Kinase Inhibitor Specificity in VEGF-activated Primary Human Retinal Microvascular Endothelial Cells

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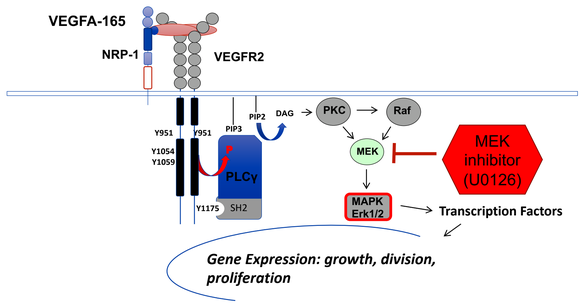
Introduction

- Diabetic Retinopathy is among one of the most common diseases to cause blindness.[1]
- Vascular Endothelial Growth Factor A 165b-isoform (VEGFA-165b) is necessary for normal retina health. In diseases like Diabetic Retinopathy, there is a switch from a prevalence of the 165b-isoform to the 165a isoform.
- The Mitton lab elucidates the mechanisms of $VEGFA_{165}$ isoform activity in primary Human Retinal Microvascular Endothelial Cells (HRMEC's). Kinase inhibitors are valuable research tools but most researchers fail to confirm kinase dose-specificity. We characterized two popular inhibitors of the AKT and MEK1/2 pathway.

Aims and Objectives

- How does the inhibition of either or both MAPK and AKT signaling pathways affect VEGF impact on human retinal endothelial cell migration and proliferation? To what extent do two isoforms of VEGFA (VEGFA-165 and VEGFA-165b) use these two pathways to cause cell growth and proliferation
- To compare proliferation and migration of Human Retinal Endothelial Cells (HRECs) stimulated by equal amounts of VEGFA-165 and VEGFA-165b.
- To use inhibitors of MAPK activation and AKT activation to discover the contributions of each pathway to HREC's migration and proliferation.



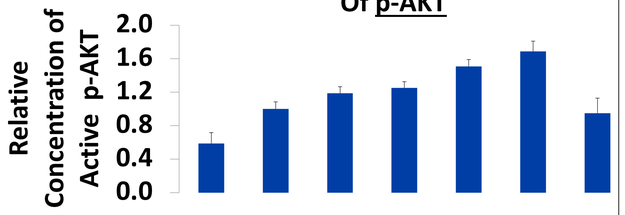
Methods

- An In-cell western blot with a 96 well plate setup was used. Primary human retinal proliferative endothelial cells were used. they were seeded in 96 wells, grown in 5% FBS (HC) Endogrow for 3 days. 3000 cells were seeded in each well. There were about 20,000 cells per well once confluence was reached. Each well was pretreated with either an inhibitor (of various concentrations) or media alone. A control was included as well that had no primary antibody. The inhibitor (AKT or MEK1/2 inhibitor)[3] was added first for 30 minutes and then a solution of inhibitor (various concentrations) and VEGFA165a (5000 pM) for another 10 minutes. Then the cells were fixed, permeabilized and blocked. Then the primary and secondary antibodies were added. The primary antibody is p-AKT, ser473 by Cell signaling (catalog # 4058). The secondary antibody was goat anti-rabbit IRdye 800 (CW). Then the plates were scanned with an odyssey classic imager to measure the fluorescence levels. The in situ label targets only the active forms of these kinases. The VEGF concentrations were chosen to ensure activation based on our known previous dose-response studies.[4]

Results

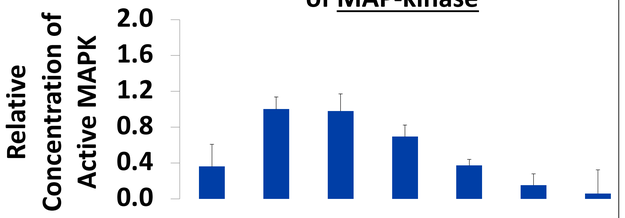
- All concentrations of the AKT inhibitor MK2206(ED50=0.01uM) decreased VEGFA165 activation of AKT. MK2206 (10 um) caused significant activation of p38-MAPK by 38%.
- U0126 (inhibitor of ERK1/2 activation) significantly decreased VEGFA165 induced activation of ERK1/2 and p38-MAPK while increasing VEGFA165 mediated activation of AKT over a range of doses from 0.1-10uM(ED50=0.3uM).

U0126 (MEK inhibitor) Effects on VEGFA165a Dependent Activation Of p-AKT



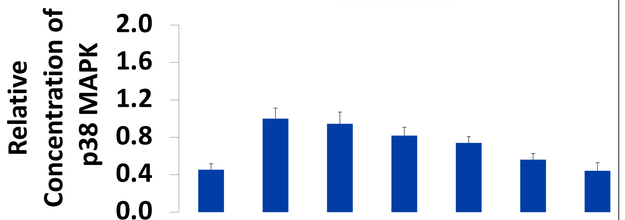
$VEGFA_{165}a$	-	+	+	+	+	+	-
U0126 (pM)	-	-	.01	.1	1.0	10	10
Significant (p<0.05)	n/a	n/a		*	*	*	

U0126 (MEK inhibitor) Effects on VEGFA165a Dependent Activation of MAP-kinase



$VEGFA_{165}a$	-	+	+	+	+	+	-
U0126 (pM)	-	-	.01	.1	1.0	10	10
Significant (p<0.05)	n/a	n/a		*	*	*	*

U0126 (MEK inhibitor) Effects on VEGFA165a Dependent Activation of p38 MAPK



$VEGFA_{165}a$	-	+	+	+	+	+	-
U0126 (pM)	-	-	.01	.1	1.0	10	10
Significant (p<0.05)	n/a	n/a		*	*	*	*

Conclusions

- While commonly used for inhibition of ERK1/2 activation, U0126 has strong effects on AKT activation when used at doses to completely block ERK1/2 activation.
- U0126 is best used at 0.5uM to significantly inhibit ERK1/2 activation with minimal effects on AKT activation.
- The AKT pathway inhibitor MK2206 can be used at levels below 10um without significantly affecting off-target pathways (ERK1/2 and p38-MAPK). Levels of 0.1 uM are sufficient to reduce VEGFA 165a induced AKT to basal levels.

References

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3. Burkhard K, Shapiro P. Use of inhibitors in the study of MAP kinases. *Methods Mol Biol*. 2010;661:107-122. doi:10.1007/978-1-60761-795-2_6
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