Fluid Shear Stress Promotes Placental Growth Factor Upregulation in Human Syncytiotrophoblast Through the cAMP–PKA Signaling Pathway

Edouard Lecarpentier, Anthony Atallah, Jean Guibourdenche, Marylise Hebert-Schuster, Sarah Vieillefosse, Audrey Chissey, Bassam Haddad, Guillaume Pidoux, Daniele Evain-Brion, Abdul Barakat, Thierry Fournier, Vassilis Tsatsaris

Abstract—The effects of fluid shear stress (FSS) on the human syncytiotrophoblast and its biological functions have never been studied. During pregnancy, the syncytiotrophoblast is the main source of placental growth factor (PIGF), a proangiogenic factor involved in the placental angiogenesis and the vascular adaptation to pregnancy. The role of FSS in regulating PIGF expression in syncytiotrophoblasts is unknown. We investigated the impact of FSS on the production and secretion of the PIGF by the human syncytiotrophoblasts in primary cell culture. Laminar and continuous FSS (1 dyn cm⁻²) was applied to human syncytiotrophoblasts cultured in a parallel-plate flow chambers. Secreted levels of PIGF, sFlt-1 (soluble fms-like tyrosin kinase-1), and prostaglandin E2 were tested by immunologic assay. PIGF levels of mRNA and intracellular protein were examined by RT-PCR and Western blot, respectively. Intracellular cAMP levels were examined by time-resolved fluorescence resonance energy transfer cAMP accumulation assay. Production of cAMP and PIGF secretion was significantly increased in FSS conditions compared with static conditions. Western blot analysis of cell extracts exposed to FSS showed an increased phosphorylation of protein kinase A substrates and cAMP response element-binding protein on serine 133. FSS-induced phosphorylation of cAMP response element-binding protein and upregulation of PIGF were prevented by inhibition of protein kinase A with H89 (3 μmol/L). FSS also triggers intracellular calcium flux, which increases the synthesis and release of prostaglandin E2. The enhanced intracellular cAMP in FSS conditions was blocked by COX1/COX2 (cyclooxygenase) inhibitors, suggesting that the increase in prostaglandin E2 production could activate the cAMP/protein kinase A pathway in an autocrine/paracrine fashion. FSS activates the cAMP/protein kinase A pathway leading to upregulation of PIGF in human syncytiotrophoblast. (Hypertension. 2016;68:1438-1446. DOI: 10.1161/HYPERTENSIONAHA.116.07890.) ● Online Data Supplement

Key Words: calcium signaling ■ cell extracts ■ phosphorylation ■ placental growth factor ■ trophoblast

During pregnancy, the syncytiotrophoblast is the main source of placental growth factor (PIGF). PIGF is a member of the vascular endothelial growth factor (VEGF) family and is crucial in initiating and perpetuating placental angiogenesis. PIGF also induces vasodilation of uterine and myometrial arteries contributing to uterine vascular remodeling and is associated with the systemic maternal cardiovascular adaptations to pregnancy. In preeclampsia, which affects 2.5% to 3.0% of pregnancies because of excessive circulating levels of soluble fms-like tyrosin kinase-1 (sFlt-1), the bioavailability of PIGF is reduced, which prevents its interactions with endothelial cell surface receptors leading to endothelial dysfunction. The reduction in PIGF has been noted as early as the first trimester in those women who will go on to develop preeclampsia subsequently in pregnancy. Increasing circulating PIGF by the administration of recombinant human PIGF improves clinical parameters in a primate animal model of experimental preeclampsia. The biological effects of PIGF are pleiotropic and have been extensively studied in different organs. In contrast, few...
results have been published on the cellular regulation of the production of PlGF. Factors that regulate PlGF production by trophoblast are not well known. Relative decreases on oxygen tension decrease PlGF expression in trophoblast but increase PlGF expression in other cell types. Endothelial cells respond to fluid shear stress (FSS) by changing their morphology, ultrastructure, function, and gene expression. FSS upregulates the PlGF secretion in human coronary artery endothelial cells. In the human placenta, the syncytiotrophoblast is in direct contact with maternal blood, which exerts FSS on the apical membrane. Although the syncytiotrophoblast is usually likened to an endothelium, the effects of FSS on the human syncytiotrophoblast and its biological functions have never been studied before. The aim of this study was to explore the effects of physiological FSS on PlGF production by the human syncytiotrophoblast in primary cell culture.

Methods

Ethics Statement
The local ethics committee (Comité de Protection des Personnes Ile de France 3) approved the human primary cell culture studies. All women who donated placental tissue gave their written informed consent for participation in this study.

Human Villous Trophoblastic Primary Cell Culture
Third-trimester placentas were obtained immediately after planned cesarean section from healthy mothers who gave birth at 37 to 39 weeks of gestation. Cytotrophoblasts (CTs) were isolated as previously described. CTs were plated at 150 000 cells cm⁻² on microslides (Ibitreat Ibidi) and incubated at 37°C under 10% CO₂. Syncytiotrophoblasts were obtained by aggregation and fusion of CTs after 48 hours of culture. Culture supernatants were stored in Perinat Collection (ANR-10-EQPX-0010) until assayed.

FSS Experiments
After 48 hours of culture in static conditions, the microslides were placed in a parallel-plate, homemade flow chamber connected to a pump system (Ibidi) generating a flow rate of 5.19 mL min⁻¹. Syncytiotrophoblasts were exposed to steady unidirectional laminar FSS of 1 dyn cm⁻² for varying duration (online-only Data Supplement). Static control cells were exposed to the same solution and duration as sheared cells but without exposure to FSS.

Calcium Imaging
Syncytiotrophoblasts were washed with PBS 1x and incubated with the fluorescent calcium indicator 5 μmol/L Fluo-4 AM (F14201, Invitrogen) in phenol red-free Opti-MEM (ThermoFisher Scientific) for 15 minutes at 37°C. Flow chambers containing syncytiotrophoblasts and fluidic units were placed in an environment maintained at 37°C, 5% CO₂ for imaging. Cells in the laminar flow chamber were maintained under no shear to confirm that baseline (Ca₂⁺) was stable for 5 minutes. The pump rate was then abruptly increased to produce a FSS of 1 dyn cm⁻² for 2 minutes. Successive images were collected at 1-s time intervals for 120 s using VisionStage 1.6 software (AllianceVision, France) on an Olympus BX60 epifluorescence microscope equipped with a Sony 3CCD DYC.950P color camera. FSS was applied 20 s after initiation of image acquisition and, thus, represents static culture at start time. Videos were compiled using ImageJ software (National Institutes of Health) using 3 frames/s for visualization and subsequently analyzed for integrated intensity using manual selection of individual cell boundaries.

Biochemical Assay
Medium was processed for measurement with the prostaglandin E2 (PGE2) Parameter Assay Kit (ref: KGE004B, R&D Systems) and the Prostacyclin Assays Kit (ADI-900-025 kit ENZO). Free PIGF and sFlt-1 levels were assessed in cell culture supernatants on a Cobas analyzer (Roche Diagnostics) using the ElectroChemiluminescence Immunoassay (ref: 05144671 for PIGF and ref: 05109523 for sFlt-1). The immunoassay of PIGF is specific and does not cross-react with recombinant VEGF-A (ref: 293-VE-010, R&D Systems; data not shown). VEGF was assessed using an immunoassay that recognizes VEGF-A (Human VEGF Quantikine R&D Systems). All assays were done in duplicate according to the manufacturer’s instructions. The protein levels were calculated using a standard curve derived from known concentrations of the respective recombinant proteins.

Time-Resolved Fluorescence Resonance Energy Transfer cAMP Accumulation Assay
Syncytiotrophoblasts were preincubated with pan-phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 2.5 mmol/L, Sigma-Aldrich) for 1 hour to prevent cAMP degradation. Syncytiotrophoblasts were exposed to FSS (1 dyn cm⁻²) or static culture conditions for 45 minutes. Total intracellular cAMP was measured using the LANCE cAMP detection kit (ref: AD0262, PerkinElmer) according to the manufacturer’s directions. Cells were lysed by the addition of 10 μL lysis buffer (prepared according to the PerkinElmer LANCE cAMP detection kit manual). The assay plate was incubated for another 2 hours at room temperature, then time-resolved fluorescence resonance energy transfer was detected by a Victor3 1420 multilabel plate reader (PerkinElmer) in 384-well white OptiPlate (PerkinElmer). Data for cAMP curves were fitted to the sigmoidal dose-response equation (Graph Pad Prism), and the concentration of cAMP in each sample was then determined by interpolation from the standard curve.

Reagents
Several intracellular pathways were stimulated or inhibited by pharmacological agents. Syncytiotrophoblasts were preincubated in Dulbecco’s modified Eagle medium containing the reagents for 60 minutes at 37°C. Intracellular calcium was sequestered with 10 μmol/L cell-permeable bis-(o-aminophenoxy)-tetracetic acid-ace toxyethyl (BAPTA-AM; Sigma-Aldrich). Intracellular calcium was increased with 0.5 μmol/L of calcium ionophore ionomycin (Sigma-Aldrich) or 10 μmol/L adenine triphosphate (Sigma-Aldrich). IBMX (2.5 mmol/L, Sigma-Aldrich) was used as a pan-phosphodiesterase inhibitor. PGE2 production was inhibited by 0.1 μmol/L SC560 (COX1 [cyclooxygenase] inhibitor, Santa-Cruz) or 1 μmol/L CAY10404 (COX1/COX2 inhibitor, Cayman Chemical). The stabilized synthetic analog PGE2 (sc201225A, Santa-Cruz) was applied at 10 μmol/L. H89 (Sigma-Aldrich) was applied at 3 μmol/L to inhibit protein kinase A (PKA) activity. Forskolin (Sigma-Aldrich) was administered at 15 μmol/L, as an adenyl cyclase activator to stimulate cAMP production. 8-Br-cAMP (Sigma-Aldrich) was applied at 10 μmol/L and was used as a positive control. Trypan blue assays were routinely performed to check syncytiotrophoblast viability.

Quantitative Reverse-Transcription Polymerase Chain Reaction
Each sample was analyzed in duplicate, and a calibration curve was run in parallel in each analysis. Raw fold changes in PlGF expression (ΔCT) were calculated by transforming the difference in CT values of FSS versus static conditions: 2−(FSS CT−Static CT). Fold changes in target gene expression were then normalized to 3 housekeeping genes (HKGs) via the comparative 2−ΔΔCT method using the formula: ΔΔCT = (CT_hkg−CT_hkg)FSS−(CT_hkg−CT_hkg)Static. The complete protocol and primers used (Eurogentec) are described in Data Supplement S1.

Immunocytochemistry
The protocol and the antibodies are described in the Data Supplement S1 section. The controls, obtained by excluding the primary antibody or applying the nonspecific IgG of the same isotype, were all negative.
Immunoblotting
Intracellular proteins were quantitatively detected using bicinchoninic acid method (Pierce BCA Protein Assay Kit). Equal amounts of proteins were separated on 4% to 8% SDS/PAGE and transferred to nitrocellulose membrane (BioRad) by electrophoretic Transfer System. The primary/secondary antibodies are described in the Data Supplement S1 section. The quantification of protein bands using densitometry was performed with the Image J software. A normalization with β-actin and GAPDH was performed for the comparison between static and dynamic conditions.

Statistical Analysis
All quantitative data were presented as mean±SEM. Significant differences were determined by ANOVA and the 2-tailed Student t test. All the experiments were repeated 5× with 5 different primary cell cultures (n=5), except the experiments with FSS of 0.5 dyn cm⁻² (n=3). The difference was considered significant when the P value was <0.05. The tests were performed using GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA).

Results

PIGF Expression
In primary human cell cultures, the multinuclear layer of syncytiotrophoblasts is a syncytium formed by aggregation and fusion of CTs. During this process of differentiation, trophoblasts secrete increasing levels of PIGF, and the mean values±SEM were 85.4±33.3, 1001.2±449.0, and 2223±950.1 pg mL⁻¹ at 24, 48, and 72 hours, respectively (n=5). We checked that the culture medium completed with fetal calf serum was free of PIGF (undetectable levels, data not shown).

After 6 hours of cell culture in dynamic conditions (FSS 1 dyn cm⁻², n=5), we found increases in PIGF mRNA (Figure 1A) and intracellular levels of PIGF protein (Figure 1B). Between 48 and 72 hours of cell culture, 24 hours of FSS (1 dyn cm⁻²) significantly increased the secretion of PIGF compared with static conditions (secreted PIGF normalized to total intracellular proteins: 9.81±2.1 versus 4.24±0.89 pg mL⁻¹ μg⁻¹; P<0.05; n=5; Figure 1C; normalized to PIGF secretion in static conditions between 24 and 48 hours: 8.46±1.29 versus 2.88±0.18 pg mL⁻¹ μg⁻¹; P<0.05; n=5; Figure 2). sFlt-1 secretion was not significantly modified by FSS (data not shown).

Activation of PIGF by FSS was unlikely because of an increase in VEGF-A secretion and the activation of PKA.10 We hypothesized that FSS-induced PIGF production in the syncytiotrophoblast was mediated by the activation of the cAMP–PKA pathway. We found a significant increase in intracellular cAMP levels in FSS conditions (values normalized to the total intracellular proteins: 2.89×10⁻⁹±2.91×10⁻¹⁰ versus 1.05×10⁻⁹±1.46×10⁻¹⁰ mol/g; P<0.05; n=5; Figure 3A). The accumulation of extracellular cAMP was also significantly greater in FSS conditions (data not shown). PKA activity is dependent on cellular levels of cAMP.20 Western blot analysis of cell extracts from syncytiotrophoblasts exposed to FSS (1 dyn cm⁻², 6 h) showed an increase of IC PIGF normalized by the amounts of IC β-actin. C and D. Secretion of PIGF and soluble fms-like tyrosin kinase-1 (sFlt-1) in the supernatant after 48 and 72 h of cell culture. The results are normalized to total IC proteins. Comparison between static and dynamic conditions (FSS; 1 dyn cm⁻²; 24 h; n=5).

Figure 1. Fluid shear stress (FSS) promotes placental growth factor (PIGF) upregulation in the human syncytiotrophoblast. A. Expression of PIGF mRNA in syncytiotrophoblasts studied by relative quantitative reverse-transcription polymerase chain reaction. Comparison between static and dynamic conditions. A laminar and continuous FSS of 1 dyn cm⁻² was applied for 3 and 6 h (n=5). B. Relative expression of intracellular (IC) PIGF proteins in syncytiotrophoblasts. Western blot analysis of cell extracts from syncytiotrophoblasts exposed to FSS (1 dyn cm⁻², 6 h) showed an increase of IC PIGF normalized by the amounts of IC β-actin. C and D. Secretion of PIGF and soluble fms-like tyrosin kinase-1 (sFlt-1) in the supernatant after 48 and 72 h of cell culture. The results are normalized to total IC proteins. Comparison between static and dynamic conditions (FSS; 1 dyn cm⁻²; 24 h; n=5).
releases catalytic subunits that phosphorylate on serine 133, the downstream target cAMP response element-binding protein (CREB). Western blot analysis of cell extracts from syncytiotrophoblasts exposed to FSS (1 dyn cm\(^{-2}\); 24 h) compared with static conditions. Syncytiotrophoblasts were exposed to the same concentrations of vehicle (DMSO [dimethylsulfoxide]) or PKA inhibitor (H89 3 \(\mu\)mol/L), selective COX1 inhibitor (cyclooxygenase; SC560 0.1 \(\mu\)mol/L), COX2 inhibitor (CAY10404 1 \(\mu\)mol/L) in static and dynamic conditions. Values were normalized to PlGF secretion in static conditions between 24 and 48 h (n=5 independent experiments (n=3 with FSS of 0.5 dyn cm\(^{-2}\))). 2-tailed unpaired Student t test: \(**P<0.01\). FSS-enhanced PlGF upregulation was significantly blocked by the PKA inhibitor (H89 3 \(\mu\)mol/L) and partially blocked by the selective COX1 inhibitor (SC560 0.1 \(\mu\)mol/L). Forskolin (FSK; 15 \(\mu\)mol/L) activated adenylyl cyclase and was used as a positive control. In static conditions, 8-Br-cAMP (10 \(\mu\)mol/L), FSK (15 \(\mu\)mol/L), and prostaglandin E2 (PGE2; 10 \(\mu\)mol/L) significantly increased PGE2 secretion.

Figure 2. Fluid shear stress (FSS) promotes placentation growth factor (PIGF) upregulation in human syncytiotrophoblasts via the cAMP–protein kinase A (PKA) signaling pathway. Syncytiotrophoblasts were exposed to FSS (1 dyn cm\(^{-2}\); 24 h) compared with static conditions. Syncytiotrophoblasts were exposed to the same conditions of vehicle (DMSO [dimethylsulfoxide]) or PKA inhibitor (H89 3 \(\mu\)mol/L), selective COX1 inhibitor (cyclooxygenase; SC560 0.1 \(\mu\)mol/L), COX2 inhibitor (CAY10404 1 \(\mu\)mol/L) in static and dynamic conditions. Values were normalized to PGE2 secretion in static conditions between 24 and 48 h (n=5 independent experiments (n=3 with FSS of 0.5 dyn cm\(^{-2}\); 15 minutes)). Western blot analysis of cell extracts from syncytiotrophoblasts exposed to FSS (1 dyn cm\(^{-2}\); 15 minutes) showed a significant increase of phosphorylation of CREB on serine 133 (\(P<0.05\); n=5; Figure 3C). CREB phosphorylation by FSS was significantly reduced by H89 (3 \(\mu\)mol/L), an inhibitor of PKA. The immunocytochemistry results confirmed the increased phosphorylation of CREB and a largest subcellular localization of P-CREB in the syncytiotrophoblast nuclei in FSS conditions (1 dyn cm\(^{-2}\); 15 minutes; n=5; Figure 3D).

**Intracellular Calcium**

Cytosolic phospholipase A2 activity is regulated by intracellular calcium concentrations (Ca\(^{2+}\)). Increases in (Ca\(^{2+}\)) enhance translocation of cytosolic phospholipase A2 to cellular membranes, where it releases arachidonic acid. We hypothesized that FSS stimulates calcium flux upstream of PGE2 production in the syncytiotrophoblast. Live cell imaging with Fluo-4AM confirmed spikes of intracellular calcium signaling in FSS-stimulated cells, as determined by increased intensity in cytoplasmic fluorescence (n=5 syncyti; Figure 5). To test whether the FSS-induced increase in (Ca\(^{2+}\)), is necessary for PGE2 release, cells were pretreated with the intracellular Ca\(^{2+}\) chelator (BAPTA-AM, 10 \(\mu\)mol/L), and FSS-mediated PGE2 concentration was measured in the media. BAPTA-AM inhibited the FSS-induced increase in PGE2 concentration in the media but remained greater than in static controls (n=5; Figure 4). Moreover, the enhanced intracellular Ca\(^{2+}\) in FSS conditions was significantly blocked by BAPTA-AM (n=5; Figure 3A).

**FSS, PIGF Expression, and PGE2–cAMP–PKA Signaling Pathway**

In static conditions, PGE2 (10 \(\mu\)mol/L), forskolin (15 \(\mu\)mol/L), and 8-Br-cAMP (10 \(\mu\)mol/L) caused a significant increase of PIGF secretion in the culture medium within 24 hours (reported values of PIGF secretion in static conditions between 24 and 48 hours: 10.97±1.50, 17.76±5.69, and 40.35±16.67, respectively; \(P<0.05\); n=5). These results confirm that the synthesis and secretion of PIGF by syncytiotrophoblasts are potentially under the control of the PGE2–cAMP–PKA signaling pathway (Figure 2). Moreover, the FSS-induced increase of PIGF was prevented by inhibition of PKA with H89 (3 \(\mu\)mol/L; 3.81±0.66). These results suggest that the cAMP–PKA signaling pathway is required for the FSS-induced PIGF upregulation. Inhibition of COX1 (SC560 0.1 \(\mu\)mol/L; 4.84±1.17) also decreased the FSS-induced increase in PIGF. PGE2 production could stimulate the cAMP–PKA signaling pathway in an autocrine/paracrine fashion.

**Discussion**

To our knowledge, this is the first description of a biological effect of FSS on human syncytiotrophoblasts. On the basis of a previous study, we applied a laminar continuous FSS of 1 dyn cm\(^{-2}\). Our main result is that expression and secretion of PIGF by human syncytiotrophoblasts are significantly increased in fluid flow that mimics physiological conditions.
compared with static conditions. Human PI GF is essentially expressed in the placenta, more precisely in the syncytiotrophoblast. PlGF is a potent stimulator of placental angiogenesis. These new data provide 1 mechanism by which biomechanical forces induced by maternal blood flow, that is FSS, could modulate the angiogenic potential of the syncytiotrophoblast. Vascular functions are controlled by biochemical mediators and the autonomic nervous system. It is although well established that the biomechanical forces generated by blood flow and blood pressure regulate vascular functions. Endothelial cells responses to shear stress play a critical role in blood flow–dependent phenomena, including angiogenesis, vascular remodeling, and atherosclerosis. The placenta is a noninnervated organ. The biomechanical forces exerted by the maternal blood on the syncytiotrophoblast can potentially affect the placental angiogenesis, the regulation of fetal blood flows, and potentially the placental exchange functions. Previous studies have shown that mechanical forces can increase the expression of PI GF, which have already been reported. In a model of primary bronchial airway epithelial cells cultured on a deformable silicoelastic membrane, Mohammed et al found that cyclic stretch induces PI GF expression in bronchial airway epithelial cells via nitric oxide release. Rashdan et al found that FSS (12.4 dyn cm$^{-2}$) upregulates by 40% the secretion of PI GF in cocultured vascular cells (human coronary artery endothelial cells and smooth muscle cells). Their findings show that FSS may regulate the expression of PI GF in the vessel wall, which is a potent stimulator of collateral growth.

Figure 3. Fluid shear stress (FSS) enhances the cAMP–protein kinase A (PKA)–cAMP response element-binding protein (CREB) pathway. A, FSS increases intracellular (IC) cAMP levels. Syncytiotrophoblasts were preincubated with pan-phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 2.5 mmol/L; 60 min) and then exposed to FSS (1 dy cm$^{-2}$, 45 min) compared with static conditions. Total IC cAMP accumulation was measured by time-resolved fluorescence resonance energy transfer and normalized to total IC proteins. Total IC cAMP per microgram of protein was significantly increased in FSS conditions compared with static controls ($n=5$ independent experiments, 2-tailed unpaired Student $t$ test: $*P<0.05$, $**P<0.01$). The FSS-induced increase of IC cAMP was blocked by the selective COX1 inhibitor (cyclooxygenase; SC560, 0.1 mmol/L), the COX1/COX2 inhibitor (CAY10404 1 mmol/L) and the IC Ca$^{2+}$ chelator (bis(o-aminophenoxy)-tetraacetic acid-acetoxyethyl [BAPTA-AM] 10 mmol/L). In static conditions, prostaglandin E2 (10 mmol/L) significantly increased IC cAMP per microgram of cellular protein ($n=5$ independent experiments, 2-tailed unpaired Student $t$ test: $*P<0.05$). Forskolin (FSK, 15 mmol/L) activates adenyl cyclase and was used as a positive control. B, FSS promotes activation of PKA. Western blot analysis of cell extracts from syncytiotrophoblasts exposed to FSS (1 dy cm$^{-2}$, 15 min) showed increased phosphorylation of PKA substrate motifs normalized by the amounts of IC $\beta$-actin. FSK (15 mmol/L, 15 min) was used as a positive control. C, Western blot analysis of cell extracts from syncytiotrophoblasts exposed to FSS (1 dy cm$^{-2}$, 15 min) shows increased phosphorylation of CREB on serine 133 (P-CREB S133) normalized by the amounts of IC GAPDH. FSK (15 mmol/L, 15 min) was used as a positive control. CREB phosphorylation by FSS was significantly reduced by inhibition of PKA (H89 3 mmol/L). D, Largest subcellular localization of P-CREB S133 in the syncytiotrophoblast nuclei in FSS conditions (1 dy cm$^{-2}$, 15 min). Syncytiotrophoblast plasma membranes were immunolabeled with desmoplakin (DPK, green), and syncytiotrophoblast nuclei were labeled with 6-diamidino-2-phenylindole (DAPI, blue).
degradation. Moreover, the antagonists of COX inhibited the FSS-induced increase of intracellular cAMP. In the human placenta, the PGE2 receptor EP2 is expressed mainly in the syncytiotrophoblast. We found that PGE2 was responsible for an increase of cAMP in syncytiotrophoblasts, probably via stimulation of EP2 receptor, which is Gαs-linked and activates adenylyl cyclase. We plan to study whether upregulation of PlGF by PGE2 released in response to FSS is mediated through the EP2 receptor, using antagonists and agonists specific for EP receptor-mediated signaling. The FSS-induced increase of intracellular cAMP via the autocrine/paracrine action of released PGE2 has already been reported in hematopoietic stem cells (5 dyn cm−2), osteocytes (16 dyn cm−2), and renal epithelial collecting duct cells (0.4 dyn cm−2). Wu et al have demonstrated that PGE2 increases the expression of PlGF in human synovial fibroblasts. Their results demonstrate the upregulation of PlGF by 15-LOX (lipoygenase) activation in human synovial fibroblasts. Activation of 15-LOX results in the production of 15-(S)-HETE (hydroxyeicosatetraenoic acid), which increases COX2 expression and PGE2 production. During preeclampsia, the urinary excretion of PGE2 is reduced, but the correlation between circulating levels of PlGF and PGE2 has never been studied.

cAMP as a second messenger plays a critical role in directing the differentiation of trophoblastic cells. The binding of cAMP to proteins such as PKA and exchange protein activated by cAMP explains most of its functional activities. Although H89 has nonspecific effects, it is a tool widely used to assess the role of PKA in vitro and in vivo. In our model, PKA inhibition by H89 (3 μmol/L) limited FSS-induced CREB phosphorylation and PlGF upregulation, suggesting that PKA may be the cAMP effector in this process.

Two functional cAMP responsive elements in the PlGF promoter have been identified, and CREB contributes to the regulation of PlGF gene expression. Our results show CREB phosphorylation and nuclear translocation of phospho-CREB under FSS conditions. This phosphorylation allows interaction with the coactivator CBP (CREB-binding protein)/p300 and recruitment of
the ternary transcription complex. Moreover, we found that CREB phosphorylation was reduced by inhibition of PKA. These results strongly suggest that FSS activates the cAMP–PKA–CREB signaling pathway, which results in upregulation of the expression of PIGF. Activation of the cAMP–PKA–CREB pathway by FSS has been observed in diverse cell types, including chondrocytes, osteocytes, and hematopoietic stem cells. Diaz et al showed that the effects of FSS on hematopoiesis are mediated, in part, by a cascade that involves calcium efflux and stimulation of the PGE2/cAMP/PKA signaling pathway.

Recently, Miura et al showed in BeWo cells that FSS induces Ca influx and (Ca2+), increase via mechanosensitive activation of transient receptor potential vanilloid family type-6, which regulates microvillus formation through the functional activation of ezrin via Ca-dependent Akt phosphorylation. The kinetics of (Ca2+) increase are identical to those we describe for syncytiotrophoblasts. Berryman et al have shown that ezrin is a major protein component of human placental microvilli, comprising ≥5% of the total protein mass and present at about one quarter of the molar abundance of actin. Stumpf et al observed that the mature glycosylated transient receptor potential vanilloid family type-6 channel is expressed in microvillus apical membranes of the human syncytiotrophoblast. As for BeWo cells, the transient receptor potential vanilloid family type-6 channel might be responsible for the FSS-induced rise in (Ca2+), but further investigations are needed to confirm this hypothesis.

This study illustrates the fact that the FSS-induced biological response of syncytiotrophoblast is dynamic and requests different time exposure. Short responses, such as calcium fluxes and phosphorylation processes, are detected within the first few minutes of the FSS exposition. Transcription processes and intracellular proteins synthesis induced by FSS are detected after 3 to 6 hours of dynamic culture. The assays of angiogenic factors (PIGF and sFlt-1) into the supernatants represent an accumulation in the extracellular space. We, therefore, performed the assays after 24 hours of culture.

Placental hypoperfusion, in addition to the relative hypoxia caused by the decline in the total supply of oxygen, might cause a significant decrease in the FSS exerted on the syncytiotrophoblast. Schlembach et al found that maternal serum PIGF levels are negatively correlated with uterine artery Doppler pulsatility index in women with preeclampsia and intrauterine growth retardation. They concluded that in addition to an increased secretion of sFlt-1, which may bind free PIGF, reduced uteroplacental blood flow might have downregulated PIGF protein expression and production.

We present here the first study of the effects of laminar and continuous low FSS (1 dyn cm−2) on biological functions of human syncytiotrophoblasts in primary cell culture. The production of PIGF by syncytiotrophoblasts is significantly increased in flow conditions. Our results also show that FSS triggers intracellular calcium flux, increases the synthesis and release of PGE2, and increases the production of intracellular cAMP, which leads to PKA activation and CREB phosphorylation. These results suggest that FSS triggers an autocrine/paracrine action of PGE2 leading to the activation of the cAMP/PKA/CREB pathway and upregulation of PIGF.

**Perspectives**

The syncytiotrophoblast of the human placenta is a mechano-sensitive tissue. Our results suggest a dose–effect relationship between the intensity of FSS and the syncytiotrophoblast biological response. The shear stress–sensing mechanisms are yet unknown. Research on shear stress mechanoperception and mechanotransduction will help to understand the mechanism by which the syncytiotrophoblast detects blood flow to optimize the maternal–fetal exchange function.

PIGF is demonstrated to be part of the physiological sequence controlling blood pressure and proteinuria as a consequence of placental ischemia in preeclampsia. Alterations of the PIGF rates in preeclampsia correlate with the diagnosis and adverse outcomes, particularly when the disease presents prematurely (<34 weeks). According to our results, pharmacological agents capable of interacting with the PGE2/PKA/CREB pathway could increase the production and the release of PIGF in the maternal circulation, correct the angiogenic imbalance, and have a therapeutic impact in preeclampsia.

**Acknowledgments**

We thank Bernard Le Bonniec (INSERM, UMR-S 1140, PRES Sorbonne Paris Cité, Université Paris Descartes, Paris, France) for his help in the design and construction of the flow chambers; René Lai Kuen (Plateau Technique d’Imagerie Cellulaire et Moléculaire, IFR71-IMTCE, Faculté des Sciences Pharmaceutiques et Biologiques, Université Paris Descartes, Paris, France) for the surface electron microscopy; Centre d’Investigation Clinique (CIC P0901 Mère Enfant, INSERM, Hôpital Cochin, Assistance Publique Hôpitaux de Paris, Université Paris Descartes, Paris, France) for information on the women who donated placentas and for the recovery of the placentas; Maternité de l’Institut Mutualiste Montsouris, Maternité de l’Hôpital Privé d’Anthony, Maternité de l’Hôpital Trousseau (Assistance Publique Hôpitaux de Paris), Maternité de l’Hôpital Bichat (Assistance Publique Hôpitaux de Paris) for information on the women who donated placentas and for the recovery of the placentas; and PremUp Foundation for the storage of culture supernatant in PerinatCollection (ANR-10-EQPX 0010) pending assay.

**Sources of Funding**

This study was supported by INSERM and PremUp Foundation. This work was partially funded by a permanent endowment in Cardiovascular Cellular Engineering from the AXA Research Fund (to A. Barakat).

**Disclosures**

J. Guibourdenche and V. Tsatsaris are consultants for Roche Diagnostics. The other authors report no conflicts.

**References**


What Is New?

• The syncytiotrophoblast of the human placenta is a mechanosensitive tissue.
• Shear stress–sensing mechanisms, which are yet unknown, trigger intracellular calcium fluxes in the syncytiotrophoblast.
• A physiological low fluid shear stress (FSS; 1 dyn cm−2) triggers an autocrine/paracrine action of prostaglandin E2 leading to the activation of the cAMP/protein kinase A/cAMP response element-binding protein pathway.
• A physiological low FSS (1 dyn cm−2) enhances the production and secretion of placental growth factor (PlGF) by the syncytiotrophoblast.

What Is Relevant?

• We have demonstrated a new regulation mechanism for the PlGF expression by the syncytiotrophoblast.
• During preeclampsia, low concentrations of circulating PlGF released by the placenta contribute to systemic endothelial dysfunction and the development of the maternal hypertensive syndrome. Interacting with the prostaglandin E2/protein kinase A/cAMP response element-binding protein pathway in the syncytiotrophoblast to increase PlGF in the maternal circulation could be a therapeutic strategy for preeclampsia.

Summary

We investigated the impact of FSS on the production and secretion of the PlGF by the human placental syncytiotrophoblast in primary cell culture. Physiological FSS (1 dyn cm−2) was applied to syncytiotrophoblast cultured in a parallel plate flow chambers. Our main result is that expression and secretion of PlGF by human syncytiotrophoblast are significantly increased in fluid flow that mimics physiological conditions compared with static conditions. FSS triggers intracellular calcium flux, which increases the synthesis and release of prostaglandin E2. Prostaglandin E2 production activates the cAMP/protein kinase A/cAMP response element-binding protein pathway in an autocrine/paracrine fashion leading to upregulation of PlGF in human syncytiotrophoblast.

Novelty and Significance


Fluid Shear Stress Promotes Placental Growth Factor Upregulation in Human Syncytiotrophoblast Through the cAMP–PKA Signaling Pathway
Edouard Lecarpentier, Anthony Atallah, Jean Guibourdenche, Marylise Hebert-Schuster, Sarah Vieillefosse, Audrey Chissey, Bassam Haddad, Guillaume Pidoux, Daniele Evain-Brion, Abdul Barakat, Thierry Fournier and Vassilis Tsatsaris

Hypertension. 2016;68:1438-1446; originally published online October 3, 2016; doi: 10.1161/HYPERTENSIONAHA.116.07890

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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SUPPLEMENTAL MATERIAL

FSS experiments
After 48 hours of culture in static conditions the microslides were placed in a parallel plate homemade flow chamber connected to a pump system (Ibidi®) generating a flow rate of 5.19 mL.min⁻¹. STBs were exposed to steady unidirectional laminar FSS of 1 dyn.cm⁻² (τ= 6μQ/[h².b]; b=2.5 cm; μ=0.0077 Poise; h=400 μm) for varying durations (5 minutes for calcium imaging, 15 minutes for PhosphoPKA/CREB immunoblotting, 45 minutes for measurements of extracellular PGE2 and cAMP intracellular accumulation assays, 3-6 hours for relative quantitative RT-PCR, 6 hours for PlGF immunoblotting, 24 hours for extracellular PlGF/VEGF/sFlt-1 biochemical assays). Static control cells were exposed to the same solution and duration as sheared cells, but without exposure to FSS. STB viability (trypan blue assays) and early apoptotic events (immunocytochemistry with caspase-cleaved cytokeratin 18 M30 CytoDEATH Sigma-Aldrich®) were measured after 24 h of FSS (1 dyn.cm⁻²).

Relative quantitative RT-PCR (RT-qPCR)
Total RNA was extracted from STB using an Arcturus® PicoPure® RNA Isolation Kit (Ref: KIT0204, Applied Biosystems®). RNA concentration was measured with a Nanodrop® ND-1000 spectrophotometer. cDNA was synthesized from total RNA (1 mg) using the Superscript II Reverse Transcriptase kit (Invitrogen®). All RT-qPCR reactions were performed in an ABI Prism 7300 Sequence Detection system (Applied Biosystems®) with the SYBRH Green PCR kit (Applied Biosystems®). The PCR was performed in a 384-well clear optical reaction plate 7900 (Applied Biosystems®). 18S RNA, hypoxanthine-guanine phosphoribosyltransferase (HGPRT), succinate dehydrogenase complex subunit A (SDHA) were used as endogenous RNA controls (housekeeping genes).
Primers used for relative quantitative RT-PCR

<table>
<thead>
<tr>
<th>Position</th>
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<th>Size PCR product</th>
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<tr>
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<tr>
<td>(726-707) +/-</td>
<td>5'-CTCGCTGGGGTACTCGGACA-3'</td>
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<tr>
<td>h18S rRNA</td>
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<tr>
<td></td>
<td>rRNA Endogenous Control (Applied Biosystems)</td>
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<td>hSD HA (229-248)</td>
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<tr>
<td>(314-293) +/-</td>
<td>5'-CCACCACTGCAATCAATTCATG-3'</td>
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<tr>
<td>hHP RT (578-598)</td>
<td>5'-TGACACTGGAAACAATGCA-3'</td>
<td>94 bp</td>
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<tr>
<td>(671-651) +/-</td>
<td>5'-GGTCCTTTTACCAGCAAGCT-3'</td>
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**Immunocytochemistry**

To detect desmoplakin, CREB and Phospho-CREB, cultured cells were rinsed with PBS, fixed and permeabilized in methanol at -20°C for 8 min. The cells were incubated with 1% BSA in TBS for 60 min at room temperature. The primary antibodies were applied overnight at 4°C, followed by an Alexa 488-labeled donkey anti-mouse antibody or an Alexa 594-labeled goat anti-rabbit antibody (Molecular Probes®, ThermoFischer Scientific®, 1/400) for 60 min at room temperature in the dark. STB nuclei were labeled with 6-diamidino-2-phenylindole (DAPI).

**Immunoblotting**

Following aspiration of the culture medium, the cells were washed once in PBS 1X and subsequently scraped off. The pellets were immediately snap-frozen in liquid nitrogen and stored at -80°C. Destruction of cell membranes was performed with lysis buffer (1% NP40, Cell Signaling Technology® 9803) containing protease inhibitor (Calbiochem® Cat 539131) and phosphatase inhibitor (Calbiochem® Cat 524629).

The specific band was detected by fluorescence (LI-COR® Odyssey Scanner) after incubation with an anti-rabbit or anti-mouse IR-Dye®-coupled antibody according to the primary antibody (IRDye® 800CW Donkey anti-rabbit 0.1 μg/mL, IRDye® 680CW Goat anti-mouse 0.1 μg/mL, LI-COR®).
Primary antibodies used for immunocytochemistry and immunoblotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>Isotype</th>
<th>Species</th>
<th>Concentration</th>
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<td>Rabbit</td>
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