GeroScience (2019) 41:619–630
https://doi.org/10.1007/s11357-019-00074-2

ORIGINAL ARTICLE

Nicotinamide mononucleotide (NMN) treatment attenuates oxidative stress and rescues angiogenic capacity in aged cerebromicrovascular endothelial cells: a potential mechanism for the prevention of vascular cognitive impairment

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Received: 11 May 2019 / Accepted: 16 May 2019 / Published online: 29 May 2019

Abstract Age-related impairment of angiogenesis likely has a critical role in cerebromicrovascular rarefaction and development of vascular cognitive impairment and dementia (VCID) in the elderly. Recently, we demonstrated that aging is associated with NAD^+ depletion in the vasculature and that administration of NAD^+ precursors exerts potent anti-aging vascular effects, rescuing endothelium-mediated vasodilation in the cerebral circulation and improving cerebral blood supply. The present study was designed to elucidate how treatment with nicotinamide mononucleotide (NMN), a key NAD^+ intermediate, impacts age-related impairment of endothelial angiogenic processes. Using cerebromicrovascular endothelial cells (CMVECs) isolated from young and aged F344xBN rats, we demonstrated that compared with young cells, aged CMVECs exhibit impaired proliferation, cellular migration (measured by a wound-healing assay using electric cell-substrate impedance sensing [ECIS] technology), impaired ability to form capillary-like structures, and increased oxidative stress.

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NMN treatment in aged CMVECs significantly improved angiogenic processes and attenuated H_2O_2 production. We also found that pre-treatment with EX-527, a pharmacological inhibitor of SIRT1, prevented NMN-mediated restoration of angiogenic processes in aged CMVECs. Collectively, we find that normal cellular NAD^+ levels are essential for normal endothelial angiogenic processes, suggesting that age-related cellular NAD^+ depletion and consequential SIRT1 dysregulation may be a potentially reversible mechanism underlying impaired angiogenesis and cerebromicrovascular rarefaction in aging. We recommend that pro-angiogenic effects of NAD^+ boosters should be considered in both preclinical and clinical studies.

Keywords Senescence · Endothelial dysfunction · Vascular contributions to cognitive impairment and dementia · Microcirculation · NAD^+ precursor

Introduction

The brain is the most energy-demanding organ, yet, it lacks energy stores. Normal neuronal function is therefore critically dependent on adequate supply of nutrients and oxygen through a dense network of over 600 km of cerebral microvessels. In the brain, the number of endothelial cells is very similar to that of neurons (Garcia-Amado and Prensa 2012) and nearly every neuron is supplied by its own capillary, with an average distance of 8–20 μm between the neuron and the microvessels. Aging-induced functional and structural impairments of the cerebral microcirculatory network have a critical role in the pathogenesis of age-related cognitive decline (Zlokovic 2011; Toth et al. 2013, 2017; Tucsek et al. 2014a, 2014b; Tarantini et al. 2016; Csiszar et al. 2017).

The dynamic balance between angiogenesis (new capillary formation from pre-existing microvessels) and microvascular regression is critical for the maintenance of a healthy cerebral microcirculatory network. Advanced aging is associated with a progressive deterioration of cerebromicrovascular homeostasis, at least in part, due to a significant impairment of endothelial angiogenic processes (Ingraham et al. 2008; Murugesan et al. 2012; Ungvari et al. 2018a). This results in cerebromicrovascular rarefaction/decreased capillary density in the aged brain, which contributes to a decline in cerebral blood flow compromising oxygen and nutrient delivery to the active neurons (Hagstadius and Risberg 1989; Martin et al. 1991; Kawamura et al. 1993; Moeller et al. 1996; Sonntag et al. 1997; Krejza et al. 1999; Lynch et al. 1999; Schultz et al. 1999; Bentourkia et al. 2000; Farkas and Luiten 2001; Khan et al. 2001; Pagani et al. 2002; Riddle et al. 2003; Mitschelen et al. 2009) and the formation of ischemic foci, neuronal dysfunction, demyelination, and, ultimately, to neurodegeneration (Sonntag et al. 1997, 2000; Khan et al. 2001; Ingraham et al. 2008; Warrington et al. 2011, 2012).

Sprouting angiogenesis, which is initiated by VEGF in poorly perfused hypoxic areas of the brain, is critical to satisfy the metabolic requirements of the neuronal tissue. Previous ex vivo studies provide strong evidence that cell-autonomous mechanisms contribute to age-related impairment of sprouting angiogenesis, compromising cellular angiogenic processes induced in response to VEGF in cerebromicrovascular endothelial cells (including endothelial cell proliferation and directed migration, tubulogenesis) (Ungvari et al. 2013; Csiszar et al. 2014). However, the molecular mechanisms, by which aging impairs VEGF-induced endothelial angiogenic processes, remain elusive (Lahteenvuo and Rosenzweig 2012).

NAD^+ acts as a coenzyme in electron transfer reactions, as a donor of ADP-ribose moieties in ADP-ribosylation reactions, as a precursor of the second messenger molecule cyclic ADP-ribose, and as a substrate for the longevity assurance factor sirtuin enzymes. Maintenance of NAD^+ levels is critical for normal cellular proliferation and function, regulation of mitochondrial metabolism and cellular bioenergetics, adaptive stress responses, and normal activation of pro-survival, anti-aging pathways. With advanced age, there is decreased availability of cellular NAD^+ (Massudi et al. 2012; Gomes et al. 2013; Yoshino et al. 2018), which may be a fundamental, evolutionarily conserved contributor to aging processes across tissues. Aging-induced NAD^+ depletion was suggested to predispose to a wide range of chronic diseases and pathological conditions associated with old age (Yang et al. 2007; Garten et al. 2009; Bonkowski and Sinclair 2016; de Picciotto et al. 2016; Imai and Guarente 2016; Schultz and Sinclair 2016; Das et al. 2018; Csiszar et al. 2019), including endothelial dysfunction (Csiszar et al. 2019). There is strong preclinical evidence that restoration of cellular NAD^+ levels in aged rodents by administration of NAD^+ precursors exerts potent anti-aging effects, reversing age-related organ dysfunction (Gomes et al. 2013; Mills et al. 2016; Johnson et al. 2018) and increasing mouse lifespan

(Zhang et al. 2016). Recently, we demonstrated that treatment of old mice with nicotinamide mononucleotide (NMN), a key NAD^+ intermediate, restores vascular NAD^+ levels, rescues endothelium-mediated vasodilation in the cerebral circulation, and improves cerebral blood supply (Tarantini et al. 2019).

The present study was designed to elucidate how NMN treatment impacts age-related impairment of endothelial angiogenic processes. Using cerebromicrovascular endothelial cells (CMVECs) isolated from young and aged F344xBN rats, we tested the hypothesis that chronic treatment of aged endothelial cells with NMN improves angiogenic capacity, including proliferation, migration, and ability to form capillary-like structures.

Materials and methods

Animals and endothelial cell isolation

We used Fischer 344x Brown Norway (F344xBN) rats as a model of aging, since this strain has a lower incidence of age-specific pathology than other rats. In F344xBN rats, the primary effects of aging can be studied without complications caused by age-related pathology. Male, 3- and 24-month-old F344xBN rats were obtained from the National Institute on Aging. All animals were disease-free with no signs of systemic inflammation and/or neoplastic diseases. The rats were housed in an environmentally controlled vivarium under pathogen-free conditions with unlimited access to food and water and a controlled photoperiod (12 h light:12 h dark). All experimental animals were maintained according to National Institutes of Health guidelines, and all animal use protocols were approved by the Institutional Animal Care and Use Committees of the participating institutions. The animals were euthanized with CO_2. The brains were rapidly dissected to establish primary cerebromicrovascular endothelial cell (CMVEC) cultures as described.

Establishment and characterization of primary cerebromicrovascular endothelial cell cultures

To assess the effects of NMN on endothelial angiogenic capacity, we measured the effects of NMN on cell proliferation, migration, and tube formation ability in cultured primary CMVECs. The establishment and characterization of the CMVEC strains have been recently reported. In brief, to establish primary cultures of CMVECs, the brains of the 3- and 24-month-old F344xBN rats were removed aseptically, rinsed in ice cold PBS, and minced into ≈ 1 mm^2. The tissue was washed twice in ice cold 1X PBS by low-speed centrifugation (50*g*, 2–3 min). The diced tissue was digested in a solution of collagenase (800 U/g tissue), hyaluronidase (2.5 U/g tissue), and elastase (3 U/g tissue) in 1 mL PBS/100 mg tissue for 45 min at 37 °C in a rotating humid incubator. The digested tissue was passed through a 100-μm cell strainer. The single-cell lysate was centrifuged for 2 min at 70*g*. After removing the supernatant, the pellet was washed twice in cold PBS supplemented with 2.5% fetal calf serum (FCS), and the suspension was centrifuged at 300*g* for 5 min at 4 °C. To create an endothelial cell–enriched fraction, the cell suspension was centrifuged using an OptiPrep gradient solution (Axi-Shield, PoC, Norway). Briefly, the cell pellet was resuspended in Hanks' balanced salt solution (HBSS) and mixed with 40% iodixanol thoroughly (final concentration 17% (*v*/*v*) iodixanol solution; $\rho = 1.096$ g/mL). Two milliliters of HBSS was layered on top and centrifuged at 400*g* for 15 min at 20 °C. Endothelial cells, which banded at the interface between HBSS and the 17% iodixanol layer, were collected. The endothelial cell–enriched fraction was incubated for 30 min at 4 °C in the dark with anti-CD31/PE (BD Biosciences, San Jose, CA, USA) and anti-MCAM/FITC (BD Biosciences, San Jose, CA, USA). After washing, the cells twice with MACS Buffer (Milltenyi Biotech, Cambridge, MA, USA) anti-FITC and anti-PE magnetic bead labeled secondary antibodies were used for 15 min at room temperature. Endothelial cells were collected by magnetic separation using the MACS LD magnetic separation columns according to the manufacturer's guidelines (Milltenyi Biotech, Cambridge, MA, USA). The endothelial fraction was cultured on fibronectin coated plates in Endothelial Growth Medium (Cell Application, San Diego, CA, USA) for 10 days. Endothelial cells were phenotypically characterized by flow cytometry (GUAVA 8HT, Merck Millipore, Billerica, MA, USA). Briefly, antibodies against five different endothelial specific markers were used (anti-CD31-PE, anti-erythropoietin receptor-APC, anti-VEGF R2-PerCP, anti-ICAM-fluorescein, anti-CD146-PE), and isotype specific antibody

labeled fractions served as negative controls. Flow cytometric analysis showed that after the third cycle of immunomagnetic selection, there were virtually no $CD31^-$, $CD146^-$, $EpoR^-$, and $VEGFR2^-$ cells in the resultant cell populations. All antibodies were purchased from R&D Systems (R&D Systems, Minneapolis, MN, USA).

Primary CMVECs were cultured in custom-made Rat Brain Endothelial Cell Growth Medium (Cell Applications, Inc.) with reduced nicotinamide concentration (11.04 μM). Since the results of the assays investigating the endpoints used are affected by the number of viable cells, cell viability of each population was determined as described. To assess the direct effects of NMN on endothelial phenotype, primary CMVECs derived from aged rats were treated with NMN (Santa Cruz, Dallas, TX) in vitro (5×10^{-4} mol/L; for 1 to 5 days).

Cell proliferation assay

Cell proliferation capacity was assessed in CMVECs using the flow cytometry–based Guava CellGrowth assay (Guava Technologies, Inc., Hayward, CA) as previously reported. Briefly, cells were collected, resuspended in PBS containing 0.1% BSA, and stained with 16 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE) for 15 min at 37 °C. This dye diffuses into cells and is cleaved by intracellular esterases to form an amine-reactive product that produces a detectable fluorescence and binds covalently to intracellular lysine residues and other amine sources. Upon cell division, CFSE divides equally into the daughter cells halving the CFSE concentration of the mother cell; therefore, there is an inverse correlation between the fluorescence intensity and the proliferation capacity of the cells. After incubation, unbound dye was quenched with serum-containing medium. Then, cells were washed three times and incubated for 24 h with 100 ng/mL VEGF. Finally, cells were collected, washed, stained with propidium iodide (to gate out dead cells), and analyzed with a flow cytometer (Guava EasyCyte 8HT; Millipore, Billerica, MA). The inverse of the fluorescence intensity was used as an index of proliferation.

Assessment of cell migration by ECIS-based wound-healing assay

Electric cell-substrate impedance sensing technology was used to monitor the migration of CMVECs in a wound-healing assay as reported (Applied BioPhysics Inc., Troy, NY). Briefly, CMVECs (2.5×10^5 cells/well) were seeded in 96-well array culture dishes (electric cell-substrate impedance sensing (ECIS), 96W1E) and placed in an incubator (37 °C), and changes in resistance and impedance were continuously monitored. When impedance reached a plateau, cells in each well were subjected to an elevated field pulse ("wounding") of 5 mA applied for 20 s at 100 kHz, which killed the cells present on the small active electrode due to severe electroporation. The detachment of the dead cells was immediately evident as a sudden drop in resistance (monitored at 4000 Hz) and a parallel increase in conductance. VEGF (100 ng/mL) was immediately added to each well. CMVECs surrounding the active electrode that had not been subjected to the wounding then migrated inward to replace the detached dead cells resulting in resistance recovery (continuously monitored at 4000 Hz for up to 24 h). The time to reach 50% resistance recovery (corresponding to 50% confluence on the active electrode) was determined for cells in each experimental group, and this parameter and the known physical dimensions of the electrode were used to calculate the migration rate (expressed as μm/h).

Tube formation assay

To investigate the influence of age and NMN on tube formation ability, young, aged, and NMN-treated aged CMVECs were plated on Geltrex Reduced Growth Factor Basement Membrane Matrix (Invitrogen, Carlsbad CA) in Medium 200PRF (Invitrogen, Carlsbad CA). To inhibit sirtuin activity, half of the aged control cells and NMN-treated aged cells were pre-treated with EX-527 (Active Motif Inc., Carlsbad, CA). EX-524 is a potent and selective sirtuin 1 (SIRT1) inhibitor (IC50 38 nM). Briefly, 150 μL/well of Geltrex was distributed in ice-cold 24-well plates. The gel was allowed to solidify while incubating the plates for 30 min at 37 °C. CMVECs were then seeded at a density of 5×10^4 cells/well and placed in the incubator for 24 h. Microscopic images were captured using a Nikon Eclipse Ti microscope equipped with a ×10 phase-contrast objective (Nikon Instruments Inc., Melville, NY). The extent of tube formation was quantified by measuring total tube length in five random fields per well using NIS-Elements microscope imaging software (Nikon Instruments Inc.), as recently reported. The mean of

the total tube length per total area imaged (μm tube/mm^2) was calculated for each well. Experiments were run in quadruplicates. The experimenter was blinded to the groups throughout the period of analysis.

Measurement of cellular H_2O_2 production

To assess cellular peroxide production, we used the cell-permeant oxidative fluorescent indicator dye CM-H_2DCFDA (5 (and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate-acetyl ester, Invitrogen, Carlsbad, CA) as we previously reported. Cells were washed with warm PBS and incubated with CM-H_2DCFDA (10 μM, at 37 °C, for 30 min). CM-H_2DCFDA fluorescence was assessed by flow cytometry.

Data analysis

Statistical analyses were performed using one-way ANOVA. $p < 0.05$ was considered statistically significant. Data are expressed as means ± S.E.M.

Results

NMN treatment improves proliferative capacity of aged CMVECs

Proliferation represents a key step in angiogenesis. Proliferative capacity of young and aged CMVECs was compared after incubation with VEGF for 24 h. We found that CFSE fluorescence was significantly increased in aged CMVECs as compared with young CMVECs, indicating that proliferation capacity is impaired by aging (Fig. 1). NMN treatment rescued proliferative capacity of aged CMVECs (Fig. 1).

NMN treatment improves migratory capability of aged CMVECs

The migratory capability of vascular endothelial cells has a pivotal role in the maintenance of microvascular integrity and angiogenesis. An ECIS-based wound-healing assay was used to assess the effect of NMN treatment on migratory capability of VEGF-treated CMVECs. We found that aged CMVECs exhibited impaired migratory capability as compared with young CMVECs (Fig. 2). In contrast, migration rate of aged CMVECs with NMN treatment did not differ significantly from that of young CMVECs (Fig. 2).

NMN treatment increases formation of capillary-like structures by aged CMVECs

When seeded onto Geltrex matrices, young CMVECs form elaborated capillary networks (Ungvari et al. 2013; Csiszar et al. 2014). Compared with young cells in aged CMVECs, formation of capillary-like structures was significantly impaired (Fig. 3a–e). The finding that treatment with NMN significantly improved formation of capillary-like structures by aged CMVECs (Fig. 3e)

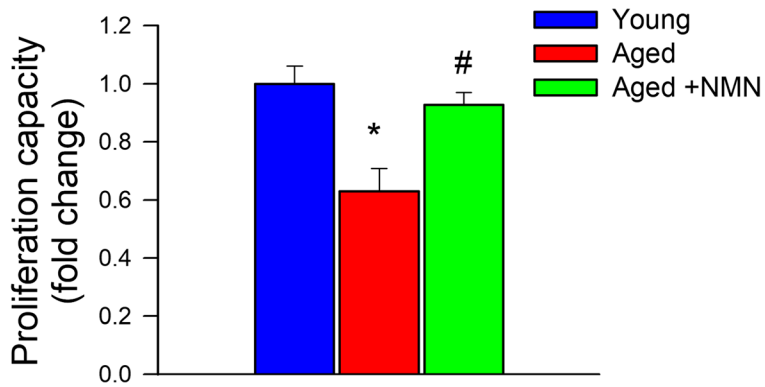

Fig. 1 NMN treatment significantly increases proliferation capacity of aged CMVECs. Cell proliferation capacity of CMVECs isolated from aged F344xBN rats is impaired as compared with that of cells isolated from young F344xBN rats, and it is significantly improved by treatment with NMN. Cell proliferation capacity was assessed in primary CMVECs stimulated with VEGF (100 ng/mL) using the flow cytometry–based *Guava CellGrowth* assay (see "Materials and Methods"). The inverse of the fluorescence intensity of the indicator dye CFSE was used as an index of proliferation capacity of the cells. Data are plotted as means ± S.E.M. ($n = 6$ in each group); $^*p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. aged

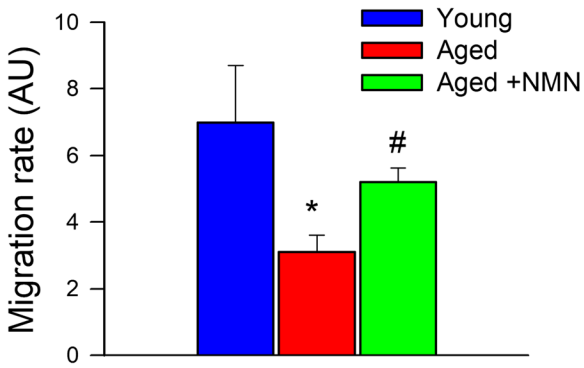

Fig. 2 NMN treatment significantly increases migration capacity of aged CMVECs. Migration capacity of CMVECs isolated from aged F344xBN rats is impaired as compared with that of cells isolated from young F344xBN rats, and it is significantly improved by treatment with NMN. VEGF (100 ng/mL)-stimulated cell migration was monitored by electric cell-substrate impedance sensing (ECIS) technology in a wound-healing assay (see "Materials and Methods"). In brief, time course of resistance recovery after wounding (electric pulse of 5 mA for 20 s at 60 kHz) was monitored at 4000 Hz. The time to reach 50% resistance recovery (corresponding to 50% confluence on the active electrode) was determined for each group, and this parameter and the known physical dimensions of the electrode were used to calculate the migration rate. Bar graph depicts the summary data for migration rate in each group. Data are plotted as means ± S.E.M. ($n = 5$ in each group); $^{*}p < 0.05$ vs. young control, $^{\#}p < 0.05$ vs. aged

suggests that age-related NAD^+ deficiency is causally linked to the impaired angiogenic capacity of aged endothelial cells. We found that pharmacological inhibition of SIRT1 significantly inhibited the formation of capillary-like structures by NMN-treated aged CMVECs (Fig. 3e).

NMN treatment attenuates oxidative stress in aged CMVECs

Age-related oxidative stress has been implicated in endothelial angiogenic dysfunction (Ungvari et al. 2013). ROS production in young and aged CMVECs was compared by assessing CM-H_2DCFDA fluorescence. We found that CM-H_2DCFDA fluorescence was significantly increased in aged CMVECs as compared with that in young CMVECs, consistent with the view that endothelial cells in the aged cerebral microcirculation exhibit increased oxidative stress (Fig. 4). NMN treatment resulted in dramatic attenuation of H_2O_2 production in aged CMVECs (Fig. 4). Recent developments in our understanding of mechanisms of aging (Deepa et al. 2017; Fang et al. 2017; Grant et al. 2017; Konopka et al. 2017; Podlutsky et al. 2017; Cunningham et al. 2018; Habermehl et al. 2018; Kim et al. 2018; Lewis et al. 2018; Masser et al. 2018; Nacarelli et al. 2018; Olecka et al. 2018; Reglodi et al. 2018) and vascular aging processes (Csiszar et al. 2017; Tarantini et al. 2017a, b; Tucsek et al. 2017; Ungvari et al. 2017a, b; Csipo et al. 2018; Fulop et al. 2018; Lee et al. 2018; Reglodi et al. 2018; Sure et al. 2018; Ungvari et al. 2018b, c) highlight the importance of in vitro screening assays that model complex physiological processes for the evaluation of the anti-aging effects of novel pharmacological interventions. The combination of the in vitro assays used in this study, based on rescue of age-related loss-of-function in endothelial cells, could correctly identify the anti-aging effects of caloric restriction (Csiszar et al. 2013, 2014) as well as neuroendocrine factors (Banki et al. 2015).

Discussion

The principal new findings of this study are that (1) age-related decline in cellular NAD^+ levels is associated with impaired angiogenic response in aged rat CMVECs, and that (2) restoration of cellular NAD^+ levels in aged CMVECs by treatment with NMN confers pro-angiogenic effects, counteracting, at least in part, the adverse effects of aging.

The formation of a new sprout growing out of existing vessels represents the first step in angiogenesis, which is mediated by VEGF-induced stalk cell proliferation and tip cell migration. VEGF also induces in endothelial cell branching and tubulogenesis to create microvascular networks. VEGF-induced proliferation and migration and tube forming capacity of CMVECs decline significantly with age, which are thought to contribute significantly to aging-induced impairment of angiogenesis and, consequentially, microvascular rarefaction (Valcarcel-Ares et al. 2012a, b; Ungvari et al. 2013; Csiszar et al. 2014; Ungvari et al. 2018a, b).

Recently, we demonstrated that age-related decline in NAD^+ levels in CMVECs can be reversed by treatment with the NAD^+ precursor NMN (Tarantini et al. 2019). This is the first study to demonstrate that treatment with NMN also improves proliferation and rescues migration and tube forming capacity of aged CMVECs. Our studies provide strong evidence that age-related NAD^+ depletion compromises endothelial angiogenic responses in the cerebromicrovasculature. Follow-up studies are needed to determine whether in vivo treatment of aged

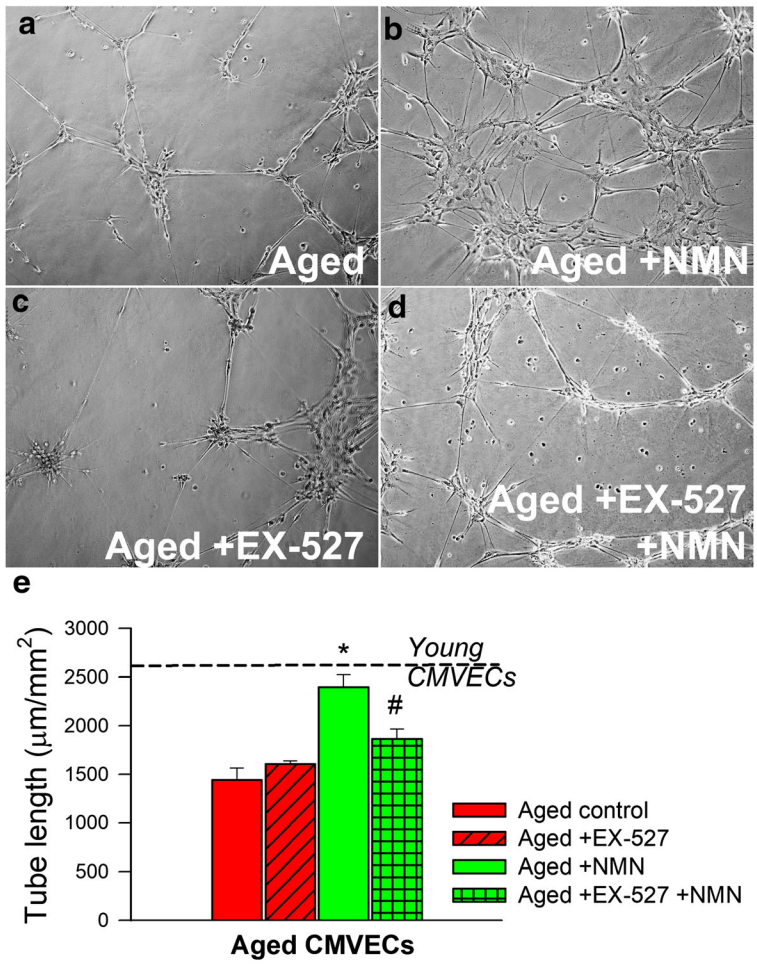

Fig. 3 NMN treatment significantly improves the tube formation ability of aged CMVECs. Tube formation ability of CMVECs isolated from aged F344xBN rats is impaired as compared with that of cells isolated from young F344xBN rats (dashed line), and it is significantly improved by NMN treatment. Inhibition of SIRT1 by EX-524 significantly impairs the ability of NMN-treated aged CMVECs to form capillary-like structures, suggesting that the protective effects of NMN are mediated by sirtuin activation. CMVECs were plated on Geltrex matrix–coated wells, and tube formation was induced by treating cells with VEGF (100 ng/mL, for 24 h). Representative examples of capillary-like structures are shown on panels **a**, **b**, **c**, **d**. Summary data, expressed as total tube length per total area scanned (μm tube/mm^2), are shown in panel **e**. Data are means ± S.E.M. ($n = 5$ in each group); *$p < 0.05$ vs. aged control, $^{\#}p < 0.05$ vs. aged + NMN

rodents with NMN restores a youthful capillary density in brain regions important for learning and memory and whether NMN positively affects cerebral angiogenesis and/or collateral formation induced by physiological (e.g., exercise, local ischemia) or pharmacological stimuli. As the protective effect of NMN on formation of capillary-like structures by aged CMVECs is prevented by disruption of SIRT1 signaling, it is likely that restoration of NAD^+ levels activates sirtuins, which confer pro-angiogenic effects. This concept is supported also by the observation that treatment of aged mice with NMN improves skeletal muscle blood flow by promoting SIRT1-dependent increases in capillary density (Das et al. 2018).

Previous studies established a causal link among age-related oxidative stress, decreased bioavailability of NO, and impaired angiogenic capacity of aged endothelial cells (Koike et al. 2003; Sadoun and Reed 2003; Bach et al. 2005; Reed et al. 2005; Ungvari et al. 2013; Ungvari et al. 2018a, b). Our previous studies demonstrate that increased cellular H_2O_2 levels promote down-regulation of Dicer-dependent angiomirs (pro-angiogenic miRNAs) in aged CMVECs (Ungvari et al. 2013). Further, induction of oxidative stress by

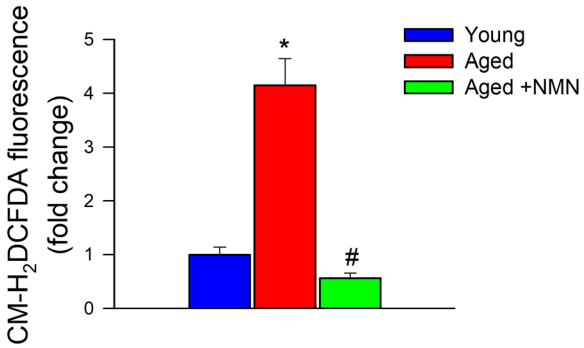

Fig. 4 NMN treatment significantly attenuates oxidative stress in aged CMVECs. Cellular peroxide production is significantly increased in cultured primary CMVECs derived from aged F344xBN rats as compared with cells isolated from young F344xBN rats, and it is significantly attenuated by treatment with NMN. Cellular peroxide production was assessed by measuring CM-H_2DCFDA fluorescence using a flow cytometry–based approach. Data are plotted as means ± S.E.M. ($n = 6$ in each group); $^{*}p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. aged

downregulation of key antioxidant systems impairs angiogenic potential of endothelial cells (Valcarcel-Ares et al. 2012a, b). Here, we demonstrate that age-related increase in endothelial H_2O_2 production is effectively attenuated by NMN treatment. This observation extends the findings of our recent studies showing that in vivo treatment with NMN treatment also attenuates age-related mitochondrial oxidative stress in CMVECs restoring NO bioavailability and improving endothelium-mediated vasodilation, suggesting a key role for these mechanisms in NAD^+-mediated endothelial protection (Csiszar et al. 2019; Tarantini et al. 2019). Mitochondria-derived O_2^- is dismutated to H_2O_2 by manganese superoxide dismutase (MnSOD). H_2O_2 can readily penetrate the mitochondrial membrane, and its increased cytosolic level is likely responsible for the anti-angiogenic effects associated with mitochondrial oxidative stress. Previous studies provide additional support to this concept by showing that attenuation of mitochondrial oxidative stress using structurally different inhibitors/scavengers of mtROS production (resveratrol, mitoTEMPO) increases cerebral capillary density and/or restores angiogenic potential in aged rodents (Oomen et al. 2009; Miura et al. 2017). Our recent studies also demonstrate that attenuation of mitochondrial oxidative stress (Ungvari et al. 2009; Toth et al. 2014; Tarantini et al. 2018, 2019) also restores endothelium-mediated vasodilation in aged mice. The synergistic functional and structural microvascular protective effects of NMN and mitochondria-targeted antioxidants likely significantly improve cerebral blood flow in aging, contributing to their beneficial effects on cognitive function (Tarantini et al. 2018, 2019). Other age-related mechanisms, which may contribute to the induction of the anti-angiogenic phenotype in CMVECs exacerbating the effects of NAD^+ depletion, include age-related IGF-1 deficiency (Sonntag et al. 1997, 2012; Ungvari and Csiszar 2012) and Nrf2 dysfunction (Valcarcel-Ares et al. 2012a, b).

Significant data are available to support the efficacy and translational relevance of NMN and other related NAD^+ boosters (e.g., nicotinamide riboside treatment; Yoshino et al. 2018) (Csiszar et al. 2019). Studies are currently underway to determine whether chronic treatment with nicotinamide riboside improves cerebral blood flow (ClinicalTrials.gov Identifier: NCT03482167) in older adults with mild cognitive impairment. If these studies yield positive results, the effects of NMN treatment of organ capillarization in elderly patients should also be investigated.

Funding information This work was supported by grants from the American Heart Association (ST, MNVA), the Oklahoma Center for the Advancement of Science and Technology (to AC, AY, ZU), the National Institute on Aging (R01-AG047879; R01-AG038747; R01-AG055395), the National Institute of Neurological Disorders and Stroke (NINDS; R01-NS056218 to AC, R01-NS100782 to ZU), the Oklahoma Shared Clinical and Translational Resources (OSCTR) program funded by the National Institute of General Medical Sciences (GM104938, to AY), and the Presbyterian Health Foundation (to ZU, AC, AY).

Compliance with ethical standards

Disclaimer The funding sources had no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

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