Science Translational Medicine
AAAS

www.sciencetranslationalmedicine.org/cgi/content/full/10/457/eaat1662/DC1

Supplementary Materials for

Hypercapnia increases airway smooth muscle contractility via caspase-7–mediated miR-133a–RhoA signaling

Masahiko Shigemura, Emilia Lecuona, Martín Angulo, Tetsuya Homma, Diego A. Rodríguez, Francisco J. Gonzalez Gonzalez, Lynn C. Welch, Luciano Amarelle, Seok-Jo Kim, Naftali Kaminski, G. R. Scott Budinger, Julian Solway, Jacob I. Sznajder*

*Corresponding author. Email: j-sznajder@northwestern.edu

Published 5 September 2018, *Sci. Transl. Med.* **10**, eaat1662 (2018)
DOI: 10.1126/scitranslmed.aat1662

The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

(available at www.sciencetranslationalmedicine.org/cgi/content/full/10/457/eaat1662/DC1)

Materials and Methods

Lung microarray

Total RNA from tissues was isolated with the miRNeasy Mini kit (Qiagen). Messenger RNA or miR profiling was performed with an Agilent SurePrint G3 8×15K mouse microarray containing 39,430 messenger RNAs or 470 microRNAs (Sanger miRbase release 9.1), in accordance with the protocol described by the manufacturer (Agilent) and as previously described by us *(42)*. Results were compared by unpaired *t* test, and gene expression was considered to be significantly different between groups when $p<0.05$. Pathway and network analyses were performed using MetaCore software (GeneGo, Thomson Reuters). When the cut-off was set at greater than 2.0-fold change in mRNA microarray, we found 157 genes (105 upregulated and 52 downregulated) in the mice exposed to HC for 7 days. The 157 genes were used for the pathway analysis. For the network analysis, we analyzed the 157 genes and miRs from the mice exposed to HC for 3 days and predicted potential enriched pathway.

Cell isolation

Primary alveolar type II cells were isolated from the lungs of mice exposed to room air or hypercapnia using techniques described elsewhere *(43)*. After the cell isolation, cells were immediately homogenized in lysis buffer (Cell Signaling) for Western blot.

Quantitative reverse transcription PCR

To isolate total RNA from cells were homogenized directly in 700 μL of lysis/binding buffer provided by the miRNeasy Mini kit (Qiagen). Complementary DNA (cDNA) to study *Mef2d* and *ribosomal protein L19* (*Rpl19*) was synthesized from 1 μg of total RNA using a qScript cDNA Synthesis kit (Quanta Biosciences), and mRNA expression was determined by quantitative PCR (qPCR) using SYBR Green chemistry (Bio-Rad). Primers were purchased from Integrated DNA Technologies. The following primer pairs were used to amplify specific gene products. *Rpl19, GAAGGTCAAAGGGAATGTGTTCAA* (forward), *TTTCGTGCTTCCTTGGTCTTAGA* (reverse); *Mef2d, AGGGAGGCAAAGGGTTAATG* (forward), *CCTGGCTGAGTAAACTTGGTG* (reverse). Relative expression of the transcripts was determined according to the $\Delta\Delta C_t$ method using *Rpl19* as reference for normalization.

Cell death detection enzyme-linked immunosorbent assay (ELISA)

Apoptosis in mouse ASM cells was assessed by detecting DNA fragmentation with an ELISA Cell Death Detection kit (Sigma), according to the manufacturer's instructions. Oligonucleosome-bound DNA was determined spectrophotometrically at 405 nm and the ratio of fragmentation versus controls was calculated. Positive and negative controls were performed as suggested by the supplier.

Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining

The lungs were excised and fixed in 10% normal buffered formalin. Paraffin-embedded sections (thickness, 5 μm) were stained with TUNEL (EMD Millipore), which was performed by the Northwestern University Mouse Histology and Phenotyping Laboratory.

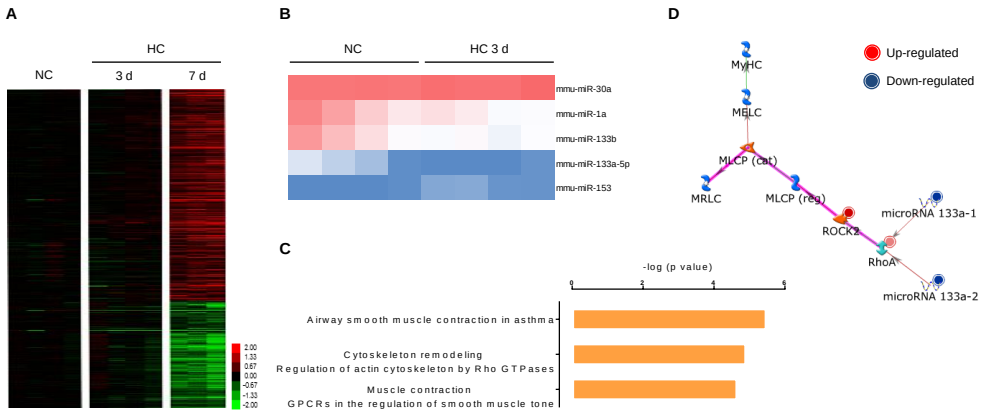

Fig. S1. Normoxic hypercapnia alters gene expression in mouse lung.
C57Bl/6J mice were exposed to 21% O_2/10% CO_2 (HC) or maintained in room air (NC) for up to 7 days. (A) Heat map showing differentially expressed mRNA in mice exposed to NC (*n*=4 mice) vs. HC for 3 (*n*=4 mice) or 7 days (*n*=3 mice). Red or green color reflects relative high or low expression, respectively, as indicated in the scale bar (log_2 transformed scale). Differentially expressed genes can be found in **table S1**. (B) Heat map showing differentially expressed miR genes in mice exposed to NC (*n*=4 mice) or HC for 3 days (*n*=4 mice). Red or blue color reflects relative high or low expression, respectively. Differentially expressed miRs can be found in **table S1**. (C) Metacore pathway analysis showing the top 3 pathways with significant p values. (D) Metacore network analysis showing the biological signaling pathways obtained using the data from 3-day exposure conditions of miR microarray and 7-day exposure conditions of mRNA microarray.

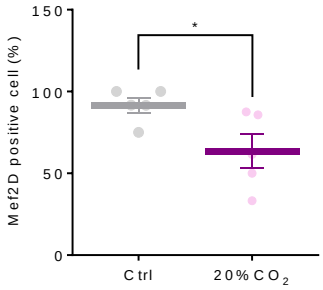

Fig. S2. Quantification of Mef2D-positive cell in mouse ASM cells.
Immunological staining of Mef2D was performed in mouse ASM cells exposed to 5% (Ctrl) or 20% CO_2 for 2 days (n>50). Mef2D-positive cells were counted in 5 different fields and the average of the positive cells from each field was statistically analyzed. All data are expressed as mean ± SEM. * $p<0.05$

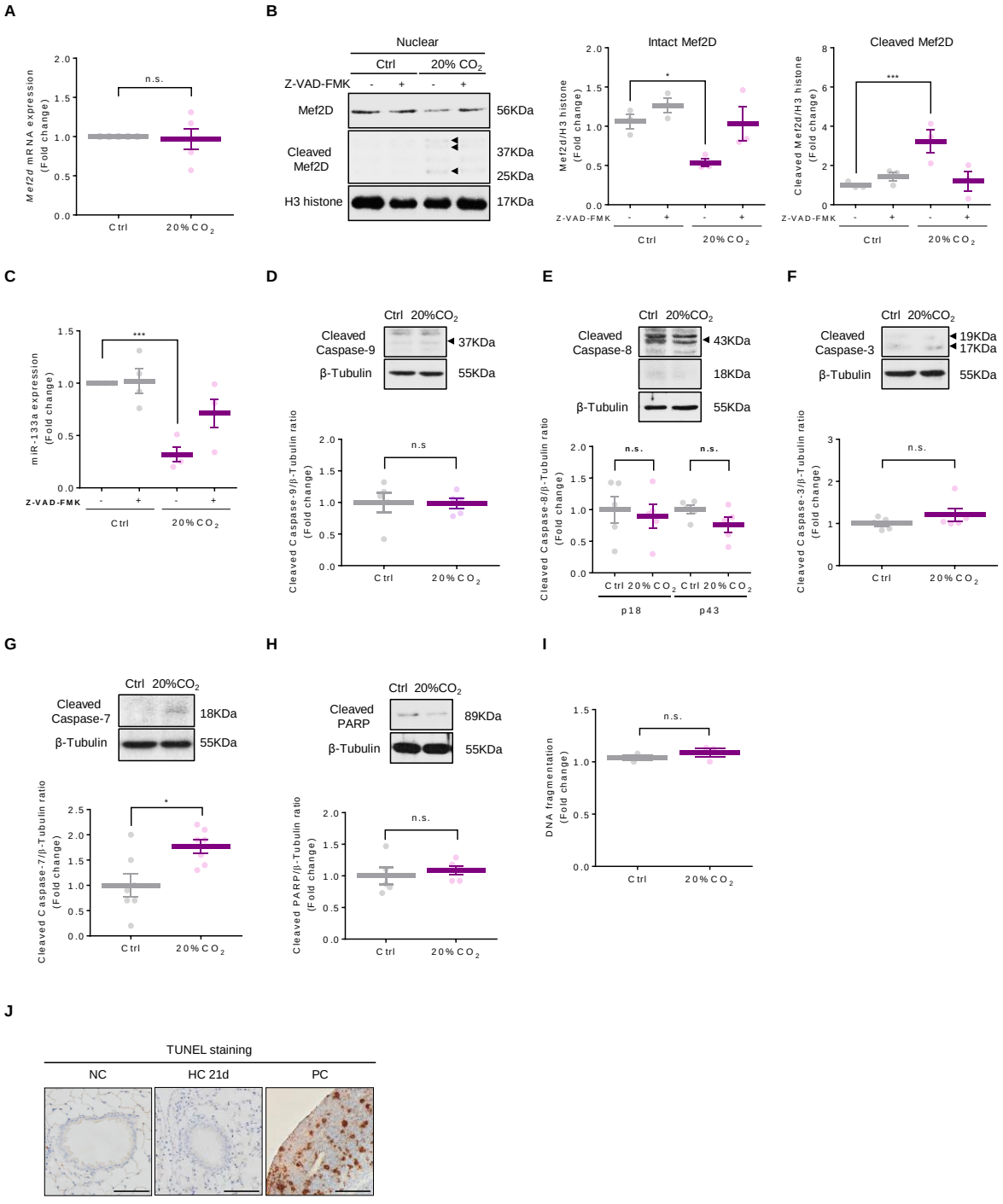
A
B
C
D
E
F
G
H
I
J
Nuclear
Ctrl
20% CO2
Z-VAD-FMK
Mef2D
Cleaved Mef2D
H3 histone
56KDa
37KDa
25KDa
17KDa
Intact Mef2D
Cleaved Mef2D
Mef2d mRNA expression (Fold change)
Mef2d/H3 histone (Fold change)
Cleaved Mef2D/H3 histone (Fold change)
n.s.
miR-133a expression (Fold change)
Cleaved Caspase-9
Cleaved Caspase-8
Cleaved Caspase-3
Cleaved Caspase-7
Cleaved PARP
β-Tubulin
37KDa
43KDa
18KDa
19KDa
17KDa
55KDa
89KDa
Cleaved Caspase-9/β-Tubulin ratio (Fold change)
Cleaved Caspase-8/β-Tubulin ratio (Fold change)
Cleaved Caspase-3/β-Tubulin ratio (Fold change)
Cleaved Caspase-7/β-Tubulin ratio (Fold change)
Cleaved PARP/β-Tubulin ratio (Fold change)
DNA fragmentation (Fold change)
p18
p43
TUNEL staining
NC
HC 21d
PC

Fig. S3. High CO_2 causes caspase-dependent cleavage of Mef2D protein, resulting in down-regulation of miR-133a, but does not induce apoptosis in mouse ASM cells.

(A-H) All experiments were performed in mouse ASM cells exposed to 5% (Ctrl) or 20% CO_2 for 2 days. (A) *Mef2d* mRNA expression (*n*=5). (B) Representative Western blot (left) and quantification of intact (middle) and cleaved (right) Mef2D in nuclear fraction from ASM cells treated with or without 50 μM the caspase inhibitor, Z-VAD-FMK (*n*=3). (C) miR-133a expression in ASM cells treated with or without 50 μM Z-VAD-FMK (*n*=4). (D-H) Representative Western blot (top) and quantification (bottom) of cleaved Caspase-9 (D, *n*=5), cleaved Caspase-8 (E, *n*=5), cleaved Caspase-3 (F, *n*=5), cleaved Caspase-7 (G, *n*=7) and cleaved poly(adenosine 5′-diphosphate–ribose) polymerase (PARP) (H, *n*=5). (I) Apoptotic DNA fragmentation (*n*=3). (J) Representative images of TUNEL staining (Scale bar, 100 μm) in lungs from C57Bl/6J mice exposed to hypercapnia (HC) or maintained in room air (NC) for 21 days (*n*=3 mice). Left, NC mice; middle, HC mice; right, mouse thymus treated with dexamethasone as a positive control (PC). All data are expressed as mean ± SEM. Statistical analysis was performed by one-sample *t* test (A) or with Bonferroni adjustment (C), or one-way ANOVA with Dunnett's post-hoc test (B). For B and C, all comparisons were made with the control group of "Z-VAD-FMK, untreated". $^{*}p<0.05$, $^{***}p<0.001$; n.s., nonsignificant.

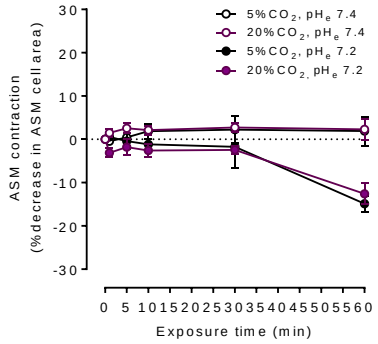

Fig. S4. Acute hypercapnia causes ASM relaxation due to hypercapnia-associated acidosis. Cell contractility in mouse ASM cells exposed for the indicated time to different CO_2 and extracellular pH (pH_e) conditions (*n*=3-4).

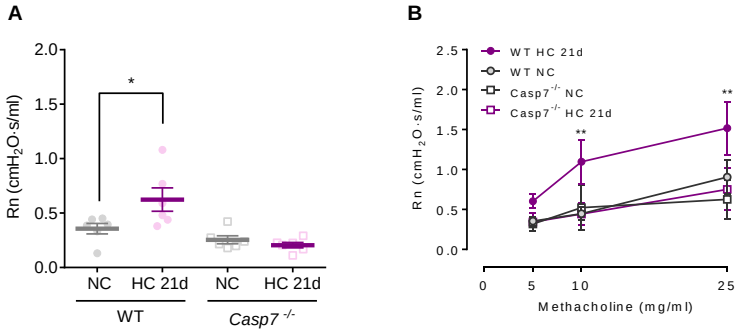

Fig. S5. Central respiratory resistance in WT and Caspase-7–null mice.

C57Bl/6J (WT) and B6.129S6-*Casp7* ^{tm1Flv}/J (*Casp7*$^{-/-}$) mice were exposed to hypercapnia (HC) or maintained in room air (NC) for 21 days. Central respiratory resistance (Rn) at baseline (A) and after methacholine challenge (B) measured on a FlexiVent instrument (*n*=6 mice). All data are expressed as mean ± SEM. Statistical analysis was performed by one-way ANOVA with Dunnett's post-hoc test (A) or Two-way ANOVA with Dunnett's post-hoc test (B). All comparisons were made with the group of "WT NC". $^{*}p<0.05$, $^{**}p<0.01$.

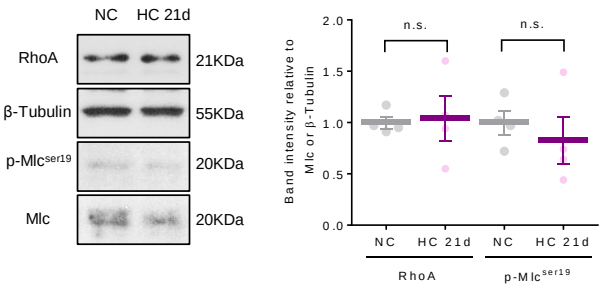

Fig. S6. RhoA protein abundance and Mlc phosphorylation in mouse primary alveolar type II cells.

Mouse primary alveolar type II cells were isolated from the lungs of C57Bl/6J mice exposed to hypercapnia (HC) or maintained in room air (NC) for 21 days. Representative Western blot (left) and quantification (right) of RhoA and Mlc phosphorylation in mouse primary alveolar type II cells (*n*=4 mice). β-Tubulin and total Mlc were used as loading controls. Data are expressed as mean ± SEM.

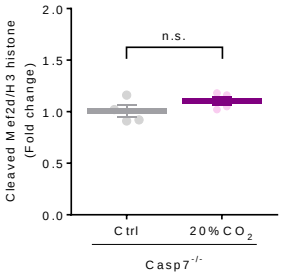

Fig. S7. Quantification of cleaved Mef2D in ASM cells from Caspase-7–null mice. Cleaved nuclear Mef2D from *Caspase-7*$^{-/-}$ (*Casp7*$^{-/-}$) ASM cells exposed to 5% (Ctrl) or 20% CO_2 for 2 days (*n*=4). All data are expressed as mean ± SEM.

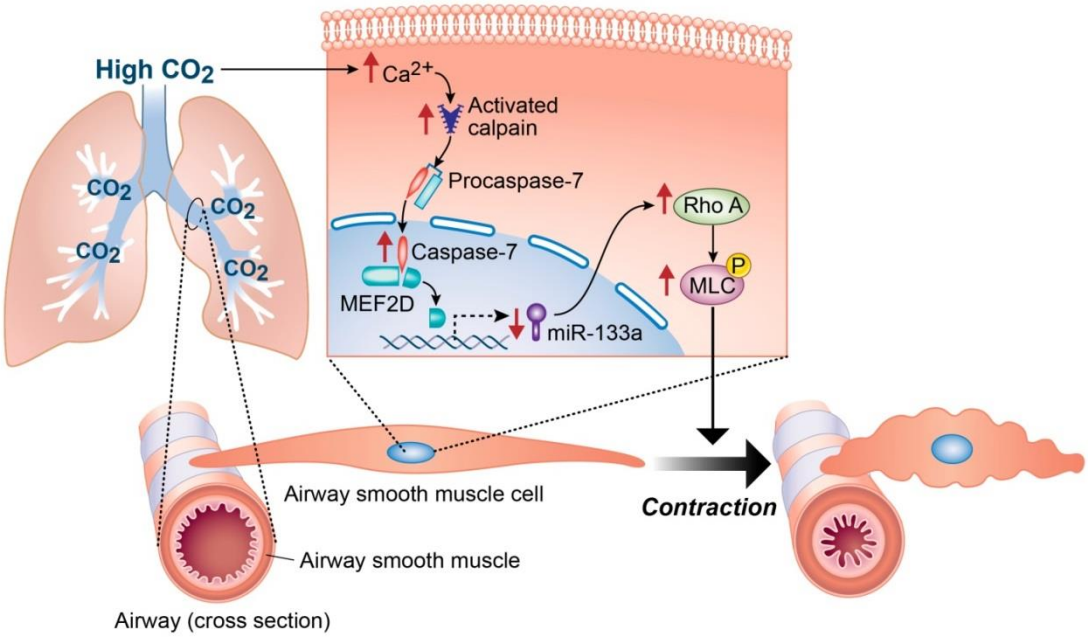

Fig. S8. Schematic of nonapoptotic role of Caspase-7 in ASM contractility during hypercapnia.
Hypercapnia promotes airway smooth muscle (ASM) contractility through an increase in intracellular Ca^{2+} and consequent activation of calpain which cleaves Caspase-7. Cleaved Caspase-7, in turn, cleaves the transcription factor myocyte-specific enhancer factor 2D (MEF2D) that reduces miR-133a expression, thereby increasing Ras homolog family member A (RhoA) abundance and myosin light chain (MLC) phosphorylation.

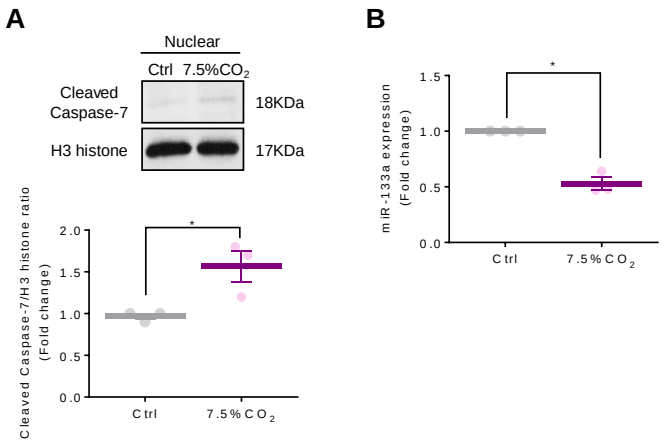

Fig. S9. Same signaling pathways activated in mouse ASM cells when exposed to similar pCO_2 values to the COPD patients.
All experiments were performed in mouse ASM cells exposed to 5% (Ctrl) or 7.5% CO_2 for 7 days. (A) Representative Western blots (top) and quantification (bottom) of cleaved Caspase-7 in nuclear fractions (n=3). (B) Expression of miR-133a (n=3). All data are expressed as mean ± SEM. Statistical analysis was performed by one-sample t test (B). $^{*}p<0.05$.

Table S1. Processed data from mRNA and miR microarray analysis of lungs isolated from C57BL/6J mice exposed to normoxic hypercapnia or room air for 3 or 7 days (Excel file).

Table S2. Primary data (Excel file).