SUPPLEMENTARY MATERIAL

Pulmonary Hypertension in Smoking Mice Over-expressing Protease-Activated Receptor-2.

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Materials and Methods

All animal experiments were conducted in conformity with the “Guiding Principles for Research Involving Animals and Human Beings” and approved by the Local Ethics Committee of the University of Siena, Siena, Italy.

Experimental Animals

Transgenic mice over-expressing PAR-2 ($FVB^{PAR\text{-}2\text{-}TgN}$) were supplied by Roche Bioscience, Palo Alto (CA). Generation of these mice has previously been described (1). Wild-type (WT) FVB/N mice were purchased from Charles River Italia (Calco, Italy).

The animals were bred at the University of Siena, Siena, Italy. Mice were housed in a light (07:00 to 19:00) and temperature controlled (18° to 22°C) environment, and food (Global Diet 2018; Mucedola, Settimo Milanese, Italy) and water were provided for consumption *ad libitum*.

Exposure to Cigarette Smoke

$FVB^{PAR\text{-}2\text{-}TgN}$ and WT mice were chronically exposed to either room air or to the smoke of 3 cigarettes/day (Virginia filter cigarettes: 12 mg of tar and 0.9 mg of nicotine) for 5 days/week for 1, 2, 4, and 7 months.

In a preliminary study, $FVB^{PAR\text{-}2\text{-}TgN}$ and WT mice were also acutely exposed to the smoke of 5 cigarettes/day for 3 consecutive days.

The methodology for cigarette smoke exposure has previously been described in detail (2).

Morphology and Morphometry

At 2, 4 and 7 months after chronic exposure to room air or cigarette smoke, 12 animals of each group were sacrificed under anaesthesia with pentobarbital (60 mg/Kg) and the lungs fixed by intra-tracheally with buffered formalin (5%) at a constant pressure of 20 cm H_2O. Lung volume (V) was measured by water displacement according to Scherle (3). Sagittal sections of each pair of lungs were cut and stained with haematoxylin/eosin. The slides were coded to prevent bias. Morphometric evaluations included determination of the average inter-alveolar distance (mean linear intercept: Lm) (4) and internal surface area (ISA) estimated by the Lm method at post-fixation lung volume by the formula 4V/Lm, where V is the postfixation lung volume (5). For the determination of the Lm for each pair of lungs, 40 histological fields were evaluated both vertically and horizontally.

Hemodynamic Analysis and Assessment of Right Ventricular Hypertrophy

$FVB^{PAR\text{-}2\text{-}TgN}$ and WT mice exposed to chronic CS or room air for 4 and 7 months were anaesthetized with sodium pentobarbital (60 mg/Kg) by intraperitoneal injection. Measurements of right ventricular systolic pressure (RVSP) were performed by an open chest technique essentially according to Lips et al. (6). RVSP was recorded during 1 minute period by using an Ultra-miniature Pressure Catheter® (Millar Instruments, Houston, TX, USA) placed into the right ventricular cavity by direct puncture of the right ventricle. The catheter was connected to an analog-to-digital converter MP-100A-CE (BIOPAC Systems, Inc., Goleta, CA, USA). Measurements carried out at the heart rate below 300 beats/min were excluded from analysis.

After heart removal, the right ventricle (RV) was dissected from the left ventricle plus septum (LV+S), and each was weighed separately. The determination of Fulton's index (RV/LV+S) was used as an index of right ventricular hypertrophy (RVH).

Radioimmunoassay (RIA) for ET-1

Plasma ET-1 assay was carried out on additional 8 $FVB^{PAR\text{-}2\text{-}TgN}$ and 8 WT mice exposed to room air or chronic CS for 4 and 7 months. Blood was withdrawn by direct cardiac puncture and collected in EDTA tubes. The samples were centrifuged at 1,600 g for 15 minutes at 4°C into centrifuge tubes containing aprotinin (Sigma, St. Louis, MO, USA) (0.6 TIU/ml of blood). Plasma ET-1 levels were determined using commercially available radioimmunoassay kit (RIA, Phoenix Pharmaceuticals INC., Belmont, CA, USA) according to the manufacturer's recommendation.

Immunohistochemical Analysis of PAR-2

For antigen retrieval, tissue sections (7 μm) were heated in a microwave for 10 min in citrate buffer 0.01 M, pH 6.0, and allowed to cool slowly to room temperature. The slides were then incubated with 3% bovine serum albumin for 30 min at room temperature to block non-specific antibody binding, and then incubated to the primary antibody, a rabbit polyclonal to PAR-2 (1:25) (Santa Cruz Biotechnology Inc, Europe) overnight at 4°C.

The sections were rinsed and incubated with goat polyclonal anti-rabbit biotinylated IgG (1:100) (Vector Labs, burlingame, CA) for 40 min. at room temperature. The staining was revealed by adding Streptavidin-alkaline phosphatase (BD Pharmingen, Buccinasco, Italy). After rinsing in 0.01 M PBS containing 0.1% Triton X-100, the alkaline phosphatase reaction was developed with NBT/BCIP stock solution (Roche Diagnostics, Milan, Italy) as chromogen diluted in 0.1 M TRIS buffer, pH 9.5, 0.05 M MgCl2, 0.1 M NaCl, 2 mM levamisole.

Immunohistochemical Analysis of alpha-Smooth Muscle Actin (α-SMA)

Muscularization of intraparenchymal vessels was determined by immunohistochemical staining with mouse monoclonal antibody (ab) to α-SMA (Sigma, St. Louis, MO, USA) diluted 1:400 using the M.O.M. immunodetection kit (Vector Laboratories, Burlinghame, CA, USA)

The Vector M.O.M. immunodetection kit is designed specifically to localize mouse primary monoclonal and polyclonal antibodies on mouse tissues by using a novel blocking agent and reducing the undesired background staining. Immunostaining was revealed by using the M.O.M. detection kit with 3,3′-diaminobenzidine tetra hydrochloride (DAB) substrate.

The degree of muscularization of small (≤ 80 μm) and medium (81-150 μm) vessels was determined with the use of a blind code in a minimum of 50 small and 15 medium vessels per animal according to Quinlan et al. (7). Vessels were classified as nonmuscular, partly muscular, or fully muscular by α-SMA staining. Partially muscular vessels were defined as those exhibiting at least one smooth muscle (SM) cell but no continuous media. Fully muscularized vessels had a continuous SM layer. The number of vessels exhibiting at least one SM cell divided by the total number of vessels yielded the percentage of muscularized vessels. Muscularized vessels were then classified as partly or fully muscularized by the criteria described above. The mean of repeated measurements from the endothelium to the outer edge of the SM layer in fully muscularized vessels was used in the formula (2 x wall thickness x 100)/external diameter to give percent vessel wall thickness (%VWT) or, for the three sizes of vessels, to analyze increases in VWT vascular remodeling (7).

Immunohistochemical Analysis of Vasoconstrictor, Vasoproliferative and Vasodilator Mediators

Tissue sections from mice exposed to room air or cigarette smoke for 2, 4 and 7 months were stained for transforming growth factor beta (TGF-beta), platelet-derived growth factor beta (PDGF-beta), vascular endothelial growth factor A (VEGF-A), endothelin-1 (ET-1), endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS).

The sections were pre-treated with 3% hydrogen peroxide for the endogenous peroxidase blocking. Antigen retrieval was performed by heating in a microwave for 20 min in 0.01 mol/L pH 6.0 citrate buffer and allowing to cool slowly to room temperature. All the sections were incubated with 3% bovine serum albumin for 30 min at room temperature to block non-specific antibody binding.

Tissues were incubated overnight at 4 °C with primary rabbit antibodies: Ab to mouse TGF-beta (Insight Biotechnology LTD., Wembley, UK) diluted 1:20; Ab to mouse PDGF-beta (Abcam, Cambridge, UK) diluted 1:100; Ab to mouse iNOS (Abcam, Cambridge, UK) diluted 1:100 and Ab to mouse ET-1 (Peninsula Laboratories, Belmont, CA, USA) diluted 1:100. For ET-1 detection no antigen retrieval was performed.

After incubation with primary Abs, tissue sections were rinsed with PBS, incubated with sheep anti-rabbit IgG for 30 min at room temperature followed by incubation with peroxidase–antiperoxidase complex, prepared from rabbit serum. Colour development was performed using DAB as chromogen. As negative controls for the immunostaining, primary Abs were replaced by non-immunised rabbit serum.

The M.O.M. immunodetection kit was used for immunodetection of mouse monoclonal to eNOS (Transduction Laboratories, Lexington, KY, USA) diluted 1:40 and to VEGF-A (Abcam, Cambridge, UK) diluted 1:100. Immunostaining was revealed with DAB substrate.

To evaluate staining, we utilized a grading system based on extent and intensity of staining reaction compared with background. Each lung small vessel (≤ 80 μm) in the histological section was examined and assigned a grade according to Wright et al. (8). Grade 0 was defined as no staining, whereas grade 3 was defined as intense and diffuse vessel staining. A final animal grade was obtained by summation of all grades and expressed as a percentage (mean ± standard deviation) of the maximum possible grade for that number of vessels. Staining was analysed on a tissue slide randomly selected from each mouse of each group at 2, 4 and 7 months after chronic exposure to CS or room air. The slides were coded to prevent bias.

Immunohistochemistry for Hypoxia-Inducible Factor-1alpha subunit (HIF-1α)

HIF-1α protein was immunohistochemically evaluated on paraffin sections using mouse monoclonal HIF-1α Ab (Abcam, Cambridge, UK) diluted 1:1000. Reaction was visualized using the M.O.M. immunodetection kit and DAB as substrate.

Proliferation and apoptosis indices

SM cells of small lung vessels undergoing apoptosis or proliferation were evaluated by terminal deoxy-nucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay or by immunostaining with proliferating cell nuclear antigen (PCNA), respectively. We examined lungs from 8 mice for each group at 1 month after CS exposure. Similar cross-sectional areas were serially sectioned at 5 mm thickness, and two slides from each mouse were subjected to TUNEL and PCNA staining. Staining procedures are as follows: two lung sections per mouse were rehydrated and incubated in an antigen retrieval solution (10mM Citric buffer, pH6.0) at 95° C for 20 min. After 1 hour of blocking, the sections were treated with an anti-PCNA mouse monoclonal antibody diluted 1:100 (Dakocytoautomation, Glostrup, Denmark) for

30min. PCNA-postitive cells were visualized by incubation with DAB substrate after using M.O.M. immunodetection kit. The ratio of PCNA-positive nuclei per a total number of nuclei in smooth muscle layer of distal blood vessels (30-80 μm outer diameter) was compared. Evaluation of smooth muscle vascular cells undergoing apoptosis was performed with TUNEL assay using the *In Situ* Cell Death Detection kit (Roche Applied Science) as described by manufacturer's manual. TUNEL-positive cells were observed and counted under fluorescent microscopy after DAPI staining (Vector Laboratories).

RNA isolation and cDNA synthesis

Total RNA was extracted from lungs of mice at 1 month after chronic exposure to room air or cigarette smoke, using TRi Reagent (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Eight mice for each group were used for RNA isolation. RNA was re-suspended in RT-PCR Grade Water (Ambion, Austin, Texas, USA) and the amount and purity of RNA was quantified spectrophotometrically by measuring the optical density at 260 and 280 nm. Integrity was checked by agarose gel electrophoresis.

Two micrograms of total RNA was treated with TURBO DNAse (TURBO DNA-free kit, Ambion, Austin, TX, USA) for 30 min and reverse transcribed using the RETROscript kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Two hundredths of the final volume of reverse transcription was used for real-time RT-PCR.

Real-time RT-PCR

Real-time RT-PCR was performed in triplicate for each sample on the MJ Opticon Monitor 2 (MJ Research Co., Waltham, MA, USA) with specific locked nucleic acid

(LNA) probes from the Mouse Universal Probe Library Set (UPL probes, Roche, Indianapolis, IN, USA).

Primers were designed by using the free online ProbeFinder software (available at the Universal ProbeLibrary Assay Design Center: www.universalprobelibrary.com) that shows a pair of specific primers for each probe from the Universal ProbeLibrary set (see below). The combination of primers and probes provides specific amplification and detection of the target sequence in the sample.

Primers Sequence and Probe Catalog Number

Transcript	Primer forward reverse	Probe
Alpha-SMA	Forward 5′-actctcttcagccatctttca-3′ Reverse 5′-ataggtggtttcgtggatgc-3′	58
Tgfb1	Forward 5′-tggagcaacatgtggaactc-3′ Reverse 5′-gtcagcagccggttacca-3′	72
Pdgfb	Forward 5′-cggcctgtgactagaagtcc-3′ Reverse 5′-gagcttgaggcgtcttgg-3′	32
ET-1	Forward 5′-tgctgttcgtgactttccaa-3′ Reverse 5′-gggctctgcactccattct-3′	50
Vegf-A	Forward 5′-gcagcttgagttaaacgaacg-3′ Reverse 5′-ggttcccgaaaccctgag-3′	4

eNOS	Forward 5′-ccagtgccctgcttcatc-3′	12
	Reverse 5′-gcagggcaagttaggatcag -3′	
iNOS	Forward 5′-ctttgccacggacgagac-3′	13
	Reverse 5′-tcattgtactctgagggctgac-3′	
HIF-1 alpha	Forward 5′-gcactagacaaagttcacctgaga-3′	95
	Reverse 5′-cgctatccacatcaaagcaa-3′	
18S rRNA	Forward 5′-aaatcagttatggttcctttggtc-3′	55
	Reverse 5′-gctctagaattaccacagttatccaa-3′	

PCR reactions were performed in a volume of 25 µl and contained 12.5 µl of FastStart TaqMan Probe Master (Roche, Indianapolis, IN, USA), 300 nmol/L forward and reverse primers (TIBMolbiol, Genova, Italy), 200 nmol/L UPL probes and 5 µl of cDNA.

Reactions were incubated at 95 °C for 10 min and then amplified for 40 cycles, each cycle comprised of an incubation step at 94 °C for 15 s followed by 60 °C for 1 min.

The real-time RT-PCR assay included a non-template control and a standard curve of four serial dilution points (in steps of 10-fold) of each of the test cDNAs.

The analysis of the results was based on the comparative Ct method (ΔΔCt) in which Ct represents the cycle number at which the fluorescent signal, associated with an exponential increase in PCR products, crosses a given threshold. The average of the target gene was normalized to 18S rRNA as the endogenous housekeeping gene (9).

Protein Isolation and Western blots

Protein was precipitated from the phenol-ethanol supernatant obtained after sedimentation of the DNA pellet according to the manufacturer's recommendations (TRI Reagent ®DNA/Protein Isolation, Ambion, Austin, TX). After a series of washes, the protein-containing pellet was solubilized in a suitable detergent-containing solvent. The protein content was measured using the Bio-Rad Dc protein assay (Bio-rad Laboratories, Hercules, CA, USA).

Samples were electrophoresed through a SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking with 5% non-fat powdered milk in Tris-buffered saline/Tween 20, the blots were incubated with a mouse monoclonal anti-eNOS/NOS type III antibody (1:500) (Transduction Laboratories, Lexington, KY, USA), a rabbit polyclonal to phospho-eNOS (ser1177) antibody (1:500) (Cell SignalingTechnology, Beverly, MA) and with a mouse monoclonal to VEGF (1:1000)

(Abcam, Cambridge, UK) at 4 °C overnight.

After washing with Tris-buffered saline/Tween 20, they were incubated with a secondary anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (1:10000) (Roche Molecular Biochemicals, Milan, Italy). The blot was developed using the enhanced chemiluminescence method (ECL-Kit Lumi-LightPlus, Roche Molecular Biochemicals, Milan, Italy) according to the manufacturer's instructions. The membranes were re-probed with rabbit polyclonal to glyceraldehyde 3 phosphate dehydrogenase (GAPDH) (1:2500) (Abcam, Cambridge, UK) or β-actin (1:5000) (Sigma-Aldrich, Missouri, USA) to normalize for protein loading. Each band was scanned by densitometry analysis and normalized to GAPDH or β-actin.

Bronchoalveolar Lavage Fluid (BALF) cell counts after Acute Cigarette Smoke Exposure

Immediately after the last exposure, 8 mice for each group (exposed or not to CS) were sacrificed under anaesthesia with pentobarbital (60 mg/Kg). The trachea was isolated and then cannulated with a 20 gauge blunt needle. With the aid of a peristaltic pump (P-1 Pharmacia) the lungs were lavaged *in situ* 3 times with 0.6 ml saline solution. The average fluid recovery was greater than 95%.

Total cell counts were performed in a hemocytometer on BALFs. Differential counts of 300 cells were done on slides stained with Diff-Quick stain.

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Supplementary Figure 1.

Representative immunohistochemical reaction for PAR2 in lung tissue slides of an air- (A) and smoke-exposed WT (B) mouse at 1 month of treatment. Note the presence of a positive reaction on the endothelium of a small pulmonary vessel (*SPV*) in (B). BL= Bronchiolar lumen; *ALV= alveolus*.

Scale Bars: (A and B) = 30 μm

Supplementary Table 1. Total and differential cell counts in BALFs.

Experimental group	N	Total cells, $x10^5$	Macrophages, $x10^5$	Neutrophils, $x10^5$	Lymphocytes, $x10^5$
Air control (WT)	8	1.22±0.11	1.09±0.10	0.08±0.02	0.05± 0.01
Cigarette smoke (WT)	8	1.66±0.17*	1.20±0.12	0.39±0.05*	0.07±0.02
Air control (PAR-2^{TgN})	8	1.46±0.16	1.26±0.15	0.14±0.01	0.06±0.01
Cigarette smoke (PAR-2^{TgN})	8	1.89±0.32*	1.37±0.22	0.44±0.08*	0.08±0.02

Data are given as mean ± SD

*P<0.05 *versus* air control