Apelin attenuates hyperoxic lung and heart injury in neonatal rats

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Online Data Supplement

Materials and Methods

Animals

Wistar rats were kept in a 12-12 h dark-light cycle and a standard chow diet (Special Diet Services, Witham, Essex, United Kingdom) and drinking water were provided *ad libitum*. Adult rats (6 months old) were exsanguinated by transection of the abdominal blood vessels after induction of anesthesia with an intraperitoneal injection of ketamine (50 mg/kg body weight, Nimatek^{TM}, Eurovet Animal Health BV, Bladel, The Netherlands) and xylazine (50 mg/kg body weight, Rompun^{TM}, Bayer AG, Leverkusen, Germany). Organs were removed directly, snap-frozen in liquid nitrogen and stored at -80 °C until isolation of RNA for real time RT-PCR. For timed pregnancies breeding pairs were allowed access for one hour at the day female rats showed very specific sexual behavior: lordosis, hopping and air-flapping. After a gestation of approximately 21.5 days pregnant rats were sacrificed by decapitation (spontaneous birth occurs 22 days after conception) and pups were delivered by hysterectomy through a median abdominal incision to ensure that the delay in birth between the first and the last pup was only 5 min. Immediately after birth, pups were dried and stimulated. Pups from four litters were pooled and distributed over four experimental groups: the oxygen (O_2), an oxygen-apelin (apelin) group, an oxygen-L-NAME (L-NAME) group, and an oxygen-L-NAME-apelin (L-NAME-apelin) group and four room air-exposed (RA) control groups injected either with saline, apelin, L-NAME or a combination of L-NAME and apelin. Pups were kept in a transparent 50 x 50 x 70 cm Plexiglas chamber for 10 days or until death occurred (survival experiments). In this way influences of the birth process within and between litters can be avoided and exposure to hyperoxia can be started within 30 min after birth. Pups were fed by lactating foster dams, which were rotated

daily to avoid oxygen toxicity. All pups delivered by the foster dam were replaced by the experimental pups, which were delivered by hysterectomy after decapitation of a pregnant rat at E21.5. Each foster dam nursed a litter of 12 pups in the experimental groups. Foster dams were exposed to 100% oxygen for 24 h and next to room air for 48 h. The oxygen concentration in the chamber was kept at 100% using a flowrate of 2.5 L/min. The oxygen concentration in the chamber was monitored daily with an oxygen sensor (Drägerwerk AG, Lübeck, Germany). Body weight, evidence of disease, and mortality were also checked daily. The research protocol was approved by the Institutional Animal Care and Use Committee of the Leiden University Medical Center.

Early concurrent treatment

In this model neonatal lung injury was induced by continuous exposure to 100% oxygen for 10 days. Hyperoxia-exposed and room-air-exposed pups were injected subcutaneously, starting on day 2, via a 0.5 mL syringe (U-100 Micro-Fine insulin 29G syringe, Becton Dickinson, Franklin Lakes, NJ, USA) at the lower back for 10 days. Because shorter C-terminal sequences of apelin–36, including apelin-13, are functionally active *in vitro* and *in vivo* (1-4) pups received either 100 µL of apelin-13 (pre-pro-apelin 65-77, Sigma, St Louis, MO, USA) in 0.9% saline or 100 µL of 0.9% saline (age-matched control). To investigate whether the nitric oxide synthase (NOS) dependent signaling pathway is involved in apelin treatment of experimental BPD, NOS activity was blocked with L-NAME, at a dose that has been shown previously to inhibit NOS activity effectively in neonatal rats (5). Pups received 100 µL of L-NAME (25 mg/kg/day, N_{ω}-nitro-L-arginine methyl ester hydrochloride, Sigma, St Louis, MO, USA) in 0.9% saline or 100 µL L-NAME plus apelin in 0.9% saline. In a pilot experiment in which hyperoxia-exposed rats were treated with 31-1,000 µg/kg/day of apelin (15.6-500 µg/kg twice a day), we found that pups treated with 125-1,000 µg/kg/day of apelin showed severe growth retardation and increased mortality. Therefore, experiments were performed with 62 µg/kg/day of apelin. Separate experiments were performed for [1] median survival studies (N=12), [2] collection of lung and heart tissue for fibrin deposition, cyclic GMP levels and RT-PCR (N=10), [3] histology (N=8), and [4] collection of bronchoalveolar lavage fluid (N=10). Median

survival was only studied in those experimental BPD groups that showed significant differences in survival at day 10 and were compared to room air controls.

Late treatment and recovery

The effect of apelin on lung injury and recovery was investigated by exposing newborn rat pups to hyperoxia for 9 days, followed by recovery in room air for 9 days. After 6 days of exposure to hyperoxia daily subcutaneous injections with 0.9% saline, 62 µg/kg/day apelin, 25 mg/kg L-NAME or a combination of L-NAME and apelin were started and continued throughout the 9-day recovery period in room air. Lung and heart tissue was collected for histology at the end of the 9-day hyperoxia period (N=8) and after the 9-day recovery period in room air (N=8).

Tissue preparation

Pups were anesthetized with an intraperitoneal injection of ketamine (25 mg/kg body weight; Nimatek, Eurovet Animal Health BV, Bladel, The Netherlands) and xylazine (50 mg/kg body weight; Rompun, Bayer, Leverkusen, Germany) on day 10. To avoid postmortem fibrin deposition in the lungs, heparin (100 units; Leo Pharma, Breda, The Netherlands) was injected intraperitoneally. After 5 min, pups were exsanguinated by transection of the abdominal blood vessels. The thoracic cavity was opened, and the lungs and heart were removed, snap-frozen in liquid nitrogen, and stored at –80°C until RNA isolation for *real-time* RT-PCR, cyclic GMP ELISA or the fibrin deposition assay. For histology studies, the trachea was cannulated (Bioflow 0.6 mm intravenous catheter, Vygon, Veenendaal, The Netherlands), and the lungs were fixed *in situ* via the trachea cannula with buffered formaldehyde (4% paraformaldehyde in PBS, pH 7.4) at 25 cm H_2O pressure for 6 min. Lungs and hearts were removed, fixed (additionally) in formaldehyde for 24 h at 4°C, and embedded in paraffin after dehydration in a graded alcohol series and xylene. To quantify the degree of right ventricular hypertrophy (RVH), hearts were harvested, followed by removal of the atria. Next, the right ventricular (RV) free wall was dissected, weighed separately from the interventricular septum (IVS)

and left ventricle (LV), frozen immediately in liquid nitrogen, and stored at –80°C for RNA isolation. As an indicator of RVH the weight ratio RV/(LV + IVS) was calculated.

Histology

Paraffin sections of the right lower lung lobe (5 μm) were cut and mounted onto SuperFrost plus-coated slides (Menzel, Braunschweig, Germany). Slides used for the different staining procedures mentioned below were chosen at random. After deparaffinization, lung sections were stained with hematoxylin and eosin, with monoclonal 59D8, which specifically recognizes β-fibrin (6;7), with polyclonal anti-APJ and anti-apelin, with monoclonal anti-ED-1 antibody that specifically recognizes rat monocytes and macrophages (8), with polyclonal (rabbit) anti-myeloperoxidase (MPO) antibody, a marker for neutrophilic granulocytes (9), with monoclonal anti-alpha smooth muscle actin (ASMA) to visualize the pulmonary medial arterial walls, or with polyclonal (rabbit) anti-von Willebrand Factor (vWF) as a marker for pulmonary blood vessels. Heart sections were stained with hematoxylin and eosin. For immunohistochemistry, sections were incubated with 0.3% H_2O_2 in methanol to block endogenous peroxidase activity. After a graded alcohol series, sections were boiled in 0.01 M sodium citrate (pH 6.0) for 10 min. Sections were incubated overnight with monoclonal 59D8 (Oklahoma Medical Research Foundation, Oklahoma City, OK, USA; diluted 1:5,000), polyclonal anti-APJ (H-001-79, Phoenix Pharmaceuticals, Belmont, CA, USA; diluted 1:5,000), polyclonal anti-apelin (H-057-15, Phoenix Pharmaceuticals, Belmont, CA, USA; diluted 1:5,000), monoclonal anti-ED-1 (diluted 1:5), polyclonal anti-MPO (RB-373-A1, Thermo Fisher Scientific, Fremont, CA, USA; diluted 1:1,500), monoclonal anti-ASMA (A2547, Sigma-Aldrich, St. Louis, MO, USA; diluted 1:10,000) or polyclonal anti-vWF (A0082, Dako Cytomation, Glostrup, Denmark; diluted 1:4,000), stained with EnVision-HRP as recommended by the manufacturer (Dako, Glostrup, Denmark) using the chromogenic substrate NovaRed as recommended by the manufacturer (Vector, Burlingame, CA, USA), and counterstained briefly with hematoxylin. Primary antibodies were diluted in PBS/1% bovine serum albumin, fraction V (Roche Diagnostics, Almere, The Netherlands).

For morphometry of the lung, an eye piece reticle with a coherent system of 21 lines and 42 points (Weibel type II ocular micrometer; Paes, Zoeterwoude, The Netherlands) was used. We used different

(immuno)histochemically stained lung sections for each quantification, except for mean linear intercept (MLI) and septum thickness, which were performed on the same hematoxylin and eosin stained section and alveolar crest and pulmonary arteriolar wall thickness which were determined on the same ASMA stained section. To investigate alveolar enlargement in experimental BPD we studied in addition to mean linear intercept (MLI) also the number of alveolar crests to exclude potential effects of heterogenous alveolar development. MLI and the number of alveolar crests were determined on different lung sections of the same animal. MLI an indicator of mean alveolar diameter (10), determined on hematoxylin and eosin stained sections, and the number of alveolar crests (11), determined on lung sections stained immunohistochemically for ASMA, were assessed in 10 non-overlapping fields at a 200x (MLI) or 400x (alveolar crest) magnification for each animal. Alveolar crests were normalized to field or to tissue ratio. The number of points of the eye piece reticle that fell on tissue and on secondary crests was expressed as secondary crest/tissue ratios. The density of ED-1 positive monocytes and macrophages or MPO-positive neutrophilic granulocytes was determined in the alveolar compartment by counting the number of cells per field. Results were expressed as cells per mm^2. Per experimental animal 20 fields in one section were studied at 400x magnification. Pulmonary alveolar septum thickness was assessed in HE-stained lung sections at a 400x magnification by averaging 100 measurements per 10 representative fields. Capillary density was assessed in lung sections stained for vWF at a 200x magnification by counting the number of vessels per field. At least 10 representative fields per experimental animal were investigated. Results were expressed as relative number of vessels per mm^2. Pulmonary arteriolar wall thickness was assessed in lung sections stained for ASMA at a 1000x magnification by averaging at least 10 vessels with a diameter of less than 30 μm per animal. Medial wall thickness was calculated from the formula "percent wall thickness = $\frac{2 * wall \cdot thickness}{external \cdot diameter} * 100$". Fields containing large blood vessels or bronchioli were excluded from the analysis. Thickness of the right and left ventricular free walls and interventricular septum (IVS) was assessed in a transversal HE-stained section taken halfway the long axis at a 40x magnification by averaging 6 measurements per structure. For morphometric studies in lung and heart at least 6 rat pups per experimental group were studied. Quantitative morphometry was

performed by two independent researchers blinded to the treatment strategy using the NIH Image J program.

In situ hybridization

Paraffin sections (7 μm) from the left upper lobe were cut and mounted onto SuperFrost plus coated slides (Menzel-Gläzer, Braunschweig, Germany). The in situ hybridization procedure was based on the method described by Wilkinson (12). Sections were deparaffinized, digested with proteinase K (Roche, Almere, The Netherlands), postfixed in 4% paraformaldehyde, acetylated with acetic anhydride dissolved in triethanolamine, and hybridized with ^{35}S-CPT and ^{35}S-UTP double labeled probe ($2x10^6$ cpm per section) in hybridization buffer overnight at 55 °C. The hybridization buffer contained 50% formamide, 2xSSC, 1x Denhardt's solution and 10% dextran sulphate, cRNA probe ($2x10^6$ cpm per 100 μl) and 100 ng/μl yeast RNA. After hybridization, high stringency washing was performed in 50% formamide/2x SSC at 55 °C. Sections were treated with RNase A, rinsed in 2x SSC and 0.2x SSC, dried and immersed in LM-1 Hypercoat emulsion RPN40 (Amersham Biosciences). Development took place after an exposure time of 3 weeks. Sections were briefly counterstained with haematoxylin, dehydrated in a graded ethanol series and xylene and mounted in pertex. As a negative control RNase pretreated sections were used prior to hybridization, which did not give any staining at all.

^{35}S-CTP and ^{35}S-UTP double labeled anti-sense cRNA probes to rat APJ and CC10 were made by in vitro transcription using pCR4-TOPO as a vector, containing a 920 bp APJ and a 416 bp CC10 fragment. Primers used to generate PCR fragments for APJ and CC10 from a first strand cDNA rat lung (10 days after birth) sample (see real-time RT-PCR section) were designed with primer3 software (frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are listed in Table 1. PCR reactions were performed under the following conditions: 95°C for 5 min (1 cycle), 95°C for 1 min, 60°C for 1 min, and 72 °C for 1 min (40 cycles) and 72°C for the last 30 min, in the presence of Amplitaq gold (Invitrogen, Paisley, England) on a Perkin Elmer GeneAmp PCR system 9600 (Perkin Elmer, Boston, MA, USA). PCR fragments were subsequently cloned into pCR4-TOPO vector exactly as described

by the manufacturer (Invitrogen, Breda, The Netherlands) and checked by restriction fragment analysis and sequencing.

Fibrin detection assay

Lung tissue homogenates for quantitative fibrin deposition by Western blotting were pretreated as described previously (6). Tissue samples, dissolved in reducing sample buffer (10 mM Tris pH 7.5, 2% SDS, 5% glycerol, 5% β-mercaptoethanol, and 0.4 mg/mL of bromophenol blue) were subjected to SDS-PAGE (7.5% gel; 5% stacking gel) and blotted onto PVDF membrane (Immobilon-FL, Millipore, Bredford, MA, USA). The 56-kDa fibrin β-chains were detected with monoclonal 59D8 (diluted 1:1000), infrared labeled (IRDye 800CW; Licor Biosciences, Lincoln, NE, USA) goat-anti-mouse secondary antibody (Dako Cytomation, Glostrup, Denmark, diluted 1:5000), and quantified using an infrared detection system (Odyssey infrared imaging system, Licor Biosciences). Fibrin deposition was quantified in lungs of at least 10 rats per experimental group using rat fibrin as a reference.

Bronchoalveolar lavages

Pups were anesthetized with an intraperitoneal injection of ketamine and xylazine and injected intraperitoneally with heparin on day 10. A cannula (Bioflow 0.6 mm intravenous catheter, Vygon, Veenendaal, The Netherlands) was positioned in the trachea, and the pups were exsanguinated by transection of the abdominal blood vessels. Lungs were slowly lavaged two times with 500 μL 0.15 M NaCl, 1 mM EDTA (pH 8.0), without opening the thorax. Samples were pooled, stored temporarily at 4°C, and centrifuged for 10 min at 5,000 rpm. Supernatants were stored at –20 ºC until further use.

Protein assay

Total protein concentration was measured in bronchoalveolar lavage fluid (BALF) using the Dc protein assay (Bio-Rad, Veenendaal, the Netherlands), according to the manufacturer's

instructions, using bovine serum albumin (fraction V; Roche Diagnostics, Almere, The Netherlands) as a standard. The detection limit was 31 μg/mL.

Cyclic GMP assay

Lung tissue samples were homogenized in 10 volumes of 5% trichloroacetic acid (TCA) at 4 ºC. Samples were centrifuged at 1,500 g for 10 min. TCA was extracted from the supernatant by adding 5 volumes of water-saturated ether for 3 times. Residual ether was removed from the aqueous layer by heating at 70ºC for 10 min. Cyclic GMP was detected in non-acetylated samples using a cyclic GMP EIA Kit (581021, Cayman Chemical Company, Ann Arbor, MI, USA) according to manufacturer's instructions.

Real-time RT-PCR

Total RNA was isolated from lung and heart tissue homogenates using guanidium-phenol-chloroform extraction and isopropanol precipitation (RNA-Bee, Tel-Test Inc, Bio-Connect BV, Huissen, the Netherlands). The RNA sample was dissolved in RNase-free water and quantified spectrophotometrically. The integrity of the RNA was studied by gel electrophoresis on a 1% agarose gel, containing ethidium bromide. Samples did not show degradation of ribosomal RNA by visual inspection under ultraviolet light. First-strand cDNA synthesis was performed with the SuperScript Choice System (Life Technologies, Breda, the Netherlands) by oligo(dT)12-18 priming as described previously (6). For *real-time* quantitative PCR, 1 μL of first-strand cDNA diluted 1:10 in RNase-free water was used in a total volume of 25 μL, containing 12.5 μL 2x SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 200 ng of each primer. Primers, designed with the Primer Express software package (Applied Biosystems), are listed in Table 2. Hyperoxia-induced lung injury induces alterations in inflammation, coagulation, fibrinolysis, alveolar enlargement, and edema. Therefore, we studied differential expression of key genes of these pathways, previously characterized in this rat model for experimental BPD (6). PCR reactions were performed at the following conditions: 95°C for 10 min (1 cycle), 94°C for 15 s, and 60°C for 1 min (40 cycles) on an ABI Prism 7900 HT Fast Real Time PCR system (Applied Biosystems) of the Leiden Genome Technology Center (Leiden,

The Netherlands). Data were analyzed with the ABI Prism 7900 sequence detection system software (version 2.2) and quantified with the comparative threshold cycle method using β-actin as a housekeeping gene reference (13). In a DNA array experiment we demonstrated that β-actin was not differentially expressed in lungs of hyperoxic rat pups compared to room air controls (6). In addition β-actin was not differentially expressed in left and right ventricle in both control and experimental rat pups.

Statistical analysis

Values are expressed as mean ± SEM. Differences between groups (≥ 3) were analyzed with analysis of variance (ANOVA), followed by Tukey's multiple comparison test. For comparison of survival curves, Kaplan-Meier analysis followed by a log rank test was performed. GraphPad Prism 5 (GraphPad Software, Inc, La Jolla, CA, USA) was used for statistical analysis. Differences at p values <0.05 were considered statistically significant.

Results

Relative mRNA expression of apelin and APJ

In adult rats highest apelin mRNA expression was observed in the heart, lung and brain (330-fold, 290-fold, and 210-fold above background, respectively), moderate expression in ovary, testis, kidney and liver (20-140-fold above background) and at background levels in thymus and spleen (Figure E1A). Highest APJ mRNA expression was observed in heart and lung (410-fold and 210-fold above background, respectively), moderate expression in kidney, ovary, and brain (10-50-fold above background) and at background levels in liver, testis, thymus and spleen (Figure E1B).

Effects of apelin on pulmonary medial wall thickness.

Early concurrent treatment

After normal neonatal lung development on day 10 the wall of small arterioles is thin (Figure E3A). Treatment with apelin (Figure E2B), L-NAME (C) or apelin and L-NAME (D) for 10 days had no adverse effect on medial wall thickness. Oxygen exposure for 10 days (E) resulted in a significant increase in medial wall thickness compared to room air-exposed controls, which was significantly reduced after apelin treatment for 10 days (F). L-NAME had no additional effect compared to oxygen-exposed controls (G), but L-NAME completely abolished the protective effect of apelin on medial wall thickness (H).

Effects of apelin on pulmonary vascular development.

Late treatment and recovery

Continuous neonatal exposure to hyperoxia for 9 days resulted in enlarged alveoli and a reduction in blood vessel density (Figure E3B) compared to room air controls (A). Apelin treatment during the last 3 days of the injurous hyperoxic period improved alveolarization and vascular development by increasing blood vessel density (C). A recovery period of 9 days in room air after hyperoxia-induced lung injury had only a minor beneficial effects on blood vessel density (E), but treatment with apelin increased blood vessel density significantly compared to non-treated experimental BPD pups at the end of the recovery period.

Effects of apelin on lung inflammation

Early concurrent treatment

Treatment with apelin for 10 days (Figure E4B), L-NAME (C) or apelin and L-NAME (D) had no effect on the influx of macrophages, compared to room air controls (A). Oxygen exposure for 10 days (E) resulted in edema, enlarged air-spaces which were surrounded by septa with increased thickness and an overwhelming influx of macrophages compared to room air-exposed controls. Apelin treatment reduced the influx of macrophages (F) compared to oxygen exposure for 10 days. L-NAME had no additional effect compared to oxygen-exposed controls (G), but L-NAME completely abolished the protective effect of apelin on macrophage influx alveolarization (H).

Effects of apelin on right ventricular hypertrophy.

Early concurrent treatment

Because an increase in medial wall thickness is associated with pulmonary hypertension and right ventricular hypertrophy we studied ventricular free wall thickness of the heart in experimental BPD as well. On neonatal day 10 the right ventricular free wall is relatively thin compared to the left ventricular free wall (Figure E4A). Treatment with apelin (B), L-NAME (C) or apelin and L-NAME (D) for 10 days had no adverse effect on right ventricular free wall thickness. Oxygen exposure for 10 days (E) resulted in a significant increase in right ventricular free wall thickness compared to room air-exposed controls, which was significantly reduced after apelin treatment for 10 days (F). L-NAME had no additional effect compared to oxygen-exposed controls (G), but L-NAME completely abolished the protective effect of apelin on medial wall thickness (H).

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Legends

Figure E1

Relative apelin (A) and APJ (B) mRNA expression in the adult brain (Br), thymus (Th), heart (He), lung (Lu), liver (Li), spleen (Sp), kidney (Ki), ovary (Ov) and testis (Te). Data are mean ± SEM (N=6).

Figure E2

Lung sections stained for alpha smooth muscle actin of room-air (RA, A-D) and O_2-exposed pups (E-H) daily injected with either saline (A, E), apelin (B, F), L-NAME (C, G) or apelin and L-NAME (D, H) after early concurrent treatment on day 10 (N=8).

Figure E3

Lung sections stained for von Willebrand factor on days 9 (A-C) and 18 (D-F) of room-air (RA, A, D) and O_2-exposed pups (B, E) and O_2-exposed pups daily injected with apelin (C, F) after late treatment and recovery (N=8).

Figure E4

Lung sections stained for the ED-1 epitope on macrophages of room-air controls (RA, A-D) and O_2-exposed pups (E-H) daily injected with either saline (A, E), apelin (B, F), L-NAME (C, G) or apelin and L-NAME (D, H) after early concurrent treatment on day 10 (N=8).

Figure E5

Heart sections of room-air (RA, A-D) and O_2-exposed pups (E-H) daily injected with either saline (A, E), apelin (B, F), L-NAME (C, G) or apelin and L-NAME (D, H) after early concurrent treatment on day 10 (N=8).

Table E1. Sequences of oligonucleotides for forward and reverse primers for PCR

Gene Product	Forward Primer	Reverse Primer
APJ	5'-TGTGGCTGACTTGACCTTTG -3'	5'-CCACAAGGGTTTCTTGGCTA-3'

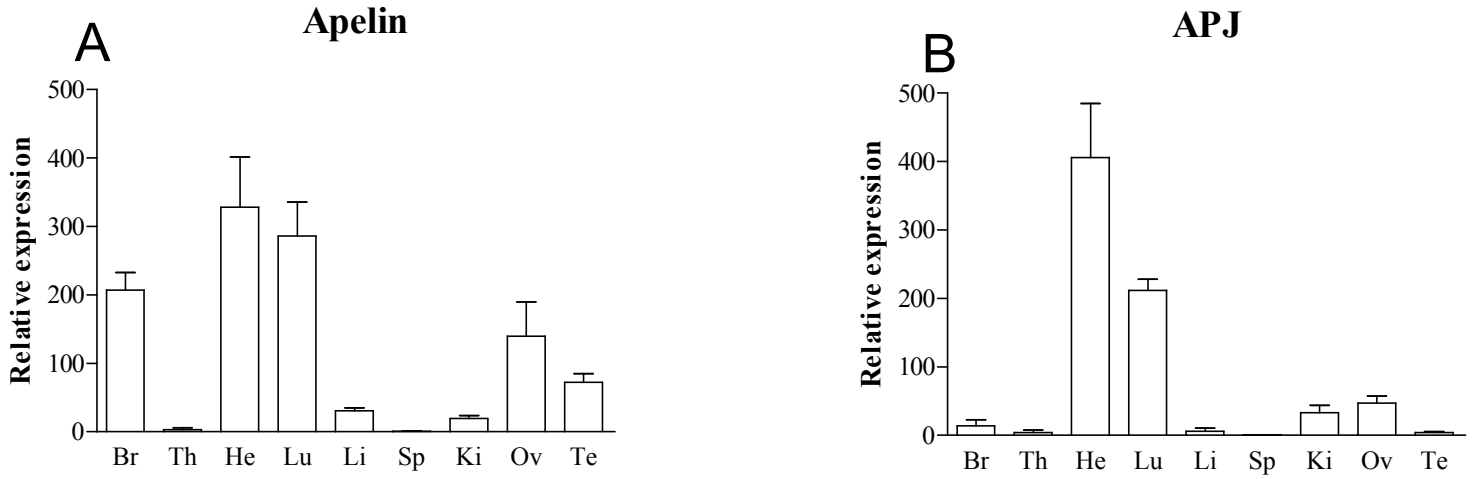
Table E2. Sequences of oligonucleotides for forward and reverse primers for real-time RT-PCR

Gene Product	Forward Primer	Reverse Primer
Amphiregulin	5'-TTTCGCTGGCGCTCTCA-3'	5'-TTCCAACCCAGCTGCATAATG-3'
Apelin	5'-CAGGGAGGCAGGAGGAAATT-3'	5'-AGGCATGGGTCCCTTATGG-3'
APJ	5'-GGATCCCTGCCAGAGATGGT-3'	5'-GACATGACATGTGCCATTGGA-3'
BNP	5'-GAAGCTGCTGGAGCTGATAAGAG-3'	5'-TGTAGGGCCTTGGTCCTTTG-3'
eNOS	5'-CCCTGCCAACGTGGAGAT-3'	5'-ATCAAAGCGGCCATTTCCT-3'
PAI-1	5'-AGCTGGGCATGACTGACATCT-3'	5'-GCTGCTCTTGGTCGGAAAGA-3'
PDE5	5'-TGCTGATGACTGCCTGTGATC-3'	5'-CGAGTTCTGCTATCCGTTGTTG-3'
TF	5'-CCCAGAAAGCATCACCAAGTG-3'	5'-TGCTCCACAATGATGAGTGTT-3'
VEGFa	5'-GCGGATCAAACCTCACCAAA-3'	5'-TTGGTCTGCATTCACATCTGCTA-3'
VEGFR2	5'-CCACCCCAGAAATGTACCAAAC-3'	5'-AAAACGCGGGTCTCTGGTT-3'
β-actin	5'-TTCAACACCCCAGCCATGT-3'	5'-AGTGGTACGACCAGAGGCATACA-3'

Table E3. Cardiac characteristics

	RA				O_2			
Apelin*	–	+	–	+	–	+	–	+
L-NAME†	–	–	+	+	–	–	+	+
RV free wall thickness ($\mu m/\sqrt[3]{BW}$)	103 ± 5 ***	103 ± 5 ***	96 ± 4 ***	93 ± 3 ***	144 ± 5	112 ± 5 *	142 ± 13 ΔΔΔ	136 ± 6 Δ
IVS wall thickness ($\mu m/\sqrt[3]{BW}$)	295 ± 6	291 ± 11	276 ± 10	263 ± 5	291 ± 8	288 ± 7	278 ± 10	275 ± 12
LV free wall thickness ($\mu m/\sqrt[3]{BW}$)	276 ± 8	280 ± 11	271 ± 13	264 ± 4	300 ± 6	303 ± 7	299 ± 10	292 ± 13
RV/LV ratio	0.38± 0.02 **	0.37 ± 0.01 **	0.36 ± 0.02 **	0,35 ± 0.01 ***	0.48 ± 0.02	0.37 ± 0.02 **	0.47 ± 0.3 ΔΔ, $	0.47 ± 0.04 Δ
RV weight (mg/g)	1.04 ± 0.03 ***	0.95 ± 0.04 ***	0.99 ± 0.04 ***	1.12 ± 0.04 ***	1.74 ± 0.04	1.45 ± 0.05 ***, ΔΔΔ	1.78 ± 0.11 ΔΔΔ, $$	1.67 ± 0.03 ΔΔΔ
LV + IVS weight (mg/g)	4.05 ± 0.10 *	3.90 ± 0.06 **	3.68 ± 0.06 ***	3.83 ± 0.06 **	4.42 ± 0.10	4.40 ± 0.09 Δ	4.69 ± 0.11 ΔΔΔ	4.30 ± 0.08

* apelin concentration was 62 µg/kg/day and † L-NAME concentration was 25 mg/kg/day.
* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus age-matched O_2 exposed controls. $^{\Delta}$ $p < 0.05$ and $^{\Delta\Delta\Delta}$ $p < 0.001$ versus own age-matched room-air exposed controls. $^{\$}$ $p < 0.05$, $^{\$\$}$ $p < 0.01$ and $^{\$\$\$}$ $p < 0.001$ versus apelin treated O_2 exposed pups. BW = body weight.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Te

Figure E1

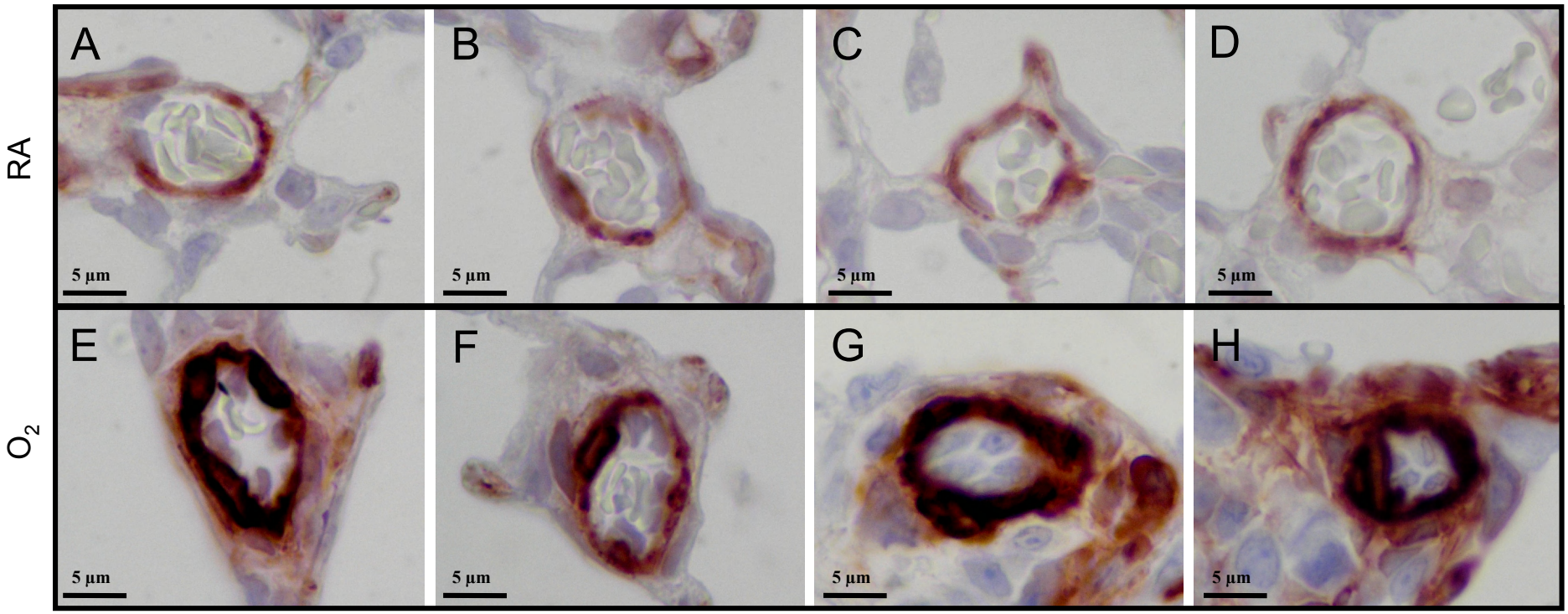

Figure E2

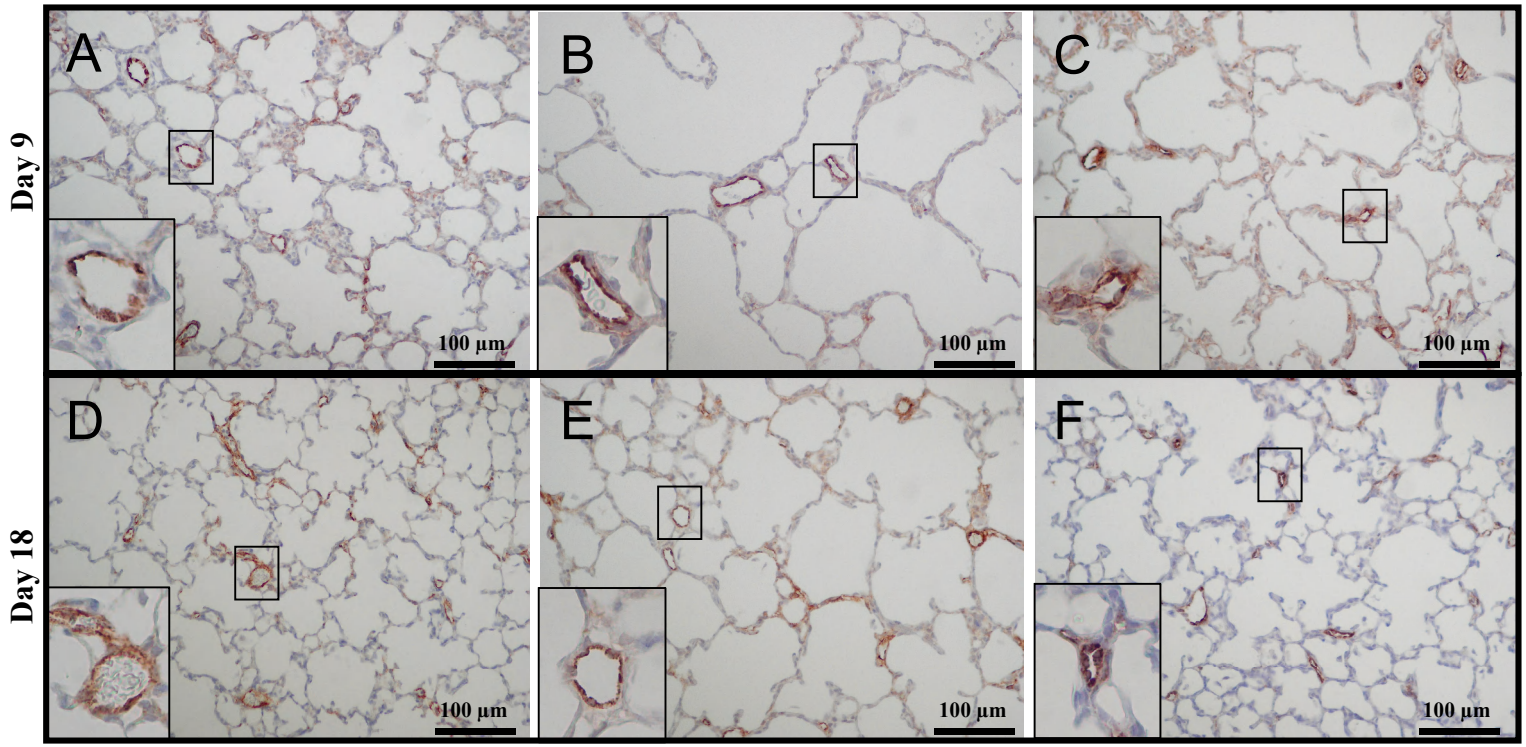

Figure E3

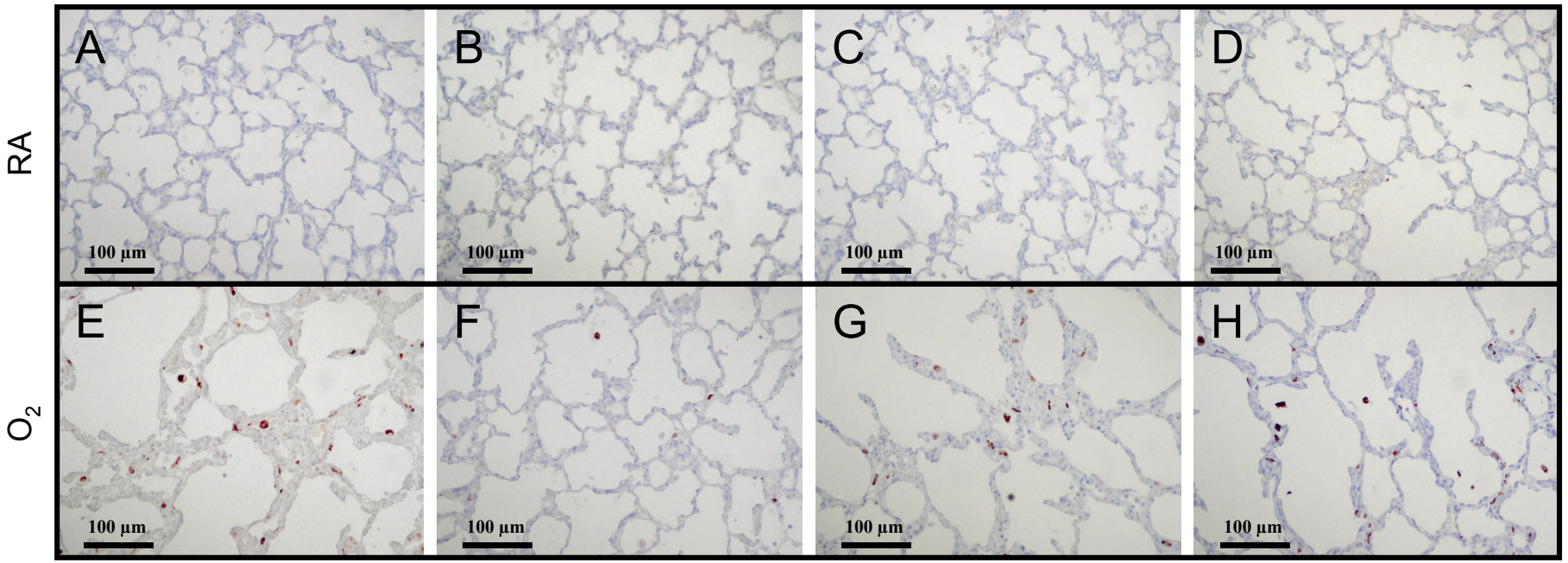
RA
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Figure E4

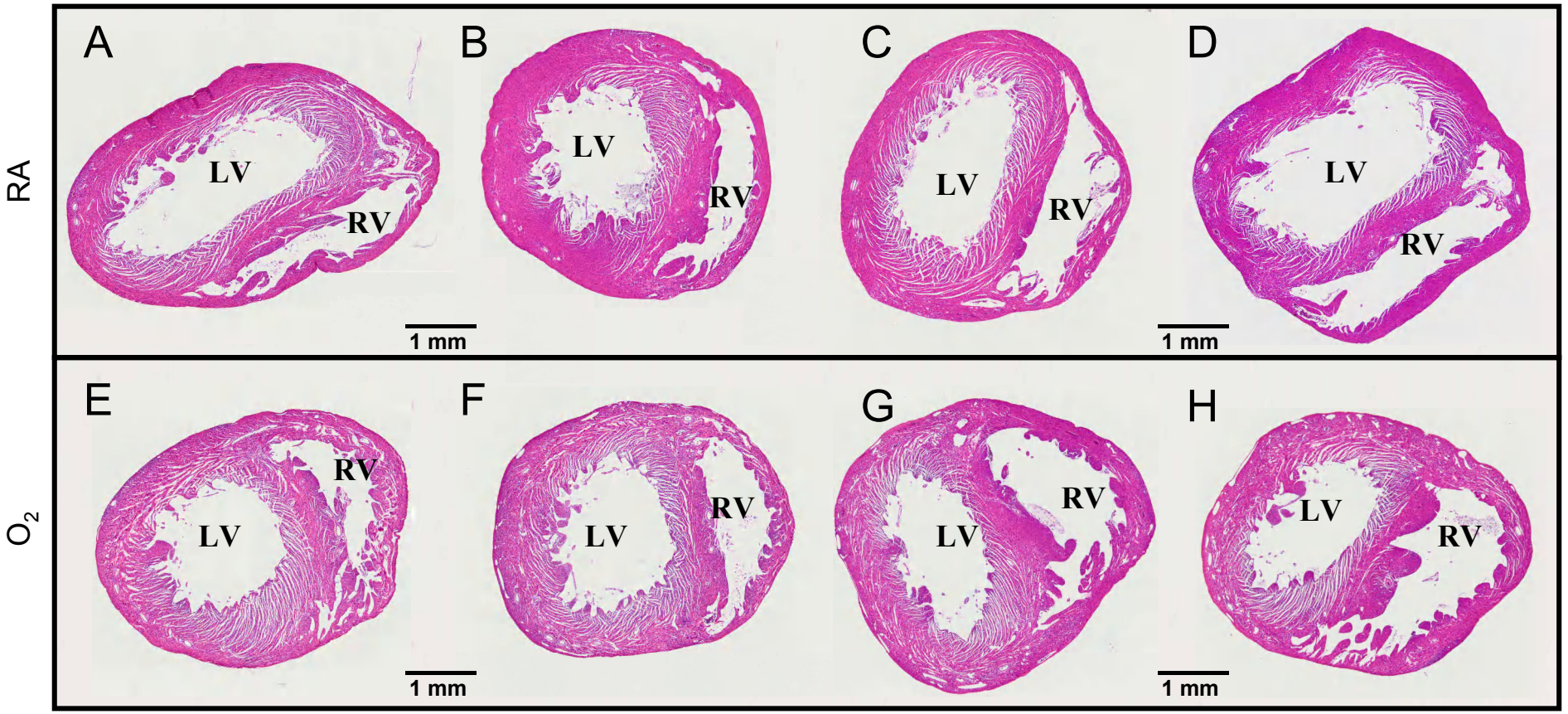

Figure E5