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Reagents: For genotyping, ethylenediaminetetraacetic acid, sodium dodecyl sulphate, proteinase K, acrylamide/ bisacrylamide, ammonium persulfate and N,N,N', N –tetramethylethylenediamine were purchased from Sigma-Aldrich (Oakville, ON). Tris–HCl, KCl, $MgCl_2$, dNTP's and JumpStart™ Taq Polymerase were all obtained from Invitrogen (Burlington, ON). For cell isolation, culture and characterization, Dulbecco's modified Eagle's medium, antibiotic-antimycotic solution, L-glutamine, ethylenediaminetetraacetic acid (EDTA), trypsin, collagenase, bovine serum albumin, dispase, DMEM/F12 HAM, fetal bovine serum (FBS), dialyzed FBS, GS-I lectin, rabbit anti-human von Willibrand factor VIII antibody, Dulbecco's phosphate buffered solution (PBS), horseradish peroxidase-conjugated goat anti-rabbit IgG antibody, and 3,3-diaminodenzidine solution were obtained from Sigma-Aldrich. Endothelial growth supplement was purchased from BD Biosciences (Bedford, MA). Magnetic beads and the magnetic particle concentrator were from Dynal (Lake Success, NY) and heparin was from Leo Laboratories (Ajax, ON). For adenovirus purification, ViraBind™ Adenovirus Purification Mega Kit (# VPK-101) was purchased from Cell Biolabs (San Diego, CA). For adenovirus immunotitration assay, QuickTiter™ Adenovirus Titer Immunoassay Kit (# VPK-109) was also from Cell Biolabs. For RT-PCR, chloroform, isopropyl alcohol, ethanol, DEPC water and DNASe I were from Sigma-Aldrich. Trizol, random primers and reverse transcriptase SuperScript II were from Invitrogen and Quantifast SYBR Green was from Qiagen (Toronto, ON). For immunofluorescence, rabbit polyclonal anti-Cx40 antibody was from Alpha Diagnostics (San Antonio, TX), whereas rabbit polyclonal anti-Cx40 (mid) antibody (#365000) and Alexa 488-conjugated or Alexa 555-conjugated secondary antibodies were from Invitrogen. We also used the rabbit anti-Cx40 antibodies from Zymed (catalogue number 36-4900, Burlington, ON) and from Chemicon (catalogue number AB1726, Etobicoke, ON). Hoechst 33342 stain was from Sigma-Aldrich. For western blotting, protease inhibitor cocktail was from Roche (Mannheim, Germany), iBlot Dry Blotting System was from Invitrogen, the rabbit anti-Cx40 antibody was from Zymed, mouse anti-GAPDH antibody (catalogue number MAB374) was from Millipore Chemicals (Billerica, MA), whereas anti-rabbit Alexa Fluor 680 antibody and anti-mouse IRdye 800 antibody were from LI-COR Biosciences (Lincoln, Nebraska).

Mouse strains: The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 65-23). Experiments were approved by the Animal Use Subcommittee of the Council on Animal Care at the University of Western Ontario. Several Cx40 knockout mice were kindly provided by Dr. D. Paul (Harvard University, Boston, MA). Additional male $Cx40^{-/-}$ mice were produced by crossing $Cx40^{-/-}$ males with wild type C57BL/6 females and by breeding the heterozygous offspring. Using a standard genotyping procedure, we confirmed the absence of Cx40 gene in $Cx40^{-/-}$ mice. Male $Cx40^{-/-}$ littermates (progeny of heterozygous $Cx40^{+/-}$ parents) between the ages of 2-6 months were used after two generations of backcrossing with wild type (WT) C57BL/6 mice. These mice were obtained from the Jackson Laboratory (Bar Harbor, ME).

Mouse microvascular endothelial cells (MMEC): We isolated endothelial cells from male mice as previously described (Bolon et al., 2005; Lidington et al., 2000). Briefly, after mouse anesthesia with ketamine (80 mg/kg) and xylazine (4 mg/kg) i.p., the hindlimb muscle was excised and cut into ~ 0.5 mm fragments and then digested in an enzyme solution. The mouse was euthanized with ketamine/xylazine overdose. Digested tissue was filtered through a 100 μm nylon mesh and cells were collected and washed in Dulbecco's modified Eagle's medium. Cells were grown to confluence and

then purified by immunoseparation using GS-I lectin coated magnetic beads. MMEC were cultured in standard incubator conditions and used between passages 6 and 15 as detailed by us (Bolon et al., 2008). Endothelial cell phenotype and purity at these passages was confirmed by the presence of von Willebrand factor VIII and GI-1 antigens as described previously (Wilson et al., 1996), showing purity near 100%.

Electrophysiology: Intercellular coupling was assessed by determining intercellular resistance (as an inverse measure of coupling) based on an electrophysiological approach previously described by us (Bolon et al., 2005; Lidington et al., 2000). In this approach, the cell monolayer is considered to be an infinite sheet of cytoplasm bounded on both sides by a membrane. The electrotonic spread is predicted by a relationship based on a Bessel function: $\Delta E_m(d) = (I_o R_i / 2\pi t)\, K_o(x)$, where $\Delta E_m(d)$ is the change in membrane potential (V) at distance d (cm) from the injecting electrode, I_o is the injected current (A), R_i is the resistivity (Ω cm) of the intercellular pathway (includes both cytoplasmic and junctional resistivity), t is the thickness (cm) of the monolayer and x is the distance, d, divided the space constant, λ. $K_o(x)$ is a zero order Bessel function of the second kind with imaginary argument (Jahnke and Emde, 1960). For the interelectrode distances used (i.e., 50 - 300 μm), x ranged between 0.02 and 0.3, the exponential portion of the Bessel function. Because the spatial decay of electrotonic potential is exponential for the range of interelectrode distances employed, a semilogarithmic plot of voltage ($\Delta E_m(d)$) versus log distance (d) produces a straight line through the experimental values. Here, R_i is directly proportional to the slope of the line. Using a statistical program (SAS System, Cary, NC) R_i was calculated by dividing the regression slope of the experimental plot by the plotted slope of the Bessel function (exponential portion) and the constant parameters of the model ($I_o/2\pi t$). Once R_i was determined, it was then substituted back into the Bessel function relationship so that λ could be calculated. This method was verified by comparing the theoretical plot to an eye fit of the experimental data (Shiba, 1971): resulting values for R_i and λ agreed well with those determined by the statistical program. Intercellular resistance, r_i (Ω), was calculated by dividing R_i by t. Transmembrane resistivity (R_m; Ωcm^2) was determined from the space constant, defined as $\lambda = (R_m\, t/2R_i)^{1/2}$.

In our experiments, cells were grown to a confluent monolayer on a glass coverslip. The monolayer was injected with 2 - 4 hyperpolarizing constant-current pulses (25 nA, 100 ms duration, at 1 s intervals). The resulting spread of current in the monolayer caused E_m deflections (range: 2 - 30 mV, measured in absolute values) in cells at various distances along the monolayer (i.e., distances between the injecting and recording electrodes). Based on these deflections, the monolayer thickness of MMEC (1.9 μm) (Bolon et al., 2008), and the Bessel function model described above, we computed r_i and transmembrane resistivity. Since electrophysiology was done in room air (cell chamber heated to 37 ^oC), cells were covered by culture medium including 25 mM HEPES (to maintain pH at 7.3).

There was a large day-to-day variability in baseline r_i. To cope with this variability, our experimental protocol was designed to measure the change in r_i relative to the concurrently measured baseline r_i. Thus, for each experimental day, we prepared only one cell line grown under identical conditions to form monolayers on up to 10 coverslips (i.e., for each coverslip, there was the same number of days from cell seeding to confluency, the same passage number, culturing medium, etc.). On the experimental day, these coverslip were then randomly split into 2 groups, up to 5 coverslips for control (i.e., the baseline) and up to 5 for one particular treatment. We compared r_i results from the treatment group against its own control group from that day. We prepared separately these cell lines: non-infected $Cx40^{-/-}$ cells, or $Cx40^{-/-}$ cells infected with various Cx40 adenoviruses or with AdV-β-gal. Coverslips covered with these cells were then randomly assigned to (i) control and LPS treatment, (ii) control and H/R treatment, or (iii) control and concurrent LPS+H/R treatment. Control and treatment

coverslips were studied in parallel. No coverslip was used for both control and treatment conditions. To confirm our previous electrophysiological results with WT cells (Bolon et al., 2007), we have similarly prepared WT cell monolayers and exposed them to conditions (i) - (iii) above.

The spread of injected current in the monolayer and the resulting E_m deflections are governed by fundamental physical principles (i.e., Ohm's and Kirchhoff's laws). However, the manner of this spread and the pattern these deflections along the monolayer may not be intuitive. To this end, we present a simple model of a string of cells into which a constant direct current is injected (Fig. S1). Although the current spreads one-dimensionally along the string, rather than two-dimensionally in the cell monolayer, the same fundamental principles govern its current spread and pattern of E_m deflections as in the monolayer. When the model assumes realistic values of intercellular and transmembrane resistances under control and treatment (e.g., LPS) conditions, it yields useful predictions for both the E_m deflections pattern and the treatment-induced changes in this pattern in the monolayer.

Treatment of MMEC with LPS, H/R, or LPS+H/R: We used the same experimental models of cellular exposure to LPS and H/R as those described in our previous study (Bolon et al., 2008). Briefly, one hour prior to all experiments, the maintenance medium of cells was replaced by a 5 % dialyzed serum medium (DSM) for the duration of the experiment. For exposure to LPS, we used 10 µg/ml (final concentration) in DSM for 1 h. Prior to H/R experiments, hypoxic DSM was prepared by bubbling with 100 % N_2 for 5 min. For hypoxia, cells were covered by ~2 mm thick layer of hypoxic DSM and placed into a hypoxic incubator (5 % CO_2, 0.1 % O_2 and 94.9 % N_2 at 37 ^oC). At the end of hypoxia (1 h for all experiments), hypoxic medium was replaced with normoxic DSM for 5 - 20 min (duration of reoxygenation). For LPS+H/R, the same protocol for H/R was carried out with LPS added to all media. In control experiments, cells were exposed to 5 % DSM.

Quantitative PCR Analysis: Total RNA of $Cx40^{-/-}$ cells, of $Cx40^{-/-}$ cells separately infected with AdV-Cx40, AdV-$Cx40_{\Delta 237-358}$ or AdV-$Cx40_{\Delta 345-358}$, and of WT cells, were isolated with extraction reagent (Trizol); 1 µg of total RNA was treated with DNAse-I to remove DNA contamination and then reverse-transcribed using the Superscript II reverse transcription kit. cDNA reactions were then amplified using gene-specific primers for Cx40 (5'- CTGGCTCACTGTCCTGTTCA- 3', 5'-GCAACCAGGCTGAATGGT-3') and ß-actin (5'- ATCGTGGGCCGCTCTAGGCACCA- 3', 5'-GTTGGCCTTAGGGTTCAGGGGGG- 3'). The Cx40 primer targeted a sequence in the cytoplasmic loop of Cx40, permitting detection of WT Cx40 and the truncated Cx40 mutants. Samples were analyzed in triplicates. Target sequences were denatured for 5 minutes at 95°C, and then amplified for 40 cycles using 95°C/10 sec, 60°C/30 sec and 80°C/30 sec cycling sequence. Amplified DNA product was detected using the DNA-binding fluorophore SYBR Green. Quantification was determined by comparing the cycle threshold value (Ct, the number of cycles required to cross a certain threshold of fluorescence) of the gene of interest (Cx40) to the Ct of the reference gene (ß-actin). The difference in these Ct values (ΔCt) for WT cells was then compared to ΔCt for adenovirus-infected $Cx40^{-/-}$ cells. We used the difference between these ΔCt values ($\Delta\Delta$Ct) to compute the increase of Cx40 mRNA expression in infected cells relative to the Cx40 mRNA expression in WT cells, such that the fold increase equaled to $2^{\Delta\Delta Ct}$.

Immunofluorescence: $Cx40^{-/-}$ cells were grown on glass coverslips. At 10 - 20 % confluence $Cx40^{-/-}$ cells were infected with Adv-Cx40 or AdV-$Cx40_{\Delta 345-358}$ and incubated for 24 h. At this time point, cells reached semiconfluence. WT cells were similarly grown on coverslips without infection. Cells were then fixed with 4 % paraformaldehyde at 4°C for 20 min and then rinsed with PBS and prepared for

immunostaining. Briefly, the $Cx40^{-/-}$ cells, $Cx40^{-/-}$ +AdV-Cx40 cells and WT cells were permeabilized and blocked with PBS containing 2 % bovine serum albumin (BSA) (w/v) for 1 h, immunolabeled with primary anti-Cx40 antibody targeting Cx40CT (Alpha Diagnostics) at 1:300 dilution for 1 h at room temperature, washed with PBS, and then labeled with Alexa 488-conjugated secondary antibody (1:5000) for 1 h at room temperature in the dark. Alternatively, the $Cx40^{-/-}$, $Cx40^{-/-}$ +AdV-$Cx40_{\Delta 345\text{-}358}$ and WT cells were similarly fixed, blocked, and immunolabeled with anti-Cx40 antibody targeting the cytoplasmic loop of Cx40 (Invitrogen) at 1:100 for 1 h at room temperature, and then labeled with Alexa 488-conjugated secondary antibody (1:500) for 1 h at room temperature in the dark. All cells were then washed in PBS and the nuclei stained with 0.1 % Hoechst 33342 for 10 min followed by washes with double distilled H_2O. The coverslips were mounted on slides before storage at 4°C. The cells were imaged using a Zeiss LSM 510 META confocal microscope (Thornwood, NY). Fluorescent signals were captured after excitation with 488 and 730 nm laser lines. Digital images were prepared using Zeiss LSM and Adobe Photoshop 7.0 software.

We also probed for Cx40 in WT cells subjected to control and LPS conditions (10 µg/ml, 1 h). Cells on coverslips were fixed with 10 % formalin at room temperature for 30 min and then rinsed with PBS and prepared for immunostaining. The permeabilized cells were blocked with PBS containing 2 % BSA (w/v), 0.1 % Triton-100 for 30 min, immunolabeled with primary anti-Cx40 antibody (Chemicon or Zymed) at 1:100 for 1 h at room temperature, washed with PBS, and then labeled with Alexa 555-conjugated secondary antibody (for Chemicon Ab) or Alexa 488-conjugated secondary antibody (for Zymed Ab) at 1:500 for 1 h at room temperature. Cells were similarly stained with Hochest 33342, mounted on slides and viewed on the confocal microscope.

Western blotting: Cells were homogenized in sodium dodecyl sulfate lysis buffer with protease inhibitor cocktail. After centrifugation, protein concentrations were determined using a Bradford protein assay. Equal amounts of protein were loaded on a 10% polyacrylamide gel followed by gel electrophoresis and transfer to a nitrocellulose membrane using the iBlot Dry Blotting System. The membrane was blocked with 3% bovine serum albumin for 1 h, followed by incubation with primary rabbit anti-Cx40 antibody (1: 2,500; Zymed) together with mouse anti-GAPDH antibody (1: 5,000) at 4 °C overnight. The membrane was then washed and further incubated with secondary anti-rabbit Alexa Fluor 680 antibody together with secondary anti-mouse IRdye 800 antibody (both 1:10,000) for 1 h at room temperature. The membrane was then washed and imaged on an Odyssey Infrared Imaging System (LI-COR Biosciences), which permits concurrent visualization of both fluorochromes.

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