Online Supplement

Thrombin-Induced Podocyte Injury is Protease Activated Receptor (PAR)-Dependent

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SUPPLEMENTAL METHODS

Antibodies, Peptides, and Reagents

Antibodies against PARs were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), as follows: anti–PAR1 (human and rat: ATAP-2), anti–PAR2 (human and rat: SAM-11), anti–PAR3 (human: 8E8; rat: 8E8 or M-20), and anti–PAR4 (human and rat: H-120). For the human kidney section immunofluorescence, primary antibodies were goat anti-synaptopodin (P-19) sc-21537 (also from Santa Cruz); secondary antibodies were donkey anti-goat FITC (A16006) and donkey anti-rabbit TRITC (A25466), both from Novex (Thermo Fisher Scientific Inc., Waltham, MA). Human PAR-activation peptides (AP) were purchased from Peptides International (Louisville, KY, USA); Rat PAR-APs were purchased from Ohio Peptide, LLC (Columbus, OH, USA). Specifically, human PAR-APs included PAR1 AP (TFFLRNPNDK), PAR2 AP (SLIGRL); PAR3 AP (TFRGAP); PAR4 AP (AYPGKF) and a scrambled control peptide (FSLLRN).[1, 2] Rat PAR-APs included PAR1 AP (SFFLRN), PAR3 AP (SFNGNE), PAR4 AP (GFPGKP).[3] Rat PAR2 AP was the same as that for human PAR2 AP. Rabbit polyclonal thrombin antibody (bs-1914R; raised against the thrombin heavy chain) was purchased from Antibodies-Online, Inc. (Atlanta, GA, USA). Mouse monoclonal synaptopodin (SYNPO) antibody (10R-S125A) was purchased from Fitzgerald Industries International (Acton, MA, USA). Anti-rabbit IgG conjugated to Alexa Flour 594 and anti-mouse IgG Alexa Flour 488) were purchased from Invitrogen (Carlsbad, CA, USA). Human α-thrombin was purchased from MP Biomedicals LLC (Santa Ana, CA, USA). Human plasma-derived AT (Kybernin® P) was a kind gift from CSL Behring (Marburg, Germany). Hirudin was purchased from Bachem Americas, Inc. (Torrance, CA). Alexa Fluor 488 Phalloidin was purchased from Molecular Probes, Inc. (Eugene, OR, USA). FITC and APC antibody conjugation kits were purchased from Abcam (Cambridge, MA, USA). Matched, isotype controls for flow cytometry were as follows: mouse IgG1-FITC, mouse IgG1-APC, mouse IgG2a-APC, mouse IgG2b-APC, and rabbit IgG-APC; matched to the SYNPO and PAR1 – PAR4 antibodies, respectively (all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA).

Urine Analysis

Urine protein-to-creatinine ratios were determined by Antech GLP (Morrisville, NC) using standard techniques that are fully compliant with the Good Laboratory Practice regulations and other regulatory requirements, as previously described and are reported as mg protein/mg creatinine (mg/mg).[4]

Enzyme-Linked Immunosorbent Assays

Plasma and urinary concentrations of thrombin-antithrombin (TAT) complex (Enzygnost; Siemens, Tarrytown, NY, USA) and prothrombin fragment 1.2 (F1.2; My Biosource, San Diego, CA, USA) were measured by ELISA assay kits, validated for use in the rat, as previously described.[4]

Cell Culture

Conditionally immortalized human and rat podocyte cell lines, which have recently been comprehensively characterized and verified to be pathogen free, were cultured as previously described.[5-8] In brief, human podocytes were grown on plates coated with collagen type 1 at 33°C. Under these conditions, podocytes proliferate and remain undifferentiated; differentiation was subsequently induced by incubation at 37°C. Experiments were performed after 14 days of differentiation, which was confirmed by determining expression of *SYNPO* and Nephrin (*NPHS1*) mRNA.[6] Rat podocytes were grown in Dulbecco's modified Eagle's/F-12 media containing 10% fetal bovine serum (Fisher, USA), penicillin (100 U/ml), streptomycin (100 mg/ml), and 0.1% Insulin-Trans-Cel-X (Gibco BioChemical, Grand Island, NY, USA) at 33 °C. They were differentiated under 'restrictive conditions' at 37°C in 5% CO_2 for ≥4 days. Differentiation was confirmed by demonstrating presence of *SYNPO* mRNA.[6] All experiments were performed on differentiated human and rat podocytes.

RNA Extraction and Polymerase Chain Reaction

Podocytes were lysed in RLT buffer (Qiagen,Valencia, CA) containing 1% β-Mercaptoethanol. Total RNA and subsequently cDNA were isolated from podocytes using the RNeasy kit (Qiagen) and Protoscript First Strand cDNA Synthesis kit (New England Biolabs; Ipswich, MA), respectively, according to the manufacturer's instructions. Purity and yield of RNA and cDNA were confirmed by measuring the absorbance at 260 and 280 nm. cDNA was amplified by polymerase chain reaction (PCR); 0.5 µL cDNA (diluted in a total volume of 11

μL RNase-free H_2O), 0.5 uL forward (fwd) and reverse (rvs) primers (below), and 12.5 uL HotStarTaq Plus Master Mix (Qiagen). Primers were obtained from Invitrogen (Carlsbad, CA). Primers were obtained from Invitrogen (Carlsbad, CA). PCR was performed as follows: 1 cycle at 95 °C for 5 min, 40 cycles at 94 °C for 30 sec and 55 °C for 30 sec and 72 °C for 30 sec, with a final extension of 72 °C for 10 min. PCR products were electrophoresed on a 1.8% agarose gel, and visualized using a Universal Hood II GelDoc and accompanying Quantity One 1-D analysis software (Bio-Rad; Hercules, CA).

Human Podocyte primer pairs (5' → 3'):

β-actin (*ACTB)*: (fwd) ACAGAGCCTCGCCTTTGCCG; (rvs) ACAGAGCCTCGCCTTTGCCG

Synaptopodin (*SYNPO*): (fwd) AGAGTGGCCCAGAAACCAG; (rvs) TGGCTCTCCAAGGTGAACTC

Nephrin (*NPHS1*): (fwd) CCCATGGAGGAGACAGTCAT; (rvs) ACGTTCAGGATGAGCGACTT

PAR1 (*F2R*): (fwd) CCTGGCTGACACTCTTTGTCC; (rvs) ACTGCCGGAAAAGTAATAGCTG

PAR2 (*F2RL1*): (fwd) TTCCCAGCC TTCCTCACAG; (rvs) TCTTTGAGGTGAGGGATAC

PAR3 (*F2RL2*): (fwd) TCCTGGTGTTTGTAGTTGGTGT; (rvs) CCAGTTGTTCCCATTGAGATGA

PAR4 (*F2RL3*): (fwd) CTGGGCAACCTCTATGGTG; (rvs) GCACCTTGTCCCTGAACTCG

Rat Podocyte primer pairs (5' → 3'):

β-actin (*Actb*): (fwd) CGGTCCACACCCGCCACC; (rvs) CTTGCTCTGGGCCTCGTCGC

Synaptopodin (*Synpo*): (fwd) CAAACCCAACACTCCACGCG; (rvs) TGCATGCCAATGAGCAGAGA

PAR1 (*F2r*): (fwd) AATTGGCAAGGGAGGGGATG; (rvs) CGGTTTAGCTGATAGGCCGT

PAR2 (*F2rl1*): (fwd) GAACGAAGAAGAAGCACC; (rvs) GGAACAGAAAGACTCCAATG

PAR3 (*F2rl2*): (fwd) ACAGCTGCGTAGACCCTTTC; (rvs) TAATGAAGGTCGCGCCAAGT

PAR4 (*F2rl3*): (fwd) GCTGCGTAGACCCTTTCATC; (rvs) AGGGTTCAGGAGGGACAGTT

Western Blotting

Western Blot experiments were performed with human and rat podocyte protein lysates for the detection of PAR protein expression. Adherent human and rat podocytes were washed with ice-cold phosphate buffered saline (PBS) and lysed with 450 μL MPER lysis buffer (Thermo Fisher Scientific Inc., Waltham, MA) containing

protease and phosphatase inhibitor cocktails (Fisher). Protein concentration of lysates was determined using the bicinchoninic acid (BCA) protein assay reagent (Fisher). Appropriate amounts of protein (30-60 µg) were separated by 10% SDS-PAGE and transferred to PVDF membranes, blocked for 1 h with 5% milk in PBS-T (PBS containing 0.1% Tween 20), and incubated with desired PAR primary antibodies (1:1000 in 2% PBS blotto) overnight at 4°C. Membranes were washed with TBST and incubated with anti-mouse IgG (1:2000), anti-rabbit IgG (1:10000), or anti-goat (1:5000) horseradish peroxidase conjugated antibodies, as appropriate. Proteins were detected on x-ray film using the ECL chemiluminescence reagent (Fisher), and the density of each band was quantified using Image J software. Accuracy of protein loading was confirmed by GAPDH (1:10000; Millipore) Western Blot.

For the co-immunoprecipitation assays, BS3 (bis(sulfosuccinimidyl)suberate; Thermo Fisher), a water soluble covalent protein linker, was utilized during podocyte thrombin exposure to enhance interaction stability during the aqueous phases of the protocol. Thrombin-dependent PAR homo- and hetero-dimerization is dependent upon interactions between hydrophobic PAR transmembrane domains.[9, 10] Similarly, BS3 has thus been utilized to enhance oligomer detection of another G protein-coupled receptor (M_2 Muscarinic Cholinergic Receptor).[11] Briefly, thrombin (20 nM) was added to differentiated podocyte cultures at time zero, 2.5 minutes later 2 mM BS3 (bis(sulfosuccinimidyl)suberate; Thermo Fisher) was added, followed by protein lysate preparation at 5 minutes. PAR antibodies were adsorbed to sepharose beads for the respective pull-down conditions and incubated overnight with 500 µg of total protein lysates. Beads were then washed 5 times in RIPA buffer, resolved in sample buffer, and boiled for 5 minutes at 95 °C for elution of bound protein complexes, which were subjected to Western Blot for the respective target PARs using the conditions described above. The membranes were subsequently stripped and re-blotted with the respective pull-down antibodies to confirm appropriate immunoprecipitation (data not shown).

Antibodies against total and phosphorylated ERK1/2 MAPK (137F5 and 20G11, respectively) were purchased from Cell Signaling Technology (Danvers, MA). Western Blot experiments were performed as above on human and rat podocyte protein lysates obtained following exposure to thrombin with or without PAR antibody blockade. Total protein (30 µg) was loaded onto 12% gels. Total and phosphorylated ERK1/2 primary

antibodies were used at 1:1000 in 2% PBS blotto overnight at 4°C. Membranes were washed with TBST and incubated with anti-rabbit IgG (1:5000) horseradish peroxidase conjugated antibody. Relative levels of protein were quantified by optical density analysis using ImageJ open source software (National Institutes of Health; Bethesda, MD).

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assays

Differentiated podocytes were serum-starved overnight in serum-free medium. After 36 hours of exposure to thrombin or PAR activation peptides (AP), podocytes were fixed in 4% neutral buffered formalin, washed in PBS, and injury was determined using TUNEL assay (Roche Diagnostics; Indianapolis, IN, USA), according to the manufacturer's directions and as previously described.[1, 12] Briefly, podocytes were incubated with terminal deoxynucleotidyl transferase in the presence of fluorescein-labeled dUTP (60 minutes at 37 °C) and counterstained with Hoechst 33258 (3.5 µg/mL). Random images were captured with a Ziess 510 META confocal microscope (Zeiss; Thornwood, NY, USA) coupled to a Zeiss LSM 700, and the frequency (%) of TUNEL positive podocytes was determined by a blinded investigator (RS). Varying concentrations of thrombin (from 0 – 50 nM) were used to evaluate thrombin-mediated exacerbation of PAN-induced podocyte injury. Thrombin (20 or 30 nM) without PAN was evaluated for PAN-independent podocyte injury. Subsequent experiments were conducted using 20 nM thrombin (final concentration), which was added every 12 hours.[12] For anti-PAR experiments, podocytes were pretreated with anti–PAR antibodies (10 µg/mL) for 30 minutes before thrombin exposure.[1] In separate experiments, podocytes were exposed to PAR APs and scrambled (control) peptide at 20 µM (final concentration).[1]

Viability and F-actin Rearrangement Assays

Podocyte viability was assessed using the MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide; Sigma) assay, as described previously.[13] Briefly, podocytes were differentiated in 6-well plates, serum starved overnight, then exposed to thrombin (20 nM) for 48 hours in the presence or absence of pan-caspase inhibitor (Z-VAD-FMK, R&D Systems Inc., Minneapolis, MN, USA) pretreatment (40 µM). Afterward, the podocytes were incubated with MTT (500 µg/ml) for 4 hours at 37 °C. MTT formazan crystals were extracted in dimethyl sulfoxide containing 0.01 M glycine and absorbance was measured at 570 nm on a SpectraMax M2 plate

reader (Molecular Devices, Sunnyvale, CA). Control cells (100% viable) were not exposed to Z-VAD-FMK or thrombin, whereas Triton X-100 treated cells were used as negative controls (0% viable). Each experiment consisted of 3 wells per treatment and experiments were repeated 5-11 times. In preliminary experiments, 40 μM Z-VAD-FMK was determined to be the minimal concentration necessary to maximally inhibit caspase-3 activity in differentiated human podocytes exposed to PAN (30 μg/mL; data not shown).

To assess F-actin cytoskelatal rearrangement, differentiated podocytes were grown on coverslips. Following exposure to thrombin (20 nM) for 72 hours, the coverslips were stained with phalloidin and DAPI. Images of random individual cells that were well spaced from their neighbors were collected with a BZ900 inverted confocal microscope. Each cell was subsequently scored as follows: (a) Filament thickness: uniform thickness (1 point); mixture of thick and thin filaments (2 points), predominantly thin filaments (3 points); (b) Filament pattern: organized (0 points), disarrayed (1 point); (c) Filament condensation: none (0 points), condensed clumps or clouds (1 point). Thus, each cell was scored on a 5 point scale.

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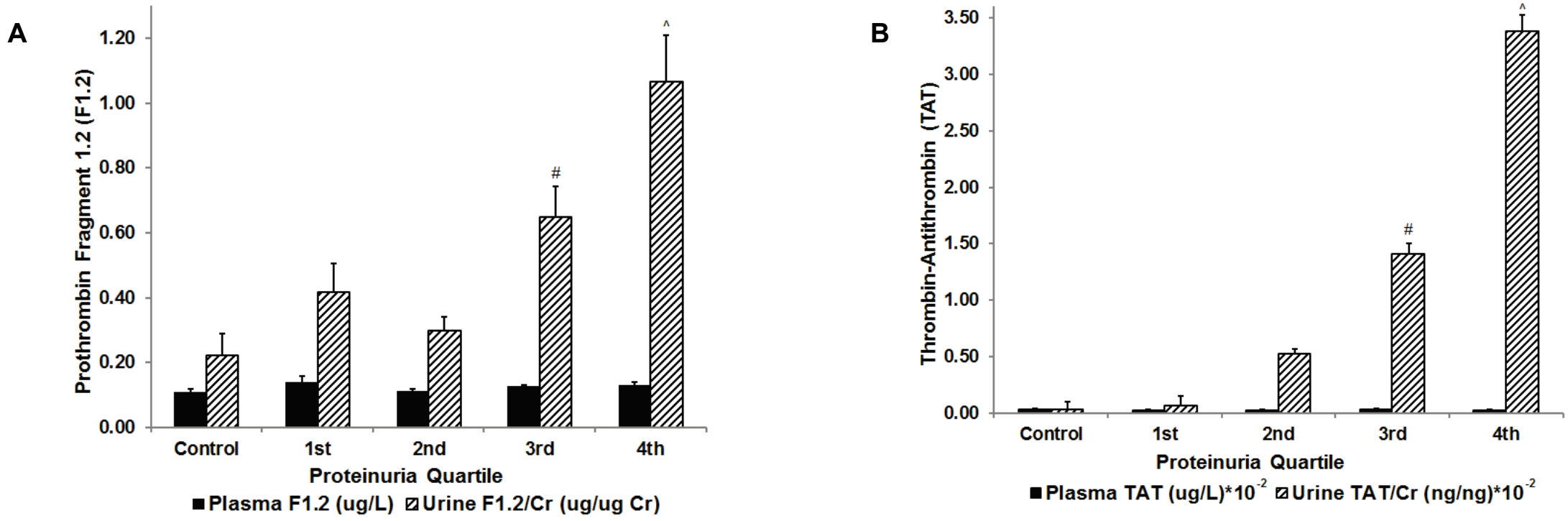
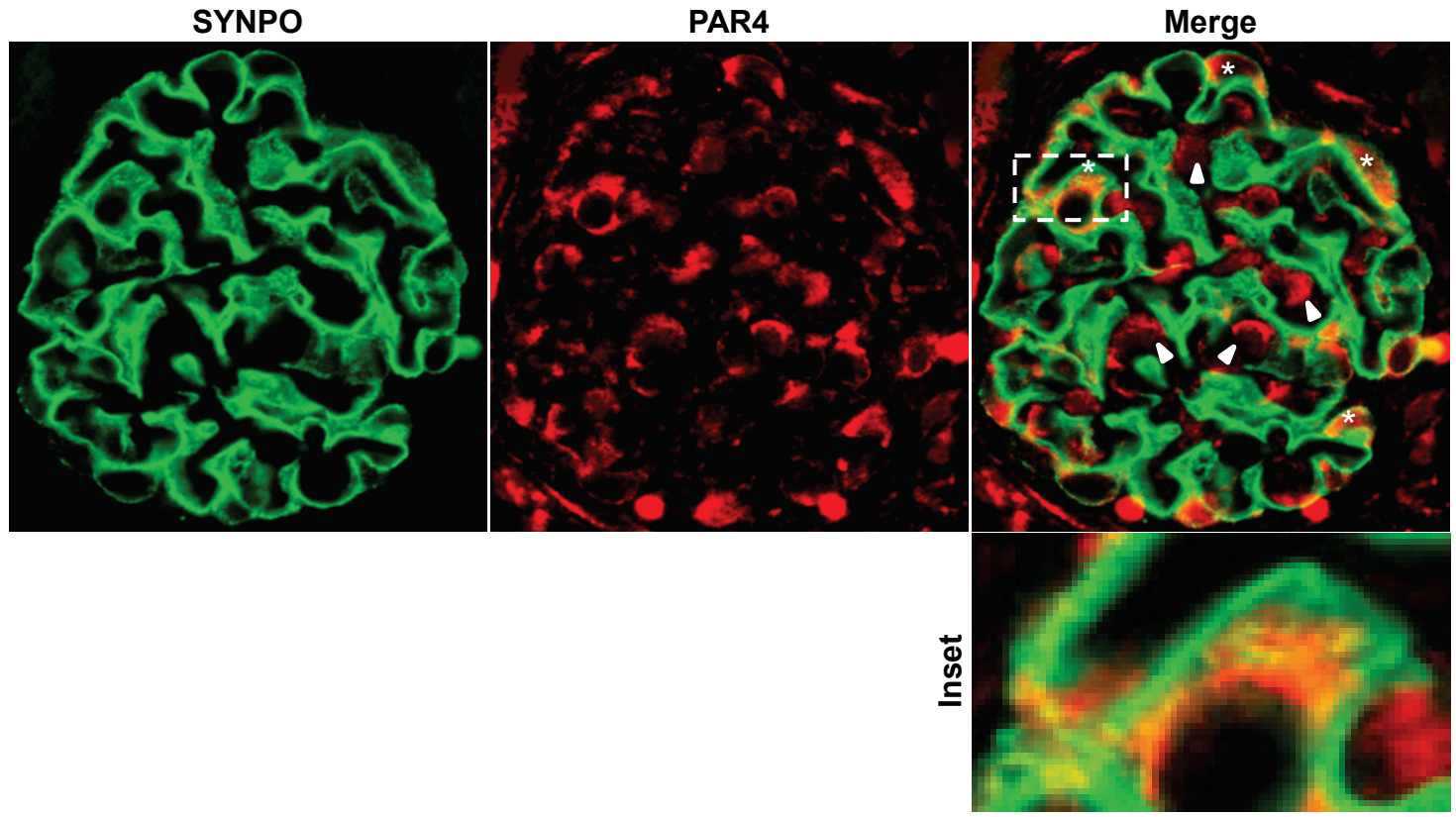
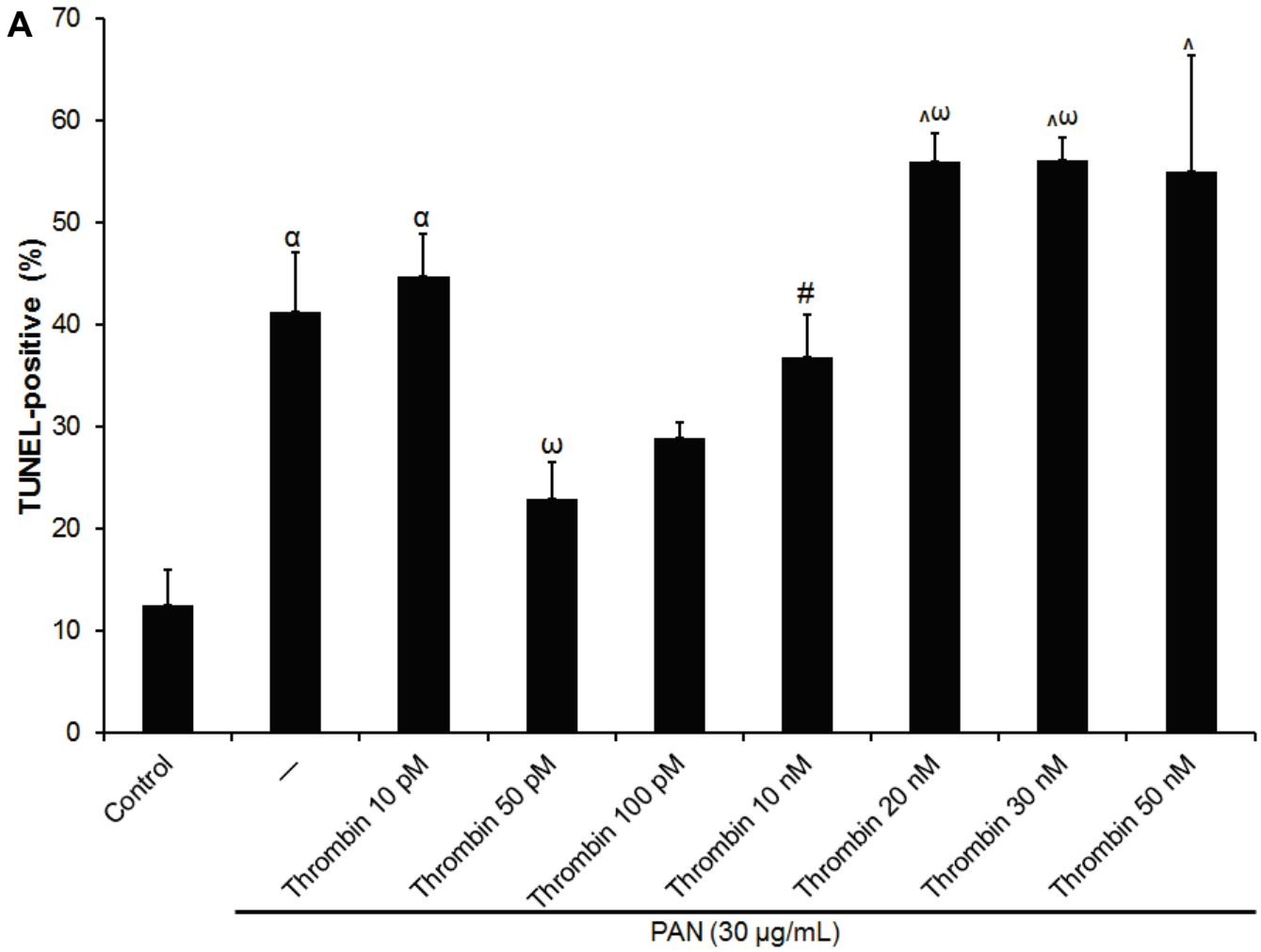
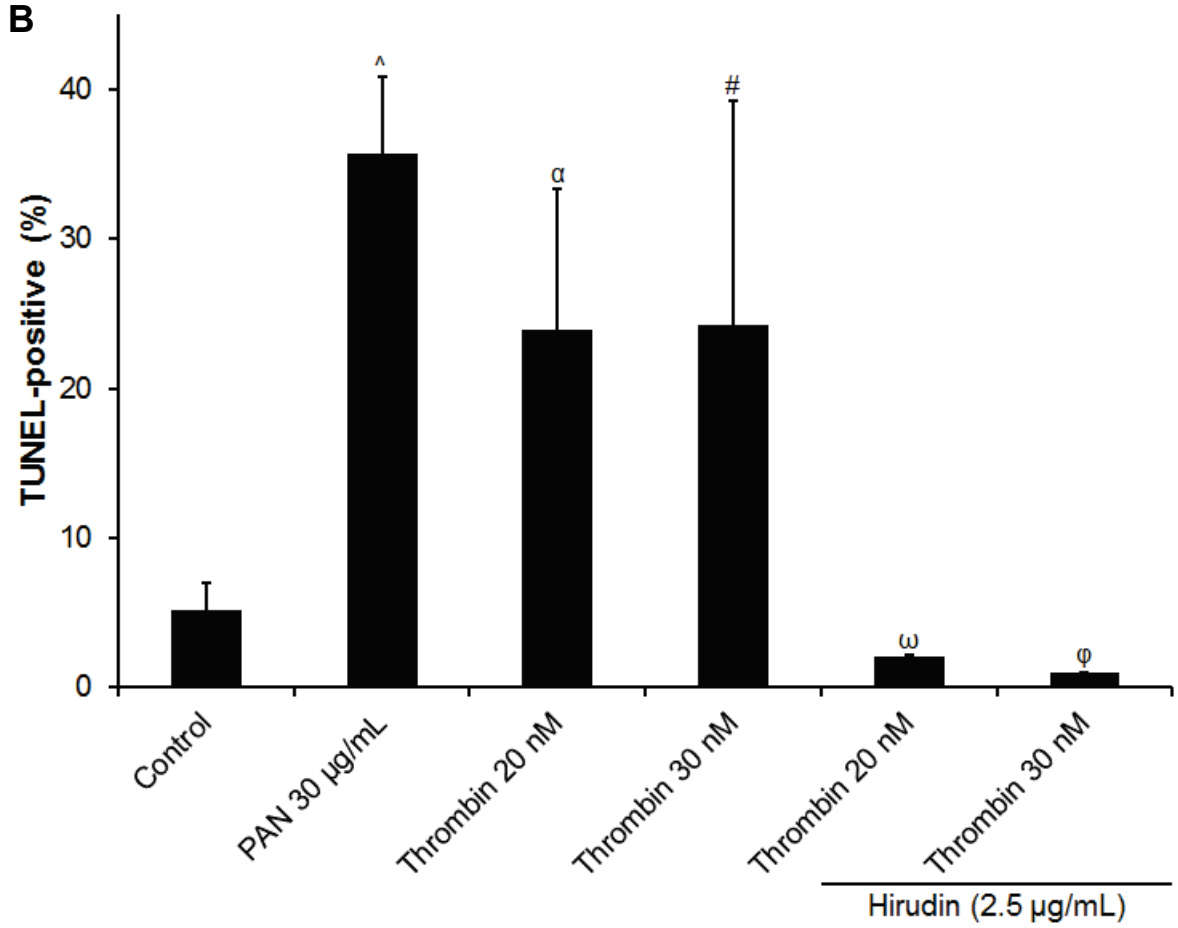


Supplemental Figure 1: During PAN-Induced Rat Nephrosis Urinary Markers of Thrombin Activation and Regulation Increase. At day 9 of PAN-nephrosis, urinary byproducts of thrombin activation, F1.2 (**A**), and regulation, thrombin-antithrombin complex (TAT; **B**), increase directly with proteinuria severity while plasma levels remain stable (*n*=30 rats administered varying PAN doses 0-150 mg/kg). $^{\#}P<0.05$ vs. control; $^{\wedge}P<0.001$ vs. control.

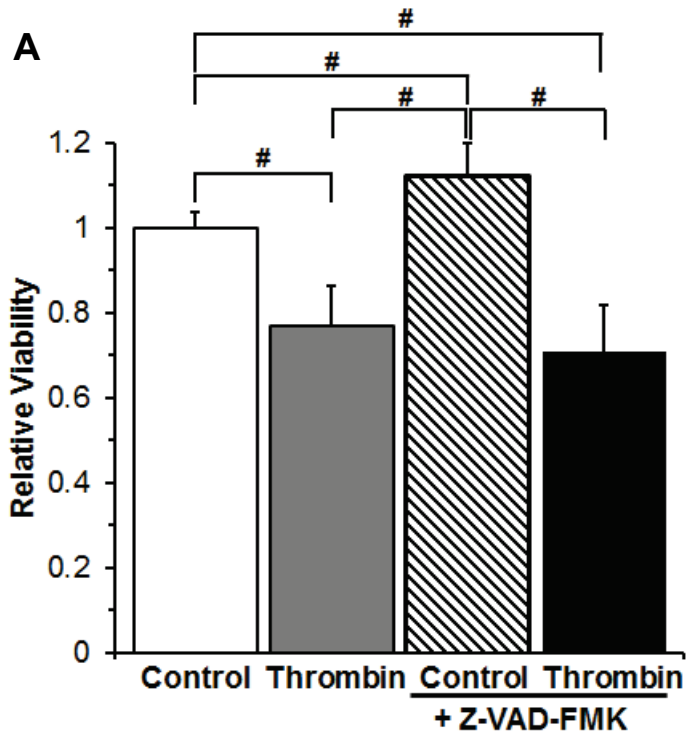


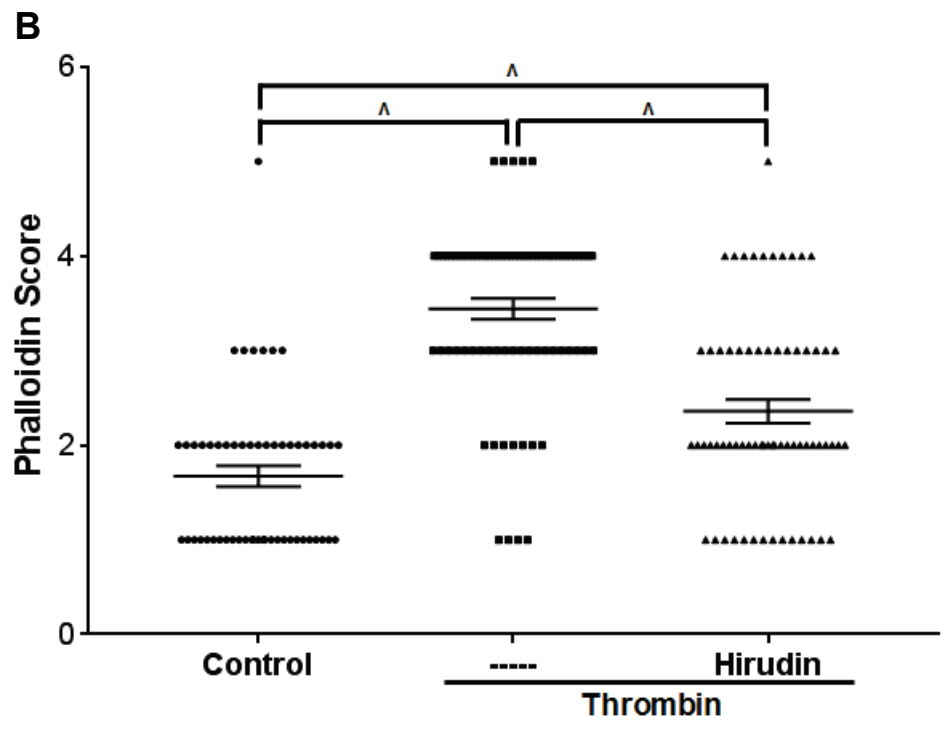
Supplemental Figure 2: Human Podocytes Express PAR4 *In Vivo*. Immunofluorescent histology of representative human glomeruli (60x) demonstrating predominantly cell body (asterisks) colocalization (yellow) of synaptopodin (green; FITC) with PAR4 (orange; TRITC) on podocytes, whereas glomerular endothelial cells (arrow heads) express only PAR4 (orange;TRITC); representative of 4 tissue sections with 1-2 glomeruli per section from normal adult human kidney sections.

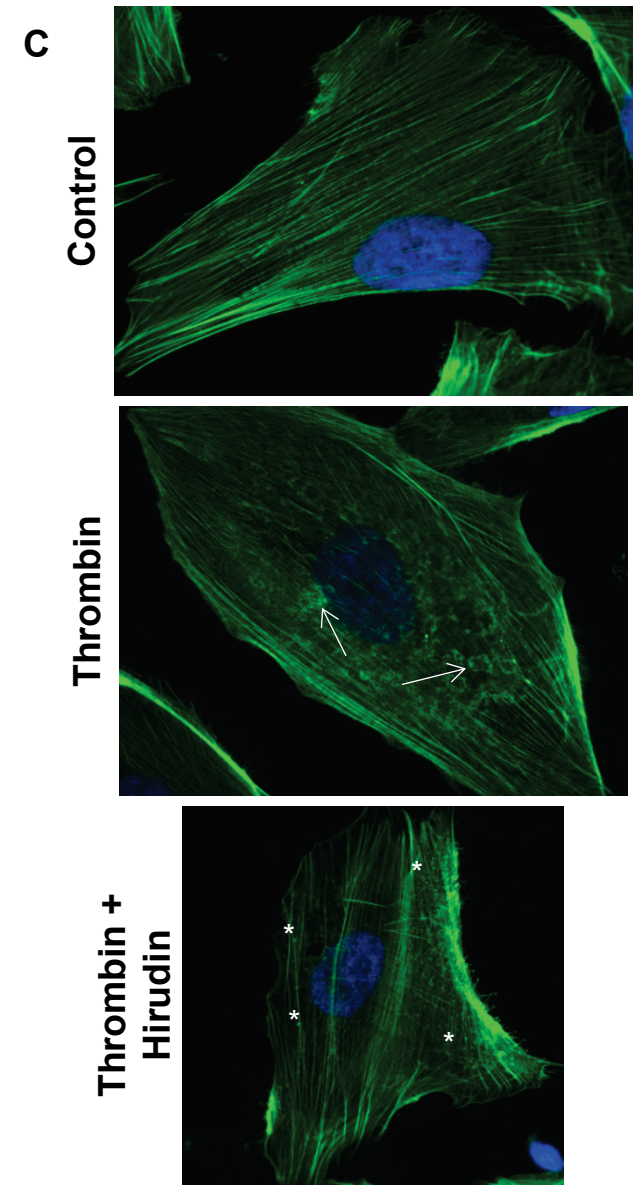




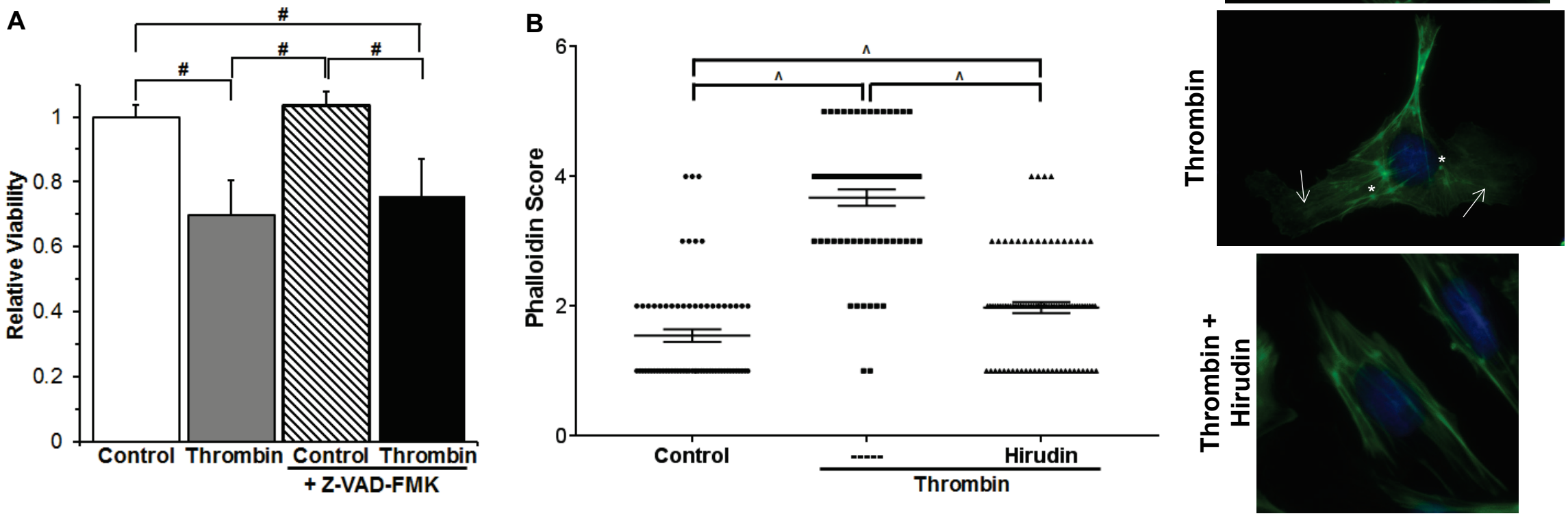
Supplemental Figure 3: Thrombin Alters PAN-Induced Human Podocyte Injury in a Concentration-Dependent Manner with Maximal Effects at 20 nM. (**A**) Thrombin diminishes podocyte injury at low concentration (50 pM) and exacerbates injury at concentrations ≥20 nM. (**B**) Thrombin induces podocyte injury, independently of PAN at concentrations ≥20 nM ($^{\#}P<0.05$, $^{\alpha}P<0.01$, $^{\wedge}P<0.001$ vs control; $^{\omega}P<0.05$, $^{\varphi}P<0.01$ vs PAN; n=3-4 per condition).



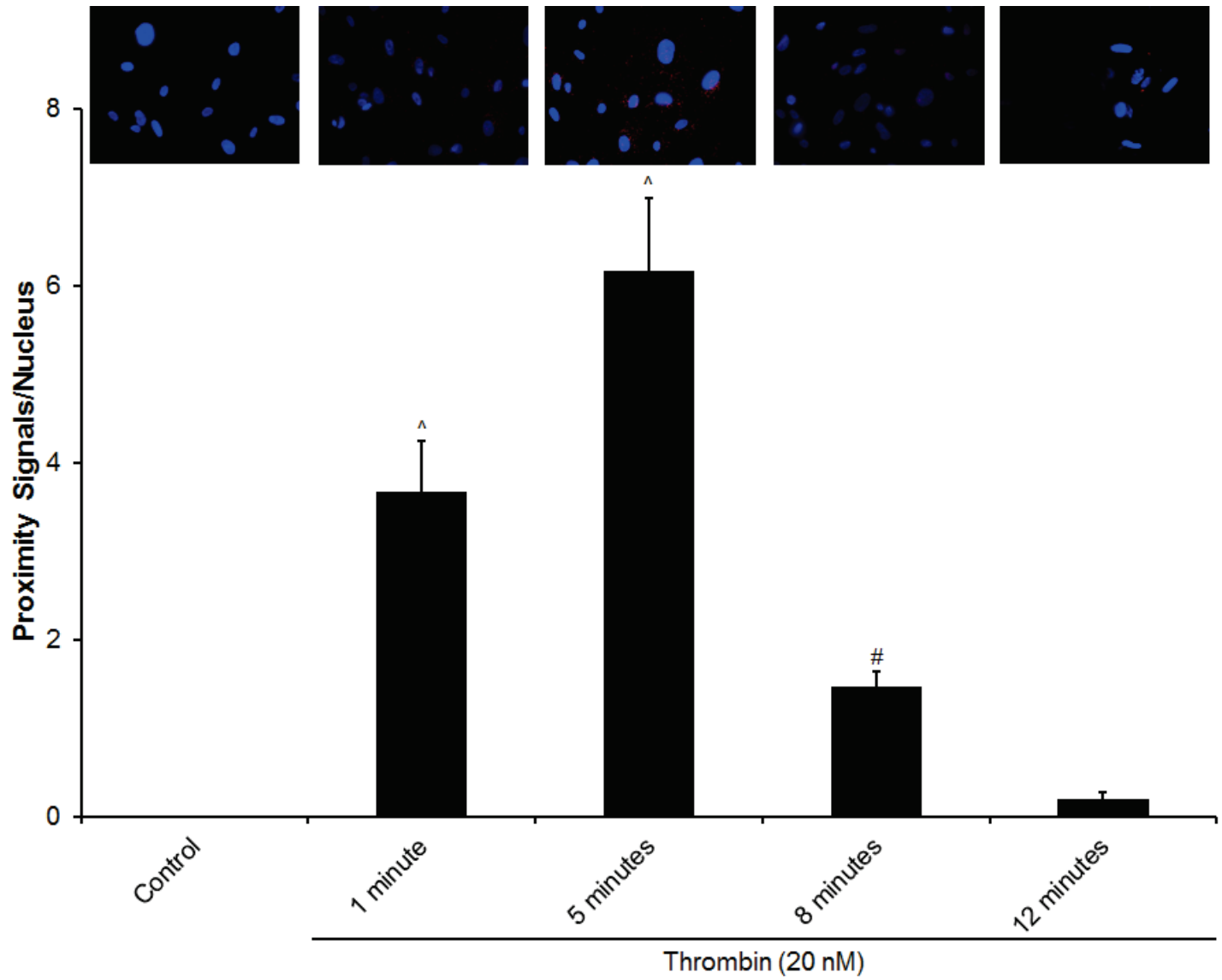




Supplemental Figure 4: Thrombin-Induced Human Podocyte Injury. (**A**) Cell viability (MTT assay) is reduced by thrombin (20 nM) and is not rescued by pancaspase-inhibition (40 μM Z-VAD-FMK; $^{\#}P<0.05$; n=5-9 per condition). (**B**) Phalloidin staining scores of individual cells (symbols) and mean ± SEM showing F-actin cytoskeletal rearrangement by thrombin (20 nM), partially rescued by hirudin (n=55-75 cells per condition; $^{\wedge}P<0.001$). (**C**) Representative phalloidin stained cells: (Top) Control cell with well-organized, mixed fiber thickness; score=2, (Middle) Thrombin-exposed cell with thin, organized fibers with condensed clouds (arrows); score=4, (Bottom) Thrombin+Hirudin-exposed cell with organized, mixed thickness fibers and clumps (asterisks); score=3.



Supplemental Figure 5: Thrombin-Induced Rat Podocyte Injury. (**A**) Cell viability (MTT assay) is reduced by thrombin (20 nM) and is not rescued by pancaspase-inhibition (40 μM Z-VAD-FMK; $^{\#}P<0.05$; n=9-11 per condition). (**B**) Phalloidin staining scores of individual cells (symbols) and mean ± SEM showing F-actin cytoskeletal rearrangement by thrombin (20 nM), partially rescued by hirudin (n=64-91 cells per condition; $^{\wedge}P<0.001$). (**C**) Representative phalloidin stained cells: (Top) Control cell with organized, normal fiber thickness and condensed clumps (asterisk); score=2, (Middle) Thrombin-exposed cell with a mixed thickness, disorganized fibers and condensed clumps (asterisk) & clouds (arrows); score=4, (Bottom) Thrombin+Hirudin-exposed cell with thin, organized fibers; score=3.



Supplemental Figure 6: Thrombin Induces Maximal PAR3/PAR4 Interactions in Human Podocytes at 5 Minutes. Mean ± SEM number of proximity signals per nucleus over time with representative images in upper panels ($^{\#}P<0.05$, $^{\wedge}P<0.001$ vs. control; $n=3$ per condition).