Manipulating the quality control pathway in transfected cells: low temperature allows rescue of secretion-defective fibrinogen mutants

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Design and Methods (*Supplementary Section*)

Cell culture and transfection

COS-7 cells were grown in a humidified incubator at 37°C and in 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal serum and penicillin/streptomycin. Cells were transiently transfected with FuGENE6 reagent (Roche Applied Science, Basel, Switzerland), according to the manufacturer's instructions.

Cell treatment with chemical chaperones or low temperature

For cell treatment with chemical chaperones, COS-7 cells in 10 cm-Petri dishes were transfected with either 7.5 μg of empty pcDNA3.1/V5-His TOPO vector (Invitrogen, Basel, Switzerland), or 3×2.5 μg of the three normal or mutant fibrinogen constructs previously generated.[7,9,11,12] Six hours after transfection, complete medium was replaced with complete medium without supplement, or supplemented with 10 mM 4-phenylbutyrate (4-PBA; Merck, Nottingham, UK), 2% dimethyl sulfoxide (DMSO; Sigma, Buchs, Switzerland) or 100 mM trimethylamine N-oxide dihydrate (TMAO; Sigma). The medium was removed 24 h post-transfection and the cells incubated for an additional 24 h in serum-free DMEM containing the same chemical compound as the day before. For incubation at low temperature, the culture medium was replaced 24 h after transfection with serum-free DMEM, and the cells incubated at 27°C for an additional 24 h.

Western blot analysis

Cells and culture media were harvested, and western blots performed as previously described,[11] using polyclonal rabbit anti-human fibrinogen antibodies (Dako, Baar, Switzerland) or mouse anti-actin IgG (Millipore, Billerica, MA, USA). Purified human plasma fibrinogen (Hyphen Biomed, Neuville-sur-Oise, France) was loaded as a control.

Clotting assay

COS-7 cells in six-well plates were transfected with 1.5 μg of empty pTRIDENT2 vector, wild-type or mutant tricistronic fibrinogen-containing constructs. The clotting assay was modified from previous studies.[20-22]Twenty-four hours after transfection, cells were washed three times with phosphate-buffered saline and incubated for 24 h at 27°C in 1 mL of methionine cysteine glutamine-free DMEM (Invitrogen), supplemented with 100 μCi/mL [^{35}S]-labeled methionine and cysteine (Hartmann Analytic, Braunschweig, Germany) and 2 mM L-glutamine (Sigma). Culture media were harvested and centrifuged 10 min at 110 g to pellet down the cell debris. Ten microliters of a pool of citrated human plasma was added for each milliliter of medium with or without 3 mM iodoacetamide (Sigma). Samples were incubated 30 min or 3 h at 37°C or 27°C (four conditions in total). The clot formed was recovered by centrifugation for 10 min at 15700 g at 4°C, while the supernatant was collected for immunoprecipitation. Clots were washed three times with RIPA buffer, solubilized in reducing Laemmli loading buffer and denatured by boiling at 100°C for 5 min.

For immunoprecipitation, Dynabeads coated with protein A (Invitrogen) were washed twice with wash buffer (0.1 M sodium phosphate, pH 8.1; 0.1% bovine serum albumin) and incubated for 40 min with rotation with polyclonal rabbit anti-human fibrinogen antibodies (0.14 μg per μL of beads). Coupled beads were washed three times before adding 20 μL of immunoglobulin-bead complexes to 1 ml of supernatant. Binding was performed by rotating samples 2 h at room temperature. The complexes were washed four times with RIPA buffer. Samples treated with iodoacetamide were resuspended in non-reducing loading buffer while non-treated samples were resuspended in reducing Laemmli buffer. Immunoprecipitated proteins and immunoglobulins were released from the beads by boiling.

All samples were resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). To increase the detection of the autoradiography signal, gels were soaked twice with DMSO for 30 min, impregnated 45 min with DMSO+10% 2,5-diphenyloxazole (Sigma), rinsed four times with water and dried. After autoradiography exposure, gels were stained with Bio-Safe Coomassie stain (Bio-Rad, Reinach, Switzerland).

Plasmin digestion of fibrinogen

Twenty-four hours after transfection of COS-7 cells in 10 cm-Petri dishes with 3 μg of plasmids, culture medium was replaced with serum-free DMEM and the cells incubated at 27°C for 24 h. Conditioned medium was centrifuged, concen-

trated using Amicon Ultra-4 5 kDa (Millipore) and adjusted to 50 mM Tris pH 7.4, 0.15 M NaCl.

When indicated, samples were pre-incubated at room temperature for 15 min with 10 mM EDTA, 5 mM CaCl2, or 10 mM EDTA and 4 mM Gly-Pro-Arg-Pro (GPRP) peptides (Anaspec, San Josè, CA, USA). In these cases, conditioned media from one given condition were distributed into three tubes corresponding to the three pre-incubations. Then, 2.8 µL plasmin 0.2 µg/µL (Chromogenix, Milano, Italy) per 100 µL were added, and samples were incubated at 37°C or 27°C for the time indicated. Reactions were stopped by the addition of non-reducing loading buffer and boiling. Purified fibrinogen diluted in DMEM was treated in parallel in the same manner. Samples were resolved by SDS-PAGE and visualized by Western blot using anti-fibrinogen antibodies.