Online supplemental material

Supplementary methods

Isolated-perfused rat lung model

The isolated lung preparation has been described in detail previously (1-3). Briefly, the lungs and heart of anesthetized rats were removed en bloc. The pulmonary artery and left atrium were catheterized and perfused continuously with a solution of 3% bovine serum albumin (BSA) in buffered physiological salt solution (135.5 mM Na^{+}, 119.1 Cl^{-}, 25 mM HCO_3^{-}, 4.1mM K^{+}, 2.8 mM Mg^{+}, 2.5 mM Ca^{+2}, 0.8 mM SO_4^{-2}, 8.3 mM glucose). Trace amounts of FITC-albumin was also added to the perfusate. The recirculating volume of the constant pressure perfusion system was 90 ml; arterial and venous pressures were set at 12 and 0 cm H_2O respectively. The vascular pressures were recorded every 10 seconds with a multichannel recorder (Cyber Sense Inc. Nicholasville, KY). The lungs were immersed in a "pleural" bath (100 ml) filled with the same BSA solution. The entire system was maintained at 37 °C in a water bath. Perfusate pH was maintained at 7.40 by bubbling with a gas mixture of 95%O_2/5%CO_2. The lungs were then instilled via the tracheal cannula in two sequential phases with a total of 5 ml volume of the BSA solution containing 0.1mg/ml EBD-albumin, 0.02 μCi/ml of $^{22}Na^{+}$ and 0.12 μCi/ml of ^{3}H-mannitol. Samples were taken from the instillate, perfusate, and bath solutions after an equilibration time of 10 minutes from the instillation and again 60 minutes later. To ensure a homogenous sampling of the instillate, a volume of 2 ml was aspirated and reintroduced into the airspaces three times before removing each sample. All samples were centrifuged at 3000 g for 10 minutes. Absorbance analysis of the supernatant or EBD albumin was performed at 620 nm in a Hitachi model U2000 spectrometer (Hitachi,

San Jose, CA). Analysis of FITC-albumin (excitation 487 nm and emission 520 nm) was performed in a Perkin-Elmer fluorometer (model LS-3B, Perkin-Elmer, Oakbrook, IL). Scintillation counts for $^{22}Na^{+}$ and ^{3}H-mannitol were measured in a Beckman beta counter (model LS 6500,Beckman Instruments Inc., Fullerton, CA).

To assess whether isoproterenol can prevent the hypercapnia-induced decrease in AFR, isolated rat lungs were first perfused for 1 h with 40 mmHg CO_2 (pH_e 7.4) in the absence and subsequently in the presence of isoproterenol (1 μM, 1 h) after which perfusion was changed to 60 mmHg CO_2 (pH_e 7.2) and AFR was measured. To determine whether the cAMP analog, 8Br-cAMP can ameliorate the hypercapnia-induced inhibition of AFR, isolated rat lungs were first perfused for 1 h with 40 mmHg CO_2 (pH_e 7.4) in the absence and subsequently in the presence of 8Br-cAMP (100 μM, 1 h) after which perfusion was changed to 60 mmHg CO_2 (pH_e 7.2) and AFR was measured. To assess whether isoproterenol can overcome the hypercapnia-induced decrease in AFR isolated rat lungs were first perfused for 1 h with 40 mmHg CO_2 (pH_e 7.4) after which perfusion was changed to 60 mmHg CO_2 (pH_e 7.2) for an additional hour in the absence and subsequently for 1 h in the presence of isoproterenol (1 μM) and AFR was measured.

Hematoxylin and eosin (HE) staining

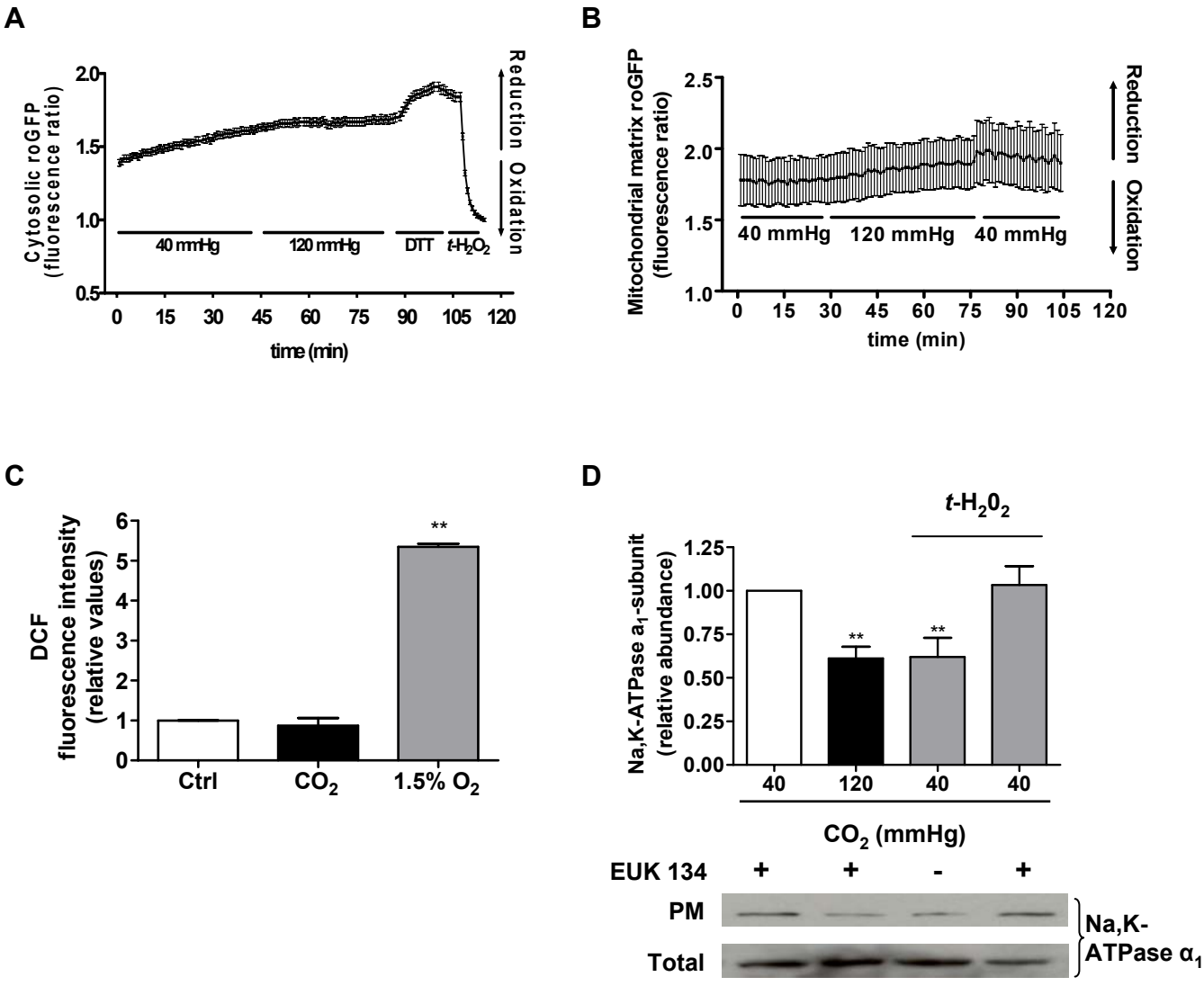
Lung tissues were rinsed in ice-cold PBS and fixed in 4% paraformaldehyde overnight. Lungs were embedded in paraffin, and cut into 4 μm lung tissue sections, which were placed on glass slides. Slides were deparaffinized in xylene for 5 min (3 times) and then rehydrated in 100%, 95%, 70% ethanol and PBS. Hematoxylin and eosin (H&E) staining was performed. Briefly, slides were stained in hematoxylin for 3 min, rinsed in tap water,

dipped in acid-alcohol 8–12 times, and finally rinsed in tap water. Next, slides were stained with eosin for 30 s and then dehydrated with 95% ethanol, 100% ethanol, and xylene. Images were observed with a Olympus Vanox-s equipped with an Olympus Japan 138132 objective and were captured using a Nikon Digital Camera System.

Determination of reactive oxygen species

Intracellular ROS generation was assessed using 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR) as previously described (4). Data were normalized to values obtained from normocapnic controls. Cells exposed to hypoxia (1.5 % O_2) were used as a positive control. Real-time determination of ROS was assessed using a r*o*GFP expressed in ATII cells, as previously described (5, 6). The r*o*GFP was expressed in ATII cells using modified pEFGP-N1 as expression vector and Lipofectin as transfection reagent. After 24 h of incubation at 37 °C in culture medium, the cells were washed twice with Hank's balanced salt solution buffer. Cell images were obtained with a multi-mode inverted microscope (Nikon TE2000, Nikon Instruments Inc, Melville, NY) and a Cascade camera CCD650 controlled by MetaFluor Software (Molecular Devices Corp. Downingtown, PA). During the experiment, the cells were continuously perfused with culture media containing CO_2 of 40 or 120 mmHg and the fluorescence images from excitation at 400 and 484 nm (emission 535 nm) were recorded every 60 s. Oxidant stress was assessed in the cytosol using a non-targeted expression construct, and in the mitochondrial matrix using a r*o*GFP targeted to that compartment with the targeting sequence from cytochrome oxidase subunit IV. The roGFP2 mutant (GFP with mutations C48S, S147C, S65T and Q204C) was used in all studies.

Supplementary Figure 1



Supplementary Figure 1. CO_2-induced Na,K-ATPase endocytosis is independent of ROS.

(A) Cytosolic ROS were measured in real time as the change in fluorescence intensity of ATII cells expressing a non targeted r*o*GFP and exposed for the indicated times to 40 and 120 mmHg CO_2 (pHe: 7.4). Dithiothreitol (DTT) and *tert-butyl*-hydroperoxide (*t*-H_2O_2) were used as positive controls for reduced and oxidized states, respectively. (B) Mitochondrial ROS were measured as in A in ATII cells expressing a r*o*GFP targeted to the mitochondrial matrix compartment and exposed for the indicated times to 40 and 120 mmHg CO_2 (pHe: 7.4). (C) ATII cells were loaded with 10 M DCFH-DA, exposed for 30 min to 40 or 120 mmHg CO_2 (pHe: 7.4, 21% O_2) or to 40 mmHg CO2 (pHe: 7.4, 1.5% O_2) as a positive control, and fluorescence was measured in the cell lysates. (mean ± SEM, n=3). (D) ATII cells were exposed to 40 or 120 mmHg CO_2 (pHe: 7.4) for 30 min in the presence of Eukarion 134 (EUK 134). Na,K-ATPase protein abundance was determined as described. Incubation with 100 μM *t*-H_2O_2 at 40 mmHg (pHe: 7.4) was used as positive control (grey bars). mean ± SEM, n= 5.
PM: plasma membrane. WB: Western blot. ** $p<0.01$.

References

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