Online Data Supplement

Urokinase Plasminogen Activator Regulates Pulmonary Arterial Contractility and Vascular Permeability in Mice

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Materials and Methods

Materials. The PAI-1 derived peptides Ac-EEIIMD-amide (6 aa) was synthesized by Peptisyntha (Brussels, Belgium and San Diego, CA**),** as described previously (1, 2). Anti-LRP and receptor associated protein (RAP) were obtained from (American Diagnostica, Stamford, CT)**.** MK-801 and glutamate were purchased from Sigma (Rehovot, Israel). Anti-NMDA-R1 antibody was purchased from Biotest (Tel-Aviv, Israel). Polyclonal anti-uPA was provided by UMTEK (Moscow, Russia). Recombinant uPAs were synthesized and purified as described below.

Human uPA PAI-I docking site (DS) mutant ($^{179-184}$RHRGGS→AAAAAA). To generate the uPA PAI-I DS mutant ($^{179-184}$RHRGGS→AAAAAA), uPA cDNA was amplified by PCR using the primer pair: 5'-GCG GCC ATC TAC CGC GCT GCC GCC GCT GCC GCT GTC ACC TAC GTG TGC GGA GGC AG-3' and 5'-CTG CCT CCG CAC ACG TAG GTG ACA GCG GCA GCG GCG GCA GCG CGG TAG ATG GCC GC-3' in pMT/BiP/V5/HisA-uPA plasmid under the conditions recommended in The QuikChange Site-Directed Mutagenesis Kit manual (Stratagene, La Jolla, CA). The resulting plasmid was sequenced to confirm the mutations were introduced correctly and that the remainder of the wild type sequence was intact. The protein was produced in the Drosophila S2 Expression System and purified from the media using an anti-uPA affinity column. The product migrated at a single band, Mr~54 kDa, on 4-12% SDS-PAGE under non-reducing and reducing conditions.

Catalytically inactive human uPA. WT and catalytically inactive (uPA-S^{356}A) were synthesized and characterized as previously described (3). The final products migrated as single bands on SDS-PAGE at the expected sizes, and their plasminogen activator activity was confirmed using the plasmin chromogenic substrate SpectrazymePL, as described (4). Proteins were stored at -70^{o}C or lyophilized prior to use.

Contractile response of isolated pulmonary and aortic rings. All experimental protocols involving the use of vertebrate animals were approved by the Israeli Board for Animal Experiments. Sprague-Dawley rats (Harlan Laboratories, Jerusalem, Israel) (average weight 250-275 g) were killed by exsanguination as described previously (3, 5). Pulmonary arteries and thoracic aorta were removed

with care to avoid damage to the endothelia, dissected free of fat and connective tissue and cut into transverse rings 4 mm in length. To record isometric tension, the rings were mounted in a 10-mL bath containing oxygenated (95% O_2; 5% CO_2) solution of Krebs-Henseleit (KH) buffer (144 mM NaCl, 5.9 mM KCl, 1.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 11.1 mM D-glucose). Aortic rings were equilibrated for 1.5 hrs at 37°C and maintained under a resting tension of 1.5 g throughout the experiment. The resting tension in the pulmonary rings was fixed at 0.75 g throughout the experiment. Each pulmonary or aortic ring was then contracted by adding phenylephrine (PE) in stepwise increments (from 10^{-10} M to 10^{-5} M). In other experiments, WT and variant uPAs were added 15 min before adding PE. Isometric tension was measured using a force displacement transducer and was recorded online using a computerized system (ExperimentiaÆ, Budapest, Hungary). The half-maximal effective concentration (EC_{50}) was calculated by measuring the response of the rings (y-axis) to increasing concentrations of PE (x-axis). The lines that intersect with the y- and x-axes were drawn to determine the concentration of PE that induces 50% of its maximal effect.

Pulmonary vascular permeability. Adult male, 8–10-week old WT C57BL/6J mice (5) (average weight of 20–25 g), were anaesthetized with an intraperitoneal injection of ketamine (85 mg/kg-1) and xylazine (10 mg/kg^{-1}). uPA (1 mg/kg) was injected into the vain tail of the anesthetized mice (estimated plasma concentration 20 nM). No effect on mean arterial blood pressure was observed. Thirty min later, Evans blue dye (20 mg/kg^{-1}) in 250 µl of 0.9% saline was infused into the left internal jugular vein of anesthetized mice and allowed to circulate for 60 min. The animals were sacrificed by exsanguination under anaesthesia. A tracheotomy was performed, and the trachea was cannulated with a 20-gauge i.v. catheter, which was sutured in place. Bronchoalveolar lavage (BAL) was performed using 1.5 mL of warmed sterile Hanks balanced salt buffer (30°C). BAL fluid was collected, centrifuged at 14000 rpm for 20 min at 4°C, the supernatant was removed and the optical density at 620 nm was measured.

Co-immunoprecipitation.
Pulmonary arterial rings isolated from $uPA^{-/-}$ mice were incubated in an oxygenated (95% O_2-5% CO_2) Krebs-Ringer bicarbonate solution (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 1.3, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11, Na_2-EDTA 0.05). Where indicated, WT uPA (uPA), WT uPA with PAI-1 derived peptide EEIIMD (EEIIMD or the uPA variant that lacks a functional docking site (ΔDSuPA) were added. The rings were homogenized in 5 volumes of cold RIPA buffer composed of 1% NP40, 0.5% sodium deoxycholate, 10% sodium lauryl sulfate, 0.5 mM PMSF, in phosphate-buffered saline (PBS) in the absence of protease inhibitors at 4°C for 30 min in order to prevent possible interference by endogenous uPA.

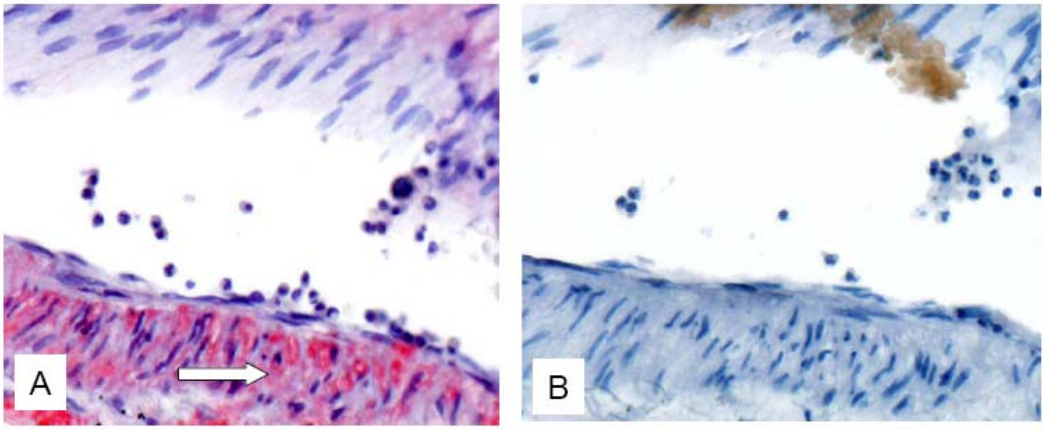
The lysates were cleared by centrifugation; the supernatant fractions were then pre-cleared with protein A-agarose beads that had been pre-blocked with 1% BSA. The supernatants were then incubated for 2 h with beads containing anti-NMDA-R1 IgG or irrelevant IgG. The beads were washed five times with PBS, the proteins were eluted by three additions of 0.1 glycine buffer for 5 min each and centrifuged, and the supernatant was analyzed by Western blotting. Samples were applied to nitrocellulose membranes. The membranes were blocked with horse serum, incubated initially with anti-uPA antibodies, and then with a species-specific secondary antibody conjugated to horseradish peroxidase (HRP). All experiments were performed in triplicate and were repeated a minimum of three times. Irrelevant IgG was added in lieu of specific primary antibodies as a control. Western blotting to identify the NR1 subunit of the NMDA-R1 was performed as described(6). Briefly, immunoprecipitates were electrophoresed using an 8–10% SDS glycine polyacrylamide gel. Separated proteins were electroblotted onto nitrocellulose membranes. The membranes were blocked with low-fat dry milk in 10 mM Tris·HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween 20, and the NMDA-R1 subunit was detected with the same antibodies. The membranes were incubated with secondary antibodies conjugated with peroxidase and developed with the appropriate colorometric substrates.

Immunohistochemistry. Formalin-fixed, paraffin-embedded archived normal human lung tissues were used for NMDA-R immunostaining. Three unstained recuts were obtained from tissue blocks of normal-appearing human lungs. Immunostains were performed using avidin-biotin-peroxidase complex. Sections were cut at 4 μm, deparaffinized using Clear-Rite 3 (Richard-Allan Scientific, Kalamazoo MI) followed by a series of graded alcohol incubations, treated with methanolic H_2O_2 to quench endogenous peroxidase activity, and rehydrated. Sections were incubated with a rabbit polyclonal antibody against NMDA-R1 (Abcam, Cambridge, MA; 1μg/ml) or normal rabbit IgG overnight at 4^{o}C. The slides were washed in PBS, incubated with Streptavidin-labeled polyclonal anti-rabbit IgG (Equitech-Bio; Kerrville, TX) and washed in PBS. Streptavidin detection was performed using the BioGenex (San Ramon, CA). The sections were rinsed in PBS, incubated with chromogenic substrate for 2 min, rinsed again in PBS and incubated with Mayers Hematoxylin. The slides were rinsed again with water and incubated with Scott's Bluing Reagent for 10 sec and dried in a fume hood. After one dip in xylene, coverslips were added using Permount solution.

Supplementary Results

Expression of NMDARs in human lung. To examine the clinical relevance of our findings, we asked whether NMDA-R1s are expressed in the vasculature of human lungs. Figure 1 shows intense staining for the presence of NMDA-R1 in the vascular smooth muscle of human blood vessel from normal lung tissue.

Figure 1:



Supplemental Figure 1: Expression of NMDA-R1 in human normal lung blood vessels. Panel A. High power (600x) images of a blood vessel in normal lung tissue showing NMDAR-1 immunopositivity in vascular smooth muscle cells (arrow) (NMDAR-1; 600x). **Panel B.** A negative control with irrelevant IgG (MIgG; 40x). Results are representative of sections taken from three separate sources of lung tissue.

References

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