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Transient receptor potential vanilloid 1 is expressed in human cytotrophoblasts: Induction of cell apoptosis and impairment of syncytialization

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ABSTRACT

The normal development of placenta relies essentially on a balanced proliferation, differentiation and apoptosis of cytotrophoblasts. These processes are tightly regulated by several hormones, cytokines, lipids and other molecules and anomalies in these events are associated with gestational complications. The cation channel transient receptor potential vanilloid 1 (TRPV1) is expressed in several organs and tissues and it participates in cellular events like nociception, inflammation and cell death. However, the expression and importance of this receptor in human placenta still remains unknown. In this work, we found that TRPV1 is expressed in human cytotrophoblasts and syncytiotrophoblasts. Furthermore, the TRPV1 agonists capsaicin and anandamide decreased cytotrophoblast viability and induced morphological alterations, such as chromatin condensation and fragmentation, which suggest the occurrence of apoptosis. Also, both TRPV1 agonists induced a loss of mitochondrial membrane potential and an increase of caspase 3/7 activity and production of reactive species of oxygen and nitrogen. Furthermore, capsaicin (10 μM) impaired the spontaneous *in vitro* differentiation of cytotrophoblasts into syncytiotrophoblasts by triggering TRPV1, as observed by the decrease in placental alkaline phosphatase activity and in human chorionic gonadotropin secretion. On the other hand, anandamide decreased placental alkaline phosphatase activity via a TRPV1-independent mechanism but did not influence the secretion of human chorionic gonadotropin. In conclusion, we showed that TRPV1 is expressed in human cytotrophoblasts and syncytiotrophoblasts and also reported the involvement of this receptor in cytotrophoblast apoptosis and differentiation.

1. Introduction

The transient receptor potential vanilloid 1 (TRPV1) is a non-selective cation channel with six- transmembrane-domain that was identified for the first time in 1997, in sensory neurons (Caterina et al., 1997). TRPV1 is sensitive to several stimuli such as noxious, heat, low pH and several endogenous and exogenous molecules (Vriens et al., 2009). It is mainly involved in temperature sensing and nociception, but TRPV1 also participates in other cellular processes such as apoptosis (Contassot et al., 2004; Pan et al., 2013; Song et al., 2013), muscle contraction (Charrua et al., 2007; Matsumoto et al., 2009; Shimizu et al., 2007), autophagy (Farfariello et al., 2012; Li et al., 2014) and inflammation (Fernandes et al., 2012; Trevisani et al., 2004; Vigna et al., 2011). Some of the endogenous ligands of TRPV1, the endovanilloids, are the endocannabinoids anandamide (AEA) and *N*-arachidonoyldopamine (NADA), the oleoylethanolamide (OEA), protons, prostaglandins and lipoxygenase (LOX) or cytochrome P450 (CYP450) products (Vriens et al., 2009). TRPV1 is present in several organs and tissues, though its expression in human placenta has not been reported yet.

Placenta is an exclusive organ of pregnancy that supports, feeds and protects the foetus. Its formation requires a proper proliferation, differentiation and apoptosis of its main cell type, the trophoblast. Trophoblast cells are subdivided in four types: (i) the villous cytotrophoblasts (CTs), which proliferate and differentiate

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into (ii) syncytiotrophoblasts (STs), multinucleated cells involved in biosynthesis of several hormones and proteins elemental for the progression of gestation; (iii) extravillous trophoblasts (EVTs), which invade the maternal spiral artery and uterine tissues; and iv) giant trophoblast cells. Although there is no information about the expression and function of TRPV1 in human placenta, TRPV1 has already been described in rat placenta (Cella et al., 2008; Fonseca et al., 2012), where it mediates AEA-induced increase in nitric oxide synthase activity (Cella et al., 2008). Nonetheless, other types of vanilloid receptors (TRPV5 and TRPV6) were already described in human placenta (Bernucci et al., 2006; Moreau et al., 2002) and, apparently, they are important for the calcium uptake by the syncytiotrophoblast. Calcium is an important second messenger in the cytotrophoblast differentiation and EVTs migration and changes in its homeostasis are associated with preeclampasia and intrauterine growth restriction (IUGR) (Baczyk et al., 2011). Although TRPV1 is not a selective cation channel, it has preference for calcium ions, so it may be relevant for calcium signalling in cytotrophoblasts. Moreover, since TRPV1 also mediates apoptosis, it may also participate in cytotrophoblast turnover. A coordinated proliferation, differentiation and apoptosis of cytotrophoblasts is essential for the placental development and alterations in these processes are associated with pregnancy disorders (Crocker et al., 2003; Roje et al., 2011; Smith et al., 1997).

Here, we investigated the expression of TRPV1 in human cytotrophoblasts and syncytiotrophoblasts of normal term placentas. Also, we studied the influence of the TRPV1 exogenous agonist capsaicin (CPS), a component of hot chilli pepper, and of the endovanilloid AEA in human primary cytotrophoblasts apoptosis and differentiation into syncytiotrophoblasts, to study the role of this receptor in cytotrophoblast cells turnover.

2. Material and methods

2.1. Primary cultures of human cytotrophoblasts

All the procedures performed with human placentas were conducted in accordance with the Ethical Committee of Hospital S. João, Porto. Term placentas of normal pregnancies were immediately collected after delivery. Then, human cytrotrophoblast cells (hCTs) were isolated, as previously described (Keating et al., 2007). Briefly, villous tissue was dissected and digested in a trypsin and DNAse I-containing solution and cells were separated in a discontinuous percoll gradient (GE Healthcare, Buckinghamshire, UK). Cytotrophoblasts were collected and seeded in 96 and 24-well plates, 21 cm^2 dishes or 8-well chamber slides, at densities 1.35×10^5, 1×10^6, 1×10^7 or 6×10^5, respectively, in DMEM/F12 medium (Sigma–Aldrich Co., St. Louis, MO, USA) supplemented with 10% (v/v) of FBS and an antibiotic–antimycotic solution (100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericine B) (Gibco/Invitrogen Corporation, Carlsbad, CA, USA) and were incubated at 37 °C and 95% air/5% CO_2 humidified atmosphere. After 12 h of adhesion, for the cell death related-assays, cell culture medium was replaced by fresh medium supplemented with 1% FBS. For differentiation studies, cells were kept in medium supplemented with 10% FBS, since it is an experimental requirement for the spontaneous *in vitro* differentiation of cytotrophoblasts into syncytiotrophoblasts.

2.2. Expression of TRPV1 at 12 and 72 h of culture

Human cytotrophoblasts and syncytiotrophoblasts (at 12 and 72 h of culture, respectively) were collected from 21 cm^2 plates in TRIzol® reagent (Gibco/Invitrogen Corporation, Carlsbad, CA, USA). Total RNA and protein was extracted according to manufacturer's instructions. RNA was quantified in the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and its quality assessed with the Experion RNA StdSens Kit and analysed with the Experion analytical software (Bio-Rad Laboratories, USA). RNA was reverse transcribed into cDNA with the iScript™ Select cDNA Synthesis (Bio-Rad Laboratories, USA). For the analysis of *TRPV1* gene transcription, cDNA was amplified with KAPA SYBR® FAST qPCR Master Mix 2x Kit (Kapa Biosystems, Woburn, MA, USA), according to the kit protocol, in MiniOpticon Real-Time PCR Detection System (Bio-Rad Laboratories, USA). Specific primers for human TRPV1 amplification were used (sense 5′-CAAGAACATCTGGAAGCTGC-3′; antisense 5′-CTTCTCCCCGGAAGCGGCAGG-3′ (Ludanyi et al., 2008)) and the annealing temperature was set at 62 °C. The specificity of the amplification PCR product was evaluated by the melting curve analysis.

For Western Blot, protein samples were loaded in a 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were transferred onto nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). After blocking the nonspecific binding sites with a blocking solution (5% dry milk in PBS with Triton™ X-100 0. 1%), membranes were incubated with anti-TRPV1 (goat; 1:100; Santa Cruz Biotechnology, Dallas, Texas, USA; sc-12500) overnight, at 4 °C. Then, they were incubated with peroxidase-conjugated secondary antibody (rabbit anti-goat, 1:1000; Santa Cruz Biotechnology, Dallas, Texas, USA) for 1 h, at room temperature. Lastly, membranes were exposed to WesternBright™ ECL (Advansta, Menlo Park, USA) and then to x-ray film (Kodak XAR; Eastman Kodak, Rochester, NY). Membranes were stripped and re-incubated with anti-β-tubulin antibody (rabbit; 1:500; Santa Cruz Biotechnology, Dallas, Texas, USA), for loading control. Rat brain was used as positive control. The signal intensity of the bands was analysed by densitometry (BIO-PROFIL Bio-1D2; Vilber Lourmat, Marne-la-Vallée, France) and normalized for the corresponding β-tubulin band; the results were expressed in arbitrary units.

For immunocytochemistry studies, cells at 12 and 72 h of culture seeded in 8-well chamber slides were fixed with a 4% paraformaldeylde solution. For immunohistochemistry, deparaffined slides of human placenta (4 μm thick) were used. The expression of TRPV1 was analysed by an avidin–biotin alkaline phosphatase complex immunohistochemical technique (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA). The non-specific binding sites were blocked and slides were incubated with anti-TRPV1 antibody (1:100) overnight at 4 °C. Slides were then incubated with biotinylated secondary antibody followed by incubation with Vectastain ABC-AP reagent, according to the manufacturer's instructions. The reaction was developed with Sigma Fast Red™ tablets (Sigma–Aldrich Co., St. Louis, MO, USA), slides were counterstained with Mayer's hematoxylin solution (Sigma–Aldrich Co., St. Louis, MO, USA) and mounted in Aquamount medium (BDH Laboratory Supplies, Poole, England). Negative control was performed by the replacement of the primary antibody by goat IgG.

2.3. Cell viability assays

For cell viability assessment, MTT assay was used. hCTs were plated in 96-well plates and, after adhesion, cells were incubated with or without capsaicin (Tocris Bioscience, Bristol, UK) (1-25 μM) for 12 and 24 h. The involvement of TRPV1 in the effects induced by CPS and anandamide (AEA; Tocris Bioscience, Bristol, UK) was evaluated after a preincubation with 200 nM Capsazepine (CPZ) or 20 nM 5'-Iodoresiniferatoxin (5-iRTX) (Tocris Bioscience, Bristol, UK), two TRPV1 antagonists, 30 min before the addition of CPS or AEA (25 or 15 μM, respectively). At the end of incubation period, the yellow tetrazole MTT (0. 5 mg/ml final concentration) was added to the cells for 2 h 30 min, at 37 °C. The purple formazan formed was extracted by a solution of Dimethylsulfoxide (DMSO):isopropanol