***Streptococcus pneumoniae* induced regulation of cyclooxygenase-2 in human lung tissue**

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Supplementary Material

MATERIALS AND METHODS

Human lung tissue

Fresh lung explants were obtained from patients undergoing lung resection at local thoracic surgeries. The lung pieces used in the study have been obtained in the periphery of the resected lobes. In total 60 patients were included in the study. Most of the patients suffered from bronchial carcinoma. Half of the patients had the clinical diagnosis COPD and also half of the patients have stated to be smokers. Oral steroid therapy has been confirmed for less than 10%. Patients with pulmonary or systemic inflammation, tuberculosis, HIV or other chronic infections were excluded from the study. Written informed consent was obtained from all patients and the study was approved by the ethic committee at the Charité clinic (protocol number EA2/050/08 and EA2/023/07). Tumour-free normal lung tissue was stamped into small cylinders (~ 8x8x8 mm) and weighed. Afterwards, pieces were incubated for 24 h in 4 mL RPMI 1640 with 10% (vol·vol-1) heat-inactivated FCS (except for bacterial growth), before being infected. 200 µL of prepared control or infection medium per 100 mg tissue was injected, thereby assuring thorough stimulation of the tissue. After respective time points lungs were fixed for immunohistochemistry or shock frozen in liquid nitrogen and stored at -80 °C for further processing. Homogenization of lung tissue was done using the FastPrep-24 homogenizer on dry ice (MP Biomedicals, Illkirch, France). Depending on the assay 80-120 mg lung tissue was used in appropriate extraction buffer.

Patients with acute pneumonia

Lung tissue samples of 3 patients with acute pneumonia and 1 control patient were randomly selected from routine cases diagnosed at the Institute of Pathology, Charite in 2010/2011. After reviewing the archived H&E sections, the corresponding tissue blocks with prominent acute inflammatory infiltrates were selected and used for immunohistochemical COX-2 staining. The selected patients had the following diagnoses:

Patient #1: Pneumonia in organization with a strong acute leucocytic inflammatory infiltrate.

Patient #2: Alveolar lung tissue with moderate acute pneumonia.

Patient #3: Lung lobe resection with strong acute, abscess-forming pneumonia.

Control patient: Lung lobe resection with a bronchiolo-alveolar carcinoma. Surrounding normal alveolar lung tissue was without inflammation. All patients had given prior consent to their tissue specimens being used in scientific studies.

Western Blot

In order to investigate COX-1, COX-2 and EP_4 receptor protein expression or phosphorylation of MAP kinases ERK and p38, human lung tissue was infected with *S. pneumoniae* for the indicated times and doses. For protein extraction, 80-120 mg shock frozen tissue was weighed and put into 8 volumes of lysis buffer [1] following disruption by the FastPrep-24 homogenizer. Afterwards, 100 µg of protein was heated in Laemmli buffer at 95º C, subjected to SDS-PAGE and blotted on Hybond ECL membrane (Amersham Biosciences, Freiburg, Germany). After transfer of proteins, membranes were blocked with Odyssey blocking buffer (LI-COR Inc., Bad Homburg, Germany) diluted 1:2 in PBS for 1 h at room temperature. Proteins were detected with specific polyclonal antibodies, which were applied to the membrane overnight at 4º C. Following antibodies were used for detection: COX-1, COX-2, ERK2, actin, (Santa Cruz Biotechnology, Santa Cruz, CA, USA), EP_4 receptor (Cayman Chemical Company, Ann Harbor, MI, USA), phosphorylated ERK or p38 (Cell Signaling Technology Inc., Danvers, MA, USA). All antibodies were diluted 1:1000 in blocking buffer. In all experiments ERK-2 or actin were detected on the same membrane to ensure equal protein load. Visualization of proteins was achieved by incubation with appropriate secondary IRDye 800- or Cy5.5-labelled antibodies (all diluted 1:2000 in blocking buffer) for 1 h at room temperature in the dark and subsequent scanning of the membrane with an infrared scanner (Odyssey infrared imaging system; LI-COR Inc.).

Immunohistochemistry

After infection of human lung tissue with *S. pneumoniae* for 24 h, specimen were fixed in 3% formalin, embedded in paraffin and routinely processed for histology. Lung sections were heated to 60 ºC overnight, deparaffinised with 60 ºC in Roticlear for 3 x 15 min and subsequently rehydrated through a descending alcohol series. After three washes in 0,01M PBS, slides were boiled in citrate buffer for 30 min (10 mM, ph 6,0). After permeabilising with Triton X-100 1% (in 0,01 M PBS) for 15 min and three PBS washes, sections were blocked with protein block (DAKO, Hamburg, Germany) for 30 min and primary monoclonal COX-2 antibody (Cayman Chemical Co.) diluted 1:500 in antibody diluent solution (Zymed, San Francisco, CA, USA) was applied to the sections overnight at 4 º C. After three PBS

washes, the biotinylated anti-mouse secondary antibody from the Super Sensitive Link Label Detection System (Biogenex, Fremont, CA, USA) was applied to the sections for 20 min. After three times washing in PBS, the slides were incubated with streptavidin conjugated alkaline phosphatase for further 20 min. Following three PBS washes Fast Red (Sigma Aldrich, Munich, Germany) was utilised to visualise antibody binding. Mayer's hematoxylin (Carl Roth, Karlsruhe, Germany) was used to counterstain nuclei. Finally, slides were mounted with Aquatex (Merck, Darmstadt, Germany). Secondary antibody specificity was verified by incubating with non-immune mouse serum instead of primary antibody. For confocal imaging of COX-2 immunofluorescence was performed. Instead of an alkaline phosphatase detection system, secondary antibodies labelled with Alexa Fluor 488 or 594 (Invitrogen, Darmstadt, Germany) were used (all diluted 1:2000 in antibody diluent). Type I epithelial cells were detected with anti-caveolin 1 antibody staining (Santa Cruz Biotechnology) (1:200), whereas type II pneumocytes were labelled with anti-PRO-SP-C antibodies (Millipore, Billerica, MA, USA) (1:800). Slides were mounted with Mowiol (1 $g \cdot mL^{-1}$ glycerol, 0.4 $g \cdot mL^{-1}$ Mowiol 4-88 (Carl Roth), 25 $mg \cdot mL^{-1}$ 1,4-Diazabicyclo[2.2.2]octan, 130 mM Tris-HCl, pH 8.5). Antibody specificity in double staining experiments was checked by incubating each primary antibody with both secondary antibodies. *S. pneumoniae* was stained with anti-*S. pneumoniae* (kind donation by S. Hammerschmidt, University of Greifswald, Germany) and all slides were analysed using a Zeiss Axioskop 2 mot [2] or a Zeiss LSM 780 confocal microscope (Objectives Plan-Apochromat 40x/1,3 Oil Dic, Plan Neofluar 40x/1,3 Oil Dic, Plan Neofluar 20x/0,5) (Zeiss, Jena, Germany).

Mass Spectrometry

Human lung tissue was infected with *S. pneumoniae* for 16 h and supernatants were analysed for free and lipid matrix bound eicosanoids. A solid phase extraction method was used as formerly described [3]. 100 µL supernatant was mixed with 10 ng internal standard, 500 µL methanol and 2 mL acetat buffer. The sample was centrifuged and the clear supernatant was extracted.

HPLC conditions

An HPLC system Agilent 1200SL series with binary pump, autosampler and column thermostat were used. The analysis was done on an analytical column Phenomenex Kinetex C-18 150 x 2.1 mm, 2.6 µm using solvent system acetonitrile/0.1 % formic acid. The gradient

elution was started with 10 % acetonitrile, increased within 10 minutes up to 90% and held for 8 minutes. The flow rate was 0.4 $mL \cdot min^{-1}$, injection volume was 7.5 µL, column temperature 40 °C.

MS conditions

HPLC was coupled with Agilent 6460 Triplequad mass spectrometer with electrospray ionisation source. Analysis was performed with Multiple Reaction Monitoring in negative mode. Drying/Sheath Gas Temp were 250 / 400 °C, nitrogen stream 11/11 $L \cdot min^{-1}$. Nozzel and capillary voltage were set at 1500 / 4000 V. The MRMs and MS conditions are shown in the table [4].

Quantification

The calibrated range extends from 0.1 to 50 ng absolute. A linear regression with blank offset and 1/x weighting was calculated by the Agilent Mass Hunter software.

Compound Name	Precursor Ion	MS1 Resolution	Product Ion	MS2 Resolution	Fragmentor [V]	Collision Energy [v]
TXB_2	369.2	Wide	195	Wide	100	9
PGE_2-D4	355.3	Wide	319	Wide	110	4
PGE_2	351.3	Wide	315	Wide	110	4
6-keto-$PGF_{1\alpha}$	369.2	Wide	245.1	Wide	130	22
TXB_2	369.2	Wide	195.1	Wide	100	9
6-keto-$PGF_{1\alpha}$	369.2	Wide	163.1	Wide	130	24
PGE_2-D4	355.3	Wide	319.2	Wide	110	4
2,3-dinor-6-keto $PGF_{1\alpha}$	355.2	Wide	243.2	Wide	90	13
2,3-dinor-6-keto PGF1α	355.2	Wide	187.1	Wide	90	11
PGE_1	353.2	Wide	317.2	Wide	80	4
$PGF_{2\alpha}$	353.2	Wide	309.2	Wide	130	13
PGE_1	353.2	Wide	235.2	Wide	80	4
$PGF_{2\alpha}$	353.2	Wide	193.1	Wide	130	22
PGE_2	351.3	Wide	315.2	Wide	110	4
PGH_2	351.2	Wide	271.2	Wide	110	10

Quantitative PCR

For total RNA extraction, shock frozen lung tissue was weighed and put into 8 volumes of TRIZOL solution (Invitrogen). The tissue was disrupted using the FastPrep-24 homogenizer and further processing of samples was carried out according to the manufacturer's manual. 1-5 µg total RNA were reverse transcribed into cDNA using a mixture of oligo dT and random

nucleotide primers and a H Minus M-MuLV Reverse Transcriptase (Fermentas, St. Leon Rot, Germany). Hot start real-time PCR for the quantification of each transcript was carried using 2 x Maxima SybrGreen qPCR mix (Fermentas), 0.25 µM of each primer and 2.5 µl - 5 µL of cDNA which was diluted 1:10. PCR was performed with an initial enzyme activation step at 95°C for 10 min, followed by 50 cycles of denaturation at 95°C for 30 sec, annealing at 57°C (GAPDH) or 63°C (EP-receptors) for 30 sec and extension at 72°C for 1 min in a real-time DNA thermal cycler (iCycler™, 20 µl reaction volume or CFX96™, 10 µl reaction volume, BIO-RAD, Munich, Germany). The following forward and reverse primers were used for EP1: 5'-TCGCTTCGGCCTCCACCTTCTTTG and 5'-CGTTGGGCCTCTGGTTGTGCTTAG; EP2: 5'-CGAGACGCGACAGTGGCTTCC and 5'-CGAGACGCGGCGCTGGTAGA; EP3: 5'-CGGGGCTACGGAGGGGATGC and 5'-ATGGCGCTGGCGATGAACAACGAG; EP4: 5'-TCGCGCAAGGAGCAGAAGGAGACG and 5'-GGACGGTGGCGAGAATGAGGAAGG; GAPDH: 5'-TGATGACATCAAGAAGGTGG and 5'-TTACTCCTTGGAGGCCATGT. For the calculation of EP receptor copy numbers, plasmids with cloned cDNAs coding for EP-receptors and GAPDH were used as templates to prepare standard curves with defined copy numbers.

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