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Original Contribution

Impairment of biliverdin reductase-A promotes brain insulin resistance in Alzheimer disease: A new paradigm

Eugenio Barone [a,b], Fabio Di Domenico [a], Tommaso Cassano [c], Andrea Arena [a], Antonella Tramutola [a], Michele Angelo Lavecchia [d], Raffaella Coccia [a], D. Allan Butterfield [e,*], Marzia Perluigi [a,*]

[a] Department of Biochemical Sciences "A. Rossi-Fanelli", Sapienza University of Rome, Piazzale A. Moro 5, 00185 Roma, Italy
[b] Universidad Autónoma de Chile, Instituto de Ciencias Biomédicas, Facultad de Salud, Avenida Pedro de Valdivia 425, Providencia, Santiago, Chile
[c] Department of Clinical and Experimental Medicine, University of Foggia, Via Napoli 20, 71122 Foggia, Italy
[d] Department of Physiology and Pharmacology "V. Erspamer", Sapienza University of Roma, Piazzale A. Moro 5, 00185 Roma, Italy
[e] Department of Chemistry, Markey Cancer Center, and Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY 40506-0055, USA

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ABSTRACT

Clinical studies suggest a link between peripheral insulin resistance and cognitive dysfunction. Interestingly, post-mortem analyses of Alzheimer disease (AD) subjects demonstrated insulin resistance in the brain proposing a role for cognitive deficits observed in AD. However, the mechanisms responsible for the onset of brain insulin resistance (BIR) need further elucidations. Biliverdin reductase-A (BVR-A) emerged as a unique Ser/Thr/Tyr kinase directly involved in the insulin signaling and represents an upstream regulator of the insulin signaling cascade. Because we previously demonstrated the oxidative stress (OS)-induced impairment of BVR-A in human AD brain, we hypothesize that BVR-A dysregulation could be associated with the onset of BIR in AD. In the present work, we longitudinally analyze the age-dependent changes of (i) BVR-A protein levels and activation, (ii) total oxidative stress markers levels (PC, HNE, 3-NT) as well as (iii) IR/IRS1 levels and activation in the hippocampus of the triple transgenic model of AD (3xTg-AD) mice. Furthermore, *ad hoc* experiments have been performed in SH-SY5Y neuroblastoma cells to clarify the molecular mechanism(s) underlying changes observed in mice. Our results show that OS-induced impairment of BVR-A kinase activity is an early event, which starts prior the accumulation of Aβ and tau pathology or the elevation of TNF-α, and that greatly contribute to the onset of BIR along the progression of AD pathology in 3xTg-Ad mice. Based on these evidence we, therefore, propose a new paradigm for which: OS-induced impairment of BVR-A is firstly responsible for a sustained activation of IRS1, which then causes the stimulation of negative feedback mechanisms (i.e. mTOR) aimed to turn-off IRS1 hyper-activity and thus BIR. Similar alterations characterize also the normal aging process in mice, positing BVR-A impairment as a possible bridge in the transition from normal aging to AD.

1. Introduction

During the last years a growing number of observations highlighted a close interconnection between Alzheimer disease (AD) and common diseases of modern adulthood, including obesity and type 2 diabetes mellitus (T2DM) [1,2]. Furthermore, epidemiological studies showed that hallmarks of peripheral metabolic disorders, such as glucose intolerance and/or impairment of insulin secretion, are associated with a higher risk to develop dementia or AD [2–5], whereas patients with AD more frequently present with an impaired glucose metabolism or T2DM [6,7].

This clinical evidence raised doubts about the correct functioning of insulin signaling especially in light of the neurotrophic actions mediated by insulin [8]. Indeed, the activation of insulin signaling cascade does not induce a significant glucose uptake in the brain as it does in peripheral tissues [9,10], but, rather, it modulates other important functions through the activation of the two main pathways downstream to the insulin receptor (IR): (i) the phosphoinositide-3 kinase (PI3K) pathway, which is involved in the maintenance of synaptic plasticity and memory consolidation [11,12]; and (ii) the mitogen-activated protein kinase (MAPK) cascade, which is responsible both for the induction of several genes required for neuronal and synapse growth,

* Corresponding authors.
E-mail addresses: dabcns@uky.edu (D.A. Butterfield), marzia.perluigi@uniroma1.it (M. Perluigi).

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maintenance and repair processes, as well as serving as a modulator of hippocampal synaptic plasticity that underlies learning and memory [13].

Interestingly, human postmortem studies have convincingly shown that a dysregulation of the insulin signaling with reduced downstream neuronal survival and plasticity mechanisms are consistent and fundamental abnormalities in AD [9,14,15]. In particular, AD brain is characterized by a phenomenon known as brain insulin resistance (BIR) – broadly defined as the inadequate response to insulin by target cells [13] – due to reduced insulin receptor (IR) activation and increased levels of inhibitory phosphorylation of the insulin receptor substrate-1 (IRS1) on specific serine (Ser) residues [9,14,15].

Consistent with prior studies from the Butterfield group and others [20–23], some of the common clinical signs and symptoms of T2DM and AD could arise from an impairment of the activity of biliverdin reductase-A (BVR-A). BVR-A, the main isoform of BVR [24], is a pleiotropic enzyme primarily known for its canonical activity (reductase activity) named for the reduction of heme-derived biliverdin (BV) into the powerful antioxidant and anti-nitrosative molecule bilirubin (BR) [25,26]. But BVR-A also has a unique serine/threonine/tyrosine (Ser/Thr/Tyr) kinase directly involved in insulin signaling [22,27]. Indeed, BVR-A is a direct target of IR, which stimulates BVR-A kinase activity (thereafter indicated as BVR-A activation) via Tyr phosphorylation [22]. Once activated, BVR-A is able to phosphorylate IRS1 on Ser inhibitory domains, thus representing an upstream regulator in the insulin signaling cascade [22].

We have previously reported the oxidative stress-induced impairment of BVR-A in the hippocampus of AD and amnestic mild cognitive impairment (aMCI) subjects due to reduced Tyr phosphorylation and increased 3-nitrotyrosine (3-NT) modifications, thus questioning about the real neuroprotective role of BVR-A [20,21,28]. Based on these results and given the above background, decreased BVR-A activation would have deleterious effects including the inhibition of the insulin signaling pathway.

In the present work, we longitudinally analyze the age-dependent changes of BVR-A protein levels and activation, as well as IR/IRS1 levels and activation in the hippocampus of the triple transgenic model of AD (3xTg-AD) mice, which develop both Aβ and tau pathologies in an age-dependent manner [29]. The hippocampus is one of the brain regions mostly affected by amyloid beta (Aβ) and tau pathologies in the 3xTg-AD mice and whose alterations have major functional impact in AD symptoms. Further, we profile the molecular mechanisms responsible for the onset of BIR, by focusing on the contribution of oxidative/nitrosative stress. Our data suggest that BVR-A integrates both oxidative/nitrosative stress- and insulin-mediated signaling, both mechanisms dysregulated in AD brain.

2. Materials and methods

2.1. Animals

3, 6, 12 and 18 months-old 3xTg-AD male mice ($n=6$ per group) and their wild-type (WT) male littermates ($n=6$ per group) were used in this study. The 3xTg-AD mice harbour harbour 3 mutant human genes (APP_{Swe}, $PS1_{M146V}$, and tau_{P301L}) and have been genetically engineered by LaFerla and colleagues at the Department of Neurobiology and Behavior, University of California, Irvine [29]. Colonies of homozygous 3xTg-AD and WT mice were established at the vivarium of Puglia and Basilicata Experimental Zooprophylactic Institute (Foggia, Italy). The 3xTg-AD mice background strain is C57BL6/129SvJ hybrid and genotypes were confirmed by PCR on tail biopsies [29]. The housing conditions were controlled (temperature 22 °C, light from 07:00–19:00, humidity 50%–60%), and fresh food and water were freely available. All the experiments were performed in strict compliance with the Italian National Laws (DL 116/92), the European Communities Council Directives (86/609/EEC). All efforts were made to minimize the number of animals used in the study and their suffering. Animals were sacrificed at the selected age and the hippocampus was extracted, flash-frozen, and stored at −80 °C until total protein extraction and further analyses were performed.

2.2. Immunohistochemistry

Briefly both 3xTg and WT mice at 3, 6, 12 and 18 months of age ($n=3$ per group, per genotype) were intra-cardioventricularly perfused with saline followed by fixation solution (4% paraformaldehyde in PBS 0.1 M, pH 7.4) at a flow rate of 36 ml min^{-1} [30]. Brains were post-fixed in the fixation solution for 1 day and then transferred in 0.02% sodium azide in PBS. Free-floating coronal sections of 50 μm thickness were obtained using a vibratome slicing system (microM, Walldorf, Germany) and stored at 4 °C in 0.02% sodium azide in PBS. The endogenous peroxidase activity was quenched for 30 min in 0.3% H_2O_2. Sections were then pretreated in 90% formic acid and incubated overnight at 4 °C either with the monoclonal 6E10 antibody (1:3000, Signet Laboratorie–Covance, Emeryville, CA, USA, #sig-39320) for Aβ staining, or with the human-specific anti-tau antibody, HT7 (1:2000, Thermo Scientific Pierce Product, Rockford, IL, USA, #MN1000). After removing the primary antibody in excess, sections were incubated with the appropriate secondary antibody and developed with diaminobenzidine substrate using the avidin-biotin horseradish peroxidase system (Vector Laboratories, Inc, Burlingame, CA, USA, #SK-4100; #PK-6100). All stained slices were viewed using a Nikon 80i Eclipse microscope equipped with a DS-U1 digital camera, and NIS-elements BR software (Nikon, Tokyo, Japan). The intensity of Aβ and tau immunostaining was measured semi-quantitatively as regional optical density using the Scion Image software, as previously reported [30–32]. Per each animal, measurements were obtained in at least 3 consecutive sections containing the region of interest. The averaged optical densities of non-immunoreactive regions of each section were used for background normalization.

2.3. Cell culture and treatments

The SH-SY5Y neuroblastoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (20 units/ml) and streptomycin (20 mg/ml), (GIBCO, Gaithersburg, MD, U.S.A.). Cells were maintained at 37 °C in a saturated humidity atmosphere containing 95% air and 5% CO_2. Cells were seeded at density of $40 \times 10^3/cm^2$ in 6 wells culture dishes. After 24 h medium has been replaced with DMEM with 1% FBS and cells have been treated with (i) insulin, (ii) H_2O_2 in separate sets of experiments as following described. To test the responsiveness of our cellular model to insulin signaling the experiments have been performed as previously described with minor modifications [33]. Briefly, SH-SY5Y neuroblastoma cells were pre-treated with insulin (humulin®R, Ely-Lilly, Inadianapolis, IN, USA) 0.1 μM or vehicle (PBS) for 24 h. Insulin concentration has been selected based on previous reports [22,33]. Then, medium was discarded, cells were washed twice with PBS, and rechallenged with DMEM with 1% FBS containing insulin (0.1–0.5–1–5 μM) or vehicle (PBS) for an additional hour to mimic insulin over-exposure. To test the effects of oxidative/nitrosative stress on the insulin signaling, cells have been treated with peroxynitrite ($ONOO^-$, 50–500 μM) or hydrogen peroxide (H_2O_2, 1–50 μM) (Sigma-Aldrich, St Louis, MO, USA, #16911) or vehicle (PBS) for 24 h. To test the effects produced by