Neurobiology of Aging 50 (2017) 25–29

Contents lists available at ScienceDirect

Neurobiology of Aging

journal homepage: www.elsevier.com/locate/neuaging

Brief communication

Old age potentiates cold-induced tau phosphorylation: linking thermoregulatory deficit with Alzheimer's disease

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ARTICLE INFO

Article history:
Received 9 April 2016
Received in revised form 9 September 2016
Accepted 29 September 2016
Available online 12 October 2016

Keywords:
Aging
Thermoregulation
Tau
Alzheimer's disease
Body temperature

ABSTRACT

Thermoregulatory deficits coincide with a rise in the incidence of Alzheimer's disease (AD) in old age. Lower body temperature increases tau phosphorylation, a neuropathological hallmark of AD. To determine whether old age potentiates cold-induced tau phosphorylation, we compared the effects of cold exposure (4 °C, 24 hours) in 6- and 18-month-old mice. Cold-induced changes in body temperature, brown adipose tissue activity, and phosphorylation of tau at Ser202 were not different between 6- and 18-month-old mice. However, following cold exposure, only old mice displayed a significant rise in soluble tau pThr181 and pThr231, which was correlated with body temperature. Inactivation of glycogen synthase kinase 3β was more prominent in young mice, suggesting a protective mechanism against cold-induced tau phosphorylation. These results suggest that old age confers higher susceptibility to tau hyperphosphorylation following a change in body temperature, thereby contributing to an enhanced risk of developing AD.

1. Introduction

Aging is by far the most important risk factor for Alzheimer's disease (AD) (Alzheimer's Association, 2015; Querfurth and LaFerla, 2010; Rocca et al., 2011; Ziegler-Graham et al., 2008). Even familial forms of the disease are rarely expressed clinically before the fifth decade of life (Ridge et al., 2013). Thus, a better understanding of how old age contributes to AD pathogenesis will likely provide critical clues on potential treatments. Strikingly, thermoregulatory deficits appear at the same time in life when the incidence of AD greatly increases (Degroot and Kenney, 2007; Florez-Duquet and McDonald, 1998). In fact, mean body temperature in the elderly has been long known to be lower than in young people and below 37 °C (Gomolin et al., 2005; Hoshino et al., 2007; Howell, 1975), increasing their risk of hypothermia (Fox et al., 1973; Whittington et al., 2010). Furthermore, several other parameters linked to thermoregulation are affected in the elderly: energy metabolism (Cunnane et al., 2011; Frisard et al., 2007), thermogenic activity of brown adipose tissue (BAT) (Cypess et al., 2009), ability to recover after cold exposure (Florez-Duquet and McDonald, 1998; Sugarek, 1986), and circadian cycles of body temperature (Weinert, 2010).

Preclinical investigations in animal models have brought potential mechanisms coupling thermoregulatory deficits to AD pathogenesis. Most prominently, hypothermia in mice has repeatedly been shown to increase tau phosphorylation and pathology in the brain (Planel et al., 2004, 2009; Vandal et al., 2016), one of the main neuropathological markers of AD (Cowan and Mudher, 2013; Frost et al., 2014; Tremblay et al., 2007). Although the link between lower body temperature and tau phosphorylation was evidenced in multiple experiments, to our knowledge, no study investigated the role of age in this mechanism.

Since the appearance of thermoregulation deficits coincides with a higher incidence of AD and that a lower body temperature has repeatedly been shown to increase tau phosphorylation (Arendt, 2003; Planel et al., 2007; Whittington et al., 2010), we aimed to verify whether this post-translational mechanism was potentiated in old mice compared with younger mice. Using 6- and 18-month-old mice exposed to 4 °C during 24 hours, we found that cold exposure led to a higher increase in soluble phosphorylated tau in old compared to young mice. In parallel, only young mice displayed higher levels of the inactivated form of glycogen synthase kinase 3β (GSK3β) in the cortex following

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http://dx.doi.org/10.1016/j.neurobiolaging.2016.09.024

cold exposure, suggesting that GSK3β inactivation is a potential protective mechanism against tau hyperphosphorylation that is lost in old animals.

2. Methods

2.1. Animals and cold exposure

All experiments were performed in accordance with the Canadian Council on Animal Care and were approved by the Institutional Committee of the Centre Hospitalier de l'Université Laval. 6- and 18-month-old C57BL6/129SvJ mice produced at our animal facility were used in an equal number of males and females in each group. One animal per cage was housed at 4 °C or 22 °C during 24 hours. Body temperature was determined rectally with an electronic thermometer coupled with a rectal probe before and after exposure at the same hour of the day (8 AM). All mice were kept under their housing temperature (4 °C or 22 °C) until sacrifice by intracardiac perfusion as described elsewhere (Julien et al., 2009). Although it is known that anesthesia has an effect per se on body temperature (Lenhardt, 2010), all mice were put under deep anesthesia with ketamine/xylazine injection (100 mg/kg ketamine, 10 mg/kg xylazine) for ethical reasons. Rapidly, interscapular BAT and brain were dissected and frozen at −80 °C until processing for Western blot. In a separate experiment, we found that exposure to 4 °C for 24 hours before anesthesia significantly aggravated the ketamine-induced decrease in body temperature (−3.23 ± 0.64 °C, n = 7) compared to exposure to room temperature (−1.61 ± 0.59 °C, n = 7).

2.2. Protein extraction and Western immunoblotting

The protein extraction method results in a TBS-soluble intracellular and extracellular fraction (cytosolic fraction), a detergent-soluble fraction (membrane fraction), and a detergent-insoluble fraction solubilized in formic acid as previously described (Ishihara et al., 1999; Lebbadi et al., 2011). The detailed method for Western immunoblotting is described elsewhere (Vandal et al., 2014). The list of primary antibodies used in our experiments is available in Supplementary Table 1. All brain homogenates from the same experiment were put on the same gel, but consecutive bands were not taken for all representative photo examples.

2.3. Quantitative real-time PCR

The expression level of uncoupling protein 1 (*UCP1*) messenger RNA was determined in BAT using a reverse transcription real-time quantitative polymerase chain reaction (qPCR). Frozen BATs were powderized, and 10 mg of each sample was homogenized in 1 mL QIAzol lysis reagent (Qiagen, Valencia, CA, USA) to which was added 200 μL chloroform (J.T. Baker, Center Valley, PA, USA). RNA was extracted with an RNeasy lipid tissue mini kit (Qiagen, Valencia, CA, USA). The High Capacity cDNA Reverse Transcription kit with random hexamer primers and MultiScribe Reverse Transcriptase (Life Technologies, Burlington, ON, Canada) was used to synthesize complementary DNA according to the instructions from the manufacturer. Then, TaqMan qPCR primers were used to amplify mouse *UCP1* (TaqMan Gene Expression Assays, Mm01244861_m1 from Life Technologies) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, Mm99999915_g1, Life Technologies) as the housekeeping gene. Reactions were prepared in triplicates and cycle threshold (Ct) values were measured using a real-time qPCR cycler (MxPro-Mx3005P, Agilent Technologies, Mississauga, ON, Canada) in 96-well optical plates covered with adhesive optical film. Each run contained a "no template" control for both genes. The delta delta Ct (ddCT) method was used to evaluate differences in relative gene expression between groups using *GAPDH* as a control gene. Results are presented as ratios of *UCP1*/*GAPDH* complementary DNA on the control group.

2.4. Statistical analysis

Data are presented as means ± standard error of the mean or as relative change observed in cold-exposed mice compared with control mice. Statistical analysis and number of mice per group are specified in each figure. Bartlett's tests were used to rule out inequality of variances between the groups. One-way (one independent variable) or 2-way analysis of variance (ANOVA) (2 independent variables) were used when more than 2 groups were compared. ANOVAs were followed by Tukey's post hoc analysis in case of equal variance or by a Dunnett's post hoc analysis in case of unequal variances. An unpaired Student *t* test was performed when only 2 groups were compared, with a Welch correction when variances were not equal. Correlations between variables were investigated using linear regression analyses. All statistical analyses were performed with Prism 5 (GraphPad software, San Diego, CA, USA) or JMP (Version 12.1.0; SAS Institute Inc, Cary, IL, USA) software, and statistical significance was set at $p < 0.05$.

3. Results

3.1. Enhanced cold-induced phosphorylation of soluble tau in old mice compared to young mice

Cold exposure at 4 °C is a classical method to study nonshivering thermogenesis in rodents (Cannon and Nedergaard, 2010; Lim et al., 2012). Thus, we first confirmed that cold exposure induced a decrease in body temperature and an increase in thermogenesis, as indexed by the enhanced ratio of *UCP1* on *GAPDH* expression in BAT compared to animals maintained at 22 °C. However, no significant difference in body temperature or BAT activation was detected between 6- and 18-month-old mice (Fig. 1A and B).

Given the importance of tau phosphorylation in AD pathogenesis (Cowan and Mudher, 2013; Frost et al., 2014; Querfurth and LaFerla, 2010; Tremblay et al., 2007), we then assessed the extent by which cold exposure potentiates the phosphorylation of key epitopes. We found that 18-month-old mice displayed a greater hyperphosphorylation of tau in response to cold exposure. More specifically, although cold exposure increased the phosphorylation of tau at epitopes pSer396/404 in young mice (+40%) and pSer202 in both 6- and 18-month-old mice (+101% and +132%, respectively), only old mice displayed an increase in pThr181 and pThr231 (+41% and +61%, respectively; Fig. 1C and D). Two-way ANOVA further confirmed the upregulating effect of a lower temperature on phosphorylated tau species in the soluble fraction ($p = 0.0001$ for pSer202; $p = 0.026$ for pThr231, and $p = 0.031$ for pThr181).

Interestingly, the phosphorylation of soluble tau in response to cold exposure was correlated with body temperature suggesting that both events are linked. In fact, we found that tau pSer202 was correlated with body temperature in both 6- and 18-month-old mice ($r^2 = 0.16$ and 0.43, respectively; Fig. 1E). However, phosphorylation levels of soluble tau at pThr231 and pThr181 were correlated with body temperature only in old mice ($r^2 = 0.29$ and $r^2 = 0.47$, respectively), not in young ones (Fig. 1F and G).

3.2. GSK3β inactivation in young but not in old mice

Since tau phosphorylation is regulated by kinases and phosphatases (Wang et al., 2013), we next measured several of these key enzymes in cortex homogenates of young and old mice. No significant changes were found in main phosphatases and kinases