Online Data Supplement

ERM are Phosphorylated in Response to 2-methoxyestradiol and Modulate Endothelial Hyperpermeability

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Measurement of transendothelial permeability. Transendothelial electrical resistance (TER) was measured using the highly sensitive biophysical assay with an electrical cell-substrate impedance sensor (ECIS) (Applied Biophysics, Troy, NY) as described previously (15). Cells were grown to confluence on gold microelectrodes (to the resistance of approximately 1000 Ohm). Media were changed to fresh complete or basal media 1h prior the experiment. Permebility of FITC-dextrans was measured as described before (16).

Endothelial cells (EC) Imaging. For immunofluorescence experiments, EC were plated on gelatin-covered coverslips. Media was changed to basal media 1h prior the experiment. Before immunostaining, cells were briefly washed with phosphate-buffered saline (PBS) and fixed in 4% formaldehyde dissolved in PBS. After permeabilization with 0.25% triton X-100 and blocking, cells were stained with specific antibodies, fluorescent secondary antibodies and/or phalloidin conjugated with Alexa594 or Alexa488. After mounting in anti-fade mounting media, the coverslips were viewed and photographed with Zeiss Axio Observer Video Imaging system using Zeis Axiovision software.

Western immunoblotting. To obtain cell lysates, cells were grown in 12-well or 6-well plates; media was changed to basal media 1h prior the experiment. After pretreatment and stimulation, cells were rinsed with ice-cold PBS and lysed with PBS containing 1% SDS, antiprotease cocktail and 20mM NaF. After freezing-thawing and aspiration through 25 g needle, samples were supplemented with Western blot loading buffer and boiled.

To obtain lung extracts, dry-blotted lungs were snap-frozen in liquid nitrogen and stored at -80^{o} C until homogenization. The lungs were pulverized using Besson tissue pulverizer and lysed with PBS containing 1% SDS, antiprotease cocktail and 20mM NaF. After freezing-thawing in liquid nitrogen and sequential aspiration through 18g and 23g needles, protein concentration was assessed with BCA kit (Pierce). Samples were equalized in protein, supplemented with Western blot loading buffer and boiled.

Protein extracts were separated on 4-20% gels and transferred to nitrocellulose membrane. After staining with specific antibodies, membranes were developed and scanned using Kodak MI imaging system.

Depletion of endogenous ERM in EC. To reduce the expression of endogenous ERM proteins, HPAEC plated in 12-well plates (with or without coverslips) or in ECIS chambers were treated with specific siRNA or non-specific non-silencing RNA. Transfection with 50nM siRNA

(individual protein knockdown) or a mixture of 25nM siRNAs for ezrin, radixin, and moesin (triple ERM knockdown) was performed using Dharmafect1 transfection reagent (Dharmacon Research, Lafayette, CO) when cells reached 60% confluence. Cells were used in the experiments 48h post-transfection.

Cloning of human endothelial ERM proteins. Total RNA from HPAEC was extracted with Trizol; first strand of cDNA was synthesized by reverse transcription with both random hexamer and oligo (dT) primers. PCR primers containing a specific cloning site for each ERM protein were used to amplify the entire coding sequence of each ERM protein. Amplified cDNA was subcloned into pcDNA3.1/V5-HisA (Invitrogen) mammalian expression vector, and the sequence was analyzed at the Georgia health Sciences University sequencing facility to confirm the insert orientation, in-frame V5 position, and the absence of RT PCR-generated mutation. The specific point mutations (Thr567Ala for ezrin, Thr564Ala for radixin, and Thr558Ala for moesin) were generated by GenScript (Piscataway, NJ) using ERM-coding constructs as template.

EC transfection. To overexpress ERM proteins, suspended cells were nucleofected with a mixture of 1μg/ml of individual plasmids (wild-type ezrin, radixin, and moesin, or mutant ezrin, radixin, and moesin) using Amaxa nucleofection technology (Lonza). Cells were plated in ECIS chambers and allowed to grow for 48h. Efficiency of transfection with ERM plasmids, assessed by immunostaining with V5 antibody, did not exceed 20%.

Statistical analysis was performed on the data pooled from parallel experiments using one-way ANOVA test; results with $p<0.05$ were considered significantly different.