Inhibition of Src but not MEK/ERK Signaling Supports HUVEC Monolayer Integrity

Amanda Mauro, Nauman Khurshid, MD, Ian Bird, PhD, Derek Boeeldt, PhD.
Dept. Ob/Gyn, Perinatal Research Laboratories, UW-Madison, Madison, WI 53715

Abstract

Endothelial dysfunction is a hallmark of preeclampsia (PE), a pregnancy-specific syndrome characterized by hypertension, for which there is currently no treatment available. Dysfunctional endothelial cells present with disrupted Ca2+ signaling and weakening of cell-cell connections, resulting in decreased nitric oxide production (reduced vasodilation) and monolayer breakdown (vascular leakiness), in PE, inappropriate levels of circulating factors have been reported. We have shown that growth factors and cytokines such as VEGF, bFGF, EGF and TNFα inhibit Ca2+ signaling capacity in endothelial cells, via Src and ERK signaling. To investigate a more complete picture of endothelial dysfunction we experimentally applied these results to monolayer integrity, hypothesizing that treatment with growth factors and cytokines will negatively impact the monolayer and inhibition of Src and/or ERK will offer relief.

Human umbilical vein endothelial cells (HUVECs) were grown to confluence on 96 well plates for monolayer integrity assessment of electrical resistance across the monolayer by electric cell-substrate impedance sensing (ECIS). A 3 hour serum withdrawal allowed resistance to recover to pre-serum starve levels before pretreatment with PP2 or U0126. 30 minutes later, 10ng/mL VEGF, bFGF, EGF and TNFα treatments were added and the experiment continued for 24 hours. Statistical analysis was by students t-test.

VEGF treatment led to an early increase in resistance (2.15hr, p<0.05) which was brought closer to control with PP2 pretreatment. Treatment with bFGF resulted in a continuous rise in resistance (1hr-24 hr, p<0.05), which was steadied with PP2 pretreatment. EGF treatment was not significantly different from control, but PP2 pretreatment lead to a modest increase in resistance (2.5-6.4, 6.7-24hr p<0.05). Treatment with TNFα resulted in decreased resistance (-4.3-24 hr, p<0.05) which returned to control levels at 22 hours with PP2 pretreatment. Across the board, U0126 pretreatment significantly reduced resistance.

For endothelial monolayer integrity there is no broad negative effect for growth factor and cytokine treatment, as we have shown is the case for Ca2+ signaling. The opposite occurs for VEGF and bFGF which instead provide support for the monolayer. TNFα is inhibitory for both monolayer integrity and Ca2+ signaling, PP2 is consistently a positive influence on monolayer integrity, offering stability if not rescue, whereas U0126 is consistently deleterious to monolayer integrity in HUVECs. This further highlights Src inhibition as a viable strategy for combating the endothelial side of PE, as Src inhibition offers increases in Ca2+ signaling and stability to the monolayer, while also revealing a role for MEK/ERK as an important pathway for support of the monolayer.

Methods

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were collected from vessel segments harvested from umbilical cords from normal pregnancies within 30 minutes of delivery. Cells were harvested after passage 2 and used for imaging at passage 3. The Institutional Review Boards of the University of Wisconsin Hospital and Clinics and Mariner Hospital (both located in Madison, WI) have approved this study. Patients meeting the inclusion criteria for the study were identified by the nurses and house staff and patients were consented for the study. The patient was provided with a written consent and verbal description of the study.

Monolayer Integrity

HUVECs were seeded into gelatin-coated, stabilized 96 well plate for resistance measurement by ECIS and grown to confluence. Complete media was removed and replaced with serum free media for ~3 hours. Cells are pretreated with 10uM PP2, U0126, or CLA Mix 30 minutes before treatment with 10ng/mL EGF, bFGF, TNFα, or VEGF for 24 hours after growth factor/cytokine treatment the experiment is stopped.

Background

Inhibition of Src but not ERK induced ATP-adenized Ca2+ bursts in growth factor or cytokine treated HUVECs. HUVECs treated with 10ng/mL EGF, TNFα, and bFGF were also increased (p<0.05), but there was no significant increase with the addition of PP2 or U0126. HUVECs pretreatment with 10ng/mL EGF was increased (p<0.05), but there was no significant increase with the addition of PP2 or U0126. HUVECs pretreatment with 10ng/mL TNFα was increased (p<0.05), but there was no significant increase with the addition of PP2 or U0126. HUVECs were pretreated with 10ng/mL bFGF and TNFα for 24 hours, followed by an ATP burst assay. HUVECs monolayer integrity was measured using ECIS (100kΩ). To enable statistical analysis, Ca2+ bursts were measured to those with a stable shift in AEIS for 1 min on average of 10-50 Ω. HUVECs monolayer integrity was measured using ECIS (100kΩ). To enable statistical analysis, Ca2+ bursts were measured to those with a stable shift in AEIS for 1 min on average of 10-50 Ω.

Results

• VEGF maintained monolayer
• Significantly improved monolayer integrity 2-5 hours after treatment (p<0.05) at 10ng/mL
• bFGF drastically improved monolayer integrity
• 10ng/mL dose continually increased resistance, significant beginning at hour 6 (p<0.05)
• 1ng/mL treatment led to a more modest but still significant increase hours 9-22 (p<0.05)
• EGF exerted a modest but significant decrease in monolayer integrity at only the 10ng/mL dose beginning at hour 5 (p<0.05)
• TNFα had a negative effect on monolayer in a dose-dependent manner
• 10ng/mL treatment led to a significant decrease in resistance beginning at hour 4 (p<0.05)
• 1 ng/mL treatment led to a decrease in resistance hours 7-18 (p<0.05)
• PP2 supported the monolayer
• When used alone increased resistance hours 14-24 (p<0.05)
• When used with VEGF, EGF, bFGF it kept resistance close to control (until hour 18 for bFGF - p<0.05)
• When used with TNFα it was able to rescue the monolayer from TNFα insult, with resistance statistically not different from control hours 17-24 (p<0.05)
• U0126 reduced a negative effect on the monolayer (p<0.05) beginning at 7 hours when used alone, around 2-4 hours with VEGF, EGF and TNFα, although bFGF was able to balance this out compared to the other factors

Conclusions

• Treatment with bFGF and TNFα results in drastic changes to monolayer integrity at the 10ng/mL dose, with less of an effect seen as dose decreases
• Treatment with VEGF or EGF, although significantly at times, had a marginal impact on the monolayer
• Src inhibition offers monolayer stability overall, and offers rescue in the case of TNFα-mediated insult
• MEK/ERK Inhibition generally had a negative impact on the monolayer, except in the case of bFGF
• Because Src inhibition has proven to improve Ca2+ bursting and stabilize the monolayer it could be viable potential strategy for combating endothelial dysfunction in PE

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