SUPPLEMENT MATERIAL

Materials and Methods

PAR-2 Cleavage Assay: HEK 293 cells were cultured in 24 well tissue culture plates to approximately 70% confluency. The cells were then transiently transfected with a PAR2-ALP reporter construct (1 μg/well) in antibiotic-free Opti-MEM medium using Lipofectamine (Invitrogen, Carlsbad, CA) following the protocol previously described [1]. After 6 hours the media was changed and the cells were incubated an additional 48 hours. The cells were then incubated with varying concentration of PR3 or thrombin (0.01 to 1 U/ml) for 4 hours. In some treatments, the PR3 was first incubated 15 minutes with 2 μM elafin before being added to the cells. Conditioned media was collected, centrifuged to remove cell debris and analyzed for ALP activity using the SensoLyte luminescent ALP reporter assay kit (AnaSpec, San Jose, CA) following the manufacturer's instructions. Results are expressed as the mean ±SEM of four separate experiments.

Rac1-GTP and RhoA-GTP detection: HUVECs cultured on 12 well plates were serum-starved overnight at 37°C in 5% CO_2. The cells were then treated with the PAR1 agonist peptide (AP) (SFLLRN, 10 μM), PAR2 agonist peptide (SLIGKV, 10 μM) or PR3 (1 U/ml) for 5-15 minutes. The cells were then washed with ice-cold PBS, followed by lysis buffer to remove the cells and then flash frozen in liquid nitrogen. Quantification of Rac1-GTP and RhoA-GTP was done using the G-LISA kit (Cytoskeleton, Inc; Denver, CO) following the manufacturer's instructions. The relative quantity of RhoA-GTP and Rac1-GTP in the cell lysates was determined using a fluorescent plate reader at 490 nM. This was then expressed as a percentage (% of max) of the

RhoA and Rac1 positive control proteins. Results are expressed as the mean ±SEM of four separate experiments.

Results

PR3 cleaves PAR2 of the cell surface

In cells transiently transfected with PAR2-ALP we found that PR3 could efficiently cleave PAR2, promoting the release of ALP that was detected by our assay (supplemental Figure I). However, if PR3 was first inactivated by elafin we did not detect any significant increase in PAR2 cleavage compared to untreated cells. Thrombin, another serine protease, was unable to significantly cleave PAR-2 from the surface of HEK 293 cells. Therefore, PR3 can cleave cell surface expressed PAR2 and this is inhibited by elafin.

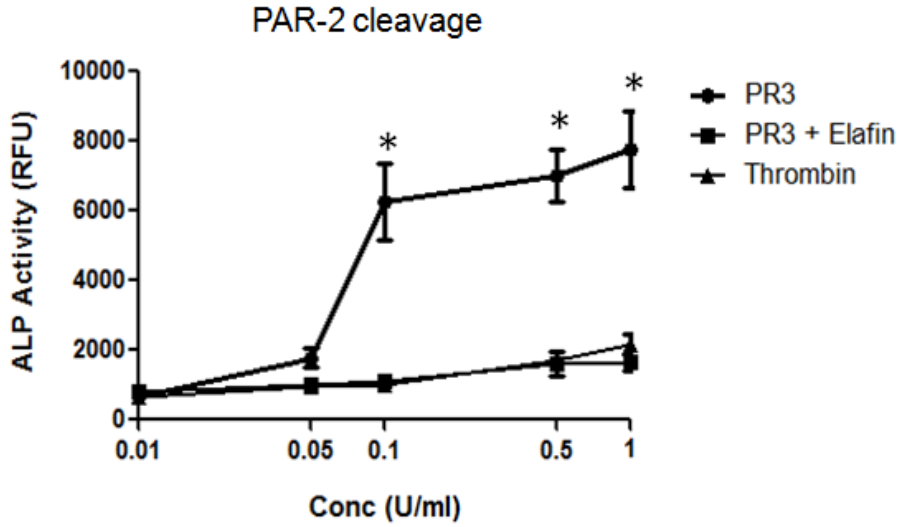
PR3 promotes Rac1 activation in endothelial cells

In endothelial cells treated with a PAR2 agonist peptide or PR3 we observed a significant increase in Rac1-GTP activity compared to untreated cells (supplemental Figure II), but no significant increase in RhoA-GTP. In contrast, we found that endothelial cells treated with a PAR1 agonist peptide had a significant increase in RhoA-GTP activity; however, PR3 nor PAR2 agonist peptide were able to significantly increase RhoA-GTP at the time points we used. These results demonstrate that PR3 can promote Rac1-GTP activity while RhoA-GTP activation is not significantly increased.

Reference

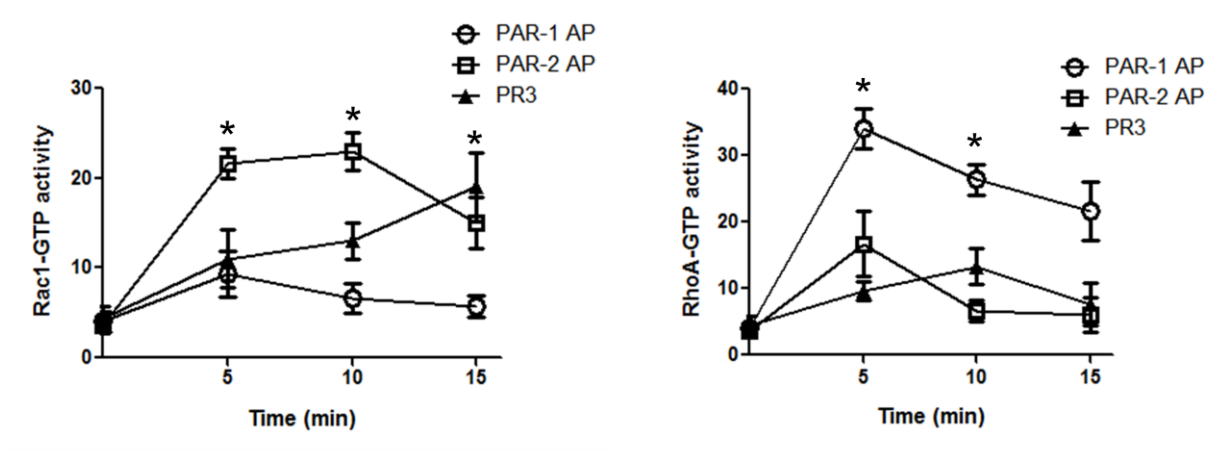
(1) Bae JS, Yang L, Rezaie AR. Lipid raft localization regulates the cleavage specificity of protease activated receptor 1 in endothelial cells. *J Thromb Haemost* 2008;6:954-961.

Supplemental Figure I



Supplemental Figure I. PR3 cleaves PAR-2 expressed on the cell surface. HEK 293 cells were transiently transfected with a PAR-2 alkaline phosphatase (ALP) containing reporter construct. These cells were then incubated with PR3, PR3 treated with elafin (2 μM) or thrombin for 4 hours. Supernatants were then collected and analyzed for ALP activity. Conditioned media from cells treated with PR3 had significantly increased ALP activity compared to media from untreated cells. PR3 inactivated by elafin was not able to cleave PAR-2. (* = $p<0.01$ compared to untreated cells) Results represent the mean ±SEM of four separate experiments.

Supplemental Figure II



Supplemental Figure II. PR3 promotes Rac1-GTP activity. Serum starved endothelial cells in 12 well tissue culture plates were incubated with PR3 (1 U/ml), PAR-1 agonist peptide (SFLLRN, 10 μM) or PAR-2 agonist peptide (SLIGKV, 10 μM). Cell lysates were then collected and analyzed for Rac1- and RhoA-GTP binding compared to a positive control protein. Results are expressed as the percent of maximum compared to the positive control protein. Both PR3 and PAR-2 agonist peptide were able to promote a significant increase in Rac1-GTP activity compared to untreated cells. In contrast, only the PAR-1 agonist peptide was able to significantly increase RhoA-GTP activity at the time points we observed. (* = $p<0.01$ compared to untreated cells) Results represent the mean ±SEM of four separate experiments.