UPREGULATION OF PROTEASE-ACTIVATED RECEPTORS IN VASCULAR SMOOTH MUSCLE IN RESPONSE TO INJURY AND THE COOPERATIVITY BETWEEN PAR 2 AND 4

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Overarching Theme & Hypothesis

Vascular Injury

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Initial Injury cascade

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Upregulation and activation of proteins that could potentially be involved in pathological VSM growth

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Arteriolosclerosis & Negative Homeostasis

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More Vascular Injury

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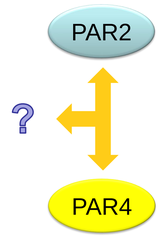
Cardiovascular disease And Death



Hypothesis 1

Vascular Injury (balloon stretch model)

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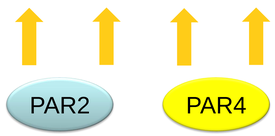
Upregulation of PAR 2 and PAR 4



Hypothesis 2

Vascular Injury

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Neutrophil and Macrophage Attraction

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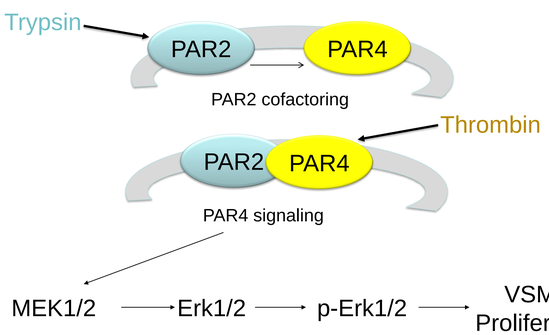
Release of Trypsin and other Serine Proteases



Results

Figure 1: Expression and Activity of PAR2/PAR4 after Injury

A: Significant Increase in PAR2 expression after injury. B: Trending increase in PAR4 expression. C: Trending increase in activity of PAR2 and PAR4

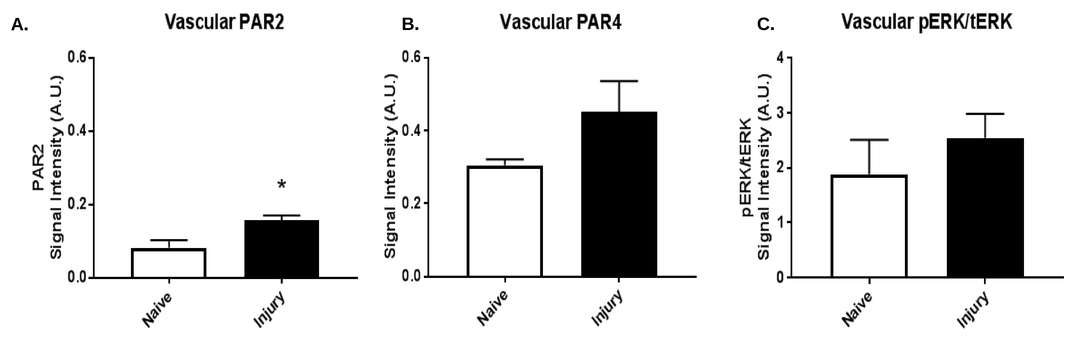
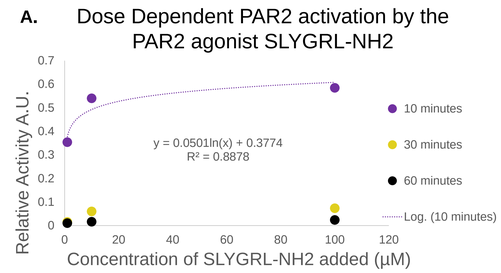
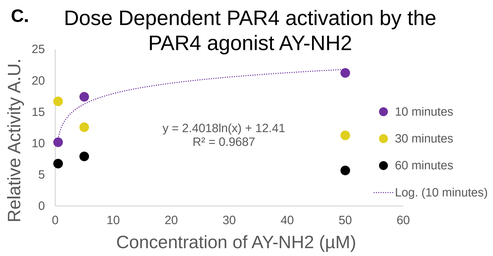


Figure 2: Characterization and validation of exogenous PAR2 and PAR4 agonists and PAR2 antagonist





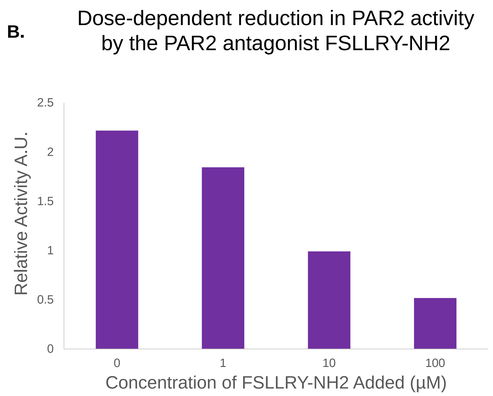
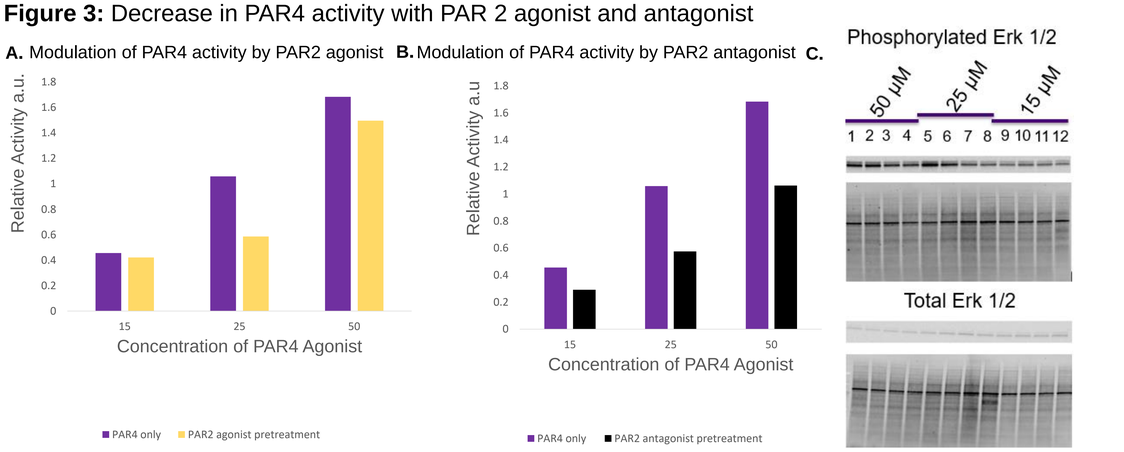


Figure 3: Decrease in PAR4 activity with PAR 2 agonist and antagonist



Materials and Methods

Cell culture: Following established procedures, thoracic aorta VSMCs were harvested from male Sprague-Dawley rats (100-125 grams) and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) and Primocin (100mg/L) at 37ºC in 95% air/5% CO_2. Cells were split and propagated through passage 11.

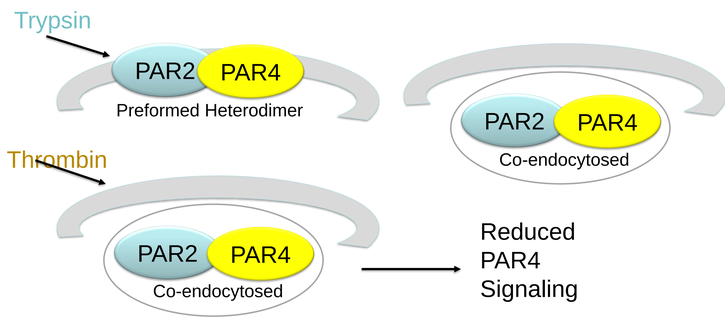
Western Blotting: Following cell lysis, samples were snap frozen in -80˚C overnight. Following BCA protein quantification, 12 µg protein were loaded into wells of Criterion™ TGX (Tris-Glycine eXtended) Stain-Free™ precast gels. Proteins were separated at 200V for 45-50 min. Proteins were transferred to PVDF membranes and placed in 5% dry milk to block non-specific binding. To visualize immunoblot, ECL was incubated for 5 minutes and then placed into the ChemiDOCIT system.

Injury Model: Sprague-Dawley rats (400-450 grams) were anesthetized and their left common carotid artery was ligated proximal to the bifurcation. A 2 French balloon catheter was inserted and used to mechanically damage the luminal lining of the vessel. Thirty minutes after injury animals were euthanized and ipsilateral left and uninjured right carotid arteries were harvested for evaluation of PAR2/4 expression and activity.

Conclusions

- PAR2/PAR4 are involved in the growth response to arterial injury.
- Pharmacologic modulation of PAR2 decreases PAR4 activity in primary VSMCs.
- Based on these observations, a PAR2/PAR4 partnership in VSM is implicated for control of vascular growth during cardiovascular disorders.

Future Directions



- Evaluate dimerization and/or endocytic feedback mechanisms in fractionated tissue and cell samples
- Determine expression and activity of PAR2/PAR4 during adaptive remodeling after arterial injury
- Characterize the pathological VSM growth through migration studies
- Characterize the biological relationship and inter-dependency between PAR2 and PAR4
- Assess the biological impact of PAR2 on PAR4 activity using florescent intracellular calcium assays

Acknowledgements

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Figure 1: Western blot analyses of PAR2/PAR4 expression and PAR activity 30 min post-balloon injury. Uninjured (naïve) and balloon-injured common carotid arteries were homogenized 30 min following injury and tissues were lysed and protein extracted. **A:** Western blot analysis yielded a significant ($p < 0.05$) increase in PAR2 expression compared to naïve vessels (n=3/group). **B.** Western blot shows an increase in PAR4 expression compared to naïve tissues (n=3/group). **C.** Indirect readout of PAR2/PAR4 activity through phosphorylated/total Erk1/2 30 min after injury. Data suggest that activity of PAR2/PAR4 was higher following injury compared to naïve vessels, implying a role for PAR2/PAR4 in the VSM proliferative response to in vivo arterial injury.

Figure 1: A. Vascular smooth muscle cells (VSMCs) were treated for 10 - 60 min with varying concentrations of the synthetic PAR2 agonist SLYGRL-NH2, and activity of PAR2 was assessed through phospho/total Erk1/2 after 10 min. **B.** VSMCs were incubated with varying concentrations of the synthetic PAR2 antagonist FSLLRY-NH2 for 60 min prior to addition of 10 μM SLYGRL-NH2 for 10 min. Cells were collected, lysed, and Erk phosphorylation was evaluated. Relative reductions of 17%-77% in phospho/total Erk1/2 were observed. **C.** Cells were treated for 10 - 60 min with varying concentrations of the synthetic PAR4 agonist AY-NH2, and PAR4 activity was assessed through phospho/total Erk1/2 after 10 min.

Figure 2: A. VSMCs were pretreated with the PAR2 agonist SLYGRL-NH2 (10 μM, 10 min) 60 min prior to addition of the synthetic PAR4 agonist AY-NH2 at varying concentrations. Cells were collected, lysed, and phosphorylated/total Erk1/2 was evaluated and compared to groups that received sole PAR4 agonist. Activity was made relative to control cells that received no PAR4 agonist. **B.** Cells were incubated with the PAR2 antagonist FSLLRY-NH2 (10 μM , 60 min) prior to addition of PAR4 agonist. Cells were collected, lysed, and phosphorylated and total Erk1/2 was evaluated and compared to groups that just received PAR4 agonist. Activity was made relative to control cells that received no PAR4 agonist. **C.** Immunoblot images of Western blots for phosphorylated Erk1/2, total Erk1/2, and total protein.