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ORIGINAL ARTICLE

Fusogenic liposomes effectively deliver resveratrol to the cerebral microcirculation and improve endothelium-dependent neurovascular coupling responses in aged mice

Tabea Wiedenhoeft · Stefano Tarantini · Ádám Nyúl-Tóth · Andriy Yabluchanskiy · Tamas Csipo · Priya Balasubramanian · Agnes Lipecz · Tamas Kiss · Anna Csiszar · Agnes Csiszar · Zoltan Ungvari

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Abstract Adjustment of cerebral blood flow (CBF) to the increased oxygen and nutrient demands of active brain regions via neurovascular coupling (NVC) has an essential role in maintenance of healthy cognitive function. In advanced age, cerebromicrovascular oxidative stress and endothelial dysfunction impair neurovascular coupling, contributing to age-related cognitive decline. Recently we developed a resveratrol (3,4′,5-trihydroxystilbene)-containing fusogenic liposome (FL-RSV)-based molecular delivery system that can effectively target cultured cerebromicrovascular

Tabea Wiedenhoeft, Stefano Tarantini, Ádám Nyúl-Tóth and Andriy Yabluchanskiy contributed equally to this work.

T. Wiedenhoeft · S. Tarantini · Á. Nyúl-Tóth · A. Yabluchanskiy · T. Csipo · P. Balasubramanian · A. Lipecz · T. Kiss · A. Csiszar · Z. Ungvari
Vascular Cognitive Impairment and Neurodegeneration Program, Reynolds Oklahoma Center on Aging/Oklahoma Center for Geroscience, Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

T. Wiedenhoeft · A. Csiszar
Institute of Complex Systems, ICS-7: Biomechanics, Forschungszentrum Jülich GmbH, 52425 Jülich, Germany

S. Tarantini · T. Csipo · A. Lipecz · Z. Ungvari
International Training Program in Geroscience, Doctoral School of Basic and Translational Medicine/Department of Public Health, Semmelweis University, Budapest, Hungary

Á. Nyúl-Tóth
Institute of Biophysics, Biological Research Centre, Szeged, Hungary

T. Csipo
International Training Program in Geroscience, Division of Clinical Physiology, Department of Cardiology/ Kalman Laki Doctoral School, Faculty of Medicine, University of Debrecen, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

T. Kiss · A. Csiszar
International Training Program in Geroscience,Theoretical Medicine Doctoral School/Department of Medical Physics and Informatics, University of Szeged, Szeged, Hungary

A. Csiszar · Z. Ungvari
Doctoral School of Basic and Translational Medicine/Institute of Clinical Experimental Research, Semmelweis University, Budapest, Hungary

Z. Ungvari
Department of Health Promotion Sciences, College of Public Health, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

Z. Ungvari (✉)
Reynolds Oklahoma Center on Aging, Department of Geriatric Medicine, University of Oklahoma Health Sciences Center, 975 NE 10th Street, BRC 1311, Oklahoma City, OK 73104, USA
e-mail: zoltan-ungvari@ouhsc.edu

endothelial cells, attenuating age-related oxidative stress. To assess the cerebromicrovascular protective effects of FL-RSV in vivo, aged (24-month-old) C57BL/6 mice were treated with FL-RSV for four days. To demonstrate effective cellular uptake of FL-RSV, accumulation of the lipophilic tracer dyes in cells of the neurovascular unit was confirmed using two-photon imaging (through a chronic cranial window). NVC was assessed by measuring CBF responses (laser speckle contrast imaging) evoked by contralateral whisker stimulation. We found that NVC responses were significantly impaired in aged mice. Treatment with FL-RSV significantly improved NVC responses by increasing NO-mediated vasodilation. These findings are paralleled by the protective effects of FL-RSV on endothelium-dependent relaxation in the aorta. Thus, treatment with FL-RSV rescues endothelial function and NVC responses in aged mice. We propose that resveratrol containing fusogenic liposomes could also be used for combined delivery of various anti-geronic factors, including proteins, small molecules, DNA vectors and mRNAs targeting key pathways involved in microvascular aging and neurovascular dysfunction for the prevention/treatment of age-related cerebromicrovascular pathologies and development of vascular cognitive impairment (VCI) in aging.

Keywords Fusogenic liposomes · Resveratrol · Oxidative stress · Aging · Cerebral circulation · Vascular cognitive impairment · Endothelial dysfunction · Functional hyperemia · Endothelium

Introduction

Normal functioning of the brain requires tight coordination between neuronal activity and cerebral blood flow, to ensure an adequate supply of oxygen and nutrients as well as effective washout of harmful metabolites (Tarantini et al. 2017a). During periods of intense neuronal activity, there is a requirement for prompt adjustment of oxygen and glucose delivery to the increased demands of active neurons through rapid adaptive increases in regional blood flow. This is ensured by a homeostatic mechanism known as neurovascular coupling. The resulting functional hyperemia is essential to maintain an optimal humoral microenvironment around firing neurons and thereby enabling their normal functioning.

There is a scientific consensus that microvascular contributions to cognitive impairment in elderly patients are critical (Tarantini et al. 2017a). Importantly, neurovascular coupling responses are impaired both in older adults (Zaletel et al. 2005; Topcuoglu et al. 2009; Stefanova et al. 2013; Fabiani et al. 2013) and in preclinical animal models of aging (Toth et al. 2014; Park et al. 2007), which contributes to the age-related cognitive decline (Tarantini et al. 2017a; Sorond et al. 2013; Sorond et al. 2011; Tarantini et al. 2015). Recent studies demonstrate that the age-related molecular and cellular mechanisms that contribute to neurovascular un-coupling include increased mitochondrial oxidative stress and mitochondrial dysfunction in endothelial cells and impairment of endothelial NO-mediated vasodilation (Tarantini et al. 2018; Tarantini et al. 2019). Preclinical studies demonstrate that pharmacological interventions that attenuate endothelial oxidative stress and improve NO mediation have the potential to increase neurovascular coupling responses and cerebral blood flow and improve cognitive function in rodent models of aging (Tarantini et al. 2018; Tarantini et al. 2019). Inspired by these findings, our long-term goal is to develop innovative therapeutic interventions to rescue cerebromicrovascular endothelial function and restore functional hyperemia in elderly patients to delay cognitive impairment (Toth et al. 2014). In order to achieve that goal, we have developed an innovative fusogenic liposome-based method for effective delivery of anti-geronic factors to cerebromicrovascular endothelial cells (Csiszar et al. 2010, 2014; Hersch et al. 2016; Kleusch et al. 2012; Kolasinac et al. 2018; Kube et al. 2017). The cellular uptake of conventionally pharmaceutically applied liposomes is mediated by either clathrin-dependent or clathrin-independent endocytosis, limiting the uptake efficiency to a small percentage. Our innovative liposomal carrier system delivers its cargo by protein-independent fusion with the cell membrane, allowing a delivery more efficient and less affected of lysosomal cargo degradation in contrast to aforementioned liposomes. Using such fusogenic

liposomes (FLs) we successfully delivered the anti-geronic compound resveratrol with significant neurovascular protective effects (Baur et al. 2006; Lagouge et al. 2006; Smith et al. 2009) as shown in Fig. 1.

There is strong preclinical evidence that treatment with high doses of resveratrol exerts significant vasoprotective effects in aged mice and mice with accelerated vascular aging (Ungvari et al. 2007a; Pearson et al. 2008; Zhang et al. 2009). Resveratrol, a plant-derived polyphenolic stilbene, was shown to attenuate mitochondrial oxidative stress, improve mitochondrial function and increase NO bioavailability in cultured endothelial cells (Csiszar et al. 2014; Csiszar et al. 2009; Csiszar et al. 2012; Toth et al. 2015a; Ungvari et al. 2010; Ungvari et al. 2009; Ungvari et al. 2007b; Ungvari et al. 2007c). Treatment with resveratrol can further increase the activation of the nuclear factor (erythroid-derived 2) factor 2 (Nrf2) in vitro and in rodent models (Csiszar et al. 2014; Ungvari et al. 2010). Nrf2 is an inducible nuclear transcription factor that adjusts the redox balance in the cell and positively regulates expression of antioxidant enzymes

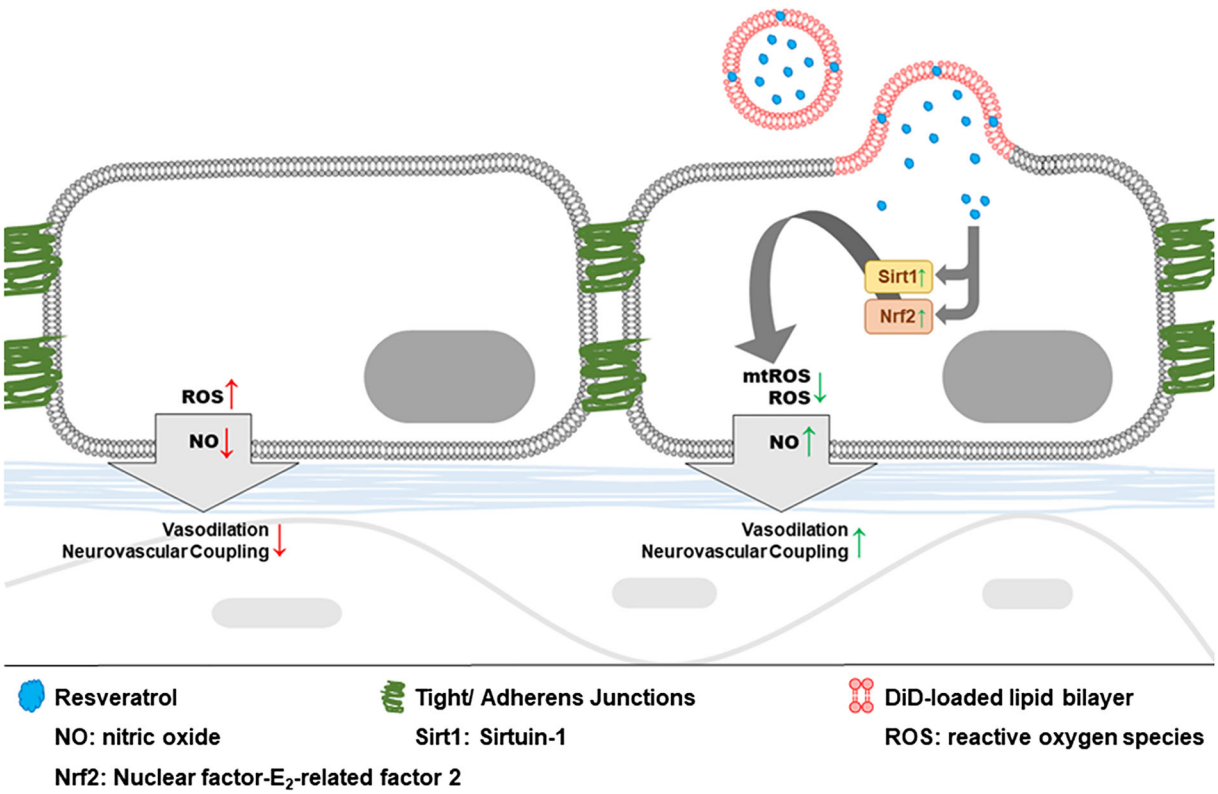


Fig. 1 Proposed model for neurovascular protection by enhanced delivery of resveratrol to cells of the neurovascular unit in aged mice using fusogenic liposomes. The scheme depicts the enhanced membrane fusion between resveratrol-containing fusogenic liposomes and aged cerebromicrovascular endothelial cells (CMVECs) and the complex anti-aging neurovascular protective effects of increased cellular resveratrol levels. Note that synergistic interaction of the neutral lipid DOPE, the positively charged lipid DOTAP, and the aromatic resveratrol and DiD, results in an effective fusogenic mixture that readily delivers resveratrol to vascular cells (Csiszar et al. 2014). Resveratrol is highly lipophilic and is deeply located in the lipid bilayer. Only 10% of resveratrol molecules is found in the aqueous phase (Brittes et al. 2010). The model predicts that increased delivery of resveratrol encapsulated in fusogenic liposomes exerts anti-aging protective effects in cells of the neurovascular unit by activating sirtuins and Nrf2 and thereby attenuating age-related mitochondrial production of ROS (mtROS) and restoring bioavailability of vasodilator NO. We propose that resveratrol containing fusogenic liposomes could be used to deliver a complex cocktail of anti-geronic factors, which include proteins, small molecules, DNA vectors and miRNAs targeting key pathways involved in microvascular aging and neurovascular dysfunction

like mitochondria and NADPH oxidases (Kovac et al. 2015). Furthermore, resveratrol promotes allosteric activation of NAD-dependent deacetylase sirtuin-1 (SIRT-1), a member of the sirtuin family of regulatory enzymes involved in life span extension mechanisms (Hubbard et al. 2013; Howitz et al. 2003). Genetic depletion of SIRT-1 in rodent cells was shown to diminish the beneficial antioxidative effects of resveratrol in multiple tissues (Price et al. 2012). Previous studies by us and other laboratories provide proof-of-concept that treatment of aged rodents with resveratrol exerts protective effects on the cerebral microcirculation (Toth et al. 2014; Oomen et al. 2009). Despite significant advances in our understanding of the endothelial protective and antigeronic effects of resveratrol, its low aqueous solubility, relatively low bioavailability, rapid metabolism in the liver and rapid systemic elimination are barriers to its clinical application. Previously we confirmed that the uptake of resveratrol delivered by fusogenic liposomes (FL-RSV) to endothelial cells is substantially enhanced (Csiszar et al. 2014). Specifically, we demonstrated treatment of primary cerebromicrovascular endothelial cells isolated from aged rats with FL-RSV results in rapid and effective cellular uptake of resveratrol-containing liposomes, increasing cellular resveratrol levels and attenuating cellular production of reactive oxygen species (Csiszar et al. 2014).

The present study was designed to test successful targeting of the cerebral microcirculation by resveratrol-containing fusogenic liposomes and to assess the cerebromicrovascular and endothelial protective effects of in vivo treatment with FL-RSV. To achieve this goal, aged (24-month-old) C57BL/6 mice were treated with FL-RSV for four days. To demonstrate effective cellular uptake of FL-RSV, the animals were equipped with chronic cranial window, and accumulation of the lipophilic tracer dyes, DiD or DiO respectively, in cells of the neurovascular unit was monitored using two-photon imaging. Neurovascular coupling was assessed by measuring cerebral blood flow (CBF) responses (laser speckle contrast imaging) evoked by contralateral whisker stimulation. Endothelial function was also assessed in isolated aorta ring preparations.

Methods

Preparation of fusogenic liposomes

1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-3-trimethylammonium-propane, chloride salt (DOTAP) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA) while the fluorescent lipophilic tracers 1,1′-dioctadecyl-3,3,3′,3’-Tetramethylindotricarbocyanine iodide (DiD) and 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) were purchased from ThermoFisher Scientific (Waltham, MA, USA). The method of liposomal preparation was modified from previously described protocols (Csiszar et al. 2014). For the preparation of fusogenic liposomes (FL), the lipids DOPE and DOTAP (25 mg/ml in chloroform) were mixed with DiD or DiO 1/1/0.05 or 1/1/0.1 (mol/mol), respectively. The lipophilic fluorophores DiD and DiO differed in fluorescence excitation and emission, yet showed similar physicochemical properties. Resveratrol (RSV; Sigma-Aldrich, St.Louis, MO, USA) was disolved in ethanol and added to the lipid solution in a ratio of 2/3 (*w*/w). The solvents were evaporated in vacuum, and the lipid film was hydrated using 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma-Aldrich, St. Louis, MO, USA) to a final lipid concentration of 2 mg/ml at pH 7.4. After brief vortexing, lipid film hydration was followed by sonication in an ultrasonic bath (Sonocool, Bandelin electronic GmbH, Berlin, Germany) at 5 °C for 20 min. Those with higher fluorophore content were chosen for intravital imaging and histology.

Animals, treatment with FL-RSV

Young (3 months, $n = 10$) and aged (24 months, $n = 20$) male C57BL/6 mice were purchased from the aging colony maintained by the National Institute on Aging at Charles River Laboratories (Wilmington, MA). Animals were housed under specific pathogen-free barrier conditions in the Rodent Barrier Facility at University of Oklahoma Health Sciences Center under a controlled photoperiod (12 h light; 12 h dark) with unlimited access to water and were fed a standard AIN-93G diet (ad libitum). Mice in the aged cohort were assigned to two groups ($n = 10$ each group).

Mice of each group were retro-orbitally injected for four days with 2 mg/kg/day of RSV encapsulated in

fusogenic liposomes (FL-RSV) diluted in phosphate-buffered saline (PBS). PBS and empty fusogenic liposomes (FL) were used as vehicle control. Four hours after the last injection, mice of the aged cohort were used for experiments determining relative change of cerebral blood flow in the barrel cortex. Subsequently, all mice were transcardially perfused with PBS and decapitated, then dissected. The thoracic aortae of aged mice were removed directly after decapitation for the aortic ring assay, and brains of all mice were removed and fresh-frozen for measurement of the cortex fluorescence intensity. All procedures were approved by the Institutional Animal Use and Care Committees of the University of Oklahoma Health Sciences Center.

Chronic cranial window preparation, two-photon imaging

A group of C57BL/6 mice was equipped with a chronic cranial window for in vivo imaging according to the published protocols of Mostany and Portera-Cailliau (Mostany and Portera-Cailliau 2008a; Mostany and Portera-Cailliau 2008b). Briefly, mice were anesthetized with 2% isoflurane from a Surgivet Classic T3 vaporizer (Smiths Medical, Minneapolis, MN, USA) and the depth of anesthesia was monitored during the whole process by inspection of the toe pinch reflex. The subject was placed on a warming blanket-covered bench, and the head was fixed in a stereotaxic frame. Eye ointment was applied to both eyes to prevent them from drying out. After local disinfection, hair and then skin were removed from the skull, and the bone surface was cleared. A ~3 mm diameter circle was drawn with a pneumatic dental drill (Foredom, Blackstone Industries, Bethel, CT) until the bone had become thin enough to perform craniotomy. A glass coverslip (Thomas Scientific, Swedesboro, NJ, USA) previously soaked in 70% ethanol was rinsed in PBS and applied on the dura mater. The window was stabilized with liquid instant adhesive and secured by Jet Set-4 dental acrylic resin (Lang Dental, Wheeling, IL, USA). After the surgery, the animals were allowed at least ten days to stabilize before intravital microscopy.

For intravital microscopy, a FluoView 1000 MPE (Olympus, Tokyo, Japan) two-photon microscope coupled with a MaiTai HP DeepSee-OL 690 nm–1040 nm (Spectra-Physics, San Jose, CA, USA) laser and a XLPLN25XWMP 25× water immersion objective (Olympus, Tokyo, Japan) was used. Subjects were illuminated with 800–910 nm laser light adjusted to fluorophores. Mice were anesthetized with isoflurane (2% for initiation and then 1.5% for maintenance) then fixed in a stereotaxic frame. FL-RSV(DiD), or –(DiO) was administered 30 min before the microscopy. 100 μl FITC- labeled dextran (500 kDa) (Sigma-Aldrich, St. Louis, MO, USA) or Texas red- labeled dextran (70 kDa) (Thermo Fischer Scientific, Waltham, MA, USA) tracer was also injected along with FL-RSV(DiD), or –(DiO) retro-orbitally. The animals were imaged before and after injection. First, meningeal vessels were detected and the imaging depth was set to them. Cerebral vessels were examined ~0–200 μm deep. To detect the movement of FL-RSV 150 μm × 150 μm (x, y) region of interests (ROIs) were recorded for 30 s repeatedly, for several days in indicated time points (at least 3 ROIs per animal). For 3D volume scanning 508 μm × 508 μm × 50 μm (x, y, z) ROI was recorded. The images were analyzed using ImageJ 1.52i version (National Institutes of Health). Imported images were assembled to time-stacks or z-stack (maximum projection), and were cropped for presentation.

Measurement of neurovascular coupling responses

Mice in each group were anesthetized with isoflurane (4% induction and 1% maintenance), endotracheally intubated and ventilated (MousVent G500; Kent Scientific Co, Torrington, CT) as described previously (Tarantini et al. 2018; Tarantini et al. 2017b; Ungvari et al. 2017). A thermostatic heating pad (Kent Scientific Co, Torrington, CT) was used to maintain rectal temperature at 37 °C (Toth et al. 2014). End-tidal CO_2 was controlled between 3.2% and 3.7% to keep blood gas values within the physiological range, as described previously (Tarantini et al. 2015; Toth et al. 2015b). Cannulation of the right femoral artery was performed for arterial blood pressure measurement (Living Systems Instrumentations, Burlington, VT) (Toth et al. 2014). The blood pressure was within the physiological range throughout the experiments (90–110 mmHg). Mice were immobilized and placed on a stereotaxic frame (Leica Microsystems, Buffalo Grove, IL), the scalp and periosteum were pulled aside and the skull

was gently thinned using a dental drill while cooled with dripping buffer. Recent advances in our understanding of cellular aging processes (Kim et al. 2018; Lee et al. 2018; Masser et al. 2018; Nacarelli et al. 2018; Reglodi et al. 2018; Sarker and Franks 2018) and mechanisms underlying vascular aging strongly suggest that age-related decline in neurovascular function (Tarantini et al. 2017b; Ungvari et al. 2017; Tarantini et al. 2017c; Fulop et al. 2018) and cognitive performance can be reversed. To assess neurovascular coupling responses a laser speckle contrast imager (Perimed, Järfälla, Sweden) was placed 10 cm above the thinned skull, and to achieve the highest CBF response the right whiskers were stimulated for 30 s at 10 Hz from side to side as described (Tarantini et al. 2017c). Differential perfusion maps of the brain surface were captured. Changes in cerebral blood flow (CBF) were monitored above the left barrel cortex in six trials in each group, separated by 5–10 min intervals. To assess the role of NO mediation, CBF responses to whisker stimulation were repeated 20 min after administration of the nitric oxide synthase inhibitor N^{ω}-Nitro-L-arginine methyl ester (L-NAME, Sigma-Aldrich, St. Luis, MO). For data evaluation, the relative change in the CBF signal was compared between the baseline of the region of interest (ROI) and during stimulation. To rule out an unspecific increase of cerebral blood flow, the difference between the relative change for both lateral ROI's was used for further evaluation to determine specific changes for the contralateral somatosensory cortex. In each study, the experimenter was blinded to the treatment of the animals.

Assessment of endothelial function in the aorta

To assess the specific effect of FL-RSV treatment on endothelial function, endothelium-dependent vasorelaxation was assessed in isolated aorta ring preparations as described previously (Pearson et al. 2008). In brief, aortas were cut into ring segments 1.5 mm in length and mounted in myographs chambers (Danish Myo Technology A/S, Inc., Denmark) for measurement of isometric tension. The vessels were superfused with Krebs buffer solution (118 mM NaCl, 4.7 mM KCl, 1.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.1 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 5.6 mM glucose; at 37 °C; gassed with 95% air and 5% CO_2).. After an equilibration period of 1 h during which an optimal passive tension was applied to the rings (as determined from the vascular length-tension relationship), they were pre-contracted with 10^{-6} M phenylephrine until reaching a plateau phase, and subsequent relaxation in response to increasing doses of acetylcholine was measured.

Demonstration of effective endothelial targeting by fusogenic liposomes in the aorta using confocal microscopy

The segmented aorta of a young C57BL/6 mouse treated with 10 mg/kg FL-RSV(DiD) was isolated four hours after a single dose injection, embedded in cryogel and frozen at −80 °C, then sectioned in 50 μm slices and transferred to glass. After counterstaining with 4′,6-diamidine-2′-phenylindole (DAPI) and mounting with ProLong™ diamond antifade mountant (ThermoFisher Scientific, Massachusetts, USA), sections were imaged using an SP8 confocal microscope (Leica, Hessen, Germany) and a water immersion objective (20x HC PL APO 0.75). DAPI and DiD were stimulated with a 405 nm UV laser and a white light laser system with a notch filter at 633 nm. Images were acquired at 8 bits.

Demonstration of effective targeting of the brain by fusogenic liposomes: Fluorescence intensity measurement in cortical samples

A longitudinal section of the fresh frozen brain of young C57BL/6 mice after four-day treatment was homogenized using an ultrasonic cell disruptor homogenizer in 1% Triton X-100 containing PBS (*w*/w; 1:10). The homogenized sample was centrifuged for 20 min at 4 °C and 15.000 rpm. The supernatant was divided into three wells of 200 μL in a flat black 96-well-plate, and fluorescence intensity was measured using a Spark® plate reader (Tecan Life Sciences, Maennedorf, Switzerland). For data evaluation, the blank intensity of 1% Triton X-100 in PBS was removed from the intensity of other samples.

Statistical analysis

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

Results

Effective uptake of FL-RSV into cells of the neurovascular unit and aortic endothelial cells

Our delivery system is schematically presented in Fig. 1. To confirm effective cellular uptake of fusogenic liposomes, we demonstrated the accumulation of the lipophilic tracer dyes DiD and DiO in cells of the neurovascular unit using two-photon microscopy (Figs. 2 and 3). As shown in Fig. 2c, d and 3d, after retro-orbital administration of FL-RSV a significant increase in DiD or DiO-stained cells in the neurovascular unit (both in endothelial and paravascular astrocyte-like and pericyte-like cells) was evident. For semi-quantitative assessment of cellular liposomal uptake, we compared background-corrected DiD fluorescence in the brain tissue after administration of empty fusogenic liposomes (FL) and FL-RSV. Previously we showed that the fusion efficiency of this lipid mixture could be modulated by incorporation of RSV in vitro (Csiszar et al. 2014). We hypothesize that the large delocalized π electron systems of resveratrol promotes destabilization of the lipid bilayer in the cell membrane, thereby significantly increasing uptake of liposomes via membrane fusion with endothelial cells in vitro (Csiszar et al. 2014). As expected on the basis of the *in vitro* studies (Csiszar et al. 2014), cellular incorporation of DiD upon in vivo administration of empty fusogenic liposomes was low, whereas cellular incorporation of DiD upon administration of FL-RSV was significantly increased (Fig. 2e).

To demonstrate effective endothelial uptake of FL-RSV, we also assessed DiD incorporation in the endothelial layer of the aorta. Figure 2f shows that increased DiD fluorescence was localized to the luminal cellular layer of the aorta of FL-RSV treated mice, indicating effective delivery of resveratrol to endothelial cells.

To determine the liposomal clearance from the cerebral circulation, FL-RSV particles were monitored 6d post injection. As Fig. 3c shows, the number of circulating liposomes substantially decreased after one day consistent with the effective uptake of the liposomes from the systemic circulation. After several days of tracking DiO-labeled cells appeared in and around of the mouse microcirculation (Fig. 3d).

Treatment with FL-RSV rescues neurovascular coupling responses in aged mice by restoring NO mediation

CBF responses in the whisker barrel cortex elicited by contralateral whisker stimulation were significantly decreased in aged mice compared to young animals indicating impaired neurovascular coupling in aging (representative laser speckle contrast images and CBF tracings are shown in Fig. 4a and b, summary data are shown in Fig. 4c) (Park et al. 2007; Tarantini et al. 2018; Tarantini et al. 2019). We found that four-day treatment with FL-RSV significantly increased CBF responses induced by contralateral whisker stimulation in aged mice, restoring neurovascular coupling to levels observed in young mice (Fig. 4c). In young animals administration of the NO synthase inhibitor L-NAME significantly decreased neurovascular coupling responses, eliminating the differences between the age groups (Fig. 4c). In aged animals treated with the liposomal controls, administration of L-NAME was without effect (Fig. 4c). In contrast, in FL-RSV treated aged mice L-NAME significantly decreased CBF responses elicited by whisker stimulation (Fig. 4c), suggesting that FL-RSV treatment restored the NO mediation of neurovascular coupling in aged animals.

Treatment with FL-RSV improves endothelial function in aged aortas

To further ascertain the endothelial protective effects of FL-RSV, endothelium-dependent vasodilator responses were tested in aorta ring preparations. In young vessels administration of acetylcholine resulted in significant relaxation, whereas these responses were significantly attenuated in vessels derived from aged mice (Fig. 5). Treatment

of aged mice with FL-RSV significantly improved acetylcholine-induced vasorelaxation, restoring responses to the level observed in vessels of young mice. To assess the role of endothelium-derived NO, L-NAME was applied. L-NAME significantly inhibited acetylcholine-induced responses, eliminating the differences between the three groups. These finding suggests that FL-RSV significantly improves endothelial function by restoring endothelial NO mediation in aged vessels (data not shown).

Discussion

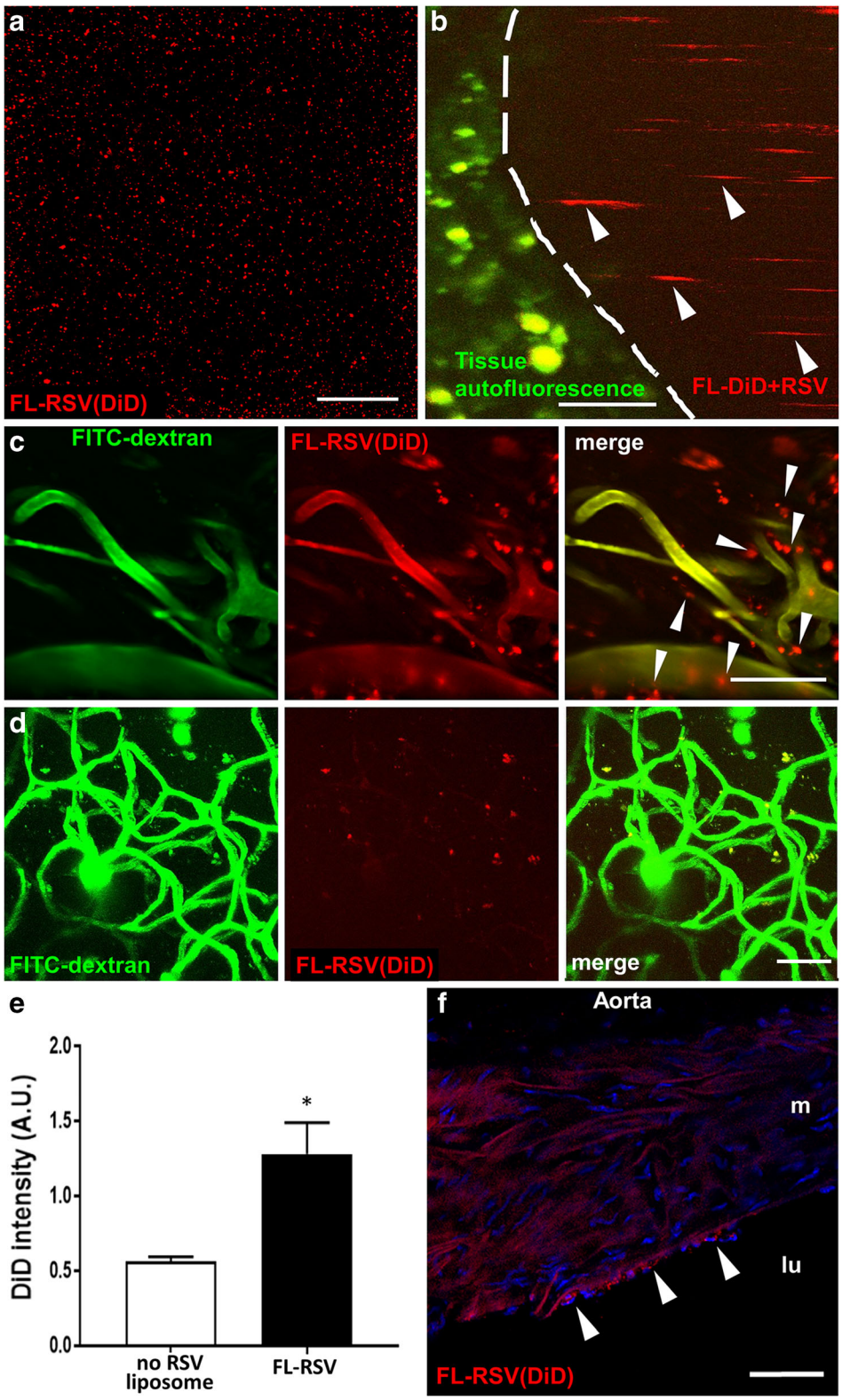
The key finding of his study is that treatment with FL-RSV effectively targets the neurovascular unit and rescues endothelium-dependent neurovascular coupling responses in a mouse model of aging that recapitulates cerebromicrovascular dysfunction and cognitive deficits manifested in elderly patients.

Our previous studies demonstrated that resveratrol present within fusogenic liposomes significantly enhances their fusion to endothelial cell membranes and thus can facilitate the uptake of resveratrol into cultured cells (Csiszar et al. 2014). Here, we demonstrate for the first time that this lipophilic delivery system is also an effective approach to deliver resveratrol in vivo to the cells of the neurovascular unit, including vascular endothelial cells.

During the past three decades various liposomal carrier systems have been developed in pharmaceutical research, which enabled remarkable progress in the development of drugs with difficult-to-formulate active ingredients. While resveratrol is historically has been used as a dietary supplement, its tissue distribution after oral absorption is low, and high efficacies in vitro cannot be sufficiently translated to in vivo applications. Also, systemic use has been proven difficult, the lipophilic structure of resveratrol forbids a solubility >0.05 g/l in water (Robinson et al. 2015). Applying resveratrol in our newly developed liposomal carrier system FL-RSV facilitates the application of higher systemic doses if necessary. In our experiments, FL-RSV contained 0.3 g/l to apply a dose of 2 mg/kg. Using FL-RSV, we could show that the pharmacological effect of resveratrol in the cerebral microcirculation was comparable to previous data of orally applied resveratrol (Toth et al. 2014), yet the treatment period and dose could be reduced for comparable rescue of neurovascular responses.

Age-related impairment of neurovascular coupling responses manifests in older adults (Zaletel et al. 2005; Topcuoglu et al. 2009; Stefanova et al. 2013) and has been causally linked to cognitive decline (Sorond et al. 2013; Sorond et al. 2011). Our previous research has confirmed that advanced aging in mice is also associated with significant neurovascular dysfunction, characterized by diminished CBF changes in response to increased

Fig. 2 Fusogenic liposomes effectively deliver their content to cells of the neurovascular unit as well as endothelial cells of large arteries. Panel A: Resveratrol-containing fusogenic liposomes labeled with DiD [FL-RSV(DiD)] in suspension, visualized by two-photon microscopy. Scale bar: 100 μm. Panel B: Circulating FL-RSV(DiD) observed in the cerebral circulation with two-photon microscopy after retro-orbital injection. Note that imaging FL-RSV(DiD) can be used to assess cerebral blood flow along with the microvascular network. Arrows point to the moving FL-RSV(DiD) particles in the vessel lumen. Tissue autofluorescence (green) is shown for orientation; the dashed line indicates the vessel wall on the time-stack. Scale bar: 20 μm. Panel C: Real-time liposomal delivery of resveratrol was monitored by detecting the incorporation of the fluorescent tracer DiD (red) in cells of the neurovascular unit using two-photon microscopy. After retro-orbital injection of FITC-dextran and FL-RSV(DiD) the cerebral vasculature was imaged by two-photon microscopy. Vessels lumens (FITC-dextran; green) and fusogenic liposomes (DiD, red) are visible on the time stack images. Note the early (within 4 h) incorporation of DiD delivered by the fusogenic liposomes in the lipid membranes of cells of the neurovascular unit (arrows). Scale bar: 50 μm. Panel D: Early (within 4 h) accumulation of DiD delivered by the fusogenic liposomes in the lipid membranes of cells of the neurovascular unit. Z-stack image was recorded from the upper 0–100 μm depth of the cortex. Scale bar: 50 μm. Panel E: Comparison of the incorporation of DiD-labeled resveratrol containing fusogenic liposomes [FL-RSV] and DiD-labeled liposomes that did not contain resveratrol [FL] into the cerebral microcirculation. Note the significant increase in the intensity of incorporated tracer dye DiD in brain homogenates upon administration of FL-RSV(DiD) as compared to empty fusogenic liposomes and PBS treatment (see Methods). Data are mean ± S.E.M., $*P < 0.05$ vs. empty fusogenic liposomes (FL). Panel F: Liposomal delivery of resveratrol to arterial endothelial cells was visualized by detecting the fluorescent tracer DiD in frozen sections of the aorta. Note the increased DiD signal in the plasma membrane of the inner luminal endothelial cell layer upon delivery of resveratrol incorporated in positively charged liposomes (arrows; lu: lumen; m: media)


a
FL-RSV(DiD)
b
Tissue autofluorescence
FL-DiD+RSV
c
FITC-dextran
FL-RSV(DiD)
merge
d
FITC-dextran
FL-RSV(DiD)
merge
e
DiD intensity (A.U.)
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1.5
1.0
0.5
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no RSV liposome
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lu
FL-RSV(DiD)

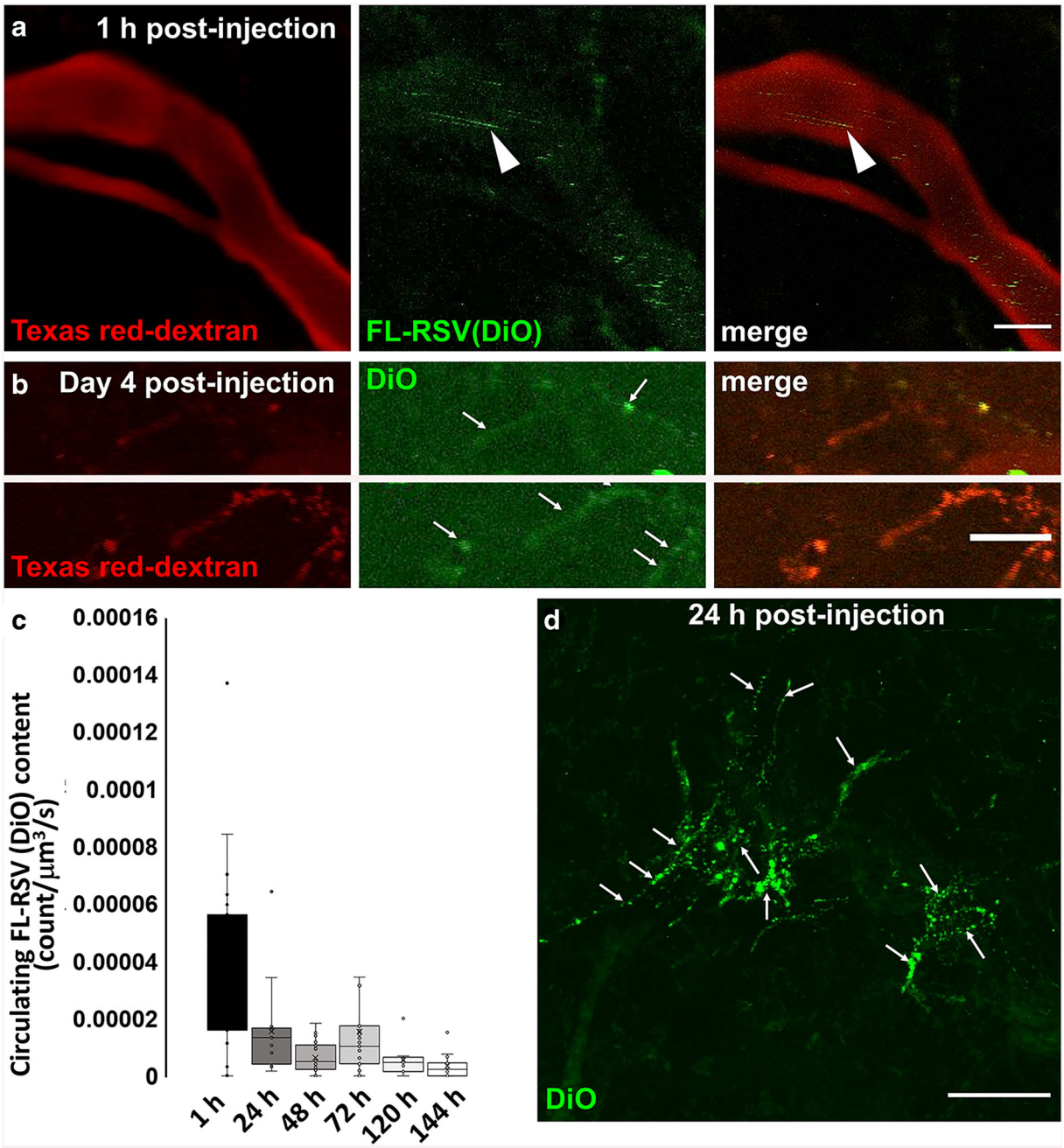

Fig. 3 Long time tracking of fusogenic liposomes in the cerebral microcirculation. Panel A: Circulating fusogenic liposomes labeled with DiO [FL-RSV(DiO)] observed in the cerebral microcirculation with two-photon microscopy after retro-orbital injection of FL-RSV(DiO) and Texas red-dextran (to visualize vessel lumens). Arrow: moving FL-RSV(DiO) particles in the vessel lumen. Scale bar: 10 μm. Panel B: DiO labeled cells in the cerebral microcirculation. Four days after injection of a single dose of fusogenic liposomes, circulating FL-RSV(DiO) were virtually absent in the microvasculature. DiO labeling in the cells of the neurovascular unit was evident (arrows). Scale bar: 20 μm. Panel C: Time course of uptake of circulating FL-RSV(DiO). Shown is the relative count of circulating DiO-labeled FL-RSV particles in the cerebral microcirculation at the indicated time points post-retro-orbital injection. Calculated quantities of intraluminal circulating FL-RSV(DiO) particles are expressed as count/μm^3/s. FL-RSV(DiO) are eliminated from the cerebral microcirculation within 24 h, suggesting daily dosing for efficacy testing of resveratrol incorporated in FL-RSV. Data ($n \geq 9$ measurements) are presented as a box plot. *$P < 0.05$ vs. 1 h. Panel D: Intensive DiO staining is evident in the cells of the neurovascular unit at 24 h post-retro-orbital injection of FL-RSV(DiO). Arrows: DiO staining in the wall of cerebral microvessels. Scale bar: 50 μm

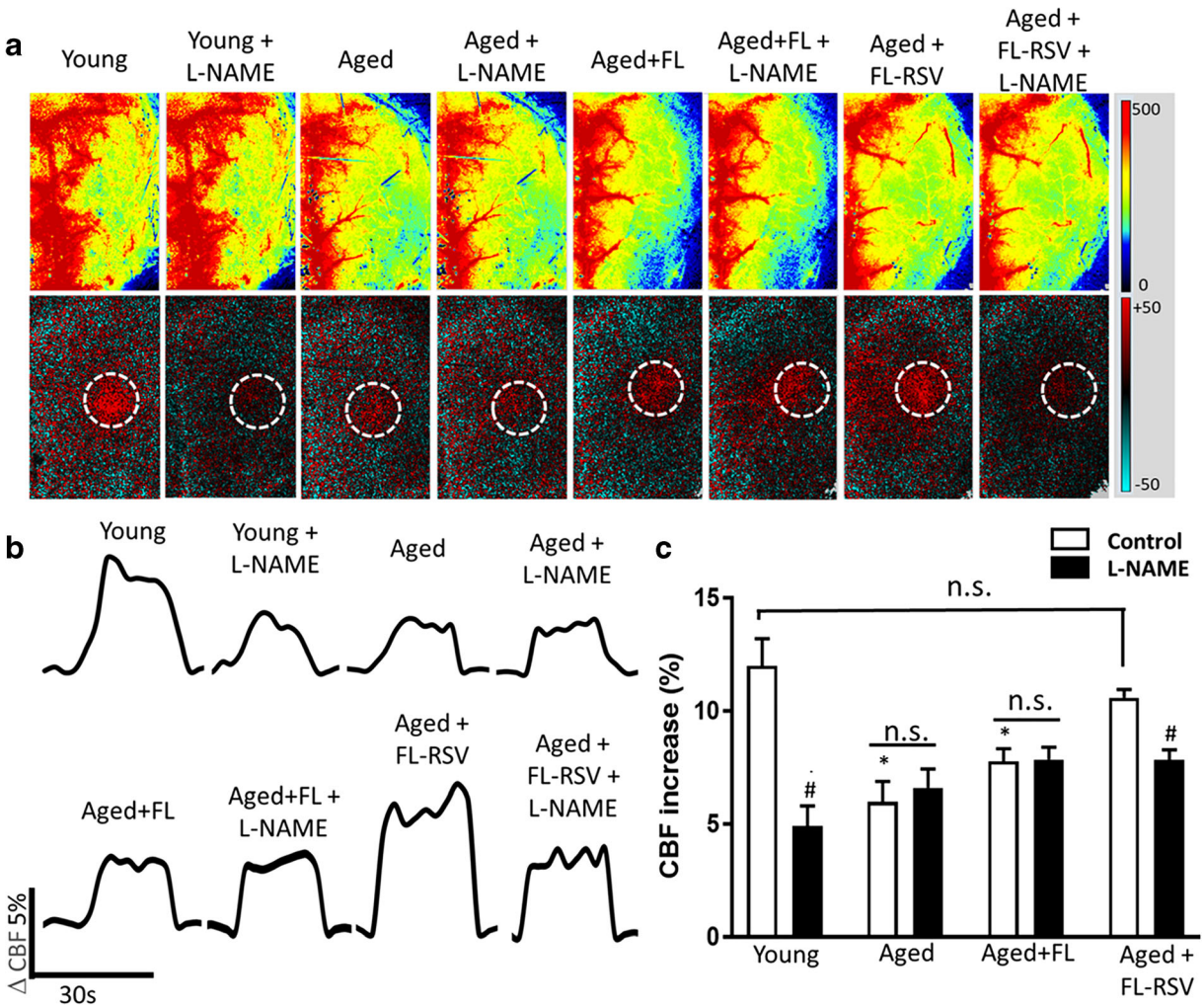

Fig. 4 Treatment with resveratrol encapsulated in novel fusogenic liposomes rescues endothelium-dependent neurovascular coupling responses in aged mice. Panel A: Representative pseudocolour laser speckle flowmetry maps of baseline CBF (upper row) and CBF changes in the whisker barrel field (circle) relative to baseline (differential images are shown in the bottom row) during contralateral whisker stimulation (30 s, 5 Hz) in untreated young (3 month old) and aged (24 month old) mice and aged mice treated with fusogenic liposomes (FL) and resveratrol containing fusogenic liposomes (FL-RSV), before and after administration of the NO synthase inhibitor L-NAME. Color bar represents CBF as percent change from baseline. Panel B shows the time-course of CBF changes in the whisker barrel cortex (see circle in Panel A) after the start of contralateral whisker stimulation (horizontal bars). Summary data are shown in panel C. Data are mean ± S.E.M. ($n = 6–8$ in each group), *$P < 0.05$ vs. Young; #$P < 0.05$ vs. Aged+FL-RSV. (one-way ANOVA with post-hoc Tukey's tests)

neuronal activation (Tarantini et al. 2017a; Toth et al. 2014; Tarantini et al. 2018; Tarantini et al. 2019). Here, we demonstrate for the first time that age-related impairment of neurovascular coupling responses is rescued by delivery of resveratrol using our innovative fusogenic liposome-based delivery system. Rescue of a critical homeostatic mechanism that matches nutrient and oxygen delivery to the increased needs of active neuronal tissue is expected to have beneficial effects on cognitive function in aging (Oomen et al. 2009; Zhao et al. 2013; Liu et al. 2012). This should be experimentally tested in future studies.

Endothelium-derived NO contributes importantly to neurovascular coupling responses (Tarantini et al. 2017a; Toth et al. 2014; Tarantini et al. 2015; Tarantini et al. 2018; Tarantini et al. 2019; Toth et al. 2015c). We find that administration of FL-RSV to aged animals restores NO mediation of neurovascular coupling responses, supporting the concept that potent endothelial

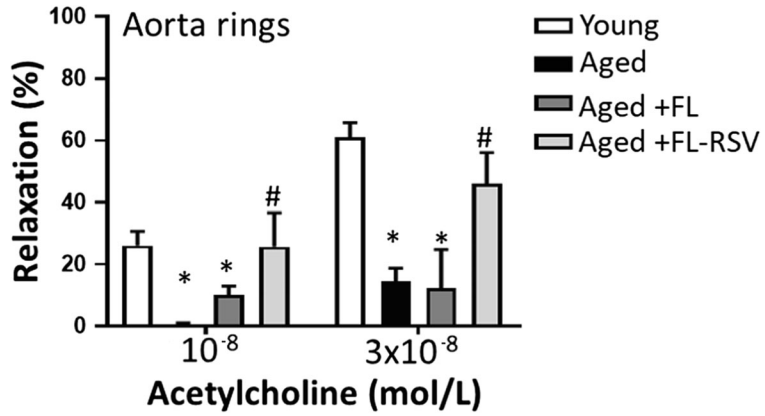

Fig. 5 Treatment with resveratrol encapsulated in fusogenic liposomes rescues endothelium-dependent relaxation in aged mouse aortas. Acetylcholine-induced, L-NAME sensitive relaxation was measured in aorta ring preparations isolated from untreated young (3-month-old) and aged (24-month-old) mice and aged mice treated with fusogenic liposomes (FL) and resveratrol-containing fusogenic liposomes (FL-RSV). Data are mean ± S.E.M. ($n = 5–8$ for each data point).*$P < 0.05$ vs. Young; $^{\#}$ $P < 0.05$ vs. Aged. (one-way ANOVA with posthoc Tukey's test)

protective effects of resveratrol play a key role in its anti-aging action (Ungvari et al. 2007a; Pearson et al. 2008; Ungvari et al. 2007c; Bernier et al. 2016; Csiszar et al. 2008; Csiszar et al. 2006; Zhang et al. 2010) The available evidence suggests that the mechanism(s) by which advanced age impairs cerebromicrovascular endothelial function involves an increased breakdown of NO by elevated levels of ROS (Toth et al. 2014; Tarantini et al. 2018; Tarantini et al. 2019). Restoration of endothelial function in aged peripheral arteries has also been reported using structurally different mitochondria-targeted antioxidants, including MitoQ, MitoTEMPO and SS-31 (Tarantini et al. 2018; Tarantini et al. 2019; Gioscia-Ryan et al. 2014; Kiss et al. 2019), suggesting that ROS production by dysfunctional mitochondria critically contributes to age-related endothelial oxidative stress. In that regard it is important that, resveratrol was shown to effectively inhibit mitochondrial ROS production in endothelial cells (Ungvari et al. 2009; Ungvari et al. 2007b). On the basis of the observation that administration of FL-RSV significantly inhibits oxidative stress in aged CMVECs *in vitro (*Csiszar et al. 2014*)*, we posit that in aged mice fusogenic liposome-mediated delivery of resveratrol to the aged neurovascular unit restores NVC responses and endothelial function by preventing ROS-mediated scavenging of NO in the endothelial cells. The mechanisms by which resveratrol, in part via sirtuin activation, may attenuate mtROS production in aging are likely multifaceted. Age-related loss of efficiency in mitochondrial electron transport likely increases electron leak and mtROS production, which could be reversed by resveratrol- and sirtuin-induced up-regulation of mitochondrial electron transport chain subunits (Tarantini et al. 2019; Csiszar et al. 2009; Kiss et al. 2019; Dai et al. 2012; Ungvari et al. 2008). In addition, there is preclinical evidence that resveratrol also activates the antioxidative transcription factor Nrf2 in vascular cells, up-regulating antioxidant enzymes and promoting glutathione metabolism (Csiszar et al. 2014; Csiszar et al. 2012; Ungvari et al. 2010). It is likely that activation of Nrf2-dependent pathways (Fulop et al. 2018) contribute to the endothelial and neurovascular protective effects of FL-RSV treatment as well (Ungvari et al. 2010).

Our findings have important translational relevance that extends beyond resveratrol-mediated neurovascular protection. Our studies provide direct evidence that the cerebral microcirculation and specifically the neurovascular unit can be successfully targeted with systemically applied fusogenic liposomes. Our recent studies demonstrate that fusogenic liposomes are also ideal molecular carrier systems for effective delivery of other cargo as well, e.g. proteins and nucleic acids (Kube et al. 2017; Hoffmann et al. 2019). We propose that resveratrol containing fusogenic liposomes could be used to deliver a complex cocktail of anti-geronic factors, which include proteins, small molecules (Nacarelli et al. 2018; Moore et al. 2017; An et al. 2017; Urfer et al. 2017; Perrott et al. 2017; Grimmig et al. 2017), DNA vectors and miRNAs targeting key pathways involved in microvascular aging and neurovascular dysfunction (Ungvari et al. 2018).

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