Supplementary Information

Activation of TRPC6 channels is essential for ischemia–reperfusion-induced lung edema in mice.

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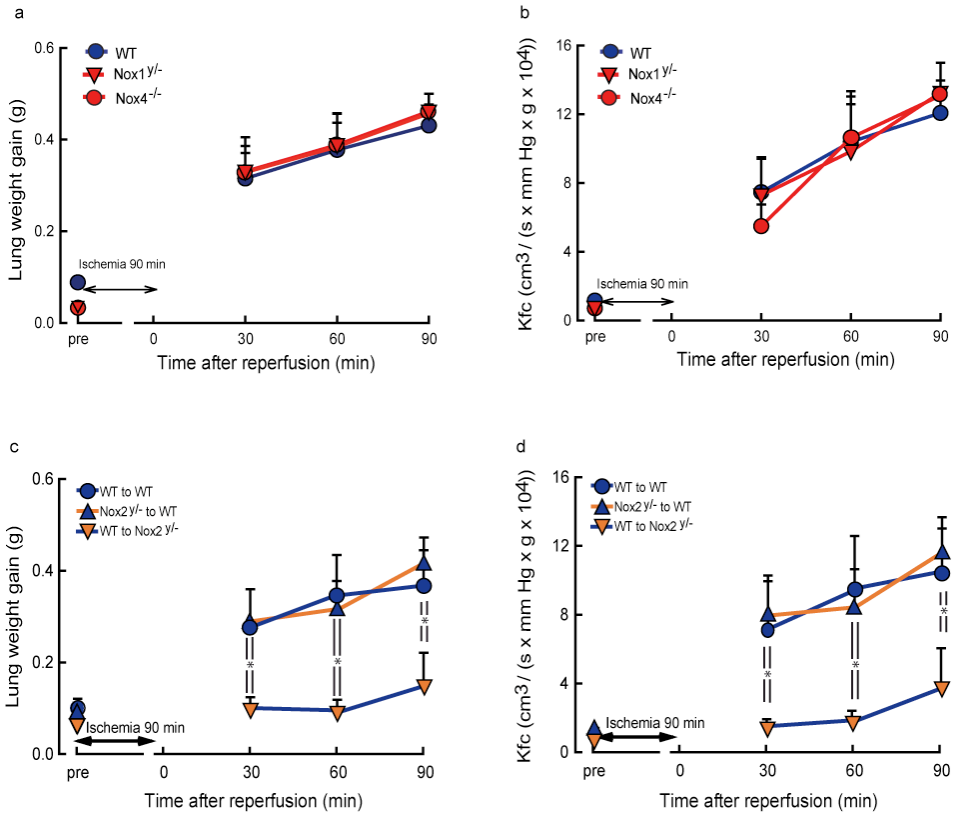
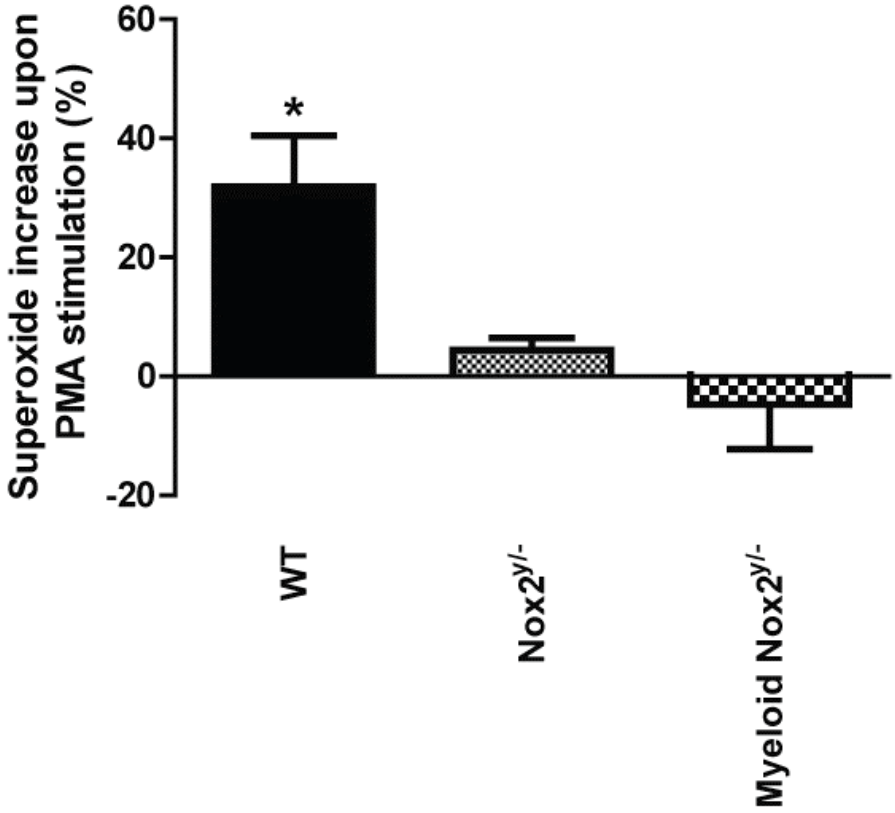
Supplementary Figures S1 to S7

Supplementary Methods

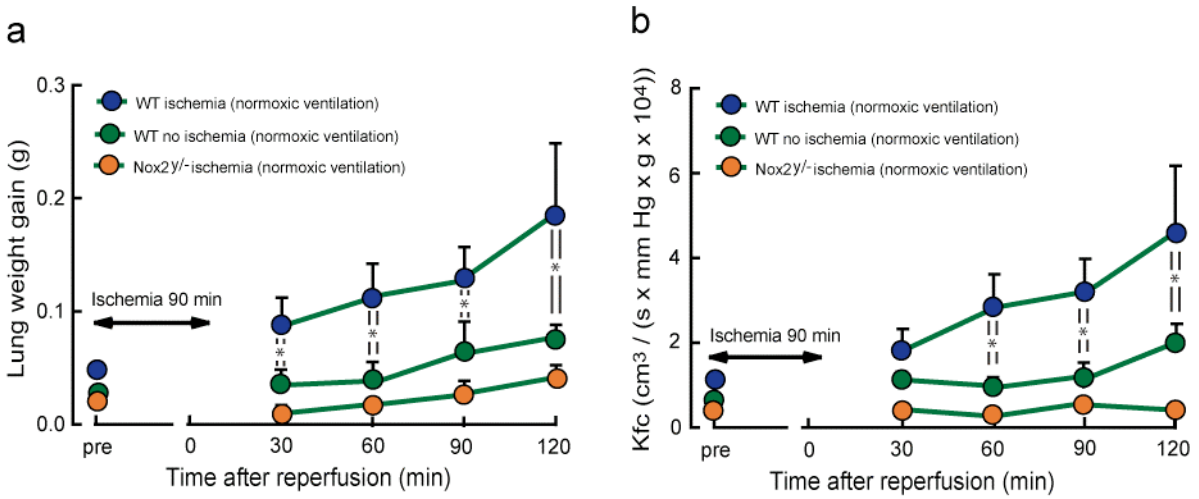
Supplementary References



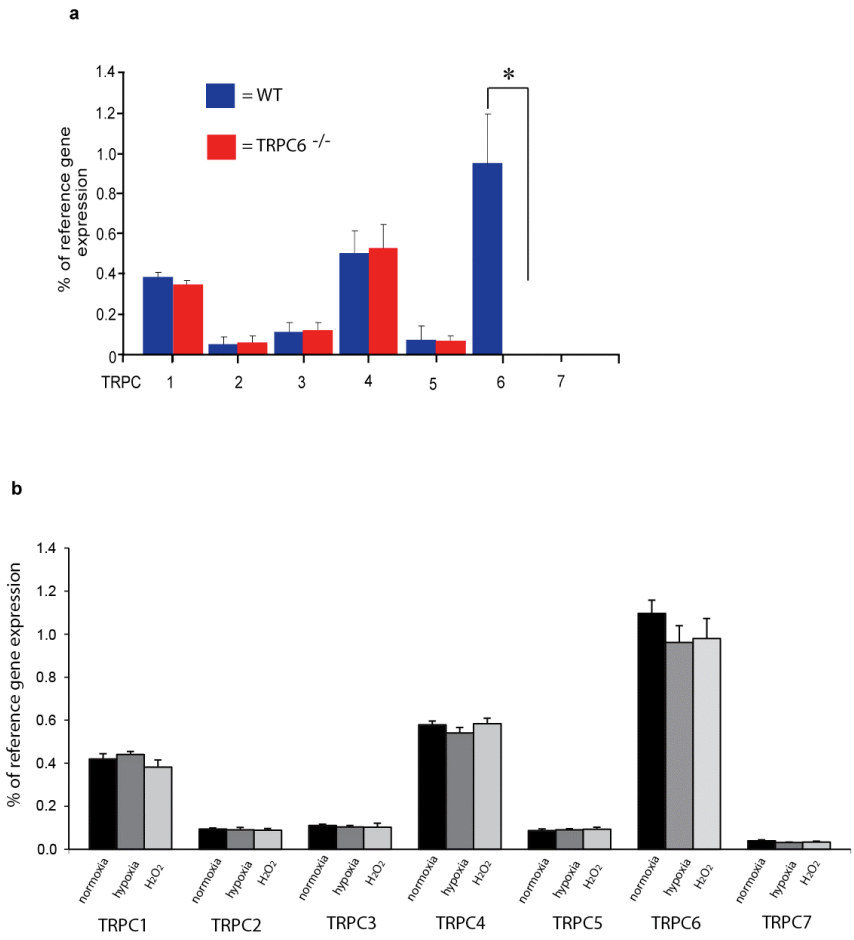
Supplementary Figure S1 | Analysis of lung ischemia–reperfusion-induced edema (LIRE) in wild-type (WT) mice and *Nox*-deficient lungs (Nox1$^{y/-}$, Nox2 $^{y/-}$, Nox4$^{-/-}$). (**a** and **c**) Lung weight gain was calculated as the weight difference before and after each hydrostatic challenge maneuver. (**b** and **d**) The lung capillary filtration coefficient (Kfc) was determined before ischemia and at 30 min intervals after re-establishing perfusion. (**a-b**) Post-ischemic vascular leakage in isolated lungs of WT, *Nox1*$^{y/-}$, or *Nox4*$^{-/-}$). mice. (**c-d**) Post-ischemic vascular leakage in isolated lungs of chimeric mice where bone marrow cells of WT or *Nox2*$^{y/–}$ mice were transplanted to irradiated WT or *Nox2*$^{y/–}$ mice. n = 4-6. All statistical data were assessed using one-way ANOVA with the Student-Newman-Keuls test and are presented as mean ± s.e.m.; *P < 0.05.



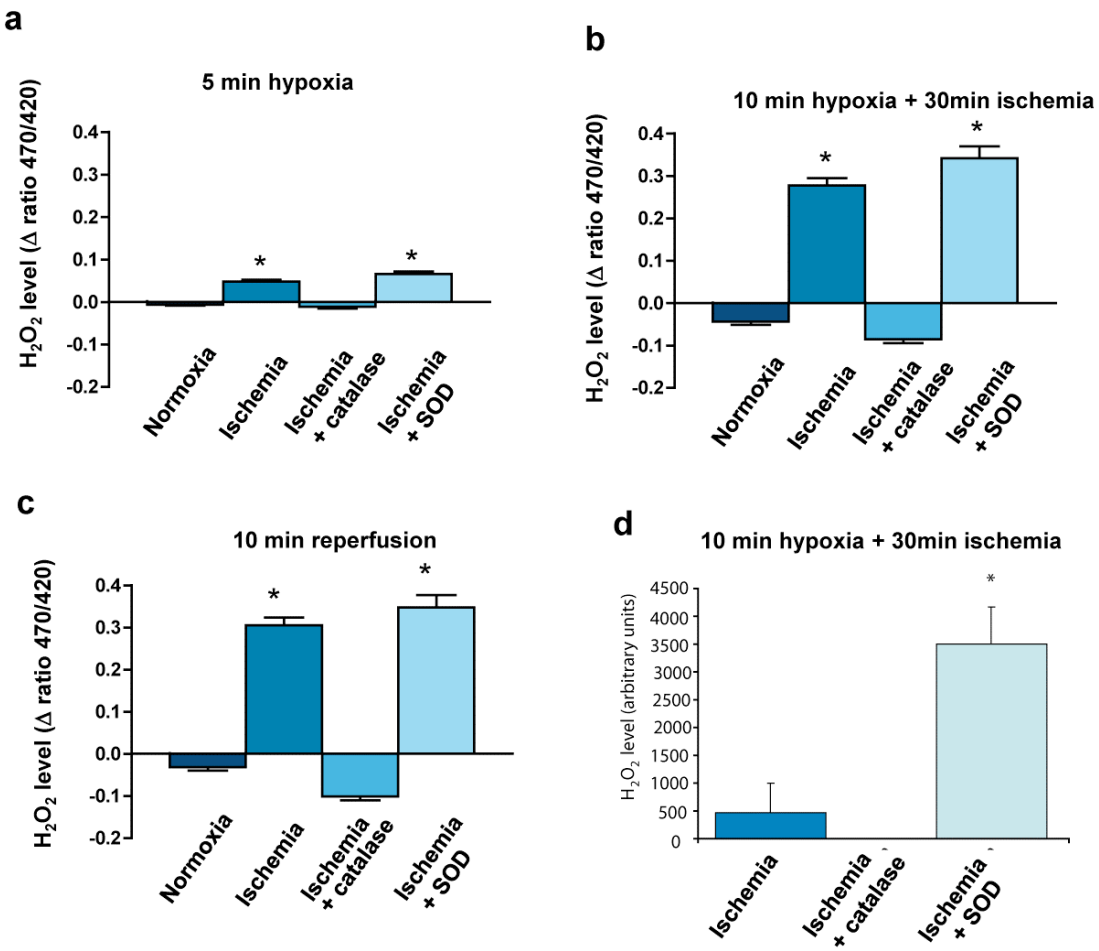
Supplementary Figure S2 | Superoxide generation by bronchoalveolar lavage (BAL) cells of wildtype (WT), *Nox2*$^{y/-}$ and myeloid *Nox2*$^{y/-}$ mice. Samples were either untreated or treated with 12-phorbol myristate 13-acteate (PMA) for 30 min. Superoxide measurement was performed by ESR spectroscopy. Data are given as changes of superoxide production upon PMA stimulation in percent of their respective non-stimulated control. n = 4-6. Data were statistically analyzed by one-way ANOVA with the Student-Newman-Keuls test and are presented as mean ± s.e.m.; *$P < 0.05$.



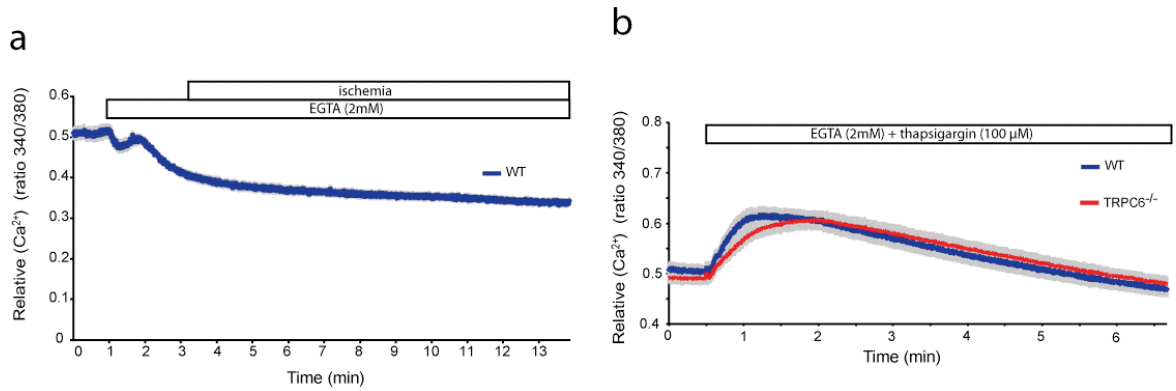
Supplementary Figure S3 | Effects of normoxic ventilation on ischemia-reperfusion-induced lung edema in isolated perfused and ventilated mouse lungs. (**a**) Lung weight gain in WT ischemia (normoxic ventilation), WT no ischemia (normoxic ventilation; no ischemia), *Nox2*$^{y/-}$ ischemia (normoxic ventilation), was calculated as the weight difference before and after each hydrostatic challenge maneuver. (**b**) The lung capillary filtration coefficient (Kfc; WT, ischemia (normoxic ventilation); WT no ischemia (normoxic ventilation, no ischemia); *Nox2*$^{y/-}$ ischemia (normoxic ventilation); was determined before ischemia and at 30-min intervals after re-establishing perfusion. (**a, b**) n = 5. Data were statistically analyzed by ANOVA with the Student-Newman-Keuls test and are presented as mean ± s.e.m.; *P < 0.05.



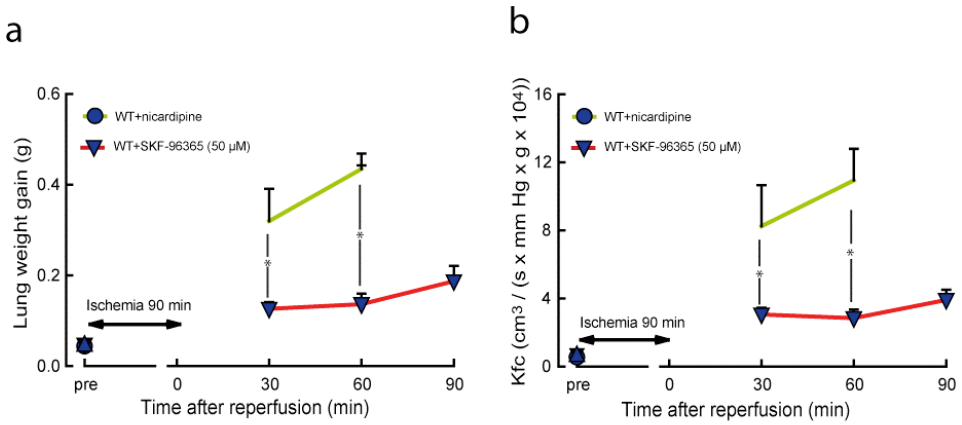
Supplementary Figure S4 | Characterization of TRPC expression murine lung endothelial cells (MLEC) from wild-type (WT) and *TRPC6*$^{-/-}$ mice. (**a**) Total RNA was prepared from MLEC of wild-type and *TRPC6*-deficient mice and reverse-transcribed. Products of the first strand synthesis were analyzed for the presence of amplification products obtained with primer pairs listed in Methods[1]. mRNAs coding for TRPCs and β-actin (as reference gene) were quantified with the aid of a light cycler. Values are presented as percentage of reference mRNA expression (β-actin mRNA expression). (**b**) Quantitative RT-PCR of wild-type lung endothelial cells after exposure to normoxia (90 min), hypoxia (90 min) or H_2O_2 (100 µM for 30 min) with TRPC-specific oligonucleotides described above. n = 3-5. All statistical data were assessed using Student´s *t*-test with Welsh's correction and are presented as mean ± s.e.m.; *P < 0.05.



Supplementary Figure S5 | H_2O_2 levels in WT MLEC detected intracellularly by the HyPer sensor molecule (a-c) or extracellularly by amplex red (d). SOD (150 U/ml) or catalase (300 U/mL) were added 10 min prior to ischemia. Data were assessed after 5 min of hypoxia (**a**), 10 min of hypoxia, followed by 30 min of stopping perfusion during hypoxia (**b**) as well as after a subsequent 10 min period of normoxic reperfusion (**d**) (**a-d**) n = 16-28 cells. Data represent mean ± s.e.m.; **P* < 0.05 at the indicated time points. n = 4-6. Data were statistically analyzed by ANOVA with the Dunnett´s post test compared to normoxic (a-c) or ischemic control (d) and are presented as mean ± s.e.m.; **P* < 0.05.



Supplementary Figure S6 | Ischemia- and thapsigargine-induced changes of $[Ca^{2+}]_i$ in MLEC in Ca^{2+}-free extracellular medium (2 mM EGTA). (**a**) Absence of ischemia-induced influx of Ca^{2+} in WT MLEC observed after removal of extracellular Ca^{2+}. (**b**) Thapsigargin-induced changes in $[Ca^{2+}]_i$ in WT and *TRPC6*$^{-/-}$ MLEC in Ca^{2+}-free extracellular medium (2 mM EGTA). Thapsigargin (100 µM) was added after starting the experiment and was present during the remainder of the experiment. (**a,b**) n = 33-45 cells. All statistical data were assessed using Student´s *t*-test with Welsh's correction and are presented as mean ± s.e.m.; **P* < 0.05.



Supplementary Figure S7 | Effects of nicardipine and SKF-96365 on ischemia-reperfusion-induced lung edema in isolated perfused and ventilated mouse lungs. Lungs were exposed to ischemia for 90 min. Nicardipine (10 µM) or SKF-96365 (50 µM) were added to the perfusate 5 min prior to ischemia (**a** and **b**). (**a**) Lung weight gain values (WT) were calculated as the weight difference before and after each hydrostatic challenge manoeuvre. (**b**) Lung capillary filtration coefficients (Kfc) were determined before ischemia and at 30 min intervals after re-establishing perfusion. (**a,b**) *n* = 5. All statistical data were assessed using Student´s *t*-test with Welsh's correction and are presented as mean ± s.e.m.; **P* < 0.05. If 90 min values are not displayed experiments had to be terminated before due to excessive edema formation.

Supplementary Methods

Isolated Mouse Lung Preparation. Mice were deeply anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (8 mg/kg) and anticoagulated with heparin (500 IU/kg body weight). Animals were then intubated via a tracheostoma and were ventilated with room air (tidal volume, 300 µL; respiratory rate, 90 breaths/min; positive end–expiratory pressure, 3 cm H_2O) with a piston pump (Minivent Type 845; Hugo Sachs Elektronik, March-Hugstetten, Germany). After midsternal thoracotomy, the ribs were spread, the heart was incised at the apex, the right ventricle was incised, and a fluid-filled perfusion catheter was immediately placed into the pulmonary artery, with the catheter already being perfused, and secured with a ligature. Perfusion was done with a REGLO Digital MS-4/12 pump (Ismatec SA, Labortechnik-Analytik, Glattbrugg, Switzerland) with sterile ice-cold Krebs-Henseleit solution (Serag-Wiessner, Naila, Germany). In parallel with the onset of artificial perfusion, ventilation was changed from room air to a pre-mixed normoxic normocapnic gas mixture of 21% O_2, 5.3% CO_2, balanced with N_2 (Air Liquide, Deutschland GmbH, Ludwigshafen, Germany). Next, the trachea, lungs and heart were excised *en bloc* from the thorax (without interrupting ventilation and perfusion) and were freely suspended from a force transducer to monitor lung weight gain. A second perfusion catheter with a bent cannula at its tip was introduced via the left ventricle into the left atrium. Meanwhile, the flow was slowly increased from 0.2 to 2 mL/min (total system volume: 15 mL). After rinsing the lungs with >20 mL buffer to wash out blood, the perfusion circuit was closed for recirculation. Left atrial pressure was set at 2.0 mm Hg. The isolated, perfused lung was placed in a temperature-equilibrated housing chamber, and the whole system (perfusate reservoirs, tubing,

housing chambers) was heated to physiological mouse body temperature. Pressures in the pulmonary artery, the left atrium and the trachea were registered by means of pressure transducers connected to the perfusion catheters.

Ischemia-reperfusion-induced lung edema protocol. The capillary filtration coefficient (Kfc) and lung weight gain were used to quantify lung vascular permeability. Kfc was determined gravimetrically from the slope of the lung weight gain curve induced by a 7.5 mm Hg step elevation of the venous pressure for 8 min, as described[50]. All Kfc values were referenced to 1 g predicted lung weight on the basis of the ratio of lung weight to body weight as described[51]. Lung weight gain was calculated as the weight difference before and after each hydrostatic challenge maneuver.

After a control hydrostatic challenge, the lungs from respective gene-deficient or corresponding WT mice, were exposed to ischemia for 90 min by stopping the perfusion. The arterial and venous parts of the perfusion tubing were both clamped to maintain a positive intravascular pressure. During ischemia, the lungs were continuously ventilated with an anoxic gas mixture (94.7% N_2, 5.3% CO_2; Air Liquide, Deutschland GmbH, Ludwigshafen, Germany). At the end of the ischemic period, ventilation was returned to normoxia (21% O_2, 5.3% CO_2, 73.7% N_2; Air Liquide), and perfusion was re-established by increasing the flow stepwise over 3 min. Hydrostatic challenges were repeated 30, 60, and 90 min after the onset of reperfusion. For experiments in the Fig. S3 lungs were ventilated with normoxia during ischemia and non-ischemic conditions. These experiments demonstrate that ischemia with normoxic ventilation increased permeability but in a somewhat slower time course in WT lungs. The fact that $Nox2^{y/-}$ mice are similarly protected in the altered setup

underscores similar effects of ischemia-reperfusion in anoxic and normoxic ventilation.

With regard to repetitive Kfc measurements it has to be taken into account that in injured lungs preceding capillary pressure elevations can affect subsequent Kfc and lung weight values. Although such interfering effects can only be excluded for the first Kfc measurement after reperfusion subsequent values are displayed as they nevertheless allow quantifying the severity of damage.

Where indicated, the NADPH oxidase inhibitor, apocynin (500 µmol/L; Sigma-Aldrich, Steinheim, Germany), the PLC inhibitor U-73122 (5 µmol/L; Calbiochem, Darmstadt, Germany), the non-active PLC inhibitor U-73343 (5 µmol/L; Calbiochem), the DAG kinase inhibitor R59949 (30 µmol/L; Calbiochem) or DAG analogue OAG (90 µmol/L, Sigma-Aldrich) were admixed to the perfusion buffer of WT mouse lungs 5 min before the onset of anoxic ischemia. In the time-matched non-ischemic controls untreated lungs from WT mice were continuously perfused and normoxically ventilated throughout the experiment. Where indicated the substances were added to the perfusion buffer at time points corresponding to those of the ischemia-reperfusion protocol.

Generation of Chimeric Mice. Donor mice (6 weeks old) were anticoagulated with an intraperitoneal injection of heparin (500 IU/kg) and euthanized in deep anesthesia. Bone marrow (BM) cells were collected by flushing femurs and tibias with RPMI 1640 containing 1% FCS, 100 U/mL penicillin and 1000 U/mL streptomycin. About 5×10^7 mononuclear cells were harvested from each donor. Recipient mice were BM depleted by lethal irradiation with an 1100-rad (11 Gray) ^{60}Co irradiator at a dose rate of ~0.6 Gray/min. Within 6–8 hr of irradiation, $2–5 \times 10^6$ donor BM cells in a volume of 100 µL sterile medium were injected into the lateral tail vein of the warmed

recipient. BM reconstitution was allowed to occur for 10 weeks. During this time, chimeric mice were housed individually in sterile filter-isolator cages. Six different groups of chimeras were generated. To control for BM transplantation procedures, WT to WT chimeras (i.e., WT animals that received BM cells from WT mice) were generated, thus preserving TRPC6 of Nox2 function in all cells. In *TRPC6*$^{-/-}$ to WT chimeras, BM cells from *TRPC6*$^{-/-}$ mice were transplanted into WT mice, resulting in selective inactivation of *TRPC6* in immune cells. In WT to *TRPC6*$^{-/-}$ chimeras, BM cells harvested from WT animals were transferred to TRPC6$^{-/-}$ mice, resulting in selective reconstitution of *TRPC6* in immune cells. In *Nox2*$^{y/-}$ to WT chimeras, BM cells from *Nox2*$^{y/-}$ mice were transplanted into WT mice, resulting in selective inactivation of *Nox2* in immune cells. In WT to *Nox2*$^{y/-}$ chimeras, BM cells harvested from WT mice were transferred to Nox2$^{y/-}$ animals, resulting in selective reconstitution of *Nox2* in immune cells.

Successful reconstitution of recipient mice with the donor cells was verified in genomic DNA from blood cells by PCR at 10 weeks after bone marrow transplantation as described for mice with different genotypes in the literature[52-53]. Genomic DNA obtained from peripheral blood cells was amplified by PCR. For Nox2-deficient chimeric mice common, wild-type and mutant primers (as described by Jackson Laboratory, Bar Harbor, USA) were used. Primer pair oIMR0517 (5'- AAg AgA AAC TCC TCT gCT gTg AA -3') and oIMR0518 (5'- CgC ACT ggA ACC CCT gAg AAA gg -3') amplifies a 240-bp fragment from the wild-type allele. Primer pair oIMR0519 (5'- gTT CTA ATT CCA TCA gAA gCT TAT Cg -3') and oIMR0518 amplifies a 195-bp fragment from the disrupted Nox2 allele. TRPC6 chimeric mice were analyzed as described[54].

Quantitative RT–PCR analysis. Total RNA from MLEC was isolated using the TriFast Reagent (PeqLab, Erlangen, Germany).Real time PCR was done using the 2x master mix from the Absolute QPCR SYBR-Green kit (Thermo Scientific-Epsom, UK) containing a HotStar Taq polymerase, buffer, nucleotides, 5 mM $MgCl_2$ (final 2.5 mM) and SYBR Green. Ten pmol of each primer pair and 0.2 µl from the first strand synthesis were added to the reaction mixture and PCR was carried out in a light-cycler apparatus (Roche, Mannheim, Germany) using the following conditions: 15 min initial activation and 45 cycles of 12 sec at 94 °C, 30 sec at 50 °C, 30 sec at 72 °C and 10 sec at 80 °C each. Fluorescence intensities were recorded after the extension step at 80 °C after each cycle to exclude fluorescence of primer dimers melting at temperatures lower than 80°C. All primers were tested by using diluted cDNA from the first strand synthesis (10-1000 fold) to confirm linearity of the reaction. Samples containing primer dimers were excluded by melting curve analysis and identification of the products by agarose gel electrophoresis. Crossing points were determined by the software program. The relative gene expression was quantified using the formula: $(2e^{(\text{Crossing point GAPDH - Crossing point X})}) \times 100 = \%$ of reference gene expression. The following primers pairs were used for the amplification of specific fragments from the first strand synthesis: TRPC1: *C1F* (5'-TGG GCC CAC TGC AGA TTT CAA) and *C1R* (5'-AAG ATG GCC ACG TGC GCT AAG GAG); TRPC2: *C2F* (5'-TTG CCT CCC TCA TCT TCC TCA CCA) and *C2R* (5'-CCG CAA GCC CTC GAT CCA CAC CT), TRPC3: *C3F* (5'-AGC CGA GCC CCT GGA AAG ACA C) and *C3R* (5'-CCG ATG GCG AGG AAT GGA AGA C); TRPC4: *C4F* (5'-GGG CGG CGT GCT GCT GAT) and *C4R* (5'-CCG CGT TGG CTG ACT GTA TTG TAG); TRPC5: *C5F* (5'-AGT CGC TCT TCT GGT CTG TCT TT) and *C5R* (5'-TTT GGG GCT GGG AAT AAT G); TRPC6: *C6F* (5'-GAC CGT TCA TGA AGT TTG TAG CAC) and *C6R* (5'-AGT ATT CTT TGG GGC CTT GAG TCC), TRPC7: *C7F* (5'-GTG GGC GTG CTG GAC

CTG) and *C7R* (5'-AGA CTG TTG CCG TAA GCC TGA GAG); $Ca_V1.2\alpha_1$: *α1CF* (5'-GAC GTT CCC CCA GGC TGT GTT ACT) and *α1CR* (5'-GTG ATG GGG ACC GAG GAT AGA CC) and β-actin (*ACTF, ACTR*) primer as described above.

The following primer pairs were used for the amplification of specific fragments of DAG kinases from the first strand synthesis: DAG kinase α: forward 5'-GTT CGA CCT GAA GGA TGG TC, reverse 5'-CTA GAA CCC AGC CTA CTG TGC; DAG kinase β: forward 5'-TGG CGT GCT TGC AAA GTA T, reverse 5'-AAG GTG GGC GGT GAA ATC, DAG kinase γ: forward 5'-GCA GTA TGA CCC ACA CAA GC, reverse 5'-CCA GGA AGA GGT GTG TGC TC; DAG kinase η_{common}: forward 5'-AAA AGG AAA AGG CTC AGA AAC A, reverse 5'-GGG TTC CGT TCA AGC TCT.

$[Ca^{2+}]_i$-imaging and Mn^{2+}-quench experiments in MLEC. MLEC from WT or *TRPC6*$^{-/-}$ mice harvested from at least three independent preparations of mice (5 WT or 5 *TRPC6*$^{-/-}$ mice in each preparation) were analyzed. MLEC were loaded with fura-2 acetoxymethyl ester (5 μM) in HEPES-Ringer buffer (HRB, in mM: NaCl 140, KCl 2.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.3, HEPES 25, glucose 2.5 and 0.1% BSA, pH 7.4) at 37 °C for at least 60 min. Coverslips were then placed on the microscope in a low-volume glass-covered recording chamber, perfused (0.5 ml/min) with HRB without BSA saturated with 21% O_2 (normoxia, pO_2 ~ 150 mmHg) and maintained at 32± 0.2 °C by heating both the HRB and the chamber. Ischemia was induced by application of a classical ischemia–reperfusion protocol[55]. Hypoxic medium (HRB, pH 7.4, without glucose) equilibrated with 100% N_2 before and during the experiment was perfused to the cells. Perfusion was stopped and afterwards normoxic medium (HRB, pH 7.4; with glucose) was perfused (see Figure 3e, f). Drugs were diluted in HRB in separate reservoirs connected to a manifold. MLEC were analyzed using a Polychrome IV monochromator and an IMAGO CCD camera (Till Photonics,

Martinsried, Germany) coupled to an inverted microscope (Olympus IX70, Hamburg, Germany) at 340 and 380 nm for measuring $[Ca^{2+}]_i$. Mn^{2+} quench experiments were done as described[56].

ROS measurements from bronchoalveolar lavage. Mice were anaesthetised and bronchoalveolar lavage fluid (BALF) was obtained by flushing the lung with 700-1000 µl 0.9 % NaCl (Laboratori Diaco Biomedicali, Italy). The procedure was repeated up to 10 times per lung. BALF was collected and centrifuged at 320xg for 5 min. Supernatant was discarded and the sediment was resuspended and washed two times in ESR- Krebs HEPES buffer (Noxygen Science Transfer & Diagnostics GmbH, Germany). Number of cells was counted and similar cell numbers were adjusted in each sample. Samples were then incubated for 2 hours at 37 °C. For determination of the superoxide dismutase inhabitable portion of ROS in BALF, parallel samples were investigated in the presence and absence of polyethylene-conjugated superoxide dismutase (PEG-SOD, 15U/ml, Sigma-Aldrich, Germany). After incubation of all samples for 30 min at 37 °C with the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH, Noxygen Science Transfer & Diagnostics GmbH, Germany) measurements were performed using the Bruker EMX micro Spectrometer (Bruker Biospin, Steinheim, Germany). For calculation of the PMA (6µM, Sigma-Aldrich, Germany)-stimulated superoxide production parallel samples were investigated in the presence or absence of PMA.

H_2O_2 detection by amplex red. After washing MLEC in MEM without phenolred (PAA, Cölbe, Germany) cells were incubated in the presence or absence of 300 U/ml catalase (Sigma-Aldrich) or 150 U/ml superoxide dismutase (Sigma-Aldrich). After 10 minutes 50 µM amplex red (Invitrogen, Darmstadt, Germany) was added and MLEC

were transferred into an ischemic atmosphere (hypoxic glove box, Toepffer Lab Systems, Göppingen, Germany; 0% O_2, 5% CO_2) at 37 °C. In parallel, experiments were performed in the presence of 2 U/ml horse radish peroxidase (1 U/µl stock in PBS, Sigma-Aldrich). Moreover, all experiments were performed without cells for calculation of background fluorescence.

After 30 minutes fluorescence of supernatants was measured in a 96-well plate by a multimode microplate reader (TECAN, Männedorf, Switzerland) at 540 nm excitation and 580 nm emission. After subtraction of background fluorescence the difference between fluorescence with and without horse radish peroxidase was calculated to determine the signal derived from H_2O_2 production.

Electrophysiology. To investigate changes in macroscopic cationic currents caused by ischemia in MLEC, the perforated patch-clamp technique was used. In a cell culture dish mounted to a small perfusion chamber, adherent MLEC were superfused with the extracellular solution (in mM): NaCl 140, CsCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10, HEPES 10, adjusted to pH 7.4 with NaOH. The chloride channel blocker 5-nitro-2-(4-phenylpropylamino) benzoic acid (NPBB) was added to the extracellular solution and the extracellular solution was gassed with N_2 for at least 30 min. Data of perforated-patch recordings were acquired at a frequency of 5 kHz after filtering at 1.67 kHz with an EPC10 patch clamp amplifier (HEKA, Lambrecht, Germany) using the Pulse software v8.7 (HEKA). Patch pipettes of resistances of 3.0 to 4.5 MΩ caused series resistances of 6 to 11 MΩ. The tip of this patch electrode was first filled with the amphotericin B-free solution (in mM: CsCl 120, NaCl 10, $MgCl_2$ 1, buffered at 100 nM free Ca^{2+} with 10 mM BAPTA and 10 mM HEPES titrated to pH 7.2 with CsOH) by aspiration to a column length of 500 µm. Then the pipette was backfilled with the same solution additionally containing 300 µg ml^{-1} water-soluble amphotericin

B (Sigma-Aldrich, Deisenhofen, Germany). The liquid junction potential was +4.0mV and offset corrections were made by the Pulse software. The perforation started shortly after seal formation and reached a steady-state level within 1–2 min. The osmolarity of all solutions was 300±5 mOsm kg^{-1} measured using the vapor osmometer Vapro 5520 (Wescor, Logan, USA). MLEC were held at a potential of −60 mV, and current–voltage relations were obtained from triangular voltage ramps from −100 to +60 mV with a slope of 0.4 V s^{-1} applied at a frequency of 1 Hz. Experiments were performed at room temperature.

***In vitro* assay for DAG kinase activity.** In vitro analysis of heterologously expressed DAG kinase isoforms was essentially done as described[57]. In brief, the assay was performed in a 50-µl reaction volume containing 100 mM Tris-Cl, pH 7.4, 1 mM sodium deoxycholate, 0.5 mM dithiothreitol, 1 mM diolein, 1.6 mM [g-32P]ATP (5000 cpm/nmol), 5 mM magnesium chloride, and 50 µg of protein lysate from COS-7 cells heterologously expressing the DAG-kinase isotype. The stock diolein solution (0.25 mM) (Avanti Polar Lipids, Alabaster, AL) was freshly prepared by sonicating on ice in 100 mM Tris-Cl, pH 7.4, containing 2.5 mM sodium deoxycholate and 1.25 mM dithiothreitol. An aliquot (20 µl) of this solution was added to 50 µg of the protein lysate from COS-7 cells in 20 µl of 100 mM Tris-Cl. All additions were made at 4 °C. The reaction was initiated by adding 10 µl of a 5 X solution containing 8 mM [γ-32P]ATP and 25 mM magnesium chloride. Samples were incubated at 30 °C for 10 min. Concentrated hydrochloric acid (50 µl) was added to stop the reaction. The lipids were extracted by adding 0.5 ml of water and 0.33 ml of butanol. After vortexing, the tubes were centrifuged for 3 min at 2000 rpm. The upper layer was transferred to a new tube and washed with an equal volume of butanol-saturated water. An aliquot of the upper layer (50 µl) was assayed on a scintillation counter.

The mean value obtained from samples containing protein-lysates from untransfected COS-7 cells as controls were subtracted as background levels.

Supplementary References:

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