Supporting Information: Collagenolytic matrix metalloproteinases antagonise proteinase-activated receptor-2 activation, providing insights into extracellular matrix turnover

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SUPPORTING METHODS

Materials — All Fmoc-amino acids (Fmoc-Lys(Boc)-OH, Fmoc-Gly-OH, Fmoc-Thr(^{t}Bu)-OH, Fmoc-Val-OH, Fmoc-Ser(^{t}Bu)-OH, Fmoc-Ala-OH·H_2O, Fmoc-Phe-OH, Fmoc-Glu(O^{t}Bu)-OH, Fmoc-Asp(O^{t}Bu)-OH, Fmoc-His(Trt)-OH, Fmoc-Asp(O^{t}Bu)-(Dmb)Gly-OH, Fmoc-Ile-OH, Fmoc-Leu-OH and Fmoc-Arg(Pbf)-OH) and COMU were purchased from MerckMillipore. DIEA was obtained from Kokusan Chemical Co. Ltd. (Tokyo, Japan). Other reagents and solvents were of commercially available grade.

Experimental procedure of synthesis of PAR-2 (42mer peptide) — PAR2$^{31-72}$ peptide was synthesised using Microwave Supported Solid Phase Peptide Synthesis (MW-SPPS) in a 5 mL LibraTube using a MWS-1000 microwave synthesizer (EYELA, Tokyo, Japan). MALDI-TOF MS analyses were performed using an Autoflex instrument (Bruker, Germany) with DHB as the matrix. HPLC was performed on a Hitachi HPLC system with an L-7100 intelligent pump and an L-7405 UV detector.

PAR2$^{31-72}$ was assembled on H-Lys(Boc)-HMPB-ChemMatrix resin (HiPep Laboratories, Japan). The resin (0.039 mmol) was swelled in DMF for 3 hours before use. Peptide synthesis was performed over three stages. The first stage (Gly^{71} to Ser^{65}) used SPPS, and was performed with cycles of: Fmoc deprotection with 1 ml of 20% piperidine in DMF for 10 minutes at room temperature before washing the resin three times with DMF, and coupling the next Fmoc-amino acid (0.195 mmol) with COMU (0.195 mmol) and DIEA (0.195 mmol) in 1 ml of DMF for 40 minutes at room temperature, then washing the resin three times with DMF. Finally, acetyl capping for unreacted amines was performed with 20% acetic anhydride in DMF for 5 minutes at room temperature and then washing the resin three times with DMF.

The second stage, (Phe^{64} to Val^{53}) used MW-SPPS method, and was performed with cycles of: Fmoc deprotection with 1 ml of 20% piperidine in DMF for 2 minutes under microwave irradiation (max 100W, 50°C), before washing the resin three times with DMF, and coupling the next Fmoc-amino acid (0.195 mmol) with COMU (0.195 mmol) and DIEA (0.195 mmol) in 1 ml of DMF for 5 minutes under microwave irradiation (max 60W, 50°C) then washing the resin three times with DMF. Finally, acetyl capping for unreacted amines was performed with 20% acetic anhydride in DMF for 3 minutes at room temperature and then washing the resin three times with DMF.

The third stage (Gly^{52} to Arg^{31}) used MW-SPPS method, and was performed with cycles of: Fmoc deprotection with 1 ml of 20% piperidine in DMF for 4 minutes under microwave irradiation (max 100W, 50°C) before washing the resin three times with DMF, and coupling the next Fmoc-amino acid (0.195 mmol) with COMU (0.195 mmol) and DIEA (0.195 mmol) in 1 ml of DMF for 10 min under microwave irradiation (max 60W, 50°C) then washing the resin three times with DMF. Finally, acetyl capping for unreacted amines was performed with 20% acetic anhydride in DMF for 3 minutes at room temperature and then washing the resin three times with DMF.

For introduction of His and Arg, coupling reactions were performed twice. The introduction of Asp^{42}-Gly^{43}

was accomplished using a dipeptide synthon, Fmoc-Asp(O^{t}Bu)-(Dmb)Gly-OH.

The resulting resin was washed with dichloromethane, dried and treated with 2 ml of TFA/TIS/water (95:2.5:2.5 v/v/v) for 3 hours at room temperature. The resin was washed with TFA three times and and the combined removed TFA was concentrated using a nitrogen blow down evaporator. The resulting solution was precipitated with 10 ml ice-cold ether and centrifuged at 5,000g for 5 minutes at 4°C. The precipitate was washed three times by the same procedure, dried and purified by reversed-phase HPLC using an Inertsil WP300-C8, (ϕ10x250 mm) coulmn at 40°C with a flow rate of 4 ml/min (detection: 220 nm) with a gradient of 5% to 35% acetonitrile containing 0.1% TFA over 50 minutes.

Buffers utilised for PAR-2 (42mer peptide) digestion —
MMP-1, -8, -13 buffer: 100 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$, 0.05% (w/v) Brij 35, 0.01% (w/v) PEG 6000.

Matripase, Hepsin buffer: 100 mM Tris pH 8.5, 150 mM NaCl, 0.01% Brij-35

Neutrophil elastase, Cathepsin G buffer: 100 mM Tris pH 7.5, 150 mM NaCl, 0.01% Brij-35

Analytical RP-HPLC — To analyse enzyme digest products and collect peptides for further analysis by MS, analytical reversed-phase HPLC was performed using an Agilent 1100 HPLC system fitted with a 2.1 X 150 mm ACE 3 C18 analytical column (Hichrom Ltd, UK), operating at a flow rate of 0.2 ml/min. The standard gradient conditions were 5-60% buffer B over 27 minutes. Buffer A: 0.1% formic acid in water; Buffer B: 0.1% formic acid in acetonitrile. Fractions were collected manually (typically 20 – 200 mAU @ 214 nm with 30 second peak width) at the expected elution times and either used directly for electrospray MS or stored at -20°C until required.

Infusion Electrospray MS Analysis — Infusion spray analyses were performed by supplementing peptide fractions in 30% acetonitrile and 0.1% formic acid (aq) and then spraying using medium 'nanoES' spray capillaries (ThermoFisher) in positive ion mode at 1.4 kV. Electrospray data was acquired using an LTQ-FT mass spectrometer (ThermoFisher) with a FT-MS resolution setting of 100,000 at m/z = 400 and an injection target value of 1,000,000.

FOOTNOTES

The abbreviations used are: COMU, (1-Cyano-2-ethoxy-2-oxoethylideneaminooxy)dimethylaminomorpholinocarbenium hexafluorophosphate; DIEA, *N*,*N*-diisopropylethylamine; DHB, 2,5-dihydroxybenzoic acid; DMF, Dimethylformamide; Fmoc, 9-Fluorenylmethyloxycarbonyl; TIS, Triisopropylsilane

TABLES

MMP	Retention time (min)	Mass (Da)	Sequence
Control	16.8	4323.3	RSSKGRSLIGKVDGTSHVTGKGVTVETVFSVDEFSASVLTGK
MMP-1	18.4	3564.9	LIGKVDGTSHVTGKGVTVETVFSVDEFSASVLTGK
MMP-8	16.3	3924.1	RSSKGRSLIGKVDGTSHVTGKGVTVETVFSVDEFSASV
MMP-8	18.4	3564.9	LIGKVDGTSHVTGKGVTVETVFSVDEFSASVLTGK
MMP-13	17.9	3165.6	LIGKVDGTSHVTGKGVTVETVFSVDEFSASV
MMP-13	18.4	3564.9	LIGKVDGTSHVTGKGVTVETVFSVDEFSASVLTGK

Table S1: Observed masses Masses observed by electrospray MS corresponding to the HPLC peaks identified in Figure 2, and the responsible peptide fragment sequences

FIGURES

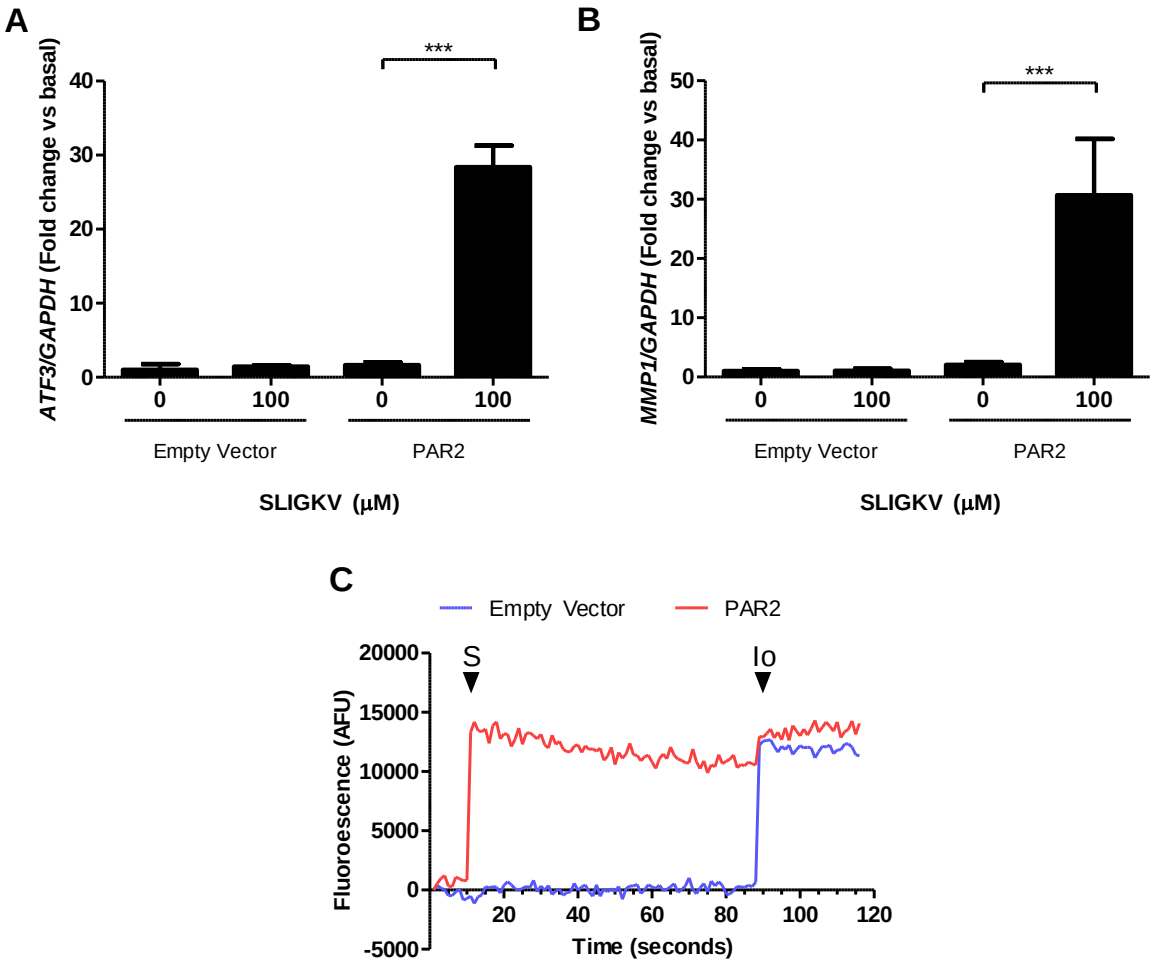

Figure S1: Activation of SW1353-PAR2 cells is dependent on PAR2 overexpression. SW1353-PAR2 cells or empty vector control cells were stimulated with 100 μM SLIGKV-NH_2 or SFM for 90 minutes (A) or 24 hours (B) and RT-qPCR performed for *ATF3* (A) and *MMP1* (B). Data are expressed relative to *GAPDH* and presented as fold-change compared with basal expression (mean ± S.D., n = 4 for A and n = 6 for B), representative of three (A) or two (B) independent experiments. SW1353-PAR2 and control cells were loaded with Rhod-4-AM fluorescent calcium probe and stimulated with 50 μM SLIGKV-NH_2 (S) followed by 5 μM ionomycin (Io) (C), N = 2 independent experiments. Selected statistical comparisons utilised student's two-tailed unpaired t tests against basal, where ***, p <0.001.