

# Alteration of calcium homeostasis in primary preeclamptic syncytiotrophoblasts: effect on calcium exchange in placenta

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## Abstract

Preeclampsia (PE) is characterized by maternal hypertension, proteinuria, oedema and, in 30% of cases, by intrauterine growth retardation. Causes are still unknown; however, epidemiological and clinical studies have suggested alterations in maternal calcium metabolism. We suggested that in PE, calcium transport by the syncytiotrophoblast (ST) is disturbed. From total placental tissues, we studied the expression of: calcium channels (TRPV5, TRPV6 [transient receptor potential vanilloid]), calcium binding proteins (CaBP-9K, CaBP-28K), plasma membrane calcium ATPase (PMCA)1,2,3,4 pumps, ATP synthase, genes implicated in Ca<sup>2+</sup> release [inositol-1,4,5-triphosphate receptor (IP3R)1,2,3; Ryanodine receptor (RyR)1,2,3] and replenishment (SERCA1,2,3 [sarcoendoplasmic reticulum Ca<sup>2+</sup> ATPases]) from endoplasmic reticulum, channels implicated in mitochondrial Ca<sup>2+</sup> accumulation (VDAC1,2,3 [voltage-dependent anion channels]) and a marker of oxidative stress (hOGG1 [Human 8-oxoguanine-DNA glycosylase 1]), as well as the influence of these variations on calcium transport in primary ST cultures. The mRNA and protein levels were thereby examined by real-time PCR and Western blot analysis, respectively, in two different groups of pregnant women with similar gestational age: a normal group ( $n = 16$ ) and a PE group ( $n = 8$ ), diagnosed by a clinician. Our study showed a significant decrease in calcium transport by the ST cultured from preeclamptic placentas. We found a significant ( $P < 0.05$ ) decrease in mRNA levels of TRPV5, TRPV6, CaBP-9K, CaBP-28K, PMCA1, PMCA4, ATP synthase, IP3R1, IP3R2, RyR1, RyR2 and RyR3 in PE group compared to normal one. We also noted a significant decrease in protein levels of TRPV5, TRPV6, CaBP-9K, CaBP-28K and PMCA1/4 in PE group. In contrast, SERCA1, SERCA2, SERCA3, VDAC3 and hOGG1 mRNA expressions were significantly increased in PE placentas. Calcium homeostasis and transport through placenta is compromised in preeclamptic pregnancies and it appears to be affected by a lack of ATP and an excess of oxidative stress.

**Keywords:** calcium transport • calcium homeostasis • ATP • oxidative stress • placenta • preeclampsia • syncytiotrophoblast • trophoblast

## Introduction

Preeclampsia (PE) represents a major factor for maternal and perinatal morbidity and mortality [1] and it affects 7–10% of pregnancies worldwide [2]. PE is characterized by maternal syndromes

such as gestational hypertension, proteinuria, oedema and foetal syndromes like reduced amniotic fluid, abnormal oxygenation and, in 30% of cases, intrauterine growth retardation (IUGR). The precise factors involved in the pathogenesis of PE remain unclear and it is considered as a multisystem disorder [1]. PE is principally characterized by impaired placentation and would be the result of an impaired differentiation of villous trophoblasts, which leads to an abnormal remodelling of the spiral arteries [3]. Diabetes, obesity, hypertension, renal disease, primiparity, maternal age, sperm and oocyte donation are all known predisposing factors for PE [1]. The oxidative stress, resulting from deficient remodelling of spiral arteries, is an important consequence of PE. It induces the placenta

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to release various factors, as inflammatory cytokines, apoptotic wastes and anti-angiogenic factors [4]. These secreted soluble factors are then thought to alter endothelial metabolic status, mitochondrial integrity and vascular functions [5]. Oxidation of DNA bases by endogenously generated and environmental reactive oxygen species (ROS) leads to mutations. Guanine is particularly prone to oxidation by ROS. 8-Oxo-deoxyguanine (8-oxo-dG) is a quantitatively major form of oxidative DNA damage [6, 7], inducing mainly G (guanine) to T (thymine) and A (adenine) to C (cytosine) substitutions [8]. Human 8-oxoguanine-DNA glycosylase 1 (hOGG1) is the main enzyme that excises 8-oxo-dG from damaged DNA via the base-excision repair pathway [9]. It was suggested that cells under oxidative stress may require increased expression of hOGG1 to protect them from oxidative damage-induced mutations [10].

In addition, during PE, the oxidative stress exposes the placenta to fluctuating oxygen concentration [11] and this periodic hypoxia will deplete placental cells from ATP. More precisely, it was observed that lower ATP levels in PE placental cells are due to an important change in expression of ATP synthase gene [12], an important mitochondrial enzyme synthesizing ATP from ADP.

The most effective treatment for PE is delivery itself; however, several randomized trials reported the effective use of various methods to reduce the rate or severity of PE [1], such as calcium ( $\text{Ca}^{2+}$ ) supplementation, but results showed minimum to no benefit. Several alterations in maternal  $\text{Ca}^{2+}$  homeostasis were identified in PE, such as low urinary  $\text{Ca}^{2+}$  excretion and low circulating level of 1,25-dihydroxyvitamin  $\text{D}_3$ , parathyroid hormone-related peptide and calcitonin gene related peptide [13, 14]. Epidemiological data suggest an inverse correlation between dietary  $\text{Ca}^{2+}$  uptake and incidence of hypertensive disorders of pregnancy in diverse populations [15]. Even if the results of two large clinical trials demonstrated disparity of the resulting benefits with respect to  $\text{Ca}^{2+}$  supplementation in prevention of PE [16, 17],  $\text{Ca}^{2+}$  supplementation did reduce PE risk in high-risk pregnancies as well as for women with a low baseline dietary  $\text{Ca}^{2+}$  uptake [18].

$\text{Ca}^{2+}$  is a universal intracellular second messenger involved in many processes such as signal transduction, neurotransmission, enzyme and hormone secretion, cell cycle regulation and programmed cell death. It is a crucial element for adequate foetal development and prenatal programming of future diseases.  $\text{Ca}^{2+}$  is actively transported across the placenta at the rate of 140 mg/kg/day [19] to reach about 25–30 mg of  $\text{Ca}^{2+}$  at term in the foetus. About 80% of the total foetal  $\text{Ca}^{2+}$  is accumulated during the last trimester of pregnancy [20]. This  $\text{Ca}^{2+}$  transfer allows adequate foetal skeleton mineralization [21] and various cellular functions. There are two pathways for  $\text{Ca}^{2+}$  entry in the foetus. Paracellular diffusion, active in perfused placental cotyledons, enables  $\text{Ca}^{2+}$  to cross the placental barrier and represents 66% of the total maternal foetal transfer [22]. The second process involves transepithelial transport, which is mediated by the syncytiotrophoblast (ST). The ST is a polynucleated structure [23] formed during implantation and represents the most important maternal-foetal barrier [24]. The villous trophoblast cells are con-

tinuously incorporated, by syncytial fusion, into the ST. The ST is characterized by a brush border membrane facing maternal circulation and a basal plasma membrane facing the foetal circulation. Besides transport, the multifunctional properties of the ST include absorption, exchanges and specific hormonal secretion such as human chorionic gonadotropin (hCG) and human placental lactogen (hPL). Cytotrophoblasts isolated from human term placenta undergo spontaneous ST-like morphological and biochemical differentiation *in vitro* and are thought to resemble *in vivo* ST [25]. In a previous experiment, we quantified secretion of hCG, a well-known marker of this differentiation process for a period of 7 days [25], and interestingly, we observed that maximal hCG secretion was at day 4 of culture and paralleled the peak in  $\text{Ca}^{2+}$  transport [26]. Intracellular  $\text{Ca}^{2+}$  signalling pathway can be briefly described as extracellular stimulations of tyrosine kinase and G-protein-coupled receptors launching cascade reactions leading to the production of the second messenger IP<sub>3</sub> (inositol1–4, 5-triphosphate) [27, 28]. IP<sub>3</sub> will bind to its receptor (IP<sub>3</sub>R) and  $\text{Ca}^{2+}$  will be released from the endoplasmic reticulum (ER), triggering  $\text{Ca}^{2+}$  entry through the different plasma membrane store-operated channels. Ryanodine receptors (RyRs) are multimeric  $\text{Ca}^{2+}$  channels in the ER membrane, which also participate in the release of  $\text{Ca}^{2+}$  from the ER [29, 30].  $\text{Ca}^{2+}$  release from ER must be followed by  $\text{Ca}^{2+}$  replenishment via sarcoendoplasmic reticulum  $\text{Ca}^{2+}$  ATPases (SERCAs) [31, 32]. In addition, the mitochondria constitutes another  $\text{Ca}^{2+}$  storage organelle which can control the amplitude, length, localization and propagation of cytosolic calcium elevations, and the  $\text{Ca}^{2+}$  recycling back to the ER.  $\text{Ca}^{2+}$  accumulation by the mitochondria implicates VDACs (voltage-dependent anion channels) [33]. Roles of VDACs and SERCAs are essential in maintaining a low resting intracellular calcium concentration [ $\text{Ca}^{2+}$ ]<sub>i</sub> [34].

The  $\text{Ca}^{2+}$  transepithelial transfer through the ST is a passive-active transport and requires various proteins. The  $\text{Ca}^{2+}$  diffuses from the maternal blood to the cytoplasmic compartment of the syncytium through specific channels named 'transient receptor potential vanilloid' (TRPV) on the brush border membrane using the electrochemical gradient. Then, calcium-binding proteins (CaBP) bind  $\text{Ca}^{2+}$  and shuttle it to the foetal face of the ST where it is actively excreted by pumps (PMCA) [35, 36].  $\text{Ca}^{2+}$  signalling pathways in the placenta are still under investigation and there is very little information concerning the expression of these proteins in PE placental tissues.

Because PE is potentially associated with an impaired maternal  $\text{Ca}^{2+}$  homeostasis, we suggested that transplacental  $\text{Ca}^{2+}$  exchange by ST is disturbed in PE. Our objectives were to: (i) characterize the placental  $\text{Ca}^{2+}$  transfer in PE; (ii) identify altered gene expression of  $\text{Ca}^{2+}$  handling proteins involved in transplacental  $\text{Ca}^{2+}$  transport, such as  $\text{Ca}^{2+}$  channels TRPV5 and 6, CaBP-9K, CaBP-28K and PMCA1 to 4; (iii) investigate  $\text{Ca}^{2+}$  intracellular signalling in PE placentas, involving ER and mitochondria, by studying the expression pattern of IP<sub>3</sub>R, RyRs, SERCAs and VDACs and (iv) to find what may be the cause of such changes in PE, as inadequate ATP and ROS levels.

**Table 1** Clinical characteristics of mothers and babies

Characteristics	Normal group ( <i>n</i> = 16)	PE group ( <i>n</i> = 8)
Maternal age (years)	33.7 (±5)	34 (±4)
Gestational age (weeks)	37.9 (±1.7)	36.7 (±2)
Body mass index (kg/m <sup>2</sup> )	21.2 (±3)	26.9(±4)
Placental weight (g)	533(±115)	518(±130)
Babies birth weight (kg)	3.1 (±0.36)	2.9 (±0.5)
Mode of delivery	Vaginal	Vaginal
Smoking	No	No

## Materials and methods

### Placental tissues

Placental tissues were obtained from human placentas in accordance with the established guidelines of the ethical committee of St. Luc Hospital of the Centre Hospitalier Universitaire de Montréal and of Université du Québec à Montréal, (Montréal, QC, Canada). Placentas were obtained immediately after vaginal delivery, from normal term pregnancies (*n* = 16) and from PE pregnancies (*n* = 8). In order to avoid inter-ethnic variability of 1,25(OH)<sub>2</sub>D<sub>3</sub> and Ca<sup>2+</sup> metabolism, only Caucasian women were invited to participate to this study. The sample contained women without pre-existing clinical risk factors of gestational hypertension. The following conditions were excluded from this study: pre-pregnancy diabetes mellitus, gestational diabetes, foetal physical or chromosomal abnormalities, untreated hypo/hyper thyroidism, tobacco and alcohol use, renal diseases with altered renal function, active and chronic liver disease, cancer, collagen diseases, congenital rickets and other malformations. The control group was represented by women without diagnosed pathologies, with no drugs intake. PE was diagnosed by a clinician and classified according to strict criteria recommended by the Society of Obstetricians and Gynaecologists of Canada [37]: a systolic blood pressure of 140 mmHg or higher or a diastolic blood pressure of 90 mmHg or higher on two occasions at least four hours apart, occurring after 20 weeks of gestation in a pregnant women with previously normal blood pressure, and detectable proteinuria (≥0.3 g/24 hrs). This group also includes the late onset of PE characterized by the late manifestation of PE and excludes pregnancy with IUGR, SGA (small for gestational age) foetus or HELLP (haemolysis, elevated liver enzymes, low platelet count) syndrome. Four of our PE patients were treated with magnesium sulphate (MgSO<sub>4</sub>), safe for both mother and foetus [38], which prevents seizures by altering membrane permeability and increasing seizure threshold [39]. Interestingly, MgSO<sub>4</sub> infusion caused significant increases in ionized Mg<sup>+2</sup> levels, while serum ionized Ca<sup>2+</sup> concentrations were unchanged [40], suggesting that the effect of MgSO<sub>4</sub> is not exerted through modulations of ionized calcium levels [41]. Also, it was observed that in the normal pregnant women, ionized Ca<sup>2+</sup> levels were higher in foetal blood and that this same condition was found in PE women treated with MgSO<sub>4</sub> [42], suggesting that this treatment has no effect on Ca<sup>2+</sup> transplacental transfer. Characteristics of pregnancies are summarized in Table 1. From the placentas, maternal and foetal membranes were removed and small pieces of tissue from several cotyledons

(5–10) were collected to have a good representation of the total placenta [43]. For RNA sample, tissues were embedded in RNA later stabilization reagent (Qiagen, Mississauga, Ontario, Canada), frozen in liquid nitrogen and stored at –80°C. Tissue samples for protein extraction were directly frozen into liquid nitrogen and subsequently transferred into sterile tubes for storage at –80°C.

### RNA extraction and cDNA synthesis

RNA was isolated from total placenta (30 mg) using RNeasy mini kit extraction columns (Qiagen) and reverse-transcribed. After RNA isolation, 1 µg of RNA was reverse transcribed into cDNA at 37°C for 1 hr using 10 µM poly(dT) (Roche Applied Science, Laval, Qc, Canada), 0.5 mM dNTP and 4 U of Omniscript reverse transcriptase (Omniscript RT kit) (Qiagen) in a 20 µl final volume. Exceptionally for the CaBP-28K gene, after reverse transcription, cDNA were first preamplified with the LightCycler RNA Pre-Amplification Kit (Roche Applied Science) before real-time PCR analyses.

### Real-time PCR

Quantification of the mRNA coding for TRPV5, TRPV6, CaBP-9K, CaBP-28K, PMCA1, PMCA2, PMCA3 and PMCA4 was performed by real-time PCR using the Roche LightCycler 480 PCR (Roche Applied Science). All primer sequences (Table 2) were generated through Primer3 Input (<http://fokker.wi.mit.edu/primer3/input.htm>), verified for specificity by BLAST analyses and designed to span from two different exons. PMCA2 and PMCA3 primers were purchased from Qiagen, Inc. Real-time PCR reactions were performed in a volume of 10 µl containing 1 µl of cDNA, 1 µM of each primer and 5 µl of SYBR Green I Master (Roche Applied Science). Quantification of the endogenous ribosomal gene hypoxanthine phosphoribosyltransferase (HPRT)1 was performed to normalize variations in cDNA content among different samples. The expression of HPRT1 remained stable under our experimental conditions. Results were analysed with the LightCycler 480 software version 1.5, and melting curves were used to verify the specificity of the amplified products. Quantification of PCR products was performed through a standard curve generated by simultaneously amplifying serial dilutions of the corresponding cDNA. Values obtained for each gene were normalized to HPRT1 in the same sample.

### Western blots analysis

Tissues were initially washed with lymphocyte lysis buffer containing 1.7 mM Tris-HCl (pH 7.3), 1.44 mM NH<sub>4</sub>Cl and protease inhibitor cocktail in tablets and then proteins were purified by the addition of the extraction buffer containing 125 mM Tris-HCl (pH 8.0), 2 mM CaCl<sub>2</sub>, 1.4% Triton X-100 (v/v) and protease inhibitor cocktail in tablets (Roche Applied Science). Protein concentrations were evaluated with BCA (bicinchoninic acid) assay (Pierce, Brockville, Ontario, Canada). Cellular proteins (100 µg) were solubilized in Laemmli buffer and denatured by heating at 95°C for 5 min. Lysates were resolved in 10% SDS-PAGE and proteins were electro-blotted on a polyvinylidene difluoride (PVDF) membrane (Millipore, Cambridge, Ontario, Canada) at 17 V for 30 min. Membranes were blocked for 1 hr at room temperature in TBS-T (20 mM Tris (pH 7.6),

**Table 2** Real-time PCR primer sequences

Gene	Primer sequence sense antisense	Product size (bp)
CaBP-9k	5' ATA TGC AGC CAA AGA AGG TG 3' 5' TGG ACC TTT GAG TAA ACT GGG 3'	94
CaBP-28k	5' TCA GGA CGG CAA TGG ATA CA 3' 5' AAG AGC AAG ATC CGT TCG GT 3	166
TRPV5	5' AGA CAA GGA GGA TGA CCA GGA 3' 5' CCC AGG GTG TTT TGA CGA AG 3'	168
TRPV6	5' TCT GCG GAC GGG AGT ATG G 3' 5' CCT GTG CGT AGC GTT GGA T 3'	96
PMCA1	5' CAG CAG GAG AAC CAG AAC CA 3' 5' ATT CCA GCC CTC TGA CAC TT 3'	139
PMCA2	Hs_ATP2B2_1_SG QuantiTect Primer Assay (Qiagen, Mississauga, Ontario, Canada)	220
PMCA3	Hs_ATP2B3_1_SG QuantiTect Primer Assay (Qiagen)	149
PMCA4	5' TCAGGAATCCCAACGGTG 3' 5' TCGATGACAGTGCCTACC3'	148
ATPase	5' CGC CAC CCT AGC AAT ATC AA 3' 5' TTA AGG CGA CAG CGA TTT CT 3'	98
IP3R1	5' TGA CGA GAA CCT GCC CTA T 3' 5' TCC TTT CGC CAT CTT GCT 3'	430
IP3R2	5' GCA ATC GTG TCT GTT CCA 3' 5' TCT TCA AGT CTC AGC ATC G 3'	332
IP3R3	5' GCC TAC TAT GAG AAC CAC ACG 3' 5'CAG AAG AGC AAT GAG ATG AGA G 3'	389
RyR1	5' TGA CTA CCA TCA GCA CGA CA 3' 5' ACG AAG ACG GCA GGA AAT A 3'	297
RyR2	5' TTT ACC AGC ACC CTA ATC TC 3' 5' CAG CCA AAT AAC GAA CTA CC 3'	348
RyR3	5' TCT GCT GTC TGG GTC TAT CTC 3' 5' CCA AAT GTC TTA TGC GTC AC 3'	262
SERCA1	5' GTG ATC CGC CAG CTA ATG 3' 5' CGA ATG TCA GGT CCG TCT 3'	361
SERCA2	5' CGC TAC CTC ATC TCG TCC A 3' 5' TCG GGT ATG GGG ATT CAA 3'	406
SERCA3	5' GAT GGA GTG AAC GAC GCA 3' 5' CCA GGT ATC GGA AGA AGA G 3'	409
VDAC1	5' CAC CAC GTA TGC CGA TCT TG 3' 5' TGC TTG AAA TTC CAG TCC 3'	340
VDAC2	5' CAC CTC ATA TGC TGA CCT TGG C 3' 5' AGC CTC CAA CTC CAG GGC 3'	340
VDAC3	5' CAC CAC ACC AAC GTA CTG TG 3' 5' AGC TTC CAG TTC AAA TCC 3'	340
hOGG1	5' TGG AAG AAC AGG GCG GGC TA 3' 5' ATG GAC ATC CAC GGG CAC AG 3'	164
HPRT1	5' GAC CAG TCA ACA GGG GAC ATA A 3' 5' AAG CTT GCG ACC TTG ACC 3'	149

**Table 3** Antibodies information

Names	Companies	Dilution
TRPV5	Alpha Diagnostic International, San Antonio, TX, USA	1/1000
TRPV6	Alomone Labs, Jerusalem, Israel	1/1000
CaBP-9k	Santa Cruz, Santa Cruz, CA, USA	1/1000
CaBP-28k	Sigma-Aldrich, Oakville, Ontario, Canada	1/1000
PMCA1/4	Santa Cruz	1/1000
Antimouse-IgG HRP-conjugated	Millipore, Billerica, MA, USA	1/3000
Anti-rabbit-IgG HRP-conjugated	Cell Signaling, Danvers, MA, USA	1/2500
Cytokeratin-7 FITC-conjugated	Abcam, Cambridge, MA, USA	1.25 µg per 10 <sup>6</sup> cells

137 mM NaCl and 0.1% Tween-20) containing 5% skimmed milk. Membranes were then incubated with the appropriate primary antibody (see Table 3 for details on antibodies) in TBS-T–3% bovine serum albumin (BSA) overnight at 4°C, washed three times with TBS-T and probed with horseradish peroxidase-conjugated secondary antibodies for 2 hrs at room temperature. Blots were washed three times with TBS-T and the detection was performed with the BM Chemiluminescence (POD) system (Roche Applied Science) and visualized on Kodak(tm) X-Omat™ Blue XB-1 autoradiography films from Perkin Elmer (Waltham, MA, USA). PVDF membranes were stripped with a stripping solution containing 25 mM glycine-HCl (pH 2) and 1% SDS at room temperature for 10 min., rinsed twice with PBS (10 mM sodium phosphate (pH 7.2) and 0.9% NaCl) and blocked for 1 hr before reprobing with another antibody. Precisely, we loaded eight samples/gel (eight normal + eight PE) and transferred two gels/membrane. After exposure, membranes were stained with Amido Black Staining Solution (Sigma) to correct variations in protein content among samples. Densitometric analysis of band intensities was made with QuantityOne Bio-Rad (Hercules, CA, USA) software and results were normalized with protein content obtained by Amido Black.

## Cell cultures

For  $\text{Ca}^{2+}$  transport assay, primary cytotrophoblast cells were prepared from placentas obtained immediately after vaginal delivery, from normal term pregnancies ( $n = 5$ ) and from PE pregnancies ( $n = 4$ ) (see characteristics in Table 1). Cytotrophoblast cells were isolated using the trypsin (Sigma)-DNase (Roche Applied Science)/Percoll (Sigma) method as described by Kliman *et al.* [25], with some modifications [44]. Following cell isolation, cytotrophoblast cells were seeded at a density of  $1.5 \times 10^6$  cells per well in 24-well plate (Corning, Acton, USA) and maintained in Dulbecco's modified Eagle's medium (high glucose) (DMEM-HG) (Sigma) containing 10% FBS (Cansera International Inc, Etobicoke, Ontario, Canada), 2 mM glutamine (Gibco-Invitrogen, Burlington, Ontario, Canada), 25 mM HEPES (Sigma) and PSN, a penicillin-streptomycin-neomycin antibiotic mixture (Gibco-Invitrogen). The medium was refreshed daily. The purity of the cytotrophoblast cell preparation was evaluated by flow cytometry using FITC-conjugated monoclonal antibody against cytokeratin-7 as previously described [44].

## MTT Assay

The cells viability was measured by the MTT assay (Sigma). Cells were cultured with DMEM-HG without phenol red and were incubated with 100  $\mu\text{l}$  MTT per well each day. Plates were incubated for 1 hr at 37°C and 200  $\mu\text{l}$  of DMSO were added to each well. After mixing, the optical density was measured at 570 nm.

## $\text{Ca}^{2+}$ transport assay

$\text{Ca}^{2+}$  transport studies were performed on trophoblast cells after 4 days of culture. Briefly, cells were washed twice with the  $\text{Ca}^{2+}$  uptake buffer (HBSS [Hanks' balanced salt solution from Sigma] containing 1.26 mM  $\text{CaCl}_2$ , 10 mM HEPES, and 0.1% BSA [from Roche Applied Science]) and allowed to equilibrate in the same buffer (250  $\mu\text{l}$ ) for 10 min. Thereafter, cells were incubated at 37°C for different intervals of time after the addition of 250  $\mu\text{l}$  of uptake buffer containing  $^{45}\text{CaCl}_2$  (5  $\mu\text{Ci}/\text{well}$ ) (MP Biomedicals, Inc., Irvine, CA, USA). The incubation was stopped by aspiration of the uptake buffer. The cells were washed three times with 1 ml of ice-cold PBS containing 4 mM ethylene glycol tetraacetic acid (EGTA) (to eliminate the nonspecific component of the uptake), then solubilized in 0.1 M NaOH. The cell-associated radioactivity was measured by a  $\beta$ -scintillation 1400 Wallace counter. The cellular proteins content of each well was evaluated by spectrophotometric quantification, using the BCA assay. The  $\text{Ca}^{2+}$  transport is expressed as nmole of  $\text{Ca}^{2+}$  (from specific activity) per milligram of cellular proteins.

## Statistical analyses

Data were expressed as the mean  $\pm$  S.E.M. and were analysed using GraphPad Prism software for windows, version 5.0. One-way ANOVA followed by the Tukey's test was used to analyse  $\text{Ca}^{2+}$  transport and nonparametric Mann-Whitney U-test was used to determine differences in mRNA and protein levels between normal and PE placentas. A  $P$ -value  $< 0.05$  was considered statistically significant.

## Results

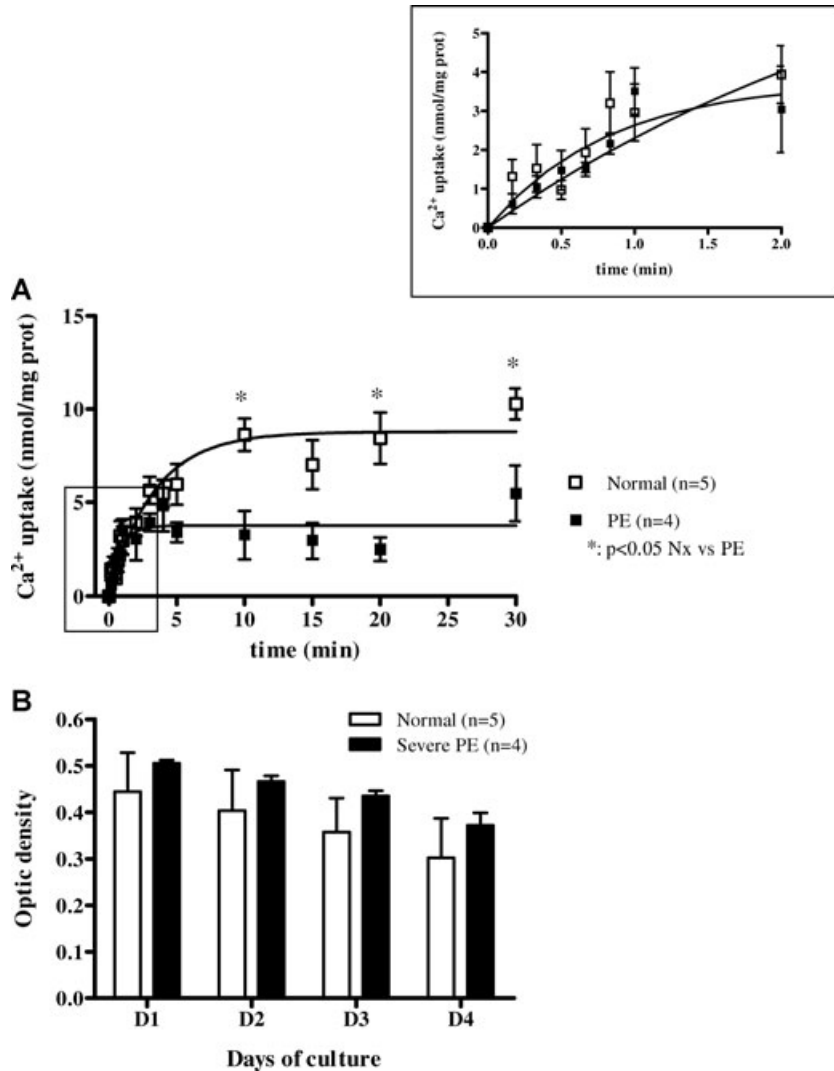
### $\text{Ca}^{2+}$ transport is less efficient in ST from PE placentas than in normal ST

To assess whether  $\text{Ca}^{2+}$  homeostasis is affected in PE, we first examined the  $\text{Ca}^{2+}$  transport in primary ST, obtained from normal and PE placentas. The  $\text{Ca}^{2+}$  transport assay was performed by means of a radioactive form of calcium ( $^{45}\text{CaCl}_2$ ) for different time intervals (10, 20, 30, 40, 50 sec., 1, 2, 3, 4, 5, 10, 15, 20, 30 min.). As we described earlier, isolated trophoblasts undergo morphological changes during culture to form the functional multinucleated syncytium, which is properly developed after 96 hrs of culture. Therefore, we measured the cell-associated radioactivity after 4 days of culture, based on  $\beta$ -scintillation. The  $\text{Ca}^{2+}$  transport was expressed as nmole of  $\text{Ca}^{2+}$  (from specific activity) per milligram of cellular proteins. The  $\text{Ca}^{2+}$  uptake from normal and PE ST is rapid and there is no difference during the first 2 min. with an initial velocity ( $V_i$ ) of  $4.39 \pm 0.73$  nmol/mg/min for PE and  $5.38 \pm 1.04$  nmol/mg/min for normal group. Then, the  $\text{Ca}^{2+}$  transport reaches a plateau, which represent a balance between the  $\text{Ca}^{2+}$  entry and exit, showing a decrease of 60% in PE group ( $3.75 \pm 0.31$  nmol/mg protein) compared to normal group ( $8.99 \pm 0.48$  nmol/mg protein) at 10, 20 and 30 min. The plateau is reached earlier for the PE group (after 2 min. of treatment) than for the normal group (after 10 min. of treatment). The plateau is generally known to reflect cells  $\text{Ca}^{2+}$  entry and exit (Fig. 1A). To make sure that the  $\text{Ca}^{2+}$  transport in PE cultures was not affected by a probable higher rate of apoptosis, we measured the cells viability in the two cellular populations and there was no difference between normal and PE trophoblast cells (Fig. 1B). These observations suggest strongly that in PE,  $\text{Ca}^{2+}$  transfer by ST is compromised.

To study whether the decreased calcium transport in placentas from PE women is due to a decrease of calcium-handling protein in ST, we extracted mRNA and proteins from total placental tissues and we compared the changes in protein and mRNA levels, by Western blot and real-time PCR, of several genes implicated in placental  $\text{Ca}^{2+}$  transport in normal and PE placentas.

### TRPV5 and TRPV6 expression is decreased in PE

To establish if the entry of calcium in the placenta is disturbed during PE, we examined the protein and mRNA expression of TRPV5 and TRPV6, gatekeepers of  $\text{Ca}^{2+}$  transport. We found that the protein expression of TRPV5 was significantly decreased in PE compared to normal placental tissues (Fig. 2A), as its mRNA expression (Fig. 2B). Similarly, TRPV6 protein and mRNA expression decreases in PE compared to normal group (Fig. 2C, D). These findings suggest that the decrease in the  $\text{Ca}^{2+}$  transport in ST is partially due to an impaired entrance in the placenta.



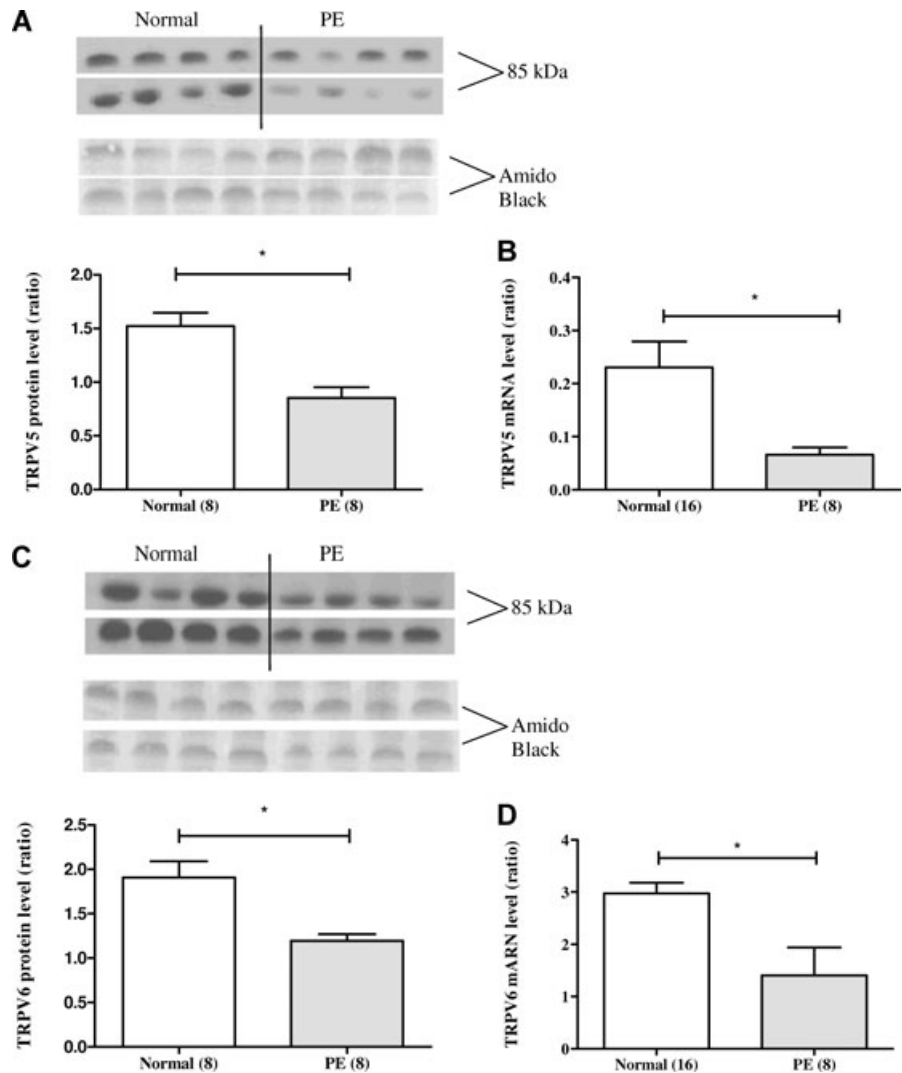
**Fig. 1** The calcium transport decreased in trophoblasts coming from PE placentas. **(A)** This calcium transport analysis was performed after 4 days of culture with radioactive calcium (<sup>45</sup>CaCl<sub>2</sub>) and was performed for different time intervals. The Ca<sup>2+</sup> transport is expressed as nmole of Ca<sup>2+</sup> (from specific activity) per milligram of cellular proteins. The results are the mean ± SEM of experiment performed in duplicates on cell preparations from five normal and four PE placentas. **(B)** The viability of trophoblasts from day 1 to day 4 of culture is not significantly decreasing in PE cultured cells compared to normal. The viability was measured by the MTT assay and the results are shown as optic density measured at 570 nm of the blue formazan crystals formed.

**CaBP-9K and CaBP-28K expression is decreased in PE**

Immediately after its penetration in the ST, Ca<sup>2+</sup> moves toward the foetal side, where it is extruded, or it is buffered to avoid reaching lethal concentration for the cell. Those tasks are accomplished by calcium binding proteins, as CaBP-9K and CaBP-28K. To verify if this system is altered in PE, we analysed their protein and mRNA expression. We found that the protein expression of CaBP-9K is decreased in the PE placental tissue compared to normal ones (Fig. 3A) as well as mRNA expression (Fig. 3B). For the gene CaBP-28K, the protein and mRNA expression is also decreased in PE placentas (Fig. 3C, D). These observations suggest that Ca<sup>2+</sup> buffering is altered in PE placental cells.

**PMCA expression is decreased in PE**

Once arrived on the other side of the cell, Ca<sup>2+</sup> has to cross over a last membrane to reach the foetal circulation. This expulsion is mainly achieved by the ATPase pumps PMCA, which respond to foetal specific needs in calcium. To determine if an impaired Ca<sup>2+</sup> exit from ST could be responsible for the impaired placental transfer seen in Fig. 1, we studied protein and mRNA levels of 4 isoforms of PMCA (PMCA1–4). For the protein expression, we used an antibody that detects PMCA1 and PMCA4 together. Thus, we observed that the protein expression of PMCA 1/4 (Fig. 4A) decreases significantly in PE compared to normal group. We observed a similar pattern for PMCA1 and PMCA4 mRNA expression (Fig. 4B, C). In contrast, PMCA2 and PMCA3 mRNA expressions show no variation (data not shown). Collectively, these



**Fig. 2** Protein and mRNA expression of calcium channels TRPV5 and TRPV6 in placentas coming from normal women compared to women with PE. **(A)** The protein expression of TRPV5 is significantly decreased in placentas PE compared to normal ones ( $P = 0.0008$ ). **(B)** TRPV5 mRNA expression is decreased in PE compared to normal placentas ( $P = 0.0044$ ). **(C)** The protein expression of TRPV6 is significantly decreased in placentas with PE compared to normal ones ( $P = 0.0029$ ). **(D)** The mRNA expression of TRPV6 is significantly decreased in PE compared to normal placentas ( $P = 0.0156$ ). The number of placentas tested for the mRNA expression is shown in parentheses for **(B)** and **(D)**. For protein and mRNA expression, data were normalized according to amido black total protein levels and to HPRT1 housekeeping gene, respectively.  $*P < 0.05$ .

findings indicate that the  $\text{Ca}^{2+}$  extrusion towards the foetal side is compromised in PE.

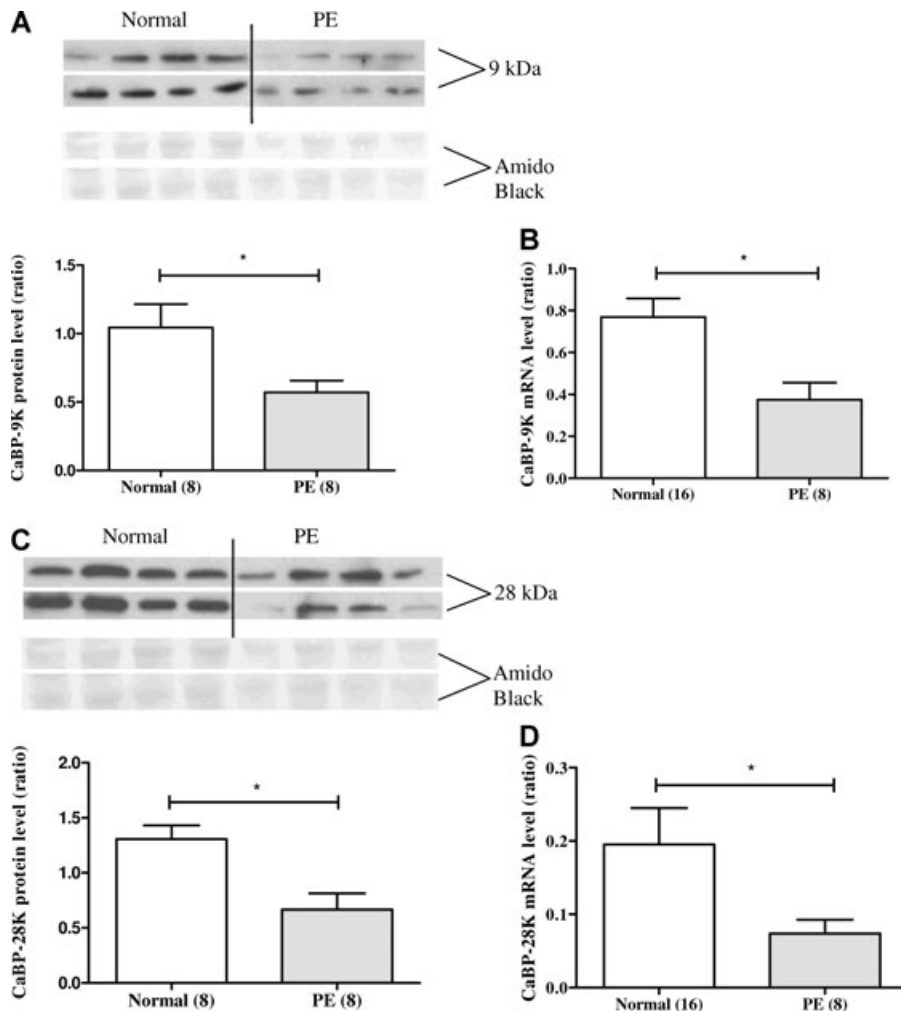
### ATP synthase level is decreased in PE

PMCA are ATPase pumps, thus they need ATP to function properly. As it was observed that lower ATP levels in PE placental cells is due to an important change in expression of ATP synthase gene [12], we wanted to verify if that phenomenon was present in our tissue samples and if it could be the cause of the reduction of PMCA. Therefore, we analysed mRNA levels of the mitochondrial ATP synthase gene in normal and PE placental tissues (Fig. 5). We observed that its mRNA expression was drastically decreased PE compared to normal. This result sug-

gests, as we suggested, that depletion of ATP by hypoxia affect PMCA expression in PE tissues.

### $\text{Ca}^{2+}$ intracellular signalling implicating mitochondria and ER is altered in PE

Following the decrease of CaBPs (Fig. 3) and PMCA (Fig. 4) expressions, respectively, responsible for  $\text{Ca}^{2+}$  buffering and extrusion, we suggested that placental cells may suffer of  $\text{Ca}^{2+}$  overload. Therefore, we analysed the mRNA expression of IP3R and RyR, molecules in charge of the  $\text{Ca}^{2+}$  release from the ER, increasing  $\text{Ca}^{2+}$  intracellular concentration. As we show in Figs 6 and 7, IP3R and RyR mRNA expressions are decreased in PE placentas compared to normal ones. In parallel, we also analysed



**Fig. 3** Protein and mRNA expression of CaBP-9K and CaBP-28K in placentas coming from healthy women compared to women with PE. **(A)** The placental protein expression of CaBP-9K is significantly decreased in PE placentas compared to normal ones ( $P = 0.0268$ ). **(B)** CaBP-9K mRNA expression is significantly lower in PE compared to normal placentas ( $P = 0.0465$ ). **(C)** The protein expression of the CaBP-28K gene is decreased in placentas with PE ( $P = 0.0055$ ). **(D)** The mRNA expression of CaBP-28K is significantly decreased in placentas with PE compared to normal placentas ( $P = 0.0383$ ). The number of placentas tested for the mRNA expression is shown in parentheses for **(B)** and **(D)**. For protein and mRNA expression, data were normalized according to amido black total protein levels and to HPRT1 housekeeping gene, respectively. \* $P < 0.05$ .

mRNA expression of SERCA, transferring  $\text{Ca}^{2+}$  from the cytosol of the cell to the lumen of the ER, to see if it responded to that impending  $\text{Ca}^{2+}$  overload by diminishing  $[\text{Ca}^{2+}]_i$ . Unsurprisingly, we obtained an increase in mRNA of SERCA gene in PE placentas (Fig. 8). Likewise, the mitochondria is another important organelle that stores  $\text{Ca}^{2+}$  and as its replenishment implicates VDAC gene, we analysed its mRNA expression and also observed a significant increase in PE placentas (Fig. 9). These results support our hypothesis about the alteration of intracellular  $\text{Ca}^{2+}$  signalling in placentas with PE, more precisely of  $\text{Ca}^{2+}$  overload.

### Presence of high hOGG1 mRNA expression: PE placentas undergo severe oxidative stress

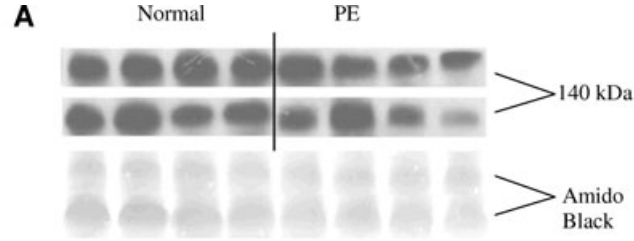
hOGG1 is an enzyme repairing DNA oxidation damages by excising oxidized-guanine. As the presence of elevated levels of hOGG1 correlates with elevated ROS levels, we analysed its mRNA

expression to determine, as we suggested, if PE placentas are more affected by oxidative stress than normal ones. Interestingly, we observed a huge increase of hOGG1 mRNA expression in PE placentas (Fig. 10). This result confirms that in PE, placental cells suffer from severe oxidative stress, which surely alters many cellular mechanisms, as  $\text{Ca}^{2+}$  signalling.

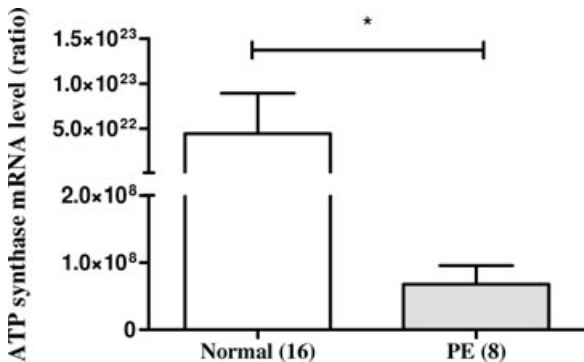
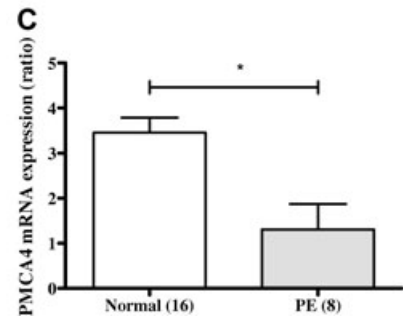
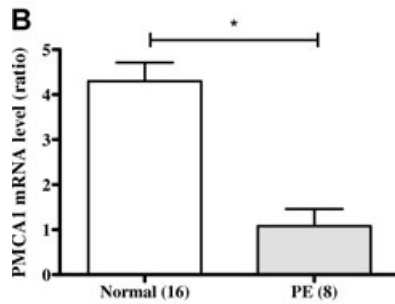
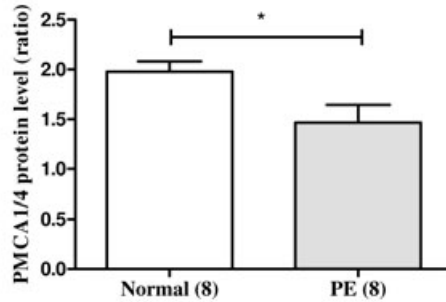
## Discussion

In this study, we demonstrated for the first time a significant decrease in  $\text{Ca}^{2+}$  transport in primary cultures of ST from PE placentas compared to uncomplicated ones. As we show in Fig. 1,  $\text{Ca}^{2+}$  transport is about 60% lower in primary PE ST cells than in normal. Earlier, we and others demonstrated that, after 4 days of culture, there was a significant reduction of 40% in cell fusion in trophoblasts isolated from PE placentas when compared to





**Fig. 4** Protein and mRNA expression of calcium pumps (PMCA) isoforms 1 and 4 in normal and PE placentas. **(A)** This graph shows that the protein expression of both PMCA1 and 4 is significantly decreased in placentas PE compared to normal placentas ( $P = 0.0256$ ). **(B)** The mRNA expression of PMCA1 is significantly decreased in PE placentas compared to normal group (0.0007). **(C)** The mRNA expression of PMCA 4 is significantly decreased in PE compared to normal placentas ( $P = 0.0082$ ). The number of placentas tested for the mRNA expression is shown in parentheses for **(B)** and **(C)**. For protein and mRNA expression, data were normalized according to amido black total protein levels and to HPRT1 housekeeping gene, respectively. \* $P < 0.05$ .



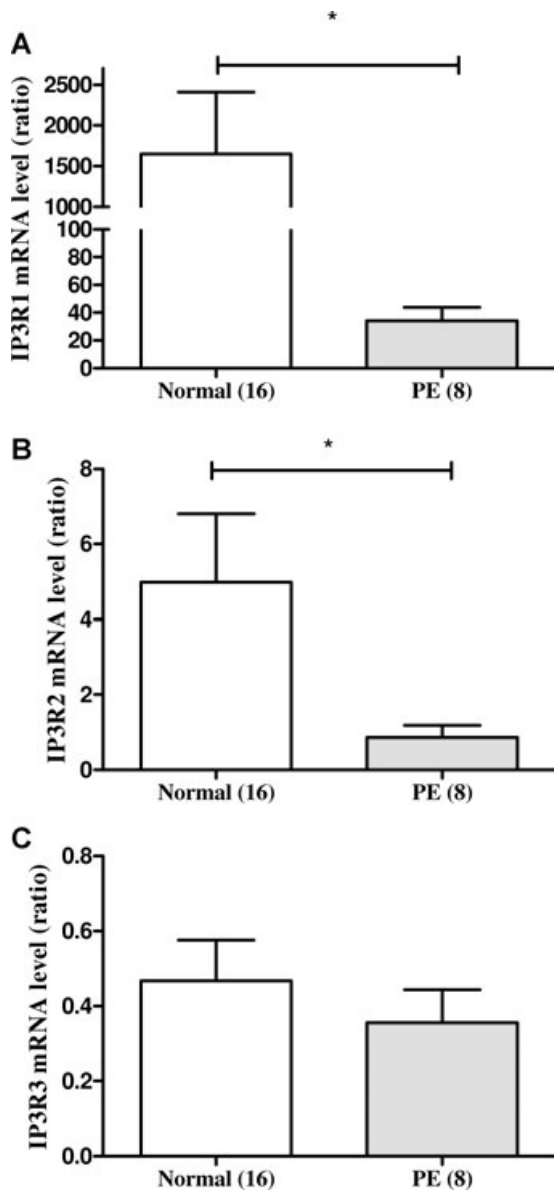
**Fig. 5** mRNA expression of the ATP synthase gene in normal and PE placentas. The mRNA expression is significantly decreased in PE compared to normal placentas ( $P = 0.0098$ ). The number of placentas tested for the mRNA expression is shown in parentheses. For mRNA expression, data were normalized according to HPRT1 housekeeping gene. \* $P < 0.05$ .

trophoblasts obtained from normal patients (unpublished data). Thus, the decrease in  $Ca^{2+}$  transport from ST is only partially due

to the 40% reduction of syncytial volume seen in primary ST from PE (unpublished data). To explain what may also be responsible for the altered  $Ca^{2+}$  transfer and to clarify the mechanism involved, we investigated the expression of different proteins involved in  $Ca^{2+}$  homeostasis in placentas from PE pregnancies. Until now, there is no information on the variation of the  $Ca^{2+}$  handling proteins and the  $Ca^{2+}$  transfer in PE placentas. Importantly, for the first time, we successfully quantified the placental mRNA expression of CaBP-28K by real-time PCR. Herein, we demonstrated that the  $Ca^{2+}$  homeostasis is perturbed in PE placentas, most likely caused by a high oxidative stress level and lack of ATP.

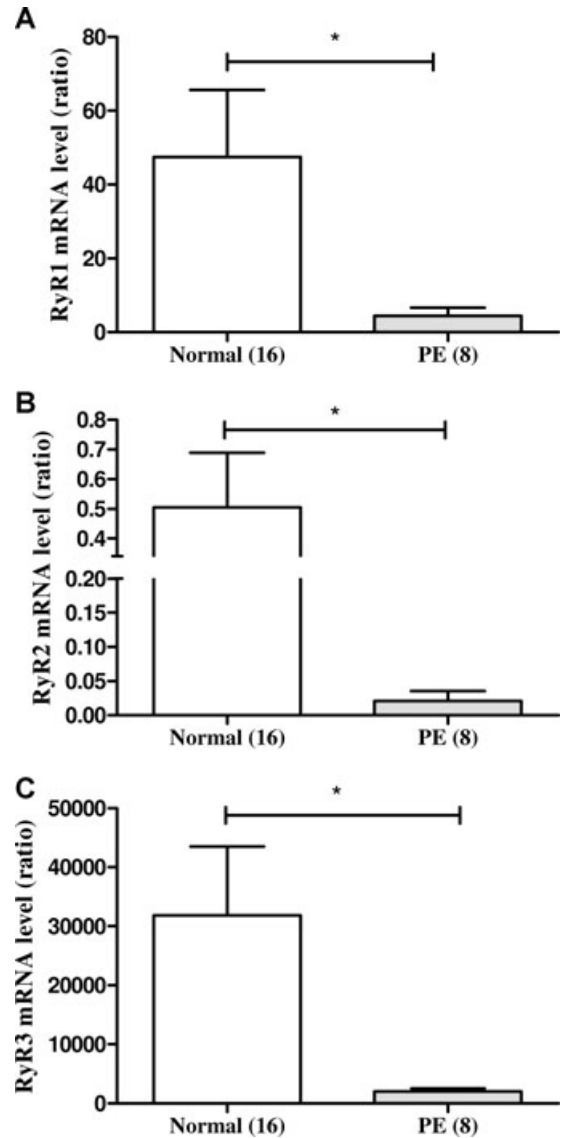
### Decreased expression of TRPVs is accompanied with a decreased expression of CaBPs

A recent study shown that the  $Ca^{2+}$  concentration in foetal blood and amniotic fluid is significantly lower in murine TRPV6 knock-out (KO) fetuses than in wild-types [45]. The same team also demonstrated that the transport activity of radioactive  $Ca^{2+}$  ( $^{45}Ca^{2+}$ ) from mother to fetuses was 40% lower in TRPV6 KO



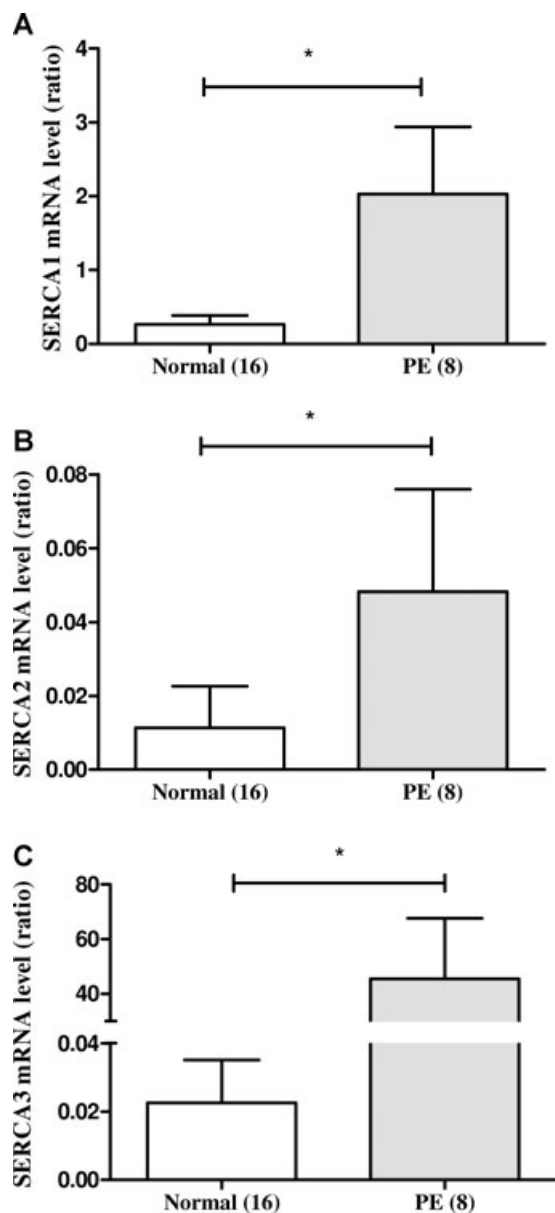
**Fig. 6** mRNA expression of 3 isoforms of the IP3R gene in normal and PE placentas. The mRNA expression is significantly decreased in PE compared to normal placentas for (A) IP3R1 ( $P = 0.0287$ ) and (B) IP3R2 ( $P = 0.0366$ ). (C) The mRNA expression of IP3R3 is not altered in PE placentas. The number of placentas tested is shown in parentheses. For mRNA expression, data were normalized according to HPRT1 housekeeping gene. \* $P < 0.05$ .

foetuses than in wild-types, that TRPV6 and CaBP-9K co-localized in the yolk sac and that CaBP-9K mRNA expression was decreased in TRPV6 KO mice placentas [45]. Also, TRPV6 expression has been shown to increase with the induction of CaBP-9K [46]. These data suggest an important role for the TRPV6 channel in  $Ca^{2+}$  homeostasis and placental transfer, and suggest that TRPV6 and CaBP-9K are cooperatively involved in maternal-foetal  $Ca^{2+}$



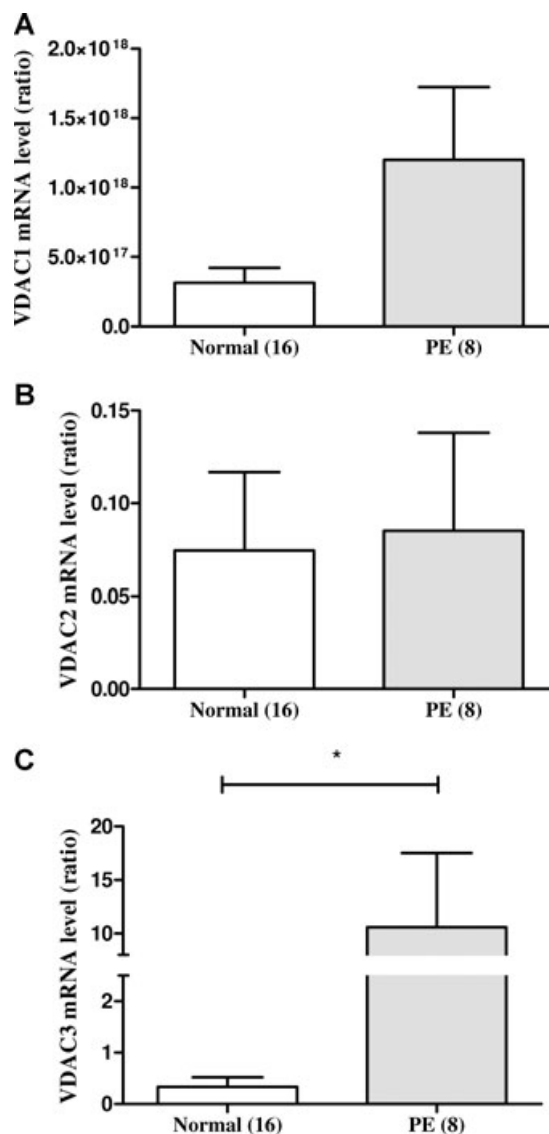
**Fig. 7** mRNA expression of 3 isoforms of the RyR gene in normal and PE placentas. The mRNA expression is significantly decreased in PE compared to normal placentas for (A) RyR1 ( $P = 0.0336$ ), (B) RyR2 ( $P = 0.0320$ ) and (C) RyR3 ( $P = 0.0179$ ). The number of placentas tested is shown in parentheses. For mRNA expression, data were normalized according to HPRT1 housekeeping gene. \* $P < 0.05$ .

transport. This matches with our results, as we obtained a decrease in transport of  $^{45}Ca^{2+}$  in ST from PE placentas (Fig. 1A), accompanied by a decrease of TRPV6 (Fig. 2D) and CaBP-9K (Fig. 3B) protein and mRNA expression. Mice TRPV5 KO have shown a defective  $Ca^{2+}$  homeostasis, limited  $Ca^{2+}$  transfer to the foetus, as well as a CaBP-28K mRNA down regulation [47]. Moreover, a previous report showed that CaBP-28K translocates towards the plasma membrane and directly associates with TRPV5, facilitating high calcium transport rates by preventing



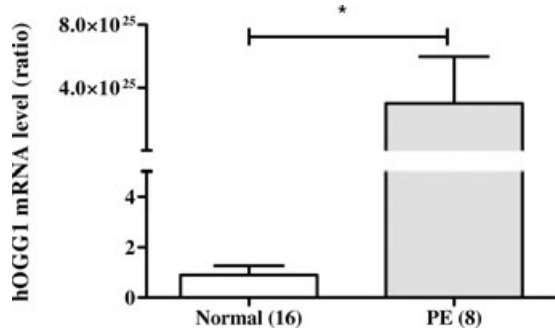
**Fig. 8** mRNA expression of 3 isoforms of the SERCA gene in normal and PE placentas. The mRNA expression is significantly increased in PE compared to normal placentas for (A) SERCA1 ( $P = 0.0147$ ), (B) SERCA2 ( $P = 0.0225$ ) and (C) SERCA3 ( $P = 0.0092$ ). The number of placentas tested is shown in parentheses. For mRNA expression, data were normalized according to HPRT1 housekeeping gene. \* $P < 0.05$ .

channel inactivation [48]. In our study, we also observed a decreased mRNA and protein expression of TRPV5 (Fig. 2A, B) accompanied by a decreased mRNA and protein expression of CaBP-28K (Fig. 3C, D). It has been demonstrated that TRPV5 and TRPV6 are considered as gatekeepers of transcellular  $Ca^{2+}$  transport as they are inversely correlated with the intracellular  $Ca^{2+}$



**Fig. 9** mRNA expression of 3 isoforms of the VDAC gene in normal and PE placentas. The mRNA expression of (A) VDAC1 and (B) VDAC2 is not altered in PE placentas, while it is significantly increased for (C) the VDAC3 isoforms compared to normal ones ( $P = 0.0253$ ). The number of placentas tested is shown in parentheses. For mRNA expression, data were normalized according to HPRT1 housekeeping gene. \* $P < 0.05$ .

concentration [49]. Effectively, it was demonstrated that in condition of high  $Ca^{2+}$  concentration, both TRPV5 and TRPV6 exhibit calcium-dependant inactivation to avoid elevation in intracellular calcium to toxic levels [46]. This is the first time that the correlation of the expression of TRPVs with CaBPs is observed in human placentas, suggesting a fundamental role of CaBPs in the regulation of TRPVs. Moreover, it has also been demonstrated that CaBP-28K binds to the amino and carboxyl end of both TRPV5 and TRPV6 in low calcium concentration and that the disruption of this



**Fig. 10** mRNA expression of the hOGG1 gene in normal and PE placentas. The mRNA expression is significantly increased in PE compared to normal placentas ( $P = 0.0126$ ). The number of placentas tested for the mRNA expression is shown in parentheses. For mRNA expression, data were normalized according to HPRT1 housekeeping gene. \* $P < 0.05$ .

association results in a decrease of  $Ca^{2+}$  buffering at the entry gate, followed by impairment of transcellular  $Ca^{2+}$  transport [48]. Finally, a synchronized rise in the expression of both TRPV5/TRPV6 and intracellular CaBP-9K/28K is essential to achieve maximal  $Ca^{2+}$  influx at the apical side, which seems to be deficient in placentas with PE.

### PE placentas demonstrate a decreased expression of PMCA1 and 4 as well as an increase of ATP synthase and hOGG1 genes

In  $Ca^{2+}$  transport, the importance of CaBPs is also highlighted by its co-expression with PMCA1 [48]. In human placentas, PMCA1 are the principal proteins implicated in  $Ca^{2+}$  extrusion to the foetal side from the ST. Their function is to eliminate intracellular calcium in excess, and, at a resting state, maintain cytosolic calcium concentration to prevent toxic surcharge [50]. To be functional, PMCA1 need the presence of ATP. It was shown that ATP level in PE placental cells is lower due to an important change in ATP synthase gene [12] and we demonstrated that the ATP synthase level in our tissue samples was significantly decreased in PE (Fig. 5). Moreover, it was shown that changes in PMCA1 expression was correlated with its activity and ATP content in rat cells [51]. This agrees with our results where we observed a significant decrease in mRNA and protein expression of PMCA1 and 4 (Fig. 4). This lack of ATP is possibly attributed to hypoxia in PE placenta, caused by oxidative stress, which would deplete cellular ATP. To determine if there was a real increased of ROS in our samples, we analysed the mRNA expression of hOGG1, a marker of oxidative stress. Effectively, mRNA expression on hOGG1 is extremely higher in PE placental tissue than in normal (Fig. 10). Indeed, oxidative stress, resulting of deficient spiral artery remodelling, is an element of PE acting as a key intermediary event in the pathology [52]. Thus, based on the reduced mRNA and protein expression of PMCA1 and 4 in PE placentas, we suggest that this could

result in inefficient calcium exclusion from ST by PMCA1. Moreover, the oxidative stress in PE placenta promotes the formation of lipids peroxides, which alter cell membranes, where, as we known, are located the PMCA1 [52]. It was recently demonstrated the PMCA1 activity is very sensitive to the level of lipid peroxidation of the plasma membrane [53] and that the reduced activity of PMCA1 in PE women could not be associated with a defective enzyme but rather with a high level of lipid peroxidation [54]. It also has been demonstrated that oxidative stress induces a  $Ca^{2+}$  overload response and inhibition of PMCA1 in rat pancreatic cells [55]. A recent study indicated that the inhibitory effect of both oxidative stress and mitochondrial inhibitors on PMCA1 activity was explained by ATP depletion due to the inhibition of mitochondria [56]. That information could suggest that PMCA1 are negatively affected by the depletion of ATP caused by oxidative stress in PE, which in consequence will increase the intracellular calcium concentration. This rise in  $Ca^{2+}$  content could affect mitochondria and contribute to the rise of apoptotic events, accentuating the ATP depletion.

### Avoiding $Ca^{2+}$ overload by the action of ER and mitochondria

Besides the action of CaBPs, the major mechanisms known to avoid excessive calcium concentrations in the cell are the buffering action of the ER and the mitochondria. It is well known that ER, a specialized calcium store organelle, is intimately involved in regulating  $Ca^{2+}$  movements within cells. IP3Rs and RyRs participate in the release of  $Ca^{2+}$  from the ER [29, 30] and we observed that the mRNA expression of IP3R1,2 (Fig. 6) and RyR1,2,3 (Fig. 7) was decreased in PE placentas. Thus, we suggest that this decrease is a consequence of elevated  $[Ca^{2+}]_i$ . In contrast, we observed that SERCA1,2,3 (Fig. 8) mRNA expression was increased in PE placenta, which functions to pump  $Ca^{2+}$  from the cytosol into the ER store. Its action, in combination with  $Ca^{2+}$  efflux mechanisms, is essential in maintaining a low resting  $[Ca^{2+}]_i$  [34]. In parallel, we also observed that the mRNA expression of VDCA3 (Fig. 9), channel implicated in mitochondrial  $Ca^{2+}$  accumulation, increased in PE placentas.

In conclusion, the important finding of this study is the alteration of the calcium transfer in PE placentas compared with normal placentas. Another important finding in this work was the successful quantification of the CaBP-28K mRNA by real-time PCR. Based on our results and recent works, we can hypothesize that oxidative stress in PE is a determinant factor affecting calcium transfer in ST. As we demonstrated, the decrease of PMCA1 expression could be attributed to the ATP depletion, probably caused by oxidative stress, and in consequence, elevates intracellular  $Ca^{2+}$  concentration, which in turn down-regulates CaBPs, TRPVs. We also propose that IP3Rs and RyRs expression decrease, while SERCAs and VDCA3 increase in order to avoid a  $Ca^{2+}$  overload. In perspectives, as placental insufficiency could lead to IUGR in 30% of the cases [2], which is associated with lower cord blood calcium concentration [57], it could be interesting to compare the

mechanisms of calcium transfer in PE placentas with or without IUGR. Working on strategies to protect placentas against reduced expression of PMCA may be very useful to weaken symptoms related to oxidative stress and intracellular calcium overload, and to enhance  $\text{Ca}^{2+}$  transfer to the foetus. By elucidating factors involved in the placenta  $\text{Ca}^{2+}$  transport in pregnancy, we will be able to determine one of the possible mechanisms responsible for foetal predisposition to adult diseases related to PE. This fundamental approach will not have an immediate impact in clinical practice, but it will help to characterize the  $\text{Ca}^{2+}$  transfer process from mother to foetus in these pregnancies and evaluate if ultimately it will be possible to improve antenatal placental transfer using nutritional intervention, pharmacological or hormonal agents. This project is the first step of a project of great

magnitude. Future studies are needed to investigate the mechanism of placental  $\text{Ca}^{2+}$  transport in PE and in several years, we shall perhaps be able to intervene to develop strategies before birth to improve foetal and neonatal health using nutritional, pharmacological or hormonal intervention.

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