SUPPLEMENTARY METHODS FOR

Live dynamic imaging of caveolae pumping antibody rapidly and specifically across endothelium in the lung

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Supplementary methods.

Isolation of rat lung luminal endothelial cell plasma membranes and caveolae. Rat lungs were processed using the silica coating technique[1,2] to isolate luminal endothelial cell plasma membranes and caveolae. These lung tissue subfractions have been extensively characterized previously[1-3] as enriched 20-fold in endothelial and caveolar markers while being 20-fold depleted in markers found elsewhere.

Generation of monoclonal antibodies. The luminal endothelial cell membranes isolated from rat lungs were used to immunize Balb/c mice for hybridoma generation and to screen monoclonal antibodies as in our past work[4]. J310 was selected by additional ELISA screening against purified recombinant rat APP. TX3.833 was also generated as above and has been partially characterized[4].

Cloning of TX3.833 antigen. Protein from total rat lung homogenate was affinity purified on a TX3.833-coupled Affi-Gel Hz column (BioRad). The eluted proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted using TX3.833. The band corresponding to the isolated TX3.833 protein was cut out of the filter, sent to the Harvard Microchemistry Facility for microsequencing. Five peptide sequences were identified but did not match any known sequences in GenBank at the time. To clone the rat cDNA for TX3.833 antigen, degenerate oligonucleotides derived from the peptide sequences were synthesized and used for polymerase chain reaction to amplify larger cDNA fragments from a rat lung cDNA library. The PCR products were used to screen a rat lung cDNA library (Clontech) by hybridization and one 1.5kb positive clone, 12H1, was identified. 12H1 contained an open reading frame of 500 amino acids as well as sequences corresponding to 3 of the original peptides. This sequence was used to search GenBank, resulting in the identification of four ESTs, one of which, RODY89 (TIGR/ATCC Special Collection of Rat cDNA Clones, Manassas, VA), contained the 5' end and which also contained sequences corresponding to the remaining two peptides. The 3' end of the cDNA was obtained by utilizing rtPCR and 3' rapid amplification of cDNA ends (RACE) on rat lung mRNA. A search of the GenBank database using this amino acid sequence revealed considerable identity to human and mouse membrane-bound Aminopeptidase P. The full-length cDNA was cloned into the *Bgl*II/*Not*I sites of pDisplay (Invitrogen, Carlsbad, CA) mammalian expression vector deleted for the myc tag and PDGFR transmembrane domain to generate the expression construct, pDdlMP-APP. To confirm that the isolated APP cDNA corresponded to the

TX3.833 antigen, pDdlMP-APP was transfected into CHO cells using Effectene (Qiagen,) grown to confluency, solubilized with CLB buffer (2M Urea, 3 mM EDTA pH8, 3% sodium dodecyl sulphate, 1.2% 2-mercaptoethanol in 0.15 M Tris-HCl pH6.8), separated by SDS-PAGE, and electrotransferred to nitrocellulose filters for immunoblotting with TX3.833 as described[3].

MS identification of antigens. The appropriate SDS-PAGE gel bands were excised and in-gel trypsin digested to yield an extracted complex peptide mixture that was separated using a reversed phase C-18 microcolumn connected to an HPLC solvent delivery system before elution over 60 minutes via a binary gradient directly into the electrospray ion trap mass spectrometer (LCQ Deca XP, ThermoFinnigan, San Jose, CA). Automated analysis of ion spectra was performed using SEQUEST software to designate acquired MS/MS spectra to peptide spectra of known proteins in available public rat databases.

Immunohistochemical staining of rat tissues. Frozen rat tissues were cut (5μm) on a Microm HM505E cryomicrotome. Sections were fixed with neutral buffered formalin for 5 min at room temperature then incubated for one hour at room temperature in blocking solution (5% FBS, 0.1% Tween 20 in PBS). After a 2-hour incubation at room temperature in primary antibodies (diluted in blocking solution) the sections were washed then treated with the appropriate biotin-conjugated secondary antibody (KPL Laboratories, Gaithersburg, MD) for 1 hour at room temperature, washed again then treated with a streptavidin-conjugated horseradish peroxidase (KPL Laboratories, Gaithersburg, MD) for 1 hour at room temperature. Immune complexes were detected using a Liquid DAB staining kit from BioGenex (San Ramon, CA). Formalin-fixed, paraffin-embedded sections (5μm) were cut on a Microm HM340E microtome. Antigen retrieval was performed using acid citrate buffer following standard procedures. After the sections were washed in water to remove the citrate buffer, they were blocked and immunostained as above.

Tissue subfractionation and 2D gel analysis. 2D gel electrophoresis was performed to resolve proteins in whole lung homogenate or luminal endothelial cell plasma membranes (P) isolated from heart, lung, liver, kidney, and brain using the silica-coating technique as in our past work[1,2]. The gel was silver stained or subjected to Western analysis using TX3.833[2,3].

Immunofluorescence microscopy. Rat lung frozen and paraffin-embedded sections were incubated with caveolin-1 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA)) and G278 (a pan-endothelial marker antibody generated in-house recognizing podocalyxin) followed by labeling with fluorophore-conjugated secondary antibodies (anti-rabbit IgG-Alexa

488 (caveolin-1; green signal) and anti-mouse IgG-Alexa 568 (G278; red signal); Molecular Probes, Inc. (Eugene, OR)). The washed sections were fixed, incubated with unlabeled mouse IgGs (Southern Biotech, Birmingham, AL) as a quench before incubation with Cy5-conjugated TX3.833 (blue pseudocolor) and examination using a Nikon E800 microscopy fitted with a Perkin Elmer Wallac UltraView confocal head and a triple line laser (Perkin Elmer Wallac, Gaithersburg, MD).

Electron Microscopy

Preparation of gold nanoparticle solution

10 nm gold nanoparticles were prepared by modification of the procedure of Mühlpfordt[5]. Briefly, to a solution of 10 mg (0.03 mmol) of $HAuCl_4$ in 80 mL of distilled water at 60ºC was added a solution of 40 mg of trisodiumcitrate (0.14 mmol) and 80 μL of 1% tannic acid in 20 mL of distilled water. After 5 minutes the red solution was warmed up rapidly to 95ºC followed by cooling in an icebath. The solution was adjusted to pH 8.5 with NaOH. TEM showed discrete nanoparticles 10–15nm in diameter.

mAPP conjugation to gold nanoparticles

1 mg of antibody at 0.1 mg/mL was dialyzed overnight against 2mM boric acid adjusted to pH 8.5 with NaOH. This was added dropwise to a strongly stirred solution of the above prepared nanoparticles in a 50mL plastic conical tube. After 25 min 50mg of PEG 20000 was added and the solution stirred for a further 5 min. The solution was then centrifuged at 13,500 rpm and 4º C for 35 min to remove aggregates. The supernatant was decanted and spun at 22,000 rpm for 1h, 10min at 4º C. The resulting soft pellet was collected, made up to 2mL of phosphate buffer (10 mM, pH 8) and dialyzed against 50mM Tris.HCl overnight. A saturated NaCl solution was then added by syringe pump over 24 h such that the final NaCl concentration was 150mM. 1.5 mL of a deep red solution was obtained after filtration through a 0.22μm filter to remove a small amount of solid. OD_{520} of final perfused solution was 5.

Electron microscopy of tissue after vascular perfusion of antibody-gold nanoparticles

As described[4], antibody-gold (antibody-Au) complexes (3 mL; OD_{540} = 8.5 and 2.7 for rat #1 and #2, respectively) were delivered to two separate rats via pulmonary artery into a cranial lobe of rat lung by perfusion in situ at 15mm Hg. After flushing and then perfusion fixation in situ with 30 mL of 4% paraformaldehyde/2.0 % glutaraldehyde in 0.1 M Na cacodylate, pH 7.4, the tissue was cut into ~1 mm^3 and incubated for 2 hrs at 4° C in the same fixative. The fixed samples were

washed with 0.1 M Na cacodylate – HCl buffer pH 7.4 (3 x 15 min.) and post fixed in 1% OsO_4 in 0.1 M Na cacodylate buffer, pH 7.0 for 60 min. on ice. After a final wash with water the samples were embedded in EMbed-12 (EM Sciences Cat. No 14120). 60 nm sections were cut on Leica Ultracut UCT ultramicrotome and stained with 2% uranyl acetate followed by lead citrate[6]. Sections were viewed and photographed using Morgagni 268 D electron microscope equipped with MegaView III digital camera.

Immunogold labeling of frozen tissue sections and electron microscopy

Immunogold staining of tissue was performed essentially as described[7]. In brief, 60 nm sections of frozen rat lung tissue were cut with diamond cryo 35^{o} knife (Diatome) on EM FC-S low temperature sectioning system (Leica). They were picked from knife with 2.3 M sucrose, transferred to formvar-carbon-coated nickel grids and then floated on 1% albumin (Sigma, Cat No.A5378) in 0.1 M Na-cacodylate buffer for at least one hour before incubation with antibody (50 μg/mL) at RT for one hour. Sections were then washed eight times with 0.1 % albumin in the same buffer and incubated with 10 nm Au coupled to protein A (Cell Microscopy Center, University Medical Center Utrecht, The Netherlands; Dr G. Posthuma). The wash step was repeated before fixation with 1% glutaraldehyde. The sections were stained with mixture of uranyl acetate and methyl cellulose (25 centipoises, Sigma M-6385) in water at final concentration of 1.3 % each, for 10 min. at RT. Images were obtained with Morgagni 268 D electron microscope equipped with MegaView III digital camera at 100kV.

^{99m}Tc labeling. As previously described[8], mAPP (0.4 mg) was modified for 3 hrs with succinimidyl 6-hydrazinonicotinate (HYNIC:Antibody molar ratio of 8 or 16), dialyzed overnight against PBS (pH 7.4), and radiolabeled with ^{99m}Tc pertechnetate (supplied by Central Pharmacy (Birmingham, AL, USA)) using tricine as the transfer ligand before separation from non-bound ^{99m}Tc by G-25 Sephadex chromatography[9]. Protein concentrations of the collected fractions were determined by the method of Lowry. Protein bound ^{99m}Tc was greater than 99%, as determined by thin layer chromatography. The ^{99m}Tc-labeled, HYNIC-modified mAPP had specific activities ranging from 100–371 MBq/nmole.

Intravital Microscopy Image Processing

Intravital Microscopy Image Filtering

IVM Images were filtered according to the scheme described below to extract a more useful image, or stream of images, from the output of the original Intravital Microscope system. Image

filtering of original images was carried out in two main steps, an image enhancement step followed by noise compensation.

Image Enhancement

Image enhancement was carried out to increase the dynamic range of the original images. In this step, the intensity resolution was increased and the full 8-bit intensity range of the output image was utilized. The mean pixel value of the original images was ~*55*. The average minimum pixel value was ~*5*, and the average maximum pixel value was ~*95*. These values correspond to an average dynamic range of 19.8 dB. Also, the resolution of the original image was 0.0105 of the maximum pixel value. Enhancement utilized the following equation (1):

(1) $$I_f(m,n) = \alpha(I_o(m,n)-\mu)+128,$$

where $I_f(m,n)$ and $I_o(m,n)$ represent the final and original image pixel values respectively, μ represents the mean of the original image, and α represents the enhancement constant. The enhancement constant was calculated using equation (2):

(2) $$\alpha = 1.6*128*(1/\max[\, abs(\max(I_o(m,n))-\mu),\ abs(\min(I_o(m,n))-\mu)\,]).$$

The constant *1.6* in (2) was determined experimentally. With this constant set to *1.0*, the enhancement block of the filtering system linearly maps the original image pixel values to a set of pixel values centered about 128, and bounded by the minimum value of 0 and the maximum value of 255. This scenario maximizes the dynamic range of the enhanced image without any clipping due to values mapped outside of the [0–255] intensity range. Setting the constant in the above equation to a value >1.0 causes the mapped pixel values to be clipped and therefore leads to a certain loss of information. However, this results in a cleaner and more aesthetic filtered image at the output of the overall system. The dynamic range of the enhanced images was increased to 24.1 dB. The resolution was decreased to 0.0039 of the maximum pixel value.

Noise Compensation

The noise compensation step of the overall filtering system was designed to manipulate the original image in order to minimize the effects of additive image noise. There were two main correctable sources of noise in the IVM system, the lateral and vertical movements of the animal in relation to the microscope. The former occurred when the relative position of the microscope along the x-axis in the output images changed due to movement of the animal. In the latter case, movement of the animal was directed along axis of the microscope lens.

The algorithms used to compensate for the additive noise due to motion of the animal were based on finding the optimal magnitude multiplication constants and the optimal x-axis offset constants. The grainy nature of the output images made this task difficult. Therefore, both algorithms were preceded by lowpass filtering of the images along the x-axis.

Motion of the animal along the axis of the microscope lens resulted in adjacent rows of the output image (lines parallel to the x-axis) being uncalibrated in relation to each other. Differences in the distances between the animal and the lens surface resulted in varying attenuation of the florescence light waves as they propagated to the microscope lens. Thus, the shape of the normalized intensity curve at any given time was not affected by the vertical motion of the animal, though the magnitude of the curve was affected. Therefore, in order to compensate for the additive noise caused by the vertical motion of the animal, the optimal magnitude of each reading in time was determined. This optimal magnitude was calculated using forwards- and backwards- prediction. The following equation was used to determine the magnitude for the m^{th} reading in time:

$$Magnitude(m) = (1/(2k+1))* \sum_{\tau=-k}^{\tau=k} [max(image(m+\tau,1:N))].$$

This *Mx1* vector of optimal magnitudes was saved for future application.

Lateral motion of the animal in relation to the microscope resulted in adjacent readings in time being offset by a certain number of pixels. The X-axis Spatial Offset algorithm was based on finding the optimal offset of each reading time. This was done by minimizing the following cost function:

$$Cost = \sum_{\tau=1}^{k} \sum_{n=1}^{N} [image(m,n) - \beta^{\tau} * image(m-\tau,1:n)]^2 .$$

The cost function was minimized for each reading in time (for each row of the image), and the determined offset values were applied to the low-filtered image. The total change in the image intensity was then calculated. The above steps were repeated until the change in image intensity converged to a local minimum. The 1xN vector of optimal offset values determined was saved for future application. The final step in the Noise Compensation Block of the overall system

was to apply the amplitude normalization and spatial adjustment values to the original (unfiltered) image.

A custom MATLAB script, *clean_image( )*, was written for this task, and included an abundance of constants used in the filtering process. Many of these constants were not optimized in closed form, but were instead optimized experimentally. Thus, adjustments of constants can result in various results. The derivation of the filtering system described in this paper was done with only two sample images. Thus, it is important to "fine tune" these constants to a larger database of sample images before deciding upon optimal values.

Specifically, it is very important to tune the constants relating to the X-axis Spatial Offset Adjustment algorithm. This algorithm is only designed to converge to a local extremum, and the algorithm can diverge from the desired result.

Polar Plot Image Processing Algorithm:

The fluorescence scan values were exported to a matrix, and the intensity values were scaled to the output matrices. The maximum scan values in distance were then normalized to fit a low-pass version of the intensity vs. time curve. The value of each reading in time *m* was scaled by the value *M(m)*, as described below:

$$M(m) = (1/(2k+1))*\left(\sum_{\tau=-k}^{\tau=k} [\max(\text{image}(m+\tau,1:N))]\right) / [\max(\text{image}(m,1:N))].$$

Next, the filtered scan lines were made into polar plots. Finally, these polar plots were concatenated into an ".avi" video file. The vessel position and radius were approximated from the video file. The image intensity located outside of the approximated vessel was summed, and this value was plotted in time. This curve represents the amount of fluorescence at each point in time. The derivative of this curve is related to the flux of fluorescence through the vessel wall.

Supplementary references

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