A ROLE FOR ATP IN MODULATING VASOMOTION DURING HYPOXIA IN UMBILICAL CORD BLOOD VESSELS

By

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ABSTRACT

Previous studies have associated intracellular calcium ($[Ca^{2+}]_i$) oscillations in vascular smooth muscle cells (SMC) with vasomotion in multiple species. In normal and pre-eclamptic pregnancies, there is strong evidence to suggest that the intrauterine environment is hypoxic. The aim of this study was to investigate whether ATP and $[Ca^{2+}]_i$ oscillations play a role in modulating vasomotion during hypoxia in human umbilical blood vessels.

The results obtained from *in vitro* studies using firefly luciferase assay and quinacrine staining indicated that human umbilical artery and vein endothelial cell (HUAEC, HUVEC respectively) constitutively released ATP and, in HUVEC at least, the release was accentuated by hypoxia (7.6 mmHg O_2, 30 min). This release is dependent on the PI_3K/ROCK pathway, and on normal vesicular transport. Further, application of ATP to human umbilical artery SMC induced dose-dependent $[Ca^{2+}]_i$ oscillations, which is mediated by $P2Y_4$ receptor. Moreover, e*x vivo* data from freshly isolated umbilical artery rings showed that acute hypoxia increased the frequency of vasomotion.

It is therefore proposed that the findings of the present study is important in the understanding of the behaviour of human umbilical vessels in normal pregnancy, but may also have implications in the pathophysiology of complicated pregnancy such as pre-eclampsia.

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PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS THESIS

The effect of hypoxia on ATP release from human umbilical endothelial cells. Wilson King Lim To, Yuchun Gu, Prem Kumar, and Janice M Marshall. FASEB J. March 17, 2011 25:670.12.

Calcium oscillations induced by ATP in human umbilical cord smooth muscle cells. Fei Meng, Wilson To, Jackson Kirkman-Brown, Prem Kumar, Yuchun Gu. J. Cell. Physiol. 2007 Oct;213(1):79-87.

Poster presentation at the Physiology 2011, The Physiological Society, University of Oxford, July 2011, PC 35.

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ABBREVIATIONS

2-APB	2-aminoethoxydiphenyl borate
A23187	5-(Methylamino)-2-[[2*R*,3*R*,6*S*,8*S*,9*R*,11*R*) -3,9,11-trimethyl-8-[(1*S*)-1-methyl-2-oxo-2-(1*H*-pyrrol-2-yl)-ethyl]-1,7-dioxaspiro[5.5]undec-2-yl]methyl]-4-benzoxazolecarboxylic acid
AA	Arachidonic acid
ACh	Acetylcholin
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BK	Large conductance potassium
CCE	Capacitative calcium entry
CO_2	Carbon dioxide
COX	Cyclooxygenase
DAG	Diacylglyverol
EC	Endothelial cell
EET	Epoxyeicosatrienoic acid
EN	Ectonucleotidase
EDTA	Ethylenediaminetetraacetic acid
EDCF	Endothelium-derived contracting factor
DERF	Endothelium-derived relaxing factor

ET	Endothelin
FACS	Flow-activated cell sorting
GPCR	G-protein coupled receptor
H2O2	Hydrogen peroxide
HFPV	Hypoxic fetoplacental vasoconstriction
HPV	Hypoxic pulmonary vasoconstriction
Hr	Hour(s)
I/R	Ischaemia-reperfusion
IK_{Ca}	Intermediate conductance Ca^{2+}-activated K^+ channels
IP_3	Inositol trisphosphate
K_M	Michaelis-Menten constant
L-NMMA	N^G-monomethyl-L-arginine
La^{3+}	Lanthanum ion
LY294002	2-(4-Morpholinyl)-8-phenyl-4*H*-1-ben zopyran-4-one
Min	Minute(s)
NO	Nitric oxide
OAG	1-oleoyl-2-acetyl-sn-glycerol
O_2^-	Superoxide
$ONOO^-$	Peroxynitrite
PE	Pre-eclampsia
PBS	Phosphate buffered saline
PET	Polyethylene terephthalate
PI_3K	Phosphatidylinositol 3 kinase

PLC	Phospholipase C
PG	Prostaglandins
PPADS	Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid
PGI_2	Prostacyclin
ROI	Region of interest
RT	Room temperature
ROCC	Receptor-operated calcium channels
S	Second(s)
SK_{Ca}	Small conductance Ca^{2+}-activated K^+ channels
SMC	Smooth muscle cell
SNAP	S-Nitroso-N-acetyl-D,L-penicillamine
SOCC	Store-operated calcium channels
SOD	Superoxide dismutase
SERCA	Sacroplasmic reticulum Ca^{2+} ATPase
TG	Thapsigargin
TRP	Transient Receptor Potential
TXA_2	Thromboxane
U73122	1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione
V.S.	Versus
VOCC	Voltage-operated Calcium channel
VSM	Vascular smooth muscle
Y27632	*Trans*-4-[(1*R*)-1-Aminoethyl]-*N*-4-pyridinylcyclohexanecarboxamide dihydrochloride

CHAPTER 1

GENERAL INTRODUCTION

1.1 OVERVIEW

An adequate O_2 and nutritional supply via the umbilical vasculature is important for the normal development and growth of the placenta and fetus. Indeed, reduced or impaired blood flow patterns in the umbilical cord blood vessels is found in many pathological conditions that are associated with a hypoxic intrauterine environment (Jouppila & Kirkinen, 1984; Gudmundsson & Marsal, 1988; Ferguson & Dodson, 2009). Vascular vasomotion is the Ca^{2+}-dependent rhythmic oscillation of vascular tone found in many blood vessels including the human umbilical blood vessels. It has been argued that vasomotion improves vascular conductance to a tissue in a manner that does not require an increased cardiac output, and can help to meet metabolic demands of and improve oxygenation to the tissue. It has also been argued that spontaneous vasomotion in the placenta and umbilical vessels is important in normal pregnancy, because it creates ischemia / reperfusion and helps to up-regulate antioxidant enzymes so protecting the fetus against oxidative stress (Hung & Burton, 2006; Jauniaux *et al.*, 2006). Few studies have explored the linkage between vasomotion and intrauterine hypoxia, but evidence suggests that the pattern of vasomotion is altered in abnormal pregnancies that lead to miscarriages or pre-eclampsia (Rosen *et al.*, 1990; Sweeney *et al.*, 2008).

The extracellular nucleotide, ATP, has been implicated in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) oscillations that occur in vascular smooth muscle cells (VSM) and vasomotion in blood vessels (Mahoney *et al.*, 1993). There is also evidence that ATP can act on endothelial cells (EC) to cause release of vasoactive substances (Olsson & Pearson, 1990). Further, there is evidence that hypoxia and other stimuli can induce ATP release from VSM and EC, which then acts in an autocrine or paracine fashion on those cells and affects Ca^{2+} signalling

(Gerasimovskaya *et al.*, 2002). It has already been shown that ATP can induce $[Ca^{2+}]_i$ oscillations in SMC (Bergner & Sanderson, 2002), which may drive vascular vasomotion. However, evidence that hypoxia can induce ATP release from VSM or EC of umbilical blood vessels is still lacking. It can be hypothesised that the Ca^{2+} signals induced by ATP could subsequently mediate the production and release of important vasoactive substances such as nitric oxide (NO), prostacyclin (PGI_2), endothelium-derived hyperpolarising factors (ENHF) and endothelins (ET). Thus, purinergic signalling in umbilical blood vessels may well have important physiological consequences in the control of vascular tone and vasomotion.

The particular aims of the present study were therefore (1) to determine whether hypoxia can induce ATP release from human umbilical artery endothelial cells (HUAEC), and from human umbilical venous endothelial cells (HUVEC) *in vitro*; (2) to quantify the amount of ATP released; (3) to examine the effect of ATP stimulation on Ca^{2+} signalling in umbilical EC and SMC and (4) to investigate the consequences of hypoxia and ATP stimulation on human umbilical blood vessels *ex vivo*.

In view of these aims, the discussion that follows covers aspects of the human umbilical circulation and its functions, the ability of the endothelium to synthesise and release vasoactive substances, the known vascular ATP-releasing pathways, cellular responses to ATP stimulation, and aspects of vascular vasomotion.

1.2 HUMAN UMBILICAL CIRCULATION AND ITS FUNCTIONS

1.2.1 Need for a Circulatory System

Single cells and simple organisms do not require a circulatory system. Their metabolic needs can be met by the simple process of diffusion and convection. Through evolution, as the structures of organisms increased in size and complexity with the oxygenation of the Earth's atmosphere (Catling *et al.*, 2005), there came a time when diffusion of O_2 was no longer sufficient to meet metabolic demands: nutrients cannot reach centrally-located cells and nor could waste products be eliminated. Hence, the metabolic demand of multicellular organisms was the limiting factor for an increase in organismal size and complexity. At some stage in evolution, further development of complexity and size was supported by development of circulatory physiology. Animals such as birds and mammals, at higher levels of the food chain, developed a sophisticated circulatory system for the effective distribution and elimination of nutrients and waste products respectively. This ensures centrally-located cells are within a distance of vascular capillaries that is sufficiently short from the source of nutrients for material exchange. Indeed, the exchange of gases is so important that a dual circulatory system was evolved: the blood returns from the body tissues to the heart, and then enters the pulmonary circulation for oxygenation before re-entering the heart for distribution around the systemic circulation. Thus, the pulmonary and systemic circulations are connected in series, and for every heart beat the full cardiac output is delivered to the respiratory exchange surfaces. Further, the evolutionary consequence of the heart as an active dual pump, is that the circulatory system provides a fast convection rate which maintains a steep concentration for the uptake of O_2 and excretion of CO_2 in the lungs, as well as for other non-volatile substances on other equilibration surfaces. At the cytosolic level, delivery of O2 is driven by the concentration gradient generated by O2 consumption in the mitochondria.

Multiple studies have attempted to measure the PO_2 at different levels in the vasculature from the alveoli to the cytosol by O_2-sensitive microelectrodes and phosphorescence quenching methids, and these levels have recently been reviewed (Ward, 2008) and can be found in the *Appendices* section.

Thus, the primary function of the circulation system is to distribute molecules important for cellular survival, growth and repair. Secondary functions have evolved from the presence of the circulatory system and these include hormonal and neurotransmitter distribution; thermoconduction; and distribution of the effectors of the central and peripheral immune system.

1.2.2 Fetal Life Support System

1.2.2.1 Need for a Fetal Life Support System

In humans, embryonic and fetal development essentially recapitulates the evolution of the adult cardiovascular system: an effective circulatory system begins to develop as the fetus increases in size and complexity (Falkowski *et al.*, 2005). The system that provides O_2 and nutritional support for the developing fetus comprises its own heart and vascular system and, importantly, the umbilical circulation which supplies blood to and from the placental and uterine circulations by the umbilical arteries and veins. Together with the uterine and umbilical circulations, the placenta makes up the fetal life support system (Schneider, 1991).

From conception through pregnancy, it is the uterine blood supply that is important first, and this is followed by the placenta and the umbilical cord as the fetus develops. The fetal

cardiovascular system develops concomitantly and becomes connected to the placental circulation at around eight weeks of gestation (Schoenwolf & Larsen, 2009).

1.2.2.2. Adaptations During Gestation

Not surprisingly, the maternal circulation as a whole shows many adaptive changes to the state of pregnancy, such as an increase in circulating blood volume, an increase in cardiac output (due to both increased stroke volume and heart rate), and a fall in peripheral vascular resistance. The details of these adaptations and their physiological basis are outside the remit of the present thesis and were reviewed elsewhere (Duvekot & Peeters, 1994).

1.2.2.3. Uterus

1.2.2.3.1. Structure

The uterus, which contains the placenta and the developing embryo or fetus during pregnancy, is normally a pear-shaped organ in the pelvic cavity, supported by multiple muscles and ligaments. The uterus serves as a site for the reception, retention and nutrition of the fertilised ovum. It comprises three main layers: the outer peritoneum, which lines most of the internal viscera in the human body, the muscular myometrium, which constitutes the bulk of the uterus and comprises involuntary muscle fibres and major branches of the blood vessels, lymphatic vessels and nerves, and finally the mucous membrane endometrium, which undergoes extensive changes through the menstrual cycle in response to the ovarian hormones (Verralls, 2004). During pregnancy, the implanting blastocyst induces the thickening of the endometrium, to become a nutrient-packed vascular tissue called the decidua. It is vital in meeting the nutritional need of the developing fetus during the first trimester before the onset of blood-facilitated maternal-fetal exchange (Burton *et al.*, 2002).

1.2.2.3.2. Blood Supply

The main arterial supply of the uterus is the uterine artery, which is a branch of the internal iliac artery. The ovarian artery also supplies the fundus region of the uterus. The venous drainage largely follows the arteries. As mentioned above, multiple vascular adaptations occur during pregnancy. Indeed, the blood vessels supplying the uterus enlarge in parallel with the growing uterus and fetus. In particular, the uterine arteries become less tortuous and this change is accompanied by an increase in lumen size. The ovarian veins also become considerably larger and able to deal with the extra blood flow from the uterus (Moore & Dalley, 1999; Verralls, 2004). Importantly, during early placentation, spiral arteries in the maternal myometrium undergo extensive morphological changes, with the loss of SMC and elastic lamina from the vessel wall and thus significant dilation, forming a low resistance flaccid conduit of the uteroplacental circulation (Burton *et al.*, 2009).

1.2.2.4. Placenta

1.2.2.4.1 The Origin, Development and Functions of the Placenta

The placenta is responsible for the transfer of the bulk of the substance between maternal and fetal circulations including oxygen (O_2), carbon dioxide (CO_2), water and all the nutrients required for the development and growth of the placenta itself and of the fetus. On one side of this exchange interface are the spiral arteries and endometrial veins of the maternal circulation, and on the other side are the fetal capillaries which ultimately pool to form the umbilical vessels which are connected to the fetal cardiovascular system (Schoenwolf & Larsen, 2009).

It is during the third week post-conception, that the first morphological signs of vasculogenesis that supports placental growth can be seen: precursor EC join together with either desmosomes or tight junctions and begin to form capillary tubes in the secondary chorionic villi of the trophoblast of the developing embryo. The processes of vasculogenesis and angiogenesis are tightly controlled and have been reviewed (Demir *et al.*, 2007). These villi, which contain the fetal blood vessels, continue to invade deeper into the maternal endometrium and towards the end of the first trimester begin to rupture maternal blood vessels, starting from the central area of the placenta. The trophoblastic plugs progressively become dislodged. This results in the placental villi bathing in the maternal blood-filled intervillous space, which allows nutrients and O_2 to pass from the maternal blood across the layers of the villus wall into the fetal blood and waste products such as CO_2, uric acid and bilirubin to pass from the fetal blood to the maternal blood. This transitional phase lasts approximately 2-3 weeks such that by the 14th week of pregnancy, the placenta is fully formed and occupies about one-third of the uterine wall (Schoenwolf & Larsen, 2009).

1.2.2.4.2. The Hormonal Function of the Placenta

During the first trimester of pregnancy, human chorionic gonadotrophin (hCG) produced by the trophoblast of the blastocyte, stimulates secretion of oestrogen and progesterone from the corpus luteum of the ovaries, thereby maintaining the thick lining and blood supply of the endometrium in which the zygote develops. Towards the end of the first trimester, the corpus luteum degenerates into a corpus albicans. The syncytiotrophoblast in the placenta, which starts to produce oestrogen and progesterone in the 8th week of pregnancy, then takes over the hormonal role of maintaining the pregnancy (Schoenwolf & Larsen, 2009).

1.2.2.4.3. The Microstructure of the Material Exchange Barrier

During gestation, the placental capillary network develops to yield a nutrient exchange system of a staggering 550 km in length and 15m^2 in surface area (Burton & Jauniaux, 1995). There is also a progressive thinning of the placental exchange barrier toward term which may help to increase the efficiency of transport of nutrients (Jones & Fox, 1991). The exchange barrier comprises the syncytiotrophoblast, which is a continuous syncytial epithelium covering the entire surface of the chorion, and the fetal endothelium (Fig 1.1): these two cell layers have to be crossed to effect maternal-fetal nutrient exchange in a mature placenta (Fuchs & Ellinger, 2004).

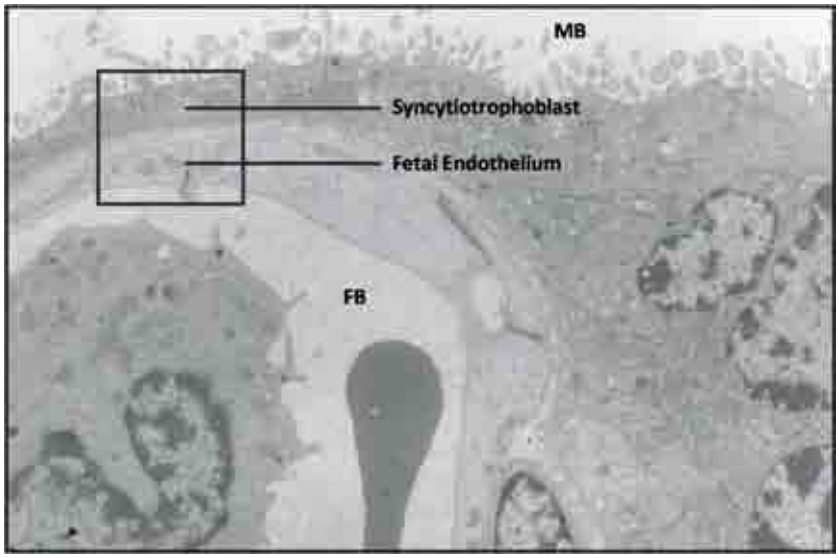


Figure 1.1. Electron transmission electron microphragh of a chorionic villus.

The 'Placental Barrier' consists of the maternal syncytiotrophoblast and the Fetal Endothelium. MB = Maternal Blood. FB = Fetal Blood. Adapted from (Fuchs & Ellinger, 2004)

Within this barrier, the most important anatomical barriers are considered to be the maternal blood-facing plasma membrane and the fetal endothelium-facing basal plasma membrane of

the syncytiotrophoblast (de Virgiliis *et al.*, 1982). Placental transfer has been the subject of many reviews e.g.(Fuchs & Ellinger, 2004; Desforges & Sibley, 2009) and simplistically it can be divided into diffusion, which is dependent on the molecular radius of the diffusate, and specialist transcellular mechanisms.

1.2.2.5. Exchange at the Placenta

O_2 and CO_2 are lipophilic molecules and therefore, as in the adult mammalian alveolar endothelium, they can diffuse readily through the exchange surface of the syncytiotrophoblast plasma membrane in the placenta. Thus, the transfer of O_2 and CO_2 is flow-limited. There is a large placental surface area available for diffusion, and their net transfer is therefore dependent on maternal-fetal gaseous concentration difference, which is in turn dependent on the flow rates of the uterine and umbilical circulation (Desforges & Sibley, 2009). It is important to note that the solubility of O_2 is much lower than CO_2 and as a consequence of this, O_2 limitation is more likely to occur than CO_2 accumulation. It is not surprising then, that impeded blood flow in the umbilical cord is associated with a hypoxic intrauterine environment, which in turn is associated with the pathophysiology of many pathological conditions of pregnancy including intrauterine growth retardation (IUGR) and pre-eclampsia (PE) (Jouppila & Kirkinen, 1984; Gudmundsson & Marsal, 1988; Ferguson & Dodson, 2009).

Intuitively, it would be expected that an adequate O_2 supply to the placenta and fetus is essential for their normal development and growth. However, in many placental mammals including humans, the period of organogenesis, and therefore early pregnancy, is primarily supported by nutrient-rich secretions from the uterine glands of the female reproductive tract.

This is known as the histiotrophic phase of fetal development. It is followed by a period when exchange of blood-borne materials between the maternal and fetal circulation can occur, known as the haemotrophic phase (Burton *et al.*, 2002).

Compared to other mammals, conceptus implantation occurs much earlier in humans. The prevailing dogma has been that the earlier implantation, as well as its complete embedment in the endometrium, enable the earlier onset of the haemotrophic exchange, which in turn supports higher development and confers an evolutionary advantage (Burton & Jaunaiux, 2001). Therefore, in the past it was assumed that maternal-fetal circulation is established shortly post-conception, the implication being that it is normally continuous and not subjected to interruption during gestation (see e.g.(Larsen *et al.*, 2001)).

However, in recent years it has become apparent that human pregnancy has a much longer histiotrophic developmental phase than previously thought, with two distinct corresponding periods with regards to O_2 tension within the intrauterine environment. Before the second trimester, there is only limited communication between the invading placental trophoblast and the maternal endometrium as explained above (Hustin & Schaaps, 1987) and the O_2 level in the early fetal-placental unit is low, with historical measurements from rodents and humans uterus reporting pO_2 averages of less than 20 mmHg (Yedwab *et al.*, 1976; Ottosen *et al.*, 2006). Evidence that in normal pregnancy, placental and fetal tissues have the capacity to tolerate such an extreme environment (reviewed by (Schneider, 2009)) indirectly support these measured levels of PO_2. The low O_2 environment may be beneficial to early placental development, as it promotes angiogenesis through transcriptional and post-transcriptional regulation of important growth factors (Charnock-Jones, 2002). The ability of the fetus to

tolerate a considerable level of hypoxia also allows the fetus to survive the inherently dangerous events surrounding natural birth (Mortola, 1999; Singer, 1999).

As described in *Section 1.2.2.4.1*, it is at the interphase between the first and second trimester when the aggregating cells that effectively plug the maternal spiral arteries loosen and the maternal-fetal circulation becomes established (Fig 1.2). As a consequence, the intrauterine O_2 level increases relatively rapidly (Rodesch *et al.*, 1992), to about 55 mmHg at 16 weeks gestation, which enables conversion of uterine gland nutrient-dependence of placental and fetal development to haematrophic nutrition (Burton & Jaunaiux, 2001).

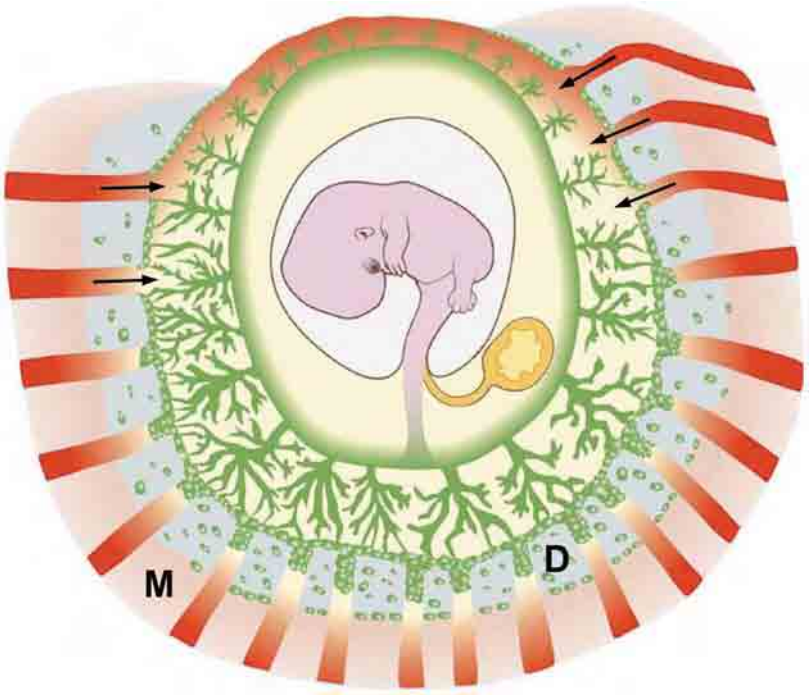


Figure 1.2. Diagrammatic representation of transition from histiotrophic to haemotrophic phase of fetal development.

Around the end of the first trimester, aggregates of invading trophoblast become unplugged from the tips of spiral arteries, resulting in onset of maternal blood flow (arrows) into the intervillous space. D, ducidua; M, myometrium. Taken from (Burton, 2009)

1.2.2.6. Oxidative Stress and Pregnancy

Overwhelming evidence suggest that in the placenta, the rate of reactive oxygen species (ROS) production is proportional to the prevailing O_2 tension (Burton, 2009). Therefore, it is not surprising evidence is accumulating that they may play an important role during the transitional period of establishing of the placental exchange surface structure and the provision of maternal blood and therefore O_2 to the placenta. This has recently been reviewed (Burton, 2009). If ROS generation exceeds the capacity of cellular antioxidant defences, then oxidative stress occurs. Indeed, there is substantial evidence of signs of oxidative stress, including increased xanthine oxidase, catalase and superoxide dismutase (SOD) activities, and heat shock proteins expression, in placental tissues specifically during the transitional period between the histiotrophic and haematrophic phases of normal fetal development (Watson *et al.*, 1997; Watson *et al.*, 1998; Jauniaux *et al.*, 2000).

It has been argued that oxidative stress occurs, not simply because PO_2 rises during the transition phase, but also because perfusion of the placenta is intermittent in this phase due to as yet, incomplete invasion of the maternal endometrium during the transitional period of gestation. This could at least in part be due to spontaneous contraction (vasomotion) of intrauterine vessels such as the spiral arteries (Jauniaux *et al.*, 2006). This is discussed further in Section 1.2.2.7 below. The resultant intermittent placental perfusion may therefore represent a repetitive ischaemia-reperfusion (I/R) insult. As I/R is a potent stimulus for ROS generation via activation of xanthine oxidase and increased electron leakage from the mitochondrial respiratory chain (Hung & Burton, 2006), it is argued that I/R is a physiological

phenomenon in normal pregnancy as it provides an important stimulus for the generation of antioxidant enzymes before the onset of full oxygenation (Jauniaux *et al.*, 2006).

However, excessive oxidative stress or failure to appropriately match antioxidant defence mechanisms to the prevailing level of oxidative stress would be expected to lead to trophoblastic degeneration. As the syncytiotrophoblast is responsible for the synthesis and transport of the essential placental hormones (*see Section 1.2.2.4.2 & 1.2.2.4.3*), pregnancy failure would be expected to rapidly ensue. In fact, there is evidence that the ability of the placenta and fetus to adapt to and cope with oxidative stress is a determinant of the success or otherwise of pregnancy (Jauniaux *et al.*, 2000; Hempstock *et al.*, 2003). Further, if early trophoblastic invasion of the myometrium is defective, as is the case in PE, and therefore the conversion of spiral arteries is incomplete, the arteries retain SMC with their wall and hence vasoreactivity. The resultant pulsatile blood flow to the intervillous space can lead to low grade chronic I/R injury, and the resultant abnormality in oxidant-antioxidant balance is believed to play a significant role in the aetiology of PE (Hung & Burton, 2006).

1.2.2.7. Umbilical Circulation

The umbilical cord is the convoluted bound product of the connecting stalk and the vitelline, enclosed by the amniotic membrane and extends from the fetal side of the placenta to the umbilical area of the fetus (Schoenwolf & Larsen, 2009). The capillaries in the primitive chorionic villi form anastomoses and eventually converge to form the umbilical arteries and vein (Verralls, 2004). As discussed in *Section 1.2.2.1*, fetal development essentially recapitulates the evolution of the adult cardiovascular system. Thus, the umbilical circulation is a specialised set of vasculature, analogous to the adult pulmonary circulation; a normal

umbilical cord contains two umbilical arteries and one umbilical vein. The former carry CO_2 and other waste products towards the exchange surface in the placenta, and the latter conveys O_2 and other nutrients towards the fetus. Accordingly, the measured PO2 in the umbilical vein is consistently higher than that of the umbilical artery: umbilical vein: 28-60 mmHg; umbilical artery: 11-38 mmHg (Rizzo *et al.*, 1996; Lackman *et al.*, 2001; Armstrong & Stenson, 2007; Kotaska *et al.*, 2010).

Traditionally, the umbilical vessels were regarded as a passive connection between maternal and fetal circulation. They are now increasingly seen as important regulating components of placental material exchange; recent data show that they play a role in both normal and abnormal fetal growth (Ferrazzi *et al.*, 2000; Di Naro *et al.*, 2001; Todros *et al.*, 2002). For example, absent end-diastolic flow in the umbilical arteries is associated with fetal hypoxia in severe cases of IUGR (Kingdom & Kaufmann, 1997). Blood pressure in the umbilical circulation is variable, and *in vivo* measurements in sheep umbilical vein and artery gave values in the region of 84-39 mmHg and 51-14 mmHg respectively (Barcroft & Barron, 1945).

Interestingly, it was found that freshly isolated human umbilical arteries and veins both exhibit rhythmic contractions and relaxations *ex vivo* in uncomplicated pregnancies (Garcia-Huidobro *et al.*, 2007). As is discussed in Section 1.5.1 below, vasomotion is accompanied by fluctuations in vessel conductance and downstream O_2 tension. Therefore, it is possible that vasomotion in the umbilical vessels may play a role in the regulation of ROS and I/R-type injury in both normal and complicated pregnancies. Indeed, a recent study has shown that hypoxia induced vasodilatation in human umbilical vein in an endothelium-dependent manner (Mildenberger *et al.*, 2003), strongly suggesting that the umbilical vessels are

sensitive to prevailing PO_2. However, the effects of hypoxia on vasomotion have not been studied (see Chapter 7). Given that vasomotion occurs in both umbilical artery and vein, it is a reasonable hypothesis that factors that influence the pattern of vasomotion in the umbilical blood vessels may have implications for the supply of O_2 and nutrients, as well as for the oxidative status of the placenta and the fetus.

1.3 REGULATION OF THE FETAL-PLACENTAL CIRCULATION

It is generally recognised that as the fetal-placental circulation has negligible or sparse innervation (Reilly & Russell, 1977; Fox & Khong, 1990), the control of its vascular tone is likely not to be under the influence of autonomic nervous system. Control by locally-released vasoactive substances is therefore of great importance in these blood vessels (Boura & Walters, 1991). The control mechanisms of the uterine and umbilical circulations are highly complex and mainly represent the interactive influences of substances derived from the endothelium. These have been reviewed in the past (Chaudhuri & Furuya, 1991; Poston *et al.*, 1995). The discussion below describes the most important of these and, when appropriate, their relevance to the modulation of vascular tone in hypoxia.

1.3.1 Endothelium-Derived Vasoactive Substances

In 1980, Furchgott and Zawadzhi investigated the apparent anomaly that acetylcholine could not always elicit vasodilatation *in vitro* (Furchgott & Zawadzki, 1980). From these seminal experiments it was found that blood vessels isolated with an intact endothelium reliably demonstrated ACh-induced vasodilation, and that removal of endothelial cells (EC) by rubbing the intimal surface of the vessels abolished this response. Further, it was shown that ACh, acting on muscarinic receptors on EC, stimulated release of a substance(s) that caused

vasodilation of vascular smooth muscle. It was then that the importance of EC in vascular biology became apparent and the concept of the EC producing mediators that could influence vascular function was first illustrated. As a consequence, following numerous studies in the 80s, many more mediators of vascular tone were found to act in similar ways to ACh. They also confirmed that the endothelium serves important roles in vascular function in both health and disease. Indeed, it is now well recognised that the EC synthesises and releases a host of heterogeneous vasoactive substances which are important in cardiovascular homeostasis. The synthesis and release processes are controlled by sophisticated systemic and local, often both interactive and complex mechanisms. These are described below.

The vasoactive mediators can be broadly categorised into the *endothelium-derived relaxing factors* and *contracting factors* (EDRF and EDCF respectively). Some examples of the EDRFs are nitric oxide (NO), Prostacyclin (PGI_2), endothelium-derived hyperpolarizing factor (EDHF), while EDCFs include endothelin (ET), angiotensin, thrombin, superoxide anions (Feletou & Vanhoutte, 2009). The purines adenosine triphohphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine may function as EDRFs or EDCFs.

The actions of these factors involve a range of mechanisms via specific receptors and second messenger systems which eventually modulate the contractile status of SMC. Moreover, these endothelium-derived substances can act directly on EC themselves to produce further mediators in an autocrine, as well as directly on the VSM in a paracrine fashion. It is therefore important to realise that the distinction between the two groups of endothelium-derived factors is not unambiguous and an endogenously produced mediator can often have

different effects in different vascular beds. Complex patterns of interactions are at play and no one single mediator may be solely responsible for a given physiological process. The control mechanisms of the uterine and umbilical circulations reflect this highly complex interaction. In the section below, the important endothelium-derived vasoactive substances: NO, EDHF, ET, adenosine and the purine nucleotides are discussed in more detail and indications are given when possible of their known roles in the umbilical cord vessels during hypoxia.

1.3.2. Nitric Oxide

1.3.2.1. Diverse Importance of NO

NO is synthesised and released from a wide variety of cell types such as vascular endothelium, smooth muscles, leukocytes, platelets and neurones. Amongst other important roles such as regulation of the immune system (Bogdan, 2001), apoptosis (Kim *et al.*, 1999), inflammation (Laroux *et al.*, 2000), central nervous system signalling (Garthwaite & Boulton, 1995; Murphy, 2000), most notable is the involvement of NO in the regulation of systemic blood pressure and in local vascular control. This has been reviewed by (Arnal *et al.*, 1999).

In the cardiovascular system, NO has been heavily implicated in inhibition of platelet aggregation, encouraging platelet disaggregation, and inhibition of platelet adhesion; it thereby helps to maintain an anti-atherogenic state of the endothelium under resting, non-pathological conditions (Schafer & Bauersachs, 2008). These beneficial effects are promoted because, *in vivo*, NO is constitutively released from vascular EC by shear stress generated by flow of blood (Rubanyi *et al.*, 1986) In normal human physiology, shear flow ranges from 2-10 dyne/cm^2.

However, the continuous release of NO caused by shear-stress and by pulsatile stretching of the vascular wall is also of primary importance in producing tonic NO-induced vasodilatation (Fleming & Busse, 2003). This serves to promote local blood flow and maintain low peripheral vascular resistance. The exact details of this mechanotransduction mechanism is still unknown, but the four candidate for the putative mechanoreceptor(s) are integrin-matrix interactions, specialised membrane microdomains, ion channels, and G protein (Traub & Berk, 1998). Further details of the signalling pathway is considered in *Section 1.3.2.3* below. In addition, numerous agonists, including ACh, bradykinin, serotonin, adenosine, ADP, ATP, histamine, thrombin, as well as physiological and pathophysiological stimuli such as hypoxia and increased shear stress, can increase NO synthesis and/or release (Sun & Reis, 1992; Xu *et al.*, 1995; Arnal *et al.*, 1999). In the systemic vascular tree, NO production is greatest in resistance vessels and it has been shown that it is involved in the modulation of regional blood flow distribution (Griffith *et al.*, 1987). Indeed, relative to other EDRFs, NO plays a particularly important role in controlling peripheral vascular tone (Vallance *et al.*, 1989). Thus, NO is an essential component of the regulation of blood flow in response to systematically induced changes in blood flow and tissue metabolic demands.

1.3.2.2 NO Synthesis

As indicated above, the release of NO from the endothelium and its effect on vascular tone was first experimentally demonstrated by Furchgott and Zawadzki in 1980. However, its identity as the gaseous molecule NO was not revealed until several years later (Ignarro *et al.*, 1987); the journey of its discovery has often been reviewed (Furchgott & Vanhoutte, 1989; Moncada & Higgs, 1993). The biosynthesis pathway for NO is now well established.

Together with obligate co-substrates NAHPH and molecular oxygen (O_2), the amino acid substrate L-arginine is the natural substrate oxidised for NO synthesis in a reaction catalysed by NO synthase (NOS), and that produces NO and L-citrulline (Stuehr, 1999) (Fig 1.3).

Despite the fact that intracellular L-arginine concentration (~100µM to 2mM in freshly isolated EC) being much higher than the Michaelis-Menten constant (K_M) of isolated eNOS (~2µM), multiple studies have shown that NO-mediated biological effects are dependent on extracellular L-arginine concentration e.g. (Creager *et al.*, 1992). This is referred to the 'Arginine paradox' and although several explanations have been proposed to explain this phenomenon, there is as yet no consensus.

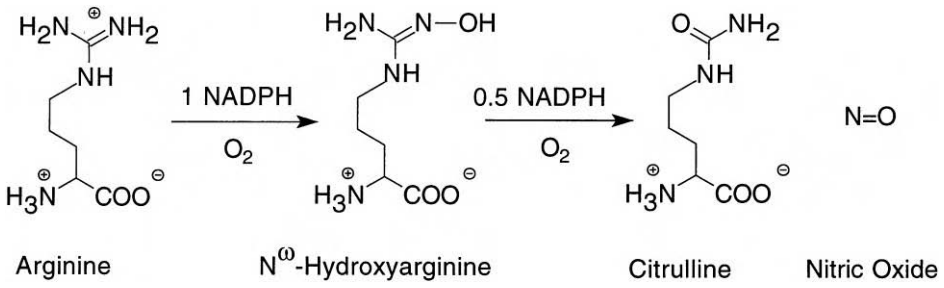


Figure 1.3. NO synthesis from L-arginine.

The hydroxylation of L-arginine forms N-hydroxy-L-rginine as an intermediate, which in the second step is concerted to form L-citrulline and NO. Adapted from (Stuehr, 1999).

Three NOS isoforms have been described: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS. For NOS catalysis several redox-active cofactors are also required, including tetrahydrobiopterin (BH4), heme, (FMD) flavin mononucleotide and FAD (flacin adenone dinucleotide) (Fleming & Busse, 2003). The three isoforms of NOS are derived

from separate genes and are regulated by diverse signalling pathways (Griffith & Stuehr, 1995). The isoforms nNOS and eNOS are constitutively expressed in cells and are primarily regulated by Ca^{2+} influx and subsequent binding of Ca^{2+} with calmodulin (CaM) (Stuehr, 1999). By contrast, iNOS expression is induced by inflammatory cytokines (eg. IL1, IL2, TNF-, LPS). The resultant iNOS is fully active at normal $[Ca^{2+}]_i$ levels and is therefore considered Ca^{2+} -independent (Stuehr, 1999).

1.3.2.3. Molecular Regulation of eNOS

Endothelial NO is synthesised by eNOS, which is characterised by a constitutive activity responsible for maintaining low peripheral vascular resistance (see above). It also has a restricted pattern of expression, which includes myocytes, bronchiolar epithelium, endothelium, platelets and the placenta (Vallance & Hingorani, 1999). The regulation of eNOS is complex, and involves a range of transcriptional and post-transcriptional mechanisms. The present thesis is primarily concerned with the effect of acute events such as hypoxia on vascular function, so only post-transcriptional mechanisms are discussed below.

1.3.2.3.1. Ca^{2+}

An increase in $[Ca^{2+}]_i$ is generally a key event in the activation of eNOS, as it permits the binding of Ca^{2+}-CaM to eNOS, leading to the dissociation of the inhibitory eNOS-caveolin complex and to an activated CaM-eNOS complex that is capable of NO generation (Michel *et al.*, 1997). Agonists (such as those mentioned above) can activate eNOS by increasing $[Ca^{2+}]_i$.

However, eNOS production can also be activated without a sustained increase in $[Ca^{2+}]_i$. Indeed, it has been shown that shear stress-induced NO formation can be mediated by such a

pathway (Fleming *et al.*, 1998). In this so called 'Ca^{2+}-independent pathway', shear stress increases eNOS activity via the activation of phosphatidylinositol 3 kinase (PI_3K) and the subsequent activation of serine kinase Akt and protein kinase A (PKA), which lead to the phosphorylation of eNOS (see below). Although this process is often referred to as a 'Ca^{2+}-independent pathway', a basal level of $[Ca^{2+}]_i$ is essential, as chelating of $[Ca^{2+}]_i$ abolished the shear stress-induced increase in eNOS activity (Fleming & Busse, 2003).

1.3.2.3.2. Phosphorylation

Phosphorylation is an important aspect of the regulation of eNOS activity. There are many putative phosphorylation sites on eNOS, but most is known about the functional consequences of phosphorylation of a serine residue of eNOS (human sequence Ser^{1177}) in the reductase domain and a threonine residue (human sequence Thr^{495}) within the CaM-binding domain (Fleming, 2010). In un-stimulated, cultured ECs, for example, Ser^{1177} is normally not phosphorylated. As indicated in *Section 1.3.2.3.1*, fluid shear stress can quickly *activate* eNOS via the activation of PI_3K, Akt and PKA. On the other hand, Thr^{495} is constitutively phosphorylated (by PKC), and phosphorylation at this site causes a decrease in enzyme activity (Michell *et al.*, 2001). It has been suggested that this results from conformational changes within eNOS, which interferes with the binding of CaM to the CaM-binding domain. There are numerous sites on the eNOS enzyme that can be phosphorylated in response to a wide variety of humoral, mechanical and pharmacological stimuli, and the kinases and phosphatises involved in the activation of eNOS vary depending on the nature of the stimuli applied. These have been extensively reviewed by (Mount *et al.*, 2007; Fleming, 2010).

1.3.2.3.4. Intracellular Translocation and Associated Proteins

The consequences of enzyme activation, or activation of the signal transduction pathway can be determined to a large extent by following the intracellular localisation of the signalling complex. Although evidence suggests that distinct intracellular pools of eNOS are present which respond preferentially to varying physiological and agonist stiumulation, there is little consensus on these issues. The only common consent at present is that stimulation of ECs induces eNOS translocation between different cellular compartments, namely, the plasma membrane, the plasmalemmal caveolae, the Golgi apparatus, and possibly the nucleus (Fleming & Busse, 2003). Within these different compartments, eNOS are co-localised with a host of proteins, together forming what is known as the 'eNOS signalling complex'. Association with calmodulin (CaM) and caveolin, in particular, has profound influence on the intracellular localisation and activity of eNOS (Fleming, 2010). Other eNOS-associated proteins include Hsp90, PECAM-1, Gab1, soluble guanylyl cyclase and hynamin. Concepts in relation to the regulation of eNOS activity by alterations in the localisation and association of protein mediators are constantly changing and are outside the remit of this project.

1.3.2.4. Action of NO

The non-polar NO diffuses freely across the cell membrane and acts on underlying VSM, activating its major receptor, soluble guanylate cyclase, which increases intracellular cyclic GMP concentration (Vallance & Hingorani, 1999). The increase in cGMP levels may reduce vascular tone by a number of mechanisms, including lowering $[Ca^{2+}]_i$. This is in turn achieved by a number of mechanisms. The understanding of these is ever-evolving, but they include inhibition of voltage-operated Ca^{2+} channels (VGCC) and activation of large conductance potassium (BK) channels (Francis *et al.*, 2010).

The cGMP can also reduce vascular tone via phosphorylation of heat shock protein 20 (Hsp20), which binds thin actin filaments and inhibits cross-bridge cycling (Rembold *et al.*, 2000). Also, cGMP can activate cGMP-dependent kinase (PKG), so leading to phosphorylation of myosin light chain kinase (MLCK) and therefore to its inactivation (Nishikawa *et al.*, 1984; Hathaway *et al.*, 1985), thereby causing relaxation of vascular smooth muscle. Of the mechanisms by which NO induces VSM relaxation, it is likely that no single pathway acts exclusively or independently, although accumulating evidence suggests that the relative importance of the various pathways vary phenotypically and regionally. The cGMP-dependent cellular responses were reviewed in detail (Francis *et al.*, 2010).

1.3.2.5. Physiological Roles of NO in the Cardiovascular System

The importance of NO in the physiological control of vascular function has been demonstrated by studies in which NO production was nullified: For example, specific inhibition of NO production by L-arginine analogues such as N^G-monomethyl-L-arginine (L-NMMA), or by genetic knock-out, showed a marked increase in central blood pressure in several species. These respective approaches have been recently reviewed (Moncada & Higgs, 2006; Tsutsui *et al.*, 2009). NO can also reciprocally attenuate action of many vasoconstrictors including noradrenaline, angiotensin II, and vasopressin and can inhibit the production and action of the most potent endogenous vasoconstrictor, ET (Boulanger & Luscher, 1990; Kourembanas *et al.*, 1993; Ahlborg & Lundberg, 1997). The interaction between NO and ET is discussed in *Section 1.3.5.2*. It has long been suggested that a healthy balance between the production of the various vasoactive substances is one of the bases on which the vascular system maintains homeostasis e.g.(Vanhoutte, 2000). Certainly, a decrease in NO production or bioavailability and endothelial dysfunction have been

implicated in the pathophysiology of many cardiovascular diseases, such as atherosclerosis, stroke, kidney disease, pulmonary hypertension, essential hypertension and preeclampsia (Bian *et al.*, 2008).

Intuitively, it might be expected that NO would be of an even greater importance in the control of vascular tone in the umbilical circulation, since the umbilical arteries and veins have spare or no innervations (Reilly & Russell, 1977; Fox & Khong, 1990), and thus rely on regulation by autocoids (Boura & Walters, 1991). Certainly, HUVECs have been commonly used as EC model in a wide range of experimental assays, including the study of angiogenesis, inflammatory cellular communication, thrombosis and control of vascular tone. Moreover, isolated human umbilical cord vein endothelial cells (HUVEC) have a functional NO system (Tsukahara *et al.*, 1993). Indeed, by using direct NO measurement with an amperometric NO-sensor, the authors showed that agonist-induced release of NO from HUVEC are sustained and displayed an oscillatory pattern (Tsukahara *et al.*, 1993). Thus, NO may represent an important step in vasomotion of the human umbilical vessels *in vivo*. This possibility has so far not been addressed experimentally. Further, although the mechanisms that determine the regulation of the fetoplacental circulation, including the umbilical arteries and vein, have so far received relatively little attention, in a study of perfused intact human umbilical artery and vein, it was shown by using exogenous NO and PGI_2 that NO is a much more potent vasodilator than PGI_2 *ex vivo* (Chaudhuri *et al.*, 1993). This suggests that NO may be more important than PGI_2 for maintenance of low vascular tone in fetoplacental vessels.

1.3.2.6. Hypoxia and NO

Interestingly, a reversible phenomenon similar to the vasoconstriction that occurs in the pulmonary circulation in response to hypoxia, hypoxic pulmonary vasoconstriction (HPV), occurs in the placenta: this is known as hypoxic fetoplacental vasoconstriction (HFPV). *In vitro* data showed that, in freshly isolated human placental cotyledon (the natural extension to the umbilical circulation), vascular resistance increased with hypoxia proportionally indicating vasoconstriction (Ramasubramanian *et al.*, 2006). Further, it was found that L-NAME (a L-arginine analogue that acts as a NO synthase inhibitor) was able to inhibit this vasoconstrictor response to hypoxia (Byrne *et al.*, 1997), suggesting that NO mediates HFPV. It has been hypothesised that HFPV is responsible for matching fetal to maternal blood flow within the placenta (Hampl & Jakoubek, 2009). This is supported by the finding that HFPV only occurs in small, but not large (>1mm), placental blood vessels (Hampl *et al.*, 2002), which suggests that, whilst embryologically the uteroplacental circulation as a whole is of the same origin, their mechanism of vascular control may well be region-specific.

Indeed, in a more recent study, it was shown that a over a range of physiologically relevant PO_2 (5-104 mmHg), hypoxia caused a graded *vasodilatation* in freshly isolated human umbilical vein (Mildenberger *et al.*, 2003). However, this was reversed to graded *vasoconstriction* by L-NAME or by removal of the endothelium.

Taken together, it would seem that in umbilical vein, a reduction in NO availability promotes HFPV, and that endothelial dysfunction leads to impairment of hypoxia-induced vasodilatation.

1.3.2.7. NO and Reactive Oxygen Species (ROS)

In EC, O_2 readily accepts unpaired electrons to produce superoxide (O_2^-), which is a main source of a range of deleterious free radicals and ROS. NO produced from eNOS or iNOS can deactivate free O_2^- and hence exert protective actions. Indeed, ROS are the major determinants of NO breakdown (Kelm, 1999). The product of this reaction, peroxynitrite (ONOO-), is itself a further source of NO, but it also can become a toxic radical when its levels exceed endogenous antioxidant and scavenging capacity (Henderson, 2001).

In situations when there is an inadequate supply of the substrate L-arginine, or of the cofactor BH_4 (dietary), NO production by eNOS becomes uncoupled. In this state, electron flow through the enzyme dimer results in reduction of O_2 at the prosthetic heme site rather than normal formation of NO, generating a mixture of NO and O_2^- (Stuehr *et al.*, 2001). If the oxidative state of the cell is already increased, for example by increased NADPH oxidase expression or activity, this can itself prevent BH_4 from being regenerated, further exacerbating the level of oxidative stress (Griendling *et al.*, 2000). This therefore implies a positive feedback of increasing oxidative stress, unless compensated by the upregulation of endogenous antioxidants such as superoxide dismutase (Henderson, 2001).

Thus, the production and bioavailability of NO in the placenta and fetus might be expected to be highly dependent on the prevalent oxidative status, and this in turn may be dependent on the pattern of vasomotion in the umbilical blood vessels (see *Section 1.2.2.7*). As discussed in *Section 1.2.2.6*, the balance between levels of oxidative stress and anti-oxidative defence mechanisms is an important determinant of the success or otherwise of pregnancy, and overwhelming evidence now demonstrate that ROS plays an important role in the pathophysiology of several pregnancy-related disorders (Steinert *et al.*, 2009). In particular,

the potential role of ROS in leading to endothelial dysfunction in the aetiology of PE has received much research interest. It has been shown that shear-stress mediated vasodilator responses are blunted in the systemic circulation of women suffering from PE when compared to women in the control group. Some older studies concluded that the defect was mainly NO-dependent e.g.(Cockell & Poston, 1997).

1.3.3. PROSTAGLANDINS

1.3.3.1. Synthesis and Breakdown

Prostaglandins (PG) are products of arachidonic acid (AA), which is formed from action of phospholipases (PLC and PLA_2) on phospholipids of the cell plasma membrane (Fig 1.4). Cyclooxygenases (COX-1 and COX-2) act on AA to produce PG endoperoxides such as PGH_2, which, depending on the cell type, leads to further synthesis of PG subtypes (e.g. PGD_2, PGE_2 and PGF_2), prostacyclin (PGI_2), and thromboxane (TXA_2) via PGI_2 synthase and thromboxane synthase (Moncada & Vane, 1978). Of these, the effect of PGI_2 on vascular function has been most extensively studied. PGI_2 synthase acts on PGH_2 to produce PGI_2 and this is the most abundant form of PG synthesised in the endothelium (Valdes *et al.*, 2009). VSM does not produce PGI_2 under normal conditions (MacIntyre *et al.*, 1978).

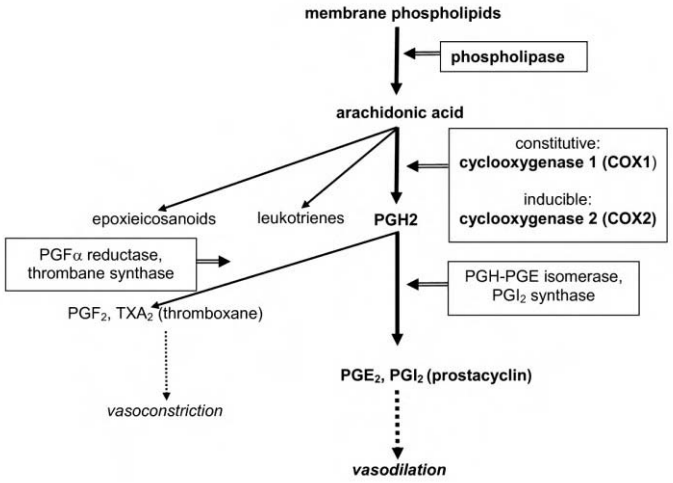


Figure 1.4. Production of prostonoids from membrane phospholipids. Taken from (Valdes *et al.*, 2009)

PGI_2 is rapidly catabolised to a stable metabolite 6-keto-$PGF_{1\alpha}$ in blood: at 37ºC its half life is only 2-3 minutes. Unlike other prostanoids, it is not metabolised in the pulmonary circulation. This is an important difference from other vasodilatory PGs, such as PGE_1 and PGE_2. In fact, PGI_2 is equipotent whether given intra-arterially or intravenously (Moncada & Vane, 1978).

1.3.3.2. Physiological Role of Prostanoids

PGI_2 is constitutively released from the endothelium and its effects are mediated by cell surface PGI_2 (IP) receptors and/or intracellular peroxisome proliferator-activated receptors (PPAR)β (Mitchell *et al.*, 2008). PGI_2 is a potent vasodilator. In studies in whole animal and human, *in vivo* in single organs, and in isolated blood vessels, infusion of PGI_2 or AA (which produces mainly PGI_2 in isolated vascular tissue) caused a drop in blood pressure and vasodilatation, which was abolished by selective PGI_2 synthase inhibitors, such as 15-HPAA (Moncada & Vane, 1978). On the other hand, TXA_2 is a vasoconstrictor, and this contrast in their opposing actions on vascular tone may be important in maintaining cardiovascular

homeostasis. As discussed in the next section, this vascular control by prostanoids is also important in the uteroplacental unit during pregnancy (Valdes *et al.*, 2009).

The PGI_2 system is widely expressed in the mammalian systems. In a study of normal human umbilical arteries *in vitro* it was found that the cyclooxygenase inhibitor, indomethacin, enhanced agonist-induced contractions to serotonin and bradykinin, whereas nitro-L-arginine, a selective inhibitor of endogenous NO biosynthesis, did not (Klockenbusch *et al.*, 1992). This suggests that PGI_2 seemed to a more important EDRF than NO in regulating umbilical arteries. This contrasts to an extent with the findings of Chaudhuri et al (1993) which, as discussed *Section 1.3.2.5*, indicated that NO plays a larger role than PGI_2 in maintaining tonic dilatation in umbilical and placental circulation. However, it has been shown that the vascular effects of PGI_2 and NO are additive rather than synergistic (Mitchell *et al.*, 2008).

During pregnancy, up-regulation of PGI_2 synthesis contributes to the maintenance of a low resistance placental circulation (Goodman *et al.*, 1982). Indeed, pulsatile stress and constant shear stress caused PGI_2 release from HUVEC *in vitro* (Frangos *et al.*, 1985). Indeed, hypoxia-induced dilatation in umbilical vein was sensitive to cyclogenase inhibition by indomethacine (Mildenberger *et al.*, 2004b). In the human umbilical artery, hypoxia also caused significantly increased release of PGI_2 (Bjoro *et al.*, 1987), which in turn caused a dose-dependent relaxation of isolated artery strips at concentrations less than 10^{-6} M (Pomerantz *et al.*, 1978). This raises the possibility that tonic release of PGI_2 may contribute to a tonic vasodilatory influence on umbilical vessels in normal pregnancy, which is accentuated in hypoxia. Indeed, it was found that PGI_2 activity was significantly lower in umbilical and placental vessels in pre-eclamptic women than from women with

uncomplicated pregnancy (Remuzzi *et al.*, 1980). Unfortunately, like so many of the experiments carried out in the active period of PGI_2 research in 70s and 80s, the experiments were carried out at in organ baths equilibrated with 95% O_2, which give a much higher *P*O2 than the appropriate physiological level of PO_2 ~30 mmHg. Therefore, the results must be interpreted with a suitable degree of caution.

As indicated above, TXA_2 is the major vasodilator PG. Evidence is gathering which suggest that an imbalance in prostanoids control may contribute to PE and other pregnancy-related disorders. Multiple studies have consistently found that in PE, impaired PGI_2 production is associated with an increase in TXA_2 production in placental tissues e.g. (Walsh, 1985). Further, a raised TXA_2/PGI_2 ratio excreted in urine is associated with PE where uteroplacental perfusion is impaired (Mills *et al.*, 1999), suggesting TXA_2 contributed to vasoconstriction, thus impairing O_2 delivery. More recently, by using trophoblasts isolated from normal and PE placentas, it was found that exposure to AA caused a much greater release of TX metabolites than 6-keto-$PGF_{1\alpha}$ in PE tissue, suggesting the increased TX production may play a role in increased placental contractility in PE (Zhao *et al.*, 2008).

It has been suggested that this imbalance between PGI_2 and TXA_2 production was caused by oxidative stress, which, as discussed in *Section 1.2.2*.6, is a feature of normal pregnancies, but especially prominent in pregnancies complicated by PE (Walsh, 2004). Oxidative stress may cause this imbalance by the increased level of lipid peroxides, which stimulates COX activity but at the same time inhibits PGI_2 synthase, therefore increasing TXA_2 synthesis and inhibiting PGI_2 synthesis. Indeed, the TXA_2 / PGI_2 ratio, as well as lipid peroxides, are higher

in the placental exchange surface in PE pregnancies than in those of normal pregnancies (Valdes *et al.*, 2009).

1.3.4. EDHF

1.3.4.1. Candidates for EDHF

In addition to NO and PGI_2, endothelial control of vascular tone also includes the production of another EDRF whose action involves the hyperpolarisation of the underlying VSM. Originally, this non-NO, non-PGI_2 mediated relaxation was thought to be due to an endothelium-derived hyperpolarising factor(s) that diffuses across the myo-endothelial space to activate K^+ channels (e.g. (Taylor *et al.*, 1988; Chen & Suzuki, 1989)). When investigated in the presence of inhibitors of NO synthases and COXs, evidence has been obtained indicating that the putative EDRF may be K^+, an endogenous vasodilator typically released in hyperaemia in skeletal muscle and in active hyperaemia of the brain; or an epoxyeicosatrienoic acid (EET), which is a metabolite from the endothelial cytochrome P450 pathway generated from AA. These and other possibilities, such as EDHF being hydrogen peroxide (H_2O_2), are reviewed by (Feletou & Vanhoutte, 2006).

More recent evidence also indicates a role for gap junction coupling the electrical activity (hyperpolarisation) from EC to VSM. In this pathway, small and intermediate conductance Ca^{2+}-activated K^+ channels (SK_{Ca}, IK_{Ca} respectively) are activated in the EC by a rise in $[Ca^{2+}]_i$, so hyperpolarising these cells (Sankaranarayanan *et al.*, 2009). This hyperpolarisation then spreads to the VSM via gap junctions. Thus, this pathway does not involve the release of a factor(s) from EC. The VSM hyperpolarisation leads to lowering of the open probability of voltage-operated Ca^{2+} channels (VOCC), a reduction in the turnover rate of intracellular

phosphatidylinositides, or less VOCC activation of sarcoplasmic reticulum, thus decreasing $[Ca^{2+}]_i$ in the VSM (Nelson *et al.*, 1990; del Valle-Rodriguez *et al.*, 2003). One study showed that in ACh-induced endothelium-dependent responses, the release of NO is mediated by the M2-subtype, whereas the release of EDHF is mediated by the M1-subtype (Komori & Vanhoutte, 1990).

Interestingly, "EDHF"-induced hyperpolarisation of EC in turn favours further Ca^{2+} entry into these cells by increasing the driving force for this ion via non-selective cation ion channels such as Transient Receptor Potential (TRP) ion channels (Busse *et al.*, 1988). As discussed above, $[Ca^{2+}]_i$ increase can activate eNOS. Therefore, the EDHF pathway also favours the agonist- and shear stress- evoked synthesis of NO (Sheng & Braun, 2007).

1.3.4.2. Physiological Role of EDHF

Apart from specialised vascular beds such as the coronary and renal circulation in which EDHF has been shown to play a major role even in the conduit arteries (Busse *et al.*, 2002), EDHF apparently contributes more significantly to vasodilatation as the vessel size decreases, and its role in mediating vasodilatation is therefore most prominent in resistance vessels (Busse *et al.*, 2002). Another evidence which indicates the apparent importance of EDHF, specifically gap junction, in endothelium-dependent vasodilatation in small, resistance vessels is an increasing number of myo-endothelial gap junctions as the vessel diameter becomes smaller (Sandow & Hill, 2000). In multiple, transgenic mice models, in which expression of a number of the K^+ channels thought to be responsible for the EDHF response were individually or collectively abolished, an increase in arterial blood pressure and left ventricle hypertrophy were found (Feletou & Vanhoutte, 2009). These findings suggest the

fundamental roles these K^+ channels may play in the EDRF-mediated hyperpolarisation and consequent vascular response.

It is important to note that EDHF has not consistently been shown to mediate shear stress- or agonist- induced vasodilation, the fundamental mechanisms underlying the regulation of basal blood flow and distribution responses to local stimuli e.g. (Passauer *et al.*, 2003). Rather, it is thought that the EDHF component of vasodilatation becomes especially important when the NO- and prostanoids- dependent mechanisms is depressed under pathological situations including atherosclerosis, hypercholesterolemia and ischemia (Hecker, 2000). Indeed, EDHF-mediated responses are clearly altered in various pathological conditions such as hypertension (Fujii *et al.*, 1992; Mori *et al.*, 2006), atherosclerosis (Urakami-Harasawa *et al.*, 1997) and PE (Kenny *et al.*, 2002). It has been shown that this is partly because EDHF is less sensitive to degradation by ROS generated by oxidative stress (Csanyi *et al.*, 2006).

Evidence also suggests that in normal pregnancy EDHF function is up-regulated, and this up-regulation is impaired in PE-complicated pregnancy (Kenny *et al.*, 2002). As there is evidence to suggest that EDHF may be H_2O_2 (Feletou & Vanhoutte, 2006), it is possible that the apparent lack of anti-oxidative mechanism in PE (e.g. catalase and SOD, see *Section 1.2.2.6*), which normally reduces superoxide (O_2^-) to H_2O_2, is responsible for this phenomenon.

1.3.5. ENDOTHELIN

1.3.5.1. ET Structure

Endothelin (ET) is an endothelium-derived, 21-amino acid residues peptide, first purified and identified in 1988 (Yanagisawa *et al.*, 1988). Shortly after its discovery, two other isoforms were found by screening for the gene coding for the peptide; due to the similarity of the peptide sequences, the isoforms were named ET-1, ET-2 and ET-3. They are endogenously and differentially expressed in various tissues (Rubanyi & Polokoff, 1994). All forms of the synthetically produced ETs produce vasoconstrictor and pressor responses (Inoue *et al.*, 1989).

1.3.5.2. ET Production

ET is generated as the precursor preproendothelin, which is converted first to big endothelin by furin-like endopeptidase and then further cleaved by a family of ET-converting enzymes, into ET which can be released by the EC (Kedzierski & Yanagisawa, 2001) (Fig 1.5).

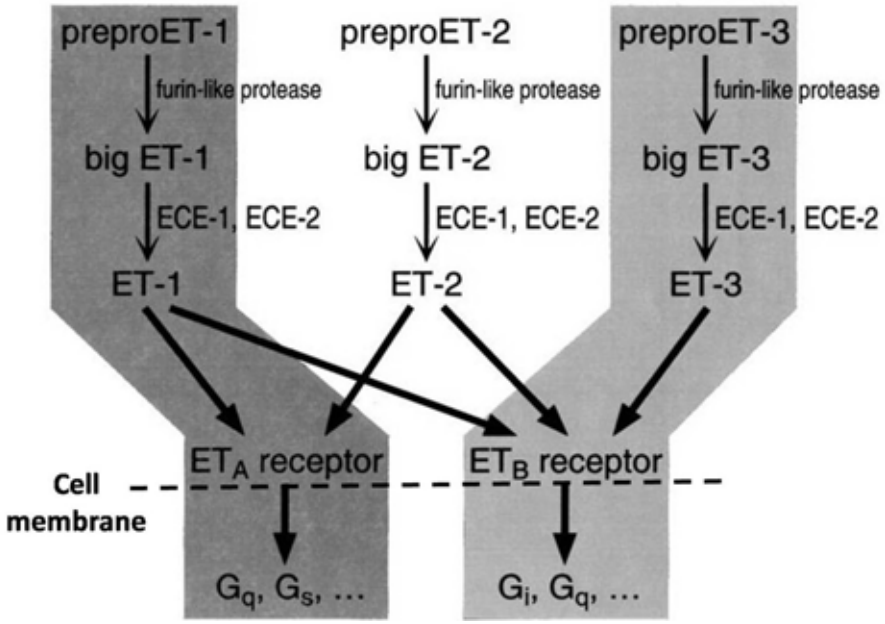


Figure 1.5. The Endothelin pathway.

Preproendothelin, is converted first to big ET by furin-like endopeptidase and then cleaved by a family of ET-converting enzymes, into three physiologically active ETs. The two common mammalian ET receptors, ETA and/or ETB receptors, are linked to various G-proteins. Adapted from (Kedzierski & Yanagisawa, 2001).

ET-1 is released in a dual secretory pathway: on the one hand, it is continuously released under the constitutive pathway, which acts on underlying VSM to maintain basal vascular tone. On the other hand, under physiological or pathophysiological situations, ET-1 is also released from EC-specific storage granules the Weibel-Palade bodies (Russell *et al.*, 1998). The functional consequences of the different release pathways are discussed below. ET-2 and ET-3 are also expressed in human tissues, with ET-3 displaying a preferential affinity for specific subtypes of ET receptors, but these ET isoforms are less well characterised (Davenport, 2002).

Much of the regulation of ET production occurs at pre-transcriptional level. In EC, ET-1 mRNA is initially upregulated, then down-regulated by shear stress and pulsatile stretching, whereas hypoxia upregulates ET-1 mRNA expression. On the other hand, NO and PGI_2 decrease ET-1 mRNA level in EC. ET also acts on ET receptors (ET_B; see below) on EC, which is linked to the production of NO (Fig 1.6). Conversely, exogenous NO or the liberation of NO within EC inhibits ET-converting enzyme and thereby dampens down ET production (Vanhoutte, 2000). Hence, a negative feedback mechanism is in place to keep the relative production of two major opposing endothelium-derived vasoactive substances in check, a balance that is normally skewed towards NO production. Interestingly, ET_A, the ET-receptor that is mainly responsible for the vasoconstrictor effect of ET (see *Section 1.3.5.4*), expression is upregulated by NO in VSM. These complex interactions of local ET regulation are reviewed in (Kedzierski & Yanagisawa, 2001).

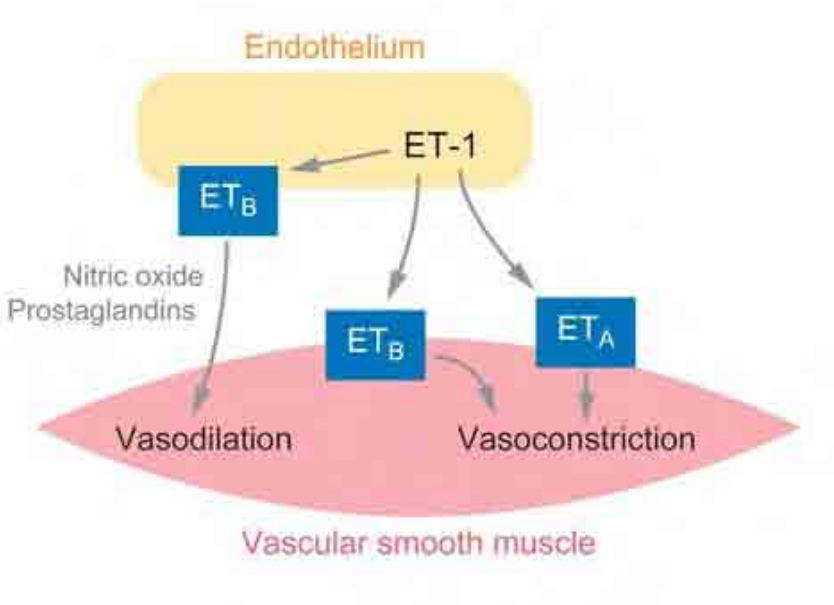


Figure 1.6. ET control of vascular contractility. Taken from (Schneider *et al.*, 2007).

1.3.5.3. ET Receptors and Downstream Regulation

By cloning complementary DNA sequences, three receptor subtypes have been found: ET_A, ET_B and ET_C receptors (Arai *et al.*, 1990; Sakurai *et al.*, 1990), although to date no mammalian form of ET_C receptor have been identified (Schneider *et al.*, 2007). The receptors have differential regional expression and affinity for the three ET isoforms differs; thus the ET control system is complex. The ET receptor signal transduction mechanisms have been reviewed (Douglas & Ohlstein, 1997). Although the precise intracellular mechanism responsible for the action of ET is still under active investigation, it is commonly accepted that the two common mammalian subtypes of ET receptors activate phospholipase C via G-proteins, releasing IP_3 and thereby increasing $[Ca]_i$ by both intra- and extracellular source mobilization. However, a study on transfected ovary cells shows that ET_A and ET_B differ with regards to their effect on cellular cAMP concentration: ET_A receptor activation leads to cAMP accumulation, whereas ET_B receptor activation leads to a decrease in cAMP levels

(Aramori & Nakanishi, 1992). It is as yet unclear whether this is the case in vascular cells (Masaki, 2004), but as mentioned above, the phosphorylation status of MLCK in VSM is affected by cellular cAMP concentration. Therefore, the direction of cellular response elicited by ET receptor activation in a particular blood vessel may be determined by the ET receptors subtype present and the relative affinities of the ET isoform to each receptor (Kedzierski & Yanagisawa, 2001).

This situation is further complicated by the fact that activation of ET_B receptor on EC stimulates the production of NO and prostaglandins (Kedzierski & Yanagisawa, 2001), which, as described above, have vasodilator effects on VSM. It is therefore not surprising that results from studies on the effect of ET receptor antagonists on the vasculature lack consistency. Indeed, the predominant influence of endogenous ET on vascular tone and basal blood pressure remains somewhat contentious.

1.3.5.4. Physiological Roles of ET

The ET system has a diverse expression pattern in mammals, in both vascular and nonvascular structures, including the brain, kidneys, lungs, heart and blood vessels. ET is the most ubiquitously expressed and potent constrictor of human vessels, and has an unusually long duration of action (Yanagisawa *et al.*, 1988). It is well established that the physiological plasma concentration of ET is generally much lower than the pharmacological threshold (~1pM for ET-1; even lower for ET-2 and -3) (Battistini *et al.*, 1993), suggesting that ETs function as local autocrine and paracrine mediators and not as regulatory hormones under normal conditions.

As indicated above, ET-1 is the principle isoform in the human cardiovascular system and 80% of the ET-1 released is from the basal side of EC (Howard *et al.*, 1992; Wagner *et al.*, 1992). When released, the ET-1 acts on ET_A on vascular VSM, and on ET_B found on both vascular VSM and EC. The vasoconstriction is mediated mainly through action on the VSM ET_A receptor, but also via the VSM ET_B receptor, whilst interaction with the endothelial ET_B receptor induces the release of vasodilators (see *Section 1.3.5.3*; (Masaki, 2004)). The vascular action of ET is complex as it depends on the relative contribution from the ET receptor types present, which varies between different vascular beds. Basal release of ET may therefore contribute to the maintenance of normal vascular tone, without appearing in plasma, and under conditions that stimulate further release of ET, it could produce further vasoconstriction. Stimuli that increase release of ET include hypoxia and noradrenaline (Yanagisawa *et al.*, 1988), suggesting that ET may modulate vascular tone under conditions in which there is hypoxia and / or vasoconstriction. The plasma ET level is also raised under pathological conditions such as pulmonary arterial hypertension, essential hypertension, chronic heart failure and subsequent to hemorrhage (Schneider *et al.*, 2007).

Many studies on humans and laboratory mammals have shown that administration of an ET_A antagonist or ET_B antagonist, or a combination of these, caused a lowering of resting blood pressure, that was not due to other regulatory mechanisms such as the angiotensin and sympathetic systems (McMahon *et al.*, 1991; Haynes & Webb, 1994; Veniant *et al.*, 1994; Haynes *et al.*, 1996; Wenzel *et al.*, 1998). This implies a net vasoconstrictor effect of ET released from the basolateral surface of EC acting on VSM receptors. ET receptor antagonists have also been used clinically to treat patients with hypertension (Krum *et al.*, 1998), in accord with the raised levels of ET in hypertension (see above). However, ET receptor

antagonists are now restricted mainly to the treatment of pulmonary arterial hypertension, as other traditional anti-hypertensive drugs are have lower toxicology profiles (especially in the liver) and are better tolerated.

As indicated above, vasodilator influences predominate in the umbilical circulation under normal circumstances. However, pregnancy-related diseases such as PE and IUGR are associated with decreased production of NO and PGI_2. Moreover, as discussed above, NO inhibits the production and action of ET. Therefore, not surprisingly these diseases are also associated with higher plasma level of vasoconstrictors such as ET-1 (Taylor *et al.*, 1990; Ihara *et al.*, 1991; Nisell *et al.*, 1991). The data for the umbilical circulation is limited, but it has been shown that ET contracts both uterine and umbilical arteries via an action on ET receptor on VSM (Bodelsson *et al.*, 1992; Stjernquist *et al.*, 1995). As discussed in *Section 1.3.2.6*, human umbilical vein displays vasodilatation in response to hypoxia, which was reversed by the removal of endothelium i.e. vasoconstriction happens at low PO_2. Interestingly, in these endothelium-denuded vessel, blockade of ET_B receptor, but not ET_A receptor, restored hypoxia-induced vasodilatation (Mildenberger *et al.*, 2003), suggesting a role of ET_B receptor in mediating vasoconstriction in hypoxia. It is reasonable to hypothesise that these changes lead to elevated sensitivity to vasoconstrictor influences, to impaired blood flow in the umbilical circulation and may play a part in the aetiology of PE. However, as solid evidence is lacking, more studies are needed to improve our understanding of the role of ET in the umbilical circulation.

1.3.6. PURINERGIC REGULATION

1.3.6.1. Purinergic Substances are Vasoactive

For many years ATP was strictly regarded as an intracellular molecule, the existence of it in the extracellular space being accounted for purely by uncontrolled release from ageing and necrotic tissue. In 1972, Burnstock provided the first direct evidence that purines were released as a transmitter from nerve fibres under physiological conditions and caused smooth muscle contraction (Burnstock *et al.*, 1972). For a review of the evidence at that time see (Burnstock, 1972). It is now well established that nucleotides and nucleosides are important extracellular signalling molecules in both neuronal and non-neuronal tissues (Ralevic & Burnstock, 1991, 1998).

Early data showed that ATP, ADP and AMP can all cause vasodilatation of dog femoral arteries, and that the responses to ATP and ADP were endothelium-dependent (De Mey & Vanhoutte, 1981). Since then, it has been shown that the endothelium-dependent dilatation to ATP and ADP is NO-dependent and that ATP can also cause the release of PGI_2 from endothelium (Olsson & Pearson, 1990). A recent study has shown that the nucleotides ATP, UTP and ADP can induce eNOS phosphorylation (da Silva *et al.*, 2009). In addition, it has been shown that adenosine, which is formed as a breakdown product of the nucleotides, can cause NO release from EC, including those of umbilical vein origin (Li *et al.*, 1995; Sobrevia *et al.*, 1997; Ray & Marshall, 2006). Interestingly, both ATP and adenosine are implicated in the systemic vasodilator response to systemic and local hypoxia (Burnstock & Kennedy, 1986; Buck, 2004; Ellsworth, 2004; Adair, 2005; Ray & Marshall, 2006). Indeed, *purinergic signalling* is now a major widely accepted principle in a wide range of physiological conditions. The evolutionarily conserved actions of ATP and its metabolites as paracrine /

autocrine signalling molecules contribute to the regulation of basal level of activation for signal transduction pathways and mediate a range of responses including tissue blood flow (Corriden & Insel, 2010).

1.3.6.2. Purinergic Modulation of Vascular Tone

The detailed characterisation and downstream cellular signalling pathways of purinoceptor subtypes is discussed in later sections (*1.3.6.3 & 1.3.6.4*). Briefly, these consist of P2 receptors for ATP, ADP and UTP, and P1 receptors for adenosine. P2 receptors have been subdivided into P2X ligand-gated ion channel receptors and P2Y G-protein coupled receptor families. Presently the most convincing model of purinergic control of vascular tone is the one proposed in 1990 (Burnstock, 1990). This model argues that ATP modulates vascular tone via two different mechanisms. On the one hand, ATP is co-released with noradrenaline from sympathetic perivascular nerves and acts on P2X receptors located on vascular smooth muscle to cause vasoconstriction. On the other hand, ATP released from EC and red blood cells (RBC) can act on P2Y receptors located on EC to elicit release of NO and PGs, which subsequently cause dilatation of the underlying VSM. However, it should be noted that ATP-induced NO-*in*dependent vasodilatation has also been documented in human forearm (Rongen *et al.*, 1994). It has been proposed that the endothelium-derived purinergic substances may be of greater significance in modulating response to local changes, while perivascular nerves may be concerned with integration of blood flow in the whole organism (Burnstock & Ralevic, 1994). In the umbilical circulation where there is little or no autonomic innervations, it is reasonable to propose that purinergic signalling may be of a greater importance than in systemic vascular beds.

Since the human umbilical cord is at most sparsely innervated, it is logical to postulate that the principle action of intraluminal ATP on umbilical vessel tone is dilator (via an action on EC P2Y receptors). However, In the umbilical vein EC, both receptor families are expressed (Wang *et al.*, 2002). Moreover, the distinction between the expression of P2X and P2Y receptors on VSM and EC is not always mutually exclusive. In a recent mouse study (Yamamoto *et al.*, 2006), it was shown that $P2X_4$ receptor knockout in mouse led to an elevation of blood pressure, and it was shown that the shear stress-induced $[Ca^{2+}]_i$ elevation in the endothelium of mesenteric arteries was largely due to $P2X_4$ receptor. The influence of adenosine is also not straightforward. Adenosine is released from hypoxic tissues, and is generated by metabolism of extracellular ATP (Berne, 1963). It can cause vasodilation or vasoconstriction via P1 receptors on VSM, or it can cause vasodilatation via EC. The P1 receptors have also been divided into subtypes: A_1, A_{2A}, A_{2B} and A_3, but there is significantly more evidence to support the role of A_1 and A_{2A} in controlling vascular responses (Marshall, 2002; Tawfik *et al.*, 2005). It is unclear exactly how either ATP or adenosine modulates the tone of umbilical arteries and veins. But clearly, the purinergic regulation of vascular tone is generally more complicated than is currently understood.

1.3.6.3. ATP and the Endothelial Cells

A brief description of the expression of P2X and P2Y receptors on human umbilical vein EC and their relevance in purinergic control of vascular tone has been given in *Section 1.3.6.2*. Pharmacological studies and molecular cloning have further subdivided these ligand-gated ion channel and G protein-coupled purinoceptors families into 7 and 8 subtypes, respectively. A detailed review of known distribution and roles of these receptor subtypes has been published (Burnstock, 2004). It would be inappropriate to discuss these multiple receptors in any detail

here. What follows below is a discussion of findings that are relevant to the present thesis. It should be noted that one of the underlying problems in understanding the functional role of P2 receptos is that there are no selective receptor antagonists or agonists for the various receptor subtypes. As will be discussed in Chapter 3 Section 3.4.4 and Chapter 6 Section 6.4.1, the general purinergic receptor suramin, for example, has no effect on $P2X_{4,6}$ or $P2Y_4$ receptors, even at high concentration. Similarly, neither $P2X_4$ or $P2Y_{2,4}$ receptors are sensitive to pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), although it is generally used experimentally as a selective P2X receptor (Charlton *et al.*, 1996; Burnstock, 2007). Further, purinergic receptors are susceptible to desensitisation on repeated agonist stimulation (Communi *et al.*, 1996; Burnstock, 2007). Thus the selective P2X receptor agonist, αβ-methylene ATP, is in fact often used as an antagonist.

The purinergic control of physiological responses is mediated by the purinoceptors located on the cell surface. As indicated above, upon release from nearby cells, ATP can act directly on the purinoceptors on both EC and VSM and nucleotide stimulation of P2Y receptors on EC can produce PGI_2 and NO, two potent vasodilators (Olsson & Pearson, 1990). Until relatively recently, the physiological function of P2X receptor subtypes in the control of vascular tone has received relatively little attention. It is important to note that multiple studies have shown that *both* purinoceptor families, not just P2Y receptors, and their respective members can be co-expressed in the same cell, and therefore presumably could mediate the effects of adenine nucleotides (Khakh & North, 2006). For example, $P2X_1$, $P2Y_2$ and $P2Y_6$ receptors are expressed in human internal mammary artery VSM, while $P2X_4$, $P2Y_{11}$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and P2Y6 receptors are expressed in HUVEC (Kunapuli & Daniel, 1998; Wang *et al.*, 2002).

$P2X_4$, $P2X_5$, and $P2X_7$, as well as $P2Y_1$ and $P2Y_2$ receptors are expressed in bovine aortic EC and EC from other origins. (Pirotton *et al.*, 1996; Ramirez & Kunze, 2002).

The intracellular pathways of P2Y receptors have been reviewed (Boarder & Hourani, 1998; Kunapuli & Daniel, 1998). The $P2Y_1$ and $P2Y_2$ receptors are coupled to separate arms of the G-protein but both are linked to PLC. Upon receptor stimulation they cause an increase in cellular [IP_3]. The subsequent IP_3 -induced increase in $[Ca^{2+}]_i$ activates phospholipase A_2 (PLA2), which is responsible for liberating AA from the membrane phospholipids as the initial step in PGI_2 synthesis. An alternative $[Ca^{2+}]_i$ -independent pathway involving phosphorylation of mitogen-activated protein kinase (MAPK) has also been shown (Patel *et al.*, 1996). The currently accepted view is that following activation of P2 receptors on EC, phosphorylation of MAPK activates PLA_2, and that raised $[Ca^{2+}]_i$ is required to translocate the activated enzyme (PLA_2) to the membrane for access to its substrate (Boarder & Hourani, 1998). It has also been shown that both $P2Y_1$ and $P2Y_2$ receptors in bovine EC are coupled to the activation of eNOS, which, as for PGI_2 production, is protein kinase-dependent (Brown *et al.*, 1996). These findings have recently been confirmed in HUVEC (da Silva *et al.*, 2009).

Considering the P2X receptor subtypes, a recent study showed that antisense oligonucleotides targeted to the $P2X_4$ receptor abolished shear stress–induced Ca^{2+} response in HUVEC (Yamamoto *et al.*, 2000b). Furthermore, the authors showed that expression of $P2X_4$ in a human embryonic cell line conferred the cells ability to sense shear stress (Yamamoto *et al.*, 2000a), indicating that shear stress-induced $[Ca^{2+}]_i$ increase in human umbilical vein EC is mediated by $P2X_4$ receptors. Later, by using $P2X_4$ knockout mice, it was confirmed that $P2X_4$ receptor mediated vascular dilatation *in vivo* is NO-dependent (Yamamoto *et al.*, 2006).

Data from another group supported these findings by using real-time PCR and Western-blotting and showed that P2X4 is by far the most highly expressed purinoceptor in HUVEC (Wang *et al.*, 2002).

This latter group and others also showed that $P2Y_6$ and $P2Y_{11}$ receptors, as well as $P2Y_1$ and $P2Y_2$, are expressed in HUVEC and have quantified their expression (Wang *et al.*, 2002). The finding that $P2Y_{11}$ was the most highly expressed purinoceptor is of special interest as the receptor had never before been described in the cardiovascular system. Because there are no specific agonists or antagonists available for the $P2Y_{11}$ receptor, its potential functional role cannot be demonstrated; it may account for some of the vascular effects previously ascribed to $P2Y_1$ and $P2Y_2$ receptors (Buvinic *et al.*, 2006).

Further, as is discussed in Section 1.4.2, there are enzymes expressed on the surface of EC that catalyse the sequential breakdown of ATP into nucleotides and nucleosides, which in turn may activate their respective purinoceptors. Indeed, by using selective adenosine receptor agonists and antagonists, it was shown that adenosine challenge could induce eNOS activation through the activation of P1 (A_2) receptors, which is mediated by increased L-arginine transport (Sobrevia *et al.*, 1997). It may also act via P1 receptor-independent pathway to activate essential kinases in these ECs (da Silva *et al.*, 2006). As a much higher level of adenosine is found in the umbilical vein and other placental tissues of PE-afflicted pregnancies compared to normal pregnancy, it has been hypothesised that increased adenosine concentration may account for the reduced perfusion in the feto-placental circulation exhibited in PE (Escudero & Sobrevia, 2008; von Versen-Hoynck *et al.*, 2009). Indeed, exposure of villous explants to hypoxia (2% O_2) increased the expression of A_{2A} receptor by

50% (von Versen-Hoynck *et al.*, 2009), strongly indicating an association between hypoxia, adenosine and PE. Further, human placental syncytiotrophoblast has specialised nucleoside transporters, responsible for the transport of adenosine across placenta and into EC (Britton *et al.*, 1991; San Martin & Sobrevia, 2006). However, this is outside the remit of the present thesis and will not be discussed further.

1.3.6.4. ATP and Vascular Smooth Muscle Cells

Previous studies have identified the expression and sometimes the functional role of different purinoceptors on vascular smooth muscle and isolated SMC from different species, and have indicated the wide variation in vascular control by in different vessels and species (Boarder & Hourani, 1998).

In contrast to skeletal muscle, vascular smooth muscle (VSM) contraction is induced by interaction between $[Ca^{2+}]_i$ and calmodulin (CaM), although a rise in $[Ca^{2+}]_i$ is the principle trigger in both cases. The Ca^{2+}-calmodulin complex activates myosin light chain kinase (MLCK) which in turn phosphorylates the regulatory myosin light chain (MLC) located on each myosin head (Hathaway *et al.*, 1991). Phosphorylation of MLC allows myosin to interact with actin, producing contraction. Relaxation occurs when myosin light chain phosphatase dephosphorylates the MLC. Thus, besides $[Ca^{2+}]_i$, the activity of MLCK is important in the modulation of VSM contraction. Intracellular cyclic AMP and cyclic GMP are also important in smooth muscle tone control. They activate cAMP-dependent kinase (PKA) and cGMP-dependent kinase (PKG), which phosphorylate MLCK, leading to its inactivation (Nishikawa *et al.*, 1984; Hathaway *et al.*, 1985). As a consequence, the affinity

of MLCK to CaM decreases, limiting the phosphorylation of MLC. This in turn stabilises the inactive form of myosin and therefore prevents contraction (Boron & Boulpaep, 2009).

In innervated vascular beds, vasoconstriction in response to ATP/ADP stimulation is mediated mainly by the $P2X_1$ receptor on VSM, activation of which causes the $[Ca^{2+}]_i$ elevation that is important for contraction (Burnstock, 2002). However, a detailed *in situ* hybridization and RT-PCR study of the expression of P2X receptor subtypes in the rat cardiovascular system also demonstrated the presence of the $P2X_4$ receptor in VSM (Nori *et al.*, 1998). Its functional role is unclear as there are no selective $P2X_4$ agonists or antagonists. The $P2X_4$ receptor is also insensitive to the non-specific P2 receptor antagonists suramin, PPADS and Reactive blue 2, which are able to abolish ATP-induced responses (Evans & Kennedy, 1994; Burnstock, 2006).

Further, in a study of isolated, pressurised rat cerebral arterioles, it was found that ATP induced initial constriction, which was followed by secondary dilatation (Horiuchi *et al.*, 2003). By applying specific P2Y antagonists extraluminally, it was found that these ATP-induced constrictor and dilator responses were mediated by $P2Y_2$ and $P2Y_1$ receptors, respectively.

Interestingly, both of these receptor subtypes are expressed in the human umbilical cord artery and vein VSM, as well as EC (Buvinic *et al.*, 2006). Moreover, the activation of these purinoceptors with their preferential agonists, 2-MeSADP and UTP respectively, was able to induce contraction in both isolated umbilical artery and vein, both with or without intact endothelium, indicating that $P2Y_2$ and $P2Y_1$ receptors that evoke contraction are present on

the VSM. Interestingly, it has been shown that hyperoxia-induced vasoconstriction of umbilical vein was abolished by functional adrenergic denervation of the vessel by pre-treatment in 6-OH-dopamine, and that the effect of 6-OH-dopamine was absent in endothelium-denuded vessels (Mildenberger *et al.*, 2004a). As it is well known that ATP is often co-released with noradrenaline from nerve terminals (Burnstock, 1972), it raises the possibility that ATP released from EC may cause hyperoxia-induced vasoconstriction via its action on VSM.

1.4. ATP RELEASE

1.4.1. Sources of Vascular Extracellular ATP

Notwithstanding its release from perivascular nerves, the major source of ATP (and its metabolites) as a paracrine or autocrine vasoactive substance is EC, whose activation by stimuli such as agonists, shear stress and hypoxia causes ATP release (Pearson & Gordon, 1979; Bodin *et al.*, 1991; Burnstock, 1999). It has been shown that cultured HUVEC consistently released ATP in response to a repeated shear stress challenge (Milner *et al.*, 1990). The mechanisms responsible for ATP release from EC are considered below. Limited data also suggest that ATP may be released from guinea pig hearts in hypoxia (Hopwood *et al.*, 1989), or from cultured VSM and ECs under certain agonist stimulation, such as thrombin and collagenase (Pearson & Gordon, 1979), and that release of ATP from cultured VSM may be constitutive (Prosdocimo *et al.*, 2009). ATP can also be released from aggregating platelets (Ingerman *et al.*, 1979; Beigi *et al.*, 1999) and from erythrocytes during periods of hypoxia, when haemoglobin is deoxygenated as blood flows through metabolising tissues such as in skeletal muscle during exercise (Bergfeld & Forrester, 1992; Ellsworth, 2004).

Hence, purinergic signalling has the potential to contribute to the control of local vascular tone under physiological conditions as well as under pathological situations.

The concentration of extracellular ATP is difficult to determine, as it is rapidly broken down by ectonucleotidases (ENs) such as nucleoside triphosphate diphosphohydrolases, nucleotide pyrophosphatases, alkaline phosphatase and 5'-nucleotidase (Zimmermann, 2000). They are also considered in detail in the *Section 1.4.4*. As a result of the presence of these ENs, extracellular ATP has a short half-life and therefore, in line with the model proposed by (Burnstock & Ralevic, 1994), is likely to evoke immediate vascular responses to changes in local conditions, rather than mediating long-term events such as vasculogenesis and angiogenesis. However, in recent years, the *status quo* has been challenged by new evidence which suggests that extracellular ATP is highly implicated in processes such as angiogenesis and chronic inflammation (see *Chapter 8*).

1.4.2. Hypoxia-induced ATP release

It is well documented that ATP is released from endothelial cells by shear force (Milner *et al.*, 1990; Bodin *et al.*, 1991; Bodin & Burnstock, 1995). However, one of the key concepts in the hypothesis that underlies the present project is that a hypoxic intrauterine environment, often associated with pregnancy-related disorders such as IUGR and PE, may be able to elicit release of ATP in the umbilical vessels. It is proposed that this ATP can initiate vascular vasomotion, which may represent intermittent placental blood supply, thus affects the oxidative status of the fetus.

It has been indicated in *Section 1.4.1* that erythrocytes are able to release ATP during periods of hypoxia in association with O_2 being offloaded from haemoglobin (Ellsworth, 2004). It is often hypothesised, or even assumed, that hypoxia is also able to induce ATP release from vascular ECs and VSM e.g.(Gerasimovskaya *et al.*, 2002; da Silva *et al.*, 2006). In fact, it is only recently that data suggesting that hypoxia *per se* is able to induce ATP release from EC has emerged (Woodward *et al.*, 2009). These data were obtained from an animal model (passaged EC from bovine pulmonary vaso vasorum). Direct data from freshly isolated cells of human origin has not been reported. In this respect, the only data available to date is from a study performed on HUVEC (Bodin & Burnstock, 1995). These authors reported that hypoxia had a synergistic relationship with increased shear stress in stimulating ATP release from these cells, but this was only the case with freshly isolated HUVEC, not with HUVEC when it had been cultured. They reported that hypoxia (5 min) alone had no effect on the basal release of ATP from EC. However, it should be noted that solutions bubbled with a 95% O_2 / 5% CO_2 gas mixture and 95% N_2 /5% CO_2 gas mixture were considered to be 'nomoxic' and 'hypoxic' conditions, respectively. These data may be suggestive, but these levels of oxygenation are very different from those experienced by HUVEC *in vivo* in the umbilical environment. A 95% N_2 / 5% CO_2 gas mixture was likely to have produced a PO_2 of >500 mmHg, whereas the normal level for the umbilical vein is ~44 mmHg (Armstrong & Stenson, 2007). On the other hand, it is not clear how low the PO_2 value achieved with the 95% N_2 / 5% CO_2 gas actually was, for no measurements were made and the PO_2 achieved would have depended on the extent to which the buffer was equilibrated with the gas mixture and open to room air.

1.4.3. Known ATP Release Pathways

As indicated above, in addition to its fundamentally important role in cellular energy storage and transfer, compelling evidence for the physiological release of ATP and its metabolites from cells in a large variety of tissues, organs and species has been documented. This has been reviewed (Burnstock & Knight, 2004). However, the mechanisms of release are poorly understood and there is still no consensus for the non-lytic mechanisms by which ATP is secreted in any particular cell type (Lazarowski *et al.*, 2003). Because of the size and charge of the molecule, ATP cannot simply diffuse across the plasma membrane (Glynn, 1968; Dieterle *et al.*, 1978). There is considerable evidence that the ATP release from nerve fibres is a vesicular exocytotic mechanism (e.g.(Bodin & Burnstock, 2001b)). For non-neuronal cells such as EC and SMC, vesicular exocytosis of ATP in response to shear stress has also been proposed (Bodin & Burnstock, 2001a). This vesicular-mediated ATP release pathway is discussed further in *Chapter 4 Section 4.4.3* and *Chapter 5 Sections 5.4.2 & 5.4.3*. However, several other mechanisms have attracted a great deal of attention, such as ATP-binding cassette (ABC) transporters, ATP-permeable anion channels, volume-regulated anion channels (VRAC), and connexin or pannexin hemichannels. One of the subtypes of P2 receptors, the $P2X_7$ purinoceptor, has also been implicated (Pellegatti *et al.*, 2005). The description below provides the details of these better known pathways.

1.4.3.1. ABC Transporters and VDAC

ABC transporters have perhaps attracted the most attention amongst the various candidate mechanism and pathways for ATP release, most existing data for this having been derived from studies that have been performed on epithelial cells. The ABC transporters are members of a protein family that is widely expressed in all cells, including the plant and bacterial kingdom and their structures are high conserved (Dean & Allikmets, 1995). The basic

structure consists of two cytoplasmic ATP-binding sites and two hydrophobic domains, each made up of six putative transmembrane segments (Higgins, 1995) (see Fig 1.7). ABC transporters utilise the energy from the hydrolysis of ATP to facilitate movement of molecules, including ATP, across the plasma membrane (Higgins, 1995). The most common mammalian ABC transporters are the cystic fibrosis transmembrane conductance regulator (CFTR), the P glycoprotein and the sulfonylurea receptor (i.e. the ATP-sensitve K^+ channel); ATP transport across the plasma membrane has been documented for all of these proteins (Higgins, 1995).

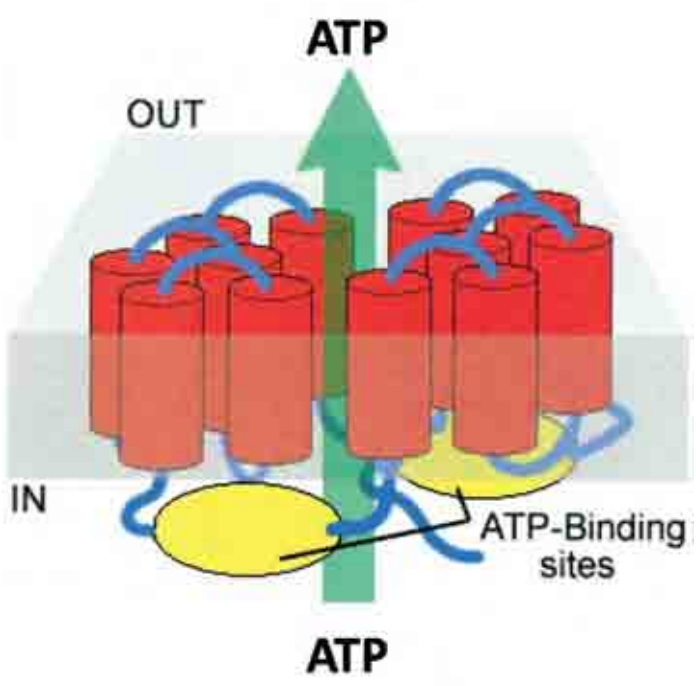


Figure 1.7. The structure of ABC transporter. Taken from (Bodin & Burnstock, 2001b)

Although expression of ABC-transporters is documented at multiple locations and species, mostly in epithelial tissues and at the blood brain barrier (Schwiebert, 1999; Scherrmann, 2005), to date there is little evidence to support the notion that they mediate ATP transport in vascular EC and SMC (Schwiebert, 1999). HUVEC do express CFTR, but there is as yet no

evidence to indicate its role in the release of ATP (Tousson *et al.*, 1998). Likewise, a number of studies on rabbit macula densa and mammary cell line showed that a voltage-dependent anion channel (VDAC) was ATP-conductive (Sabirov *et al.*, 2001; Bell *et al.*, 2003), and transgenic study on murine airway epithelial cells have suggested that the VDAC may be the splicing variant of the mitochondrial ATP porin VDAC-1 (Okada *et al.*, 2004). However, there are presently no *in vivo* data available demonstrating that functional presence of VDAC in mediating trans-membrane ATP transport in vascular SMC or EC, or indeed in any cell type (De Pinto *et al.*, 2010).

1.4.3.2 VRAC

Numerous studies have demonstrated that cell swelling (for example, induced by hypotonic stress) activates a robust ATP release in a range of cells including EC (Okada *et al.*, 2001). Interestingly, cellular swelling could also activate VRAC (Strange *et al.*, 1996). Indeed, this has prompted the hypothesis that VRAC are involved in ATP transport. The main evidence for this is a study on bovine aortic EC in which pharmacological inhibition of VRAC by glibenclamide, verapamil, tamoxifen, and fluoxetine also inhibited ATP release. By using the patch-clamp technique, the authors showed that the VRAC current is inhibited not only by ATP, but also other extracellular nucleotides UTP, GTP, CTP and ADP, in a permeating blocker model (Hisadome *et al.*, 2002). Interestingly, the study also showed that inhibition of VRAC also inhibited the hypotonic stress-induced $[Ca^{2+}]_i$ oscillations in and NO production from the EC. Further, a related study on the same cell type showed that the hypotonic stress-induced ATP release, as well as actin reorganisation, is tyrosine kinase and Rho-kinase dependent, although the ATP and actin responses had no causal relationship (Koyama *et al.*, 2001). More recently, it has been shown that this is also the case for HUVEC, with the

further information that tyrosine kinase activation is a subsequent event to Rho-kinase activation (Hirakawa *et al.*, 2004). To date there is no direct evidence to show that VRAC can release ATP, and the precise role of VRAC in mediating ATP release from EC remains unclear.

1.4.3.3. Hemichannels and P2X7 Purinoceptor

The gap junction molecules, connexins, can form gap junctions between cells and it is well established that they are involved in intercellular communication, for example in Ca^{2+} wave propagation in epithelial cell, vascular EC and SMC (Sanderson *et al.*, 1990; Domenighetti *et al.*, 1998; Billaud *et al.*, 2009). Accordingly, using a luminometric ATP detection assay, it has recently been shown that connexin mimetic peptides for connexin 43 inhibited constitutive release of ATP from an immortalised endothelial cell line HMEC-1 (Faigle *et al.*, 2008). Importantly, connexin channels have also been implicated in vascular vasomotion in the cerebral blood vessels (Haddock *et al.*, 2006). In this study, it was also shown with immunohistochemical techniques that the connexin protein expression was predominantly localised to the myoendothelial junctions, suggesting their importance in coupling ECs and VSM, an important feature of vasomotion. Indeed, connexin 43 is expressed in umbilical and chorionic vessels, and application of three structurally different inhibitors, Gap 27, 18α-glycyrrhetinic acid or octanol, inhibited vasomotion amplitude in these vessels (Garcia-Huidobro *et al.*, 2007).

The other principle model for intercellular signal propagation is a pathway that involves cellular ATP release. This mechanism, which has been demonstrated in HUVEC and calf pulmonary artery EC, involves regulated ATP release which acts on purinoceptors on the

neighbouring cells, the activation of which leads to further ATP release from these cells (Bodin & Burnstock, 1996; Moerenhout *et al.*, 2001). It has been shown in bovine corneal EC that this extracellular ATP-mediated method of intercellular communication can occur even when there is no direct physical cell to cell contact, for Ca^{2+} wave propagation occurred even across a cell-free zone (Gomes *et al.*, 2005). Interestingly, increased expression of connexins has been shown to facilitate greater increase in ATP release following purinergic receptor activation by UTP. It has thus been speculated that the connexins may form a regulated exit pathway for ATP, and hence play a central role in propagation of Ca^{2+} waves (Cotrina *et al.*, 1998). Indeed, for non-excitable cells such as human astrocytes, lung epithelial cells and HUVEC, data from real-time visualisation of cellular ATP release (using extracellular buffer with a mixture containing luciferase and its substrate luciferin) and expression system showed that regulated physiological ATP release is responsible for Ca^{2+} wave propagation, and that it was mediated by at least in part mediated connexins which form hemichannels in the plasma membrane (Arcuino *et al.*, 2002; Stout *et al.*, 2002).

This potential role of connexin in mediating ATP release in VSM, however, is still a subject of controversy. A number of research groups have found that, although connexin channels were implicated, in contrast to astrocytes or EC (Arcuino *et al.*, 2002), ATP-hydrolysing enzymes (e.g. apyrase) or purinoceptor blockers (e.g. suramin) had no effect on the transmission of the Ca^{2+} wave in primary murine VSM or cultured human uterine SMC (Young *et al.*, 2002; Hanner *et al.*, 2008).

Very recently the family of pannexin channels has also entered the field of ATP release (Shestopalov & Panchin, 2008). Several properties of the channel, such as activation by

membrane depolarisation, mechanosensitivity and ATP-permeability (Locovei *et al.*, 2006; Shestopalov & Panchin, 2008), suggest that it is a suitable candidate for an ATP-releasing channel. It was mentioned in *Section 1.4.3* that the $P2X_7$ purinoceptor has been implicated in ATP release. This proposal was based on evidence that expression of P2X7 receptor in a cell line conferred the cell the ability to release ATP in response to BzATP, a potent $P2X_7$ agonist (Pellegatti *et al.*, 2005). However, there had been no molecular data to suggest that the receptor itself is pore-forming (Praetorius & Leipziger, 2009), although recent evidence suggests that pannexin channels may be the pore-forming component of P2X7 purinoceptor (Shestopalov & Panchin, 2008). However, all the data on the properties of pannexin channels have been derived from expression systems, and presently there is no evidence to suggest their expression or function in vascular SMC or EC. Their potential role in mediating intercellular communication via ATP release in the vasculature *in vivo* is therefore unclear.

1.4.4. Fate of Extracellular ATP

1.4.4.1. Ectonucleotidase (EN)

Intercellular signalling systems generally require mechanisms of signal inactivation such as receptor desensitisation and receptor down-regulation. In addition to these mechanisms, ATP released from vascular EC, SMC and blood cells is rapidly inactivated by hydrolysis (Pearson & Gordon, 1985). It was assumed that single enzymes are responsible for the hydrolysis of either ATP or ADP, or both (e.g. (Coade & Pearson, 1989)). However, following molecular cloning and functional characterisation of several families of ectoenzymes, it has now become clear that this is not the case. In fact, multiple enzyme families exists that catalyse the sequential reactions of ATP ⇨ ADP ⇨ AMP ⇨ adenosine (see Fig 1.8). These enzymes

have differential but often overlapping substrate specificities and tissue distributions, thereby conferring broad functional diversity (Zimmermann, 2000).

The known ENs are divided to the following groups: (i) the E-NPP Family (Ecto-Nucleotide Pyrophosphatase/Phosphodiesterase Family); (ii) the E-NTPDase Family (Ecto-Nucleoside Triphosphate Diphosphohydrolase Family); (iii) Ecto-5'-Nucleotidase; and (iv) Alkaline Phosphatases. Some of these ENs have distinct patterns of distribution in different cell types and are regulated during physiological and pathophysiological processes, probably in association with purine and pyrimidine signalling (Zimmermann, 2000). The dominant EN expressed by vascular EC is the E-NTPDase family, and also ecto-5'-Nucleotidase that completes the enzymatic reaction initiated by the E-NTPDases (see Fig 1.8) (Atkinson *et al.*, 2006). The presence of purinergic control of vascular function suggests the important functional significance of the ENs. Thus, the biological effect of released ATP depends on the local activities of EN expressed on EC and SMC cell surface (Pearson *et al.*, 1980; Gordon, 1986).

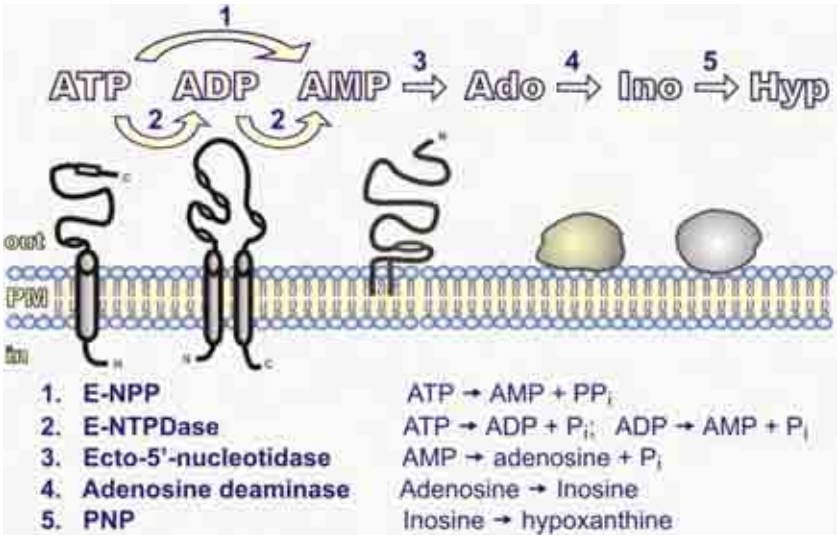


Figure 1.8. Extracellular metabolism of nucleotides. The numbers indicate the type of enzyme involved in the reaction.

The nucleotide-hydrolysing pathway comprises at least three ectoenzyme families E-NPP, E-NTPDase and Ecto-5'-Nucleotidase. The resulting adenosine can be further deaminated through sequential adenosine deaminase and PNP reactions. E-NPP, ecto-nucleotide phosphodiesterase; E-NTPDase, ecto-nucleoside triphosphate diphosphohydrolase; PNP, purine nucleotide phosphorylase; Pi, inorganic phosphate; PPi, inorganic pyrophosphate. Taken from (Yegutkin, 2008).

Extracellular nucleotide inter-converting activities such as nucleoside dikinases have also been described e.g. (Lazarowski *et al.*, 2000). Moreover, cleaved and soluble forms of EN exist and are referred to as exonucleotidases, but there is currently little experimental evidence suggesting their existence outside of the nervous system. In general, EN are membrane bound, with the catalytic site facing the extracellular medium. Hence, the combined actions of ENs regulate the concentrations of the individual extracellular and circulating vasoactive nucleotides and can also generate vasoactive adenosine (Pearson & Gordon, 1985; Robson *et al.*, 2001). Indeed, it was shown that in resting, non-secretory cells the concentration of extracellular ATP remains constant through time, but that it is actively re-circulated by the cells, which indicates that the resting level of extracellular nucleotides is the

result of constitutive nucleotide release balanced by the concerted activities of the ENs (Lazarowski *et al.*, 2001).

The maximum catalytic activity of the individual ENs is adapted to the extracellular condition and requires the presence of divalent cations such as Ca^{2+} or Mg^{2+} and an alkaline pH. In most cases the K_m-values are in the lower micromolar range (Zimmermann, 2000). In addition to their catalytic activity, there are also reports of ENs functioning as cell adhesion molecules or as transmembrane receptors (Lennon *et al.*, 1998).

1.4.4.2. Regulation of EN

By monitoring the metabolism of radioactive ATP, ADP and adenosine on the surface of HUVEC, it was shown that exposure to a combination of hypoxanthine and xanthine oxidase, which generates O_2^- and H_2O_2, increased the half time of disappearance of these substrates significantly (Aalto & Raivio, 1993). Similarly, by expressing a recombinant human EN gene in a model cell line, it was found that hypoxanthine, xanthine oxidase as well as H_2O_2 were able to almost completely abolish the inhibitory effect of EN on exogenously applied ADP-induced platelet aggregation (Kaczmarek *et al.*, 1996). Further, it was shown that oxidative stress in EC resulted in persistent immune-reactive, but functionally inactive E-NTPDase, and that recovery from the insult was possible only after new protein synthesis (Robson *et al.*, 2001). Taken together, these independent studies demonstrated that exposure to ROS can potently impair the catabolism of extracellular ATP by EN.

As discussed above, impeded umbilical blood flow as well as excessive oxidative stress are often associated with complications of pregnancy including IUGR and PE (see *Section*

1.2.2.6). There is also some evidence, albeit limited, that acute hypoxia induces ATP release from at least some EC (Woodward *et al.*, 2009). Thus, it might be expected that hypoxia would simply increase extracellular ATP concentration. However, paradoxically, several studies have shown that chronic hypoxia (3%; 2-4 weeks) or I/R injury increased the expression of EN in pulmonary EC and perfused heart, thereby indirectly lowering prevailing local levels of ATP (*Section 1.6* Fig *1.9*) (Gerasimovskaya *et al.*, 2002; Van Linden & Eltzschig, 2007; Eltzschig *et al.*, 2009). Further, it was shown that hypoxia (30mmHg; 18hr) was able to increase the expression as well as the activity of Ecto-5'-Nucleotidase in aortic EC, the other major family of EN expressed by EC, and thereby has the potential to increase adenosine production from ATP released from cell membrane (Ledoux *et al.*, 2003). Thus, chronic hypoxia may lead to lower levels of ATP in umbilical vessels that might be expected from effects of acute hypoxia and may also lead to increased levels of adenosine.

1.5. VASCULAR VASOMOTION

Vascular vasomotion refers to spontaneous rhythmic oscillations of blood vessel tone or diameter in the range of 1-20 min^{-1}, that is independent of heart rate (Aalkaer & Nilsson, 2005). It has been reported in both *in vivo* and *ex vivo* experimental studies.

1.5.1. The Physiological Role of Vasomotion

The physiological consequence of vasomotion is not well understood. Several ideas have been put forward and have been reviewed recently (Nilsson & Aalkjaer, 2003). For example, it has been argued that vasomotion increases flow conductance without a need for a change in perfusion pressure (Meyer *et al.*, 2002); and that the oscillations of oxygen tension which are caused by vasomotion provides better tissue oxygenation than that obtained with a steady O_2

delivery (Tsai & Intaglietta, 1993). In relation to this idea, Rucker *et al* (2000) used NADH fluorescence to study the metabolic status of several tissues adjacent to tissue in rat hind limb. They found that under critical perfusion conditions induced by stepwise reduction of blood flow to the whole hind limb by restriction of the femoral artery, vasomotion developed in the skeletal muscles and that this was able to confer beneficial effects on oxygenation of the adjacent tissues, including the periosteum, subcutis and skin (Rucker *et al.*, 2000). Another group studied the effect of vasomotion in patients suffering from traumatic brain injury and suggested that vasomotion in the middle cerebral artery is a physiological phenomenon that compensates for variability in mean arterial pressure. Indeed, it was argued that the unaltered middle cerebral artery flow that occurred as a consequence of vasomotion was beneficial for maintaining brain tissue oxygenation (Turalska *et al.*, 2008). At a microcirculatary level, it was proposed that chaotic pattern of vasomotion is essential in promoting oxygenation of surrounding tissue (Pradhan *et al.*, 2007), although this was based on theoretical study and to date, data from physiological situation is scarce. The results of these studies are consistent with the generally accepted view that metabolic challenge e.g. low blood flow induces vasomotion. Indeed, many studies have shown that a reduction in blood flow or haematocrit potently increases the incidence, frequency and relative amplitude of vasomotion (Rucker *et al.*, 2000; Lee *et al.*, 2005). Hence, it can be argued that vasomotion is a response to, and may be, a protective mechanism against, tissue hypo-perfusion or hypoxia.

On the other hand, theoretical modelling on data from mesenteric arteries from Sprague-Dawley rats suggested that vascular resistance is higher when a vessel displays vasomotion than when it does not e.g. (Gratton *et al.*, 1998). The calculation of vascular resistance of a vessel displaying vasomotion was based on a *mean* radius. It has since been pointed out that

time-dependent variability in vessel radius absolutely affects volume flux of the vessel, and if the effect of time-dependent change in radius was taken into consideration when modelling, then vasomotion would in fact increase conductance (decrease resistance) (Meyer *et al.*, 2002). Clearly, more experimental data is needed to test these theories more fully. Experimental data on the physiological role of vasomotion in the human umbilical circulation is scarce.

Notwithstanding, vasomotion does occur in both umbilical artery and vein, as well as in vessels of placenta. Indeed, *in vitro* measurements showing that the amplitude in the change in vascular wall tension of spontaneous vasomotion oscillation was ~11% of the maximum contractile tension, leading the authors to suggest that vasomotion is important in regulating blood flow, oxygen and nutrients to the fetus (Garcia-Huidobro *et al.*, 2007). Moreover, *in vivo*, umbilical blood flow has been shown to remain constant (Jensen *et al.*, 1999) or even increase from 436 to 491 ml/min during placental hypoxia, even though the mean arterial and venous pressure and vascular resistance did not significantly change (van Huisseling *et al.*, 1991). In contrast, IUGR is associated with a fall in umbilical blood flow (Ferrazzi *et al.*, 2000; Ferrazzi *et al.*, 2002). This raises the possibility that vasomotion may provide a self protecting mechanisms in umbilical blood vessels, which can be evoked under hypoxia to improve blood flow for the maintenance of O_2 supply to the fetus. Further, as discussed in *Section 1.2.2.7*, vasomotion in the umbilical vessels may have an important role in mediating ROS status and I/R-type injury in normal and complicated pregnancies. Taken together, and taking into consideration the fact that intrauterine hypoxia is a key feature of normal and complicated pregnancies, it is reasonable to propose vasomotion in the feto-placental circulation may play a key role in both normal and abnormal placental and fetal development.

Elucidation of the mechanisms underlying the effect of hypoxia on vasomotion would facilitate our understanding of fetal blood supply and health.

1.5.2. Mechanisms Underlying Vasomotion

Although vasomotion has been widely observed in a variety of blood vessels e.g. coronary artery (Kawasaki *et al.*, 1981; Kawasaki *et al.*, 1985), radial artery (Stojnic *et al.*, 2006), pial artery (Gokina *et al.*, 1996), cerebral artery (Vinall & Simeone, 1987), pulmonary artery (Bonnet *et al.*, 2001; Guibert *et al.*, 2005) and umbilical artery and vein (Garcia-Huidobro *et al.*, 2007), the mechanisms underlying vasomotion remain unclear. One of the reasons for this is probably that there are multiple mechanisms underlying vasomotion, which are difficult to address experimentally (Aalkaer & Nilsson, 2005). Nevertheless, $[Ca^{2+}]_i$ oscillations, have been observed in synchronisation with the rhythmic blood vessel contractions, in arterial SMC (Aalkaer & Nilsson, 2005), and aortic and corneal EC (Hisadome *et al.*, 2002; Gomes *et al.*, 2005), both *in vitro* and in whole blood vessels (Gustafsson, 1993; Aalkaer & Nilsson, 2005). The increase in $[Ca^{2+}]_i$ precedes each increase in wall tension and these $[Ca^{2+}]_i$ oscillations in SMC and are therefore considered to drive vasomotion (Aalkaer & Nilsson, 2005). In umbilical vein, equilibration in Ca^{2+}-free buffer for >15 min reduced basal tension and reduced amplitude of spontaneous vasomotion by 70%, whereas transient removal of external Ca^{2+} had little effect. In contrast, increasing the $[Ca^{2+}]_i$ by blockade of sarcroplasmic ATPase increased the amplitude of the vasomotion and basal tension. The authors therefore suggested that vasomotion is critically dependent on intracellular Ca^{2+} stores in VSM (Garcia-Huidobro *et al.*, 2007). On the other hand, as discussed in S*ection 1.3.2.5* the release of NO from HUVEC has been shown to be oscillatory, which may represent another mechanism by which vasomotion occurs.

A number of oscillator models have been proposed to explain the phenomenon; including a sarcolemma oscillator, a membrane potential oscillator and a metabolic oscillator related to glycolysis (Peng *et al.*, 2001; Aalkaer & Nilsson, 2005). Synchronization of the oscillation from cell to cell may involve changes in the cell membrane potential. In freshly excised umbilical artery and vein strips, it was reported that amplitude of vasomotion was three times greater in umbilical vein than artery, and that the vasomotion lasted for up to 8 hr (Garcia-Huidobro *et al.*, 2007). Removal of endothelium from the umbilical vein reduced the vasomotion amplitude by ~50%, but frequency by only 10%. As discussed in *Section 1.4.3.3*, blockade of gap functions by Gap 27, 18α-glycyrrhetinic acid or octanol reduced the amplitude, but had no effect on frequency of the vasomotion. Interestingly, blockade of K_{ATP} channel in umbilical vein with glibenclamide caused a dose-dependent inhibition of vasomotion and basal tension, indicating an important role for K_{ATP} channel in vasomotion activity (Garcia-Huidobro *et al.*, 2007).

1.5.3. Vasomotion and Pre-eclampsia

Given the apparent functional significance of vasomotion on tissue oxygenation and oxidative status, it is not surprising that it has been implicated in PE, where it is generally accepted that oxidative stressed is increased. As discussed in *Section 1.3.2.3*, $[Ca^{2+}]_i$ is important for the activation and release of NO from EC, which may represent a mechanism by which vasomotion is mediated. Consistent with this theme, it was shown that serum from PE pregnancy induced higher frequency $[Ca^{2+}]_i$ oscillations in HUVEC than that from normal pregnancy (Matsubara *et al.*, 1998), suggesting that a factor(s) is present in the systemic circulation of women with PE, which could alter vascular reactivity. Indeed, it was reported

that vasomotion is exaggerated even in epigastric, omental and skeletal arterioles from women with PE or hypertensive rats (Ebeigbe & Ezimokhai, 1988; Boegehold, 1993).

Although impaired endothelial dependent relexation is the hallmark of PE, there are relatively little studies on the feto-placental circulation. A more recent study in vessels from women with PE showed that changes to oscillation pattern in response to TXA_2 challenge differed between the feto-placental circulation and the systemic circulation: the chorionic arteries (from the feto-placental circulation) displayed reduced vasomotion amplitude, while myometrial arteries (from the systemic circulation) displayed increased vasomotion frequency (Sweeney *et al.*, 2008). The evidence therefore suggests that placental vessels from women with PE displayed less vasomotion than those from normal pregnancies, whereas in systemic vessels of women with PE, a hyper-sensitivity is apparent. There is little consensus regarding the functional status of the feto-placental circulation in PE, but studies have shown that basal $[Ca^{2+}]_i$, $[Ca^{2+}]_i$ response and NO production to AA and histamine challenges were significantly altered in HUASMC from PE tissue when compared to control (Steinert *et al.*, 2002; Steinert *et al.*, 2003). Given that $[Ca^{2+}]_i$ in VSM is considered to drive vasomotion (*Section 1.5.2*), it is reasonable to suggest that the altered vasomotion amplitude or pattern may play a role in the pathophysiology of PE.

1.6. GENERAL HYPOTHESIS

In general, then, vasomotion is an intrinsic phenomenon that can occur in most blood vessels including the umbilical cord artery and vein under certain conditions which seem to include low flow and low PO_2. Although vasomotion has been proposed to be a protective

mechanism against tissue hypoxia, few studies have been carried out to investigate the effect of hypoxia upon vasomotion in umbilical cord.

As discussed above in *Section 1.4.2*, there has been some limited evidence to suggest that EC may release ATP in response to hypoxia. Moreover, extracellular ATP, as a regulatory metabolite, has been suggested to mediate $[Ca^{2+}]_i$ oscillations via activation of P2 receptors in multiple cell types including skeletal muscls, pulmonary VSM, lymphatics, chondrocytes and model cell lines (Mahoney *et al.*, 1992; Mahoney *et al.*, 1993; Wyskovsky, 1994; Gao *et al.*, 1999; Pauvert *et al.*, 2000; Visegrady *et al.*, 2001; Zhao & van Helden, 2002; Kono *et al.*, 2006). As discussed above, oscillations in $[Ca^{2+}]_i$ certainly seem to drive vascular vasomotion. The possibility that hypoxia can elicit ATP release from EC and VSM of human umbilical artery and/or vein and induce $[Ca^{2+}]_i$ oscillations and hence vasomotion in these vessels is therefore an attractive possibility for vascular control in the umbilical circulation in hypoxia (see Fig 1.9).

In *Section 1.2.2.6*, it was indicated that oxidative stress caused by intermittent perfusion of the placenta is implicated in normal and pathological human gestation, but the possibility was also raised that vasomotion of the umbilical vessels may affect the ROS status of the fetus and hence determine the success or otherwise of pregnancy. The ability of ATP to initiate $[Ca^{2+}]_i$ oscillations in umbilical SMC and drive vasomotion may therefore act as a mechanism that maintains adequate blood flow in the umbilical circulation, the functioning of which may be impaired in the uteroplacental circulation in PE (see ■ in Fig 1.9).

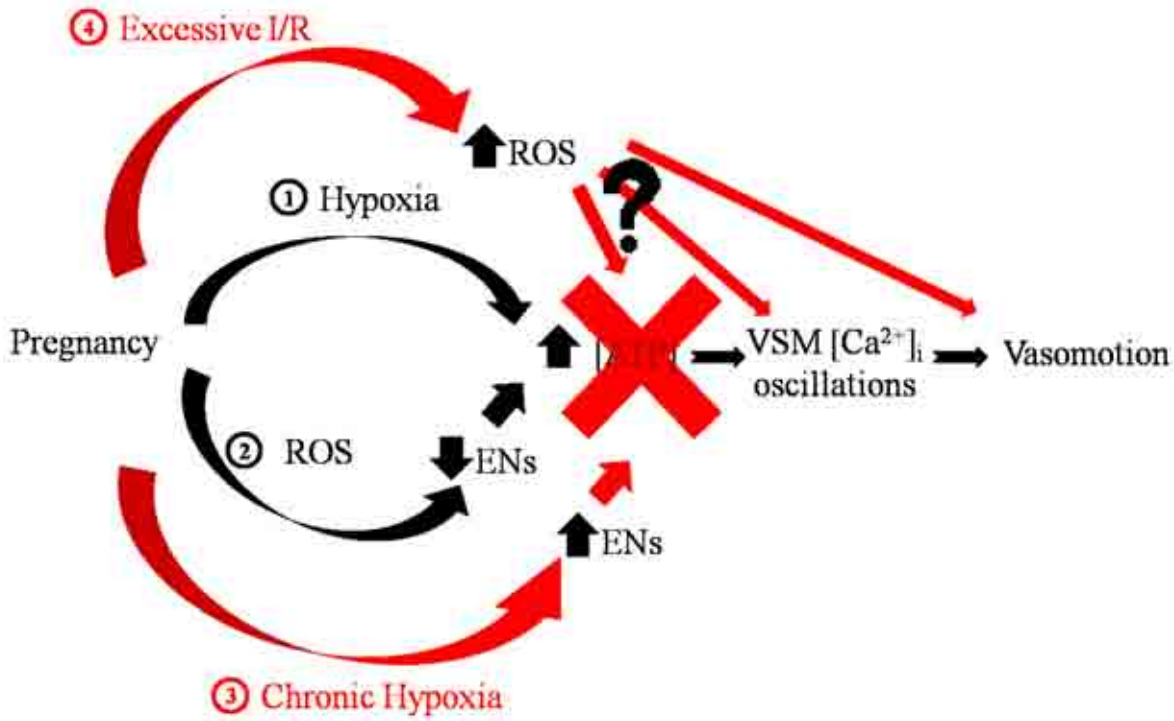


Figure 1.9. Schematic hypothesis showing a modulation of vascular vasomotion in human umbilical vessels during pregnancy.

■: Physiological levels of intrauterine hypoxia induces ATP release from vascular EC and/or SMC and induces $[Ca^{2+}]_i$ oscillations, which drive vasomotion. ■: Physiological levels of ROS from intermittent placental perfusion inhibits EN, leading to accumulation of ATP and facilitating vasomotion. ③: In chronic hypoxia, increased EN expression reduces extracellular [ATP] so abrogating vasomotion. ④: Increased ROS caused by excessive I/R-type injury and ROS generation in PE may affect pattern of vasomotion in the uteroplacental circulation via unknown mechanisms. Key: ROS = Reactive Oxygen Species; [ATP] = Extracellular ATP concentration. Black arrows indicates physiological phenomenon, whereas red arrows indicate potential deleterious consequences of chronic hypoxia or excessive I/R.

In *Section 1.4.4.2*, the evidence that ROS inhibit ENs and can thereby increase the prevailing level of ATP was discussed. Thus, the inhibitory effect of ROS on the activity level of EN may be a mechanism by which vasomotion is facilitated at times when hypoxia or I/R-type insult occurs (see ■ in Fig *1.9*).

On the other hand, evidence of a paradoxical increase in EN expression with chronic hypoxia was discussed in *Section 1.4.4.2*. Therefore, it is reasonable to propose that this would lead to a decreased level of extracellular ATP, potentially impairing normal vasomotion function (③ in Fig 1.9). As abnormal intrauterine hypoxia is associated with PE (Soleymanlou *et al.*, 2005), and vasomotion is attenuated in the uteroplacental circulation in PE (Sweeney *et al.*, 2008), a lack of ATP may play a role in the impaired perfusion seen in PE. These mechanisms, if proved to be correct, would clearly be of important scientific and clinical interest with regards to our understanding of normal and pathological fetal development and growth.

Finally, although it is well established that incomplete conversion of spiral arteries in the placenta, as found in PE, leads to excessive I/R injury and ROS generation through retained vasoreactivity (see *Section 1.2.2.6*), it is not known how this leads to the clinical symptom of fetal hypo-perfusion. Given that vasomotion is believed to facilitate downstream tissue oxygenation, it is thus a reasonable hypothesis that hypoxia-induced ROS may affect the pattern of vasomotion in the umbilical vessels (④ in Fig 1.9).

Given the lack of experimental evidence of how vasomotion may play a role in the control of umbilical circulation in normoxia and in hypoxia, the aims of the present study were to examine umbilical artery and vein from normal pregnancy. It would focus on evidence that hypoxia releases ATP from EC and on evidence that it induces or modulates vasomotion in artery and vein, by acting on EC or SMC. Therefore, preparations of EC, arterial SMC and whole umbilical artery would be used.

CHAPTER 2

GENERAL METHODS AND MATERIALS

In this chapter, the general protocols of each technique are described. The protocols for each study performed in this project are described in the relevant Results Chapter. Experiments were performed on human umbilical arteries, and on VSM and EC cultured from these vessels and from human umbilical veins. These vessels were isolated from umbilical cords obtained from full term placentae from normal uncomplicated pregnancies, from either natural birth or elective Caesarean section. Full informed consents were obtained from the patients as per local ethic committee policy. Following delivery by vaginal or caesarean sections at the maternity unit of the Birmingham Women's Hospital, the placentae were stored at 4°C until they were collected. They were transported to the laboratory in the Medical School at the University of Birmingham, 5 minutes away and were used on the same day as delivery.

2.1. CELL ISOLATION AND CULTURE

2.1.1 General Conditions

Cellular work was performed in the laboratory using the following protocols under sterile conditions provided by laminar flow hoods. For ATP release experiments (*Chapter 4*), cells were first grown to confluence in culture flasks before being transferred onto 24 well culture inserts or culture plates (BD Falcon). Antimicrobial additives in the culture medium were withdrawn 24 hr prior to these and quinacrine staining experiments (*Chapter 5*), as it is well known that they affect the behaviour of ion channels (Goldhill *et al.*, 1996), which could potentially mediate ATP transport (see *Chapter 1 Section 1.4.3*). For Ca^{2+} imaging experiments (*Chapter 3 & 6*), freshly isolated cells were plated directly onto pre-prepared sterilized EPSE-coated ø13 mm glass coverslips, on which the changes in $[Ca^{2+}]$ were examined. For quinacrine staining experiments, HUVECs were prepared in the same way but were used up to the 2nd passage. All experiments were carried out when confluence was

reached. *All cells were cultured in a conventional 37 °C humidified incubator of 5% CO_2 at atmospheric pressure unless otherwise indicated*.

2.1.2. Cell culture apparatus and their preparation for cell attachment

In order to facilitate adhesion of cells to culture plates, different techniques were used to prepare the surfaces of the culture plates. This was particular important when the experimental protocol required the cells to be exposed to shear-stress or mechanical disruption. For the initial ATP release experiments (*Chapter 4*), cells were grown in 24-well culture plate (BD Falcon) which has been pre-coated with Type B gelatine (Sigma-Aldrich, U.K.). Each culture plate was coated with 100 µl of ready-made 2% gelatine solution for 10 min, the excess solution was aspirated and the culture plate was allowed to dry for 2 hr under sterile condition before introduction of culture medium and cells. For subsequent ATP release experiments that examined the degree of polarised released from apical and basolateral cellular membrane, cells were directly grown on untreated cell inserts (see *section 2.2* for details).

For quinacrine staining and Ca^{2+} imaging experiments (*Chapter 3, 5 & 6*), cells were grown on ø13 mm glass coverslips (WPI, U.K.) of thickness #1.5 (0.16-0.19 mm). Prior to use, they were first coated with 3-aminopropyltriethoxysilane (APES; Sigma-Aldrich, U.K.). This involved the following steps: First, new coverslips were submerged in laboratory grade nitric acid in a glass bottle overnight. On the following day, they were rinsed gently with running tap water for 3 hr. Each individual coverslip was dried on tissue paper, which was then transferred to a plastic 50ml bottle (BD Falcon, U.K.). The coverslips were then submerged in laboratory grade acetone. The bottle containing the glass coverslips was manually inverted

for approximately 30 s, after which the acetone was drained off. This step was repeated twice, to ensure all traces of oil and water were removed. A 4% APES solution was prepared in fresh acetone. The glass coverslips were submerged in the APES-acetone solution in the 50ml bottle, which was then manually inverted continuously for 30 s. This old APES-acetone solution was then drained off and replaced with fresh APES-acetone solution. The 50ml bottle containing the coverslips in APES-acetone solution was then left on an automatic rotator for 1 hour, after which it was left in the dark overnight. Next day, the coverslips were rinsed in acetone twice as per the protocol for the previous day. They were then rinsed once with distilled water, before being spread out individually to allow drying. They were finally autoclaved as per normal laboratory practice.

2.1.3. Subcultivation

Prior to detaching cells, culture medium was aspirated from the 25cm^2 culture flask, and cells were washed in room temperature (RT) 0.02% ethylenediaminetetraacetic acid solution (EDTA; Sigma-Aldrich, U.K.) to remove the Ca^{2+} in any residual culture medium. This was then aspirated and replaced by 2.5ml of a specially weak trypsin-EDTA solution (0.05% porcine trypsin; Sigma-Aldrich, U.K.) at RT. The cap of the culture flask was then replaced and cells were examined under a microscope. When the cells started to detach, the culture flask was tapped gently to loosen the cells. 2.5ml of RT trypsin inhibitor (from Glycine max; Sigma-Aldrich, U.K.) was then added to the culture flask and this was gently agitated. The content of the culture flask was next transferred to a 14ml test tube, in which the cells were spun down at 220 x g. The supernatant was discarded, a suitable amount of the corresponding culture medium was used to re-suspend the cells by gently pipetting up and down. Cells were

then placed in new receptacles (i.e. culture flask/insert/glass coverslip) containing the pre-warmed culture medium. These were then re-introduced into the incubator (37 °C, 5% CO_2).

2.1.4. Human Umbilical Vein Endothelial Cells

The primary endothelial cultures were isolated from the veins of human umbilical cords as described in (Cooke *et al.*, 1993) with some modification. The veins of cords were cannulated with custom-made glass pipettes and washed thoroughly with sterile phosphate buffered saline (PBS), supplemented with 100 units/ml penicillin, 0.1 mg/ml streptomycin and 5.6 µg/ml amphotericin B in order to remove any blood clots. The vessel was then infused with 1 mg/ml (125 U/ml) of type I collagenase (Sigma-Aldrich, UK) in Ca^{2+}- and Mg^{2+}- free PBS, and incubated at 37°C for 15 min. Detached EC were removed from the cord with two separate boluses of PBS, washed, centrifuged at 1800 rmp and re-suspended in EGM® Endothelial Cell Growth Medium (for Ca^{2+} imagine experiments; Lonza, Switzerland) or Endothelial Cell Growth Medium (for ATP-release experiments; Promocell, UK), which were supplemented with penicillin, streptomycin and amphotericin B at the concentrations indicated above. The composition of the respective mediums can be found in the *Appendices* of the present thesis. Cells were seeded in the appropriate cell culture apparatus detailed in *Section 2.1.2* with pre-warmed culture medium. Removal of residual blood cells was achieved by a complete change of medium after 2 hr of cell plating. Cells were fed were fed every 2-3 days.

2.1.5. Human Umbilical Artery Endothelial Cells

HUAEC were isolated using a protocol similar to that for HUVEC. The vein of the human umbilical cords was first washed to aid cannulation of the artery. Next, one end of the artery

was carefully isolated by surgical dissection from the Wharton's Jelly and the artery was cannulated with a 20G animal feeding needle. The artery was infused with type I collagnease, incubated and harvested as with HUVEC (see *Section 2.1.4*). Isolated EC were seeded in Endothelial Cell Growth Medium (Promocell, UK) and in cell culture apparatus as discussed in Section 2.1.2, and were cultured as for HUVEC (see above).

2.1.6. Human Umbilical Artery Smooth Muscle Cells

HUASMC were explanted from the arteries of umbilical cords as described by (Kocan *et al.*, 1980). The arteries were excised from the human umbilical cord into segments of approximately 3 cm and then cut into 1 mm rings. 6–10 rings were put into plastic culture dishes with Smooth Muscle Cell Growth Medium (Promocell, UK) containing 100 units/ml penicillin, 0.1mg/ml streptomycin and 5.6µg/ml amphotericin B, 5.0% FBS, 0.5 ng/ml epidermal growth factor, 2.0 ng/ml basic fibroblast growth factor and 5.0 µg/ml human recombinant insulin. Rings remained undisturbed in culture for 3–5 weeks until substantial SMC migration onto culture plastic occurred. Confluent primary SMC were dispersed using trypsin/EDTA (2.5 mg/ml; 0.01%, respectively), washed and re-plated and allow to grow to confluence. 4 days before experiments, SMC were re-plated on sterilized ø13 mm glass coverslips on 24-well culture plates and starved in low serum DMEM (0.25% FCS; Sigma-Aldrich, U.K.) to recover the contractile phenotype (Rainger *et al.*, 2001). SMC were isolated from two individual donors and used up to the fifth passage.

2.1.6.1. Chronic Hypoxia Treatment

After being starved for 2 days (see *Section 2.1.6*), HUASMC on coverslips were cultured in a 1% O_2 hypoxic incubator with 5% CO_2 at 37 °C for 72 hr before Ca^{2+} imaging experiments.

2.1.7. Validation of cell phenotype

The isolation of ECs from the human umbilical vein and artery using the methods described above is a well-documented protocol. Nevertheless, as the umbilical cord is also made of other cell types other than EC, namely SMC of the blood vessels, and also fibroblasts and SMC which are constituents of the Wharton Jelly (Takechi *et al.*, 1993), validation of cell phenotype with immunohistochemistry was warranted. The fluorescence-activated cell sorting (FACS) technique was used.

2.1.7.1. FACS

FACS is a specialised form of flow cytometry, in which cells are sorted according to florescence characteristics of each cell. Specific light scattering was conferred to each cell by fluorescence-tagged antibodies which bind to the EC-specific cell surface protein CD31 (Baldwin *et al.*, 1994).

HUVEC and HUAEC were isolated and cultured as described above. Cells were grown in 24-well plates and when confluent were washed with 0.02% EDTA solution (as described in *Section 2.1.3*). 200 µl of the 0.05% trypsin at RT was then added to each well and when the cells start to detach, typically after 1-2 min, 500 µl of pre-cooled (4ºC) trypsin inhibitor was added to each well. The solution in each well was agitated by pipetting it several times and this helped to dislodge any cells that remained attached. The content of each well was transferred to pre-labelled sterilised polyurethane tubes. For each cell type, 4 separate tubes were prepared: CD31 staining, an isotype control, unstained, and a blank. These would contain cells with anti-CD31 antibodies, cells with a non-specific Ig-G immunoglobulin, cells

without antibodies, and without cells, respectively (see below). 2 ml of 4ºC 2% bovine serum albumin (BSA; pre-made in PBS; Sigma-Aldrich, U.K.) was added to each of these tubes containing the detached cells. These were centrifuged at 4ºC 1500 rpm for 5 min.

Supernatant was aspirated after centrifugation and the cell pellet in each tube (except *blank*) was dislodged by carefully agitating the tubes and re-suspended in the following solutions: For the *CD31 staining* tube, 2 µl of a phycoerythrin (PE)-conjugated mouse monoclonal IgG1 anti-human CD31 antibodies diluted in 98 µl of the BSA solution (1:50 dilution from 25 µg/ml; R&D Systems, U.S.A.); For the *isotype control* tube, 2 µl of a PE-conjugated mouse monoclonal IgG1 non-specific antibodies diluted in 98 µl of the BSA solution (1:50 dilution; ImmunoTools, Germany); For the *unstained* tube, 100 µl of BSA; For the blank, 100 µl of BSA. These were then leaving on ice for 30 min to allow antibodies-antigen binding.

After the Incubation period, 3ml of the BSA solution was added into each tube and these were centrifuged at 4ºC 1500 rpm for 5 min. This was repeated again to remove excess, unbound antibodies. The cells are then fixed by adding 200 µl 2% formaldehyde solution (pre-made from 40% stock in PBS; Sigma-Aldrich, U.K.) into each tube, agitated, and these were left at RT for 20 min and then cooled to 4ºC.

The tubes containing the suspended cells were processed using a Dako CyAn™ FACS analyser according to granulation (side scatter) and volume (forward scatter). Flow cytometry data was analysed with FlowJo software (Tree Star, Inc).

2.2. HYPOXIA-INDUCED ATP RELEASE

Freshly isolated cells were first grown to confluence in 25cm^2 culture flasks (see above), before being transferred onto 24 well culture inserts (BD Falcon). The inserts had a membrane made of translucent PET (polyethylene terephthalate), with pore size of 0.4 μm and generally with pore density of (100 ± 10) x 10^6 / cm^2. The volumes of culture medium used were 200 μl and 700 μl for inside and outside the inserts, respectively, in order to maintain a neutral hydrostatic pressure across the membrane. The difference in volume between the two compartments is important when comparing the concentration of ATP released from the apical and basolateral side of the cells (see *Section 4.3.3*). In viability studies which required direct visualisation of cells by phase contrast light microscopy, transparent inserts were used. These had a lower pore density of (2.0 ± 0.2) x 10^6 / cm^2.

2.2.1. Induction of Hypoxia

Cell were plated at equal density between hypoxic and control cultures. Both cultures were grown to confluence in a 37 °C humidified incubator of 5% CO_2 and ATP release experiments were carried out typically 48 hr post seeding. The hypoxic and control cell cultures were always transported together in order to eliminate any potential unintended side-effects of changing temperature, pH, and mechanical disruption on ATP release. Immediately prior to exposure to 21% O_2 or hypoxia, the cells were carefully rinsed with normal Krebs' solution.

For hypoxic treatment, cells on culture inserts or 24 well culture plates were placed in a humidified incubator containing 1% O_2, 5% CO_2, balanced with N_2 at 37 °C, whilst the control culture was put in a conventional 37 °C humidified incubator containing 5% CO_2.

The O_2 % present in the hypoxic incubator was constantly monitored by a sophisticated real-time O_2 sensor, with a 0.1% O_2 sensitively (New Brunswick Scientific). Introducing the culture plates to the incubator necessitated the introduction of atmospheric air into the incubator and O_2% rose briefly (Fig 2.1). When the incubator was closed again it took approximately 10 min for the O_2 level to return to 1.0 % as detected by the O_2 sensor. The cells were incubated for 30 min from when the O_2 level in the incubator reached 1.0 + 0.1%. After this both the hypoxic and control cultures were taken from the respective incubators and concentration of the ATP in the medium was measured.

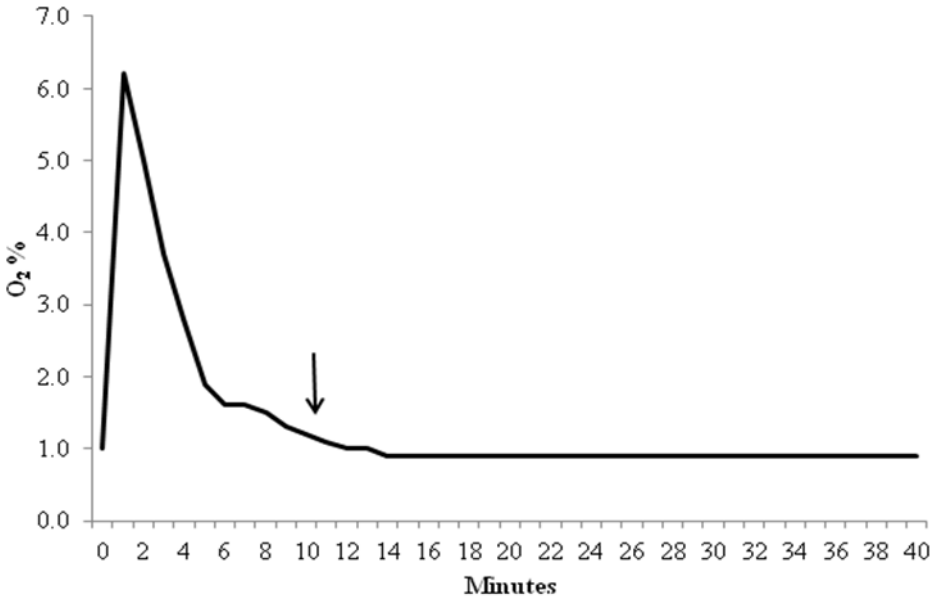


Figure 2.1. Continuous record of O_2 % in incubator. Cells were introduced into incubator at 0 min. Arrow denotes beginning of the 30 min hypoxic period.

2.2.2. ATP Measurement

2.2.2.1. Principle

ATP released from HUVEC and HUAEC was measured by using a conventional luciferin-luciferase assay: CellTiter-Glo® Luminescent Cell Viability Assay (Promega, U.K.). The assay contains cell lysis buffer, enzyme inhibitors (ATPase inhibitors) and a stable form of firefly luciferase, and was originally designed for viability study, in which the lysis buffer lyses the cells, the released cellular content of ATP reacts with the luciferase to produce a luminescent signal which is recorded, whilst simultaneously the endogenous enzymes as well as ectonucleotidases released by cell lysis are inhibited. The primary principle of this assay is that the strength of the luminescent signal is linear to the ATP content, and hence to the number of cells. In the present study, the assay was adapted to measure the ATP released by cultured cells.

2.2.2.2. Choice of assay

This particular assay product was chosen because, according to the manufacturer, it utilises a stable form of luciferase, based on the gene of the firefly *Photuris pennylvanica* (LucPpe2), which is less sensitive to chemical environment such as pH and detergents than the historical firefly luciferase purified from *Photinus pyralis* (LucPpy). In addition, the luminescent signal of this thermostable luciferase has a half-life of more than 5 hr, which minimizes the deleterious effect of any time lag between collecting samples and analysis (such as RT equilibration). The protocol developed in this project required careful manipulation of pipette for aspiration of culture medium without disturbing the monolayer of cells, which was a time-consuming process. Therefore, although more sensitive ATP-luciferase assays are available on the market, the relative stability of the CellTiter-Glo® Assay was considered as a more

important feature as far as the present project is concerned. Further, it contains ATPase inhibitors, which minimises interference with the accurate measurement of ATP concentration in the medium.

2.2.2.3. Exposure to hypoxia

Immediately prior to introduction to hypoxia, the culture medium was removed from both compartments of the culture inserts or the culture plates, and the cells were rinsed briefly with PBS containing Ca^{2+} and Mg^{2+}. This was then aspirated and replaced with fresh control culture medium or that containing the pharmacological agents. For culture inserts, 200 µl and 700 µl of growing medium were added to the inside and outside of the inserts respectively. For culture plates, 500µl of growing medium was added to each well. Triplicates of cells from at least three individual donors on culture inserts or plates were exposed to 30 min of 1% or 21% O_2 as described above. Wells containing medium but without cells were used as controls for background luminescence.

After 30 min of exposure to 1 % O_2 (~ 7.6 mmHg pO_2), 50 µl of medium from each compartment was collected for the luciferin-luciferance assay. For consistent results, the medium collected was equilibrated to room temperature (RT) for 30 minutes. It is because the rate of decay of the luminescent signal from the assay depends on the rate of the luciferase reaction, which in turn depends on environmental factors such as temperature. The medium collected from each well was then mixed with an equal volume of CellTiter-Glo® Reagent in separate wells of opaque-walled multiwell plates (Costar). These wells were selected because they have higher signal intensity and less cross talk between wells. The plates containing the medium/reagent mixture were incubated at RT for 10 min to stabilise the luminescent signal.

Luminescence was recorded using a luminometer (Perkin Elmer), with a measurement time of 1.0 s duration per sample.

2.2.2.4. ATP standards

In order to find out the actually concentration released by the cells, ATP disodium (Sigma-Aldrich, U.K.) was dissolved in UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, U.K.) to prepare 10.0 µM concentration stock solution, from which tenfold serial dilutions of ATP were made. Multiwell plates containing these concentrations of standard ATP solution were mixed with equal volume of CellTiter-Glo® Reagent. The plate was incubated at RT for 10 minutes to stabilize luminescent signal. Luminescence was recorded using the luminometer. A curve of Luminescence against ATP concentration was generated for each experiment (e.g. Fig 2.2), from which the concentration of ATP released from cells was calculated.

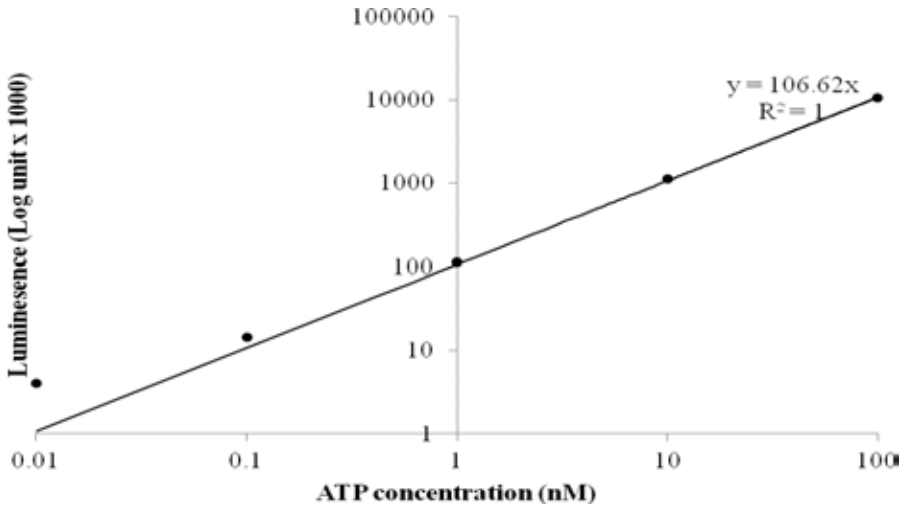


Figure 2.2. Example of an ATP standard curve on a logarithmic scale. This was repeated for each experiment.

2.2.3. Viability study

Lytic release ATP from dying cells may confound the results. Therefore, viability of cells was monitored with Trypan Blue (Sigma-Aldrich, U.K.), which could not penetrate the plasma membrane of intact cells. Following exposure to 21% O_2 or hypoxia, the medium in the cell culture was replaced with PBS containing 0.1% Trypan Blue. This was left for 10 min at RT before being replaced with normal PBS. The cells were then examined using a phase-contrast optical microscope, and the number of blue stained cells was counted. For positive control, cells were left to air dry for 10 min before being treated with Trypan Blue.

2.2.4. Visualisation of quinacrine-stained HUVEC

HUVECs are isolated and prepared on coverslips as described in *Section 2.1.1, 2.1.2 & 2.1.4*. Quinacrine is an acridine derivative and because of its high affinity to ATP, has been used experimentally as an indicator of intracellular ATP (see *Chapter 5* Section 5.1 and 5.4.1 for further discussions regarding mechanisms and specificity). Quinacrine was prepared freshly on the day in Krebs-Ringer buffer of the following composition (mM): NaCl 125, KCl 5.0, $CaCl_2$ 2.0, KH_2PO_4 0.7, $MgSO_4$ 1.0, HEPES 25.0, and glucose 6.0. Cells were prepared for confocal microscopy by pre-incubation in 1.5 μM quinacrine for 60 min, and carefully rinsed twice before mounted on normal glass microscopy slides with VECTASHIELD® Mounting Medium with DAPI. These were examined with a confocal microscope (Zeiss LSM510). Digital reconstruction of spinning disk confocal z stacks was done by Zeiss ZEN software and data was analysed with Zeiss LSM Image Browser.

To examine the effect of hypoxia on staining, HUVEC were stained with quinacrine in either 21% O_2 or hypoxia (for protocol see *Chapter 5 Section 5.2.2*), mounted on microscope slides

in mounting medium without DAPI and were immediately examined under an UV light microscope (Axioskop 2 *plus*, Zeiss), with excitation at 494 nm and emission captured at 518 nm, and images recorded by a AxioCam MRc camera (Zeiss) and stored on a PC. To examine the effect of vesicle trafficking inhibitors on quinacrine staining, HUVEC was incubated in brefeldin A (10 μM), monensin (100 μM), or vehicle (1:1000 DMSO) for 60 min before the medium was change to quinacrine for a further 5 min, all in a conventional culture chamber. 30 μM for 5 min was determined as the optimal quinacrine staining conditions as it provided the most even staining in HUVEC out of out of 1, 3, 10 and 30 μM with 5, 30 and 60 min combinations in preliminary experiments. The cells were carefully rinsed twice with Krebs' before mounted as above. All direct comparisons of fluorescence intensity were made between images taken at identical exposure time and gain settings. These are indicated in each Figure.

2.3. CALCIUM IMAGING

2.3.1. Principle

Real time changes in $[Ca^{2+}]_i$ in HUASMC and HUVEC were measured using conventional UV fluorescence imaging technique. There are single- and double-wavelength fluorescence indicators available on the market, Calcium Green-1 AM and Fura-1 AM (both Invitrogen, UK). The former works on the principle that when bound to its parameter of interest, in this case Ca^{2+}, it emits fluorescence at a given wavelength. The greatest concern for using a single-wavelength probe is that the level of fluorescence emission is dependent on the amount of the dye present, which in turn depends on variables such as the rate of uptake of the dye by the cells, and the rate of decline of the quantum yield of the probe, as the dye is repeatedly

exposed to UV light during the course of the experiment (a process called photo-bleaching). This could lead to difficulties in interpreting changes in measured fluorescence.

Fura-2 is a double-wavelength UV light-excitable indicator, first developed in the 1980s. It was developed from the Ca^{2+} chelator BAPTA, which is essentially a double aromatic analogue of EGTA, with fluorophores incorporated into the molecule (Grynkiewicz *et al.*, 1985). In contrast to single-wavelength indicators, upon binding Ca^{2+}, it exhibits an absorption shift around the excitation spectrum of 360 nm, which is visible when being monitored at the emission wavelength of 510 nm (O'Connor & Silver, 2007).

Thus, the fluorescent intensity at excitation below 360 nm is *positively* correlated to free Ca^{2+}, whereas at excitation above 360 nm it is *negatively* correlated. The ratio of the emitted fluorescence are used to determine the $[Ca^{2+}]_i$. Hence, the fluorescence measurements made with dual-wavelength excitation probes is independent of the amount of dye present, yet correlated to $[Ca^{2+}]_i$. The use of a dual-excitation ratiometric indicator, as opposed to a single-wavelength excitation indicator, gives several additional advantages, in the form of normalisation of artefact from the recording environment such as uneven cell thickness, compartmentalisation and instrumentation noise.

Fura-2 is hydrophilic and is therefore membrane-impermeable. Negative charges on the molecule are masked by acetoxymethyl (AM) esters in order to make it lipophilic. In this form, the dye can passively diffuse across the cell membrane. When it is in the intracellular space, endogenous non-specific esterases cleaves off the hydrolysable AM ester, leaving the ionised form of the dye trapped inside the cell, ready to bind Ca^{2+}.

2.3.2. Fluorescence indicator loading protocols

$[Ca^{2+}]_i$ in HUASMC and HUVEC was measured using the non-ratiometric or radiometric dye, Calcium Green-1 AM or Fura-2 AM, respectively (both Invitrogen, UK). 50 μg of the dye was dissolved in 20 μl 20% pluronic acid (0.01 g in 50 μl DMSO) as the stock concentration making a final concentration of 12.5 μM. Before each experiment, the growing medium was removed and replaced by 200 μl Krebs solution containing the respective dye. The HUVEC, on sterilized EPSE-coated ø13 mm glass coverslips on 24-well culture plates, were left at room temperature in atmospheric air for 30 min before incubation in a 37 °C humidified incubator of 5% CO_2 and balanced air for a further 30 minutes. HUASMC were incubated for 60 minutes without the room temperature incubation period. Immediately prior to placing the coverslips into the recording chamber, they were rinsed in normal Krebs solution to remove residue dye.

For HUASMC that has undergone chronic hypoxic treatment, Calcium green was loaded onto the cells in the hypoxic incubator for 60 min prior to experiments, which were carried out immediately after the cells were re-introduced to atmospheric air.

2.3.3. Cytosolic free calcium measurement

Cells on glass coverslip (see *Section 2.1.1*) were placed into a diamond-shape cuvette which enabled laminar flow. The cuvette chamber was sealed by a glass coverslip and was perfused with Krebs' buffer maintained at 37 °C bubbled with either 95% air/5% CO_2 (normoxia), or 95% N_2/5% CO_2 (hypoxia). The perfusion was provided by a peristaltic pump (MINIPULS® 3 by Gilson, Inc.) at a constant rate of 22.5 ml/min unless otherwise stated. The level of O_2

was measured by a polarographic mini clark style O_2 electrode (Diamond General Development Corp.; product no. 733) connected to a dissolved O_2 meter (VWR International; product no. ISO2-A) at the chamber outflow, and consistently measured between PO_2 7.6 – 9.9 mmHg in the hypoxic solution. The O_2 electrode was calibrated before each series of experiments by following the manufacturer's instructions. Lead time was less than 10 s upon switching from normoxic to hypoxic solution. The cuvette containing the glass coverslip was placed in the stage of an Olympus IX71 inverted microscope. For HUASMC, fluorescence changes of Calcium Green-1 AM were measured with excitation at 488 nm and emission at 528 nm. For HUVEC, Fura-2 AM fluorescent dye was excited alternatively at 340 and 380 nm (F_{340}, F_{380}) and emissions were captured at 510 nm (*F 510*) with a CCD camera (Hamamatsu Ltd). Images were collected at the resolution of 1344 x 1024 pixels every 3, 5 or 10 seconds, with 10 second intervals reserved for protocols exceeding 30 min to minimize the effect of photo bleaching. Background subtraction was used and the change in $[Ca^{2+}]_i$ were expressed either as: $\Delta F/F$, where F was the fluorescence intensity when cells were at rest and ΔF was the change in fluorescence during stimulation (HUASMC), or as F_{340}/F_{380} (HUVEC). Images were analysed offline using WASABI Imaging Software (Hamamatsu Photonics GmbH, Germany), when changes in $[Ca^{2+}]_i$ in single cells or groups of cell were delineated.

Modified Krebs' solution for HUVEC $[Ca^{2+}]_i$ measurement contained (mM): NaCl 118, KH_2PO_4 1.2, KCl 4.7, $CaCl_2$ 1.8, $MgSO_4$ 0.8, D-glucose 5.5, $NaHCO_3$ 23.8. Ca^{2+}-free solution contained (mM): NaCl 118, KH_2PO_4 1.2, KCl 4.7, $MgSO_4$ 0.8, D-glucose 11.1, $NaHCO_3$ 23.8, EGTA 0.2. High K^+ Krebs solution for HUVEC $[Ca^{2+}]_i$ measurement contained: NaCl 52.7, KH_2PO_4 1.2, KCl 70, $CaCl_2$ 1.8, $MgSO_4$ 0.8, D-glucose 11.1, $NaHCO_3$ 23.8. Earle's balanced salt solution (EBSS) for HUASMC $[Ca^{2+}]_i$ measurement contained

(mM): NaCl 116.3, NaH_2PO_4 1, KCl 5.3, $MgCl_2$ 1, $CaCl_2$ 1.8, $NaHCO_3$ 13.8, D-glucose 11.1. High K^+ EBSS for HUASMC $[Ca^{2+}]_i$ measurement contained (mM): NaCl 66.3, NaH_2PO_4 1, KCl 45.3, $MgCl_2$ 1, $CaCl_2$ 1.8, $NaHCO_3$ 23.8, D-glucose 11.1. The pH of all solutions was adjusted to 7.3 with HCl (10 mM) and NaOH (3 mM).

2.4. MYOGRAPHY

Vascular vasomotion was measured using myography. The sections below describe the isolation and validation of human umbilical arteries, the equipment, data acquisition techniques, and calibration of both hard- and softwares.

2.4.1. Isolation and Validation of Human Umbilical Blood Vessels

Full term placentae from normal pregnancies delivered by vaginal or caesarean sections at the maternity unit of the Birmingham Women's Hospital, located within 5 minute walk from the laboratory, were transported from the delivery suite to the laboratory within 2 hr of parturition. Upon arrival at the laboratory, segments of umbilical arteries derived from the cord segment closest to the fetus (usually within 10cm) were dissected in ice cold buffer, carefully avoiding endothelial and smooth muscle damage. 4 arterial rings of 1.0 – 1.5 mm in width were prepared microscopically from each umbilical artery. Each ring was mounted between supporting pins and isometric contractions from the circular layer of these vessels were recorded (see below).

Initially the traditional 'blunt dissection' technique was employed to isolate the umbilical arteries. However, it was found that vasomotion was not consistently present in the blood vessels isolated using this method (spontaneous vasomotion was present in only 26.0% of

vessels). After some eight weeks' of trial and error, a new set of dissection method was developed, which involved the retention of more connective tissue, a change to new ice-cold buffer every 5 minutes during the isolation procedure. In addition, vessels were stretched to resting tension (see below) before the incubation period in the myograph chamber. Blood vessels isolated by using this protocol demonstrated a markedly higher rate of vasomotion (62.5%), which provided a more reliable model of studying the vascular phenomenon.

For the myograph experiments, the arterial rings were placed in Krebs-Ringer buffer of the following composition (mM): NaCl 118, KCl 5.4, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 0.8, $NaHCO_3$ 23.8, and glucose 11.1. High K^+ Krebs-Ringer buffer contained (mM): NaCl 53.4, KCl 70, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 0.8, $NaHCO_3$ 23.8, and glucose 11.1. The buffers were maintained at 37 °C bubbled with a 95% O_2 / 5% CO_2 mixture, final pH 7.4.

2.4.2. Calibration of Myography

Before each series of myograph experiments the myograph system was fully calibrated as per manufacturer's instructions with suitable adjustments for the use of supporting pins. The full calibration protocol is described here: The support mounting pins were moved apart in all the myograph chambers and the units were placed on the interface with the cables connected. The chambers were then filled with double distilled water. Next, the heating element of the myograph plateform was turned on. This was important as temperature is one of the variables of the force transducer output. Typically, the system reached the target set temperature (37ºC) after ~20 min.

Next, the calibration bridge, balance and weight were set up and pre-warmed on the myograph units. It was important that the calibration kit was pre-warmed together with the myograph unit to minimize temperature variation of the chambers, which would consequently affect the force transducer output. Once the system has been warmed up to the pre-set temperature (verified by a temperature probe placed in the chamber), the Calibration Program of the Myo-Interface was initiated.

In brief, the tip of the transducer arm on the balance was placed between the closest pin and the force transducer and the calibration bridge was moved carefully until the tip of the transducer arm was as close as possible to the pin but without touching it. It was imperative that the force transducer was not subjected to any force as this stage. A relative force of zero was then registered on the Myo-Interface Calibration Program. To generate the other point on the calibration curve, a 2.0 g weight was carefully placed on the pan of the calibration bridge to mimic the stretch created by a contraction of a mounted vessel ring preparation. When the relative force reading was stable, the force was again registered on the Myo-Interface Calibration Program. Force transducer 1 was now weight-calibrated to an output of 9.81 mN. The weight, balance and calibration bridge were then carefully removed from myograph unit 1. Force transducer 2, 3 and 4 were also calibrated using the same methods described above.

2.4.3. Data Acquisition

Force generated by the vessel rings were recorded by a myograph machine (Multi Myograph Model 610 M; Danish Myo AB). Mounting support pins instead of jaws were used because of the relative large luminal size (>500nm). Each vessel ring was mounted between the two supporting pins, one of which is riveted on the myograph unit; the other supporting pin was

connected directed to a force transducer. Therefore, any contraction response of the vessel rings could be accurately monitored and recorded. Extreme care was taken when mounting the vessel sections so as to minimise potential damage to both the luminal surface and the outer smooth muscle layer of the vessels. The mounting pins were adjusted as closely as possible to each other (without touching) so no force is exerted on the vessel.

In order to study the isometric response of the blood vessels, they were stretched to a normalised basal tone as determined by the DMT Normalization software, which calculates the internal diameter of the blood vessels when exposed to the *in vivo* blood pressure, which was set as 50 mmHg. In this normalization technique, the vessel rings were manually stretched stepwise using a myograph micrometer and the contractile force response was recorded. For every stretch the tension of the vessel increased sharply, was allowed to stabilise, before the vessel was again manually stretched. The force generated on the vessel rings were constantly monitored and recorded. The DMT Normalization software uses the micrometer reading and contractile force values for each stop in the recording to generate a tension vs. internal circumference curve, which was then used to determine the internal circumference. The vessel rings were stretched to their respective internal circumferences, and was generally approximately 10 mN. This was taken as the basal tension. The vessel rings were then left for 1 hr, with a change of buffer every 15 min, before the start of the experiments. Change of buffer was an automated process via a vacuum pump, and pharmacological agents were either dissolved in pre-warmed Krebs-Ringer buffer or introduced as concentrated stock solution directly into the chamber.

The superfusate was constantly bubbled with 21% O_2 / 5% CO_2 and balanced N_2. Hypoxia was simulated by bubbling the superfusate with 95% N_2 / 5% CO_2, with a gentle flow of argon above the recording chamber. PO_2 levels were recorded with a blood gas machine (IL1640; Instrumentation Laboratories, Warrington, UK), and were found to be 160 mmHg and 50 mmHg for normoxia and hypoxia, respectively.

At the beginning and end of each experiment the vessel rings were challenged with 70 mM KCl, to evoke a strong contraction of the circular smooth muscle layer. Data recorded from vessel rings that did not develop a KCl contracture of at least 10 mN were discarded. Recording from freshly isolated human umbilical cord artery were compared to other recorded vasomotion in the literature (Gokina *et al.*, 1996; Peng *et al.*, 2001; Garcia-Huidobro *et al.*, 2007), to ensure that their properties were consistent with each other. In particular, frequency and amplitude of vasomotion were compared.

Data acquisition was carried out using a Powerlab. The LabChart software (ADInstruments) was used to record the data for post-experimental analysis on a Mac computer.

2.4.4. Immunohistochemistry

In order to assess the role of endothelium in vasomotion, half of the vessel from a number of donors were treated with gentle injection of air thorough the vessel lumen with a syringe connected to a cannula. After the myography experiments, the vessel rings were removed from the supporting pins. They were orientated on a cork disc in OCT gel (VWR International) and immersed in pre-chilled isopentane, as per normal specimen freezing

technique. They were then further cooled in liquid nitrogen, and subsequently stored in at -80ºC.

Before sectioning, the frozen sample on cork disc were warmed up to -24ºC in the cryostat compartment for 15 min. They were then sectioned at between 10-20 µm thickness (specified at respective figures). The sections were mounted on pre-labelled Polysine® slide (VWR International) and stored at -80ºC.

For immunohistochemistry, sections on slides were warmed up to RT for 30 min. A wax circle was drawn around each sample on the slides and left to dry for 15 min. They were then fixed in 4% formaldehyde (VWR International) made in PBS (Oxoid Ltd, UK). After 30s, they were rinsed twice in normal PBS for 3 min each. Excess fluid was removed from each slide using tissue and they were place in a humidified incubation chamber. The samples were covered with blocking solution (PBS with 0.05% Tween 20, 2% BSA and 15% normal goat serum, the former two from Sigma-Aldrich, U.K. and latter from Fitzgerald Industries International, U.S.A., respectively). After 20 min, the slides were drained of blocking solution, and were replaced by either 1:20 dilution PE-conjugated mouse monoclonal IgG1 anti-human CD31 antibodies, or 1:20 dilution PE-conjugated mouse monoclonal IgG1 non-specific antibodies as negative control (see *section 2.7.1*). The antibodies were prepared in PBS with Tween 20 (0.05%) and BSA (2%). The slides were incubated at 4ºC for 16 hr.

After 16 hr, the slides were warmed to RT for 30 min. The slides were washed in PBS 3 times, for 3 min each. The slides were drained of excess fluid, and mounted on normal glass microscopy slides with VECTASHIELD® Mounting Medium with DAPI. The slides were

examined under an inverted UV light microscope (Axioplan 2 *imaging*, Zeiss), with excitation at 488 nm and emission captured at 578 nm for PE; excitation at 358 emission captured at 461nm for DAPI. Images were recorded by AxioCam HRc (Zeiss), and dual staining images were superimposed offline using Adobe® Photoshop. Importantly, when direct comparisons were made, photos were captured at identical exposure and gain settings. Preliminary experiments in the present study showed that only the anti-CD31, but not the non-specific IgG1 antibodies stained positive for the endothelium.

2.5. Statistical methods

For the present thesis. a number of different experimental approaches were used, including $[Ca^{2+}]_i$ imaging, luciferin-luciferase ATP release measurement, confocal and conventional microscope, immunohistochemistry and myograph. Some of these techniques were performed on multiple tell types, namely HUASMC, HUAEC and HUVEC. As a result, different statistical methods were chosen for the individual sets of experiments. The specifics of the protocols and respective statistical method of analysis are included in the end of the *Methods section* of each individual chapter.

CHAPTER 3

THE EFFECT OF ATP ON $[Ca^{2+}]_i$ IN HUMAN UMBILICAL ARTERY SMOOTH MUSCLE CELLS

3.1. INTRODUCTION

The need for a sophisticated fetal life support system, which includes the umbilical vessels, for a normal and healthy pregnancy was discussed in *Chapter 1 Section 1.2.1*. Vasomotion is the rhythmic contraction of blood vessels independent of heart rate. It is displayed by many vascular beds and importantly has recently been studied *in intro* in human umbilical vessels (Garcia-Huidobro *et al.*, 2007). The body of evidence seems to suggest that, under under-purfused conditions, vascular vasomotion could result in an improvement of downstream blood flow and tissue oxygenation (see *Chapter 1 Section 1.5.1*).

Separately, it has long been hypothesised that hypoxia is able to induce ATP release from the endothelium (see *Chapter 1 Section 1.4.2*), but there has been no direct evidence to support this (see *Chapter 4*). Therefore, the experiments in this study were designed to investigate firstly whether primary SMC derived from the human umbilical artery (HUASMCs) display $[Ca^{2+}]_i$ oscillations, which have previously been observed in synchronisation with vasomotion and are believe to drive vasomotion (Aalkaer & Nilsson, 2005), and secondly whether exogenously applied ATP increases the frequency of these $[Ca^{2+}]_i$ oscillations, as they would be expected to increase the frequency of vascular vasomotion.

Having established that exogenous ATP could indeed induce $[Ca^{2+}]_i$ oscillations in HUASMC, experiments were carried out which were designed to establish the source of Ca^{2+} that underlies the $[Ca^{2+}]_i$ oscillations. Namely, the removal of extracellular Ca^{2+} together with the Ca^{2+} chelator EGTA, and the depletion of intracellular Ca^{2+} stores with the sacroplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor thapsigargin (TG).

In *Chapter 1 Section 1.3.6*, it was discussed that P2 receptors are activated by extracellular ATP and are responsible for the vascular actions of ATP. However, the molecular identity of the ion channels or receptors subtypes which mediate the ATP-induced $[Ca^{2+}]_i$ oscillations in VSM remains unknown. In the present study, α,β-methylene ATP was used as a specific agonist of P2X receptors (Kitajima *et al.*, 1993; Zhao & van Helden, 2002), and UTP was used as a selective agonist of $P2Y_{2,4}$ receptors when it was found that P2X receptors could not explain the action of ATP (Charlton *et al.*, 1996). They were used at concentrations previously shown to be effective at increasing $[Ca^{2+}]_i$ in other cell types (Kitajima *et al.*, 1993; Charlton *et al.*, 1996; Zhao & van Helden, 2002).

The potential role of P2 receptors in $[Ca^{2+}]_i$ oscillations and their mechanism of action was also tested using a pharmacological approach. P2Y receptor activation leads to the activation of phospholipase C (PLC), which generates inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) and leads to further Ca^{2+} influx (Burnstock & Knight, 2004). The mechanism of this Ca^{2+} influx is complex and is currently a subject of much attention. A number of mechanisms have been proposed. First, there are the store-operated Ca^{2+} channels (SOCC), which include some of the subtypes of the transient receptor potential cation (TRPC) channels. These are activated by the emptying of intracellular Ca^{2+} stores via IP_3R, which subsequently conduct Ca^{2+} influx (capacitative Ca^{2+} entry) (Parekh & Putney, 2005). Second, there are receptor-operated Ca^{2+} channels (ROCC) which are activated by DAG (from GPCR and PLC activation) and include some other subtypes of TRPC channels (Birnbaumer, 2009). Third, the well-characterised voltage-gated calcium channels (VGCC) that are ubiquitous in excitable cells and forms the important link between membrane excitability and physical contractility; and finally Na^+-Ca^{2+} exchanger when working in reverse mode. Experiments

were therefore carried out to explore the presence of these Ca^{2+} influx mechanisms in HUASMC.

As discussed in *Chapter 1 Section 1.2.2.6*, intrauterine hypoxia is a feature in both normal and complicated human pregnancy human. The evidence of evidence of paradoxical increase in EN expression with chronic hypoxia was also discussed in *Section 1.4.4.2.* Accordingly, in a set of experiments HUASMC were exposed to chronic hypoxia for 72 hr to simulate *in-vitro* hypoxia (see *Section 2.1.6*) and the $[Ca^{2+}]_i$ response to ATP was recorded and compared to those without prior hypoxic treatment.

3.2. METHODS

All experiments in the studies described in the present chapter were carried out on cultured SMC isolated from human umbilical arteries as described in *Chapter 2 Section 2.1.6*. The details of the methodology were described in *Chapter 2 Section 2.1.3, 2.1.6 and 2.3*. The description below provides the details of the protocols. For clarification, HUASMC were loaded with Calcium Green-1 AM (Invitrogen, UK) for Ca^{2+}-imaging, the exact protocols of which could be found in *Chapter 2 Section 2.3.2*. The cells were isolated from two separate donors. The number of coverslips (N) and the number of cells (n) used in each protocol are indicated in the appropriate figure legends. All protocols began with a challenge with EBSS containing 45 mM K^+ followed with a 10 min wash to validate that cells were viable.

Protocols

3.2.1. Group 1: $[Ca^{2+}]_i$ oscillations at rest

HUASMC on glass coverslips were placed in the recording chamber and changes in $[Ca^{2+}]_i$ under control condition were initiated recorded using fast-frame (200 ms intervals). To conserve storage space, images were subsequently recorded at 3s intervals. $[Ca^{2+}]_i$ changes in individual cells were monitored (see *Chapter 2 Section 2.3.3*). n > 200, N = 6.

3.2.2. Group 2: Effect of exogenous ATP

After 4 min of recording at basal condition, 10 – 1000 μM ATP were added to the HUASMC at random with 10 min washout intervals. n > 200, N = 6.

3.2.3. Group 3: Source of Ca^{2+} and effect of changing extracellular $[Ca^{2+}]$

In order to test the hypothesis that extracellular Ca^{2+} is important for the initiation or maintenance of ATP-induced $[Ca^{2+}]_i$ oscillations, extracellular Ca^{2+} in the EBSS was replaced with the Ca^{2+} chelator EGTA (200 uM) for 2 min, after which the HUASMC were challenged with ATP, again in the absence of extracellular Ca^{2+} (with EGTA). n = 80, N = 3.

In other experiments in Ca^{2+}-free EBSS (with EGTA), in order to assess the role of intracellular Ca^{2+} in ATP-induced $[Ca^{2+}]_i$ oscillations, internal Ca^{2+} stores in the HUASMC were first depleted by applying TG (1 μM) in the presence of EGTA, then bath application of ATP with Ca^{2+} was added to the cells whilst the fluorescence was being recorded. n = 70, N = 3.

In a separate set of cells, the effect of varying the concentration of external $[Ca^{2+}]$ on the frequency of $[Ca^{2+}]_i$ oscillations was assessed by changing the $[Ca^{2+}]$ in the EBSS bathing the

HUASMC in the presence of ATP (100 μM). $[Ca^{2+}]$ at various concentrations (0.9, 1.8 and 3.5 mM) with ATP were applied at random order, with 10 min wash intervals. n = 80, N = 3.

3.2.4. Group 4: Receptor identity

To identify the receptor responsible for mediating the ATP-induced $[Ca^{2+}]_i$ oscillations in HUASMC, the $[Ca^{2+}]_i$ response to the specific P2X receptors agonist α,β-methylene ATP (20 μM; n = 90, N = 2), or the selective $P2Y_{2,4}$ receptors agonist UTP (10 μm; n = 60, N = 2), were tested as described for ATP in *Group 2*.

3.2.5 Group 5: Modulation of $[Ca^{2+}]_i$ oscillations

In other experiments, $[Ca^{2+}]_i$ oscillations were induced by ATP (100 μM) as in *Group 2*. Once induced, the general purinoceptors suramin (100 μM), the non-specific IP_3R and SOCC/ROCC inhibitor 2-Aminoethoxydiphenyl borate (2-APB; 50 μM), the specific PLC inhibitor U-73122 (5 μM), the VOCC inhibitor nifedipine (10 μM), or the combination of suramin, 2-APB and nifedipine, were added at random order. The cells were washed for 10 min before re-application of ATP to evoke new $[Ca^{2+}]_i$ oscillations, on which a different inhibitor was tested. n > 100, N = 4.

3.2.6. Group 6: SOCC and ROCC

1-oleoyl-2-acetyl-sn-glycerol (OAG), a cell-permeable analogue of DAG, is a known direct activator of ROCC (believed to be a number of the TRPC family; see *Section 3.4.5*). HUASMC was stimulated with 100 μM OAG, a concentration previously shown to activate a TRPC current using patch clamp technique (Chen *et al.*, 2009), and the changes in $[Ca^{2+}]_i$ was recorded.

In order to test the functional presence of SOCC in HUASMC, the intracellular Ca^{2+} stores of a separate set of cells were depleted by bathing the cells in 0 Ca^{2+} EGTA (200 μM) solution with TG (1 μM), as in *Group 3*. The 0 Ca^{2+} EGTA EBSS was subsequently replaced by normal Ca^{2+}-containing EBSS with TG. If SOCC are functionally active, the introduction of external Ca^{2+} should evoke a $[Ca^{2+}]_i$ elevation. Further, a range of widely used inhibitors of SOCC were used to test if they were able to interfere with any $[Ca^{2+}]_i$ elevation following store depletion. These were 2-APB, the lanthanide lanthanum (La^{3+}), and the combined SOCC / ROCC inhibitor SKF-96365 (Facemire & Arendshorst, 2005; Smani *et al.*, 2008).

3.2.7. Group 7: Chronic hypoxia

After 2 days of starvation, HUASMC on sterilised ø13.0 mm glass cover slips on 24-well culture plates were placed in a humidified hypoxic chamber with 1% O_2, 5% CO_2, balanced N_2 at 37 °C for 72 hr, before loaded with Calcium green for 60 min. $[Ca^{2+}]_i$ in the cells were measured immediately after cells were re-introduced to atmospheric air. The details of the hypoxic chamber and dye loading protocol was described in the relevant sections of *Chapter 2*. The cells were tested as in *Group 1 & 2*.

3.2.8. Analysis of results

Post hoc analysis was carried out using OpenLab software. Individual cells or group of cells were outlined and analyzed as the region of interest (ROI) and expressed as n. The same experiments were repeated on different coverslips (N) over different isolation batches from different donors, which were indicated at each result figure. Data are presented as mean ± S.E.M., and findings made under different conditions were compared using Student's

paired *t*-test, taking $P < 0.05$ as significant. Oscillation frequency of each ROI was calculated by dividing the number of recorded $[Ca^{2+}]_i$ spikes (using WASABI Imaging Software as detailed in *Chapter 2 Section 2.3.3*) by the duration of observation and expressed in Hz (10^{-3}).

3.3. RESULTS

3.3.1. Group 1

Only a small percentage of HUASMC displayed spontaneous $[Ca^{2+}]_i$ oscillations (about 1%). The amplitude of these spontaneous $[Ca^{2+}]_i$ elevations were small; less than 5% $\Delta F/F$, and the frequency varied from 3 to 10 x 10^{-3} Hz (data not shown).

3.3.2. Group 2

In the initial experiments of this type, ATP (10, 50, 100 & 200 μM) caused regular $[Ca^{2+}]_i$ oscillations in ~70% of HUASMC (Fig 3.3.1), no $[Ca^{2+}]_i$ oscillations was observed in the remaining 30% of cells. The amplitude and frequency of the $[Ca^{2+}]_i$ oscillations were dependent on the [ATP] applied (Fig 3.3.2); below 100 μM ATP, an increase in [ATP] enhanced both amplitude and frequency, whereas over 100 μM ATP, an increase in [ATP] decreased the amplitude and frequency. The $[Ca^{2+}]_i$ oscillations induced by 100 μM ATP were quenched by application of 1000 μM ATP (Fig 3.3.3).

3.3.3. Group 3

When HUASMC were bathed in 0 Ca^{2+} with EGTA, a rapid increase in $[Ca^{2+}]_i$ was observed when ATP was applied (100 μM; Fig 3.3.4). The amplitude of this $[Ca^{2+}]_i$ elevation was comparable to that seen in the presence of extracellular Ca^{2+}, but this was not followed by $[Ca^{2+}]_i$ oscillations (Fig 3.3.4). After depletion of intracellular Ca^{2+} stores with 1 μM TG and

EGTA in a separate set of cells, stimulation with 100 μM ATP in Ca^{2+}-containing bath solution induced $[Ca^{2+}]_i$ oscillations (Fig 3.3.4). In fact, in normal, Ca^{2+}-containing EBSS, the frequency of the $[Ca^{2+}]_i$ oscillations induced by 100 μM ATP showed a linear relationship with external $[Ca^{2+}]$ ($R^2 = 0.9863$; Fig 3.3.5).

3.3.4. Group 4

When HUASMC were exposed to the specific P2X receptor agonist α,β-methylene ATP (20 μM), a rapid transient $[Ca^{2+}]_i$ elevation was recorded, but not $[Ca^{2+}]_i$ oscillations (fig 3.3.6). In contrast, when the cells were exposed to the selective $P2Y_{2,4}$ receptors agonist UTP (10 μM), $[Ca^{2+}]_i$ oscillations similar to those induced by ATP were observed (fig 3.3.6).

3.3.5. Group 5

$[Ca^{2+}]_i$ oscillations were induced in HUASMC by ATP (100 μM). Once induced, suramin (100 μM) increased the oscillation frequency, whereas nifedipine (10 μM) decreased it (Fig 3.3.7). 2-APB (50 μM) and U-73122 (5 μM) did not affect the oscillations (Fig 3.3.7). However, a combination of suramin, 2-APB and nifedipine, almost completely inhibited the $[Ca^{2+}]_i$ oscillations, whilst this did not change basal $[Ca^{2+}]_i$ significantly (Fig 3.3.7). The averaged results are summarised in Fig 3.3.8.

3.3.6. Group 6

OAG (100 μM), the cell-permeable direct activator of ROC, caused a significant increase in $[Ca^{2+}]_i$, and 2-APB (100 μM), the non-specific SOCC antagonist, inhibited 67 $\pm$ 13% of this OAG-induced $[Ca^{2+}]_i$ elevation (Fig 3.3.9).

In a separate set of cells, return of Ca^{2+} to HUASMC that had intracellular Ca^{2+} store depleted with O Ca^{2+} EBSS and EGTA (200 μM) evoked a significant $[Ca^{2+}]_i$ elevation, which was amenable to inhibition by 2-APB, La^{3+} and SKF-96365 to varying degrees (Fig 3.3.10).

3.3.7. Group 7

After incubation of HUASMC at 1% O_2 for 72 hr, approximately 1% of cells exhibited spontaneous $[Ca^{2+}]_i$ oscillations, a similar proportion to those recorded from control cells without hypoxic treatment. The frequency vs. concentration curve was apparently left shifted relative to that observed in normoxic cells (Fig 3.3.11). Thus, at 10 μM, ATP caused a greater $[Ca^{2+}]_i$ oscillation frequency in chronically hypoxic cells, whereas at concentrations > 100 μM, the oscillation frequency was lower in hypoxic cells than normoxic cells. With regards to amplitude of the ATP-induced $[Ca^{2+}]_i$ oscillations, those of the chronically hypoxic cells were greater at all ATP concentrations tested (Fig 3.3.11).

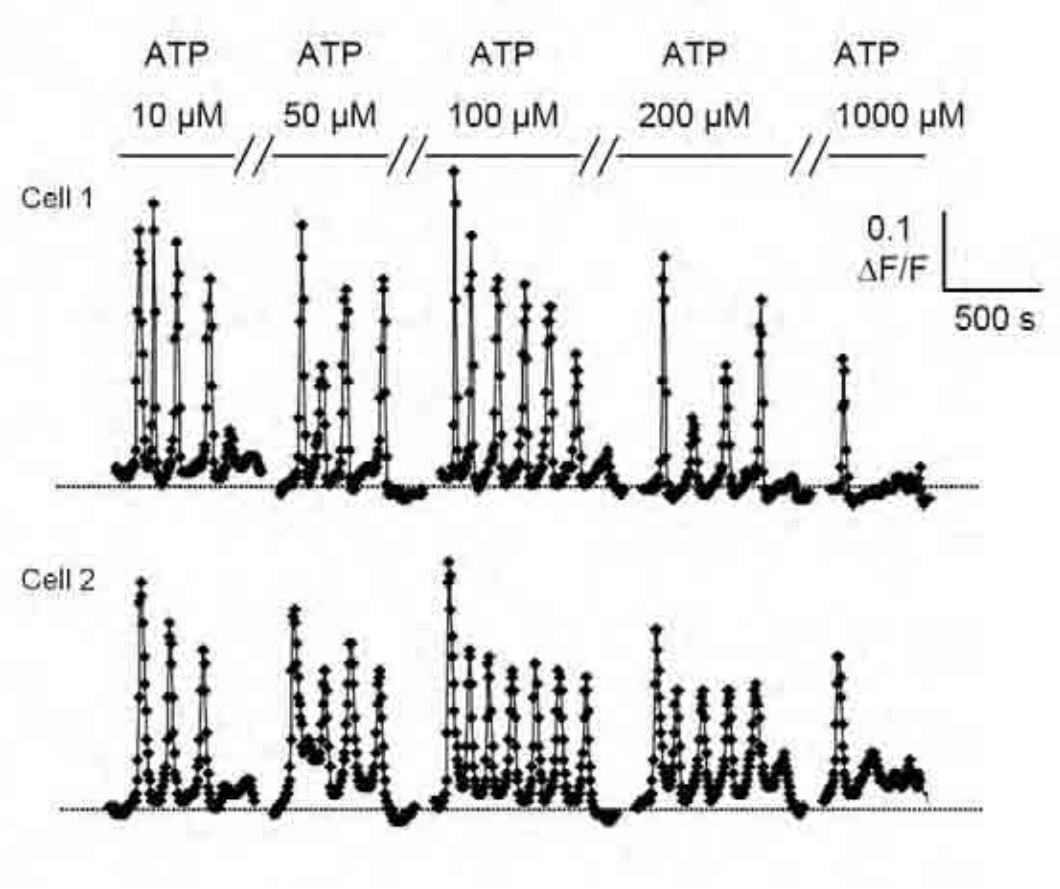


Figure 3.3.1. $[Ca^{2+}]_i$ oscillations induced in two HUASMC by micromolar concentrations of ATP.

Concentrations of ATP were applied in random with 10 minus wash intervals but are displayed in incremental order for ease of interpretation. At concentrations < 100 µM, ATP enhanced the amplitude and frequency of the $[Ca^{2+}]_i$ oscillations; > 100 µM, ATP decreased $[Ca^{2+}]_i$ oscillations amplitude and frequency.

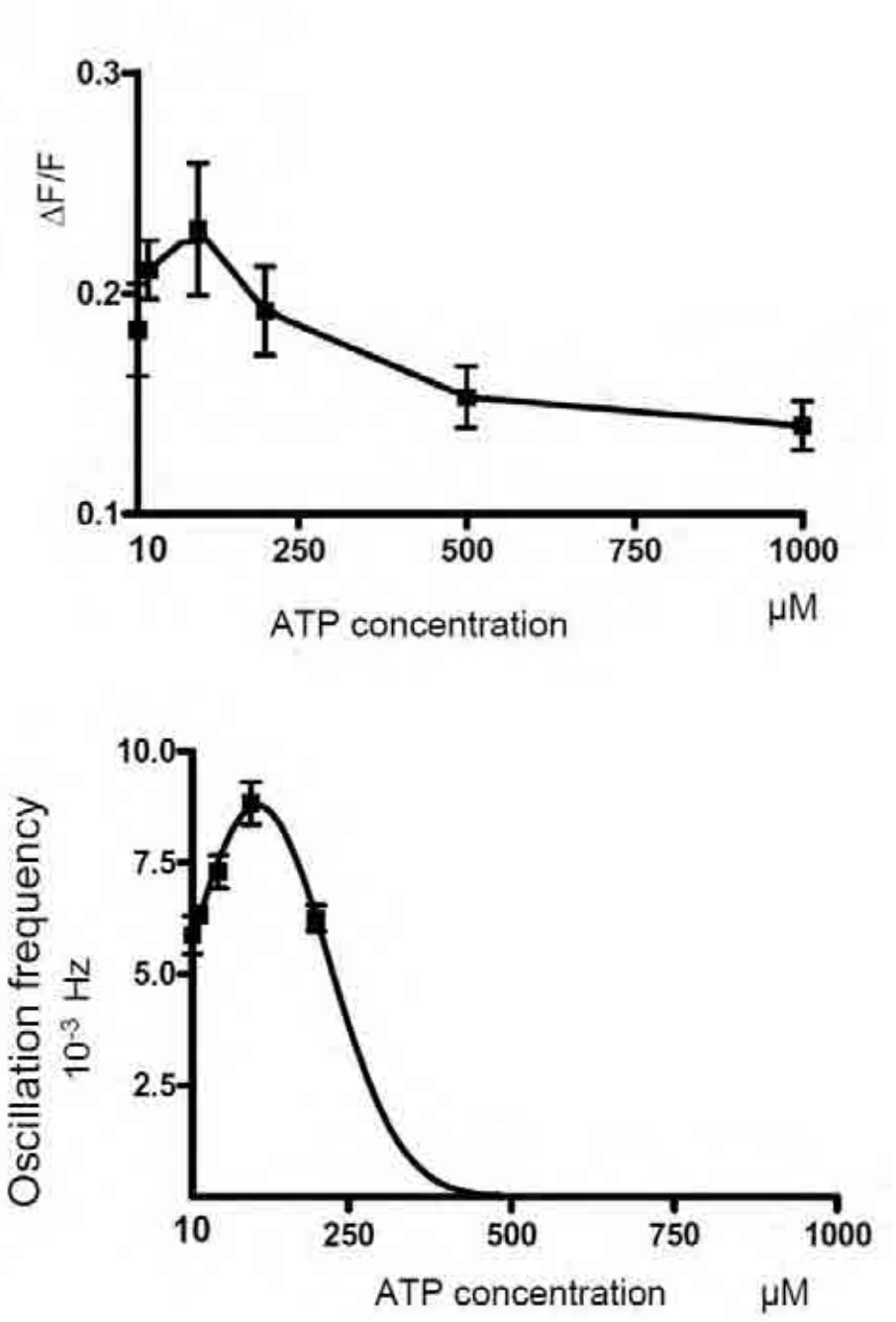


Figure 3.3.2. The amplitude (above) and frequency (below) of $[Ca^{2+}]_i$ oscillations induced by different concentrations of ATP.

The amplitude and frequency of the of $[Ca^{2+}]_i$ oscillations were dependent on [ATP]. < 100 μM, ATP enhanced both amplitude and frequency; > 100 μM, ATP decreased the amplitude and frequency. Frequency-concentration data was fitted by Gaussian distribution. Maximum amplitude and frequency occurred at 100 μM ATP. Data ± S.E.M, n > 200, N = 6 (n=number of cells, N= number of coverslips tested).

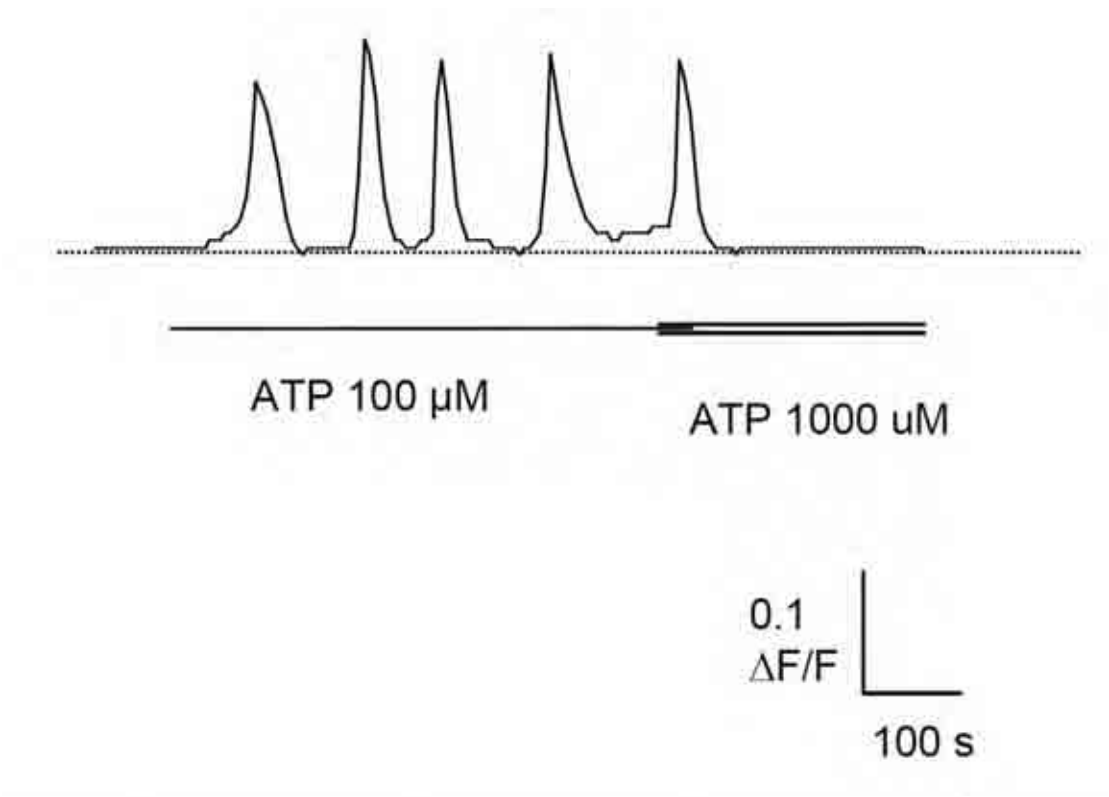


Figure 3.3.3. Original recording of $[Ca^{2+}]_i$ oscillations in HUASMC.

100 µM ATP induced $[Ca^{2+}]_i$ oscillations in HUASMC, which was quenched by addition of 1mM ATP. Recording is representative of n = 80, N = 4 (n=number of cells, N= number of coverslips tested from 2 patches).

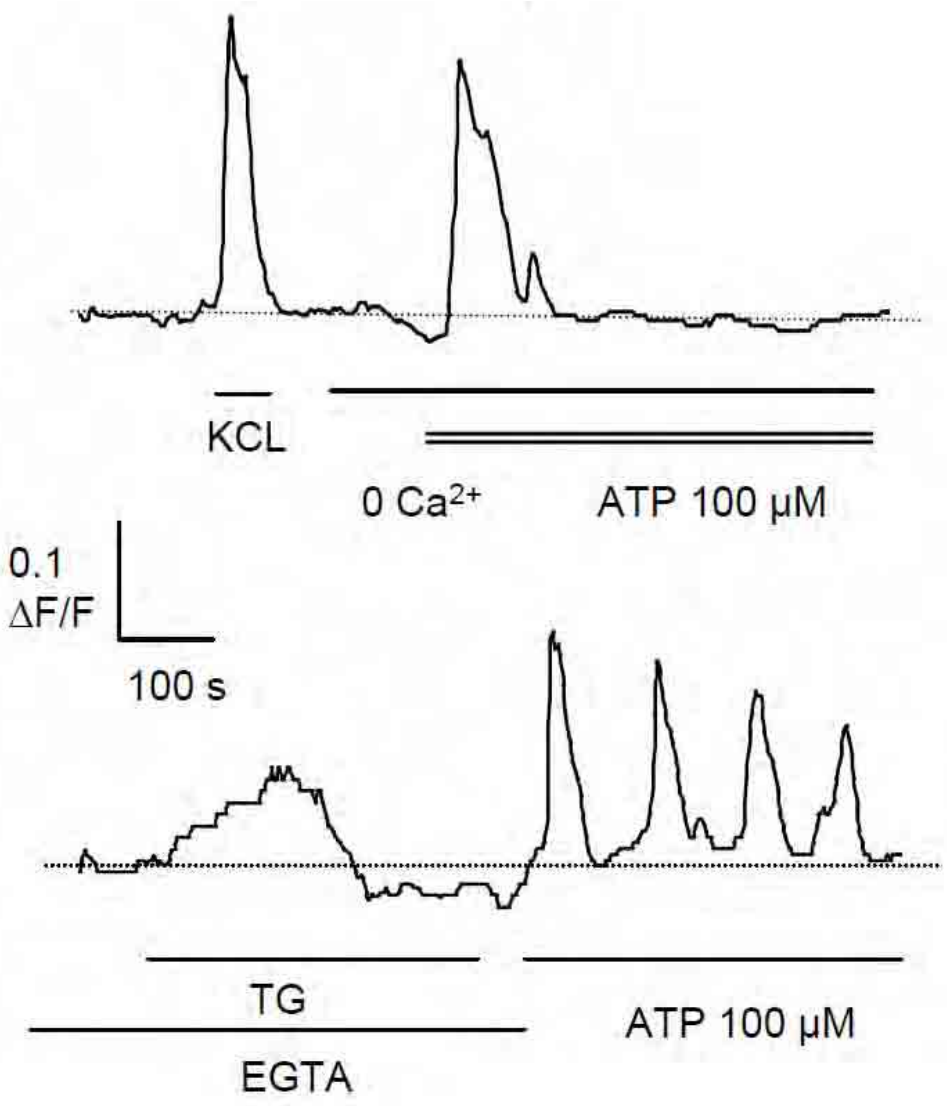


Figure 3.3.4. Original recording of $[Ca^{2+}]_i$ in HUASMC showing the source of ATP.

Upper: HUASMC depolarised by 45 mM KCl showed a strong $[Ca^{2+}]_i$ increase. After HUASMC were incubated in Ca^{2+}-free solution for 2 min, stimulation with ATP (100 µM) caused a significant $[Ca^{2+}]_i$ increase, which was not followed by $[Ca^{2+}]_i$ oscillations. This is representative of n = 80, N = 3 from two donors (n=number of cells, N= number of coverslips tested).

Lower: Upon intracellular Ca^{2+} depletion with TG (1 µM) and EGTA, stimulation with ATP (100 µM) in the presence of Ca^{2+} induced $[Ca^{2+}]_i$ oscillations in HUASMC. Recording is representative of n = 70, N = 3 from two donors (n=number of cells, N= number of coverslips tested).

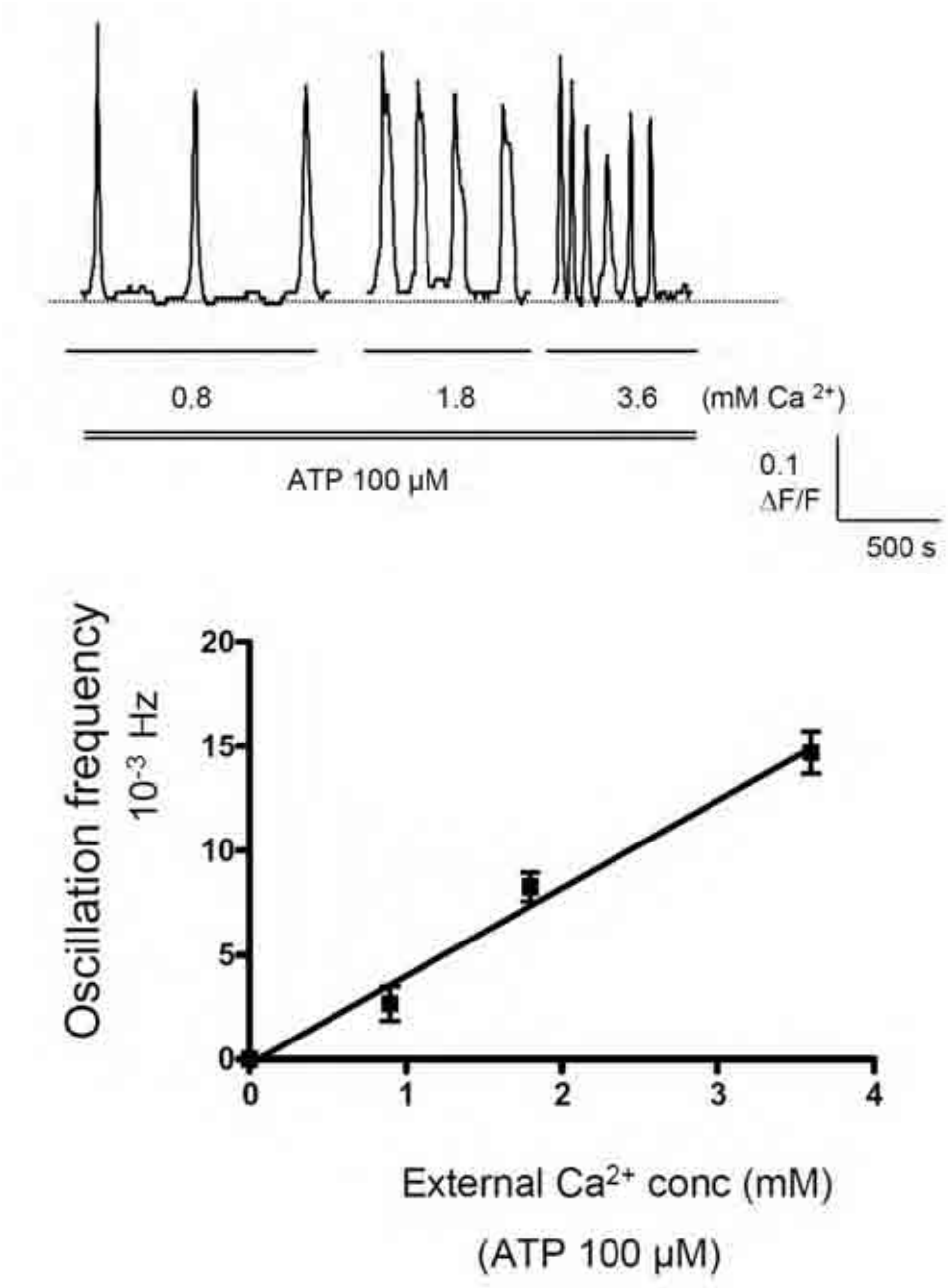


Figure 3.3.5. The effect of changing extracellular [Ca^{2+}] on [Ca^{2+}]$_i$ oscillation frequency

Upper: Original recording of [Ca^{2+}]$_i$ in HUASMC. The frequency of ATP-induced [Ca^{2+}]$_i$ oscillations varies with external [Ca^{2+}]. Bath solutions of different [Ca^{2+}] were applied at random order with 10 min intervals but are shown incrementally for ease of interpretation.

Lower: Averaged [Ca^{2+}]$_i$ oscillation frequency ± S.E.M. in bath solution with various external [Ca^{2+}]. Line fitted with r^2 = 0.9863. ([Ca^{2+}] = 0.9 mM: n=120, N=3; [Ca^{2+}] = 1.8 mM: n=240, N=6; [Ca^{2+}] = 3.6 mM: n=140, N=4; [Ca^{2+}] = 0 mM: n=80, N=3; n=number of cells, N= number of coverslips tested, all from 2 donors).

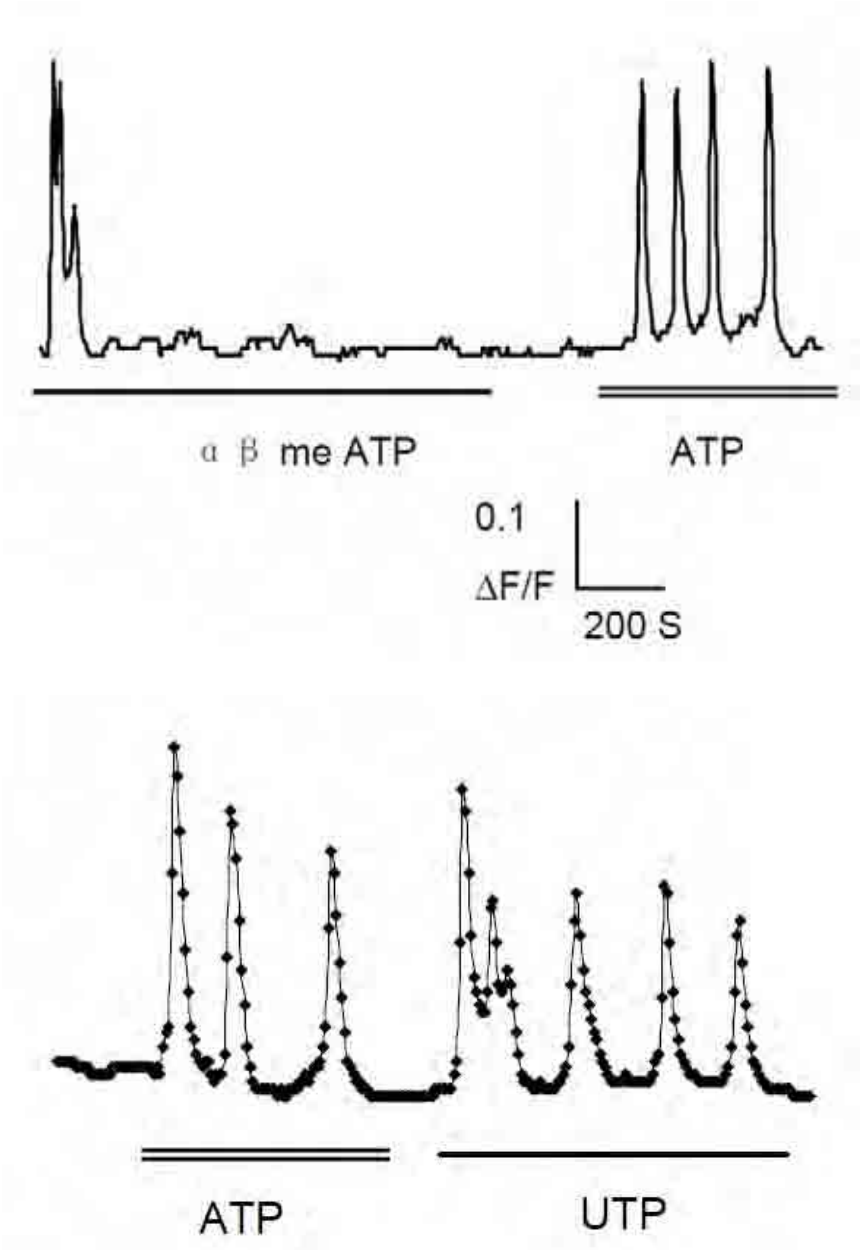


Figure 3.3.6. Original recording of $[Ca^{2+}]_i$ in HUASMC when challenged with α,β-methylene ATP or UTP.

Upper: α,β-methylene ATP (20 μM) induced a transient $[Ca^{2+}]_i$ elevation in HUASMC, but not $[Ca^{2+}]_i$ oscillations. In contrast, ATP induced clear $[Ca^{2+}]_i$ oscillations in the same cell. Recording is representative of n = 90, N = 3 from 2 donors (n=number of cells, N= number of coverslips tested).

Lower: UTP (10 μM) induced $[Ca^{2+}]_i$ oscillations similar to that induced by ATP (100 μM). Recording is representative of n = 60, N = 2 from 2 donors (n=number of cells, N= number of coverslips tested).

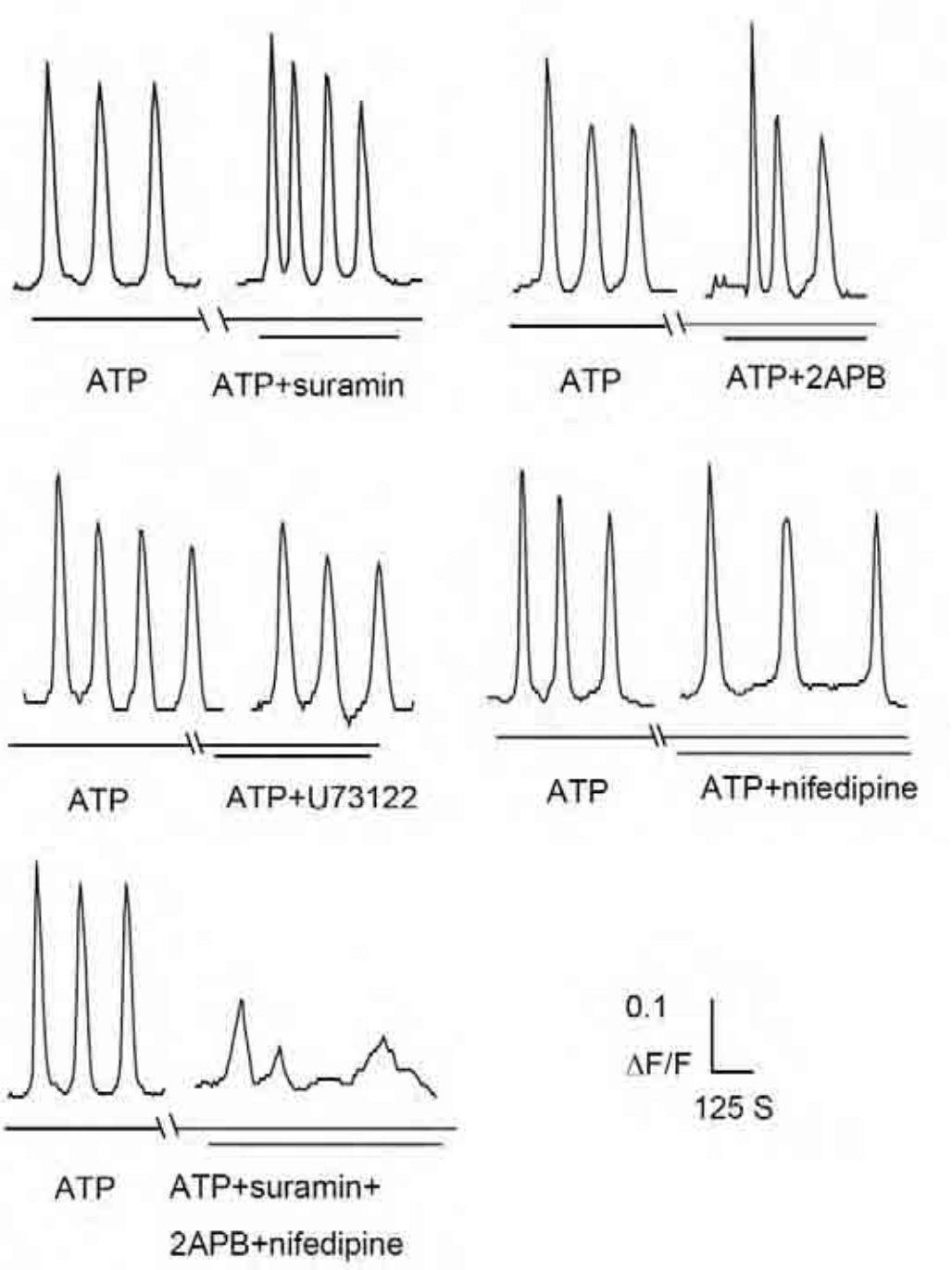


Fig 3.3.7. Modulation of $[Ca^{2+}]_i$ oscillations.

Original recording of ATP-induced $[Ca^{2+}]_i$ oscillations in HUASMC when challenged with suramin (100 µM; upper left), 2-APB (50 µM; upper right), U-73122 (5 µM; middle left), nifedipine (10 µM; middle right), and the combination of suramin, 2-APB and nifedipine (bottom left). Recordings are representative of all cells that displayed $[Ca^{2+}]_i$ oscillations upon ATP (100 µM) challenge.

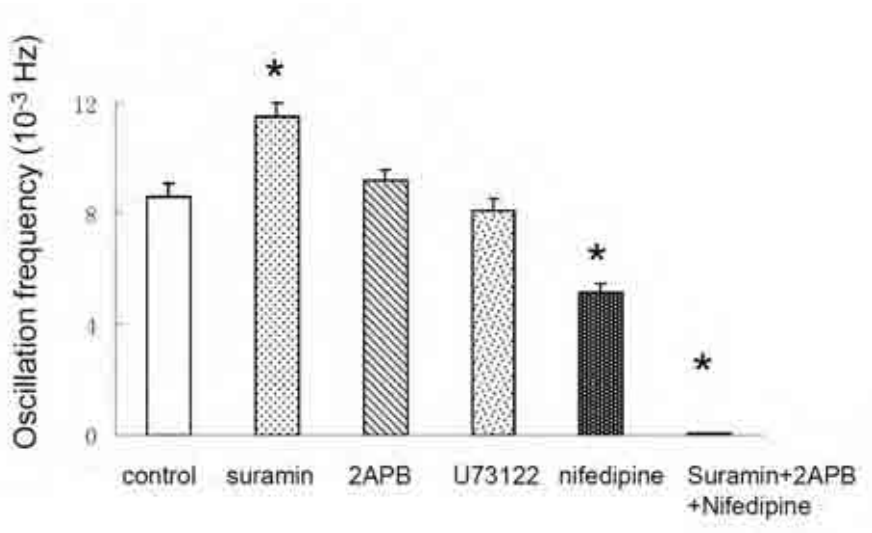


Fig 3.3.8. Modulation of $[Ca^{2+}]_i$ oscillations.

The effects of suramin, 2-APB, U-73122, nifedipine, or the combination of suramin, 2-APB and nifedipine on frequency ± S.E.M. of $[Ca^{2+}]_i$ oscillations shown in columns. The frequency of $[Ca^{2+}]_i$ oscillations induced by ATP (100 µM) is designated as control. Suramin increased the frequency whilst nifedipine decreased it. 2-APB and U-73122 had no effect. A combination of suramin, 2-APB and nifedipine almost completely inhibited the oscillations. *$P < 0.05$, n > 100 for each antagonist tested, N = 4 from 2 donors (n=number of cells, N= number of coverslips tested).

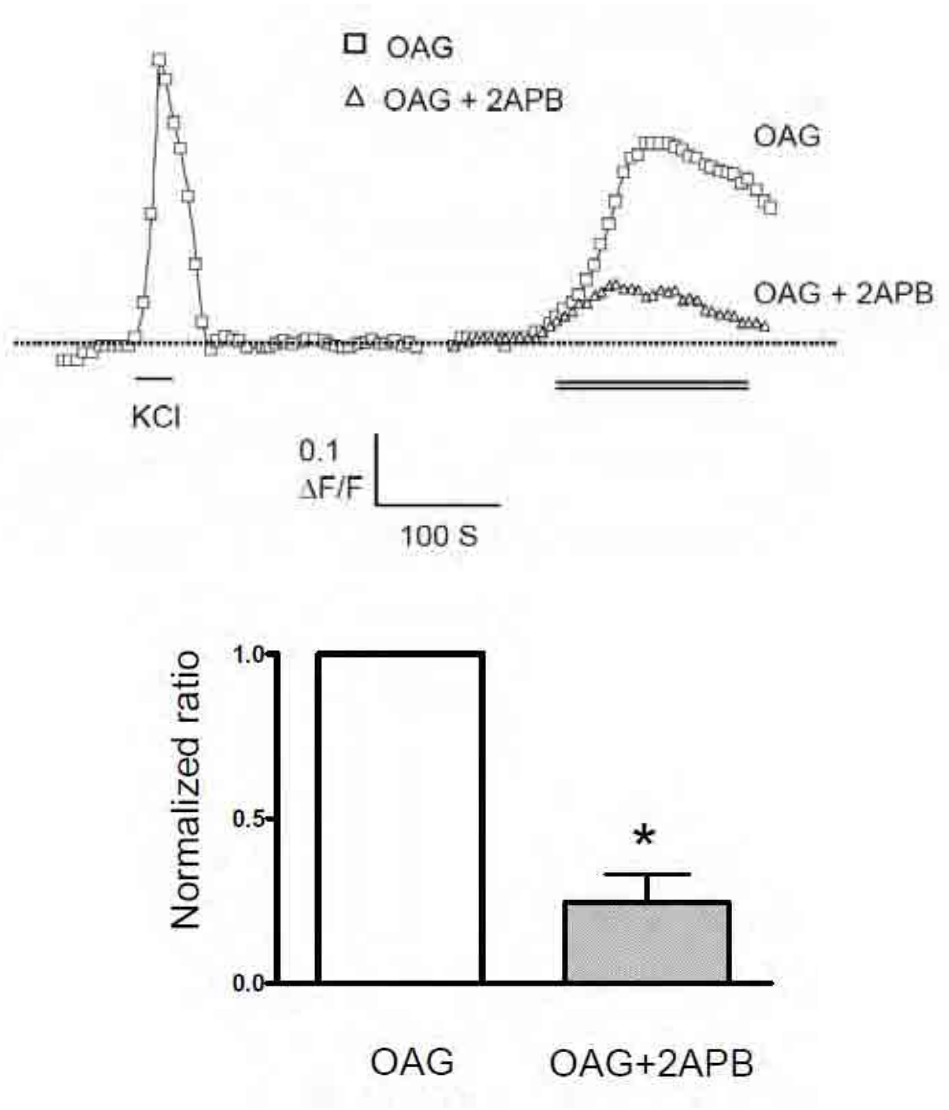


Figure 3.3.9. OAG stimulation of HUASMC and inhibition with 2-APB.

Upper: Depolarisation induced by 45 mM KCL evoked a strong $[Ca^{2+}]_i$ elevation in HUASMC. In the same cells, OAG (100 µM) caused a significant increase in $[Ca^{2+}]_i$, which could be inhibited by 2-APB (100 µM).

Lower: Results from upper shown in column ± S.E.M. Results were normalised to $[Ca^{2+}]_i$ response to OAG. *P < 0.05, n = 100, N = 4 from 2 donors (n=number of cells, N= number of coverslips tested).

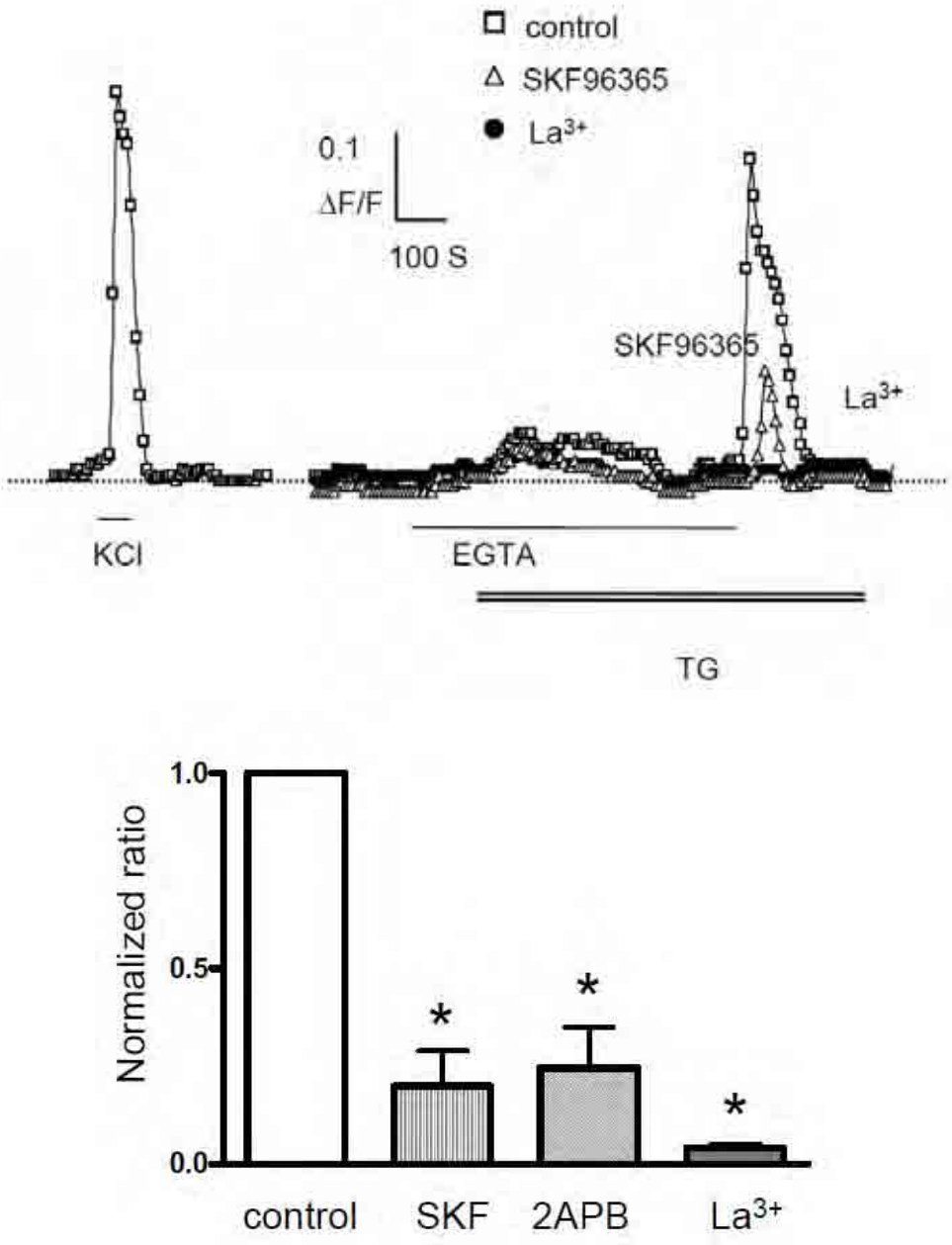


Figure 3.3.10. $[Ca^{2+}]_i$ elevation in HUASMC mediated by SOCC.

Upper: When external Ca^{2+} was re-introduced, a clear SOCC-mediated Ca^{2+} entry followed the emptying of internal Ca^{2+} stores in 0 Ca^{2+} EGTA bath with TG (1 μM).

Lower: Results from upper shown in columns ± S.E.M. Results were normalised to control $[Ca^{2+}]_i$ entry. SKF-96365 and 2-APB caused 78 ± 11% and 73 ± 12% inhibition, respectively, and La^{3+} almost completely blocked all Ca^{2+} entry. *P < 0.05; SKF96365: n = 80, N = 2; 2-APB: n = 60, N = 2; La^{3+}: n = 60, N = 2 (n=number of cells, N= number of coverslips tested from 2 patches).

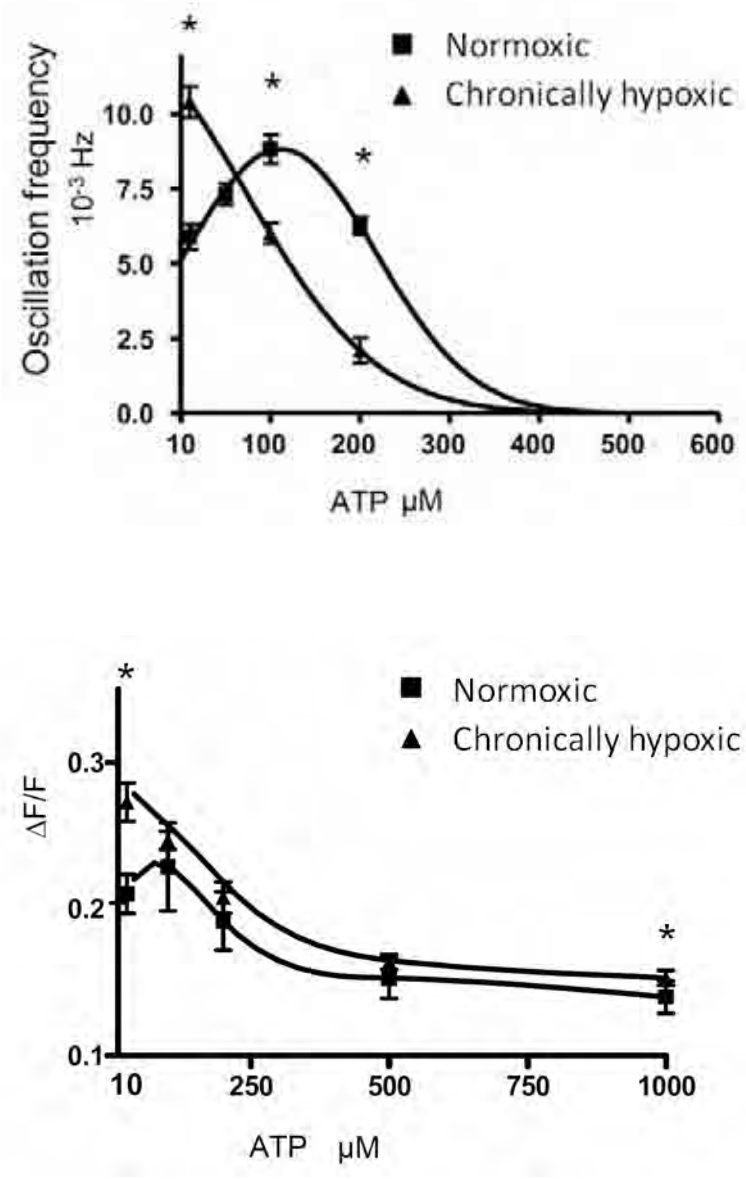


Figure 3.3.11. Comparison of effects on ATP-induced $[Ca^{2+}]_i$ oscillations in normoxic cells and in HUASMC cells made chronically hypoxic in 1% O_2 for 12 hr.

(i) The $[Ca^{2+}]_i$ oscillations frequency vs. [ATP] curve. (■): The $[Ca^{2+}]_i$ oscillation frequency and [ATP] relationship is biphasic. (▲): Effect of chronic hypoxia on frequency of ATP-induced $[Ca^{2+}]_i$ oscillations. The $[Ca^{2+}]_i$ oscillations induced by ATP in cells treated with 72h of 1% O_2 were enhanced at concentrations < 100 µM, but they were reduced at concentrations > 100 µM. The $[Ca^{2+}]_i$ oscillations frequency vs. [ATP] curve effectively left shifted (n=80, N=3 from 2 donors, ± = S.E.M; n=number of cells, N= number of coverslips tested, *=P<0.05 in post-hoc Student's t-test).

(ii) The $[Ca^{2+}]_i$ oscillations amplitude vs. [ATP] curve. Compared to normoxic cells (■), chronic hypoxia treatment caused an elevated $[Ca^{2+}]_i$ oscillations amplitude (▲) at all concentrations tested (n=80, N=3 from 2 donors, ± = S.E.M; (n=number of cells, N= number of coverslips tested, *=P<0.05 in post-hoc Student's t-test).

3.4. DISCUSSION

The experiments described in this Chapter showed that HUASMC respond to exogenous ATP stimulation with $[Ca^{2+}]_i$ oscillations, the frequency and amplitude of which were dependent on the concentration of ATP applied. The oscillations were insensitive to inhibition by suramin, 2-APB, U73122 or nifedipine when applied separately. However, a combination of suramin, 2-APB and nifedipine abolished the oscillations. Application of α,β-methylene ATP to HUASMC resulted in only a short-lasting increase in $[Ca^{2+}]_i$, whereas UTP induced $[Ca^{2+}]_i$ oscillations similar to that evoked by ATP. Application of the ROCC activator OAG, or emptying of intracellular Ca^{2+} stores resulted in an increase in $[Ca^{2+}]_i$, which was sensitive to inhibition by the SOCC inhibitors 2-APB, La^{3+} and SKF96365. Further, chronic hypoxia altered the oscillation frequency and enhanced the $[Ca^{2+}]_i$ oscillations.

3.4.1. $[Ca^{2+}]_i$ oscillations at rest and the effect of exogenous ATP

Under basal conditions, only 1% of HUASMC showed $[Ca^{2+}]_i$ oscillations. However, in accord with the working hypothesis, exogenous ATP at concentration of 10 – 200 μM induced $[Ca^{2+}]_i$ oscillations in 70% of cells. Somewhat surprisingly, the relationship between ATP concentration and $[Ca^{2+}]_i$ oscillation amplitude and frequency in HUAVSM was biphasic (Fig 3.3.2). At 10 to 100 μM, ATP enhanced the amplitude and frequency, whereas > 100 μM, ATP decreased both $[Ca^{2+}]_i$ oscillation frequency and amplitude. This biphasic relationship was not due to desensitization of the purinoceptors and therefore an artefact of the protocol, because different ATP concentrations were applied to any given batch of HUASMC in random order. However, $[Ca^{2+}]_i$ oscillations induced by low, μM ATP were quenched by application of ATP at > 1mM, which suggests that the receptors are prone to desensitisation and consistent with the idea that the initiation or continuation of $[Ca^{2+}]_i$

oscillations was by P2 receptors, for high concentration of ATP would be expected to saturate and desensitise these receptors (Khiroug *et al.*, 1997).

3.4.2. Ca^{2+} entry or Ca^{2+} release?

It was clear that when extracellular Ca^{2+} was replaced with the Ca^{2+} chelator EGTA from the EBSS bathing the HUASMC, ATP caused a single transient elevation in $[Ca^{2+}]_i$, but not $[Ca^{2+}]_i$ oscillations (Fig 3.3.4). This suggests that Ca^{2+} influx is important for the initiation or maintenance of $[Ca^{2+}]_i$ oscillations. However, when extracellular Ca^{2+} and ATP were present, a linear relationship between the frequency of $[Ca^{2+}]_i$ oscillations and external Ca^{2+} concentration was observed (Fig 3.3.5). This is consistent with previous studies in some cell types, but not others e.g.(Shuttleworth & Thompson, 1996). There are two schools of thought that try to explain this phenomenon. On the one hand, it has been argued that since external Ca^{2+} concentration only affected the frequency but not the amplitude of the oscillations, the individual Ca^{2+} spikes represented release from internal store, and that the rate of external Ca^{2+} entry affects the rate of replenishment of these stores (Kawanishi *et al.*, 1989; Berridge, 1993). On the other hand, it has been argued that the capacitative entry pathway only plays a limited role in submaximal, physiological responses, and that the rate of Ca^{2+} entry determines the probability of the low level of IP_3 causing repetitive Ca^{2+} release from internal stores (Shuttleworth & Thompson, 1996). In fact, in the present study, after intracellular Ca^{2+} stores were depleted by inhibiting SERCA with TG (1 μM) in the presence of EGTA, application of ATP (100 μM) in Ca^{2+}-containing EBSS caused $[Ca^{2+}]_i$ oscillations in HUASMC (Fig 3.3.4. Upper). This finding therefore suggests that the latter IP_3 receptor instability model for the generation of $[Ca^{2+}]_i$ oscillations does not hold as the underlying mechanism for ATP-induced $[Ca^{2+}]_i$ oscillations in HUASMC.

The general debate regarding the mechanisms underlying $[Ca^{2+}]_i$ oscillations is further complicated by the fact that even within one cell type, the effect of Ca^{2+} entry on the frequency of $[Ca^{2+}]_i$ oscillations varies with the agonist (Yule *et al.*, 1991). However, more recently a theoretical model has emerged, which can explain these and other conflicting experimental data. It is suggested that it is the *total amount* of Ca^{2+} in the cell, called Ca^{2+} load, which is largely determined by membrane transport, that influences whether the cell is in an oscillatory state or not (Sneyd *et al.*, 2004). A cell can fire a Ca^{2+} spike only if its Ca^{2+} load is above threshold. In this model, Ca^{2+} is lost at the peak of each oscillation (the high $[Ca^{2+}]_i$ concentration is removed by the plasma Ca^{2+}-ATPase pump), and an increase in Ca^{2+} influx achieved by increasing extracellular $[Ca^{2+}]_i$ will increase oscillation frequency because it decreases the rate at which the Ca^{2+} lost during each spike is regained, or increases the rate at which the Ca^{2+} lost during each spike is regained. This is an especially strong model because, not only is it compatible with the other older models of $[Ca^{2+}]_i$ oscillations, but its functioning does not depend on the exact details of the pumps and release processes found in various cell types. The data presented in the present Chapter seem to be consistent with this model. Lack of Ca^{2+} influx prohibited the oscillations induced by ATP (Fig 3.3.4. Upper); oscillations are not possible because the Ca^{2+} load is below the threshold. Re-introduction of external Ca^{2+} caused $[Ca^{2+}]_i$ oscillations (Fig 3.3.4. Lower); increased Ca^{2+} influx drives the Ca^{2+} load over the threshold and a spike occurs. This cycle repeats and results in $[Ca^{2+}]_i$ oscillations.

3.4.3. P2 receptors responsible

As there are no selective antagonists of different subtypes of P2 receptors, an attempt was made to identify the receptor stimulated by ATP in HUASMC by comparing responses to

agonists known to activate particular receptor subtypes. When the cells were bathed in α,β-methylene ATP, a specific agonist of P2X receptors, a transient elevation in $[Ca^{2+}]_i$ was recorded, but no oscillations. In contrast, UTP, the specific $P2Y_{2,4}$ agonist, induced $[Ca^{2+}]_i$ oscillations similar to that evoked by ATP. These data suggest that the ligand-gated P2X channels play no role or a very limited role in ATP-induced $[Ca^{2+}]_i$ oscillations in HUASMC. Rather, it seems that the GPCR P2Y receptor, specifically, the $P2Y_2$ and $P2Y_4$ receptor subtypes, are likely to be the major mediator. As discussed in *Chapter 1 Section 1.3.6.4*, the study by Buvinic *et al* identified the mRNA coding for $P2Y_2$ in the umbilical artery, and for $P2Y_4$ in the chorionic and cotyledon vessels (Buvinic *et al.*, 2006). The potential presence of the mRNA coding for $P2Y_4$ in the umbilical circulation has not been explored, but it is commonly expressed in epithelial and placental tissue e.g. (Valdecantos *et al.*, 2003; da Silva *et al.*, 2006). Thus, to-date there is no direct evidence that HUASMC express $P2Y_4$.

Nevertheless, on the basis of the present findings it seems reasonable to propose that activation of $P2Y_{2/4}$ and subsequent release of Ca^{2+} from the ER store is important for ATP-induced $[Ca^{2+}]_i$ oscillations in HUASMC, although external Ca^{2+} seems to be essential for maintaining the $[Ca^{2+}]_i$ oscillations (Fig 3.3.4).

3.4.4. Maintenance of $[Ca^{2+}]_i$ oscillations

Once ATP-induced $[Ca^{2+}]_i$ oscillations was established in HUASMC, separate inhibition of the P2X, P2Y and SOCC/ROCC by suramin (100 μM), 2-APB or the PLC inhibitor U-73122 did not inhibit the oscillations; in fact suramin increased the oscillations. This could be explained by the fact that suramin may inhibit ENs (Hourani & Chown, 1989), thereby increasing extracellular [ATP]. Further, when used alone, the VOCC inhibitor nifedipine (10

μM) reduced the frequency of the oscillations, but had no effect on the amplitude. However, the combination of suramin, 2-APB and nifedipine completely abolished ATP-induced $[Ca^{2+}]_i$ oscillations. This inhibition of $[Ca^{2+}]_i$ oscillations is unlikely to be due to a change in cell viability, as basal $[Ca^{2+}]_i$ during incubation with the antagonists remained similar to that at rest. Taken together, the data suggest that a combination of Ca^{2+} influx mechanisms is needed to sustain the Ca^{2+} load that is required to maintain the oscillations induced in HUASMC by activation of $P2Y_{2,4}$ receptors. This is in agreement with the experimental data and theoretical model for $[Ca^{2+}]_i$ oscillations discussed in some detail above (Sneyd *et al.*, 2004).

It should be noted that the inability of suramin to prevent $[Ca^{2+}]_i$ oscillations provides further evidence for the receptor subtype responsible for oscillation initiations. Although suramin is a widely used broad-spectrum P2 antagonist, it has no effect on $P2Y_4$ receptor even at high concentration (Charlton *et al.*, 1996; Wildman *et al.*, 2003). Thus, the fact that UTP, the specific $P2Y_{2,4}$ agonist, was able to induce $[Ca^{2+}]_i$ oscillations, and that suramin was unable to prevent the ATP-induce oscillations, is consistent with $P2Y_4$ receptors being functionally expressed in HUASMC and responsible for the initiation of ATP-induced $[Ca^{2+}]_i$ oscillations.

3.4.5. ROCC, SOCC and VOCC in HUASMC

P2Y receptor activation leads to liberation of second messengers IP_3 and DAG, which can then cause further Ca^{2+} influx via SOCC and ROCC pathways (see *Section 3.1*). Application of the synthetic DAG analogue OAG bypasses the effect of IP_3 and Ca^{2+} store depletion, thereby led to direct activation of ROCC. Previous studies have shown that these may be $TRPC_{3,6,7}$ (Birnbaumer, 2009). In the present study, since OAG induced a $[Ca^{2+}]_i$ elevation

that was sensitive to the general SOCC/ROCC antagonist 2-APB, this suggests that ROCC are functionally expressed in HUASMC.

After depletion of internal Ca^{2+} store in HUASMC with EGTA and the SERCA inhibitor TG, re-introduction of Ca^{2+} into the bath caused a clear $[Ca^{2+}]_i$ elevation. This is unequivocal evidence that SOCC is functionally active in HUASMC. Further, as this capacitative Ca^{2+} entry was sensitive to inhibition by two SOCC antagonists, SKF-96365 and La^{3+}, this is further evidence that SOCC is present in HUASMC. Previous studies suggest that these may be $TRPC_{1,4,5}$ and this has been reviewed (Parekh & Putney, 2005).

A further complication is that these ROCC and SOCC are not Ca^{2+}-selective, and in fact behave as non-selective cation channels (Birnbaumer, 2009). Therefore, upon their activation by the GPCR-Gq-PLCβ signalling pathway, they lead to membrane depolarisation. In the present study, depolarisation of the membrane by 45 mM KCl caused increases in $[Ca^{2+}]_i$, presumably mediated by VOCC. Membrane depolarisation and entry of Na^+ may also activate the reverse form of Na^+/Ca^{2+} exchanger, causing further Ca^{2+} entry (Lee *et al.*, 2001). Therefore, it seems VOCC and/or Na^+/Ca^{2+} exchanger are functionally active in HUASMC.

Thus, the findings of this chapter showed that exogenous ATP induced $[Ca^{2+}]_i$ oscillations in HUASMC, the frequency and amplitude of which were dependent on the concentration of the ATP applied. The $[Ca^{2+}]_i$ oscillations were dependent on the activation of a P2Y receptor, most probably the $P2Y_4$ subtype, but Ca^{2+} entry was also important in maintaining the oscillations. It was shown that SOCC, ROCC and VGCC and/or Na^{2+}/Ca^{2+} exchanger were

functionally active in HUASMC, and the combination of these Ca^{2+} entry mechanisms were needed to sustain the Ca^{2+} load needed for ATP-induced $[Ca^{2+}]_i$ oscillations.

3.4.6. Effect of chronic hypoxia

It was discussed in some detail in *Chapter 1 Section 1.2.2.6.* that a hypoxic intrauterine environment is a common feature in both normal pregnancies, and that hypoxia is accentuated in PE (Soleymanlou *et al.*, 2005). In the studies described in the present chapter, it was shown that chronic hypoxia (1% O_2 for 72 hr) induced a change in the HUASMC response to ATP: $[Ca^{2+}]_i$ oscillations induced by ATP in cells treated with chronic hypoxia were enhanced at concentrations < 100 μM, but they were reduced at concentrations > 100 μM, resulting in a left shift in the $[Ca^{2+}]_i$ oscillations frequency vs. [ATP] curve (Fig 3.3.11). This contradicts the hypothesis set out in *Chapter 1 Section 1.6*, that chronic hypoxia increases the expression of ENs, thus lowering the concentration of ATP in the extracellular space, and thereby leading to impaired vasomotion function. In fact, the results from the present study suggest that ion channel expression or activity may be modulated by hypoxia and lead to *increased* Ca^{2+} activity in response to low concentration of ATP, which drives vasomotion at higher frequency. The mechanism underlying this is unclear and requires investigation in future studies.

As discussed in the General Introduction Chapter, the umbilical artery is an important component of the fetal life support system and abnormalities in its functioning are strongly associated with fetal pathologies (Morris *et al.*, 2011). Evidence in the literature (see *Chapter 1 section 1.4.2*), and that presented in *Chapter 4*, suggest that hypoxia can accentuate ATP

release from various vascular cells, notably HUVEC. If the same were also true for HUAEC, then ATP released by acute hypoxia may be able to induce $[Ca^{2+}]_i$ oscillations as described in this Chapter, and thus drive vasomotion. Chronic hypoxia may then enhance the effect of ATP that is released by acute hypoxia or other stimuli, on $[Ca^{2+}]_i$ oscillations and thereby vasomotion. Thus, the findings of this Chapter are consistent with the hypothesis that hypoxia, a common feature in normal and complicated pregnancies, is able to release ATP to modulate the oscillation pattern of the umbilical artery, so play a role in the control of blood flow between the fetus and mother. Whether or not hypoxia can release ATP from HUAEC and HUVEC was explored in *Chapter 4 & 5*.

CHAPTER 4

THE EFFECT OF HYPOXIA ON ATP RELEASE FROM HUAEC AND HUVEC

4.1. INTRODUCTION

As discussed in *Chapter 1 (section 1.4.1)*, the endothelium is an important source of extracellular ATP. It has been shown both *in vivo* and *in vitro* that a variety of stimuli can induce release of ATP from the endothelium, including osmotic, shear, mechanical, oxidative, ischemic and pH challenges. It has previously been shown that both ATP and adenosine signalling are involved in hypoxia-mediated vasodilatation (see *section 1.3.6.1*). However, the direct effect of hypoxia on the release of ATP from ECs is largely unknown.

In this respect, the only direct evidence to date originate from Gerasimovskaya's group in Colorado whose primary interest was in the angiogenic effect of extracellular ATP on the highly specialist vessels of calf pulmonary artery vasa vasorum. They recently reported that hypoxia was able to induce ATP release from the EC of these vessels via a vesicular mechanism (Woodward *et al.*, 2009). EC derived from the human umbilical vessels, especially HUVEC, are much more commonly used as an EC model in scientific studies. It was also discussed in *Chapter 1* that intrauterine hypoxia is a feature in both normal and complicated pregnancies. Hence, given the effect of extracellular ATP on vascular tone shown in *Chapter 3*, the effect of hypoxia on ATP release from HUVEC and HUAEC is of clear relevance to the physiological control of vascular tone in general, as well as to the processes of normal pregnancy and complicated pregnancies such as pre-eclampsia and IUGR. Accordingly, the experiments in the present chapter were designed to address the hypothesis that hypoxia can induce ATP release from both HUAEC and HUVEC.

To this end, preliminary experiments were carried out to assess whether hypoxia (30 min 1% O_2) affected ATP release from both HUVEC and HUAEC. When it had been established that

hypoxia was able to cause ATP release, this process was examined in HUVEC and HUAEC that were grown on a high-density pore membrane in order for the release from apical and basolateral membranes to be differentiated. This is an important extension to the study of Woodward *et al* (2009), as apically and basolaterally released ATP have the potential to act on different cell types and receptors and consequently different effects (see *Section 1.3.6.3 and 1.3.6.4*). The other important difference is that only cells in the first passage was used in the present study, as preliminary experiments, as well as previous studies, suggested that with sub-culturing cells lose their sensitivity to natural external stimuli (Bodin & Burnstock, 1995). The roles of PI_3K, Rho-associated protein kinase and vesicular transport were tested due to their known sensitivity to PO_2 and the evidence that they are implicated in ATP release from vaso vasorum EC in (Woodward *et al.,* 2009). In addition, the role of $[Ca^{2+}]_i$ was also explored, as it is the principle temporal trigger for regulated exocytosis (Barclay *et al.*, 2005).

It was discussed in *Section 1.3.6.1* that adenosine is able to induce NO release from EC, including that of the umbilical origin. More recent evidence suggested that NO released from EC by shear stress or agonists can cause release of adenosine from EC, by competing with O_2 for their binding site on cytochrome oxidase: release of adenosine by this mechanism is accentuated in hypoxia, forming a positive feedback loop (Edmunds *et al.*, 2003). The question therefore arises as to whether it is adenosine that is released as such, or whether it is in fact ATP that is released which is then metabolised to adenosine by ENs. Therefore, an NO donor was used in the present study to test whether NO *per se* is able to induce ATP release from HUVEC.

4.2. METHODS

EC from the human umbilical vein and artery were isolated as described in *Chapter 2 Section 2.1.4* and *2.1.5* respectively for each cell type. The details of the methodology used in the present Chapter are described in *Chapter 2 section 2.1.7 & 2.2.*

PROTOCOLS

4.2.1. Group 1 & 2: CD31 staining in HUAEC and HUVEC

To validate cell phenotype, four 24 wells confluent cells were stained for the EC-specific surface protein CD31 and analysis by FACS as described in *Chapter 2 Section 2.1.7.1*. Isotype and negative control were also performed as described on cells isolated from the same donor. Cells were selected according to granulation (side scatter) and volume (forward scatter), which were then analysed for PE-tagged CD31 staining.

4.2.2. Group 3: Effect of hypoxia on ATP release from HUVEC and HUAEC

In these preliminary experiments, primary HUVECs and HUAECs were isolated from 3 separate donors and mixed. These were grown on 24 well culture plates as described in *Chapter 2 Section 2.1.2* until confluent. The culture medium was replaced with fresh ones immediately before the experiment. In order to test the hypothesis that hypoxia could increase ATP release from EC, 6 wells were placed in a humidified incubator containing 1% O_2, 5% CO_2, balanced with N_2 at 37 °C, while another 6 wells of control culture was put in a conventional 37 °C humidified incubator containing 5% CO_2. The cells were incubated for 30 min from the time at which the O_2 level in the hypoxic incubator reached 1.0 + 0.1%, which took typically 10 min (see *Chapter 2 Section 2.2.1*). In order to assess background luminescence resulting from ATP in the culture medium, 2 wells without cells but with

medium were added to each of the hypoxic and normoxic group. After the incubation period, 50 µl of medium from each well was withdrawn by careful aspiration without disrupting the cell monolayer. This was immediately mixed with the CellTiter-Glo® luciferin-luciferance assay and the level of luminescence from each sample was measured using a luminometer (see *Chapter 2 Section 2.2.2.3*). The concentration of ATP was determined by calibrating the level of luminescence to those of a serial dilution of stock ATP freshly made on the day of experiment (see *Chapter 2 Section 2.2.2.4*).

To assess changes in viability, the remainder of the medium was replaced by 0.1% trypan blue in PBS for 10 min. As a positive control, medium was taken out from one of the wells from each group and left to air dry for 10 min before introducing the trypan blue solution (see *Chapter 2 Section 2.2.3*). The trypan blue solution was replaced with normal PBS before the cells were examined under a phase contrast light microscope and the number of stained cells was counted. This procedure was performed at the end of each of the protocols described below.

4.2.3. Group 4: Effect of hypoxia on ATP release from HUVEC on inserts

HUVEC were isolated from three different donors and prepared on culture inserts as described in *Chapter 2 Section 2.2*. In contrast to *group 3*, cells from individual donors remained separate (see *Section 4.2.7* for statistical methods). The protocol for exposing the cells to hypoxia and measurement of ATP concentrations were same as *Group 3*. For a viability study, cells were grown on two transparent inserts in both normoxic and hypoxic groups, at the same density as those for ATP measurement (see *Chapter 2 Section 2.2*). The release experiments were carried out typically 48 hr post seeding, as it has been shown that a

significant trans-endothelial resistance was achieved in HUVEC grown in a similar configuration to the present study by measuring with a Voltmeter (Schwiebert *et al.*, 2002).

4.2.4. Group 5: Effect of PI_3K and ROCK inhibition on HUVEC

The protocol is essentially the same as *Group 4*, except that the number of individual donors was increased to 6. HUVEC was pre-incubated for 60 min in medium with LY294002 (20 μM), Y27632 (10 μM), at concentrations shown to be highly specific PI_3K and ROCK inhibitors (Vlahos *et al.*, 1994; Ishizaki *et al.*, 2000), or vehicle, and exposed to 21% O_2 or hypoxia. ATP measurements were taken as above. LY297002 and Y27632 were purchased from Tocris Bioscience, U.K. They were dissolved in DMSO (Sigma-Aldrich, U.K.) or distilled water, respectively, as 1000x stock in 50 μl aliquots and stored at -20ºC. The chemicals were thawed immediately prior to use.

4.2.5. Group 6: Effect of vesicular transport inhibition on HUVEC

The effect of the vesicular transport inhibitors brefeldin A (20 μM) and monensin (10 μM) on constitutive and hypoxia-induced ATP release from HUVEC was test as in *Group 5*. These agents were both purchased from Enzo Life Sciences, U.K., dissolved in DMSO as 1000x (brefeldin A) or 10000x (monensin) stock solution in 50 μl aliquots and stored at -20ºC. They were thawed immediately prior to use. The concentrations at which agents were used have been shown to inhibit vesicular transport processes in multiple cell types (see *Section 4.4.3*).

4.2.6. Group 7: Effect of Ca^{2+} ionophore and NO donor on HUVEC

The effect on the Ca^{2+} ionophore A23187 (10 μM) and NO donor S-Nitroso-N-acetylpenicillamin (SNAP; 100 μM) on ATP release from HUVEC was tested as in *Group 4*.

A23187 was purchased from Tocris Bioscience, U.K. and SNAP from Sigma-Aldrich, U.K. A23187 was dissolved in DMSO as stock solution as in *Group 5 & 6*, whereas SNAP was freshly prepared in DMSO (1:1000) on the day of experiments. They were used at concentrations previously shown to be effective in *in vitro* experiments (see *section 4.4.3*).

4.2.7. Statistical analysis

In initial experiments (*Group 3*), cells from 3 different donors (N) were mixed and each coverslip was designated 1 n. In all subsequent experiments cells isolated from each donor remained separate and results from each insert was therefore considered as n=1. Data was presented as means ± S.E.M., and comparisons were made using Student's unpaired *t*-test, taking $P < 0.05$ as significant. When cells were incubated in different pharmacological agents and their vehicle, repeated measures ANOVA analysis was carried out and when this indicated significance, post hoc Fisher PLSD tests were used to measure the degree of significance. The methods used are clearly stated in each Figure legend.

4.3. RESULTS

4.3.1. Group 1 & 2

HUVECs were selected according to similar granulation (side scatter) and volume (forward scatter), which were analysed for PE-tagged CD31 staining. FACS analysis showed that there was only one population of cells in each samples of cells (Fig 4.3.1 upper left and right). When cells were analysed for PE-tagged CD31, isotype control showed a limited level of fluorescence, whereas cells in the CD31 group showed a significant level of fluorescence (Fig 4.3.1 lower left). The results obtained from HUAECs were very similar to those just

described for HUVECs and again indicate only a single population of cells is present in the sample which is CD31 positive (Fig 4.3.2).

4.3.2. Group 3

In 21% O_2, HUVEC released ATP into medium at concentrations that were greater than that recorded in medium with no cells; this release was accentuated by exposure to 1% O_2 (Fig 4.3.3.). HUAEC showed a similar release of ATP in 21% O_2, and in $^4/_6$ wells there was an increase in ATP release in hypoxia, but this did not reach statistical significance (Fig 4.3.3). Visualisation of cells after trypan blue staining revealed no staining in either HUVEC or HUAEC, confirming that all cells were viable. However, for the HUAEC there were often large areas of the culture plates which were devoid of cells in both normoxic and hypoxic groups, suggesting that some cells were lost during the washing process: the area that was covered with cells varied between wells.

All of the cells in both the positive control groups of HUVEC and HUAEC, which were air dried were stained blue, indicating, as expected, that they were not viable.

Also, at the end of each of the protocols described below, trypan blue staining revealed that all cells were viable.

4.3.3. Group 4

In 21% O_2, HUVEC released ATP from both apical and basolateral membrane (Fig 4.3.4; 0.46 $\pm$ 0.08 and 0.05 $\pm$ 0.001 nM, respectively). Consistent with results from *Group 3*, the release was accentuated by hypoxia in both cases (3.04 $\pm$ 0.91 and 0.11 $\pm$ 0.01 nM,

respectively). In 21% O_2, apical release was apparently about 10 times larger than basolateral release. In hypoxia, apical release was about 30 times larger than basolateral release. However, when the difference in the total volume of medium present in the apical and basolateral compartments is taken into consideration (200 vs. 700 µl), the real ratio difference for apical:basolateral release is about 3 times and 10 times in 21% O_2 and hypoxia, respectively (i.e., one can effectively multiply all the basolateral concentrations by 3.5 in Fig 4.3.4, 4.3.6, 4.3.8, 4.3.9 & 4.3.10 to get an impression of the relative release from the two sides).

4.3.4. Group 5

Consistent with the results from *Group 3 & 4*, un-stimulated HUVEC showed constitutively release of ATP from both apical and basolateral membrane (17.9 $\pm$ 5.08 and 0.129 $\pm$ 0.01 nM, respectively), that was accentuated by hypoxia (26.0 $\pm$ 6.13 and 0.170 $\pm$ 0.03 nM, respectively). When HUVEC were pre-incubated with the PI_3K inhibitor LY294002 (20µM; 60 min), or the ROCK inhibitor Y27632 (10µM; 60 min), the hypoxia-induced ATP release from apical and basolateral membrane was attenuated (Fig 4.3.5; 4.3.6, respectively; apical: to 17.1 $\pm$ 7.84 nM and 18.1 $\pm$ 6.19 nM, respectively; basolateral: to 0.12 $\pm$ 0.02 nM and 0.13 $\pm$ 0.02 nM, respectively), but neither antagonist affected constitutive release. Further, vehicle (DMSO; 1:1000 dilution) did not affect either the constitutive or hypoxia-induced ATP release (data not shown).

4.3.5. Group 6

As in the groups described above, un-stimulated HUVEC showed a constitutive release of ATP from both apical and basolateral membrane (Fig 4.3.7 & 4.3.8: 3.97 $\pm$ 0.90 and 0.16 $\pm$

0.02 nM, respectively), and this release was accentuated by hypoxia (to 11.0 ± 2.94 nM and 0.23 ± 0.03 nM, respectively). When HUVEC were pre-incubated with the vesicular transport inhibitors brefeldin A (20μM; 60 min) or monensin (10μM; 60 min), the hypoxia-induced ATP release was attenuated from both apical and basolateral membrane (Fig 4.3.7 & 4.3.8; apical: to 6.83 ± 2.18 nM and 7.57 ± 1.94 nM, respectively; basolateral: to 0.14 ± 0.01 nM and 0.15 ± 0.01 nM, respectively), but neither affected the constitutive release (Fig 4.3.7 & 4.3.8).

4.3.6. Group 7

Un-stimulated HUVEC showed constitutive release of ATP from both apical and basolateral membrane (2.62 ± 0.38 and 0.22 ± 0.01 nM, respectively) and this release was accentuated by the Ca^{2+} ionophore A23187 (10 μM; Fig 4.3.9; to 61.7 ± 10.3 and 0.26 ± 0.01 nM, respectively), whereas the vehicle (DMSO) had no effect (2.68 ± 0.67 and 0.21 ± 0.01 nM, respectively). As the concentration of ATP released under normoxic conditions from the apical and basolateral membranes in this group was consistent with that measured in *Group 4 – 6* above (see Fig. 4.3.4, 4.3.5, 4.3.6, 4.3.7 & 4.3.8), it appears that A23187 caused much greater release of ATP from the apical membrane than from the basolateral membrane and much greater release than was evoked from the apical membrane by hypoxia

The NO donor SNAP (100 μM) had no effect on either apical or basolateral release of ATP in HUVEC (Fig 4.3.10; 2.52 ± 0.57 and 0.22 ± 0.001 nM, respectively).

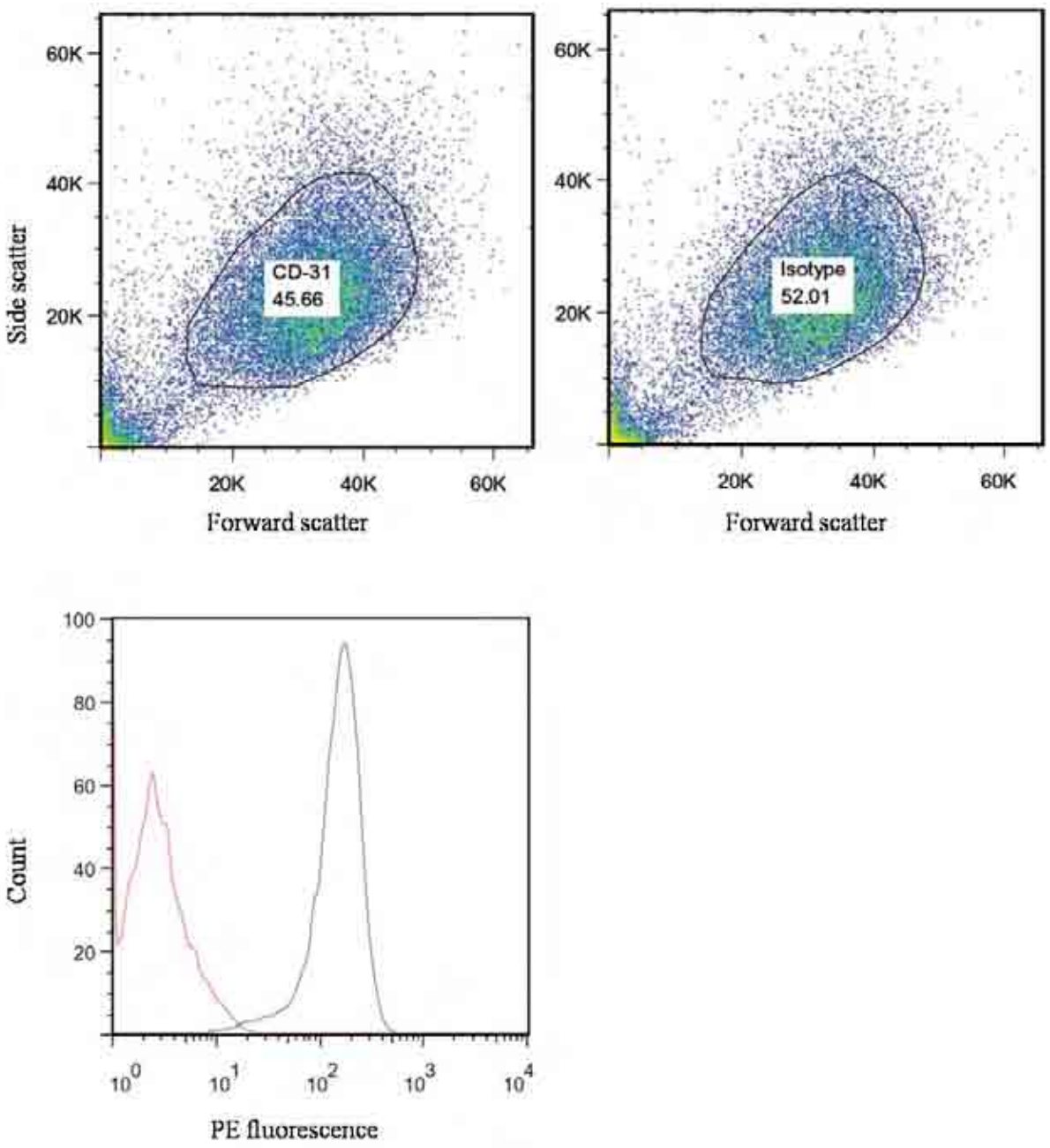


Figure 4.3.1. FACS analysis of CD31 stained cells isolated from the human umbilical vein.

Cells were sorted according to granulation property and volume by FACS. Cells from the CD31 group (upper left) and isotype group (upper right) both showed only one population of cells. Analysis of level of PE fluorescence showed a limited level of fluorescence in the isotype group (lower left; red), whereas the CD31 group displayed a significant level of fluorescence (lower left; black).

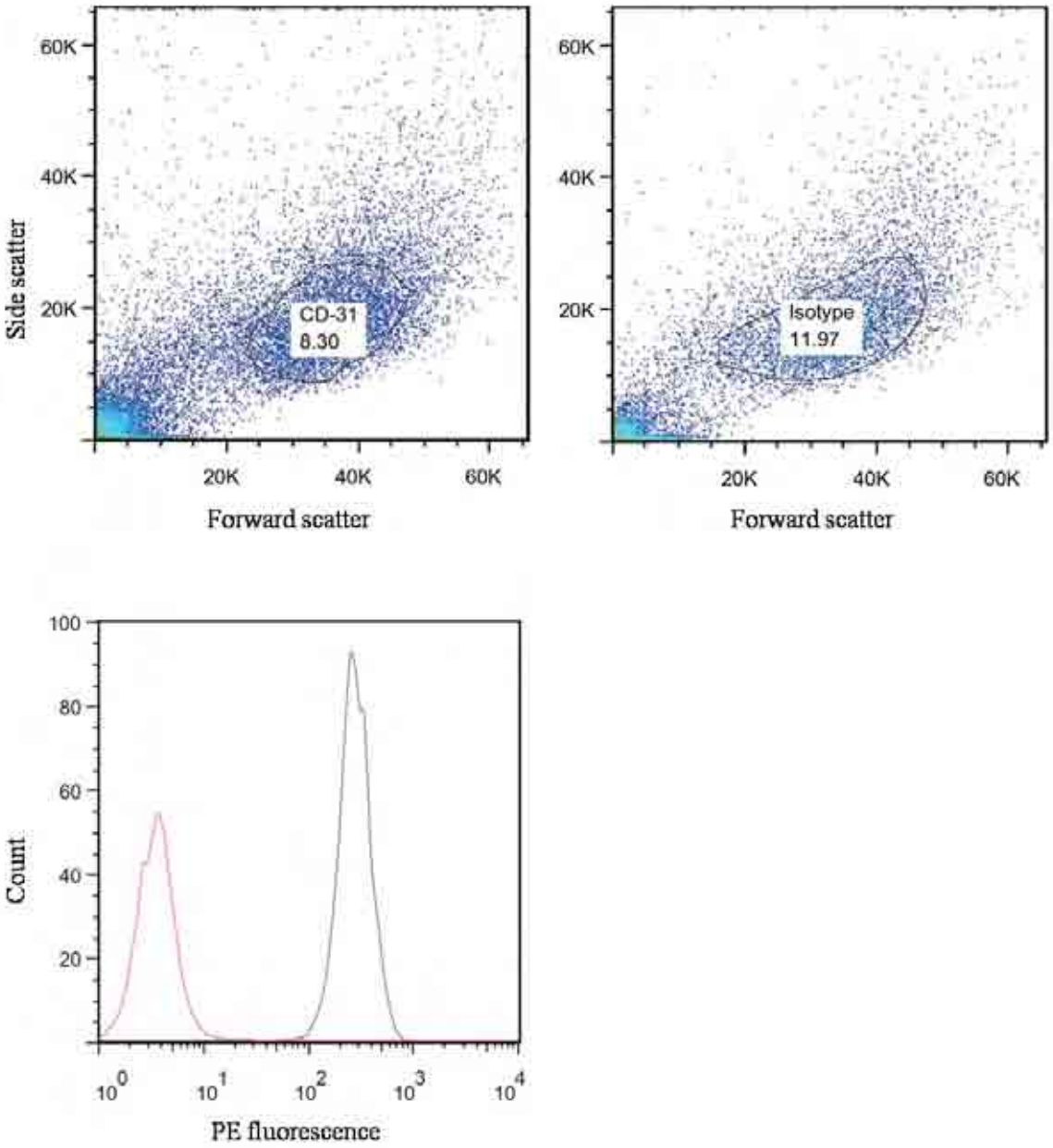


Figure 4.3.2. FACS analysis of CD31 stained cells isolated from the human umbilical artery.

Cells were sorted according to granulation property and volume by FACS. Cells from the CD31 group (upper left) and isotype group (upper right) both showed only one population of cells. Analysis of level of PE fluorescence showed a limited level of fluorescence in the isotype group (lower left; red), whereas the CD31 group displayed a significant level of fluorescence (lower left; black).

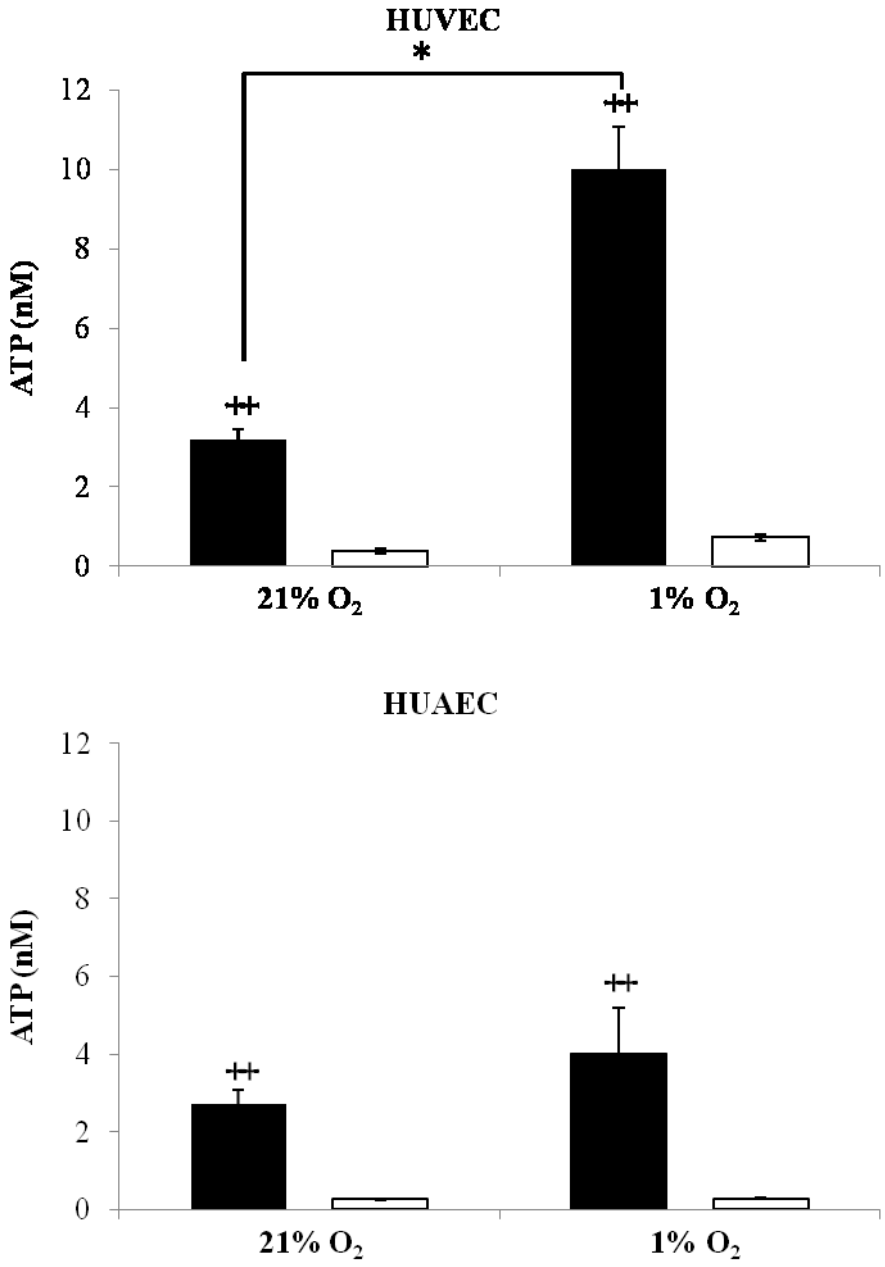


Figure 4.3.3. ATP release from HUVEC (above) and HUAEC (below) as determined by the luciferin-luciferance assay.

In normoxia (21% O_2, 5% CO_2 37°C; solid bars), HUVEC showed greater released of ATP than culture medium alone (open bars). This release was accentuated by hypoxia (1% O_2; solid bars). By contrast HUAEC showed a release of ATP in normoxia but this was not accentuated in hypoxia. Statistical difference in Student t-test: *P < 0.05 for HUVEC and p = 0.17 for HUAEC. ++ : cell vs medium P < 0.01. ± = S.E.M. n=6, N=3 for each cell types (n=number of wells, N=number of donors).

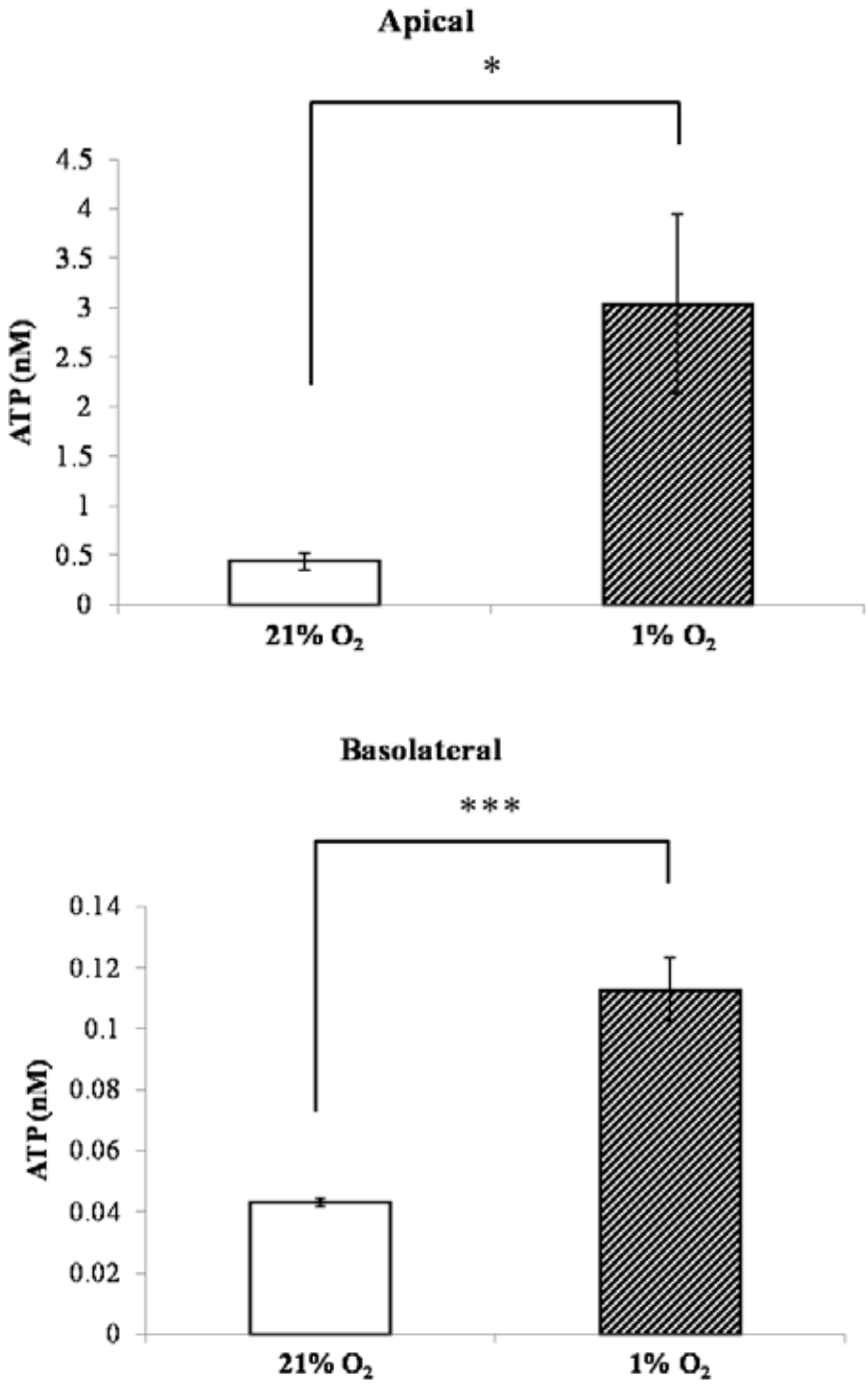


Figure 4.3.4. Effect of hypoxia on apical and basolateral release of ATP from HUVEC.

In normoxia (21% O_2, 5% CO_2 37°C), HUVEC released ATP from both apical (upper) and basolateral (lower) membrane. Release was accentuated by 30 min hypoxia (1% O_2, 5% CO_2 37°C). *P < 0.05, *** P < 0.01, ± = S.E.M., n=9, N=3 (n=number of inserts, N=number of donors). NB: different scales used for apical and basolateral release (see text).

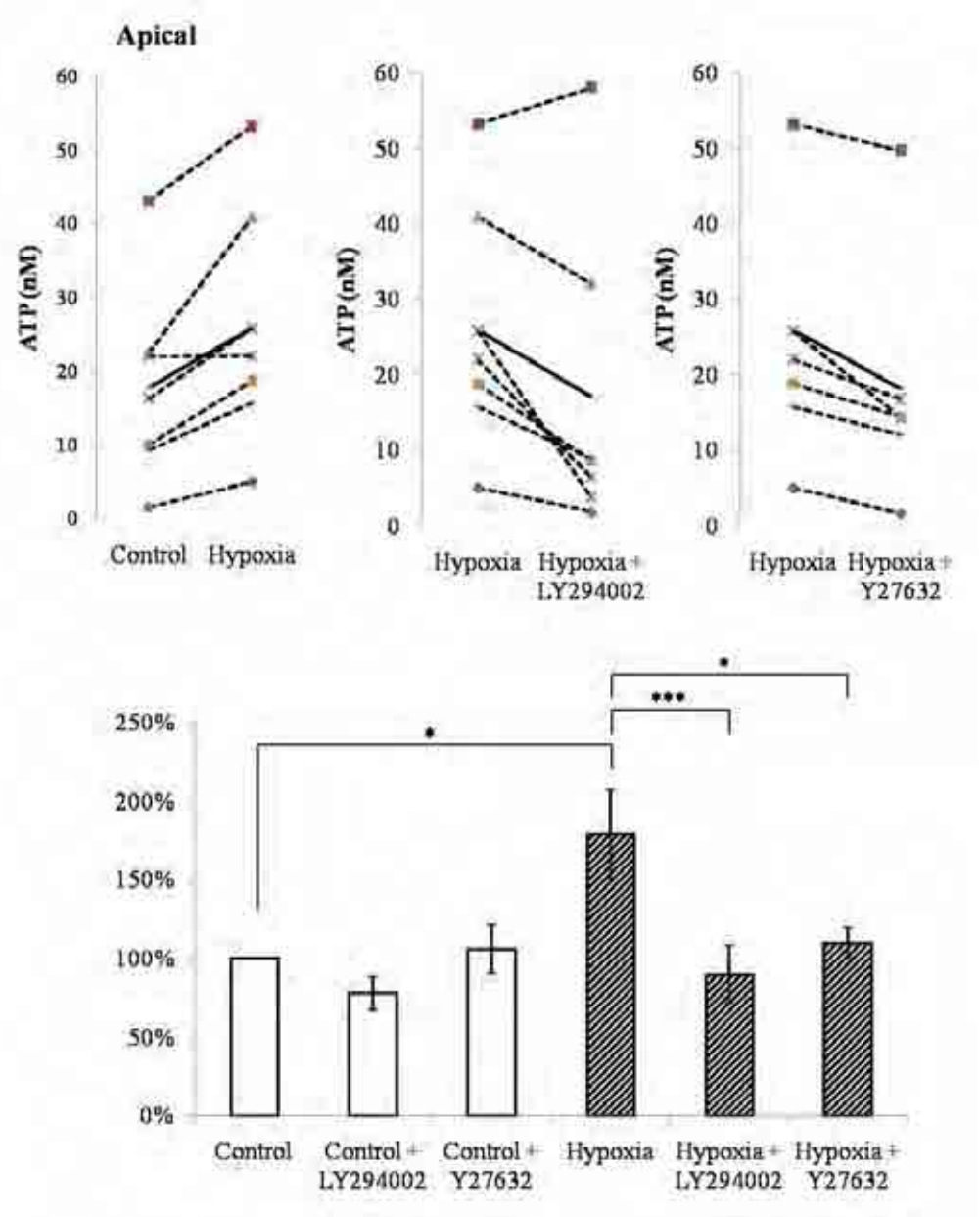


Figure 4.3.5. The role of PI_3K and ROCK in hypoxia-induced release of ATP from the apical membrane of HUVEC.

Upper: In each panel, dotted lines join data points measured in cells from individual donors, and solid line joins the average data for all 6 donors.

Lower: Columns show ATP release ± S.E.M relative to that measured in normoxic conditions taken as 100%. ATP release in normoxia was accentuated by 30 min hypoxia (1% O_2, 5% CO_2 37°C) but normoxic release was unaffected by LY294002 (20µM) or Y27632 (10µM). In contrast, hypoxia-induced ATP release was attenuated by LY294002 and by Y27632. *P < 0.05, * < 0.01, n=18, N=6 (n=number of inserts, N=number of donors). Repeated measures ANOVA with post hoc Fisher PLSD.**

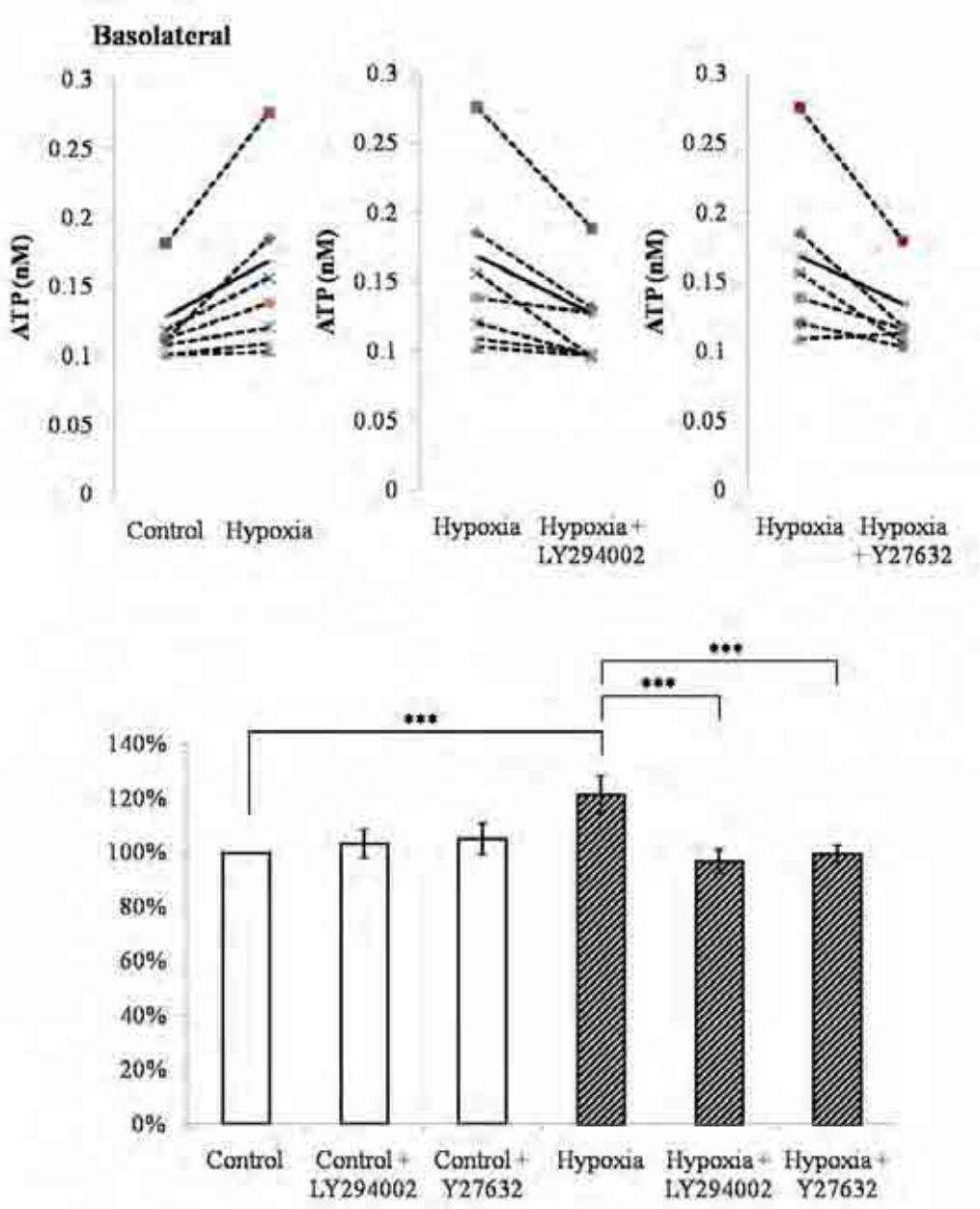


Figure 4.3.6. The role of PI_3K and ROCK in hypoxia-induced release of ATP from the basolateral membrane of HUVEC.

Upper: In each panel, dotted lines join data points measured in cells from individual donors, and solid line joins the average data for all 6 donors.

Lower: Columns show ATP release ± S.E.M relative to that measured in normoxic conditions taken as 100%. ATP release in normoxia was accentuated by 30 min hypoxia (1% O_2, 5% CO_2 37°C) but normoxic release was unaffected by LY294002 (20µM) or Y27632 (10µM). In contrast, hypoxia-induced ATP release was attenuated by LY294002 and by Y27632. *P < 0.05, * < 0.01, n=18, N=6 (n=number of inserts, N=number of donors). Repeated measures ANOVA with post hoc Fisher PLSD.**

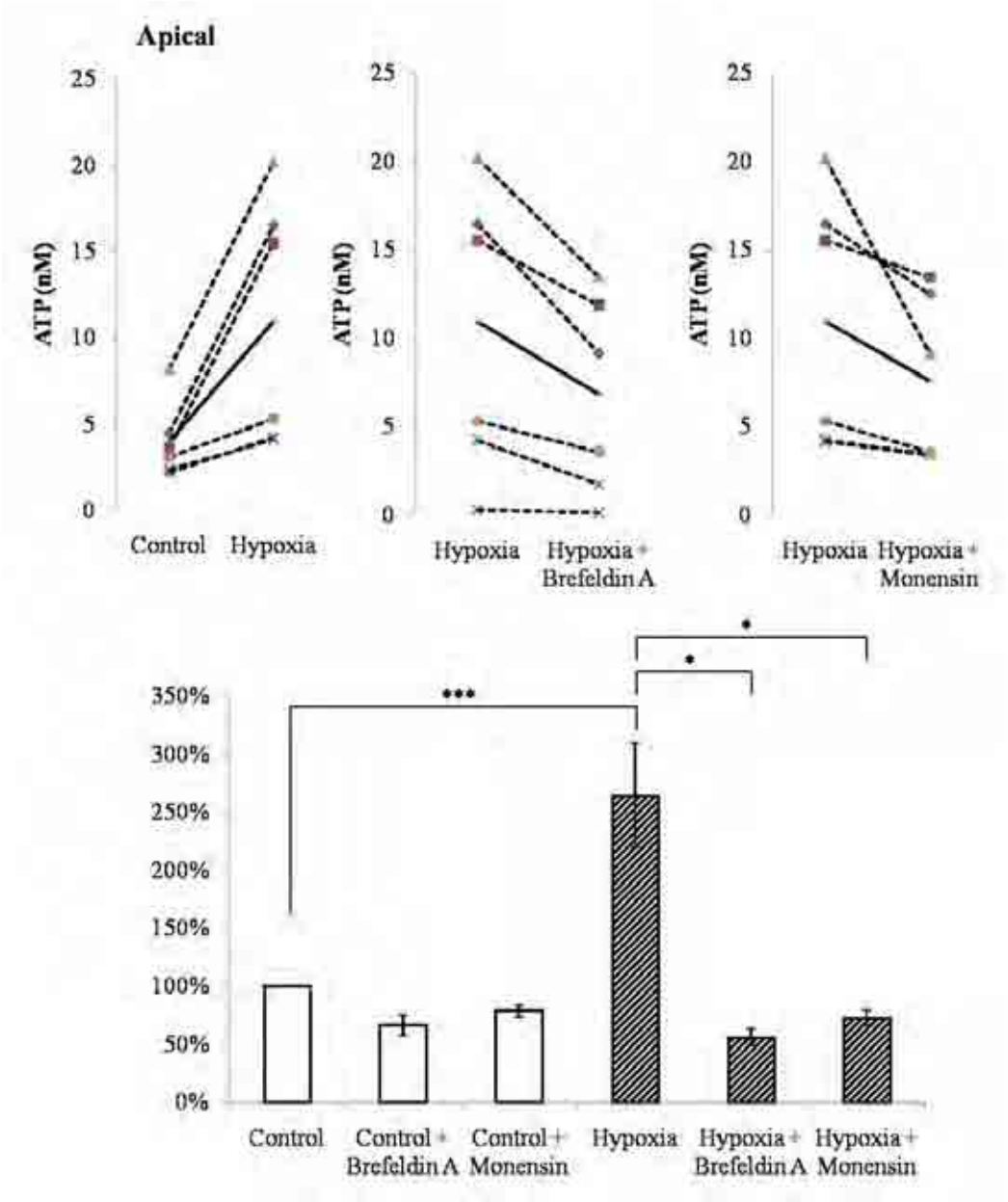


Figure 4.3.7. The effect of brefeldin A and monensin on constitutive and hypoxia - induced release of ATP from the apical membrane of HUVEC.

Upper: In each panel, dotted lines join data points measured in cells from individual donors, and solid line joins the average data for all 6 donors.

Lower: Columns show ATP release ± S.E.M relative to that measured in normoxic conditions taken as 100%. ATP release in normoxia was accentuated by 30 min hypoxia (1% O_2, 5% CO_2 37˚C) but normoxic release was unaffected by brefeldin A (20µM) or monensin (10µM). In contrast, hypoxia-induced ATP release was attenuated by LY294002 and by Y27632. *P < 0.05, * < 0.01, n=18, N=6 (n=number of inserts, N=number of donors). Repeated measures ANOVA with post hoc Fisher PLSD.**

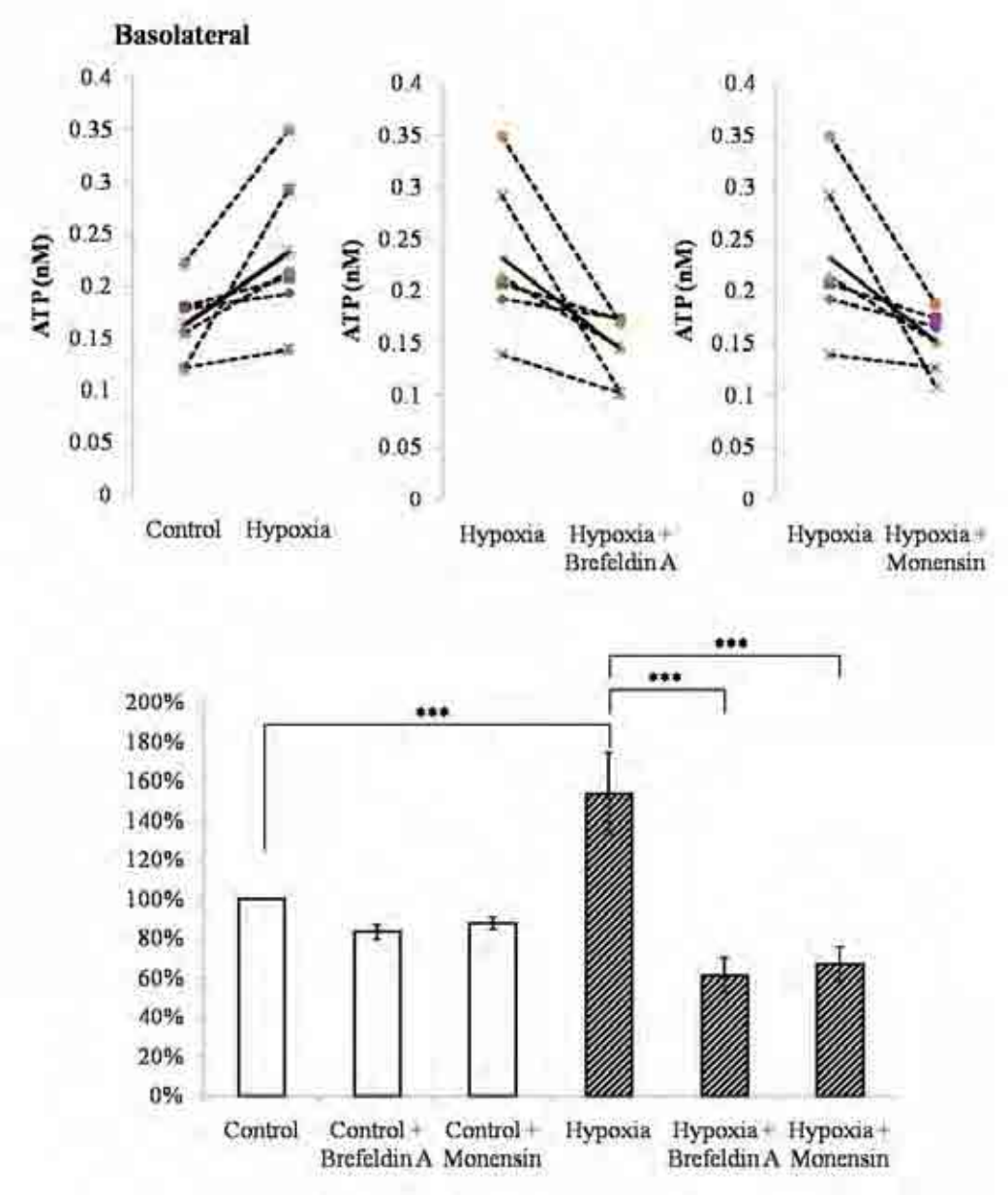


Figure 4.3.8. The effect of brefeldin A and monensin on constitutive and hypoxia - induced release of ATP from the basolateral membrane of HUVEC.

Upper: In each panel, dotted lines join data points measured in cells from individual donors, and solid line joins the average data for all 6 donors.

Lower: Columns show ATP release ± S.E.M relative to that measured in normoxic conditions taken as 100%. ATP release in normoxia was accentuated by 30 min hypoxia (1% O_2, 5% CO_2 37°C) but normoxic release was unaffected by brefeldin A (20µM) or monensin (10µM). In contrast, hypoxia-induced ATP release was attenuated by LY294002 and by Y27632. *P < 0.05, * < 0.01, n=18, N=6 (n=number of inserts, N=number of donors). Repeated measures ANOVA with post hoc Fisher PLSD.**

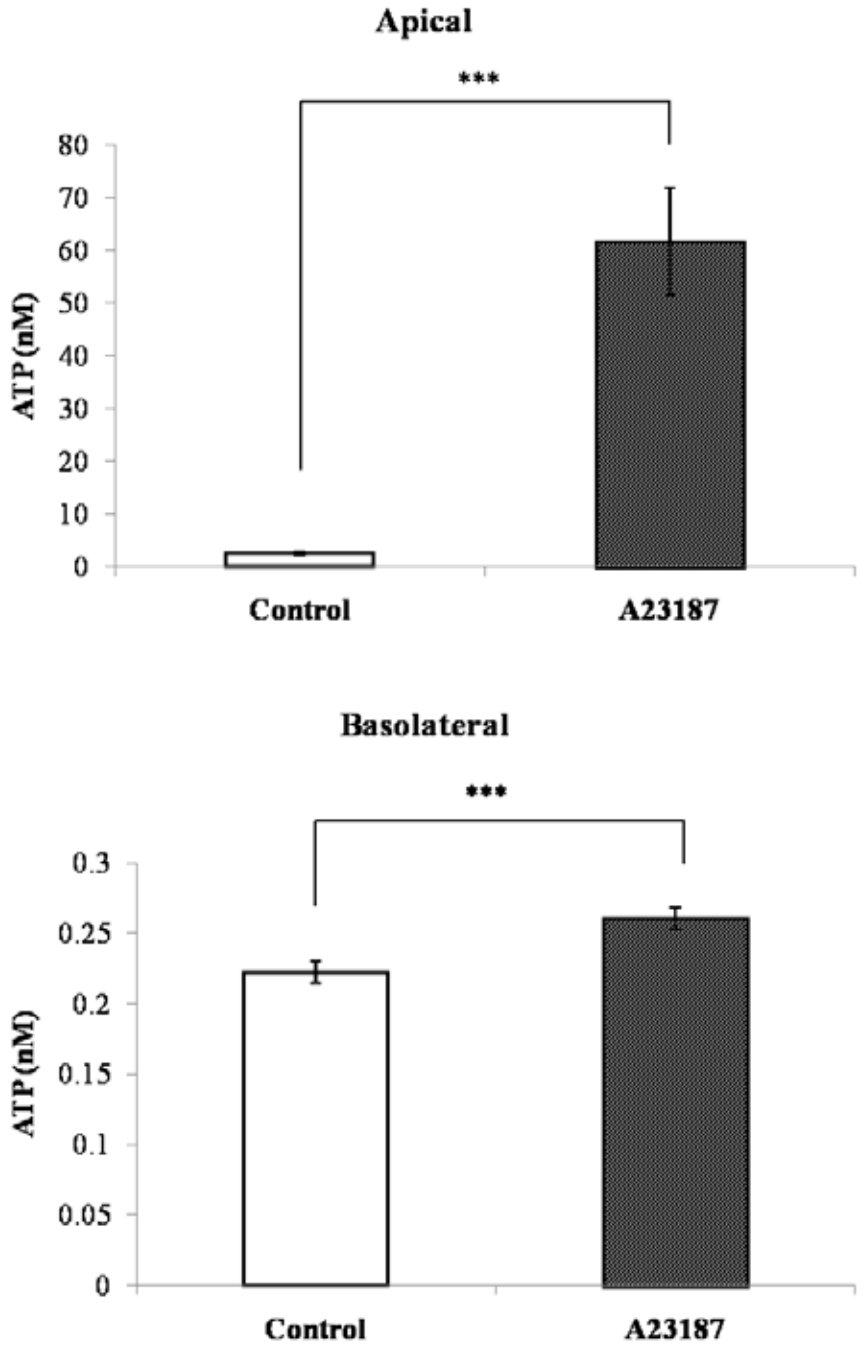


Figure 4.3.9. The effect of Ca^{2+} ionophore on apical and basolateral release of ATP from HUVEC.

Un-stimulated HUVEC released ATP from both apical (upper) and basolateral (lower) membrane (control). The release was accentuated by A23187 (10 µM). The constitutive release of ATP are at concentrations consistent to those of the other groups, so it appears that A23187 affected the release from the apical membrane in particular. *** $P < 0.01$, ± = S.E.M., n=9, N=3 (n=number of inserts, N=number of donors).

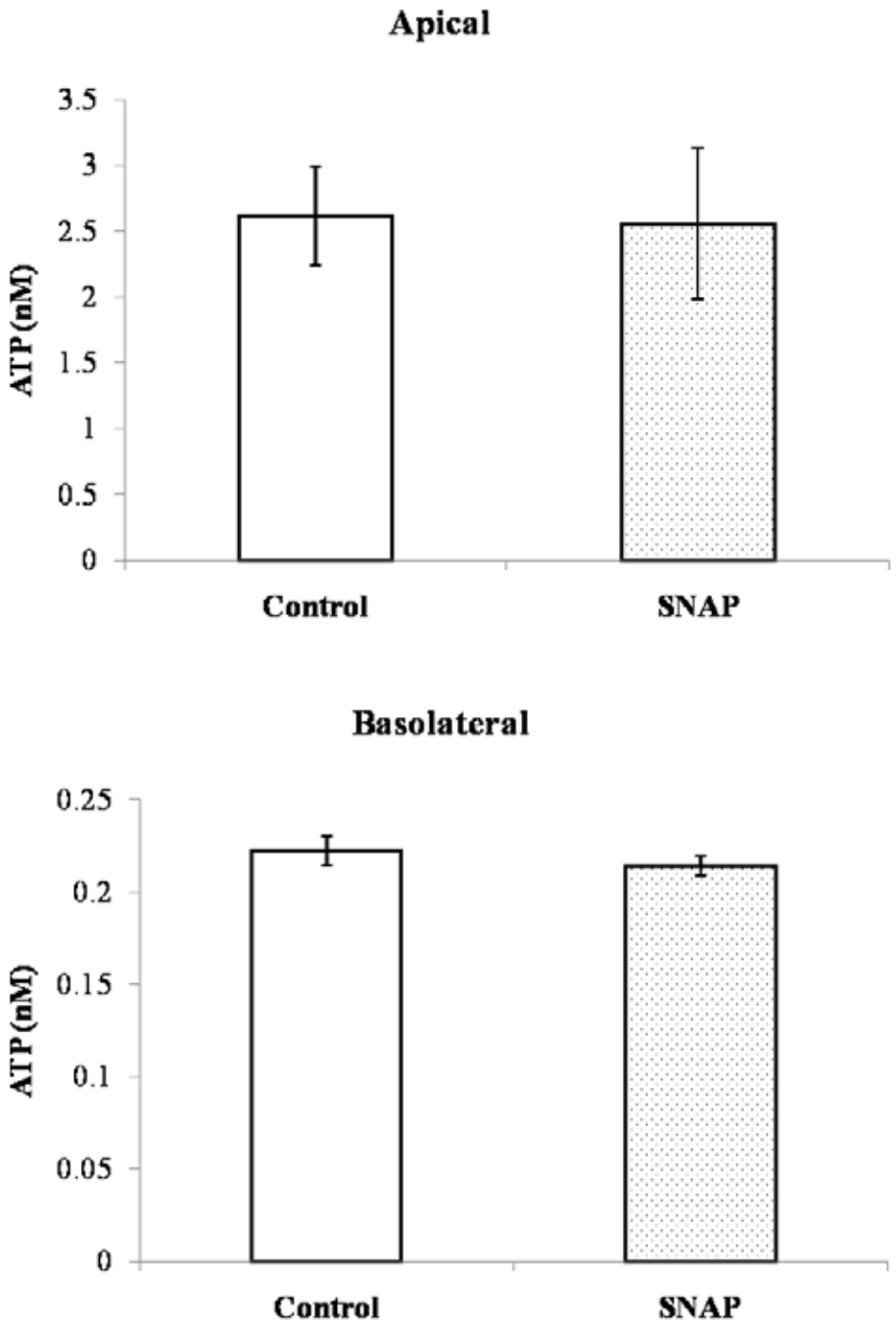


Figure 4.3.10. The effect of NO on apical and basolateral release of ATP from HUVEC.

Un-stimulated HUVEC released ATP from both apical (upper) and basolateral (lower) membrane. SNAP (100 µM), the potent NO donor, had no effect on the release. For apical *P* = 0.87 and for basolateral *P* = 0.62, ± = S.E.M., n=9, N=3 (n=number of inserts, N=number of donors).

4.4. DISCUSSION

The EC contribute to regulation of vascular tone via the release of vasoactive substances such as NO, PG, EDHF and ATP. It is well established that EC release ATP under basal conditions, and this release is accentuated by increasing the shear stress to which the EC are exposed (Milner *et al.*, 1990; Bodin *et al.*, 1991). Further, it was shown that shear stress-induced ATP release was potentiated by acute hypoxia (Bodin & Burnstock, 1995), although, as explained in *Chapter 1 Section 1.4.2*, in that study the gas mixtures used as normoxic and hypoxic conditions were 95% O_2 / 5% CO_2 and 95% N_2 / 5% CO_2 respectively. No measurements of PO_2 were made in that study, but it is likely that these conditions actually represented *hyperoxia* vs. hypoxia. More recently, using more physiological PO_2 values (equilibration with 21% vs. 1% O_2), it was reported that hypoxia *per se* could increase release of ATP from EC derived from the highly specialised vessels of calf pulmonary artery vaso vasorum (Woodward *et al.*, 2009). In the present study, equilibration with these same levels of O_2 (see *Fig 2.1* in *Chapter 2*) were used to test the hypothesis that hypoxia *per se* can induce the release of ATP from freshly isolated EC from human umbilical vessels, which are commonly used as models for EC.

4.4.1. Constitutive and hypoxia-induced ATP release in EC

In preliminary experiments where cells were grown in 24 well plates, it was shown that both un-stimulated HUVEC and HUAEC constitutively released ATP. It was noted that 30 min hypoxia at 1% O_2 accentuated this release in HUVEC, but in HUAEC any additional release did not reach statistical significance. The elevated levels of ATP measured in the hypoxia-treated HUVEC and HUAEC must have come from a regulated release source rather than from lysed cells, as the viability test with trypan blue revealed no staining. On the other hand,

all of the cells in the positive control group were strongly stained, indicating that trypan blue is a valid method of assessing cell viability.

Concerning the lack of a statistically significant effect of hypoxia on HUAEC, the trypan blue staining also revealed that there were regions of coverslip where HUAEC were absent, whereas this was not the case for HUVEC. This suggests that HUAEC did not tolerate the washing steps in the protocol, and that some of the cells became detached prior to the experiment protocol. Clearly, it is likely that their detachment and the fact that the number of cells in the normoxic and the hypoxic well was variable would have introduced variability into both sets of data. Therefore, without a suitable matrix on which HUAEC can firmly adhere, it is not possible to accurately evaluate ATP release from HUAEC or to compare directly with the release from HUVEC. If a suitable matrix could be found, it seems likely that it would be possible to demonstrate that hypoxia also induces ATP release from HUAEC.

In the main experiments of the present study, experiments were performed to differentiate the apical and basal release from EC by culturing them on inserts with an ultra-high pore density ($100 \pm 10 \times 10^6$ / cm^2). It was found that in 21% O_2, HUVEC released ATP from both apical and basolateral membrane. Further, when the difference in the culture medium volume between the apical and basolateral compartments is taken into consideration (see *Chapter 2 Section 2.2*), the apical membrane released approximately three times as much ATP as the basolateral membrane (see *Fig 4.3.4*). This polarity of ATP release is consistent with previous experiment of a similar design, which when measuring apical and basolateral constitutive ATP release from EC derived from blood vessels from a number of vascular beds, found that the majority of ATP was released from the apical membrane of cells (Schwiebert *et*

al., 2002). In the present study, it was shown that ATP release from both apical and basolateral membrane was accentuated by hypoxia. Taken together, these data demonstrate that hypoxia *per se* releases ATP from HUVEC, i.e. in the absence of shear stress. This is a novel finding for HUVEC and is in addition to the synergistic effect of hypoxia on shear stress-induced release of ATP in HUVEC (Bodin & Burnstock, 1995). The present finding is in agreement with results of Woodward *et al* (2009) on EC of calf pulmonary artery vaso vasorum. However, instead of using freshly isolated cells, they used passaged cells (no. 2 – 7) and they did not differentiate release from apical and basolateral membrane. Thus, the present study has provided the first evidence that hypoxia *per se* causes greater proportional increase in release of ATP from apical then basolateral surface of HUVEC (i.e. 79% vs. 21% increase, respectively; see *Fig. 4.3.5 & 4.3.6*).

Depending on the methodology and gestational age at the point of sampling, PO_2 values in the human umbilical vein in normal pregnancies range from 22 - 60 mmHg (Rizzo *et al.*, 1996; Lackman *et al.*, 2001; Armstrong & Stenson, 2007). However, in confirmed cases of IUGR and PE, where the intrauterine space is under-perfused, PO_2 values in the human umbilical vein are typically significantly lower, in the range of 5-16 mmHg (Nicolaides *et al.*, 1986; Rizzo *et al.*, 1996; Matsuo *et al.*, 2009). As indicated above, the O_2 levels used in the present study were 21% and 1% O_2 for normoxia and hypoxia, respectively, which equates to PO_2 valves of ~160 and 7.6 mmHg respectively. Although the PO_2 value for normoxia seems much higher than that found physiologically, it should be noted that the O_2 *content* in normal umbilical cord blood would be much high than in the medium used in the present study due to the presence of haemoglobin in the RBC in the fetal blood. On the other hand, according to the oxygen dissociation curve, fetal haemoglobin carries very little O_2 at very low PO_2 levels.

Therefore, it is reasonable to argue that the change from normoxia to the hypoxic level used in this present study (1%; 7.6 mmHg) does give an indication of the O_2 levels that the HUVEC might be exposed to in instances where intrauterine perfusion is compromised. Therefore, it can also be argued that the present study gives an indication that additional ATP would be released from the apical and basolateral surfaces of HUVEC, but particularly from the former, when umbilical vein PO_2 levels are compromised by IUGR or PE.

As discussed in the *Chapter 1*, the vasoactive substances released from endothelium can act in an autocrine way (on EC) and / or in a paracrine way (on SMC). Thus, the present results are also consistent with the idea that in hypoxia, ATP released from the apical surface of EC may act as an autocrine signal, activating P2Y receptors and EC and release potent vasodilators such as PGI_2, NO and EDHF (Chen & Suzuki, 1990; Olsson & Pearson, 1990), whereas ATP released from the basolateral surfaces may act as an autocrine as well as an paracrine signal, activating P2X and P2Y on SMC (see *Chapter 3 Section 3.4.3*). In addition, there is the possibility that hypoxia-induced ATP released into the lumen of the vessel can interact with circulating cells and platelets. The potential implication of this is discussed in *Chapter 8*.

4.4.2. Intracellular transduction pathways

Considering the present new finding that hypoxia releases ATP from HUVEC, several questions arise: what is the O_2 sensor and what are the intracellular signal transduction pathways between O_2 sensing and ATP release.

A number of important cellular functions, notably growth and survival, cytoskeleton remodelling and intracellular organelles trafficking depend on phosphoinositide 3-kinases

(PI_3K) together with their downstream effectors protein kinase C (PKC) and Rho-associated protein kinase (ROCK) (De Camilli *et al.*, 1996; Katso *et al.*, 2001; Koyasu, 2003). In the present study, either the PI_3K inhibitor or ROCK inhibitor, LY294002 and Y27632 respectively, attenuated the hypoxia-induced ATP release from HUVEC, but had no effect on constitutive release in 21% O_2. Previous studies have provided strong evidence that PI_3K is activated by shear stress in EC e.g. (Dimmeler *et al.*, 1999). However, a static model was used in the present study. Thus, given that neither antagonist significantly affected constitutive release of ATP in 21% O_2, it can be deduced that hypoxia *per se* is able to mediate ATP release via the activation of the PI_3K pathway, and that constitutive release is dependent on a different mechanism. This is consistent with recent evidence from studies in rat liver epithelium and calf pulmonary aorta vaso vasorum EC which suggested that hypoxia may be able to activate PI_3K and subsequently mediate ATP release via PKC and ROCK (Woodward *et al.*, 2009; Feranchak *et al.*, 2010). Thus, it seems that the mechanisms underlying the hypoxic release of ATP are similar across different tissue cells in different species.

4.4.3. Is vesicular transport responsible?

PI_3K, PKC and ROCK are associated with trafficking of intracellular organelles (Slomiany *et al.*, 1998; Kaibuchi *et al.*, 1999; Zhao *et al.*, 2007), and it was shown that intracellular transport vehicles contributed to ATP release from oocytes and osteoblasts (Maroto & Hamill, 2001; Orriss *et al.*, 2009). Thus, experiments were performed in the present study to test the hypothesis that the hypoxia-induced ATP release from HUVEC was mediated by vesicular transport. Brefeldin A and monensin are two commonly used inhibitors which prevent vesicular transport by targeting different stages of the intracellular vesicle trafficking process.

The former inhibits transport from the endoplasmic reticulum (ER) to the Golgi apparatus (Lippincott-Schwartz *et al.*, 1989), whereas the latter is a monovalent ion-selective ionophore that is able to disrupt the H^+ gradient of intracellular organelles such as Golgi apparatus, lysosomes and secretory vesicles (Mollenhauer *et al.*, 1990). Here, it was shown that both brefeldin A and monensin were able to functionally inhibit hypoxia-induced ATP release from HUVEC, providing strong evidence that this ATP release from HUVEC is mediated by regulated exocytosis. Again, these data are consistent with the findings on EC derived from calf pulmonary artery vaso vasorum (Woodward *et al.*, 2009), and suggest that vesicular transport may mediate hypoxia-induced release of ATP by EC across different vascular beds in different species.

The NO donor SNAP did not affect ATP released from either apical or basolateral surfaces in HUVEC. As hypoxia-induced adenosine release from EC is NO-dependent (see *Section 4.1*), this data suggest that the release mechanism of ATP release from EC is distinct from that of adenosine. Further, as it was shown that NO inhibited ATP release from RBC (Ellsworth *et al.*, 2009), this data also suggests that the mechanisms underlying release of ATP from EC is distinct from that of RBC.

It is generally accepted that Ca^{2+}-dependent, regulated exocytosis is responsible for the release of ATP and other transmitters from neuronal and secretory cells (Barclay *et al.*, 2005). However, in the last few years, evidence is accumulating which suggests that this may also be the case in non-excitable cells such as epithelial cells, fibroblasts and EC when they are stimulated with cell swelling, agonists or mechanically challenged (Boudreault & Grygorczyk, 2004; Praetorius & Leipziger, 2009), although these mechanisms are currently

far from well-understood. In the present study, when $[Ca^{2+}]_i$ in HUVEC was raised by using the Ca^{2+} ionophore A23187, this alone caused a substantial release of ATP from the apical and basolateral surfaces, but particularly from the apical surface. A previous study showed that addition of ionomycin, another Ca^{2+} ionophore, caused a sustained release of ATP from HUVEC but did not differentiate apical and basolateral release (Schwiebert *et al.*, 2002).

The amount of ATP released by A23187 from the basolateral surface was in similar proportion to that of hypoxia-induced ATP release (20-50% greater than constitutive release), whereas that released from the apical surface was much greater (~600% vs. 200-300% greater than constitutive release). Assuming that the hypoxia-induced ATP release was indeed mediated by vesicular processes, this result suggests that there is a bigger pool of ATP-rich vesicles close to the *apical* membrane than to the basolateral membrane. A further deduction can be made from this data. Given that ATP can act on purinergic receptors on EC to increase $[Ca^{2+}]_i$ (see *Chapter 1 Section 1.3.6.3*), hypoxia-induced ATP release from HUVEC may cause further ATP release. The phenomenon of ATP-induced ATP released from HUVEC has previously been shown by adding exogenous ATP to cultured cells and measuring ATP present in the extracellular space for up to 20 min (Bodin & Burnstock, 1996). The present findings raise the possibility that $[Ca^{2+}]_i$ elevation plays a role in this phenomenon. The question also arises as to whether hypoxia-induced ATP release is mediated by a rise in $[Ca^{2+}]_i$. These issues are addressed in *Chapter 6*.

In summary, by using the luciferin-luciferance assay, it was demonstrated that HUVEC constitutively release ATP from both apical and basolateral membrane, and this release was accentuated in hypoxia via a PI_3K / ROCK dependent pathway. Further, the present results

suggest that the hypoxia-induced component of the ATP release is mediated via regulated exocytosis. In the studies described in the next chapter, the location and behaviour of the putative ATP vesicles in HUVEC were investigated using a combination of quinacrine staining and fluorescence microscopy.

CHAPTER 5

VISUALISATION OF ATP VESICLES IN HUVEC AND THE EFFECT OF HYPOXIA

5.1. INTRODUCTION

In Chapter 4, functional data obtained by using the luciferin-luciferance assay demonstrated that HUVEC constitutively released ATP from both the apical and basolateral membrane, and this release was accentuated in hypoxia via a PI_3K / ROCK dependent pathway. Further, the hypoxia-induced component of ATP release was shown to be inhibited by antagonists that are known to inhibit the process of intracellular vesicle trafficking.

If the ATP release is indeed mediated via regulated exocytosis, one would expect vesicles containing ATP to be present in HUVEC. Further, given the fact that release of ATP from the apical membrane was consistently higher than that from the basolateral membrane (see *Chapter 4 Section 4.3.4*), one would expect the morphology of the cells to reflect the physiology i.e. that the putative ATP vesicles would be concentrated in regions close to the apical membrane.

In order to address these questions in the present study, HUVECs were stained using the fluorescent dye quinacrine, an acridine derivative. Quinacrine has a high affinity to ATP, produces a concentration-dependent fluorescence, and has been used to label intracellular ATP in a number of cell types, including HUVEC (Mitchell *et al.*, 1998; Bodin & Burnstock, 2001a; Sorensen & Novak, 2001; Pangrsic *et al.*, 2007). Confocal and conventional fluorescent microscopy were used to access the behaviour of the ATP vesicles of HUVEC in 21% O_2, after exposure to hypoxia, and after treatment with antagonists that interfere with intracellular vesicle trafficking processes.

5.2. METHODS

The details of the methodology used in the studies described in this chapter are described in *Chapter 2 Section 2.1, 2.2.1 & 2.2.4*.

PROTOCOLS

5.2.1. Group 1: Confocal imaging

Primary HUVECs from 2 donors were isolated from human umbilical vein, subcultured and grown on EPSE-coated ø13 mm glass cover slips as described in section *Chapter 2 Section 2.1.1, 2.1.2 & 2.1.3*. Confluent HUVEC monolayers were incubated in quinacrine (1.5 µM) for 60 min, rinsed in Krebs-Ringer buffer, then mounted in VECTASHIELD® mounting medium with DAPI (Vector Laboratories ltd, U.K.) and examined with confocal microscopy (see *Chapter 2 Section 2.2.4*).

5.2.2. Group 2: Fluorescent imaging and the effect of hypoxia

HUVECs isolated from the two separate donors were cultured separately. To examine the effect of hypoxia on quinacrine fluorescence, cells from each donor were divided into two groups: a normoxic and a hypoxic group. Cells in both groups were then incubated in 0.5 µM quinacrine (Sigma-Aldrich, U.K.; freshly made in Krebs-Ringer buffer) for 60 min at 37 ºC in either 5% CO_2 / 95% air (normoxia), or 1% O_2 / 5% CO_2 / 94% N_2 (hypoxia; in hypoxic chamber described in *Chapter 2 Section 2.2.1)*. On leaving the respective incubators, the cells were rinsed twice in Krebs' and mounted on microscope slides in mounting medium without DAPI. The cells were immediately examined with FITC-fluorescent microscopy (within 5 min; see *Chapter 2 Section 2.2.4* for details of the methodology). Cells were compared to those from the same donor and passage.

5.2.3. Group 3: Fluorescent imaging and the effect of vesicular inhibitors

HUVECs were isolated and cultured as in *Group 2*. In order to examine the effect of the vesicle trafficking inhibitors, cells were first incubated in brefeldin A (10 μM), monensin (100 μM), or vehicle (1:1000 DMSO) for 60 min in 21% O_2, before incubation with quinacrine (30 μM) for 5 min together with the same inhibitors. Brefeldin A and monensin were made up from frozen stock solutions as in *Chapter 4 Section 4.2.5*. They were then carefully rinsed and mounted as in *Group 2*. As for *Group 2*, cells were compared to those from the same donor and passage. This combination of quinacrine concentration and incubation time was determined by preliminary experiments using 1, 3, 10 and 30 μM with 5, 30 and 60 min combinations (see *Chapter 2 section 2.2.4*).

5.2.4. Analysis of results

Comparison of intensity of fluorescence were made between cells from the same donor and of the same passage. Direct comparisons of fluorescence intensity *in Group 2 & 3* were made with identical power of magnification, exposure and gain settings (indicated at each Figure).

5.3. RESULTS

5.3.1. Group 1

Confocal imaging study revealed that cellular ATP stores labelled with quinacrine were not uniform. Rather, they assume a punctate pattern (Fig 5.3.1). Moreover, the clusters of ATP fluorescence were mainly concentrated in the para-nuclear space; not all cells contained ATP fluorescence. Each locus of fluorescence measured 0.5-1.4 μm in diameter. The monolayers of HUVEC were approximately 4.0 μm in thickness. Images captured at 1.0 μm intervals in

the *z* plane showed no obvious differential apical and basolateral distribution of ATP fluorescence (Fig 5.3.1).

5.3.2. Group 2

In HUVEC incubated in 21% O_2, fluorescent imaging of the monolayer by conventional microscopy showed similar punctate distribution to that of the confocal imaging study (Fig 5.3.2 A). When the HUVEC monolayers were visualised with the additional aid of phase contrast bright-field light, it was apparent that the fluorescence loci were concentrated in the para-nuclei space away from the cell borders (Fig 5.3.2 A & B right). Real-time observation of the monolayers showed that the loci of fluorescence were not stable. Rather, there was an occasional non-recurring transient increase in fluorescence intensity at particular locus followed by rapid disappearance (data not shown).

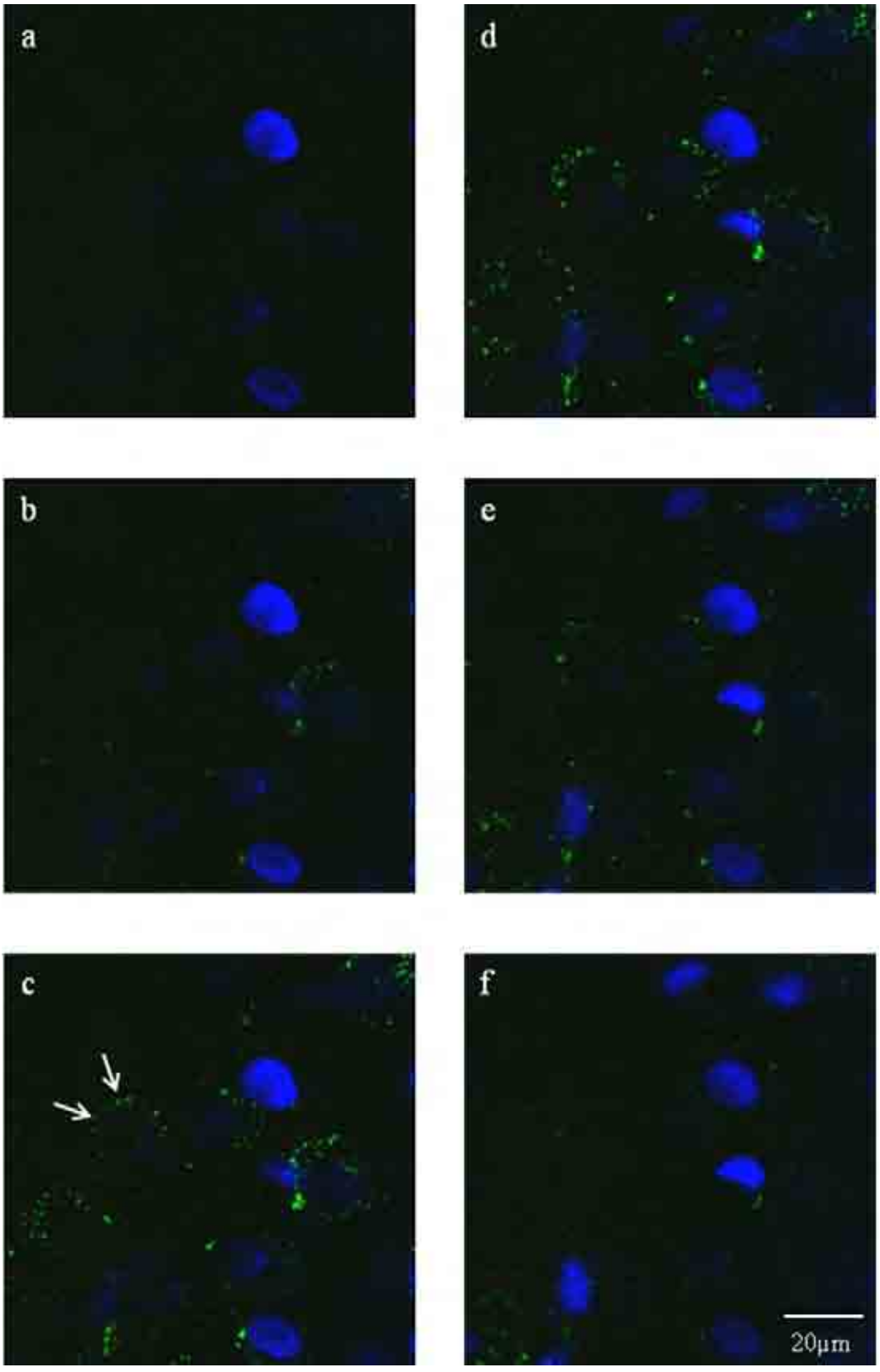
When HUVEC was incubated in hypoxia (1% O_2, 60 min), the fluorescence intensity was markedly attenuated (Fig 5.3.2 B).

5.3.3. Group 3

HUVEC incubated with quinacrine in vehicle for the antagonists (1:1000 DMSO) showed typical punctate fluorescence staining, similar to that of *Group 2* (Fig 5.3.3A). Brefeldin A, which inhibits vesicular trafficking, caused an increase in the areas of fluorescence within the cells (10 μM for 60 min; Fig 5.3.3B). In contrast, monensin, another agent that inhibits vesicular trafficking, decreased the intracellular levels of quinacrine fluorescence (100 μM for 60 min; Fig 5.3.3C).

Figure 5.3.1. Confocal imaging of HUVEC monolayer dual-labelled with quinacrine and DAPI.

a-f are *z stack* images of HUVEC, at 1.0 µm interval. The nuclei appear blue and the ATP vesicles appear green. The ATP vesicles were found mainly in the cytoplasm, in the para-nuclei space, but not in the nuclei (white arrows). HUVEC are only ~4.0 µm in thickness and it was not apparent whether the ATP vesicles were distributed more apically or basolaterally. Under these staining conditions, not all cells appear to contain ATP vesicles. Images are representative of 4 coverslips tested from 2 donors.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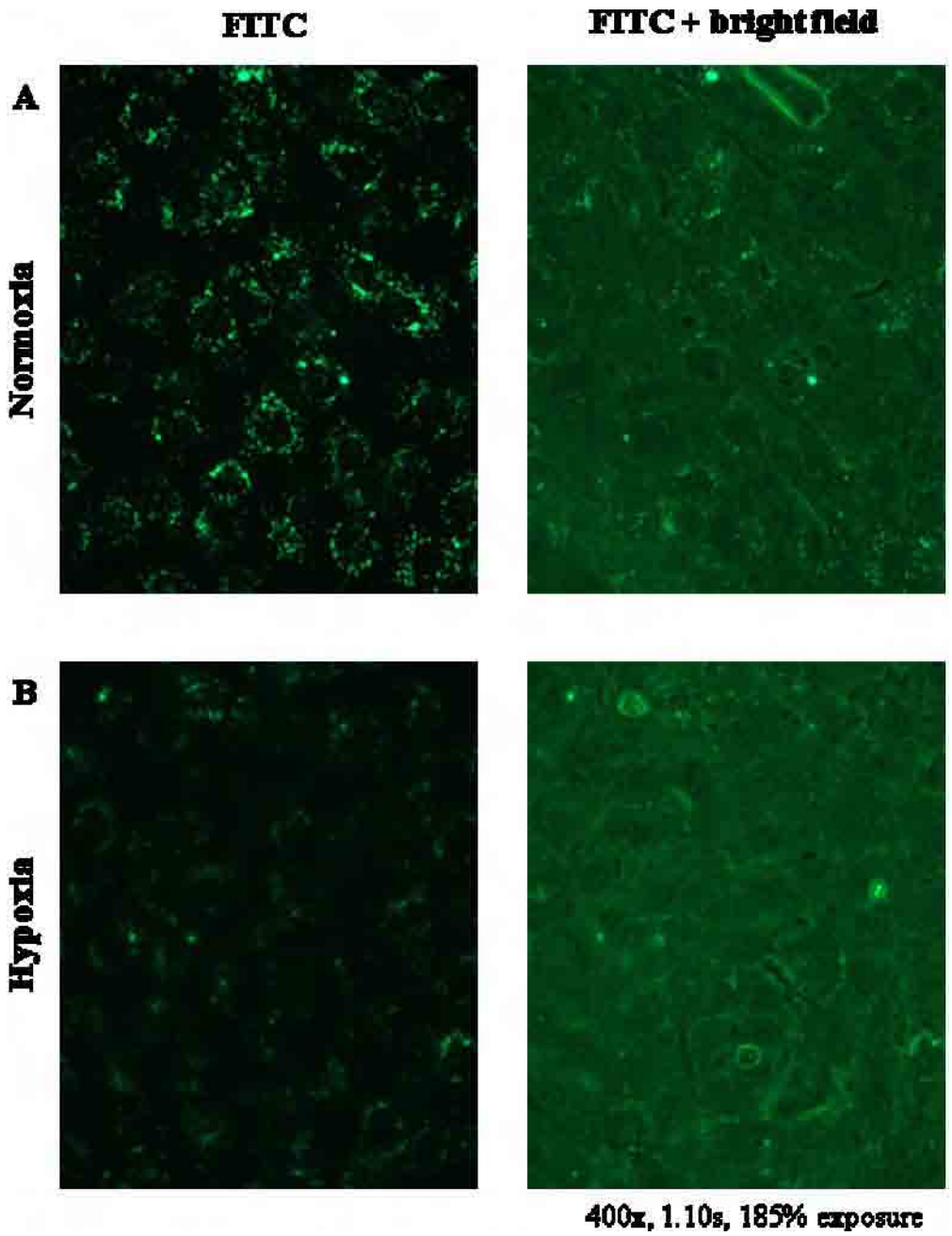
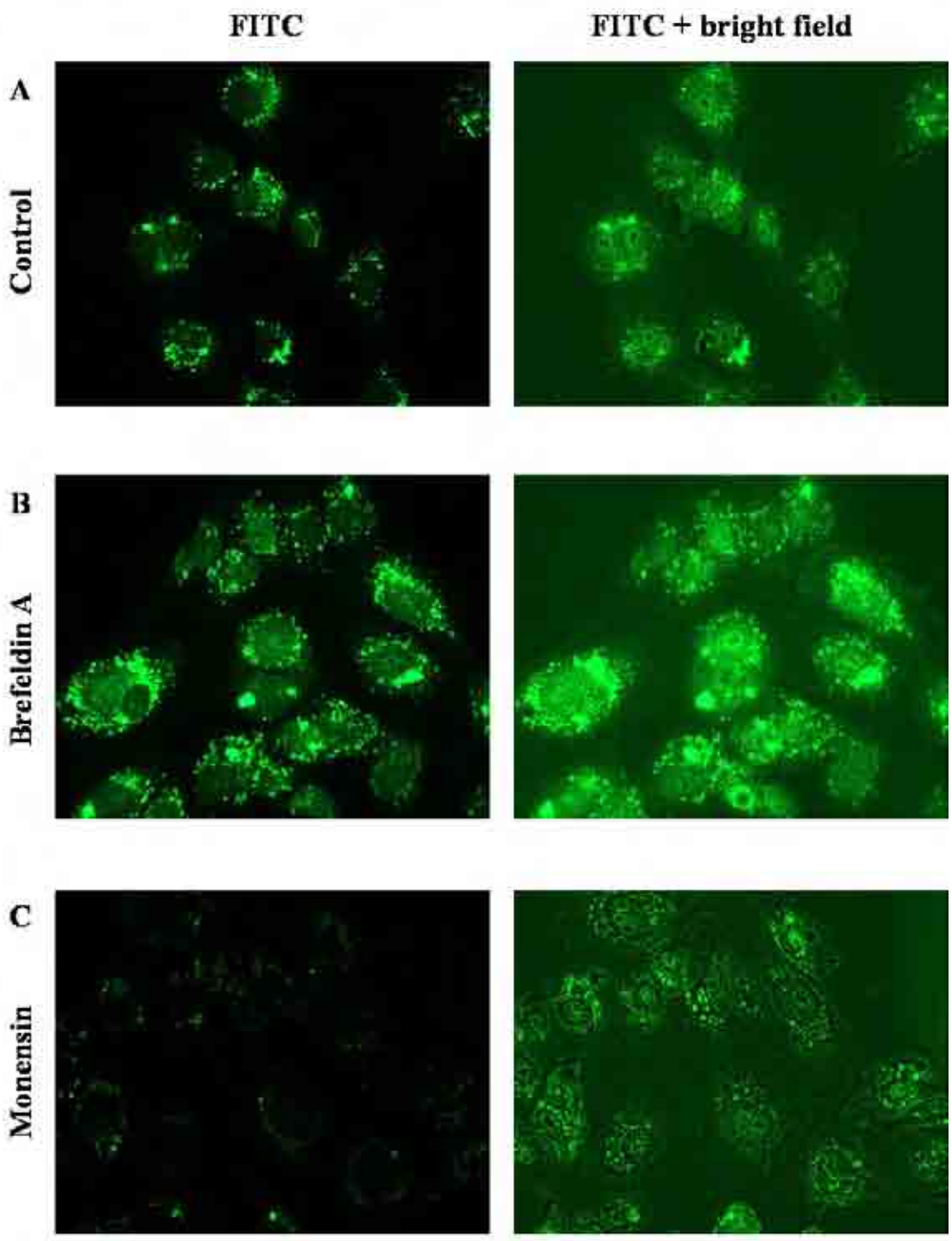


Figure 5.3.2. Representative fluorescent and bright field images of HUVEC incubated in quinacrine in 21% or 1% O_2.

A: In 21% O_2, HUVEC showed strong punctate staining in the cytoplasm. When visualised together with bright field illumination, it can be seen that the fluorescent staining are mainly in the para-nuclei space, and are not present in all HUVEC. B: Hypoxia (1% O_2; 60 min) caused a pronounced attenuation of the fluorescent intensity. n = 3, N=2 for both 21% and 1 % O_2 (n=number of coverslips, N=number of donors).

Figure 5.3.3. Typical representation of fluorescent and bright field images of quinacrine-stained HUVEC after treatment with brefeldin A, monensin or vehicle.

A: In control, HUVEC showed strong punctate staining in the cytoplasm. B: Brefeldin A (10 µM) increased the areas of fluorescence within the cell, away from the cell membrane (cf A). C: Monensin (100 µM), by contrast, decreased the intracellular levels of quinacrine fluorescence (cf A). n = 3, N=2 for each conditions tested (n=number of coverslips, N=number of donors).


FITC
FITC + bright field
A
Control
B
Brefeldin A
C
Monensin
400x; 237ms; 100% exposure

5.4. DISCUSSION

5.4.1. Quinacrine staining of HUVEC

As discussed in *Chapter 1 Section 1.4.3*, a number of mechanisms for the release of ATP have been proposed. In the studies described in *Chapter 4*, results obtained using luciferin-luciferance assay for ATP and pharmacological agents suggested that hypoxia-induced ATP release from HUVEC is mediated by vesicular pathways. In the present study, staining of HUVEC with the fluorescent dye quinacrine revealed a punctate granular pattern, that is reminiscent of ATP localisation within intracellular secretory vesicles. Further, real-time observation of quinacrine-stained HUVEC in 21% O_2 revealed that the vesicles were not in a steady state, but sometimes displayed a non-recurring transient increase in fluorescence intensity followed by rapid disappearance. This point-source burst release is consistent with fusion of ATP-containing vesicles with the plasma membrane and exocytotic release of quinacrine/ATP into the extracellular space, similar to that described by others in liver epithelium and astrocytes (Bowser & Khakh, 2007; Feranchak *et al.*, 2010). This spontaneous release may contribute to the constitutive ATP release by HUVEC in normoxia discussed in *Chapter 4*.

Interestingly, in a previous study it was reported that point-source burst pattern of ATP release from HUVEC was associated with propodium uptake (Arcuino *et al.*, 2002). The authors associated this with an increase in membrane permeability, and on this basis they speculated that the ATP release was conducted though ATP-permeable channels, possibly a form of connexin hemichannel.

Quinacrine is an acridine derivative that has long been used as an experimental tool for localisation of intracellular ATP owing to its property of high affinity to ATP as well as its ability to produce fluorescence that is dependent on the concentration of ATP (see *Section 5.1*). The specificity of this technique has never been established, and it is possible that, being a weak base, it accumulates in intracellular acidic stores, and hence acts as an acidic store marker. However, parallel staining with established acidic store markers, acridine orange and LysoTracker Red, revealed only partial overlapping staining pattern with quinacrine staining (Sorensen & Novak, 2001), indicating a degree of preferential binding of quinacrine for ATP. Further, the observations made with confocal microscopy in the present Chapter revealed that the fluorescence loci in HUVEC were between 0.5-1.4 μm in diameter, which is consistent with intracellular vesicles. Recently, a novel technique of monitoring intracellular level has been developed by utilising genetically-encoded fluorescence resonance energy transfer (FRET), which, when coupled with intracellular compartment signals, was shown to be capable of indicating ATP concentrations in different subcellular compartments (nucleus and mitochondria) in an immortalised cell line (Imamura *et al.*, 2009). Potentially, the identification of ATP-rich vesicles in HUVEC could be further investigated by adapting this technique to target Golgi-associated vesicles.

5.4.2. Effect of hypoxia

In the present study, it was shown that hypoxia markedly reduced the level of fluorescence of the quinacrine-stained loci, suggesting ATP was released from these putative vesicles in response to hypoxia. It is possible that hypoxia could affect the binding of quinacrine to ATP in vesicles. As this has to-date never been reported in the literature, it seems an unlikely possibility. This data is consistent with previous report in HUVEC, which showed that the

fluorescence of these loci decreased following a shear stress challenge (Bodin & Burnstock, 2001a). Together with the data of *Chapter 4*, these findings are fully consistent with the view that hypoxia *per se* i.e. in the absence of shear stress, releases ATP from HUVEC via a regulated exocytotic mechanism. Interestingly, the results of the studies described in the present Chapter are very similar to that shown obtained in rat osteoblasts (Orriss *et al.*, 2009), and suggest that at least some ATP release mechanisms are universal between different tissues across different species.

5.4.3. Effects of vesicular inhibitors

Intracellular trafficking involves multiple complex processes that mediate multi-directional movement of molecules between the ER, Golgi apparatus, secretory vesicles and lysosomes. As discussed in *Chapter 4*, brefeldin A interrupts the transport between ER and Golgi apparatus. In the present study, it was shown that incubation of HUVEC in brefeldin A together with quinacrine increased the number of fluorescence loci when compared to incubation with quinacrine alone. It is well documented that brefeldin A interrupts the vesicular pathway by inducing retrograde transport from the trans Golgi network towards the ER which often results in an accumulation of itinerant molecules (Klausner *et al.*, 1992; Rosa *et al.*, 1992; Vetterlein *et al.*, 2003). The present finding therefore suggests that the anterograde transport of ATP was interrupted by brefeldin A and resulted in an accumulation of intracellular ATP, presumably in the *cis* Golgi network and ER. This is in contrast to a previous study on liver epithelial cells, in which exposure to brefeldin A reduced hypotonic-induced ATP *release*, but it decreased the number of quinacrine-stained vesicles under basal conditions (Feranchak *et al.*, 2010). The reason for this apparent discrepancy is not absolutely clear. However, it should be noted that the liver epithelial cells were incubated in brefeldin A

for as long as 4-6 hr, as compared with 30 min for HUVEC in the present study. Thus, it would not be surprising if the accumulation of ATP in ER and Golgi apparatus was disrupted to a larger extent in liver epithelium. Alternatively, it could be that there are differences in intracellular trafficking processes between different tissue of different species, or between primary cultures and immortalised cell lines.

On the other hand, it was shown in the present study that monensin, another widely used investigative tool for studying intracellular trafficking pathways, reduced the quinacrine-stained vesicles in HUVEC under basal conditions, as it did in liver epithelial cells (Feranchak *et al.*, 2010). As discussed in *Chapter 4*, monensin is a monovalent ion-selective ionophore whose primarily action is facilitation of Na^+/H^+ transport, thus collapsing the membrane potential difference generated in the trans Gogi network and secretory vesicles (Mollenhauer *et al.*, 1990).

Therefore, taken together the findings discussed above are consistent with the conclusion that ATP vesicles are present in HUVEC under basal condition. In future studies, it would be important to take further steps to establish that hypoxia-induced release of quinacrine-stained ATP is mediated from vesicles by monitoring ATP release with time.

5.4.4. Formation of ATP vesicles in HUVEC

The presence of ATP vesicles has been described in a number of cell types and tissues including chromaffin, acinar, epithelial, endothelial cells and astrocytes (Sorensen & Novak, 2001; Arcuino *et al.*, 2002; Bowser & Khakh, 2007; Sawada *et al.*, 2008; Feranchak *et al.*, 2010). The precise mechanism of formation of ATP vesicles is far from understood.

Essentially there are two recognised processes for vesicular formation in cells. Several studies, including the present one, which utilised the intracellular transport inhibitor brefeldin A have found that it inhibited ATP release (Abdipranoto *et al.*, 2003; Woodward *et al.*, 2009; Feranchak *et al.*, 2010). This suggests that ATP vesicles originate from a Golgi – ER – vesicle source.

However, it is also widely accepted that ATP vesicles are formed just like synaptic vesicles in specialised secretory cells (Pangrsic *et al.*, 2007). It is believed that H^+ accumulates in synaptic vesicles via vesicular ATPase (V-ATPase), the resultant electrochemical gradient then provides energy to drive a recently identified H^+/ATP exchanger SLC17A9, thus forming ATP-rich vesicles (Sawada *et al.*, 2008). Accordingly, it is possible that both mechanisms are functionally active in HUVEC. Therefore, it would be very important in future studies to perform experiments on HUVEC which involve observation of changes in ATP vesicles after incubation in bafilomycin, the general inhibitor of V-ATPase (Hanada *et al.*, 1990), or silencing of SLC17A9 by small interference RNA. Indeed, strong evidence indicates that multiple mechanisms simultaneously orchestrate the release of ATP in other cell types such as astrocytes and lymphocytes (Stout *et al.*, 2002; Coco *et al.*, 2003; Tokunaga *et al.*, 2010). To complicate matters even further, astrocytes incubated in FM dye showed that agonists and ischemia (KCN)-induced release of ATP was from a lysosomal source, which was distinct from the secretory vesicles (Zhang *et al.*, 2007). It will be important to determine whether these different populations of organelles are recruited in HUVEC under different conditions.

In summary, by using quinacrine staining, it was shown here that ATP vesicles are present in HUVEC in 21% O_2. Interference of the intracellular trafficking pathway with brefeldin A and

monensin affected the staining in different but explainable ways. Further, hypoxia caused a pronounced attenuation of the number of these punctuate fluorescent vesicles. These findings add another dimension of evidence in support of the findings in *Chapter 4*, that HUVEC release ATP in hypoxia via a vesicular pathway. The consequences of the action of ATP on the HUASMC were considered in *Chapter 3*. In the next Chapter, the effects of ATP on HUVEC are presented.

CHAPTER 6

THE EFFECT OF EXOGENOUS ATP AND HYPOXIA ON $[Ca^{2+}]_i$ IN HUVEC

6.1. INTRODUCTION

The endothelium lines the blood vessels, arguably the ideal location for sensing the metabolic signal generated from surrounding organs. It is well documented that shear stress produces an endothelium-dependent vasodilatation via the release of PGI_2, EDHF, but also by release of NO via the action of purines (see *Chapter 1 section 1.3.3.2, 1.3.4.2 & 1.3.6.3*). The direct effect of hypoxia on endothelium, however, is far from well understood. The studies described in *Chapters 4 & 5* indicated that HUVEC and HUAEC constitutively release ATP in 21% O_2, and importantly, the release from HUVEC at least, was accentuated in hypoxia. The resultant ATP may act on SMC (*Chapter 3*), or it may act on the ECs themselves to activate purinoceptors and release a range of endothelium-derived vasoactive substances, NO, PGs, EDHF and ET, which were discussed in some detail in *Chapter1 section 1.3*. It is apparent that intracellular Ca^{2+} plays at least some role in the action of such substances, as well as in the mechanism that releases ATP. Indeed, as shown in *Chapter 4 section 4.3.5*, elevating $[Ca^{2+}]_i$ in HUVEC with ionophore caused ATP release, presumably at least in part through release of ATP-containing vesicles. Therefore, in the studies described in the present chapter, the effects of hypoxia, as well as those of exogenous ATP on $[Ca^{2+}]_i$ HUVEC were explored.

6.2. METHODS

The details of the methodology used to prepare the HUVEC are described in *Chapter 2 section 2.3.* For clarification, HUVEC were loaded with Fura-2 AM (Invitrogen, UK) for Ca^{2+}-imaging, the exact protocols of which could be found in *Chapter 2 Section 2.3.2.*

PROTOCOLS

6.2.1. Group 1: Exogenous ATP

Umbilical cords from 6 individual donors were divided into 2 sets, and freshly isolated HUVEC within each set (3 separate donors) were mixed and plated on APSE-coated glass coverslips as described in *Chapter 2 Section 2.1.1*. Fura-2 AM was loaded by incubating the cells in 200 µl of 12.5 µM of the dye for 30 min at RT and then incubated in a 37 °C humidified incubator of 5% CO_2 and balanced air for a further 30 min. They were then rinsed in normal Krebs' before mounted in an air-tight cuvette as described in *Chapter 2 Section 2.3.3*. This loading procedure was performed for all of the following protocols. To test if shear stress in the present system affected $[Ca^{2+}]_i$ in HUVEC in the present set up, cells was first exposed to static, then laminar flow, by controlling the peristaltic pump. ATP (10 µM; Sigma-Aldrich, UK; dissolved in normal Krebs' on the day of experiment) was applied to the HUVEC monolayer for a period of 4 min before being washed off. For the purpose of standardisation, ATP applications in all of the protocols described below are of 4 min in duration unless otherwise specified.

Next, in order to avoid desensitisation of the purinoceptors in subsequent experiments, it was necessary to establish the length of wash period between consecutive ATP challenged. To this end, an increasing length of wash period was used, starting with 2 min then increasing at 2 min increments, until two identical $[Ca^{2+}]_i$ responses were produced by repeated challenge with ATP (10 µM). The same procedure was done for ATP at 100 µM.

An ATP:$[Ca^{2+}]_i$ dose-response curve was generated by comparing the maximum $[Ca^{2+}]_i$ response of concentrations of ATP to that produced by 100 µM ATP. The ATP

concentrations used were 1, 10, 100, 300 and 1000 μM. Because of the length of the wash period (12 min), only one concentration other than 100 μM was used in each experiment. e.g. 1 and 100 μM, 10 and 100 μM etc. To eliminate time-dependent effects, the test concentration and 100 μM ATP were applied in random order.

6.2.2. Group 2: General P2 antagonist

HUVEC was isolated from 6 umbilical cords in the same configuration as in *Group 1,* with a mixture of cells from 3 separate donors constituting each of the 2 sets of coverslips, and loaded with Fura-2 AM. To test whether the $[Ca^{2+}]_i$ response of HUVEC was mediated by ATP, cells were either challenged with ATP alone (10 μM; as in *Group 2*), or pre-incubated with the non-specific P2 receptor antagonist suramin (100 μM) for 2 min before challenged with exogenous ATP (10 μM). Both with and without suramin, ATP stimuli were tested on cells from the same coverslip. In order to eliminate time-dependent factors, the order of with or without suramin conditions was randomised. Suramin at 100 μM has been previous shown to inhibit ATP-induced Ca^{2+} response in mesenteric arteries (Lagaud *et al.*, 1996).

6.2.3. Group 3: Role of extracellular Ca^{2+}

$[Ca^{2+}]_i$ in non-excitable cells such as EC respond to agonist stimulation via mobilisation from intracellular store and Ca^{2+} influx via ion channels. The experiments described here and in *Group 4* were designed to differentiate these pathways.

2 sets of HUVECs were prepared from 6 different donors as in *Group 1*. For one set of cells, they were first exposed to Krebs' without Ca^{2+} and containing the Ca^{2+} chelator EGTA (100 μM) for 2 min, before being challenged with ATP (10 μM). The Ca^{2+}-free solution was then

replaced by normal Krebs' (with 1.25 mM Ca^{2+}) for a wash period, before the cells were again challenged with the same concentration of ATP. The same was repeated on the second sets of cells. With and without extracellular Ca^{2+} conditions was randomised in order to eliminate time-dependent factors.

The same protocol was repeated, with modified Krebs' containing 60 mM K^+ instead of Ca^{2+}-free solution, on HUVEC isolated from 6 different donors. The electrical potential inherent across any cellular membrane confers a driving force for cations, such as Ca^{2+}, to enter the cell through ion channels, such as P2X receptors (North, 2002). It was expected that the resting membrane potential of the HUVEC was therefore increased by raising the extracellular $[K^+]$.

6.2.4. Group 4: Role of intracellular Ca^{2+} stores and store-operated Ca^{2+} channels (SOCC)

Evidence from *Group 3*, specifically that return of Ca^{2+} to the extracellular milieu following activation of store release with ATP challenge caused a small increase in $[Ca^{2+}]_i$, suggested that SOCC may be present in HUEVC (see *Fig 6.3.4*). These experiments were designed to further examine this. HUVECs were prepared as in previous groups, and were challenged with ATP (10 μM) as control. Intracellular Ca^{2+} stores are normally maintained by the constitutive activity of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Mountian *et al.*, 1999). In these experiments, the intracellular stores were emptied by exposure to Ca^{2+}-free solution with EGTA, as in *Group 3*, together with the potent SERCA inhibitor thapsigargin (TG; 1 μM). The cells were then again challenged with ATP (10 μM). After a

wash period, the cells were re-exposed to normal Krebs' containing Ca^{2+}. TG at 1 μM together with Ca^{2+}-free solution and EGTA was previously shown to completely empty intracellular Ca^{2+} stores in EC (Aley *et al.*, 2005).

6.2.5. Group 5: Hypoxia

HUVECs from 6 different donors were prepared as in *Group 1*. As in previous groups, during the experiments cell were constantly perfused with normal Krebs' that was bubbled with 95% air/5% CO_2 (see *Chapter 2 Section 2.3.3*). Krebs' was then switched to one bubbled with 95% N_2/5% CO_2 (which real time PO_2 recording showed to be 7.6 – 9.9 mmHg ; see *Chapter 2 Section 2.3.3*). After 4 min, the perfusion solution was changed back to 21% O_2. The cells were washed for 2 min before another hypoxic challenge.

6.2.6. Group 6: ATP in the presence of Hypoxia

HUVECs from 3 different donors were prepared as in *Group 1*. In order to investigate the combined effect of acute hypoxia and agonist on $[Ca^{2+}]_i$, cells were exposed to ATP (10 μM; 4 min), dissolved in either normal Krebs' (21% O_2, as in *Group 1*), or bubbled with 95% air/5% CO_2 (hypoxia, as in *Group 5*; see *Chapter 2 Section 2.3.3*). Similar to previous groups, time-dependent effect was accounted for by randomising the normoxic and hypoxic ATP challenges.

6.2.7. Analysis of results

Post hoc analysis was carried out using Wassabi software. Individual cells or group of cells were outlined and analyzed as the region of interest and expressed as 1 n. The same experiments were repeated on different coverslips (N) over different isolation batches from

different donors, which were indicated at each result figure. A mixture of 3 different donors was used on each coverslips. Data was presented as mean ± S.E.M., and findings made under different conditions were compared using Student's paired *t*-test, taking $P < 0.05$ as significant. In *Group 5*, where responses of HUVEC from different cells were compared, unpaired t-test was used.

6.3. RESULTS

6.3.1. Group 1

A change from static to active perfusion (22.5ml/s) did not affect $[Ca^{2+}]_i$ in the system used in the present study (data not shown).

ATP (10 μM) consistently induced a rapid increase in $[Ca^{2+}]_i$ in HUVEC, which was followed by a sustained phase, before returning to basal level upon a wash (e.g. Fig 6.3.1 & 6.3.7 Lower). The time intervals that allowed HUVEC to show two identical $[Ca^{2+}]_i$ responses was found to be 8 and 10 min for 10 μM and 100 μM, respectively (data not shown). The ATP-induced $[Ca^{2+}]_i$ elevation in HUVEC was concentration-dependent, as shown by the [ATP]:$[Ca^{2+}]_i$ dose-response curve (Fig 6.3.2). The EC_{50} was approximately 10 μM as derived from the dose-response curve, and the saturation concentration was at 300 μM.

6.3.2. Group 2

When the non-specific P2 receptor antagonist suramin was co-applied with ATP (10 μM), the $[Ca^{2+}]_i$ response was always slower in initiation than control (Fig 6.3.3). On average 57.8 ± 5.8% of the maximum $[Ca^{2+}]_i$ elevation was inhibited (fig 6.3.3).

6.3.3. Group 3

In the absence of extracellular Ca^{2+} and with Ca^{2+} chelator EGTA (100 μM), ATP (10 μM) induced a rapid $[Ca^{2+}]_i$ increase in HUVEC. The dynamics of the Ca^{2+} increase was initially similar to that seen in the presence of extracellular Ca^{2+}, but lacked the sustained phase (Fig 6.3.4). Re-introduction of Ca^{2+} to the perfusate consistently triggered an influx of Ca^{2+} and the response to ATP then comprised the rapid *and* sustained rise in $[Ca^{2+}]_i$ (Fig 6.3.4).

Incubation of HUVEC in 60 mM K^+ did not affect the baseline $[Ca^{2+}]_i$ in HUVEC. However, co-application with ATP (10 μM) caused a brief increase in $[Ca^{2+}]_i$, which was much smaller in magnitude than that evoked by ATP in normal Krebs' (Fig 6.3.5).

6.3.4. Group 4

When intracellular Ca^{2+} stores of HUVEC was depleted by the SERCA inhibitor TG (1 μM) in Ca^{2+}-free Krebs' with Ca^{2+} chelator EGTA (100 μM), stimulation with ATP (10 μM) produced a very small $[Ca^{2+}]_i$ elevation in 3 out of 6 coverslips (Fig 6.3.6). In the remainder of coverslips, ATP stimulation had no effect on $[Ca^{2+}]_i$ (data not shown). Upon re-introduction of extracellular Ca^{2+}, HUVEC consistently showed a very strong increase in $[Ca^{2+}]_i$ in all populations (Fig 6.3.6).

6.3.5. Group 5

In 21% O_2, $[Ca^{2+}]_i$ in HUVEC was constant. Exposure to hypoxia (1% O_2) caused a small but significant increase in $[Ca^{2+}]_i$ in HUVEC, that was reversible upon return to 21% O_2 and repeatable within the same cells (Fig 6.3.7). The hypoxia-induced $[Ca^{2+}]_i$ elevation was compared to that evoked by ATP (10 μM; from *Group 1*; Fig 6.3.1).

6.3.6. Group 6

Consistent with results in all previous groups, stimulation with ATP (10 μM) caused a reversible $[Ca^{2+}]_i$ elevation in HUVEC, which had both initial and sustained phases. In contrast, when the cells were exposed hypoxia, the ATP-evoked $[Ca^{2+}]_i$ elevation was consistently attenuated (Fig 6.3.8).

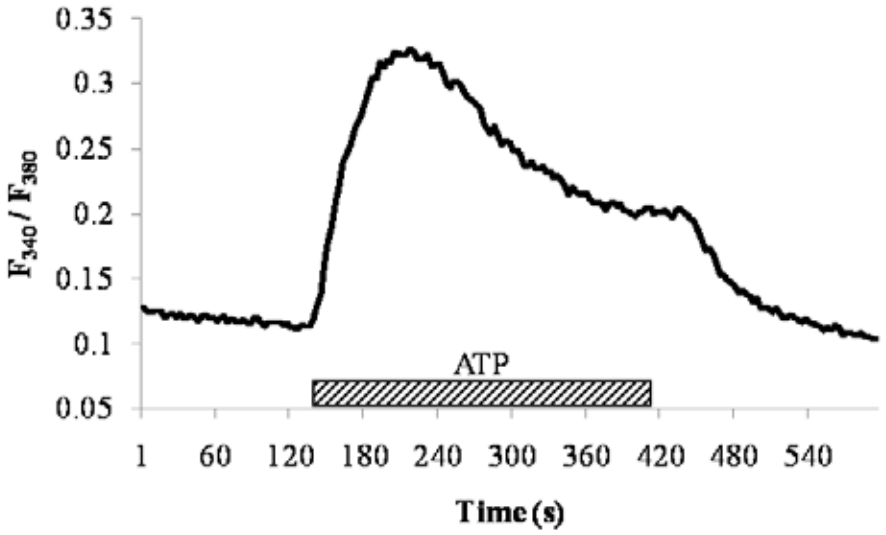


Figure 6.3.1. Original recording showing effects of ATP on $[Ca^{2+}]_i$ in HUVEC.

HUVEC showed a characteristic rapid increase in $[Ca^{2+}]_i$ followed with a sustained phase when stimulated with ATP (10 µM). Recording is representative of n > 120, N=6 from 6 different donors.

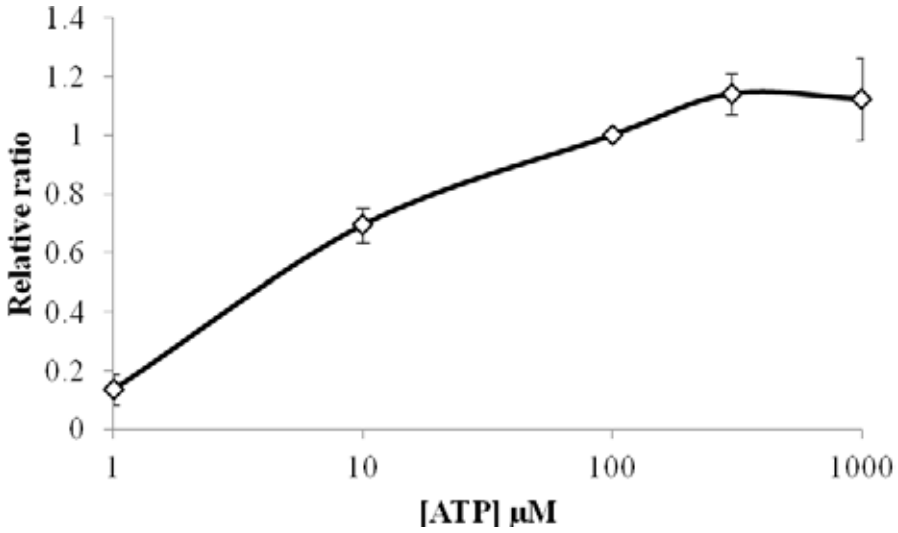


Figure 6.3.2. Dose response relationship for ATP-induced $[Ca^{2+}]_i$ elevation in HUVEC expressed relative to response evoked by 100 µM ATP in logarithmic scale.

ATP-induced $[Ca^{2+}]_i$ elevation in HUVEC was dose dependent. The higher the ATP concentration, the bigger the $[Ca^{2+}]_i$ increase, up to 300 µM. ATP concentration is shown in log scale. Responses were shown relative to 100 µM challenge. For each data point N=6 from 6 different donors, ± = S.E.M (N=number of coverslips).

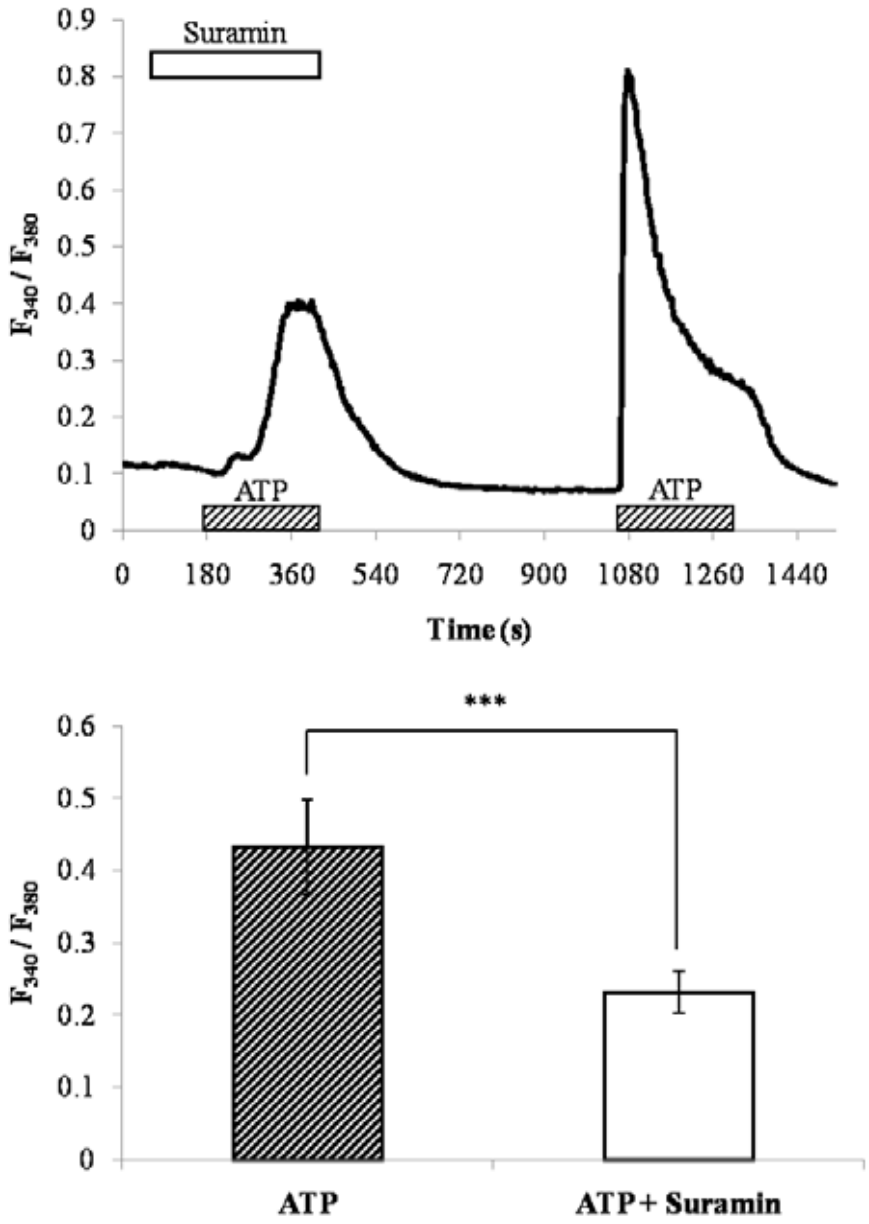


Fig 6.3.3. The effect of the non-specific P2 antagonist suramin on ATP-induced $[Ca^{2+}]_i$ elevation in HUVEC.

Upper: Original recording of $[Ca^{2+}]_i$ in HUVEC in the presence or absence of suramin. In the presence of suramin (100 µM), ATP (10 µM) stimulation of HUVEC induced a $[Ca^{2+}]_i$ elevation. When compared to control, the maximum $[Ca^{2+}]_i$ achieved is lower, and the $[Ca^{2+}]_i$ elevation showed a much slower rate of increase.

Lower: The results shown in histogram format. ***P < 0.01. ± = S.E.M. n>220, N=11 from 6 different donors (n=number of cells, N=number of coverslips).

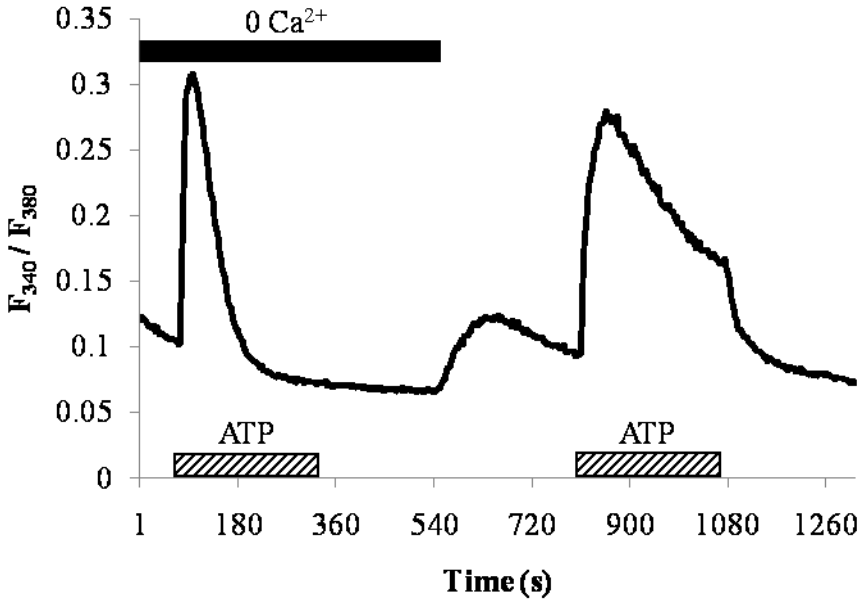


Figure 6.3.4. Original recording showing ATP-induced $[Ca^{2+}]_i$ elevation in HUVEC in the absence and presence of extracellular Ca^{2+}.

In the absence of extracellular Ca^{2+}, the dynamics of ATP-induced $[Ca^{2+}]_i$ elevation is initially similar to control, but lacked the sustained phase. At the end of the black bar, Ca^{2+} (1.25mM) was re-introduced. Recording is representative of n>120, N=6 from 3 different donors and from all coverslips tested (n=number of cells, N=number of coverslips).

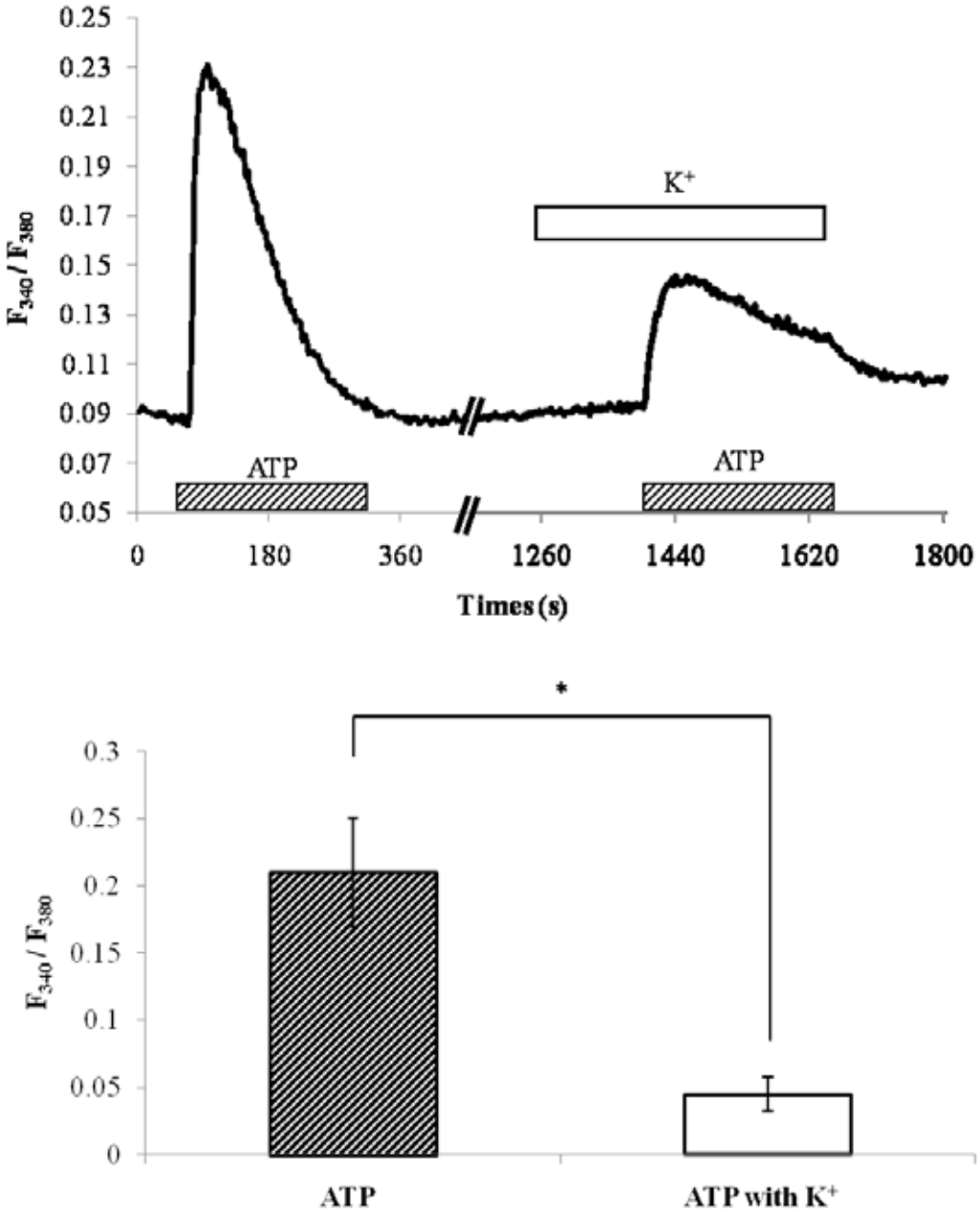


Figure 6.3.5. The effect of membrane depolarisation with 60 mM K^+ on baseline $[Ca^{2+}]_i$ in HUVEC and on ATP-induced $[Ca^{2+}]_i$ elevation.

Upper: Original trace of $[Ca^{2+}]_i$ in HUVEC when challenged with K^+ (60 mM). K^+ alone had no effect on $[Ca^{2+}]_i$. When HUVEC was stimulated with ATP (10 μM) in the presence of K^+, the resultant increase in $[Ca^{2+}]_i$ was much smaller than control.

Lower: The results shown in histogram format. *$P < 0.05$. ± = S.E.M. n>60, N=3 from 3 different donors (n=number of cells, N=number of coverslips).

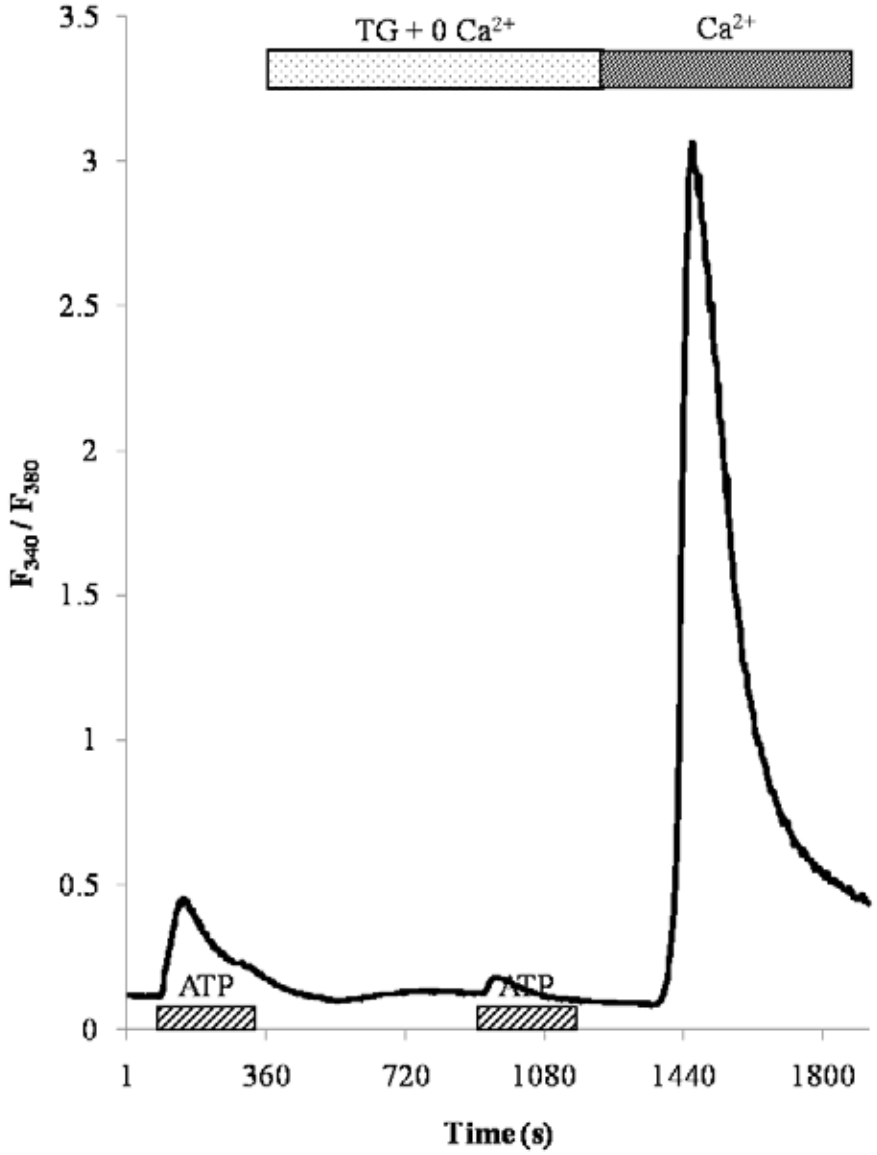


Figure 6.3.6. Original recording of ATP-induced $[Ca^{2+}]_i$ elevation in HUVEC in the presence and absence of extracellular Ca^{2+}.

In the absence of extracellular Ca^{2+} and after intracellular Ca^{2+} stores were emptied by TG (1 µM), ATP stimulation cause a very small $[Ca^{2+}]_i$ elevation when compared to control. Upon re-introduction of extracellular Ca^{2+}, HUVEC showed a significant increase in $[Ca^{2+}]_i$. Recording is representative of half of the coverslips tested. n>60, N=3 from 3 different donors (n=number of cells, N=number of coverslips).

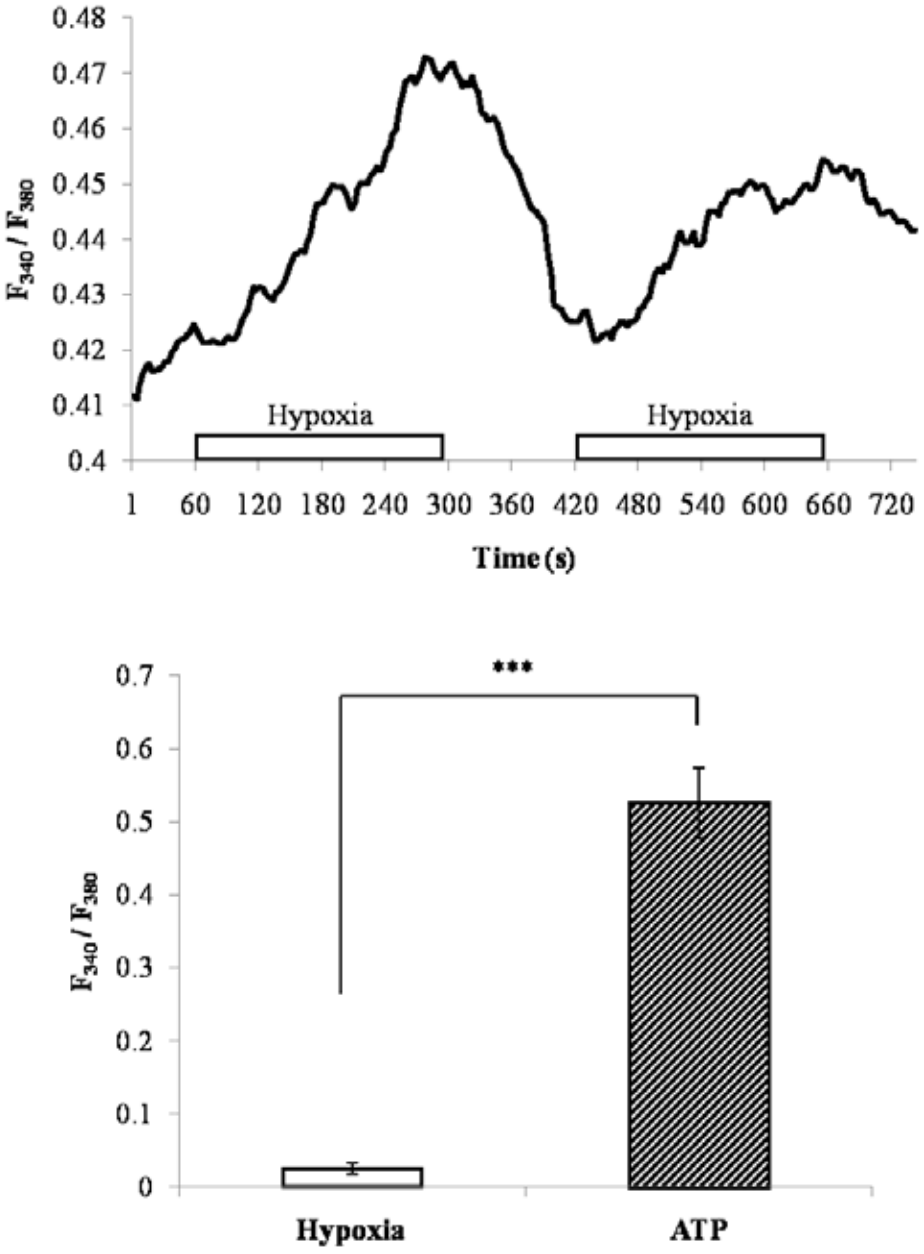


Figure 6.3.7. The effect of acute hypoxia on $[Ca^{2+}]_i$ in HUVEC.

Upper: Original recording showing effect of acute hypoxia on $[Ca^{2+}]_i$ in HUVEC. Bars indicate periods of exposure to hypoxia (1%). The $[Ca^{2+}]_i$ response evoked by hypoxia was consistently reversible and repeatable.

Lower: Columns showing the Ca^{2+} response to hypoxia ± S.E.M when compared to that evoked by ATP (10 µM; from Group 6). For response to hypoxia: n > 100, N=5 from 6 different donors (n=number of cells, N=number of coverslips). For response to ATP: see Group 6. ***P < 0.001 by Student's unpaired t-test.

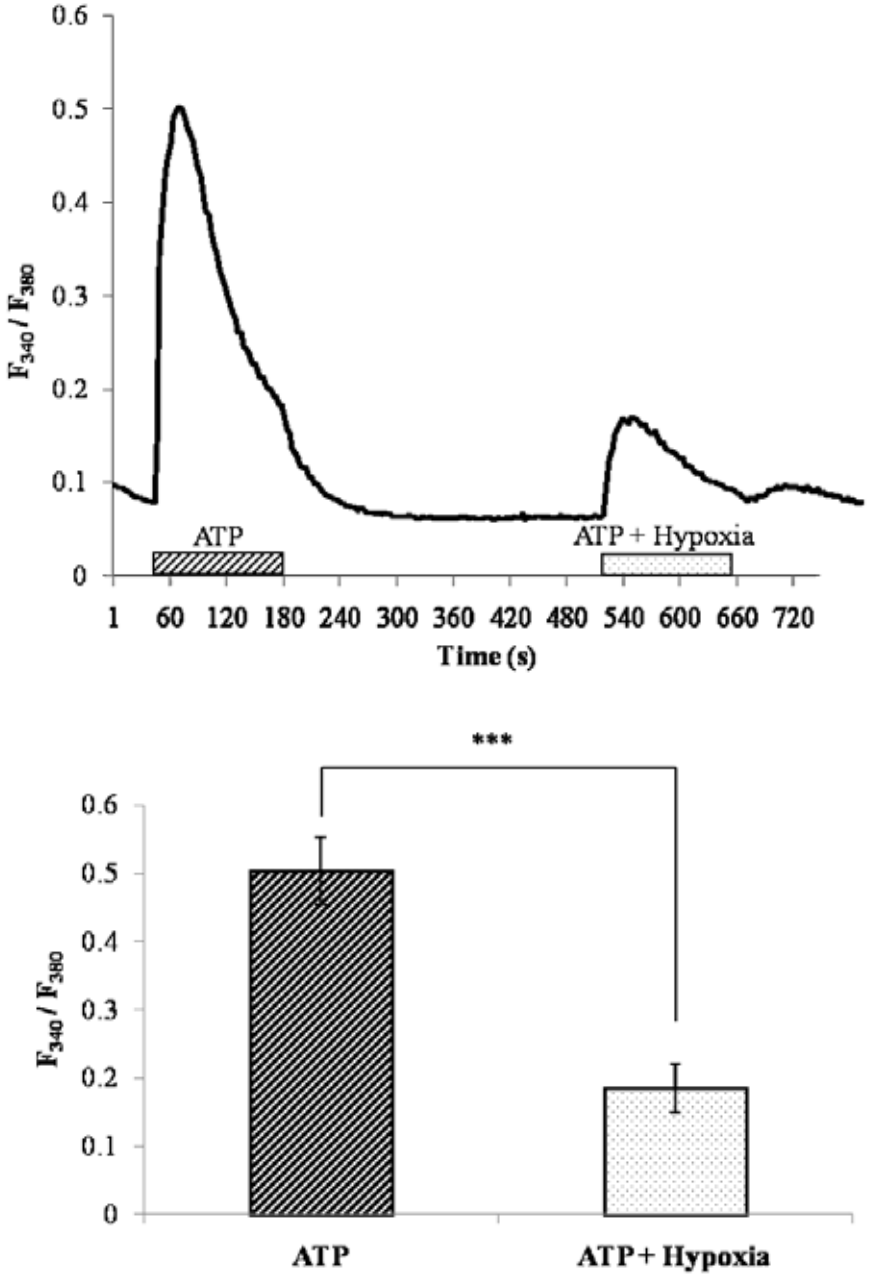


Figure 6.3.8. The effect of acute hypoxia on ATP-induced $[Ca^{2+}]_i$ elevation in HUVEC.

Upper: Original recording showing the effect of acute hypoxia on $[Ca^{2+}]_i$ in HUVEC. Bars indicate application of ATP (10 μM) in normoxic or hypoxic Krebs'.

Lower: Columns showing the Ca^{2+} response to ATP (10 μM) in normoxic or hypoxic Krebs' ± S.E.M. n > 120, N=6 from 3 different donors (n=number of cells, N=number of coverslips). ***P < 0.01.

6.4. DISCUSSION

The studies described in this chapter demonstrated that exogenous ATP induced $[Ca^{2+}]_i$ elevation in freshly isolated HUVEC. The $[Ca^{2+}]_i$ response was reversible and repeatable in the same cell, and was partially sensitive to the non-specific P2 antagonist suramin. ATP stimulation of HUVEC in Ca^{2+}-free Krebs' (with Ca^{2+} chelator EGTA) or in 60 mM K^+, which themselves did not affect $[Ca^{2+}]_i$, caused a $[Ca^{2+}]_i$ elevation that was similar to that of control, but lacked the sustained phase. Re-exposure of HUVEC to extracellular Ca^{2+} after depletion of intracellular stores with TG caused a significant $[Ca^{2+}]_i$ elevation. It was also found that acute hypoxia *per se* caused a $[Ca^{2+}]_i$ elevation, which was small when compared to that evoked by ATP (at EC_{50} concentration). Interestingly, hypoxia attenuated the $[Ca^{2+}]_i$ response to ATP.

6.4.1. Effect of exogenous ATP

As discussed in Chapter 1, the endothelium, which lines the interior surface of blood vessels, responds to physiological stimuli and releases a whole host of important vasoactive substances. In view of the findings described in *Chapter 4*, namely that HUVEC constitutively release ATP, which was amenable to accentuation by hypoxia, and of the apparent importance of $[Ca^{2+}]_i$ in mediating synthesis and release of vasoactive substances such as NO (see *Chapter 1 section 1.3.2.3*), it was essential to explore the effect of ATP stimulation on HUVEC, which is not well understood.

Consistent with studies of many other cell types, HUVEC responded to exposure to extracellular ATP with an initial rapid increase in $[Ca^{2+}]_i$, followed by a sustained phase. The Ca^{2+} increase was at least partly due to P2 receptor activation, as it was sensitive to inhibition

by the non-selective P2 receptor antagonist suramin. Interestingly, suramin (100 μM), which inhibits most P2 receptors with an IC_{50} in the lower μM range (Ralevic & Burnstock, 1998), was able to inhibit only little over 50% of the $[Ca^{2+}]_i$ response to ATP. This is perhaps not surprising, as in addition to P2Y receptors, $P2X_4$ and $P2X_6$ receptors are expressed in HUVEC (Glass *et al.*, 2002; Wang *et al.*, 2002). These receptors are at best only weakly sensitive to suramin at the concentration used in the present study (Buell *et al.*, 1996; Ralevic & Burnstock, 1998). Indeed, HUVEC treated with antisense oligonucleitides against $P2X_4$ receptors showed significantly attenuated $[Ca^{2+}]_i$ response to ATP stimulation (Yamamoto *et al.*, 2000b). Thus, the findings discussed so far are consistent with ATP acting on HUVEC via several different receptors including $P2X_{4/6}$ as well as P2Y receptors.

It is well documented that ATP acts on HUVEC to increase $[Ca^{2+}]_i$, for example to activate or permit eNOS phosphorylation (da Silva *et al.*, 2009), or to induce PGI_2 release. This may play a role in the hypoxia-induced vasodilatation in human umbilical vein and that has been shown to be mainly dependent on the presence of the endothelium (Mildenberger *et al.*, 2003; Mildenberger *et al.*, 2004b). However, the mechanism by which this happens in HUVEC is unclear. This is because the characterisation of P2 receptors has been hampered to a large extent by the lack of selective antagonists (see *Chapter 1 Section 1.3.6.3*). What is known, however, from experiments on several cell types is that the increase in $[Ca^{2+}]_i$ resulting from ATP stimulation is mediated either by the ionotropic P2X receptors (Ca^{2+} entry), or the metabotropic P2Y receptors (Ca^{2+} release from intracellular stores; see *Chapter 1 Section 1.3.6.3* for details).

6.4.2. Origin of the Ca^{2+}

When HUVEC was stimulated with ATP in the absence of extracellular Ca^{2+} (together with the Ca^{2+} chelator EGTA), the $[Ca^{2+}]_i$ response displayed an initial spike which rapidly returned to basal level, despite the continued presence of ATP. This is consistent with a previous study in HUVEC, and suggests that the $[Ca^{2+}]_i$ response is at least partly mediated by P2Y receptors (Kaczmarek *et al.*, 2005), for which the initial phase of the $[Ca^{2+}]_i$ response is dependent on Ca^{2+} release from intracellular store (see Aley *et al* 2005 and below). On the other hand, it can be surmised that the sustained phase of the ATP response is dependent on an extracellular source of Ca^{2+}.

When the resting membrane potential was raised by incubating the HUVEC in 60 mM K^+, the $[Ca^{2+}]_i$ was not affected, suggesting that VOCCs are not present in HUVEC, which is consistent with the notion that EC is a non-excitable cell type and the fact that it has to-date, never been consistently reported in the literature (Nilius & Droogmans, 2001). But, when ATP was co-applied with 60 mM K^+ on these cells, the $[Ca^{2+}]_i$ response was much less pronounced than when ATP was applied alone, confirming that Ca^{2+} entry is also important in the $[Ca^{2+}]_i$ response to ATP stimulation. This is also consistent with and supports the findings discussed above that a substantial ATP-induced Ca^{2+} response remained intact despite the presence of suramin. Taken together, the data suggest that during ATP-induced Ca^{2+} elevation, Ca^{2+} release from intracellular stores is responsible for the initial phase presumably mediated by P2Y receptors, and that this is followed by a period in which Ca^{2+} entry, presumably via $P2X_{4/6}$ channels (see above), is functionally important. Although both P2X and P2Y receptors have been separately reported in HUVEC (Yamamoto *et al.*, 2000b; Tanaka *et al.*, 2004), this is the first time that their functional presence are demonstrated in the

same cell. It should be noted that Ca^{2+}-induced Ca^{2+} release via ryanodine receptor (RyR) on the ER has been previously shown in bovine arterial EC (Mozhayeva & Mozhayeva, 1996), so it is possible that it may also play a role in HUVEC. This hypothesis is beyond the scope of the present study and was not tested.

6.4.3. Role of intracellular Ca^{2+} stores and SOCC

To further investigate the origin of the non-entry source of Ca^{2+}, intracellular Ca^{2+} stores were emptied by incubating the HUVEC with the SERCA inhibitor TG in the absence of Ca^{2+}. ATP challenge (at 10 μM) following this revealed that an extremely small proportion of the ATP-induced $[Ca^{2+}]_i$ response remained intact in 50% of the coverslips tested. This is slightly different from the finding of a previous study (Kaczmarek *et al.*, 2005), that the same conditions completely obliterated the ATP-induced $[Ca^{2+}]_i$ response in HUVEC. Several factors could have contributed to this difference. For example, the Kaczmarek *et al* 2005 study used 100 μM ATP as the standard ATP concentration, which could mean that some of the P2 receptors were desensitised at this high ATP concentration. Further, unlike the present study, Kaczmarek *et al* (2005) used HEPES-containing buffer and room temperature obtain the $[Ca^{2+}]_i$ recordings.

In nearly all cell types, depletion of ER Ca^{2+} stores using TG activates store-operated Ca^{2+} entry (Putney, 1986). Interestingly, in the present study, when extracellular Ca^{2+} was re-introduced to the medium, there was a significant reversible increase in $[Ca^{2+}]_i$, consistent with the functional presence of SOCC in HUVEC. This capacitative Ca^{2+} entry (CCE) on Ca^{2+} addition was also observed when the stores were initially depleted by application of ATP (10 μM; Fig 6.3.4). Consistent with the present findings, in saphenous vein EC, Ca^{2+} store

depletion by ATP or hypoxia (in the absence of extracellular Ca^{2+}) activated a SOCC current (Aley *et al.*, 2005). Thus, it seems that SOCC are present more generally in EC and may play a role in ATP (or hypoxia) -induced $[Ca^{2+}]_i$ response. CCE is well documented, and has been shown to occur via the members of the transient receptor potential canonical (TRPC) family (Abdullaev *et al.*, 2008). Interestingly, it was recently shown that oxidative stress, induced by artificial ROS generation, was able to profoundly inhibit CCE in calf pulmonary EC (Florea & Blatter, 2008). The significance of this is discussed below in *Section 6.4.5*. In the next Chapter, studies are described in which the potential role of TRPC channels in regulating vascular vasomotion was explored in intact umbilical arteries.

6.4.4. The effect of hypoxia on $[Ca^{2+}]_i$

It is now commonly accepted that ATP and its breakdown products ADP, AMP and adenosine play an important role in vascular control, and this is termed purinergic signalling (*Chapter 1 Section 1.3.6*). Given its physical location in the vascular system, and the findings described in *Chapter 3, 4 & 5*, it is an important finding that acute hypoxia *per se* caused a small but significant increase in $[Ca^{2+}]_i$ in HUVEC in the present study. This observation is in similar to that of a previous study, in that a comparable level of hypoxia induced an acute $[Ca^{2+}]_i$ elevation in EC isolated from human saphenous vein (Aley *et al.*, 2005). In that study, it was demonstrated that the hypoxia-induced $[Ca^{2+}]_i$ elevation was due to ROS production from the mitochondria and that $[Ca^{2+}]_i$ was released from a RyR-gated intracellular source. Indeed, multiple studies have reported that hypoxia causes an increase in mitochondrial ROS production (Chandel & Schumacker, 2000). However, as indicated above, all of their experiments were carried out in the absence of extracellular Ca^{2+}, which is different from the present study. No comparable studies to the present study on HUVEC have been done in the

past, but other authors, while studying Ca^{2+} signalling in HUVEC in the context of anoxia and associated pathology, have reported that $[Ca^{2+}]_i$ increased substantially after exposure to up to 2 hr of anoxia (Arnould *et al.*, 1992; Aono *et al.*, 2000; Berna *et al.*, 2001; Peers *et al.*, 2006). Taking the findings described in *Chapter 4* into consideration, it would seem likely that in addition to the effects of hypoxia *per se* on $[Ca^{2+}]_i$, hypoxia may have caused a release of ATP from the HUVEC, which then acted in an autocrine manner and increased the $[Ca^{2+}]_i$ by activating P2 receptors on the cell surface. Importantly, any shear stress in the present study did not affect $[Ca^{2+}]_i$ in HUVEC in 21% O_2, further suggesting it was hypoxia *per se* that caused the increase in $[Ca^{2+}]_i$, rather than any mechanical stress. In future studies it will be important to investigate whether the effects of hypoxia on Ca^{2+} handling in HUVEC has the same characteristics as that evoked by ATP i.e. partly dependent on Ca^{2+} influx and partly on release from intracellular stores as discussed above.

6.4.5. The effect of hypoxia on ATP-induced $[Ca^{2+}]_i$ elevation

Clearly, if the model proposed above is correct, it is surprising that acute hypoxia consistently *attenuated* the $[Ca^{2+}]_i$ response to ATP stimulation in the present study. In EC isolated from human saphenous vein, it was found that, in the absence of extracellular Ca^{2+} at least, when ATP was applied during hypoxia, this *accentuated* the $[Ca^{2+}]_i$ response to ATP (Aley *et al.*, 2005). Because inhibition of NADPH oxidase (a source of ROS in EC) accentuated the Ca^{2+} response to ATP stimulation (in the absence of extracellular Ca^{2+}), the authors proposed that the facilitatory effect of hypoxia on the ATP-induced Ca^{2+} response asserted because the decrease in O_2 level caused a substrate-dependent decrease in oxidase-derived ROS, which normally tonically inhibits IP_3-dependent Ca^{2+} release that is activated by ATP (Aley *et al.*, 2005). On the other hand, Aley *et al* (2005) found that the increase in $[Ca^{2+}]_i$ induced by

hypoxia (in the absence of extracellular Ca^{2+}) was inhibited by agents that interfere with mitochondrial generation of ROS or by ryanodine. They therefore proposed that hypoxia increases $[Ca^{2+}]_i$ by generating ROS from mitochondria which stimulates RyR on Ca^{2+} stores (also see *Section 6.4.4*). Thus, if the mechanisms underlying hypoxia and ATP-mediated Ca^{2+} signalling are consistent between the two types of EC, it can be deduced that in the presence of extracellular Ca^{2+} if hypoxia increased Ca^{2+} release from intracellular stores (via the mechanisms suggested by Aley *et al* 2005), it must have also inhibited Ca^{2+} entry (and presumably Ca^{2+}-induced Ca^{2+} release) in HUVEC, thus conferring an overall *inhibitory* effect of hypoxia on ATP-induced Ca^{2+} response as found in the present study. This suggestion is consistent with the observation that Ca^{2+} entry plays a major role in the Ca^{2+} response to ATP in HUVEC (see *Fig 6.3.4*). In fact, oxidative stress, induced by the ROS generator tert-butyl-hydroperoxide, was found to profoundly inhibit agonist (bradykinin or ATP) -induced CCE in calf aortic and pulmonary EC (Elliott & Doan, 1993; Florea & Blatter, 2008). Thus, the ROS released from the mitochondria in the presence of hypoxia (Chandel & Schumacker, 2000; Aley *et al.*, 2005) may not only stimulate RyR but also inhibits ATP-evoked Ca^{2+} entry through P2 receptors (see above).

In other words, the effect of ATP released in hypoxia (Chapter 4) or of exogenous ATP in hypoxia (the present Chapter) on HUVEC may depend on the balance between the effect of a decrease in NADPH oxidase ROS generation by hypoxia to increase Ca^{2+} release via IP_3R, the effect of increased mitochondrial ROS generation by hypoxia to increase Ca^{2+} release via RyR, and the ability of mitochondrial of ROS to inhibit CCE subsequent to P2 receptor activation. The balance could depend on the level of hypoxia and/or concentration of ATP available to act on P2 receptors. Importantly, the present study demonstrated the effect of

hypoxia on ATP-induced $[Ca^{2+}]_i$ response in HUVEC under physiological conditions i.e. when Ca^{2+} is present in the extracellular medium.

6.4.6. Functional role of hypoxia-induced ATP release from HUVEC

The EC_{50} of the dose-response curve for ATP vs. $[Ca^{2+}]_i$ in the present study was approximately 10 μM ATP, which is almost an order of magnitude higher than the concentration measured from the apical side of HUVEC in *Chapter 4.* However, as ATP released from the membrane of the EC is rapidly hydrolysed by EN and therefore has a short half-life (see *Chapter 1 section 1.4.4*), the actual concentration at the local level close to the receptors could well have reached 10 μM. Further, as discussed in *Chapter 1 section 1.4.1*, other cell types in the vascular environment, most notably RBC, also release significant amount of ATP in response to a fall in PO_2 (Ellsworth, 2004), suggesting that in the condition of hypoxia, EC may well be exposed to much higher concentration of ATP than measured in *Chapter 4*. On the other hand, it is possible that this dose-response curve gives an 'exaggerated' impression of the response induced by ATP in HUVEC under hypoxic situations because, as argued above, hypoxia has an overall inhibitory effect on the $[Ca^{2+}]_i$ increase induced by submaximal concentrations of ATP. Nevertheless, the present findings are fully consistent with the hypothesis that ATP released from the endothelium by hypoxia may act on EC in an autocrine manner, to induce a $[Ca^{2+}]_i$ elevation and cause subsequent release of vasoactive substances from EC.

Interestingly, in a previous study incubation of HUVEC with a cocktail designed to eliminate ATP, ADP, AMP and adenosine (a mixture of hexokinase, apyrase and adenosine deaminase), lowered the basal $[Ca^{2+}]_i$, and this was reversible after a wash (Schwiebert *et al.*, 2002). This

suggested that constitutive release of ATP from the endothelium serves to maintain the basal $[Ca^{2+}]_i$ level in the EC themselves. In light of the results of *Chapter 4* and of the present study, it is suggested that in hypoxia, more ATP is released which serves to raises the $[Ca^{2+}]_i$ of the EC, and presumably in turn to mediate Ca^{2+}-dependent production and release of vasoactive substances such as NO, PG, EDHF and ATP. It is possible that this forms part of a positive feedback mechanism, as an increase in $[Ca^{2+}]_i$ in EC itself causes significant ATP release (see *Chapter 4 Section 4.3.6*). As umbilical blood flow is usually constant despite changes in the prevailing PO_2 (Jensen *et al.*, 1999), this mechanism may play an important role in maintaining adequate fetal perfusion during intrauterine hypoxia. In future studies it would be important to monitor release of the vasodilator (e.g. NO and PGI_2) induced by ATP under normoxic and hypoxic conditions that compare to PO_2 values present in the umbilical vein *in situ*.

In summary, it was shown that ATP induced a $[Ca^{2+}]_i$ elevation in HUVEC. The Ca^{2+} response was initially dependent on release from intracellular stores, via P2Y receptors while Ca^{2+} entry, possibly via $P2X_{4/6}$, was responsible for the sustained phase. Store depletion by either ATP, or TG, activated CCE. On its own, hypoxia induced a $[Ca^{2+}]_i$ increase in HUVEC, which was much smaller than that induced by ATP (10 μM). Moreover, hypoxia had an inhibitory effect on the ATP-induced Ca^{2+} response. It is proposed that this was likely due to inhibition of CCE by ROS generated in mitochondria.

CHAPTER 7

VASOMOTION IN THE HUMAN UMBILICAL ARTERY AND THE EFFECT OF HYPOXIA

7.1. INTRODUCTION

In the studies described in *Chapter 4 & 5*, it was shown that HUVEC and HUAEC released ATP constitutively, and that in HUVEC at least ATP release was accentuated by hypoxia. On the other hand, it was shown in studies described in *Chapter 3* that exogenous ATP induced $[Ca^{2+}]_i$ oscillations in HUASMC which, as described in *Chapter 1 Section 1.5.2*, are believed to drive vasomotion. Thus, the experiments in the present chapter were designed to test the hypothesis in preparations of whole umbilical artery rings that ATP released from endothelium acts in an autocrine manner, via the release of vasoactive substances from EC, or in a paracrine manner to activate purinoceptors on underlying SMC to cause vasomotion. As indicated in *Chapter 3 & 6*, extracellular Ca^{2+}, purinoceptors and SOCC/ROCC are all implicated in $[Ca^{2+}]_i$ oscillations in HUASMC, and in the Ca^{2+} response to ATP stimulation in HUVEC. Thus, the potential role of these in umbilical artery vasomotion was also explored. As discussed in *Chapter 1 Section 1.4.4.1*, ATP in the extracellular space is readily broken down by EN to ADP, AMP, adenosine and inorganic phosphate. Hence, experiments were also designed to explore the potential role of these purines in the arterial vasomotion.

It is generally accepted that the rhythmic contractions and relaxations of arterial vessels result in enhanced O_2 delivery to surrounding tissues. The fact that the number of incidence, frequency and relative amplitude of vasomotion was increased following a reduction in blood flow or haematocrit (Rucker *et al.*, 2000; Lee *et al.*, 2005) led to the present hypothesis that during hypoxia, more ATP is released from EC or other source, which in turn causes, or changes the property of background, vasomotion (*Chapter 1 Section 1.6*). This was tested in the studies described below. Clearly, the endothelium plays a central role in this hypothesis. Therefore, some of the experiments described below involved attempts to denude the

umbilical artery rings. The umbilical artery is part of a specialised circulation which does not show endothelium-dependent relaxation in response to acetylcholine (Lovren & Triggle, 2000). Hence, an immunohistochemistry approach was taken to assess the physical integrity of the blood vessel.

7.2. METHODS

The details of the methodology of dissection, normalisation and calibration are described in *Chapter 2 section 2.4.*

PROTOCOLS

7.2.1. Group 1: Development of an experimental model for vasomotion

A total of 42 umbilical arteries were isolated from 42 individual donors using the dissection methods developed in this study (see *Chapter 2 Section 2.4.1*). 4 vessel rings were dissected from each vessel, mounted on supporting pins, and normalised to resting tension (see *Chapter 2 Section 2.4.3*). After an hour of equilibrations, they were challenged with modified Krebs-Ringer buffer with 70mM K^+. Changes in isometric tension were recorded for offline analysis.

In 17 vessel rings that did not display spontaneous vasomotion after an hour of incubation, they were challenged with ATP (0.01, 0.03, 0.1, 0.3, 1, 3, 10 mM; 10 min), each challenge separated with a 20 min wash interval.

7.2.2. Group 2: Role of extracellular Ca^{2+}

Human umbilical artery rings were prepared as in *Group 1*. In order to access the role of extracellular Ca^{2+} in vasomotion, extracellular Ca^{2+} in the Krebs-Ringer buffer was replaced

with the Ca^{2+} chelator EGTA (200 μM) when vasomotion was established. After 20 min, buffer was change to normal Krebs-Ringer (with Ca^{2+}). This was tested on 4 arterial rings from 2 separate donors.

7.2.3. Group 3: Role of endothelium

In order to assess the role of the endothelium in vasomotion, the endothelium was removed from half of the vessel from each donor by gentle injection of air thorough the vessel lumen with a syringe connected to a cannula, before mounting on supporting pins (see *Chapter 2 Section 2.4.3*). It has previously been shown by immunohistochemiscal technique that this process was successful in denuding umbilical arteries (Bodelsson & Stjernquist, 1994). After the myograph experiments, vessel rings from both intact and denuded groups (2 each) from 3 separate donors were frozen and stained for the endothelium-specific marker CD31 (see *Chapter 2 section 2.4.4*).

7.2.4. Group 4: Role of purinoceptors

Human umbilical artery rings were prepared as in *Group 1*. To test if vasomotion was mediated by purinoceptors, the non-specific P2 receptor antagonist suramin (100 μM) was applied to vessel rings displaying vasomotion. After 15 mins, the incubating buffer was changed to normal Krebs-Ringer. 100 uM suramin has been shown to inhibit ATP-induced contraction in rat mesenteric arteries. It was tested on 7 vessel rings isolated from 2 separate donors.

7.2.5. Group 5: Role of IP_3R and SOC / ROC channels

Human umbilical artery rings were prepared as in *Group 1*. In order to test for a role for IP_3R and SOC / ROC channels in vasomotion, the non-specific inhibitor 2-APB (100 μM), was used as in *Group 4*, in the same manner as suramin (see above). IP_3R and SOCC / ROCC have been previously shown to be sensitive to inhibition by 100 μM or lower concentrations of 2-APB (Bootman *et al.*, 2002; Facemire & Arendshorst, 2005).

7.2.6. Group 6: Role of adenosine

Human umbilical artery rings were prepared as in *Group 1*. In order to test whether adenosine, a downstream product of ATP breakdown, plays a role in vasomotion, the P1 receptor antagonist 8-SPT was used as in *Group 4*, in the same manner as suramin (see above). 100 μM 8-SPT has been shown to completely reverse the effect of adenosine in an organ bath situation not dissimilar to that used in the present study (Ziganshin *et al.*, 2009). 8-SPT was purchased from Sigma-Aldrich, U.K. and was made up as 1000x frozen stock solution in DMSO before dilution to the working concentration on the day of experiments.

7.2.7. Group 7: Effect of hypoxia

Human umbilical artery rings were prepared as in *Group 1*. In order to explore the effect of hypoxia on vasomotion, once vasomotion was established, the superfusate was bubbled with 95% N_2 / 5% CO_2, with a gentle flow of argon above the recording chamber (see *Chapter 2 Section 2.4.3*). After 15 min, the air was to bubble the superfusate was returned to normal 21% O_2 / 5% CO_2 with balances N_2. This was tested on 6 vessel rings from 2 separate donors.

7.2.8. Group 8: Effect of apyrase

Human umbilical artery rings were prepared as in *Group 1*. In order to test the hypothesis that ATP or ADP is responsible for vasomotion, apyrase (2 U/ml; 30 min) was applied to vessel rings that were displaying vasomotion. This concentration of apyrase was shown to inhibit ATP-induced Ca^{2+} response in HUVEC (Nejime *et al.*, 2008). After 30 min, the vessel rings were exposed to hypoxia (50 mmHg; 15 min) as in *Group 7*, in the presence of apyrase. The frequency and amplitude of vasomotion during the three periods: PO_2 160mmHg, apyrase, and hypoxia in the presence of apyrase, were monitored and recorded. The apyrase used was purchase from Aigma-Aldrich, U.K. and has a mixture of high and low ATPase/ADPase ratio. It was dissolved in normal Krebs-Ringer buffer on the day of experiment as described in *Chapter 2 Section 2.4.3*.

7.3. RESULTS

7.3.1. Group 1

Spontaneous contractions and relaxations were found in 62.5% of the arterial rings (Fig 7.3.1). The frequency and amplitude of the rhythmic contractions and relaxations varied between different rings, averaging 1.32 ± 0.10 min^{-1} and 2.74 ± 0.55 mN respectively. The duration also differed, with some lasting over 12 hr if left uninterrupted (data not shown).

In vessel rings that did not display spontaneous contractions and relaxations, stimulation of exogenous ATP (0.01, 0.03, 0.1, 0.3, 1, 3, 10 mM; 10 min) all caused a short-lasting contraction but not vasomotion (e.g. Fig 7.3.2 for 10 μM; not all data shown).

7.3.2. Group 2

When extracellular Ca^{2+} was replaced with Ca^{2+} chelator EGTA (200 μM), vasomotion was inhibited, and the basal tension decreased. After 20 min, when normal Krebs-Ringer buffer (with Ca^{2+}) was re-introduced to the vessel rings, vasomotion re-appeared (Fig 7.3.3).

7.3.3. Group 3

In the vessel rings that have not been denuded, the luminal surface showed positive staining for CD31 (Fig 7.3.4 Upper). Further, in the vessel rings that have undergone the denuding process, the luminal space still showed positive staining for CD31 (Fig 7.3.4 Lower).

7.3.4. Group 4

In human umbilical artery rings that displayed vasomotion, addition of suramin (100 μM; 15 min) did not change either the frequency or amplitude of established vasomotion in human umbilical artery rings (Fig 7.3.5).

7.3.5. Group 5

In human umbilical artery rings that displayed vasomotion, addition of 2-APB (100 μM; 10 min) did not change either the frequency or amplitude of established vasomotion in human umbilical artery rings (Fig 7.3.6).

7.3.6. Group 6

In human umbilical artery rings that displayed vasomotion, addition of 8-SPT (100 μM; 20 min) did not change either the frequency or amplitude of established vasomotion in human umbilical artery rings (Fig 7.3.7).

7.3.7. Group 7

In human umbilical artery rings that displayed vasomotion, hypoxia (50 mmHg; 15 min) caused an immediate increase in the frequency but decrease in amplitude in vasomotion of human umbilical artery rings, and the effect was reversible upon returning to PO_2 160mmHg (Fig 7.3.8). Further, it is an increased in tough tension of the oscillation which resulted in decreased amplitude. The effect was consistent in all vessel rings tested (Fig 7.3.8 Lower).

7.3.8. Group 8

In human umbilical artery rings that displayed vasomotion, incubation with apyrase (2 U/ml; 30min) did not affect the frequency or amplitude of the vasomotion (Fig 7.3.9). After 30 min, the vessel wings were exposed to hypoxia (50 mmHg; 15min), which increased the frequency and decreased the amplitude of the vasomotion (Fig 7.3.9), as in the absence of apyrase (see *Group 7*).

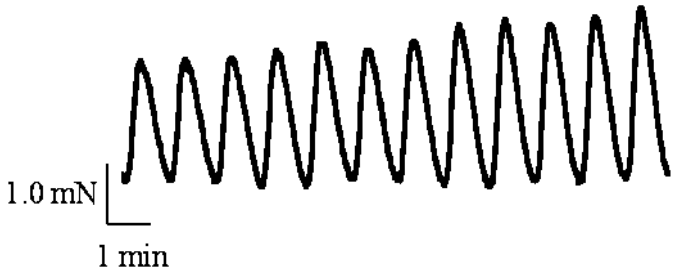


Figure 7.3.1. Original recording of isometric force generated by human umbilical artery rings.

Spontaneous rhythmic contractions and relaxations were found in 62.5% of arterial rings.

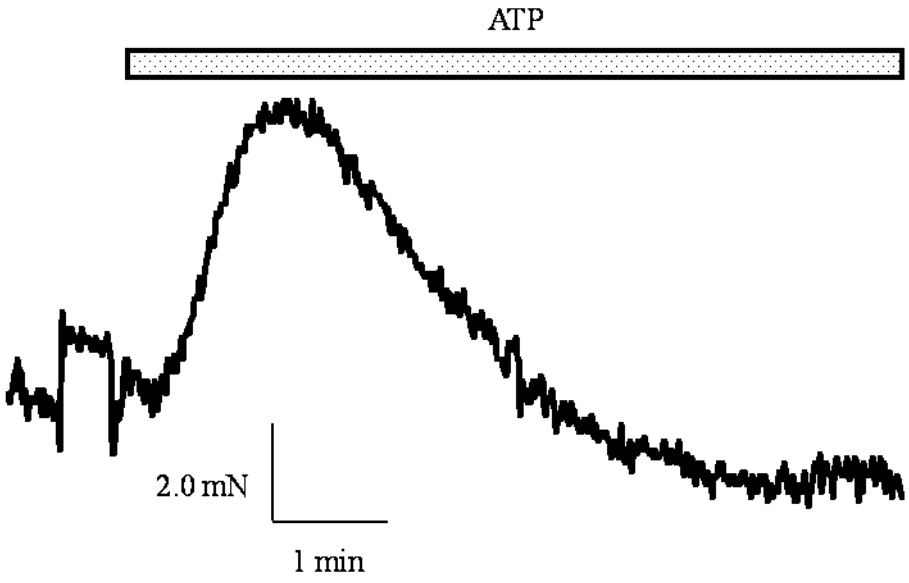


Figure 7.3.2. Original recording of isometric force generated by human umbilical artery rings.

ATP (10 µM) caused a transient contraction in umbilical artery rings that did not generate vasomotion. This is representative of 9 vessel rings from 3 different donors.

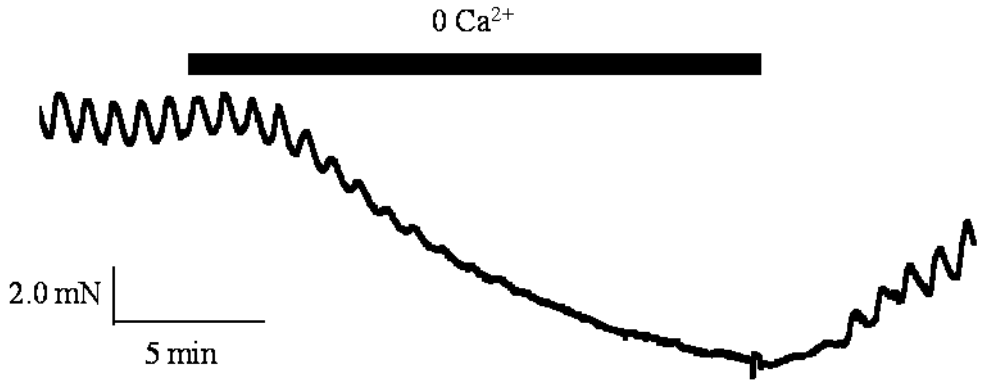


Figure 7.3.3. Original recording of vasomotion in human umbilical artery ring in the presence and absence of extracellular Ca^{2+}.

Replacement of extracellular Ca^{2+} with Ca^{2+} chelator EGTA (200 µM; 20 min) inhibited vasomotion. It also lowered basal tension of the arterial rings. Vasomotion and basal tension was recoverable following replacement of Ca^{2+} into the incubating buffer. This is representative of 4 recordings from 2 individual donors.

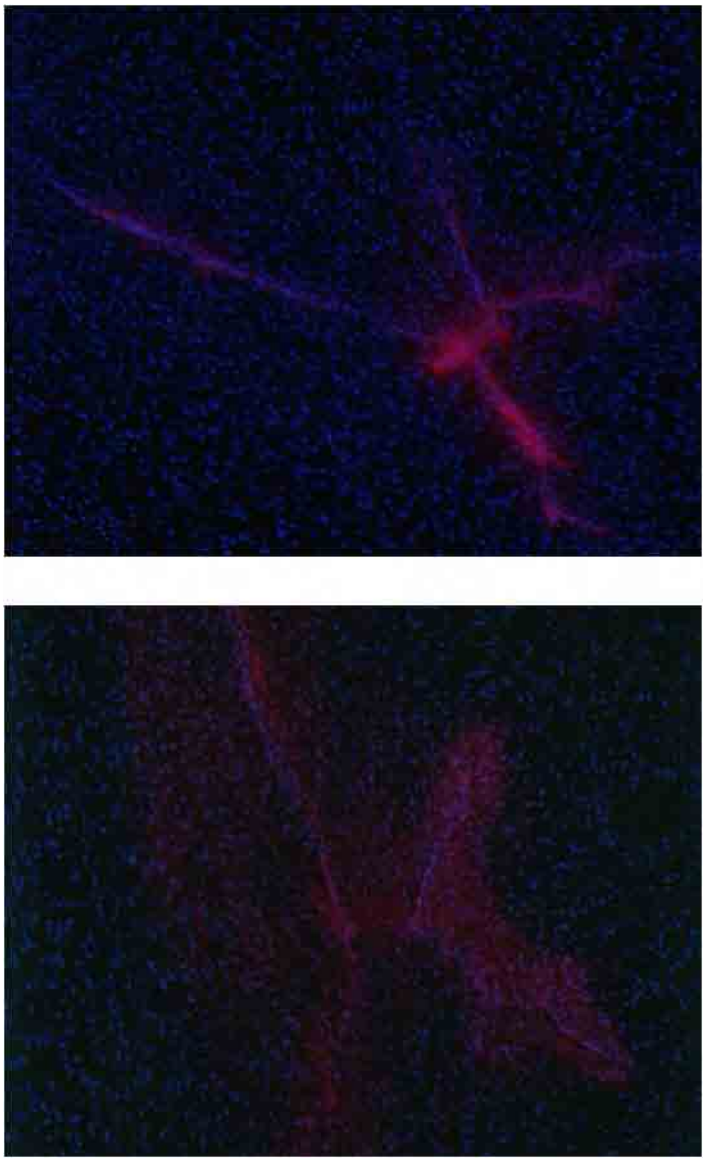


Fig 7.3.4. CD31 and DAPI co-staining of human umbilical artery rings.

Upper: Example of a 12 µm thick section of an umbilical artery ring which has not been denuded. The luminal surface was stained positive for CD31.
Lower: Example of a 12 µm thick section of an umbilical artery ring from a vessel that has under gone the denuding process. The luminal surface still showed positive staining for CD31.
Both images were taken at 252ms (CD31) and 53 ms (DAPI) at 100X magnification, and are typical of sections of their respective groups. CD31 staining appears red and DAPI staining appears blue.

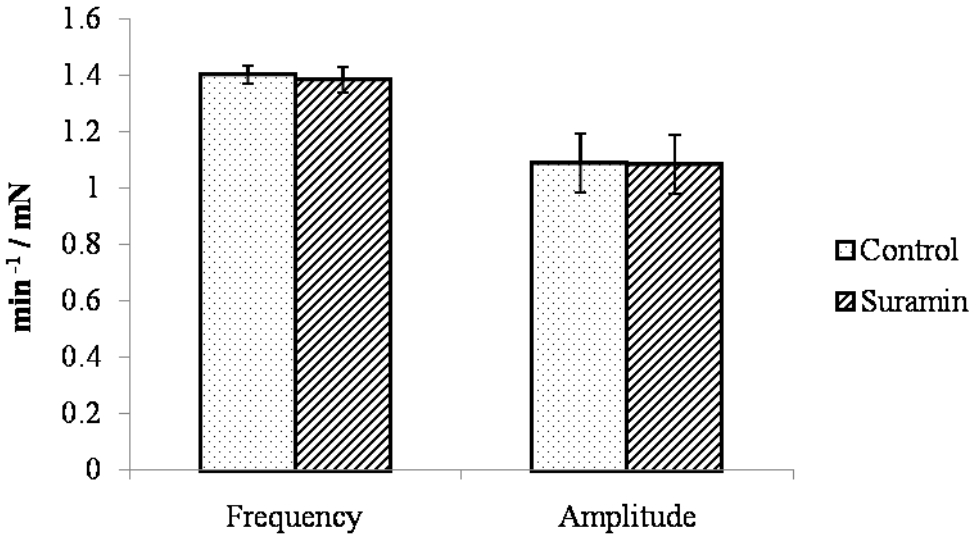


Figure 7.3.5. The role of purinoceptors in vasomotion of human umbilical artery.

Histograms showing frequency and amplitude of vasomotion in the presence and absence of suramin (100 µM; 15 min). Suramin had no effect on either frequency or amplitude of vasomotion. *P* = 0.45 (frequency) and 0.95 (amplitude). ± = S.E.M. n=7 from 2 different donors (n=number of arterial rings).

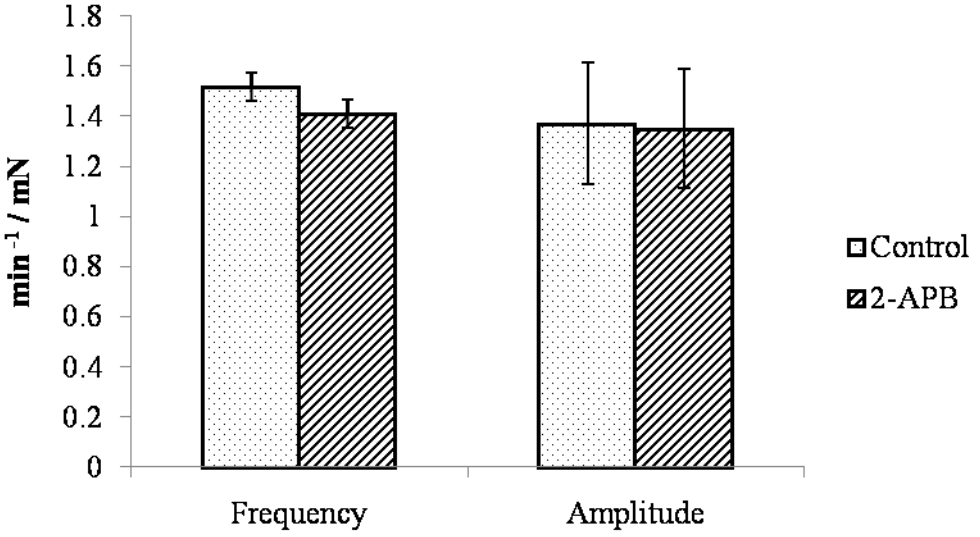


Figure 7.3.6. The role of IP3R, SOC and ROC channels in vasomotion of human umbilical artery.

Histograms showing frequency and amplitude of vasomotion in the presence and absence of 2-APB (100 µM; 10 min). 2-APB had no effect on either frequency or amplitude of vasomotion. *P* = 0.08 (frequency) and 0.84 (amplitude). ± = S.E.M. n=7 from 2 different donors (n=number of arterial rings).

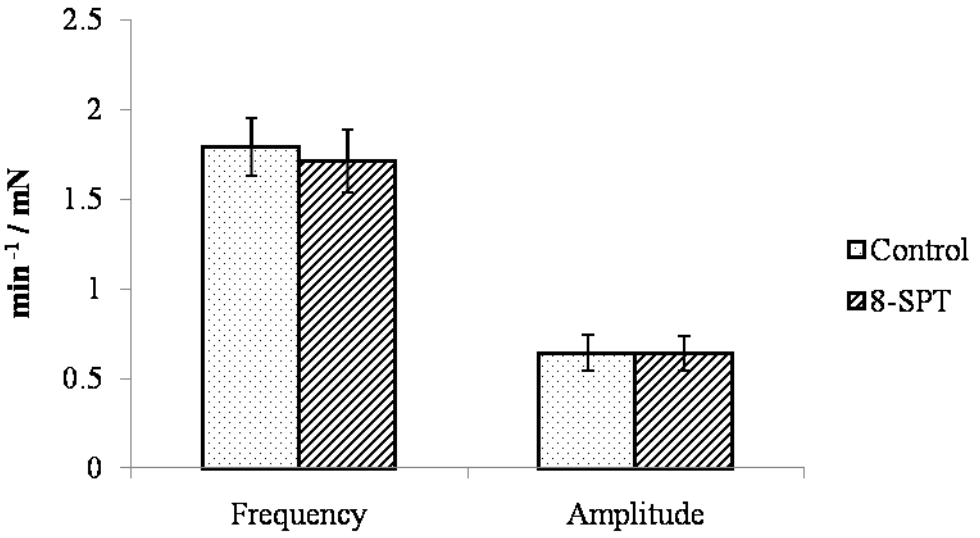


Figure 7.3.7. The role of adenosine in vasomotion of human umbilical artery.

Histograms showing frequency and amplitude of vasomotion in the presence and absence of 8-SPT (100 µM; 20 min). 8-SPT had no effect on either frequency or amplitude of vasomotion. *P* = 0.23 (frequency) and 1.00 (amplitude). ± = S.E.M. n=7 from 2 different donors (n=number of arterial rings).

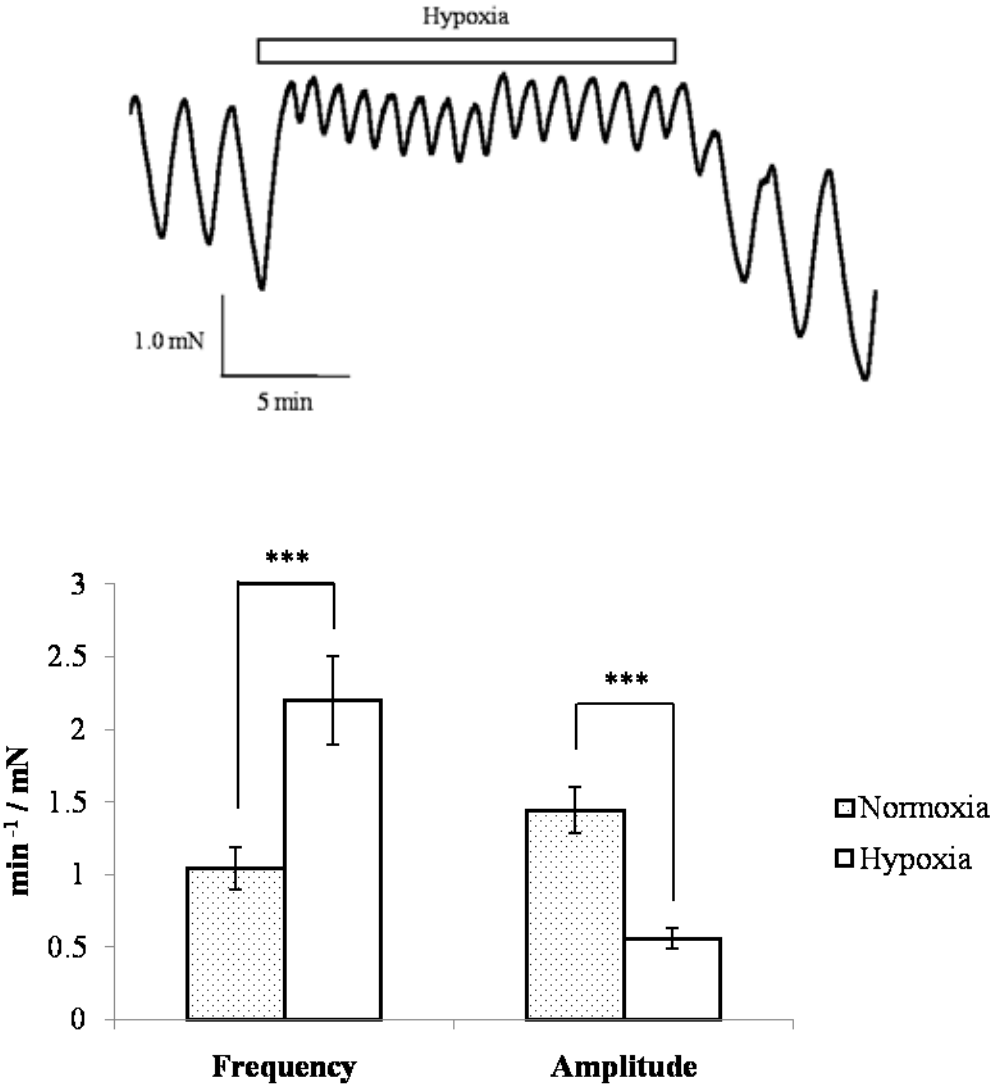


Figure 7.3.8. The effect of acute hypoxia on $[Ca^{2+}]_i$ in human umbilical artery.

Upper: Original recording showing effect of acute hypoxia on vasomotion in a vessel ring. Bar indicates period of hypoxia (PO_2 50mmHg). As can be seen the response evoked by hypoxia reversed quickly and was repeatable.
Lower: Columns showing the frequency and amplitude of vasomotion ± S.E.M in PO_2 160mmHg and hypoxia. Hypoxia consistently increased the frequency but decreased the amplitude of vasomotion. n=6 from 2 separate donors (n=number of arterial rings). ***$P < 0.01$.

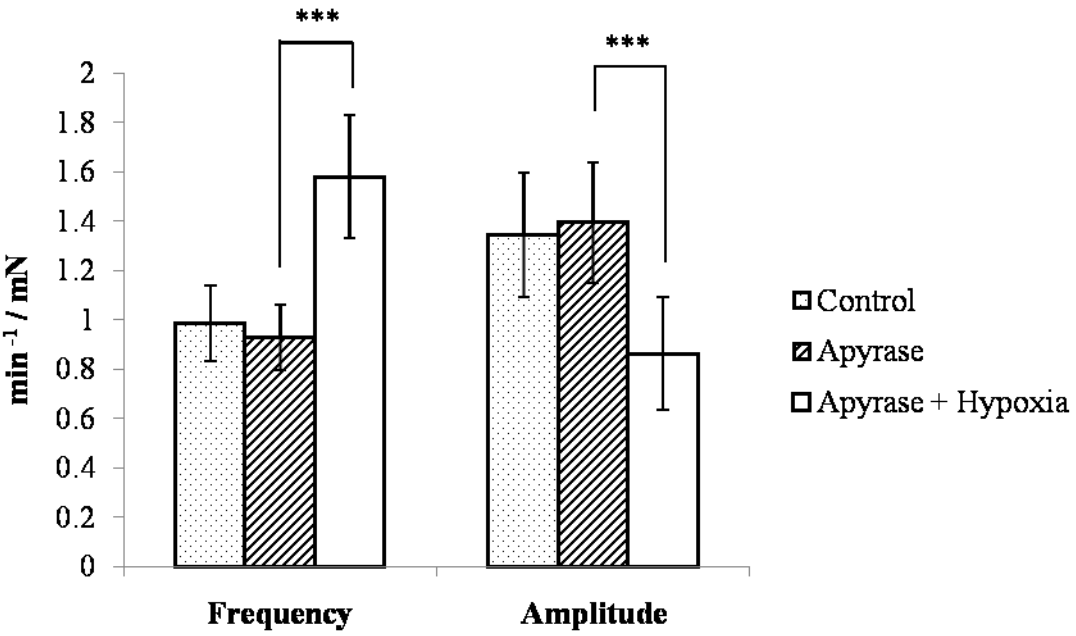


Figure 7.3.9. The effect of apyrase on vasomotion of human umbilical artery rings in normoxia and hypoxia.

Bar indicates the frequency and amplitude ± S.E.M of vasomotion in normoxia, apyrase (2 U/ml), and hypoxia in the presence of apyrase (50 mmHg; 2 U/ml). Vasomotion was not affect by incubation of the vessel rings in apyrase (2 U/ml; 30 min). After the pre-incubation and in the presence of apyrase (2 U/ml; 30 min), hypoxia increased the frequency and decreased the amplitude of vasomotion. n = 6 from 2 different donors (n=number of arterial rings). ***P < 0.05.

7.4. DISCUSSION

The studies described in the present chapter demonstrated that spontaneous vasomotion occurred in a majority of freshly isolated human umbilical arterial rings. The vasomotion was insensitive to separate blockade by the non-specific P2 antagonist suramin, the general IP_3R and SOCC/ROCC inhibitor 2-APB, or the adenosine antagonist 8-SPT. In contrast, replacement of extracellular Ca^{2+} with Ca^{2+} chelator EGTA abolished vasomotion. Exogenous ATP induced contraction but not vasomotion in vessel rings that did not display spontaneous vasomotion. Furthermore, acute hypoxia (PO_2 50 mmHg) increased the frequency but lowered the amplitude of vasomotion. Pre-incubation of the isolated umbilical artery rings in apyrase, which hydrolyses extracellular ATP to downstream nucleotides and inorganic phosphate, did not affect vasomotion *per se* or the effect of hypoxia.

7.4.1. Characteristics of the vasomotion

Using the isolation technique developed in the present study (see *Chapter 2 Section 2.4.1*), 62.5% of the arterial rings developed spontaneous rhythmic contractions and relaxations. According to the definition of Nilsson et al (2003), these variations in vascular tone can be considered as vasomotion, as they clearly fulfilled two important criteria: that they were neither a consequence of heartbeat or respiration, nor a result of neuronal input. Further, the fact that the frequency and amplitude of the rhythmic contractions and relaxations are consistent to those previously reported (Garcia-Huidobro *et al* 2006, but also see *Chapter 1 Section 1.5.2*), and that the oscillations persisted for >12 hr if uninterrupted, strongly suggests that they were what is generally referred to as physiological vasomotion. Importantly, the frequencies of the vasomotion in the human umbilical arterial rings and that of ATP-induced $[Ca^{2+}]_i$ oscillations in HUASMC (*Chapter 3 Section 3.3.1*) were broadly similar (~0.01Hz),

which supports findings from previous studies which also demonstrated synchronicity between vasomotion and $[Ca^{2+}]_i$ oscillations when theses were simultaneously recorded (Aalkaer & Nilsson, 2005).

In a previous study on vasomotion in umbilical and placental vessels, it was found that removal of the endothelium cause a modest reduction in the amplitude (umbilical vein) or frequency (chorionic vein); the effect on the umbilical artery vasomotion was not tested (Garcia-Huidobro *et al.*, 2007). In the present study, an attempt was made to test the role of the endothelium in the umbilical artery, but the endothelium remained intact in the present study despite the denuding procedure of injecting air. It is difficult to understand why this procedure failed for it was previously shown to succeed in removing the endothelium in previous studies in human umbilical artery rings (Bodelsson & Stjernquist, 1994). It would be important to properly test the role of endothelium in future studies on umbilical artery.

7.4.2. Role of extracellular Ca^{2+}

As discussed in *Chapter 1 Section 1.5.2*, the mechanisms underlying vasomotion are currently poorly understood. In the present study, it was found that replacement of extracellular Ca^{2+} with the Ca^{2+} chelator EGTA inhibited vasomotion in human umbilical arterial rings. This is consistent with the paradigm that $[Ca^{2+}]_i$ plays a significant role in the contractions and relaxations, as $[Ca^{2+}]_i$ oscillations are reported to be in temporal synchronisation with the variation in vessel tone in a number of blood vessels (Aalkaer & Nilsson, 2005). If this is indeed the case, then the theoretical model proposed by Sneyd *et al* (see *Chapter 3 Section 3.4*) would suggest that the Ca^{2+} entry into the HUASMC as discussed in *Chapter 3* is essential in mediating the vasomotion observed in the arteries of the present study.

In the studies described in the present chapter, it was found that neither the non-specific P2 receptor antagonist suramin, nor the IP_3R and SOC/ROC channel inhibitor 2-APB, nor the adenosine antagonist 8-SPT affected vasomotion once it was established. Considering the findings in *Chapter 4* which showed that HUAECs constitutively release ATP from both their apical and basolateral surfaces, and that exogenous ATP induced concentration-dependent $[Ca^{2+}]_i$ oscillations in HUASMC (*Chapter 3*), it was logical to hypothesise that ATP, once released, may act directly on the underlying SMC, or indirectly through the action of endothelial active substances (see *Chapter 1 Section 1.3.6.3 & 1.3.6.4*). Taken at face value, the findings presented here suggest that this may not be the case in human umbilical arteries. Notwithstanding, three important facts should be noted.

First, the antagonists of ATPs mechanism of action were applied in the Krebs' incubating the arterial rings which were mounted on supporting pins. As a significant amount of connective tissue was deliberately retained on the external surface of the arterial ring preparation in order to facilitate vasomotion (see *Chapter 2 Section 2.4.1*), it may be that the various antagonists used in the present study did not reach the VSM effectively from the abluminal surface and did not reach myo-endothelial border; they may even have been confined to the apical surface of EC. Notably, we cannot be sure that the antagonists penetrated through the endothelial basolateral surface and internal layer to reach the VSM of the media from the adluminal surface. If this is the case then the present results would suggest that the autocrine actions of ATP released from the apical membrane of HUAEC was not important in vasomotion. However, they do not eliminate the possibility that ATP released from the basolateral surface of the EC was acting in a paracrine manner on underlying HUASMC to facilitate vasomotion.

Second, it should be noted that the antagonists were applied when vasomotion was already established. It was reported in *Chapter 3* that ATP (100 μM) induced $[Ca^{2+}]_i$ oscillations in HUASMC, and these could not be inhibited by separate application of suramin, 2-APB or the VOCC inhibitor nifedipine, but were inhibited by a combination of these antagonists. Therefore, the findings described here, that separate inhibition of the individual Ca^{2+} entry pathways did not inhibit established vasomotion, do not preclude the possibility that a combination of these antagonists may have inhibited vasomotion as was the case in $[Ca^{2+}]_i$ oscillations in HUASMC (*Chapter 3 Section 3.3.4*). Nor do they preclude the possibility that these pathways were important in the *generation* of vasomotion. In future studies, co-application of a combination of the inhibitors of these pathways *during* vasomotion would be warranted, although the access problem discussed above may still be an issue.

Third, as discussed in *Chapter 3 Section 3.4.3 & Chapter 6 Section 6.4.1*, not all P2 receptors are sensitive to inhibition by suramin. Indeed, the findings in *Chapter 3* strongly suggested that ATP-induced $[Ca^{2+}]_i$ oscillations in HUASMC were mediated by $P2Y_4$ receptors, which are insensitive to suramin even at high concentration (Charlton *et al.*, 1996; Wildman *et al.*, 2003). Thus, when a $P2Y_4$ receptor antagonist becomes available, its effect on vasomotion in human umbilical artery rings should be tested.

7.4.3. Role of ATP

In the present study, exogenous application of ATP to quiescent umbilical arterial rings caused contraction, and contrary to the hypothesis set out in *Chapter 1*, did not evoke vasomotion. As indicated above, one of the more obvious limitations of the preparation is

that the site of action of any drugs is not clear. It is not clear whether ATP produced this effect by acting on abluminal receptors on VSM, and/or apical receptors on EC and/or whether it reached adluminal surface of VSM. Whichever combination is the case, this does not reflect the in vivo situation where ATP might be released from EC or RBCs. It would have been helpful if it had been possible to perform the same experiment with prior removal of the endothelium, to isolate the site of action of ATP to SMC alone. Certainly, a role for ATP in vasomotion in human umbilical artery cannot be excluded on the basis of the present results: ATP receptors that are important in generating vasomotion could be on the surface of VSM that faces basolateral membrane of the endothelium and these may not have been reached by exogenous ATP. Clearly, experiments on the umbilical artery to investigate the potential role of ATP should be done with and without the endothelium, and with and without inhibitors of the transporter uptake mechanisms for ATP and other purines and of ENs, for the endothelium can act as a metabolic barrier for the diffusion of ATP to VSM.

It is interesting to note that incubation with apyrase, which hydrolyses extracellular ATP and ADP into their downstream nucleotides and inorganic phosphate, also did not affect vasomotion in umbilical artery rings. As apyrase was dissolved in the Krebs' bathing the arterial rings, this might suggest that constitutive release of ATP from the apical surface of EC is not important in the maintenance of vasomotion. However, as discussed above for the other antagonists applied to the vessel preparation, it is not clear whether apyrase crossed the endothelial barrier or the surrounding supporting tissue to the underlying SMC. Therefore, the lack of effect of apyrase does not exclude the possibility that ATP released from the basolateral surface of EC acted on the underlying SMC to affect vasomotion.

7.4.4. Effect of hypoxia

A novel finding of the present study is that when umbilical arterial rings that were displaying vasomotion were exposed to acute hypoxia (a change of 150 to 50 mmHg PO_2), the pattern of the vasomotion immediately altered such that background tone was increase and vasomotion was changed to one with significantly higher frequency and lower amplitude. This is in direct contrast to a comparable study on umbilical vein, where separate recordings in 20% or 95% O_2 perfused solution did not affect vasomotion (Garcia-Huidobro *et al.*, 2007). According to the findings presented in the present thesis, it can be proposed that hypoxia caused contraction and increased the frequency of vasomotion by elevating the concentration of ATP present in the myo-endothelial junction, by increasing ATP release from EC, such that ATP acted on P2X and $P2Y_4$ receptors on VSM to increase the frequency of $[Ca^{2+}]_i$ oscillations in HUASMC as discussed in *Chapter 3*. The fact that these higher frequency oscillations in vasomotion were superimposed on tonic contraction (Fig 7.3.8) is more difficult to explain, but which could also be an consequence of increased $[Ca^{2+}]_i$ due to P2 receptor activation.

As discussed in *Chapter 1 Section 1.5.1*, the physiological consequences of vasomotion are poorly understood, but it is now generally accepted that it increases tissue oxygenation of surrounding tissue and organs. This view is largely based on mathematical modelling of capillary beds, whereas the umbilical artery, which conducts deoxygenated blood from the fetus to the placenta, is clearly a conduit vessel. Nevertheless, studies in sheep umbilical artery indicated that a significant proportion of arteriovenous pressure gradient (30 ± 6%) occurred across the umbilical arteries (Adamson *et al.*, 1992). This suggests that, in contrast to other systemic arterial vessels, and in line with the current view that the umbilical circulation represents a unique and separate vascular system, the umbilical arteries *do* provide

significant vascular resistance. Hence, changes in its contractility induced by hypoxia may well have considerable impact on the delivery of fetal blood to placenta for oxygenation and therefore oxidative status of the fetus.

Pre-incubation of umbilical artery rings in apyrase did not alter vasomotion in PO_2 160mmHg or the effect of hypoxia on vasomotion. This might be surprising, if the effect of hypoxia were mediated by ATP. However, as argued above, the apyrase may not have reached the myo-endothelial junction area to act on additional ATP released from the basolateral surface of the EC towards VSM. Further, it is possible that once vasomotion is induced, it relies on a self-perpetuating mechanism which does not require the continual presence of ATP in the extracellular space.

In order to further our understanding of the mechanisms underlying vasomotion in human umbilical artery, a number of improvements to the technique used in the present study seems paramount. Since it was not possible to identify the site of action of any of the pharmacological agents on the arterial ring preparation used in the present study, it is essential to develop a reliable technique for removing the endothelium. For the same reason, it would be sensible to repeat some of the experiments in a pressurised myography system, where the application of pharmacological agents can be directed specifically to the intra- or extraluminal surfaces of the arterial rings. Further, an underlying assumption for the studies described in the present chapter is that $[Ca^{2+}]_i$ oscillations in HUASMC drive vasomotion. This could be examined more closely by simultaneously measuring vascular tension and Ca^{2+} signalling, as has been done in several vascular beds such as rat mesenteric artery (Jensen *et al.*, 1993).

CHAPTER 8

GENERAL DISCUSSION

8.1. GENERAL DISCUSSION

In the present thesis, data obtained by using a number of techniques and cell types have been included. The findings have been discussed in the individual chapters. The aim of the present chapter is to integrate these concepts (Fig 8.1).

It is well recognised that intermittent perfusion of the placenta, which involves changes in O_2 delivery, around the end of the first trimester produces I/R-type injury, and that ROS and intrauterine hypoxia are features of all normal pregnancies. Further evidence suggests that it is the balance between the anti-oxidant mechanisms and ROS levels that determine the success or failure of pregnancies. In PE, conversion of spiral arteries is incomplete, which leads to their retained exaggerated contractility. Consequently, the variability of PO_2 at the placental exchange surface is increased, leading to excessive ROS. It is proposed that this contributes to the fetal and maternal symptoms of PE.

Clearly, the umbilical vessels are part of the uteroplacental circulation. The role of vasomotion, and the effect of hypoxia on vasomotion on these vessels has received relatively little attention. A main hypothesis of this study was that in hypoxia, ATP would be released from EC of the umbilical artery and vein, and act on the underlying VSM to induce or modulate vasomotion, thus affecting the blood flow and O_2 delivery to and from the fetus and the placenta. A second hypothesis was that ATP may act on EC to modulate release of endothelial-derived vasoactive substances.

The ATP release experiments of *Chapter 4* clearly showed that constitutive release of ATP occurred in 21% O_2, and the release was greater from the apical than the basolateral surface of HUAEC and HUVEC (Fig 8.1). Further, in HUVEC at least, the release recorded was

accentuated in hypoxia, and this was similarly polarised (Fig 8.1). It was apparent that this may also be the case in HUAEC, but technical difficulties with adhesion of HUAEC to the culture inserts meant that the difference in ATP release from in 21% O_2 and hypoxia did not reach statistical significance. Previous studies in the context of hypotonic stress have similarly demonstrated that a number of EC, including HUVEC and HUAEC, constitutively release ATP, and the release was accentuated by hypotonic challenge (Schwiebert *et al.*, 2002). They too, showed that release of ATP is predominantly from the apical membrane, but also from the basolateral membrane. Interestingly, blockade of PI_3K was able to inhibit hypoxia-induced release of ATP in the present study as it did whether it was shear stress-, hypotonic-, or hypoxic-induced, as in the present study. However, it seems that the downstream pathway involved are different; shear stress: Akt/PKB; hypotonic: MAPK/ERK1/2; hypoxia: Rho/ROCK [Chapter 4 and (Li *et al.*, 2005; Nandigama *et al.*, 2006; Woodward *et al.*, 2009)]. This suggests that PI_3K may be a common pathway which is involved in ECs detecting changes in their external milieu. Indeed, as discussed in Chapter 1, it was shown that shear stress-induced release of ATP from HUVEC was accentuated by hypoxia (Bodin & Burnstock, 1995).

Evidence was later provided by using brefeldin A and monensin which showed that shear stress-induced release of ATP from HUVEC is mediated by vesicles (Bodin & Burnstock, 2001a). The mechanisms mediating cellular ATP release in response to hypoxia was not investigated, but recently in calf vaso vasorum EC, it was reported that ATP release was vesicular on the basis that antagonists that targeted vesicular transport reduced hypoxia-induced release of ATP (Woodward *et al.*, 2009). The present study showed that this is also the case for HUVEC, but importantly, in addition to quantifying ATP release with luciferase

(Chapter 4), the present study provided direct evidence by using quinacrine, which has a high affinity to ATP, and showed that ATP are contained in vesicles in HUVEC, and that ATP is released from the vesicles in hypoxia (Chapter 5; Fig 8.1). Furthermore, by artificially elevating [Ca^{2+}] in HUVEC, which is the principle trigger of vesicular release in neurones and secretory cells, it was shown that ATP release from both apical and basolateral membrane of HUVEC was significantly accentuated (Chapter 4; Fig 8.1). The lack of effect of NO donor on release of ATP showed that the mechanisms underlying adenosine and ATP release are different, and that NO generation by HUVEC in response to ATP does not feedback to generate more ATP release. These data provided vital new evidence to support a role of vesicular release in ATP from EC in hypoxia, which has obvious physiological consequences, which were discussed in *Chapter 1 Section 1.3.6*.

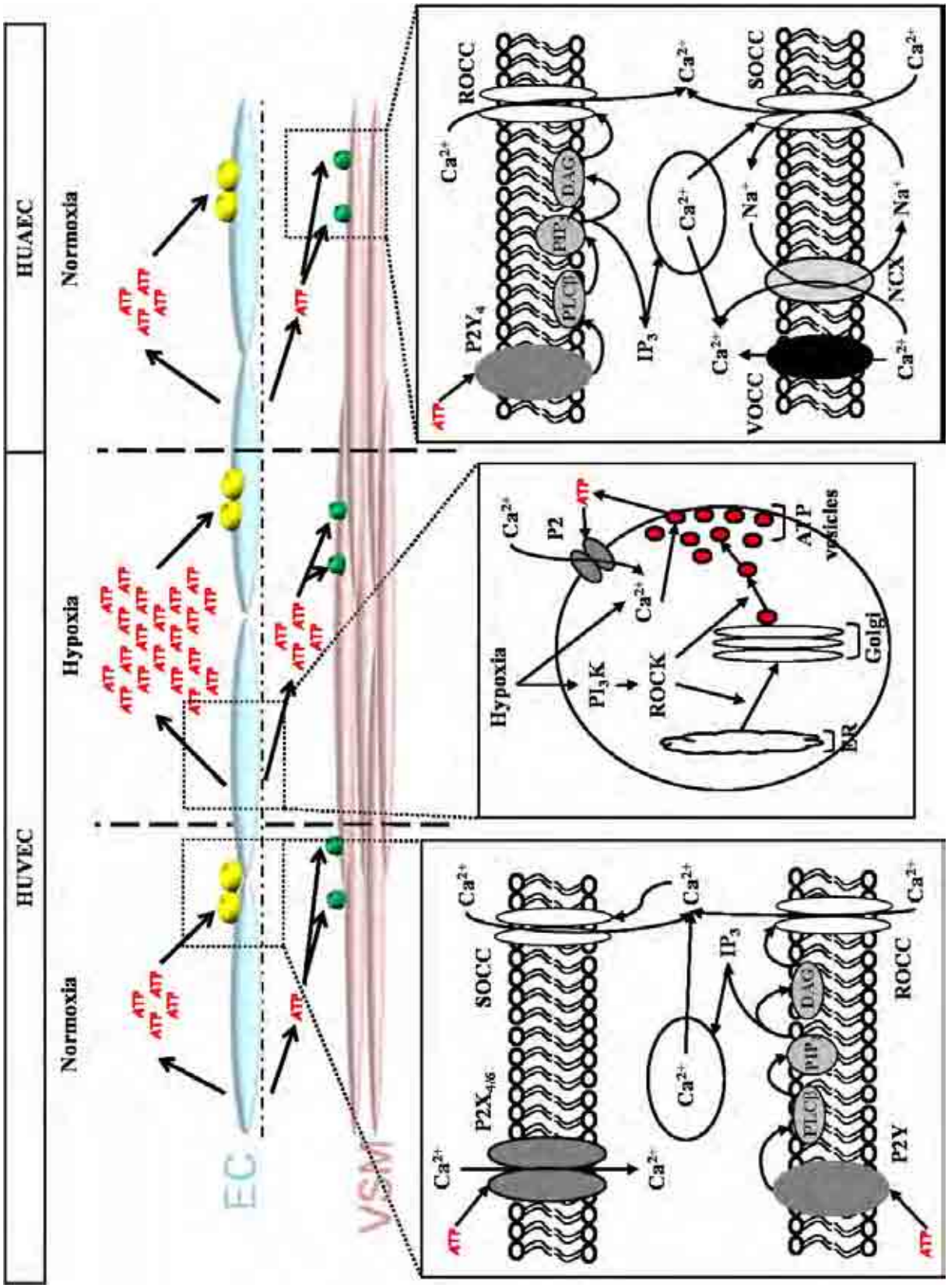


Figure 8.1. Schematic diagram of the major findings of the present thesis.

The findings described in *Chapter 3* showed that extracellular ATP caused $[Ca^{2+}]_i$ oscillations in HUASMC where the frequency and amplitude were dose-dependent. By using UTP and suramin, evidence was presented which showed that $P2Y_4$ receptors are especially important in initiating the $[Ca^{2+}]_i$ oscillations, but that multiple Ca^{2+} entry pathways participate in maintaining the Ca^{2+} load necessary for oscillations (Fig 8.1). Such $[Ca^{2+}]_i$ oscillations are considered to drive vasomotion (see *Chapter 1 Section 1.5.2*).

Indeed, freshly isolated umbilical arteries also displayed spontaneous vasomotion (Chapter 7), and the frequency of these was in accordance with the frequency of the $[Ca^{2+}]_i$ oscillations in HUASMC, especially between 10-50uM [ATP] range (cf Fig 3.3.2 & 7.3.8), consistent with the idea that ATP released from HUAEC might be responsible for initiating vasomotion in the umbilical artery rings. Vasomotion in the umbilical artery rings was similarly dependent on extracellular Ca^{2+}, and attempts were made in experiments of *Chapter 4* to link ATP with generation or maintenance of vasomotion in umbilical artery. Separate antagonism of P2, SOCC / ROCC or P1 receptors did not affect vasomotion, although a combination of these was not tested (the combination considerably inhibited ATP-induced $[Ca^{2+}]_i$ oscillations in HUASMC). Moreover, incubation of the blood vessel in apyrase, which was intended to metabolise endogenous ATP and ADP, or 8-SPT, a P1 (adenosine) receptor antagonist, did not affect vasomotion. However, in the myograph experiments apyrase or 8-SPT was applied into the Krebs' incubating intact vessel rings. Therefore, it is possible that the antagonists did not have adequate access to ATP released at active site, such as the myo-endothelial junction, which it is important for vasomotion.

Attempts were also made to remove the endothelium as a source of ATP by bubbling of air into the lumen of the vessels. However, this did not denude the endothelium, even though this technique has previously been shown successful (Bodelsson & Stjernquist, 1994). Hence, in order to properly investigate the role of EC, the expected source of ATP, in vasomotion, chemical removal of the endothelium, such as by saponin, would be a crucial experiment (Graser *et al.*, 1988).

Interestingly, however, hypoxia did increase the frequency of the vasomotion, whilst decreasing the amplitude (Chapter 7). It is possible that hypoxia released larger quantities of ATP basolaterally from HUAEC, which acted in a paracrine manner on HUASMC and increased the Ca^{2+} load in these cells, leading to the effect on vasomotion.

Results from Chapter 6 showed that, in 21% O_2, exogenous ATP increased $[Ca^{2+}]_i$ in HUVEC. The initial increase was likely due to activation of P2Y receptors, while the sustained phase was due to $P2X_{4/6}$ receptors (Fig 8.1). In addition, it was shown that SOCC/ROCC are functionally active, and contributed to the ATP-evoked $[Ca^{2+}]_i$ increase in HUVEC. Hypoxia *per se* induced a small $[Ca^{2+}]_i$ elevation, and taking the results of *Chapter 4* in consideration, it seems likely that the Ca^{2+} response was at least in part due to release of ATP from the apical membrane in response to hypoxia, which acted in a autocrine way on P2 receptors on HUVEC (Fig 8.1). Further, since increasing the Ca^{2+} in HUVEC by the ionophore A23187 also induced ATP release (presumably via vesicular release; see Chapter 6), it is proposed that hypoxia-induced ATP release can lead to further release of ATP, forming a positive feedback loop (Fig 8.1). The finding that the combination of ATP with hypoxia led to a smaller increase in $[Ca^{2+}]_i$ than ATP in 21% O_2 is not inconsistent with that suggestion. It simply

suggests that *in vivo* there is a balance between all the effects acting on HUVEC simultaneously.

As discussed, mitochondrial ROS production is known to be increased in hypoxia, and it was shown in calf pulmonary EC that ROS inhibited CCE entry (Chandel & Schumacker, 2000; Florea & Blatter, 2008). Since CCE and extracellular Ca^{2+} are clearly important in ATP-induced Ca^{2+} elevation in HUVEC (Chapter 4 Section 6.4.2 & 6.4.3), it is reasonable to suggest that ROS-induced inhibition of CCE in HUVEC might have led to the depressed Ca^{2+} response to ATP reported in the present thesis. The data presented in the present these therefore provides an important indication of how HUVEC may respond to ATP stimulation *in vivo*, when it is likely to be hypoxia, and where the $[Ca^{2+}]_i$ level would be important in activating release of endothelium-derived vasoactive substances such as NO and PGI_2. In future studies, it would be important to test if antioxidants were able to influence the effect of hypoxia on ATP-induced Ca^{2+} response in HUVEC.

As ATP release from HUVEC is dependent on $[Ca^{2+}]_i$ (see Chapter 4 Section 4.4.3), it is possible that ROS generated in hypoxia impairs or blunts hypoxia-induced ATP release from EC. Interestingly, it was reported that the $[Ca^{2+}]_i$ response to histamine was attenuated in HUVEC from PE pregnancies compared to normal pregnancies (Steinert *et al.*, 2002). However, it was shown that it is the plateau phase of the induced response that was absent in PE-cells. As the plateau phase of agonist-induced $[Ca^{2+}]_i$ response is sustained by Ca^{2+} entry (see Chapter 4 Fig 6.3.4), and that ROS is elevated in PE due to excessive I/R-type injury (Hung & Burton, 2006), it may be that impaired CCE by ROS may play an even greater role in impairing Ca^{2+} response to agonists in EC in PE. This is in accordance with the general

hypothesis set out in *Chapter 1 Section 1.6* ( in Chapter 1 Fig 1.9), and suggests that excessive I/R-type injury in PE may lead to decreased ATP release from EC, leading to attenuated or absent vasomotion in the utero-placental circulation. This may provide an explanation for the aetiology of PE.

In the present study, the [ATP] released measured from HUVEC and HUAEC were in the lower nM range at the end of 30 min period of exposure to hypoxia (PO_2 7.6 mmHg). On the other hand, the EC_{50} for evoking $[Ca^{2+}]_i$ elevation in HUVEC was in the region of 10 μM. This apparent discrepancy can perhaps be reconciled by the fact that, as discussed in *Chapter 1 Section 1.4.4*, nucleotides in the extracellular space are rapidly hydrolysed by ENs. A list of measured extracellular [ATP] in many different studies was composed by (Lazarowski *et al.*, 2003), and indicated that [ATP] measured by off line techniques, such as the firefly luciferase or HPLC, are broadly similar, and concentrations measured in the present study are consistent with these and other measurements. The major disadvantage of the off-line measurement techniques is that they do not provide any information on the time course of release. More recently, new attempts have been made to quantify [ATP] at the cell surface by genetically modifying the luciferase gene to anchor it to the plasma membrane. Expression of this gene in the model cell line HEK, or ACN neuroblastoma cells, revealed that they released ATP at concentrations of 100-200 μM upon stimulation. Thus, it is likely that estimates made in the present study were underestimated. It may also be noted, as discussed in *Chapter 4 Section 1.4.1*, *in vivo* and *in vitro* experiments have shown that erythrocytes also releases significant concentration of ATP in hypoxia, at least in the region of 10^{-6} M (Jagger *et al.*, 2001; Ellsworth, 2004). Thus, taken together, there is strong evidence to suggest that during

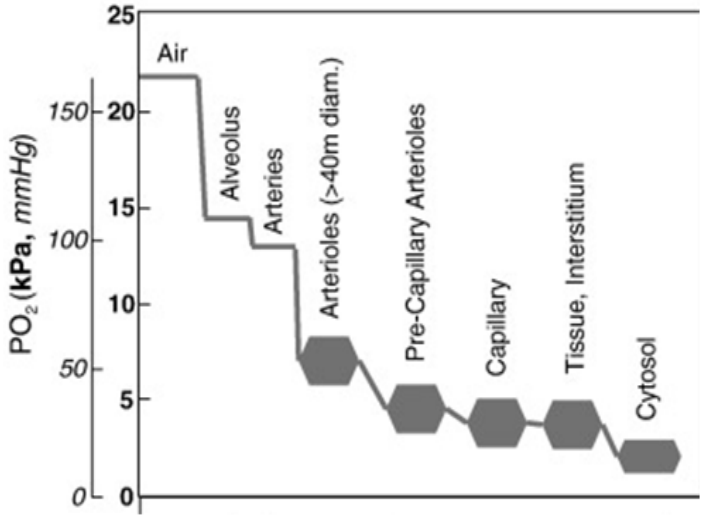
hypoxia, HUVEC and indeed HUAEC may well be exposed to the concentrations of ATP tested in *Chapter 6*.

It was hypothesised in Chapter 1 Section 1.6.1 that chronic hypoxia in PE may lead to reduced vasomotion due to increased expression of ENs. In accordance with this, HUASMC were incubated in 1% O_2 for 72 hr. In these cells it was found that the frequency of ATP-induced $[Ca^{2+}]_i$ oscillations was increased at concentrations <100 μM ATP, whereas at concentration > 100 μM ATP, their frequency was reduced (Chapter 3). As discussed earlier, it is not clear exactly what the physiological extracellular [ATP] is under different conditions, but high micromolar concentrations of ATP can be expected to be present at surface of cells in acute hypoxia. It is clear that the role of ENs need to be investigated. Whether the effect of chronic hypoxia on the frequency of ATP-induced $[Ca^{2+}]_i$ oscillations described in *Chapter 3* plays a part in the depressed vasomotion in the utero-placental circulation in PE required is a fertile ground for future investigation. In particular, it would be important to investigate the effect of chronic hypoxia on Ca^{2+} signalling in venous SMC.

In summary, in the present study, it was shown that exogenous ATP induced $[Ca^{2+}]_i$ oscillations in HUASMC, the frequency of which is dependent on the [ATP] applied. It is likely that $P2Y_4$ is responsible for the initiation of the $[Ca^{2+}]_i$ oscillations, and multiple Ca^{2+} signalling pathways, including SOCC, ROCC, VGCC and/or Na^+/Ca^{2+} exchanger, contributed to maintaining the Ca^{2+} load necessary for the continuation of the oscillations. Vasomotion of a similar frequency was found in freshly isolated human umbilical artery rings, the frequency of which was increased in hypoxia. In vitro data showed that HUVEC and HUAEC constitutively released ATP from both their apical and basolateral surfaces, and hypoxia

accentuated the release, at least in HUVEC, but this was also likely the case in HUAEC. Furthermore, the hypoxia-induced ATP release was mediated by a PI_3K/Rho/ROCK/vesicular pathway. These results were supported by positive quinacrine staining of ATP vesicles, where hypoxia decreased the staining fluorescence, and antagonists that modulate the vesicular pathway altered the staining pattern in a predictable manner. Finally, it was found that hypoxia and exogenous ATP induced $[Ca^{2+}]_i$ elevation in HUVEC itself, the latter via $P2X_{4/6}$ activation. It was also found that the ATP-induced Ca^{2+} response of HUVEC was attenuated in hypoxia, providing novel information on the $[Ca^{2+}]_i$ response of EC to hypoxia, and ATP, *in vivo*. These novel findings are summarised in *Fig 8.1*.

APPENDICES



Measured PO_2 levels in the vasculature from the airways to the cytosol. Adopted from a recent review article (Ward, 2008).

Composition of EGM® Endothelial Cell Growth Medium (Lonza, Switzerland)

- 10 ng/ml human recombinant epidermal growth factor
- 1.0 mg/ml hydrocortisone
- 3 mg/ml bovine brain extract
- 2% fetal bovine serum
- 0.3mM L-Arginine

Composition of Endothelial Cell Growth Medium (for ATP-release experiments; Promocell, UK).

- 0.1 ng/ml human recombinant epidermal growth factor
- 1.0 µg/ml Hydrocortisone
- 0.4% endothelial cell growth supplement
- 1.0 ng/ml human recombinant basic fibroblast growth factor
- 90 µg /ml heparin

- 2% fetal calf serum
- 0.3mM L-Arginine

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