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Age-related changes in pial arterial structure and blood flow in mice

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ABSTRACT

Age-related cerebral blood flow decreases are thought to deteriorate cognition and cause senescence, although the related mechanism is unclear. To investigate the relationships between aging and changes in cerebral blood flow and vasculature, we obtained fluorescence images of young (2-month-old) and old (12-month-old) mice using indocyanine green (ICG). First, we found that the blood flow in old mice's brains is lower than that in young mice and that old mice had more curved pial arteries and fewer pial artery junctions than young mice. Second, using Western blotting, we determined that the ratio of collagen to elastin (related to cerebral vascular wall distensibility) increased with age. Finally, we found that the peak ICG intensity and blood flow index decreased, whereas the mean transit time increased, with age in the middle cerebral artery and superior sagittal sinus. Age-related changes in pial arterial structure and composition, concurrent with the observed changes in the blood flow parameters, suggest that age-related changes in the cerebral vasculature structure and distensibility may induce altered brain blood flow.

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1. Introduction

The cerebral vessels play an important role in maintaining brain perfusion to meet the dynamic needs of normal brain functions. Age-related degeneration of the brain vasculature may reduce the blood flow and stem cells' neurogenic potentials, which leads to reduced cognition (Farkas and Luiten, 2001; Meltzer et al., 2000). Using a variety of imaging methods, investigators have reported that cerebral blood flow (CBF) decreases significantly with age in rodents, non-human primates, and humans (Farkas and Luiten, 2001; Lartaud et al., 1993; Martin et al., 1991; Noda et al., 2002). Knox and Oliveira (1980) have reported similar numbers of arterioles in strips of cortex that extend from the pia to the white matter of rats at 3 and 24 months of age. However, the arteriole density on the cortical surface was recently found to be nearly 40% lower in senescent animals (compared with that in young adults), which suggests that surface vessels and possibly the cortical vasculature are affected by aging (Hutchins et al., 1996; Sonntag et al., 1997). However, only a few reports have addressed the cerebral pial arterial blood flow and vasculature in the aged brain.

Use of the indocyanine green (ICG) fluorescent tracer can significantly enhance the signal-to-noise ratio of imaging evaluations. Kinetic information after administering an ICG bolus, such as filling and wash-out patterns, enhances the detection of deep organs and of tissue blood flow in both experimental animals (Kang et al., 2009; Keller et al., 2003; Kuebler et al., 1998) and humans (Kang et al., 2010). A recent study has also demonstrated that a combination of near-infrared ICG fluorescence imaging and a time-series analysis of molecular dynamics can be used as a tool for measuring blood flow in the brain (Ku and Choi, 2012). ICG also accurately and reliably identifies arteries and veins within the field of view, based on hemodynamic differences (Hong et al., 2014). Therefore, the use of ICG-based optical imaging provides 2 benefits: estimating blood flow dynamics and providing 2-dimensional images of blood vessels. In our previous study, we compared pial arterial trees and used ICG to measure the arterial blood flow in the brains of different mouse strains (Kang et al., 2015a) and CBF in the ischemically injured brain (Kang et al., 2015b).

In the present study, we used ICG dynamics to estimate the cortical CBF in young (2-month-old) and old (12-month-old) mice. We also visualized the cortical pial arterial trees in these mice and used the ICG intensities to investigate changes in the cerebral cortex arterial vasculature. Finally, we compared the blood flow in the middle cerebral arteries (MCAs) and superior sagittal sinuses (SSS) of young and old mice, using ICG imaging to evaluate the age-related changes.

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2. Materials and methods

2.1. Animal preparation

Six-week-old male BALB/c mice were purchased from Daehan Biolink (Gyeonggi-do, Republic of Korea). Mice were initially housed at 3–4 animals per cage, in a controlled environment (21 °C, 40%–50% humidity) with a 12/12-hour light/dark cycle. Food and tap water were available ad libitum throughout the experiment. Because male BALB/c mice are aggressive and can fight other males if housed together (Southwick and Clark, 1966), we separated dominant males that exhibited aggressive behaviors. All animals were housed in the same room of an animal facility that is dedicated to aging experiments. As the median life span of a male BALB/c mouse ranges from 13.2 to 21.4 months (Research Model Data Sheets at Harlan, http://www.harlan.com/education_and_resources/literature/research_model_data_sheets.hl), we used 2-month-old mice to represent young mice ($n = 12$, average body weight: 21–23 g) and 12-month-old mice to represent old mice ($n = 16$, average body weight: 30–32 g) (9 young and 12 old mice for the imaging experiments, 5 young and 6 old mice for the Western blot analysis, 4 young and 6 old mice for the immunohistochemistry, and 3 young and 4 old mice for the gelatin stamp ink experiment). All procedures were approved by the Institutional Animal Care and Use Committee of Kyung Hee University and were conducted according to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (http://www.nc3rs.org/ARRIVE).

2.2. ICG imaging

Animals were anesthetized using ketamine (0.1 mg/g body weight) and xylazine (0.01 mg/g body weight), and we measured their blood pressure before and after anesthesia to determine its effect on resting CBF (Supplementary Fig. 1). Body temperatures were monitored using a rectal probe and were maintained at 37 °C using a temperature-controlled heating pad (Harvard Apparatus, Holliston, MA, USA). An incision was made to expose the entire skull regions, after which the mouse was placed under a charge-coupled device camera with an 830-nm band-pass filter. A solution of ICG (0.5 μg/μL solution and 1 μg/g body weight) was injected manually into the tail vein using a 31-gauge needle, whereas 760-nm overhead lights provided illumination. Time-series ICG fluorescence signals were acquired every 160 ms for 2 minutes. The initial 100 frames were used to generate blood flow maps via customized software that was provided by the manufacturer (Vieworks Co, Ltd, Anyang, Republic of Korea). These maps were calculated using the methods from a report by Ku and Choi (2012). The T_{rising} value was calculated as the duration between $T_{arrival}$ (time of first appearance of ICG fluorescence, i.e., arrival of the bolus) and T_{max} (time to maximum intensity). The slope of the first peak (usually equal to T_{max} in CBF) in the time-intensity curve was used to calculate the blood flow index (BFI) by dividing the relative fluorescence intensity at T_{peak} by T_{rising}. The mean transit time (MTT) was calculated as the center of gravity of the dynamic curve (Gobbel et al., 1991).

2.3. Cerebral angiography via gelatin/stamp ink perfusion

Gelatin/stamp ink perfusion was performed using the methods that were described by Xue et al. (2014), with some modifications. Young and old BALB/c mice were anesthetized using ketamine and xylazine, as described previously. After complete anesthetization, the chest was opened, a syringe needle (23 gauge) was inserted into the left ventricle, and a small slit was cut in the right atrium. Next, 50% stamp ink with 1% gelatin in 1 mL of prewarmed phosphate-buffered saline (PBS) was injected manually using a 1-mL disposable medical syringe at a rate of 1.8–2 mL/minute. After the injection, we immediately collected the brain for photographic imaging.

2.4. Western blot analysis

Cerebral cortical tissues were isolated under a stereomicroscope and homogenized in cold RIPA buffer (Thermo Fisher Scientific, Inc, Waltham, MA, USA) that contained a protease inhibitor cocktail, Na_3VO_4, and NaF (all Sigma-Aldrich, St Louis, MO, USA). After centrifugation (12,000 rpm, 4 °C for 20 minutes), the supernatant was used for immunoblotting. Proteins were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline with Tween-20 (TBS-T) for 1 hour at room temperature and incubated with one of the following primary antibodies at 4 °C overnight: anti-mouse α-smooth muscle actin (α-SMA, 1:1000 dilution, SC-53015; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or α-tubulin (1:10,000, T5168; Sigma, St Louis, MO, USA) in TBS-T or anti-goat elastin (1:1000, SC-17581; Santa Cruz Biotechnology) or collagen (1:1000, SC-8784; Santa Cruz Biotechnology) in 5% BSA TBS-T. After washing, the membrane was incubated with a peroxidase-labeled secondary antibody against goat or mouse immunoglobulin (Vector Laboratories, Burlingame, CA, USA) at room temperature for 1 hour. The Western blot was then developed using an enhanced chemiluminescent western detection system (Thermo Fisher Scientific, Inc). Bands at 43 kDa (α-SMA), 70 kDa (elastin), 70–90 kDa (collagen), and 55 kDa (α-tubulin) on the blots were subjected to quantitative densitometric analysis. Images were scanned and analyzed semiquantitatively using the ImageJ analysis software package (National Institutes of Health, Bethesda, MD, USA).

2.5. Immunohistochemistry

For the immunohistochemistry sample preparation, animals were anesthetized as described previously and transcardially perfused with 0.05 M PBS, followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and postfixed overnight at 4 °C in the same paraformaldehyde solution and cryoprotected in 0.05 M PBS with 30% sucrose. Serial 30-μm-thick coronal sections were cut on a freezing microtome (Leica, Nussloch, Germany) and stored in cryoprotectant (25% ethylene glycol, 25% glycerol, 0.05 M phosphate buffer, and pH 7.4) at −20 °C until use. For the immunohistochemical detection of CD31, free-floating sections were incubated for 15 minutes in 1% H_2O_2 and then overnight at 4 °C in 0.3% Triton X-100 with 0.5 mg/mL BSA and a rat polyclonal anti-CD31 antibody (1:200 dilution, 553370; BD Biosciences, San Diego, CA, USA). Sections were incubated for 2 hours with anti-rat secondary antibodies as appropriate (1:200; Vector Laboratories), followed by treatment with an avidin-biotin-peroxidase complex (1:100; Vector Laboratories) for 1 hour at room temperature. Peroxidase activity was visualized by incubating the sections with 0.02% 3,3'-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 in 0.5 M TBS (pH 7.6). After several rinses with PBS, the sections were mounted on gelatin-coated slides, dehydrated, and cover slipped with Histomount medium (National Diagnostics, Atlanta, GA, USA). Slices were visualized using a light microscope (LSM 510 META; Zeiss Axiovert, Jena, Germany).

2.6. Stereological analysis

For the analysis of the microvascular parameters, every 10th coronal section throughout the somatosensory cortex (Bregma

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