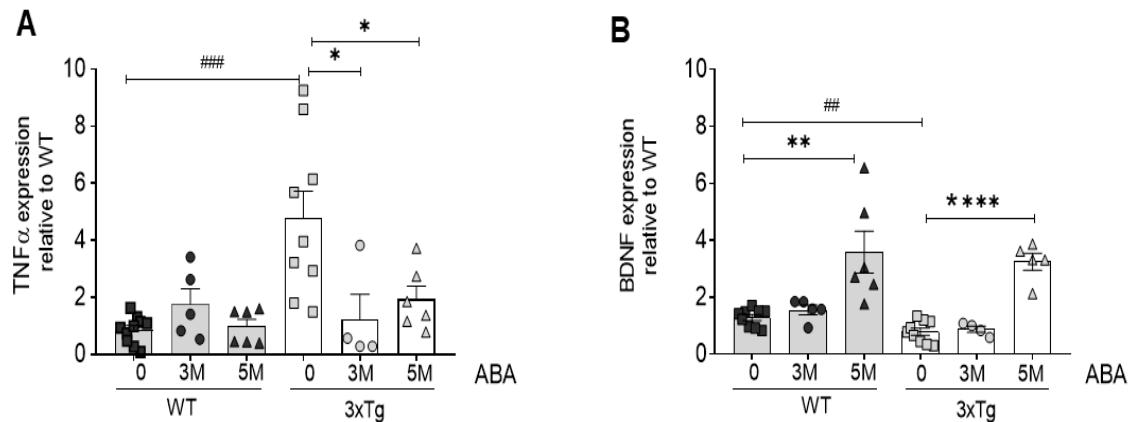


Figure 1. ABA treatment (A) reduces hippocampal TNF and (B) increases BDNF expression as measured by qPCR. Data are represented as the mean \pm of at least 5–9 independent subjects in triplicate. Data were analyzed by Student t-test (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$ for ABA effect; ## $p < 0.01$ and ### $p < 0.001$ for genotype effect).

2.2. ABA Treatment Increases mRNA Levels of Insulin Substrate Receptors 1 and 2 (IRS1/2)

Next, we determined the levels of IRS1 and IRS2 at 3 and 5 months of ABA treatment (Figure 2). We observed that, at 8 months of age, the IRS2 levels (Figure 2A) were significantly lower in the hippocampi of the 3xTg mice compared to the wild type (## $p = 0.0075$; $t = 3.34$; $df = 10$). Early ABA treatment (5 months duration) rescued the levels of hippocampal IRS2 mRNA expression in the 3xTg mice compared to untreated mice (* $p = 0.043$; $t = 2.35$; $df = 9$). The later ABA treatment (3 months) increased the expression but did not reach statistical significance. In the wild-type mice, the short ABA treatment induced a significant increase in the IRS2 mRNA expression (*** $p = 0.0003$; $t = 5.372$; $df = 8$). The increases in IRS2 mRNA obtained with the long ABA treatment (5 months) did not reach statistical significance. On the contrary, the IRS1 levels were not different in the untreated 3xTg mice compared to the wild-type mice at 8 months of age. In the wild-type mice, ABA treatment significantly increased the IRS1 mRNA levels, both the short (* $p = 0.0215$; $t = 2.642$; $df = 12$) and long (* $p = 0.0311$; $t = 2.44$; $df = 12$) treatments, while the increase observed in the 3xTg mice with 3 months of ABA treatment was not significant, but the long treatment induced significant growth (* $p = 0.0115$; $t = 2.980$; $df = 12$).

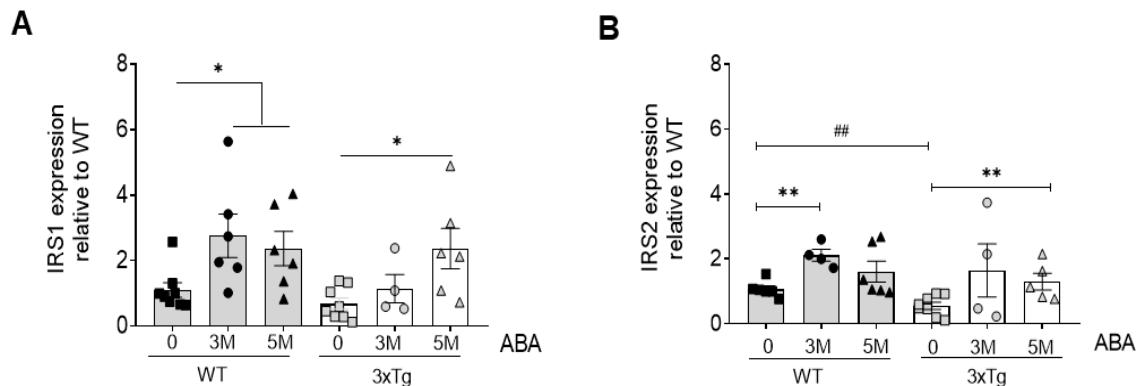


Figure 2. ABA administration increases IRS mRNA expression in hippocampi as measured by qPCR. (A) IRS1 expression increases in WT mice as soon as 3 months of ABA treatment, but in 3xTg mice, only at 5 months. (B) IRS2 increases in WT animals at 3 months of treatment in WT animals. Data are represented as the mean \pm of at least 5–9 independent subjects in triplicate. Data were analyzed by Student t-test (* $p < 0.05$ and ** $p < 0.01$ for ABA effect, and ## $p < 0.01$ for genotype effect).

3. Discussion

In this study, using the 3xTg mice model of AD, we confirmed that the 8-month-old mice displayed higher levels of TNF α and lower levels of BDNF mRNA, as expected. Interestingly, the short ABA treatment (3 months) that started when the mice were 5 months old was sufficient to significantly decrease the TNF α mRNA levels in the 3xTg mice to control levels. However, the earlier ABA treatment started at 3 months old; thus, longer ABA exposure was required to observe an increase in the BDNF mRNA levels, suggesting that, in this model, the BDNF levels are regulated by inflammation. The relationship between BDNF and inflammation is bidirectional, and other studies have shown that reductions in inflammatory markers are a consequence of BDNF increase [15]. Because our previous studies [13] have shown that memory improvement by ABA in this model requires longer treatment, the present data are in line with the fact that lowering inflammation is not sufficient, but it is likely a mandatory step to rescue BDNF and cognitive function.

The mechanism by which ABA exerts its effects has not been completely elucidated, and some studies indicate that ABA may be a PPAR- γ agonist (brain tissue [16]; in T-cells [17]), or indirectly activate PPAR- γ expression via LANC-2 (microglia cells [18]). However, other studies indicate that ABA's function may be PPAR- γ -independent (macrophages) [19]. This discrepancy may be due to the different cell types analyzed, and it requires further research to fully elucidate the ABA mechanism of action. Our data would be in accordance with ABA as a PPAR- γ agonist, as PPAR- γ can decrease the TNF α mRNA levels through NFkB activation and, in parallel, activate the BDNF promoter [20]. Moreover, this action is associated with improving brain injury, as demonstrated in a neuropsychiatric systemic lupus erythematosus [21], and with alleviating memory impairment in different rat models of neuroinflammation, either by LPS administration [22] or metabolic syndrome [23].

We also analyzed the IRS1 and IRS2 mRNA levels, given the importance of insulin resistance in AD. We found that the IRS2 (but not IRS1) mRNA levels were reduced in the 8-month-old 3xTg mice compared to the wild-type controls. This is concomitant with the reduction in spatial memory that we previously observed in this model [13]. These data agree with the hypothesis stating that IRS2 deficiency could be the link between type II diabetes [24] and AD (for review, see [25,26]). Moreover, these findings are in accordance with our previous studies in which we found that the IRS2 but not IRS1 gene expression was reduced in the brain of a rat metabolic syndrome model [23]. Interestingly, we also found that physiological aging is accompanied by a reduction in both the IRS1 and IRS2 levels [27], which agrees with the notion that AD accelerates aging pathology.

Although some investigations suggest that a reduction in IRS2 [28], and specifically in IGF1R/IRS2 signaling [29,30], can ameliorate AD, our current data are in line with other studies showing that IRS2 decline correlates with impaired spatial memory and emotional responses [31]. Furthermore, our findings agree with the pivotal role of IRS2 in NMDA-receptor-dependent synaptic transmission [32]. Moreover, the beneficial effects observed with ABA administration effects are supported by those reported for exendin (an anti-diabetic molecule) administration in a mice model of Alzheimer's disease [33]. Furthermore, **thiazolidinediones (TZD), which are known PPAR- γ agonists, also increase IRS2 [34] and IRS1 [35] expression**, which supports that the action mechanism of ABA may be via PPAR- γ . However, there is no direct evidence to suggest that PPAR- γ directly activates the IRS2 promoter, but it indirectly interacts with transcription factors that bind and regulate the IRS2 promoter (for example, transcription factor Sp1) [36]. Likewise, the IRS1 promoter can be activated by Sp1 [37], and we indeed observed that ABA also increased the IRS1 gene expression, both in the wild-type and 3xTg mice, which could agree with the common activating mechanisms. Because 3xTg mice do not show brain IRS1 expression alteration compared to the wild type, it is unclear whether the ABA effect on IRS1 contributes to the improvement in the 3xTg mice memory function.

Interestingly, the short ABA treatment elevated the IRS1 and IRS2 mRNA levels in the wild-type mice, whereas 5 months of treatment was required to influence the 3xTg mice, suggesting a slower promoter activation in these mice.

Further studies are required to understand whether the key factor is the early intervention or the long exposure time. In conclusion, our data support that early ABA administration is a promising intervention to reduce inflammation-induced brain damage in Alzheimer's models, effectively targeting the gene expression regulation of key molecules (BDNF, IRS1/2) for neuronal survival and optimal function.

4. Materials and Methods

4.1. Mice and Treatment

Mice were treated as in [13]. The procedures followed directive 86/609/EEC of the European Community on the protection of animals used for experimental and other scientific purposes. The experiments were approved by the Ethics Committee of the University Jaume I (approval number 2014/VSC/PEA00209). Briefly, control and transgenic mice were divided randomly into three experimental groups: ABA 3M and ABA 5M animals were supplemented with ABA (Fernandez-Rapado, Spain) in their drinking water (20 mg/L) for 3 or 5 months, respectively. Control groups were administered vehicle for 3 or 5 months (and pooled together for the analysis). Mice were fed ad libitum for 8 months. After the behavioral tests, mice were anesthetized with pentobarbital (120 mg/kg Eutanax, Barcelona, Spain) and sacrificed by quick decapitation. Brains were quickly removed, snap-frozen, and kept at -80 °C until RNA extraction.

4.2. RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted from the mouse hippocampus ($n = 5-9$) using the RNeasy LipidTissue Mini Kit (Qiagen), according to the manufacturer's protocol. We disrupted and homogenized fatty tissue in 1 mL QIAzol Lysis Reagent using the TissueRuptor. Then, the homogenate was incubated at room temperature (15–25 °C) for 5 min, and after this, 200 μ L of chloroform was added. After incubating the sample for 2–3 min and centrifuging it (12,000 $\times g$ for 15 min at 4 °C), the upper (aqueous phase) was transferred to a new tube with 1 volume of 70% of ethanol. An amount of 500 μ L of the sample was transferred to an RNeasy Mini spin column in a 2 mL collection tube, and after centrifugation (15 s at 8000 $\times g$), the flow-through was discarded. This step was performed twice. An amount of 700 μ L of Buffer RW1 was added to the RNeasy column and was centrifuged at 8000 $\times g$, and the flow-through was discarded. After, we added 500 μ L Buffer RPE to the RNeasy column twice to dry membrane, and the flow-through was discarded. Finally, the RNA samples were resuspended in 40 μ L of nuclease-free water.

RNA concentration and quantification of total RNA was performed using Thermo Scientific Nanodrop 2000c, with the OD260/OD280. Genomic DNA was removed using DNase I, RNase-free (Life Technologies, Carlsbad, CA, USA), for 30 min at 37 °C. The reaction was stopped by the addition of 1 μ L of EDTA for 10 min at 65 °C. The first strand of cDNA was synthesized using the Prime-Script™ RT Reagent Kit (Perfect Real Time) (Takara Bio Inc., Shiga, Japan). For each reaction, 1 μ g of RNA was used for reverse transcription, in a mixture of 4 μ L of PrimeScript Buffer, 1 μ L of PrimeScript RT, 1 μ L of Oligo dT Primer (50 μ M), and 1 μ L of random primer (100 μ M). Enzyme mix was adjusted to a final volume of 20 μ L at room temperature. The mixture was incubated at 37 °C for 15 min and heated at 85 °C for 5 min to terminate the reaction. DNA concentration and quantification of total DNA was performed using Thermo Scientific Nanodrop 2000c, with the OD260/OD280, and the cDNA was subsequently stored at -20 °C.

Primers were designed using the Primer3 software tool (<http://primer3.ut.ee/> (accessed on November 2018) (Table 1). RT-PCR was performed in a volume of 10 μ L with 5 μ L of Maxima SYBR Green/ROX qPCR Master Mix (2X) (Applied Biosystems Life Technologies, Carlsbad, CA, USA), 1 μ L of primer, and 1 μ L of cDNA. All PCR reactions were performed under the following conditions: initial cycle at 98 °C for 10 min, followed by 40 cycles at 98 °C for 10 s, 60 °C for 10 s, and 72 °C for 20 s. Gene expression in the hippocampus was quantified using a StepOnePlus Real-Time PCR system (Applied Biosystems Life Technologies). Each sample was tested in triplicate, and data were analyzed

using the comparative critical threshold method, with the amount of target gene normalized to the housekeeping gene GAPDH. Relative gene expression was calculated using $2^{-\Delta\Delta Ct}$ relative to control.

Table 1. Primer sequences [23].

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
IRS1	CCTGACATTGGAGGTGGGTC	TGGGGATCTCTGGGCCATA
IRS2	GCAGCCAGGAGACAAGAACT	AGCGCTTCACTCTTCACGA
BDNF	TACCTGGATGCCGCAAACAT	AGTTGGCCTTGGATACCGG
TNF α	GACCCTCACACTCAGATCA	TGCTACGACGTGGCTACG
GAPDH	TGCCCCCATGTTGTGATG	TGGTGGTGCAGGATGCATT

4.3. Statistics

Data were expressed as mean \pm SEM using Graph Pad Prism version 8. Data were subjected to an unpaired Student t-test, two-tail, with a confidence of 95%, for the identification of the statistical differences between means. Gaussian distribution was monitored with the Shapiro–Wilk normality test.

Author Contributions: Conceptualization, A.M.S.-P.; methodology, V.E.-F.; software, L.A.B. and A.M.S.-P.; formal analysis, A.M.S.-P.; investigation, L.A.B., A.C.R., and V.E.-F.; data curation, V.E.-F., L.A.B., and A.M.S.-P.; writing—original draft preparation, A.M.S.-P., writing—review and editing, L.A.B. and A.C.R.; visualization, L.A.B.; supervision, A.M.S.-P.; project administration, A.M.S.-P.; funding acquisition, A.M.S.-P. All authors have read and agreed to the published version of the manuscript.

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