Online Supplement

Intrabronchial activated protein C enhances lipopolysaccharide-induced pulmonary responses

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METHODS

Assays

Cell differentials were determined in BAL fluid (BALF) on cytospins stained with a modified Giemsa stain (Diff-Quick; Siemens Healthcare Diagnostics, Marburg, Germany). For immune assays, BALF was centrifuged at 300 *g* for 10 minutes. Blood was centrifuged at 1500 *g* for 10 minutes. Supernatants were stored at -80ºC until analysis. RhAPC concentrations in BALF samples containing 90 mM (final concentration) benzamidine were measured using a chromogenic assay as described[1]. TATc (TAT Enzygnost, Siemens Healthcare Diagnostics, Marburg, Germany), soluble thrombomodulin (Diaclone, Gen-Probe, San Diego, CA), tissue-type plasminogen activator (tPA antigen; Hyphen BioMed, Andrésy, France) and plasmin-antiplasmin complexes (DRG Diagnostics, Marburg, Germany) were measured using commercial available enzyme-linked immunosorbent (ELISA) kits according to the manufacturers' instructions. Protein C activity was measured by a kinetic assay (Coamatic Protein C, Chromogenix, Mölndal, Sweden). Total protein S and von Willebrand factor antigen levels were determined with ELISAs developed in our laboratory using antibodies from Dako (Glostrup, Denmark). Free protein S was measured by precipitating the C4b-

binding protein-bound fraction with polyethylene glycol 8000 and measuring the concentration of free protein S in the supernatant. Antigen levels of protein C inhibitor and plasmin activator inhibitor type-1 (PAI-1) antigen were measured by ELISA as described earlier[2-4]. Rh-TFPI concentration was measured by an enzyme-linked immunosorbent assay (ELISA), using a mouse monoclonal antibody directed against the human TFPI Kunitz-2 domain (amino acids 88–160; Sanquin, Amsterdam, the Netherlands) as capture antibody, polyclonal rabbit anti-rh-TFPI (kind gift of Dr Walter Kisiel, University of New Mexico, Albuquerque, NM, USA) as detection antibody and rh-TFPI as standard. This assay detects both full-length and cleaved TFPI. Levels of the following cytokines, chemokines and other inflammatory markers were measured by a multiplex assay (Luminex, Austin, TX): chemokine (C-C motif) ligand (CCL) 1, 2, 3, 4, 8, 13, 14a, 15, 17, 19, 20, 21, 24, 26, 27; chemokine (C-X-C motif) ligand (CXCL) 1, 5, 6, 7, 9, 10, 11, 12a+b, 13; chemokine (C-motif) ligand (XCL) 1; interleukin (IL)-11, -12p40, -16, -17, -20, -21, -23, -28A, -29, -33; leukemia inhibitory factor (LIF); granulocyte colony-stimulating factor (G-CSF); macrophage colony-stimulating factor (M-CSF); stem cell factor (SCF) ; thrombopoietin (TPO); thymic stromal lymphopoietin (TSLP) and TNF-related apoptosis-inducing ligand (TRAIL). Tumor necrosis factor (TNF)-α, CXCL8, IL-1β, -6, -10 and -12p70 were measured using a cytometric bead array (CBA; BD Biosciences, San Jose, CA) in accordance with the manufacturers' instructions. For the *in vitro* experiments human CXCL8 and TNF-α were measured with a commercially available ELISA kit (R&D systems, Minneapolis, MN) according to the manufacturers' instructions.

Reference List

[1] Liaw PC, Ferrell G, Esmon CT. A monoclonal antibody against activated protein C allows rapid detection of activated protein C in plasma and reveals a calcium ion

dependent epitope involved in factor Va inactivation. *J Thromb Haemost* 2003; **1:**662-670.

[2] Elisen MG, Maseland MH, Church FC, Bouma BN, Meijers JC. Role of the A+ helix in heparin binding to protein C inhibitor. *Thromb Haemost* 1996; **75:**760-766.

[3] Zeerleder S, Schroeder V, Hack CE, Kohler HP, Wuillemin WA. TAFI and PAI-1 levels in human sepsis. *Thromb Res* 2006; **118:**205-212.

[4] Portielje JE, Kruit WH, Eerenberg AJ, Schuler M, Sparreboom A, Lamers CH *et al.* Interleukin 12 induces activation of fibrinolysis and coagulation in humans. *Br J Haematol* 2001; **112:**499-505.