Hyperinsulinemia Stimulates Angiogenesis of Human Fetoplacental Endothelial Cells: A Possible Role of Insulin in Placental Hypervascularization in Diabetes Mellitus

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Context: The insulin/IGF system regulates fetal and placental growth and development. In a pregnancy complicated by maternal diabetes, placentas are hypervascularized and fetal insulin levels are elevated. In the fetal circulation, insulin can act on the placenta through insulin receptors present on the fetoplacental endothelial cells.

Objective: We hypothesized that insulin exerts proangiogenic effects on the fetoplacental endothelial cells, thereby contributing to the placental hypervascularization in diabetes.

Design: The effect of insulin on angiogenesis and proliferation of human fetoplacental endothelial cells was investigated by a 2-dimensional network formation assay, staining for actin fibers, automatic cell counting, and cell cycle analysis. The signaling pathways involved were identified using antibodies against activated signaling proteins and pharmacological inhibitors.

Results: Insulin enhanced network formation by 23% (P < .05%) and caused actin reorganization. Insulin stimulated (P < .05) phosphorylation of insulin receptor (+320%), and insulin receptor substrate-1 (+140%), Akt (+177%), glycogen-synthase kinase-β3 (+70%), and endothelial nitric oxide synthase (eNOS; +100%) increased nitric oxide production and activated Ras-related C3 botulinum toxin substrate 1 (Rac1). Insulin did not induce ERK1/2 phosphorylation or proliferation. Inhibition of phosphatidylinositol 3-kinase, eNOS, and Rac1 signaling abolished the effects on network formation.

Conclusions: Elevated fetal insulin levels may contribute to the placental hypervascularization in diabetes via the phosphatidylinositol 3-kinase/eNOS pathway and involve Rac1. However, insulin does not stimulate proliferation and may need to cooperate with other growth factors.

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The insulin/IGF system is indispensable for proper fetal and placental growth and development (1, 2). In pregnancies complicated by maternal diabetes, the placenta undergoes alterations in gene expression, morphology, and function (3–6). As a consequence of maternal and the ensuing fetal hyperglycemia, fetal insulin levels are increased. The fetoplacental vessels are in contact with the fetal circulation and thus can be affected by altered hormone and growth factor levels in fetal blood (7). The fetoplacental endothelium is rich in insulin receptors, en-
able fetal insulin to exert a direct effect on it (2). Placental hypervascularization resulting from increased angiogenesis is one of the features of placentas in diabetes (1, 5, 6, 8). Circumstantial evidence suggests a contribution of fetal insulin: changes in hypervascularization are more pronounced when fetuses are strongly hyperinsulinemic (9), and placental capillary length correlates with fetal insulin levels (10). However, the role of insulin in placental vascularization and hypervascularization has not yet been determined.

The development of new blood vessels is a complex process that depends on interactions of endothelial cells (ECs) with the surrounding environment, such as growth factors and basement membrane (11). Angiogenesis comprises a series of biological events including lamellipodia formation, cell motility, changes in cell shape, adhesion, and differentiation. All of them depend on cytoskeletal rearrangements, mostly driven by activation of several members of the RhoGTPases family (12) including the Ras-related C3 botulinum toxin substrate 1 (Rac1). Rac1 plays an essential role in ECs by activating downstream signaling effectors for actin reorganization (13).

Endothelial nitric oxide synthase (eNOS) is another key player in endothelial function. Participation of eNOS in the development of vascular trees by promoting angiogenesis was demonstrated in eNOS-deficient mice, which showed defective development of the lung vasculature (14). Endothelial NOS catalyzes the conversion of L-arginine to L-citrulline, generating the endothelium-derived vasodilator nitric oxide (NO) (15, 16), which is essential for the activity of proangiogenic mediators, such as vascular endothelial growth factor (VEGF) and angiopoietin, to modulate angiogenesis (17, 18). Akt/protein kinase B plays a central role as upstream regulator and consequently activation of eNOS. It phosphorylates eNOS at Ser1177, leading to a calcium-independent eNOS activation (19). Akt activity per se is induced after the activation of phosphatidylinositol 3-kinase (PI3K) in growth factor signaling effectors for actin reorganization (13).

Lately glycogen-synthase kinase (GSK)-3β was identified as a further key player in angiogenesis by controlling EC migration and differentiation by β-catenin stabilization (22) and by stimulating cell cycle progression (23).

Here we hypothesized that insulin regulates angiogenesis in fetoplacental ECs. If proven true, fetal hyperinsulinemia would contribute to placental hypervascularization in diabetic pregnancies. Therefore, we investigated whether and by which pathways insulin can stimulate proliferation and in vitro network formation, both biological processes involved in angiogenesis, in primary human fetoplacental ECs isolated from third-trimester placentas. These cells keep their endothelial phenotype in culture and thus are an excellent tool for in vitro studies (24).

Materials and Methods

Isolation and culture of third-trimester human fetoplacental ECs

The study was approved by the Ethical Committee of the Medical University of Graz. Informed consent of the patients was obtained. Primary fetoplacental ECs were isolated from third-trimester human placentas as described (24). In brief, arterial chorionic blood vessels were dissected, washed with Ca2+-, Mg2+-free Hanks’ balanced salt solution (HBSS; Gibco). Subsequently cells were isolated by perfusion of the arteries with HBSS containing 0.1 U/mL collagenase, 0.8 U/mL dispase (Roche), and antibiotics (Gibco), prewarmed to 37°C for 7 minutes. Cells were centrifuged (200 g for 5 min), resuspended in endothelial basal medium (EBM; Clonetics; Lonza) supplemented with the EGM-MV BulletKit (Clonetics; Lonza) containing gentamicin/amphotericin, hydrocortisone, human epidermal growth factor (EGF), bovine brain extract, and 5% fetal calf serum (FCS) and plated on culture plates precoated with 1% gelatin. This medium was also used for cell expansion. All cell preparations were subjected to immunocytochemical characterization for identity, purity, and functionality (24). Cells were used up to passage 7. For experiments, different concentrations of FCS, but no further supplements, were added. Cells were grown at 37°C and 21% oxygen.

Treatments

For the experiments, insulin (Calbiochem) or VEGF (Sigma) was added to a final concentration of 10 nM and 650 pM, respectively. HBSS was used as vehicle control. Both insulin and VEGF concentrations are 100-fold higher than the physiological levels in cord blood (25, 26). At 10 nM, insulin will activate the insulin receptor (EC50 0.9 nM) but not the IGF-I receptor (EC50 > 30 nM) to a significant extent (27).

Insulin stimulation of angiogenesis

Angiogenesis was analyzed by a 2-dimensional (2D) Matrigel network formation assay. Fetoplacental ECs were detached with trypsin/EDTA (Clonetics; Lonza) from 75-cm2 flasks, centrifuged, and resuspended in EBM containing 2% FCS. Insulin, VEGF, and HBSS were added and cells (8000/well) were seeded on 96-well plates precoated with 50 μL of growth factor-reduced Matrigel (BD Biosciences) according to the manufacturer’s instructions, incubated for 24 hours, and monitored in real time in a Zeiss Cell Observer microscope (Carl Zeiss) with an AxioCam HRm camera and an A-Plan ×5/0.12 Ph0 objective using the AxioVision software (Carl Zeiss). Images of the 2D networks were taken hourly and quantified [total tube length (microns), number of branching points, and number of meshes] using the AngioJ-Matrigel assay plug-in (28), which was developed and adapted for the ImageJ software (National Institutes of Health) by Diego Guidolin (University of Padova, Padova, Italy).
Insulin effect on proliferation and cell cycle

Fetoplacental ECs (300,000 per 75 cm² flasks) were cultured in EB containing 5% FCS for 24 hours. Then insulin, VEGF, and HBSS were added and cells were incubated for another 24 hours. For cell cycle analysis, 5-bromo-2’-deoxyuridine (BrdU; BD Biosciences) was added to a final concentration of 10 μM, and cells were incubated for 2 hours, harvested with trypsin/EDTA, and centrifuged at 170 × g for 6 minutes. Cells were fixed, permeabilized, and processed following the instructions of the fluorescein isothiocyanate BrdU flow kit (BD Biosciences). Samples were analyzed using a BD FACS Calibur system (BD Biosciences) with BD CellQuest Pro software (BD Biosciences). For measuring proliferation and viability, cells were harvested after 24 hours of treatment, resuspended in 10 mL fully supplemented EB, and counted using CASY1 (Scherfe System) as described (29). This system allows determination of viable and dead cells in one measurement run.

Insulin-stimulated signal transduction

Fetoplacental ECs (180,000 cells/well) were seeded in 6-well plates and cultured for 12 hours in EB containing 5% FCS, followed by 6 hours of serum starvation and stimulation with insulin, VEGF, or HBSS for 2, 5, 10, and 15 minutes. After stimulation, plates were placed on ice. For the phospho-multiplex assay, protein was isolated, concentrations measured, and phosphorylation of the insulin signaling components determined using the Bio-Plex cell lysis kit, DC protein assay, Bio-Plex phosphoprotein singleplex assays for phosphorylated forms of Akt (Ser473), ERK1/2 (Thr202/Tyr204, Thr185/Tyr187), GSK3 (Ser21/Ser9), insulin receptor-β (Y1146), and insulin receptor substrate (IRS)-1 (Y612, Y632, Y636, Y639), and the Bio-Plex phosphoprotein detection kit (Bio-Rad Laboratories) according to the manufacturer’s instruction. The multiplex assay allows simultaneous quantification of the phosphoproteins in one single well in a 96-well format. The assays were run in triplicates of three independent experiments with three different cell isolations. The values were normalized to total protein.

The eNOS and GSK3α/β phosphorylation after insulin stimulation was determined by immunoblotting as described (29). Membranes were incubated overnight at 4°C with antibodies against pSer1177-eNOS (1:500; Cell Signaling), pSer21/Ser9GSK3 (1:10,000; Cell Signaling), or pSer1177-eNOS (1:5000; Cell Signaling). After washings, membranes were incubated 1 hour at room temperature with the secondary antibodies (Bio-Rad Laboratories) goat antirabbit (1:1000, for phospho-eNOS and phospho-GSK3) or goat antimouse (1:7000, for β-actin). Signals were normalized to β-actin.

Intracellular NO levels

Transient intracellular NO levels were determined using the fluorescent probe 4-aminomethylmethylene-2’,7’-difuorofluorescein (Sigma) as described (30). Fetoplacental ECs (20,000 cells/well) were seeded in 96-well black plates in 100 μL EB with supplements and incubated overnight. Cells were then serum starved in EB without supplements for 5 hours. Diaminofluorescein-FM diacetate (2 μM) was added together with L-arginine (100 μM) for 30 minutes. Thereafter insulin or HBSS was added for 15 minutes and fluorescence intensity was measured using a fluorimeter (FLUOstar OPTIMA; BMG Labtech) with 485 nm excitation and 520 nm emission wavelength.

Rac1 activity assay

Rac1 activation by GTP was measured using the luminescence based G-LISA Rac1 Activation Assay Biochem Kit (Cyto-skeleton) according to the manufacturer’s instruction. Fetoplacental ECs (150,000 cells/well) were seeded in gelatin-coated 6-well plates in fully supplemented EB. After 24 hours, the medium was replaced by EB supplemented with 0.5% FCS for 8 hours, followed by complete starvation in EB without supplements overnight. Cells were then treated for 30 minutes with insulin or HBSS. Nonstarved cells served as positive control for normal F-actin distribution. In the Rac1 activity assay, equal amounts of protein were loaded on the assay plates, which are coated with anti-GTP-Rac1 antibodies. The amount of captured activated, ie, GTP bound Rac1, was quantified by incubation with Rac1 antibodies and subsequent horseradish peroxidase-conjugated secondary antibodies. The luminescence signal was then measured in a microplate reader (FLUOstar OPTIMA; BMG Labtech).

Pathway analysis of insulin-induced angiogenesis using pharmacological inhibitors

Prior to insulin treatment, detached cells were treated for 1 hour with inhibitors for PI3K (wortmannin, 100 nM; Calbiochem), eNOS [N-omega-nitro-L-arginine methyl ester (LNAME), 400 μM; Sigma], Rac1 activation (NSC23766, 200 μM; Calbiochem), and dimethylsulfoxide (vehicle; Merck). Insulin, VEGF, HBSS, and the NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP; 100 μM; Sigma) were then added. Subsequently cells (8000/well) were used for the angiogenesis assay as described above. To demonstrate the blocking effect of NSC23766 on Rac1 activation, an immunoblot with antibodies against the active Rac1-GTP (1:750, mouse; Abcam) was performed as described above after pretreatment with NSC23766 in the presence or absence of insulin.

Insulin stimulation of actin reorganization and F-actin immunofluorescence staining

Fetoplacental ECs (50,000 cells/well) were seeded in 1% gelatin-coated chamber slides in EB containing 5% FCS. After 12 hours, medium was replaced by EB containing 0.5% FCS. After 24 hours cells were serum starved for another 24 hours in EB alone. Insulin or HBSS was then added for 30 minutes. Starved cells treated for 30 minutes with 20 ng/mL EGF, and nonstarved cells were used as positive controls for F-actin distribution (31). All subsequent steps were performed at room temperature and all washings with PBS. Monolayers were washed and fixed with 3.7% formaldehyde in PBS for 10 minutes. After three washings, cells were permeabilized with 0.1% Triton X-100 in PBS for 25 minutes. Slides were washed 3 times and blocked with 1% BSA in PBS for 25 minutes. After washing, slides were incubated with 1 U per 200 μL methanolic phalloidin-Texas Red (Molecular Probes, Invitrogen) for 20 minutes in the dark to stain for F-actin. After 3 further washings, slides were mounted with fluorescent mounting medium (ProLong Gold antifade reagent; Invitrogen) containing 4’,6’-diamin-2-phenylindole to identify nuclei. After overnight drying, F-actin organization was observed in a Zeiss Axioplan fluorescence microscope with ×200 magnification using the AxioVision software (Carl Zeiss Imaging Solutions).
Pathway analysis of insulin effects on actin assembly using pharmacological inhibitors

Fetoplacental ECs (50,000 cells/well) were seeded and starved as described above. Cells were pretreated for 1 hour with inhibitors for Rac1 activation (NSC23766, 200 μM, 29262 M), PI3K (wortmannin, 100 nM), ERK1/2 activation (UO126, 10 μM; Calbiochem), eNOS (L-NAME, 400 μM), and dimethylsulfoxide (vehicle). Cells were incubated for 30 minutes with insulin or HBSS. Nonstarved and starved cells treated with 20 ng/mL EGF for 30 minutes were used as positive controls (31). Cells treated with inhibitors alone served as control for inhibitor effects. After treatments, monolayers were stained for F-actin.

Statistical analysis

Data are expressed as mean ± SD. Raw data for viability, cell number, and BrdU incorporation were tested by Wilcoxon signed rank test. A Student’s t test was applied for the phosphorylation Bio-Plex assays (Bio-Rad Laboratories), NO levels, and Rac1 activity after testing for normal distribution (Kolmogorov-Smirnov test). Two-way ANOVA with Holm-Sidak method as a post hoc test was used for testing the time-course experiments. Significance was accepted when P ≤ .05.

Results

Insulin stimulates angiogenesis in human fetoplacental ECs

To test whether insulin stimulates angiogenesis in primary human fetoplacental ECs, a 2D in vitro network formation assay was performed. VEGF was used as a positive control. Figure 1A shows cells immediately and 6 hours after seeding on Matrigel (BD Biosciences). To analyze in vitro network formation, tube length and branching points were quantified (Figure 1A). Insulin increased the tube length of the network (+21%; P < .05) comparable with VEGF (Figure 1B). Similar effects were found when the number of branching points (+29%; P < .05) (Figure 1C) or meshes were counted (+31% from 73.0 ± 7.6 in the controls to 95.7 ± 7.4 in the insulin treatment; P < .05; not shown).

Insulin does not stimulate cell cycle progression and proliferation of human fetoplacental ECs

Angiogenesis in vivo requires proliferation and insulin is a potent mitogen. However, insulin did not stimulate proliferation after 24 hours, as reflected by the absent increase in viable and total fetoplacental ECs (Figure 2A). Similar results were obtained after 48 hours (not shown). To confirm these data, the insulin effect on cell cycle progression was measured using BrdU incorporation combined with flow cytometry. This allows determining the proportion of cells in the G0/G1, S, and G2/M phases of the cell cycle. Insulin increased (P = .001) the proportion of cells in the S phase by about 24% (Figure 2C) but without changing the proportion of cells in the G2/M phase (Figure 2D). The proportion of cells in the G0/G1 phase was reduced by insulin, but variation of the data precluded significance (Figure 2B). The failure of insulin to increase the proportion of cells in the S and G2/M phases corroborates the proliferation measurements. VEGF as positive control increased the proportion of cells in the S and G2/M phases by approximately 100% and 50%, respectively (P < .001, Figure 2D), confirming its role as a potent EC mitogen.

Insulin induces eNOS activation and GSK3 phosphorylation via the IRS/PI3K/Akt signaling pathway but does not activate the ERK1/2 pathway

To delineate signaling pathways involved in the above-mentioned insulin effects, fetoplacental ECs were treated for 5, 10, and 15 minutes with insulin. Time-course anal-
Analysis showed that highest phosphorylation levels were reached after 5 minutes stimulation (not shown). Therefore, this time point was used for further experiments. Insulin increased insulin receptor autophosphorylation at Tyr1446 by approximately 300% (P < .01) and, consequently, IRS1 phosphorylation by 100% (P < .05) (Figure 3, A and B). Downstream of IRS1, insulin stimulated Akt phosphorylation of eNOS phosphorylation (Ser1177). Insulin increased eNOS phosphorylation after 15 minutes (ANOVA: P < .01). Immunoblot (G) and graph (H) for phosphorylated GSK3 isoforms distinguished both isoforms by their molecular mass, ie, GSK3α (51 kDa) and GSK3β (47 kDa). I, NO levels after 15 minutes of insulin treatment. Data are means ± SD of 3 different cell isolations, each measured in triplicates. A–E and I, Students t test; F, 2-way ANOVA with Holm-Sidak method as post hoc test. C, control.
and GSK3 phosphorylation, both by approximately 60% (Figure 3, C and D). Because the phosphoprotein assay detected both GSK3 isoforms, ie, GSK3α and -β, immunoblotting determined which isoform accounted for the increase in GSK3 phosphorylation. GSK3β (47 kDa) was significantly phosphorylated by insulin (+10%; P < .05), whereas phosphorylated GSK3α was also increased but without reaching significance (Figure 3, G and H). Insulin had no effect on ERK1/2 phosphorylation, neither at 5 minutes nor at any other time point up to 30 minutes (Figure 3E). VEGF, however, a well-established potent activator of ERK1/2 signaling, increased ERK1/2 phosphorylation after 5 minutes (Figure 3E).

eNOS activation is central in EC signaling along the IRS1/PI3K/Akt pathway. Insulin stimulated phosphorylation of eNOS at Ser1177 after 15 minutes (Figure 3F), later than the upstream proteins. Transient NO levels demonstrated that the observed eNOS phosphorylation indeed causes NO production after 15 minutes (P < .001, Figure 3G).

Insulin stimulates angiogenesis via eNOS activation

To study whether the activation of the PI3K/Akt/eNOS pathway or Rac1 is involved in insulin-induced angiogenesis, in vitro network formation was measured in the presence of pharmacological inhibitors. The blocking effect of wortmannin and NSC23766 on the PI3K/Akt pathway and Rac1, respectively, was confirmed. Pretreatment with wortmannin indeed blocked the insulin-stimulated phosphorylation of Akt as described previously (8). The well-established eNOS substrate analog L-NAME blocked the insulin effect on NO bioavailability (Figure 4A). Furthermore, the NO donor SNAP was used as a positive control to demonstrate the role of NO in the in vitro network formation of fetoplacental ECs. Pretreatment with NSC23766 blocked the insulin-stimulated increase of Rac1-GTP (Figure 4B).

VEGF and the NO donor SNAP served as positive controls. Quantification of the network showed that pretreatment with wortmannin and L-NAME reduced insulin effects on tube length (Figure 4, A and B), the number of branching points (not shown), and the number of meshes (not shown) to levels similar to control. Pretreatment with NSC23766 also blocked the insulin effect, but even the inhibitor alone reduced total tube length (Figure 4C), branching point number (not shown), and meshes (not shown) to lower than control levels. This confirms the important role of Rac1 in placental angiogenesis but precludes conclusions on its specific function in insulin signaling effects. These findings demonstrate that insulin-induced angiogenesis in fetoplacental ECs involves the PI3K/eNOS pathway. In fact, the addition of the NO donor to the culture medium in the absence of insulin increased angiogenesis by approximately 25% (Figure 4, A–C).

Insulin effect on actin organization and cell shape is Rac1 mediated

Lamellipodia formation as the first step of cell motility and network formation depend on actin polymerization and rearrangement. Herein Rac1 has a key role (32). Thus, we
tested whether insulin activates Rac1 and induces actin rearrangement. Insulin indeed stimulates Rac1 activity \((P = .003; \text{Figure 5A})\). Staining F-actin fibers with phalloidin demonstrated actin rearrangements. After Rac1 inactivation by serum starving for 48 hours, cells were pre-treated for 1 hour with wortmannin, UO126, NSC23766, or vehicle followed by insulin stimulation for 30 minutes. The blocking effect of UO126 on the ERK1/2 pathway in fetoplacental ECs had already been demonstrated (8). Under normal growing conditions (nonstarved cells), actin organization can be observed as F-actin stress fibers arranged in parallel and well organized across the cell. In this condition barely any membrane ruffles are occurring (Figure 5, A and B, arrows). Cell starvation leads to Rac1 inactivation (32). Thus, cells present less intense F-actin fibers, acquire a round, shrunken shape, and form membrane ruffles (Figure 5C, filled arrows). Insulin stimulation of starved cells restored actin distribution and the original cell shape, suggesting insulin participation in actin stabilization (Figure 5D). Treatment of starved cells with inhibitors of insulin signaling pathways, ie, wortmannin, NSC23766, and UO126, similarly resulted in shrunken cells and membrane ruffles (Figure 5, E–G, filled arrows). Pretreatment with wortmannin and NSC23766 abolished the insulin effect on actin stabilization, consequently causing actin cytoskeleton disorganization with the formation of membrane ruffles (Figure 5, H and I, filled arrow). However, in the presence of the ERK1/2 inhibitor UO126, insulin improved the cell shape and the actin distribution (Figure 5J), confirming absence of ERK1/2 activation by insulin. Because Rac1 is a main mediator of actin organization and because the PI3K inhibitor wortmannin abolished the insulin effects, our data suggest that insulin activates Rac1 via the PI3K/Akt pathway.

**Summary of proposed signaling pathways by which insulin stimulates angiogenesis in human fetoplacental ECs**

The combined signaling pathways insulin activates to induce angiogenesis in fetoplacental ECs tested in the present study are shown in Figure 6. Activation of eNOS and production of NO as well as actin rearrangement induced by Rac1 activation seems central events in insulin induced angiogenesis. PI3K seems central for the activation of these pathways, whereas the activation of the ERK1/2 pathway, commonly regarded as one of the two main insulin signaling pathways, was not detected here. GSK3β may contribute to the stimulation of angiogenesis by inducing cell cycle progression.

**Discussion**

Here we hypothesized that insulin stimulates angiogenesis in primary human fetoplacental ECs which may, at least partially, explain the hypervascularization observed in
angiogenesis.

insulin on actin reorganization and its effects on stimulation of inhibitors wortmannin, L-NAME, and NSC23766 confirmed the role of insulin-induced angiogenesis in placental ECs. The pharmacological production. NO generation and Rac1 activation will contribute to the cycle progression. Akt also phosphorylates eNOS, leading to NO production. NO generation and Rac1 activation will contribute to the insulin-induced angiogenesis in placental ECs. The pharmacological inhibitors wortmannin, L-NAME, and NSC23766 confirmed the role of insulin on actin reorganization and its effects on stimulation of angiogenesis.

placentas from diabetic pregnancies. The main findings of this study are as follows: 1) insulin stimulates in vitro network formation but does not stimulate proliferation; 2) insulin-induced network formation involves the IRS1/PI3K/Akt signaling pathway and downstream eNOS and Rac1 activation, and 3) insulin induces actin rearrangements involving PI3K, eNOS, and Rac1 signaling. These are the first results to show the proangiogenic effect of insulin on primary human fetoplacental ECs and to provide mechanistic evidence for insulin contribution to placental hypervascularization in maternal diabetes.

The formation of new vessels depends on the interaction of ECs with the surrounding environment. The latter comprises a variety of soluble factors, such as VEGF and fibroblast growth factors; their receptors; membrane-bound factors, such as integrins and adhesion molecules; matrix metalloproteinases; and ECM (11). Recently insulin has emerged as a potential proangiogenic factor and our data support this notion.

To observe and quantify angiogenesis in our cell model, we used 2D network formation as a widely used in vitro angiogenesis assay (33). Thus, we demonstrated that insulin increases angiogenesis of human fetoplacental ECs. These primary cells provide an excellent tool to mimic the in vivo situation as closely as possible. However, primary ECs are not only notoriously difficult to transfect, but they also lose their typical angiogenic property after transfection (Bilic J. and U. Hiden, unpublished data). Therefore, we had to resort to widely used and validated pharmaceutical inhibitors for key signaling molecules to identify pathways involved in insulin-stimulated angiogenesis.

Inhibition of PI3K abolished insulin-induced angiogenesis, confirming the central role of the PI3K/Akt-pathway herein. eNOS is one of the downstream targets of PI3K/Akt signaling pathway (34), and NO modulates endothelial function by regulating processes such as vasodilation and angiogenesis (15). We showed that insulin activates the IRS1/PI3K/Akt pathway and leads to phosphorylation and activation of the downstream eNOS, which mediates the increase in angiogenesis upon insulin treatment. This concept is supported by using L-NAME, a pharmacological eNOS inhibitor, which blocked the insulin effect, and by the increase of network formation in the presence of an NO donor. GSK3β is downstream of PI3K/Akt and was also phosphorylated after insulin stimulation. GSK3β may promote angiogenesis via regulation of β-catenin function (22) or via stimulating cell cycle progression (22).

Angiogenesis depends on a cascade of events including changes in cell shape, cell adhesion, and migration. All of these processes require cytoskeleton rearrangements, in which Rac1 actively participates (12). Here we demonstrate that inhibition of Rac1 activation by the pharmacological inhibitor NSC23766 reduces insulin stimulated angiogenesis. NSC23766 alone also reduced angiogenesis. FCS in the culture medium also contains soluble growth factors and growth factor-reduced Matrigel used here still contains small amounts of growth factors such as basic fibroblast growth factor, EGF, and basement membrane proteins, which will also activate Rac1 (31, 35, 36). We found a basal activity of Rac1, which is required for normal, unstimulated angiogenesis and may thus have been inhibited by NSC23766, even in the absence of insulin.

Despite the effect on angiogenesis, insulin did not stimulate proliferation, which was surprising but not without precedence (37). Even though neither an increase in ERK1/2 phosphorylation nor an effect on proliferation was found, insulin stimulated cell cycle progression from the G1/S phase to the G2 phase. In several other cells, the PI3K/GSK3β pathway is activated during G1/S transition and is indispensable for DNA synthesis (38) and for stabilization...
of key regulators for G1/S progression (38–40). Insulin activation of the PI3K/GSK3β pathway reported here could thus explain the increased proportion of cells in the S phase. However, cell cycle progression is governed by the cooperation of different intracellular signaling events. Hence, insulin may require cooperation with other growth factors, such as fibroblast growth factor 2 that have been established to regulate fetoplacental angiogenesis in sheep (41) to promote proliferation.

The pathways studied here were selected because they currently are the best-established insulin-activated pathways (17, 22). However, they do not fully cover all insulin signaling pathways. Other pathways, which could contribute to the observed effects, would involve the protein kinase C (42), mammalian target of rapamycin (43), or p38 MAPK (44) but were not studied here.

Given the role of Rac1 in crucial processes for angiogenesis and because the inhibitor of Rac1 activation, NSC23766, reduced fetoplacental EC basal angiogenesis, we investigated whether insulin stimulation caused Rac1 activation and had an effect on actin cytoskeleton assembly and organization. Our results show that insulin activates Rac1 and actin reassembly. By using inhibitors for PI3K and Rac1 activation, we demonstrated that insulin effects occur via the PI3K/Akt pathway and require Rac1 activation because the inhibitors abolished insulin effects in restoring and keeping actin organization and cell shape.

Collectively these data strongly argue for a role of insulin as proangiogenic factor in placental angiogenesis. This may explain excessive placental vascularization in conditions of fetal hyperinsulinemia because it often occurs in diabetic pregnancies and maternal obesity. Cord blood insulin levels strongly correlate with birth weight (45) and with placental capillary volumes (10). Fetal hyperinsulinemia can occur already early in pregnancy (46), and this may coincide with the onset of insulin receptor expression on the fetoplacental ECs (2). Thus, insulin may exert its effect described here even earlier in gestation at which period for obvious reasons the placenta is not available for cell isolation. Regardless of the particular phase in pregnancy, when insulin starts to affect and alter placentale development, insulin would represent a fetal signal regulating vasculatization of the placenta by signaling through the PI3K/Akt/eNOS/NO pathway and by activating downstream Rac1 (Figure 6). All these findings support our concept of fetal insulin as a contributor to placental hypervascularization in diabetes.

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References


