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Differential Activation of Multiple Signalling Pathways Dictates eNOS Upregulation by FGF2 but not VEGF in Placental Artery Endothelial Cells

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Abstract

Fibroblast growth factor (FGF2), but not vascular endothelial growth factor (VEGF), upregulates endothelial nitric oxide synthase (eNOS) protein expression, at least partially, via activation of extracellular signal-regulated kinase 2/1 (ERK2/1) in ovine fetoplacental artery endothelial (oFPAE) cells. Herein we further investigated the temporal effects of FGF2 and VEGF on other signalling pathways including members (Jun N-terminal kinase JNK1/2 and p38MAPK) of mitogen-activated protein kinases (MAPK), phosphatidylinositol-3 kinase/*v*-akt murine thymoma viral oncogene homologue 1 (PI3K/AKT1), and the tyrosine kinase c-SRC, and examined if either one or more of these pathways play a role in the differential regulation of eNOS by FGF2 and VEGF. We first confirmed that in oFPAE cells, FGF2, but not VEGF, increased eNOS protein. FGF2 stimulated eNOS protein in a time- and concentration-dependent manner, which also depended on cell density. FGF2 provoked sustained (5 min to 12 h) whereas VEGF only stimulated transient (5 min) ERK2/1 phosphorylation. FGF2 was 1.7-fold more potent in stimulating ERK2/1 phosphorylation than VEGF. FGF2 and VEGF only transiently activated JNK1/2 and AKT1 within 5 min; however, FGF2 was a stronger stimulus than VEGF. FGF2 and VEGF did not significantly activate p38MAPK at 5 min; however, VEGF stimulated p38MAPK phosphorylation at 60 min. VEGF but not FGF2 significantly stimulated c-SRC phosphorylation. Inhibitors of MEK-ERK2/1 (PD98059), JNK1/2 (SP600125) and PI3K (wortmannin), but not p38MAPK (SB203580) and SRC (PP2), decreased the FGF2-increased eNOS protein expression. Thus, the FGF2-induced eNOS protein expression requires activation of multiple signalling pathways including ERK2/1, JNK1/2 and PI3K/AKT1. Differences in intensity and temporal patterns of activation of these pathways by FGF2 and VEGF may account for their differential effects on eNOS expression in OFPAE cells.
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Keywords: FGF2; VEGF; Signalling pathways; eNOS protein; Endothelial cells; Placenta

1. Introduction

Normal pregnancy is associated with dramatic increases in uteroplacental and fetoplacental blood flows directly correlating with fetal growth, survival and neonatal outcomes [1]. During the third trimester of gestation, a substantial rise in placental blood flow is believed to result from angiogenesis and vasodilatation, of which both are controlled by locally and systematically produced factors [2,3]. During late human and ovine pregnancy, the maternal and fetal compartments produce large quantities of angiogenic factors, including fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor (VEGF) [1,4]. Increased FGF2 and VEGF expressions have been implicated in placental angiogenesis at a time when fetal weight and blood flows increase exponentially at the maternal–fetal interface during pregnancy [1–3]. Concomitantly, production of the potent vasodilator nitric oxide

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(NO) is dramatically increased in association with enhanced vascular endothelial expression of endothelial NO synthase (eNOS) within the uteroplacental and fetoplacental vascular beds [1,5]. For example, in accordance with increased NO production, expression of eNOS protein in ovine fetoplacental and uterine arteries increases 3.5-fold from days 110 to 130 of gestation [6]. Inhibition of NO production leads to fetal growth restriction via decreases in fetoplacental blood flow [7]. Thus, complex interplays between angiogenic factors and the eNOS–NO system apparently play a critical role in the regulation of placental angiogenesis and blood flow [1–3].

Increased NO production during pregnancy is, at least in part, due to upregulation of endothelial eNOS protein by angiogenic factors [1,6]. Many *in vitro* studies have shown that both FGF2 and VEGF increase eNOS expression in various endothelial and non-endothelial cells [8–10]. In ovine fetoplacental artery endothelial (oFPAE) cells we have previously reported that FGF2, but not VEGF, upregulates eNOS expression, at least partially, via activating mitogen-activated protein kinase (MAPK)–extracellular signal-regulated kinases (ERK2/1) [11]. However, whether other signalling pathways are involved and why FGF2 but not VEGF stimulate oFPAE cell eNOS expression are currently unknown.

Gene expression regulation upon FGF2 and VEGF stimulation is initiated by ligand binding to their respective specific tyrosine kinase receptors resulting in activation of signalling networks that regulate nuclear transcription, *de novo* protein synthesis, and/or protein degradation. In oFPAE cells, FGF2 and VEGF activate common pathways, including phosphatidylinositol 3 kinase/v-akt murine thymoma viral oncogene homologue 1 (PI3K/AKT1) and ERK2/1 [11,12]. ERK2/1 activation is necessary but not sufficient for eNOS upregulation by FGF2 in oFPAE cells [11]. In various endothelial cells, many signalling pathways have been shown to mediate eNOS upregulation by various stimuli [9–11,13–18]. Among them, ERK2/1 and/or PI3K/AKT1 mediate the upregulation of eNOS by most stimuli including VEGF, FGF2, oestrogen, and insulin [10,11,13,17].

Both FGF2 and VEGF activate many other MAPK pathways (Jun N-terminal kinase JNK1/2 and p38MAPK) and c-SRC tyrosine kinase in a variety of endothelial and non-endothelial cells [19,20]. Of note, they may activate the same signalling pathway but potentially with different intensities as well as with unique spatial and temporal activation patterns in one cell type or different signalling pathways in parallel in another cell type. More recently, we have shown that a complex signalling cross-talk among ERK2/1, AKT1 and endogenous NO is critical for FGF2- and VEGF-induced oFPAE cell proliferation [12]. In this study, we hypothesised that differential activation of these pathways dictates why FGF2, but not VEGF, stimulates eNOS expression in oFPAE cells. Our objectives were to determine the temporal effects of VEGF and FGF2 on multiple signalling pathways (ERK2/1, JNK1/2, p38MAPK, PI3K/AKT1 and c-SRC) and to delineate their role(s) in the differential regulation of eNOS protein expression by FGF2 but not VEGF in placental endothelial cells.

2. Materials and methods

2.1. Materials and reagents

Human recombinant FGF2 157aa and VEGF 165aa, both carrier free, were purchased from R & D systems (Minneapolis, MN), diluted to a stock concentration of 10 μg/ml in sterile PBS with 0.1% bovine serum albumin (BSA) and stored at −70 °C until use. Mouse monoclonal antibodies (mAb) against eNOS and panERK2/1 were from BD Biosciences (San Jose, CA) and β-actin mAb was from Ambion (Austin, TX). Rabbit polyclonal antibodies (pAb) against phospho-ERK2/1 (Thr202/Tyr204), phospho-JNK1/2 (Thr183/Tyr185), phospho-p38MAPK (Thr180/Tyr182), phospho-AKT1 (Ser473), AKT1, and phospho-c-SRC (Tyr416) were from Cell Signaling (Danvers, MA). Rabbit pAbs against p38MAPK and c-SRC were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

The following pharmacological inhibitors were from Sigma (St. Louis, MO): PD98059 (MEK1/ERK/12), SP600125 (JNK1/2), SB203580 (p38MAPK), wortmannin (PI3K/AKT1) and PP2 (c-SRC). All inhibitors were dissolved in DMSO at a stock concentration of at least 2000× of the final treatment concentration to avoid adding vehicle (DMSO) more than 0.05% into the treatment media, which did not affect eNOS protein expression and phosphorylation of signalling molecules tested (data not shown). All cell culture medium and reagents were from Invitrogen/Gibco (San Diego, CA) and all other chemicals were from Sigma (St. Louis, MO), unless indicated elsewhere.

2.2. Cell culture, experimental conditions and preparation of total cell extracts

Four primary oFPAE cell preparations were isolated by collagenase digestion from 2nd degree fetoplacental arteries obtained from late pregnant sheep placentas (D120–130, term ~145) and validated before use as previously described [21]. All cells were tested positive for the presence of FGF receptor 1 (FGFR1), VEGF receptor 1 (fms-related tyrosine kinase 1 or Flt1) and VEGFR2 (kinase insert domain receptor or KDR), eNOS and von-Willebrand factor. The animal use protocol was approved by the University of California San Diego Animal Subjects Committee, and we followed the National Research Council's Guide for the Care and Use of Laboratory Animals throughout the study. Cells were grown in MCDB 131 supplemented with 10 mM L-glutamine, 10% FBS and 1% antibiotics. All cells used in this study were at passages 8–10. Before cell stimulation, subconfluent cells were serum starved in treatment medium (Phenol red-free M199 containing 0.1% BSA, 25 mM HEPES, 1% FBS and 1% antibiotics) for 18 h. The medium was then replaced with fresh treatment media. Inhibitors or growth factors were added for the time periods as described in the figure legends. Concentrations of MAPK and PI3K inhibitors used did not cause significant cytotoxicity in oFPAE cells (<10% cytotoxicity). Cell stimulation was terminated by aspiration of the medium. To harvest total protein extracts, cells were lysed in non-denaturing lysis buffer and kept on ice for 15 min. The extracts were vortexed and clarified by centrifugation (13,000 × *g*, 5 min). The protein content was measured by a Bradford procedure using BSA as the standard [22].

2.3. Cell density/proliferation assay

To study cell density effects on eNOS expression, cells were seeded at 200,000, 400,000, and 600,000 cells per 60 mm dish and allowed to attach and recover for 24 h before treatment in order to generate cell confluences of approximately 50%, 70% and 90% at the time of cell stimulation. Complete media was then replaced with starvation media with or without FGF2/VEGF (10 ng/ml) for an additional 24 h. The cells were trypsinised and counted using a haemocytometer to determine mitogenic responses. Additional cells in 60-mm dishes were plated and treated similarly and protein levels of eNOS and β-actin were measured at 24 h of growth factor stimulation.

2.4. SDS–PAGE and immunoblotting

Western blotting was performed as previously described [12,22]. Briefly, the protein samples were heat denatured in Laemmli buffer, separated on