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A role for GPR55 in human placental venous endothelial cells

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Abstract

Endocannabinoids and their G protein-coupled receptors have been suggested to play a key role in human pregnancy, by regulating important aspects such as implantation, decidualization, placentation and labour. G protein-coupled receptor 55 (GPR55) was previously postulated to be another cannabinoid receptor, since specific cannabinoids were shown to act independently of the classical cannabinoid receptors CB1 or CB2. Current knowledge about GPR55 expression and function in human placenta is very limited and motivated us to evaluate human placental GPR55 expression in relation to other human peripheral tissues and to analyze spatiotemporal GPR55 expression in human placenta. Gene expression analysis revealed low GPR55 levels in human placenta, when compared to spleen and lung, the organs showing highest GPR55 expression. Moreover, expression analysis showed 5.8 fold increased placental GPR55 expression at term compared to first trimester. Immunohistochemistry located GPR55 solely at the fetal endothelium of first trimester and term placenta. qPCR and immunocytochemistry consistently confirmed GPR55 expression in isolated primary placental arterial and venous endothelial cells. Incubation with L- α -lysophosphatidylinositol (LPI), the specific and functional ligand for GPR55, at a concentration of 1 μ M, significantly enhanced migration of venous, but not arterial endothelial cells. LPI enhanced migration was inhibited by the GPR55 antagonist O-1918, suggesting a role of the LPI-GPR55 axis in placental venous endothelium function.

Keywords

Human placenta; Primary placental endothelial cells; Cannabinoid receptors; G protein-coupled receptor 55

Introduction

Endocannabinoids and their G protein-coupled receptors, CB1 and CB2, have been suggested to play a key role in human pregnancy, by regulating important aspects such as implantation, decidualization, placentation and labour (Chan et al. 2013; Fonseca et al.

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Conflict of interest

The authors declare they have no conflict of interest

2013). Both CB1 and CB2 are expressed in human decidual cells and placental villous tissue, where they are detected in the syncytiotrophoblast and the underlying villous cytotrophoblast throughout gestation. Moreover, CB1 is expressed in the endothelium of placental blood vessels within the villous stroma at term (Fugedi et al. 2014; Taylor et al. 2011). However, it is becoming increasingly evident that elevated levels of endocannabinoids rather have adverse effects on pregnancy. Activation of CB1 inhibits human decidualization and stimulates apoptosis of human endometrial stromal cells by a cAMP dependent mechanism (Moghadam et al. 2005). Adverse effects of endocannabinoids are also reported for trophoblast viability. Treatment of primary cytotrophoblasts and the trophoblast cell line BeWo with anandamide (*N*-arachidonylethanolamine, (AEA)), an endogenous cannabinoid, prevents proliferation and mediates morphological features of apoptosis through CB1 and CB2 receptor activation (Costa et al. 2015; Habayeb et al. 2008). While AEA and 2-arachidonoylglycerol (2-AG) are the best studied endocannabinoids, other endogenous compounds binding cannabinoid receptors have been identified. However, since some compounds were shown to act independently of CB1 or CB2, although initially thought to be specific for these receptors, the existence of a third cannabinoid receptor has been suggested.

One candidate was G protein-coupled receptor 55 (GPR55), although only sharing approximately 14% sequence homology with both cannabinoid receptors and lacking their typical cannabinoid binding pocket (Baker et al. 2006; Kotsikourou et al. 2011). GPR55 is a classical intronless seven-transmembrane G-coupled receptor, consisting of 319 amino acids (Sawzdargo et al. 1999). The assumption that GPR55 could be a cannabinoid receptor was based on *in silico* screenings, revealing potential interactions of GPR55 with some cannabinoid receptor agonists and antagonists (Brown 2007). Low homology with CB1 and CB2, together with an altered cannabinoid binding site and controversial pharmacological data, however, left some doubt on the postulation GPR55 may act as a cannabinoid receptor. Meanwhile, several studies have established that the non-cannabinoid bioactive lipid, L- α -lysophosphatidylinositol (LPI), is the specific and functional ligand for GPR55 (Henstridge et al. 2009; Kargl et al. 2013; Oka et al. 2007). LPI is synthesized by cytosolic phospholipase A2 and released from the cell by ATP-binding cassette transporter C1 (ABCC1) / multidrug resistance protein 1 (MRP1) (Ruban et al. 2014). This mechanism not only enables a paracrine, but also an autocrine action of LPI, which upon GPR55 activation acts as a key modulator of cell proliferation, migration, survival and tumorigenesis (Pineiro et al. 2011; Ruban et al. 2014).

In healthy individuals, GPR55 is expressed in various regions of the human brain, but is also detected in a wide range of peripheral tissues including adrenals, jejunum, ileum, spleen, and bone (Sanger 2007; Sawzdargo et al. 1999; Whyte et al. 2009). Recently, GPR55 has been detected in metabolically important tissues such as liver, adipose tissue and pancreas. While the function of GPR55 in liver has to be determined yet, its function in adipocytes comprises induction of lipogenic enzymes and upregulation of peroxisome proliferator activated receptor γ (PPAR γ), a key regulator of adipocyte differentiation and lipid storage (Moreno-Navarrete et al. 2012). In pancreatic β -cells activation of GPR55 induces insulin secretion, which together with functions in adipose tissue suggests a role of the receptor in energy homeostasis (Liu et al. 2015). While this interesting concept can be accepted for adipose

tissue and pancreas, very little is known about expression and function of GPR55 in other tissues involved in energy homeostasis. In human pregnancy, the placenta contributes as a temporal organ to regulation of energy balance of the mother and the growing fetus. The current knowledge about GPR55 expression and function in placental tissue is restricted to one gene expression profile of twenty human peripheral tissues and a functional study in rat (Fonseca et al. 2011; Henstridge et al. 2011). In rat uterine tissue, GPR55 expression peaks between gestational days twelve and fourteen and is detected in decidual cells, uterine natural killer (uNK) cells and giant trophoblast cells. Based on functional studies with primary rat decidual stroma cells, which showed decreased cell viability in response to AM251, a specific agonist of GPR55, the authors suggested that GPR55 could be involved in decidual regression by inducing apoptosis of decidual cells (Fonseca et al. 2011).

The aim of the present study was to reevaluate human placental GPR55 expression in relation to other human peripheral tissues and to analyze spatiotemporal GPR55 expression in human placenta, by comparing expression and localization of placental GPR55 in first trimester and at term of gestation. Since spatiotemporal expression analysis localized GPR55 at the fetal endothelium, a subsequent aim of this study was to determine effects of LPI on cell viability and angiogenic properties of primary placental endothelial cells.

Materials and methods

Human placental tissue samples

The study was approved by the ethics committee of the Medical University of Graz and written informed consent was obtained from each woman. Term placentas were obtained immediately after delivery from women with singleton pregnancies (>37 weeks of gestation). Pregnancies complicated by clinical evidence of infection, steroid treatment, AIDS, alcohol and/or drug abuse were excluded. First trimester placental tissues (6 to 12 weeks of gestation) were obtained from women undergoing elective pregnancy terminations.

Isolation and culture of primary endothelial cells from term placentas

Primary endothelial cells were isolated from term placental tissues as described previously (Lang et al. 2008). Briefly, arterial and venous chorionic blood vessels were washed with Hank's balanced salt solution (HBSS; Gibco, Invitrogen) and perfused with HBSS containing 0.1 U/ml collagenase and 0.8 U/ml dispase (both from Roche), supplemented with 300 IU/ml penicillin and 300 µg/ml streptomycin for 7 min at 37°C. The obtained cell suspension was centrifuged at $200 \times g$ for 5 min and resuspended with EBM medium (Endothelial Basal Medium, Lonza). Thereafter, cells were seeded on culture plates precoated with 1% gelatin and cultured in EBM medium supplemented with the EGM-MV BulletKit (Microvascular Endothelial Growth Medium; Lonza). Primary endothelial cells were scrutinized for identity and purity by positive staining for the classical endothelial marker van Willebrandt factor and absence of markers against fibroblasts (CD90) and smooth muscle cells (smooth muscle actin and desmin). Cells were only used at a purity of >99% between passages 4 and 6 to avoid phenotypic drift. For treatments EBM medium supplemented with the EGM-MV BulletKit without FBS was used. Human umbilical artery endothelial cells (HUAEC) and human umbilical vein endothelial cells (HUVEC) were

purchased from PromoCell (Heidelberg, Germany) and were cultured in the same medium as primary placental endothelial cells.

Immunohistochemistry

For immunohistochemistry villous tissue of first trimester (n=10) and term (n=10) placentas and umbilical cord tissue of 6 term placentas were fixed in formalin and embedded in paraffin. Serial tissue sections (5 µm) were mounted on Superfrost Plus slides (Menzel, Braunschweig, Germany). Sections were deparaffinized and rehydrated according to standard protocol. Antigen retrieval was performed by cooking slides in antigen retrieval buffer (pH 9, Eubio) under pressure for 7 min at 120°C. Immunohistochemistry was performed using the Ultravision LP detection system (Thermo Scientific, Fremont, USA) as described previously. In brief, endogenous peroxidase was blocked using the hydrogen peroxidase block for 10 min at RT. Thereafter, slides were washed thrice with TBS including 0.05% Tween 20 (TBS/T; Merck), followed by a 5 min background blocking step with Ultra Vision Protein Block at RT. Polyclonal anti-human GPR55 antibody (No. 10224, Cayman Chemical) was diluted 1:50 (5 µg/ml working concentration) in Antibody Diluent (Dako) and incubated on slides for 45 min at RT. For identification of fetal endothelium, monoclonal anti-CD34 Class II antibody (1:1000, 45 ng/ml, clone QBEnd-10, DAKO) was incubated on adjacent serial sections. Incubation with primary antibody was followed by three TBS/T washing steps and incubation with Primary Antibody Enhancer for 10 min at RT. Thereafter slides were washed and detection achieved by incubation with the anti-mouse/rabbit UltraVision HRP-labelled polymer system (15 min) and 3-amino-9-ethylcarbazole (AEC, Thermo Scientific), according to the manufacturer's instructions. Sections were counterstained with hemalaun and mounted with Kaiser's glycerol gelatin (Merck, Vienna, Austria). For negative controls, slides were incubated with rabbit Ig (Neo Markers) at the same concentration as mentioned above. Moreover, specificity of polyclonal anti-human GPR55 antibody was evaluated by an antibody pre-adsorption approach. For this purpose polyclonal anti-human GPR55 antibody (5 µg/ml) and GPR55 Blocking Peptide (40 µg/ml, No. 10225, Cayman chemical) were mixed in Antibody Diluent and incubated with gentle shaking 1 h at RT. A mixture containing solely anti-human GPR55 antibody was incubated in parallel and served as control. After incubation immunohistochemistry was performed as described above.

Immunocytochemistry

For immunocytochemistry, pellets of primary cells were subjected to formalin fixation and paraffin embedment. Cell pellets were obtained from isolated primary placental arterial endothelial cells (PAEC), primary placental venous endothelial cells (PVEC) and HUVEC. Confluent cells ($3 - 4 \times 10^6$ / 75 cm² flask) were harvested, centrifuged (310 × g, 5 min), washed and fixed in formalin for 30 min. After further washing and centrifugation steps, cells were incubated with 5% gelatin for 45 min at 37°C. Thereafter, gelatin embedded cell pellets jelled at 4°C, followed by a last fixation step. Fixed gelatin embedded cell pellets were subsequently embedded in paraffin by standard procedure and subjected to immunostaining as described above.

Quantitative gene expression analysis of GPR55

Total RNA from placental tissue and primary cells was isolated using the peqGOLD TriFast reagent (Peqlab, Erlangen, Germany) according to the manufacturer's protocol. For tissue RNA, small pieces of first trimester and term placental tissue (5mg moist mass) were rinsed in HBSS and homogenized using a tissue homogenizer and the TriFast reagent according to the manufacturer's protocol. For RNA extraction from primary cells, 1×10^6 cells/well were cultured in 6 well plates and lysed with TriFast reagent after 48 h culture. Besides placental tissue and cell RNA, the Human total RNA Master Panel II (TaKaRa, Clontech) was used to compare GPR55 expression within different human tissues. RNA quality was assessed on 1.5% denaturing agarose gels (Biozym, Vienna, Austria) and staining with GelRed™ Nucleic Acid Gel Stain (Biotium). RNA quantity and purity were determined using a NanoDrop1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). After quality check, 0.5 - 2 µg of total RNA was reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies). Reverse Transcription was performed with and without Reverse Transcriptase to check contamination with genomic DNA. cDNA was subjected to quantitative real time PCR using TaqMan Gene Expression Assays for human GPR55 (Hs00271662_s1, (Wilhelmsen et al. 2014)), and ribosomal protein L30 (RPL30, Hs00265497_m1) and the TaqMan Universal PCR Mastermix (Applied Biosystems). Kit components and cDNA were mixed in 20 µl total volume/well (96 well plates, Roche, Mannheim, Germany) according to the manufacturer's instructions and amplified using a Bio-Rad CFX96 Real-Time PCR System. Ct values were automatically generated by the CFX Manager 2.0 Software (Bio-Rad). Relative quantification of gene expression was calculated by standard Ct method using the expression of RPL30 as reference. Data are presented as mean of 2^{-Ct} values.

Cell migration Assay

The influence of LPI on endothelial cell migration was assessed by the colorimetric, transwell based migration assay CytoSelect™ (24-Well Cell Migration Assay, CytoSelect™ Cell Biolabs, Inc.). 3×10^5 cells/well were seeded in 300µl serum-free medium into upper wells, whereas lower wells contained complete endothelial culture medium (EGM-MV). Endothelial cells were pre-incubated with or without O-1918 (10 µM) in serum-free medium for 30 minutes. After pre-incubation, LPI was supplemented to the cells at a final concentration of 1 µM, resulting in either co-incubation of cells with LPI and O-1918 or incubation with LPI alone. Cells were incubated with culture medium supplemented with equal volumes of DMSO alone and served as solvent control. After 4 h incubation non-migrating cells were removed from the upper side of the membranes, while the cells on the lower side of the membrane were stained with provided Cell Stain Solution for 10 min at RT. Thereafter membranes were incubated with Extraction Solution for 10 min while shaking. Absorption of extracted solution was measured at 560 nm using a microplate reader.

Assessment of cell cytotoxicity

Cytotoxic effects of components were assessed by determination of LDH activity in supernatants of endothelial cells using an LDH Cytotoxicity Detection Kit (Takara Bio Inc., Eubio). In brief, 6000 cells/well were seeded in 96 well plates one day before experimental

start. Endothelial cells were treated as described for migration assays, but incubated for 24h. Culture supernatants of treated cells were centrifuged at $6080 \times g$ for 10 min to remove cellular debris. Thereafter, clear culture supernatants were subjected to LDH activity assay according to the manufacturer's instructions.

Assessment of cell viability

Cell viability was assessed by measuring the mitochondrial activity in metabolically active cells. For this purpose the reduction of the MTS tetrazolium compound was determined using the CellTiter 96 AQueous One Solution Cells Proliferation Assay Kit (Promega) according to the manufacturer's instructions. 10 000 cells/well were seeded in 96 well plates. Next day, cells were treated as described for migration assays, but incubated for 24h. Thereafter, 20 μ l of CellTiter 96 One Solution Reagent were added to cells, cultured in 100 μ l of culture medium, and incubated for 2 h at 37°C. Afterwards absorption of supernatants was measured at 490 nm in a microplate reader.

Statistical analysis

Data were analyzed using SigmaPlot 12.5 and are presented as means $\pm$ SD. Data were subjected to Normality Test (Shapiro-Wilk test) and Equal Variance Test. In case of normally distributed data differences between groups were tested using two-tailed t-test. Otherwise Mann-Whitney Rank Sum Test was applied. For multiple comparison procedure One Way Repeated Measures Analysis of Variance was followed by Holm-Sidak method to isolate groups that differ from the others. A p-value of less than 0.05 was considered statistically significant.

Results

GPR55 mRNA is expressed in human placenta

So far, available data on placental GPR55 expression have been restricted to a study in rat (Fonseca et al. 2011). In order to extend this knowledge, GPR55 expression was analyzed in term placenta tissues and compared with a panel of different human tissues. Quantitative gene expression analysis revealed highest GPR55 expression in spleen and lung, followed by salivary gland, trachea, small intestine and thymus (table 1). Compared to these tissues, GPR55 expression was relatively low in human term placenta and accounted for only 1.8 and 2.3%, when compared to spleen and lung, respectively. However, the level of placental GPR55 expression was in the same range as observed in prostate, thyroid gland, heart, uterus and adrenal gland. Lowest GPR55 expression was detected in skeletal muscle and cerebellum. To get a picture of putative changes of placental GPR55 expression over gestation, first trimester placental tissue was compared with term. Quantitative gene expression analysis revealed a 5.8 fold ($p < 0.001$) increase in placental GPR55 expression at term, when compared to first trimester (Fig1).

GPR55 protein is located at the fetal endothelium of human placenta

After gene expression analysis, the spatiotemporal expression of GPR55 in human placenta was analyzed by immunohistochemistry. Before starting a detailed immunohistochemical survey of human first trimester and term placenta sections, antibody specificity was

evaluated by a pre-adsorption approach. While immunohistochemistry for GPR55 showed distinct staining, pre-incubation of the primary antibody with a recombinant blocking peptide completely abolished staining of adjacent serial sections (Fig2). After validation of the antibody, spatiotemporal expression of GPR55 in human placenta was analysed by staining tissue sections from first trimester (n=10) and term (n=10) placentas.

In human first trimester placenta, GPR55 was detected in the fetal endothelium, whereas no staining was observed in the villous stroma and the villous trophoblast compartment, including cell columns (Fig3a). Interestingly, GPR55 was not only expressed in well established, large vessels in the center of villi, but was also detected in newly emerging capillaries just underneath the villous trophoblast layer. Staining of adjacent serial sections with anti-CD34II antibody confirmed identity of the fetal endothelium and enabled identification of small, inconspicuous vessels (Fig3b). In human term placenta, a similar staining was observed, showing GPR55 in the fetal endothelium (Fig3c). Noteworthy, differential endothelial expression of GPR55 was repeatedly detected in the central arteries and veins located in stem villi with a clear staining in the arterial endothelium and a weak to absent staining of the venous endothelium. Like in first trimester, no staining was detected in the villous stroma, in villous cytotrophoblasts and syncytiotrophoblast. In umbilical cord very weak GPR55 staining was detected in the endothelium of arteries and veins (Fig3e).

GPR55 is expressed in primary placental endothelial cells

In order to confirm immunohistochemistry data, we next isolated primary arterial (PAEC) and venous endothelial cells (PVEC) from the chorionic plate of healthy human term placentas and compared GPR55 expression with human umbilical artery endothelial cells (HUAEC) and human umbilical vein endothelial cells (HUVEC). Gene expression analysis revealed a trend towards increased GPR55 expression in placental endothelial cells, compared to endothelial cells from umbilical cord (Fig4a). Accordingly, GPR55 expression was 2.6 fold increased in PVEC, when compared to HUVEC, which however did not reach statistical significance ($p=0.062$). Like in venous cells, GPR55 expression tended to be higher in PAEC (1.6 fold increased, $p=0.161$), when compared to HUAEC.

Next, expression and localization of GPR55 was determined in PVEC on a cellular level by immunohistochemistry. PVEC cell pellets were formalin fixed and paraffin embedded (FFPE) and stained as placental tissue sections. Immunohistochemistry of FFPE PVEC sections localized GPR55 staining at the plasma membrane of a considerable proportion of cells (Fig4b).

Effect of LPI on endothelial cell viability and migration

LPI has previously been suggested to enhance a migratory response through GPR55 not only in human breast cancer cells, but also in human endothelial cells (Ford et al. 2010). Based on this observation, the effect of LPI on migration of primary placental endothelial cells was analyzed. Incubation of PAEC with LPI at a concentration of 1 μM enhanced cell migration towards complete medium by 26.2%, which however, did not reach statistical significance ($p=0.177$). Co-incubation of PAEC with LPI and the GPR55 antagonist O-1918 (10 μM) partly, but not significantly, reduced this effect (Fig5a). However, in PVEC, the effect of LPI

on cell migration was more pronounced and showed an increase by 42.5%, when compared to control. Co-incubation with LPI (1 μ M) and O-1918 (10 μ M) reduced LPI enhanced migration by 37.5% (Fig5b).

In order to determine any cytotoxic effects of LPI and O-1918 on placental endothelial cells, the release of LDH activity into the culture media of PAEC (Fig5c) and PVEC (Fig5d) was analyzed after 24h incubation and showed no difference between treatments and controls. Moreover, potential alterations in viable cell number in response to treatments were tested with MTS assays after 24h culture. Neither PAEC (Fig5e) nor PVEC (Fig5f) showed a significant difference in viable cell number between treatments.

Discussion

Placental GPR55 expression seems to be low when compared with spleen, the organ showing highest expression of the receptor. This observation is in line with previous gene expression analysis of primary peripheral tissues (Henstridge et al. 2011). High expression in the spleen may be explained by the white pulp consisting of lymphatic tissue, mostly lymphocytes known for its high GPR55 expression. Pulmonary capillaries may account for high GPR55 expression in lung, since microvascular lung endothelial cells have been shown to express the receptor (Kargl et al. 2013). Herein, salivary gland, trachea, and thymus have been identified as other organs highly expressing GPR55. While T lymphocytes, or so called thymocytes, and macrophages may most likely be responsible for high GPR55 expression in thymus, the underlying cell populations expressing the receptor in salivary gland and trachea remain to be identified.

Immunohistochemistry data suggest placental endothelial cells to be the sole cell type accounting for GPR55 expression in human placenta. This assumption is substantiated by immunocytochemistry of isolated primary placental endothelial cells, showing membrane associated staining for the receptor. Immunostaining results can be accepted as reliable, since the antibody has been successfully used in several other studies (Bouskila et al. 2013; Li et al. 2013; Lin et al. 2011) and antibody specificity was critically evaluated by a pre-adsorption approach in the current study. Increasing villous vascularization over gestation (Kaufmann et al. 2004), together with the fact that GPR55 is localized solely in placental endothelium, explains the significant increase of GPR55 expression in human placenta towards term. While GPR55 expression in placental villous tissue increases over gestation, protein levels in decidual tissue may peak in midtrimester. This was shown for uterine rat tissue, where highest GPR55 protein levels in decidual cells on day 14 were suggested to be associated with important uterine remodeling processes (Fonseca et al. 2011). Thus, placental villous tissue and decidua may have different peaks in GPR55 expression during gestation. Localization of GPR55 in human placental endothelial cells is in line with the fact that primary endothelial cells from multiple vascular beds, including endothelial cells from lung, dermis, brain, liver and coronary arteries express the receptor (Wilhelmsen et al. 2014; Zhang et al. 2010). Furthermore, HUVEC and their derived cell lines have previously been shown to express GPR55 (Waldeck-Weiermair et al. 2008; Wilhelmsen et al. 2014). Our immunohistochemistry and qPCR data confirm these observations and moreover suggest

higher GPR55 expression in the placental than in umbilical cord endothelium, making placental endothelial cells an interesting cell type for pharmacological studies.

Endothelial GPR55 expression and its activation are suggested to be associated with angiogenesis and endothelial wound-healing capacity (Kargl et al. 2013; Zhang et al. 2010). However, for human placenta nothing is known about a role of GPR55 as regulator of endothelial function. Our experiments suggest an effect of LPI on placental venous endothelial cell migration, whereas no significant effect was observed in arterial endothelial cells. This may be explained by differential GPR55 expression in venous, compared to arterial endothelial cells. Indeed, physiological differences and a different degree of maturity have previously been described between these two cell types. While PAEC have a fully differentiated arterial phenotype, PVEC show a juvenile, less differentiated phenotype with a high degree of plasticity. (Lang et al. 2008). Moreover, the two cell types show a distinct difference in global DNA methylation level, with the PVEC hypomethylated relative to PAEC (Joo et al. 2013). Interestingly, DNA methylation is inversely correlated to CB₁ expression in human colon cancer cells (Di Francesco et al. 2014). However, whether different DNA methylation levels accounted for differential GPR55 expression in placental endothelial cells remains unresolved at this point.

Beside cell migration, other processes such as cell proliferation and survival are suggested to be regulated by the LPI-GPR55 axis. Here, no significant effect of LPI on placental endothelial cell viability was detected. This may be explained by the relatively low, but nevertheless physiologic, concentration of LPI used in our study. No effect of LPI at a concentration of 1 μ M was also observed in human dermal microvascular endothelial cells, whereas concentrations of 0.01 and 0.1 μ M significantly increased proliferation. In contrast, LPI at a concentration of 10 μ M considerably decreased proliferation compared to control (Zhang et al. 2010). Thus, depending on cell type, LPI at suprapharmacological concentrations of 10 μ M or higher may have adverse effects. In human, circulating LPI levels range between 0.3 and 1.5 μ M, and show increased levels in obese patients and a positive correlation with fat percentage and body mass index in women (Moreno-Navarrete et al. 2012; Sutphen et al. 2004). Notably, alterations of the levels and composition of plasma lysophospholipids correlate well with the glycemic state of pregnant women. A decrease of various LPIs, in particular those with long-chain poly-unsaturated fatty acids have been shown in women with gestational diabetes mellitus (GDM) (Dudzik et al. 2014). Although cord blood LPI levels have not been determined yet, it is conceivable that the metabolic state in pregnancies, complicated by obesity and/or GDM, may alter fetal LPI levels, which in turn could affect GPR55 mediated placental endothelial functions. While LPI is now considered as the endogenous ligand for GPR55, the role of endocannabinoids in regulating GPR55 is rather controversial and poorly understood. Recently, the endocannabinoids AEA and virodhamine were suggested to act as partial agonists, by having no to very weak response on their own, but enhancing the LPI effect at low concentrations yet inhibiting it at high concentrations (Sharir et al. 2012). Thus, depending on plasma levels, fetal endocannabinoids may interfere with LPI mediated GPR55 function in the placental endothelium. Indeed, AEA has been detected in cord blood, showing significant higher levels in the umbilical vein compared with the umbilical artery (Marczylo et al. 2010). Besides regulating angiogenesis, the LPI-GPR55 axis may act as an

endothelium dependent vasodilator. This was shown in small mesenteric rat arteries, which showed a concentration dependent relaxation in response to LPI (AlSuleimani and Hiley 2015).

In summary, GPR55 is expressed in human placenta, located solely at the placental endothelium. LPI, the endogenous ligand of GPR55, increases the migratory activity of venous, but not arterial placental endothelial cells, suggesting a role of the LPI-GPR55 axis in placental venous endothelium function. Altered LPI levels, as shown in obesity and GDM, may affect venous endothelium functions in human placenta.

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Fig.1. Analysis of GPR55 expression in human first trimester and term placenta tissue
Quantitative gene expression analysis showed a 5.8 fold increase of GPR55 mRNA expression in term (n=7) compared to first trimester (n=8) placental tissues. Values are given as mean $\pm$ SD. ***p 0.001

Fig.2. Evaluation of the used anti-GPR55 antibody

Specificity of the antibody was evaluated on serial first trimester placenta sections using an antibody pre-adsorption approach and negative control rabbit IgG. **(a)** While incubation with the antibody alone showed distinct staining of fetal vessels (arrowheads), **(b)** pre-adsorption of the antibody with recombinant GPR55 Blocking Peptide completely abolished staining. **(c)** Incubation with negative control rabbit IgG revealed no staining. Scale bar represents 100 μ m.

Fig.3. Immunohistochemical localization of GPR55 in human placenta

(a) In human first trimester placenta, GPR55 was detected in the fetal endothelium (arrowhead), whereas no staining was observed in the villous trophoblast compartment (arrow) and cell columns (asterisks). (c) Like in first trimester, GPR55 was observed in the fetal endothelium (arrowhead) of human term placental villi, whereas no staining was detected in the villous stroma, in villous cytotrophoblasts and syncytiotrophoblast (arrows). (e) In umbilical cord, very weak GPR55 staining was detected in the endothelium of arteries

and vein. (**b**, **d**, and **f**) Adjacent serial sections were stained with anti-CD34II antibody and confirmed identity of the fetal endothelium. Scale bar represents 100 μm .

Fig.4. GPR55 expression in primary placental endothelial cells

(a) Primary arterial (PAEC, n=8) and venous endothelial cells (PVEC, n=8) were isolated from healthy human term placentas and expression of GPR55 compared with human umbilical arterial (HUAEC, n=3) and human umbilical venous endothelial cells (HUVEC, n=3). (b) Immunohistochemistry of formalin fixed and paraffin embedded sections of PVEC pellets localized GPR55 at the plasma membrane. (c) Incubation with control rabbit IgG revealed no staining. Values are given as mean $\pm$ SD. Scale bar represents 50 μ m.

Fig.5. Effect of LPI on primary placental endothelial cell migration and viability

PAEC (n=3) and PVEC (n=3) were incubated with LPI (1 μ M) in presence or absence of GPR55 antagonist O-1918 (10 μ M). Cells incubated without compounds, but in presence of solvent control DMSO served as control. Cell migration of PAEC (a) and PVEC (b) was analyzed after 4h incubation. Cytotoxic effects were determined by measurement of LDH activity in supernatants of PAEC (c) and PVEC (d) after 24h incubation. Cell viability of

PAEC (**e**) and PVEC (**f**) in response to treatments was analyzed by MTS assays after 24h incubation. Values are given as mean $\pm$ SD. *p < 0.05

Table 1
Quantitative gene expression analysis of GPR55 in human placenta and various other human tissues

Expression of GPR55 was analyzed in pooled total RNA from indicated numbers of tissues given in parentheses. Data are presented as mean $\pm$ SD of fold change compared to placenta

Human Tissue	Fold change
Spleen (15)	54.74 $\pm$ 9.80
Lung (3)	44.06 $\pm$ 4.06
Salivary gland (24)	19.97 $\pm$ 1.85
Trachea (22)	18.95 $\pm$ 4.40
Small intestine (5)	17.64 $\pm$ 1.31
Thymus (2)	12.40 $\pm$ 0.75
Prostate (12)	1.68 $\pm$ 0.23
Thyroid gland (64)	1.65 $\pm$ 0.30
Heart (3)	1.64 $\pm$ 0.28
Uterus (8)	1.40 $\pm$ 0.29
Adrenal gland (64)	1.40 $\pm$ 0.10
Placenta, term (3)	1.00 $\pm$ 0.12
Skeletal muscle (7)	0.67 $\pm$ 0.22
Brain, cerebellum (10)	0.65 $\pm$ 0.04