ONLINE DATA SUPPLEMENT

ROLE OF ACID SPHINGOMYELINASE AND IL-6 AS POTENTIAL NOVEL MEDIATORS OF ENDOTOXIN-INDUCED PULMONARY VASCULAR DYSFUNCTION.

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MATERIAL AND METHODS

The investigation conforms the Royal Decree 1201/2005 and 53/2013 on the Care and Use of Laboratory Animals and all procedures were approved by our institutional Ethical Committee and reported following ARRIVE guidelines[1]. Male Wistar rats (weight, 275 to 325 g; age, 12 weeks) were obtained from Harlan Laboratories (Barcelona, Spain). Animals were kept under standard conditions of temperature 22±1°C and 12:12 hour dark/light cycle with free access to food and water.

The Human Research Ethics Committee of the Hospital Universitario de Getafe (Madrid, Spain) approved the use, after informed consent, of lung tissue discarded by pathologists following thoracic surgery. Lung tissue was obtained from 4-13 adult patients with lung carcinoma surgery.

Vessel and cell isolation.

Pulmonary arteries (PA) were isolated from male Wistar rats or human lung tissue, as previously described.[2-6] Secondary branches of the PA were carefully dissected in Ca^{2+}-free physiological salt solution (PSS) of the following composition (in mmol/L): NaCl 130, KCl 5, $MgCl_2$ 1.2, glucose 10, and HEPES 10 (pH 7.3 with NaOH) and cut into rings (1.7-2 mm length).

Freshly isolated PA smooth muscle cells (PASMCs) used for immunocytochemistry were obtained by digestion of endothelium-denuded vessels in a Ca^{2+}-free PSS containing (in mg/ml): papain 1, dithiothreitol 0.8 and albumin 0.7 for 5-7 min. After enzymatic incubation tissues were washed in Ca^{2+}-free PSS and disaggregated using a wide bore, smooth-tipped pipette, as previously described.[2 3 6]

Cell culture.

Rat PASMCs primary cell cultures were used in this study. Briefly, PASMCs were isolated from endothelium-denuded vessels digested in a Ca^{2+}-free PSS containing (in mg/ml): collagenase 1.125, elastase 0.1, albumin 1. PAs were digested for 4 min at 4ºC followed by 1 min at 37ºC. Following digestion, tissues were washed in Ca^{2+}-free PSS and disaggregated using a wide bore, smooth-tipped pipette, to make a cell suspension of PASMCs. Cell suspension was plated onto 35 mm petri dishes and

incubated in a humidified atmosphere of 5% CO_2 in air at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 20% heat-inactivated foetal calf serum (FCS), pyruvate (0.11 mg/ml), 1% non-essential amino acids, streptomycin (0.1 mg/ml), penicillin (100 U/ml) and amphotericin B (250 ng/ml) for 1 week. Cells were subcultured in 75 cm^2 sterile flasks in DMEM supplemented with 10% FCS and used within passages 2-3.

PASMCs were seeded in 96 well plates at a seeding density of 25 x 10^3 cells / well. Cells were treated for 24 hours in 10% FCS DMEM in the absence (control) or presence of LPS (1 µg/ml; *Escherichia coli* 055:B5) or bacterial SMase (0.1 U/ml; *Bacillus cereus*). In some experiments, cells were pre-treated for 45-60 min with the following pharmacological inhibitors prior to the addition of the stimulus: the TLR4 receptor inhibitor TAK-242 (Ethyl- (6R) – 6 - (N- (2-chloro-4-fluorophenyl) sulfamoyl) cyclohex-1-ene-1-carboxylate; $5x10^{-8}$-$1x10^{-6}$ M), the glucocorticoid dexamethasone (10^{-9}-10^{-6} M), the inducible nitric oxide synthase (iNOS) inhibitor 1400W (N-[[3-(aminomethyl) phenyl] methy] ethanimidamide; 10^{-5}-10^{-4} M), the transforming growth factor β-activated kinase 1 (TAK1) 5Z-7-oxozeaenol ((3*S*,5*Z*,8*S*,9*S*,11*E*)-3,4,9,10-tetrahydro-8,9,16-trihydroxy-14-methoxy-3-methyl-1*H*-2-benzoxacyclotetradecin-1,7 (8*H*)-dione; 10^{-9}-10^{-6} M), the aSMase inhibitors D609 (tricyclodecan-9-yl xanthogenate; 10^{-5}-10^{-4} M) and desipramine (3-(5,6-dihydrobenzo[b][1]benzazepin-11-yl)-N-methylpropan-1-amine; 10^{-6}-10^{-5} M). 24 hours later, culture media was collected and frozen for ELISA determinations and cell viability was evaluated by MTT assay.

Human and rat pulmonary artery organ culture.

Rat PA rings were incubated for 24 hours in 0% FCS DMEM in the absence (control) or presence of LPS (1 µg/ml; *Escherichia coli* 055:B5), bacterial SMase (0.001 U/ml; *Bacillus cereus*) or recombinant rat IL-6 (30 ng/ml; R&D Systems, USA). In some experiments, rings were pre-treated with the following pharmacological inhibitors prior to the addition of the stimulus: the inducible nitric oxide synthase (iNOS) inhibitor 1400W (10^{-4} M), the aSMase inhibitor D609 (10^{-4} M), goat IgG (0.56 µg/ml) and neutralizing anti-IL-6 antibody (0.56 µg/ml; R&D Systems, USA).

Human PA rings obtained from lung tissues surplus were isolated and incubated for 24 hours in 0% FCS DMEM in control conditions or following treatment with LPS (1 µg/ml; *Escherichia coli* 055:B5) or D609 (10^{-4} M) plus LPS.

Analysis of IL-6 release.

IL-6 release was measured in culture medium (diluted 1 in 10) by specific enzyme-linked immunosorbent assay (ELISA) (Rat IL-6 DuoSet ELISA Development Systems from R&D Systems, USA).

Determination of NO production.

NO production by PA rings was estimated by the accumulation of its oxidation product, nitrite, in culture medium of PA rings, using the Griess reaction as previously described.[7] Nitrite levels were quantified after incubation for 48 horas in the absence (control) or presence of bacterial SMase (0.1 U/ml) or LPS (1 µg/ml). In some experiments, cells were pre-treated for 40-60 minutes with selected pharmacological inhibitors.

Assessment of cell viability by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT).

Cell viability was assessed after all treatments by measuring the reduction of MTT to formazan.

Ceramide content detection by immunofluorescence.

Freshly isolated PASMCs were allowed to settle in a chamber slide system (Lab-Tek II Chamber Slide 154534, Thermo Fisher Scientific, USA) for 30 minutes at 4ºC. Then, cells were incubated for 30 minutes at room temperature in the absence (control) or presence of LPS (1µg/ml) or exogenous SMase (0.1 U/ml) in a Ca^{2+}-physiological salt solution and rapidly fixed with 4% formaldehyde. Ceramide content was quantified by immunocytochemistry using an anti-ceramide antibody (15B4), as previously described.[6] FITC-fluorescence intensity relative to cell surface was quantified using ImageJ (vs. 1.49) by a blinder operator.

siRNA transfection.

Two different small interfering RNAs (siRNAs) duplexes against the gene coding aSMase (Smpd1 Trilencer-27 Rat siRNA from OriGene, USA) were transfected by a cationic lipid-based transfection reagent (siTran1.0 transfection reagent from OriGene, USA), following manufacturer´s instructions. A non-silencing RNA (random siRNA) was used as a negative control.

siRNA duplexes with a final concentration of 10 nM and siTran1.0 transfection reagent were diluted in Opti-MEM (Life Technology, USA) supplemented with 2% FCS and 1% antibiotic/antimycotic solution. The mixture of siRNA/siTran1.0 was kept at room temperature for 10 min to form the transfection complexes and then added to PASMCs. After incubation for 18 hours at 37 ^{o}C in a humidified incubator (95% O_2/5% CO_2), transfection complexes were replaced with complete growth media (DMEM supplemented with 10% FCS and 1% antibiotic/antimycotic solution) and kept for 6 hours. Cells were then treated with LPS (1 µg/ml) or exogenous SMase (0.1 U/ml) for 24 hours. At the end of the 48-hours period, culture medium was collected and kept frozen until further analysis and cell viability was determined by MTT assay. In parallel experiments, siRNA efficiency was measured by RT-PCR, confirming a reduction in SMPD1 mRNA levels by 75 %–85%, as compared to cells exposed to random siRNA.

Recording of vascular reactivity.

PA rings from organ culture (1.7–2 mm long, ~0.8 mm internal diameter) were mounted in a wire myograph with Krebs solution bubbled with 21% O_2-5% CO_2-74% N_2 (normoxia). After equilibration, arterial rings were first stimulated with KCl (80 mM) and then every vessel was exposed to two hypoxic challenges (by bubbling the chambers with 95 % N_2-5 % CO_2) of 15 min duration each, with a 30 min recovering period in normoxia between hypoxic challenges. Afterwards, dose-response curves to serotonin (5-HT, $3x10^{-8}$ to $3x10^{-5}$ M), phenylephrine (Phe, 10^{-9} to 10^{-5} M) or endothelin-1 (ET-1, $3x10^{-11}$ to $3x10^{-8}$ M) were performed by cumulative addition. Endothelial function was assessed in PA rings pre-contracted with serotonin and subjected to a dose-response curve to acetylcholine (ACh, 10^{-9} to 10^{-5} M).

Animal model of LPS-induced lung injury.

Adult male Wistar rats were anesthetized (80 mg/kg ketamine plus 8 mg/kg xylacine i.p.) and treated with vehicle (DMSO) or D609 (50 mg/Kg body weight; i.p.) for 30 minutes before intratracheal (I.T.) administration of LPS (3 mg/Kg body weight) or saline solution (control, Ctrl). Animals were randomly allocated to 3 experimental groups: 1) control group (n=9), i.p. administration of DMSO and I.T. instillation of saline solution; 2) DMSO-LPS group, i.p. administration of DMSO and I.T. instillation of LPS (n=9); and 3) D609-LPS group, i.p. administration of D609 and I.T. instillation of LPS (n=6).

Pressure measurements.

Four hours following I.T. instillation, rats were anaesthetized with 80 mg/kg ketamine (Merial, Lyon, France) plus 8 mg/kg xylazine (KVP Pharma und Veterinär-Produkte GmbH, Kiel, Germany) and ventilated with room air (tidal volume 9 ml/kg, 60 breaths/min, positive end-expiratory pressure 2 cm H_2O). Pulmonary arterial pressure was registered in open-chest rats with a pressure transducer via a catheter advanced through the right ventricle into the pulmonary artery. Systolic, diastolic and mean pulmonary arterial pressures (SPAP, DPAP and MPAP) were then measured as previously reported.[2 8]

Oxygenation saturation following partial airway occlusion.

Partial airway occlusion of the larger airways was induced by tracheal instillation of 100 µl of saline solution to induce ventilation-perfusion mismatching, as previously reported, [9] and oxygen saturation was monitored with a pulse oximeter (MouseOx®Plus, Starr Life Sciences).

Analysis of inflammatory cytokines.

Levels of IL-6, IL-1β or endothelin-1 present in plasma and whole lung homogenates were quantified by specific Rat DuoSet ELISA Development Systems (R&D System, USA) or by Endothelin-1 Quantikine ELISA Kit (R&D System, USA).

Mieloperoxidase activity assay.

Myeloperoxidase (MPO) activity was measured in frozen lung tissue, homogenized in phosphate buffer and centrifuged. As we have previously described,[8] pellets were resuspended and subjected to three cycles of freezing and thawing prior to a final centrifugation step. The supernatants were collected and assayed in triplicate for MPO activity in a reaction buffer containing 50 mM potassium phosphate buffer, 0.167 mg/ml of O-dianisidine dihydrochloride and 0.0006% H_2O_2 and using kinetic readings over 7 min at 450 nm.

Acid sphingomyelinase activity assay.

Acidic sphingomyelinase (aSMase) activity was measured using a commercial fluorimetric assay kit (Cayman). Briefly, lung tissue was homogenized using an aSMase solution provided with the kit (50 mM sodium acetate, pH=5), incubated on ice for 30 min and centrifuged. The supernatant was collected and sonicated for 5 seconds. Supernatants were assayed in triplicate for aSMase activity using kinetics readings over 30 min at 590 nm (excitation wavelength: 535 nm) using a fluorometer Varioskan (Thermo Fisher Scientific, USA).

Reagents.

Drugs and reagents were obtained from Sigma-Aldrich (Spain), unless otherwise stated, except for: desipramine (Tocris, UK), D609 (Santa Cruz Biotechnology, USA), TAK-242 (Calbiochem, Germany), rat recombinant IL-6 (R&D Systems, USA), neutralizing anti-IL-6 antibody (R&D Systems, USA), primary antibody anti-ceramide (MID 15B4 Enzo Life Sciencies, France), sheep FITC-conjugated anti-mouse secondary antibody (Jackson InmunoResearch, Europe Ltd., UK) and goat polyclonal anti-aSMase primary antibody (Santa Cruz Biotechnology). All drugs were dissolved in water with the exceptions of D609, dexamethasone and TAK-242 which were disolved in DMSO. Final vehicle concentration was ≤ 0.1% and did not affect tissue or cell viability.

Statistical analysis.

Results are expressed as mean ± SEM. Technical replicates were averaged to provide a single data point before any further analysis. Statistical analysis was performed using GraphPad Prism 5 as detailed in each figure legend. One-way

ANOVA (for normally distributed data) followed by Bonferroni's post hoc test or non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test were used to compare three or more datasets. Repeated measures ANOVA was used to compare dose-response curves and one sample t test to evaluate normalized datasets. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Table 1. Effects of TAK-242 on cell viability.

% of control	CONTROL		LPS 1 µg/ml		SMase 0.1 U/ml	
	Mean±SEM	n	Mean±SEM	n	Mean±SEM	n
CONTROL	100,0±3,5	11,0	97,0±1,9	12,0	104,1±1,6	12,0
TAK-242 $5x10^{-8}M$	106,4±2,6	12,0	102,0±2,2	12,0	97,4±4,6	12,0
TAK-242 $1x10^{-7}M$	110,7±1,3	12,0	104,1±2,4	12,0	99,8±3,3	12,0
TAK-242 $5x10^{-7}M$	106,6±2,6	12,0	103,6±4,5	12,0	101,4±2,8	12,0
TAK-242 $1x10^{-6}M$	106,5±2,8	12,0	104,4±3,6	12,0	95,5±4,5	12,0

Table 2. Effects of Dexamethasone on cell viability.

% of control	CONTROL		LPS 1 µg/ml		SMase 0.1 U/ml	
	Mean±SEM	n	Mean±SEM	n	Mean±SEM	n
CONTROL	100,0±0,8	9,0	104,7±3,3	15,0	91,3±5,2	12,0
Dexa $1x10^{-9}M$	104,0±1,2	9,0	114,2±9,7	15,0	109,3±3,4	12,0
Dexa $1x10^{-8}M$	95,1±1,9	9,0	107,1±9,3	15,0	103,6,±2,5	12,0
Dexa $1x10^{-7}M$	82,1±2,9	9,0	95,4±7,6	15,0	93,8±2,1	12,0
Dexa $1x10^{-6}M$	77,8±5,6	9,0	79,6±3,5	9,0	94,6±2,5	12,0

Table 3. Effects of 1400W on cell viability.

% of control	CONTROL		LPS 1 µg/ml		SMase 0.1 U/ml	
	Mean±SEM	n	Mean±SEM	n	Mean±SEM	n
CONTROL	100,0±1,3	12,0	100,3±1,0	12,0	99,3±4,4	15,0
1400W $1x10^{-5}M$	103,6±2,9	9,0	93,8±3,7	9,0	100,4±4,7	12,0

Table 4. Effects of 5z-7-Oxozeaenol on cell viability.

% of control	CONTROL		LPS 1 µg/ml		SMase 0.1 U/ml	
	Mean±SEM	n	Mean±SEM	n	Mean±SEM	n
CONTROL	100,0±1,0	9,0	106,0±3,1	15,0	106,5±8,6	15,0
Oxo $1x10^{-9}$M	103,9±0,7	6,0	101,4±1,1	6,0	118,0±5,6	15,0
Oxo $1x10^{-8}$M	105,2±1,3	9,0	115,2±6,6	15,0	123,3±6,5	15,0
Oxo $1x10^{-7}$M	99,8±1,0	9,0	109,5±7,4	15,0	113,9±6,9	15,0
Oxo $1x10^{-6}$M	86,4±1,9	9,0	88,1±2,4	15,0	90,3±5,4	15,0

Table 5. Effects of D609 on cell viability.

% of control	CONTROL		LPS 1 µg/ml		SMase 0.1 U/ml	
	Mean±SEM	n	Mean±SEM	n	Mean±SEM	n
CONTROL	100,0±1,0	12,0	97,5±0,8	12,0	96,5±0,9	12,0
D609 $1x10^{-5}$M	96,2±1,4	11,0	96,6±0,8	12,0	96,8±1,8	12,0
D609 $5x10^{-5}$M	100,2±2,2	11,0	102,5±1,9	12,0	102,5±1,3	12,0
D609 $1x10^{-4}$M	97,0±1,3	11,0	94,8±1,6	12,0	91,7±1,7***	12,0

Table 6. Effects of Desipramine on cell viability.

% of control	CONTROL		LPS 1 µg/ml		SMase 0.1 U/ml	
	Mean±SEM	n	Mean±SEM	n	Mean±SEM	n
CONTROL	100,0±3,0	8,0	95,0±8,8	6,0	115,9±7,2	9,0
Desi $1x10^{-6}$M	101,3±1,8	6,0	107,3±1,7	6,0	100,5±2,1	6,0
Desi $5x10^{-6}$M	100,9±1,9	6,0	101,2±1,3	6,0	91,7±1,2	6,0
Desi $1x10^{-5}$M	97,8±4,3	8,0	94,2±2,4	6,0	100,2±7,7	8,0

Table 7. Effects of siRNA on cell viability.

% of random control	CONTROL		LPS 1 µg/ml		SMase 0.1 U/ml	
	Mean±SEM	**n**	**Mean±SEM**	**n**	**Mean±SEM**	**n**
Random	100,0±2,4	9,0	95,3±2,3	9,0	96,3±4,0	8,0
SMPD1B	83,5±5,5	9,0	87,8±4,6	9,0	90,9±6,8	9,0
SMPD1C	70,0±5,7***	10,0	71,0±7,0***	9,0	65,8±4,2	9,0***

Data were analyzed with one-way ANOVA followed by Dunnett's multiple comparison. ***p<0.01 versus control/Random control.

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SUPPLEMENTARY FIGURE LEGENDS.

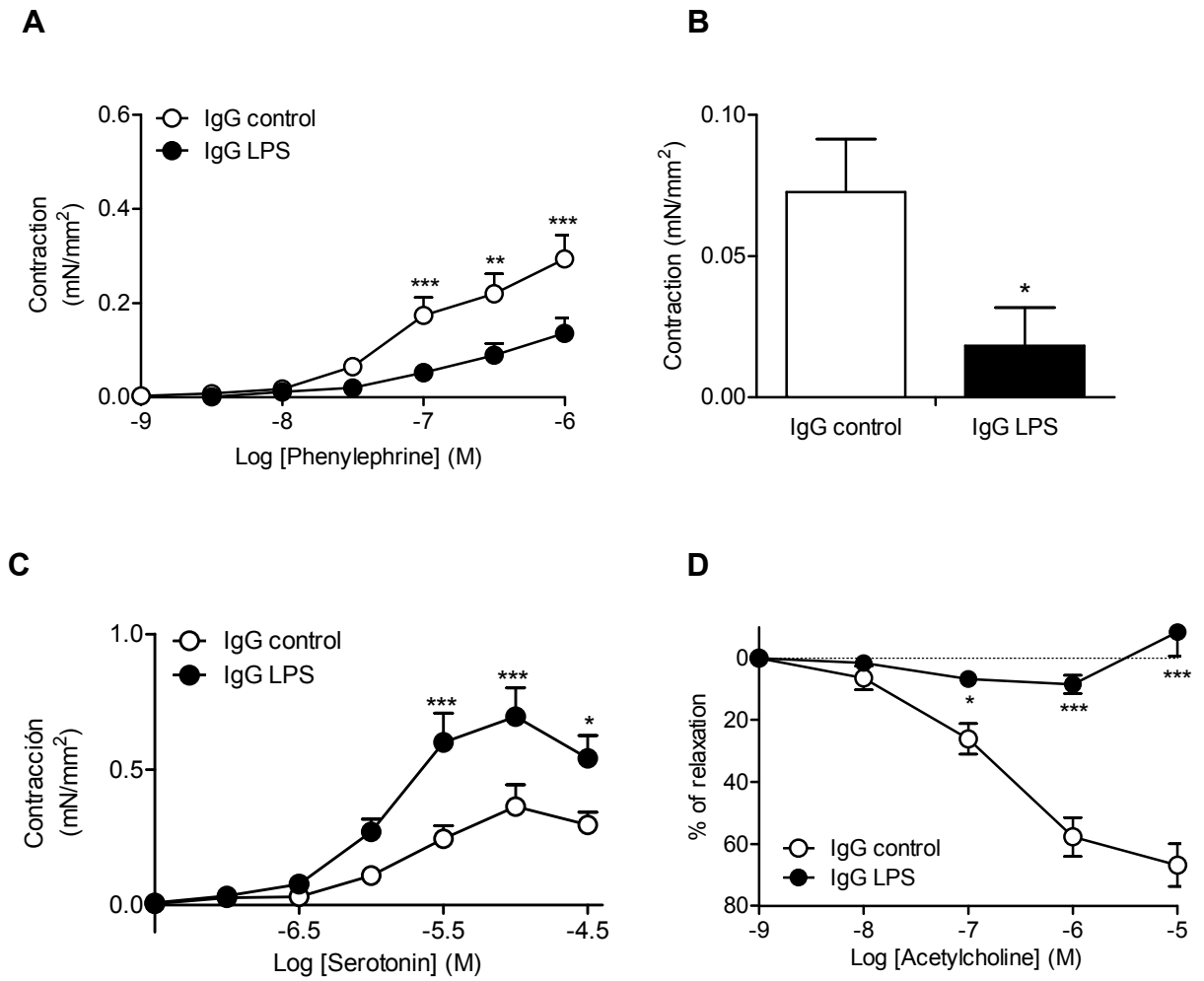
Figure S1. IgG does not interfere with the effects induced by LPS in isolated rat PA. Mean contractile responses to phenylephrine (A; n=11-13), hypoxia (B; n=11-13) and serotonin (C; n=12-13) in isolated rat PA incubated 24 hours with goat IgG (0.56 µg/ml) in the absence (control) or presence of LPS (1 µg/ml). (D) Relaxation induced by acetylcholine in rat PA incubated with goat IgG under control conditions and exposed to LPS (n=12-13). Data are shown as active effective pressure (mN/mm^2) or percentage of relaxation. *, ** and *** $p<0.05$, $p<0.01$ and $p<0.001$ versus control (by t-test or repeated measures ANOVA followed by Bonferroni´s post hoc test, as appropriate).

Figure S2. Contractile responses to endothelin-1 are resistant to LPS and D609. (A) Mean contractile responses to endothelin-1 in isolated rat PA incubated 24 hours in the absence (control; n=13) or presence of LPS (1 µg/ml; n=13). (B) Effects of D609 (10^{-4} M; n=6) on endothelin-1 induced contraction. Data are shown as active effective pressure (mN/mm^2). Data were analysed by repeated measures ANOVA followed by Bonferroni´s post hoc test.

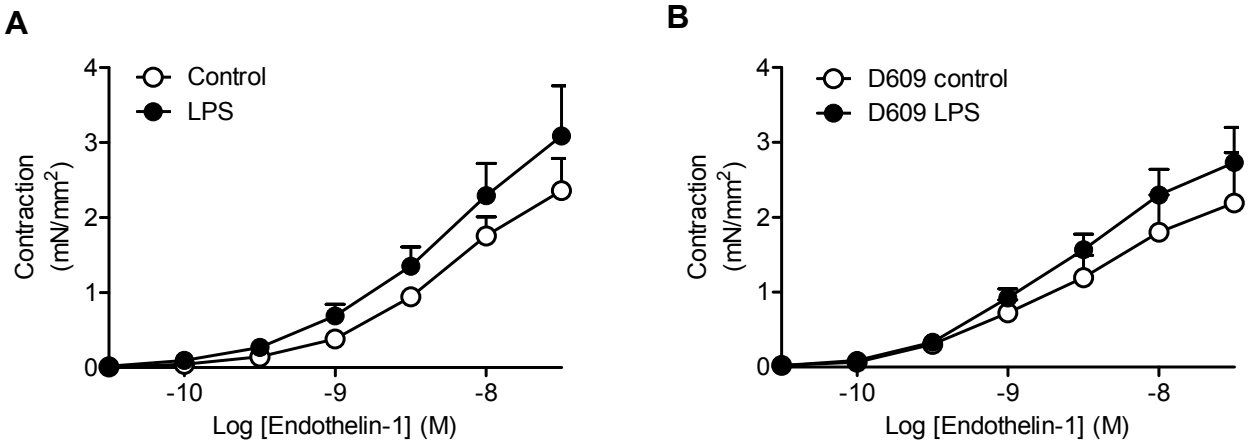
Figure S3. Concentration-dependent protective effects of D609 on HPV impairment and endothelial dysfunction induced by LPS. (A) Mean contractile responses to hypoxia in rat PA incubated under control conditions (white bars; n=35), in the presence of LPS (1 µg/ml; black bars; n=25) or following treatment with LPS and increasing concentrations of D609 ($5x10^{-5}$ and 10^{-4} M; dashed bars; n=9-18). (B) Averaged data of the relaxation induced by acetylcholine in rat PA in the absence (Control; n=35) or the presence of LPS (1 µg/ml; n=25) following treatment with D609 ($5x10^{-5}$ and 10^{-4} M; n=6-17). Data are shown as active effective pressure (mN/mm^2).

*, ** and *** p<0.05, p<0.01 and p<0.001 versus control and † and ††† p<0.05 and p<0.001 versus LPS control (analysed by t-test or repeated measures ANOVA, as appropriate, followed by Bonferroni´s post hoc test).

Figure S4. Effects of D609 on pulmonary and systemic levels of IL-6, IL-1β and following intratracheal instillation of LPS. (A) Plasma levels of IL-6 and (B) linear regression analysis between IL-6 levels and mean pulmonary artery pressure (PAP) in control, LPS- or D609 plus LPS-treated rats. (C-F) Concentration of IL-1β in lung (C) and plasma (E) and linear analysis between pulmonary (D) or plasma (F) IL-1β and mean PAP in control, LPS and D609 plus LPS-treated rats. (G-J) Concentration of ET-1 in lung (G) and plasma (I) and linear analysis between pulmonary (H) or plasma (J) IL-1β and mean PAP in control, LPS and D609 plus LPS-treated rats. Data are shown as the mean ± S.E.M (n=3-8). Data were analysed with one-way ANOVA followed by Bonferroni's post hoc test (A, C, G) or the non-parametric Kruskal-Wallis test followed by Dunn´s test (E, I).



Suplementary figure S1



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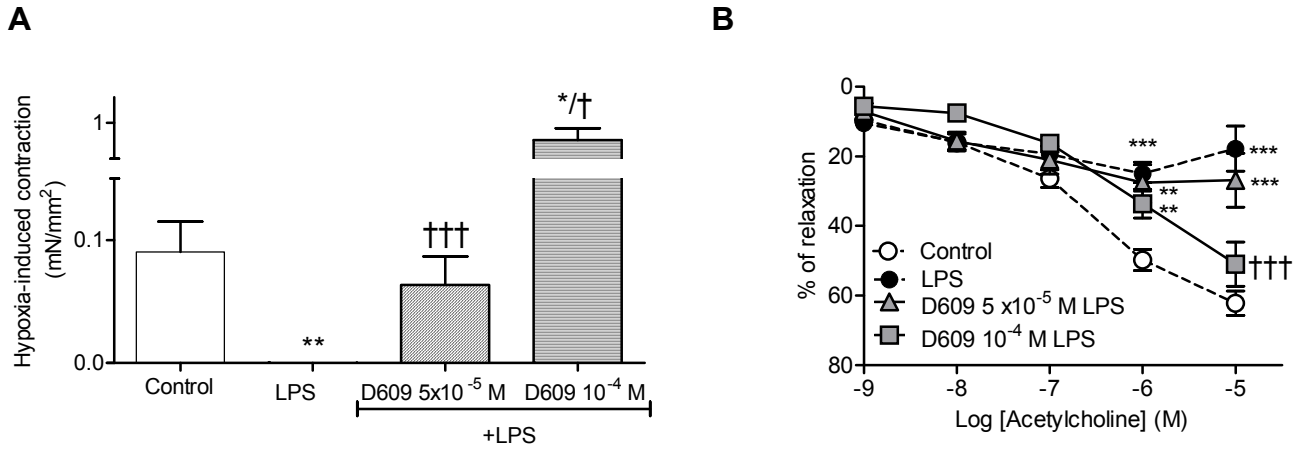

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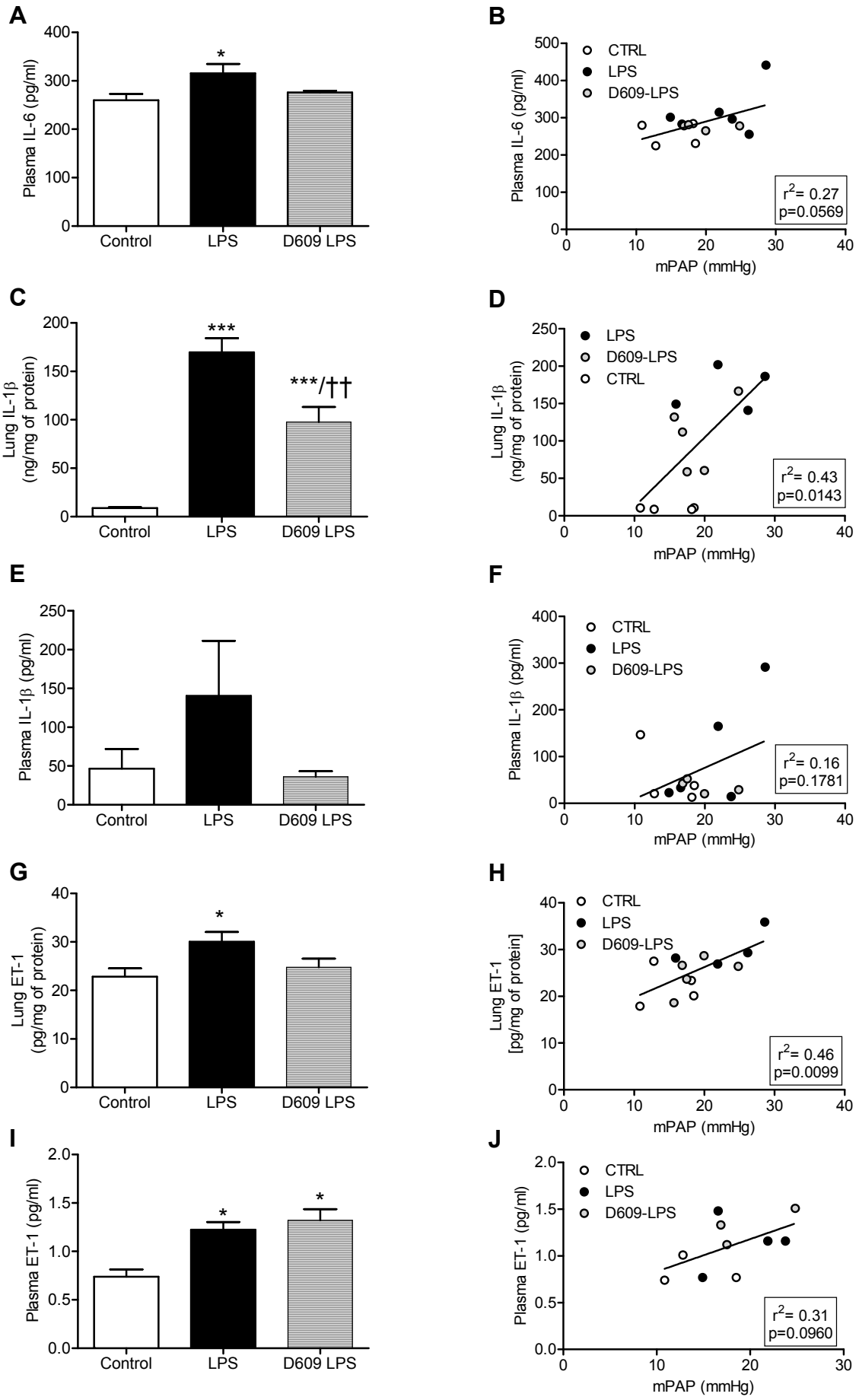

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