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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 (PlGF), a proangiogenic factor involved in the placental angiogenesis and the vascular adaptation to pregnancy. The role of FSS in regulating PlGF expression in syncytiotrophoblasts is unknown. We investigated the impact of FSS on the production and secretion of the PlGF 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 PlGF, sFlt-1 (soluble fms-like tyrosin kinase-1), and prostaglandin E2 were tested by immunologic assay. PlGF 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 PlGF 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 PlGF 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 PlGF 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 (PlGF).[1] PlGF is a member of the vascular endothelial growth factor (VEGF) family and is crucial in initiating and perpetuating placental angiogenesis.[2] PlGF also induces vasodilation of uterine and myometrial arteries contributing to uterine vascular remodeling[3] and is associated with the systemic maternal cardiovascular adaptations to pregnancy.[4] 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 PlGF is reduced, which prevents its interactions with endothelial cell surface receptors leading to endothelial dysfunction.[5,6] The reduction in PlGF has been noted as early as the first trimester in those women who will go on to develop preeclampsia subsequently in pregnancy.[7] Increasing circulating PlGF by the administration of recombinant human PlGF improves clinical parameters in a primate animal model of experimental preeclampsia.[8]

The biological effects of PlGF are pleiotropic and have been extensively studied in different organs.[9] In contrast, few

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From the INSERM, UMR-S 1139, Paris, France (E.L., A.A., J.G., M.H.-S., S.V., A.C., B.H., G.P., T.F., V.T.); PRES Sorbonne Paris Cité, Université Paris Descartes, Paris, France (E.L., A.A., J.G., M.H.-S., S.V., A.C., T.F., V.T.); Port Royal Maternity, Department of Gynecology Obstetrics I, Centre Hospitalier Universitaire Cochin Broca Hôtel Dieu, Groupe Hospitalier Universitaire Ouest, Assistance Publique-Hôpital de Paris, France (E.L., V.T.); DHU Risques et grossesse, Paris, France (E.L., J.G., T.F., V.T.); PremUP Foundation, Paris, France (E.L., J.G., D.E.-B., T.F., V.T.); Laboratoire d'Hydrodynamique (LadHyX), CNRS, École Polytechnique, Palaiseau, France (A.B.); SDBA Centre Hospitalier Universitaire Cochin Broca Hôtel Dieu, Groupe Hospitalier Universitaire Ouest, Assistance Publique-Hôpital de Paris, France (M.H.-S.); Department of Obstetrics and Gynecology, Centre Hospitalier Intercommunal de Créteil, CRC CHI Creteil, University Paris Est Creteil, France (B.H.); Service d'hormonologie Centre Hospitalier Universitaire Cochin Broca Hôtel Dieu, Groupe Hospitalier Universitaire Ouest, Assistance Publique-Hôpital de Paris, France (J.G.); and INSERM, UMR-S 1180, Université Paris-Sud, Université Paris-Saclay, F-92296, Châtenay-Malabry, France (G.P.).

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Correspondence to Edouard Lecarpentier, INSERM, UMR-S 1139, Physiopathologie et Pharmacotoxicologie Placentaire Humaine, Faculté de pharmacie de Paris, 4 Ave de l'Observatoire, 75006 Paris, France. E-mail edouard.lecarpentier@wanadoo.fr

results have been published on the cellular regulation of the production of PlGF.[10–12] Factors that regulate PlGF production by trophoblast are not well known. Relative decreases on oxygen tension decrease PlGF expression in trophoblast[13] but increase PlGF expression in other cell types.[14] 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.[15] In the human placenta, the syncytiotrophoblast is in direct contact with maternal blood, which exerts FSS on the apical membrane.[16] 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.[17] CTs were plated at 150 000 cells cm^{-2} on microslides (Ibitreat Ibidi) and incubated at 37°C under 10% CO_2. 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 1× and incubated with the fluorescent calcium indicator 5 µmol/L Fluo-4AM (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% CO_2 for imaging. Cells in the laminar flow chamber were maintained under no shear to confirm that baseline $(Ca_2)i$ was stable for 5 minutes. The pump rate was then abruptly increased to produce an 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 PlGF and sFlt-1 levels were assessed in cell culture supernatants on a Cobas analyzer (Roche Diagnostics) using the ElectroChemiLuminescence Immunologic Assay (ref: 05144671 for PlGF and ref: 05109523 for sFlt-1). The immunoassay of PlGF 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 detection 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)-tetraacetic acid-acetoxymethyl (BAPTA-AM; Sigma-Aldrich). Intracellular calcium was increased with 0.5 µmol/L of calcium ionophore ionomycin (Sigma-Aldrich) or 10 µmol/L adenosine 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 adenylyl 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 $2^{-\Delta\Delta CT}$ method using the formula: $\Delta\Delta CT = (CT_{PlGF} - CT_{HKG})_{FSS} - (CT_{PlGF} - CT_{HKG})_{Static}$. 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 electroblotting (Trans-Blot Turbo 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

PlGF 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 PlGF, 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 PlGF (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 PlGF mRNA (Figure 1A) and intracellular levels of PlGF protein (Figure 1B). Between 48 and 72 hours of cell culture, 24 hours of FSS (1 dyn cm^{-2}) significantly increased the secretion of PlGF compared with static conditions (secreted PlGF 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 PlGF secretion in static conditions between 24 and 48 hours: 8.46±1.29 versus 2.88±0.18 pg mL^{-1} g^{-1}; $P<0.05$; n=5; Figure 2). sFlt-1 secretion was not significantly modified by FSS (38.43±14.67 versus 42.74±10.79 pg mL^{-1} μg^{-1}; n=5; Figure 1D). FSS-induced PlGF increase in the supernatant was unlikely because of an increase in the soluble receptor sFlt-1 that binds to the VEGF and the PlGF and acts as an efficient specific antagonist.[18] The immunoassay we used to measure PlGF is specific and does not cross-react with recombinant VEGF-A (data not shown). VEGF-A was not detectable in the supernatant in our cell culture conditions (O_2 21%/CO_2 5%). The affinity of sFlt-1 was greater for VEGF-A than for PlGF,[19] and FSS-induced increase in PlGF was unlikely because of an increase in VEGF-A secretion and a displacement of PlGF/sFlt-1 binding. There was no significant difference in cellular viability and apoptosis between the static and FSS groups (data not shown).

cAMP–PKA–cAMP Response Element-Binding Protein Pathway

cAMP is known to be a strong activator of PlGF gene expression in human trophoblasts, and this effect is mediated through activation of PKA.[10] We hypothesized that FSS-induced PlGF 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: $2.89\times10^{-9}\pm2.91\times10^{-10}$ versus $1.05\times10^{-9}\pm1.46\times10^{-10}$ 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 syncytiotrophoblast exposed to FSS (1 dyn cm^{-2}, 15 minutes) showed a significant increase of phosphorylation of PKA substrates ($P<0.05$; n=5; Figure 3B). The binding of cAMP to PKA

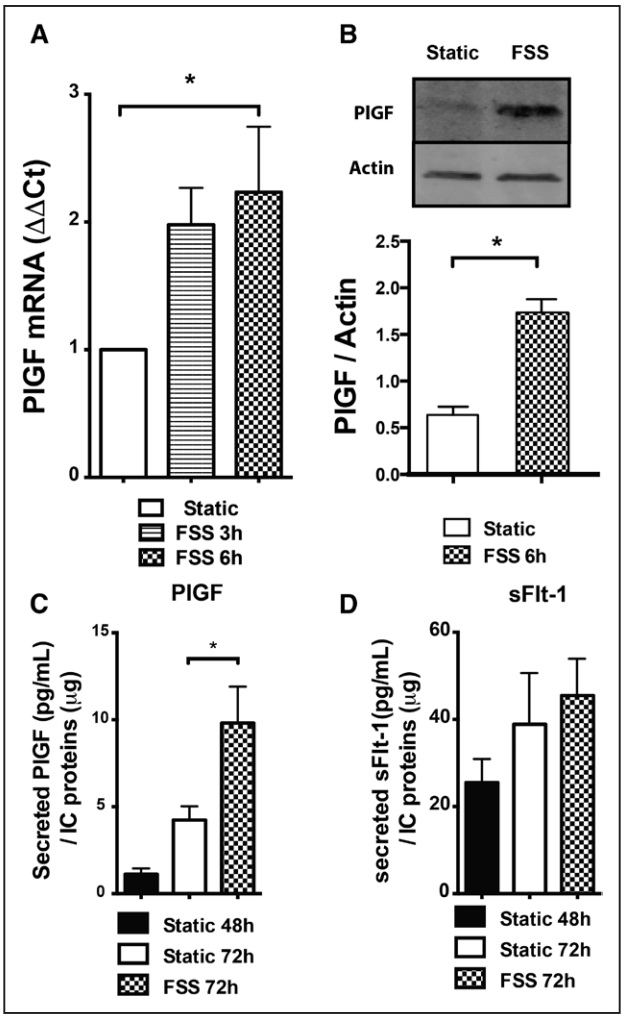

Figure 1. Fluid shear stress (FSS) promotes placental growth factor (PlGF) upregulation in the human syncytiotrophoblast. **A**, Expression of PlGF mRNA in syncytiotrophoblasts studied by relative quantitative reverse-transcription polymerase chain reaction. Comparison between static and dynamic conditions. 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) PlGF 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 PlGF normalized by the amounts of IC β-actin. **C** and **D**, Secretion of PlGF 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).

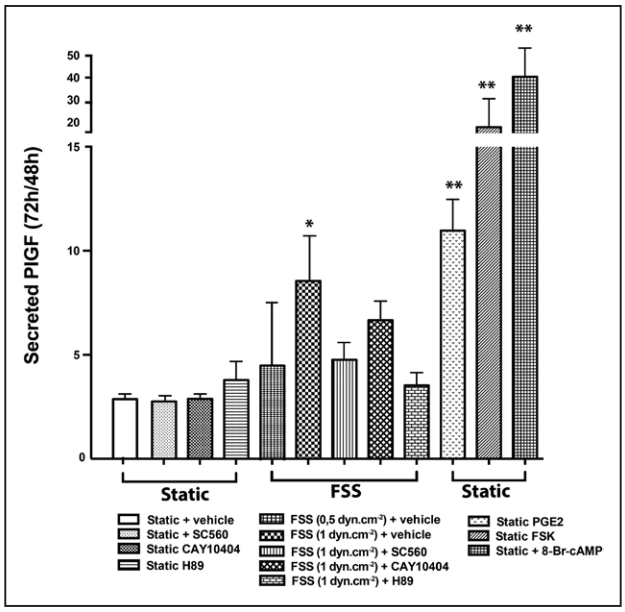

Figure 2. Fluid shear stress (FSS) promotes placental growth factor (PlGF) upregulation in human syncytiotrophoblasts via the cAMP–protein kinase A (PKA) signaling pathway. Syncytiotrophoblasts were exposed to FSS (1 dyn cm^{-2}, 24 h) compared with static conditions. Syncytiotrophoblasts were exposed to the same concentrations of vehicle (DMSO [dimethylsulfoxyde]) or PKA inhibitor (H89 3 μmol/L), selective COX1 inhibitor (cyclooxygenase; SC560 0.1 μmol/L), COX2 inhibitor (CAY10404 1 μmol/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*<0.05; ***P*<0.01). FSS-enhanced PlGF upregulation was significantly blocked by the PKA inhibitor (H89 3 μmol/L) and partially blocked by the selective COX1 inhibitor (SC560 0.1 μmol/L). Forskolin (FSK; 15 μmol/L) activated adenylyl cyclase and was used as a positive control. In static conditions, 8-Br-cAMP (10 μmol/L), FSK (15 μmol/L), and prostaglandin E2 (PGE2; 10 μmol/L) significantly increased PlGF secretion.

releases catalytic subunits that phosphorylate on serine 133, the downstream target cAMP response element-binding protein (CREB).[21] Western blot analysis of cell extracts from syncytiotrophoblast 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 μmol/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).

Prostaglandin

It has been reported that FSS stimulates the cAMP–PKA signaling axis by increasing the synthesis/release of PGE2, which activates adenylyl cyclase in an autocrine or paracrine fashion.[22,23] We found that PGE2 secretion by syncytiotrophoblasts was significantly increased in FSS conditions compared with static conditions (values normalized to the total intracellular proteins: 0.14±0.03 versus 0.37±0.07 pg·mL^{-1} μg^{-1}; *P*<0.05; n=5; Figure 4). This enhanced PGE2 production was blocked by the selective COX1 (SC560 0.1 μmol/L) and COX2 (CAY10404 1 μmol/L) inhibitors (Figure 4), suggesting that the increase of PGE2 in the conditioned medium is not secondary to release of the initial intracellular pool by multiple drug resistance–associated protein 4 channels.[24] In static conditions, the addition of PGE2 (10 μmol/L) led to a significant increase in the accumulation of intracellular cAMP (*P*<0.05; n=5; Figure 3A). The enhanced intracellular cAMP in FSS conditions was blocked by the selective COX1 inhibitor (0.14±0.03 pg mL^{-1} μg^{-1}) and the COX2 inhibitor (0.09±0.02 pg mL^{-1} μg^{-1}; n=5; Figure 3A). We found no FSS-increased synthesis/release of prostacyclin (data not shown).

Intracellular Calcium

Cytosolic phospholipase A2 activity is regulated by intracellular calcium concentrations $(Ca^{2+})_i$. Increases in $(Ca^{2+})_i$ enhance translocation of cytosolic phospholipase A2 to cellular membranes, where it releases arachidonic acid.[25] 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 syncytia; Figure 5). To test whether the FSS-induced increase in $(Ca^{2+})_i$ is necessary for PGE2 release, cells were pretreated with the intracellular Ca^{2+} chelator (BAPTA-AM, 10 μmol/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 cAMP in FSS conditions was significantly blocked by BAPTA-AM (n=5; Figure 3A).

FSS, PlGF Expression, and PGE2–cAMP–PKA Signaling Pathway

In static conditions, PGE2 (10 μmol/L), forskolin (15 μmol/L), and 8-Br-cAMP (10 μmol/L) caused a significant increase of PlGF secretion in the culture medium within 24 hours (reported values of PlGF 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 PlGF by syncytiotrophoblasts are potentially under the control of the PGE2–cAMP–PKA signaling pathway (Figure 2). Moreover, the FSS-induced increase of PlGF was prevented by inhibition of PKA with H89 (3 μmol/L; 3.81±0.66). These results suggest that the cAMP–PKA signaling pathway is required for the FSS-induced PlGF upregulation. Inhibition of COX1 (SC560 0.1 μmol/L; 4.84±1.17) also decreased the FSS-induced increase in PlGF. FSS-enhanced PGE2 production could stimulate the cAMP–PKA signaling pathway in an autocrine/paracrine manner.

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,[16] we applied a laminar continuous FSS of 1 dyn cm^{-2}. Our main result is that expression and secretion of PlGF by human syncytiotrophoblasts are significantly increased in fluid flow that mimics physiological conditions

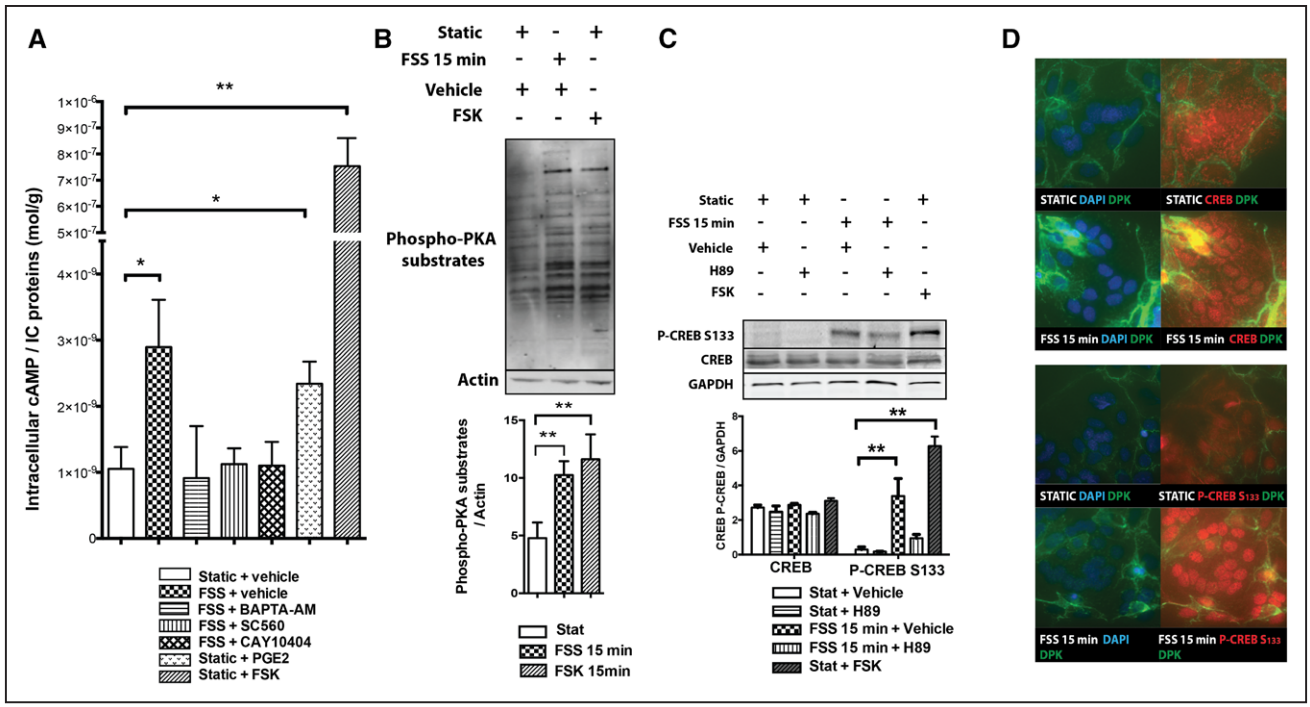

Figure 3. Fluid shear stress (FSS) enhances the cAMP–protein kinase A (PKA)–cAMP response element-binding protein (CREB) pathway. **A**, FSS increases intracellular (IC) cAMP levels. Syncytiotrophoblasts were preincubated with pan-phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 2.5 mmol/L; 60 min) and then exposed to FSS (1 dyn 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 μmol/L), the COX1/COX2 inhibitor (CAY10404 1 μmol/L) and the IC Ca^{2+} chelator (bis-(o-aminophenoxy)-tetraacetic acid-acetoxymethyl [BAPTA-AM] 10 μmol/L). In static conditions, prostaglandin E2 (10 μmol/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 μmol/L) activates adenylyl 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 dyn cm^{-2}, 15 min) showed increased phosphorylation of PKA substrate motifs normalized by the amounts of IC β-actin. FSK (15 μmol/L, 15 min) was used as a positive control. **C**, Western blot analysis of cell extracts from syncytiotrophoblasts exposed to FSS (1 dyn cm^{-2}, 15 min) shows increased phosphorylation of CREB on serine 133 (P-CREB S133) normalized by the amounts of IC GAPDH. FSK (15 μmol/L, 15 min) was used as a positive control. CREB phosphorylation by FSS was significantly reduced by inhibition of PKA (H89 3 μmol/L). **D**, Largest subcellular localization of P-CREB S133 in the syncytiotrophoblast nuclei in FSS conditions (1 dyn 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).

compared with static conditions. Human PlGF is essentially expressed in the placenta,[26] more precisely in the syncytiotrophoblast.[1] PlGF is a potent stimulator of placental angiogenesis.[3,27] 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.[28,29] 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 PlGF, which have already been reported. In a model of primary bronchial airway epithelial cells cultured on a deformable silicoelastic membrane, Mohammed et al[30] found that cyclic stretch induces PlGF expression in bronchial airway epithelial cells via nitric oxide release. Rashdan et al[15] found that FSS (12.4 dyn cm^{-2}) upregulates by 40% the secretion of PlGF in cocultured vascular cells (human coronary artery endothelial cells and smooth muscle cells). Their findings show that FSS may regulate the expression of PlGF in the vessel wall, which is a potent stimulator of collateral growth.

cAMP is known to be a strong activator of PlGF gene expression in human trophoblasts, and this effect is mediated through activation of PKA.[10] The action of forskolin and 8-Br-cAMP on PlGF secretion by syncytiotrophoblasts that we observed confirms that the cAMP–PKA signaling pathway is required for PlGF upregulation. Our results support the hypothesis that the FSS-induced synthesis/release of PGE2 could activate adenylyl cyclase, thus increasing intracellular cAMP. We observed an increase in intracellular cAMP levels under FSS. These experiments were performed with inhibition of phosphodiesterases, suggesting that FSS increases the production of cAMP by adenylyl cyclase activation rather than a decrease of cAMP

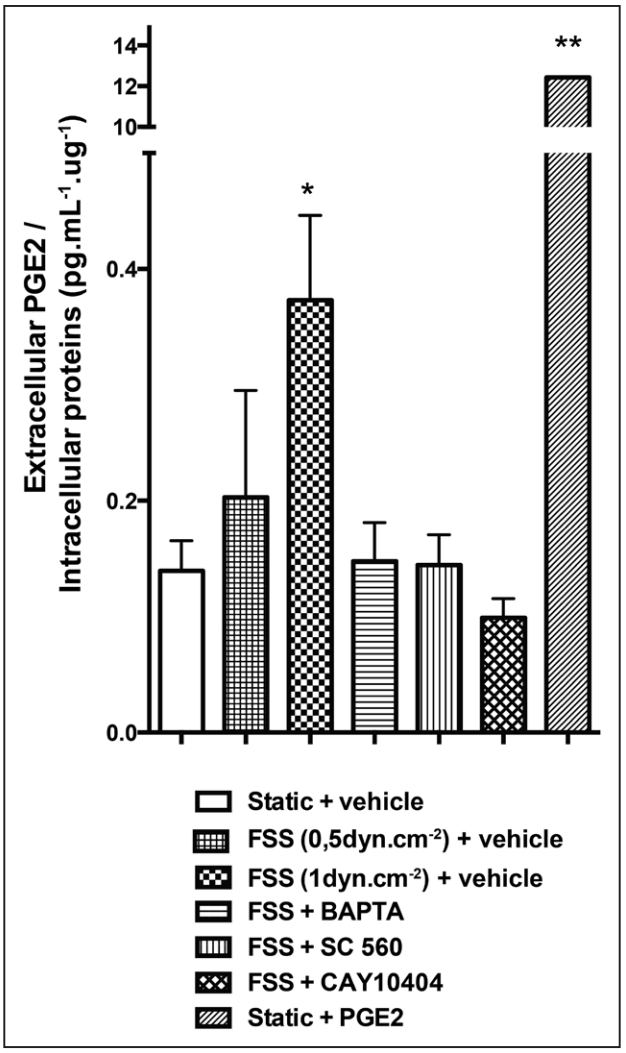

Figure 4. Fluid shear stress (FSS) increases extracellular prostaglandin E2 (PGE2) levels. Syncytiotrophoblasts were exposed to FSS (1 dyn cm^{-2}, 45 min) compared with static conditions. Total extracellular PGE2 was measured by enzyme-linked immunosorbent assay and normalized to total intracellular proteins. Total extracellular PGE2 per microgram of protein was significantly increased in FSS conditions (1 dyn cm^{-2}) compared with static controls (0.14±0.03 vs 0.37±0.07 pg mL^{-1} μg^{-1}; n=5 independent experiments, 2-tailed unpaired Student *t* test: **P*<0.05, ***P*<0.01). The FSS-enhanced PGE2 was blocked by the selective COX1 inhibitor (cyclooxygenase; SC560, 0.1 μmol/L) and the COX1/COX2 inhibitor (CAY10404 1 μmol/L). Treatment of syncytiotrophoblasts with bis-(o-aminophenoxy)-tetraacetic acid-acetoxymethyl (BAPTA-AM; 10 μmol/L) reduced FSS-induced PGE2 secretion compared with untreated sheared cells but remained greater than in static controls. In static conditions, PGE2 (10 μmol/L) was used as a positive control.

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.[31] We found that PGE2 was responsible for an increase of cAMP in syncytiotrophoblasts, probably via stimulation of EP2 receptor, which is $G\alpha_s$-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}),[22] osteocytes (16 dyn cm^{-2}),[32] and renal epithelial collecting duct cells (0.4 dyn cm^{-2}).[23] Wu et al[33] have demonstrated that PGE2 increases the expression of PlGF in human synovial fibroblasts. Their results demonstrate the upregulation of PlGF by 15-LOX (lipoxygenase) 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,[34–36] 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.[37] 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,[38] 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.[10] 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

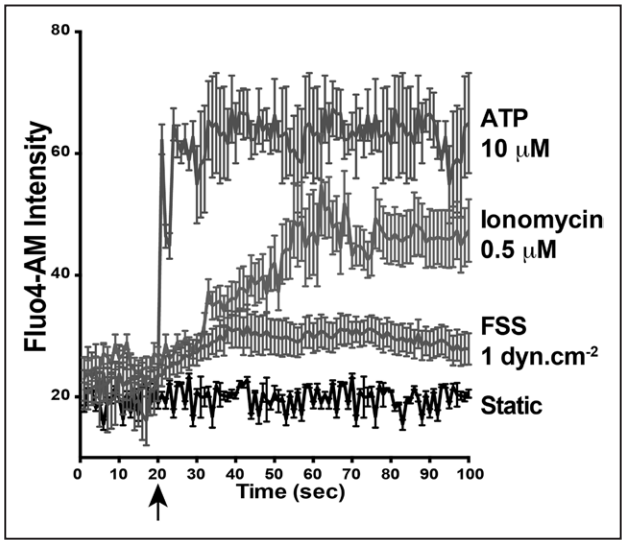

Figure 5. Fluid shear stress (FSS) triggers intracellular Ca^{2+} flux. Syncytiotrophoblasts were preincubated with Fluo-4AM (5 μmol/L, 15 min) in phenol red-free Opti-MEM and then exposed to FSS (1 dyn cm^{-2}, 2 min). Quantification of Fluo-4AM intensity by Image J captured multiple spikes in calcium flux after application of FSS/ionomycin (0.5 μmol/L)/adenosine triphosphate (ATP; 10 μmol/L) at 20 s. The arrow on the horizontal axis represents the injection of ionomycin/ATP or the start of the FSS. Traces represent the mean intensity of values recorded from individual syncytia (n=5).

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 PlGF. Activation of the cAMP–PKA–CREB pathway by FSS has been observed in diverse cell types, including chondrocytes,[39] osteocytes,[32] and hematopoietic stem cells.[22] Diaz et al[22] 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[40] showed in BeWo cells that FSS induces Ca^{2+} influx and $(Ca^{2+})_i$ increase via mechanosensitive activation of transient receptor potential vanilloid family type-6, which regulates microvillus formation through the functional activation of ezrin via Ca^{2+}-dependent Akt phosphorylation. The kinetics of $(Ca^{2+})_i$ increase are identical to those we describe for syncytiotrophoblasts. Berryman et al[41] 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[42] 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 $(Ca^{2+})_i$, 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 (PlGF 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.[16] Schlembach et al[43] found that maternal serum PlGF 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 PlGF, reduced uteroplacental blood flow might have downregulated PlGF 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 PlGF 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 PlGF.

Perspectives

The syncytiotrophoblast of the human placenta is a mechanosensitive 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.

PlGF 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 PlGF 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 PlGF in the maternal circulation, correct the angiogenic imbalance, and have a therapeutic impact in preeclampsia.

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Disclosures

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

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Novelty and Significance

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.