Chorionic Gonadotropin Stimulation of Angiogenesis and Pericyte Recruitment

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Context: During the periimplantation period, human chorionic gonadotropin (hCG) plays a key role by increasing the uterine blood flow through uterine vessel vasodilatation but also through angiogenesis. Indeed, we previously demonstrated that hCG contributes to endothelial cell recruitment and vessel formation.

Objective: In this study, hCG was proposed as an arteriogenic factor that could promote perivascular cell recruitment and vessel stabilization.

Design: The aortic ring assay, a three-dimensional ex vivo angiogenesis system mimicking all the steps of the angiogenesis process was used to study the impact of hCG on pericyte recruitment and vessel maturation.

Setting: The study was conducted at a university hospital laboratory.

Main Outcome Measures: Perivascular cell proliferation, migration, and apposition were quantified by computerized image analysis.

Results: Physiological concentrations of hCG (10–400 IU/ml) significantly enhanced pericyte sprouting and migration and gave rise to the maturation and coverage of endothelial capillaries. In a three-dimensional coculture model of endothelial and perivascular cells, hCG enhanced vessel tube formation and endothelial/mural cell adhesion. In addition, hCG stimulated the proliferation of human umbilical vein endothelial cells and smooth muscle cells. The specificity of these effects was determined by using an anti-hCG blocking antibody. Signaling pathways implicated on this hCG effect is protein kinase A and phospholipase C/protein kinase C dependent for the proliferative effect but only phospholipase C/protein kinase C for the migrative process.

Conclusions: Our findings highlight a novel paracrine role of this early embryonic signal in vessel maturation by stimulating perivascular cell recruitment, migration, and proliferation. (J Clin Endocrinol Metab 94: 4567–4574, 2009)
nullating factor release, 2) tissue remodeling by increasing matrix metalloproteinase-9 production, and 3) angiogenesis by inducing vascular endothelial growth factor (VEGF) up-regulation (1, 2). LH/hCG receptor (LH/hCGR) was first described on endometrial and myometrial vascular smooth muscle cells and thereafter in vascular endothelial cells (ECs) of the uterine vessels (3, 4). In vivo administration of hCG reduced vascular resistance in human uterus and decreased in vitro the formation of vasoconstrictor eicosanoids of the vascular wall (4, 5). Zygmunt et al (6), showed that hCG was able to promote angiogenesis by stimulating EC migration and capillary sprout formation. We previously showed that the angiogenic activity of hCG is mediated through a direct effect on ECs and through a paracrine dialogue between ECs and endometrial epithelial cells (1). Upon hCG binding to its LH/hCGR at the surface of endometrial ECs, VEGF synthesis and secretion is up-regulated, thereby activating the angiogenic switch in ECs. The endometrial epithelium is thus sensitive to specific embryonic signals, leading to the production of VEGF, the main angiogenic factor (7). As classically observed in pathological conditions (8), VEGF-driven angiogenesis results in the formation of highly abnormal vessels deficient in mural/pericyte coverage. The putative effect of hCG on perivascular cell recruitment and hence on vessel maturation is currently unknown. To address this issue, we used in this study the aortic ring assay, which mimics the angiogenic process including EC proliferation, migration, and differentiation into capillaries (9). In addition, we evaluated the effect of hCG on perivascular cell outgrowth and EC coverage by mural cells. The effects of hCG was also investigated in monocultures and cocultures of ECs and smooth muscle cells (SMCs). In this study, we provide evidence for a promoting effect of hCG on vessel maturation through the recruitment of perivascular cells. These novel findings underline a much broader spectrum of activities for hCG than initially anticipated.

Materials and Methods

Materials

Reagents tested were purified urinary hCG (Pregnyl, Organon, Roseland, NJ), recombinant hCG (Ovitrelle; Serono, Geneva, Switzerland), protein kinase A (PKA) inhibitor H89 dihydrochloride (Calbiochem, La Jolla, CA), phospholipase C (PLC) inhibitor U73122 (Calbiochem), protein kinase C (PKC) inhibitor bisindolylmaleimide I (BIM; Calbiochem), anti-hCG neutralizing antibody (Sc-7821; Santa Cruz Biotechnology, Santa Cruz, CA), cell tracker Green 5-chloromethylfluorescein diacetate (CMFDA; Molecular Probes, Invitrogen, Carlsbad, CA), and CellTracker red CMRA (Molecular Probes, Invitrogen).

Animals

Wistar rats (±250 g) and C57/Bl 6 mice (10–12 wk old) were provided by the Animal Center of the University of Liège. Experiments were conducted with the approval of the local ethical committee for the care of experimental animals. Mice were maintained with a 12-h light, 12-h dark cycle, with food and water available ad libitum.

Aortic ring assay

Rat and mice aortic rings were cultured in three-dimensional type I collagen gels as previously described (10, 11). Effects of different compounds were tested by addition to culture medium at d 0. For quantification, computerized image analysis was performed on a Sun SPARC30 workstation with the software Visilog 5.0 (Noesis, St. Aubin, France) according to Blacher et al. (10). The following parameters were determined: number of microvessels; maximal microvessel length, total number of branching in microvessels, number of perivascular cells (Nf), and perivascular cells maximal migration distance from the explant (Fmax).

Whole-mount immunostaining of aortic rings

At d 9 of culture, aortic fragments embedded in collagen gels were washed three times in PBS for 10 min followed by a fixation in 4% paraformaldehyde for 10 min. Samples were washed again in PBS three times for 10 min, and then nonspecific antibody binding was blocked with milk 1.5% for 20 min. Samples were incubated overnight at room temperature with a mix of a lectin and a primary antibody: Griffonia Simplifolia isoelectin-B4/Alexa Fluor 488 at 5 µg/ml (Molecular Probes; I21411) and rabbit anti-NG2 chondroitin sulfate pro-

FIG. 1. Effect of hCG on pericyte recruitment in the aortic ring assay. A, Photomicrographs of aortic rings cultured in the absence (0) or presence of increasing concentrations of recombinant hCG (rhCG; 0–100 IU/ml). Endothelial capillaries are depicted by the arrow, and fibroblast like-cells are indicated by the dotted arrow. B, Quantification of the Nf and Fmax (C) in response to increasing rhCG concentrations (10–400 IU/ml) after 9 d of culture. Data are mean ± S.D of one representative experiment, each condition running at least four rings. *, P < 0.05; ***, P < 0.001.
teoglycan antibody (Chemicon, Temecula, CA; AB5320) at 10 μg/ml. After three washes in PBS for 10 min each, the tissues were incubated at room temperature with a secondary goat anti-rabbit biotin antibody (Dako, Glostrup, Denmark; E432) at 1.9 μg/ml. Finally, an incubation with streptavidin Cy3 (Sigma, St. Louis, MO; S6402) was performed for 1 h at room temperature at 1 μg/ml. Samples were mounted with Vectashield-Dapi mounting medium (Vector Laboratories, Burlingame, CA; H-1200), after three washes in PBS for 10 min at room temperature.

Electron transmission microscopy
Aortic three-dimensional gels culture were stopped after 10 d, and gels were fixed at 4°C with 2.5% glutaraldehyde in a Sörensen 0.1 M phosphate buffer (pH 7.4). Gels were postfixed for 30 min with 1% osmium tetroxide. After dehydration, samples were embedded into Epon. Ultrathin sections, obtained with a Reichert Ultracut S ultramicrotome (Leica, Germany), were contrasted with uranyl acetate and lead citrate. Observations were done with a Jeol 100 CX II transmission electron microscope at 60 kV (Peabody, MA).

Cell culture
Human umbilical vein endothelial cells (HUVECs) were grown on 0.2% gelatin-coated dishes in MCDB 131 medium (Life Technologies, Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), HEPES (25 mM), glutamine (2 mM), endothelial cell growth supplement (12 ng/ml; BD Biosciences, Bedford, MA), heparin (2.5 mg/ml), and antibiotics (penicillin-streptomycin, 100 U/ml); for experiments, cells at passages 2–10 were used. Human aortic SMCs (AoSMC) purchased from Lonza (Basel, Switzerland; CC-2671) were grown in complete smooth muscle medium containing 10% FBS (SmBm; Lonza; CC-3181). Cells were at passages 6–10 used for experiments. Mouse SMCs C3H/10T1/2, clone 8 cell line (American Type Culture Collection, Manassas, VA; CCL-226) were grown in basal medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin/streptomycin (100 μg/ml), HEPES (15 mM), and bicarbonate. All cell types were cultured in 5% CO2 at 37°C, and media were replaced every 2 d.

10 T1/2 cell migration
To evaluate SMC migration, the wound assay model was used as described by Savani et al. (12). 10 T1/2 cells (10^5) were seeded on 24-well microplates and grown overnight in 10% FBS basal medium. A cross-shape scratch was made with a tip in cell monolayers. After 4 and 9 h, pictures were acquired at the same magnification and location in the bottom of the dish. Quantification was done using ImageJ software (National Institutes of Health, Bethesda, MD) by measurements of wounded areas at time 0, 4, and 9 h.

Proliferation assays
Proliferation tests were performed on AoSMCs, 10 T1/2 cells, and HUVECs cultured for 24 h. Cells (5 × 10^3) were seeded and allowed to adhere in 96-well microplates. For HUVEC proliferation, 96-well microplates were coated with gelatin at 2 mg/ml. Cells were treated with or without hCG (10–1000 U/ml), H89 (10 μM), U73122 (10 μM), or BIM (1 μg/ml) alone or combined with hCG (100 IU/ml) in their respective culture media containing 2% FBS. Cell proliferation was evaluated using 5-bromo-2’-deoxyuridine incorporation into cells followed by a cell proliferation colorimetric assay (Roche Applied Science, Penzberg, Germany) as previously described (13).

Boyden chamber migration assay
AoSMC cell migration was assessed using Boyden chamber assay. Cells were seeded on top of polycarbonate filters (8 μm pore) of Transwell Permeable Support (Costar, Corning Inc., Lowell, MA). Cells in stock culture were trypsinized, suspended in serum-free medium containing 0.1% BSA, and placed on the upper compartment of the chamber (7.10^4 cells/filter). The lower compartment of the chamber was filled with serum-free medium containing 1% BSA, supplemented with or without hCG, anti-hCG, H89, U73122, and BIM. After an incubation period of 24 h at 37°C, filters were fixed in methanol. Cells on filters were stained with crystal violet solution 0.1% (Sigma). Nonmigrated
cells at the upper surface of the filters were wiped away with a cotton swab. Quantification of the migration assay was done by colorimetric measurement (\(\lambda = 560\) nm) of cells at the lower surface of the filter.

HUVEC and AoSMC coculture assays

AoSMC and HUVEC coculture assays were performed in a modified matrigel overlay assay as previously reported (14). Briefly, HUVEC cells (15 \(\times 10^3\) CMFDA green labeled) were seeded on top of a 300 \(\mu l\) matrigel layer (15) in 24-well microplates and allowed to form tubular structures. Six hours later, AoSMCs (15 \(\times 10^3\) CMRA red labeled) were overseeded on HUVECs, and 100 IU/ml hCG were added or not. After 24 h, cultures were stopped, rinsed with PBS, and fixed with 4% paraformaldehyde until microscopic examination. To analyze the AoSMC distribution around HUVEC tube network, an original computer-assisted quantification was developed by implementing an algorithm using the image analysis tool box of MATLAB 7.1 (The Mathworks) using Student’s \(t\) test or using Wilcoxon test, with regard to heteroscedasticity. In the other experiments, Mann-Whitney tests were used for statistical analysis. \(P < 0.05\) was considered as statistically significant.

Statistical analyses

Statistical analysis were conducted with GraphPad Prism software (GraphPad Inc., La Jolla, CA). Aortic rings experiments including four explants per condition were repeated at least twice. Student’s \(t\) test was used to evaluate whether differences among groups were significant. For computerized image analysis, statistical analysis were performed with the statistics toolbox of MATLAB 7.1 (MathWorks) using Student’s \(t\) test or using Wilcoxon test, with regard to heteroscedasticity. In the other experiments, Mann-Whitney tests were used for statistical analysis. \(P < 0.05\) was considered as statistically significant.

Results

hCG promotes perivascular cell recruitment in the aortic ring assay

According to our previous results (1), the incubation of rat aortic fragments embedded in a collagen gel with recombinant hCG induced an angiogenic response characterized by enhanced capillary formation (Fig. 1 A). Interestingly, dispersed cells spread out, suggesting a possible mobilization of perivascular cells in addition to that of EC. These cells have been quantified through a computerized quantification (10) and were identified as mural cells by immunostaining. The addition of hCG (10–400 IU/ml) to the aortic ring culture media resulted in an increase of Nf (Fig. 1B) and distance of cell migration from the aortic explant (Fmax) (Fig. 1C). Similar results were obtained with urinary hCG. For next experiments, urinary hCG (uhCG) was used at its optimal concentration (100 IU/ml).

Whole-mount immunostainings of mice aortic ring cultures (d 9 of culture) were performed to identify the ECs through Isoclectin B4 staining and pericytes through NG2 detection. They revealed the pericyte phenotype of perivascular cells that were growing out in the presence of stimulatory dose of hCG (100 IU/ml) (Fig. 2). In control conditions, in the absence of hCG, two types of capillary-like structures were detected, one composed of naked endothelial cells and the other one composed of pericytes (Fig. 2, C and E). In sharp contrast, on hCG treatment, pericyte proliferation and migration were drastically stimulated (Fig. 2D), and endothelial tubes displayed a mature phenotype with a pericyte coating (Fig. 2, F and H). Observations by electron transmission microscopy confirmed the presence of perivascular cells upon treatment with hCG (Fig. 2H). In sharp contrast, in the absence of hCG,
Capillaries were formed of a single layer of naked ECs delimiting a lumen (Fig. 2G).

**hCG promotes EC and SMC interactions in a three-dimensional in vitro coculture model of HUVECs and human AoSMCs**

The interactions between EC and human AoSMCs were then investigated in vitro in a coculture system on matrigel. When HUVECs were seeded on matrigel, they organized into tube-like structures. AoSMCs were then seeded on top of the HUVEC culture to study pericyte-endothelial cell interactions (Fig. 3). Under hCG treatment, pericytes became more closely apposed to the ECs (Fig. 3A). An original computer-assisted method of quantification was used to measure the distance separating pericytes from ECs (Fig. 3B). The density of mural cells apposed to ECs (distance \( \leq 5 \mu m \)) was 20% higher under hCG treatment (*, \( P < 0.05 \)) (Fig. 3B). In contrast, the density of mural cells present at a distance higher than 50 μm from ECs was 1.5-fold lower on hCG treatment (**, \( P < 0.05 \)).

**hCG promotes SMC and EC proliferation through PKA/PKC signaling pathways**

We previously reported that hCG can directly modulate the proliferation of ECs (1). To evaluate the putative direct effect of hCG on perivascular cells, we next determined the impact of hCG on the proliferation of human AoSMCs. Increasing concentrations of hCG (10–500 IU/ml) stimulated AoSMC proliferation in a dose-dependent manner (Fig. 4A). This dose-response curve displayed a bell shape with maximal effect of hCG between 50 and 100 IU/ml. Similar results were obtained by using murine smooth muscle cells (10 T1/2) (Fig. 4B). The specificity of this effect was assessed by the addition of an anti-hCG-blocking antibody together with hCG (Fig. 4C).

Cell signaling pathways involved in the proliferative effects exerted by hCG on ECs and SMCs have next been investigated by inhibiting different pathways putatively involved in LH/hCGR signaling (Fig. 4, D and E). Addition of a PKC inhibitor (1 g/ml BIM) and a PLC inhibitor (10 M U73122) abolished the effect of hCG on HUVEC proliferation (Fig. 4D). Addition of a PKA inhibitor (10 M H89) and a PLC inhibitor (10 M U73122) abolished the effect of hCG on AoSMCs (Fig. 4E).

**hCG induces SMC migration**

A wound healing in vitro assay was first applied to determine the effect of hCG on SMCs (10T1/2). Recolonization of wounded areas was evaluated after 4 and 9 h by measuring the width of the gap in the same areas. At each time point, the size of the gap was measured, normalized to each native wound surface (time 0) and data were averaged (n = 24). Already after 4 h, hCG induced cell migration at a higher extent than in control condition (Fig. 5, A and B). After 9 h the recolonization was increased 1.4-fold (Fig. 5, A and B). A Boyden migration chamber assay was next used to further determine the promigratory effect of hCG on both smooth muscle cells.
Incubations of a stimulatory dose of hCG (100 IU/ml) resulted in a significant enhancement of both HUVEC (Fig. 6A) and AoSMC migration (Fig. 6B). HUVECs and AoSMCs seeded on Boyden chambers filters were incubated for 24 h with hCG (100 IU/ml) alone or in combination with signaling pathway inhibitors. Whereas hCG-induced migration of HUVECs was abolished by PKA (H89) or PLC (U73122) inhibitor (Fig. 6C), only the PLC and PKC inhibitor (BIM) was able to block the hCG-stimulated migration of AoSMCs (Fig. 6, D and E). All biological effects described above were observed in the presence of urinary and recombinant hCG.

Discussion

hCG is a key actor of placental vascularization (16–18), which is one of the crucial local adaptations to successful pregnancy. hCG stimulates directly angiogenesis through EC recruitment, enhanced proliferation, and migration (19). It also stimulates the release of VEGF by human epithelial endometrial cells that causes a paracrine stimulation of ECs. VEGF-mediated angiogenesis results in the formation of naked, dilated, and leaky capillaries resulting in tissue ischemia. Indeed, normal efficient blood circulation requires the coverage of newly formed endothelial tubes by vascular SMCs. In this study, through the use of five different models (aortic ring, cell proliferation assay, wound healing assay, Boyden chamber assay, and tube formation on matrigel), we report a potent effect of recombinant and urinary hCG on various SMC activities including proliferation, migration, and capacity to cover EC capillaries.

Because of the complexity of the mechanism underlying the angiogenic reaction and vessel maturation through pericyte recruitment, a panel of complementary models (9) has been used to investigate the mechanisms of action of hCG. The aortic ring assay comprises three different cell types (ECs, fibroblastic cells, and SMCs) and mimics four steps of the angiogenic process: 1) EC proliferation, 2) EC migration, 3) EC differentiation into capillaries, and 4) maturation by pericyte coverage (20). hCG was able to enhance the sprouting of both IB4-positive EC and NG2-positive pericytes. Interestingly, both their number and distance of migration were stimulated on hCG treatment. Of great interest is the finding that hCG promoted the coverage of endothelial capillaries by pericytes, whereas in its absence, EC and pericytes spread out in an independent manner. The impact of hCG on EC/mural cell interactions was then confirmed in a three-dimensional coculture model leading to tube-like structure formation. When EC and pericytes were cocultured on matrigel, hCG stimulated an affixing of close pericytes to ECs, which mimics the vascular maturation process. Our data are in accordance with clinical observations performed on hCG-rescued human corpus luteum. Indeed, hCG treatment of women increased endothelial cell proliferation and pericyte recruitment indicating an increase rate of angiogenesis together with vessel stabilization (21).

The presence of LH/hCGRs has been evidenced in vivo in the endothelium and smooth muscle of uterine blood vessels. Their expression levels are significantly increased in the intramyometrial segment (3, 4). In line with these findings, immunostaining of LH/hCGR revealed positivity on AoSMCs (data not shown). Our previous work reported that hCG has a direct stimulating effect on ECs (1). Accordingly, vascular uterine endothelial cells expressing LH/hCGR responded to physiological doses of hCG with increased capillary formation in vitro (6). The LH/hCGR-dependent effect of hCG is further supported by the application of the aortic ring assay to LH/hCGR-deficient mice (22). This genetic approach clearly demonstrated an abrogation of hCG angiostimulation in the absence of LH/hCGR.

To further dissect the mechanism of hCG action, more simple models consisting of monocultures of EC or pericytes/SMCs were used to determine whether hCG can exert an effect on cell proliferation and/or cell migration. hCG induced a strong proliferative effect on both ECs and
SMCs from murine or human origin, which is consistent with previous reports (23–25). Blocking antibody addition and use of recombinant hCG further confirmed the specificity of this effect. This effect was dose dependent with an optimal effect at 50–100 IU/ml corresponding to the physiological hCG concentration achieved in the first 2 wk of pregnancy. Such hCG concentration has proven a similar optimal angiogenic response in terms of endothelial cell proliferation and sprouting in different in vitro models (1, 19) and led to a stimulation of trophoblast migration in vitro (26).

We previously reported that hCG-mediated proliferative effect was PKA dependent (1). In the present study, we provide additional data that the stimulation of HUVEC proliferation by hCG is also sensitive to PKC and PLC inhibition. AoSMC proliferation was affected by PKA and PLC pathway inhibitions under hCG treatment. This is in line with previous reports showing that in porcine myometrial cells, LH/hCGR activation is coupled with two different signaling systems: adenylate cyclase and PLC (27).

SMC-induced migration was further studied in a wound healing assay in which hCG enhanced 10T1/2 migration already after 4 h of treatment and further after 9 h. This early promigrative effect signed a direct effect on migration property and was not reflecting an enhancement of cell proliferation in the wounded area. hCG caused a 2.5-fold increase of AoSMC migration in a Boyden chamber assay and a 2-fold increase of HUVEC migration. The effect of hCG on EC migration was clearly more potent when uterine microvascular endothelial cells were used (6). The present study demonstrates for the first time that hCG enhances pericyte/SMC migration. To specify the signaling pathways involved in this process, PKA and PLC inhibitors were tested in combination or not with hCG in the Boyden chambers model. We demonstrated that HUVEC-hCG induced migration signals through PKA and PLC pathways, whereas it involves only the PLC pathway in AoSMCs. Interestingly, this suggests that the signaling pathways involved in hCG’s promigrative property are cell type dependent.

In summary, our results support the complex and key role played by hCG in the angiogenic process occurring at the implantation site. They provide evidence for a novel function of hCG in vessel maturation through the stimulation of pericyte recruitment.

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