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Docosahexaenoic acid stimulates tube formation in first trimester trophoblast cells, HTR8/SVneo

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ABSTRACT

Angiogenesis is a key factor in the placentation process and vascular remodeling that involves several growth factors such as vascular endothelial growth factor (VEGF) and angiopoietin-like protein 4 (ANGPTL4). PPARs are involved in the placentation process but not much information is available on whether their ligands such as fatty acids have any effects on these processes. We therefore investigated the effect of fatty acids (arachidonic acid, 20:4 n-6(ARA), eicosapentaenoic acid, 20:5 n-3(EPA), docosahexaenoic acid, 22:6 n-3 (DHA) and oleic acid, 18:1 n-9 (OA)) on tube formation (as a measure of angiogenesis) on matrigel in the first trimester trophoblast cells, HTR8/SVneo. In addition we also investigated the effects of fatty acids on expression of genes involved in angiogenesis (VEGF and ANGPTL4) and lipid metabolism in these cells. Gene expression was determined after incubating these cells with different fatty acids for 24 h using real-time qRT-PCR, whereas VEGF and ANGPTL4 proteins were measured by respective ELISA kits. Of all the fatty acids tested, DHA increased tube formation to the greatest extent. DHA-induced increase in tube length was 583%, 247% and 70% over control, OA and EPA, respectively ($p < 0.05$). In addition, DHA stimulated cell proliferation by 150% of these cells. Of all fatty acids investigated, only DHA stimulated VEGF mRNA expression and protein secretion compared with control. Unlike DHA, other fatty acids (OA, EPA, ARA) stimulated ANGPTL4 mRNA expression and protein secretion in these cells. An inhibitor of VEGF decreased DHA stimulated tube formation in these cells. Altogether these data indicate that DHA may potently influence the placentation process by stimulating tube formation and this effect may be mediated in part via VEGF in first trimester trophoblast cells.

1. Introduction

Invasive extravillous trophoblasts of the human placenta are critically involved in successful pregnancy outcome. They remodel the uterine spiral arteries to increase blood flow and oxygen delivery to the placenta and the developing fetus [1,2]. This invasive behavior of extravillous trophoblasts (EVTs) follows a precise chronology of vascular events during the first trimester of gestation. Defective invasion of the uterine spiral arteries is directly involved in pre-eclampsia, a major and frequent complication of human pregnancy with serious fetal and maternal consequences [3]. The human trophoblastic invasion, unlike tumor invasion, is precisely regulated. It is temporally restricted to early pregnancy, and it is spatially confined to the endometrium, the first third of the myometrium, and the associated spiral arterioles [3]. Several factors are involved in this angiogenic process, including vascular endothelial growth factor (VEGF), angiopoietin-like protein 4 (ANGPTL4), platelet-derived growth factor (PDGF) and platelet-activating factor (PAF) [1,4]. VEGF strongly induces angiogenesis and increases permeability of blood vessels [5]. VEGF and its receptors are expressed in both the endometrium and in trophoblast cells [6]. VEGF expression is up-regulated in placental tissues by hypoxia, associated with early placental development, whereas PlGF (placental growth factor), another angiogenic factor, is down-regulated [7]. Surprisingly, placental VEGF mRNA was reduced in pre-eclamptic pregnancies, despite the prolonged hypoxic condition associated with pre-eclampsia [8].

Studies in mice established that two nuclear hormone receptors, peroxisome proliferator-activated receptor γ (PPARγ) and retinoid X receptors (RXRs) are essential for placental development and vascularization [9,10]. A major role for PPARγ in the

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control of human trophoblast invasion during early placental development has been suggested. Previous studies have observed that PPARα and PPARγ inhibit both angiogenesis and VEGF-induced angiogenesis [11,12]. PPARβ/δ has an opposite effect, protecting endothelial cells from oxidative stress mediated apoptosis, and leading to increases in proliferation and angiogenesis [13]. Certain fatty acids may act as ligands for PPARs and thus may influence placentation [14]. During intra-uterine life, the human placenta selectively transfers ARA and DHA from the maternal circulation to the fetus [15]. Placental delivery of these fatty acids is important for fetal growth and development but little information is available on their roles in the placentation processes. We used an immortalized EVT cell line, the HTR8/SVneo in this study. These cells have similar properties to highly invasive extravillous trophoblast (EVT) cells [16] and form tubes under appropriate experimental conditions [17]. We investigated the effects of fatty acids on tube formation, proliferation and expression of relevant genes in these cells.

In this paper we report that DHA stimulates tube formation more potently compared with other fatty acids in HTR8/SVneo cells. DHA stimulated VEGF mRNA expression and protein secretion, whereas OA, EPA, ARA and GW501516 (PPARδ ligand) stimulated ANGPTL4 mRNA expression and protein secretion. We therefore suggest that DHA increases the tube formation process by stimulating the secretion of VEGF in first trimester trophoblast cells.

2. Materials and methods

2.1. Materials

Trypsin–EDTA solution, VEGF receptor tyrosine kinase (SU5416), 3-(4,5-dimethyl thiazol-2-yl)-2,5 diphenyl tetrazoliumbromide (MTT), penicillin–streptomycin solution and RPMI-1640 medium were obtained from Sigma–Aldrich Oslo, Norway AS. Unlabeled fatty acids were from Cayman Chemicals, Ann Arbor, USA. LDH assay kit was obtained from Roche Molecular Biochemicals, Mannheim, Germany. Matrigel kit was obtained from BD Biosciences, Bedford, USA. PPAR ligands: Rosiglitazone/BRL49653 (71740-5-CAY) and Wy14643 (70730-5-CAY) were purchased from AH Diagnostics, Oslo, Norway. GW501516 was purchased from Alexis Biochemicals, San Diego, USA. ELISA kits for Human ANGPTL4 DuoSet (DY3485) and human VEGF Quantikine ELISA kit (DVE00) were purchased from R&D Systems, Minneapolis, USA. All other chemicals and solvents were high purity commercial materials obtained from Sigma–Aldrich Oslo, Norway AS.

2.2. Cell culture

The HTR8/SVneo trophoblast cell line was kindly provided by Dr. C.H. Graham, Queen's University, Kingston, Ontario, Canada. The HTR8/SVneo cells were maintained in RPMI-1640 medium supplemented with 5% fetal calf serum, 2 mM L-glutamine, penicillin (50 units/ml) and streptomycin (50 μg/ml) at 37 °C in 5% CO_2 [16].

2.3. Preparation of fatty acids

Stock solutions of 6 mM fatty acids were complexed with fatty acid-free bovine serum albumin (BSA) (6%). The fatty acids were dissolved in 0.1 M NaOH for 10 min before adding pre-warmed (37 °C) 6% fatty acid-free BSA in Hams F12. The fatty acid-BSA solution was incubated at 37 °C for 10 min to allow fatty acid–albumin complex formation.

2.4. Tube formation assay

Cellular angiogenesis was measured *in vitro* based on tube formation on an extracellular matrigel in a 24-well plate, as described [18]. Briefly, 24-well plates were coated with 40 μl cold liquid matrigel (8 mg/ml) per well and incubated at 37 °C for 30 min to promote solidification. HTR8/SVneo cells were grown for 48 h in FCS free media prior to incubation with different fatty acids in the presence of 5% FCS media. Cells were seeded at a density 70,000 cells/well. After 16 h incubation, the wells were photographed by an inverted microscope at 4× magnification (Nikon TS 100F, Japan). Images were captured from the central view of at least five different fields per well and extreme edges were excluded due to gel meniscus formation. Adobe Photoshop (version CS4) was used to quantify tubule length of the capillary network formation [19]. The length of each tubule was determined by drawing a line over each tubule and the mean length of the lines (pixels) drawn in each image was calculated. Quantification of tubular network was measured by counting the total length of tubes in pixels. The results were expressed as % over control using the formula: % over control = the mean length of total tubes (assay groups) × 100/mean length of tubes (control groups). The numbers of capillary connections or branch points between cells were counted manually. Independent experiments were performed in triplicate to reduce intra-assay variability. The average numbers and length of connections were calculated for each fatty acid treatments.

2.5. Effects of fatty acids and PPAR agonists on gene expression

HTR8/SVneo cells were incubated for 24 h with 100 μM of fatty acid (OA, AA, EPA and DHA) or synthetic agonists for PPARγ (BRL49653; 1 μM), PPARδ (GW501516; 100 nM) and PPARα (WY14643; 100 μM) in RPMI-1640 medium containing 10% FCS, L-glutamine and P/S. The cells were then washed in ice cold PBS and lysed in RNA lysis buffer (Applied Biosystems, Foster City, CA) and total RNA was isolated using ABI 6100 (Applied Biosystems) according to the manufacturer's instruction.

For quantitative reverse transcription-PCR (qRT-PCR), cDNAs were synthesized from extracted total RNA using high-capacity cDNA Reverse Transcription kit according to the manufacturer's instructions and analyzed using TaqMan Gene Expression Assays (listed in Table 1) and TaqMan Gene Expression Master Mix on the 7900HT Real-Time PCR System (all Applied Biosystems). Briefly, a 20 μl reaction mix consisting of 1 μl cDNA, 10 μl gene expression master mix (2×), 1 μl gene expression assay and 8 μl H_2O was amplified in a 96 well clear plate under the standard thermal cycling conditions as instructed by the manufacturer (50 °C for 2 min followed by an initial denaturation step at 95 °C for 10 min, then 40 cycles at 95 °C for 10 s and 60 °C for 1 min). Cycle threshold (Ct) values were calculated using the SDS software v.2.3 using automatic baseline and threshold settings. Ct values above 35 were considered to be below the detection level of the assays, therefore only genes with a Ct value at 35 or below were included in the expression analysis. The Ct value of an endogenous control gene TBP (TATA binding protein) was subtracted from the corresponding Ct value for the target gene resulting in the delta Ct value which was used for relative quantification of gene expression by the comparative Ct method ($2^{-\Delta\Delta Ct}$) [20].

2.6. Enzyme-linked immunosorbent assay (ELISA)

The protein concentrations of ANGPTL4 and VEGF in the medium from HTR8/SVneo cells were measured after cells were incubated with fatty acids or PPAR ligands for 24 h. VEGF concentration was also measured in cell-secreted media on matrigel after the treatment of DHA. The samples were assayed in duplicates according to the manufacturer's instructions using ELISA kits. Independent experiments were performed in duplicate to reduce intra-assay variability.

2.7. Cytotoxicity of fatty acids in HTR8/SVneo cells

The cytotoxicity of fatty acids on HTR8/SVneo cells was determined by measuring LDH release after incubating the cells with different fatty acids (OA, AA, DHA and EPA) for 24 h.

2.8. Cell proliferation and migration assay

HTR8/SVneo cells were seeded in a 96-well microtitre plate and grown until 90–95% confluence. The cells were then incubated with different concentrations of fatty acids as indicated in the figure legends. After 16 h incubation the medium was removed and replaced with 100 μl of fresh serum-free culture medium, as described

Table 1
TaqMan assays used for gene expression analysis.

Symbol	Gene name	Assay ID	Amplicon (bp)
ADRP	(Adipose differentiation -related protein)	Hs00605340_m1	139
CAV-1	(Caveolin-1)	Hs00971716_m1	66
COX-2	(Prostaglandin G/H synthase & cyclooxygenase)	Hs00153133_m1	75
FABP4	(Fatty acid binding protein 4)	Hs00609791_m1	105
ANGPTL4	(Angiopoietin-like 4)	Hs01101127_m1	92
VEGFA	(Vascular endothelial growth factor A)	Hs00173626_m1	77
HIF1α	(Hypoxia inducible factor 1)	Hs00153153_m1	76
TIMP-1	Tissue inhibitor metalloproteinases 1	Hs00171558_m1	104
GPR40	(Free fatty acid receptor 1)	Hs03045166_s1	56
GPR120	(G protein-coupled receptor 120)	Hs00699184_m1	86
TBP	(TATA binding protein)	Hs99999910_m1	127