Potassium channel functions in human placental syncytiotrophoblast

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ABBREVIATIONS

$[Ca^{2+}]_i$	intracellular calcium concentration
4-AP	4-aminopyridine
^{86}Rb	^{86}-rubidium
ATP	adenosine triphosphate
Ba^{2+}	barium
BK_{Ca}	large conductance calcium-activated potassium channel
BM	syncytiotrophoblast basal plasma membrane
BSA	bovine serum albumin
Ca^{2+}	calcium
Cl^-	chloride
CV	coefficient of variation
DCEBIO	5, 6-dichloro-1-ethyl-1, 3-dihydro-2H-benzimidazol-2-one
DMEM	Dulbecco's modified Earle's medium
DMSO	dimethyl sulfoxide
ELISA	enzyme-linked immunosorbent assay
E_m	membrane potential
FCS	fetal calf serum
FGR	fetal growth restriction
FITC	fluorescein isothiocyanate
GPX	glutathione peroxidase
H_2O_2	hydrogen peroxide
HBSS	Hank's balanced salt solution
hCG	human chorionic gonadotropin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IK_{Ca}	intermediate conductance calcium-activated potassium channel
K^+	potassium
K_{2P}	two-pore domain potassium channel
K_{ATP}	ATP-sensitive potassium channel
K_{Ca}	calcium-activated potassium channel
K_{IR}	inwardly rectifying potassium channel
K_V	voltage-gated potassium channel
LDH	lactate dehydrogenase
Mg^{2+}	magnesium
MVM	syncytiotrophoblast microvillous plasma membrane
Na^+	sodium
NADPH	nicotinamide adenine dinucleotide phosphate
NCS	newborn calf serum
NO	nitric oxide
NOS	nitric oxide synthase
NSCC	non-selective cation channel
O_2	molecular oxygen
O_2^-	superoxide anion
OH^-	hydroxyl anion

$ONOO^-$	peroxynitrite
PBS	phosphate-buffered saline
PE	pre-eclampsia
PFA	paraformaldehyde
PI	propidium iodide
pO_2	partial pressure of oxygen
RNS	reactive nitrogen species
ROS	reactive oxygen species
RVD	regulatory volume decrease
SIN-1	3-morpholinosydnonimine hydrochloride
SK_{Ca}	small conductance calcium-activated potassium channel
SOD	superoxide dismutase
STB	syncytiotrophoblast
TALK	TWIK-related alkaline pH-activated potassium channel
TASK	TWIK-related acid-sensitive potassium channel
TBS	tris-buffered saline
TEA	tetraethylammonium
THIK	tandem pore, halothane-inhibited potassium channel
TRAM-34	1-[(2-chlorophenyl) diphenylmethyl]-1H-pyrazole
TREK	TWIK-related potassium channel
TRESK	TWIK-related spinal cord potassium channel
TWIK	tandem pore, weak inwardly rectifying potassium channel
VGCC	voltage-gated calcium channel
XDH	xanthine dehydrogenase
XO	xanthine oxidase

ABSTRACT

The functions of the human placenta depend on the syncytiotrophoblast (STB), a highly specialised multinucleated epithelium. STB transfers nutrients to the fetus and secretes hormones that are required to support fetal development. Throughout normal pregnancy STB is renewed by cell turnover which involves proliferation, migration and fusion/differentiation of cytotrophoblasts, balanced by apoptosis/autophagy. Dysregulation of STB renewal underlies pre-eclampsia (PE), a serious pregnancy disease, but the mechanisms remain to be elucidated. Potassium (K^+) channels facilitate fusion and differentiation, cellular events critical for tissue renewal. K^+ channels are regulated by partial pressure of oxygen (pO_2), reactive oxygen (ROS) and nitrogen (RNS) species. K^+ channel function could be modulated in PE which is characterised by altered placental pO_2 and increased oxidative and nitrative stress. This thesis tested the hypotheses that K^+ channels participate in STB renewal and human chorionic gonadotropin (hCG) secretion and that dysregulation of K^+ channel function, by pO_2 and/or elevated ROS/RNS, could contribute to the abnormal STB turnover and endocrine secretion that is a feature of PE. Three aims were pursued:

1) *To determine whether pO_2 and ROS modulate STB hCG secretion through an effect on K^+ channels.* Placental villous tissue was cultured in 1%, 6% and 21% pO_2 and K^+ channel activity (^{86}-rubidium (^{86}Rb) efflux) and hCG secretion were determined. STB voltage-gated K^+ channel (K_V) activity and hCG secretion was greater in 21% than 6% pO_2 and K_V blockers reduced hCG secretion at 21% but not 6% pO_2. ROS had pO_2-dependent effects on both hCG secretion and STB K^+ channel activity but causal links between the two remain to be established. Altered pO_2 and elevated ROS in PE could modulate K_V channel activity leading to altered STB hCG secretion and compromised STB renewal;

2) *To determine whether intermediate conductance calcium-activated K^+ channels (IK_{Ca}) participate in cytotrophoblast morphological and/or biochemical differentiation.* Cytotrophoblasts were isolated from normal term placentas and studied at 15/42h (mononucleate) and 66h (multinucleate) of culture. Cytotrophoblasts expressed IK_{Ca} protein, and IK_{Ca} was activated by the opener DCEBIO, or hyposmotic cell swelling, but was normally quiescent. Chronic activation of IK_{Ca} by DCEBIO inhibited cytotrophoblast multinucleation and hCG secretion, an effect which may be mediated by altered cytotrophoblast volume homeostasis. Inappropriate activation of IK_{Ca} could compromise STB turnover and volume homeostasis in PE;

3) Having demonstrated that pharmacological activation of IK_{Ca} inhibits cytotrophoblast differentiation *in vitro*, the aim was *to determine whether IK_{Ca} is activated by nitrative stress*. Acute exposure to nitrative stress reversibly activated TRAM-34 (IK_{Ca} blocker)-sensitive ^{86}Rb efflux from cytotrophoblasts. Chronic nitrative stress inhibited cytotrophoblast hCG secretion but whether this reduction is through an effect on IK_{Ca} remains to be confirmed. This suggests that under conditions of increased nitrative stress in PE, IK_{Ca} could be activated contributing to dysregulated STB renewal.

Overall the results confirm the hypotheses that K^+ channels participate in processes of STB renewal and that their dysregulation by altered pO_2 and/or ROS/RNS could contribute to abnormal STB renewal in PE. K^+ channels play a fundamental role in regulating a wide range of cellular functions and knowledge of K^+ channels and their regulation in STB is fundamental to understand placental physiology and pathophysiology.

DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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PUBLICATIONS AND PRESENTATIONS

Publications and presentations arising from the work described in this thesis:

Manuscripts in preparation

1) **P. Díaz**, C.P. Sibley, S.L. Greenwood. Oxygen-sensitive K^+ channels modulate human chorionic gonadotropin secretion from human placental trophoblast
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1) **P. Díaz**, C.P. Sibley, S.L. Greenwood. Nitrative stress inhibits human chorionic gonadotropin (hCG) secretion by placental cytotrophoblasts. Accepted; to be published in Pediatr Res (2013)
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3) **P. Díaz**, A.M. Wood, C.P. Sibley, S.L. Greenwood. Activation of intermediate conductance calcium activated potassium channels (IK_{Ca}) impairs cytotrophoblast syncytialisation *in vitro*. Placenta (2012); 33: A31, P1.32
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5) **P. Díaz**, C.P. Sibley, S.L. Greenwood. Peroxynitrite stimulates ^{86}Rubidium efflux from cytotrophoblast cells: A role for intermediate conductance Calcium-activated Potassium channels. Placenta (2011); 32: A48, P1.73
6) **P. Díaz**, C.P. Sibley, S.L. Greenwood. Nitrative stress increases K^+ (^{86}Rb) efflux from human placental syncytiotrophoblast. Proc Physiol Soc 24 (2011); PC32
7) **P. Díaz**, C.P. Sibley, S.L. Greenwood. Syncytiotrophoblast potassium channels: possible targets of reactive nitrogen species. Reprod Sci (2011); 18. No. 4 (Supplement): 168A: O-043
8) C. Abad, D.F. Antczak, J. Carvalho, L.W. Chamley, Q. Chen, S. Daher, A.E. Damiano, V. Dantzer, **P. Díaz**, *et al*. IFPA Meeting 2010 Workshop Report I: Immunology; Ion transport; Epigenetics; Vascular reactivity; Epitheliochorial placentation; Proteomics. Placenta (2011); 32, Supplement B, Trophoblast Research, Vol. 25: S81-S89

9) **P. Díaz**, C.P. Sibley, S.L. Greenwood. Oxygen sensitive potassium channels modulate human chorionic gonadotropin (hCG) secretion in human term syncytiotrophoblast. Placenta (2010); 31: A119, P3.45

Congress communications

1) **P. Díaz**, C.P. Sibley, S.L. Greenwood (2013). A role for the intermediate conductance Ca^{2+}-activated K^+ channel (IK_{Ca}) in renewal of human placental syncytiotrophoblast. In: Institute of Human Development PGR Showcase, University of Manchester. 26th June 2013; Manchester, United Kingdom
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3) **P. Díaz**, C.P. Sibley, S.L. Greenwood (2011). Activation of syncytiotrophoblast K+ channels by reactive nitrogen species: Identification of possible targets". In: Royal Veterinary College (RVC) Young Physiologist Symposium. 31st August 2011; London, United Kingdom

Presentations and attendance to Conferences

1) Aspen/Snowmass Perinatal Biology Symposium, 24-27 August 2013, Snowmass (CO), U.S.A. Poster presentation
2) International Federation of Placenta Associations (IFPA) Meeting 2012, 18-21 September 2012, Hiroshima, Japan. Poster presentation and Workshop Oral presentation
3) Federation of European Physiological Societies (FEPS) Meeting 2012, 8-11 September 2012, Santiago de Compostela, Spain. Poster presentation
4) Federation of European Physiological Societies (FEPS) Young Investigator Symposium, 8th September 2012, Santiago de Compostela, Spain. Poster presentation
5) International Federation of Placenta Associations (IFPA) Meeting 2011, 14-17 September 2011, Geilo, Norway. Poster presentation
6) The Physiological Society: Epithelia & Membrane Transport Themed Meeting, 1-3 September 2011, London, United Kingdom. Poster presentation
7) Royal Veterinary College (RVC) Young Physiologist Symposium, 31st August 2011, London, United Kingdom. Poster presentation
8) Physiology 2011: Main Meeting of the Physiological Society, Oxford, United Kingdom, 11-14 July 2010
9) Society for Gynecological Investigation (SGI) Annual Meeting 2011, 16-19 March 2011, Miami (FL), U.S.A. Oral presentation
10) International Federation of Placenta Associations (IFPA) Meeting 2010, 19-22 October 2010, Santiago, Chile. Poster presentation and Workshop Oral presentation
11) The Physiological Society: Cardiac & Respiratory Physiology Themed Meeting, 1-3 September 2010, Birmingham, United Kingdom

12) Physiology 2010: Main Meeting of the Physiological Society, Manchester, United Kingdom, 30 June-2 July 2010

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"*Beneath the current of our existence and within it, there is another current flowing in the opposite direction. In this life we go from yesterday to tomorrow, but there we go from tomorrow to yesterday. The web of life is being woven and unravelled at the same time. And from time to time we get breaths and vapours and even mysterious murmurs from that other world, from that interior of our own world. The inner heart of history is a counter-history; it is a process which inverts the course of history. The subterranean river flows from the sea and back to its source*."

Miguel de Unamuno, Niebla (Mist)

THE AUTHOR

I graduated from the University of Chile with a BSc and professional title of Medical Technologist with a major in Histopathology and Cytodiagnosis, receiving best graduate award in 2004.

As a last year undergraduate, I started a technician position at Prof. Gloria Riquelme's laboratory (former Membrane electrophysiology laboratory, University of Chile) where I initiated and standardised histological-immunohistochemical techniques to study term human placenta through optical, fluorescence and confocal microscopy analysis.

Afterwards, I started investigating the placenta from a transport point of view being a research associate at Prof. Riquelme's lab. Our interest was focused on syncytiotrophoblast ion channels and the characterisation of the regulation and biophysical properties of ion channels in syncytiotrophoblast plasma membranes and syncytiotrophoblast membrane lipid microdomains. I received training in electrophysiology-voltage clamp technique and using purified syncytiotrophoblast apical or basal membranes transplanted into *Xenopus laevis* oocytes, I studied conductive pathways in the syncytiotrophoblast focusing on potassium channels. During this time I also gained expertise in syncytiotrophoblast membrane and microdomain isolation methods, enzyme assays for assessing purification and enrichment and Western blotting for analysing different membrane markers. In parallel, I obtained a MSc in Cell Biology (first class degree) in 2007. My thesis was entitled "Characterisation of total currents from syncytiotrophoblast apical membrane from human placenta transplanted into *Xenopus laevis* oocytes" finding that exogenous currents from a specific domain of placental syncytiotrophoblast apical membrane transplanted into *Xenopus* oocytes were mainly Na^+-sensitive tetraethylammonium (TEA)-sensitive currents. Part of this work lead to a first author paper "Barium, TEA and sodium sensitive potassium channels are present in the human placental syncytiotrophoblast", published in 2008 (Diaz, Vallejos et al. 2008). Over this period I attended and presented my work at several national and international meetings, including the Physiological Society.

In 2009, after obtaining a 4-year scholarship form CONICYT (Chilean government research funding), I started my PhD studies in the Maternal and Fetal Health Research Centre, University of Manchester, supervised by Dr. Susan Greenwood and Prof. Colin Sibley. I had an important participation in the design of my research proposal "The effects of oxidative stress on potassium channels in the term human placental syncytiotrophoblast". Since then, I have been studying the role of potassium channels in modulating syncytiotrophoblast function and studying these channels *in vitro* under pathophysiological conditions such as altered oxygenation and increased free radicals, features of pregnancy disease. During my PhD I assisted in the supervision of an undergraduate project which results has led to a manuscript to be published.

My future goals are to establish and lead an independent research group in a position that will allow me to sustain international collaboration. My future research interests are to gain understanding of syncytiotrophoblast transport function and the intracellular/extracellular mechanisms that contribute to its regulation and how does dysregulation of syncytiotrophoblast transport mechanisms contribute to pregnancy disease.

1. INTRODUCTION

1.1. Overview

The human placenta is a transient organ that serves many functions during pregnancy, being a communicating interface between mother and fetus. To achieve these functions successfully, the placenta must develop adequately throughout pregnancy.

However, in a significant number of cases, pregnancy needs to be interrupted before term, since both mother and fetus are at a high health risk; many of these cases are associated with an altered development and function of the placenta.

One of these serious diseases is pre-eclampsia (PE), which occurs in approximately 2-8% of pregnancies (1988). Although this is a multifactorial disease, it arises mainly from the maternal response to abnormal placental development (Hladunewich, Karumanchi et al. 2007; Redman and Sargent 2009).

To date, many hypotheses have tried to explain the origin of PE; one of them relates an inadequate remodelling of the maternal spiral arteries with consequent intermittent blood flow into the intervillous space leading to altered partial pressure of oxygen (pO_2) and increased oxidative-nitrative stress.

The syncytiotrophoblast (STB) is the transporting epithelium of the placenta which is continuously renewed by cellular turnover. In PE this turnover is altered, limiting nutrient transport to the fetus and necrotic material is released into the maternal blood stream triggering the maternal systemic syndrome of the clinical disease. Unfortunately, therapeutic strategies are difficult to develop because the causes and consequences of abnormal placental STB development and function in PE are still poorly understood.

In non-placental tissues, potassium (K^+) channels regulate cellular renewal, including differentiation, fusion, cellular migration and among many other processes, hormone secretion. Moreover, increasing evidence shows that K^+ channels are targets of pO_2 and oxidative-nitrative stress. This thesis focuses on the role of K^+ channels in the modulation of STB turnover events, in particular syncytialisation and endocrine secretion. In addition, this work explores the regulation of STB K^+ channels by pO_2 and oxidative-nitrative stress, important features of pregnancy disease, particularly in PE.

1.2. Placenta structure and development

1.2.1. Placenta structure at term

The human placenta at term is of the hemochorial type, implying that maternal blood is in direct contact with the trophoblast layer (Benirschke 1994). Term placenta is discoid, with a diameter of ~22cm and ~2.5cm thick, weighing an average of 500g. Macroscopically, the placenta presents two structurally and functionally independent surfaces: a maternal surface embedded within the uterine wall, characterised by the presence of various cotyledons; and a fetal surface, covered completely by the chorionic plate in contact with the amniotic liquid (Figure 1.1.A). The umbilical cord inserts into the fetal surface of the placenta and contains two arteries and one vein (Figure 1.1.B). The chorion is localised between both surfaces, immersed in the intervillous space (Benirschke 1998; Sadler 2009) (Figure 1.1.B).

1.2.2. Placenta development

The first cell lineage to differentiate in the blastocyst is the trophoblast. This is the epithelium responsible for the formation of the human placenta and fetal membranes.

The pre-implantation stage of placental development is characterised by the invasion of the uterine epithelia by the trophoblast (Huppertz 2008). This trophoblast undergoes a first differentiation step, by fusion of mononuclear cells in order to generate the first multinucleated STB (Pötgens, Schmitz et al. 2002); the remaining mononuclear inner trophoblast, now called cytotrophoblasts, remain separated from the maternal tissues (Huppertz 2008). The subsequent STB enlargement, its maintenance and turnover are achieved through the incorporation of single mononuclear trophoblast cells into the existing STB, from a subset of cytotrophoblasts which act as a stem cell (Pötgens, Schmitz et al. 2002; Huppertz 2008).

The pre-lacunar and lacunar stages of placental development are characterised by the formation of fluid filled spaces within the STB which then coalesce to form lacunas. The remaining STB between them forms trabecules. At these stages, three zones of the placenta are distinguishable: the chorionic plate facing the embryo, the lacunar system with trabecules developing into the intervillous space and the villous trees, and the primitive basal plate in contact with the maternal endometrium (Huppertz 2008) (Figure 1.1.B).

The onset of maternal blood circulation in the placental bed is initiated when cytotrophoblasts of the chorionic plate penetrate into the STB mass of the trabecules, and

reach the maternal side of the placenta. These cells will differentiate into extravillous trophoblast. A subset of extravillous trophoblast (endovascular trophoblast) reaches and invades the walls of the maternal spiral arteries, finally entering the lumen of the vessels (Huppertz 2008). When these cells penetrate, they occlude the lumen of the arteries restricting blood transfer to the placenta. This implies that placental and fetal development until week 10 of gestation occurs in a low pO_2 environment (<15mmHg; <2% pO_2) as maternal blood flow is restricted by the plugs of endovascular trophoblast (Jauniaux, Watson et al. 2000). The smooth muscle normally present in these arteries is then progressively replaced by the endovascular trophoblast with a non-contractile matrix (Figure 1.2). The physiological result of this event is a high flow, low resistance vascular conduit available to perfuse the intervillous space. At the end of the first trimester (week 10-12), it is thought that the trophoblast plugs have been displaced; consequently, the developing villous tree of the placenta becomes bathed with oxygenated maternal blood flowing continuously from the modified spiral arteries at a reduced speed rate of 10cm/s (Burton, Woods et al. 2009), raising the pO_2 to 50-60mmHg (6-8% pO_2) (Jauniaux, Watson et al. 2000).

During the villous stage of placental development, the trabecules begin to develop side branches, which are called primary villi. These now protrude into the intervillous space (previously called lacunas). Subsequently, the extraembryonic mesodermal cells of the chorionic plate follow the cytotrophoblasts and penetrate partially into the trabecules forming trophoblastic cell columns. In parallel, mesodermal cells penetrate into primary villi constituting secondary villi. Within the mesoderm of secondary villi, haematopoietic progenitor cells develop and start to differentiate transforming the respective villi into tertiary villi (Huppertz, Kaufmann et al. 2002; Huppertz 2008) (Figure 1.1.C, 1.2).

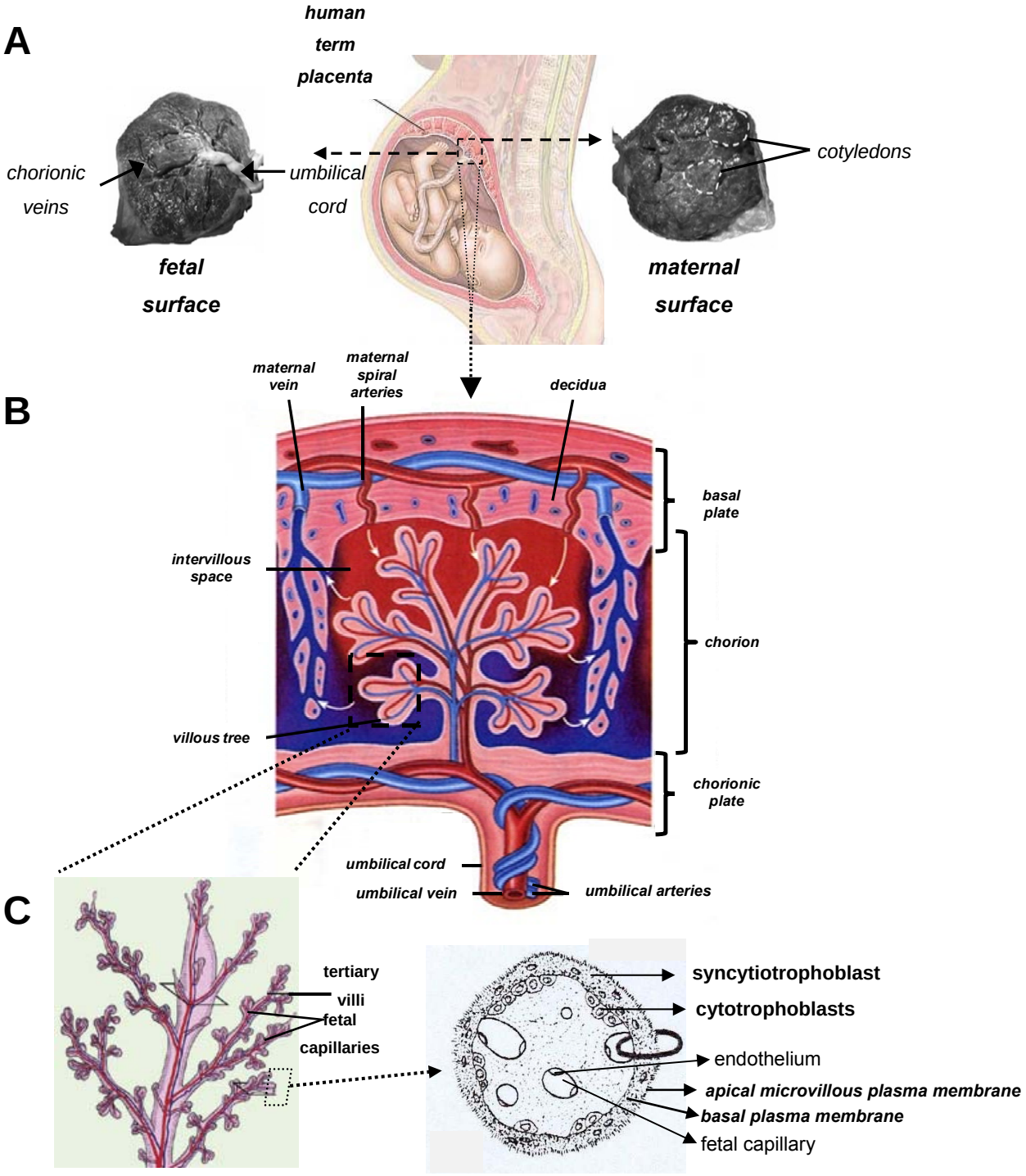

Figure 1.1: Normal human term placenta.
A: Centre: anatomical location of the human placenta at term of pregnancy. Left: fetal placental surface with umbilical cord insertion and maternal surface (right), with visible cotyledons. ***B***: Magnification of a diagrammatic section of term placenta showed in ***A***. Decidual maternal spiral arteries protrude into the intervillous space allowing maternal blood to bathe placental villous trees. The fetal umbilical vessels branch to finally submerge into the placental bed to form the villous trees. ***C***: Magnification of a schematic representation of a placental villous tree. The cross transversal section of a tertiary villous show detailed trophoblast and mesenchymal components at term pregnancy. Adapted from A.D.A.M. Medical Encyclopedia (Vorvick and Storck 2008), Huppertz (2008) (Huppertz 2008), Cornell University website (Cornell 2012), Lecuit *et al*. (Lecuit, Nelson et al. 2004) and Sadler (2000) (Sadler 2000).

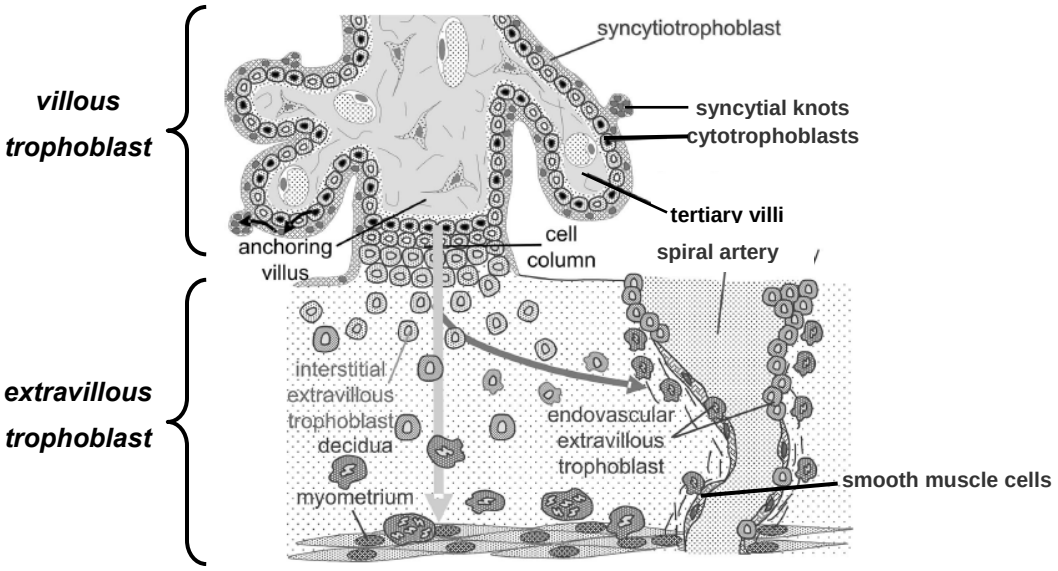

Figure 1.2: Representation of the types of trophoblast: villous and extravillous. Bottom of the diagram shows the extravillous trophoblast invading the maternal decidua. A subset of extravillous trophoblast (endovascular trophoblast) invades and replaces the existing smooth muscle from the maternal spiral arteries initiating remodelling process which will allow adequate maternal blood perfusion and oxygenation into the placental intervillous space. Villous trophoblast is showed at the top of the diagram, showing an example of tertiary villous surrounded by a monolayer of cytotrophoblasts and overlaying, the STB epithelium. Adapted from Huppertz *et al*. (2006) (Huppertz, Kadyrov et al. 2006).

1.3. Syncytiotrophoblast cellular turnover and renewal

1.3.1. Syncytiotrophoblast as an epithelium

Placental functions during pregnancy are performed by the villous STB, a highly specialised multinucleated epithelial cell layer without lateral plasma membranes, containing multiple nuclei, which surrounds the placental villi containing the fetal capillaries (Figure 1.1.C). The STB forms a physical, immunological, and endocrine interface between the maternal and fetal circulations (Huppertz, Kaufmann et al. 2002). It has an apical microvillous plasma membrane (MVM) in contact with the maternal blood and a basal plasma membrane (BM) facing the fetal capillaries (Figure 1.1.C). Due to the lack of lateral plasma membranes, STB differs from other epithelia and all solutes that are transported from the mother to the fetus and vice versa, must pass through both MVM and BM; therefore the STB constitutes the main barrier and effector for materno-fetal interchange (Shennan, Davis et al. 1986).

The STB is a polarised epithelium; thus, a variety of transporter proteins, receptors and enzymes are strategically positioned in the maternal blood facing MVM, the BM facing the villous core, or both, in order to direct and regulate materno-fetal exchange (Shennan and Boyd 1987; Challier 1989; Stulc 1997; Atkinson, Boyd et al. 2006; Cleal and Lewis 2008).

1.3.2. Syncytiotrophoblast turnover and renewal

STB has a short life span and is continuously renewed throughout pregnancy by a process of cellular turnover. One theory is that proliferative mononucleate cytotrophoblasts exit the cell cycle, differentiate and fuse with the overlying syncytial layer and obsolete nuclei are deported into the maternal blood maintaining the multinucleated STB (Huppertz 2008).

Cytotrophoblasts are reported to be mitotically active (Galton 1962; Kliman, Nestler et al. 1986). A variety of cell proliferation markers have been shown to be expressed in this trophoblast cell type (Bamberger, Sudahl et al. 1999; Genbacev, McMaster et al. 2000). After proliferation, cytotrophoblast daughter cells fuse to the overlying STB, whereas cytotrophoblast parent cells preserve their stem cell phenotype. Cytotrophoblast proliferation rate declines with the progression of pregnancy. At term, only ~15% of the villous trophoblast volume is constituted by cytotrophoblasts (Huppertz, Kaufmann et al. 2002).

Early experiments using ^{3}H-thymidine incorporation revealed that DNA synthesis does not occur within the STB; however, a more recent report shows evidence for transcriptional

activity in the STB (Ellery, Cindrova-Davies et al. 2009). Therefore, if nuclei in the STB do not replicate and transcriptional activity is in general low, the fusion of cytotrophoblasts into the STB becomes an essential mechanism in order to preserve STB function throughout pregnancy, as it enables the transfer of cytotrophoblast-derived nuclei, cellular organelles, proteins, RNA, cytoplasm and membranes into the STB (Gauster, Moser et al. 2009).

Syncytial fusion of cytotrophoblasts with the overlying STB *in vivo* is a tightly controlled event which leads to the generation of multinucleated STB. Syncytial fusion of trophoblasts is thought to consist of two plasma membranes from neighbouring cells which approach, interact with each other and dissolve in order to allow transfer of cytoplasmic content and organelles (Huppertz and Gauster 2011). It is hypothesised that the differentiation of cytotrophoblasts, which become separated from the pool of proliferative cells, is a prerequisite for fusion to occur; however, to date it is unclear whether the specific signals that trigger fusion come from cytotrophoblasts, the STB or a combination of both (Pötgens, Drewlo et al. 2004; Huppertz and Gauster 2011).

In order to maintain trophoblast homeostasis there must be a balance between the incorporation of fresh cellular components into the STB and the disposal of aged cytosolic content. Throughout the third trimester, STB nuclei are morphologically found in clusters called syncytial nuclear aggregates. These nuclei are aggregated into knots which are released from the MVM and extruded as tightly sealed corpuscular structures into the maternal circulation (Huppertz, Frank et al. 1998; Coleman, Gerza et al. 2013). However, it is also possible that these nuclear aggregates could continue to accumulate and remain in the STB until the end of pregnancy (Burton and Jones 2009). Despite the fact that it has been shown that apoptosis occurs primarily in cytotrophoblasts (Longtine, Chen et al. 2012), it still a matter of controversy whether STB nuclei undergo apoptosis or other form of degeneration (Coleman, Gerza et al. 2013).

The importance of STB turnover is highlighted by the fact that its dysregulation is evident in complications of pregnancy associated with maternal and/or fetal mortality and morbidity; particularly PE (Lim, Zhou et al. 1997; Crocker, Tansinda et al. 2004) fetal growth restriction (FGR) (Smith, Baker et al. 1997; Crocker, Tansinda et al. 2004) and maternal obesity (Higgins, Mills et al. 2012). Therefore an appropriate regulation of the events of proliferation, differentiation, fusion and apoptosis to continually renew the STB is essential for the normal progression of pregnancy.

1.3.2.1. In vitro models to study trophoblast renewal

Due to the nature of pregnancy, placental development and function have to be studied *ex vivo* and/or *in vitro* because of ethical considerations that limit experimentation *in vivo*.

Trophoblast cellular renewal has been extensively studied using *in vitro* models in health and disease: placental villous tissue maintained in explant culture (Siman, Sibley et al. 2001), cytotrophoblasts isolated from term human placenta maintained in primary culture (Kliman, Nestler et al. 1986; Greenwood, Brown et al. 1993) and choriocarcinoma cell lines (Ringler and Strauss 1990) are *in vitro* models used to study the regulation of villous trophoblast turnover and dysregulated renewal.

Placental explants/fragments consist of several villous tress as shown in Figure 1.1.C. Culture of villous explant tissue over several days represents a physiological model allowing study of chronic regulation of placental function, where the cellular architecture is maintained and all cell types and intercellular interactions are present. Previous characterisation of this model at term shows loss of the STB layer over days 0-2 of culture, followed by regeneration of a new STB by day 4, a process that resembles STB renewal throughout pregnancy (Siman, Sibley et al. 2001; Turner, Roulstone et al. 2006).

Isolated cytotrophoblasts from term placentas maintained in primary culture are mitotically inactive and show a very low rate of proliferation (~2%) (Kliman, Nestler et al. 1986). After 15-18h culture these cells are predominantly mononucleate, secreting small amounts of human chorionic gonadotropin (hCG) (detailed in section 1.3.3.1.) and at 42h they begin to migrate, aggregate and fuse (syncytialise) to become multinucleated and differentiated at 66h concomitant with a several-fold increase in hCG secretion, which is reminiscent of the STB *in vivo* (Kliman, Nestler et al. 1986; Douglas and King 1989; Douglas and King 1990). This is an established cellular model and it is illustrated from the results obtained in this thesis (Figure 1.3).

Choriocarcinoma cell lines, in particular BeWo cells, are the most extensively used model to study trophoblast fusion. These cells do not form a syncytium *in vitro*; however, they can be induced to fuse and syncytialise by the addition of cAMP analogs or factors that increase intracellular cAMP (e.g. cholera toxin or forskolin). Consequently, these cells represent a suitable model in which to study initial stages of trophoblast differentiation. Conversely, when terminally differentiated, BeWo cells differ in their gene and protein expression profile compared to normal STB (Handwerger 2010).

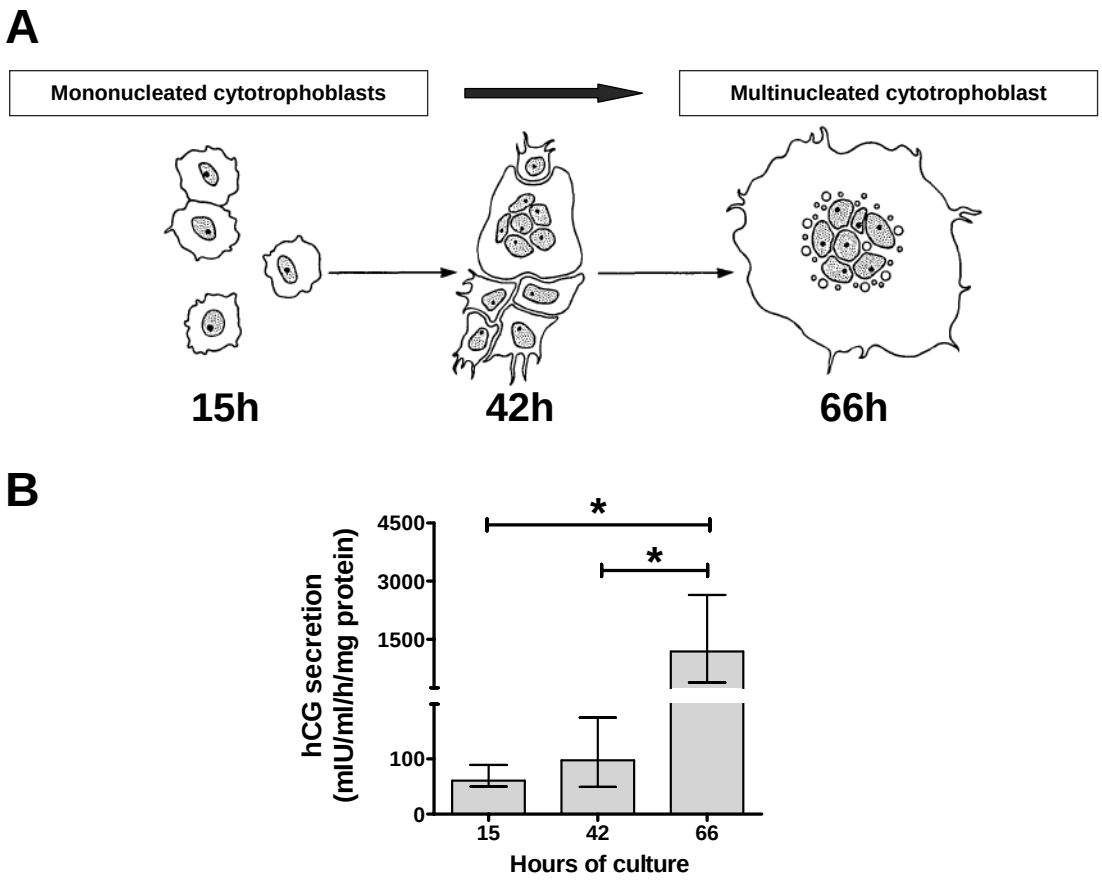

Figure 1.3: Isolated cytotrophoblasts from term placenta maintained in primary culture.

A: Isolated cytotrophoblasts from normal term pregnancy under primary culture. The cartoon illustrates morphological differentiation of cytotrophoblasts under culture from 15 to 66h. Adapted from Kliman *et al*. (1986) (Kliman, Nestler et al. 1986). ***B***: Concomitant biochemical differentiation of cytotrophoblasts under primary culture showing a 20-fold increase in β-hCG secretion (detailed in section 1.3.3.1.) from 15 to 66h; *$p<0.05$, Friedman's test with Dunn's post hoc test. Number of placentas= 16. Data are median ± interquartile range (IQR).

1.3.3. Regulation of syncytiotrophoblast renewal

STB turnover by cellular renewal is a highly co-ordinated process and regulated by several factors. Particularly, trophoblast fusion is regulated, amongst many others, by hormones, growth factors, protein kinases, transcription factors, gap junction proteins and intracellular proteases (Huppertz and Gauster 2011; Huppertz and Gauster 2011).

Placental hormones such as hCG (Shi, Lei et al. 1993; Yang, Lei et al. 2003) and human placental lactogen (hPL) (Morrish, Bhardwaj et al. 1987) increase STB formation; and hCG in particular has been widely used as a marker of trophoblast differentiation (detailed in section 1.3.3.1.). Epidermal growth factor (EGF) has been described to induce syncytialisation of cytotrophoblasts *in vitro* in parallel with the stimulation of hCG and hPL (Morrish, Bhardwaj et al. 1987). Other growth factors and cytokines which induce similar effects are transforming growth factor alpha (TGF-α) (Lysiak, Han et al. 1993; Yang, Lei et al. 2003), vascular endothelial growth factor (VEGF) (Crocker, Strachan et al. 2001), leukaemia inhibitory factor (LIF) (Yang, Lei et al. 2003), among others. On the contrary, tumor necrosis factor alpha (TNF-α) and transforming growth factor beta (TGF-β) impaired syncytium formation *in vitro* and also inhibited hCG and hPL secretion (Morrish, Bhardwaj et al. 1991; Crocker, Tansinda et al. 2004; Leisser, Saleh et al. 2006; Haider and Knofler 2009). In addition, the activation of intracellular downstream pathways promote STB differentiation, including mitogen-activated protein kinases (MAPKs) p38 and extracellular signal-regulated kinase 1/2 (ERK1/2) (Daoud, Amyot et al. 2005) and protein kinase A (PKA) (Knerr, Schubert et al. 2005), which have been shown to be positive regulators of STB formation.

As previously described, forskolin induces fusion in BeWo cells by raising cAMP intracellular concentration. Forskolin also upregulates transcription factor glial cell missing 1 (GCM1) (Knerr, Schubert et al. 2005). Interestingly, the expression of GCM1 is restricted to differentiated cytotrophoblasts (Baczyk, Satkunaratnam et al. 2004), the subpopulation of trophoblasts which fuse to the STB and one target of GCM1 is reported to be fusogenic protein syncytin 1 (Yu, Shen et al. 2002).

Membrane proteins have been shown to be important for STB formation. In particular, syncytin 1 (Mi, Lee et al. 2000) and syncytin 2 (Lee, Keith et al. 2001) are endogenous retroviral envelope fusogenic proteins encoded by HERV-W and HERV-FRD genes respectively, which are the only trophoblasts fusogens described and main players in syncytial fusion (Mi, Lee et al. 2000; Frendo, Olivier et al. 2003; Vargas, Moreau et al. 2009). Syncytin 1 is highly expressed in the STB and cytotrophoblasts (Blond, Lavillette et al. 2000; Mi, Lee et al. 2000; Lee, Keith et al. 2001; Yu, Shen et al. 2002), whereas syncytin 2 expression is located to cytotrophoblasts (Malassine, Blaise et al. 2007;

Esnault, Priet et al. 2008). Syncytin 1 induces syncytium formation (syncytialisation) by interacting with the D-type mammalian retrovirus receptor, a neutral amino acid transporter (ASCT2) (Blond, Lavillette et al. 2000; Chen, Wang et al. 2006). The role of syncytins as fusogenic proteins has been partly demonstrated by using antisense oligonucleotide to syncytin 1 which impaired syncytium formation and reduced hCG expression (Frendo, Olivier et al. 2003), however, their role as fusogens in trophoblast fusion is still unclear and needs to be further elucidated.

CD98, another membrane protein which is involved in amino acid transport, is also relevant for trophoblast fusion. Knockdown of CD98 expression partially inhibits forskolin-stimulated cell fusion and suppresses hCG secretion and system L-amino acid transport in BeWo cells (Kudo, Boyd et al. 2003).

Connexins are a family of gap junction proteins involved in trophoblast fusion, and particularly connexin 43 is expressed in cytotrophoblasts and STB. Cytotrophoblasts treated *in vitro* with connexin 43 antisense showed impaired fusion (Frendo, Cronier et al. 2003).

The coordination of trophoblast fusion events is essential to control STB renewal. Indeed, its dysregulation has been reported to be involved with the pathophysiology of pregnancy complications such as PE (Huppertz, Kaufmann et al. 2002; Huppertz and Kingdom 2004).

In addition, other regulatory factors are important for STB renewal as they acquire a pathophysiological relevance when altered in pregnancy disease by contributing to dysregulated STB turnover. These include pO_2 (Alsat, Wyplosz et al. 1996; Huppertz, Kingdom et al. 2003; Kudo, Boyd et al. 2003; Crocker, Tansinda et al. 2004; Heazell, Moll et al. 2007; Heazell, Taylor et al. 2009) and free radicals (Heazell, Moll et al. 2007; Kharfi Aris, Leblanc et al. 2007; Moll, Jones et al. 2007; Heazell, Taylor et al. 2009) (detailed in sections 1.3.3.2. and 1.5. respectively).

1.3.3.1. Human chorionic gonadotropin

The primary function of hCG during the first trimester of pregnancy is to promote the maintenance of progesterone production by the corpus luteum (Cole 2010; Cole 2012).

At term hCG is synthesised and secreted by the STB and is one of the most important endocrine factors regulating STB turnover (Yang, Lei et al. 2003). hCG is a glycoprotein hormone with a heterodimeric structure, with two subunits α and β in α1β1 stoichiometry. The α-subunit is common to all glycoprotein hormones (i.e. luteinizing hormone, follicle stimulating hormone and thyroid stimulating hormone); in contrast, the β-subunit confers biological specificity. α-hCG is produced in excess compared to β-hCG; therefore, the β-subunit is the limiting step in the production of a functional hCG dimer (Daniels-McQueen,

McWillians et al. 1978; Hussa 1980) which is synthesised only by differentiated STB in normal term pregnancy.

The highest rate of hCG secretion into the maternal circulation occurs during the first trimester of pregnancy (Yoshida 2005) in a hypoxic environment (~2.5% pO_2) before 10 weeks of gestation, and falls during the second and third trimester. Although this peak in maternal serum concentration of hCG occurs when there is limited oxygenation in the intervillous space (Jauniaux, Watson et al. 2000), it has been proposed that low pO_2 promotes oxidising conditions which facilitate assembly of hCG subunits *in vitro* (Xing, Williams et al. 2001).

At present, two specific β-hCG subunits been described: type 1 and 2. Recent evidence indicates that both types 1 and 2 β-hCG RNA expression is elevated in hypoxia. Therefore, this also might contribute to the high levels of hCG found in maternal serum during first trimester compared to term pregnancy (Cocquebert, Berndt et al. 2012).

During the second and third trimester of pregnancy, the STB continues to synthesise and secrete hCG which acts locally (Shi, Lei et al. 1993). Cytotrophoblasts and the STB express hCG receptors and hCG produced by STB has autocrine/paracrine effects on cytotrophoblasts to promote their differentiation and fusion, thereby supporting continued STB renewal (Shi, Lei et al. 1993). As a result, hCG secretion is commonly used as an endocrine marker for trophoblast differentiation/syncytialisation.

hCG at term can be secreted by a constitutive process (Yoshida 2005) and by calcium (Ca^{2+})-dependent exocytosis (Meuris, Polliotti et al. 1994). Therefore, the regulated component of hCG secretion is modulated by factors that influence intracellular Ca^{2+}, including ion channels (Long and Clarson 2002; Williams, Fyfe et al. 2008) (detailed in section 1.7.1.1.). In addition to regulation by hormones (e.g. gonadotropin-releasing hormone (Lin, Roberts et al. 1995)), hCG secretion is also modulated by pO_2 (section 1.3.3.2.) and free radicals (section 1.5.1.1.).

In addition, there is a glycosylated variant of hCG (hyperglycosylated hCG), which differs from regular hCG in being secreted by stem cytotrophoblasts and not differentiated STB. Hyperglycosylated hCG has been linked with trophoblast invasion (implantation) and malignancy (choriocarcinoma) (Cole and Khanlian 2007).

1.3.3.2. Partial pressure of oxygen

pO_2 is one of the important physicochemical factors regulating trophoblast function and differentiation.

As previously described, placental and fetal development until the end of the first trimester occurs in a low pO_2 (Jauniaux, Watson et al. 1999). At week 10-12 of gestation, perfusion

of the placental bed by maternal blood is gradually established and the placenta faces an increase in pO_2, rising to 50-60mmHg (6-8% pO_2) (Jauniaux, Watson et al. 2000).

Amongst different mechanisms, the placenta normally adapts to these changes in pO_2 by modulation of hypoxia-inducible factor (HIF-1), a transcription factor (Majmundar, Wong et al. 2010), and by increasing mRNA concentration and activities of antioxidant defences (Carter 2000; Jauniaux, Watson et al. 2000) which will become available to scavenge an increase in the generation of free radicals.

It is thought that maternal oxygenated blood entering the intervillous space through spiral arteries is initially ~11% pO_2 (80-100mmHg) (Burton and Caniggia 2001) which varies as it circulates towards the periphery of the placenta and mixes with deoxygenated blood before returning to the endometrial veins (45mmHg); however, in areas of poor blood flow (periphery) a pO_2 of 35-40mmHg is expected. This implies that a gradient of 40-50mmHg could be found across a placental lobule (Burton and Caniggia 2001). Indeed, umbilical vein measurements during late pregnancy show an average pO_2 of 40 mmHg at 40 weeks (5-6% pO_2) (Soothill, Nicolaides et al. 1986; Burton and Caniggia 2001; Lackman, Capewell et al. 2001).

Human placentation studies to specifically assess the effects of pO_2 or altered pO_2 have used *in vitro* models. However, a difficulty encountered when using *in vitro* models is to match the physiological or pathophysiological pO_2 found *in vivo*. This is still a matter of controversy (Burton, Charnock-Jones et al. 2006), and it has been suggested villous explants after 12 weeks of gestation should be maintained at ~8% pO_2 in order to assess physiological normoxia (Miller, Genbacev et al. 2005). Overall, between 5-8% pO_2 is used at present to mimic physiological pO_2 for third trimester placentas from uncomplicated pregnancies (Tuuli, Longtine et al. 2011; Chen, Longtine et al. 2013). In addition, studies focused on comparisons between the effects of exposing trophoblast *in vitro* models to conditions of potential pO_2 stress, have used pO_2 ranging from 1% to 21% pO_2.

pO_2 has diverse effects on trophoblast turnover events *in vitro*. In short term cultured explants (24h) hypoxia (1% pO_2), hyperoxia (21% pO_2) and hypoxia-reoxygenation activate pathways and mechanisms involved in trophoblast metabolism and cell death (Reti, Lappas et al. 2007).

Syncytial fusion has also been shown to be affected by pO_2, as hypoxia (1% pO_2) downregulates placental syncytin expression in BeWo cells and in *ex vivo* perfused placental cotyledons (Knerr, Weigel et al. 2003). In agreement, forskolin-induced BeWo cell fusion was suppressed at 2% pO_2 compared to 20% pO_2, concomitant with reduced hCG secretion (Kudo, Boyd et al. 2003). In addition, syncytin receptor expression, the amino acid transporter ASCT2, is also downregulated by hypoxia (Kudo, Boyd et al. 2003) indicating low pO_2 impairs BeWo cell syncytialisation. On the contrary, recent evidence

suggests cytotrophoblast fusion *in vitro*, measured as syncytin 1 mRNA expression, is not affected by low pO_2 (2.5% pO_2) (Depoix, Barret et al. 2013).

Term villous explants cultured for 4 days and exposed to 1% pO_2, exhibit a reduction in cytotrophoblast proliferation and enhanced apoptosis and necrotic cell death compared to normoxia (6% pO_2) and 20% pO_2. Moreover, the expression of apoptosis regulators such as p53 was increased in hypoxia (Heazell, Lacey et al. 2008).

As described earlier, STB contains nuclear aggregates termed syncytial knots that are hypothesised to be shed into the maternal blood. Phenotypic changes of placental villi cultured for 4 days in response to hypoxia (1% pO_2) and hyperoxia (20% pO_2) *in vitro* showed an increased formation of syncytial knots, which was unaffected in normoxia (6% pO_2) (Heazell, Moll et al. 2007).

The role of pO_2 in regulating hCG secretion has been demonstrated in term placental villous explants of normal placentas maintained up to 11 days (Crocker, Tansinda et al. 2004) and in cytotrophoblasts in primary culture (Alsat, Wyplosz et al. 1996), where reducing pO_2 inhibits hCG secretion. In addition, decreased hCG secretion in villous explants at low pO_2 was associated with reduced cytotrophoblast proliferation at 17% pO_2 compared to hypoxia (3% pO_2) together with increased cell death and elevated caspase 3, marker of apoptosis (Crocker, Tansinda et al. 2004).

These data highlight the importance of pO_2 for the regulation of trophoblast turnover. Indeed, altered placental pO_2 and dysregulated trophoblast renewal are features observed in common pregnancy pathologies such as PE (detailed in section 1.5.).

1.4. Free radicals and placenta

1.4.1. What are free radicals?

Eukaryotic cells utilise molecular oxygen (O_2) in order to produce adenosine triphosphate (ATP) as an energy source. As a result of aerobic metabolism, free radicals are produced. These are chemical species with one or more unpaired electrons and therefore, highly reactive and unstable. They can accept or donate electrons to assume a more stable state (Burton 2009).

In biological systems the most important sources of free radicals are O_2 and nitrogen (Burton and Jauniaux 2011). Superoxide (O_2^-), hydroxyl anions (OH^-) and their non radical intermediates such as hydrogen peroxide (H_2O_2) are examples of O_2 free radicals known as reactive oxygen species (ROS) (Burton and Jauniaux 2011). Nitric oxide (NO) metabolism gives origin to a further series of compounds, collectively known as reactive nitrogen species (RNS) (Patel, McAndrew et al. 1999). The terms oxidative and nitrative

(or nitrosative) stress are used to describe the cellular injury produced by ROS or RNS respectively; i.e. O_2^-, OH^-, H_2O_2 produce oxidative stress and NO-derived metabolites such as peroxynitrite ($ONOO^-$), and $ONOO^-$-derived compounds: nitrogen dioxide (NO_2) and carbonate (CO_3^-) producing nitrative stress (Lancaster 2006).

The effects of ROS and RNS are limited by their diffusion distance; they can interact with biomolecules present in their immediate vicinity i.e. cell membrane, enzyme systems and DNA. At physiological concentrations, free radicals regulate a variety of cell functions, particularly transcription factors (Burton, Hempstock et al. 2003).

Figure 1.4 shows the main pathways for the generation of free radicals (ROS and RNS).

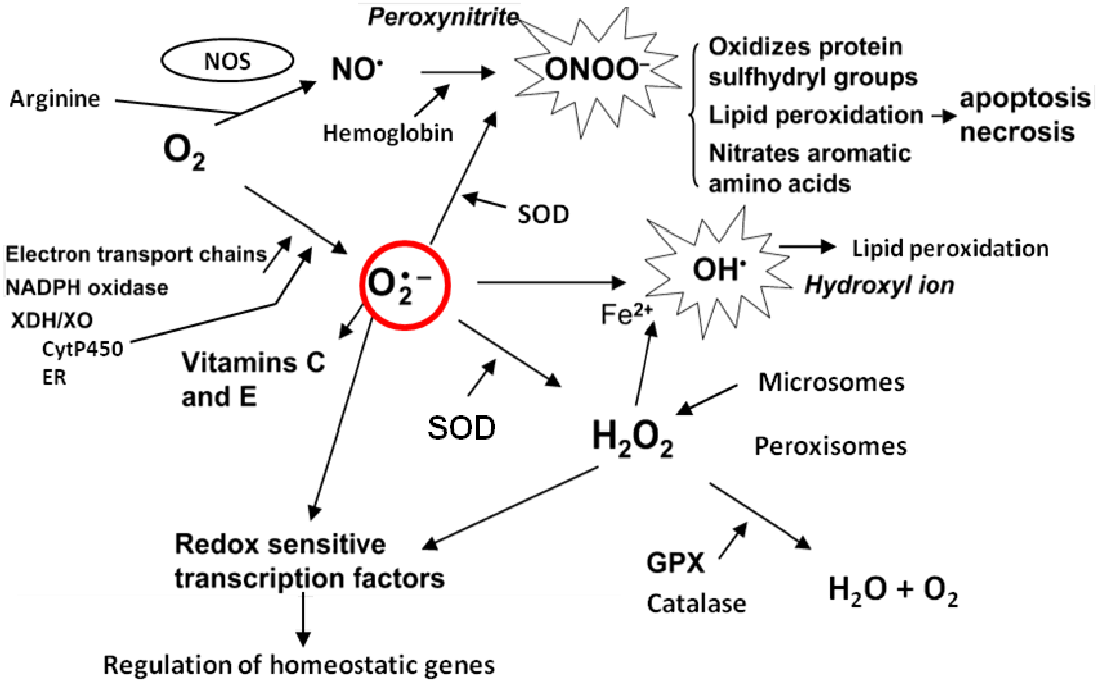

Figure 1.4: Diagrammatic representation of the main pathways for generation of ROS, RNS and antioxidant defences.
NOS: nitric oxide synthase; O_2^-: superoxide; OH^-: hydroxyl anion; H_2O_2: hydrogen peroxide; $ONOO^-$: peroxynitrite; NADPH: nicotinamide adenine dinucleotide phosphate; XDH: xanthine dehydrogenase; XO: xanthine oxidase; GPX: glutathione peroxidase. Adapted from Burton (2009) (Burton 2009).

Under physiological conditions, the main source of free radicals, and particularly O_2^-, is the mitochondria electron transport chain; however, many cytosolic processes also generate free radicals such as the endoplasmic reticulum shorter electron transport chain, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, cytochrome P450 and other oxido-reductases (Figure 1.4).

The rate of formation of O_2^- depends on the number of electrons present in the mitochondria; therefore, it is elevated under conditions of hyperoxia. Conversely, it is also increased under hypoxia, as electrons accumulate because there is low O_2 available to act as a final acceptor (Burton and Jauniaux 2011). O_2^- is the most abundant free radical formed as the first step in one-electron reduction of O_2, and itself has a short half-life with a diffusion distance of 0.4μm in one half-life (Myatt 2010). This limits O_2^- actions to be localised within the cell of synthesis. In addition, because of its negative charge, O_2^- is membrane impermeable and mainly remains in the mitochondrial matrix; however, much of the toxicity of O_2^- arises from synthesis of the more reactive OH^- and H_2O_2 (Myatt and Cui 2004; Burton 2009) (Figure 1.4).

Xanthine dehydrogenase (XDH) is an important donor of O_2^- under pathophysiological conditions. XDH degrades purines, xanthine and hypoxanthine to uric acid and, under normal conditions, uses nicotinamide adenine dinucleotide (NAD^+) as electron recipient. However, under hypoxia, XDH is proteolytically cleaved to an pO_2-dependent oxidase form (xanthine oxidase; XO), which donates electrons to O_2, promoting O_2^- formation (Burton and Jauniaux 2011) (Figure 1.4).

H_2O_2, a non-radical intermediate, is produced in microsomes and peroxisomes (Cindrova-Davies 2009). H_2O_2 is non-polar and therefore able to diffuse into cells and organelle membranes acting as a second messenger in signal transduction pathways. H_2O_2 reacts with free ferrous ions to generate OH^- through the “Fenton reaction” (Figure 1.4). OH^- is a highly reactive free radical which can react with any surrounding molecule in a diffusion-limited manner and has a half-life of 10^{-9}s (Burton and Jauniaux 2011). Polyunsaturated fatty acids are prone to the effects of OH^- and as a result generate isoprostanes and lipid peroxides (Radi 2004). Lipid peroxidation products affect cellular function by changing membrane fluidity, permeability and cell membrane potential (Ohta, Mohri et al. 1989).

NO, formed from arginine and O_2 by nitric oxide synthase (NOS), has a number of physiological functions, e.g. mediating endothelium-dependent vasodilatation, anticoagulant factor among others (Matsubara, Matsubara et al. 2010). The diffusion distance of NO is 100μm; this allows NO to act in a paracrine manner, affecting cells in the vicinity (Myatt 2010). Importantly, NO can react with O_2^- in a non-enzymatic reaction to form $ONOO^-$, a potentially very strong long lived (pro) oxidant, capable of a diffusion distance of 5μm, which is able to cross cell membranes and therefore affects surrounding

target cells (Pacher, Beckman et al. 2007; Szabo, Ischiropoulos et al. 2007) (Figure 1.4). The location within a cell where $ONOO^-$ is formed is associated with the sources of O_2^- (plasma membrane NADPH oxidase and mitochondrial electron transport chain) (Szabo, Ischiropoulos et al. 2007). Experimentally, it is possible to continuously release $ONOO^-$ *in vitro* by using 3-morpholinosydnonimine hydrochloride (SIN-1); SIN-1 releases O_2^- and NO in an aqueous solution in a 1:1 stoichiometry, which then rapidly react to form $ONOO^-$ (Feelisch, Ostrowski et al. 1989; Hogg, Darley-Usmar et al. 1992).

On the other hand, when tissues are undergoing an inflammatory stimuli, sepsis or ischemia-reperfusion injury amongst other conditions, they are induced to produce simultaneously NO and O_2^-, thereby increasing $ONOO^-$ levels (Myatt and Cui 2004). The rate constant for formation of $ONOO^-$ is faster compared to the rate of formation of O_2^- ($6.7x10^9$ versus $2x10^9$ $M^{-1}s^{-1}$ respectively (Myatt, Eis et al. 1997)). Consequently, in a situation where NO is being produced at a high concentration, it will compete with superoxide dismutase (SOD; O_2^- scavenger) for available O_2^-. If in addition O_2^- generation increases, this will therefore favour the formation of $ONOO^-$ over the removal of O_2^- by SOD (Myatt, Eis et al. 1997).

$ONOO^-$ and $ONOO^-$-derived radicals can alter cell function affecting different biomolecules: DNA (oxidation), unsaturated fatty-acid-containing phospholipids (peroxidation) and proteins through the oxidation of thiol groups (in cysteine residues) and the nitration of aromatic amino acids (tyrosine) to produce 3-nitrotyrosine (Patel, McAndrew et al. 1999; Radi 2004; Szabo, Ischiropoulos et al. 2007; Burton 2009).

Site-specific nitration focused on particular tyrosines residues may result in modification of protein function and promote a biological effect (Radi 2004). Nitrotyrosine production can be indirectly localised by the presence of nitrotyrosine residues (Beckmann, Ye et al. 1994). Protein nitration has been recently described as a selective and reversible physiological event. Many proteins show a loss of function when nitrated (MacMillan-Crow, Crow et al. 1998; Zou, Leist et al. 1999), whereas others show a gain (Landino, Crews et al. 1996; Szabo, Zingarelli et al. 1996). Nevertheless, the occurrence of protein tyrosine nitration under disease conditions represents a shift from the signal transducing physiological actions of NO to oxidative and potentially pathogenic pathways.

1.4.1.1. Antioxidants

Under physiological conditions, the oxidant effects of free radicals are counteracted by antioxidant defences. Antioxidants are enzymatic, e.g. SOD, peroxidase and catalase; non enzymatic such as ascorbate (vitamin C) and vitamin E (as lipophilic α-tocopherol);

thiol compounds (thioredoxin); metal binding proteins such as transferrin, lactoferrin, ceruloplasmin (Halliwell 1996; Burton and Jauniaux 2011).

O_2^- is removed by manganese (Mn) (if in mitochondria) or copper/zinc (Cu/Zn) (if in cytosol) SOD, which converts it to H_2O_2. H_2O_2 is detoxified to form H_2O by the enzymes catalase and glutathione peroxidase (GPX) (Cindrova-Davies 2009) (Figure 1.4). GPX activity depends on the availability of glutathione, the main cellular thiol redox buffer, synthesised in the cytosol. The effects of NO can be counteracted by hemoglobin (Khullar, Greenwood et al. 2004) (Figure 1.4). Conversely, to date, there is no known scavenger for OH^- (Burton and Jauniaux 2011).

At physiological levels antioxidant defences are sufficient to prevent an oxidative attack from free radicals. However, in disease states, an enhanced production of free radicals and/or reduced antioxidants leads to functional abnormalities.

1.4.1.2. Oxidative and nitrative stress

Oxidative-nitrative stress is described as an imbalance in the production of free radicals (ROS/RNS) and the ability of antioxidant defences to scavenge them, which can lead to tissue injury. It can arise from increased production of free radicals and/or a decrease in antioxidant capacity (Myatt and Cui 2004).

Despite the fact that free radicals play an important role as secondary messengers in several signalling pathways, increased production of free radicals also exerts a critical role in the genesis of different diseases including vascular dysfunction e.g. diabetes mellitus, hypertension, atherosclerotic coronary artery disease and in particular, in pregnancy-related diseases such as PE (Webster, Roberts et al. 2008; Roberts, Smith et al. 2009) (detailed in section 1.5.).

1.4.2. Free radicals in placenta

The amount and/or type of ROS/RNS generated within the placenta, and their effects, will be directly influenced by placental pO_2. In fact, in normal pregnancy there is a dramatic increase in the level of ROS/RNS when the uteroplacental blood flow is established by the end of the first trimester, correlating with an increased pO_2 (Jauniaux, Hempstock et al. 2003).

To date, the pathways leading to the production of free radicals in the placenta are the mitochondrial transport chain, XO, NADPH oxidase, NOS and heme oxygenase (Myatt and Cui 2004).

The most common ROS in the placenta is O_2^- (Hung and Burton 2006). The participation of the mitochondrial transport chain in the formation of O_2^- is not clear during early

pregnancy; however, it has been reported that the mitochondrial mass in the placenta increases with gestational age, suggesting an increased mitochondrial contribution to the generation of free radicals throughout pregnancy (Wang and Walsh 1998).

As previously described, another contributor to the generation of O_2^- is XO. In pathophysiological conditions, such as ischaemia-reperfusion injury, the formation of this pO_2-dependent enzyme is promoted. Indeed, *in vitro* exposure of normal placental tissues to hypoxia followed by a period of reperfusion, showed to increase trophoblast nitrotyrosine staining (Hung, Skepper et al. 2002). It was initially described that XO expression was low during first trimester compared to third trimester pregnancies (Many, Westerhausen-Larson et al. 1996; Many, Hubel et al. 2000); however, more recent evidence suggests a greater XO activity during early weeks of gestation (Poston and Raijmakers 2004). During labour the placenta is also exposed to repeated episodes of ischaemia-reperfusion, thereby increasing oxidative stress and elevated XO activity has been associated with this process (Many and Roberts 1997).

NADPH oxidase, another ROS-generating system, has been shown to produce ROS throughout gestation. In placentas from uncomplicated pregnancies, NADPH oxidase activity was initially located to the STB MVM (Matsubara and Sato 2001). More recent evidence showed the generation of O_2^- by NADPH oxidase by placental homogenates from uncomplicated pregnancies (Poston and Raijmakers 2004).

NO is commonly known as an active vasodilator in the fetal placental vasculature (Myatt, Brewer et al. 1991) and its activity is limited by the interaction with O_2^- (Moncada, Palmer et al. 1991). The endothelial NOS (eNOS) isoform was first shown to be localised in villous vascular endothelium and the STB (Eis, Brockman et al. 1995). Moreover, eNOS expression increases with differentiation from cytotrophoblasts to STB (Eis, Brockman et al. 1995).

Under conditions where there is increased generation of NO and O_2^-, they react together to form $ONOO^-$, which has a diffusion distance of 5μm, equivalent to the thickness of the trophoblast (Myatt 2010). An increased formation of $ONOO^-$ is reported in PE (detailed in section 1.5.).

Carbon monoxide is a vasodilator in the placenta (Lyall, Barber et al. 2000) synthesised by the enzyme heme oxygenase. Heme oxygenase-1 isoform is found in small amounts within the placenta (Lyall, Barber et al. 2000; McLean, Bowman et al. 2000; Yoshiki, Kubota et al. 2000; Barber, Robson et al. 2001), whereas heme oxygenase-2 is present in the vascular endothelium and villous/extravillous trophoblast (Barber, Robson et al. 2001).

1.4.3. Antioxidants in placenta

During first trimester in pregnancy, before the uteroplacental blood flow is established in the intervillous space, there are low concentrations and activities of antioxidant enzymes within the placenta (Jauniaux, Hempstock et al. 2003).

According to the degree of oxygenation and the direction of the maternal blood flow from the spiral arteries, there are regional differences in the expression of antioxidant enzymes in the placenta. The central region of the placental maternal face is well-oxygenated compared to the periphery (Hempstock, Bao et al. 2003). On the other hand, the central villi are morphologically and enzymatically immature compared to peripheral villi. Activity and mRNA expression of catalase and GPX are higher in central than in peripheral villi, but there is no difference in total SOD (Hempstock, Bao et al. 2003). However at term, all the major antioxidant systems including Mn and Cu/ZnSOD, catalase, GPX, glutathione and vitamins C and E are present in the placenta (Myatt, Eis et al. 1997; Myatt and Cui 2004).

The two existing SOD isoforms show a cell-specific expression pattern in the placenta (Myatt and Cui 2004) and their expression has been reported to increase throughout gestation (Takehara, Yoshioka et al. 1990; Miyagami, Koide et al. 2013). Mitochondrial MnSOD isoform is faintly expressed in STB but very intensely in fetal vascular endothelium whereas cytosolic Cu/ZnSOD isoform is intensely expressed in the villous stroma (Myatt, Eis et al. 1997). It has been suggested a differential physiological role for both SOD isoforms. MnSOD scavenges O_2^- and prevents its deleterious effects in placental vasculature but also could control the half-life of endothelium-derived NO (by regulating the levels of O_2^-) (Holcberg, Kossenjans et al. 1995); whereas Cu/ZnSOD in villous stroma is thought to be important for scavenging the O_2^- generated by fetal macrophages (Myatt, Eis et al. 1997). In addition, little or no SOD expression has been found in trophoblast; conversely, a transient expression of MnSOD has been associated with cytotrophoblast differentiation *in vitro* (Church, Farmer et al. 1992).

1.4.4. Oxidative and nitrative stress in placenta

Term pregnancy per se is a state of oxidative stress (Wisdom, Wilson et al. 1991) arising from the increased metabolic activity in placental mitochondria and reduced antioxidant scavenging power (Myatt and Cui 2004; Roberts, Smith et al. 2009). Serum and urine levels of oxidative-nitrative stress related molecules, such as lipid peroxides and malondialdehyde, markers of oxidative stress formed by the degradation of polyunsaturated lipids, and 8-hydroxydeoxyguanosine (8-OHdG), a marker of oxidised DNA bases, have been found to be higher in pregnant women than in non-pregnant

women (Morris, Gopaul et al. 1998; Hung, Lo et al. 2010). In addition, there is a significant increase in the expression and activities of different antioxidant enzymes, especially towards the end of pregnancy, compared to non-pregnant women (Hung, Lo et al. 2010).

Angiographic studies of the uterine vasculature of the *Rhesus* monkey have shown that blood flow from the spiral arteries into the intervillous space is intermittent in normal pregnancies (Martin, McGaughey et al. 1964) explaining the baseline level of oxidative-nitrative stress seen in normal placentas. pO_2 in the intervillous space will therefore fluctuate on a regional basis, although overall oxygenation to the fetus is preserved, allowing normal growth (Hung and Burton 2006).

1.5. Free radicals, partial pressure of oxygen and placental disease

During the first trimester of pregnancy, between 8-10 weeks, intervillous pO_2 is 2-3 times lower than after 12 weeks of gestation (Jauniaux, Watson et al. 2000; Jauniaux, Watson et al. 2001). Indeed, placental hypoxia protects the developing fetus from potential deleterious and teratogenic effects of free radicals (Jauniaux, Poston et al. 2006). However, after trophoblast invasion and transformation of the spiral arteries by the end of the first trimester, pO_2 in the intervillous space raises and consequently there is a burst of oxidative-nitrative stress (Jauniaux, Watson et al. 2000). These changes are also associated with increased mRNA concentration and activities of antioxidant enzymes in normal pregnancy (Jauniaux, Watson et al. 2000).

These controlled changes would normally stimulate remodelling and normal placental functions such as nutrient transport and hormone synthesis (Carter 2000; Schneider 2000), but also they can constitute a factor in the pathogenesis of pregnancy diseases particularly in PE but also FGR and early pregnancy failure (Jauniaux, Watson et al. 2000). It is thought that these pathologies arise from a maladaptation to a changing pO_2 in the placental environment (Jauniaux, Poston et al. 2006).

At present, there are different hypotheses to explain how oxidative-nitrative stress abnormally arises in PE.

The first is that this stress is secondary to reduced uteroplacental arterial flow, which is less than it should be, implicating that the placenta is chronically hypoxic (Burton 2009) and indeed, the expression of different markers in PE suggest a prevailing hypoxic environment (Burton and Caniggia 2001). However at present there is no direct measurement of intraplacental pO_2 to support this idea. Conversely, in pregnancies at high altitude, where environmental pO_2 decreases to 53mmHg compared to 106mmHg at

the sea level (Espinoza, Sebire et al. 2001), placentas are reported to be healthy and do not show any morphological signs of PE (Zamudio 2003). Under these conditions, the activities of antioxidant enzymes decrease but this is not associated with increased oxidative stress, as markers of oxidative stress are in fact lower (Zamudio, Kovalenko et al. 2007). This evidence suggests that the placenta is able to adapt to chronic low pO_2.

The second and most accepted view to explain how PE arises is the hypoxia-reoxygenation injury model, which is related to fluctuations in pO_2 due to intermittent maternal blood flow rather than hypoxia alone (Burton and Hung 2003; Hung and Burton 2006). It has been described that the origin could be an incomplete or absent conversion of the spiral arteries which retain smooth muscle cells within their walls, so that some vasoreactivity persists in 30-50% of the placental vascular bed; consequently, placental perfusion is not only reduced but more importantly, spiral arteries maintain their contractile properties leading to an intermittent blood flow (Hung, Skepper et al. 2001; Hung and Burton 2006). If blood flow from the spiral arteries is intermittent, this will affect O_2 delivery to the placenta. Normally, each spiral artery delivers oxygenated blood into the centre of each placental lobule and then blood drains peripherally into the villi; however, when there is a transient reduction in blood flow, peripheral arteries are not able to compensate this event and as a result, there will be a fraction of time where reduced or absent arterial inflow will lead to low pO_2 within the affected lobule. However, O_2 will continue to be extracted from the intervillous space by the trophoblast. When the maternal arterial inflow is restored to the lobule (reperfusion), the local pO_2 will raise sharply. These fluctuations in pO_2 could provide the basis for the hypoxia-reoxygenation injury and as a consequence, lead to the generation and accumulation of high concentrations of free radicals (Hung and Burton 2006).

To date no *in vivo* measurements have been performed in the placental intervillous space in PE or FGR. However, there is theoretical evidence suggesting that the placenta could be exposed to hyperoxia (Kingdom and Kaufmann 1997) in FGR as in fetal hypoxia, the fetus fails to extract O_2 through the feto-placental blood circulation, therefore the intervillous space pO_2 could be higher and reach a pO_2 similar to the maternal spiral arteries. Recently, Huppertz *et al*. (2013) (Huppertz, Weiss et al. 2013) reviewed previous evidence which support higher values of intra-placental pO_2 in the particular case of FGR instead of hypotheses that support hypoxia. These correspond to uterine vein pO_2 measurements during Caesarean sections used as a measure of intervillous pO_2, which have been previously obtained and showed to be higher compared to controls at the same gestational age (Sibley, Pardi et al. 2002); and, in addition, measurements of the concentration of oxygenated hemoglobin which showed to be increased in FGR compared to normal fetuses (Kawamura, Kakogawa et al. 2007; Kakogawa, Sumimoto et al. 2010).

However, to date, this hypothesis supporting a hyperoxic environment in the placenta in disease is still contentious.

In the specific case of PE, it is thought that oxidative stress is the key intermediate step which leads to the release of pro-inflammatory cytokines and/or angiogenic factors that cause activation of the maternal endothelial cells (Roberts and Hubel 1999; Redman and Sargent 2005). Indeed, inducing hypoxia-reoxygenation *in vitro* in placental villous explants induces elevated secretion of pro-inflammatory cytokines (Cindrova-Davies, Spasic-Boskovic et al. 2007). It is also necessary to consider that a reduced placental perfusion has also been linked to other events such as increased production of cytokines (Schipper, Bolte et al. 2005), anti-angiogenic factors (Maynard, Venkatesha et al. 2005), STB microparticles (Redman and Sargent 2005) and blood-derived products (Mellembakken, Aukrust et al. 2002) which are activated in the intervillous space and together constitute part of the pathogenesis of PE.

1.5.1. Oxidative-nitrative stress and pre-eclampsia

PE, a human pregnancy specific disorder whose incidence varies between 2% and 10% (depending on the definition used and population studied), is commonly diagnosed by the new development of hypertension (≥140/90mmHg) and significant proteinuria (≥300mg/24h urine collection) after week 20 of gestation. It is a leading cause of perinatal morbidity and mortality (1988; Staff, Benton et al. 2013).

Evidence has shown that the placenta serves as the centrepiece in the pathogenesis of PE. In fact, the only intervention that effectively reverses this syndrome is delivery (Staff, Benton et al. 2013).

According to the hypoxia-reoxygenation injury model, the increased formation of free radicals in PE could be partly explained by an altered mitochondrial function. During the hypoxic period, there is almost no O_2 available to act as a final recipient in the mitochondria electron transport chain, leading to increased formation of O_2^-. When O_2 is reintroduced into the intervillous space (reperfusion), a much greater burst of O_2^- formation takes place due to the sudden increase in the availability of O_2 for the electrons accumulated in the mitochondria electron transport chain to leak on to (Hung and Burton 2006).

It is well established that the PE placenta is affected by oxidative and nitrative stress (Wang and Walsh 1998; Staff, Ranheim et al. 1999; Many, Hubel et al. 2000; Wang and Walsh 2001; Zusterzeel, Rutten et al. 2001; Takagi, Nikaido et al. 2004; Vanderlelie, Venardos et al. 2005). However to date, it is not clear if the reduction in antioxidant defence capacity is a primary or secondary event to the excessive depletion through the

increased generation of free radicals (Serdar, Gur et al. 2002). In addition, clinical trials have been conducted to determine whether early supplementation with vitamins C and E is beneficial in women at risk of PE; however, these supplements were without effect preventing PE (Poston, Igosheva et al. 2011).

PE placentas show increased activity of XO (Many, Hubel et al. 1996). As previously described, during hypoxia, the XO enzyme form is enhanced with a consequent increase in the production of O_2^-. In parallel, the enzyme substrate hypoxanthine accumulates due to breakdown of ATP. When O_2 is reintroduced during reoxygenation, again a burst of O_2^- is produced (Hung and Burton 2006). Together with XO, it has been proposed that NADPH oxidase also contributes to the generation of increased O_2^- in PE (Raijmakers, Peters et al. 2004). Interestingly, generation of O_2^- by NADPH oxidase is increased by hormone regulation through upregulation of NADPH oxidase subunit(s) expression, being angiotensin II the most important regulator (Poston and Raijmakers 2004).

Increased free radical generation leads to the formation of lipid peroxides which alter cell membrane fluidity by incorporating cholesterol and by oxidising free-fatty acids and low-density lipoproteins (Hubel, Roberts et al. 1989). These lipid oxidation-derived products include malondialdehyde and isoprostanes (Poston and Raijmakers 2004). Lipid peroxides are direct modulators of maternal endothelial dysfunction through increasing the production of thromboxane-A2 and the expression of cell adhesion molecules in the placental and the maternal peripheral vasculature (Uotila, Tuimala et al. 1993; Walsh and Wang 1995; Poranen, Ekblad et al. 1996).

Other macromolecules affected by oxidative stress which have been measured in PE placentas include oxidised DNA bases which are quantified as the formation of 8-hydroxydeoxyguanosine (Poston and Raijmakers 2004). Oxidative stress also induces chemical modifications in proteins which include formation of carbonyl residues (Levine 2002; Poston and Raijmakers 2004).

The generation of cytotoxic free radicals including O_2^- and NO at levels exceeding the capacity of antioxidant defences, leads to the production of $ONOO^-$, which nitrates tyrosine residues in proteins altering their function. The formation of nitrotyrosine residues in proteins is an example of the effects of RNS collectively named nitrative stress. It has been shown that PE placentas show elevated levels of nitrotyrosine residues (Myatt, Rosenfield et al. 1996; Myatt and Cui 2004) indicative of excessive $ONOO^-$ production.

Maternal serum levels of H_2O_2 are higher in late onset PE than in normal pregnancy (Kharfi, Giguere et al. 2005). This increase has been correlated with elevated plasma hCG in PE (46.5μM H_2O_2 in PE compared to 33.6μM H_2O_2 in normal pregnancy) (Kharfi, Giguere et al. 2005) and also with an increased formation of protein carbonyl residues (Tsukimori, Yoshitomi et al. 2008).

1.5.1.1. Effects of free radicals and altered partial pressure of oxygen on syncytiotrophoblast renewal

Placental villous explants from pregnancies complicated by PE show altered kinetics in response to hypoxia (3% pO_2) such as increased necrotic cell death compared to explants from normal pregnancies at the same pO_2. Apoptotic cell death, located to trophoblast and stromal compartments in placental villous tissue, was also initially elevated in PE compared to normal placentas; however, the rate of apoptosis was further increased in PE placentas when exposed to 3% pO_2. Cytotrophoblast proliferation was elevated under hypoxia in villous explants from normal placentas; in contrast, PE explants remained unaffected to lowering pO_2 throughout culture (Crocker, Tansinda et al. 2004).

Abnormalities of STB reminiscent of those observed in PE can be induced *in vitro* by the application of ROS to induce oxidative stress, suggesting that free radical damage could underlie altered STB development and function (Heazell, Moll et al. 2007; Kharfi Aris, Leblanc et al. 2007; Moll, Jones et al. 2007). Heazell *et al*. (2007) showed an increase in the formation of syncytial nuclear aggregates by exposing placental villous explants to ROS (1μM-1mM H_2O_2) in normoxia for term placental villous tissue (6% pO_2) (Heazell, Moll et al. 2007), suggesting that elevated ROS could be an explanation for the increased shedding of necrotic material seen in PE placentas. Also, at the same pO_2, H_2O_2 disrupts trophoblast cell turnover (in concentrations up to 1mM) increasing apoptotic index and reducing cytotrophoblast proliferation in placental villous explants (Moll, Jones et al. 2007). These effects in STB renewal induced by H_2O_2, did not affect hCG secretion (Moll, Jones et al. 2007).

Chronic exposure of placental villous explants from normal pregnancies to 17% pO_2 revealed regeneration of the STB as demonstrated by an increased secretion of hCG after the second day in culture. However, at the same pO_2, STB regeneration was further increased in PE placentas. On the other hand, at 3% pO_2, PE placentas still showed increased hCG secretion compared to normal placentas in culture (Crocker, Tansinda et al. 2004). In addition, cytotrophoblasts exposed to low H_2O_2 concentrations (ranging 1-50μM) show an effect on differentiation due to an increase in hCG secretion; however, when H_2O_2 is used at higher concentrations (>50μM), it reduces hCG secretion (Kharfi Aris, Leblanc et al. 2007) suggesting a complex relationship between oxidative stress and STB endocrine secretion.

Despite the fact that it is evident that pO_2 and ROS modulate hCG secretion, the mechanism that links them is unexplored. As both conditions dysregulate STB turnover events *in vitro* (Crocker, Tansinda et al. 2004; Moll, Jones et al. 2007; Heazell, Lacey et al. 2008), altered hCG production and secretion might be a consequence of abnormal STB

differentiation. Alternatively, hypoxia/hyperoxia and ROS could directly affect hCG synthesis and/or the secretory process which in turn influences the paracrine/autocrine regulation of STB renewal.

1.5.2. Dysregulated syncytiotrophoblast renewal in pre-eclampsia

The importance of STB turnover for normal placenta function and successful pregnancy outcome is highlighted by the observation that abnormal STB cell turnover underlies the pathogenesis of PE (Arnholdt, Meisel et al. 1991; Lim, Zhou et al. 1997; Crocker, Tansinda et al. 2004; Crocker 2007; Roberts and Escudero 2012).

PE is associated with placental insufficiency characterised by abnormal trophoblast renewal, abnormal endocrine secretion and abnormal nutrient transfer. Dysregulated STB turnover results in the maternal syndrome, the clinical manifestation of PE, with excessive STB deportation and release of inflammatory factors into the maternal circulation causing widespread activation of the maternal endothelium (Redman and Sargent 2003; Roberts and Hubel 2009). Indeed, these trophoblast fragments are locally detectable in uterine veins at Caesarean sections and are increased in PE compared to uncomplicated pregnancies (Johansen, Redman et al. 1999). However, these fragments are not found in the maternal peripheral circulation, instead, it has been proposed the STB sheds microparticles from subcellular STB fractions which, because of their reduced size, can freely circulate. The amount of these microparticles is significantly increased in PE compared to normal pregnancy (Knight, Redman et al. 1998). Artificially generated STB fragments are capable of inducing the signs of inflammation seen in PE, such as inhibition of lymphocyte proliferation and increased lymphocyte apoptosis, together with endothelial disruption (Sargent, Germain et al. 2003).

It has been hypothesised that alterations to trophoblast total volume and thickness in PE are induced by alterations in proliferation of cytotrophoblasts, syncytial fusion and/or STB loss. The STB in PE exhibits increased apoptosis, revealed by morphological observation of condensed chromatin and DNA fragmentation, or by the immunolocalisation of products of the apoptotic cascade (Allaire, Ballenger et al. 2000; Leung, Smith et al. 2001; Ishihara, Matsuo et al. 2002; Crocker, Tansinda et al. 2004); concomitant with increased cytotrophoblast proliferation (Arnholdt, Meisel et al. 1991; Crocker, Tansinda et al. 2004; Brown, Lacey et al. 2005), detected through bromodeoxyurinine incorporation and Ki-67 or Mib-1 antigen immunodetection, suggesting STB turnover is enhanced in PE (Huppertz and Kingdom 2004).

On the other hand, the syncytial fusion of cytotrophoblasts it is hypothesised facilitates the delivery of the machinery participating in apoptosis in the STB, together with delivering other components necessary maintaining STB function. As described in section 1.3.3.,

syncytial fusion is regulated by several factors (Huppertz and Gauster 2011). Amongst them, fusogenic proteins syncytin 1 (Mi, Lee et al. 2000) and syncytin 2 (Lee, Keith et al. 2001) induce syncytium formation (Mi, Lee et al. 2000; Frendo, Olivier et al. 2003; Vargas, Moreau et al. 2009). However, the downregulation of syncytin 1/2 expression has been reported in PE, leading to reduced cytotrophoblast fusion (Lee, Keith et al. 2001; Keith, Pijnenborg et al. 2002; Langbein, Strick et al. 2008; Vargas, Toufaily et al. 2011) and resulting in dysregulated turnover. This reduced fusion in pregnancy disease is also supported by evidence indicating a decreased STB total volume in pregnancies complicated by PE and FGR (Mayhew 2009). Together, this evidence supports the hypothesis that STB turnover could be reduced in PE.

Therefore, understanding trophoblast turnover in normal pregnancy is essential to understand pregnancy disease, and research into the mechanisms and regulation of cellular turnover in non-placental tissues can give insights about what could be occurring in the placenta. Amongst the proteins that regulate cellular homeostasis in non-placental tissues are potassium (K^+) channels. Different K^+ channel protein families are encoded by approximately 80 identified genes and their expression and function is essential for a wide variety of cell functions in excitable and non-excitable tissues. In non-placental epithelia, K^+ conduction underlies different cellular processes including membrane potential (E_m) regulation and the participation mechanisms that maintain tissue homeostasis such as regulation of intracellular Ca^{2+}; control of cell volume; cell proliferation and apoptosis; cell migration, fusion and differentiation and endocrine secretion (Ashcroft and Gribble 1999; Ekhterae, Platoshyn et al. 2001; Elliott and Higgins 2003; Lang, Kaiser et al. 2003; Lang, Gulbins et al. 2004; Burg, Remillard et al. 2006; Shepherd, Duffy et al. 2007; Yun, Park et al. 2010; Su, Wang et al. 2011; Schwab, Nechyporuk-Zloy et al. 2012). Moreover, K^+ channels are regulated by pO_2 and free radicals (Archer, Weir et al. 2000; Crawford, Jovanovic et al. 2003; Tang, Garcia et al. 2004; Duprat, Girard et al. 2005) features that become dysregulated in a variety of diseases including pregnancy-related pathology.

This raises the possibility that K^+ channels could be important for maintaining trophoblast renewal in normal pregnancy but also that dysregulation of K^+ channel expression and/or activity could be a pathophysiological mechanism underlying pregnancy disease.

The following is an overview on K^+ channel structure and function.

1.6. Potassium channels: Structure and function

K^+ channels are integral membrane proteins that selectively conduct K^+ ions across the cell membrane. Under physiological conditions, intracellular K^+ concentration is higher than of the extracellular, producing a chemical gradient which drives K^+ ion efflux from the

cell. As a result, K^+ movement out of the cells is hyperpolarising (Korn and Trapani 2005). In most cells, the resting membrane potential is similar to the K^+ equilibrium potential (membrane potential obtained when electrical and chemical gradients are equal and opposite; thus there is no net flux for a specific ion).

K^+ channels are known to control the extent and shape of action potentials in excitable cells (Korn and Trapani 2005). However, K^+ channels are also present in non-excitable tissues including epithelia, where they play roles setting the membrane potential (E_m; differential of electrical potential across the cell membrane) and maintaining the driving forces for electrogenic transport (Warth 2003). Other roles for K^+ channels in epithelia include transepithelial K^+/solute efflux, cell volume regulation and cellular turnover (proliferation, differentiation, fusion and apoptosis) among others (O'Grady and Lee 2005).

K^+ channels respond to changes in E_m, to different intracellular signals and to mechanical stretch (Korn and Trapani 2005). The biophysical properties that characterise each K^+ channel are single channel conductance, activation threshold (depending on the stimulus) and single channel kinetics (Korn and Trapani 2005). K^+ channel kinetics are reflected by three protein conformational changes: a) an open/activated (conducting) state, where the channel is activated by changes in E_m (voltage-gated) or the concentration of a specific ligand (ligand-gated) (MacKinnon 2003); b) a closed/deactivated (non-conducting) state, upon removal of the activating stimulus and c) an inactivated state, where the channel does not conduct even in the presence of an activating stimulus (Korn and Trapani 2005).

The diversity of functions of over 150 existing K^+ channels (Goldstein, Bayliss et al. 2005; Gutman, Chandy et al. 2005; Kubo, Adelman et al. 2005; Wei, Gutman et al. 2005) is achieved through genes encoding the pore forming α-subunits and their assemblage. K^+ channels can also combine with auxiliary regulatory β-subunits, which modify α-subunit characteristics such as ion selectivity, gating kinetics, surface expression and pharmacology (Korn and Trapani 2005).

According to their function, K^+ channels can be classified into four main families namely voltage-gated (K_V), Ca^{2+}-activated (K_{Ca}), inwardly rectifying (K_{IR}), and two-pore domain (K_{2P}) K^+ channels.

1.6.1. Potassium channel families

1.6.1.1. Voltage-gated potassium channels

K_Vs are the largest family of K^+ channels (Gutman, Chandy et al. 2005). They are characterised by their sensitivity to changes in E_m and are activated upon membrane depolarisation (Korn and Trapani 2005). Structurally, K_V α-subunits are composed of six

transmembrane domains (S1-6), S4 being the voltage sensor, containing positively charged amino acid residues which confer the voltage sensitivity to these channels (Aggarwal and MacKinnon 1996). S5 and S6 transmembrane domains are connected by a pore loop, forming the pore conduction pathway (Korn and Trapani 2005).

There are twelve K_V subfamilies (K_V1-12), being tetramers of four α-subunits (homotetramers or heterotetramers) associated with auxiliary β-subunits (Gutman, Chandy et al. 2005) which modulate K_V activity (Li, Um et al. 2006) and contribute to the diversity of K_V currents. When associated to α-subunits, K_V β-subunits confer rapid inactivation (Rettig, Heinemann et al. 1994), blockade of α-subunits acting as a channel blocker (De Biasi, Wang et al. 1997), assemblage of α-subunits and transport to the plasma membrane (Martens, Kwak et al. 1999), pO_2 and redox sensitivity (Shimoda and Polak 2011) (detailed in section 1.6.3. and 1.6.4.), among others.

The window of activation/opening voltage in K_Vs is found within hyperpolarised potentials (e.g. Shaker (K_V1.x) subfamily) at ~-10mV; however, some members of the K_V1.x subfamily and other K_Vs are activated within more depolarised potentials (Gutman, Chandy et al. 2005). K_Vs are inhibited by broad spectrum K^+ channel blockers such as 4-aminopyridine (4-AP; at concentrations ranging 10μM-20mM), tetraethylammonium (TEA; at higher concentrations, ranging 0.1mM-0.6M), barium (Ba^{2+}; at extracellular concentrations ranging 5-30mM) and by specific toxins such as dendrotoxin, tityustoxin (Burg, Remillard et al. 2006), agitoxin-2, correolide, margatoxin, stromatoxin-1 (Kiernan, Barrie et al. 2010), among others. On the other hand, K_V2.1 is activated (opened) by linoleic acid; K_V7 channel subfamily are activated by mefenamic acid, niflumic acid, retigabine (Gutman, Chandy et al. 2005) and flupirtine (Morecroft, Murray et al. 2009), among others.

In contrast to excitable tissues, K_Vs are expressed in epithelial cells, where they have been described to exhibit inactivation, implicating that their activities are relatively transient. K_Vs in epithelia have different functions such as regulation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), transepithelial electrolyte and nutrient transport, cell volume regulation, cell proliferation and apoptosis, pH regulation and cell migration and differentiation (O'Grady and Lee 2005). Importantly, members of the K_V family are pO_2-sensitive channels (Patel and Honore 2001) which have been implicated in pO_2-sensing mechanisms (detailed in section 1.6.3.).

1.6.1.2. Calcium-activated potassium channels

Together with the K_V family, K_{Ca}s structurally constitute the six-seven transmembrane domain group of K^+-selective channels (Gutman, Chandy et al. 2005). K_{Ca}s correspond to

the second major group of K^+ channels; they consist of four α-subunits forming a tetrameric pore. Based on their single channel conductance and pharmacological properties, they are classified into large conductance (BK_{Ca} or maxi-K_{Ca} ($K_{Ca}1.1$); >200pS), intermediate conductance (IK_{Ca} ($K_{Ca}3.1$); 50-200pS) and small conductance (SK_{Ca} ($K_{Ca}2.1$-3); 2-15pS) (Wei, Gutman et al. 2005; Burg, Remillard et al. 2006). IK_{Ca}/SK_{Ca}s are voltage-insensitive and are activated by an increase in $[Ca^{2+}]_i$ (300-700nM) (Neylon, Lang et al. 1999; Wei, Gutman et al. 2005). This increase can be the result of either Ca^{2+} influx across the plasma membrane by Ca^{2+}-permeable channels, or via the release from intracellular Ca^{2+} stores (Clapham 2007). IK_{Ca}/SK_{Ca}s do not bind Ca^{2+} directly and prior binding to calmodulin, a Ca^{2+}-binding protein constitutively bound to IK_{Ca}/SK_{Ca} C-terminus, is necessary for Ca^{2+}-sensitivity (Neylon, Lang et al. 1999). Consequently, these channels have a key role participating cellular mechanisms involving Ca^{2+}-dependent signalling (Wei, Gutman et al. 2005).

In contrast, BK_{Ca}s are activated by both changes in E_m (voltage-dependent) and $[Ca^{2+}]_I$ (≤100nM) (Wei, Gutman et al. 2005). However, at higher $[Ca^{2+}]_i$ (e.g. micromolar range), BK_{Ca} is activated at less depolarised potentials, enhancing the voltage dependency of the channel (Burg, Remillard et al. 2006). BK_{Ca}s are important regulating Ca^{2+}-entry following depolarisation, stimulated through voltage-gated Ca^{2+} channels (VGCCs), which will in turn activate BK_{Ca} leading to hyperpolarisation of E_m, closure of VGCCs and decreased Ca^{2+} influx (Burg, Remillard et al. 2006). BK_{Ca}s are involved in the regulation of cell secretion mechanisms (Kunz, Thalhammer et al. 2002).

BK_{Ca}s are blocked by TEA (0.1mM; in BK_{Ca} reconstituted or expressed in heterologous systems) and toxins such as charybdotoxin and iberiotoxin (Kaczorowski, Knaus et al. 1996), among others.

SK_{Ca}s are sensitive to block by the toxin apamin (Wei, Gutman et al. 2005). IK_{Ca}s are blocked by cotrimazole and charybdotoxin (Wei, Gutman et al. 2005; Burg, Remillard et al. 2006) and specifically blocked by 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34; (Wulff, Miller et al. 2000; Kohler, Wulff et al. 2003)) but insensitive to inhibition by TEA (Fioretti, Pietrangelo et al. 2005). IK_{Ca}/SK_{Ca}s are activated by 6, 7-dichloro-1H-indole-2, 3-dione 3-oxime (NS309) and by 5, 6-dichloro-1-ethyl-1, 3-dihydro-2H-benzimidazol-2-one (DCEBIO) (Singh, Syme et al. 2001; Sheng, Ella et al. 2009). Activation of IK_{Ca}/SK_{Ca}s by DCEBIO is the primary mechanism to stimulate chloride (Cl^-) secretion via Cl^- conductances carried by the cystic fibrosis transmembrane conductance regulator (CFTR) channel (Hamilton and Kiessling 2006). Activation by DCEBIO is more potent than its analog 1-ethyl-2-benzimidazolinone (1-EBIO). Activation of IK_{Ca}s by DCEBIO is Ca^{2+}-dependent; thus, prior elevated intracellular Ca^{2+} is needed so the activator can exert its

effect; however, the concentration of Ca^{2+} needed (100nM Ca^{2+}) is close to resting intracellular Ca^{2+} levels (Pedersen, Schroder et al. 1999).

1.6.1.3. Inwardly rectifying potassium channels

K_{IR}s are important in maintaining E_m. K_{IR}s are activated at hyperpolarised potentials (relative to the equilibrium potential for K^+) and moderate elevated extracellular K^+ concentration (7-15mM). Unlike other K^+ channels, K_{IR}s preferentially support the influx of K^+ at negative E_ms (Nelson and Quayle 1995; Gurney, Joshi et al. 2010).

Upon significant depolarisation, these channels are blocked by intracellular magnesium (Mg^{2+}) or polyamines (Korn and Trapani 2005). K_{IR}s are divided into 7 sub-families (K_{IR}1-7) depending on their sensitivity to exogenous signals. They present four α-subunits, each consisting of two transmembrane domains and a pore forming loop; the latter is homologous to that of K_V and K_{Ca}s (Kubo, Adelman et al. 2005).

Members of this family include G protein-coupled K_{IR}3 and ATP-sensitive K_{IR}6 (K_{ATP}) channels (Kubo, Adelman et al. 2005).

K_{ATP}s are structurally and functionally distinct from K_{IR}s. K_{ATP}s are not only sensitive to molecules that normally regulate K_{IR}s but in addition, they are sensitive to intracellular ATP; thus, elevated intracellular ATP inhibits K_{ATP} activity (Korn and Trapani 2005). K_{ATP}s are formed by an octameric association of four K_{IR} subunits (K_{IR}6.1 and 6.2 α-subunits) with a β-subunit which is a member of the ATP-binding cassette (ABC)-family called the sulphonylurea receptor (SUR) which confers ATP sensitivity (Tucker and Ashcroft 1998; Campbell, Sansom et al. 2003).

Ba^{2+} and cesium (Cs^+) are non-selective blockers that inhibit most K_{IR} sub-families (including G protein-coupled K_{IR}3 channels). K_{ATP}s are highly sensitive to inhibition by glibenclamide and are activated by pinacidil, cromakalim and lemakalim (Clapp and Gurney 1992; Kubo, Adelman et al. 2005), among others.

1.6.1.4. Two-pore domain potassium channels

K_{2P}s are structurally different from other K^+ channel families. They are composed of two pore forming loops in each α-subunit, which join to form a tetrameric pore. K_{2P}s do not contain voltage-sensitive units (voltage-independent) (Goldstein, Bayliss et al. 2005). As a result, K_{2P}s can be activated at all E_ms and they are thought to contribute to the resting E_m in many cell types (Gurney, Joshi et al. 2010).

K_{2P}s are blocked by external changes in hydrogen ion concentration (pH) (TWIK-1; TASK-1/2/3; TALK-1/2; TRESK-1/2), quinidine (TREK-1; TASK-2; TWIK-2; TREK-2; TALK-1; TRESK-1/2), Ba^{2+} (TREK-1; TASK-1; TWIK-2; THIK-1; TALK-1/2; TRESK-1/2) and

anandamide (TASK-1) among others. TASK-1 is pO_2-sensitive (Patel and Honore 2001) and TREK-2 is a K_{2P} activated by membrane stretch (Kang, Choe et al. 2004).

1.6.2. Roles of potassium channels in tissue homeostasis

In non-placental tissues, K^+ channels have been shown to contribute to tissue homeostasis by regulating proliferation, differentiation and fusion, cell migration, apoptosis and endocrine secretion.

1.6.2.1. Cell volume regulation

Under physiological conditions, the osmolality of the extracellular fluid is kept constant (~285mOsm/kgH_2O) and cell volume is affected by changes in intracellular rather than extracellular osmolality. These changes include transepithelial transport and nutrient uptake. However, cell volume can be affected by pathophysiological conditions and cell swelling occurs during hypoxia/ischemia, hyponatremia and increased extracellular K^+ concentrations, among others (Hoffmann, Lambert et al. 2009). In addition, cell volume changes occur during a variety of physiological processes such as cell proliferation, migration, differentiation and cell death (Okada, Maeno et al. 2001).

The fact that K^+ permeability at rest is greater than for other ions, makes K^+ flux an important regulator of cell volume and activation of K^+ conductances to regulate volume after acute cell swelling is a conserved mechanism present in a variety of cell types (Hoffmann, Lambert et al. 2009). During cellular solute uptake, if uptake exceeds solute exit, this leads to accumulation of osmolytes and to intracellular hypertonicity, water entry, and cell swelling (Hallows and Knauf 1994). This cell swelling stimulates a regulatory volume decrease (RVD) mechanism in order to return the cell its normal volume. RVD generally involves swelling-activated K^+ and Cl^- effluxes and efflux of organic osmolytes (including amino acids such as taurine, methylamines, and polyols) which results in the extrusion of water through aquaporins (water-permeable channels) (Lang, Busch et al. 1998).

An experimental approach to study K^+ channels is assessing their function due to cell swelling following exposure to a hyposmotic solution (Birdsey, Boyd et al. 1999; Gow, Thomson et al. 2005). This condition mimics hyposmotic cell swelling secondary to a raise in intracellular osmolality. Hyposmotic solutions provoke an increase in cell volume, activation of transport mechanisms extruding ions (RVD) via intracellular signalling involving Ca^{2+}, and subsequent return of cell volume to its normal value.

The activation of different types of K^+ channels have been associated with osmotic cell swelling, including K_{Ca}s (Park, Beck et al. 1994; Khanna, Chang et al. 1999; Weskamp,

Seidl et al. 2000; Wang, Morishima et al. 2003), stretch-activated K^+ channels (Sackin 1989), K_Vs (Schoenmakers, Vaudry et al. 1995) and K_{2P}s (Niemeyer, Cid et al. 2001). Generally, in most epithelial cells, RVD involves the activation of K_{Ca}s (Fernandez-Fernandez, Nobles et al. 2002; Wang, Morishima et al. 2003) whereas in non-epithelial cell types, RVD is regulated by Ca^{2+}-independent channels (Pasantes-Morales and Morales Mulia 2000).

1.6.2.2. Cell proliferation

In different cell types, the inhibition of K^+ channel function leads to a decrease in proliferation, suggesting that K^+ channels participate in cell cycle progression (Wonderlin and Strobl 1996). It has been shown that membrane hyperpolarisation is a conserved mechanism necessary for the progression of G1 phase of the cell cycle (Wonderlin and Strobl 1996; Beech and Cheong 2006); in fact, inhibition of K^+ channels causing E_m depolarisation, impairs cell cycle progression. In addition, K^+ channels regulate Ca^{2+} influx as main controllers of E_m, and therefore, $[Ca^{2+}]_i$ which is known to be an important second messenger regulating cellular signalling pathways involved in cell proliferation (Nilius, Schwarz et al. 1993). The pharmacological modulation of members of different K^+ channel families has been shown to affect cell proliferation.

The K_{ATP} channel opener minoxidil stimulates cell proliferation (Abdul and Hoosein 2002), whereas K_V blockers such as 4-AP, TEA and dendrotoxin inhibit cell proliferation (Ouadid-Ahidouch, Chaussade et al. 2000; Roderick, Reinach et al. 2003). Inhibition of IK_{Ca} with cotrimazole or the downregulation of IK_{Ca} expression with an IK_{Ca} knockdown, reduced cell proliferation in bone marrow mesenchymal stem cells via modulation of cyclin expression (Tao, Lau et al. 2008). In smooth muscle cells, upregulation of IK_{Ca} expression/function is related with a physiological switch from a contractile phenotype (where BK_{Ca}s provide the hyperpolarisation counterbalance against depolarisation to allow muscle contraction), to a proliferative phenotype, where IK_{Ca}-mediated hyperpolarisation promotes Ca^{2+} entry through voltage-independent Ca^{2+} channels. Indeed, blockade of IK_{Ca} in this phenotype, inhibits smooth muscle cell proliferation (Neylon, Lang et al. 1999; Cheong, Bingham et al. 2005).

However, recent evidence suggests that participation of IK_{Ca}s in proliferation is independent of the ion channel conductive properties (Millership, Devor et al. 2011). Using direct-site mutagenesis, Millership *et al*. (2008) demonstrated that neither affecting ion conductance (pore) nor Ca^{2+} binding sites of the ion channel affected cell proliferation; instead, when the mutation was directed against the binding site of the ion channel to kinases ERK1/2 and JNK, cell proliferation was impaired (Millership, Devor et al. 2011).

This evidence supports an additional role of IK_{Ca}s regulating cell proliferation by direct interaction with signalling pathways involved in proliferation and cell survival.

1.6.2.3. Apoptosis

Appropriate balance between cell proliferation and apoptosis is essential to maintain tissue homeostasis. Apoptosis or programmed cell death result in cell shrinkage, DNA fragmentation and finally, phagocytic death.

Cell shrinkage is characteristic at the initial stages of apoptosis, with little or no effect on intracellular organelles. This shrinkage is produced by a controlled mechanism named apoptotic volume decrease (AVD) which involves K^+, Cl^- and water efflux (Remillard and Yuan 2004). This K^+ efflux, sufficient to lower intracellular K^+ concentration, is an essential pre-requisite for initiating caspase cascade activation in many cell types.

The use of K^+ ionophores to induce K^+ loss, resulted in increased apoptosis in smooth muscle cells (Krick, Platoshyn et al. 2001), and similar effects are seen in other cell types. Alternatively, increasing $[Ca^{2+}]_i$ using Ca^{2+} ionophores also stimulates K^+ efflux via K_{Ca}s, inducing cell shrinkage, membrane blebbing (Lang, Kaiser et al. 2003) and apoptosis (Krick, Platoshyn et al. 2001; Lang, Kaiser et al. 2003; Wang, Morishima et al. 2003). On the contrary, when TEA-sensitive outward K^+ currents were inhibited, apoptosis was attenuated (Yu, Yeh et al. 1997).

The use of different pro-apoptotic agents enhances the activity of 4-AP and TEA-sensitive K^+ channels, e.g. staurosporine (Yu, Yeh et al. 1997), NO donors (Krick, Platoshyn et al. 2002) and dexamethasone (Dallaporta, Marchetti et al. 1999). In addition to K_Vs and K_{Ca}s, other K^+ channels have also been involved in these early events in apoptosis; amongst them, TALK1/2, members of the K_{2P} family, participate in free radical-induced apoptosis (Duprat, Girard et al. 2005).

After this initial apoptotic phase, an intermediate phase involving the release of cytochrome-C by the mitochondria and caspase activation, leads to the final degradation of nuclear contents and the disruption of cell plasma membrane (Burg, Remillard et al. 2006). Changes in intracellular K^+ concentration can also modulate this phase by involving ion flow via K^+ channels located on plasma and mitochondria membranes (Burg, Remillard et al. 2006). Evidence shows that activation of members of the K_{Ca} family in dermal fibroblasts, induced apoptosis through disruption of mitochondrial E_m; however, the use of a pan-caspase inhibitor did not prevent this effect, suggesting that K_{Ca}s are also involved in apoptosis in a caspase-independent manner (Yun, Park et al. 2010).

1.6.2.4. Cell differentiation and fusion

Regulation of K^+ channel expression and/or activity is involved in cell differentiation. As an example, the inhibition of K_{Ca}s impairs Ca^{2+}-induced differentiation in keratinocytes (Mauro, Dixon et al. 1997).

Much of the research in cell differentiation has focused on skeletal muscle. During myogenesis, proliferating myoblasts withdraw from the cell cycle and fuse to form large, multinucleated muscle fibers. Under culture conditions of high concentrations of serum, myogenic cell lines remain in a proliferative state (myoblast); if serum is removed from culture medium, these cells arrest proliferation and begin to differentiate. Studies have shown the involvement of different ion channels in this differentiation process including K^+ channels. Swelling-activated Cl^- currents (Voets, Wei et al. 1997) and IK_{Ca}s (Fioretti, Pietrangelo et al. 2005) are downregulated during myogenesis, remaining only during the proliferative stage. However, the experimental inhibition of IK_{Ca}s does not affect myoblast proliferation, suggesting that IK_{Ca} could be present only in undifferentiated myoblasts (Fioretti, Pietrangelo et al. 2005). On the other hand, other ionic currents are upregulated during myogenesis such as delayed rectifier K^+ currents (Lesage, Attali et al. 1992) and K_{IR} currents (Liu, Bijlenga et al. 1998).

Only a few cell lineages undergo syncytial fusion (or syncytialisation) to form a syncytium, i.e. myoblasts (to generate myotubes), macrophages (to generate osteoclasts) and cytotrophoblasts (to generate STB). In particular, myoblast syncytialisation is a Ca^{2+}-dependent process, where the sequential expression and activity of members of two K^+ channel families: first, K_V11.1 (ether-à-go-go-related gene) and second, K_{IR} (e.g. K_{IR}2.1), causes E_m hyperpolarisation, allowing Ca^{2+} entry and triggering fusion (Fischer-Lougheed, Liu et al. 2001). Indeed, inhibition of K_{IR} causes myoblast E_m depolarisation and impedes fusion (Liu, Bijlenga et al. 1998).

1.6.2.5. Cell migration

All cells migrate at some point of their lives; migration is a process essential for tissue homeostasis. Cell migration is a polarised process which is divided into two main steps which are repeated over and over: protrusion of the cell front and retraction of the cell rear (Schwab, Fabian et al. 2012). Ion transport is involved in this process through regulation of E_m, cell volume, $[Ca^{2+}]_i$ and intra-extracellular pH.

In general, K^+ and Cl^- channels, through their participation in cell volume regulation, assist to induce a localised cell shrinkage that supports the retraction of the rear part of migrating cells (Schwab, Hanley et al. 2008). This localised shrinkage facilitates the passage through the dense fibroid network of the extracellular matrix.

In particular, IK_{Ca}s are the most extensively studied K^+ channels involved in cell migration. IK_{Ca}s are expressed in all migrating cells including immune cells, osteoclasts, vascular cells and fibroblasts, among others (Schwab, Fabian et al. 2012). IK_{Ca} activity in migrating cells is highly dependent on $[Ca^{2+}]_i$ oscillations and is also location-specific. IK_{Ca} clusters more at the rear than at the front of migrating cells (Schwab, Wulf et al. 2006); indeed, blockade of IK_{Ca} with charybdotoxin inhibits cell migration only when this blocker is directed at the rear of the cell (Schwab, Gabriel et al. 1995). A transient elevation in $[Ca^{2+}]_i$ triggers IK_{Ca} activity and K^+ efflux which leads to volume loss at the rear of the cell. This is concomitant with the action of Ca^{2+}-dependent cytoskeleton proteins which will allow retraction and integrin release, necessary for cell migration (Schwab, Hanley et al. 2008; Schwab, Nechyporuk-Zloy et al. 2012).

In addition, members of the K_V family (K_V1.x, K_V10.x, and K_V11.x) have been reported to be involved in cell migration. K_V1.1 plays a role in wound healing of intestinal epithelial cells by setting E_m necessary for Ca^{2+} influx which triggers different mechanisms stimulating cell migration (Wang, Wang et al. 2000; Rao, Platoshyn et al. 2002). Inhibition of K_V1.3 impairs cell migration in immune cells (Matheu, Beeton et al. 2008; Gendelman, Ding et al. 2009). It is thought that inhibition of K_V1.3 impairs Ca^{2+} signalling that mediates integrin activation, necessary for lymphocyte migration (Matheu, Beeton et al. 2008). Together with K_V11.1, it has been reported that K_V1.3 form complexes with integrins (Pillozzi and Arcangeli 2010).

1.6.2.6. Endocrine secretion

K^+ channels regulate hormone secretion in different cell types (Ashcroft and Gribble 1999; Jacobson, Kuznetsov et al. 2007; Leung, Kwan et al. 2007).

In pancreatic β-cells, K_{ATP}s couple electrical activity with changes in plasmatic glucose concentration and insulin release. K_{ATP}s are opened and set the resting E_m in pancreatic β-cells. An increase in extracellular glucose and glucose uptake by the β-cell closes K_{ATP}s, depolarising E_m and activating VGCCs with a consequent raise in $[Ca^{2+}]_i$ which stimulates insulin release (Ashcroft and Gribble 1999). The subsequent activation of K_Vs repolarise β-cells to terminate exocytosis (Leung, Kwan et al. 2007).

Indeed, K_Vs also play a role in the regulation of pancreatic β-cell insulin release. A K_V2.1 knockout mouse shows decreased K_V current and a greater glucose-stimulated insulin secretion compared to wild type mice. Furthermore, the pharmacological inhibition of K_V2.x enhances glucose-stimulated insulin secretion in wild type mice and in human pancreatic islets but not in the K_V2.1 knockout mouse (Li, Herrington et al. 2013).

In neurons and neuroendocrine cells, SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins comprise the secretory machinery for exocytosis. It

has been shown that one particular SNARE, syntaxin 1A, physically interacts with K_V2.1 and the sulfonylurea receptor subunit of K_{ATP}s to modify their gating properties. This is relevant since the conformational changes induced by syntaxin 1A on K_Vs and K_{ATP}s can therefore modulate insulin secretion and cell excitability (Leung, Kwan et al. 2007).

On the other hand, little is known about the participation of K_{Ca}s in endocrine secretion. However, a role for IK_{Ca} has been suggested in the release of adrenocorticotrophic hormone as part of the hypothalamic-pituitary-adrenal axis. Adrenocorticotrophic hormone secretion has been shown to be regulated by ion channels expressed in the excitable cell type, corticotrophs. Outward K^+ currents in this cell type are mediated by IK_{Ca} and TRAM-34 inhibition caused increased corticotroph excitability and increased adrenocorticotrophic hormone secretion *in vitro* (Liang, Chen et al. 2011).

1.6.3. Role of potassium channels in partial pressure of oxygen sensing

K^+ channels present in specialised cell types such as carotid body type 1 cells, pulmonary artery and ductus arteriosus smooth muscle cells among others, have been shown to be important regulators in the physiological/pathophysiological adaptation to hypoxia (<50-60mmHg) (O'Grady and Lee 2005; Shimoda and Polak 2011). Exposing these cells to sustained hypoxia (e.g. chronic diseases, high altitude) directly influences transcriptional mechanisms that determine the level of K^+ channel expression. On the other hand, if exposed during brief periods to hypoxia (e.g. ischemia), this induces changes in K^+ channel activity (Wang, Juhaszova et al. 1997; Lopez-Barneo, del Toro et al. 2004; Rey-Parra, Archer et al. 2008; Shimoda and Polak 2011). In particular, acute hypoxia induces E_m depolarisation by closing specific K^+ channels and triggering cellular responses (Patel and Honore 2001).

Several K^+ channels have been described to be involved in pO_2-sensing. The K_Vs: K_V1.2, K_V1.5, K_V2.1/9.3, K_V3.1, K_V3.3 and K_V4.2 are reversibly blocked by hypoxia when expressed in heterologous systems (Patel and Honore 2001; O'Grady and Lee 2005). K_{2P} TASK-1 is also pO_2-sensitive (Patel and Honore 2001).

K^+ channel β-subunits also play a role in the modulation of K_V channel α-subunits by pO_2 (Patel and Honore 2001; O'Grady and Lee 2005). For example, HEK293 cells expressing K_V4.2 do not exhibit pO_2-sensitivity; however when co-expressed with K_Vβ1.2 subunit, the complex K_V4.2/β1.2 is inhibited by hypoxia (Perez-Garcia, Lopez-Lopez et al. 1999).

To date, there is still controversy whether the effects of hypoxia affect K^+ channels directly or indirectly. Some ion channels are intrinsically pO_2-sensitive when studied using excised membrane patches or heterologous expression systems, indicating that the pO_2-sensor is

closely associated with the pore-forming α-subunits of the channel or regulatory β-subunits (Clapp and Gurney 1992; Patel and Honore 2001; Riesco-Fagundo, Perez-Garcia et al. 2001). pO_2 can directly induce conformational changes in the K^+ channel protein between oxidising (open) or reducing (close) states. Specifically, changes in pO_2 can be sensed as alterations to cysteine-thiols or methionine residues, or oxido-reductase domains which affect K^+ channel gating in response to a redox environment (Lopez-Barneo, Pardal et al. 2001; Patel and Honore 2001).

On the other hand, other evidence suggests that pO_2-sensitivity of some K^+ channels might be modulated by interaction between the pO_2-sensing signalling molecules and pore-forming α-subunits. This second mechanisms, does not involve K^+ channel subunits but the conversion of O_2 to ROS, which then alters the redox state of K^+ channels, although producing the same inhibition effect. Two ROS-generating systems that have been reported as pO_2-sensors: NADPH oxidase and the mitochondrial electron transport chain (Archer, Weir et al. 2000; O'Grady and Lee 2005).

1.6.4. Modulation of potassium channels by free radicals

A mechanism that links changes in pO_2 to K^+ channel function is the generation and alterations caused by free radicals (ROS and RNS) and non-radical metabolites such as H_2O_2 when targeting K^+ channels. The rate of formation of free radicals, as part of aerobic metabolism, is proportional to the prevailing pO_2 (Jauniaux, Watson et al. 2000). However, independent of the existing pO_2, studies have focused on the effects of different ROS/RNS *in situ*, demonstrating that the wide variety of effects of ROS/RNS on K^+ channel activity are tissue-dependent and ROS/RNS-dependent.

In general, free radicals can alter K^+ channel expression or activity over a long time frame as well as having acute effects directly on K^+ channel proteins to alter their activity (oxidation state) (Kourie 1998; Gutterman, Miura et al. 2005). Indeed, studies in the pulmonary vasculature, a tissue exposed to extremes in pO_2, reveal that free radicals can inhibit K_V expression (Caouette, Dongmo et al. 2003; Fountain, Cheong et al. 2007) and/or activity (Duprat, Guillemare et al. 1995; Gutterman, Miura et al. 2005).

H_2O_2 has been reported to both close (Archer, Wu et al. 2004) and open (Rogers, Dick et al. 2006; Rogers, Chilian et al. 2007) K_Vs, including a H_2O_2-mediated relaxation effect via 4-AP-sensitive K^+ channels in uterine smooth muscle (Appiah, Milovanovic et al. 2009). For instance, H_2O_2 at pathophysiologically relevant concentration (100μM), accelerated the activation kinetics of $K_V1.5$ expressed in Chinese hamster ovary cell line, which were reduced by the intracellular application of SOD or catalase (Caouette, Dongmo et al. 2003). On the other hand, H_2O_2 can oxidise cysteine residues reducing channel activation

(current reduction <15% of the original size) targeting cysteine residues in the Ca^{2+}-binding domain of BK_{Ca}s (Tang, Garcia et al. 2004).

Whole cell unitary currents of K_V1.x in coronary arteries are inhibited by $ONOO^-$ contributing to impaired vasodilation. This is correlated with increased nitration of tyrosine residues in the pore-forming region of K_V1.x (specifically K_V1.2), detected by immunohistochemistry. When using ebselen, a $ONOO^-$ scavenger, this effect was partially reversed (Li, Gutterman et al. 2004; Bubolz, Wu et al. 2007).

In the presence of SIN-1, added either on the cytosolic or extracellular side of the channel, K_{Ca} open probability from tracheal smooth muscle cells increases without affecting the channel unitary conductance, suggesting that the activation of K_{Ca} is likely to be due to an acute chemical modification (oxidation in thiol groups) within the ion channel protein. The addition of charybdotoxin, a BK_{Ca}/IK_{Ca} blocker, reduced the sensitivity of the channel to SIN-1 induced-relaxation (Abderrahmane, Salvail et al. 1998).

In mesenteric arteriolar smooth muscle cells, $ONOO^-$ or $ONOO^-$-generator SIN-1 increased Ca^{2+}-activated K^+ outward currents. Interestingly, the $ONOO^-$-induced currents were inhibited by the removal of external Ca^{2+}, by the addition of nifedipine (blocker of VGCCs) or ryanodine (a sarcoplasmic reticulum ryanodine receptor blocker). These results suggest that Ca^{2+} mobilisation either external Ca^{2+} influx or Ca^{2+} release from intracellular stores, is involved in $ONOO^-$-mediated K^+ outward currents (Pan, Zhao et al. 2004).

K_{ATP}s show an increased channel activity when exposed to O_2^- in cardiac myocytes and, on the contrary, in vasculature they exhibit a decreased channel activity (Goldhaber, Ji et al. 1989; Tokube, Kiyosue et al. 1996; Armstead 1997). This disparity could be correlated with molecular differences between K_{ATP}s present in myocardium and vasculature (Liu and Gutterman 2002). H_2O_2 and $ONOO^-$ increase K_{ATP} channel activity in vasculature due to a variety of mechanisms, e.g. oxidation of sulfhydryl groups (Liu and Gutterman 2002).

Members of K_{2P}s, TALK-1/2, expressed in *Xenopus laevis* oocytes are activated by NO but not by H_2O_2 or ROS-generating systems such as SIN-1 (Duprat, Girard et al. 2005).

Overall, this evidence demonstrates that K^+ channels play a pivotal role in maintaining tissue homeostasis by promoting cellular turnover and in addition, K^+ channel modulation by pO_2 and free radicals is relevant for several cellular physiological and pathophysiological processes. It is therefore possible that pO_2 and free radical modulation of K^+ channels could be contributing to the abnormalities seen in STB turnover and function that are associated with PE, a pregnancy complication associated with placental altered pO_2 and oxidative/nitrative stress. The following section explores the existing evidence for the expression and function of K^+ channels in the STB and in addition, the roles of STB K^+ channels in regulation and dysregulation of STB turnover.

1.7. Potassium channels in the syncytiotrophoblast

The understanding of STB ion channel physiology is rudimentary compared with other epithelia; relatively little is known about the expression and/or function of K^+ channels in placenta and the involvement of K^+ channels in STB function.

The following is an overview of the main findings contributing to the identification and characterisation of K^+ channels in STB MVM. Figures 1.5 and 1.6 illustrate this evidence in placental villous tissue and multinucleated cytotrophoblasts respectively.

The expression and function of K^+ channels in STB BM is almost unexplored to date, but fluorescent measurements of E_m in isolated STB BM vesicles initially demonstrated the presence of tetramethylammonium and TEA-selective K^+ conductances (Illsley and Sellers 1992). In addition, Riquelme *et al*. (2012) reported $K_{IR}2.1$, $K_V2.1$, TASK-1 and TREK-1 protein expression in purified STB BM vesicles although, compared with MVM vesicles, the expression in BM was relatively low (Riquelme, de Gregorio et al. 2012) (Figure 1.5).

The first studies on STB MVM K^+ transport were performed in MVM vesicles isolated from placenta, showing that part of the flux is dependent on E_m, and probably takes place through a conductive pathway (Illsley and Sellers 1992). ^{86}-rubidium (^{86}Rb) permeates most K^+ selective channels and it has been used as a tracer to indirectly assess K^+ permeability (Bland and Boyd 1986; Venglarik, Bridges et al. 1990) of the STB MVM (Boyd 1983; Birdsey, Boyd et al. 1999; Siman, Sibley et al. 2001). Detailed studies of ^{86}Rb fluxes and ionic dependence of E_m have confirmed the presence of K^+ conductive pathways in the MVM in isolated placental villi, in cytotrophoblasts isolated from term placentas maintained in primary culture and in purified STB MVM vesicles in reconstituted systems (Greenwood, Boyd et al. 1993; Greenwood, Clarson et al. 1996; Birdsey, Boyd et al. 1997; Clarson, Greenwood et al. 2001; Diaz, Vallejos et al. 2008).

Over the course of pregnancy, the STB MVM E_m changes, being more negative during the first trimester (-28mV) than at term (-21mV). A Ba^{2+}-sensitive K^+ conductance contributes to this potential (Birdsey, Boyd et al. 1997) (Figure 1.5). Moreover, villous fragments maintained in explant culture up to 7 days, showed that basal ^{86}Rb efflux decreased with time in culture, indicating a progressive fall in K^+ permeability as the STB regenerated *in vitro* (Siman, Sibley et al. 2001). Exposure to hyposmotic stimuli in placental villous fragments causes a Ba^{2+}-sensitive hyperpolarisation of MVM E_m (Birdsey, Boyd et al. 1999) (Figure 1.5); however, the identities of the K^+ channels underlying the resting conductance, or the change with cell swelling, remain unknown.

Patch clamp studies in STB *in vitro* are hindered because of the syncytial nature of the tissue which precludes whole cell recording and achieving high resistance seals for single channel patch clamp has a low success rate (Brown, Greenwood et al. 1993). Therefore, isolated cytotrophoblasts from term placentas maintained in primary culture are often used as a model system. Three approaches have been used to identify K^+ conductances in these cells: patch clamp studies, E_m and ^{86}Rb efflux measurements.

Initial measurements of E_m and membrane resistance in cytotrophoblast-like cells isolated from first trimester placental villi *in vitro* demonstrated that E_m was strongly hyperpolarised in the presence of extracellular Ca^{2+} and this effect was inhibited by the VGCC blocker verapamil. In addition, this effect on E_m was only affected by extracellular K^+ concentration but not by other ions Yano (Yano, Okada et al. 1982). Together this evidence supported the functional expression of Ca^{2+}-activated K^+ conductances but also verapamil-sensitive Ca^{2+} channels in this cell type (Yano, Okada et al. 1982).

E_m measurements of mono (-48mV) and multinucleated (-40mV) cytotrophoblasts isolated from term placentas, along with estimates of intracellular K^+ concentration, showed that the electrochemical gradient for K^+ changed with time in culture suggesting changes in the expression and/or function of K^+ conductances with cytotrophoblast differentiation *in vitro* (Greenwood, Clarson et al. 1996) (Figure 1.6). Single-channel patch clamp studies identified a large conductance (200pS) K_{Ca} with typical characteristics of BK_{Ca} and an unidentified 56pS channel present in cell attached patches, which was active at the cell E_m and reversed at -40mV, consistent with K^+ selectivity (Greenwood, Brown et al. 1993) (Figure 1.6). Also whole-cell patch clamp experiments in cytotrophoblasts showed the functional expression of Ba^{2+}-sensitive K_{IR} currents typical of $K_{IR}2.1$, whose incidence increased with cytotrophoblast differentiation (Clarson, Greenwood et al. 2001) (Figure 1.6). Importantly, the stimulation of cytotrophoblasts by hyposmotic swelling (Greenwood, Sides et al. 1996) or extracellular nucleotides, promotes Ba^{2+}-sensitive ^{86}Rb efflux. This K^+ permeability is due in part to the stimulation of Ca^{2+}-activated ^{86}Rb efflux, sensitive to charybdotoxin but insensitive to apamin and iberiotoxin, implicating the participation of IK_{Ca} (Clarson, Roberts et al. 2002) (Figure 1.6). IK_{Ca} mRNA is expressed in whole placental homogenate (Jensen, Strobak et al. 1998; Chen, Gorman et al. 2004), together with BK_{Ca} (Lacey, Glazier et al. 2005).

mRNA for several K_Vs is expressed in placental homogenate i.e. K_V1.5, 1.7, 6.1, 7.1, 7.2, 7.4 (Lacey, Glazier et al. 2005), K_V9.3 (Corcoran, Lacey et al. 2008) and immunostaining for K_V1.5 and 2.1 has localised their expression to the MVM of the STB in placental villous sections (Williams, Jones et al. 2009) and the plasma membrane of isolated cytotrophoblasts (unpublished data). K_V2.1 protein expression has also been described in isolated STB MVM vesicles from term placentas (Riquelme, de Gregorio et al. 2012)

(Figures 1.5, 1.6). In addition, $K_V1.5$ mRNA expression changes with cytotrophoblast differentiation *in vitro*, being highly expressed at 18h but lower at 66h (unpublished data).

$K_{IR}2.1$ mRNA is expressed in whole placental homogenate (Lacey, Glazier et al. 2005) and its protein expression has been confirmed in mono and multinucleated cytotrophoblasts, term placental tissue (unpublished data) and purified STB MVM vesicles (Riquelme, de Gregorio et al. 2012) (Figures 1.5, 1.6). K_{ATP} ($K_{IR}6.2$ subunit) mRNA has recently been identified in whole placental homogenate, and the channel protein is expressed in STB of placental villous tissue (Lybaert, Hoofd et al. 2013) (Figure 1.5).

Bai *et al*. (2005, 2006) have shown the expression and activity of K_{2P}s. In cytotrophoblasts, mRNAs encoding TASK1/2/4/5 and TREK1 were detected; whereas only TASK-1 and TREK-1 protein expression has been confirmed in mono and multinucleated cytotrophoblasts in culture and also in isolated STB MVM vesicles from term placentas (Riquelme, de Gregorio et al. 2012) (Figures 1.5, 1.6). TASK-2 showed intracellular protein localisation in mononucleate cytotrophoblasts which was lost with multinucleation and its mRNA expression was higher in first trimester than at term pregnancy. Indeed, TASK-2 protein localisation also changed in placental villous tissue; TASK-2 was localised to the trophoblast layer during the first trimester, but only associated to stem villi at term (Bai, Greenwood et al. 2005; Bai, Lacey et al. 2006). Anandamide, a blocker of TASK-1, inhibited basal and pH 8.0-stimulated ^{86}Rb efflux in multinucleated cytotrophoblasts, consistent with a contribution of these channels to E_m and background K^+ current, in common with other tissues (Bai, Bugg et al. 2005) (Figure 1.6).

Additionally, Ba^{2+} and TEA-sensitive K^+ channels have been identified using purified STB MVM in reconstituted systems suitable for electrophysiological methods (patch clamp and two-electrode voltage clamp) (Diaz, Vallejos et al. 2008).

In addition to the identification of STB K^+ channels in uncomplicated pregnancies, to date, there is limited evidence about K^+ channel expression in pregnancy diseases, in particular in PE. An increased $K_V7.3$ and $K_V7.5$ protein expression was seen in PE compared to normal pregnancies at term, and the localisation of these channels was mainly restricted to the STB (Mistry, McCallum et al. 2011) (Figure 1.5). Additionally, recent evidence shows that protein expression of $K_V2.1$, TASK-1 (pO_2-sensitive K^+ channels), $K_{IR}2.1$ and TREK-1 is unaltered in isolated MVM vesicles from placentas of normal term pregnancies compared to PE (Riquelme, de Gregorio et al. 2012). Interestingly, $K_{IR}2.1$ is expressed in cholesterol-rich fractions (rafts) and non-raft fractions present in subdomains from MVM from normal placentas, suggesting that membrane cholesterol could play a role in regulating STB $K_{IR}2.1$ function, and this distribution remains unaffected in PE (Riquelme, de Gregorio et al. 2012).

1.7.1. Functions and regulation of syncytiotrophoblast potassium channels

The contribution of STB K^+ channels to the maintenance of E_m is important as K^+ flux provides the driving force for cell electrogenic transport such as system A, a Na^+-coupled neutral amino acid transporter present in the STB, which is important for fetal growth (Glazier, Cetin et al. 1997; Desforges, Mynett et al. 2009).

As previously described, the experimental manoeuvre of using a hyposmotic solution *in vitro* to stimulate K^+ efflux is to mimic cell swelling secondary to the rise in intracellular osmolality which occurs mainly during nutrient uptake. A hyposmotic stimulus causes an increase in cell volume and triggers RVD which involves Cl^- and K^+ efflux in order to recover original cell volume (Birdsey, Boyd et al. 1999; Siman, Sibley et al. 2001) (Figures 1.5, 1.6). Importantly, this increase is mediated by a rise in $[Ca^{2+}]_i$ (van de Put, Greenwood et al. 1996). Therefore, the role of K^+ channels in STB volume regulation, in addition to the maintenance of E_m, suggests these channels are likely to be critical in mechanisms that determine STB maintenance and renewal by cellular turnover, such as cytotrophoblast proliferation, migration, syncytialisation and apoptosis. However, to date, there is scarce evidence to support this affirmation.

Many of the functions in the STB are regulated by $[Ca^{2+}]_i$; including ion transport (K^+, in particular K_{Ca}s (Clarson, Roberts et al. 2002) and Cl^- (Kibble, Greenwood et al. 1996)), endocrine secretion (hCG (Meuris, Polliotti et al. 1994; Polliotti, Lebrun et al. 1994)), NO synthesis (Myatt, Brockman et al. 1993) and amino acid transport (Karl, Chang et al. 1988; Krishna, Gude et al. 1995), among others. However, the mechanism by which $[Ca^{2+}]_i$ is controlled or the functions regulated by Ca^{2+} in the STB are still poorly understood, and although there is evidence providing relative values for $[Ca^{2+}]_i$ (using Ca^{2+}-sensitive dyes (Bax, Bax et al. 1994; Clarson, Roberts et al. 2003)) in cytotrophoblasts *in vitro*, at present there is no direct quantification of STB $[Ca^{2+}]_i$ under basal or stimulated conditions. In addition, there is controversy on the identity and characterisation of Ca^{2+}-permeable channels leading to Ca^{2+} influx (Meuris, Polliotti et al. 1994; Moreau, Simoneau et al. 2001; Moreau, Daoud et al. 2002; Clarson, Roberts et al. 2003; Bernucci, Henriquez et al. 2006) or the release of Ca^{2+} from intracellular stores.

Previous evidence shows that the stimulation of ^{86}Rb (K^+) efflux with extracellular nucleotides in isolated cytotrophoblasts and villous explants *in vitro* (Siman, Sibley et al. 2001; Clarson, Roberts et al. 2002) is mediated by an increase in $[Ca^{2+}]_i$ (Clarson, Roberts et al. 2002) (Figures 1.5, 1.6). This effect of ATP could be of pathophysiological relevance; in PE, as a result of cell damage or death mediated by factors which include altered pO_2 and oxidative stress, large amounts of ATP are released into the extracellular milieu

(Bakker, Donker et al. 2007; Roberts, Webster et al. 2007). ATP stimulated a charybdotoxin-sensitive ^{86}Rb (K^+) efflux in cytotrophoblasts implying the participation of IK_{Ca}. This efflux was highly Ca^{2+}-dependant, since the removal of extracellular Ca^{2+} or the addition of gadolinium (blocker of non-selective cation channels (NSCCs)) reduced ATP-stimulated ^{86}Rb efflux (Clarson, Roberts et al. 2002). This evidence supports a role of IK_{Ca} mediating Ca^{2+} signalling in response to increased ATP levels in PE. Other factors that raise intracellular Ca^{2+} in STB (cell volume, angiotensin II (Siman, Sibley et al. 2001), endothelin (Cronier, Dubut et al. 1999)), are also likely to affect STB K^+ channel function. Moreover, the increase in $[Ca^{2+}]_i$ caused by factors that are dysregulated in pregnancy disease could therefore alter STB K^+ channel function.

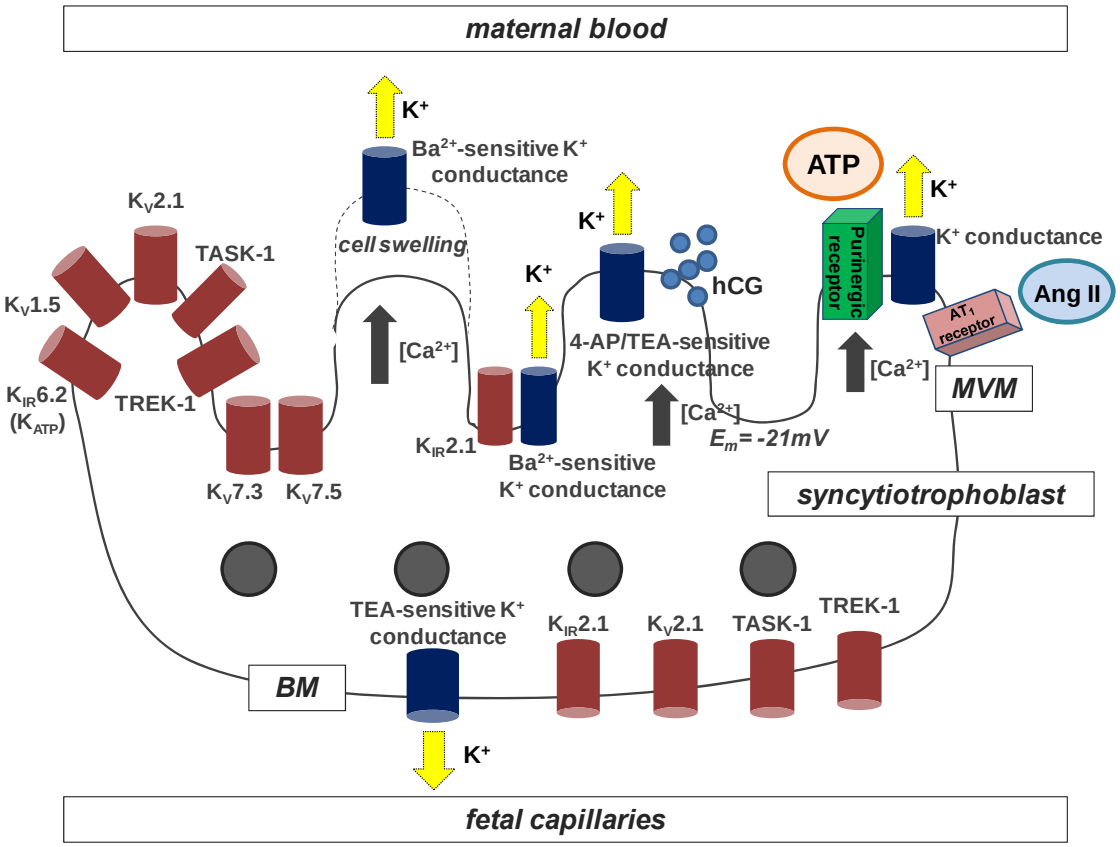

Figure 1.5: K^+ channel protein expression and functions in the STB of placental villous tissue.
Diagrammatic representation of the current understanding of K^+ channel protein expression and K^+ conductances in MVM and BM of placental STB. Blue cylinders illustrate functional studies leading to the identification of K^+ conductances in the STB MVM and BM and red cylinders illustrate K^+ channels identified in the STB at the protein level. ATP binds purinergic receptors and via intracellular signalling mechanisms, mediates an increase in $[Ca^{2+}]_i$ and consequent activation of K^+ efflux. Likewise, angiotensin II (Ang II) binding to its receptor AT_1 causing an increase in $[Ca^{2+}]_i$, thereby activating STB K^+ conductances. Information collated from Illsley and Sellers (1992) (Illsley and Sellers 1992); Simán *et al*. (2001) (Siman, Sibley et al. 2001); Birdsey *et al*. (1999) (Birdsey, Boyd et al. 1999); Williams *et al*. (2009) (Williams, Jones et al. 2009); Williams *et al*. (2008) (Williams, Fyfe et al. 2008); Mistry *et al*. (2011) (Mistry, McCallum et al. 2011); Riquelme *et al*. (2012) (Riquelme, de Gregorio et al. 2012); Lybaert *at al*. (2013) (Lybaert, Hoofd et al. 2013) and unpublished data.

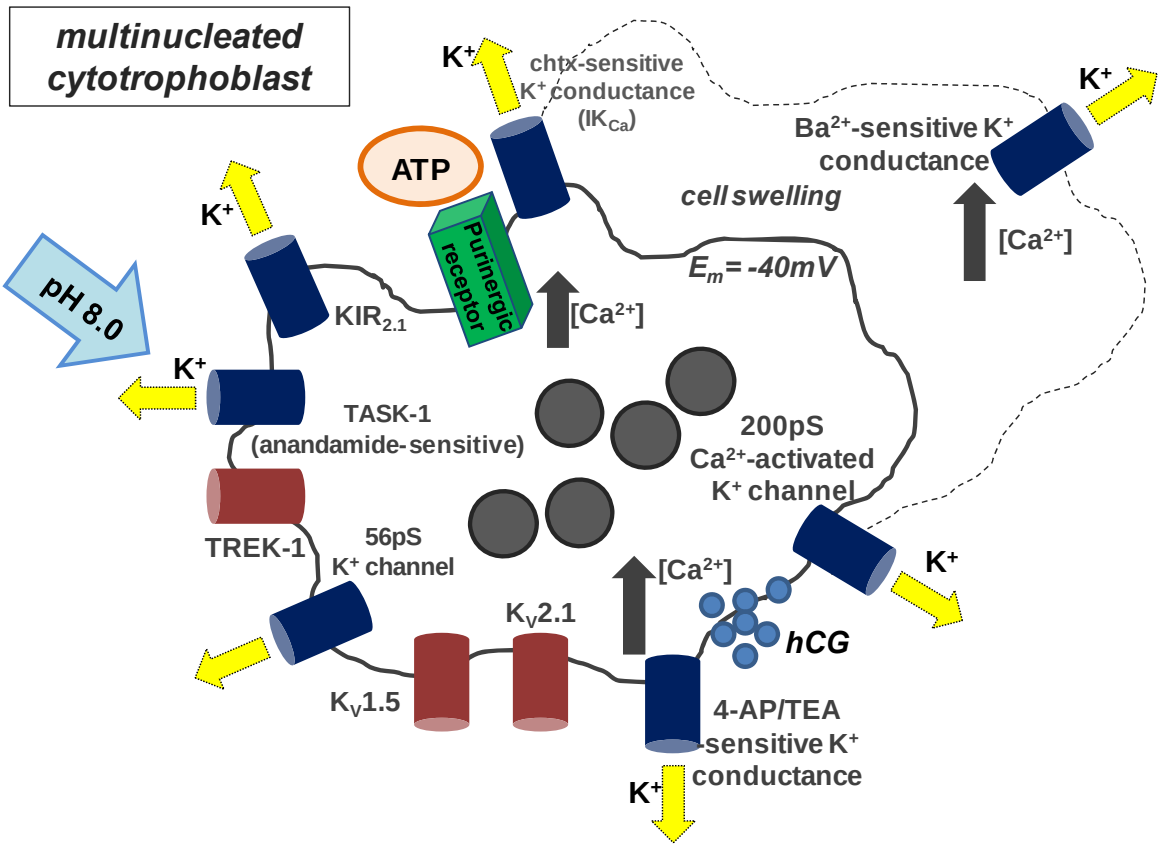

Figure 1.6: K^+ channel protein expression and functions in isolated multinucleated cytotrophoblasts.
Diagrammatic representation of the current understanding of K^+ channel protein expression and K^+ conductances in isolated multinucleated cytotrophoblasts from term placentas maintained in primary culture. Blue cylinders illustrate functional studies leading to the identification of K^+ conductances, and red cylinders illustrate K^+ channels identified in multinucleated cytotrophoblasts at the protein level. ATP binds purinergic receptors and via intracellular signalling mechanisms, mediates an increase in $[Ca^{2+}]_i$ and consequent activation of charybdotoxin (chtx)-sensitive K^+ efflux. Information collated from Greenwood *et al*. (1993, 1996) (Greenwood, Brown et al. 1993; Greenwood, Clarson et al. 1996; Greenwood, Sides et al. 1996); Van de put *et al*. (1996) (van de Put, Greenwood et al. 1996); Clarson *et al*. (2001, 2002) (Clarson, Greenwood et al. 2001; Clarson, Roberts et al. 2002); Bai *et al*. (2005) (Bai, Bugg et al. 2005) and unpublished data.

1.7.1.1. Role of syncytiotrophoblast potassium channels in human chorionic gonadotropin secretion: Implications for cell turnover

hCG secretion at term involves constitutive release (Yoshida 2005) and an exocytosis mechanism through excitation-secretion coupling which is highly Ca^{2+}-dependent (Meuris, Polliotti et al. 1994). Indeed, removal of extracellular Ca^{2+} inhibits hCG secretion from placental villous fragments (Long and Clarson 2002). In addition, hCG secretion is stimulated by hormones such as gonadotropin-releasing hormone (Lin, Roberts et al. 1995) through gonadotropin-releasing hormone receptor, a G-protein-coupled receptor, which induces intracellular Ca^{2+} mobilisation via phosphoinositide phospholipase C (Tan, Carr et al. 2013). Therefore, the regulated component of hCG secretion is modulated by factors that influence intracellular Ca^{2+}. As previously described, amongst these factors are K^+ channels.

Pharmacological blockade of STB K_Vs with 4-AP or TEA inhibited hCG secretion from placental villous explants and isolated cytotrophoblasts *in vitro* (Williams, Fyfe et al. 2008) (Figures 1.5, 1.6). 4-AP and TEA induced a significant reduction in hCG secretion, implying that K_Vs are important for regulating hCG secretion and/or production and that altered K_V expression/activity could disrupt the autocrine/paracrine regulation of STB turnover by hCG. It is hypothesised that the inhibition of K_Vs could depolarise STB E_m sufficiently to reach L-type VGCC activation threshold and consequently promote Ca^{2+} entry and hCG exocytosis (Meuris, Polliotti et al. 1994). However, to date, the mechanism and regulation of hCG secretion and/or production by the STB at term is still largely unknown.

However, direct studies have failed to identify L-type VGCCs in placental cytotrophoblasts (Bax, Bax et al. 1994; Cronier, Dubut et al. 1999). Another pathway for Ca^{2+} entry into the STB has been reported to be through NSCCs (Niger, Malassine et al. 2004). NSCCs are functionally expressed in isolated STB MVM vesicles (Llanos, Henriquez et al. 2002) and hCG secretion from placental villous fragments is inhibited when gadolinium (NSCC blocker) but not SKF96365 (store-operated Ca^{2+} channel blocker) or nifedipine (L-type VGCC blocker) is used (Long and Clarson 2002). In contrast to VGCCs, NSCCs are not activated by E_m depolarisation; Ca^{2+} entry through NSCCs is determined by the electrochemical gradient for Ca^{2+}. Consequently, E_m depolarisation decreases the driving force for Ca^{2+} entry through NSCCs.

Overall, the modulation of hCG secretion by K^+ channels implicates an important role in the regulation of placental endocrine function by contributing to the maintenance of STB renewal and homeostasis. In addition, evidence supports the fact that hCG secretion from

placental villous explants is higher in PE compared to normal pregnancies (Crocker, Tansinda et al. 2004). Therefore, it is possible that dysregulation of STB K^+ channel expression and/or activity in PE could lead to altered hCG secretion and consequently altered STB turnover.

1.8. Summary

A successful pregnancy depends on the continuous renewal of STB by a process of cell turnover which includes cytotrophoblast proliferation, migration, fusion/syncytialisation and apoptosis/autophagy. Renewal of STB is regulated by hCG which is secreted by terminally differentiated STB and has paracrine/autocrine actions to promote continued cytotrophoblast differentiation and syncytialisation. STB turnover is dysregulated in pregnancy disease, particularly in PE, which compromises STB renewal, endocrine secretion and nutrient transfer. In PE it is proposed that incomplete remodelling of the maternal spiral arteries in early placental development leads to irregular maternal blood flow into the placenta causing abnormal fluctuations in pO_2, ischaemia-reperfusion injury and an increase in the generation of free radicals. It is evident that pO_2 and free radicals dysregulate STB turnover and hCG secretion *in vitro*; however, to date, the mechanism that links them is unexplored.

In non-placental tissues, K^+ channels play a pivotal role in the maintenance of tissue homeostasis including regulation of cell volume, important for cell proliferation, apoptosis, differentiation, migration and hormone secretion. Moreover K^+ efflux across the cell membrane sets the E_m providing the driving force for the flux of other ions and solutes, particularly Ca^{2+}, thereby participating in diverse cellular signalling mechanisms. In addition, K^+ channel expression and/or activity in non-placental tissues is regulated by pO_2 and free radicals both under physiological conditions and in disease.

K^+ channel expression and function in STB is relatively poorly understood in comparison to other epithelia. However, there is evidence that K^+ channels participate in STB volume regulation and hCG secretion, suggesting that they may have a role in normal STB turnover and renewal. It is proposed that in PE, altered K^+ channel function could provide a link between pO_2 and free radicals and dysregulated hCG secretion and STB renewal.

1.9. Hypotheses and aims

This thesis addressed the overall proposal that K^+ channels participate in STB renewal and hCG secretion and that dysregulation of K^+ channels, by pO_2 and/or free radicals, could contribute to abnormal STB turnover and endocrine secretion that is a feature of PE.

To address this proposal, three specific hypotheses were tested:

Hypothesis 1:

Altered pO_2 and ROS regulate hCG secretion through an effect on STB K^+ channels

Aim: Determine whether pO_2 and ROS modulate STB hCG secretion through an effect on K^+ channels

Objectives: Using placental villous explants from term placentas:

a) To compare the effects of K^+ channel blockers on hCG secretion and ^{86}Rb efflux from explants maintained at placental normoxia (6% pO_2), hypoxia (1% pO_2) and hyperoxia (21% pO_2)
b) To investigate the effect of H_2O_2, used to generate ROS, on hCG secretion and ^{86}Rb efflux at normoxia (6% pO_2), hypoxia (1% pO_2) and hyperoxia (21% pO_2)

Hypothesis 2:

IK_{Ca} participates in the formation of multinucleate STB and;

IK_{Ca} has a role in STB volume regulation

Aims: a) Determine whether the pharmacological modulation of IK_{Ca} affects cytotrophoblast morphological and/or biochemical differentiation *in vitro* and; b) Investigate the participation of IK_{Ca} in STB RVD

Objectives: Using isolated cytotrophoblasts from term placentas maintained in primary culture:

a) To confirm IK_{Ca} protein expression
b) To test the effects of IK_{Ca} modulators on cytotrophoblast ^{86}Rb efflux, the formation of multinucleate syncytia and the secretion of hCG
c) To expose cytotrophoblasts to hyposmotic solutions and measure ^{86}Rb efflux in the presence and absence of IK_{Ca} modulators

Hypothesis 3:

IK_{Ca} are modulated by nitrative stress and;

Chronic exposure to nitrative stress regulates cytotrophoblast morphological and biochemical differentiation

Aims: a) Determine whether nitrative stress ($ONOO^-$) has an acute effect on cytotrophoblast K^+ permeability and; b) determine whether long-term exposure to nitrative stress affects cytotrophoblast morphological and/or biochemical differentiation *in vitro*

Objectives: Using isolated cytotrophoblasts from term placentas maintained in primary culture:

a) Determine the effect of $ONOO^-$ on cytotrophoblast basal ^{86}Rb efflux and efflux following altered cell volume status
b) Determine whether long-term exposure to nitrative stress in culture affects cytotrophoblast morphological and biochemical differentiation *in vitro*

2. MATERIALS AND METHODS

2.1. Placenta collection

Human placentas used in this study were obtained from St. Mary's Hospital Maternity Unit (Manchester, UK) following written informed consent as approved by the Local Research Ethics Committee. Normal term placentas (37-42 weeks gestation) were obtained from uncomplicated pregnancies following vaginal delivery or Caesarean section.

2.1.1. Chemicals

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich (Dorset, UK).

2.2. Isolation of fresh placental villous tissue

Within 30min of delivery, four chorionic villous sections (1.5cm^3) were obtained and rinsed in Tyrode's buffer (135mM NaCl, 5mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 5.6mM glucose, pH 7.4; osmolality 285mOsm/kgH_2O). Villous tissue was further dissected into fragments (3-5mm^3; as seen in Figure 1.1.C) and rinsed in Tyrode's buffer to remove excess blood.

2.2.1. ^{86}Rb efflux from fresh placental villous fragments

^{86}Rb permeates most K^+-selective channels and it has been used as a tracer to indirectly assess K^+ permeability (Bland and Boyd 1986) of the STB (Boyd 1983; Siman, Sibley et al. 2001). ^{86}Rb efflux was measured in fresh villous fragments from term human placenta using a technique previously described by Simán *et al.* (2001) (Siman, Sibley et al. 2001).

Each villous fragment was suspended on cotton threads and tied to hooks (Figure 2.1.A). Subsequently, fragments were incubated for 2h at 37°C in 1ml Tyrode's buffer containing 4μCi/ml ^{86}Rb (89.7μM; specific activity ~15mCi/mg; concentration 1μCi/ml; stock activity 1mCi; PerkinElmer, MA, USA). After incubation, fragments were washed in 15ml Tyrode's buffer (with no added isotope) twice for 5min each.

2.2.1.1. Treatments

After washing, fragments were passed through vials containing 4ml Tyrode's buffer every 2min for 16min at 37°C (control, basal ^{86}Rb efflux; Figure 2.1.B); or were subjected to

different treatments starting from min 10 onwards (experimental period; detailed in Table 2.1), with the exception of a) the free radicals H_2O_2 and $ONOO^-$, which were added directly to Tyrode's buffer at each collection period in advance, starting from min 8 onwards and, b) when using a treatment in combination with a K^+ channel activating/opening-stimulus (i.e. hyposmotic solution, DCEBIO), a pre-block with a K^+ channel modulator was performed in advance, starting from min 8 onwards. All measurements were done in triplicate.

Finally, the tissue was lysed in deionised water for 18h in order to release intracellular non-membrane bound ^{86}Rb which was then measured in the supernatant to give a measure of total ^{86}Rb remaining in the tissue at the end of the experiment (tissue ^{86}Rb; Figure 2.1.C).

Treatment		Specification
Tyrode's buffer (control)		135mM NaCl, 5mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES, 5.6mM glucose, pH 7.4; osmolality 285mOsm/kgH_2O
Hyposmotic solution	**Low strength hyposmotic**	55mM NaCl, 5mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES, 5.6mM glucose; pH 7.4; osmolality ~145mOsm/kgH_2O
	High strength hyposmotic	95mM NaCl, 5mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES, 5.6mM glucose; pH 7.4; osmolality ~220mOsm/kgH_2O
Free radicals	**ROS**	**H_2O_2** (10μM) in Tyrode's buffer
		H_2O_2 (10μM) in low strength hyposmotic solution
		H_2O_2 (10μM) in high strength hyposmotic solution
	RNS	**$ONOO^-$** (0.1mM) in Tyrode's buffer
		$ONOO^-$ (0.1mM) in low strength hyposmotic solution
		$ONOO^-$ (0.1mM) in high strength hyposmotic solution
K^+ channel modulators	**K^+ channel blockers**	**Ba^{2+}** (as $BaCl_2$, 5mM, broad spectrum K^+ channel blocker) in Tyrode's buffer
		4-aminopyridine (**4-AP**; 5mM, K_V blocker) in Tyrode's buffer
		4-AP (5mM) in high strength hyposmotic solution
		Tetraethylammonium (**TEA**; 5mM, K_V/K_{Ca} blocker) in Tyrode's buffer
		TEA (5mM) in high strength hyposmotic solution
		1-[(2-Chlorophenyl)diphenylmethyl]--1H-pyrazole (**TRAM-34**; 10μM, IK_{Ca} blocker) in Tyrode's buffer
	K^+ channel openers	5,6-Dichloro-1-ethyl-1,3-dihydro-2H--benzimidazol-2-one (**DCEBIO**; 100μM, IK_{Ca}/SK_{Ca} opener) in Tyrode's buffer

Table 2.1: Treatments used for ^{86}Rb efflux experiments in fresh placental villous fragments

For the treatments listed in Table 2.1:

i. The osmolality of Tyrode's buffer was measured in an osmometer by freezing point depression (Roebling MOD 100 Osmometer, Cambridge, UK) using 300mOsm/kgH_2O standards.

ii. H_2O_2 (authentic) was obtained from stock solution 1mM H_2O_2 (dissolved in Tyrode's buffer, protected from light). During the experimental period, 40μl from the stock were added into each vial (which already had Tyrode's buffer or hyposmotic solution) 1min in advance before submerging the respective fragments. The final concentration in each vial was 10μM H_2O_2. The rationale for using this concentration is based on an average between a) the circulating serum H_2O_2 concentration found in pregnant women (30μM) and women undergoing PE (45μM) (Kharfi, Giguere et al. 2005) and b) the low concentrations of H_2O_2 (0-50μM) that enhance hCG secretion in cytotrophoblasts isolated from term placentas. 5μM H_2O_2 produces the maximum stimulatory effect (Kharfi Aris, Leblanc et al. 2007). The half-life of H_2O_2 is 30s (Bocci 2012).

iii. $ONOO^-$ (authentic) activity can decay throughout time during storage. Consequently, the molar concentration of $ONOO^-$ stock solution (Millipore, CA, USA) was determined by diluting the stock between 100-1000-fold into 0.3M NaOH and measuring spectrophotometrically the increase in absorbance at 302nm with a given extinction coefficient of $1670M^{-1}cm^{-1}$. The concentration of the stock solution was ~110mM. $ONOO^-$ was then stored in 10mM working aliquots (diluted in 0.3M NaOH + 0.3M NaCl; 1:1 ratio respectively; protected from light) and stored at -80°C. For each ^{86}Rb efflux experiment, a 10mM $ONOO^-$ aliquot was thawed and during the experimental period, 40μl were added directly to each vial (which already had Tyrode's buffer or hyposmotic solution) 1min in advance before submerging the respective fragments. The final concentration in each vial was $10^{-4}M$ (0.1mM) $ONOO^-$. $ONOO^-$ concentration has been shown to reach nanomolar values in erythrocytes, being higher in erythrocytes from PE women (~250nM) compared to women from uncomplicated pregnancies (~200nM) (Đorđević, Babić et al. 2008). However, the rates of $ONOO^-$ production *in vivo* have been reported to reach as high as 50-100μM/min (Szabo, Ischiropoulos et al. 2007). In addition, $10^{-4}M$ $ONOO^-$ (but not 10^{-5} or 10^{-6} $ONOO^-$) induced relaxation in preconstricted placental chorionic plate arteries within 2min of exposure (Mills, Wareing et al. 2009). These findings gave the rational for the use of $ONOO^-$ at a

concentration of 10^{-4}M (0.1mM). $ONOO^-$ half-life is less that 100ms (Denicola, Souza et al. 1998; Radi, Peluffo et al. 2001).

iv. Ba^{2+} (as $BaCl_2$; BDH Laboratory Supplies, Poole, UK) is a broad-spectrum K^+ channel blocker. At a concentration of 5mM, used in this study, Ba^{2+} has previously been shown to inhibit basal ^{86}Rb efflux from placental villous explants, implicating K^+ conductances in the MVM of the STB. Basal ^{86}Rb efflux was measured at 2-day intervals (up to 7 days) and decreased with time in culture indicating a progressive fall in K^+ permeability as the STB regenerated (Siman, Sibley et al. 2001).

v. 4-AP (K_V blocker) was prepared from a 100mM 4-AP stock solution (diluted in deionised water and adjusted to pH 7.4). This stock was diluted 1:20 in Tyrode's buffer or hyposmotic solution to obtain a final concentration of 5mM 4-AP. Vials were pre-filled with this treatment for each ^{86}Rb efflux experiment. A role for STB K_Vs in regulating hCG secretion from placental villous tissue and cytotrophoblasts has been previously demonstrated with 5mM 4-AP, the concentration used in this study, producing the maximal inhibitory effect on hCG secretion without affecting tissue integrity (Williams, Fyfe et al. 2008).

vi. TEA (K_V/K_{Ca} blocker) was dissolved directly in Tyrode's buffer or hyposmotic solution, obtaining final concentration of 5mM TEA. After dissolving, initial pH 7.4 was maintained. Vials were pre-filled with these treatments for each ^{86}Rb efflux experiment. According to the specificity and the concentration of TEA used in this study, the targeted K^+ channels belong mainly to the K_V family (Coetzee, Amarillo et al. 1999; Gutman, Chandy et al. 2005). Additional evidence indicates that chronic exposure to TEA induced a concentration-dependent inhibition of hCG secretion in cultured villous explants and cytotrophoblasts. 5mM TEA, the concentration used in this study, produced the maximal inhibitory effect on hCG secretion without affecting tissue integrity (Williams, Fyfe et al. 2008).

vii. TRAM-34 (IK_{Ca} blocker) was prepared from a 10mM stock solution dissolved in dimethyl sulfoxide (DMSO). This stock was diluted 1:1000 in Tyrode's buffer to obtain a final concentration of 10μM TRAM-34. The final concentration of DMSO in Tyrode's buffer was 0.1%. Vials were pre-filled with this solution for each ^{86}Rb efflux experiment. The rationale for using 10μM TRAM-34 was based on previous evidence showing that this concentration inhibited cell proliferation in human embryonic kidney 293 cells expressing recombinant IK_{Ca} (Millership, Devor et al. 2011). In addition, 10μM TRAM-34 completely

abolished 1-EBIO-enhanced whole cell currents from placental chorionic plate arteries smooth muscle cells (Brereton, Wareing et al. 2013).

viii. DCEBIO (IK_{Ca}/SK_{Ca} opener) was prepared from a 100mM stock solution dissolved in DMSO. Before each experiment, a stock aliquot was thawed and pre-diluted 1:10 in Tyrode's buffer (10mM DCEBIO). The latter was diluted once more 1:100 in Tyrode's buffer, giving a final concentration of 100μM DCEBIO used to pre-fill the vials for each ^{86}Rb efflux experiment. The final concentration of DMSO in Tyrode's buffer was 0.1%. The rationale for using 100μM DCEBIO in this study was based on initial findings in human embryonic kidney 293 cells expressing IK_{Ca} where 100μM 1-EBIO (DCEBIO analog) activation strongly hyperpolarised Ca^{2+}-dependent cell currents (Pedersen, Schroder et al. 1999).

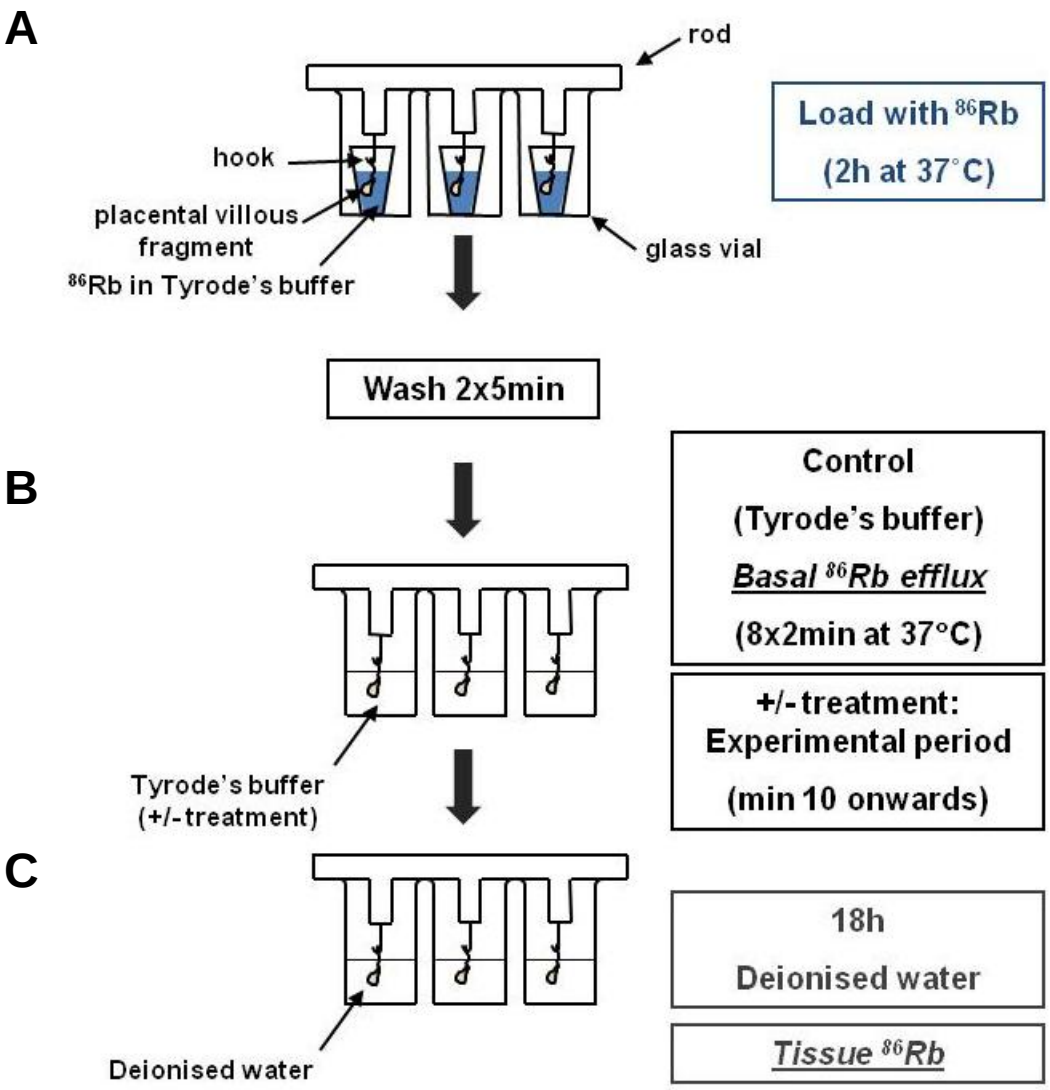

Figure 2.1: Schematic representation of the procedure for measurement of ^{86}Rb efflux in villous fragments from term human placenta

2.2.1.2. ^{86}Rb isotope counting and data analysis

^{86}Rb isotope is a high energy beta and gamma emitter. ^{86}Rb has an energy spectrum that can be identified using a beta-counter to measure the energy decay of this isotope.

Each efflux vial containing Tyrode's buffer was filled with 16ml scintillation fluid (ScintiSafe 2; Fisher Scientific, Loughborough, UK). In addition, background counts were prepared with 4ml water and filled with scintillation fluid.

Effluxed and tissue ^{86}Rb was measured in a beta-counter (Packard 2000, CA, USA). Each vial was counted for 2min. All counts recorded were at least 10 times higher than background counts.

The time course of percentage (%) ^{86}Rb efflux was calculated as:

$$\%\frac{efflux}{2min} = \left(\frac{86Rb\ effluxed}{86Rb\ in\ tissue}\right) \times 100$$

The efflux rate constant was also determined, making the assumption that, in the control, ^{86}Rb efflux at steady state reflects the loss of ^{86}Rb from a single compartment (STB) limited by the K^+ permeability of the MVM. Consequently, the loss of ^{86}Rb was measured by a first-order rate constant which was calculated over the 8min experimental period as:

$$l_n\left(\frac{86Rb\ in\ tissue\ at\ time\ t}{86Rb\ in\ tissue\ at\ start}\right)$$

2.3. Culture of placental villous explants

Term placental villous tissue maintained in explant culture is a well characterised model (Siman, Sibley et al. 2001) which has been used extensively to study the chronic effects of regulators on STB biology (Sooranna, Oteng-Ntim et al. 1999; Crocker, Tansinda et al. 2004; Turner, Roulstone et al. 2006; Heazell, Moll et al. 2007; Moll, Jones et al. 2007; Heazell, Lacey et al. 2008; Fogarty, Ferguson-Smith et al. 2013).

The method for culture of placental villous explants at term has been published elsewhere (Siman, Sibley et al. 2001; Williams, Fyfe et al. 2008). Briefly, within 30min of delivery, chorionic villous sections (1.5cm^3) were obtained and rinsed in sterile phosphate-buffered saline (PBS; with $CaCl_2$ and $MgCl_2$). Villous tissue was further dissected into explants (3-5mm^3; as seen in Figure 1.1.C) and rinsed three times in PBS to remove excess blood. Explants were then placed in 74μm-polyester mesh Netwells, in 15mm insert 12-well plates. Three explants and 1.5ml of explant culture medium (10% CMRL-1066, 100μg/ml

streptomycin sulphate, 100IU/ml penicillin-G, 0.1μg/ml hydrocortisone, 0.1μg/ml retinol acetate, 0.1μg/ml insulin, 100μg/ml L-glutamine, 2.2mg/ml $NaHCO_3$, 5% fetal calf serum (FCS), pH 7.2) were placed into each well, with the tissue supported on the mesh at the liquid-gas interface.

As previously described, the amount and/or type of free radical (ROS/RNS) generated, and their effects, will be influenced by placental oxygenation. Consequently, in this study, the effects of different pO_2 were tested in order to reflect physiological and pathophysiological pO_2 in placenta ranging from hyperoxia to hypoxia. Villous explants were cultured at 37˚C in humidified incubators at 6% pO_2 (with 5% CO_2/ balance N_2; normoxic for term placenta (Burton and Caniggia 2001; Miller, Genbacev et al. 2005; Tuuli, Longtine et al. 2011), 40-50mmHg; assuming 1atm= 760mmHg); 21% pO_2 (with 95% air/5% CO_2; hyperoxia for term placenta, 160mmHg) or 1% pO_2 (with 5% CO_2/ balance N_2; hypoxia for term placenta, 7.6mmHg) for 6 days (placenta collection= day 0; explant harvesting= day 6).

2.3.1. Treatments

Culture medium was replaced daily and fresh medium was pre-equilibrated (24h in advance) at each pO_2 before addition to explants. On days 3-5, explants were untreated (control) or treated daily with K^+ channel modulators or free radicals shown in Table 2.2.

Treatment		Specification
Free radicals	**ROS**	**H_2O_2** (10, 100μM and 1mM)
	RNS	**$ONOO^-$** (0.1mM)
		3-morpholinosydnonimine hydrochloride (**SIN-1**, 2.5mM)
K^+ channel modulators	**K^+ channel blockers**	**4-AP** (5mM, K_V blocker)
		TEA (5mM, K_V/K_{Ca} blocker)
	K^+ channel openers	**DCEBIO** (100μM, IK_{Ca}/SK_{Ca} opener)
		Flupirtine (as flupirtine maleate, 100μM; K_V7 opener)

Table 2.2: Treatments added to culture medium of placental villous explants

For the treatments listed in Table 2.2:

i. H_2O_2 (authentic) at final concentration 10, 100μM and 1mM was obtained from 1, 10 and 100mM H_2O_2 stock solutions respectively (diluted in sterile PBS). 15μl from each stock solution was added to the explant culture medium. These H_2O_2 concentrations have been previously tested in short term explants culture (4 days) to assess effects on STB turnover (Heazell, Moll et al. 2007; Moll, Jones et al. 2007). In isolated cytotrophoblasts from term placentas, hCG secretion was inhibited at high (>50μM) but markedly stimulated with low (1-50μM) H_2O_2 levels (Kharfi Aris, Leblanc et al. 2007).

ii. $ONOO^-$ (authentic) at a final concentration of 0.1mM was added from stock solution 10mM $ONOO^-$ (prepared as described in section 2.2.1.1.). 15μl from this stock was added to the explant culture medium.

iii. SIN-1 (Alexis Biochemicals, Nottingham, UK) generates O_2^- and NO which react to form $ONOO^-$. SIN-1 was prepared from a 500mM stock solution (diluted in sterile PBS). 7.5μl was added to the explant culture medium in order to obtain a final concentration of 2.5mM. The rationale for using this concentration was that SIN-1 has been reported to impair nutrient transport from isolated MVM vesicles and placental villous fragments in concentrations ranging 2.5-5mM (Khullar, Greenwood et al. 2004).

iv. K_V blockers 4-AP and TEA were both prepared as 500mM stock solutions in sterile PBS. 15μl was added to explant culture medium to obtain a final concentration of 5mM respectively. As described in section 2.2.1.1., 5mM 4-AP/TEA have been shown to produce the maximal inhibitory effect on hCG secretion from placental villous explants without affecting tissue integrity (Williams, Fyfe et al. 2008).

v. DCEBIO (IK_{Ca}/SK_{Ca} opener) and flupirtine (as flupirtine maleate, K_V opener) were both initially dissolved in DMSO to prepare a 10mM stock solution. 15μl from each stock solution was added to the explant culture medium in order to reach a final concentration of 100μM. The final concentration of DMSO was 1%; previous experiments in placental villous explants treated with DMSO as a control, have showed that it was without effect at this concentration (Williams, Fyfe et al. 2008). Flupirtine (between 10-30μM) was been previously shown to activate K_V7 subfamily members, producing acute relaxation in mice pulmonary arteries (Morecroft, Murray et al. 2009).

Explant culture medium was collected daily and stored at -20˚C before measuring hCG secretion (marker of STB differentiation; detailed in section 1.3.3.1.) and lactate dehydrogenase (LDH; released from necrotic cells and used as marker of cellular viability). On day 6 explants were dissolved in 4ml 0.3M NaOH at 37˚C for 24h to measure protein content. Otherwise explants were placed into 1.5ml water for 18h at room temperature to lyse for measurement of cell hCG/LDH. The supernatant was collected and stored at -20˚C, and explants were dissolved into 4ml 0.3M NaOH. These samples were used to measure protein content with Bio-Rad Protein Assay (Bio-Rad Laboratories, Hempstead, UK).

In addition at day 6, villous explants were fixed in 4% neutral buffered formalin (1.36g KH_2PO_4, 5.68g Na_2HPO_4 dissolved in 500ml (250ml deionised water, 200 tap water and 50ml 37% formaldehyde)) at 4˚C for 24h, following 2 washes in tris-buffered saline (TBS) prior to standard histological processing and paraffin embedding. These samples were sectioned (5μm) in a microtome, and the obtained sections were subsequently deparaffinised and rehydrated before performing standard nuclear and cytoplasmic histological staining with hematoxylin and eosin respectively. Light microscopy images were visualised using an Olympus BX41 microscope and images captured using Image-Pro Plus 7 software. Each image was morphologically assessed by two observers for general morphological preservation and categorised according to the presence of STB regeneration (yes/no) and syncytial knots (high/low).

2.3.2. Protein assay

After dissolving placental villous explants in 0.3M NaOH at day 6, samples were stored at 4°C until assaying for protein content. Before performing each assay, samples were allowed to reach room temperature.

Protein standards were prepared using bovine serum albumin (BSA) in 0.3M NaOH. Plots of absorbance against protein content were generated and analysed by linear regression to give a standard curve (Figure 2.2). Protein content was measured using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hempstead, UK).

The assay was performed using a flat-bottom 96-well plate with 20μl of each standard and samples added in duplicates. Second, 180μl of neutralizing solution (0.3M NaOH + 0.3M HCl, 1:1.25 respectively) was added to each well. Finally, 50μl of the Bio-Rad reagent was added and mixed into each well. This is a colorimetric assay adapted from the Bradford method (Bradford 1976), based on an absorbance shift in the dye Coomassie when the previous red form Coomassie reagent changes and stabilises into Coomassie blue by the binding of protein. Therefore, the amount of complex present in solution is a measure of

the protein concentration. Optical density was measured at 595nm using a VersaMax microplate reader (Molecular Devices, CA, USA).

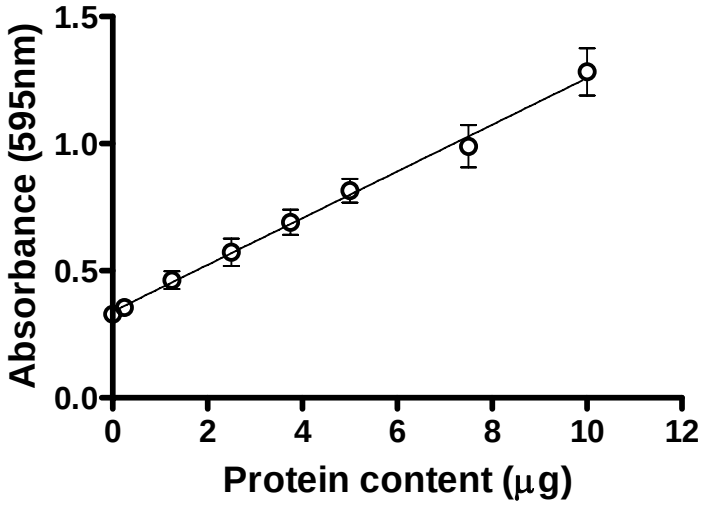

Figure 2.2: Standard curve for protein assay.
Values corresponding to optical units at 0, 0.25, 1.25, 2.5, 3.75, 5, 7.5 and 10μg BSA were analysed by linear regression to produce a standard curve. Each unknown value was interpolated from the standard curve. Correlation coefficient (r^2) 0.97; $p<0.0001$. Values are representative from one protein assay. Data are mean ± standard deviation (SD).

2.3.3. Human chorionic gonadotropin assay

hCG was assayed in daily collected explant-conditioned culture medium using an enzyme-linked immunosorbent assay (ELISA) (DRG Diagnostics, Marburg, Germany). In addition to hCG secreted during culture, cellular hCG was measured in the supernatant of villous explants lysed in water at day 6 of culture. Thawed samples were used following the instructions of the manufacturer. hCG standards are supplied with this commercial kit. Figure 2.3 shows the average standard curve obtained with the standards provided with this kit, used to calculate unknown values.

hCG hormone (both, α and β-hCG subunits) was detected using a 96-microtiter well plate kit. The wells were coated with a monoclonal antibody against a unique antigenic site on the hCG molecule. The sample was then added to these coated wells. Afterwards, the enzyme conjugate, a monoclonal antibody directed against hCG α-subunit conjugated with horseradish peroxidase, was added. After this incubation period, the unbound conjugate was washed off. The amount of bound peroxidase was proportional to the concentration of hCG in the sample. Finally, after the addition of a substrate solution, the intensity of colour developed proportional to the concentration of hCG in the sample. Optical density was measured at 450nm using a VersaMax microplate reader. hCG secretion was expressed as mIU/ml/h/mg protein.

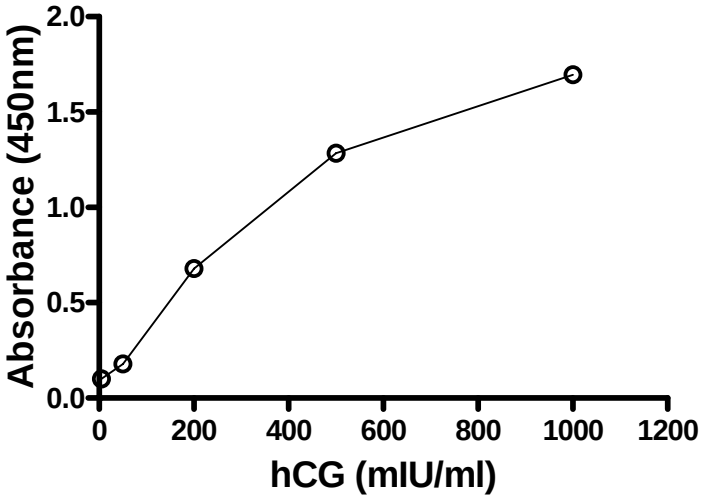

Figure 2.3: Standard curve for hCG assay.
Values corresponding to optical units at 5, 50, 200, 500 and 1000mIU/ml were extrapolated to a double logarithmic plot in order to calculate unknown values. Intra-assay coefficient of variation (CV) 3.5-4.7%. Inter-assay CV 3.3-4.3%. r^2 0.93, $p<0.0001$. Average between duplicate values from one representative hCG assay. Data are mean ± SD.

2.3.4. Lactate dehydrogenase assay

To assess cell viability in cultured placental villous explants, LDH release was measured using a cytotoxicity detection kit (Roche Diagnostics, Mannheim, Germany) in thawed explant-conditioned medium and in the supernatant of water-lysed explants (obtained at day 6).

As standards were not provided with the kit, a standard curve was generated using L-lactic dehydrogenase from rabbit muscle (initial stock concentration of 100U/ml) as an internal control, showing an established linear relationship between LDH and absorbance. This standard curve was not used to calculate the amount of LDH in each sample (i.e. LDH amount decreases with cold storage).

Briefly, each assay was performed in a flat-bottom 96-well plate. Each well was initially filled with 100μl of non-sterile explant culture medium, followed by the addition of 100μl of standards and samples. Then, 100μl of reaction mix (provided in the kit) was added to each well and incubated for 30min in the dark. In a first step, this reagent acted by a mechanism which involved the reduction of nicotinamide adenine dinucleotide (NAD^+) to $NADH/H^+$ by the LDH catalysed conversion of lactate to piruvate. In a second step, the catalyst (diaphorase) transferred H/H^+ from $NADH/H^+$ to the tetrazolium salt INT which was reduced to formazan. An increase in the amount of necrosis or plasma-membrane damaged cells resulted in an increase of the LDH enzyme activity in the sample which directly correlated to the amount of formazan produced. Therefore, the amount of colour formed in the assay was proportional to the number of necrotic/lysed cells. Finally, 50μl stop solution (1N HCl) was added to each well. Optical density was measured at two different wavelengths, 492 and 690nm using a VersaMax microplate reader. LDH levels were calculated as the difference between the absorbance measured at 492nm and 690nm. LDH release was expressed as absorbance units/mg protein/h.

2.3.5. ^{86}Rb efflux from placental villous explants

At day 6 of culture, villous explants were briefly rinsed in Tyrode's buffer and suspended and tied to hooks. Afterwards, fragments were incubated for 2h at 37°C in Tyrode's buffer containing 4μCi/ml ^{86}Rb (89.7μM) (Figure 2.1.A). After incubation, fragments were washed in 15ml Tyrode's buffer (with no added isotope) twice for 5min each. Basal ^{86}Rb efflux was then measured by changing and collecting 4ml Tyrode's buffer every 2min for 10min at 37°C (Figure 2.1.B). All measurements were done in triplicate.

Finally, villi were lysed in water for 18h to release intracellular non-membrane bound ^{86}Rb which was then measured in the supernatant to give a measure of total ^{86}Rb remaining in the tissue at the end of the experiment (^{86}Rb in tissue; Figure 2.1.C).

Effluxed and tissue ^{86}Rb was measured in a beta-counter. All counts recorded were at least 10 times higher than background counts. The counts obtained were analysed as described in section 2.2.1.2., with the exception that the loss of ^{86}Rb was measured by a first-order rate constant calculated over the 16min experimental period.

2.4. Cytotrophoblast isolation

The isolation and culture of cytotrophoblasts from normal term placentas is a well-characterised method (Greenwood, Clarson et al. 1996; Clarson, Greenwood et al. 2001; Bai, Greenwood et al. 2005; Williams, Fyfe et al. 2008) based on that of Kliman *et al*. (1986) (Kliman, Nestler et al. 1986). Briefly, full thickness samples (~2cm^3) of a placenta were taken within 30min of delivery and placed into sterile saline (0.9% NaCl) at room temperature. Placental villous tissue was dissected from each sample after removal of the chorionic plate and decidua. The tissue was further washed in saline to remove maternal blood and then finely minced, removing larger blood vessels and fibrinoid deposits. The tissue was strained using sterile gauze, weighed and 30g of villous tissue were digested in warmed 150ml Ca^{2+}/Mg^{2+}-free-Hank's balanced salt solution (HBSS; 5.3mM KCl, 0.4mM KH_2PO_4, 138mM NaCl, 0.2mM Na_2HPO_4, 5.6mM glucose) containing 1M HEPES (pH 7.4), 2.5% trypsin and 0.2mg/ml deoxyribonuclease I (DNAse; from bovine pancreas) for 30min at 37°C (in a shaking oven at 80rpm). 100ml of the supernatant were removed without disturbing the tissue and layered over 5 ml newborn calf serum (NCS). The digestion with DNAse and trypsin was repeated twice with 100 and 75ml HBSS the second and third time respectively, for 30min at 37°C (80rpm). After the second and third digestion, the supernatant was also layered over NCS. All these samples were spun using a Beckman Coulter Spinchron R-centrifuge for 10min at 2200rpm at 18-20˚C. After each centrifugation, the supernatant of each sample was discarded and the remaining pellet was resuspended

in warmed 1ml Dulbecco's modified Earle's medium (DMEM; Invitrogen, Paisley, UK). All the resuspended pellets were pooled and centrifuged for 10min at 2200rpm (18-20˚C). The supernatant was discarded and the pellet resuspended in 6ml DMEM and layered onto a discontinuous Percoll density gradient (from bottom to top: 70, 60, 55, 50, 45, 40, 35, 30 and 20% Percoll made up in HBSS) and centrifuged for 30min at 2800rpm (18-20˚C). After spinning, the bands between 35-55% Percoll corresponding to cytotrophoblasts were obtained and mixed with cell culture medium (DMEM: Ham's F-12 Nutrient Mixture (F-12; Invitrogen, Paisley, UK) diluted 1:1, supplemented with 10% FCS (heat inactivated), 1% gentamicin, 0.2% benzylpenicillin, 0.2% streptomycin and 0.6% glutamine) and then centrifuged at 2200rpm for 10min. This pellet was resuspended in 2ml of cell culture medium. A cell count of 50-100x10^6 cells/ml was typically obtained and plated in a) 16mm-glass coverslips placed inside 12-well plates (density 1-1.3x10^6 cells/ml) or, b) p35 (35mm) dishes (Nunc, Fisher Scientific, Loughborough, UK; density 1x10^6 cells/ml). The isolated cells were placed in a humidified incubator at 37°C with 95% air/5% CO_2.

2.4.1. Cytotrophoblast culture and treatment

Cytotrophoblasts plated onto coverslips were cultured for 66h. Cells were washed three times in PBS and cell culture medium was replaced with fresh medium at 15 and 42h.

Cells were untreated (control) or treated at 3, 15 and 42h with the treatments listed in Table 2.3.

Treatment		Specification
IK_{Ca} modulators	**IK_{Ca}/SK_{Ca} opener**	**DCEBIO** (100μM)
	IK_{Ca} blocker	**TRAM-34** (10μM)
Free radicals	**RNS**	**$ONOO^-$** (0.1mM)
		SIN-1 (0.5mM)

Table 2.3: Treatments added to culture medium of cytotrophoblasts

For the treatments listed in Table 2.3:

i. TRAM-34 was prepared from a 10mM stock solution dissolved in DMSO which was pre-diluted 1:10 in PBS (working solution; 1mM TRAM-34). 10μl aliquots from this working solution were added directly to cell culture medium (1:100 dilution) to obtain a final concentration of 10μM TRAM-34. The final concentration of DMSO in the cell culture medium was 0.1%.

ii. DCEBIO was prepared from a 100mM stock solution which was pre-diluted 1:10 in PBS (working solution; 10mM DCEBIO). 10μl aliquots from this working solution were added directly to cell culture medium (1:100 dilution) to obtain a final concentration of 100μM DCEBIO. The final concentration of DMSO in the cell culture medium was 0.1%.

iii. $ONOO^-$ (authentic) at a final concentration of 0.1mM was added from stock solution 10mM $ONOO^-$ (prepared as described in section 2.2.1.1.). 10μl aliquots from this stock were added to the cell culture medium.

iv. SIN-1 was prepared from a 100mM stock solution (diluted in sterile PBS). 5μl aliquots were added to the cell culture medium in order to obtain a final concentration of 0.5mM. 2.5mM, the concentration of SIN-1 previously used to treat placental villous explants, together with 1mM SIN-1, proved to be toxic when applied to cytotrophoblasts as indicated by a) impairing cell adhesion to the coverslips during the first hours of culture after treatment at 3h and/or b) the remaining cells, were unable to aggregate and maintained a mononuclear state during the remainder of time in culture. 0.5mM SIN-1 was the minimum effective concentration that did not produce these effects and therefore was used in cytotrophoblasts.

At 15, 42 and 66h of culture, cell culture medium was collected and stored at -20˚C for measurement of hCG secretion (β-hCG; to assess cytotrophoblast biochemical differentiation) and coverslips were submerged into 1ml 0.3M NaOH, cells scraped and the cell lysate was stored at 4˚C for measurement of protein content.

Also, at 15, 42 and 66h of culture, after coverslips were washed with PBS, cells were fixed in methanol (absolute; permeabilising fixative) for 20min at -20˚C or in 4% paraformaldehyde (PFA; dissolved in PBS; non-permeabilising fixative) for 15min at room temperature. These coverslips were rinsed in PBS after fixing and stored in PBS at 4˚C for immunofluorescence and analysis.

2.4.1.1. Cytotrophoblast protein assay

After cells were lysed in 0.3M NaOH at 15, 42 and 66h of culture, they were stored at 4°C until assayed for protein content.

Protein content determination was performed as previously described in section 2.3.2. using a Bio-Rad Protein Assay. A standard curve with values corresponding to absorbance at 0, 0.25, 1.25, 2.5, 3.75 and 5μg BSA were analysed by linear regression to calculate unknown values and optical density was measured at 595nm using a VersaMax microplate reader.

2.4.1.2. Cytotrophoblast β-human chorionic gonadotropin assay

β-hCG was assayed in collected cell-conditioned culture medium at 15, 42 and 66h of culture using an ELISA (DRG Diagnostics, Marburg, Germany). This assay allowed the specific detection of β-hCG subunit, which is secreted by differentiated STB.

Samples were thawed before performing the assay. The procedure resembles that described in section 2.3.3. Standards were 0, 5, 25, 50, 100 and 200mIU/ml, which were extrapolated to a double logarithmic plot in order to calculate unknown values. Intra-assay CV 3.4-8.9%. Inter-assay CV 6.6-9.9%.

Briefly, β-hCG was detected using a 96-microtiter well plate kit which specifically detected β-hCG subunit from hCG. The wells were coated with a monoclonal antibody against a unique antigenic site on the β-hCG molecule. The sample was then added to these coated wells. Afterwards, the enzyme conjugate, a monoclonal antibody directed against hCG β-subunit conjugated with horseradish peroxidase, was added. After this incubation period, the unbound conjugate was washed off. The amount of bound peroxidase was proportional to the concentration of β-hCG/hCG in the sample. Finally, after the addition of a substrate solution, the intensity of colour developed proportional to the concentration of β-hCG/hCG in the sample. Optical density was measured at 450nm using a VersaMax microplate reader. β-hCG secretion was expressed as mIU/ml/h/mg protein.

2.4.1.3. Cytotrophoblast immunocytochemistry

Cytotrophoblasts were fixed and the detection and immunolocalisation of different markers were assessed by immunofluorescence.

2.4.1.3.1. Cytotrophoblast multinucleation: desmoplakin immunostaining

Cytotrophoblast morphological differentiation was assessed by immunostaining of desmoplakin and nuclei staining. Desmoplakin is a protein associated with desmosomal

complexes forming part of intercellular junctions. The immunodetection of desmoplakin to reveal cell boundaries allows the visualisation of cytotrophoblast multinucleation (Douglas and King 1990).

Briefly, methanol-fixed cells on coverslips were placed onto microscope slides and washed 3x5min with TBS. Non-specific binding block was performed for 30min with 4% BSA in TBS at room temperature. Afterwards, cells were incubated for 1h at room temperature with mouse monoclonal (clone 2Q400) antibody to desmoplakin I+II (Abcam, Cambridge, UK), diluted 1:100 in TBS (antibody stock concentration 0.5mg/ml; final concentration 0.005mg/ml). Cells were then washed 3x5min with TBS and the secondary antibody, polyclonal rabbit anti-mouse immunoglobulin conjugated with fluorescein isothiocyanate (FITC) (Dako, Cambridgeshire, UK) diluted 1:50 in TBS, was incubated for 1h at room temperature in the dark. After washing 3x5min with TBS, coverslips were mounted onto glass microscope slides using Vectashield® mounting medium with propidium iodide (PI; Vector labs, Peterborough, UK) for nuclear counterstain. Immunofluorescent images were captured using a Zeiss AxioObserver Inverted Microscope (magnification 400x).

Based on a previously published method (Johnstone, Sibley et al. 2005; Williams, Fyfe et al. 2008), two or three observers counted the total number of nuclei per field of view and the number of multinucleated cells (multinucleated cytotrophoblast/syncytium were defined as ≥3 nuclei within desmoplakin (desmosomal) boundaries) using ImageJ 1.45 software (Wayne Rasband, National Institutes of Health, USA). The number of multinucleated cells was expressed as a percentage (%) of the total number of nuclei per field of view. For each placenta (cytotrophoblast cell isolation), two fields of view were analysed per treatment/time point and each treatment was performed in duplicate. The average of these observations was then calculated to obtain the value of multinucleation (% of nuclei in multinucleate cells).

2.4.1.3.2. Intermediate conductance Ca^{2+}-activated K^+ channel immunostaining

Cytotrophoblasts fixed at 15, 42 and 66h of culture were assessed for the expression and immunolocalisation of IK_{Ca} protein. Cells were fixed in methanol (to assess intracellular localisation of IK_{Ca}) or PFA (to assess cytotrophoblast plasma membrane IK_{Ca} localisation).

Briefly, cells on coverslips were placed onto a microscope slide and washed 3x5min with TBS. Non-specific binding block was performed for 30min with 4% BSA in TBS at room temperature. Afterwards, cells were incubated for 1h at room temperature with mouse monoclonal (clone 6C1) antibody to $K_{Ca}3.1$ (IK_{Ca}) (extracellular epitope located at the pore loop, between S5-S6 transmembrane domains; Alomone labs, Jerusalem, Israel), diluted

1:50 in 1% BSA in TBS (initial content 100μg, final concentration 5μg/μl). In parallel, the antibody specificity was confirmed using a negative control, where the incubation with primary antibody was omitted and cells were incubated with the antibody diluent. Subsequently, cells were washed 3x5min with TBS and the secondary antibody, polyclonal rabbit anti-mouse immunoglobulin conjugated with FITC diluted 1:50 in TBS, was incubated for 1h at room temperature in the dark. After washing 3x5min with TBS, coverslips were mounted onto glass microscope slides using Vectashield® mounting medium with PI for nuclear counterstain. Immunofluorescent images were captured using a Zeiss AxioObserver Inverted Microscope (magnification 400x).

2.4.2. ^{86}Rb efflux from multinucleated cytotrophoblasts

^{86}Rb efflux was measured from multinucleated cytotrophoblasts at 66h of culture. Cytotrophoblasts plated onto p35 dishes were cultured for 66h. Cell culture medium was discarded from each p35 dish and cells were briefly washed with Tyrode's buffer before being incubated with 1ml 4μCi/ml ^{86}Rb (89.7μM; specific activity ~15mCi/mg; concentration 1μCi/ml; stock activity 1mCi) for 2h at room temperature. After washing for 3min (2 x 25ml) in Tyrode's buffer (with no added isotope), ^{86}Rb efflux was measured by adding and replacing Tyrode's buffer which was collected at 1min intervals over 15min (basal ^{86}Rb efflux) and/or exposed to various treatments over 5-15min (experimental period; Table 2.4), with the exception of a) the free radicals H_2O_2 and $ONOO^-$ which were added directly to Tyrode's buffer each min, starting from min 4 onwards, b) when using treatments in combination with a K^+ channel activating/opening-stimulus (i.e. hyposmotic solution, DCEBIO), a pre-block with a K^+ channel modulator was performed in advance starting from min 4 onwards, c) when testing the reversibility of $ONOO^-$, the free radical was applied over min 5-10 and then withdrawn for the remainder of the experimental period. All measurements were done in triplicate.

Finally, the cells were lysed in 0.3M NaOH for ~1h and scraped in order to denature the cells. Intracellular and membrane bound ^{86}Rb was then measured in the lysate to give a measure of total ^{86}Rb remaining in the cells at the end of the experiment (cellular ^{86}Rb).

Treatment		Specification
Tyrode's buffer (control)		135mM NaCl, 5mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES, 5.6mM glucose, pH 7.4; osmolality 285mOsm/kgH_2O
Hyposmotic solution		55mM NaCl, 5mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES, 5.6mM glucose; pH 7.4; osmolality ~145mOsm/kgH_2O
Free radicals	**ROS**	**H_2O_2** (10μM) in Tyrode's buffer
		H_2O_2 (10μM) in hyposmotic solution
	RNS	**$ONOO^-$** (0.1mM) in Tyrode's buffer
		$ONOO^-$ (0.1mM) in hyposmotic solution
K^+ channel modulators	**K^+ channel blockers**	**Ba^{2+}** (as $BaCl_2$, 5mM) in Tyrode's buffer
		TRAM-34 (10μM) in Tyrode's buffer
		TRAM-34 (10μM) in hyposmotic solution
	K^+ channel openers	**DCEBIO** (100μM) in Tyrode's buffer

Table 2.4: Treatments used for ^{86}Rb efflux experiments in multinucleated cytotrophoblasts

For the treatments listed in Table 2.4:

i. H_2O_2 (authentic) was obtained from stock solution 1mM H_2O_2 (dissolved in Tyrode's buffer). During the experimental period, 10μl aliquots from the stock were added directly into each p35 dish (which already had Tyrode's buffer or hyposmotic solution) 1min in advance. The final concentration was 10μM H_2O_2.

ii. $ONOO^-$ (authentic) was obtained from stock solution 10mM H_2O_2 as described in section 2.2.1.1. For each ^{86}Rb efflux experiment, a 10mM $ONOO^-$ aliquot was thawed and during the experimental period, 10μl aliquots were added directly into each p35 dish (which already had Tyrode's buffer or hyposmotic solution) 1min in advance. The final concentration in each vial was 10^{-4}M (0.1mM) $ONOO^-$.

iii. TRAM-34 (IK_{Ca} blocker) was prepared from a 10mM stock solution dissolved in dimethyl sulfoxide (DMSO). This stock was diluted 1:1000 in Tyrode's buffer or hyposmotic solution to obtain a final concentration of 10μM TRAM-34. The final concentration of DMSO in Tyrode's buffer was 0.1%.

iv. DCEBIO (IK_{Ca}/SK_{Ca} opener) was prepared from a 100mM stock solution dissolved in DMSO. Before each experiment, a stock aliquot was thawed and pre-diluted 1:10 in Tyrode's buffer (10mM DCEBIO). The latter was diluted again 1:100 in Tyrode's buffer, giving a final concentration of 100μM DCEBIO. The final concentration of DMSO in Tyrode's buffer was 0.1%.

2.4.2.1. ^{86}Rb isotope counting and data analysis

Background counts were prepared with 1ml water. Effluxed and cellular ^{86}Rb were measured in a gamma-counter (Packard Cobra II Auto Gamma, CA, USA) to determine ^{86}Rb activity. Each vial was counted for 1min. All counts recorded were at least 10 times higher than background counts.

The time course of ^{86}Rb efflux was expressed as a percent per min as:

$$\%\frac{efflux}{min} = \left(\frac{86Rb\ effluxed}{86Rb\ in\ cells}\right) \times 100$$

The efflux rate constant was also determined. The loss of ^{86}Rb was measured by a first-order rate constant which was calculated over the 10min experimental period as:

$$l_n\left(\frac{86Rb\ in\ cell\ at\ time\ t}{86Rb\ in\ cell\ at\ start}\right)$$

2.5. Statistical analyses

Statistical analysis was performed using GraphPad Prism version 5 software. Prior to performing non-parametric statistics data were assessed with a Kolmogorov-Smirnov test in order to assess normality of the distribution in all cases.

2.5.1. ^{86}Rb efflux measurements

%^{86}Rb efflux (time course) from placental villous fragments/explants and multinucleated cytotrophoblasts was expressed as mean ± standard error (SE) for each time point (n= number of placentas).

Power calculations were performed based on the work of Simán *et al*. (2001) (Siman, Sibley et al. 2001) and Clarson *et al.* (2002) (Clarson, Roberts et al. 2002). Using Altman's nomogram, a standardised difference of 2.5 was considered. At a power of 90% and a 5% level of significance, the minimum number of repeats in each group needed to show statistical difference was n= 3-5 placentas.

The effects of different treatments on the %^{86}Rb efflux were assessed by calculating the area under the curve (AUC) by comparing the corresponding control (experimental period) against treatment (experimental period). AUC was obtained by adding the % of ^{86}Rb efflux for each triplicate during the experimental period (8min for villous fragments, 16min for villous explants, 10min for multinucleated cytotrophoblasts), obtaining an average between them. AUC was compared by means of the non-parametric Wilcoxon signed rank test *vs*. 100% (control). A *p* value less than 0.05 was considered statistically significant.

For all ^{86}Rb efflux experiments, significant differences between ^{86}Rb rate constants were assessed using least squares linear regression. A *p* value less than 0.05 was considered statistically significant.

2.5.2. Human chorionic gonadotropin and lactate dehydrogenase assays

Time course for hCG secretion and LDH release from control untreated explants and cytotrophoblasts were expressed as mean ± SE (n= number of placentas).

Power calculations were performed based on the work of Williams *et al.* (2008) (Williams, Fyfe et al. 2008). A standardised difference of 1.9-2.0 and 1.85 was considered for placental villous tissue and isolated cytotrophoblasts respectively. At a power of 90% and a 5% level of significance, the minimum number of repeats in each group needed to show

statistical difference was n= 5-6 placentas for placental villous explants and n=6 for cytotrophoblasts.

hCG secretion in treated explants at days 4, 5 and 6 of culture, was normalised as a percentage of control (established as a 100%), plotted as median and IQR and analysed with a Wilcoxon signed-rank test. A *p* value less than 0.05 was considered statistically significant. Accordingly, hCG secretion in cytotrophoblasts at 15, 42 and 66h of culture, was normalised as a percentage of control (100%) and analysed with Wilcoxon signed-rank test or the raw data was analysed using non-parametric Friedman's test with Dunn's post hoc test. A *p* value less than 0.05 was considered statistically significant.

2.5.3. Cytotrophoblast multinucleation

As described in section 2.4.1.3.1., the number of multinucleated cells was expressed as a % of the total number of nuclei within a given field of view. The average of these observations was then calculated to provide a value of multinucleation.

The % of nuclei in multinucleate cells and the total number of nuclei was plotted as median and IQR (n= number of placentas). Power calculations were performed based on the work of Williams *et al.* (2008) (Williams, Fyfe et al. 2008). A standardised difference of 1.8 was considered. At a power of 90% and a 5% level of significance, the minimum number of repeats in each group needed to show statistical difference was n= 6-7 placentas.

Statistical differences were assessed between untreated (control) cells and each treatment per time point with non-parametric Friedman's test with Dunn's post hoc test. Alternatively, the % of nuclei in multinucleate cells and/or the total number of nuclei was normalised as a percentage of control (established as a 100%), plotted as median and IQR and analysed with Wilcoxon signed-rank test. A *p* value less than 0.05 was considered statistically significant.

3. RESULTS

The following chapter contains the main results from this thesis organised into three manuscripts in a format suitable for submission for publication in peer-reviewed journals.

3.1. Oxygen-sensitive K^+ channels modulate human chorionic gonadotropin secretion from human placental trophoblast

To be submitted to: Placenta

Paula Díaz,[1,2] Colin P. Sibley,[1,2] Susan L. Greenwood[1,2]

Contribution of authors

Conceived and designed the experiments: PD, CPS, SLG

Performed experiments: PD

Performed data analysis: PD

Drafted manuscript and prepared figures: PD

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Running head: *p*O_2-sensitive K^+ channels and human chorionic gonadotropin secretion

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Abstract

Human chorionic gonadotropin (hCG) is a key autocrine/paracrine regulator of placental syncytiotrophoblast, the nutrient transport epithelium of the human placenta. Syncytiotrophoblast hCG secretion is modulated by oxygen (pO_2), reactive oxygen species (ROS) and potassium (K^+) channels. Here we test the hypothesis that K^+ channels mediate the effects of pO_2 and ROS on hCG secretion. Placental villous explants from normal term pregnancies were cultured for 6 days at 6% (normoxia), 21% (hyperoxia) or 1% (hypoxia) pO_2. On days 3-5, explants were treated with 5mM 4-aminopyridine (4-AP) or tetraethylammonium (TEA), blockers of pO_2-sensitive voltage-gated (K_V) K^+ channels, or ROS (10-1000μM H_2O_2). hCG secretion and lactate dehydrogenase (LDH) release, a marker of necrosis, were determined daily. At day 6, hCG and LDH were measured in tissue lysate and ^{86}Rb (K^+) efflux assessed to estimate syncytiotrophoblast K^+ permeability. hCG secretion and ^{86}Rb efflux were significantly greater in explants maintained in 21% pO_2 than normoxia. 4-AP/TEA inhibited hCG secretion to a greater extent at 21% than 6% and 1% pO_2, and reduced ^{86}Rb efflux at 21% but not 6% pO_2. LDH release and tissue LDH/hCG were similar in 6%, 21% and 1% pO_2 and unaffected by 4-AP/TEA. H_2O_2 stimulated ^{86}Rb efflux and hCG secretion at normoxia but decreased ^{86}Rb efflux, without affecting hCG secretion, at 21% pO_2. In conclusion, 4-AP/TEA-sensitive K^+ channels participate in pO_2-sensitive hCG secretion from syncytiotrophoblast. ROS effects on both hCG secretion and ^{86}Rb efflux are pO_2-dependent but causal links between the two remain to be established.

Keywords

human chorionic gonadotropin, pO_2-sensitive K_V channels, 4-aminopyridine, tetraethylammonium, hydrogen peroxide

Introduction

The functions of the human placenta during pregnancy are performed by syncytiotrophoblast, a highly specialized multinucleated epithelial cell which surrounds the placental villi containing the fetal capillaries. The syncytiotrophoblast is the interface between maternal and fetal blood and has multiple functions such as materno-fetal exchange of nutrients and endocrine secretion. The syncytiotrophoblast has a short life span and is continuously renewed throughout pregnancy by a process of cellular turnover. Proliferative mononucleate cytotrophoblasts exit the cell cycle, differentiate and fuse with the overlying syncytial layer; both apoptosis and autophagy may then play a role in completing turnover [1-2].

Syncytiotrophoblast renewal by cell turnover is a highly co-ordinated process and regulated by a number of factors. Human chorionic gonadotropin (hCG), which is synthesized and secreted by terminally differentiated syncytiotrophoblast [3], is a key autocrine/paracrine regulator of syncytiotrophoblast turnover. hCG facilitates syncytiotrophoblast renewal by promoting cytotrophoblast differentiation and fusion [4]. This role of hCG is important for healthy pregnancy as dysregulation of syncytiotrophoblast renewal is evident in pre-eclampsia [5-6], fetal growth restriction [6-7] and maternal obesity [8], complications of pregnancy associated with maternal and/or fetal mortality and morbidity.

Syncytiotrophoblast hCG secretion is modulated *in vitro* by partial pressure of oxygen (pO_2) and reactive oxygen species (ROS). The regulation of hCG secretion by pO_2 has been demonstrated in term placental villous explants of normal placentas [9] and in cytotrophoblasts in primary culture [10] where reducing pO_2 inhibits hCG secretion. The effect of ROS on hCG secretion from isolated cytotrophoblasts was investigated using hydrogen peroxide (H_2O_2) to generate oxidative stress and hCG secretion was inhibited at high (>50μM) but markedly stimulated with low (1-50μM) H_2O_2 [11]. Altered placental pO_2 and increased oxidative stress are associated with pregnancy disease [12-14], and regulation of hCG secretion by these factors is likely to be of pathophysiological significance. In pre-eclampsia, incomplete transformation of the maternal spiral arteries during early placentation is thought to cause intermittent placental perfusion resulting in hypoxia-reoxygenation injury and an increase in ROS [14]. Indeed, increased levels of markers of oxidative stress are found in placental tissue from women with pre-eclampsia [15-18] as well as elevated serum levels of H_2O_2 compared to normal pregnancies [19].

Although it is evident that pO_2 and ROS modulate hCG secretion, the mechanism that links them is unexplored. As both conditions dysregulate syncytiotrophoblast turnover events *in vitro* [9, 20-21], altered hCG production and secretion might be a consequence of abnormal syncytiotrophoblast differentiation. Alternatively, hypoxia/hyperoxia and ROS

could directly affect hCG synthesis and/or the secretory process which in turn influences the paracrine/autocrine regulation of syncytiotrophoblast renewal. hCG secretion by term trophoblast involves constitutive release [22] and Ca^{2+}-dependent exocytosis [23]. Therefore, the regulated component of hCG secretion is modulated by factors that influence intracellular Ca^{2+}, including ion channels. We have shown that pharmacological blockade of Ca^{2+} entry channels [24] and voltage-gated K^+ channels (K_V), inhibit hCG secretion from placental villous explants and isolated cytotrophoblasts [25].

The K_V channel family comprises 11 family members [26], and the expression/activity of some K_V channel subunits is acutely and chronically modulated by pO_2 [27-30]. pO_2-sensitive K_V channels close in response to lowered pO_2, raising the possibility that the reduction in hCG secretion from syncytiotrophoblast under hypoxic conditions is a result of blocking K_V channels. Furthermore, long term exposure to oxidative stress (ROS) alters K^+ channel expression/activity and acute exposure has direct effects on K^+ channel proteins to alter their activity (oxidation state) [31-32]. The effects of H_2O_2 are diverse and depend on tissue type; H_2O_2 has been reported to both close [33] and open [34-35] K_V channels. As K_V channels are modulated by ROS in non-placental tissue, it is plausible that ROS regulate syncytiotrophoblast hCG secretion through effects on K_V channels.

Here we test the hypothesis that altered pO_2 and/or ROS regulate hCG secretion through an effect on K^+ channels. Using placental villous tissue from normal term pregnancy we compared the effect of K_V channel blockers on hCG secretion and ^{86}Rb efflux (a marker of K^+ permeation through ion channels) from villous explants maintained at placental normoxia (6% pO_2; 40-50mmHg), with extreme hypoxia (1% pO_2; 7.6mmHg) and hyperoxia (21% pO_2; 160mmHg). We also investigated the effect of H_2O_2, used to generate ROS, on hCG secretion and ^{86}Rb efflux at the three different pO_2.

Materials and Methods

Materials

Unless otherwise stated, all chemicals were from Sigma-Aldrich (Poole, UK).

Placental villous explant culture

Human placentas used in this study were obtained from St. Mary's Hospital Maternity Unit (Manchester, UK) following written informed consent as approved by the Local Research Ethics Committee. Normal term placentas (37-42 weeks gestation) were obtained from uncomplicated pregnancies following vaginal delivery or Caesarean section.

Term placental villous tissue maintained in explant culture is a well characterized model [36] which has been used extensively to study the chronic effects of regulators on syncytiotrophoblast biology [9, 20-21, 37-38].

The method for culture of placental villous explants has been published elsewhere [25, 36]. Briefly, within 30min of delivery, chorionic villous sections (1.5cm^3) were obtained and rinsed in sterile PBS (with $CaCl_2$ and $MgCl_2$). Villous tissue was further dissected into explants (3-5mm^3) and rinsed three times in PBS to remove excess blood. Explants were then placed in 74μm-polyester mesh Netwells, in 15mm insert 12-well plates. Three explants and 1.5ml of explant culture medium (10% CMRL-1066, 100μg/ml streptomycin sulphate, 100IU/ml penicillin-G, 0.1μg/ml hydrocortisone, 0.1μg/ml retinol acetate, 0.1μg/ml insulin, 5% FCS, pH 7.2) were placed into each well, with the tissue supported on the mesh at the liquid-gas interface.

Villous explants were cultured at 37˚C in humidified incubators at 6% pO_2 (with 5% CO_2/ balance N_2; normoxic for term placenta, 40-50mmHg; assuming 1atm= 760mmHg), 21% pO_2 (with 95% air/5% CO_2; hyperoxia for term placenta, 160mmHg) or 1% pO_2 (with 5% CO_2/ balance N_2; hypoxia for term placenta, 7.6mmHg) for 6 days (placenta collection= day 0; explant harvesting= day 6). Culture medium was replaced daily and fresh medium was pre-equilibrated (24h in advance) at each pO_2 before addition to explants. On days 3-5, explants were untreated (control) or treated daily with pO_2-sensitive K^+ channel blockers 5mM 4-AP or 5mM TEA (these concentrations have been previously reported to produce the maximal inhibitory effect on hCG secretion without effecting tissue integrity [25]), or H_2O_2 (10, 100μM or 1mM).

Explant culture medium was collected daily and stored at -20˚C before measuring hCG secretion and lactate dehydrogenase (LDH; released from necrotic cells and used as marker of cellular viability).

On day 6 explants were dissolved in 0.3M NaOH at 37˚C for 24h to measure protein content. Otherwise explants were placed into water for 18h at room temperature to lyse for measurement of cell hCG/LDH. The supernatant was collected and stored at -20˚C, and explants were dissolved into 0.3M NaOH. These samples were used to measure protein content with Bio-Rad Protein Assay, based on the Bradford method (Bio-Rad Laboratories, Hempstead, UK).

Measurement of hCG and LDH

hCG was assayed in the explant-conditioned culture medium using an ELISA (DRG Diagnostics, Marburg, Germany). In addition to hCG secreted during culture, cellular hCG was measured in villous explants lysed in water at day 6 of culture. Thawed samples were used following the instructions of the manufacturer. Optical density was measured at 450nm using a VersaMax microplate reader (Molecular Devices, CA, USA). hCG secretion was expressed as mIU/ml/h/mg protein.

For measurement of LDH release, a cytotoxicity detection kit (Roche Diagnostics, Mannheim, Germany) was used on thawed explant-conditioned medium. As standards are not provided with the kit, a standard curve was generated using L-Lactic dehydrogenase from rabbit muscle (initial stock concentration of 100U/ml) as an internal control. LDH levels were calculated as the difference between the absorbance measured at 492nm and 690nm. LDH release was expressed as absorbance units/mg protein/h.

^{86}Rb efflux from placental villous explants

^{86}Rb is used as a tracer of K^+. ^{86}Rb efflux was measured in placental villous explants using a technique previously described [36].

Briefly, fragments were incubated for 2h at 37°C in 1ml Tyrode's buffer (135mM NaCl, 5mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES, 5.6mM glucose, pH 7.4; osmolality 300mOsm/kgH_2O) containing 4μCi/ml ^{86}Rb (89.7μM; PerkinElmer, Waltham, MA, USA). After incubation, fragments were washed in 15ml Tyrode's buffer (with no added isotope) twice for 5min each. Basal ^{86}Rb efflux was then measured by changing and collecting 4ml Tyrode's buffer every 2min for 10min at 37°C. All measurements were done in triplicate. Finally, villi were lysed in water for 18h to release intracellular non-membrane bound ^{86}Rb which was then measured in the supernatant to give a measure of total ^{86}Rb remaining in the tissue at the end of the experiment (^{86}Rb in tissue). Effluxed and tissue ^{86}Rb was measured in a beta-counter (Packard 2000, CA, USA). All counts recorded were at least 10 times higher than background counts.

The time course of $\%^{86}Rb$ efflux was calculated as ((^{86}Rb effluxed/^{86}Rb in tissue) x100). The efflux rate constant was also determined, making the assumption that, in control untreated explants, ^{86}Rb efflux at steady state reflects the loss of ^{86}Rb from a single compartment (syncytiotrophoblast) limited by the K^+ permeability of the microvillous membrane. Consequently, the loss of ^{86}Rb was measured by a first-order rate constant which was calculated over 16min experimental period as (l_n(^{86}Rb in tissue at time *t*/^{86}Rb in tissue at start)).

Expression of Results and Statistics

Statistical analysis was performed using GraphPad Prism version 5 software. Prior to performing non-parametric statistics data were assessed with a Kolmogorov-Smirnov test in order to assess normality of the distribution.

hCG secretion and LDH release from control untreated explants were expressed as mean ± SE (n= number of placentas). hCG secretion in treated explants at days 4, 5 and 6 of culture, was normalized as a percentage of control (established as a 100%) and analyzed with a Wilcoxon signed-rank test. A *p* value less than 0.05 was considered statistically significant. Data are median ± interquartile range (IQR).

$\%^{86}Rb$ efflux from placental villous explants was expressed as mean ± SE for each time point. For all ^{86}Rb efflux experiments, significant differences between ^{86}Rb rate constants were assessed using least squares linear regression. A *p* value less than 0.05 was considered statistically significant.

Results

Effect of pO_2 on hCG secretion from placental villous explants

The temporal changes in hCG secretion from term placental villous explants maintained at 21% pO_2 over a 6-day culture period (Fig. 1*A*) were similar to those previously reported and shown to be associated with syncytiotrophoblast shedding and regeneration [25, 36].
At all pO_2, hCG secretion was high at day 1, probably due to release from damaged or degenerated syncytiotrophoblast [36]. At day 2, hCG secretion fell markedly at 6%, 21% and 1% pO_2, consistent with shedding of the syncytiotrophoblast. After day 2, hCG secretion increased 4-fold by day 4 in 21% pO_2, showed a slight gradual increase towards the end of culture in 6% pO_2 but remained stable at very low values at 1% pO_2 (Fig. 1*A*). Compared to hCG secretion at 6% pO_2 (approximating normoxia for the placenta at term), secretion was significantly higher (4.1-fold) at 21% pO_2 (Fig. 1*B*) but not different at 1% pO_2 (data not shown).
LDH release is commonly used as a marker of tissue necrosis and Fig. 1*C* shows that after the first day in culture, LDH release declined in explants maintained at 6%, 21% and 1% pO_2, indicating that tissue viability and cellular integrity was maintained in all pO_2. However, the low LDH release at 1% pO_2 might be due to reduced production of the enzyme in hypoxia as cellular LDH was ~3 times lower in 1% than either 21 or 6% pO_2 (Fig. 1*D*).

Effect of pO_2-sensitive K^+ channel blockers on hCG secretion from placental villous explants

Figs. 2*A* and 2*B* show the effects of pO_2-sensitive K^+ channel blockers 4-AP (5mM) and TEA (5mM) respectively on hCG secretion from placental villous explants maintained at 6%, 21% and 1% pO_2. As previously demonstrated [37], there was marked interplacental variability in hCG secretion, and so the secretion in response to the treatments on days 4, 5 and 6 was expressed as a percentage of secretion from controls on the corresponding day of culture (100%).
In villous explants maintained at 6% pO_2, 4-AP caused a transient decrease (35%) in hCG secretion on day 5 compared to control untreated explants at the same pO_2 (Fig. 2*A*). In contrast, explants maintained at hyperoxia (21% pO_2) showed a significant reduction in hCG secretion when treated with 4-AP at days 5 (52%) and 6 (68%) of culture (Fig. 2*A*). This effect was completely suppressed under hypoxia (1% pO_2), where hCG secretion was unaffected by 4-AP (Fig. 2*A*).

hCG secretion was unaffected in explants treated with TEA and maintained in placental normoxia compared to controls at the same pO_2 (Fig. 2*B*). On the contrary, when explants were maintained at 21% pO_2, treatment with TEA caused a significant reduction in hCG secretion at day 6 of culture (41%; Fig. 2*B*). TEA had no effect on explants maintained at 1% pO_2 (Fig. 2*B*).

LDH release from placental villous explants was not affected by treatment with 5mM 4-AP or 5mM TEA compared to their corresponding controls at the same pO_2 (data not shown), indicating that tissue viability was not compromised by treatment with these pO_2-sensitive K^+ channel blockers.

Additionally, cellular hCG was measured at day 6 of culture in untreated controls and 4-AP or TEA-treated villous explants at the three different pO_2. Neither treatment with 4-AP nor TEA affected cell hCG at any of the pO_2 tested (Fig. 2*C*).

From these data it is evident that culture of term placental villous explants for 6 days in hypoxic (1% pO_2) conditions reduced hCG secretion to a very low level, inhibited the temporal recovery in hCG secretion which is associated with syncytiotrophoblast regeneration/renewal at higher pO_2, reduced the cellular production of hCG and was unaffected by 4-AP and TEA. Therefore, experiments to evaluate the effect of these K^+ channel blockers on ^{86}Rb efflux were not performed at 1% pO_2.

Basal ^{86}Rb (K^+) efflux from syncytiotrophoblast: chronic effect of pO_2

Placental villous explants were cultured for 6 days and maintained at 6% or 21% pO_2 and ^{86}Rb efflux was measured at day 6 of culture. Fig. 3*A* shows the time course for basal $\%^{86}Rb$ efflux at a steady state over a 16min period in explants maintained at 6% and 21% pO_2. $\%^{86}Rb$ efflux is higher from explants maintained at 21% than 6% pO_2. Fig. 3*B* shows the total $\%^{86}Rb$ efflux over 16min (area under the curve) from explants maintained at 21% pO_2 as a percent of efflux from explants at 6% pO_2 (100%, dotted line). Basal $\%^{86}Rb$ efflux was significantly higher in explants maintained for 6 days in hyperoxia (21% pO_2) compared to normoxia.

The ^{86}Rb efflux rate constants calculated for untreated control explants maintained at both 6% and 21% pO_2 is shown in Table 1. Rate constant analysis shows that the fall in intracellular ^{86}Rb can be described by a single exponential decline indicating that efflux is predominantly from a single tissue compartment, which we take to be the syncytiotrophoblast, in agreement with previous reports [36]. The mean rate constant for ^{86}Rb efflux was significantly lower in explants maintained at placental normoxia (6% pO_2) than hyperoxia (21% pO_2) (Table 1).

Long term effects of pO_2: effect of pO_2-sensitive K^+ channel blockers on syncytiotrophoblast K^+ permeability

^{86}Rb (K^+) permeability was assayed at day 6 in placental villous explants cultured at 6% and 21% pO_2. Explants were untreated (controls) or treated from day 3 onwards with 4-AP (5mM) or TEA (5mM).

The effect of these blockers was assessed by analyzing the differences between the rate constant of decline in intracellular ^{86}Rb for each treatment compared to control at the same pO_2 (Table 1). The efflux rate constant was significantly decreased by 4-AP and TEA in explants maintained in hyperoxia (21% pO_2) but was without effect in explants maintained in normoxia (Table 1).

Effect of H_2O_2 on basal ^{86}Rb (K^+) permeability and hCG secretion from placental villous explants

The effect of H_2O_2, used to generate oxidative stress, on hCG secretion was tested in explants maintained at 6%, 21% and 1% pO_2 over days 3-5 of culture. There was no effect of 100μM H_2O_2 at 6%, 21% or 1% pO_2 (Fig. 4). In contrast, 1mM H_2O_2 transiently increased hCG secretion by 40% in explants maintained at 6% pO_2 compared to controls (Fig. 4). 10μM H_2O_2 had no effect on hCG secretion (data not shown). Treatment with H_2O_2 did not affect LDH release from villous explants at any of the concentrations used (data not shown).

The effect of H_2O_2 on ^{86}Rb efflux was measured at day 6 in explant cultures maintained at 6% and 21% pO_2 (Table 1). H_2O_2 increased the ^{86}Rb efflux rate constant at 100μM and 1mM compared to corresponding controls in explants maintained at 6% pO_2. In contrast, treatment of villous explants with 100μM and 1mM H_2O_2 produced the opposite effect in 21% pO_2, and significantly reduced the ^{86}Rb rate constant (Table 1) compared to controls at the same pO_2. 10μM H_2O_2 had no effect on basal ^{86}Rb efflux from explants cultured at either 6% or 21% pO_2 (data not shown).

Discussion

This study confirms and extends previous observations that hCG secretion from term placental trophoblast is sensitive to pO_2 [9, 21] and ROS [11]. In villous explants prepared from the same placenta, hCG secretion was higher in 21% pO_2, and lower in 1% pO_2, than 6% pO_2 (normoxic) culture conditions. Syncytiotrophoblast K^+ permeability, estimated by ^{86}Rb efflux, was greater in explants cultured in 21% than 6% pO_2. In accordance with this, 4-AP and TEA, blockers of pO_2-sensitive K_V channels, inhibited hCG secretion and ^{86}Rb efflux to a greater extent in 21% than 6% pO_2. H_2O_2, used to induce oxidative stress, had pO_2-dependent effects, stimulating ^{86}Rb efflux and transiently increasing hCG secretion from explants cultured at 6% pO_2 but inhibiting ^{86}Rb efflux, without affecting hCG secretion, at 21% pO_2. This suggests that oxidative stress regulates endocrine secretion and syncytiotrophoblast K^+ permeability but the two may not be linked.

hCG secretion from term placental syncytiotrophoblast is pO_2-dependent

The temporal pattern of hCG secretion from term placental explants maintained at 21% pO_2 for 6 days was originally described by Siman *et al*. [36], and shown to coincide with the initial loss and then regeneration of the syncytiotrophoblast. Similar experiments to relate hCG secretion to syncytiotrophoblast regeneration over 6 days have not been performed at placental normoxia (6% pO_2) and extreme hypoxia (1% pO_2). However, in shorter term cultures (4 days) of term placental explants, hCG secretion was lower at 6% and 1% compared to 21% pO_2 and this was associated with effects on cellular turnover such as decreased cytotrophoblast proliferation and enhanced apoptosis [21]. In the present study, hCG secretion from explants maintained at 21% pO_2 was low on day 2, increased over days 2-4 and reached a plateau at days 4-6 consistent with previous observations [25, 36, 39]. hCG secretion was significantly lower at 6% than 21% pO_2, with only a small rise on day 4, and at 1% pO_2 a low hCG secretion at day 2 persisted for the duration of the culture. Cell hCG, measured following tissue lysis in water, was lower (1.7-fold) in explants maintained at 6% pO_2 compared to 21% pO_2, suggesting that pO_2 regulates hCG synthesis. However, hCG secretion at 6% pO_2 was 4.1-fold lower than at 21% pO_2, demonstrating an additional effect of pO_2 on the secretory mechanism. In contrast, hypoxia reduced cellular hCG to the same proportion as secretion, indicating that the reduced secretion in 1% pO_2 is predominantly due to altered synthesis. LDH release was unaffected by pO_2 which could be interpreted to demonstrate that extremes of pO_2 do not markedly alter tissue integrity. However, culturing villous tissue at 1% pO_2 inhibited LDH synthesis and using LDH release alone as a marker of tissue viability in hypoxia might not be reliable.

Inhibition of hCG secretion by K_V channel blockers is pO_2-sensitive

We previously demonstrated a role for K_V channels in regulating hCG secretion from placental villous tissue and cytotrophoblasts cultured at 21% pO_2. Chronic exposure to the K_V channel blockers 4-AP (0.01-5mM) and TEA (0.1-10mM) induced a concentration-dependent inhibition of hCG secretion. 5mM 4-AP and 5mM TEA, the concentrations used in the present study, produced the maximal inhibitory effect on hCG secretion without affecting tissue integrity [25].

As the activity and expression of K_V channels can be down-regulated by hypoxia [27-29, 40], we explored the possibility that the lower hCG secretion at 6% compared to 21% pO_2 is mediated by closure of K_V channels. 4-AP and TEA inhibited hCG secretion from villous explants to a greater extent in 21% than 6% pO_2 and had no effect on secretion at 1% pO_2. Neither inhibitor affected tissue integrity as LDH release was unchanged by treatment. Additionally, 4-AP and TEA inhibited the process of hCG secretion, and not production, as cellular hCG did not change after exposure to the blockers both at 21% and 6% pO_2.

Inhibition of ^{86}Rb (K^+) efflux by K_V channel blockers is pO_2-sensitive

Direct study of ion channels in the syncytiotrophoblast of intact placental villi using patch clamp methods is technically challenging as seals are hard to achieve [41] and the multinucleate nature of the tissue precludes whole cell recording. In this study we used ^{86}Rb (K^+) efflux to assess whether 4-AP and TEA inhibited K^+ conductance in the syncytiotrophoblast and whether the inhibition was pO_2-sensitive. ^{86}Rb permeates most K^+-selective channels and it has been used as a tracer to indirectly assess K^+ permeability [42] of the syncytiotrophoblast [36, 43]. We have previously shown that basal ^{86}Rb efflux from placental explants cultured at 21% pO_2 is inhibited by Ba^{2+}, a broad spectrum K^+ channel blocker [36], implicating K^+ conductances in the microvillous, maternal facing plasma membrane of the syncytiotrophoblast. In villous explants, basal ^{86}Rb efflux was measured at 2-day intervals (up to 7 days) and decreased with time in culture indicating a progressive fall in K^+ permeability as the syncytiotrophoblast regenerated [36].

In the current study, ^{86}Rb efflux measured on day 6 of culture showed that basal K^+ permeability was significantly higher in explants maintained in hyperoxia compared to placental normoxia (6% pO_2), suggesting that chronic exposure to 21% pO_2 over a 6-day period increases the activity and/or expression of syncytiotrophoblast K^+ channels. In support of this, treatment of villous explants with 4-AP and TEA (days 3-5 of culture) significantly reduced ^{86}Rb efflux when the tissue was cultured at 21% but not 6% pO_2, consistent with an inhibition of pO_2-sensitive K_V channels that are more active/more highly

expressed at 21% than at 6% pO_2. The inhibition of both ^{86}Rb efflux and syncytiotrophoblast hCG secretion by 4-AP and TEA at 21% but not 6% pO_2, implicates a role for 4-AP and TEA-sensitive K_V channels in the elevated hCG secretion at the higher pO_2.

pO_2-sensitive K^+ channel activity is acutely inhibited by low pO_2 (<50-60mmHg) in non-placental tissues and these channels also undergo chronic adaptation to altered pO_2 which directly influences transcriptional mechanisms that determine the level of channel expression [28, 44-45]. Therefore, it is possible that pO_2 regulates the expression of syncytiotrophoblast pO_2-sensitive K_V channels and this needs to be further explored. Interestingly, recent evidence shows that protein expression of $K_V2.1$, a pO_2-sensitive K^+ channel, is unaltered in isolated apical microvillous plasma membranes from placentas of normal term pregnancies compared to pre-eclampsia and fetal growth restriction [46]. Moreover, immunohistochemistry on villous tissue at term confirmed that expression of $K_V1.5$, another pO_2-sensitive K^+ channel, is not different in placentas of growth restricted compared to normally grown fetuses [47]. Therefore, under conditions of altered pO_2 and hypoxia-reperfusion injury in pregnancy pathology, such as pre-eclampsia and fetal growth restriction [14, 48], it seems that K_V channel protein expression is not altered. This raises the possibility that in syncytiotrophoblast, pO_2 might regulate K_V channel activity rather than expression.

Effect of H_2O_2 on hCG secretion and ^{86}Rb efflux

Placental oxidative stress and reduced antioxidant defenses are key features of pregnancy disease including pre-eclampsia [49] and maternal serum levels of H_2O_2 are higher in late onset pre-eclampsia than in normal pregnancy [19]. Therefore, using H_2O_2 to induce oxidative stress in placental villi is pathophysiologically relevant and in this study we explored the effects of H_2O_2 on syncytiotrophoblast hCG secretion and whether these effects could be modulated through K^+ channels. Although we did not measure oxidative stress, previous reports showed that *in vitro* treatment of placental villous tissue with 1mM H_2O_2 caused oxidative stress which was reversed by treatment with antioxidant vitamins C and E [50].

H_2O_2 (10μM-1mM) did not alter hCG secretion at 21% pO_2 but 1mM caused a transient increase in secretion at 6% pO_2. In isolated cytotrophoblasts, H_2O_2 between 0-50μM increased and >50μM decreased hCG secretion [11] when the cells were maintained at 21% pO_2. It is possible that the difference in the effective concentrations of H_2O_2 in the two situations reflects the *in vitro* model used; in placental explants, cellular interactions are maintained and tissue antioxidant defenses are available to scavenge ROS [13, 51]; as a result, it is possible that higher concentrations of H_2O_2 are required to induce an

increase in syncytiotrophoblast hCG secretion. Moreover, the effects of H_2O_2 on placental explants under culture conditions are influenced by the existing pO_2 [52] and in the current study, H_2O_2 promoted hCG secretion when the tissue was maintained in 6% pO_2, corresponding to placental normoxia.

H_2O_2 also had pO_2-dependent effects on ^{86}Rb efflux, with 100μM-1mM increasing syncytiotrophoblast ^{86}Rb efflux at 6% pO_2, but inhibiting efflux at 21% pO_2. This is consistent with the variable effects of ROS on K^+ channel activity reported on the literature [32-33, 35] and raises the possibility that in placental normoxia, K^+ channels can be activated by H_2O_2. However, it is evident that the effect of H_2O_2 on ^{86}Rb efflux and hCG secretion can be independent events and further work is required to determine which channels are activated by H_2O_2 in normoxia and whether they are involved in hCG secretion.

Mechanism of hCG secretion: Role of K^+ channels

The present work suggests a role for 4-AP and TEA-sensitive pO_2-sensitive K^+ channels in regulating hCG secretion. According to the specificity and the concentration of 4-AP and TEA used, the targeted K^+ channels belong mainly to the K_V family [26, 53].

K_V channel mRNA has been detected in whole placental homogenate i.e. K_V1.5, 1.7, 6.1, 7.1, 7.2, 7.4 [54], K_V9.3 [55] and immunostaining for K_V1.5 and 2.1 channels has localized their expression to the microvillous membrane of the syncytiotrophoblast in placental villous sections [47] and the plasma membrane of isolated cytotrophoblasts (unpublished data). K_V1.5, 2.1 are pO_2-sensitive [56] and we propose that closure of these channels, inhibited by 4-AP and TEA, could underlie the lower hCG secretion from placentas maintained in 6% compared to 21% pO_2.

At 40 weeks of gestation, pO_2 measurements obtained at cordocentesis in normal pregnancies show a value of 40mmHg (~5.3% pO_2; [57]). Thus, it has been suggested that villous explants taken from term placentas should be maintained at up to 8% pO_2 for physiological normoxia at this gestation [58]. Accordingly, we used 6% pO_2 as placental normoxia in this study.

Our data suggest syncytiotrophoblast K_V channel activity is low at normoxia and closure of K_V channels by low pO_2 could lead to a depolarized syncytiotrophoblast membrane potential. We have previously shown that hCG secretion at 21% pO_2 is stimulated by Ca^{2+} entry through non-selective cation channels (NSCCs; [59]). Membrane depolarization would inhibit Ca^{2+} entry through NSCCs and this might account for the relatively low hCG secretion under normoxic conditions. On the contrary, the elevated K^+ permeability with H_2O_2 in normoxia implicates increased K^+ channel activity which would hyperpolarize the membrane, promote Ca^{2+} entry through NSCCs and stimulate hCG secretion.

Alterations to acute or chronic pO_2-sensing by ion channels participate in the pathophysiology of different diseases [45]. Syncytiotrophoblast pO_2-sensitive K^+ channels could have a role in pregnancy complications associated with altered pO_2, such as pre-eclampsia, a disease in which the placenta is thought to be exposed to fluctuations in pO_2 due to ischaemia-reperfusion injury [14]. Whilst the range of pO_2 in the placenta in pre-eclampsia is unlikely to be as wide as that used *in vitro* in this study to test our hypothesis, our data suggest that pO_2 effects on K_V channels might be important in this pregnancy complication.

Interestingly, the highest rate of hCG secretion into the maternal circulation occurs in the first trimester of pregnancy [22] in a hypoxic environment (~2.5% pO_2) before week 10 of gestation [60]. hCG secretion from first trimester explants, although higher than at term, is not sensitive to low pO_2 [61]. This suggests that the regulation of hCG secretion is different in early pregnancy implicating a different role for pO_2-sensitive K^+ channels.

Perspectives and Significance

hCG acts as an autocrine/paracrine regulator of syncytiotrophoblast turnover, and alterations in this regulatory pathway might be involved in the placental dysfunction found in pre-eclampsia. Previous data have shown that pO_2 is important in regulating hCG secretion. The current study shows that this effect is likely to be mediated through pO_2-sensitive K^+ channels.

K^+ channels regulate membrane potential and intracellular K^+ and participate in diverse cellular events including proliferation, apoptosis, migration and fusion as well as endocrine secretion. Dysregulation of syncytiotrophoblast K^+ channel activity and/or expression though chronic exposure to altered pO_2 and/or increased ROS could lead to altered nutrient transport, altered trophoblast turnover and, as shown here, altered hormone secretion.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).

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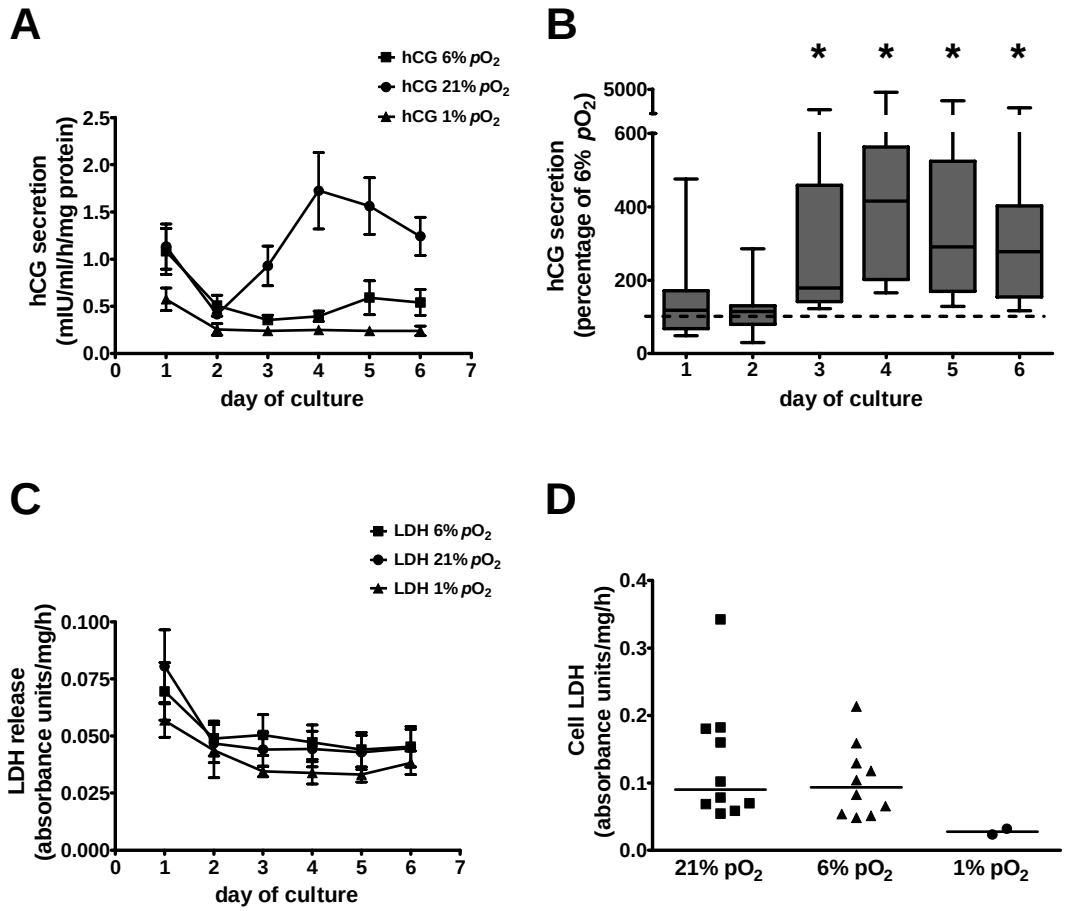

Figure 1: Effect of pO_2 on hCG secretion from villous explants.
A: time course of hCG secretion from explants maintained at 21%, 6% and 1% pO_2 during 6 days of culture. Values are mean ± SE; n= 14 placentas (n= 3 placentas maintained at 1% pO_2). ***B***: hCG secretion in explants maintained at 21% pO_2 expressed as a percentage of hCG secretion at 6% pO_2 (100%, dotted line); data are expressed as median ± IQR; n= 14 placentas, Wilcoxon signed-rank test compared to 100%, *p=0.0001. ***C***: time course of LDH release from explants maintained at 6%, 21% and 1% pO_2 during 6 days of culture. Values are mean ± SE; n= 14 placentas (n= 3 placentas maintained at 1% pO_2). ***D***: cellular LDH measured at day 6 of culture in explants maintained at 6%, 21% and 1% pO_2. Scatter dot plot shows line at median; n= 10 placentas (n= 2 placentas maintained at 1% pO_2).

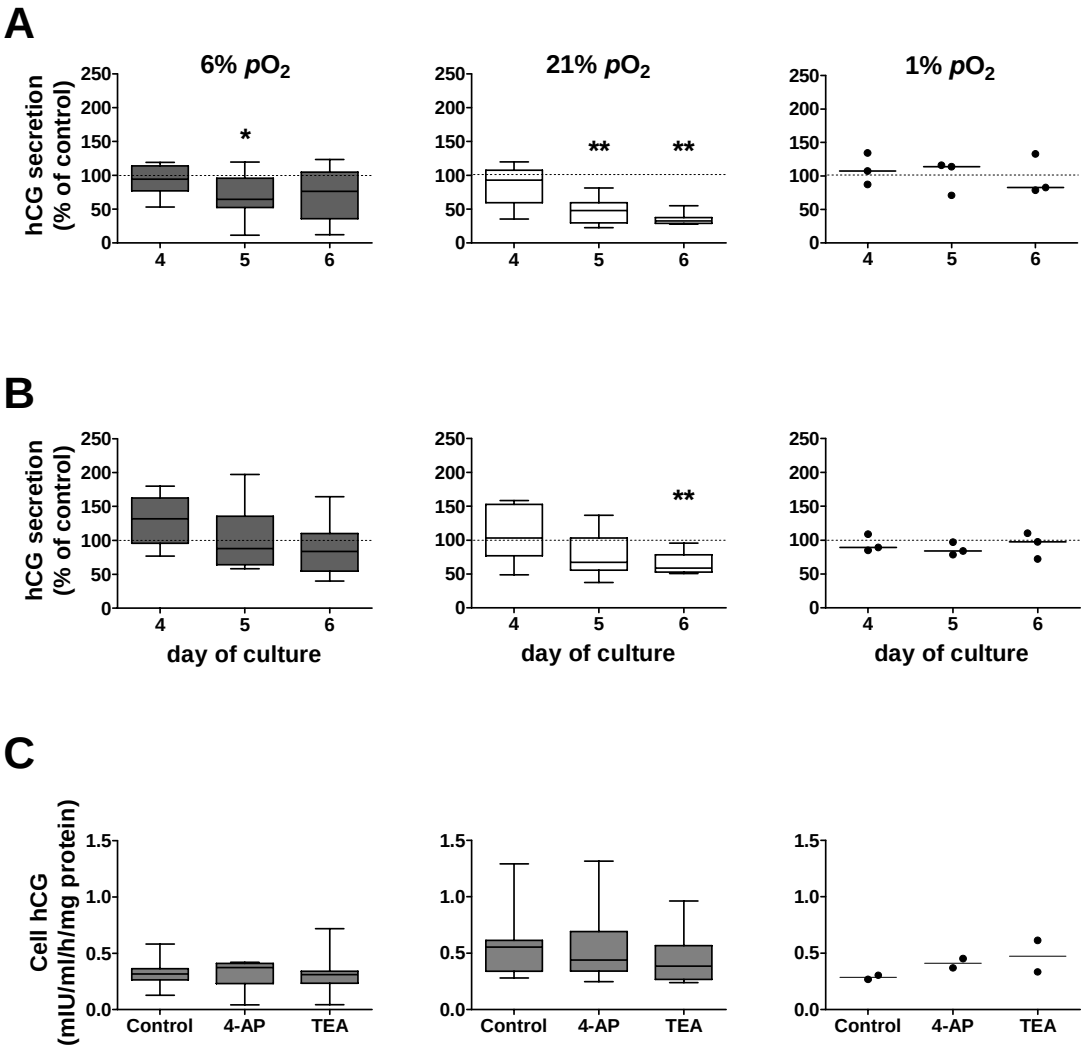

Figure 2: Effect of pO_2-sensitive K^+ channel blockers on hCG secretion from placental villous explants maintained at 6%, 21% or 1% pO_2.
hCG secretion at days 4, 5 and 6 of culture was normalized as a percentage of hCG secretion in control untreated explants at the corresponding pO_2 (dotted line, 100%); assessed by Wilcoxon signed-rank test compared to 100%. ***A***: 4-AP (**p*=0.04, ** *p*=0.008; n= 8 placentas; 1% pO_2 n= 3 placentas); ***B***: TEA (***p*=0.008, n= 8 placentas; 1% pO_2 n= 3 placentas). Data are expressed as median ± IQR (line at median in 1% pO_2). ***C***: Effect of pO_2-sensitive K^+ channel blockers on cellular hCG from placental villous explants maintained at 6, 21% or 1% pO_2. Cell hCG was measured at day 6 of culture. Data is presented as median ± IQR for 6% and 21% pO_2; n= 7 placentas. At 1% pO_2 line represents median; n= 2 placentas.

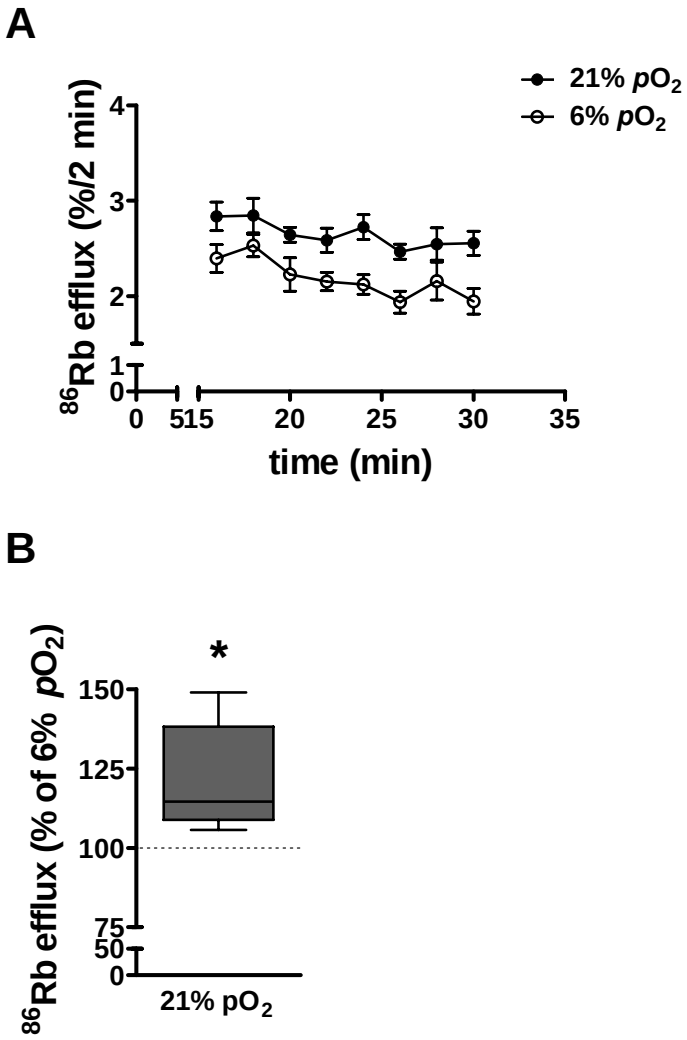

Figure 3: Effect of pO_2 on syncytiotrophoblast K^+ permeability.
A: Time course for the %^{86}Rb efflux over 16min. Untreated (control) villous explants were cultured at 6% and 21% pO_2 and basal ^{86}Rb efflux was measured at day 6. Data are expressed as mean ± SE (n= 10 placentas). ***B***: The %^{86}Rb efflux over 16min in explants maintained at 21% was normalized as a percentage of efflux in explants maintained at 6% pO_2 (dotted line). Data are median ± IQR, Wilcoxon signed-rank test compared to 6% pO_2, **p*=0.002; n= 10 placentas.

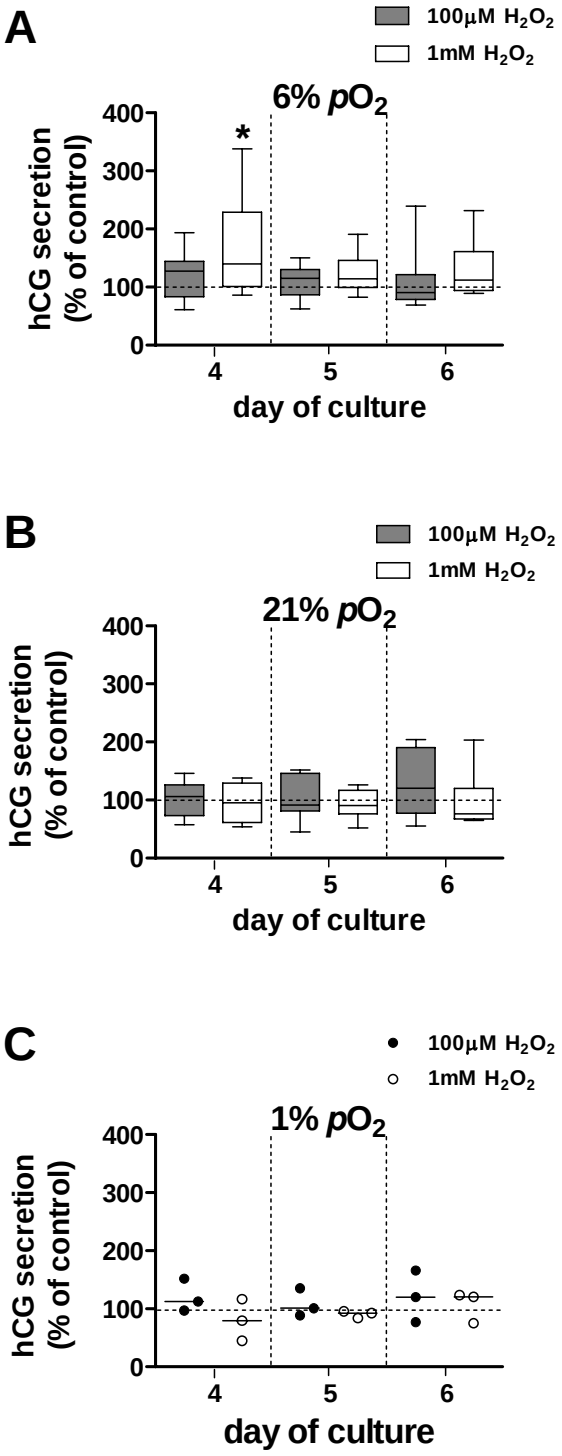

Figure 4: Effect of 100µM and 1mM H_2O_2 on hCG secretion from villous explants. hCG secretion from H_2O_2-treated explants maintained at 6% (***A***), 21% (***B***) and 1% pO_2 (***C***) at days 4, 5 and 6 of culture was expressed as a percentage of control (100%, dotted line); data are expressed as median ± IQR; n= 9 placentas for 100µM H_2O_2 (except n= 3 placentas at 1% pO_2); n= 8 placentas for 1mM H_2O_2 (except n= 3 placentas in 1% pO_2). Wilcoxon signed-rank test compared to 100%, *p=0.04.

Mean rate constants of ^{86}Rb efflux in control and treated placental villous explants

Condition	*^{86}Rb efflux rate constant (l_n ^{86}Rb (t=x)/(t=0))/min^{-1}*	*r^2*	*p value*	*n*
Control 21% pO_2	-0.0136 ± 0.0003*	0.954	<0.0001	10
Control 6% pO_2	-0.0111 ± 0.0003	0.956		10
5mM 4-AP 21% pO_2	-0.0113 ± 0.0006**	0.918	0.0007	4
5mM 4-AP 6% pO_2	-0.0114 ± 0.0009†	0.840	0.494	4
5mM TEA 21% pO_2	-0.0117± 0.0002**	0.987	<0.0001	4
5mM TEA 6% pO_2	-0.0111 ± 0.0005†	0.944	0.630	4
100µM H_2O_2 21% pO_2	-0.0122 ± 0.0004**	0.963	0.004	4
100µM H_2O_2 6% pO_2	-0.0119 ± 0.0003†	0.976	0.048	4
1mM H_2O_2 21% pO_2	-0.0130 ± 0.0005**	0.963	0.014	4
1mM H_2O_2 6% pO_2	-0.0118 ± 0.0007†	0.916	0.002	4

Table 1: Mean rate constants of ^{86}Rb efflux in control and treated placental villous explants maintained at 6% and 21% pO_2 over 16min.
Data are mean ± SE, n is the number of placentas. *p* values determined by linear regression; *compared to control 6% pO_2; **compared to corresponding controls at 21% pO_2 (-0.0144 ± 0.0006/min^{-1}; r^2 0.953); †compared to corresponding controls at 6% O_2 (-0.0107 ± 0.0005/min^{-1}; r^2 0.948).

3.2. Intermediate conductance Ca^{2+}-Activated K^+ channels modulate human placental trophoblast syncytialization

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Running head: IK_{Ca} and cytotrophoblast differentiation

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Abstract

Regulation of human placental syncytiotrophoblast renewal by cytotrophoblast migration, aggregation/fusion and differentiation is essential for successful pregnancy. In several tissues, these events are regulated by intermediate conductance Ca^{2+}-activated K^+ channels (IK_{Ca}), in part through their ability to regulate cell volume. We used cytotrophoblasts in primary culture to test the hypotheses that IK_{Ca} participate in the formation of multinucleated syncytiotrophoblast and in syncytiotrophoblast volume homeostasis. Cytotrophoblasts were isolated from normal term placentas and cultured for 66h. This preparation recreates syncytiotrophoblast formation *in vivo*, as mononucleate cells (15h) fuse into multinucleate syncytia (66h) concomitant with elevated secretion of human chorionic gonadotropin (hCG). Cells were treated with the IK_{Ca} inhibitor TRAM-34 (10μM) or activator DCEBIO (100μM). Culture medium was collected to measure hCG secretion and cells fixed for immunofluorescence with anti-IK_{Ca} and anti-desmoplakin antibodies to assess IK_{Ca} expression and multinucleation respectively. K^+ channel activity was assessed by measuring ^{86}Rb efflux at 66h. IK_{Ca} immunostaining was evident in nucleus, cytoplasm and surface of mono- and multinucleate cells. DCEBIO increased ^{86}Rb efflux 8.3-fold above control and this was inhibited by TRAM-34 (85%; $p<0.0001$). Cytotrophoblast multinucleation increased 12-fold ($p<0.05$) and hCG secretion 20-fold ($p<0.05$), between 15 and 66h. Compared to controls, DCEBIO reduced multinucleation by 42% ($p<0.05$) and hCG secretion by 80% ($p<0.05$). TRAM-34 alone did not affect cytotrophoblast multinucleation or hCG secretion. Hyposmotic solution increased ^{86}Rb efflux 3.8-fold ($p<0.0001$). This effect was dependent on extracellular Ca^{2+}, inhibited by TRAM-34 and 100nM charybdotoxin (85% ($p<0.0001$) and 43% respectively) but unaffected by 100nM apamin. In conclusion, IK_{Ca} are expressed in cytotrophoblasts and their activation inhibits the formation of multinucleated cells *in vitro*. IK_{Ca} are stimulated by syncytiotrophoblast swelling implicating a role in syncytiotrophoblast volume homeostasis. Inappropriate activation of IK_{Ca} in pathophysiological conditions could compromise syncytiotrophoblast turnover and volume homeostasis in pregnancy disease.

Keywords

syncytiotrophoblast; intermediate conductance Ca^{2+}-activated K^+ channel; trophoblast renewal; cell differentiation; cytotrophoblast fusion; cell volume; human placenta

Introduction

The syncytiotrophoblast is the transporting epithelium of the human placenta being the interface between maternal and fetal blood. This highly specialized epithelial cell also performs a number of other functions including hormone production and secretion. Syncytiotrophoblast has a short life span and is renewed by cellular turnover in a tightly regulated process where proliferative mononucleate cytotrophoblasts exit the cell cycle, differentiate and fuse with the overlying syncytial layer [1]; both apoptosis and autophagy have been hypothesized to play a role in completing turnover [2,3].

In vitro models have been used to study some of the features of syncytiotrophoblast turnover. These include cytotrophoblasts isolated from normal term placenta and maintained in primary culture [4,5]. After 15-18h of culture, cytotrophoblasts are predominantly mononucleate and secrete small amounts of human chorionic gonadotropin (hCG). Over 24-66h they migrate, aggregate and fuse to become multinucleated, a process reminiscent of syncytiotrophoblast formation *in vivo* [5,6,7]. This morphological differentiation is associated with a several-fold increase in the production and secretion of hCG. hCG, which is synthesized and secreted by terminally differentiated syncytiotrophoblast [8], acts in an autocrine/paracrine manner to facilitate syncytiotrophoblast renewal by promoting cytotrophoblast differentiation and fusion [9].

The importance of syncytiotrophoblast renewal for the progression of normal pregnancy is highlighted by the fact that its dysregulation is linked to pregnancy complications associated with maternal and/or fetal morbidity and mortality, in particular pre-eclampsia [10,11,12], fetal growth restriction [11,12,13] and maternal obesity [14]. In pre-eclampsia there is elevated cytotrophoblast proliferation [11,15,16] and apoptosis [11,17,18,19], and a greater number of syncytial nuclear aggregates [20], compared to normal pregnancy. Furthermore, there is evidence to suggest that a rate-limiting step for syncytiotrophoblast formation, cytotrophoblast fusion, is reduced in pre-eclampsia [12,21]. Cytotrophoblasts isolated from placentas of women with pre-eclampsia have a lower rate of syncytialization than those of normal pregnancy [22]. Expression of syncytin-1 [23] and syncytin-2 [24], envelope fusogenic proteins that induce syncytium formation [23,25,26] is downregulated both in isolated cytotrophoblasts and placental villous tissue from pregnancies complicated with pre-eclampsia [22,24,27,28]. Syncytiotrophoblast expression of other fusogenic proteins, for example e-cadherin [16], is also reduced in pre-eclampsia. Collectively, dysregulation of the processes contributing to syncytiotrophoblast renewal culminates in a decrease in the total volume of syncytiotrophoblast in pregnancies complicated by pre-eclampsia and fetal growth restriction [29]. This has implications for nutrient delivery to the fetus as syncytiotrophoblast volume correlates with fetal weight

[30]. However, the intracellular and extracellular signals that trigger and regulate cytotrophoblast fusion to form syncytiotrophoblast are not well understood.

In non-placental tissues, cellular proliferation, fusion and apoptosis can be regulated by members of the Ca^{2+}-activated K^+ channel (K_{Ca}) family, in particular by intermediate conductance Ca^{2+}-activated K^+ channels (IK_{Ca}; $K_{Ca}3.1$; single channel conductance 50-200pS). IK_{Ca}s are voltage-insensitive and are strongly activated by increased concentrations of intracellular Ca^{2+} ($[Ca^{2+}]_i$; 300-700nM) [31,32]. IK_{Ca} mRNA was shown to be highly expressed by human placenta over 15 years ago [33] but the functions of IK_{Ca} in the placenta have not been explored.

A major function of IK_{Ca} is that of regulating cellular volume [34,35,36,37,38]. IK_{Ca} activation induces K^+ efflux from cells, which both lowers intracellular K^+ concentration and promotes the loss of water by osmosis to induce cell shrinkage [39]. Appropriate adjustment of cell volume and/or intracellular K^+ concentration is essential for cells to undergo proliferation, migration, fusion and apoptosis [40]. Indeed, in non-placental tissues, IK_{Ca} has been shown to contribute to tissue homeostasis by regulating proliferation [31,41,42,43], differentiation/fusion [44,45], cell migration [46,47,48] and apoptosis [49]. The ability of IK_{Ca} to regulate cell volume has been revealed experimentally by exposing cells to an osmotic challenge [34,35,37,50]. When placed in hyposmotic solutions, cells initially swell but then restore their volume by a process of regulatory volume decrease (RVD). In many cells hyposmotic cell swelling elevates intracellular Ca^{2+} which activates IK_{Ca}, promotes K^+ efflux and water follows to achieve RVD [34]. However, a role for IK_{Ca} in regulating renewal of syncytiotrophoblast and/or syncytiotrophoblast volume has yet to be explored.

We tested the hypotheses that IK_{Ca} participates in the formation of multinucleate syncytiotrophoblast and that IK_{Ca} has a role in syncytiotrophoblast volume regulation. Using isolated cytotrophoblasts in primary culture we confirmed IK_{Ca} protein expression and tested the effects of IK_{Ca} modulators on ^{86}Rb efflux, the formation of multinucleate syncytia and the secretion of hCG. To investigate whether IK_{Ca} participate in syncytiotrophoblast RVD, cells were exposed to hyposmotic solutions and ^{86}Rb efflux measured in the presence and absence of IK_{Ca} modulators.

Materials and Methods

Materials

Unless otherwise stated, all chemicals were from Sigma-Aldrich (Poole, UK).

Ethics Statement

Human placentas used in this study were obtained from St. Mary's Hospital Maternity Unit (Manchester, UK) following written informed consent as approved by the Local Research Ethics Committee (North West (Haydock Park) Research Ethics Committee (Ref: 08/H1010/55). Normal term placentas (37-42 weeks gestation) were obtained from uncomplicated pregnancies following vaginal delivery or Caesarean section. The investigation conforms to the principles outlined in the Declaration of Helsinki.

Cytotrophoblast isolation

Cytotrophoblasts were isolated from term placentas using an adaptation of the method used by Kliman *et al*. [5], as previously described [4]. Briefly, full thickness placenta samples (~2cm^3) were taken within 30min of delivery and placed into sterile saline. Placental villous tissue was further dissected from each sample after removal of the chorionic plate and decidua. ~30g of villous tissue were obtained and submitted to digestion 3 times in Hank's balanced salt solution containing 2.5% trypsin and 0.2mg/ml deoxyribonuclease (DNAse I) for 30min at 37°C (80rpm). After each digestion, 100ml of supernatant were obtained, layered onto 5ml newborn calf serum and spun for 10min at 2200rpm at 20˚C. Afterwards, pellets were resuspended in 1ml Dulbecco's modified Earle's medium (DMEM; Invitrogen, Paisley, UK) and centrifuged for 10min at 2200rpm. The supernatant was discarded and the pellet resuspended in 6ml DMEM and layered onto a discontinuous Percoll density gradient and centrifuged for 30min at 2800rpm. The bands between 35-55% Percoll were obtained and mixed with cell culture medium (DMEM: Ham's F-12 Nutrient Mixture (Invitrogen, Paisley, UK) 1:1, 10% fetal calf serum (heat inactivated), 1% gentamicin, 0.2% benzylpenicillin, 0.2% streptomycin, 0.6% glutamine), before centrifugation at 2200rpm for 10min. The final pellet was resuspended in 2ml of cell culture medium. Cells were plated onto 35mm culture dishes (Nunc, Fisher Scientific, Loughborough, UK) or 16mm coverslips in 12-well culture plates at densities of 1-1.3x10^6/ml and 1x10^6/ml respectively at 37°C in a humidified incubator (95% air/5% CO_2).

Cytotrophoblast primary culture and treatment

Cytotrophoblasts plated onto 16mm coverslips were cultured for 66h. Cultures were washed 3 times with phosphate-buffered saline (PBS) and cell culture medium was replaced with fresh medium at 15 and 42h. Cells were untreated (control) or treated at 3, 15 and 42h with IK_{Ca} modulators 100μM DCEBIO (5, 6-dichloro-1-ethyl-1, 3-dihydro-2H-benzimidazol-2-one; IK_{Ca} activator) or 10μM TRAM-34 (1-[(2-chlorophenyl) diphenylmethyl]-1H-pyrazole; IK_{Ca} inhibitor). In both cases, the final concentration of dimethyl sulfoxide (DMSO) in the cell culture medium was 0.1%. Previous studies from this laboratory have shown that DMSO at 0.1% does not alter cytotrophoblast morphological or biochemical differentiation [51].

At 15, 42 and 66h of culture, cell culture medium was collected and stored at -20˚C for measurement of β-hCG (hCG β-subunit; produced by terminally differentiated syncytiotrophoblast, used to assess cytotrophoblast biochemical differentiation [51]). Coverslips were placed into 1ml 0.3M NaOH, cells scraped and the cell lysate stored at 4˚C. These samples were used to measure protein content (mg) with Bio-Rad Protein Assay, based on the Bradford method (Bio-Rad Laboratories, Hempstead, UK).

In addition, at 15, 42 and 66h of culture, cells were fixed in absolute methanol (permeabilizing fixative) for 20min at -20˚C or in 4% paraformaldehyde (PFA; non-permeabilizing fixative) for 15min at room temperature and stored in PBS at 4˚C prior to immunofluorescence staining.

Measurement of cytotrophoblast hCG secretion

The β-subunit of hCG is secreted by terminally differentiated syncytiotrophoblast and was used as an indicator of cytotrophoblast differentiation in culture [51]. β-hCG was assayed in cell-conditioned culture medium at 15, 42 and 66h of culture by ELISA (DRG Diagnostics, Marburg, Germany). Thawed samples were used following the instructions of the manufacturer. Optical density was measured at 450nm using a VersaMax microplate reader (Molecular Devices, CA, USA). hCG secretion was expressed as mIU/ml/h/mg protein.

Immunofluorescent staining

Methanol and PFA-fixed cells on 16mm coverslips were washed in tris-buffered saline (TBS). Block of non-specific binding was performed for 30min with 4% bovine serum albumin (BSA) in TBS. Cells were incubated for 1h at room temperature with mouse monoclonal antibody to desmoplakin I+II (2Q400; Abcam, Cambridge, UK), diluted 1:100

in TBS or mouse monoclonal antibody to IK_{Ca} ($K_{Ca}3.1$; clone 6C1; extracellular epitope; Alomone labs, Jerusalem, Israel), diluted 1:50 in 1% BSA in TBS. Negative control was obtained by omission of the primary antibody. Cells were washed with TBS and the secondary antibody, FITC-polyclonal rabbit anti-mouse immunoglobulin (Dako, Cambridgeshire, UK) diluted 1:50 in TBS, was applied and cells incubated for 1h at room temperature in the dark. After washing with TBS, coverslips were mounted using Vectashield mounting medium with propidium iodide nuclear counterstain (PI; Vector labs, Peterborough, UK). Immunofluorescent images were captured using a Zeiss AxioObserver Inverted Microscope (magnification 400x).

Analysis of cytotrophoblast multinucleation

Microscope images of cytotrophoblasts stained for desmoplakin and nuclei were used to assess multinucleation as a measurement of cytotrophoblast morphological differentiation. Based on a previously published method [51,52], 2-3 observers counted the total number of nuclei per given field and the number of nuclei in syncytium (multinucleated cell defined as ≥3 nuclei within desmoplakin boundaries) using ImageJ 1.45 software (National Institutes of Health, USA). The number of multinucleated cells was expressed as a percentage of the total number of nuclei within a given field (% of nuclei in multinucleate cells).

^{86}Rb efflux from cytotrophoblasts

^{86}Rb is commonly used as a tracer of K^+ and it has been previously shown that K_{Ca}s are permeable to ^{86}Rb [33]. ^{86}Rb efflux was measured in cytotrophoblasts at 66h of culture using a technique previously described [53]. Briefly, cells plated onto 35mm dishes were removed from the incubator and washed in control Tyrode's buffer (135mM NaCl, 5mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES, 5.6mM glucose, pH 7.4; osmolality ~283mOsm/kgH_2O, isotonic compared to maternal plasma at term [54]; osmolality measured by freezing point depression). Cells were incubated with 1ml 4μCi/ml ^{86}Rb (89.7μM; concentration 1μCi/ml; stock activity 1mCi) for 2h at room temperature. After washing for 3min in 2x25ml Tyrode's buffer (with no added isotope), ^{86}Rb efflux was measured by the sequential addition and removal of 1ml Tyrode's buffer at 1min intervals; samples were collected every 1min over 15min (control, basal ^{86}Rb efflux) and/or exposed to various treatments over 5-15min (experimental period): 10μM TRAM-34, 100μM DCEBIO, 100nM apamin (small conductance Ca^{2+}-activated K^+ channel (SK_{Ca}) blocker), 100nM charybdotoxin (ChTx; IK_{Ca}/large conductance Ca^{2+}-activated K^+ channel (BK_{Ca}) blocker), hyposmotic solution (55mM NaCl, 5mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 10mM

HEPES, 5.6mM glucose; pH 7.4; osmolality 145mOsm/kgH_2O), Ca^{2+}-free hyposmotic solution (extracellular Ca^{2+} was buffered by removing $CaCl_2$ and adding 0.5mM EGTA). When used together, a pre-block with TRAM-34 was performed at min 4 before adding DCEBIO-TRAM-34 or hyposmotic solution-TRAM-34. In a different set of experiments, ^{86}Rb efflux was measured in efflux buffer with osmolality ranging 283-138mOsm/kgH_2O, which was obtained by varying the NaCl concentration.

After 15min, the cells were lysed in 0.3M NaOH for ~1h and scraped in order to release intracellular ^{86}Rb which was then counted in the supernatant to give a measure of total ^{86}Rb remaining in the cells at the end of the experiment (cellular ^{86}Rb). Effluxed and cellular ^{86}Rb was measured in a gamma-counter (Packard Cobra II Auto Gamma, CA, USA). All counts recorded were at least 10 times higher than background counts.

The time course of percentage (%) ^{86}Rb efflux was calculated at each time point as ((^{86}Rb effluxed/^{86}Rb in cells) x100). The efflux rate constant was also determined making the assumption that ^{86}Rb efflux at steady state reflects the loss of ^{86}Rb from a single compartment (syncytiotrophoblast) limited by the K^+ permeability of the plasma membrane. Consequently, the loss of ^{86}Rb was measured by a first-order rate constant which was calculated over 10min experimental period as (l_n(^{86}Rb in cell at time t/^{86}Rb in cell at t_0)) where t_0 is the cellular ^{86}Rb at the start of the experiment.

Expression of Results and Statistics

Statistical analysis was performed using GraphPad Prism version 5 software. hCG secretion and multinucleation from control untreated cytotrophoblasts was expressed as mean ± standard error (SE) with n as the number of placentas. hCG secretion and multinucleation in TRAM-34 and DCEBIO-treated cells was expressed as median ± interquartile range (IQR) and analyzed with Friedman's test with Dunn's post hoc test. The relationship between ^{86}Rb efflux and extracellular fluid osmolality was analyzed comparing control *vs.* each experimental osmolality using ANOVA with Turkey Kramer multicomparison post hoc test. Each value was expressed as mean ± SE. %^{86}Rb efflux from multinucleated cytotrophoblasts was expressed as mean ± SE for each time point. The effects of all treatments on ^{86}Rb efflux were assessed for statistical significance by comparing the differences in the slopes and intercepts of the rate constants using least squares linear regression analysis. In all cases, a *p* value less than 0.05 was considered statistically significant.

Results

Cytotrophoblasts were isolated from normal term placentas and cultured for 66h. This is a well-characterized method [4,5,51,53,55,56] which recreates syncytiotrophoblast formation *in vivo*, as mononucleate cells (15h) fuse into multinucleate syncytia (66h) concomitant with elevated secretion of hCG.

Expression of IK_{Ca} in cytotrophoblasts

IK_{Ca} protein expression was confirmed in mono (Figure 1A) and multinucleated (Figure 1B, C) cytotrophoblasts using immunofluorescent staining with a specific antibody which detects an extracellular site in the pore forming domain (S5-6) of human IK_{Ca} ($K_{Ca}3.1$). Prior to immunostaining cells were fixed with methanol, a permeabilising fixative, in order to identify detectable intracellular IK_{Ca} immunostaining (Figures 1A, B) or with PFA, a non-permeabilizing fixative, to identify IK_{Ca} staining associated with cytotrophoblast surface (Figure 1C).

At 15h, IK_{Ca} staining (green) was evident in the nucleus (red; nuclear counterstain) of mononucleate cells, but also in the cytoplasm and surface of cell aggregates (Figure 1A). At 66h, IK_{Ca} was associated to both the cytoplasm (Figure 1B) and cell surface (Figure 1C) of multinucleated cytotrophoblasts. Arrows indicate specific areas were the staining was associated to the cell surface. Figure 1D corresponds to a representative negative control showing that non-specific staining was not observed.

Functional expression of IK_{Ca} was confirmed by measuring ^{86}Rb efflux, an indirect assessment of K^+ permeability, in multinucleated cytotrophoblasts after 66h of culture. The time course of $\%^{86}Rb$ efflux/min is plotted in Figure 1E and F. Basal $\%^{86}Rb$ efflux in control cytotrophoblasts showed a stable steady state over 13min (Figure 1E; black circles). DCEBIO, an IK_{Ca} activator, caused a marked rapid increase (8.3-fold) in ^{86}Rb efflux which was completely blocked by TRAM-34 (85%), an IK_{Ca} inhibitor (Figure 1E). Rate constants, taken as the slopes of the regression lines fitted over the experimental period (10min), were calculated and for all treatments the data could be fitted by a single exponential (Table 1). The fall in intracellular ^{86}Rb (slope) was significantly greater with DCEBIO compared to DCEBIO+TRAM-34 and controls. TRAM-34 had no effect on basal ^{86}Rb efflux (Figure 1F). The increase in ^{86}Rb efflux with DCEBIO confirms the functional expression of IK_{Ca} in multinucleated cytotrophoblasts.

Differentiation of cytotrophoblasts in culture

We confirmed previous reports of cytotrophoblast morphological and biochemical differentiation in culture [4,5,51]. Figures 2A-C show representative phase contrast images depicting cytotrophoblast morphology. The arrows indicate mononuclear cells at 15h, aggregates at 42h and multinucleate cytotrophoblasts at 66h (in Figures 2A, 2B and 2C respectively). Desmoplakin immunostaining (Figures 2D-F) confirmed this progression of morphological differentiation and was used to calculate the % of nuclei in multinucleate cells (multinucleation) at 15, 42 and 66h of culture. At 15h, cytotrophoblasts remained mononuclear (Figure 2D), at 42h the cells had aggregated (Figure 2E) and at 66h, cytotrophoblasts had fused to become multinucleated as indicated by the disappearance of desmoplakin staining (≥3 nuclei in syncytia; Figure 2F). Cytotrophoblast multinucleation increased 12-fold between 15 and 66h (Figure 2G). This morphological progression was accompanied by biochemical differentiation as indicated by an increase in hCG secretion (Figure 2H). Cytotrophoblast β-hCG secretion increased 20-fold between 15 and 66h (Figure 2H).

Effect of IK_{Ca} modulators on cytotrophoblast multinucleation

Cytotrophoblasts were treated at 3, 15 and 42h of culture with IK_{Ca} modulators TRAM-34 and DCEBIO and multinucleation (% of nuclei in multinucleate cells) was assessed to determine morphological differentiation. Figures 3A-F show representative images of desmoplakin immunostaining (green) and Pi (red; nuclei) in cytotrophoblasts at 15 (Figure 3A: control untreated, 3C: TRAM-34, 3E: DCEBIO-treated) and 66h (Figure 3B: control, 3D: TRAM-34, 3F: DCEBIO-treated) of culture. Compared to controls, activation of IK_{Ca} with DCEBIO significantly reduced multinucleation by 42% (median ± IQR: 26.6 *16.2/30.0* compared to 13.8 *8.6/17.2* respectively) at 66h of culture (Figure 3G). Multinucleation was unaffected by TRAM-34 (Figures 3C, D, G).

Effect of IK_{Ca} modulators on cytotrophoblast hCG secretion

Compared to controls at 66h, DCEBIO reduced β-hCG secretion by 80% (19.*5 7.1/19.5*; Figure 4A). This inhibition of differentiation was not associated with a fall in total cell protein (Figure 4B), a proxy measure of cell number, suggesting that DCEBIO did not have a generalized toxic effect. On the contrary, DCEBIO caused a transient increase in cell protein at 42h (148.8 *134.8/157.1*; Figure 4B). TRAM-34 did not affect cytotrophoblast hCG secretion (Figure 4A) or total cell protein (Figure 4B).

In addition, the total number of nuclei was unaffected by the treatment with either TRAM-34 or DCEBIO; however, treatment with DCEBIO caused a transient increase in the total number of nuclei at 15h of culture (Figure 4C).

Effect of IK_{Ca} inhibitor on swelling-activated K^+ efflux from cytotrophoblasts

A role for IK_{Ca} in regulating syncytiotrophoblast volume was explored using multinucleated cytotrophoblasts. We investigated the participation of IK_{Ca} in syncytiotrophoblast RVD by experimentally exposing cytotrophoblasts to a hyposmotic solution and measuring ^{86}Rb efflux as a marker of syncytiotrophoblast K^+ permeability.

Figure 5A shows the relationship between ^{86}Rb efflux and extracellular fluid osmolality (ranging from 283-138mOsm/kgH_2O): total ^{86}Rb efflux over 10min (experimental period) was plotted against the reciprocal value for the osmolality of the fluid bathing the cytotrophoblasts after 66h of culture. A reduction in osmolality to 218mOsm/kgH_2O (77% of control), stimulated ^{86}Rb efflux compared to control (283mOsm/kgH_2O). Reducing extracellular osmolality to 183 and 138mOsm/kgH_2O (65 and 49% of control respectively) progressively stimulated ^{86}Rb efflux over control. Consequently, the minimum extracellular osmolality required to trigger ^{86}Rb efflux from multinucleated cytotrophoblasts is between 77-65% isotonic. Therefore, the remaining experiments were performed using a hyposmotic solution with an osmolality of 145mOsm/kgH_2O.

In agreement with previous results in placental villous tissue [57], exposure of multinucleated cytotrophoblasts to a hyposmotic solution markedly increased ^{86}Rb efflux (3.8-fold; Figure 5B). The rate constant (Table 1) for ^{86}Rb efflux was significantly greater in cytotrophoblasts exposed to the hyposmotic solution than controls. In addition, swelling-activated ^{86}Rb efflux was Ca^{2+}-dependent, as removal of Ca^{2+} from the hyposmotic solution abolished the activation of ^{86}Rb efflux at 66h of culture (Figure 5B; Table 1).

Figure 5C shows that swelling-activated ^{86}Rb efflux was blocked by IK_{Ca} inhibitor, TRAM-34 (85%; Figure 5C). In parallel, rate constant analysis shows a significant difference between hyposmotic solution and TRAM-34, indicating that cytotrophoblast cell swelling activates IK_{Ca} (Table 1). Swelling-activated ^{86}Rb efflux is mediated specifically by IK_{Ca} as exposure to the SK_{Ca} blocker apamin did not affect the stimulated ^{86}Rb efflux. In contrast, exposing cytotrophoblasts to IK_{Ca}/BK_{Ca} blocker ChTx, almost completely inhibited swelling-activated ^{86}Rb efflux (Figure 5D; Table 1), suggesting that the regulation of cytotrophoblast cell volume status is through IK_{Ca}.

Discussion

This study shows that IK_{Ca} protein is expressed by mono- and multinucleate cytotrophoblasts *in vitro*. Multinucleate cells show low inherent IK_{Ca} activity as TRAM-34, an inhibitor of IK_{Ca} did not alter basal ^{86}Rb efflux. However, DCEBIO stimulated TRAM-34-sensitive ^{86}Rb efflux from multinucleate cells indicating the functional presence of IK_{Ca}. Chronic (48h) application of DCEBIO significantly inhibited both the formation of multinucleate cytotrophoblasts and their secretion of hCG. IK_{Ca}, in common with other tissues, may play a role in regulating syncytiotrophoblast volume as experimentally-induced cell swelling activated Ca^{2+}-dependent TRAM-34-sensitive ^{86}Rb efflux from multinucleated cells.

IK_{Ca} expression and function in cytotrophoblasts from term placentas

Immunofluorescent staining of cytotrophoblasts confirmed the expression of IK_{Ca} protein in mononuclear, aggregated and multinucleated cells. IK_{Ca} staining was associated with the nucleus, cytoplasm and cytotrophoblast cell surface regardless of differentiation stage. Other K^+ channels, such as K_Vs [58] and K_{Ca}s [59,60] have been localized to the cell nucleus in various cell types; it has been suggested K_{Ca}s could control Ca^{2+} release and mobilization within the cell nucleus [59]. In addition, there is evidence of intracellular localization of K_{Ca}s which may be associated with different cellular functions in non-placental cell types, e.g. in mitochondria [61], intracellular trafficking [62]. Therefore, the heterogeneous localization of IK_{Ca} could be related to diverse functions that these channels might have in cytotrophoblasts during differentiation.
The functional expression of IK_{Ca} was assessed using ^{86}Rb efflux as a tracer of K^+ efflux. The results indicate that multinucleated cytotrophoblasts express functional IK_{Ca} as exposure to the IK_{Ca} activator DCEBIO, significantly increased ^{86}Rb efflux. DCEBIO was specific for IK_{Ca} since this increase in efflux was completely blocked by TRAM-34. However, in a quiescent state IK_{Ca} are inactive as TRAM-34 did not affect basal ^{86}Rb efflux. This opens the possibility that different stimuli can activate IK_{Ca} in cytotrophoblasts under physiological/pathophysiological conditions but this remains to be determined.

Role of IK_{Ca} in cytotrophoblast multinucleation

Cytotrophoblasts isolated from term placentas subjected to trypsin-DNAse digestion and Percoll gradient separation are enriched in trophoblast markers and lack contamination from other placental cell types such as, endothelial cells, smooth muscle cells, fibroblasts, or macrophages [4,5]. After isolation and during the first hours, these cells, which are

mitotically inactive, remain mononucleated and secrete small amounts of hCG. After 24h in culture, they migrate, aggregate and syncytialize by a process of fusion. By 66h, cytotrophoblasts are predominantly multinucleated syncytial-like cells which secrete high levels of hCG reminiscent of the syncytiotrophoblast *in vivo* [4,5]. The loss of desmoplakin immunostaining was used to indicate cytotrophoblast fusion and there was, a progressive increase in the formation of multinucleated cytotrophoblasts (≥3 nuclei) after 42h in culture. Cytotrophoblast differentiation was impaired when IK_{Ca} was activated over 42-66h. DCEBIO did not alter aggregation but inhibited cytotrophoblast morphological and biochemical differentiation *in vitro* by reducing multinucleation and hCG secretion respectively. These effects were not related to toxicity as total protein was unaffected; indeed, protein levels and the total number of nuclei were higher with DCEBIO at 15-42h compared to control and this might indicate a transient improved cell viability. Conversely, TRAM-34 treatment did not affect cytotrophoblast syncytialization or hCG secretion. This IK_{Ca} inhibitor was also without effect on ^{86}Rb efflux indicating little or no IK_{Ca} activity in syncytiotrophoblast under basal conditions.

In non-placental cell types IK_{Ca} is associated with the regulation of processes that contribute to the maintenance of tissue homeostasis including proliferation [31,41,42,43], differentiation/fusion [44,45], cell migration [46,47,48] and apoptosis [49]. Particularly, a charybdotoxin (IK_{Ca} inhibitor)-sensitive K^+ channel activity is necessary for keratinocyte differentiation [44]. Here we showed that pharmacological activation of IK_{Ca} markedly reduced cytotrophoblast syncytialization implying that IK_{Ca} activation inhibits cytotrophoblast-syncytiotrophoblast fusion. In addition, this evidence suggests IK_{Ca} function could change with cytotrophoblast differentiation and therefore chronically activating these channels could lead to abnormal cytotrophoblast-syncytiotrophoblast fusion and dysregulated turnover. A reduced trophoblast fusion leading to altered syncytiotrophoblast turnover has been proposed in pregnancy complications such as pre-eclampsia as fusogenic proteins are downregulated [22,24,27,28]. However, the specific role of IK_{Ca} in this process, and the cellular signals acting in conjunction to co-ordinate trophoblast fusion, need to be addressed in future.

Despite the well established role for IK_{Ca}s in facilitating cell migration [46,47,48], it is unlikely that they have a similar role in cytotrophoblast migration *in vitro* as cell aggregation, although not assessed quantitatively, did not appear to be affected by openers/blockers of IK_{Ca} when applied from 3h after cell isolation.

Role of IK_{Ca} in syncytiotrophoblast endocrine secretion

K^+ channels participate in endocrine secretion [63,64,65,66] and hCG secretion by placental syncytiotrophoblast is modulated by voltage-gated K^+ channels (K_V) [51]. hCG is

synthesized and secreted by terminally differentiated trophoblast but the mechanism of secretion is still not fully understood. It is evident that hCG secretion is under autocrine/paracrine regulation by hCG which itself promotes cytotrophoblast cell differentiation and further hCG secretion [67]. K_Vs regulate the secretory process rather than hormone production [51]. Here we showed that the chronic activation of IK_{Ca} significantly reduced hCG secretion by cytotrophoblasts, suggesting that IK_{Ca} could inhibit the mechanism of hCG secretion. However, there is little evidence to link IK_{Ca} function with endocrine secretion, and IK_{Ca} action on hormone secretion is restricted to the central nervous system [68]. We speculate that the primary effect of IK_{Ca} is to inhibit cytotrophoblast cell fusion/terminal differentiation and, as a result, hCG production and secretion is reduced.

Role of IK_{Ca} in syncytiotrophoblast volume regulation

In many cell types, restoration of cell volume in the presence of a hyposmotic stimulus (RVD) is mediated by K^+ channels, including IK_{Ca}, in conjunction with swelling-activated anion channels [39]. In the current study, exposing multinucleated cytotrophoblasts to a hyposmotic solution increased ^{86}Rb efflux ~3.8-fold and this activated efflux was dependent on extracellular Ca^{2+}, blocked (>80%) by the IK_{Ca} blockers TRAM-34 and charybdotoxin but was unaffected by the SK_{Ca} inhibitor apamin. These data implicate IK_{Ca} in cytotrophoblast RVD. Lowering extracellular osmolality also stimulated ^{86}Rb efflux from placental villous tissue [57] and caused a Ba^{2+}-sensitive hyperpolarization of the syncytiotrophoblast microvillous membrane [69]; however, the identities of the K^+ channels underlying the resting conductance, or the change with cell swelling, remain unknown.

Exposing cells to a hyposmotic solution is an experimental maneuver often used to mimic the cell swelling which takes place secondary to a rise in intracellular osmolality as can occur following nutrient uptake [39,69,70]. In this case, activation of K^+ channels is a homeostatic process to promote water loss to restore the concentration of cytoplasmic constituents and to shrink cells back to their original size. On the other hand, in the absence of hyposmotic swelling, the activation of K^+ channels to promote water loss effects a cell volume change, and/or fall in intracellular K^+, that is essential for a variety of processes that maintain tissue homeostasis such as cell proliferation, migration, differentiation/fusion and cell death [71]. It is possible that dynamic changes in cell volume are required for normal cytotrophoblast fusion and that, in the current study, chronically activating IK_{Ca} channels induced an inappropriate change in cell volume which inhibited fusion. Cytotrophoblast fusion may be altered by promoting IK_{Ca} activity and consequently inducing water loss which alters the concentration of cytoplasmic factors that regulate

fusion. These proposals need to be investigated in future, and in particular elucidate whether the primary effect of activation of IK_{Ca} is on fusion.

Perspectives and significance

The primary stimuli for IK_{Ca} activation is an elevation in $[Ca^{2+}]_i$ and therefore factors that increase $[Ca^{2+}]_i$ will activate cytotrophoblast IK_{Ca}. To date there are relatively few studies of the regulation of $[Ca^{2+}]_i$ in syncytiotrophoblast; however, preliminary evidence indicates that hyposmotic swelling increases $[Ca^{2+}]_i$ in multinucleated cytotrophoblasts, predominantly by entry from extracellular fluid [72].

Consequently, activation of IK_{Ca} could regulate syncytiotrophoblast volume, which may change dynamically following solute uptake and/or cytotrophoblast cell fusion, an essential homeostatic mechanism to maintain nutrient transport and endocrine function respectively. In addition, we have previously shown that cytotrophoblast $[Ca^{2+}]_i$ is elevated following activation of purinergic receptors, including P2X4, by extracellular nucleotides and that this promotes ^{86}Rb efflux which is inhibited by ChTx, implicating activation of IK_{Ca}. These findings might be of relevance to the etiology of pre-eclampsia, a disease of pregnancy characterized by abnormal cytotrophoblast fusion and renewal of syncytiotrophoblast. Indeed, the expression of P2X4 by the placenta is elevated in pre-eclampsia compared to normal pregnancy [73]. It is also proposed that hypoxia/elevated reactive oxygen species release nucleotides from the trophoblast in pre-eclampsia to elevate local concentrations in the extracellular fluid [73,74]. As a result, increased activation of P2X4 would elevate $[Ca^{2+}]_i$ and activate IK_{Ca}s. The inappropriate activation of IK_{Ca} could compromise cell volume homeostasis, and impact on cytotrophoblast cell fusion and syncytiotrophoblast renewal, endocrine function and nutrient transport in pre-eclampsia.

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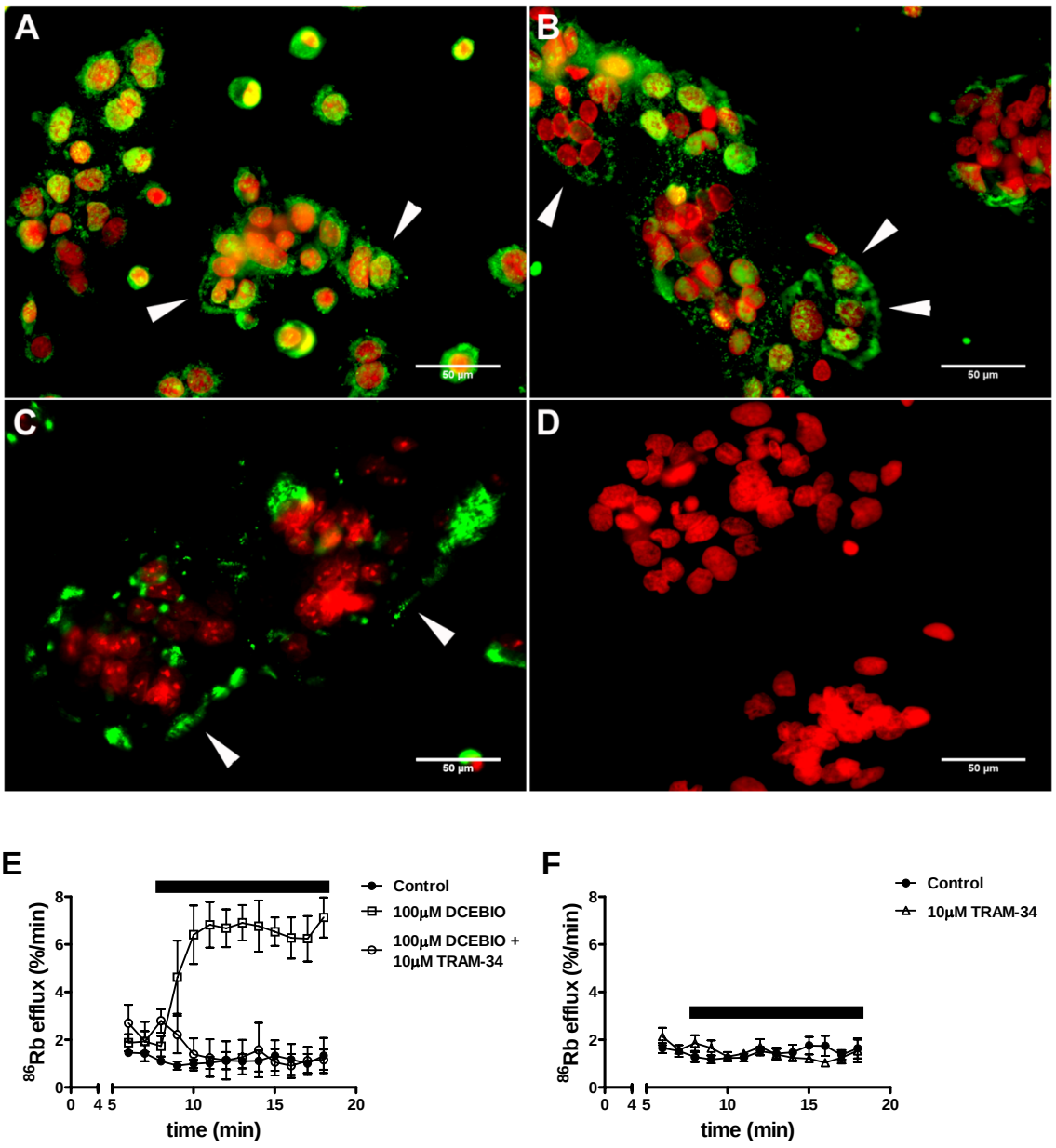

Figure 1: Expression of IK_{Ca} in placental cytotrophoblasts.
IK_{Ca} protein expression is shown in representative images of dual immunofluorescent staining for IK_{Ca} ($K_{Ca}3.1$; green) and nuclear counterstain (red) in cytotrophoblasts at 15h (***A***) and 66h (***B***, methanol-fixed cells; ***C***, PFA-fixed cells) of culture. Arrows indicate IK_{Ca} staining associated with cell surface. ***D***: Representative negative control performed in multinucleated cytotrophoblasts at 66h of culture. Scale bar 50µm. ***E***, ***F***: IK_{Ca} functional expression. Time course of $\%^{86}Rb$ efflux over 13min in multinucleated cytotrophoblasts at 66h of culture. During the experimental period (indicated by the bar) cells were untreated (control) or treated with DCEBIO, DCEBIO + TRAM-34 (***E***; n= 3 placentas), or with TRAM-34 (***F***; n= 4 placentas). Data are mean ± SE.

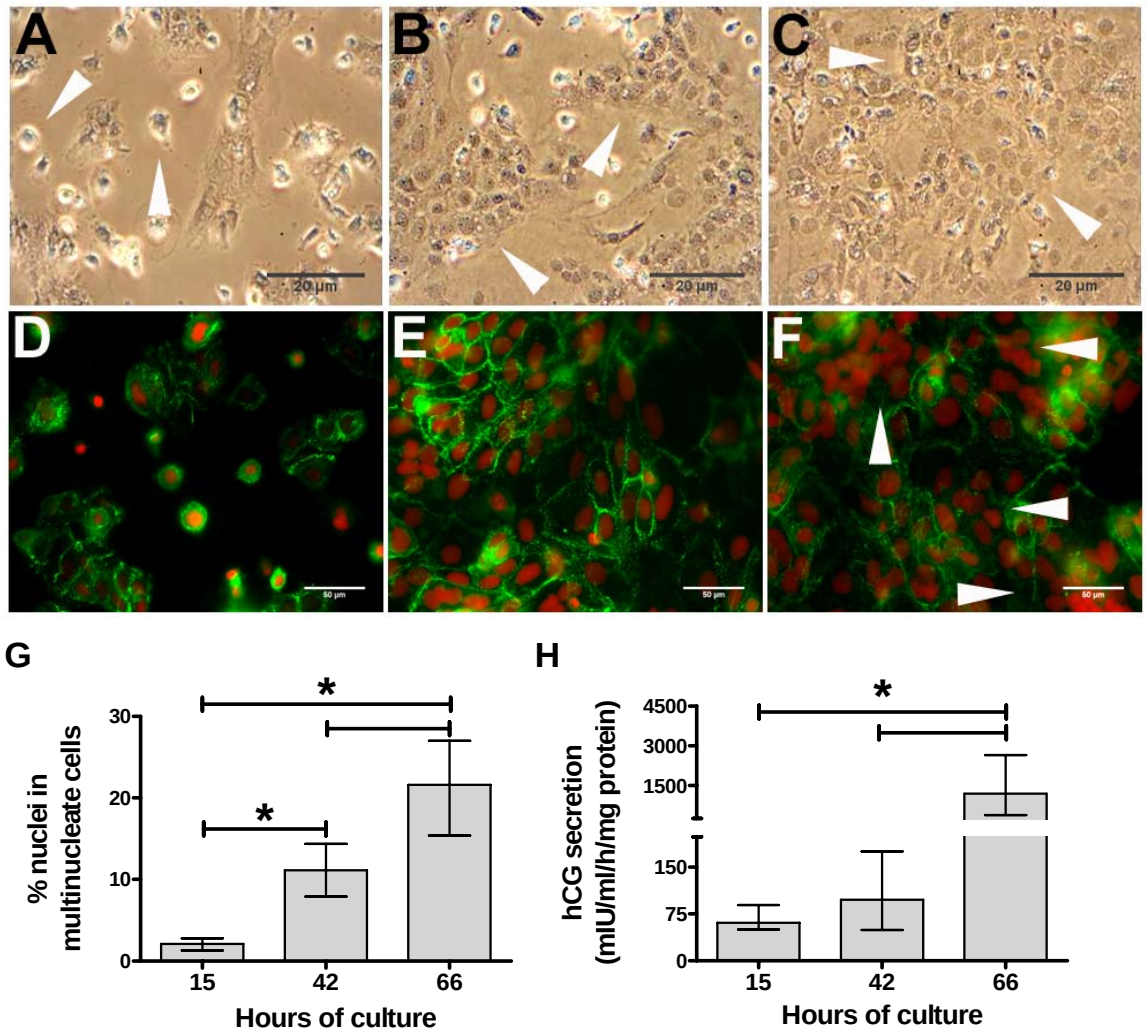

Figure 2: Characterization of placental cytotrophoblasts in culture.
Representative phase contrast images of cytotrophoblasts at (***A***) 15h, (***B***) 42h and (***C***) 66h depicting cytotrophoblast morphological differentiation in culture (scale bar 20µm), together with corresponding immunofluorescent desmoplakin-staining (green) and nuclear counterstain (red; scale bar 50µm) in ***D***, ***E***, ***F*** respectively. Arrows show different stages of cytotrophoblast differentiation: in (***A***) mononucleate cells, (***B***) multinucleate cell aggregates and (***C***, ***F***) multinucleate syncytial-type cells. ***G***: The % of cytotrophoblast nuclei in multinucleate cells depicting multinucleation and morphological differentiation (n= 15 placentas) at 15, 42 and 66h in culture. ***H***: Cytotrophoblast β-hCG secretion depicting biochemical differentiation (n= 16 placentas); **p*<0.05; Friedman's test with Dunn's post hoc test. Data are median ± IQR.

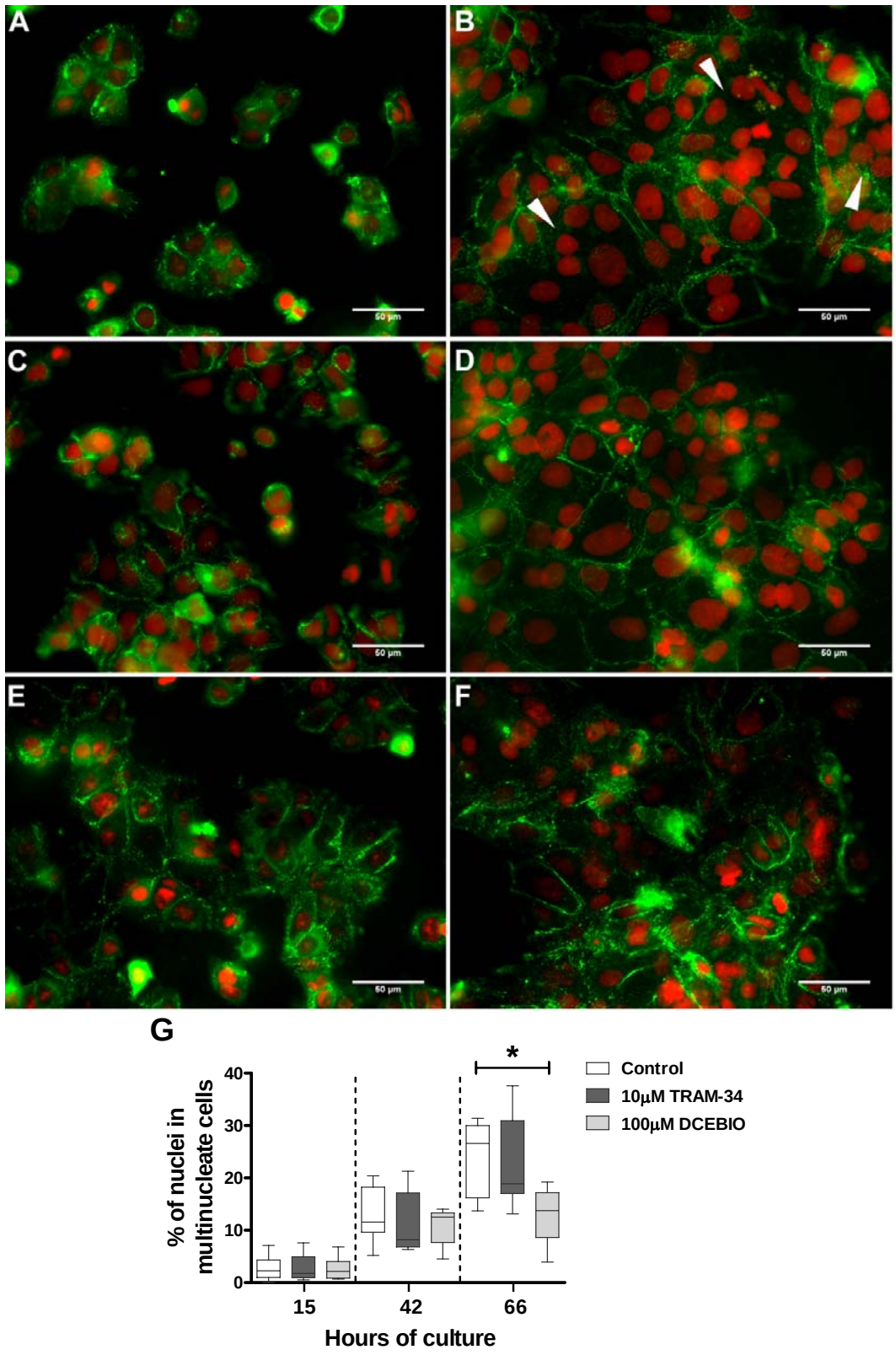

Figure 3: DCEBIO reduces cytotrophoblast multinucleation.
Representative dual immunofluorescent staining showing desmoplakin (green) and nuclear counterstain (red) in control untreated (***A***, ***B***), TRAM-34 (***C***, ***D***) or DCEBIO (***E***, ***F***) treated cytotrophoblasts at 15 (***A***, ***C***, ***E***) and 66h (***B***, ***D***, ***F***) of culture. Arrows in ***B*** indicate multinucleated cytotrophoblasts at 66h of culture. Scale bar 50µm. ***G***: The % of cytotrophoblast nuclei in multinucleate cells (multinucleation) at 15, 42 and 66h of culture (n= 6 placentas); *p<0.05; Friedman's test with Dunn's post hoc test. Data are median ± IQR.

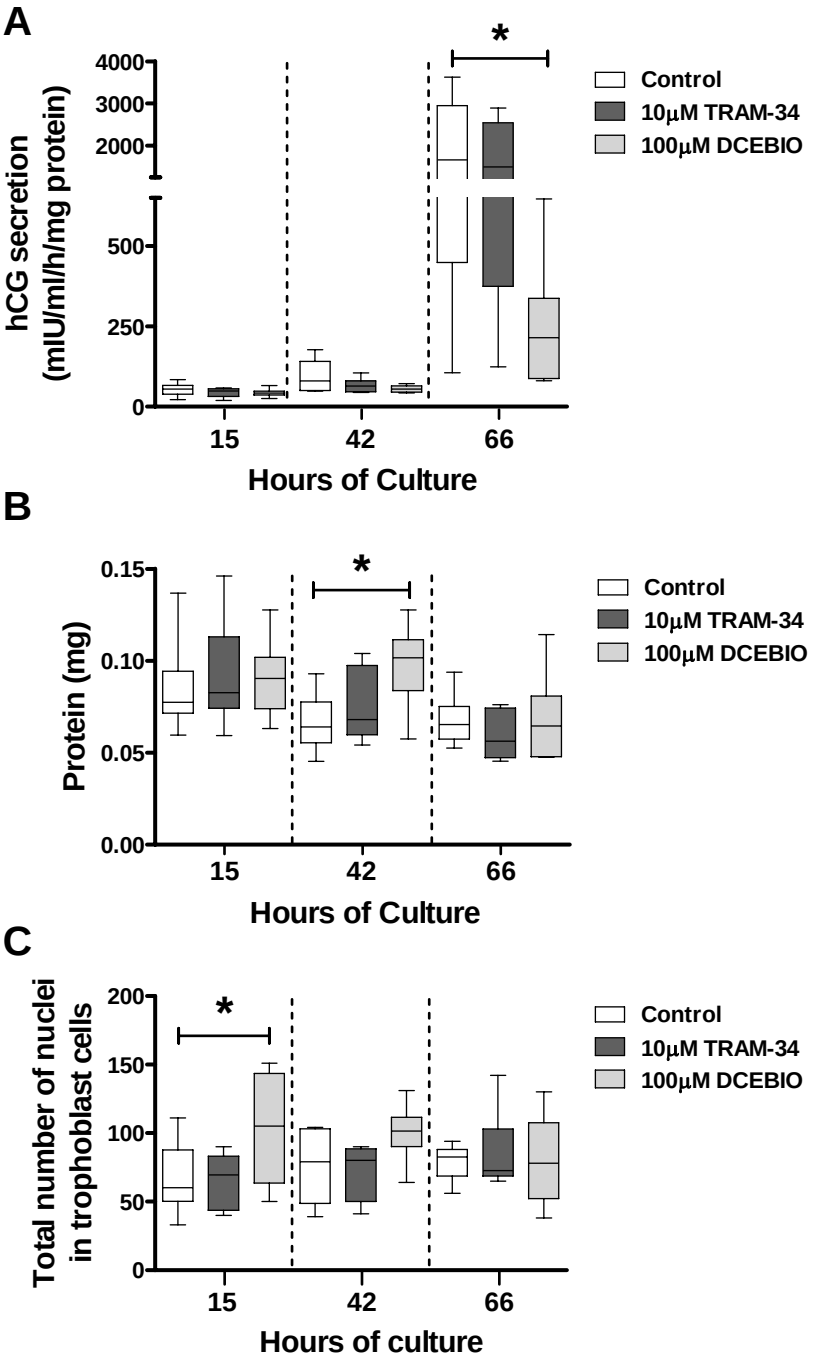

Figure 4: DCEBIO inhibits cytotrophoblast hCG secretion.
(***A***) β-hCG secretion, (***B***) cell protein and (***C***) total number of nuclei in cytotrophoblasts at 15, 42 and 66h of culture in controls and cells treated with TRAM-34 or DCEBIO; n= 6 placentas; *p<0.05; Friedman's test with Dunn's post hoc test. Data are median ± IQR.

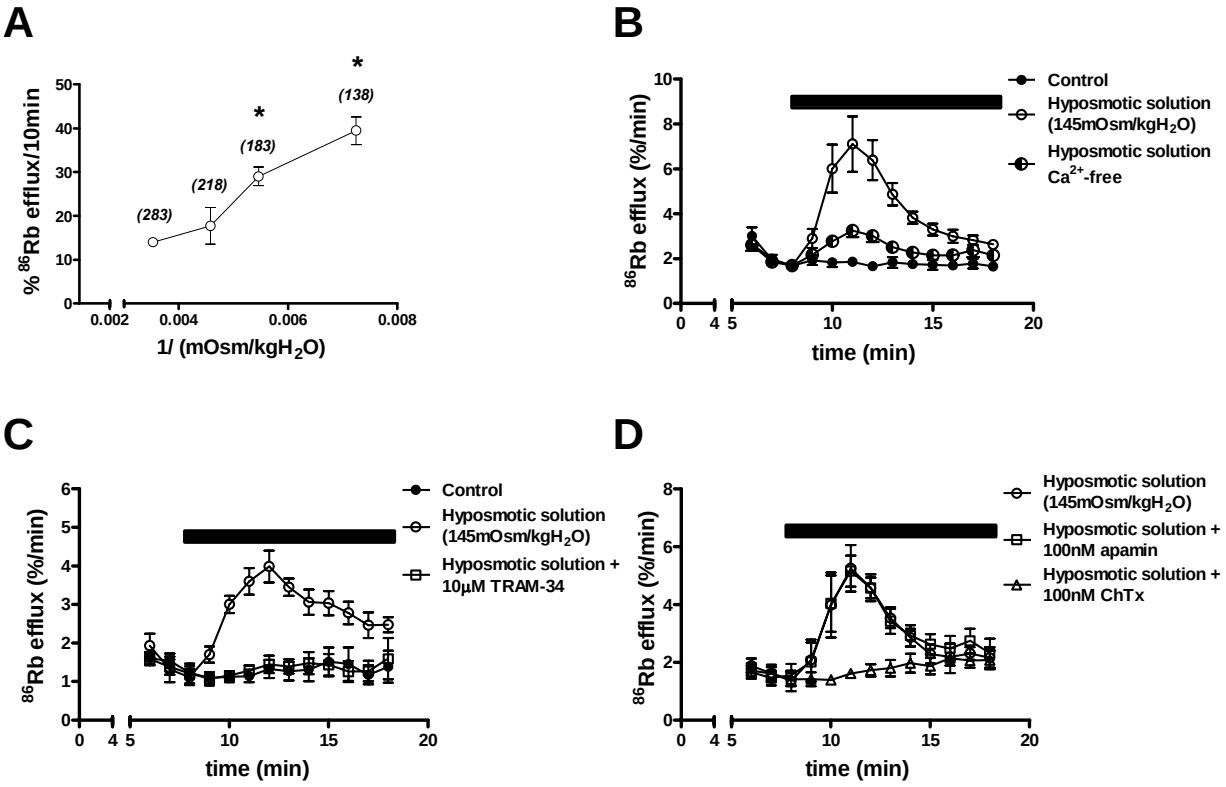

Figure 5: Hypo-osmolality activates IK_{Ca}.
A: Relationship between ^{86}Rb efflux and extracellular fluid osmolality in multinucleated cytotrophoblasts at 66h culture. The cells were bathed in Tyrode's buffer with an osmolality of 283 (control), 218, 183 or 138mOsm/kgH_2O (shown in italics) for 10min and the total ^{86}Rb efflux over this period was plotted against 1/osmolality. Values are mean ± SE (n= 4-5 placentas); *$p<0.001$ *vs.* control at 66h in culture; ANOVA with Turkey Kramer multicomparison post hoc test. ***B***-***D***: Characterization of swelling-activated ^{86}Rb efflux in multinucleated cytotrophoblasts (66h). Time course of %^{86}Rb efflux over 13min; during the experimental period (indicated by the bar) cells were (***B***) untreated (control) or exposed to hyposmotic solution (145mOsm/kgH_2O) or Ca^{2+}-free hyposmotic (n= 6 placentas); (***C***) control, hyposmotic solution or hyposmotic solution + TRAM-34 (n= 5 placentas); (***D***) hyposmotic solution, hyposmotic solution + apamin or hyposmotic solution + charybdotoxin (ChTx) (n= 5 placentas). Data are mean ± SE.

Mean rate constants of ^{86}Rb efflux in control and treated cytotrophoblasts

Condition	*^{86}Rb efflux rate constant (l_n ^{86}Rb in cell (t=x)/(t=0))/min^{-1}*	*r^2*	*p value*	*n*
Control	-0.015 ± 0.001	0.660	-	8
Control-100μM DCEBIO	-0.068 ± 0.005*	0.863	<0.0001	3
Control-100μM DCEBIO+10μM TRAM-34	-0.013 ± 0.004	0.284	0.689	3
Control-10μM TRAM-34	-0,014 ± 0.001**	0.861	0.985	4
Hyposmotic solution	-0.032 ± 0.001***	0.933	<0.0001	6
Hyposmotic solution+100nM apamin	-0.036 ± 0.003†	0.854	0.799	3
Hyposmotic solution+100nM ChTx	-0.018 ± 0.001†	0.885	<0.0001	3
Hyposmotic solution+10μM TRAM-34	-0.013 ± 0.001††	0.867	<0.0001	5

Table 1: Mean rate constants of ^{86}Rb efflux in control and treated cytotrophoblasts over 10min.
Data are mean ± SE, n is the number of placentas. *p* values determined by linear regression; *compared to corresponding control (-0.011 ± 0.002/min^{-1}; r^2 0.628) and 100μM DCEBIO + 10μM TRAM-34 (-0.013 ± 0.004/min^{-1}; r^2 0.284); **compared to control (-0.014 ± 0.001/min^{-1}; r^2 0.823); ***compared to control (-0.013 ± 0.001/min^{-1}; r^2 0.763); † compared to hyposmotic solution (-0.035 ± 0.003/min^{-1}; r^2 0.808); †† compared to hyposmotic solution (-0.032 ± 0.001/min^{-1}; r^2 0.925).

3.3. Nitrative stress modulates intermediate conductance Ca^{2+} activated K^+ channels: Implications for human placental syncytiotrophoblast differentiation

Work in progress

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Running head: IK_{Ca}, cytotrophoblast differentiation and nitrative stress

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Abstract

Placental syncytiotrophoblast (STB) is a multinucleated epithelium that transports nutrients to the fetus and secretes human chorionic gonadotropin (hCG). STB is maintained during pregnancy by fusion and differentiation of cytotrophoblasts, a process under paracrine/autocrine regulation by hCG. STB renewal is abnormal in pre-eclampsia (PE), a pregnancy disease associated with nitrative stress. We showed previously that pharmacological activation of intermediate conductance Ca^{2+}-activated K^+ channels (IK_{Ca}) inhibits cytotrophoblast fusion and hCG secretion *in vitro*. Here we test the hypothesis that IK_{Ca} is activated by nitrative stress which inhibits cytotrophoblast hCG secretion and multinucleation. Cytotrophoblasts isolated from normal term placentas were cultured for 66h to recreate STB formation *in vivo;* mononucleate cells (15h) fused into multinucleate syncytia (66h) concomitant with elevated secretion of hCG. Cells were untreated or treated over 3-66h with peroxynitrite ($ONOO^-$; 0.1mM) or the $ONOO^-$ generator 3-morpholinosydnonimine (SIN-1; 0.5mM). Medium was collected to measure hCG and cells fixed for immunofluorescence with anti-desmoplakin antibody to assess multinucleation. At 66h, ^{86}Rb efflux was measured to assess cytotrophoblast K^+ channel activity.

In untreated multinucleated cytotrophoblasts, acute application of $ONOO^-$ caused a reversible increase in ^{86}Rb efflux (2.8-fold; $p<0.0001$) which was inhibited (35%) by 10μM TRAM-34 (IK_{Ca} blocker). Acute exposure to hyposmotic buffer increased ^{86}Rb efflux (2.8-fold) which was enhanced 1.5-fold by $ONOO^-$ ($p<0.0001$) and inhibited 51% by TRAM-34. Compared to controls, SIN-1 significantly reduced hCG secretion by 23, 39 and 53% at 15, 42 and 66h respectively without altering multinucleation, the number of nuclei or cell protein. $ONOO^-$ did not affect hCG secretion or multinucleation.

Short term application of $ONOO^-$ stimulated basal ^{86}Rb efflux, and efflux following hyposmotic cell swelling, in part through an effect on IK_{Ca}. Long term exposure to $ONOO^-$ generated by SIN-1 inhibited hCG secretion from cytotrophoblasts *in vitro*. We propose that activation of IK_{Ca} by nitrative stress could inhibit hCG secretion and compromise STB renewal in PE.

Keywords

IK_{Ca}, nitrative stress, peroxynitrite, human chorionic gonadotropin

Introduction

STB is a highly specialized multinucleated epithelial cell which forms the interface between maternal and fetal blood and has functions that are essential to support normal pregnancy, such as production and secretion of hormones and transfer of nutrients to the fetus. STB has a short life span and is continuously renewed by a process of cellular turnover where proliferative mononucleate cytotrophoblasts exit the cell cycle, differentiate and fuse with the overlying syncytial layer [1]. Both apoptosis and autophagy are proposed to complete STB turnover [2-3]. STB renewal is a highly co-ordinated process and regulated by a number of factors which include human chorionic gonadotropin (hCG). hCG is synthesized and secreted by terminally differentiated STB and has an autocrine/paracrine effect to promote cytotrophoblast differentiation and fusion to form the STB [4].

The importance of STB turnover is highlighted by the fact that its dysregulation is evident in PE [5-6], a serious disease of pregnancy associated with maternal and/or fetal mortality and morbidity [7]. Evidence shows that cell proliferation [8] and apoptosis [9-10] are altered in PE and there is a reduction in cytotrophoblast fusion [11-12], thought to be a limiting step for STB formation. Although the reasons underlying altered STB turnover are not well understood, it has been hypothesized this could be induced by nitrative stress, an important feature of PE.

It is proposed that the origin of PE lies in inadequate remodeling of the maternal uterine spiral arteries during gestation which leads to intermittent blood flow into the placental bed [13-14], producing fluctuations in oxygen delivery and an increase in free radical generation including reactive oxygen and nitrogen species [14]. It is well established that placental oxidative and nitrative stress is elevated in PE [15-17]. The generation of superoxide (O_2^-) and nitric oxide (NO) exceeding the capacity of antioxidant defenses, leads to the production of $ONOO^-$, a potent and long-lived reactive nitrogen species [18]. $ONOO^-$ oxidizes thiol groups in cysteine residues [19] and also nitrates aromatic amino acids (tyrosine) to generate nitrotyrosine [19-22] and can cause gain [23-24] or loss [25-26] of protein function.

Placentas from pregnancies complicated with PE show increased formation of nitrotyrosine residues [15, 27], indicative of excessive $ONOO^-$ production, compared to normal pregnancy. The nitration of several intracellular (p38 MAP kinase [28], acetyl CoA, p53 [29]) and membrane (P2X4 purinergic receptor [30] and the taurine transporter [29]) proteins is greater in placentas of women with PE compared to normal pregnancy. Despite the evidence for elevated STB nitrotyrosine in PE, and of increased protein nitration, it is not known whether nitrative stress affects STB renewal.

We have shown that K^+ channels expressed by STB regulate processes of STB renewal. Both voltage-gated [31] and Ca^{2+}-activated K^+ channels [32] modulate hCG secretion and multinucleation of cytotrophoblasts in primary culture. Pharmacological activation of the intermediate conductance Ca^{2+}-activated K^+ channel (IK_{Ca}), expressed by cytotrophoblasts, inhibits the formation of multinucleated cells and the increase in hCG secretion normally associated with differentiated syncytial cytotrophoblasts [32]. The mechanism of this effect has not been determined but could be secondary to altered cell volume; IK_{Ca} plays a key role in maintaining cell volume in response to hyposmotic swelling (regulatory volume decrease: RVD [33-34]) and in addition IK_{Ca} promotes water loss which is required for cellular motility [35-37] and apoptosis [38]. IK_{Ca} in cytotrophoblasts are activated by a hyposmotic stimulus [39], implicating a role for these channels in cytotrophoblast volume homeostasis. However, as pharmacological activation of IK_{Ca} inhibits the morphological and biochemical differentiation of cytotrophoblasts, it is possible that they are activated inappropriately in PE and contribute to altered STB renewal. K^+ channel activity can be modulated by $ONOO^-$ [40-42] but it is unknown whether nitrative stress could promote IK_{Ca} expression/activity to dysregulate STB turnover in PE.

Here we use cytotrophoblasts isolated from normal term placentas to test the hypotheses a) that nitrative stress ($ONOO^-$) activates IK_{Ca} in multinucleated cells; b) that long-term exposure (48h) to nitrative stress downregulates hCG secretion and multinucleation.

Materials and Methods

Materials

Unless otherwise stated, all chemicals were from Sigma-Aldrich (Poole, UK).

Ethical approval

Human placentas used in this study were obtained from St. Mary's Hospital Maternity Unit (Manchester, UK) following written informed consent as approved by the Local Research Ethics Committee (North West; Haydock Park; Ref: 08/H1010/55). Normal term placentas (37-42 weeks gestation) were obtained from uncomplicated pregnancies following vaginal delivery or Caesarean section. The investigation conforms to the principles outlined in the Declaration of Helsinki.

Cytotrophoblast isolation

Cytotrophoblasts were isolated from term placentas using an adaptation of the method used by Kliman *et al*. [43], as previously described [44]. This is a well-characterized preparation [31, 45-46] which recreates STB formation *in vivo*, as isolated mononucleate cells (15h) fuse into multinucleate syncytia (66h) concomitant with elevated secretion of hCG.

Briefly, full thickness placenta samples (~2cm^3) were taken within 30min of delivery and placed into sterile saline. Placental villous tissue was further dissected from each sample after removal of the chorionic plate and decidua. ~30g of villous tissue was obtained and submitted to digestion 3 times in Hank's balanced salt solution containing 2.5% trypsin and 0.2mg/ml deoxyribonuclease (DNAse I) for 30min at 37°C (80rpm). After each digestion, 100ml of supernatant were obtained, layered onto 5ml newborn calf serum and spun for 10min at 2200rpm at 20˚C. Afterwards, pellets were resuspended in 1ml Dulbecco's modified Earle's medium (DMEM; Invitrogen, Paisley, UK) and centrifuged for 10min at 2200rpm. The supernatant was discarded and the pellet resuspended in 6ml DMEM and layered onto a discontinuous Percoll density gradient and centrifuged for 30min at 2800rpm. The bands between 35-55% Percoll were obtained and mixed with cell culture medium (DMEM: Ham's F-12 Nutrient Mixture (Invitrogen, Paisley, UK) 1:1, 10% fetal calf serum (heat inactivated), 1% gentamicin, 0.2% benzylpenicillin, 0.2% streptomycin, 0.6% glutamine), before centrifugation at 2200rpm for 10min. The final pellet was resuspended in 2ml of cell culture medium. Cells were plated onto 35mm culture dishes (Nunc, Fisher Scientific, Loughborough, UK) or 16mm coverslips in 12-well

culture plates at densities of 1-1.3x10^6/ml and 1x10^6/ml respectively at 37°C in a humidified incubator at 95% air/5% CO_2.

Cytotrophoblast primary culture and treatment

Cytotrophoblasts plated onto 16mm coverslips were cultured for 66h. Cultures were washed 3 times with phosphate-buffered saline (PBS) and cell culture medium was replaced with fresh medium at 15 and 42h. Cells were untreated (control) or treated at 3, 15 and 42h with 0.1mM $ONOO^-$ (0.005-0.1mM are the $ONOO^-$ highest concentrations in specific cellular compartments *in vivo* [19]) or the $ONOO^-$ generator 3-morpholinosydnonimine (SIN-1; 0.5mM). SIN-1 continuously generates $ONOO^-$ *in vitro* by releasing O_2^- and NO in an aqueous solution which then react to form $ONOO^-$ [47-48].
At 15, 42 and 66h of culture, cell culture medium was collected and stored at -20˚C for measurement of β-hCG (hCG β-subunit; produced by terminally differentiated STB, used to assess cytotrophoblast biochemical differentiation [31]) and coverslips were placed into 1ml 0.3M NaOH, cells scraped and the cell lysate stored at 4˚C. These samples were used to measure protein content (mg) with a Bio-Rad Protein Assay, based on the Bradford method (Bio-Rad Laboratories, Hempstead, UK). At 15, 42 and 66h of culture, cells were fixed in absolute methanol for 20min at -20˚C and stored in PBS at 4˚C prior to immunofluorescence staining.

Measurement of cytotrophoblast hCG secretion

β-hCG was assayed in cell-conditioned culture medium at 15, 42 and 66h of culture by ELISA (DRG Diagnostics, Marburg, Germany). Optical density was measured at 450nm using a VersaMax microplate reader (Molecular Devices, CA, USA). hCG secretion was expressed as mIU/ml/h/mg protein.

Immunofluorescent staining

Methanol-fixed cells on 16mm coverslips were washed in tris-buffered saline (TBS). Blocking of non-specific binding was achieved by incubating with 4% bovine serum albumin (BSA) in TBS for 30min at room temperature. Cells were incubated for 1h at room temperature with mouse monoclonal antibody to desmoplakin I+II (2Q400; Abcam, Cambridge, UK), diluted 1:100 in TBS. Cells were washed with TBS and incubated with the secondary antibody, FITC-polyclonal rabbit anti-mouse immunoglobulin (Dako, Cambridgeshire, UK) diluted 1:50 in TBS, for 1h at room temperature in the dark. After washing with TBS, coverslips were mounted using Vectashield mounting medium with

propidium iodide nuclear counterstain (PI; Vector labs, Peterborough, UK). Immunofluorescent images were captured using a Zeiss AxioObserver Inverted Microscope (magnification 400x).

Analysis of cytotrophoblast multinucleation

Fluorescent images of cytotrophoblasts stained for desmoplakin and nuclei were used to assess multinucleation as a measure of morphological differentiation. Based on a previously published method [31, 49], 2 observers counted the total number of nuclei per given field and the number of nuclei in syncytia (multinucleated cytotrophoblasts defined as ≥3 nuclei within desmoplakin boundaries) using ImageJ 1.45 software (National Institutes of Health, USA). The number of nuclei in multinucleated cells was expressed as a percentage of the total number of nuclei within a given field (% of nuclei in multinucleate cells).

^{86}Rb efflux from cytotrophoblasts

^{86}Rb efflux was measured from cytotrophoblasts, as a tracer of K^+, using a technique previously described [50]. It has been previously shown that Ca^{2+}-activated K^+ channels are permeable to ^{86}Rb [51].

^{86}Rb efflux was measured from multinucleated cytotrophoblasts at 66h of culture. Briefly, cells plated onto 35mm dishes were removed from the incubator and washed in control Tyrode's buffer (135mM NaCl, 5mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES, 5.6mM glucose, pH 7.4; osmolality 285mOsm/kgH_2O, isotonic compared to maternal plasma of pregnancy at term [52]; osmolality measured by freezing point depression). Cells were incubated with 1ml 4μCi/ml ^{86}Rb (89.7μM; concentration 1μCi/ml; stock activity 1mCi) for 2h at room temperature. After washing for 3min (2x25ml) in Tyrode's buffer with no added isotope, ^{86}Rb efflux was measured by adding and replacing control Tyrode's buffer at 1min intervals over 15min (basal ^{86}Rb efflux) or exposed to the following treatments over 5-15min (experimental period): 0.1mM $ONOO^-$ (from min 4 onwards), 10μM TRAM-34 (blocker of IK_{Ca}), hyposmotic solution (55mM NaCl, 5mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES, 5.6mM glucose; pH 7.4; osmolality 145mOsm/kgH_2O). In addition, the reversibility of $ONOO^-$ was tested; the free radical was applied over min 5-10 and then withdrawn for the remainder of the experimental period. The molar concentration of $ONOO^-$ (Millipore, CA, USA) was determined spectrophotometrically (absorbance 302nm) with an extinction coefficient of 1670$M^{-1}cm^{-1}$.

Finally, the cells were lysed in 0.3M NaOH for ~1h and scraped in order to denature the cells. Intracellular and membrane bound ^{86}Rb was then measured in the lysate to give a measure of total ^{86}Rb remaining in the cells at the end of the experiment (cellular ^{86}Rb).
Effluxed and cellular ^{86}Rb was measured in a gamma-counter (Packard Cobra II Auto Gamma, CA, USA). All counts recorded were at least 10 times higher than background counts.
The time course of %^{86}Rb efflux was calculated as ((^{86}Rb effluxed/^{86}Rb in cells) x100) and decrease in cellular ^{86}Rb was measured by a first-order rate constant which was calculated over 10min experimental period for each treatment as (l_n(^{86}Rb in cell at time t/^{86}Rb in cell at start)).

Expression of Results and Statistics

Statistical analyses were performed using GraphPad Prism version 5 software with n as the number of placentas. Cytotrophoblast hCG secretion, multinucleation and protein content in treated cells at 15, 42 and 66h of culture, was expressed as a % of control (100%) and compared to control using a Wilcoxon signed-rank test. Data were expressed as median ± interquartile range (IQR). %^{86}Rb efflux from multinucleated cytotrophoblasts was expressed as mean ± SE for each time point. For all ^{86}Rb efflux experiments, statistical differences between treatments were assessed using the ^{86}Rb rate constants by least squares linear regression. In all cases, a *p* value less than 0.05 was considered statistically significant.

Results

Effect of $ONOO^-$ on basal ^{86}Rb efflux in multinucleated cytotrophoblasts

We have previously demonstrated the expression and function of IK_{Ca} in isolated cytotrophoblast from term placentas [32]. Here we explored whether nitrative stress modulates IK_{Ca} activity in multinucleated cytotrophoblasts at 66h of culture.

Figure 1A shows the time course for $\%^{86}Rb$ efflux. Under control conditions, $\%^{86}Rb$ efflux showed a stable steady state over 13min (Figure 1A, black circles). 0.1mM $ONOO^-$, applied for the period indicated by the bar caused a marked increase (2.8-fold) in ^{86}Rb efflux which was blocked by TRAM-34 (35%). In each case, the rate constant (Figure 1B) could be fitted by a single exponential. Rate constant analysis showed that the fall in intracellular ^{86}Rb (slopes) was significantly greater in cells exposed to $ONOO^-$ than controls (Figure 1B). In addition, TRAM-34 significantly inhibited $ONOO^-$-stimulated ^{86}Rb loss. These results indicate that, in multinucleated cytotrophoblasts, $ONOO^-$ stimulates ^{86}Rb efflux in part through TRAM-34-sensitive K^+ channels.

The effect of $ONOO^-$ on basal ^{86}Rb efflux was reversible (Figure 1C), as the withdrawal of $ONOO^-$ from the efflux buffer (Figure 1C; open circles) restored the $ONOO^-$-stimulated efflux to normal basal efflux levels. This was confirmed by the rate constant analysis (Figure 1D), as the increased loss of intracellular ^{86}Rb with $ONOO^-$ was reduced by the withdrawal of $ONOO^-$.

Effect of $ONOO^-$ on swelling-activated ^{86}Rb efflux in multinucleated cytotrophoblasts

We have previously reported that swelling-activated ^{86}Rb efflux in multinucleated cytotrophoblasts is through TRAM-34 sensitive K^+ channels [39] indicating that IK_{Ca} could participate in cytotrophoblast RVD. Here we explored whether nitrative stress modulates IK_{Ca} activity which is promoted by hyposmotic cell swelling.

Figure 2A shows a time course for $\%^{86}Rb$ efflux in the presence of a hyposmotic solution (Figure 2A; black circles). As previously reported, lowering extracellular osmolality caused a marked increase in $\%^{86}Rb$ efflux (2.8-fold). This swelling-activated $\%^{86}Rb$ efflux was enhanced by $ONOO^-$ (1.5-fold) and the $ONOO^-$-induced efflux was inhibited by TRAM-34 (51.4%).

The rate constant analysis (Figure 2B) showed a significantly greater fall in cellular ^{86}Rb with $ONOO^-$ in hyposmotic solution compared to hyposmotic alone and the fall was significantly inhibited by TRAM-34. Together these data suggest that swelling-activated IK_{Ca} in multinucleated cytotrophoblasts are further stimulated by nitrative stress ($ONOO^-$).

Effect of nitrative stress on cytotrophoblast morphological differentiation

In agreement with published work [31, 43-44] and our previous data [32] isolated cytotrophoblasts remained mononuclear at 15h, secreting low amounts of hCG. At 42h, the cells migrated, aggregated and fused to form multinucleated syncytia at 66h, concomitant with a several fold increase in hCG secretion (data not shown). We then explored whether nitrative stress affects both morphological and biochemical cytotrophoblast differentiation *in vitro*.

Figures 3A-F show representative images of desmoplakin immunostaining (green) and Pi (red; nuclei) in cytotrophoblasts at 15 and 66h of culture (Figures A-C and D-F respectively), used to assess cytotrophoblast multinucleation. Multinucleation is shown in Figure 3G (% of the total nuclei in multinucleated cytotrophoblasts) expressed as % of control (100%)). Figures 3A and 3D show representative cytotrophoblasts at 15 and 66h of culture respectively. Desmoplakin immunostaining shows that at 15h (Figure 3A) cytotrophoblasts remain mononuclear, and some cells have already started to aggregate. At 66h (Figure 3D), cytotrophoblast fusion has occurred and cytotrophoblasts have formed multinucleated syncytia (with ≥3 nuclei in syncytia; indicated by arrows).

Compared to controls at 42 and 66h, multinucleation was unaffected by both $ONOO^-$ or SIN-1 (Figure 3G). However, $ONOO^-$ produced a transient increase in multinucleation at 15h of culture.

Effect of nitrative stress on cytotrophoblast biochemical differentiation

We and others [31, 43, 53] have previously reported the time course of hCG secretion by untreated cytotrophoblasts in culture [32] which shows that hCG secretion increases several fold from 15 to 66h concomitant with an increase in multinucleation.

Compared to controls, generation of $ONOO^-$ by SIN-1 caused a significant reduction in cytotrophoblast hCG secretion at 15, 42 and 66h of culture (22.6, 38.7 and 53.5% reduction respectively; Figure 4A). Cytotrophoblast treatment with native $ONOO^-$ did not affect hCG secretion. This inhibition was not caused by cytotoxicity as cytotrophoblast cell protein (Figure 4B) and total nuclei (data not shown) were not reduced with SIN-1 compared to untreated controls. On the contrary, treating cytotrophoblasts with $ONOO^-$ (15h and 66h) or SIN-1 (15h) caused a transient increase in protein content.

Discussion

In this study we showed that acute administration of $ONOO^-$ reversibly activated TRAM-34-sensitive ^{86}Rb efflux from multinucleated cytotrophoblast cells. $ONOO^-$ also enhanced volume-activated ^{86}Rb efflux which was partially inhibited by TRAM-34. These data suggest that short-term exposure to nitrative stress can activate IK_{Ca} in differentiated cytotrophoblasts. In the longer term (66h), exposing cytotrophoblasts to SIN-1, which generates $ONOO^-$, inhibited hCG secretion (biochemical differentiation) but did not affect multinucleation. Overall these data support the hypothesis that $ONOO^-$ activates IK_{Ca} and that nitrative stress could contribute to abnormal STB turnover.

$ONOO^-$ increases TRAM-34 sensitive ^{86}Rb efflux from multinucleated cytotrophoblasts

We have previously demonstrated that cytotrophoblasts express IK_{Ca} protein and that the channel is quiescent under basal conditions [32]. However, IK_{Ca} can be activated by DCEBIO, an IK_{Ca} opener, and this activation is inhibited by TRAM-34. Treating cytotrophoblasts with DCEBIO for 48h significantly reduced their multinucleation and hCG secretion, suggesting that activation of IK_{Ca} inhibits events that renew STB *in situ* [32]. As STB renewal is dysregulated in PE, a disease associated with increased nitrative stress, we hypothesized that nitrative stress could activate IK_{Ca} in cytotrophoblasts.

Current results show that basal ^{86}Rb efflux was stimulated by acute application of reactive nitrogen species (0.1mM $ONOO^-$) and this increase in efflux was partially inhibited by TRAM-34 implicating that the effect of $ONOO^-$ was mediated through IK_{Ca}. However, the mechanism by which $ONOO^-$ activates IK_{Ca} is unclear. The half-life of $ONOO^-$ is less than 100ms [54-55]; therefore, the specific chemical compound responsible for the increase in TRAM-34 mediated ^{86}Rb efflux could be $ONOO^-$ or other $ONOO^-$-derived free radicals, which are products of $ONOO^-$ dissociation in aqueous solution. For this study, fresh $ONOO^-$ was added every min during the experimental period, therefore $ONOO^-$ was likely to be the main chemical species that promoted ^{86}Rb efflux. Evidence from other cell types suggests that $ONOO^-$ could directly target IK_{Ca}, as SIN-1, added either on the cytosolic or extracellular side, increased Ca^{2+}-activated K^+ channel open probability in tracheal smooth muscle cells without affecting the ion channel unitary conductance; the addition of charybdotoxin, an IK_{Ca}/large conductance Ca^{2+}-activated K^+ channel blocker, reduced the sensitivity of the channel to SIN-1 induced-relaxation [56], which suggests that the activation of these channels is likely to be due to a direct acute chemical modification produced by $ONOO^-$ within the ion channel protein. In contrast, in common with other Ca^{2+} activated K^+ channels, IK_{Ca} is strongly dependent on intracellular Ca^{2+} [57-58] and it is

possible that the increase in cytotrophoblast ^{86}Rb efflux could be explained by an effect of $ONOO^-$ to increase intracellular Ca^{2+}. In mesenteric arteriolar smooth muscle cells, $ONOO^-$/SIN-1, increased Ca^{2+}-activated K^+ outward currents and the $ONOO^-$-induced currents were inhibited by removal of external Ca^{2+}, by the addition of nifedipine (blocker of voltage-gated Ca^{2+} channels) or ryanodine (a sarcoplasmic reticulum ryanodine receptor blocker, thereby inhibiting Ca^{2+} release from intracellular stores). These results suggest that Ca^{2+} mobilization, either external Ca^{2+} influx or Ca^{2+} release from intracellular stores, is involved in $ONOO^-$-mediated K^+ outward currents [59].

Our results show that the short-term effect of $ONOO^-$ on ^{86}Rb efflux is reversible as withdrawal of $ONOO^-$ caused ^{86}Rb efflux to return to basal levels. This suggests that $ONOO^-$ or $ONOO^-$-derived free radicals cause a transient chemical modification which could include IK_{Ca} oxidation on cysteine residues or IK_{Ca} nitration on tyrosine residues. As TRAM-34 did not completely block $ONOO^-$ stimulated ^{86}Rb efflux, it is also possible that, in addition to IK_{Ca}, other K^+ channels are activated by $ONOO^-$ and this needs to be addressed in the future.

$ONOO^-$ increases swelling-activated ^{86}Rb efflux in multinucleated cytotrophoblasts

In addition to activating basal ^{86}Rb efflux, $ONOO^-$ also enhanced the ^{86}Rb efflux from multinucleated cytotrophoblasts which was promoted by hyposmotic swelling. IK_{Ca} have a well-established role in RVD, the mechanism by which many cells restore their volume in response to a hyposmotic challenge [60]. We have previously shown that ^{86}Rb efflux increases 3.8-fold in response to a hyposmotic solution (145mOsm/kgH_2O; [39]) and that this is blocked ~85% by IK_{Ca} inhibitors TRAM-34 and charybdotoxin, implicating activation of IK_{Ca}. This swelling-activated ^{86}Rb efflux was dependent on intracellular Ca^{2+} [61]. In the current study, $ONOO^-$ potentiated ^{86}Rb efflux induced by hyposmotic swelling and this was partially inhibited by TRAM-34. In common with the stimulatory effect of $ONOO^-$ on basal ^{86}Rb efflux, this could reflect a direct effect of $IK_{Ca,}$ to increase channel open probability and/or conductance, or be mediated by a secondary action such as elevating intracellular Ca^{2+}. An additional possibility is that $ONOO^-$ could potentiate cell swelling; however previous evidence in red blood cells demonstrate that $ONOO^-$ increases K^+ permeability without affecting cell volume [62].

In non-placental cell types, volume homeostasis is known to be dysregulated by conditions which include nitrative stress [60] causing the activation of K^+ conductances and consequent abnormal cell shrinkage [60, 63-65]. Therefore in PE, elevated nitrative stress could challenge STB volume homeostasis and disrupt STB renewal. However, it is not known whether STB volume is altered in PE compared to normal pregnancy. In theory STB swelling in PE could also arise due to elevated nutrient uptake; however, limited

studies show that despite the higher fetal plasma amino acid concentration in PE compared to normal pregnancy [66], uptake of amino acids is unchanged [67] or reduced [68] in this condition. Therefore, the mechanisms underlying the effects of $ONOO^-$ on IK_{Ca} under basal conditions and following STB swelling, and how this might contribute to altered STB cell turnover, remain to be investigated.

Nitrative stress reduced cytotrophoblast biochemical differentiation

According to our previous data [32], pharmacological activation of IK_{Ca} *in vitro* leads to inhibition of cytotrophoblast morphological and biochemical differentiation. Here we showed that IK_{Ca} is acutely activated by reactive nitrogen species; thus we proposed that long-term exposure to nitrative stress, a pathophysiological condition characteristic of PE, would chronically activate IK_{Ca} leading to reduced cytotrophoblast differentiation. Although our results demonstrate that nitrative stress reduced cytotrophoblast hCG secretion but not multinucleation, it remains to be confirmed whether the effects of nitrative stress on cytotrophoblast endocrine secretion are through IK_{Ca}.

The effects of SIN-1 were specific for hCG and not related to general toxicity as cell number, cell protein and multinucleation were unaffected by treatment. SIN-1 generates O_2^- and NO which combine to form $ONOO^-$ which then nitrates tyrosine groups on proteins to form nitrotyrosine. The production of nitrotyrosine was not confirmed in the current study and it is possible that the inhibitory effects of SIN-1 on hCG secretion were caused by O_2^- and/or NO. However, results from this laboratory [69] show that long-term (48h) treatment of placental villous tissue from uncomplicated pregnancies with 1mM SIN-1 generates increased nitrotyrosine staining, indicative of nitrative stress. On the other hand, the lack of effect of $ONOO^-$ on cytotrophoblast hCG secretion could be due its short half-life [54-55], and it is possible that the effect of $ONOO^-$ is not sustained in culture.

It is important to highlight that nitrative stress could be exerting a wide range of effects which in turn reduce cytotrophoblast biochemical differentiation. Possibilities include effects on intracellular Ca^{2+} or on proteins/signaling molecules that modulate hCG secretion and this remains to be investigated. In particular, the alpha subunit of hCG in constitutively expressed but the beta subunit is only synthesized by terminally differentiated STB [70-71], which then produces and secretes hCG dimer. hCG acts in an autocrine/paracrine manner to promote further cytotrophoblast differentiation and fusion [4, 72], thereby facilitating continued STB renewal. The finding that hCG secretion was inhibited by nitrative stress, but multinucleation was unaffected, implicates a primary effect of reactive nitrogen species on the differentiation process that triggers the production of beta hCG, and/or an effect on the mechanism of hCG secretion.

It is unknown whether IK_{Ca} mediates the inhibitory effect of SIN-1 or whether the nitration of other STB proteins results in downregulation of hCG secretion. In this regard, intracellular proteins have been shown to be nitrated in PE [28-29]. Additionally, although IK_{Ca} participates in adrenocorticotrophic hormone secretion *in vitro* [73], there is little evidence to support a role for these channels in regulating endocrine production/secretion from non-excitable tissues and in the future it needs to be addressed whether activation of IK_{Ca} by nitrative stress affects hCG production, secretion or both.

Although morphological and biochemical differentiation of cytotrophoblasts occur concurrently, it has been demonstrated these can be independent processes that can be dissociated [74], which could account for the effect of nitrative stress on cytotrophoblast hCG secretion but not multinucleation. However, we have previously shown [32] that both morphological and biochemical differentiation of cytotrophoblasts *in vitro* is reduced by IK_{Ca} activation with DCEBIO. If the effects of SIN-1 were mediated by $ONOO^-$ activation of IK_{Ca}, it is possible that SIN-1 inhibited hCG secretion without affecting multinucleation because the generation of $ONOO^-$ by SIN-1 *in vitro* was transient in contrast to a sustained activation of IK_{Ca} by DCEBIO; this remains to be confirmed.

Overall, the results from this study demonstrate that free radicals have a profound effect on cytotrophoblast hCG secretion *in vitro* which could have implications for STB health in PE.

Perspectives and significance

We have previously shown that the pharmacological activation of IK_{Ca} reduces hCG secretion and cytotrophoblast multinucleation *in vitro*. In the current study, reactive nitrogen species acutely activated IK_{Ca} suggesting that exposure to nitrative stress in PE could potentially activate IK_{Ca}, impair cytotrophoblast differentiation *in vivo*, and contribute to altered STB turnover.

In addition, results from this study showed that reactive nitrogen species potentiate IK_{Ca} activity which is stimulated by STB swelling. The relevance of this event for placental physiology/pathophysiology is that IK_{Ca}, in part through its role in cell volume regulation in other tissues, facilitates proliferation, apoptosis, cell migration, cell fusion/differentiation. Therefore in pregnancy conditions such as PE, where there is increased generation of reactive nitrogen species, IK_{Ca} could be inappropriately activated and lead to impaired STB turnover.

This work also showed that chronic exposure to nitrative stress inhibited cytotrophoblast hCG secretion and, although the mechanism underlying the effects of nitrative stress remain to be determined, this reveals a potential link between elevated nitrative stress and abnormal STB renewal in PE.

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No conflicts of interest, financial or otherwise, are declared by the author(s).

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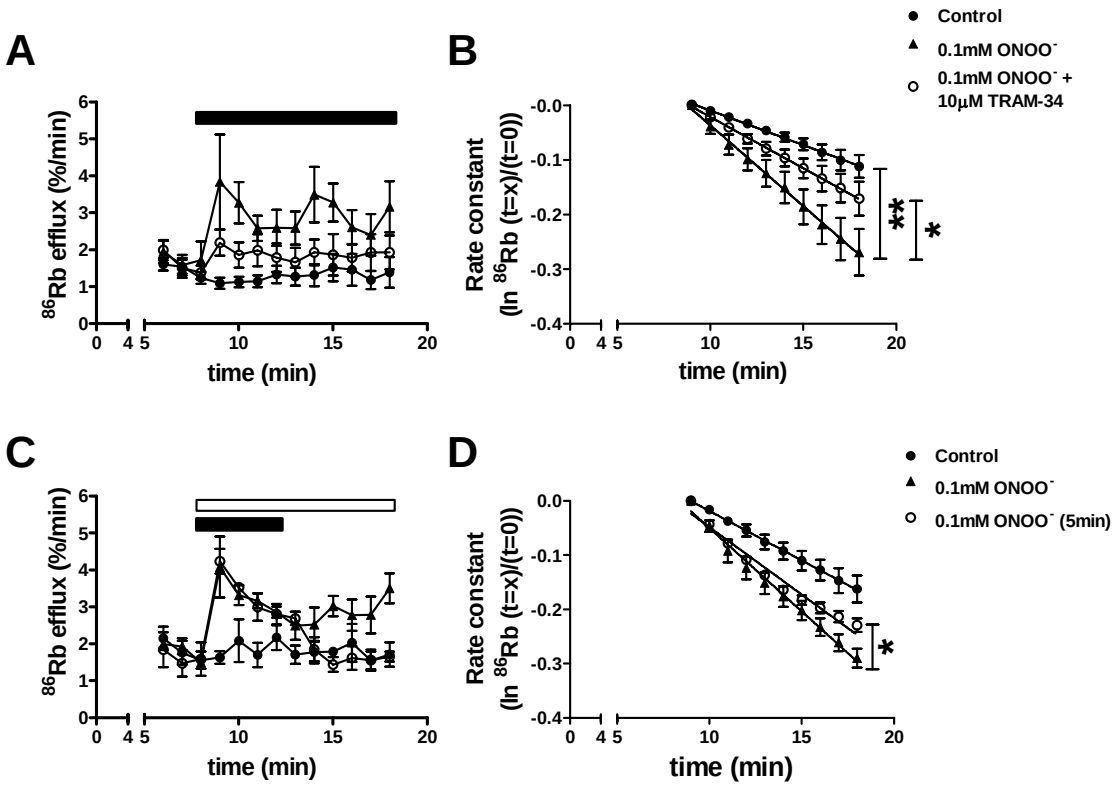

Figure 1: Effect of $ONOO^-$ on basal ^{86}Rb efflux in multinucleated cytotrophoblasts.
A: Time course for $\%^{86}Rb$ efflux over 13min in multinucleated cytotrophoblasts at 66h of culture. During the experimental period (indicated by the bar) cells were untreated (control) or exposed to $ONOO^-$ or $ONOO^-$ + TRAM-34 (n= 5 placentas). Data are mean ± SE. ***B***: Corresponding ^{86}Rb efflux first-order rate constant analysis, calculated for 10 min (experimental period). The relationship between time and rate constant was significant in all cases ($p<0.0001$). The difference in slopes between groups was $^{**}p<0.0001$, $^{*}p=0.002$ (least squares linear regression; data are mean ± SE). ***C***: Reversibility of $ONOO^-$ effects on cytotrophoblast ^{86}Rb efflux. $\%^{86}Rb$ efflux from multinucleated cytotrophoblasts; during the experimental period (open bar) cells were untreated (control) or treated with $ONOO^-$ or $ONOO^-$ for min 5-10 (5min; bar) which was then withdrawn from the efflux solution for the remain of the experimental period (n= 4 placentas). ***D***: Corresponding ^{86}Rb efflux rate constant analysis over 10min experimental period ($^{*}p=0.004$).

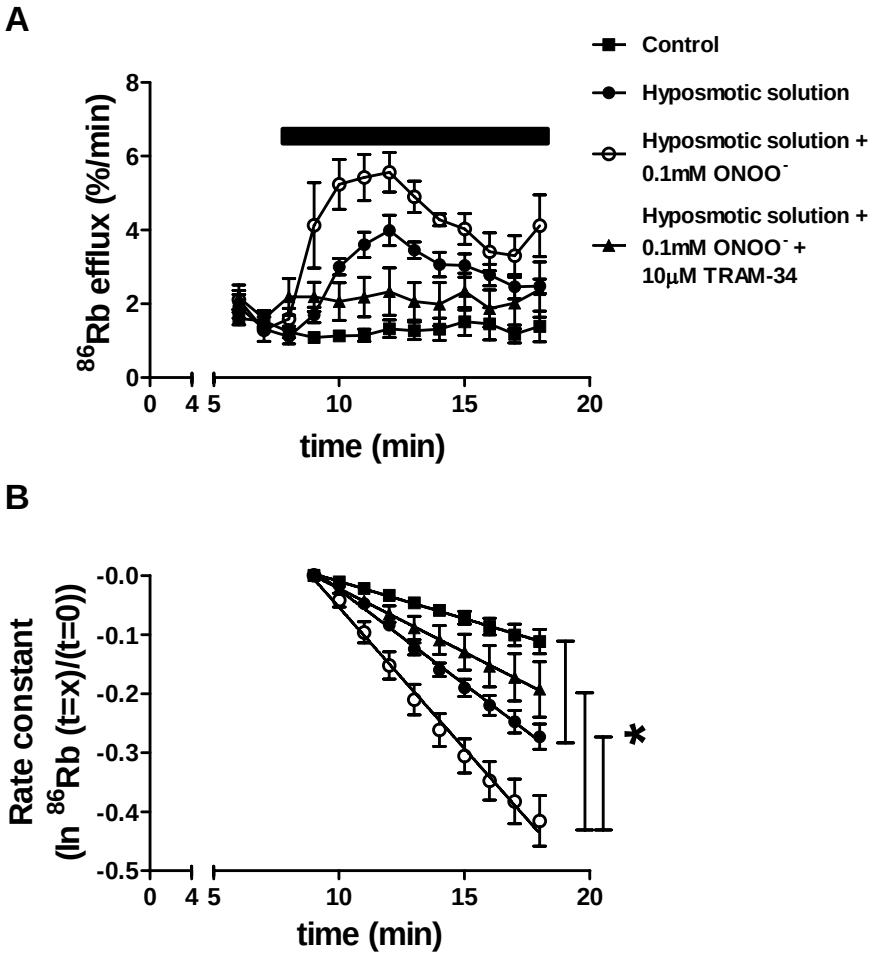

Figure 2: Effect of $ONOO^-$ on swelling-activated ^{86}Rb efflux from multinucleated cytotrophoblasts.

A: Time course for the $\%^{86}Rb$ efflux over 13min in multinucleated cytotrophoblasts at 66h of culture. During the experimental period (indicated by the bar) cells were untreated (control) or exposed to hyposmotic solution (145mOsm/kgH_2O) or hyposmotic solution + $ONOO^-$ or hyposmotic solution + $ONOO^-$ + TRAM-34 (n= 5 placentas; data are mean ± SE). ***B***: Corresponding ^{86}Rb efflux rate constant analysis over 10min experimental period (*$p<0.0001$; least squares linear regression; data are mean ± SE).

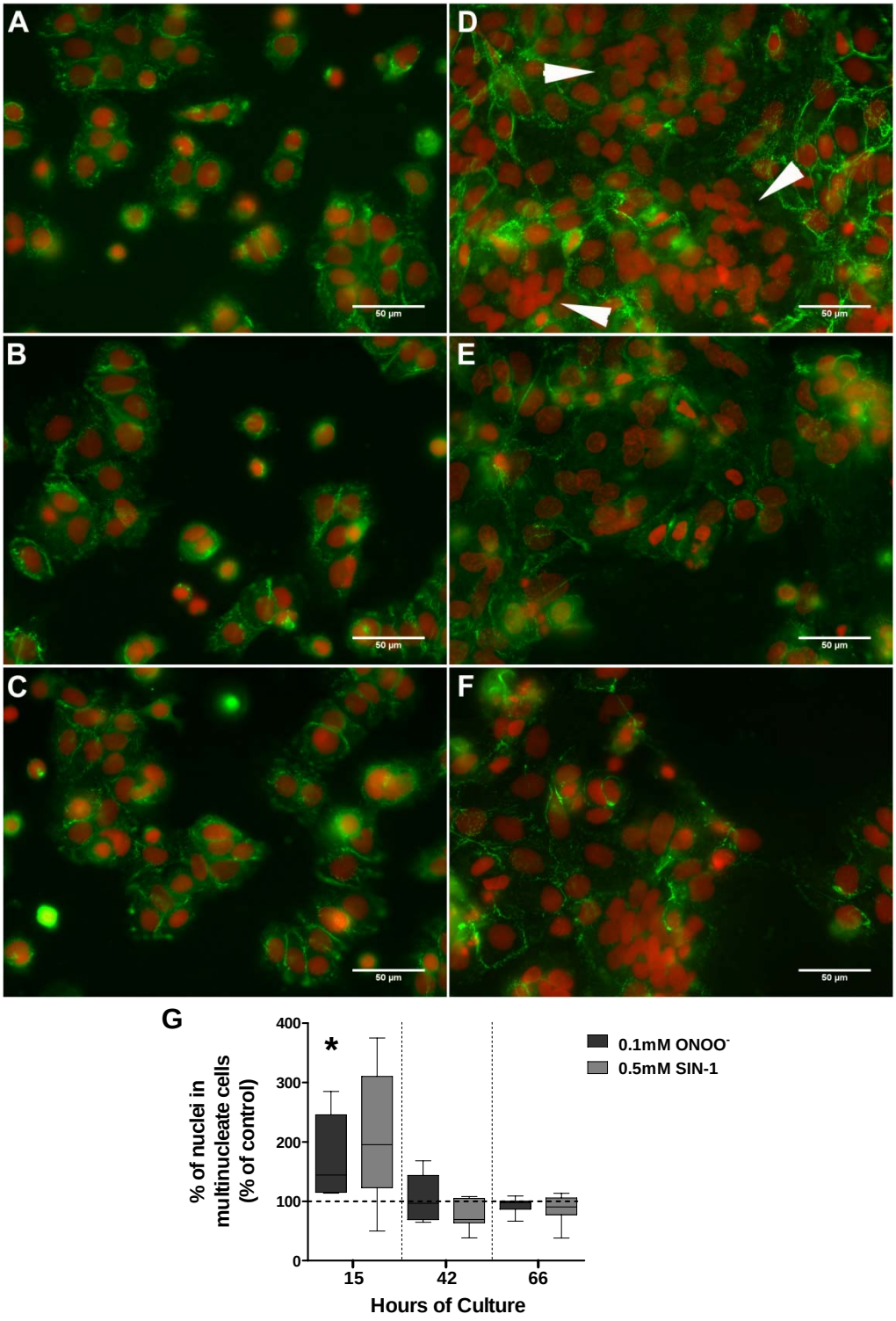

Figure 3: Effect of nitrative stress on cytotrophoblast multinucleation.
Representative dual immunofluorescent staining images showing desmoplakin (green) and nuclear counterstain (red) in untreated (control; ***A***, ***D***), $ONOO^-$ (***B***, ***E***) or SIN-1 (***C***, ***F***) treated cytotrophoblasts at 15 (***A***-***C***) and 66h (***D***-***F***) of culture. Arrows in (***D***) indicate multinucleated cytotrophoblasts at 66h of culture. Scale bar 50µM. ***G***: Average % of nuclei in multinucleate cells. Cytotrophoblast multinucleation was assessed at 15, 42 and 66h of culture in $ONOO^-$ and SIN-1 treated cytotrophoblasts and compared to controls (100%; dotted line; n= 7-10 placentas; *p=0.03; Wilcoxon signed-rank test compared to 100%). Data are median ± IQR.

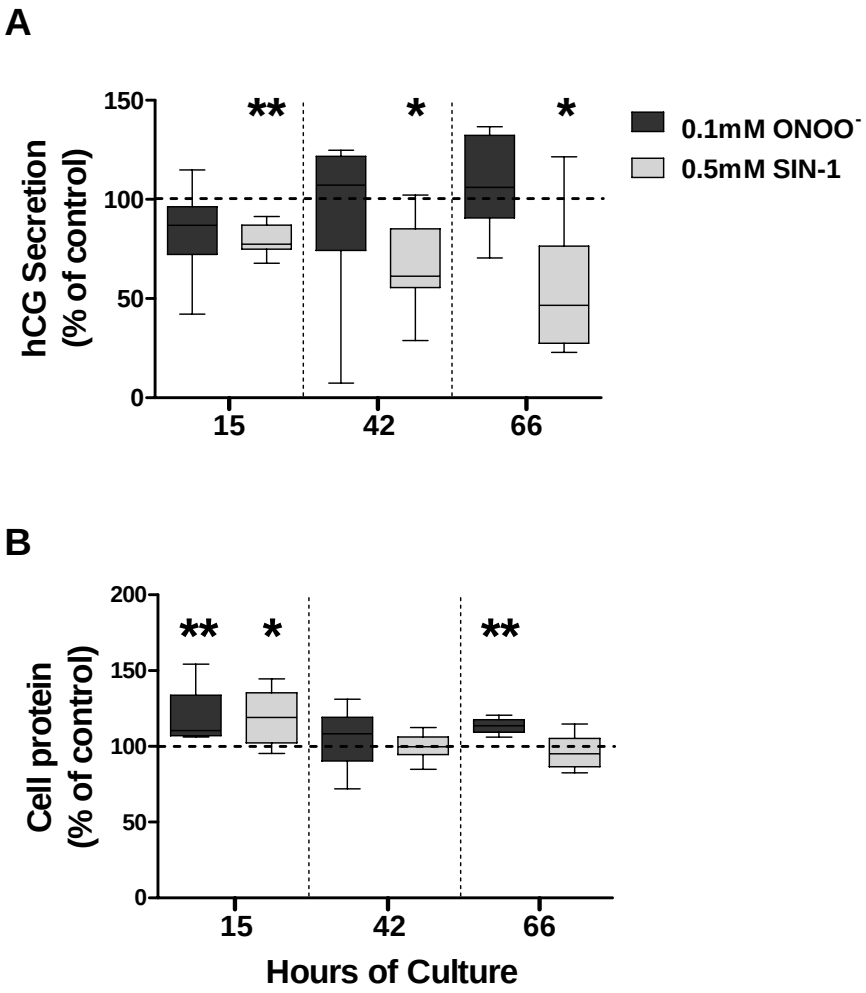

Figure 4: Effect of nitrative stress on cytotrophoblast hCG secretion.
A: hCG secretion at 15, 42 and 66h of culture in cytotrophoblasts treated with $ONOO^-$ or SIN-1 expressed as a % of controls (100%; dotted line; n= 7-10 placentas; ***p*=0.02, **p*=0.03; Wilcoxon signed-rank test compared to 100%). ***B***: Cell protein at 15, 42 and 66h of culture in cytotrophoblasts treated with $ONOO^-$ or SIN-1 expressed as a % of controls (100%; dotted line; n= 7-10 placentas; ***p*=0.03, **p*=0.05). Data are median ± IQR.

4. GENERAL DISCUSSION

In comparison with other tissues, evidence for the function of ion conductances in the STB, and in particular K^+ channels, is scarce. This thesis contributes to our understanding of K^+ channel function in the STB and how placental STB K^+ channel dysregulation could contribute to altered STB renewal which is a feature of pregnancy disease such as PE.

This final chapter presents an overview of the work reported in this thesis, provides a perspective on its significance and proposes future directions. Supplementary data, not presented in the main body of the thesis, that support the overall conclusions are provided in section 4.7.

4.1. Characterisation of potassium conductances in the syncytiotrophoblast

Direct study of ion channels in the STB of intact placental villi using patch clamp methods is technically challenging as seals are hard to achieve (Brown, Greenwood et al. 1993) and the syncytial nature of this epithelium precludes whole cell recording. Consequently, an indirect method to measure K^+ permeability in the STB was used in this thesis by measuring ^{86}Rb efflux, a tracer of K^+. ^{86}Rb is known to permeate K^+ channels (Bland and Boyd 1986; Venglarik, Bridges et al. 1990) and it has been used previously to assess K^+ permeability in the STB (Boyd 1983; Siman, Sibley et al. 2001). ^{86}Rb efflux was initially measured in fresh placental villous tissue in order to characterise K^+ conductances expressed at rest. Conversely, a disadvantage of using fresh villous fragments for measuring ^{86}Rb efflux is that the tracer may not be diffusing solely from the STB and several tissue compartments could contribute to efflux within the placental villi. However, results from this thesis show a highly significant linear correlation for the rate constant for ^{86}Rb over 18min (Figure 4.3), suggesting that ^{86}Rb is lost predominantly from a single tissue compartment, most probably the STB, in agreement with previous data (Siman, Sibley et al. 2001).

4.1.1. Ba^{2+}-sensitive potassium conductances

A broad spectrum K^+ channel blocker Ba^{2+}, previously reported to inhibit basal ^{86}Rb efflux from villous explants (Siman, Sibley et al. 2001) was used in fresh villous fragments. Results from this thesis show that 25% of basal ^{86}Rb efflux was reduced by 5mM Ba^{2+} (Figure 4.4.A, B) and the mean rate constant for ^{86}Rb efflux was significantly lower in

villous explants exposed to Ba^{2+} compared to controls (Figure 4.4.C) suggesting that basal STB efflux is partly mediated by Ba^{2+}-sensitive K^+ channels. These data are supported by previous reports showing that Ba^{2+}-sensitive conductances mediate K^+ efflux as part of the RVD mechanism in placental villous fragments (Birdsey, Boyd et al. 1999), and that Ba^{2+} also depolarises basal STB E_m (Birdsey, Boyd et al. 1999); therefore Ba^{2+}-sensitive K^+ channels and K^+ efflux normally contribute to STB resting E_m.

At present there is evidence to support the identification of the underlying conductances that contribute to this Ba^{2+}-sensitive ^{86}Rb efflux. Using whole cell patch clamp, a strong inwardly rectifying K^+ current (consistent with $K_{IR}2.1$) sensitive to Ba^{2+}, was identified in cytotrophoblasts at different stages of differentiation *in vitro* (Clarson, Greenwood et al. 2001). In addition, Díaz *et al*. (2008) using patch clamp and voltage clamp in reconstituted purified STB apical membrane vesicles, identified Ba^{2+}-sensitive K^+ conductances (Diaz, Vallejos et al. 2008). Furthermore, Bai *et al*. (2006) showed that K_{2P} channels might contribute to basal ^{86}Rb efflux, since basal efflux was inhibited by the TASK-1 blocker anandamide (Bai, Lacey et al. 2006), which is also sensitive to Ba^{2+} (Goldstein, Bayliss et al. 2005). Additionally, the Ba^{2+}-insensitive efflux is likely to result from other transport routes such as NSCCs, since ^{86}Rb efflux from cytotrophoblasts is reduced by NSCC blocker, gadolinium (150μM) (Clarson, Roberts et al. 2002). However, it is still unknown whether other Ba^{2+}-insensitive K^+ conductances contribute to STB efflux at rest.

The K^+ conductance of the STB MVM is low compared with other tissues and Ba^{+2}, which inhibits several K^+ channels, as shown in this thesis and by others (Siman, Sibley et al. 2001), only reduced basal efflux by ~25%. Additional data from the current thesis shows that the acute application of broad spectrum K_V and K_V/K_{Ca} blockers 4-AP (5mM) and TEA (5mM) respectively had no effect on basal STB ^{86}Rb efflux during the experimental period (Figure 4.5). The lack of effect of these blockers on basal efflux implies that 4-AP and TEA-sensitive K^+ channels are not open at rest. This is noteworthy as in non-placental tissues, 4-AP and/or TEA-sensitive channels (e.g. K_V family) usually exhibit an activation threshold at more depolarised potentials (~-10-20mV; (Gutman, Chandy et al. 2005)) and at term, the STB exhibits a relatively depolarised resting E_m (-21mV) compared with other cell types (Birdsey, Boyd et al. 1997). Further evidence suggests that Cl^- conductances contribute to E_m (Brown, Greenwood et al. 1993; Riquelme, Stutzin et al. 1995) such that that STB MVM E_m approaches the Cl^- equilibrium potential.

4.1.2. TRAM-34-sensitive potassium conductances

Studies of IK_{Ca} in isolated multinucleated cytotrophoblasts (Results, section 3.2) showed protein expression located to the nucleus, cytoplasm and cell surface in addition to

functional expression. There was low inherent IK_{Ca} activity in cytotrophoblasts as TRAM-34, an IK_{Ca} blocker did not alter basal ^{86}Rb efflux, but the IK_{Ca}/SK_{Ca} opener DCEBIO stimulated TRAM-34-sensitive ^{86}Rb efflux from these cells. Further work from this thesis has confirmed the functional expression of IK_{Ca} in fresh placental villous fragments (Figure 4.6). TRAM-34 did not have an effect on basal ^{86}Rb efflux (Figure 4.6.A) suggesting that, in common with cytotrophoblasts, in the quiescent state IK_{Ca} might not be active in STB; in contrast, activating IK_{Ca} with DCEBIO in villous fragments produced a marked increase in ^{86}Rb efflux, which was specifically blocked by TRAM-34 (Figure 4.6.B, C). This indicates that IK_{Ca} is functionally expressed in intact STB from term placental villous tissue.

IK_{Ca}s are voltage-insensitive K^+ channels and are activated by an increase in $[Ca^{2+}]_i$ (Neylon, Lang et al. 1999; Wei, Gutman et al. 2005). Therefore any potential stimulus that increases $[Ca^{2+}]_i$ could activate these channels. Previous evidence shows that elevated $[Ca^{2+}]_i$ is required for DCEBIO activation of IK_{Ca}s (Pedersen, Schroder et al. 1999), suggesting that in STB there is a basal free $[Ca^{2+}]_i$ sufficient to facilitate DCEBIO activation of IK_{Ca}. The minimum $[Ca^{2+}]_i$ required to produce DCEBIO-mediated IK_{Ca} activation in non-placental cells is 100nM, which is close to resting $[Ca^{2+}]_i$ level (Pedersen, Schroder et al. 1999). However, at present there is no direct measurement of free $[Ca^{2+}]_i$ in the STB under basal conditions although indirect measurements using relative values with Ca^{2+}-sensitive dyes suggest that the basal $[Ca^{2+}]_i$ is ~100nM (Bax, Bax et al. 1994).

Consequently, the results from this thesis show that STB at rest expresses Ba^{2+}-sensitive channels, but neither 4-AP nor TEA-sensitive channels. In addition, the expression of one member of the K_{Ca} family, IK_{Ca}, is relatively quiescent at rest. Although most members of the K_{IR} family are Ba^{2+}-sensitive (Kubo, Adelman et al. 2005), it is unlikely they will be open at resting STB E_m; therefore, possible candidates contributing to the basal Ba^{2+}-sensitive efflux could be members of K_V family (5mM 4-AP or TEA-insensitive; (Gutman, Chandy et al. 2005)) and K_{2P}s including TASK-1 (Bai, Lacey et al. 2006). Figure 4.1 shows a diagrammatic summary of the contribution of this thesis to understanding K^+ channel function in the STB.

4.2. Role of partial pressure of oxygen in the modulation of potassium channels in the syncytiotrophoblast

The role of pO_2 in the modulation of STB K^+ channel function and its effects on hCG secretion were explored in this thesis. Cultured placental villous explants were used as an *in vitro* model to study the effect of chronic modulation of pO_2 on STB K^+ permeability and to determine whether pO_2-sensitive K^+ channels modulate STB hCG secretion.

4.2.1. Physiological and pathophysiological partial pressure of oxygen in placenta

According to *in vivo* measurements, placental bed/intervillous space oxygenation at term pregnancy is an average of 5-8% pO_2 (Soothill, Nicolaides et al. 1986; Burton and Caniggia 2001; Lackman, Capewell et al. 2001); consequently 6% pO_2 was used throughout this thesis as placental normoxia. An important consideration when studying chronic modulation of pO_2 on placental function using *in vitro* models is that, depending on the culture system used, the pO_2 might be below or above values for the predicted atmospheric pO_2. Studies have shown that pO_2 in the pericellular region of culture medium is low compared to theoretical values (Burton, Charnock-Jones et al. 2006; Tuuli, Longtine et al. 2011). In the current thesis, explant culture medium was pre-equilibrated 24h in advance in a chamber set at 6% or 1% pO_2 before adding it to villous explants of a constant size (3-5mm^3) which were maintained at the gas-culture medium interface to ensure that the tissue was exposed to a minimum gradient of oxygenation and close to the predicted theoretical pO_2. However, it is still a matter of debate whether placentas undergoing PE and/or FGR are exposed to hypoxia (Burton and Caniggia 2001; Burton 2009), hyperoxia (Kingdom and Kaufmann 1997; Huppertz, Weiss et al. 2013) or fluctuations in pO_2 (Burton and Hung 2003; Hung and Burton 2006) and to date there is no direct *in vivo* measurement of placental pO_2 in these pregnancy complications.

In the current thesis, a range of pO_2 was implemented; 21% and 1% pO_2 were used as extremes of pO_2 and 6% pO_2 was used to recreate placental normoxia. Although it is unlikely that the range of pO_2 under pathophysiological conditions will be as wide as that used *in vitro* in this thesis, it was used to reveal the potential role of pO_2 in regulating STB endocrine secretion through effects on pO_2-sensitive K^+ channels.

4.2.2. Characterisation of pO_2-sensitive and insensitive potassium channels in the syncytiotrophoblast

Results from this thesis provide new evidence that pO_2-sensitive K^+ channels are functionally expressed in placental STB. ^{86}Rb efflux from term placental explants maintained at placental hyperoxia (21% pO_2) was higher than explants maintained at placental normoxia (6% pO_2) (Results, section 3.1). These data raise the possibility that pO_2-sensitive K^+ channel activity and/or expression is increased under exposure to higher pO_2. In support of this, treatment of villous explants with 4-AP and TEA at concentrations which inhibit members of the K_V family (5mM), significantly reduced ^{86}Rb efflux when the tissue was cultured at 21% but not at 6% pO_2 (Results, section 3.1), consistent with an

inhibition of pO_2-sensitive K_Vs that are more active/more highly expressed at 21% than at 6% pO_2.

In several cell types hypoxia (<50-60mmHg; <6% pO_2) induces depolarisation by closing pO_2-sensitive K^+ channels, in particular BK_{Ca}, TASK-1 and members of the K_V family (Patel and Honore 2001). The placenta expresses mRNA for pO_2-sensitive BK_{Ca} and K_Vs, i.e. K_V1.5 (Lacey, Glazier et al. 2005) and immunostaining for K_V1.5 and 2.1 has localised their expression to the STB in placental villous sections (Williams, Jones et al. 2009) (Figure 1.5). In addition, there is evidence of TASK-1 expression and function in isolated cytotrophoblasts (Bai, Lacey et al. 2006) (Figure 1.6). In particular, the pO_2-dependent effects of 4-AP/TEA on STB K^+ permeability indicate the presence of pO_2-sensitive K_Vs, which could include K_V1.5 and/or K_V2.1 isoforms, although this remains to be confirmed. This evidence suggests pO_2-sensitive K^+ channels are expressed in the STB and, as potential pO_2 sensors they could be contributing to regulate STB function, an epithelia exposed to physiological fluctuations in pO_2.

As previously shown in this thesis, basal ^{86}Rb efflux was unaffected by the acute application of 5mM 4-AP/TEA in fresh villous fragments (Figure 4.5); in contrast, when placental villous explants were cultured for 6 days in hyperoxia (21% pO_2), chronic treatment with these blockers caused a significant reduction in STB K^+ permeability (Results, section 3.1). The placental villi at term are exposed to 5-8% pO_2 *in vivo* (Soothill, Nicolaides et al. 1986; Burton and Caniggia 2001; Lackman, Capewell et al. 2001) and experiments on freshly isolated villi were performed at 21% pO_2 (ambient oxygenation). It is unlikely that K^+ channel mRNA/protein expression would change during the course of the experiment on fresh tissue, suggesting that K_Vs that were closed/inactive under ~6% pO_2 *in situ* remained so during the experiment. Indeed, after 6 days in culture, the long term exposure to 21% pO_2 upregulated channel expression/activity and 4-AP and TEA exerted an inhibitory effect on STB K^+ efflux. It is known that sustained hypoxia (hours to days) directly influences transcriptional mechanisms that determine the level of K^+ channel expression; in contrast, acute hypoxia (seconds to minutes) reduces K^+ channel activity (Wang, Juhaszova et al. 1997; Lopez-Barneo, del Toro et al. 2004; Rey-Parra, Archer et al. 2008; Shimoda and Polak 2011) by closing pO_2-sensitive K^+ channels and depolarising E_m (Patel and Honore 2001). However, whether an increase in K_V mRNA/protein expression or channel activity (e.g. an increase in channel open probability) would account for a higher STB K^+ permeability in hyperoxia has yet to be investigated.

IK_{Ca} are not considered to be pO_2-sensitive and in support of this, treatment with DCEBIO to chronically activate these channels increased STB K^+ permeability independent of pO_2 in placental villous explants cultured at both normoxia and hyperoxia (Figure 4.7). Additionally, this pO_2-independent increase in basal STB ^{86}Rb efflux with DCEBIO could

be reversed, as the increase in permeability reflected by $\%^{86}Rb$ efflux time course was not sustained during the total duration of the experimental period. After 8min (in explants maintained in both 6 and 21% pO_2), there was a trend to return back to basal control K^+ permeability (Figure 4.7.A, C). This suggests IK_{Ca} activity rather than expression is affected by long-term exposure to DCEBIO *in vitro*.

Additional studies explored the possibility that K_V7, a pO_2-insensitive K_V channel subfamily expressed at the protein level in placenta from uncomplicated pregnancies and in PE (Mistry, McCallum et al. 2011) (Figure 1.5), is functionally expressed in the STB. Chronic treatment of villous explants with the K_V7 opener flupirtine did not affect basal ^{86}Rb efflux (data not shown). However, some members of K_V7 subfamily are TEA-sensitive and could be inhibited at the concentration used in this thesis; therefore the possibility that K_V7s are functionally expressed in the STB needs confirmation. The K_V7 family, also known as M-channels, are characterised by their lack of inactivation (Wang, Pan et al. 1998) and evidence has suggested their participation in cell volume regulation (Jensen, Callo et al. 2005), proliferation and differentiation (Roura-Ferrer, Sole et al. 2008), indicating their expression and function could play a relevant role in STB renewal.

4.2.3. pO_2-sensitive voltage-gated potassium channels modulate syncytiotrophoblast hCG secretion

In contrast to hCG secretion in the first trimester of pregnancy, the mechanism of secretion by the STB at term is not fully elucidated (Shi, Lei et al. 1993; Meuris, Polliotti et al. 1994; Long and Clarson 2002). Previous evidence from this laboratory showed that the pharmacological blockade of STB K_Vs with 4-AP or TEA inhibited hCG secretion from placental villous explants and isolated cytotrophoblasts *in vitro* (Williams, Fyfe et al. 2008). These experiments were performed at 21% pO_2 (160mmHg) and showed that 4-AP/TEA induced a significant reduction in hCG secretion (but not production), as well as cytotrophoblast multinucleation, implying that K_Vs are important for regulating processes of STB differentiation and renewal. This effect might be specific to K_Vs as anandamide, (Williams, Fyfe et al. 2008), pinacidil/cromakalim (Williams, Fyfe et al. 2008), glibenclamide/tolbutamide (Lybaert, Hoofd et al. 2013) and flupirtine (current thesis; data not shown) did not alter hCG secretion from explants indicating that TASK-1 (Bai, Lacey et al. 2006), K_{ATP}s and K_V7s do not regulate hCG secretion.

In common with this previous study (Williams, Fyfe et al. 2008), the current thesis showed that 5mM 4-AP and TEA significantly reduced hCG secretion from villous explants maintained at 21% pO_2. In addition, this thesis showed that hCG secretion was significantly lower from explants maintained in 6% than 21% pO_2 and 4-AP/TEA did not affect hCG secretion when explants were maintained at normoxia, thereby supporting the

proposal that pO_2-sensitive K_Vs modulate hCG secretion. There was a small effect of pO_2 on hCG production, indicating that hCG synthesis was higher in 21% than 6% pO_2, but there was an additional effect of pO_2 on secretion, suggesting hCG secretion is under the control of pO_2.

It has been proposed that inhibition of STB K_Vs could depolarise E_m sufficiently to inhibit Ca^{2+} entry through Ca^{2+}-permeable NSCCs, which are functionally expressed in the STB (Llanos, Henriquez et al. 2002; Clarson, Roberts et al. 2003; Niger, Malassine et al. 2004). hCG secretion at term involves constitutive release (Yoshida 2005) and exocytosis through excitation-secretion coupling which is highly Ca^{2+}-dependent (Meuris, Polliotti et al. 1994). Indeed, removal of extracellular Ca^{2+} and also the inhibition of Ca^{2+} entry through NSCCs with gadolinium, inhibited hCG secretion from placental villous fragments (Long and Clarson 2002). Thus, a reduction in Ca^{2+} entry through NSCCs induced by K_V-dependent depolarisation could result in a decrease in $[Ca^{2+}]_i$ and reduced hCG secretion (Figure 4.1). Although activated at relatively depolarised potentials in most cell types (Catterall, Perez-Reyes et al. 2005), studies have failed to identify functional VGCCs in the STB, in particular L-type VGCCs (Bax, Bax et al. 1994; Cronier, Dubut et al. 1999; Clarson, Roberts et al. 2003), despite reports of VGCC expression in both STB MVM and BM (Bernucci, Henriquez et al. 2006). Nifedipine, a blocker of VGCC, does not affect hCG secretion from villous explants (Long and Clarson 2002) and it is therefore unlikely that VGCCs have a role in Ca^{2+} entry into the STB to modulate hCG secretion at term.

In the normal placenta at term where villi are exposed to maternal blood at ~6% pO_2, K_Vs could be downregulated/closed maintaining a relatively depolarised E_m, minimising Ca^{2+} entry through NSCC and sustaining low basal levels of STB hCG secretion (Figure 4.1). Indeed, the results from this thesis show that explants maintained at 6% pO_2 secrete significantly less hCG than explants maintained at 21% pO_2. In contrast, in pathophysiological conditions such as PE or FGR, increased fluctuations in blood flow into the placental bed could lead to altered oxygenation, implicating that the STB is exposed to a wide range of pO_2. At present it is not possible to discard the possibility that placental pO_2 could become hyperoxic under these conditions (Huppertz, Weiss et al. 2013). If this is the case, K_Vs could be activated in PE, hyperpolarising E_m, stimulating Ca^{2+} entry and promoting STB hCG secretion (Figure 4.2). In this regard it is interesting to note that maternal plasma hCG is higher in women with late onset PE compared to women having normal pregnancy (Kharfi, Giguere et al. 2005), presumably arising from elevated placental hCG secretion.

Overall, the work from this thesis implies that pO_2-sensitive K_Vs modulate hCG secretion by STB at term. As hCG secreted by differentiated STB has autocrine/paracrine actions to promote continued cytotrophoblast differentiation and fusion, changes in K_V function in

response to altered ambient pO_2 *in situ* could contribute to dysregulated STB renewal in pregnancy disease.

4.3. Role of reactive oxygen and nitrogen species in the modulation of syncytiotrophoblast potassium channels

The production of free radicals by different cell mechanisms depends on the prevailing oxygenation; while there is still a controversy, it is thought both hypoxia and hyperoxia are stimuli for increased generation of free radicals. In normal pregnancy, after the end of the first trimester, the placental bed is exposed to a range of pO_2 due to a physiological intermittency in blood flow (Martin, McGaughey et al. 1964), leading to a basal production of free radicals (Hung and Burton 2006). It is hypothesised that in PE this intermittency in blood flow increases due to impaired remodelling/transformation of the maternal spiral arteries which will create episodes of hypoxia, where there is absent blood flow into the placental bed, but also episodes of a burst in oxygenation (reoxygenation). During this transient hypoxia, there will be no O_2 to serve as substrate for the formation of free radicals leading to increased formation of O_2^-. On the other hand, when O_2 is reintroduced in the reoxygenation period, a much greater burst of O_2^- formation could take place due to the sudden increase in the availability of O_2 (Hung and Burton 2006). Indeed, it is well established that the placenta in PE is affected by both oxidative and nitrative stress which highlights the effects of an exacerbated production of free radicals, both ROS and RNS (Myatt, Rosenfield et al. 1996; Wang and Walsh 1998; Staff, Ranheim et al. 1999; Many, Hubel et al. 2000; Wang and Walsh 2001; Zusterzeel, Rutten et al. 2001; Myatt and Cui 2004; Takagi, Nikaido et al. 2004; Vanderlelie, Venardos et al. 2005). Amongst cellular proteins which have been reported to be targets of ROS and RNS in the placenta, the effects of oxidative and nitrative stress on placental transporter function are still poorly understood. However, there is evidence of effects of RNS on STB transport proteins including P2X4 purinergic receptor (Roberts, Webster et al. 2007) and the taurine transporter (Myatt 2010) and of ROS on polycystin-2 (Montalbetti, Cantero et al. 2008). It is relevant to mention that the concentrations used experimentally in this thesis and by others are mostly based on measured circulating concentrations of free radicals (Kharfi, Giguere et al. 2005; Szabo, Ischiropoulos et al. 2007). However, it is unknown whether these concentrations reproduce those *in situ* in a specific STB compartment in normal pregnancy or those in PE when an enzyme system is generating increased free radicals. In this thesis, the effects of free radicals were assessed as regards a potential role in dysregulating K^+ channel function but the effective local concentrations at which free

radicals exert their actions both during normal pregnancy and PE need to be further investigated.

In this thesis, oxidative or nitrative stress was experimentally induced to assess the effects of ROS (H_2O_2) and RNS ($ONOO^-$) on STB basal K^+ permeability. For this purpose multinucleated cytotrophoblasts and placental villous fragments from term placentas were acutely exposed to H_2O_2 or $ONOO^-$ and their effects on K^+ permeability assessed indirectly by measuring ^{86}Rb efflux. Evidence from other tissues and cell types show that ROS/RNS can directly modulate the expression (Caouette, Dongmo et al. 2003; Fountain, Cheong et al. 2007) and/or activity (Kourie 1998; Gutterman, Miura et al. 2005) of K^+ channels; however, these effects are tissue-dependent and free radical-specific.

The results showed that basal ^{86}Rb efflux was increased by acute exposure to H_2O_2 in multinucleated cytotrophoblasts (Figure 4.8.A, B). Although H_2O_2 application was sustained during the experimental period (fresh H_2O_2 was added every minute), the increase in ^{86}Rb efflux with H_2O_2 was transient. Preliminary data suggest that H_2O_2-stimulated ^{86}Rb efflux is mediated by Ba^{2+}-sensitive K^+ conductances as in 2 experiments this increase was almost completely blocked by 5mM Ba^{2+} (Figure 4.8.C, D). In contrast, the same concentration of H_2O_2 did not exert an effect on basal ^{86}Rb efflux from placental villous fragments (Figure 4.9.A); it is possible that a higher concentration of H_2O_2 could be required to obtain an effect on ^{86}Rb efflux from tissue because of unstirred layers that are likely to surround the villi. Alternatively, a reason for the lack of effect of H_2O_2 could be because the villous tissue retains antioxidant defences which can counteract the effects of free radicals (Myatt, Eis et al. 1997; Hempstock, Bao et al. 2003; Myatt and Cui 2004); for example catalase and GPX could scavenge the effects of H_2O_2.

H_2O_2 could directly target Ba^{2+}-sensitive K^+ conductances in multinucleated cytotrophoblasts through changes in the oxidation state of these channels (or associated β-subunits) which causes a transient K^+ channel activation and fast inactivation, implying that Ba^{2+}-sensitive K^+ channels could have a role in STB H_2O_2-stimulated efflux. Supporting this hypothesis, direct patch clamp experiments in a renal cell line, showed that 0.1-5mM H_2O_2 caused hyperpolarisation which was inhibited by 5mM Ba^{2+} (Filipovic and Reeves 1997). Although the identity of the conductances underlying Ba^{2+}-sensitive efflux in the STB are still unclear, K_{2P} members are activated by ROS including H_2O_2 in human pancreas (Duprat, Girard et al. 2005); thus TASK-1, which is functionally expressed in cytotrophoblasts (Bai, Lacey et al. 2006) is likely to be a candidate for H_2O_2-induced activation. It is also possible that H_2O_2 exerts its effect by targeting K^+ channel-modulator proteins which in turn activate Ba^{2+}-sensitive K^+ conductances and thereby increase ^{86}Rb efflux in cytotrophoblasts.

The RNS $ONOO^-$ also induced a marked increase in ^{86}Rb efflux from multinucleated cytotrophoblasts which was partly inhibited by IK_{Ca} blocker TRAM-34 (Results section 3.3). In contrast, ^{86}Rb efflux was unaffected by native $ONOO^-$ (0.1mM) in placental villous fragments (Figure 4.9.B), and this lack of effect could be related to the concentration used and/or the present antioxidant defences on placental villi which could acutely scavenge $ONOO^-$.

Overall, this work provides new evidence that K^+ channels in the STB could be potential mediators of the effects of ROS and/or RNS (Figure 4.2). However, the specific mechanism, direct or indirect, by which K^+ channels are activated by ROS/RNS needs to be explored in the future. Furthermore, these results suggest that in PE, which is associated with elevated placental oxidative and nitrative stress, the generation of free radicals could alter K^+ channel activity (Figure 4.2), thereby dysregulating STB renewal and function (Introduction, section 1.5.1.1.).

4.3.1. Effects of pO_2 on the modulation by reactive oxygen and nitrogen species of syncytiotrophoblast potassium channels

Additional results from this thesis provide evidence to suggest that long term effects of free radicals are pO_2-dependent. Placental villous explants maintained at normoxia (6% pO_2) or hyperoxia (21% pO_2) in culture (6 days) were treated with ROS (H_2O_2; 10μM-1mM) or RNS ($ONOO^-$; 0.1mM or SIN-1; 2.5mM) (days 3-6) to induce oxidative and nitrative stress respectively. ^{86}Rb efflux was measured at day 6 to assess the effects on basal STB K^+ permeability. The generation of oxidative stress was not confirmed but it has been previously shown that chronic treatment of villous explants with 1mM H_2O_2 causes oxidative stress which is reversed by treatment with antioxidant vitamins C and E (Cindrova-Davies 2009). Preliminary data from this laboratory (Hirst, Greenwood et al. 2012) has also shown that SIN-1 treatment increases nitrotyrosine staining in STB of explants treated for 3 days compared to control, indicative of elevated nitrative stress.

As previously shown, H_2O_2 increased the ^{86}Rb efflux rate constant at 100μM and 1mM compared to corresponding controls in explants maintained at 6% pO_2 (Results, section 3.1). In contrast, treatment of villous explants with the same concentrations of H_2O_2 produced the opposite effect at 21% pO_2, and significantly reduced the ^{86}Rb rate constant compared to controls at the same pO_2. This suggests that H_2O_2 is activating pO_2-sensitive K^+ channels that are closed/downregulated at normoxia or, alternatively, is inhibiting K^+ channels that are active/upregulated in hyperoxia. In support of this proposal is the evidence that at ambient oxygenation (~21% pO_2) H_2O_2 inhibited STB MVM polycystin-2 currents when these channels were reconstituted in lipid bilayers (Montalbetti, Cantero et

al. 2008); although there are no comparable measurements at 6% pO_2, this thesis corroborates that cationic conductances in STB MVM are modulated by free radicals and therefore could potentially affect STB function.

^{86}Rb efflux was unaffected by long-term treatment with native $ONOO^-$ in villous explants maintained at either 6 or 21% pO_2 (data not shown). The half-life of $ONOO^-$ is less than 100ms (Denicola, Souza et al. 1998; Radi, Peluffo et al. 2001), therefore it is unlikely that the effect of $ONOO^-$ (added every 24h from days 3-5) would be sustained with time in culture. In contrast, long term treatment with the $ONOO^-$ generator SIN-1, did not affect basal K^+ permeability in normoxia, but in explants maintained at 21% pO_2 it significantly reduced ^{86}Rb efflux rate constant (slope -0.0121 ± 0.0005/min^{-1}) compared to untreated controls at the same pO_2 (-0.0131 ± 0.0004/min^{-1}; n= 6 placentas; p=0.03, linear regression). Together with H_2O_2, this suggests that the treatment to generate $ONOO^-$ by SIN-1 in culture could inhibit STB K^+ conductances. This contrasts with results showing that in multinucleated cytotrophoblasts, native $ONOO^-$ activated basal ^{86}Rb efflux (Results, section 3.3), however, compared to acute effects, chronic exposure to free radicals in placental tissue is likely to modulate other cellular proteins which in turn affect K^+ channel function and this discrepancy needs to be further explored.

It is relevant to speculate that villous explants exposed to ROS/RNS in elevated pO_2 could recreate a PE environment and therefore, under conditions of chronically elevated pO_2 and raised free radicals, which could exist in PE, K^+ channels could be activated by the increased pO_2 and then become targets of RNS/ROS thereby inhibiting their activity. However, it is clear that the regulation of K^+ channels by pO_2 and RNS/ROS is complex and that both could interact to influence K^+ channel activity. In this regard, a previous study demonstrated the biophysical properties of the maxi-Cl^- channel from isolated PE MVM vesicles changed in PE (Bernucci, Umana et al. 2003) illustrating that the maxi-Cl^- channel was chronically opened in PE. These results are the first evidence of altered ion channel conductance in STB of PE. They support the hypothesis that in PE STB K^+ channels could be chronically opened by pO_2, increasing their susceptibility to elevated concentrations of free radicals.

4.4. IK_{Ca} participates in the formation of syncytiotrophoblast

Chronic activation of IK_{Ca}, achieved by treating cytotrophoblasts *in vitro* with DCEBIO, significantly inhibited both the formation of multinucleated cytotrophoblasts and their secretion of hCG (Results, section 3.2). TRAM-34 did not affect multinucleation or hCG secretion which is in agreement with the low IK_{Ca} basal activity demonstrated in both cytotrophoblasts (Results, section 3.2) and placental villous tissue (Figure 4.6). These

data suggest that IK_{Ca} quiescence is required for cytotrophoblast differentiation and that factors that inappropriately activate IK_{Ca} could compromise STB renewal.

It is important to consider that in term placentas *in vivo*, fusion only occurs between differentiated cytotrophoblasts and the STB (Huppertz and Gauster 2011); in contrast, in the experimental model used in this thesis and by others (Kliman, Nestler et al. 1986; Greenwood, Brown et al. 1993; Williams, Fyfe et al. 2008), isolated cytotrophoblasts *in vitro* fuse with each other. Others have hypothesised that cyto-cytotrophoblast fusion occurs in this model for reasons which include:

a) a change in phenotype from villous to extravillous trophoblast which *in vivo* is a phenotype that fuses to generate multinucleated giant cells (Al-Nasiry, Vercruysse et al. 2009; Huppertz and Gauster 2011); this could be confirmed through the identification of HLA-G, a specific extravillous trophoblast marker;
b) during the cytotrophoblast isolation method, the STB needs to be removed and as a result it is fragmented into smaller particles (Huppertz, Frank et al. 1999) which could indeed facilitate the first cytotrophoblast fusion *in vitro* and afterwards, once a syncytium is already formed, further fusion of cytotrophoblasts will enlarge it (Huppertz and Gauster 2011) and
c) the trypsin digestion-step in the isolation method contributes to membrane disruption and consequently results in the formation of cytotrophoblast-STB hybrids *in vitro*, therefore these hybrid cytotrophoblasts, having fragments of STB plasma membranes included in their membranes could fuse with each other (Huppertz and Gauster 2011).

Although all these are valid hypotheses, cytotrophoblasts isolated from term placentas have been shown to be enriched in trophoblast markers and lack contamination from other placental cell types (Kliman, Nestler et al. 1986; Greenwood, Brown et al. 1993), therefore this should be considered as a useful cellular model which allows the study of the processes of cytotrophoblast/STB fusion.

Cytotrophoblast differentiation was impaired when IK_{Ca} was activated over 42-66h. DCEBIO did not appear to alter aggregation, although this was not formally quantified, but inhibited morphological and biochemical differentiation *in vitro* by reducing cytotrophoblast multinucleation and hCG secretion respectively (Results, section 3.2). In agreement with this, further work from this thesis has shown that activation of IK_{Ca} in placental villous explants treated with DCEBIO (days 3-5), significantly reduced hCG secretion at day 6 of culture (Figure 4.10). This effect was pO_2-independent as treatment with DCEBIO at both 6 or 21% pO_2 exhibited a reduction in hCG secretion (Figure 4.10), confirming that opening STB IK_{Ca}, a pO_2-insensitive channel, *in vitro* also impairs STB biochemical differentiation in placental villous tissue. However, long term treatment with DCEBIO in

placental tissue caused an increase in the release of LDH, an indicator of cellular necrosis, compared to controls when explants were cultured in hyperoxia but not normoxia (data not shown), which suggests that opening IK_{Ca} with DCEBIO in hyperoxia has a toxic effect.

In non-placental cell types, IK_{Ca} is associated with the regulation of processes that contribute to the maintenance of tissue homeostasis including proliferation (Neylon, Lang et al. 1999; Cheong, Bingham et al. 2005; Tao, Lau et al. 2008; Millership, Devor et al. 2011), differentiation/fusion (Mauro, Dixon et al. 1997; Fioretti, Pietrangelo et al. 2005), cell migration (Schwab, Gabriel et al. 1995; Schwab, Wulf et al. 2006; Schwab, Fabian et al. 2012) and apoptosis (Lang, Kaiser et al. 2003). In this thesis the pharmacological activation of IK_{Ca} markedly reduced cytotrophoblast syncytialisation suggesting IK_{Ca} activation could inhibit cytotrophoblast fusion (Figure 4.2). Therefore, chronically activating these channels could lead to impaired cytotrophoblast-STB fusion and dysregulated turnover (Figure 4.2). In this regard, a reduced trophoblast fusion leading to altered STB turnover has been proposed in PE, as fusogenic proteins are downregulated (Lee, Keith et al. 2001; Keith, Pijnenborg et al. 2002; Langbein, Strick et al. 2008; Vargas, Toufaily et al. 2011) and syncytialisation of cytotrophoblasts isolated from PE placentas is impaired *in vitro* (Vargas, Toufaily et al. 2011). However, the specific role of IK_{Ca} in this process, and the cellular signals acting in conjunction to co-ordinate trophoblast fusion, need to be addressed in the future.

Although a key function of IK_{Ca} is to regulate cell motility, it is unlikely that these channels regulate the migration of cytotrophoblasts. The isolated cells were treated with DCEBIO/TRAM-34 at 3h of culture when they start to migrate before aggregating (Kliman, Nestler et al. 1986) and, although migration was not quantified, it was clear from the immunofluorescent staining that at 15h the cells were dispersed and by 42h they had moved together and aggregated to the same extent than controls (Results, section 3.2).

The activation of IK_{Ca} *in vitro* reduced cytotrophoblast hCG secretion at 66h (Results, section 3.2). hCG is secreted from differentiated STB in an autocrine/paracrine manner at term thereby promoting cytotrophoblast differentiation and fusion in order to maintain the multinucleated STB (Shi, Lei et al. 1993) (Figure 4.1). Results from this thesis and others have shown that blocking K_Vs inhibits hCG secretion in cytotrophoblasts and placental villous explants (Williams, Fyfe et al. 2008); results of this thesis also show that activating IK_{Ca} *in vitro* reduces hCG secretion from cytotrophoblasts and villous tissue. Therefore, both blocking K_Vs and opening IK_{Ca}s inhibits cytotrophoblast multinucleation. It is possible that the primary effect of blocking K_Vs is a reduction in hCG secretion and as a result, multinucleation is impaired. There is little evidence that IK_{Ca}s regulate endocrine secretion, and their activation, perhaps by altering cell volume (see below and Introduction, section

1.6.2.1) might primarily inhibit cell fusion and differentiation and secondarily reduce hCG secretion.

4.4.1. Role of IK_{Ca} in regulation of syncytiotrophoblast volume status

In many cell types, restoration of cell volume in the presence of a hyposmotic stimulus (RVD) is mediated by K^+ channels, including IK_{Ca}, in conjunction with swelling-activated anion channels (Hoffmann, Lambert et al. 2009). In the current thesis, reducing extracellular osmolality as a experimental tool to activate K^+ channels in multinucleated cytotrophoblasts markedly increased ^{86}Rb efflux and this increase was dependent on extracellular Ca^{2+} (Results, section 3.2). The results from this thesis strongly support the possibility that IK_{Ca} mediates this effect as the specific IK_{Ca} blocker, TRAM-34 but not other K_{Ca} blockers, almost completely inhibited swelling-activated ^{86}Rb efflux (Results, section 3.2). A hyposmotic solution of ~145mOsm/kgH_2O is often used (Grunnet, MacAulay et al. 2002) to induce K^+ channel activation in response to cell swelling; however, as part of this thesis, experiments were also performed using an extracellular solution with a higher osmotic strength (220mOsm/kgH_2O). Preliminary results showed that K_V blockers 4-AP (5mM) and TEA (5mM) did not have an effect on swelling-activated ^{86}Rb efflux from placental villous fragments when a hyposmotic solution of 220mOsm/kgH_2O was used (n= 3 placentas; data not shown). Further investigation is required to identify K^+ conductances underlying hyposmotic cell swelling in placental villous tissue, but also to assess whether there is a differential activation of K^+ conductances according to the osmotic strength of the stimulus.

At present is not clear whether pregnancies complicated by PE show increased STB swelling. It is likely that swelling could arise due to elevated nutrient uptake into STB in PE however, limited studies show that despite a higher fetal plasma amino acid concentration in PE (Evans, Powers et al. 2003), uptake of amino acids is unchanged (system A) (Shibata, Hubel et al. 2008) or reduced (TauT) (Desforges, Ditchfield et al. 2013) in this condition compared to normal pregnancy. Conversely, in common with other tissues and as consequence of inflammation, hypoxia and/or free radical damage (Hoffmann, Lambert et al. 2009) there is a possibility STB swelling could arise in PE and consequently any factor that induces cell swelling could potentially activate IK_{Ca} (Figure 4.2).

Additional results from this thesis showed that K^+ channel activity was enhanced by free radicals during cell swelling. In villous fragments exposed to hyposmotic solutions of 145 or 220mOsm/kgH_2O both H_2O_2 and $ONOO^-$ induced a small but significant increase in swelling activated ^{86}Rb efflux (H_2O_2: 3% increase compared to hyposmotic 220mOsm/kgH_2O, n= 7 placentas; p=0.03; $ONOO^-$: 6% increase compared to hyposmotic 145mOsm/kgH_2O, n= 8 placentas, p=0.02, Wilcoxon signed rank test *vs*. 100%; data not

shown). In addition both $ONOO^-$ (Results, section 3.3) and H_2O_2 enhanced swelling-activated ^{86}Rb efflux from multinucleated cytotrophoblasts (H_2O_2: 15% increase compared to hyposmotic solution, n= 3 placentas; *p*=0.02, linear regression comparing slope of rate constant; data not shown) and this increase was in part mediated by TRAM-34-sensitive channels (54% reduction in a preliminary experiment in H_2O_2-exposed cytotrophoblasts, data not shown) suggesting that under conditions of cell swelling and increase free radical formation such as PE, IK_{Ca} activity could be potentiated (e.g. increase in IK_{Ca} open probability) (Figure 4.2) and contribute to altered STB formation.

In addition to K^+ channel activation following a hyposmotic stimulus as part of RVD, promoting K^+ channel activity in isosmotic conditions is an essential mechanism that induces cell shrinkage and/or reduces intracellular K^+ concentration that is required for cell proliferation, migration, differentiation/fusion and death, processes that are fundamental to maintenance of tissue homeostasis (Okada, Maeno et al. 2001). The results from this thesis indicate that IK_{Ca} participates in the biochemical and morphological differentiation of STB; however it is possible that to allow adequate cyto-STB fusion, IK_{Ca} inactivity is necessary at specific time points before/during fusion in order to ensure normal progression of STB formation. On the contrary, STB IK_{Ca} activity will be necessary in order to decrease the rate of cytotrophoblast fusion when there is increased STB shedding to allow the maintenance of STB cell volume, or when STB swelling occurs due to increased nutrient uptake, in order to restore STB osmotic homeostasis. Together, this evidence suggests IK_{Ca} function needs to be tightly controlled to ensure STB renewal but also STB function in normal pregnancy.

4.5. Perspectives and significance

K^+ channels control diverse cellular processes predominantly by setting the cell E_m and by regulating intracellular K^+ concentration. In addition to influencing ion transport, K^+ channels regulate the activity of electrogenic nutrient transporters, as well as $[Ca^{2+}]_i$, an effector and second messenger that controls many essential cell functions such as ion transport, endocrine secretion, cell proliferation, cell motility and apoptosis. Therefore, knowledge of K^+ channel function and its regulation in STB is fundamental to understand placental physiology and pathophysiology. The results presented here confirm the overall hypothesis that STB K^+ channel function can influence processes that participate in the renewal of STB and reveal potential roles for K^+ channel dysregulation that could contribute to pregnancy disease, in particular in PE.

Figure 4.1 shows a model, based on published data and evidence presented in this thesis, to propose how K^+ channels contribute to STB renewal and function. In pregnancy at term, physiological fluctuations in blood flow into the placenta give an estimated average oxygenation of ~6% pO_2. This study shows that K_V channel activity is downregulated by reduced pO_2 and at 6% pO_2 STB K_Vs are likely to be relatively inactive and maintain an E_m that allows tonic levels of Ca^{2+} entry and an $[Ca^{2+}]_i$ that supports basal hCG secretion. hCG acts in an autocrine/paracrine manner to sustain STB renewal by promoting cytotrophoblast fusion, thereby maintaining normal turnover (e.g. fusion of cytotrophoblasts to STB). Additionally, in STB at rest different K^+ conductances, including Ba^{2+}-sensitive K^+ channels, will contribute to E_m and influence cell functions including the activity of electrogenic transporters and $[Ca^{2+}]_i$ among others. According to the prevailing pO_2, ROS and RNS will be generated by different intracellular enzyme systems (e.g. mitochondrial electron transport chain), maintaining the formation of free radicals at physiological levels where they exert several cellular functions. This study shows that elevated free radicals $ONOO^-$ and H_2O_2 activated IK_{Ca} activity in the STB. However, IK_{Ca} at rest remains in a quiescent state in cytotrophoblasts allowing cyto-STB fusion to occur. On the other hand, IK_{Ca} will be activated in the presence of specific physiological stimuli such as extracellular ATP, STB swelling, and will contribute to the maintenance of normal STB function.

In contrast, Figure 4.2 suggests a potential role of dysregulated K^+ channel function in STB which could contribute to some of the abnormalities evident in PE. Inadequate remodelling of maternal spiral arteries is proposed to cause intermittent blood flow into the placenta and hypoxia or hyperoxia both of which exacerbate the generation of RNS and/or ROS. Increased free radical formation, ATP levels and potentially STB swelling in PE could promote dysregulated STB K^+ channel activity, by inappropriately activating IK_{Ca} and

consequently impairing fusion of cytotrophoblasts to the STB thereby dysregulating STB turnover. In addition, hyperoxia could upregulate K_V expression/function, and through changes in E_m and $[Ca^{2+}]_i$ could promote hCG secretion *in situ*. It is unknown whether cytotrophoblast/STB susceptibility to the actions of hCG is maintained in PE or whether its autocrine/paracrine actions are impaired.

Overall this work contributes to understanding how the co-ordinated action of K^+ channels in the STB allows homeostasis of this multinucleated cell. In addition, this study presents evidence to suggest how the dysregulated action of K^+ channels could promote some of the pathophysiological events characteristic of PE. As K^+ channels are ubiquitously found in all body tissues, it is uncertain whether the pharmacological modulation of K^+ channels in the STB could represent a therapeutic alternative in the future to treat specific pregnancy complications such as PE; selective targeting might depend on identifying a K^+ channelopathy that is specific to disease in PE. However, investigating the function, regulation and cellular interactions of these proteins is essential to understand placental physiology and pathophysiology.

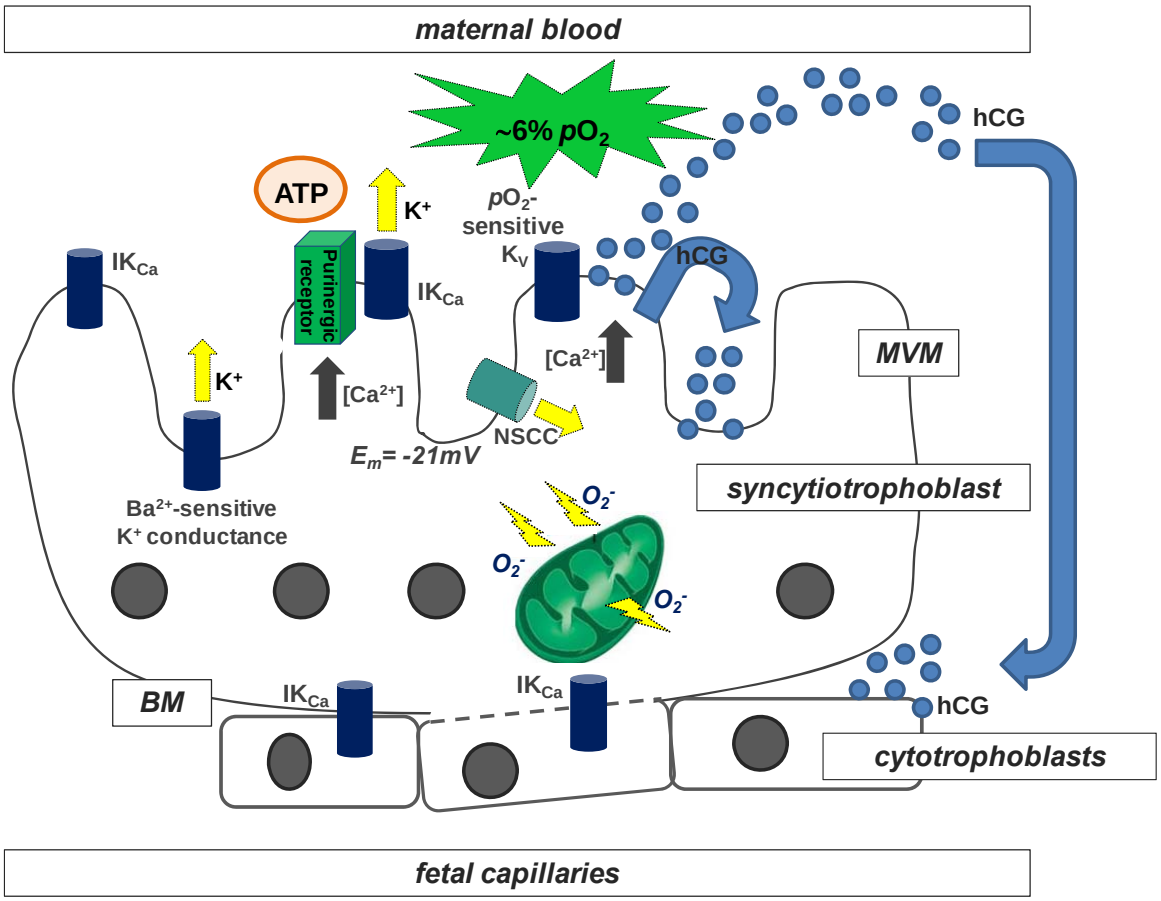

Figure 4.1: K^+ channel function contributes to STB differentiation and renewal in normal pregnancy.
A proposed model combining published findings and the results of the current thesis. The placental bed at term in normal pregnancy is exposed to a range of pO_2 (average 6% pO_2) leading to a basal production of free radicals; the mitochondrial transport chain and other enzymatic systems generate primarily O_2^- and other free radicals at physiological levels. pO_2 influences K_V activity, and at 6% pO_2, K_V will be relatively inactive, setting E_m to allow Ca^{2+} entry through NSCCs and support a basal level of hCG secretion. hCG acts in an autocrine/paracrine manner promoting cytotrophoblast fusion and therefore maintaining physiological STB turnover. In addition, in STB at rest, other K^+ conductances, including Ba^{2+}-sensitive K^+ channels, will contribute to the maintenance of E_m and, as a result, will regulate the activity of electrogenic membrane transporters and $[Ca^{2+}]_i$. IK_{Ca} remains in a quiescent state in cytotrophoblasts, thereby allowing cyto-STB fusion to occur. However, IK_{Ca} will be activated by specific stimuli such as extracellular ATP, or STB swelling, and will contribute to maintenance of STB homeostasis and function.

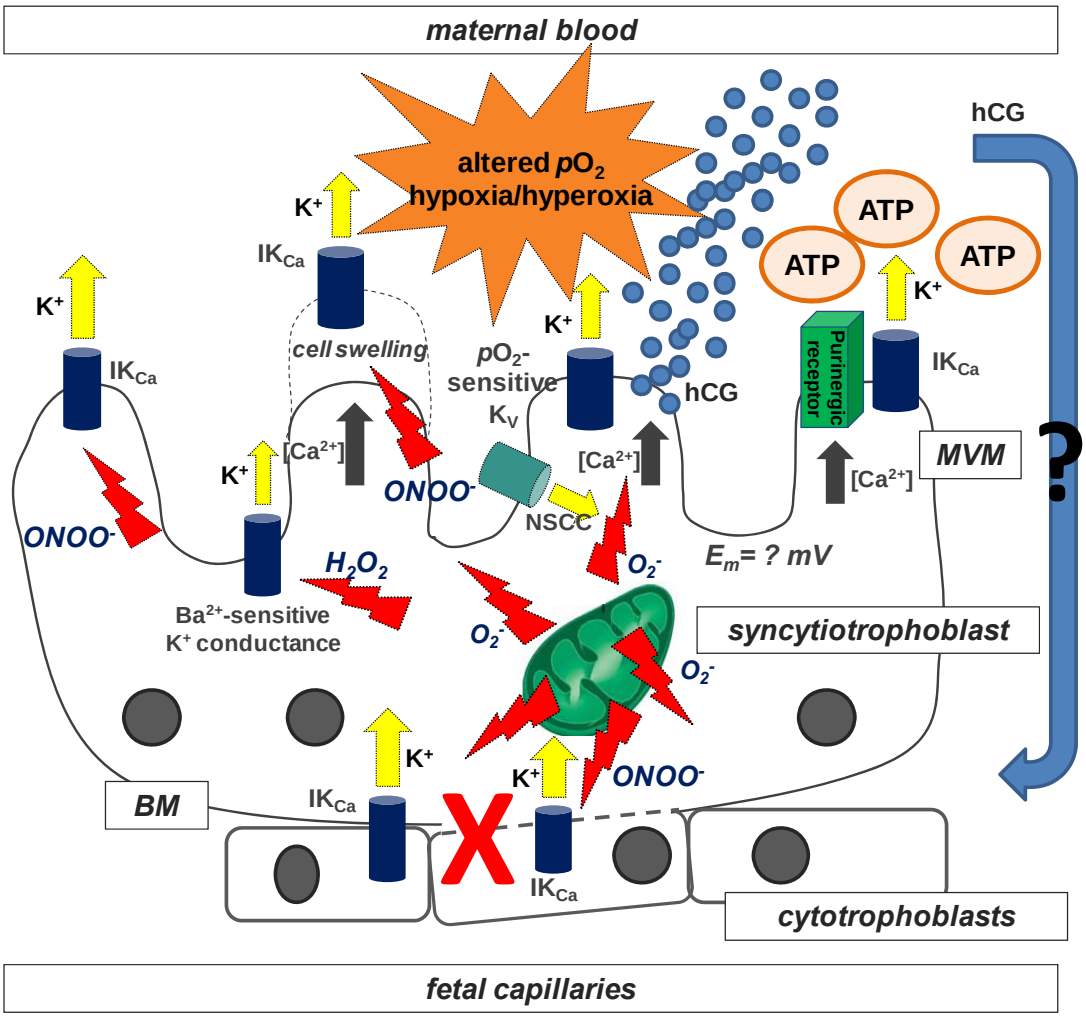

Figure 4.2: K^+ channel dysfunction contributes to dysregulated STB differentiation and renewal in PE.
A proposed model combining published findings and the results of the current thesis. In PE, it is hypothesised intermittent blood flow into the placental bed will create altered pO_2; hypoxia and hyperoxia have been suggested to exacerbate the generation of free radicals, both ROS and RNS. Increased free radical formation, potential STB swelling and elevated extracellular ATP in PE would dysregulate STB K^+ channel activity, by inappropriately activating IK_{Ca} and as a result impairing fusion of cytotrophoblasts to STB. In addition, raised pO_2 could upregulate K_V, therefore hyperpolarising E_m, stimulating Ca^{2+} entry through NSCCs and elevating $[Ca^{2+}]_i$ which would promote increased hCG secretion *in situ*. It is unknown whether regulation of cytotrophoblast/STB differentiation and fusion by hCG is maintained in PE or whether this autocrine/paracrine function of hCG is impaired.

4.6. Future work

The following outlines future work that is necessary to confirm the mechanism/s by which pO_2 modulates K^+ channels in the STB:

i. Assess differential pO_2-sensitive K^+ channel mRNA expression in cytotrophoblasts/villous explants from normal term placentas maintained at experimental normoxia/hypoxia/hyperoxia *in vitro* in order to assess long-term pO_2-modulation of K^+ channel expression.

The following outlines future work that is necessary to confirm the mechanism/s by which free radicals modulate K^+ channels in the STB and significance of this for PE:

i. Assess the underlying effects of free radicals (both ROS/RNS) on STB/cytotrophoblast K^+ efflux it is necessary to perform patch clamp experiments on cytotrophoblasts to determine whether ROS/RNS directly target K^+ channels (i.e. change specific biophysical properties) or act by an indirect mechanism such as primarily elevating $[Ca^{2+}]_i$ (see below).
ii. Determine whether free radicals, in particular $ONOO^-$, act on IK_{Ca} protein to induce nitration and/or oxidation and how this relates to acute and chronic exposure to $ONOO^-$. To elucidate this, immunoprecipitation experiments on purified STB MVM vesicles from normal placentas, PE, and following experimental exposure to $ONOO^-$ or $ONOO^-$-derivates, using IK_{Ca} and nitrotyrosine antibodies, would reveal whether IK_{Ca} is nitrated and if nitration is elevated in PE.
iii. Determine whether $ONOO^-$ increases $[Ca^{2+}]_i$ in STB of villous tissue and cytotrophoblasts by performing fluorometric measurement of $[Ca^{2+}]_i$ in response to $ONOO^-$. Perform these experiments with specific Ca^{2+}-permeable channel blockers (e.g. NSCCs, VGCCs), and modulators of Ca^{2+} stores, to determine whether $ONOO^-$ promotes Ca^{2+} entry from extracellular fluid or the release from stores.
iv. Subject cytotrophoblasts to long-term oxidative/nitrative stress *in vitro*, in order to determine whether K^+ channel activity/expression is modulated by ROS/RNS
v. Perform electrophysiological recordings in isolated cytotrophoblasts from PE placentas maintained in primary culture in order to characterise whole cell K^+

currents. It is essential to determine whether there are differences in the inherent expression/activity of K^+ channels in STB/cytotrophoblasts in PE compared to normal placentas. This will require a combination of E_m measurements, patch clamp studies of cytotrophoblasts and ^{86}Rb efflux measurements in villous tissue and measurement of $[Ca^{2+}]_i$, combined with expression studies (protein expression/gene array/immunohistochemistry).

vi. Determine a causal relationship between channel nitration/oxidation and altered cytotrophoblast/STB fusion and the mechanism(s) involved.

4.7. Supplementary data

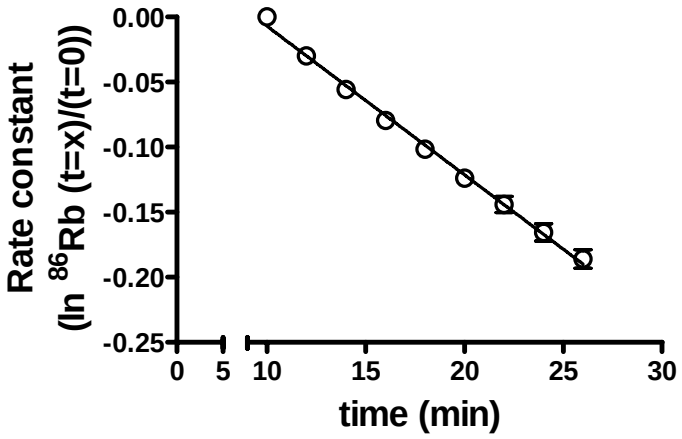

Figure 4.3: Rate constant for basal ^{86}Rb efflux from fresh villous fragments of term placentas.
The loss of ^{86}Rb was measured by a first order rate constant which was calculated over 18min. Each experimental data (x= ^{86}Rb in tissue at time t; ^{86}Rb in tissue at start) was transformed by l_n. n= 16 placentas. Slope= -0.0114 ± 0.0003/min^{-1}; r^2= 0.902 (linear regression). The relationship between time and rate constant was significant ($p<0.0001$).

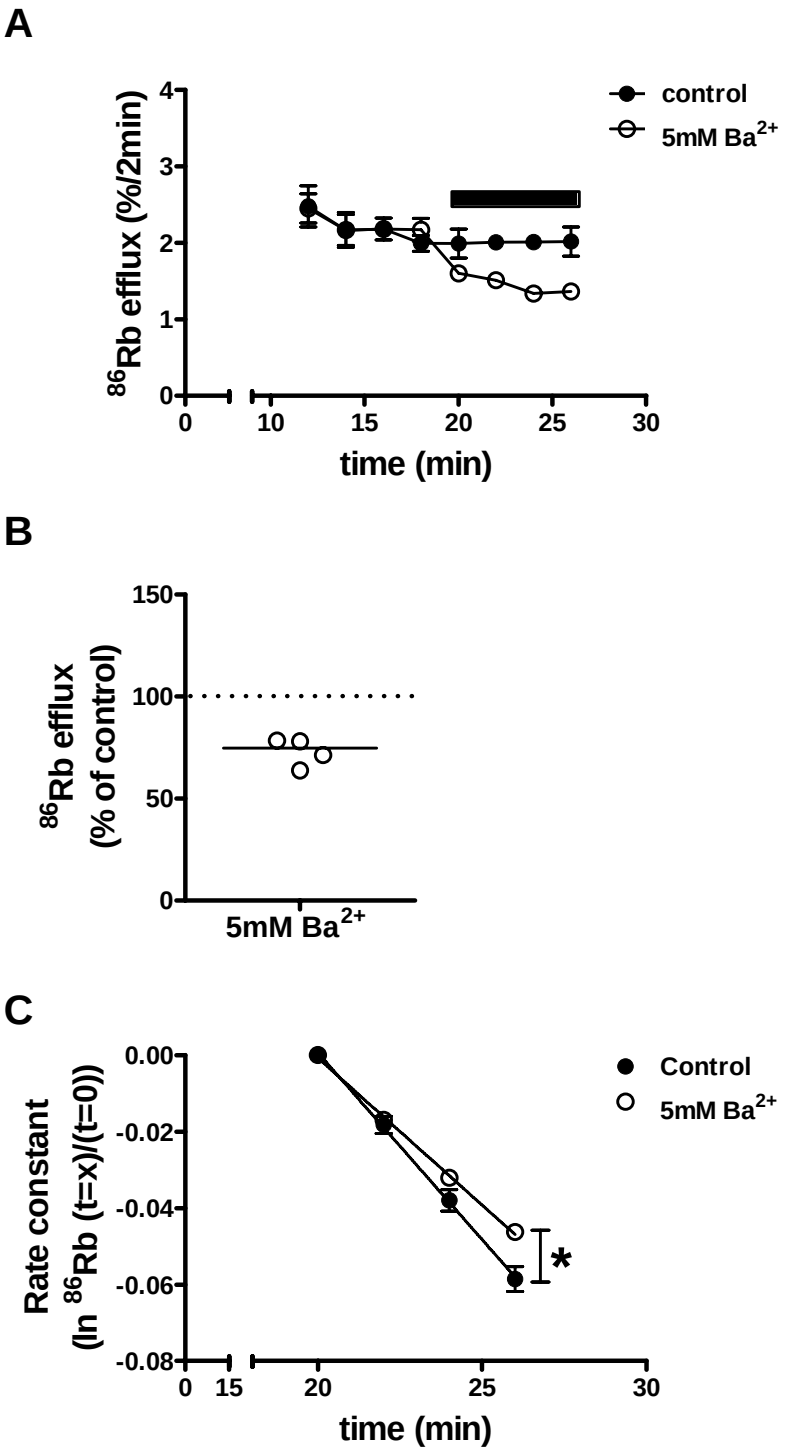

Figure 4.4: Ba^{2+} inhibits ^{86}Rb efflux from fresh villous fragments of term placentas. ***A***: $\%^{86}Rb$ efflux over 16min; ^{86}Rb efflux was measured in control conditions and with 5mM Ba^{2+} during the experimental period (indicated by the bar). Data are mean ± SE (n= 4 placentas). ***B***: $\%^{86}Rb$ efflux over the experimental period (8min) in fragments exposed to Ba^{2+} expressed as a % of control; line at median. ***C***: Corresponding ^{86}Rb efflux first-order rate constant, calculated over 8min (experimental period). The relationship between time and rate constant was significant in all cases ($p<0.0001$). The difference in slopes between groups was *p=0.0007; n= 5 placentas; mean ± SE.

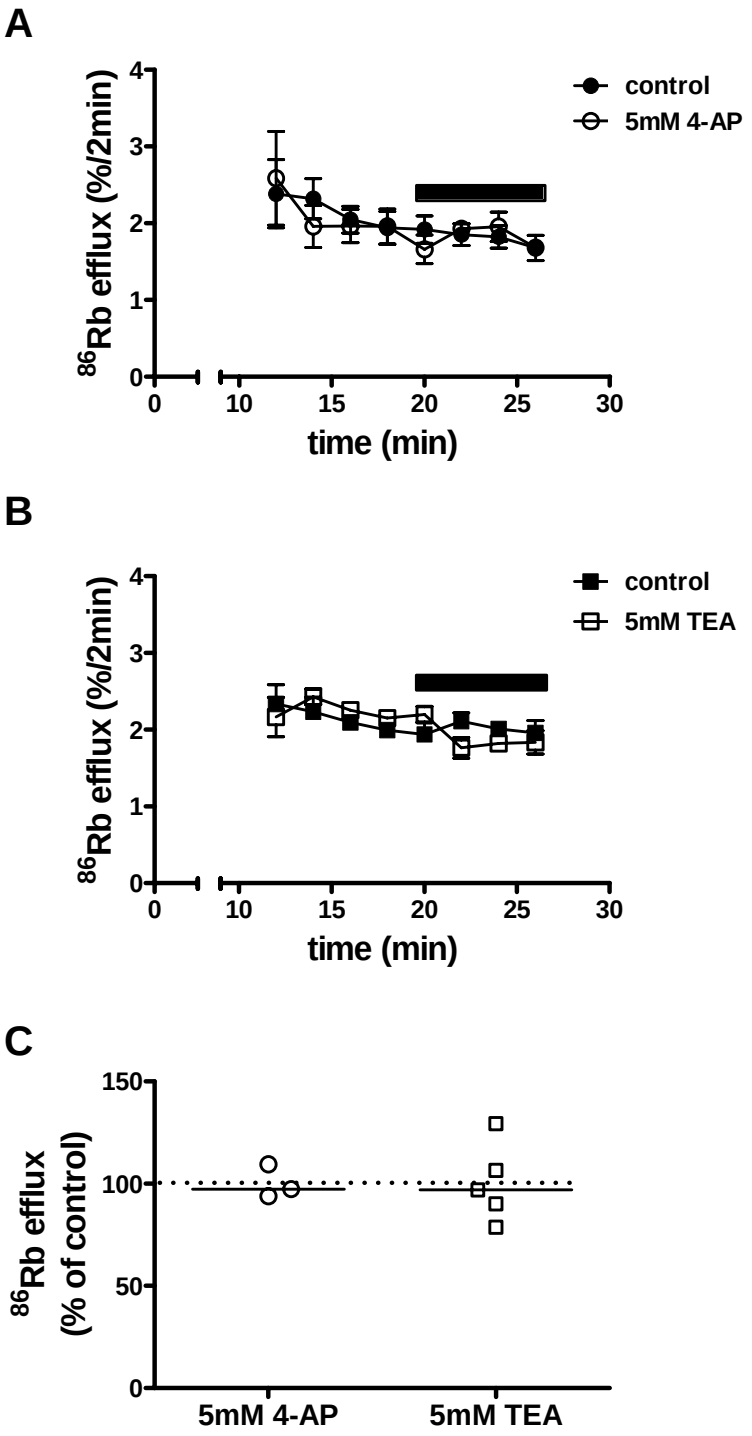

Figure 4.5: 4-AP and TEA do not affect ^{86}Rb efflux from fresh villous fragments of term placentas.

A, B: Time course for %^{86}Rb efflux over 16min. ^{86}Rb efflux was measured in control conditions or with 5mM 4-AP (***A***; n= 3 placentas) or 5mM TEA (***B***; n= 5 placentas) during the experimental period (bar). Data mean ± SE. ***C***: %^{86}Rb efflux over the experimental period (8min) in villous fragments exposed to 4-AP or TEA expressed as a percentage of efflux in control villous fragments; line at median.

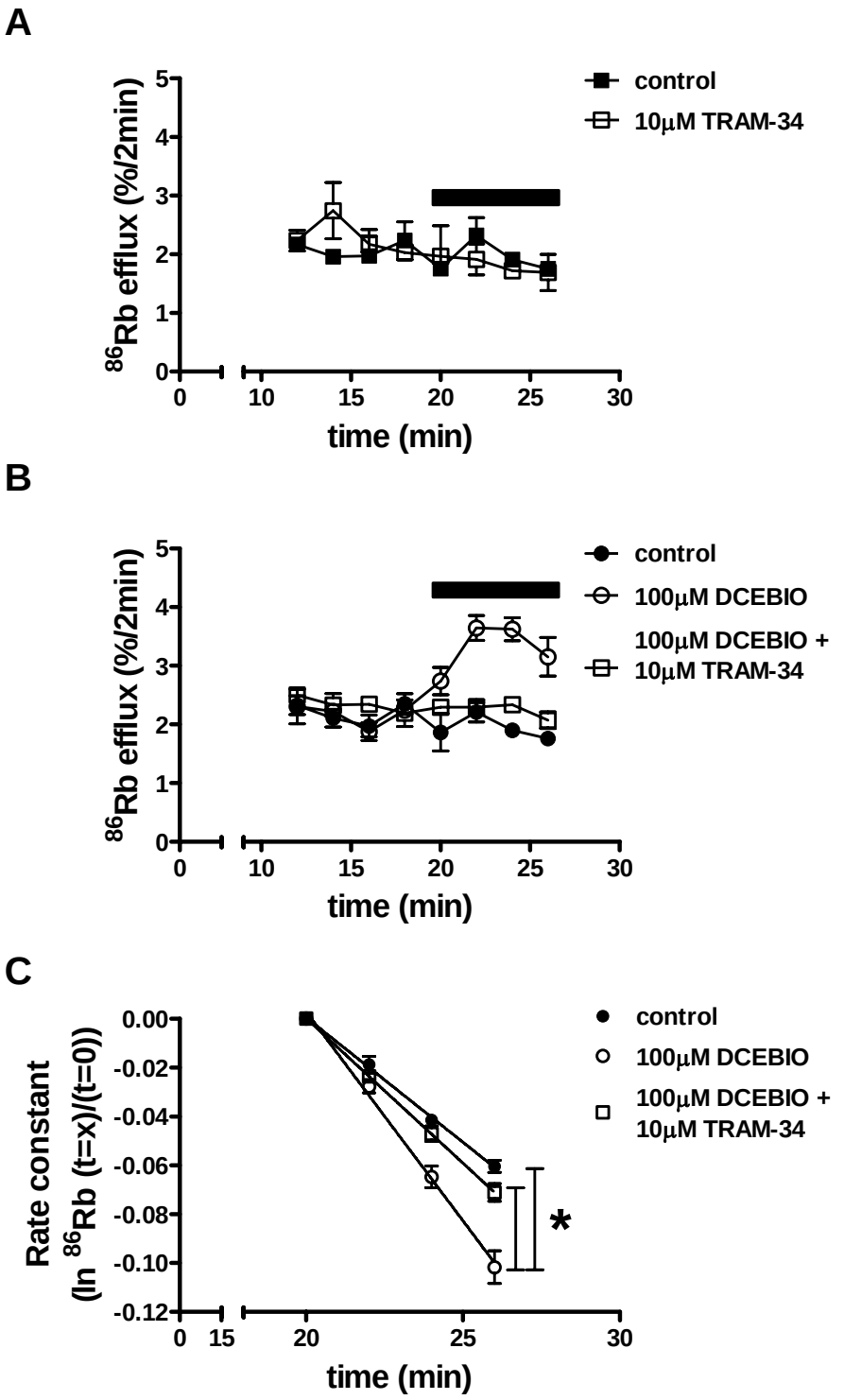

Figure 4.6: DCEBIO stimulates TRAM-34-sensitive ^{86}Rb efflux from fresh villous fragments of term placentas.

A, ***B***: Time course for %^{86}Rb efflux over 16min. ^{86}Rb efflux was measured in control and villous fragments exposed to 10μM TRAM-34 (***A***; n= 2 placentas, data are mean ± SD), 100μM DCEBIO or 100μM DCEBIO + 10μM TRAM-34 (***B***; n= 3 placentas, data are mean ± SE) during the experimental period (bar). ***C***: Corresponding ^{86}Rb efflux first-order rate constant, calculated over 8min (experimental period). The relationship between time and rate constant was significant in all cases ($p<0.0001$). The difference in slopes between groups was *$p<0.0001$; n= 3 placentas; data are mean ± SE.

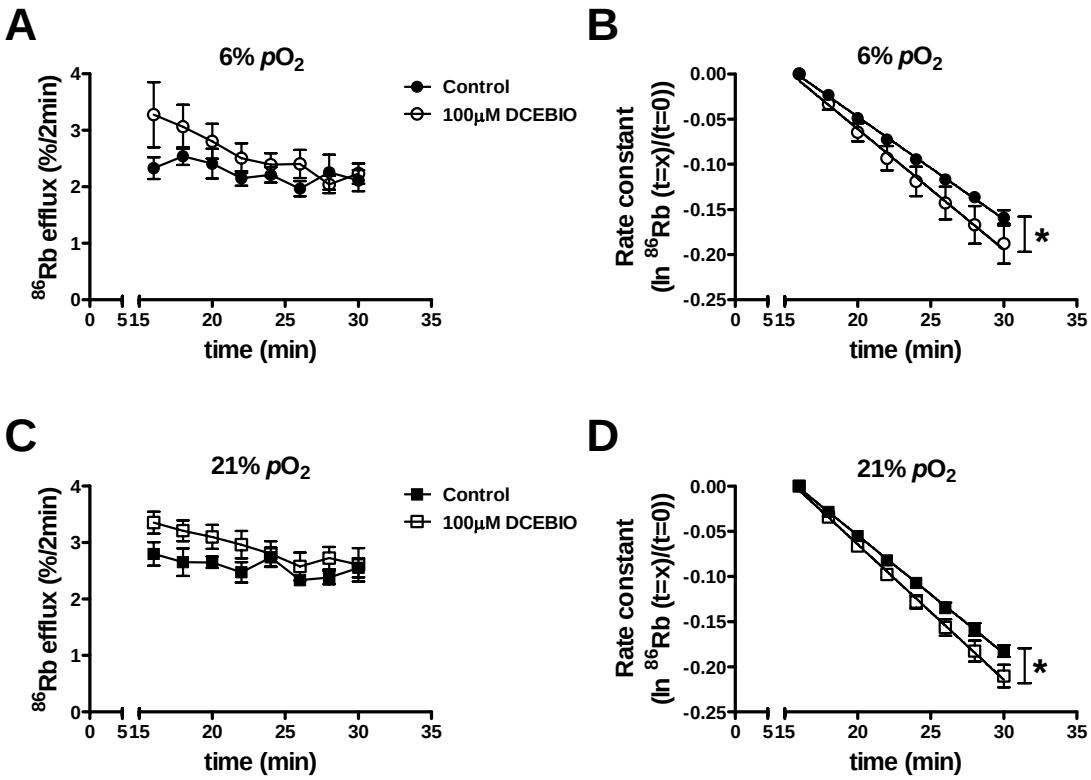

Figure 4.7: DCEBIO enhances on ^{86}Rb efflux from cultured villous explants of term placentas.

Placental villous explants were cultured for 6 days and maintained at 6% pO_2 (normoxia; ***A***, ***B***) or 21% pO_2 (hyperoxia; ***C***, ***D***) and ^{86}Rb efflux was measured at day 6 in explants untreated (control) or treated with 100μM DCEBIO (days 3-5). ***A***, ***C*** show the time course for the %^{86}Rb efflux over 16min with the corresponding ^{86}Rb efflux first-order rate constant (***B***, ***D***), calculated over 16min in explants maintained at 6% pO_2 (***A***, ***B***; n= 6 placentas; **p*=0.0004; linear regression) or 21% pO_2 (***C***, ***D***; n= 6 placentas; **p*=0.0005; linear regression). The relationship between time and rate constant was significant in all cases ($p<0.0001$). Data are mean ± SE.

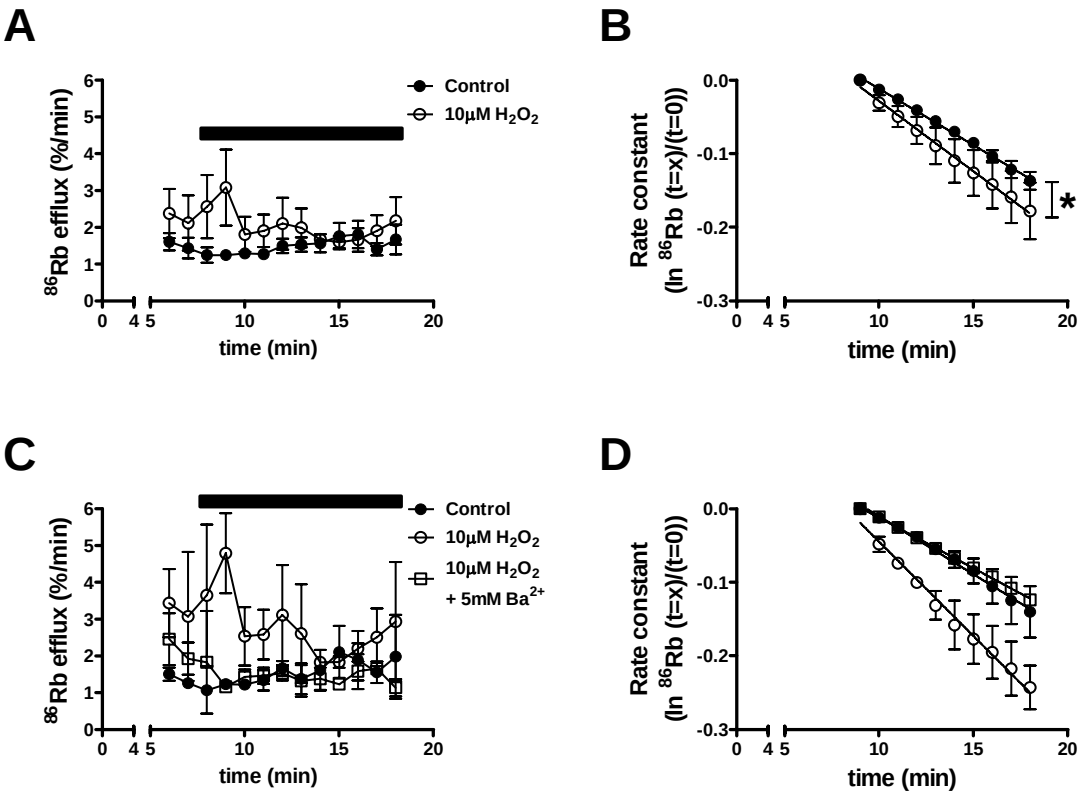

Figure 4.8: Acute application of H_2O_2 promotes ^{86}Rb efflux from multinucleated cytotrophoblasts.

A: Isolated cytotrophoblasts from term placentas were cultured for 66h and the %^{86}Rb efflux was measured in the presence or absence (control) of 10μM H_2O_2 during the experimental period (indicated by the bar); n= 4 placentas; data are mean ± SE. H_2O_2 was added from min 4 and every min during the experimental period. ***B***: Corresponding ^{86}Rb efflux rate constant calculated over 10min (experimental period). The relationship between time and rate constant was significant in all cases ($p<0.0001$). The difference in slopes between groups was *p=0.0002 (linear regression); data are mean ± SE. ***C***: Time course of %^{86}Rb efflux measured in control and under exposure to 10μM H_2O_2 or 10μM H_2O_2 + 5mM Ba^{2+} during the experimental period (bar); n= 2 placentas; data are mean ± SD. ***D***: Corresponding rate constant analysis calculated over 10min experimental period. Data are mean ± SD.

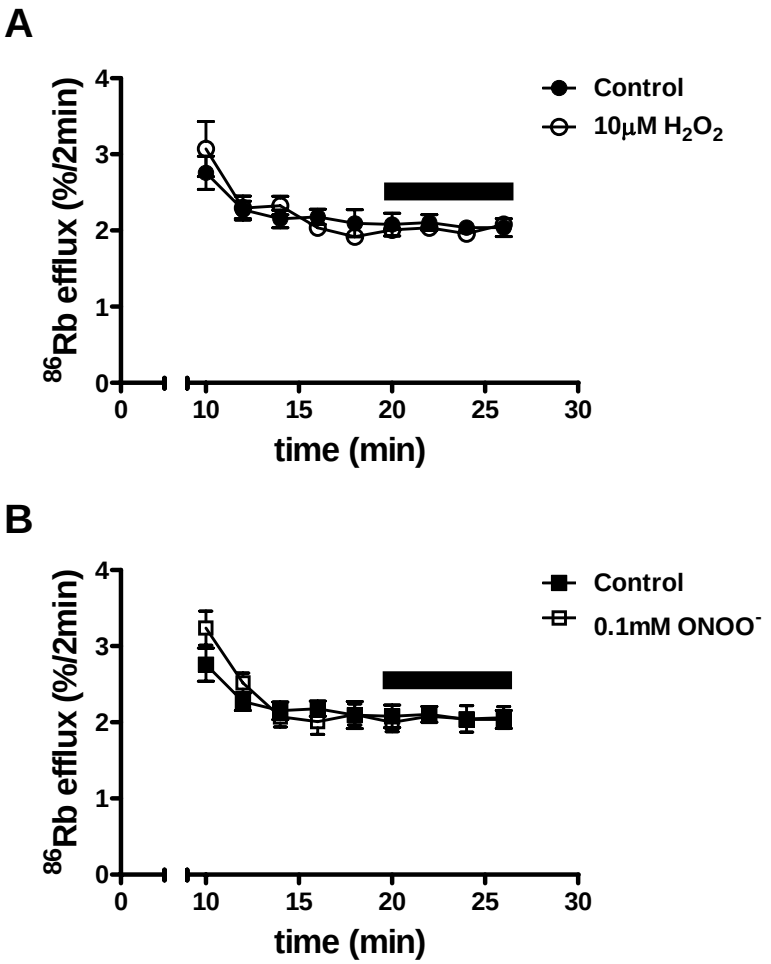

Figure 4.9: Acute application of H_2O_2 and $ONOO^-$ does not alter ^{86}Rb efflux from placental villous fragments of term placentas.
Time course of $\%^{86}Rb$ efflux measured in fresh placental villous fragments from term placentas in the presence or absence (control) of (***A***) 10μM H_2O_2 or (***B***) 0.1mM $ONOO^-$ during the experimental period (indicated by the bar). n= 6 placentas; data are mean ± SE.

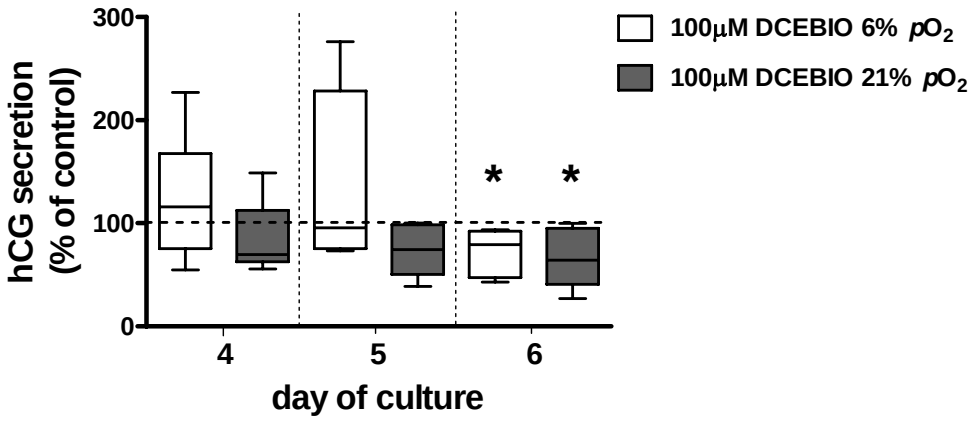

Figure 4.10: DCEBIO inhibits hCG secretion from placental villous explants of term placentas.

Placental villous explants were maintained at 6 or 21% pO_2 for 6 days and untreated (control) or treated daily with 100μM DCEBIO (days 3-5). hCG secretion from DCEBIO-treated explants maintained at 6 and 21% pO_2 at days 4, 5 and 6 of culture is expressed as a percentage of control (100%, dotted line); data are median ± IQR; n= 6 placentas; **p*=0.03; Wilcoxon signed-rank test compared to 100%.

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