GeroScience (2018) 40:513–521
https://doi.org/10.1007/s11357-018-0047-6

ORIGINAL ARTICLE

Nrf2 deficiency in aged mice exacerbates cellular senescence promoting cerebrovascular inflammation

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Received: 2 November 2018 / Accepted: 14 November 2018 / Published online: 23 November 2018

Abstract Aging-induced pro-inflammatory phenotypic alterations of the cerebral vasculature critically contribute to the pathogenesis of vascular cognitive impairment. Cellular senescence is a fundamental aging process that promotes inflammation; however, its role in cerebrovascular aging remains unexplored. The present study was undertaken to test the hypothesis that advanced aging promotes cellular senescence in the cerebral vasculature. We found that in cerebral arteries of 24-month-old mice, expression of molecular markers of senescence ($p16^{INK4a}$, p21) is upregulated as compared to that in young controls. Induction of senescence programs in cerebral arteries is associated by an upregulation of a wide range of inflammatory cytokines and chemokines, which are known to contribute to the senescence-associated secretory phenotype (SASP) in vascular cells. Age-related cerebrovascular senescence and inflammation are associated with neuroinflammation, as shown by the molecular footprint of microglia activation in the hippocampus. Genetic depletion of the pro-survival/anti-aging transcriptional regulator Nrf2 exacerbated age-related induction of senescence markers and inflammatory SASP factors and resulted in a heightened inflammatory status of the hippocampus. In conclusion, our studies provide evidence that aging and Nrf2 dysfunction promote cellular senescence in cerebral vessels, which may potentially cause or exacerbate age-related pathology.

Keywords Vascular aging · Senescence · Vascular cognitive impairment · Endothelial dysfunction · VCID

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Introduction

Aging-induced pro-inflammatory phenotypic alterations of cerebral arteries and arterioles promote atherogenesis,

dysregulation of cerebral blood flow, blood brain barrier disruption and/or exacerbation of neuroinflammation, significantly contributing to the pathogenesis of age-related vascular cognitive impairment (VCI) (Toth et al. 2017). Inflammation-related vascular contributions to cognitive impairment are common in old age and play critical roles in the pathogenesis of the entire spectrum of cognitive impairment from mild to more severe forms, including ischemic and hemorrhagic stroke, cognitive impairment associated with subclinical microvascular injury as well as Alzheimer's disease. In order to develop novel therapeutic interventions to promote cerebrovascular health preserving cognitive function in older persons, it is essential to understand the cellular and molecular mechanisms through which aging promotes vascular inflammation in the cerebral circulation.

Among the cellular and molecular mechanisms contributing to organismal aging in recent years cellular senescence has emerged as a fundamental aging process (Baker et al. 2016; Jeon et al. 2017; Tchkonia et al. 2013). Upon induction of cellular senescence, cells permanently withdraw from the cell cycle and undergo distinctive phenotypic alterations, including significant pro-inflammatory secretome changes (Freund et al. 2010). Importantly, elimination of senescent cells expressing the cell cycle regulator protein p16INK4A extends lifespan and health span in mice, reducing the inflammatory status of many organs (Baker et al. 2016). Activation of cellular senescence programs have also been suggested to contribute to vascular pathology (Regina et al. 2016; Wang et al. 2015; Yamazaki et al. 2016; Uryga and Bennett 2016; Gardner et al. 2015; Liu et al. 2018; Silva et al. 2017; Matthews et al. 2006). Despite these advances, the role of senescence in cerebrovascular aging remains unexplored.

Nrf2 (NF-E2-related factor 2) is a key redox sensitive transcription factor, which regulates the expression of detoxification and antioxidant enzymes, factors involved in repair of oxidative macromolecular damages and other cell survival pathways and thereby exerts multifaceted anti-aging vasoprotective effects (Tarantini et al. 2018a; Ungvari et al. 2010; Ungvari et al. 2011a, b; Valcarcel-Ares et al. 2012; Csiszar et al. 2012). In healthy organisms, Nrf2-mediated homeostatic responses serve to attenuate vascular oxidative stress, limit free radical-induced cellular and macromolecular damages, and protect the viability and function of endothelial cells (Ungvari et al. 2011a, b). Nrf2 was also shown to inhibit pathways involved in induction of cellular senescence (Zhou et al. 2016; Volonte et al. 2013; Kapeta et al. 2010). Several pathophysiological conditions may impair the Nrf2 system, including metabolic diseases (Tan et al. 2011). There is increasing evidence that Nrf2 dysfunction promote accelerated vascular aging by impairing cellular stress resilience, increasing oxidative stress and promoting inflammatory phenotypic alterations (Tarantini et al. 2018a; Ungvari et al. 2011c). Recent studies also demonstrate that while Nrf2 dysfunction in cerebral microvessels in the absence of an oxidative stressor in otherwise healthy young animals do not impair endothelial barrier function (Tarantini et al. 2018a; Joshi et al. 2015), it significantly exacerbates metabolic stress-induced disruption of the blood-brain barrier, contributing to increased microglia activation, neuroinflammation and neuronal dysfunction (Tarantini et al. 2018a). Despite these advances, the role of Nrf2 in protection against cellular senescencein the cerebrovasculature has not yet been explored.

The present study was undertaken to test the hypothesis that advanced aging promotes cellular senescence in the cerebral vasculature, which is exacerbated by Nrf2 deficiency. To test our hypothesis, we assessed age-related changes in expression of molecular markers of senescence in mouse cerebral arteries. Induction of senescence programs in vascular endothelial and smooth muscle cells is associated by an upregulation of a wide range of inflammatory cytokines and chemokines (Ungvari et al. 2013), termed the "senescence-associated secretory phenotype," or SASP. Thus, as an additional outcome measure, we also assessed vascular expression of pro-inflammatory SASP factors. To determine the role of Nrf2 in regulation of vascular senescence, cerebral arteries isolated from aged Nrf2 deficient ($Nrf2^{-/-}$) and wild-type mice were compared. To determine the relationship among induction of cerebrovascular senescence, vasomotor function and neuroinflammation, we assessed endothelium-mediated vasodilation in cannulated, pressurized cerebral arteries and characterized the molecular footprint of microglia activation in the mouse hippocampus.

Methods

Animal models

Male wild-type control C57BL/6J mice ($Nrf2^{+/+}$; age 3 and 24 months old) and Nrf2 knockout mice ($Nrf2^{-/-}$

[B6.129X1-Nfe2l2^{tm1Ywk}/J]; age 24 months old; purchased from Jackson Laboratories; JAX stock #017009) were used. In this study, only male mice were studied to exclude the possible confounding effects of the estrous cycle in females. The mice were housed in an environmentally controlled vivarium with unlimited access to water and a controlled photoperiod (12-h light; 12-h dark). All mice were maintained according to National Institutes of Health guidelines and all animal use protocols were approved by the Institutional Animal Care and Use Committee of the University of Oklahoma HSC.

Assessment of endothelial NO-mediated vasodilation in isolated cerebral vessels

As impaired cerebrovascular dilation plays an important role in the pathogenesis of cognitive impairment (Csiszar et al. 2017; Tarantini et al. 2017a, b; Tucsek et al. 2017; Ungvari et al. 2017a), we assessed the effects of aging and Nrf2 deficiency on endothelial NO mediation in isolated, cannulated, and pressurized segments of the middle cerebral arteries, as reported (Tarantini et al. 2018b). Dilations to acetylcholine and ATP were obtained in the absence and presence of L-NAME (3×10^{-4} mol/L, for 30 min). At the end of each experiment, the vessels were superfused with Ca^{2+}-free Krebs' buffer containing nifedipine (10^{-5} mol/L) to achieve maximal vasodilatation.

Assessment of the integrity of the blood-brain barrier

To quantify blood-brain barrier (BBB) permeability, we used the sodium fluorescein tracer assay as reported (Toth et al. 2013). In brief, in anesthetized mice, the small water-soluble tracer sodium fluorescein (5 ml/kg, 2% in physiological saline) was administered intravenously by retroorbital injection. After 30 min of circulation of the tracer, animals were transcardially perfused with 1× heparin containing PBS. Then, the mice were decapitated and the brains were removed. From each brain, the hippocampus, white matter, and the prefrontal cortex were isolated and homogenized. The extravasated sodium fluorescein was quantified spectrophotofluorometrically using a microplate reader and normalized to tissue weight.

Quantitative real-time RT-PCR

A quantitative real-time RT-PCR technique was used to analyze mRNA expression in middle cerebral arteries and hippocampal samples (collected using standard isolation protocols (Tarantini et al. 2018b)), using validated TaqMan probes (Applied Biosystems) and a Strategen MX3000 platform (Tarantini et al. 2018b).

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A p value less than 0.05 was considered statistically significant. Data are expressed as mean ± S.E.M.

Results

Nrf2 deficiency exacerbates aging-induced cellular senescence in cerebral arteries

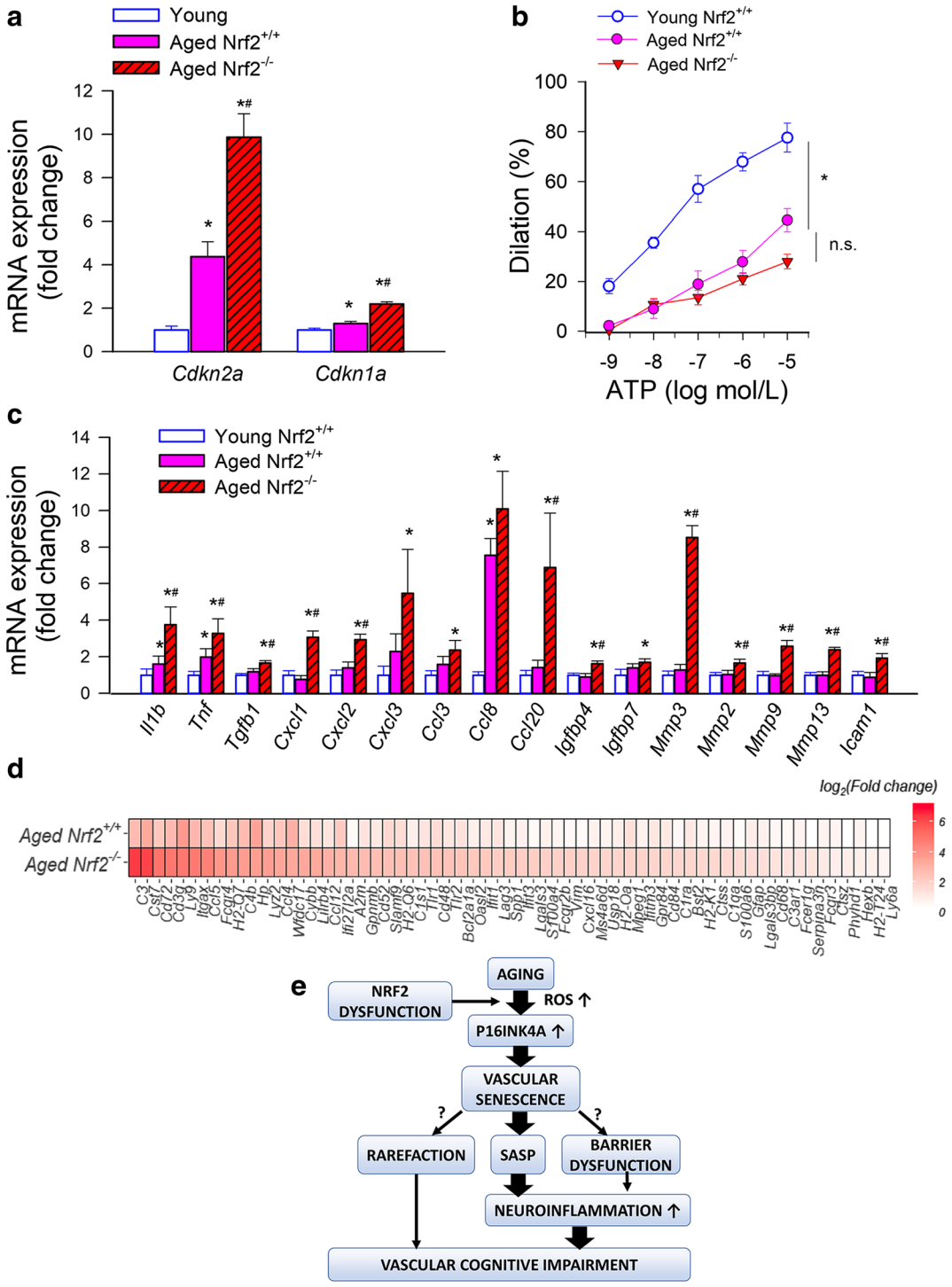
The senescence response induced by oxidative stress and DNA damage in cerebromicrovascular endothelial cells is controlled by a signaling pathway that leads to induction of cyclin-dependent kinase inhibitors p16^{INK4a} (CDKN2A) and p21 (CDKN1A) (Ungvari et al. 2013). Here, we report that aging is associated with upregulation of these molecular markers of senescence in cerebral arteries, which is exacerbated by genetic depletion of Nrf2 (Fig. 1a).

Effects of Nrf2 deficiency and aging on endothelial function in cerebral arteries

To determine how Nrf2 depletion affects endothelial function, endothelium-dependent vasodilator responses were tested in isolated, cannulated branches of the middle cerebral artery. In young vessels, administration of ATP resulted in significant dilation, whereas these responses were significantly attenuated in vessels derived from aging mice (Fig. 1b). Genetic depletion of Nrf2 tended to further impair ATP-induced vasodilation; yet, these differences did not reach statistical significance. To assess the role of endothelium-derived NO, the eNOS inhibitor L-NAME was applied. L-NAME abolished ATP-induced dilator responses, eliminating the differences between the three groups (data not shown).

Nrf2 deficiency exacerbates aging-induced inflammation in cerebral arteries

Previous studies show that senescent vascular endothelial cells and smooth muscle cells acquire a complex



phenotype that includes the secretion of many inflammatory cytokines and chemokines, termed a senescence-associated secretory phenotype (SASP) (Ungvari et al. 2013). It has been proposed that SASP in endothelial and smooth muscle cells contribute to age-related vascular inflammation (Ungvari et al.

◀ **Fig. 1** Nrf2 deficiency exacerbates cerebrovascular senescence, promoting inflammation in aging. **a** Nrf2 deficiency exacerbates age-related upregulation of mRNA expression (qPCR) of the senescence markers *Cdkn2a* and *Cdkn1a* (encoding $p16^{INK4}$ and p21, respectively) in mouse cerebral arteries. **b** Effects of aging and Nrf2 deficiency on ATP-induced dilation of cannulated middle cerebral arteries. **c** Nrf2 deficiency exacerbates age-related upregulation of mRNA expression of pro-inflammatory SASP factors in in mouse cerebral arteries. **d** Gene expression footprint of microglia activation. The heat map shows relative changes in mRNA expression (qPCR) of microglia activation-related genes in the hippocampi of aged $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice as compared to that in young mice. Data are means ± S.E.M. ($n = 6–8$ in each group), $^{*}P < 0.05$ vs. young, $\#P < 0.05$ vs. aged $Nrf2^{+/+}$. **e** Proposed scheme showing that Nrf2 dysfunction exacerbates aging-induced cerebrovascular senescence, which likely contributes to the pathogenesis of cognitive impairment by promoting neuroinflammation (due to the paracrine effects of the pro-inflammatory SASP and/or by disrupting the blood-brain barrier (Tarantini et al. 2018a)) and microvascular rarefaction (Valcarcel-Ares et al. 2012; Ungvari et al. 2013)

2018). Here, we report that increased cellular senescence is associated with pro-inflammatory phenotypic changes in aged cerebral arteries, which include upregulation of the SASP factors IL-1β and TNFα (Fig. 1c). Genetic depletion of Nrf2 exacerbated the age-related upregulation of multiple SASP factors, including cytokines, chemokines, IGF-1 binding proteins, and matrix metalloproteinases (MMPs; Fig. 1c).

Effects of aging and Nrf2 deficiency on BBB integrity and the hippocampal expression of genes involved in microglia activation and neuroinflammation

Using a sodium fluorescein tracer assay, we found that Nrf2 depletion exacerbates aging-induced fluorescein leakage in the hippocampi indicating BBB disruption (fold changes; aged $Nrf2^{+/+}$: ~1.2; aged $Nrf2^{-/-}$: ~1.9 vs. young controls). Gene expression profiling demonstrated that age-related BBB disruption and cerebrovascular inflammation is associated with a cerebral gene expression signature indicative of microglia activation (Fig. 1d), extending previous findings both in human (Berchtold et al. 2008) and mouse (Masser et al. 2014; Mangold et al. 2017) hippocampi. Previous studies suggest that pro-inflammatory vascular phenotype exacerbates neuroinflammation, in part by promoting leakage of plasma-derived factors through the damaged BBB (Toth et al. 2013), which is a potent stimulus for microglia activation (Tucsek et al. 2014). In accordance with this concept, we found that in the hippocampi of aged mice Nrf2 depletion promoted significant upregulation of microglia-enriched pro-inflammatory genes (Fig. 1d).

Discussion

Results from the present study demonstrate for the first time that advanced aging promotes cellular senescence in the cerebral arteries, as indicated by the increased expression of several independent molecular markers of senescence (Fig. 1a). Further studies are needed to determine whether p16INK4A-mediated senescence programs are primarily induced in aged endothelial cells, smooth muscle cells or in adventitial cells.

The stress-activated "cap'n'collar" transcription factor Nrf2 plays an important role in regulating the aging process by orchestrating the transcriptional response of cells to oxidative stress and DNA damage (Pearson et al. 2008a). Regulation of the expression of antioxidant enzymes and DNA repair pathways by homologs of Nrf2 is evolutionarily highly conserved and studies on model organisms demonstrate that knockdown of homologs of Nrf2 shortens lifespan (Jasper 2008). Previous studies also demonstrate that Nrf2 contributes to the anti-aging effects of caloric restriction in rodent models (Pearson et al. 2008a). Our findings that aging-induced p16INK4A-mediated cellular senescence in cerebral vessels is exacerbated by genetic disruption of the Nrf2-dependent cytoprotective pathways suggest that age-related increased oxidative stress and consequential DNA damage are likely critical inducers of the senescent program in vascular cells. These results extend those of previous studies demonstrating a critical role for increased oxidative stress in functional and phenotypic alterations in the aged cerebral vasculature (Tarantini et al. 2018b). We posit that attenuation of oxidative stress and inhibition of cellular senescence may critically contribute both to the established anti-aging (Pearson et al. 2008a) and vasoprotective effects (Ungvari et al. 2010; Valcarcel-Ares et al. 2012; Ungvari et al. 2011c) of Nrf2. This concept is supported by the findings that Nrf2 deficiency exacerbates oxidative stress and promotes accelerated cerebrovascular aging in mice exposed to high fat diet-induced metabolic stress, which is associated with increased senescence (Tarantini et al. 2018a).

From a pathophysiological standpoint, activation of senescence programs in the aged cerebral vasculature is expected to impair vasomotor function and angiogenesis and promote inflammation contributing to the development of cerebrovascular diseases and vascular cognitive impairment. This conclusion is supported by the results of previous studies showing that DNA damage-induced senescence is associated with impaired neurovascular coupling, capillary rarefaction and upregulation of inflammatory processes in a mouse models of whole brain irradiation-induced accelerated brain aging (Ungvari et al. 2013, 2017b). Advanced atherosclerotic lesions also contain senescent cells and previous investigations using genetic and pharmacological approaches to eliminate senescent cells in $Ldlr^{-/-}$ mice suggest that senescent cells promote the development of atherosclerotic vascular diseases (Childs et al. 2016). Importantly, chronic treatment with senolytic drugs was also demonstrated to improve endothelial function in mouse models of aging (Roos et al. 2016). Importantly, Nrf2 deficiency, which promotes senescence, inhibits angiogenic capacity of cerebromicrovascular endothelial cells (Valcarcel-Ares et al. 2012), exerts pro-atherogenic effects, and exacerbates endothelial dysfunction (Tarantini et al. 2018a). p16INK4A-mediated cellular senescence in vascular cells is known to be associated with the development of a highly pro-inflammatory SASP (Ungvari et al. 2013). Induction of senescence programs in aged cerebral arteries is also associated by an upregulation of a wide range of inflammatory mediators (cytokines, chemokines and MMPs), consistent with the induction of a SASP. Additionally, inflammatory foci associated with senescence vascular endothelial and/or smooth muscle cells likely disrupt the blood brain barrier (e.g., due to the upregulation of matrix metalloproteinases, which degrade the extracellular matrix). Inflammatory factors released from the vasculature and plasma-derived factors leaking through the damaged blood-brain barrier are expected to promote microglia activation and thereby exacerbate neuroinflammation. Analysis of the gene expression footprint of microglia activation in aged Nrf2 deficient mice provides preliminary evidence that induction of cerebrovascular senescence associates with increased neuroinflammation.

A number of important limitations of the present study need to be considered. First, direct demonstration of senescent cells in the cerebral vessels (e.g., using senescence reporter mice) should be performed in future studies. Second, further studies are warranted to elucidate the exact molecular mechanisms by which senescence endothelial cells contribute to BBB disruption, including dysregulation of tight junction (Yamazaki et al. 2016) and the paracrine role of SASP. Future studies are warranted to experimentally test role of cerebrovascular senescence in induction of neuroinflammation and their contribution to cognitive decline. Subsequent studies should also test the synergistic effects of aging and Nrf2 depletion on other aspects of endothelial phenotype as well, including neurovascular coupling responses, microvascular network architecture and capillary density.

In conclusion, our studies provide evidence that aging promotes cellular senescence in cerebral vessels, which is exacerbated by impaired cellular resilience linked to the depletion of the highly conserved anti-aging transcription factor Nrf2. Previous studies suggest that Nrf2 may provide a therapeutic target for cerebrovascular protection countering oxidative stress associated with aging and pathological conditions characterized by accelerated vascular aging (Tarantini et al. 2018a; Ungvari et al. 2011b, c). In that regard, it is significant that in vascular cells Nrf2 can be activated pharmacologically (Ungvari et al. 2010), which was shown to confer vasoprotection in rodent models of aging, upregulating antioxidant systems, decreasing oxidative stress and attenuating vascular inflammation (Pearson et al. 2008b). Further studies are warranted to determine whether chronic treatment with Nrf2 activators can protect against induction of cellular senescence in the cerebral circulation, mitigating its deleterious consequences in the aging brain. In recent years, microvascular contributions to neurodegeneration as well as the role of cellular senescence and the senescent secretory phenotype to brain pathologies have been increasingly recognized. Importantly, previous studies demonstrated that intact Nrf2 signaling protects against oxidative stress-mediated cellular damage and neurotoxicity in mouse models of Parkinson's disease (Rojo et al. 2010) and Alzheimer's disease (Joshi et al. 2015). Thus, further studies are warranted to determine the link between Nrf2 signaling in the vascular cells and senescence-induced sterile microvascular inflammation in neurodegenerative diseases and develop therapies targeting Nrf2 for microvascular protection and prevention of dementia.

Author contribution GF, AC, and ZU designed research; GF, TK, ST, AY, and AC performed experiments; GF, ST, AY, EF, ZU, and AC analyzed and interpreted data; GF, AC, and ZU wrote the manuscript, TK, FB, EF, ST, and AY revised the paper.

Funding information This work was supported by grants from the American Heart Association (to ST), the National Institute on Aging (R01-AG055395 to ZU, R01-AG047879 to AC; R01-AG038747), the National Institute of Neurological Disorders and Stroke (NINDS; R01-NS056218 to AC, R01-NS100782 to ZU), the NIA-supported Geroscience Training Program in Oklahoma (T32AG052363), the NIA-supported Oklahoma Nathan Shock Center (to ZU and AC; 3P30AG050911-02S1), NIH-supported Oklahoma Shared Clinical and Translational Resources (to AY, NIGMS U54GM104938), the Oklahoma Center for the Advancement of Science and Technology (to AC, ZU, AY), the Presbyterian Health Foundation (to ZU, AC, AY), the EU-funded Hungarian grant EFOP-3.6.1-16-2016-00008, and the Reynolds Foundation (to ZU and AC).

Compliance with ethical standards

Disclaimer The data were presented at the 14th International Symposium on Neurobiology and Neuroendocrinology of Aging (July 15–20, 2018, Bregenz, Austria). This paper is part of a special collection of papers published in GeroScience on the oxidation-inflammation theory of aging (Tucsek et al. 2017; Konopka et al. 2017; Aiello et al. 2017; An et al. 2017; Blodgett et al. 2017; Deepa et al. 2017; Jackson et al. 2017; Lee et al. 2017; Meschiari et al. 2017; Nikolich-Zugich and van Lier 2017; Perrott et al. 2017; Rais et al. 2017; Shobin et al. 2017; Souquette et al. 2017).

Conflict of interest The authors declare that they have no conflict of interest.

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