Sphingosine-1-phosphate inhibition of placental trophoblast differentiation through a G_i-coupled receptor response

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Abstract The failure of placental trophoblasts to differentiate properly is thought to play an important role in the cause of pregnancy disorders such as preeclampsia. We looked at the effects of the bioactive lipid sphingosine-1-phosphate (S1P) on the differentiation of primary human cytotrophoblasts (CTs) into syncytiotrophoblasts (STs) in culture. We found that S1P inhibited CT differentiation measured by human chorionic gonadotropin (hCG) secretion and the expression of placental alkaline phosphatase but had no effect on their fusion into multinucleated syncytialized cells. G-protein-linked S1P receptors 1, 2, and 3 were found in CTs by reverse transcriptase-polymerase chain reaction, and receptor 1 was found by Western blot analysis. Disruption of G_i signaling with pertussis toxin reversed the inhibitory effects of S1P. S1P reduced intracellular cAMP, and the addition of 8-bromo-cAMP reversed S1P inhibition of hCG secretion. Therefore, we suggest that S1P inhibits the differentiation of CTs into STs through G_i-coupled S1P receptor interaction(s), leading to the inhibition of adenylate cyclase and reduced production of intracellular cAMP. This is the first reported effect of S1P on placental trophoblast function.—Johnstone, E. D., G. Chan, C. P. Sibley, S. T. Davidge, B. Lowen, and L. J. Guilbert. **Sphingosine-1-phosphate inhibition of placental trophoblast differentiation through a G_i-coupled receptor response. *J. Lipid Res.* 2005.** 46: **1833–1839.**

Supplementary key words sphingosine-1-phosphate receptors • adenosine 3′,5′-cyclic monophosphate • chorionic gonadotropin • fusion • epidermal growth factor • G_i proteins

Sphingosine-1-phosphate (S1P) is a member of an important group of signaling sphingolipids now recognized to play a role in a diverse array of cellular processes, such as apoptosis, cell motility, calcium signaling, differentiation, and proliferation (1). It is particularly interesting because it appears to have dual functions inside and outside cells (2). The intracellular signaling of S1P is less well characterized because an intracellular target has yet to be identified in mammals, but this mode of action is strongly supported (1). Signaling by S1P outside the cell is mediated by extracellular G-protein-coupled membrane receptors, five of which have been identified and characterized ($S1P_1$ to $S1P_5$) (3). Each receptor signals to multiple downstream responses by coupling to different G-proteins: $S1P_1$ exclusively links to G_i, $S1P_2$ and $S1P_3$ link to G_i, G_q, and $G_{12/13}$, and $S1P_4$ and $S1P_5$ link to G_i and G_q. Downstream, $S1P_1/G_i$ inhibits adenylate cyclase (3) and stimulates the Ras small GTPase, possibly phospholipase C (4), and phosphatidylinositol 3-kinase (5). $S1P_2/G_q$ linkage leads to calcium mobilization through phospholipase C (6). In contrast to $S1P_1$ and $S1P_3$, $S1P_2$ inhibits cell migration by inhibiting Rac (7) despite coupling to the same G-proteins (G_i, G_q, and $G_{12/13}$) as $S1P_3$. It may also link to G_s, because it stimulates rather than inhibits adenylate cyclase (8). $S1P_3$ promotes cell migration through G_i (leading to Rac stimulation) and $G_{12/13}$ (leading to Rho stimulation) as well as calcium mobilization through G_q; unlike $S1P_2$, it inhibits adenylate cyclase (3).

The distribution of S1P receptors varies across cell types, with $S1P_1$ to $S1P_3$ being widespread and $S1P_4$ and $S1P_5$ being more restricted to lymphocytes, lung, and the central nervous system (7). How S1P functions through receptors 1–3 is thought to depend on the relative concentration of receptor type in individual cells and the corresponding activity of the signaling pathways triggered by their respective G-proteins (9).

The majority of work on S1P actions has centered on its roles in chemotaxis and apoptosis control. However, recently, a role was proposed in cell differentiation (10).

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The villous trophoblast separates maternal from fetal blood in the placenta and comprises immature replicative cytotrophoblasts (CTs) that fuse with and mature into functional but proliferatively senescent syncytiotrophoblasts (STs) (11). Abnormal differentiation is one of the features of the villous trophoblast of placentas from pregnancies complicated by preeclampsia and the intrauterine growth restriction that accompanies it (12–16). Platelet activation is another characteristic of preeclampsia (17) and is a major source of intravascular S1P (18). Indeed, plasma S1P levels are increased in preeclampsia patients (19).

We tested the hypothesis that altered S1P levels or signaling are responsible for the abnormal placental trophoblast differentiation seen in preeclampsia by determining whether S1P affects the differentiation of trophoblasts from uncomplicated placentas. Differentiation of highly purified villous CTs (20) was measured by three independent methods: fusion into syncytia, expression of placental alkaline phosphatase (PLAP; expressed in the apical membrane of the ST), and secretion of human chorionic gonadotropin (hCG; expressed by the ST). We show that S1P inhibits the functional markers of differentiation (PLAP and hCG), but not fusion, in a 5 day culture with epidermal growth factor (EGF). S1P inhibits through a G_i-coupled S1P receptor response that results in adenylate cyclase inhibition and lower intracellular cAMP.

MATERIALS AND METHODS

Materials

S1P (10 μM; Biomol, Plymouth Meeting, PA), lysophosphatidic acid (LPA) at 1 and 10 μM , the cell-permeable cAMP analog 8-bromo-cAMP (1 mM; Sigma, Oakville, Ontario, Canada), and pertussis toxin (200 ng/ml; Sigma) were added to cell cultures 2–4 h after cell plating. All other chemicals were of the purest grades available commercially.

Isolation, purification, and culture of pure term villous CTs

Placentas were obtained with ethics approval of the Capital Health Authority after elective cesarean or normal term delivery from uncomplicated pregnancies at the Royal Alexandra Hospital, Edmonton. Villous CTs (>99.99% pure) (20) were isolated by trypsin-DNase digestion of minced chorionic tissue and immunoabsorption onto Ig-coated glass bead columns as described previously (20, 21) using anti-CD9 (monoclonal antibody 50H.19; house preparation), anti-major histocompatibility complex (MHC) class I (W6/32; Harlan Sera-Lab, Crawley Down, Sussex, UK), and anti-MHC class II (clone 7H3; house preparation) antibodies for immunoelimination. The purified cells were routinely cryopreserved and after thawing were washed twice in Iscove's modified Dulbecco's medium (IMDM; GIBCO, Grand Island, NY) supplemented with 10% FBS (GIBCO) and antibiotics (end concentrations, penicillin, 100 U/ml; streptomycin, 100 μg/ml; Sigma). The cells were seeded at 2.5×10^6 per well per 2 ml of IMDM/FBS in 6-well tissue culture dishes (NUNC™ number 152795; GIBCO) and 1.5×10^5 per well in 96-well dishes and incubated for 4 h at 37°C in a humidified atmosphere of 5% CO_2 in air. The nonadherent cells and debris were removed with prewarmed IMDM/FBS, IMDM/FBS was readded with the experimental additions, and the dishes were returned to the incubator with additions for further culture for the times indicated for individual experiments.

R2TK fibroblast proliferation (assay for LPA)

The rat fibroblast line R2TK was obtained from David Brindley (University of Alberta) and cultured as described previously (22) with the following variations. In the last 16 h of the 24 h exposure to LPA, the cells were exposed to Bromodexyuridine (BrdU) (10 μg/ml; Sigma) and then fixed in ice-cold methanol (20 min). The cells were then treated with 4 M HCl for 30 min at 37°C, washed with PBS, and reacted with a 1:400 dilution of the mouse monoclonal antibody BU33 (Sigma) for 4 h. After washing with PBS, cells were exposed to Alexa Fluor 488 (Molecular Probes, Eugene, OR) for 30 min (to both lightly stain all nuclei and to brightly stain BrdU-labeled nuclei) and then washed again with PBS. They were visualized as described below.

Immunofluorescence

Treated cells on 96-well plates were fixed with 1% paraformaldehyde (10 min; PLAP) or methanol-acetone (−20°C, 10 min; desmoplakin, not allowed to dry) after washing once with PBS. Fixed cells were then incubated with nonimmune goat serum (Zymed Laboratories, Markham, CA) to block nonspecific binding sites for 1 h and exposed either to a primary antibody for PLAP (IgG2a monoclonal antibody, clone 8B6, 10 μg/ml; Sigma, St. Louis, MO) or desmoplakin (IgG1 monoclonal antibody, 10 μg/ml; ICN ImmunoBiologicals, Costa Mesa, CA) overnight at 4°C. The cells were then washed with PBS and exposed to 1 μg/ml streptavidin-Alexa Fluor 546 conjugate (Molecular Probes) for 30 min, then washed again with PBS and counterstained with 4′,6-diamino-phenylindole (DAPI; 100 μl of 1.4 μg/ml; Molecular Probes) for 10 min at room temperature.

Digital photography and analysis

Images were obtained with an Olympus IX2-UCB motorized inverted research microscope equipped with a Lambda DG-4 high-speed filter changer and a Cascade 16 bit digital monochrome camera (Olympus, Melville, NY). Digital images of each well were obtained with DAPI (blue) and rhodamine (red) filters. We used Slidebook 3.0 (Carsen, Markham, Ontario, Canada) as capture software and Image Pro-Plus (Media Cybernetics, Del Mar, CA) for analysis. Multinucleation was determined by manual counting of nuclei/desmoplakin-stained cells. PLAP intensity was assessed after setting the fluorescence level at the beginning of each experiment in control (untreated wells) as zero, and the relative intensity of S1P-treated cultures was assessed with Image-Pro Plus.

RT-PCR

Trophoblast RNA was isolated from three separate trophoblast cultures using Trizol (GIBCO) according to the manufacturer's instructions. Reverse transcription was performed on 2 μg of total RNA using the TaqMan reverse transcription kit (Invitrogen) containing 1× RT buffer (Invitrogen RT buffer), 5.5 mM $MgCl_2$, 500 μM deoxynucleoside triphosphate, 2.5 μM random primers, 0.4 U/μl RNase inhibitor, and 1.25 U/μl reverse transcriptase in a total volume of 40 μl. PCR was performed using the platinum-Taq polymerase kit (Invitrogen) (mixture: 1× PCR buffer, 2.5 mM $MgCl_2$, 300 μM deoxynucleoside triphosphate, and 200 nM specific primers in a reaction volume of 50 μl) using 35 cycles and annealing temperatures of 54–63°C. Primers for $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_5$ were produced at the University of Alberta according to successfully used sequences from the literature [$S1P_2$ and $S1P_3$ (23), $S1P_1$ and $S1P_5$ (24)]; the sequences for $S1P_4$, a kind gift from the Academic Unit of Child Health (Manchester, UK), were 5′-ACGGGAGGGCCTGCTCTTCA-3′ (forward) and

5′-AAGGCCAGCAGGATCATCAG-3′ (reverse). PCR products were separated on 1% agarose/ethidium bromide gels, and visualized bands of the appropriate base pair size were cut and prepared for confirmatory sequencing using a gel purification kit (Qiagen, Mississauga, Ontario, Canada). Genomic DNA was used as a positive control for $S1P_4$ and $S1P_5$, which were not detected in trophoblast cells, to confirm efficient PCR primer action.

Western blot analysis

Sample protein concentrations were determined in duplicate with Micro BCA™ Reagent (Pierce Chemical, Rockford, IL) using a serum albumin standard. Sample protein (15–20 μg/ml) was solubilized in 3× sample buffer (Sigma) by boiling for 5 min and stored until electrophoresis. SDS-PAGE on 10% gels was performed as described previously using a Mini-Protein II gel system (Bio-Rad Laboratories, Inc., Hercules, CA). After electrophoresis, gels were equilibrated for 15 min in transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol). Proteins were electrophoretically transferred onto nitrocellulose membranes (100 min, 60 V), which were then incubated with a blocking solution [5% dried skim milk in 100 mM Tris (pH 7.5) with 140 mM NaCl and 0.01% Tween 20] for a minimum of 1 h. The blots were incubated overnight at 4°C with anti-Edg1 (Biomol), washed twice with the blocking and washing solutions, incubated with diluted horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA; Southern Biotechnology Laboratories) for 1–2 h at room temperature, washed extensively, and developed using enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK) on X-ray films. Attempts to identify $S1P_2$ and $S1P_3$ using Western blotting were unsuccessful because of the failure of purchased antibodies to detect $S1P_2$ and $S1P_3$ in positive controls (human umbilical vein endothelial cells).

hCG and cAMP assays

Cell supernatants were collected, centrifuged at high speed for 5 min at 4°C, and frozen at −20°C. A colorimetric assay system (DRG Diagnostics, Marburg, Germany) was used to determine β-hCG levels with optical density determined on a 96-well plate reader (Molecular Probes).

Intracellular cAMP levels were obtained with a colorimetric assay kit (Cayman Chemicals, Ann Arbor, MI). Trophoblasts were cultured for 18 h, stimulated with forskolin (10 μM, 20 min), and then treated with or without S1P. At 1 h, trophoblasts were harvested with 1 ml of 0.1M HCl, and cAMP analysis was performed. Readings were obtained the next day.

Statistical analysis

Experiments for each figure were performed at least three times on trophoblasts isolated from two different placentas. Differences between experimental groups for the two cell types (CT- or ST-like cells) were evaluated by one-way ANOVA with pairwise multiple comparison procedures (Tukey test) using the SigmaStat program (Jandel Scientific, San Rafael, CA). Results were considered significant at $P < 0.05$.

RESULTS

Isolated term CTs (~90% pure) spontaneously mature in culture to secrete hCG, a marker of trophoblast biochemical differentiation (25), with and without FBS (26). Highly purified CTs (>99.99% purity) also spontaneously differentiate in serum-containing medium, to secrete hCG (our unpublished data) and fuse in culture to form multinucleated cells (27), although to a lesser degree than the less purified cells. The addition of S1P to CTs cultured with serum results in the inhibition of differentiation, as assessed by two markers of differentiation: hCG production and PLAP staining (**Fig. 1A**, **B**, respectively). The effect was independent of adherent cell number and apoptosis rate, which were not statistically different between the two groups (data not shown). EGF-stimulated hCG production and PLAP formation were also inhibited by S1P [59.2 ± 19.0% (n = 6 placentas) and 33.5 ± 2.1% (n = 2 placentas), respectively]. Both are very similar to S1P's inhibition of the respective spontaneous differentiation (compare with Fig. 1).

We have shown previously that CTs in culture require EGF to form extensive multinucleated syncytia but that it was not required for the upregulation of hCG production (28). However, highly purified CTs still fuse without EGF, albeit to a lesser degree. Unknown is whether S1P inhibited the fusion in association with its effects on hCG and PLAP. Therefore, we measured levels of multinucleation with EGF and S1P and found that there was no significant

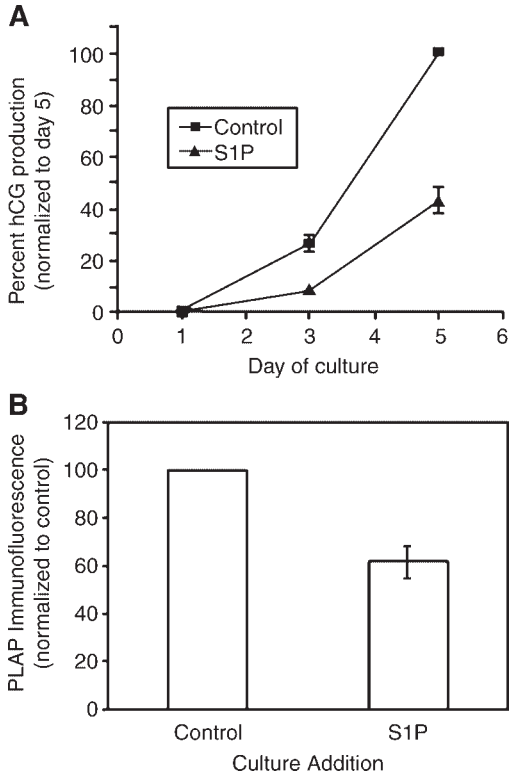

Fig. 1. Sphingosine-1-phosphate (S1P) inhibits cytotrophoblast (CT) human chorionic gonadotropin (hCG) and placental alkaline phosphatase (PLAP) upregulation. Trophoblasts were plated, washed, and cultured as summarized in Materials and Methods. Cells were cultured with 10 μM S1P, media were changed on days 1, 3, and 5, and hCG levels were measured in the media. On day 5, PLAP immunofluorescence on the cultured cells was assessed, and the staining intensity was normalized to the untreated control cells. The results shown in A are means ± SD of six independent experiments carried out with cells from five placentas. The results shown in B are means ± SD of three independent experiments carried out with cells from three placentas.

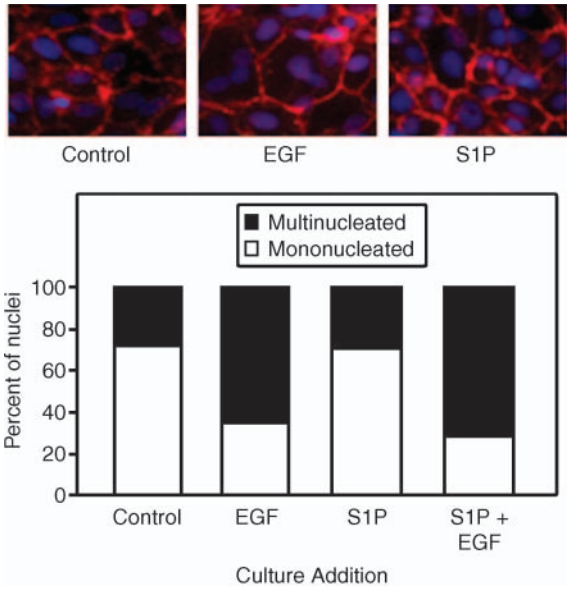

Fig. 2. S1P does not inhibit CT fusion. Trophoblasts cultured as described in the legend to Fig. 1 were exposed to epidermal growth factor (EGF; 10 μg/ml) and S1P (10 μM) with media changes every 2 days and on day 5 fixed and stained for desmoplakin (red) and 4′,6-diamino-phenylindole (blue). The number of multinucleated ($n \geq 2$) and mononucleated cells was evaluated in 16 microscope fields per experiment. Values shown are means of four independent experiments carried out with cells from three placentas. The values for control and EGF were significant different ($P < 0.05$), but those for control and S1P were not.

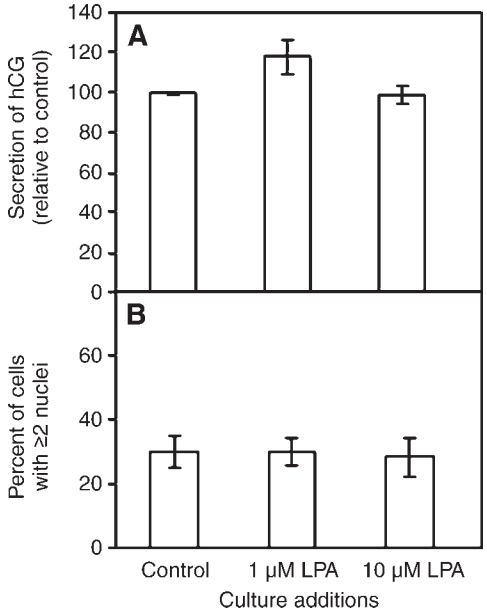

Fig. 3. Effect of lysophosphatidic acid (LPA; 1 and 10 μM) on cultured trophoblast secretion of hCG (A) and fusion (B). Supernatant hCG concentrations (see legend to Fig. 1) and multinucleation (see legend to Fig. 2) were measured on day 5 of culture. Values shown are means ± range from two of four determinations from cells derived from two placentas.

difference between S1P-treated and control cells (**Fig. 2**). In contrast, EGF strongly stimulated fusion, but S1P still did not reverse this fusion (with EGF, 64%; with S1P + EGF, 72%) (Fig. 2).

LPA also exerts its effects through a family of G-protein-coupled receptors that are broadly expressed (29). Therefore, we tested LPA at 1 and 10 μM for its effect on R2TK fibroblast proliferation (positive control) (22) and on trophoblast production of hCG and fusion into multinucleated cells. The LPA preparation was fully active (medium control, 14.8%; 1 μM LPA, 44%; 10 μM LPA, 55.2% of cells incorporated BrdU). We next showed that LPA did not decrease hCG secretion from trophoblasts at 1 and 10 μM (**Fig. 3A**) and did not change the extent of cell fusion in a 5 day treatment (Fig. 3B). We conclude that under the conditions of the culture, LPA does not affect trophoblast differentiation.

We next identified a potential mechanism of action of S1P. In **Fig. 4A**, we show that trophoblasts express $S1P_1$, $S1P_2$, and $S1P_3$ receptor mRNA but show no evidence of $S1P_4$ and $S1P_5$ mRNA. To confirm that mRNA expression of one of the S1P receptors leads to protein expression, we demonstrated the presence of $S1P_1$ protein (Fig. 4B). $S1P_1$, $S1P_2$, and $S1P_3$ couple with G_i (5). Pertussis toxin blocks G_i signaling by ADP-ribosylating the Gα subunit (5) and inhibits the actions of S1P mediated through the S1P receptor/G_i linkage (30). Pertussis toxin did not inhibit the differentiation of untreated CTs, as judged by hCG secretion; however, it reversed the inhibitory effects of S1P (**Fig. 5**). These results suggest that G_i coupling is not important in spontaneous differentiation to an hCG-secreting state but that it mediates the inhibitory action of S1P.

Activation of G_i inhibits the intracellular production of cAMP by adenyl cyclase (31). Because cAMP increases tro-

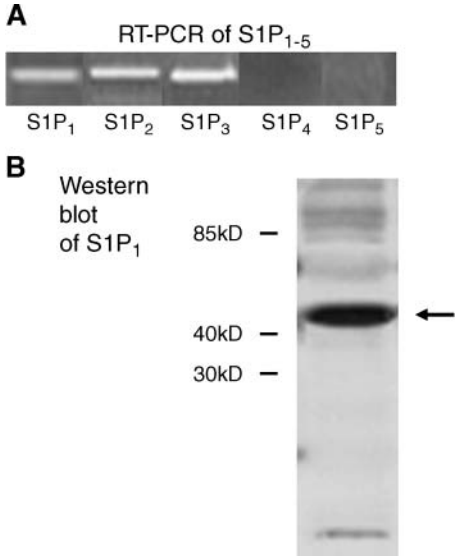

Fig. 4. Expression of S1P receptors 1, 2, and 3 on trophoblasts. A: A representative RT-PCR was carried out on total mRNA extracted from trophoblasts cultured from three placentas with the same results. Positive controls (genomic DNA) were positive for S1P receptors 4 and 5 (data not shown). B: Western blot for $S1P_1$. A representative blot of three experiments on cells from three placentas is shown. The locations of molecular mass marker proteins are shown.

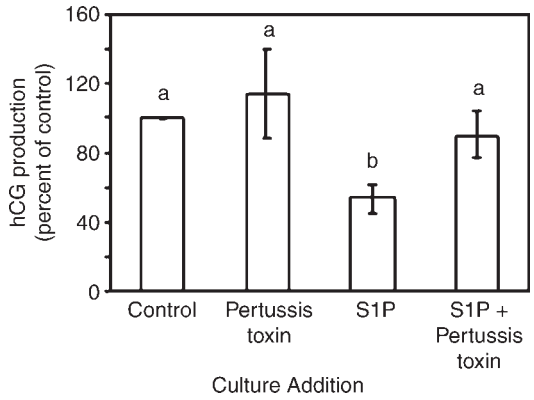

Fig. 5. Pertussis toxin reverses the inhibitory effect of S1P on hCG secretion. Trophoblasts were prepared as described in the legend to Fig. 1 and cultured with pertussis toxin (100 ng/ml) or S1P (10 μM) with medium changes every 2 days. After 5 days, the hCG levels were determined and expressed as a percentage of control. Values shown are means ± SD of four independent experiments carried out with cells from three different placentas. Bars labeled with different letters (a or b) are significantly different ($P < 0.05$).

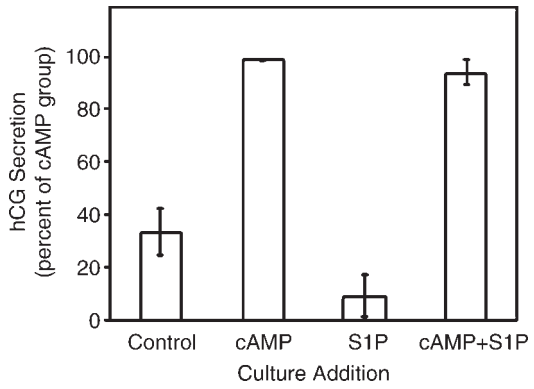

Fig. 7. Addition of 8-bromo-cAMP reverses the inhibitory effect of S1P secretion of hCG. Trophoblasts were prepared as described in the legend to Fig. 1 and cultured with or without 8-bromo-cAMP (1 mM) and S1P (10 μM), as depicted, with medium changes every 2 days. On day 5, hCG levels in the supernatant were measured and normalized to the cAMP group. Values shown are means ± SD of three independent experiments on cells from three placentas.

phoblast differentiation in vitro (32), we speculated that S1P inhibited differentiation through G_i inhibition of adenyl cyclase, resulting in reduced intracellular cAMP. To test this hypothesis, we stimulated cAMP production with forskolin and measured intracellular cAMP in the presence and absence of S1P. The addition of S1P to CTs for 1 h resulted in a reduction in intracellular cAMP levels by 46 ± 22% (**Fig. 6**). Furthermore, we found that a cell-permeable analog of cAMP, 8-bromo-cAMP, was able to reverse the inhibitory effects of S1P on hCG production when added during a 5 day culture (**Fig. 7**), showing that cAMP's prodifferentiation actions are downstream of S1P effects.

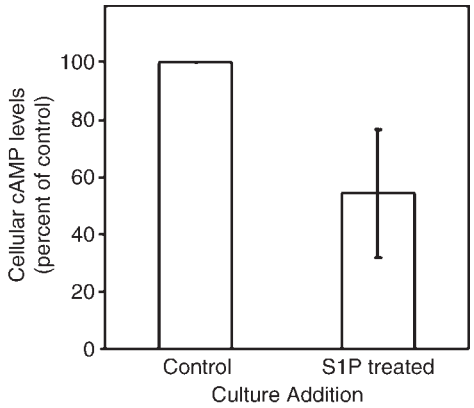

Fig. 6. S1P reduces CT intracellular cAMP levels. Trophoblasts were prepared as described in the legend to Fig. 1 and cultured with forskolin (10 μM) with or without S1P (10 μM) for 1 h. The cells were lysed, intracellular cAMP was assessed, and the results were expressed as a percentage of the control. Values shown are means ± SD of six experiments carried out with cells from three placentas. The control and S1P values were significantly different ($P < 0.001$).

DISCUSSION

To our knowledge, this is the first study to show a role for S1P in trophoblast biology. Our results suggest that this bioactive lipid may inhibit and fine-tune the differentiation of CTs into STs. Because the ST layer, which acts as the interface between maternal and fetal circulations, is incapable of dividing, it must be continuously replenished from the replicative CTs throughout pregnancy (11). Conditions that make this less likely may inhibit the replenishment process and reduce the efficiency of placental nutrient transfer, leading to the reduced fetal size that is seen in intrauterine growth restriction and some cases of preeclampsia (33). There are preliminary data that S1P is increased in maternal serum in pregnancies complicated by preeclampsia (19) but no data on fetal S1P levels. Because the majority of serum S1P comes from circulating platelets (18) and platelet function in preeclampsia is altered in both maternal (17) and fetal (34) circulations, the trophoblasts (both CTs and STs) are likely exposed to higher S1P levels in pregnancies complicated by preeclampsia.

We have demonstrated that S1P inhibited two of three markers of spontaneous and EGF-stimulated trophoblast differentiation (hCG secretion and PLAP expression but not cellular fusion; Figs. 1, 2). The expression of hCG was shown to be required for spontaneous or EGF-stimulated differentiation (measured by hCG expression) of CTs (32). PLAP expression occurs on the brush border of differentiated trophoblasts (35), although whether this is linked to hCG production or some other aspect of the differentiation pathway is unknown. The appearance of ~30% of the nuclei in multinucleated units after 5 days (without the addition of EGF) was unaffected by S1P. This may mean that the S1P inhibition of hCG secretion by 57% (or PLAP expression by 39%) did not reflect sufficient inhibition to prevent spontaneous fusion. Another possibility is that hCG secretion and PLAP expression are programmed separately from cell fusion. In support of the latter possi-

bility, we note that with EGF (which promotes greater cellular fusion), S1P still inhibits hCG production, but not fusion (Fig. 2). In a similar manner, anti-EGF receptor antibody blocked fusion but not hCG expression in cultured trophoblasts (28).

It has been suggested that CT differentiation is linked to caspase-8 activation and the onset of apoptosis (36). However, EGF both stimulates CT fusion (26, 32) and promotes CT resistance to apoptosis (37). Cellular production of S1P is stimulated by EGF, and S1P is an antiapoptotic agent for CTs (this paper). Whether EGF or S1P allows caspase-8 activation while preventing further apoptosis is currently under investigation.

S1P receptors have not previously been identified on trophoblasts. Therefore, we first screened for $S1P_1$ to $S1P_5$ mRNA by RT-PCR and identified $S1P_1$ to $S1P_3$. Because all three have been shown to link to G_i (3), we used pertussis toxin, a known inhibitor of G_i (3, 8, 30), to determine whether the observed inhibitory effect of S1P on trophoblast production of hCG was through these receptors. Pertussis toxin blocked the inhibitory effects of S1P on hCG production but did not block the spontaneous increase. We conclude that S1P (negative), but not spontaneous (positive), signaling of hCG production is coupled to G_i.

There are a number of lysophospholipids (S1P, LPA, sphingosylphosphorylcholine) that signal by binding to cell surface receptors linked to G-proteins (29). Although we are careful not to claim specificity of S1P signaling among the lysophospholipids, we have shown that LPA at 1 and 10 μM does not inhibit or stimulate trophoblast differentiation more than control cultures. We are investigating the expression of LPA receptors (LPA1 to LPA3) on primary trophoblasts, and if they are present, we will continue our investigations of the effect of LPA on trophoblast differentiation.

One of the actions of G_i is to inhibit adenylate cyclase, the rate-limiting enzyme in cAMP formation (3). When cAMP levels were stimulated with forskolin, we found that S1P reduced them. Culturing CTs with a cell-permeable form of cAMP stimulates fusion (38) and differentiation to hCG-expressing cells (32) through interaction with the cAMP response element, leading to the upregulation of hCG α transcription (39), and the SP2 α transcription factor, leading to the upregulation of hCG β transcription (40). We found that culturing CTs with 8-bromo-cAMP reversed the inhibitory effects of S1P on hCG production, indicating that S1P receptor/G_i signaling is upstream of cAMP's prodifferentiation actions.

Partly purified villous trophoblasts when cultured serum-free appear to rapidly differentiate (<48 h) into syncytia and begin to secrete hCG (25, 26). Highly purified trophoblasts also spontaneously differentiate in serum-containing medium, but much more slowly and to a lesser degree (27). However, even these highly purified cells rapidly fuse (but fail to survive) when cultured in a medium specialized for epithelial cells (41). Thus, differentiation and survival of trophoblasts in culture appear to depend partly on the presence of mesenchymal cells contaminating the preparation (probably macrophages and fibroblasts) and partly on the basal medium (including serum). These effects of mesenchyme and basal media are overlapping and partial but can be studied. The partial suppression of primary trophoblast differentiation by S1P is part of a larger study of differentiation ongoing in this laboratory.

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REFERENCES

1. Maceyka, M., S. G. Payne, S. Milstien, and S. Spiegel. 2002. Sphingosine kinase, sphingosine-1-phosphate, and apoptosis. *Biochim. Biophys. Acta.* **1585:** 193–201.
2. Spiegel, S., and S. Milstien. 2003. Exogenous and intracellularly generated sphingosine 1-phosphate can regulate cellular processes by divergent pathways. *Biochem. Soc. Trans.* **31:** 1216–1219.
3. Siehler, S., and D. R. Manning. 2002. Pathways of transduction engaged by sphingosine 1-phosphate through G protein-coupled receptors. *Biochim. Biophys. Acta.* **1582:** 94–99.
4. Okamoto, H., N. Takuwa, K. Gonda, H. Okazaki, K. Chang, Y. Yatomi, H. Shigematsu, and Y. Takuwa. 1998. EDG1 is a functional sphingosine-1-phosphate receptor that is linked via a Gi/o to multiple signaling pathways, including phospholipase C activation, Ca^{2+} mobilization, Ras-mitogen-activated protein kinase activation, and adenylate cyclase inhibition. *J. Biol. Chem.* **273:** 27104–27110.
5. Neves, S. R., P. T. Ram, and R. Iyengar. 2002. G protein pathways. *Science.* **296:** 1636–1639.
6. Kupperman, E., S. An, N. Osborne, S. Waldron, and D. Y. Stainier. 2000. A sphingosine-1-phosphate receptor regulates cell migration during vertebrate heart development. *Nature.* **406:** 192–195.
7. Ishii, I., N. Fukushima, X. Ye, and J. Chun. 2004. Lysophospholipid receptors: signaling and biology. *Annu. Rev. Biochem.* **73:** 321–354.
8. Gonda, K., H. Okamoto, N. Takuwa, Y. Yatomi, H. Okazaki, T. Sakurai, S. Kimura, R. Sillard, K. Harii, and Y. Takuwa. 1999. The novel sphingosine 1-phosphate receptor AGR16 is coupled via pertussis toxin-sensitive and -insensitive G-proteins to multiple signalling pathways. *Biochem. J.* **337:** 67–75.
9. Hla, T. 2003. Signaling and biological actions of sphingosine 1-phosphate. *Pharmacol. Res.* **47:** 401–407.
10. Kihara, A., M. Ikeda, Y. Kariya, E. Y. Lee, Y. M. Lee, and Y. Igarashi. 2003. Sphingosine-1-phosphate lyase is involved in the differentiation of F9 embryonal carcinoma cells to primitive endoderm. *J. Biol. Chem.* **278:** 14578–14585.
11. Benirschke, K., and P. Kaufmann. 2000. Pathology of the Human Placenta. 4th edition. Springer Verlag, New York.
12. Redman, C. W. 1993. The placenta, pre-eclampsia and chronic villitis. *In* The Human Placenta. C. W. Redman, I. L. Sargent, and P. M. Starkey, editors. Blackwell Scientific Publications, London. 433–467.
13. Arnholdt, H., F. Meisel, K. Fandrey, and U. Lohrs. 1991. Proliferation of villous trophoblast of the human placenta in normal and abnormal pregnancies. *Virchows Arch. B Cell Pathol.* **60:** 365–372.
14. Mayhew, T. M., C. Ohadike, P. N. Baker, I. P. Crocker, C. Mitchell, and S. S. Ong. 2003. Stereological investigation of placental morphology in pregnancies complicated by pre-eclampsia with and without intrauterine growth restriction. *Placenta.* **24:** 219–226.
15. Lee, X., J. C. Keith, Jr., N. Stumm, I. Moutsatsos, J. M. McCoy, C. P. Crum, D. Genest, D. Chin, C. Ehrenfels, R. Pijnenborg, et al. 2001. Downregulation of placental syncytin expression and abnormal protein localization in pre-eclampsia. *Placenta.* **22:** 808–812.
16. Crocker, I. P., S. Cooper, S. C. Ong, and P. N. Baker. 2003. Differences in apoptotic susceptibility of cytotrophoblasts and syncytiotrophoblasts in normal pregnancy to those complicated with

preeclampsia and intrauterine growth restriction. *Am. J. Pathol.* **162:** 637–643.
17. Trudinger, B. J. 1976. Platelets and intrauterine growth retardation in pre-eclampsia. *Br. J. Obstet. Gynaecol.* **83:** 284–286.
18. Yatomi, Y., F. Ruan, S. Hakomori, and Y. Igarashi. 1995. Sphingosine-1-phosphate: a platelet-activating sphingolipid released from agonist-stimulated human platelets. *Blood.* **86:** 193–202.
19. Hemmings, D. G., D. N. Brindley, and S. T. Davidge. 2003. Sphingosine-1-phosphate levels are elevated in plasma and serum samples from preeclamptic women. *J. Soc. Gynecol. Investig.* **10:** 280A.
20. Guilbert, L. J., B. Winkler-Lowen, R. Sherburne, N. S. Rote, H. Li, and D. W. Morrish. 2002. Preparation and functional characterization of villous cytotrophoblasts free of syncytial fragments. *Placenta.* **23:** 175–183.
21. Yui, J., M. I. Garcia-Lloret, A. J. Brown, D. W. Berdan, D. W. Morrish, T. G. Wegmann, and L. J. Guilbert. 1994. Functional, long-term cultures of human term trophoblasts purified by column-elimination of CD9 expressing cells. *Placenta.* **15:** 231–246.
22. Gomez-Munoz, A., A. Martin, L. O'Brien, and D. N. Brindley. 1994. Cell-permeable ceramides inhibit the stimulation of DNA synthesis and phospholipase D activity by phosphatidate and lysophosphatidate in rat fibroblasts. *J. Biol. Chem.* **269:** 8937–8943.
23. Hornuss, C., R. Hammermann, M. Fuhrmann, U. R. Juergens, and K. Racke. 2001. Human and rat alveolar macrophages express multiple EDG receptors. *Eur. J. Pharmacol.* **429:** 303–308.
24. Kveberg, L., Y. Bryceson, M. Inngjerdingen, B. Rolstad, and A. A. Maghazachi. 2002. Sphingosine 1 phosphate induces the chemotaxis of human natural killer cells. Role for heterotrimeric G proteins and phosphoinositide 3 kinases. *Eur. J. Immunol.* **32:** 1856–1864.
25. Kliman, H. J., J. E. Nestler, E. Sermasi, J. M. Sanger, and J. F. Strauss. 1986. Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. *Endocrinology.* **118:** 1567–1582.
26. Morrish, D. W., D. Bhardwaj, L. K. Dabbagh, H. Marusyk, and O. Siy. 1987. Epidermal growth factor induces differentiation and secretion of human chorionic gonadotropin and placental lactogen in normal human placenta. *J. Clin. Endocrinol. Metab.* **65:** 1282–1290.
27. Guilbert, L. J., B. Winkler-Lowen, R. Sherburne, N. S. Rote, H. Li, and D. W. Morrish. 2002. Preparation and functional characterization of villous cytotrophoblasts free of syncytial fragments. *Placenta.* **23:** 175–183.
28. Morrish, D. W., J. Dakour, H. Li, J. Xiao, R. Miller, R. Sherburne, R. C. Berdan, and L. J. Guilbert. 1997. In vitro cultured human term cytotrophoblast: a model for normal primary epithelial cells demonstrating a spontaneous differentiation programme that requires EGF for extensive development of syncytium. *Placenta.* **18:** 577–585.
29. Kostenis, E. 2004. Novel clusters of receptors for sphingosine-1-phosphate, sphingosylphosphorylcholine, and (lyso)-phosphatidic acid: new receptors for "old" ligands. *J. Cell. Biochem.* **92:** 923–936.
30. Lee, M. J., M. Evans, and T. Hla. 1996. The inducible G protein-coupled receptor edg-1 signals via the G(i)/mitogen-activated protein kinase pathway. *J. Biol. Chem.* **271:** 11272–11279.
31. Van Brocklyn, J. R., M. J. Lee, R. Menzeleev, A. Olivera, L. Edsall, O. Cuvillier, D. M. Thomas, P. J. Coopman, S. Thangada, C. H. Liu, et al. 1998. Dual actions of sphingosine-1-phosphate: extracellular through the Gi-coupled receptor Edg-1 and intracellular to regulate proliferation and survival. *J. Cell Biol.* **142:** 229–240.
32. Yang, M., Z. M. Lei, and C. Rao. 2003. The central role of human chorionic gonadotropin in the formation of human placental syncytium. *Endocrinology.* **144:** 1108–1120.
33. Himmelmann, K., A. Himmelmann, A. Niklasson, and A. Svensson. 1996. Hypertension in pregnancy and size at birth. *Blood Press.* **5:** 278–284.
34. Huang, S. C., and F. M. Chang. 1994. The adverse effect on fetal hemogram by preeclampsia: marked anisocytosis with normocytic, normochromic erythrocythemia as well as thrombocytopenia. *Early Hum. Dev.* **37:** 91–98.
35. Webb, P. D., and J. Todd. 1988. Attachment of human placental-type alkaline phosphatase via phosphatidylinositol to syncytiotrophoblast and tumour cell plasma membranes. *Eur. J. Biochem.* **172:** 647–652.
36. Black, S., M. Kadyrov, P. Kaufmann, B. Ugele, N. Emans, and B. Huppertz. 2004. Syncytial fusion of human trophoblast depends on caspase 8. *Cell Death Differ.* **11:** 90–98.
37. Garcia-Lloret, M., J. Yui, B. Winkler-Lowen, and L. J. Guilbert. 1996. Epidermal growth factor inhibits cytokine-induced apoptosis of primary human trophoblasts. *J. Cell. Physiol.* **167:** 324–332.
38. Keryer, G., E. Alsat, K. Tasken, and D. Evain-Brion. 1998. Cyclic AMP-dependent protein kinases and human trophoblast cell differentiation in vitro. *J. Cell Sci.* **111:** 995–1004.
39. Knofler, M., L. Saleh, H. Strohmer, P. Husslein, and M. F. Wolschek. 1999. Cyclic AMP- and differentiation-dependent regulation of the proximal alphaHCG gene promoter in term villous trophoblasts. *Mol. Hum. Reprod.* **5:** 573–580.
40. Knofler, M., L. Saleh, S. Bauer, B. Galos, H. Rotheneder, P. Husslein, and H. Helmer. 2004. Transcriptional regulation of the human chorionic gonadotrophin {beta} gene during villous trophoblast differentiation. *Endocrinology.* **145:** 1685–1694.
41. Kilani, R., L-J. Chang, D. Hemmings, and L. J. Guilbert. 1997. Placental trophoblasts resist infection by multiple HIV-1 variants even with CMV co-infection but support HIV replication after provirus transfection. *J. Virol.* **71:** 6359–6372.