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Cerebral arterioles preparation and PECAM-1 expression in C57BL/6J and ApoE$^{-/-}$ mice

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1. ABSTRACT

Exploration of molecular mechanisms involved in alterations of cerebral blood vessels in pathology such as chronic kidney disease implies cerebral vessel isolation for proteins and mRNAs quantification. We describe an updated method to isolate brain microvessels from wild type (WT) mice at 14 weeks of age. The quality of cerebral arterioles preparation was determined by measuring the enzymatic activity of gamma-glutamyl transpeptidase, an enzyme that is especially expressed in cerebral microvessels, and the quantitative expression (Western Blot) of platelet endothelial cell adhesion molecule-1 (PECAM-1), alpha-actin and gamma-enolase as markers of respectively endothelial cells, smooth muscle cells and neurons. We then assessed PECAM-1 expression in 14 and 20 weeks old ApoE$^{-/-}$ and WT mice. PECAM-1 expression was increased in 14 weeks old ApoE$^{-/-}$ mice compared to age matched WT mice. This difference in PECAM-1 expression disappeared at 20 weeks of age. These findings indicate that the present method of mice cerebral arterioles isolation is a valid method. Moreover, PECAM-1 expression, a marker of endothelial cells, changes with age in cerebral arterioles of ApoE$^{-/-}$ mice.

2. INTRODUCTION

Stroke is the third most common cause of cardiovascular death in chronic renal failure (CRF) patients. Patients with end-stage renal disease (ESRD) show a 4- to 10-fold greater risk of hospitalized ischemic and hemorrhagic stroke (1), an increased risk of cognitive impairment and dementia (2-3) and a poor long-term post-stroke prognosis (4) compared with non-ESRD individuals. Exploration of the intracellular and molecular mechanisms behind the alterations of cerebral blood vessels function and/or structure implies cerebral vessel isolation for proteins and mRNAs analysis. Isolation of brain microvessels have been described in rats (5) and mice (6). However, especially in mice, it is sometimes necessary to pool brains from several animals in order to obtain a sufficient quantity of material to perform the experiments. The goal of this study was to develop a method to isolate cerebral arterioles with an acceptable quantity and level of purity. To evaluate the purity of our preparation we 1) inspected our vessels preparation by light microscopy, 2) determined the activity of gamma-glutamyl transpeptidase, an enzyme especially present in cerebral arteries endothelial cells (7) in total brain extract and in the isolated

cerebral microvessels and 3) evaluated quantitative expression of gamma-enolase, PECAM-1 and alpha-actin as neuronal, endothelial and smooth muscle cells markers respectively in cerebral microvessels and whole brain preparations.

To assess the relevance of this method we examined the expression of platelet endothelial cell adhesion molecule-1 (PECAM-1) in both apolipoprotein E-deficient mice ($ApoE^{-/-}$) and wild type mice cerebral vessels using our preparation technique.

PECAM-1, a membrane located protein is a member of the immunoglobulin (Ig) superfamily and one of the earliest molecules expressed at the inflammation sites. PECAM-1 participates to the maintenance of vascular endothelial organisation and contributes to leukocyte extravasation during inflammation (8) and several experiments suggest that this protein plays at the same time a proinflammatory (9) and an anti-inflammatory role (10).

3. MATERIALS AND METHODS

3.1. Animals

Female $ApoE^{-/-}$ and C57BL/6J wild-type (WT) mice purchased from Charles Rivers (Lyon, France) were used in all experiments. The animals were housed in polycarbonate cages in temperature- and humidity-controlled rooms with a 12:12-hour light-dark cycle and were given standard chow (Harlan Teklad Global Diet 2018, Harlan, Oxon, UK). All mice were handled in accordance with French legislation, and the protocol was approved by an institutional animal care committee.

3.2. Isolation of cerebral arterioles

Animals were weighted, anesthetized with ketamine (80 mg/kg) plus xylazine (8 mg/kg) and perfused with 20 ml of saline through a cardiac puncture in order to wash the vessels from blood. The brain was removed and immediately frozen in nitrogen solution and stored at -80°C until used. To isolate cerebral arterioles, we adapted a technique previously described by Yamakawa *et al.* (5) to isolate rat cerebral arterioles. Briefly, isolated brains were washed in ice-cold sucrose buffer (0.32 mol/L sucrose, 3 mmol/L HEPES, pH 7.4) and, after been cleared of pia mater and choroid plexus, the brain was homogenized with a tight fitting pestle Dounce homogenizer in 5 ml of sucrose buffer. The homogenate was centrifuged at 1000g for 10 min. The supernatant containing neuronal cells was discarded and the white layer of myelin in the upper part of the pellet was removed. We resuspended the pellet again in 5 ml of the same cold buffer and centrifuged at 1000g for 10 min again. We repeated the washes three times to eliminate the rest of the myelin and free neurons. The pellet was resuspended in 3 ml of the sucrose buffer and centrifuged at 350g to eliminate detached cells. This step was repeated three times and the resulting pellet was resuspended in 1 ml of sucrose buffer and centrifuged at 75g for 5 min. The resulting supernatant was used as the cerebral microvessels preparation.

3.3. Evaluation of the cerebral microvessels preparation purity

For the development of cerebral microvessels isolation method and the evaluation of the preparation purity by Giemsa staining and gamma-glutamyl transpeptidase activity, we used cerebral microvessels isolated from 14 weeks old WT mice. For the western blot quantification of smooth muscle cells, endothelial cells and neurons markers, we used cerebral microvessels isolated from both 14 weeks old $ApoE^{-/-}$ and WT mice. Finally we evaluated PECAM-1 expression in cerebral microvessels of 14 and 20 weeks old WT and $ApoE^{-/-}$ mice.

3.3.1. Giemsa staining

We evaluated the presence of cerebral arterioles at various steps of the isolation procedure in the pellet and in the supernatant. For this, dried smears were prepared from 5 microL of homogenate in sucrose buffer (0.32 mol/L sucrose, 3 mmol/L HEPES, pH 7.4). The smears were fixed on 4% formaldehyde solution for 15 minutes. After several washes in distilled water, the slides were incubated in the Giemsa stain modified solution (Sigma Aldrich, St Louis, MO, USA) for 10 minutes. After several washes with distilled water, the slides were dried and mounted using VectaMont solution (Vector Laboratories Inc. Burlingame CA, USA). The slides were left to dry for 5 hours on the bench before being analyzed using a microscope (Axioskop 40, Carl Zeiss Jena GMBH, Jena, Germany) connected to a video system with a final magnification of 426x.

3.3.2. Gamma-glutamyl transpeptidase activity

The activity of gamma-glutamyl transpeptidase, an enzyme especially present in cerebral arteries endothelial cells (**7**), was evaluated in cerebral microvessels and whole brain preparations. For this, 50 microL of protein from each sample were incubated for 120 minutes at 34°C with 20 mmol/L glycylglycine, 10 mmol/L MgCl2, 100 mmol/L Tris, pH 9.0, and 1 mmol/L L-gamma-glutamyl-*p*nitroanilide as a substrate, in a total volume of 1.0 mL. After 120 minutes, the reaction was stopped with 500 microL of acetic acid to a final concentration of 1.0N, and 300 µL of methanol. After centrifugation for 15 minutes at 400g, the absorbance of the supernatant was measured at 415 nm.

3.3.3. Western blot detection of neuronal, endothelial and smooth muscle cells markers

We also evaluated quantitative expression of gamma-enolase, PECAM-1 and alpha-actin as neuronal, endothelial and smooth muscle cells markers respectively in cerebral microvessels and whole brain preparations. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blots were blocked in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% of nonfat dry milk for 1 hour at room temperature. For PECAM-1 and gamma-enolase, blots were incubated in the blocking solution containing an anti-PECAM-1 or anti-gamma-enolase antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1/500 dilution, at 4°c overnight. For alpha-actin, the blot was incubated in the presence of an anti-alpha-actin (Santa Cruz Biotechnology Inc) at

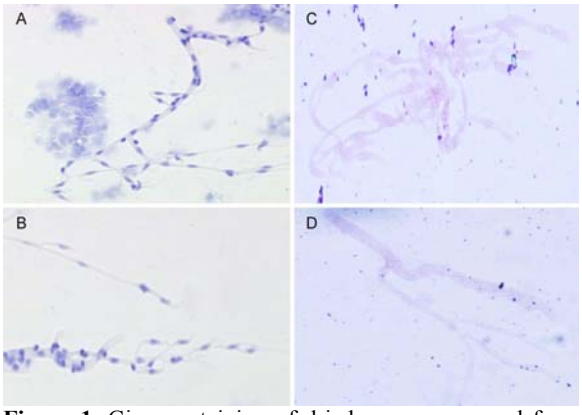

Figure 1. Giemsa staining of dried smears prepared from pellet of the 1st centrifugation step of purification (A), the 2nd centrifugation step (B) and the final centrifugation step (C and D).

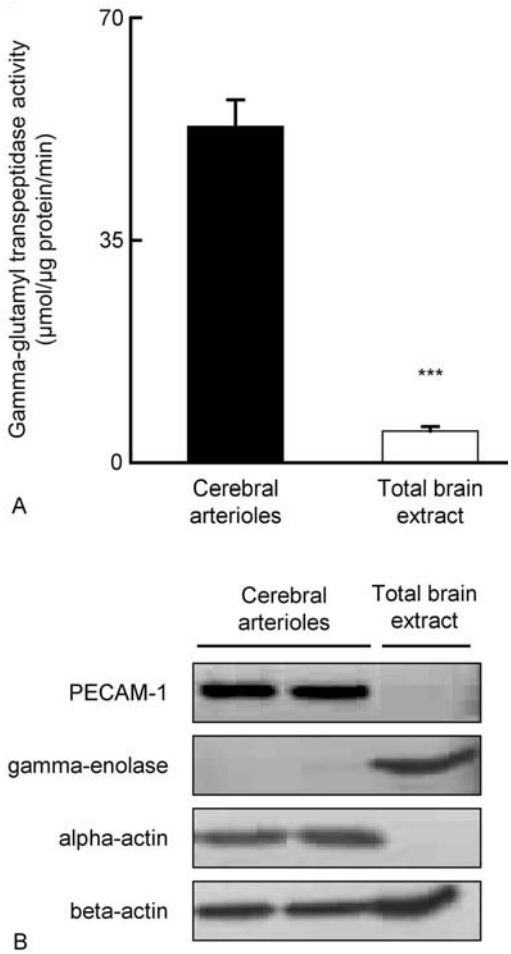

Figure 2. Cerebral microvessels preparation evaluation using: A) gamma-glutamyl transpeptidase activity measured in total brain extract (n=4) and in the isolated cerebral microvessels of WT mice (n=3) and B) Western blotting analysis of PECAM-1, alpha-actin and gamma-enolase of WT mice (n=4). Data are represented as mean ± SEM. ***: p< 0,001 total brain extract *versus* cerebral arterioles.

1/1000. After five washes of 5 min each, PECAM-1 or gamma-enolase blots were incubated in TBS-T containing a rabbit anti-goat Horse radish peroxidase (HRP) conjugated antibody at 1/5000 dilution and alpha-actin blot was incubated in TBS-T containing a goat anti-mouse IgG HRP conjugated at 1/5000. After 1 h incubation at room temperature, blots were washed extensively with TBS-T containing 0.05% Tween for 25 min. The bound antibodies were visualized by chemiluminescence using ECL Signal and exposure to X-ray films (GE-Health Care, Buckinghamshire, UK). Band densitometry was measured using the Gene Genius Gel Imaging System (Gene Tools; Syngene, Cambridge, UK).

3.4. Statistical analysis

Data are presented as mean +/- SEM. One-way ANOVA Test was performed between two groups to assess the statistical significance. $p<0.05$ was considered to be statistically significant.

4. RESULTS

4.1. Evaluation of the cerebral microvessels preparation purity

The Giemsa staining and the measure of the gamma-glutamyl transpeptidase activity were performed in the WT mice cerebral arterioles preparation. These preparations contain in large majority microvessels as shown by Giemsa staining of dried smears (Figure 1). The activity of gamma-glutamyl transpeptidase was increased approximately 10 times in the cerebral microvessels preparation compared to the whole brain preparation (Figure 2A). Gamma-enolase was almost undetectable in western blot performed with proteins from the cerebral microvessels preparation but widely present in western blot performed with proteins from the whole brain preparation (Figure 2B). In contrast, alpha-actin and PECAM-1 were widely detectable in western blot performed with proteins from the cerebral microvessels preparation and almost undetectable in western blot performed with proteins from the whole brain preparation (Figure 2B). When the present method was used to isolate ApoE$^{-/-}$ mice cerebral microvessels, the distribution of smooth muscle cell, endothelial cell and neurons markers was similar to what we observed for WT mice (data not shown).

4.2. PECAM-1 expression in cerebral arterioles of WT and ApoE$^{-/-}$ mice

Western blot analysis show that PECAM-1 expression was significantly enhanced in cerebral arterioles of 14 weeks old ApoE$^{-/-}$ mice compared to age-matched WT mice (Figures 3A and C). In contrast, PECAM-1 expression was similar in cerebral arterioles of 20 weeks old ApoE$^{-/-}$ and WT mice (Figures 3B and D).

5. DISCUSSION

There are two major findings in this study. First, the present method of preparation seems to be appropriate for obtaining purified cerebral arterioles, as attested to by the Giemsa staining on dried smears, the gamma-glutamyl transpeptidase activity and the high quantity of endothelial

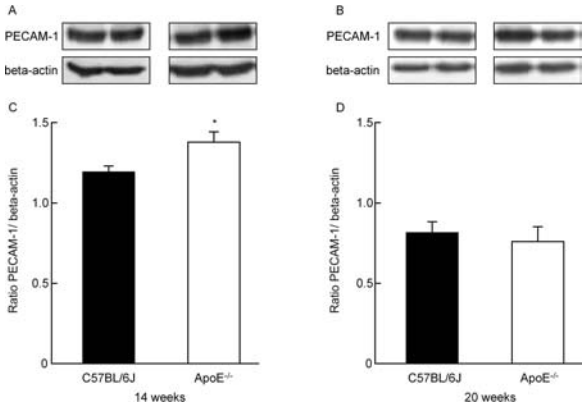

Figure 3. Quantification of PECAM-1 protein in cerebral microvessels of ApoE$^{-/-}$ and WT mice. Representative Western blots of PECAM-1 in cerebral microvessels of 14 (A) and 20 weeks (B) old WT and ApoE$^{-/-}$ mice. Densitometric value of PECAM-1 normalized to beta-actin in 14 (C) (n=8) and 20 weeks (D) (n=4) old mice. Data are represented as mean ± SEM. *: p< 0.05 WT *versus* ApoE$^{-/-}$.

cells and smooth muscle cells markers present in our preparations. Second, PECAM-1 expression is increased in 14 weeks old and similar in 20 weeks old ApoE$^{-/-}$ mice compared to WT mice indicating that endothelium integrity is preserved in cerebral arterioles of ApoE$^{-/-}$ mice at these early ages.

In the present study we report a simple method to isolate cerebral arterioles from mouse brain. Several methods have been described to isolate cerebral arterioles from bovine, rabbit and rat brain (11,12,13). We failed to adapt these techniques to isolate cerebral arterioles from mice. One possible explanation is the small size of mice cerebral arterioles that reduced their retention by nylon mesh (used in different preparation) or their complete precipitation by centrifugation at the speed indicated by the authors.To isolate cerebral arterioles, we finally adapted a centrifugation based method previously described by Yamakawa *et al.* (5) to isolate rat cerebral arterioles. We modified the method by increasing the speed of the 4th, 5th and 6th centrifugation steps (from 200g to 350g) and by adding a new centrifugation step (75g) in order to eliminate the large vessels and succeed in isolation of mice cerebral arterioles with high purity.

It is well established that PECAM-1 participates actively to the cell to cell contact in the endothelium and to the extravasations of immune cell to the site of inflammation (14). As a first application of our cerebral arterioles isolation method, we sought to compare the expression of PECAM-1 in 14 and 20 weeks old ApoE$^{-/-}$ mice and age matched WT. Our results show a significant increase of PECAM-1 expression in 14 weeks old ApoE$^{-/-}$ mice compared to age matched WT mice. This difference in PECAM-1 expression between the groups disappeared when the mice are 20 weeks old. This finding is in agreement with previous reports showing that PECAM-1 expression is modulated over period of time and in different organs such as the aortic arch and the heart of ApoE$^{-/-}$ compare to WT mice. In the aortic arch, PECAM-1 is highly expressed in young ApoE$^{-/-}$ mice (3 to 6 weeks) compared to their age matched WT and this difference disappeared when animals are 20 weeks old (15). In contrast, PECAM-1 expression in the heart of ApoE$^{-/-}$ is up-regulated in 20 weeks old mice compared to their age matched WT and to the 6 weeks old mice of both groups (14).The difference between our results and the result of the later study (14) may be due to organ specificity. The increase of PECAM-1 expression could be explained as a direct response of endothelial cells to different insults as a way to maintain cell-cell junctions and preserve the endothelium integrity (16,17).

In conclusion, we describe an updated method to isolate arterioles from mice brain. This method may facilitate the design of appropriate experiments to investigate the molecular mechanisms behind the impairment of cerebral circulation encountered during different cardiovascular diseases

6. ACKNOWLEDGEMENTS

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