Prostaglandin Signalling in the Human Endometrium

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Contents

Declaration

Except where due acknowledgement is made by reference, the studies undertaken in this thesis were the unaided work of the author. No part of this work has been previously accepted for, or is currently being submitted for another degree or professional qualification.

Dr Oliver Patrick Milling Smith

Abstract

Under the orchestration of the circulating steroid hormones, oestrogen and progesterone, the human endometrium undergoes a well-defined cycle of proliferation, differentiation and shedding (menstruation). This cyclical process has been described as a recurrent physiological injury and repair. Problems of menstruation account for a large proportion of female morbidity and many women who are referred to secondary care with a menstrual complaint undergo a hysterectomy. No differences in either circulating hormone levels or specific histological findings have been discovered to account for menstrual pathologies such as heavy menstrual bleeding. It is therefore important to examine the local mediators present in the endometrium if we are to further our understanding of aberrations of endometrial function. Disturbed angiogenesis has been implicated in many endometrial pathologies, however the mechanism for control of the endometrial vasculature is poorly understood. Prostaglandins have been implicated in both endometrial pathologies and disturbances of angiogenesis. This thesis is based on the hypothesis that menstrual dysfunction, including heavy menstrual blood loss (MBL) is due to (a) up-regulated expression/synthesis of cyclooxygenase enzymes and prostaglandin receptors, and (b) initiation of enhanced intracellular signalling pathways in response to prostaglandins.

This thesis describes the use of an endometrial epithelial cell line to explore the molecular signalling pathways involved with the activation of a prostaglandin receptor - the prostacyclin receptor. A rapid activation of ERK1/2 signalling is demonstrated with alterations in expression of angiogenic factors via crosstalk with the epidermal growth factor receptor.

By using endometrium collected from women with the complaint of heavy menstrual bleeding, the pattern of expression of the various components of the COX-prostaglandin signalling pathways present in the endometrium of women with normal and heavy MBL is elucidated. There is a significant elevation in expression of COX-1 and COX-2 mRNA in endometrium obtained from women with heavy MBL compared with endometrium obtained from women with normal MBL. Significant alterations in expression of downstream prostanoid synthase and prostanoid receptor mRNAs were also detected. Furthermore, enhanced prostaglandin stimulated

production of cyclic AMP observed in endometrium of women with heavy MBL compared with normal MBL.

By identifying the prostanoid receptors/signalling pathways that are responsible for disturbed endometrial function, this thesis aims to establish information that may result in the development of novel therapeutic targets for menstrual pathology.

Acknowledgements

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I collected all my endometrial tissue from the either the outpatient department or operating theatres of the New Royal Infirmary Edinburgh. I would therefore like to thank all the nursing staff that allowed me the opportunity to collect this tissue. In particular I would like to thank Joan Crieger for her assistance in collecting tissue and Catherine Murray for guidance in all aspects of tissue storage and record keeping as outlined by national guidance for tissue handling. I additionally would like to thank Teresa Henderson for her advice and guidance in laboratory techniques required for analysing these tissue samples.

I must thank the Medical Research Council for the support they provided and in particular I would like to thank Ted Pinner and Corinne Macleod of the graphics department for their immense assistance.

Advice on how to measure menstrual blood loss was taken from the Glasgow university research group based in the Western Infirmary and I would like to thank Dorothy Lyons for taking the time to teach me their well characterised and proven methodologies.

Finally, I would like to acknowledge the love and support of my wife during my time working as a research fellow and in particular for her understanding whilst I wrote up this thesis.

Abbreviations

AA	Arachidonic Acid
ABC	Avidin Biotin Complex
Ang	Angiopoietin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
COX	Cycooxygenase
DAB	3,3'- diaminobenzidine
DEPC	Diethylpyrocarbonate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DP	Prostaglandin D_2 receptor
DUB	Dysfunctional uterine bleeding
ECL	Enhanced chemiluminescence visualisation system
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
EG-VEGF	Endocrine gland vascular endothelial growth factor
EP	Prostaglandin E series receptor
ER	Oestrogen receptor
ERK 1/2	Extracellular related kinase 1/2
FP	Prostaglandin F series receptor
bFGF	Basic Fibroblast Growth Factor
GPCR	G protein coupled receptor
H_2O_2	Hydrogen peroxide
HB-EGF	Heparin binding epidermal growth factor
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL-8	Interleukin 8
IP	Prostacyclin receptor
IP_3	Inositol trisphosphate
MAPK	Mitogen activated protein kinases

MBL	Menstrual blood loss
MMP	Matrix metalloproteinase
NBF	Neutral buffered formalin
NK cells	Natural killer cells
NSAID	Non steroidal anti-inflammatory drug
OD	Optical density
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Tween 20
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PG	Prostaglandin
PGES	Prostaglandin E synthase
PGFS	Prostaglandin F synthase
PGI_2	Prostacyclin
PGIS	Prostacyclin synthase
PGT	Prostaglandin transporter
PI3K	Phosphatidylinositol 3-kinase
PLA2	Phospholipase A2
PR	Progesterone receptor
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide-gel electrophoresis
SPRM	Selective progesterone receptor modulators
TBS	Tris buffered saline
Tie	Angiopoietin receptor
TP	Thromboxane receptor
TRIR	Total RNA isolating reagent
TXA_2	Thromboxane
TXS	Thromboxane synthase
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells

Chapter 1

Literature Review

1.1 General Introduction

The uterus plays a crucial role in the propagation of the species. Implantation of the fertilised ovum is a critical event common to all species. Humans and other old world primates differ from most other animals in that, in the absence of pregnancy, the endometrium undergoes well-defined cycles of proliferation, differentiation and shedding (menstruation) in response to the prevailing endocrine and paracrine environment. The endometrium is therefore a very dynamic target organ, the development and function of which is orchestrated by the action of sex steroids, predominantly oestrogen and progesterone. The endocrine influences of the major sex steroid hormones exert a controlling influence upon the whole reproductive tract. It is however essential to have a detailed knowledge of the local mechanisms at work within the endometrium if we are to understand the mechanisms responsible for aberrations in endometrial function such as disorders of menstrual bleeding.

Prostaglandins are bioactive lipids produced from arachidonic acid by cyclooxygenase enzymes (COX) and specific terminal prostanoid synthase enzymes. Following biosynthesis, prostaglandins exert an autocrine/paracrine function by coupling to specific receptors to activate intracellular signalling and gene transcription. A role for COX enzymes and prostaglandins has been ascertained in reproductive tract pathology, including painful menstruation (dysmenorrhoea), heavy menstrual bleeding (menorrhagia), endometriosis and cancer (Lundstrom *et al.*, 1976; Morita, 2002; Ota *et al.*, 2001; Sales *et al.*, 2002; Smith, S. K. *et al.*, 1981a). Emerging evidence supports a role for COX enzymes, prostaglandins and prostaglandin receptor signalling pathways in a multitude of phenotypic changes within reproductive tissues including the promotion of angiogenesis and vascular function (Sales *et al.*, 2005; Tsujii *et al.*, 1998).

This thesis summarises the published data regarding prostaglandins and their receptors in the human endometrium and highlights the roles of prostaglandins in benign disorders of the endometrium. Furthermore, new data are presented that will contribute to our understanding of the role of prostaglandins in endometrial physiology.

1.2 The Human Uterus and Endometrium

Essential for reproduction, the uterus is a fibromuscular organ composed of a smooth muscle outer layer lined by endometrium. The uterus is composed of the body of the uterus and the uterine cervix. The body of the uterus is composed of smooth muscle cells that are steroid responsive, undergoing hyperplastic and hypertrophic changes at developmental milestones during a woman's reproductive life. Under the influence of oestrogen, the body of the uterus doubles in size from a pre-pubertal size approximately equal to the size of the cervix. During pregnancy, the cells undergo hyperplasia and hypertrophy under the influence of placental steroids, allowing for growth of the developing fetus.

The endometrium lines the body of the uterus and is a dynamic steroid responsive tissue that must undergo regeneration and remodelling during each menstrual cycle to prepare for possible implantation. Menstruation signals the end of one cycle and the beginning of the next. The proliferative phase follows menses and during this stage oestradiol levels rise, leading to endometrial growth and proliferation. Following ovulation the corpus luteum produces progesterone, the dominant hormone of the secretory phase. Together with oestrogen, progesterone prepares the endometrium for possible blastocyst implantation. In the absence of pregnancy, the corpus luteum regresses and sex hormone levels decline, triggering the mechanisms that lead to menstruation.

1.2.1 Endometrial Morphology

The two layers of the endometrium are the superficial functional layer, which is shed during menstruation and the basal layer from which the endometrium has always been considered to regenerate. There are three well-characterised phases of endometrial development: a preovulatory *proliferative* phase, a postovulatory *secretory* phase involving tissue differentiation and a *menstrual* phase involving tissue break down. Consequently, the endometrium may be considered a site of recurrent physiological injury and repair (Critchley *et al.*, 2001b). Markee and colleagues (Markee, JE, 1940) first described an orchestrating role for the ovarian sex steroids in regulating the changes in endometrial morphology across the menstrual cycle. Progesterone is essential for maintaining and establishing a pregnancy and this

action is dependent upon the transformation of an oestrogen-primed endometrium. Menstruation is the response of the endometrium to the withdrawal of sex steroid hormones that occurs with the demise of the corpus luteum in the absence of pregnancy (Critchley *et al.*, 2001b).

Endometrial dating has historically been related to the timing of ovulation. The series of classical morphological changes that occur in response to cyclical ovarian activity have been well detailed (Noyes RW, 1950). However, much controversy exists over endometrial dating (Coutifaris *et al.*, 2004; Murray *et al.*, 2004). There are arguments that exist which suggest histological dating according to traditional histological methods lacks both the accuracy and precision to provide a guide for clinical management within the infertility setting. More robust methods for endometrial dating are likely to involve combining histological dating with reporting of last menstrual period and quantification of circulating oestrogen and progesterone levels. Detailed gene microarray studies support this method for characterizing endometrial samples with consistency across these three parameters (Critchley *et al.*, 2006; Talbi *et al.*, 2006). It is notable that exogenous administration of steroids produces a deviation from the classical histological features of glandular structure, mitotic status of glandular cells and secretions in the lumen of the glands (Habiba *et al.*, 1998) when compared with accurately dated endometrium collected during a physiological cycle (Noyes RW, 1950).

1.2.2 Proliferative Stage

The following descriptions of endometrial morphology are based upon criteria originally described by Noyes and colleagues (Noyes RW, 1950). Oestrogen is the steroid hormone that dominates in the pre-ovulatory (follicular) half of the cycle. During the proliferative phase, the surface epithelium regenerates and short, narrow epithelial glands gradually lengthen and curve. Mitosis is evident in the epithelial and stromal cells. The stroma is compact, containing spindle like cells and stromal oedema tends to regress through the proliferative phase. Prior to ovulation, during the late proliferative phase, the surface epithelium becomes more undulant and glands become more tortuous. Nuclei appear to be pseudostratified. The stroma by this stage is moderately dense and actively growing.

1.2.3 Secretory Stage

The secretory phase of the endometrial cycle is dominated by the action of progesterone upon the oestrogen-primed endometrium. This phase of the cycle may be divided into three stages; early, mid and late, representing the widely changing physiology that occurs during this time frame.

Early Secretory Phase (Post Ovulation Days 1 to 4)

Subnuclear glycogen-rich vacuolation of the glandular epithelium becomes prominent in at least 50% of cells (CH Buckley, 1989). The nuclei lose the pseudostratification configuration with an accompanying increase in the diameter and tortuosity of the glands. Mitosis cease to be seen.

Mid Secretory Phase (Post Ovulation Days 5-9)

Acidophilic intra-luminal secretions reach a peak. Stromal oedema is characteristically marked in the mid-secretory phase, peaking about day 8-9. The stroma at this phase looks like small, dense, nuclei with only filamentous cytoplasm. The spiral arterioles, previously difficult to distinguish in the oedematous stroma, become much more prominent. These findings constitute the earliest visible pre-decidual change.

Late Secretory Phase (Post Ovulation Days 10-Menses)

Eosinophilic pre-decidual cells may be seen located around the arterioles and stromal proliferation recurs, evident by mitosis. There is a sharp increase in leukocyte infiltration. Accompanying the pre-decidual change there is stromal regression and loss of endometrial height (CH Buckley, 1989). In the absence of pregnancy, gland secretion decreases and involution of the epithelial gland occurs. The glands dilate and take on a characteristic "saw toothed" effect; but the previously tall columnar epithelium is now low, the nuclei are shrunken and the cytoplasmic edges are ragged and indistinct.

1.2.4 Menstrual Stage

The menstrual cycle commences with the first day of menstrual bleeding. The upper two thirds of the endometrium (the superficial layer) are shed. The endometrium exhibits focal areas of sub-epithelial necrosis with subsequent glandular collapse and further necrosis. As menses ceases, a typical shallow dense endometrium remains that is composed of the basal layer and residual deeper functional layer. Regeneration starts to occur from the third day and commences in the glandular and stromal elements (Smith, S. K., 2001).

1.2.5 Sex steroid hormones and their receptors

Oestrogen and progesterone are the two principal sex steroids influencing endometrial growth, differentiation and function. The development of specific monoclonal antibodies to the oestrogen receptor (ER) and the progesterone receptor (PR) has advanced immunohistochemical localisation. The ER and PR immunolocalise to the nuclear compartment of cells within the endometrium. The endometrium consists of stromal, epithelial (glandular), endothelial (vascular) and perivascular cellular compartments. The expression of endometrial sex steroid receptors varies both temporally and spatially within the endometrium (Critchley *et al.*, 2001a; Lessey *et al.*, 1988). However, with the discovery of further receptor subtypes, our understanding of their distribution and function is changing.

Oestrogen is the steroid responsible for endometrial proliferation and progesterone exerts an anti-oestrogenic affect with inhibition of endometrial growth and induction of glandular differentiation. These progesterone effects require PR to be present which paradoxically only exists in an oestrogen primed endometrium. The presence of PR is considered evidence of a functional ER pathway. Both oestrogen and progesterone receptors are upregulated in stromal and glandular tissue during the proliferative phase of the menstrual cycle, with the ER expression reaching a peak in the late proliferative/early secretary phase. During the secretory phase, ER and PR are down regulated in the glands by the action of progesterone acting at the transcriptional and post-transcriptional level (Chauchereau *et al.*, 1992). ER expression is suppressed in the stroma with moderate stromal suppression of PR (Critchley 2003).

Steroids receptors are expressed as various subtypes. The ER has two structurally related subtypes, commonly known as ER-α and ER-β. These subtypes are derived from separate genes (Enmark *et al.*, 1997). The endometrium demonstrates differing levels of ER subtype expression within all cellular compartments (Saunders *et al.*, 2002). ERα and ERβ mRNAs are present in glandular epithelial cells, stromal cells and smooth muscle cells of the uterine wall at every stage of the menstrual cycle with higher levels of ERα expression than ERβ. ERα, which may be thought of as the classical ER, does however show the greater decline within the superficial layer of the endometrium during the secretory stage (Saunders and Critchley, 2002). A similar decline in ERα protein expression has also been reported within a study that demonstrated the only oestrogen receptor expressed within the endometrial endothelial cells of the vasculature was ERβ. Both ER subtypes were however expressed in the perivascular cells (Critchley. *et al.*, 2001a).

The PR also exists as two subtypes; PRA and PRB. These structurally related gene products are expressed from a single gene as a result of transcription from two alternative promoters (Mulac-Jericevic *et al.*, 2004). The A isoform, 779 amino acids in length, is a truncated version of the B isoform which is 993 amino acids in length (Tung *et al.*, 1993). The two isoforms can display distinct transactivational properties that are specific to cell type and ligand. For the few genes regulated by both receptors, PRB appears to function as a stronger activation of transcription (Turgeon *et al.*, 2004). PRA and PRB are differentially regulated with spatio-temporal variations in expression. Both subtypes are demonstrated within the glandular and stromal compartments during the proliferative phase, with both receptor subtypes dramatically declining in the glandular cells during the secretory stage. However PRA remains the predominant progesterone receptor within the stroma during the secretory phase and would appear to mediate progesterone effects on endometrium during this phase of the cycle (Wang *et al.*, 1998).

The actions of oestrogen and progesterone may also involve membrane as well as nuclear receptors. Rapid extra-nuclear signalling has been demonstrated for both steroids (Boonyaratanakornkit *et al.*, 2004). These actions may be mediated via intra-cellular second-messenger or other non-nuclear signal transduction pathways.

1.2.6 Endometrial Vasculature

Blood supplies the uterus via the uterine arteries. In the myometrium, arcuate arteries then form by branching at 90^0 towards the endometrium to form radial arteries. These radial arteries then branch to form basal arteries and spiral arterioles. The basal arteries supply the basal layer of the endometrium and the larger spiral arterioles supply the functional layer of the endometrium (Hickey *et al.*, 2000). The endometrial microvasculature of capillaries and venous plexuses are formed downstream of the spiral arterioles.

The spiral arterioles are involved in leukocyte entry and vasoconstriction and are implicit in the process of menstruation. These vessels are under the control of steroid hormones (Perrot-Applanat *et al.*, 2000), with both growth and differentiation mediated by the presence of ER and PR within the vascular smooth muscle cells. However, direct endothelial steroid receptor expression has been difficult to prove with recent data demonstrating human endometrium endothelial cells do not express ERα or PR but do express ERβ (Critchley *et al.*, 2001a). The mechanism for control of the microvasculature is poorly understood. Endometrial microvessels consist only of endothelial cells linked by tight junctions and supported by their basal lamina. This specialised form of extracellular matrix contributes more than support, aiding vessel growth, differentiation and permeability (Hickey and Fraser, 2000).

1.3 Prostaglandins

In 1935, von Euler first proposed the name "prostaglandin" for one of the active extracts that he was studying. It was not until 30 years later that interest returned to these substances as their physiological roles were being uncovered. Prostaglandins, thromboxanes and leukotrienes, collectively known as eicosanoids, are lipid soluble, bioactive metabolites, derived from the action of COX and lipooxygenase upon arachidonic acid (AA). Much work in general has been done to elucidate the roles of COX enzymes in health and disease. Advances in knowledge have helped to evolve the commonly used non-steroidal anti-inflammatory drugs (NSAIDs) whose action is to inhibit COX enzymes. Non-selective NSAID use has been associated with gastrointestinal toxicity, ulceration, platelet dysfunction and kidney damage in at least 1% of users (Fosslien, 1998). In addition, well-publicised and unforeseen cardiovascular problems have arisen with selective COX-2 inhibitors (Krumholz *et al.*, 2007) that dramatically highlight the need for a further understanding of these enzymes, metabolites and their receptors.

The multiple roles played by COX enzymes in reproductive biology have been highlighted following establishment of COX-deficient mice. Studies in COX-1-deficient mice have shown that the gestation period is prolonged and parturition is reduced coincident with a reduction in the number of viable offspring. Interestingly, conception and fetal development are unaltered suggesting that prostanoids produced by COX-1 are not critical for ovulation, fertilization or implantation, but are essential for bringing on normal labour at term. This is confirmed by observations between wild type mice giving natural birth and COX-1 deficient females having their young delivered by caesarean section, where there are no noticeable differences in the number or size of the offspring delivered (Gross *et al.*, 1998; Langenbach *et al.*, 1995). By contrast, ablation of the COX-2 gene in mice results in multiple reproductive failures, including ovulation, fertilization, implantation and decidualization, confirming that prostaglandins produced by COX-2 play a crucial role in these processes (Dinchuk *et al.*, 1995; Langenbach *et al.*, 1999; Langenbach *et al.*, 1999; Lim *et al.*, 1997). Prior to ovulation, pituitary gonadotropins trigger the expression of COX-2 and synthesis of PGE_2 to promote follicle expansion and ovulation. In COX-2 deficient mice, absence of PGE_2 in preovulatory follicles

disrupts follicle expansion and results in anovulation. Under these conditions administration of exogenous PGE_2 has been shown to rescue ovulation (Davis *et al.*, 1999). Thus, although both COX isoforms essentially catalyze the same reaction, in the reproductive tract there are clear differences in the prostanoid profile and functions of the two COX enzymes.

The focus of this thesis is to provide insight into the role of cyclooxygenase (COX) enzymes, prostaglandins and their receptors in the human endometrium. Observations have been made over the years implicating a role for prostaglandins in benign pathologies of the endometrium such as heavy menstrual bleeding (menorrhagia), endometriosis and dysmenorrhoea. The role of prostaglandins within these endometrial pathologies will be further outlined in section 1.4.3.

1.3.1 The prostaglandin pathway

Prostaglandin is a generic term for a closely related family of C_{20} carboxylic acids. Their biochemical structure is based upon the hypothetical prostanoic acid (Figure 1.1)

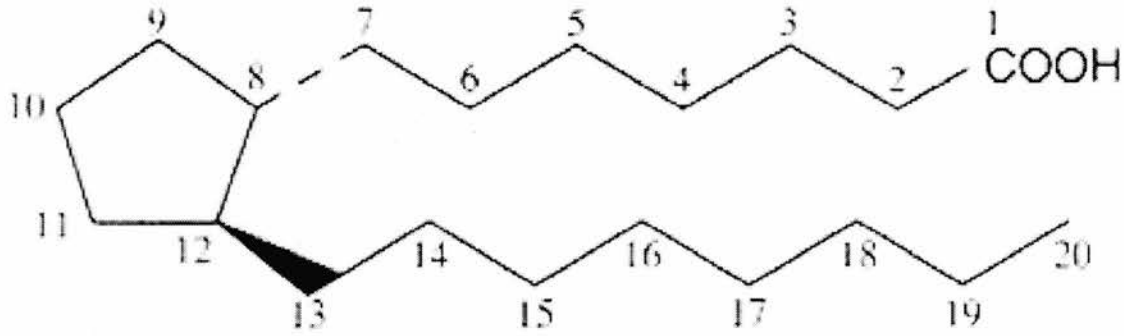


Figure 1.1 Hypothetical prostanoic acid skeleton

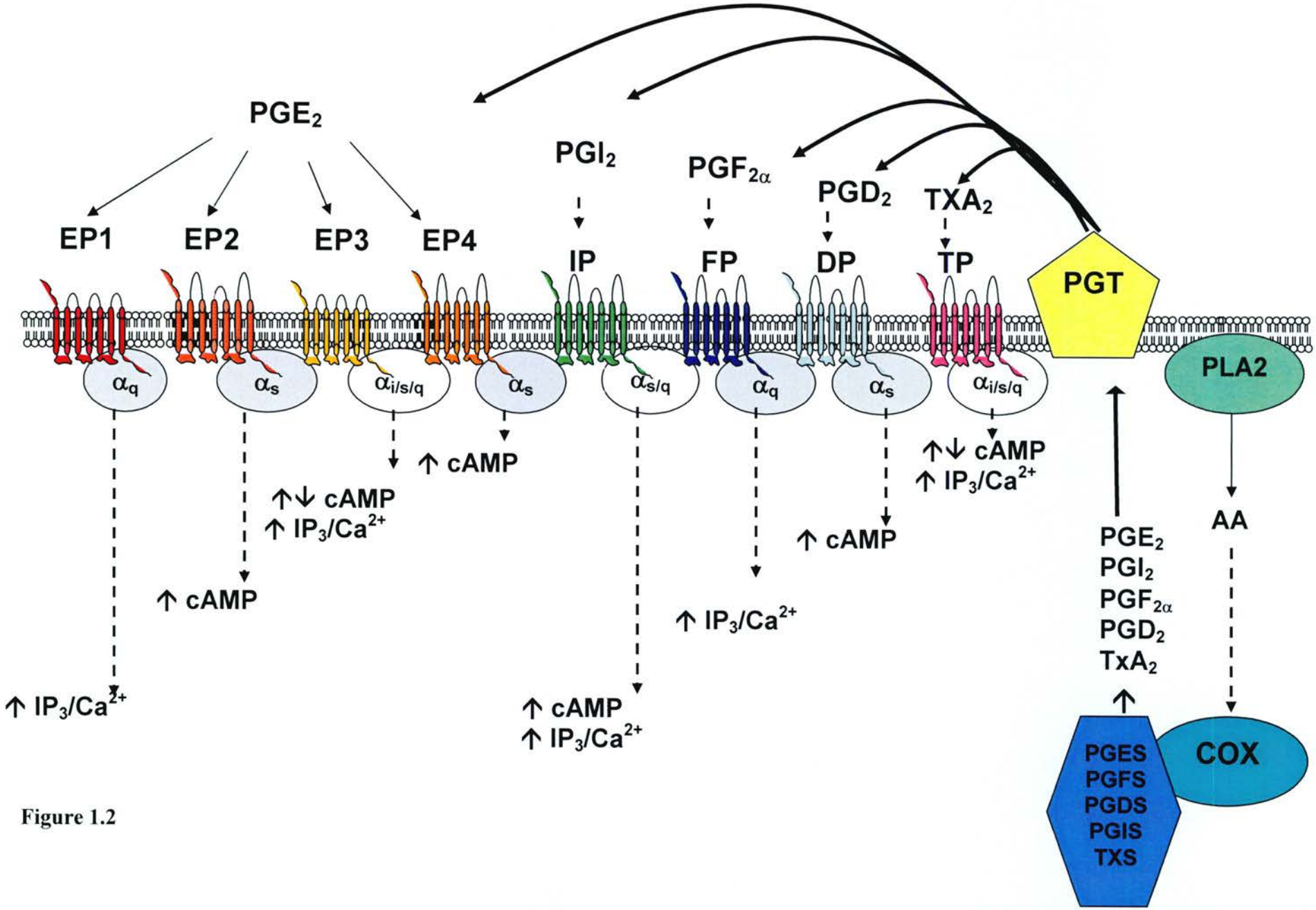
Prostaglandins may be divided into the A, B, C, D, E, F or J series and they are named according to the substituents in the cyclopentane ring. There is no significance to the letters. Prostacyclin (PGI_2) has an unusual ring structure that differs from this simplified biochemical description. The subscripted number refers to the number of double bonds in the prostaglandin. Thus PGE_2 has 2 double bonds, one originating from the 5-position and the second from the 13-position, whereas PGE_1 has only one double bond (originating from the 13-position) and PGE_3 has 3 double bonds (originating at the 5,13, and 17-positions).

Prostaglandin Biosynthesis

The kinetics of the prostaglandin biosynthetic pathway is driven by the availability of free arachidonic acid (AA). Following activation of phospholipase A2 (PLA2), AA is released from plasma membrane phospholipids or dietary fats and is cyclized and oxygenated by COX via the addition of the 15-hydro-peroxy group to form prostaglandin G_2 (PGG_2). A reduction of the hydro-peroxy group to a hydroxy group forms the intermediary prostaglandin H_2 (PGH_2). This intermediate serves as the substrate for terminal prostanoid synthase enzymes. These are named according to the prostaglandin they produce such that prostaglandin D_2 is synthesised by prostaglandin-D-synthase (PGDS), prostaglandin E_2 (PGE_2) by prostaglandin-E-synthase (PGES), prostaglandin $F_{2\alpha}$ by prostaglandin-F-synthase (PGFS), prostacyclin (PGI_2) by prostaglandin-I-synthase (PGIS) and thromboxane (TXA_2) by thromboxane synthase (TXS) (Narumiya *et al.*, 1999); figure 1.2). After biosynthesis, prostaglandins are transported out of the cell by a prostaglandin transporter (PGT) (Chan *et al.*, 1998). The expression of PGT has been shown in both bovine models (Banu *et al.*, 2003) and human endometrium (Kang *et al.*, 2005). Once released outside the cell, the prostaglandin can then act in a paracrine or autocrine manner on their cognate heptahelical transmembrane G-protein coupled receptor (GPCR). The physiological endpoint to the prostaglandin signalling pathway within specific cells and tissues, is in part dependent upon the expression of terminal synthase enzymes and respective receptors for the specific prostanoids produced.

Figure 1.2 The cyclooxygenase and prostanoid biosynthetic and signalling pathways

Arachidonic acid (AA) is released from plasma membrane phospholipids by phospholipase A2 (PLA2) and acted upon by COX enzymes and specific terminal prostanoid synthase enzymes to form prostaglandin D_2 (PGD_2), PGE_2, $PGF_{2\alpha}$, PGI_2 and thromboxane (TXA_2). These mediators are actively transported out of the cell by a prostanoid transporter (PGT). They are then able to exert an autocrine or paracrine effect by coupling to their specific heptahelical transmembrane receptors to activate intracellular signalling. (cAMP: cyclic AMP, IP_3: inositol trisphosphate. Adapted from (Jabbour et al., 2004)).


PGE2
PGI2
PGF2α
PGD2
TXA2
EP1
EP2
EP3
EP4
IP
FP
DP
TP
PGT
PLA2
αq
αs
αi/s/q
αs
αs/q
αq
αs
αi/s/q
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↑ IP3/Ca2+
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↑ IP3/Ca2+
↑ cAMP
↑↓ cAMP
↑ IP3/Ca2+
↑ IP3/Ca2+
PGE2
PGI2
PGF2α
PGD2
TxA2
AA
PGES
PGFS
PGDS
PGIS
TXS
COX

Figure 1.2

Prostanoid Receptors

PGD_2, PGE_2, $PGF_2\alpha$, PGI_2 and TXA_2 exert their biological effect through the DP, EP, FP, IP and TP prostanoid receptors respectively (Narumiya *et al.*, 1999). PGE_2 interacts with four subtypes of receptor, which have been pharmacologically divided into EP1, EP2, EP3 and EP4. These receptors are coupled to their own second messenger systems using alternate and in some cases opposing intracellular pathways (Ashby, 1998); figure 1.2). Separate genes encode for each receptor and in addition there are several splice variants for EP3, TP and FP receptors, which differ only in their C-terminal tails (Narumiya *et al.*, 1999).

In general, prostanoid receptor isoforms exhibit similar ligand binding but differ in their signalling pathways, their sensitivity to agonist-induced desensitisation and their tendency toward constitutive activity. Phylogenetic analyses indicate that receptors sharing a common signal pathway have higher sequence homology than receptors sharing a common prostanoid as their preferential ligand (Jabbour *et al.*, 2006). Among the different receptors, the IP, DP, EP2 and EP4 receptors elevate intracellular cAMP accumulation via $G_{\alpha s}$ and have been termed "relaxant" receptors since they induce smooth muscle relaxation. TP, FP and EP1 receptors induce calcium mobilisation via $G_{\alpha q}$ and constitute a "contractile" receptor group since they cause smooth muscle contractions. The remaining receptor, EP3, is generally associated with a decline in cAMP levels and has been termed the "inhibitory" receptor and usually stimulates smooth muscle contraction, however depending on the splice variant and cell type, the EP3 receptor can also elevate intracellular cAMP and mobilise calcium (Narumiya *et al.*, 1999).

Plasma membrane ligand-receptor interaction remains the principle mechanism whereby prostanoids can exert their effects. However, the picture is complicated yet further with the discovery of alternate nuclear locations for certain prostanoid receptors (Bhattacharya *et al.*, 1999; Wise, 2003). This indicates that certain prostanoids may be able to directly regulate the transcription of target genes, forgoing the requirement of intracellular signalling pathways.

In summary, prostanoids are released in high local concentrations, exerting an effect both upon the producing cell (autocrine) and local tissues (paracrine; figure 1.3).

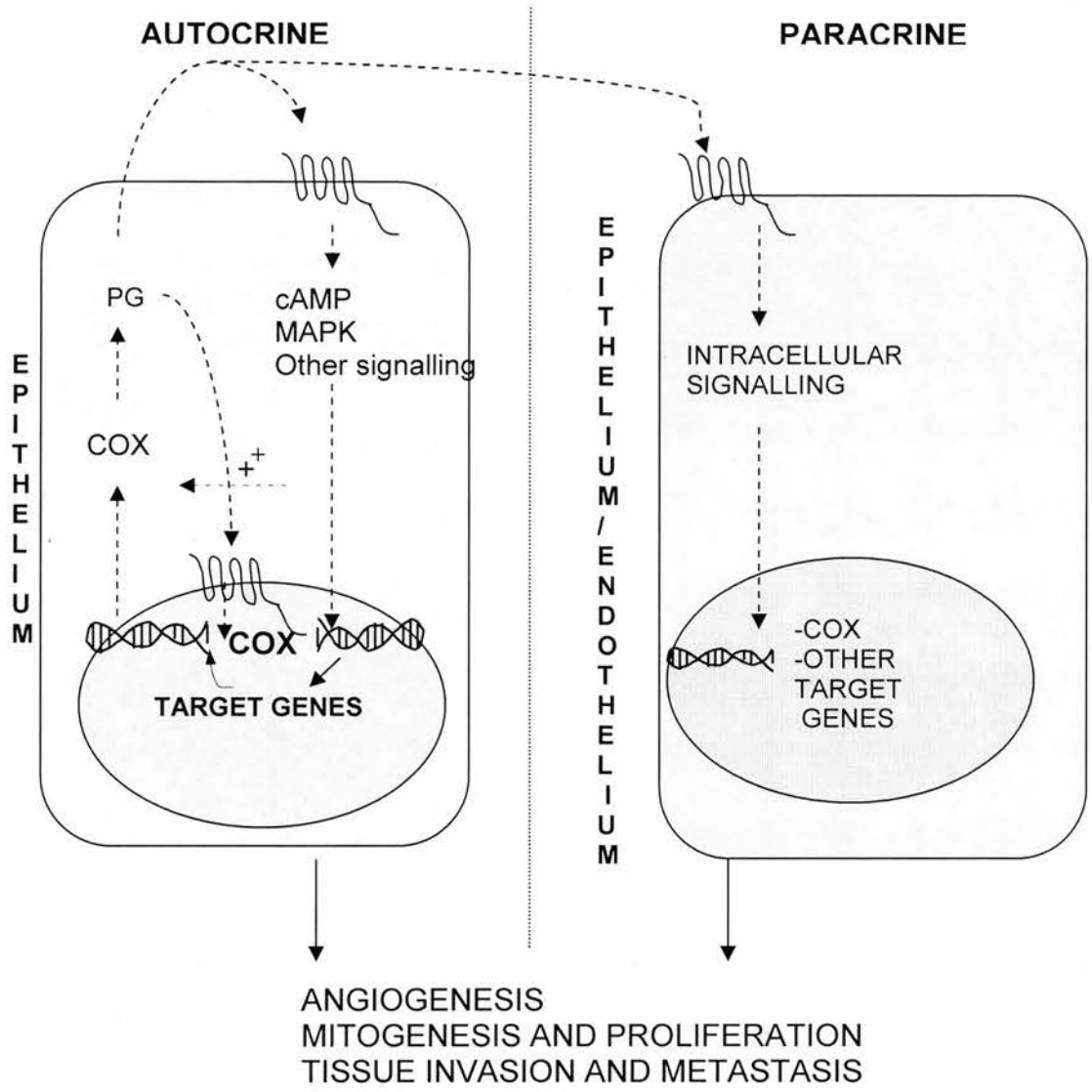


Figure 1.3 Autocrine / paracrine prostaglandin signalling mechanisms

Prostaglandin (PG) produced by the cyclooxygenase (COX) enzyme is transported out of the cell and is able to act upon locally situated heptahelical transmembrane G-protein coupled receptors (GPCRs). This initiates second messengers such as cyclic AMP (cAMP) and mitogen activated protein kinases (MAPK). Nuclear receptors for prostanoids have also been described. This diagram demonstrates a mechanism for positive feedback by prostaglandins upon COX production. (Adapted from (Sales et. al., 2003))

Receptor cross-communication

Cross-communication with the epidermal growth factor receptor (EGFR) has been demonstrated for EP2 and FP receptors (Sales *et al.*, 2004a; Sales *et al.*, 2004b). Support for these findings is provided by evidence demonstrating that prostanoid GPCRs activate receptor tyrosine kinases (RTKs) (Buchanan *et al.*, 2003; Pai *et al.*, 2002). These cross-communications result in an increased auto-phosphorylation and dimerisation of RTKs, such as the EGFR, culminating in the activation of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K), amongst other signalling mechanisms (Buchanan *et al.*, 2003; Sales *et al.*, 2004a; Sales *et al.*, 2004b). Details of the diversity in mechanisms of RTK activation, the exact intracellular mechanisms of cross talk and the significance of the functional endpoint of these cross communications have yet to be fully elucidated. Several mechanisms have been proposed which include extracellular and intracellular mechanisms (Daub *et al.*, 1996; Pierce *et al.*, 2001); figure 1.4). One of these mechanisms involves activation of transmembrane matrix metalloproteinases and extracellular release of heparin-binding EGF (HB–EGF) from its latent membrane-spanning precursor in the plasma membrane. Once cleaved, the HB–EGF ligand can associate with and activate the EGFR, and thereby induce downstream signalling events such as phosphorylation of the MAPK extracellular-signal-regulated kinases 1 and 2 (ERK1/2). In addition, several studies have shown that activation of the c-Src family of non-receptor tyrosine kinases is involved in GPCR-mediated transactivation of the EGFR (Kraus *et al.*, 2003; Pierce *et al.*, 2001).

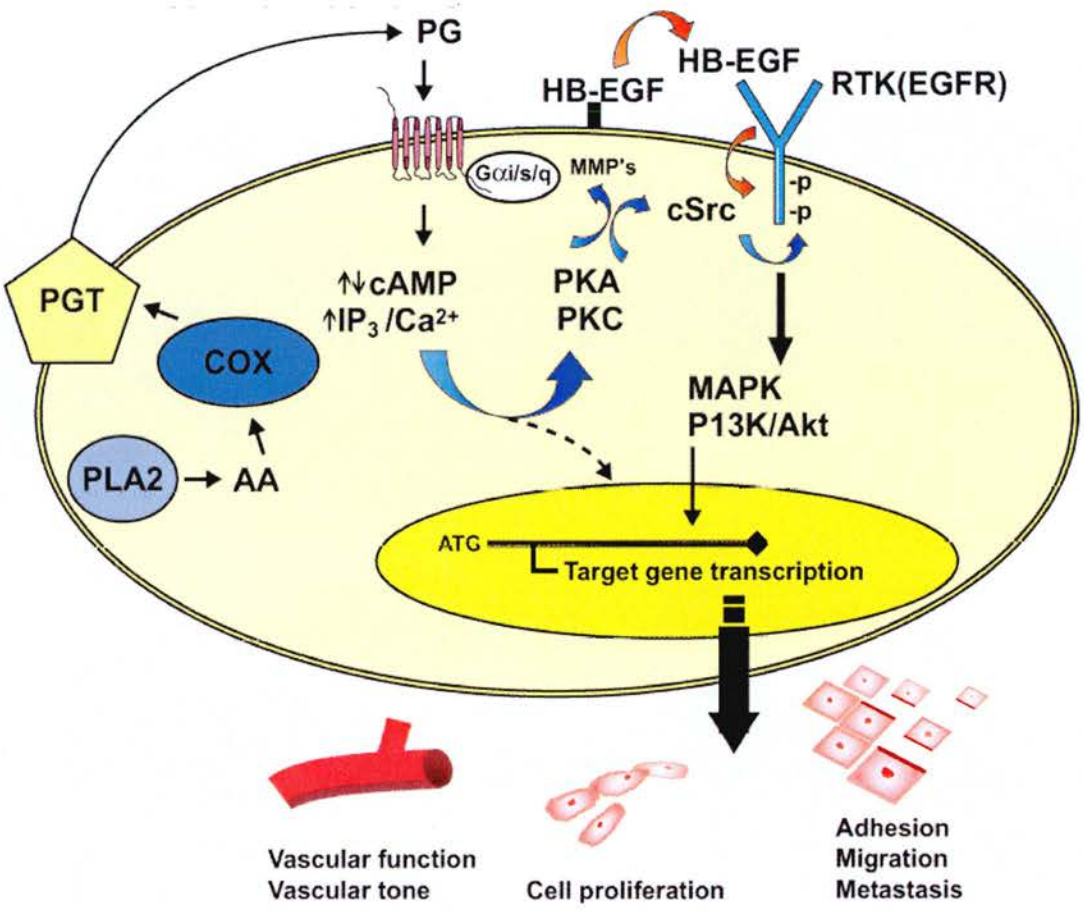


Figure 1.4 Prostanoid receptor signalling with epidermal growth factor receptor (EGFR) cross talk and the downstream effects on biological function.

Prostaglandins (PG) activate specific prostanoid receptors coupled to G proteins leading to production of the second messenger cyclic AMP (cAMP) or inositol trisphosphate (IP_3). These second messengers initiate kinase signalling (protein kinase A (PKA) and protein kinase C (PKC)) by the mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways and target gene transcription. Postulated mechanisms for prostanoid-mediated transactivation of EGFR include the release of heparin-bound EGF-like molecule (HB-EGF) to activate the receptor tyrosine kinase (RTK) directly or by intracellular mechanisms involving non-receptor tyrosine kinases such as cSrc. (AA, arachidonic acid; MMPs, matrix metalloproteinases; PLA2, phospholipase A2; PGT, prostaglandin transporter. Adapted from (Jabbour and Sales, 2004)).

1.4 Menstruation

The menstrual cycle is clinically described according to its regularity and duration of bleeding (Chiazze *et al.*, 1968; Treloar *et al.*, 1967). The average length between menses is between 28 and 32 days with the duration of bleeding lasting 4-5 days.

Menstrual problems account for much of the morbidity that occurs in women of reproductive age. Menstrual problems are a leading indication for women to undergo the major surgery of hysterectomy for which there is a lifetime risk of around 20% (Vessey *et al.*, 1992). It is this health burden, which drives intense research into the exact mechanisms of the menstrual process despite the gross physiological aspects being well established.

1.4.1 Mechanism of menstruation

Menstruation is an active process whereby the upper two thirds of the endometrium, the functional layer, is shed and regenerated on a cyclical basis. During the luteal (secretory) stage of the menstrual cycle, in the absence of fertilisation, there is regression of the corpus luteum leading to a decline of the steroid hormone, progesterone. As described in section 1.2.5, during the secretory phase, prior to the decline in circulating hormone levels, there is a decline of sex steroid receptor expression in the superficial layer of the endometrium. The epithelial glands within the superficial layer of the endometrium are essentially negative in their immunostaining for progesterone and oestrogen receptors (PR and ER). It is therefore hypothesised that the declining levels of steroids can only be directly detected in the stromal cells of the superficial layer, which persistently stain for PR (Critchley *et al.*, 2001a). Coupled with evidence demonstrating that menstruation can be blocked by progesterone add-back up to 36 hours after steroid decline (Brenner *et al.*, 2002), suggests that menstruation specifically occurs in response to the decline of progesterone levels.

Markee and colleagues were able to perform classical studies (Markee, 1940) into the mechanism of menstruation. By transplanting explants of human endometrial tissue into the anterior chamber of Rhesus monkey's eye, they were able to visualise direct events that occurred in response to progesterone withdrawal. In response to

steroid decline, they observed stromal shrinkage, increased coiling of spiral arterioles and vascular stasis. These changes were followed by a period of vasodilation and perivascular bleeding and 24 hours later, a subsequent intense vasoconstriction and tissue necrosis prior to menstruation itself.

The molecular mechanism of menstruation in response to progesterone withdrawal is a complex cascade of events that have yet to be fully elucidated. It in part involves the production of prostaglandins that are able to induce vasoconstriction leading to a reduced blood flow to the endometrium. Subsequently there is increased expression of a range of locally acting mediators including cytokines, angiogenic factors, protease enzymes and further prostaglandins. The cumulative endpoint of these changes in local mediators, together with an influx of migratory leukocytes is the process of menstruation (Brenner *et al.*, 2002; Critchley, H. O. *et al.*, 2001b; Jabbour *et al.*, 2005); figure 1.5). Interestingly, in the PRA + PRB knockout mouse, there is a dramatic influx of inflammatory leucocytes, which can not be prevented with exogenous progesterone (Lydon *et al.*, 1995). This suggests that progesterone somehow suppresses the inflammatory process within the endometrium of the wild type animal.

Figure 1.5 Hypothetical mechanism of menstruation

Coincident events of progesterone withdrawal and hypoxia. Progesterone withdrawal results in an up-regulation of inflammatory mediators, production of matrix metalloproteinases (MMPs), a leukocyte influx and expression of stromal KDR (type 2 receptor for vascular endothelial growth factor (VEGF)) in the upper endometrial zones. There is coincident hypoxia and an up-regulation of VEGF. VEGF binds to KDR and there is a paracrine/autocrine action on the up-regulation of MMP production in the same endometrial upper zone stromal cells. Menstrual sloughing takes place from the superficial regions of the endometrium. Adapted from (Critchley et al., 2001b).

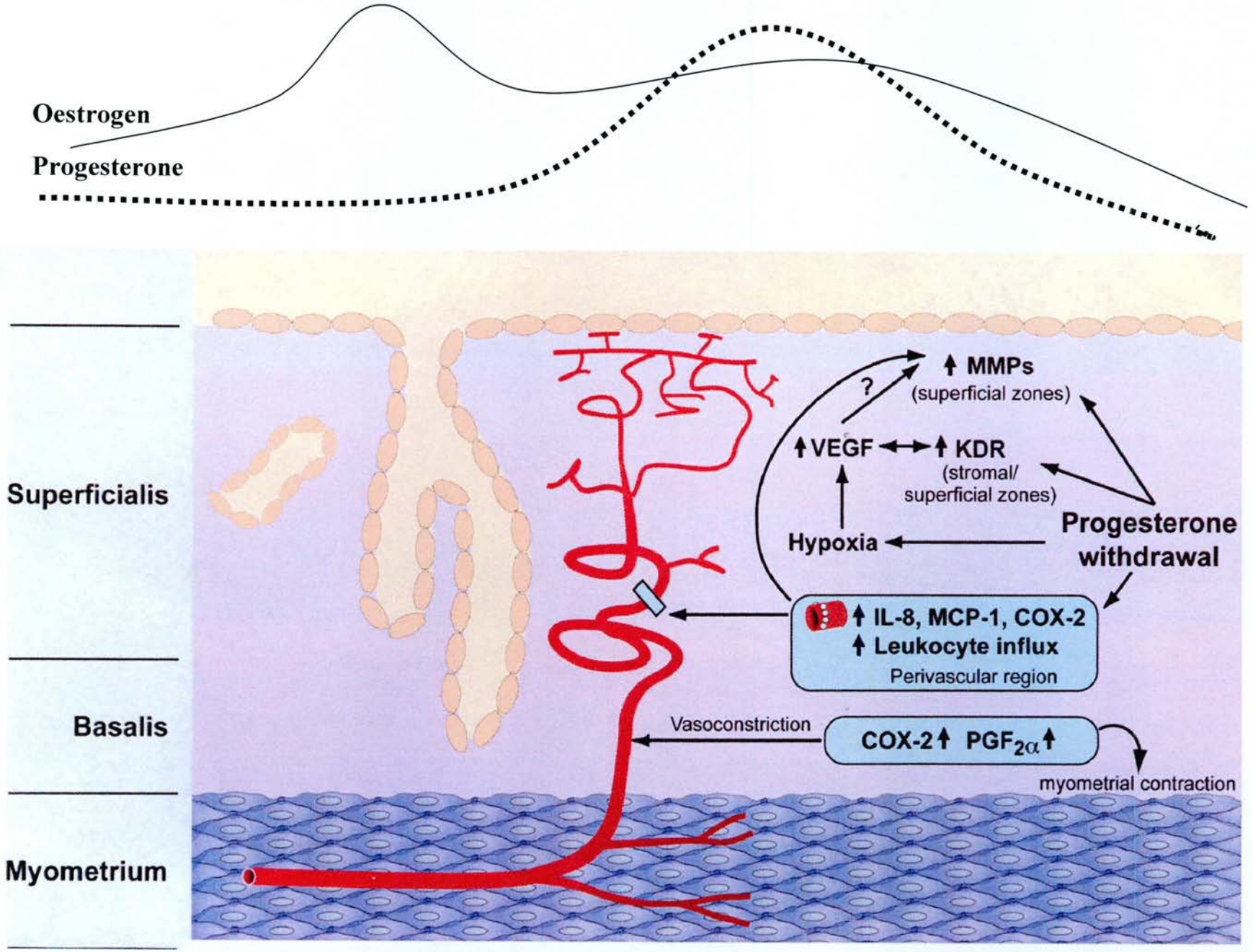

Oestrogen
Progesterone
Superficialis
Basalis
Myometrium
↑ MMPs
(superficial zones)
?
↑ VEGF ↔ ↑ KDR
(stromal/
superficial zones)
Hypoxia
Progesterone
withdrawal
↑ IL-8, MCP-1, COX-2
↑ Leukocyte influx
Perivascular region
Vasoconstriction
COX-2↑ PGF2α↑
myometrial contraction

Figure 1.5

Prostaglandins and local mediators in menstruation

Because of the leukocyte invasion and subsequent production of inflammatory mediators, menstruation has been described as an inflammatory event. Prostaglandins (see section 1.3) have been shown to play a role in menstruation (Baird *et al.*, 1996) and have been found in high concentrations within menstrual fluid (Pickles *et al.*, 1965). Regulation of prostaglandin levels by circulating steroid hormones has been demonstrated (Abel *et al.*, 1983). More recent evidence has demonstrated an increase in COX-2 expression in response to a decline in progesterone levels (Hapangama *et al.*, 2002). This work also demonstrated a decline in prostaglandin dehydrogenase (the enzyme responsible for breakdown of prostaglandins) as progesterone levels fell. COX-2, together with other inflammatory mediators such as Interleukin-8 and Monocyte Chemotactic Peptide-1 have all been localised to the perivascular region with significant upregulation in the late secretory phase (Jones *et al.*, 1997). Additionally, PGE_2 expression has been demonstrated within this perivascular area (Cheng *et al.*, 1993).

The rise of inflammatory mediators, including prostaglandins, therefore occurs subsequent to a declining progesterone level. The predominance in expression of inflammatory mediators in the perivascular areas may allow for local recruitment of leucocytes and also provides a possible mechanism for vasoconstriction of the spiral arterioles, demonstrated prior to the onset of menses. Hence, following progesterone withdrawal, there is an increase in the stromal density of a unique population of leukocytes, named NK cells (CD56 Bright CD 16-) associated with an increase in local cytokine production and matrix metalloproteinase (MMP) expression (Critchley *et al.*, 2001b; Kelly *et al.*, 2001). The increase and activation of matrix metalloproteinases (MMPs) are considered key elements of the menstrual process (Lockwood *et al.*, 1998; Salamonsen *et al.*, 1996). These enzymes are principally responsible for tissue breakdown. During the luteal phase, the circulating levels of progesterone inhibit expression and activity of MMPs in endometrial stromal cells. Sex steroid withdrawal at the end of the cycle reverses the inhibition of many MMPs, including MMP-1, which can degrade the interstitial collagens of the extracellular matrix (ECM) surrounding pre-decidual cells, especially in the superficial endometrium. There is also evidence that the focal expression of MMPs within peri-menstrual and menstrual endometrium involves local regulatory factors.

Leukocytes can directly release MMPs and there are important interactions between leukocytes, stromal and epithelial cells which result in induction and activation of MMPs (Salamonsen *et al.*, 2000).

The vascular endothelial growth factor (VEGF) receptor KDR is almost exclusively expressed by vascular endothelium. However, following progesterone withdrawal, during the premenstrual phase there is a dramatic up-regulation in expression of KDR within the stromal cells of the superficial endometrium (Nayak *et al.*, 2000). Adding back progesterone upto to 24 hours after progesterone withdrawal can reverse this stromal expression of KDR (Critchley *et al.*, 2001b; Nayak *et al.*, 2000). Progesterone withdrawal therefore leads to an up-regulation of inflammatory mediators in addition to causing a degree of tissue hypoxia as a result of the noted vasoconstriction. The hypoxic conditions have been shown to lead to an increase in stromal VEGF production (Sharkey *et al.*, 2000), which has an additional positive feedback on the expression of KDR. VEGF increase is therefore concurrent with the up-regulation of its receptor, KDR, and indeed the expression of MMPs. All these changes occur within the stromal compartment of the superficial layer of the endometrium and provide a possible mechanism whereby the menstrual cascade can be initiated (figure 1.5).

Angiogenic changes of menstruation

In addition to replenishment of the superficial epithelium, the vascular compartment of the endometrium comprising the spiral arteries and arterioles also needs to be reconstructed following menstruation. Hence, new endothelial cells are encouraged to sprout and recruit pericytes to form capillaries and smooth muscle cells to form larger vessels (Gargett *et al.*, 2001; Smith, S. K., 2001). This process involves degradation of the extracellular matrix, endothelial cell proliferation and migration and organisation of the endothelial cells into capillary networks. Several angiogenic factors have been identified to-date, which are believed to be involved in angiogenesis of the human endometrium (Gargett and Rogers, 2001). Of the known angiogenic factors, VEGF, also known as vascular permeability factor, is a major specific stimulator of endothelial cell proliferation and vascular permeability in the endometrium. VEGF acts through two tyrosine kinase receptors, VEGFR-1 and

VEGFR-2 (KDR), the latter being considered the most important in regulation of angiogenesis.

Organisation of blood vessel formation, stabilisation, maintenance and regression also involves other factors. These include the angiopoietins, Ang-1 and Ang-2, which stabilise and destabilise blood vessels respectively to facilitate angiogenesis (Maisonpierre *et al.*, 1997). Although the precise mechanism of action of the angiopoietins remains to be clarified, it is proposed that Ang-2, acting through its tyrosine kinase receptor, Tie-2, enhances the action of VEGF by reducing endothelial contact with the extracellular matrix, and hence with adjacent endothelial cell interactions. In contrast, Ang-1 acting as a competitive antagonist via Tie-2 enhances the stability of the newly formed blood vessels by recruiting pericytes (Maisonpierre *et al.*, 1997). Several other factors including basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF), endocrine gland VEGF (EG-VEGF) and their respective receptors (Battersby *et al.*, 2004b; Weston *et al.*, 2000) are also known to stimulate angiogenesis. Thus, the remodelling of the endometrial vasculature is a complex and multifactorial process, under the influence of multiple effector molecules.

As discussed, in the premenstrual phase, concomitant with the withdrawal of progesterone, COX-2 expression increases in the endometrium and lasts through the proliferative phase of the cycle (Hapangama *et al.*, 2002; Jones *et al.*, 1997). Once COX-2 expression and prostanoid biosynthesis is induced, a positive feedback system is set in motion to regulate the COX-PG biosynthetic signalling pathway and target genes involved in vascular function (figure 1.3). Auto-regulation of COX-2 expression has been reported in several *in vitro* studies (Fujino *et al.*, 2003; Maldve *et al.*, 2000; Tjandrawinata *et al.*, 1997; Tjandrawinata *et al.*, 1997). Following up-regulation of COX-2 expression, the subsequent elevated prostanoid biosynthesis and signalling can promote the expression of pro-angiogenic factors, such as VEGF, bFGF, PDGF, Ang-1 and Ang-2 (figure 1.4) (Sales *et al.*, 2002; Tsujii *et al.*, 1998) or down-regulate the expression of anti-angiogenic genes such as cathepsin-D (Perchick *et al.*, 2003).

These data suggest that endometrial angiogenesis may be under direct control of prostaglandins, produced by COX enzymes in the endometrium. Recently the role of specific prostaglandin receptors in modulating angiogenesis has been described. In

endometrial epithelial cells (Sales *et al.*, 2004a) and murine model systems (Seno *et al.*, 2002; Sonoshita *et al.*, 2001), elevated PGE_2-EP2 receptor interaction promotes the expression of pro-angiogenic genes such as VEGF. Similarly, elevated $PGF_{2\alpha}$-FP receptor interaction in endometrial epithelial cells can also elevate the expression of VEGF in a similar manner to that of PGE_2 via the EP2 receptor. This promotes angiogenesis in the endometrium via the activation of convergent intracellular signalling effector molecules and signal transduction cascades, such as the ERK1/2 cascade (Sales *et al.*, 2005).

1.4.2 Heavy menstrual bleeding (HMB; menorrhagia)

Menorrhagia is the clinical definition describing heavy menstrual blood loss. Heavy menstrual bleeding has been accepted internationally as a more accurate description for this condition and this terminology shall be used in preference to menorrhagia during the rest of this thesis (Fraser *et al.*, 2007). Unacceptable heavy menstrual blood loss affects 10-30% of women of reproductive age and up to 50% of perimenopausal women (Prentice, 1999; Prentice, 2000). In the United Kingdom, 5% of women will seek help for this symptom annually (Vessey *et al.*, 1992) and the risk of hysterectomy performed for menstrual disorders is 20% (Coulter *et al.*, 1988). In 2002-3, over 13000 surgical procedures (hysterectomy and endometrial ablation) were performed in England for complaints of heavy bleeding (Reid PC and Virtanen-Kari, 2005). An estimated 3.5million workdays are lost annually (Weeks *et al.*, 2000).

In research studies, the objective definition of menorrhagia is often based on measurement of menstrual haemoglobin content. Scandinavian studies demonstrated that the mean menstrual blood loss was around 40ml and that regular menstrual blood loss in excess of 63ml was associated with iron deficiency anaemia (Hallberg, L. *et al.*, 1966). The 90th centile for measured blood loss was 80ml and this was taken as the upper limit of normal (Hallberg, L. *et al.*, 1966). However, in the clinical setting only 40 to 60% of women with perceived heavy menstrual blood loss have an objective measurement of greater than 80 ml (Chimbira *et al.*, 1980; Fraser *et al.*, 1981).

Dysfunctional uterine bleeding (DUB) describes heavy menstrual blood loss in the absence of any pathology (Fraser *et al.*, 2007). This condition accounts for the

majority of cases of heavy menstrual bleeding and is ovulatory in 80% of cases (Cameron, 1989; Livingstone *et al.*, 2002). Other causes of heavy menstrual bleeding are pelvic pathologies such as fibroids and systemic disease including blood clotting disorders such as von Willebrand disease (Kouides, 2001; Makarainen *et al.*, 1986). 13% of women with heavy MBL have a previously undiagnosed bleeding disorder of von Willebrand disease (Lukes *et al.*, 2005). Reduced clotting is a known feature of menstruation (Livingstone and Fraser, 2002) however the exact mechanisms that lead to heavy menstrual blood loss for many systemic diseases are poorly understood. Pelvic pathologies such as fibroids are common, affecting between 20 and 25% of women and it is reported that around a third of women with fibroids complain of heavy menstrual blood loss (Buttram *et al.*, 1981).

The role of prostaglandins in the aetiology of heavy menstrual bleeding and other endometrial disorders is described in section 1.4.3.

1.4.3 The role of prostaglandins in benign pathologies of the human endometrium

Prostaglandin signalling in the normal human endometrium

Receptor-ligand interaction initiates second messenger signalling via activation and dissociation of the G-protein complex. Various soluble second messengers (including cAMP, IP3 and Ca^{2+}), whose production is dependent upon the heterotrimeric subtypes (Gαs, Gαi and Gαq; figure 1.2) lead to divergent signalling cascades. The described diversity of ligands, receptors and coupled signalling pathways may confer a delicate homeostatic control that if disrupted, could cause an imbalance of locally mediated signalling.

In the normal human endometrium, it has been demonstrated that PGE_2 activation of the EP receptors (Milne *et al.*, 2001) and PGI_2 activation of the IP receptor (Battersby *et al.*, 2004a) result in an increase in the accumulation of intracellular cAMP, whereas $PGF_{2\alpha}$–FP receptor coupling mobilizes inositol trisphosphates and intracellular Ca^{2+} (Milne *et al.*, 2003). Activation of these second messenger systems is greatest during the middle to late proliferative phase of the menstrual cycle, when levels of the prostanoid receptors are at their highest (Jabbour and Sales, 2004). However, the divergent signalling pathways that are involved in both endometrial function and dysfunction have not yet been fully elucidated.

Initial studies of menstrual fluid identified vasoactive substances with the capabilities of inducing contractions in strips of ileal muscle (Pickles, 1957). These were subsequently identified as the prostaglandins $PGF_{2\alpha}$ and PGE_2, the two most abundant prostaglandins found in human endometrium and menstrual fluid (Lumsden *et al.*, 1983). Evidence has since continued to mount, supporting a role for disturbances of prostaglandin synthesis, secretion and metabolism in several pathologies of the endometrium. Endometrial pathologies in which prostaglandins appear to have an aetiological role, are those in which the clinical symptoms of pain or bleeding disturbances are prominent. The common disorders described here are those of pain associated with menstruation (dysmenorrhoea), the troublesome pathology of endometriosis and heavy menstrual blood loss (menorrhagia).

Dysmenorrhoea

Dysmenorrhoea describes painful periods and is a significant public health burden with up to 50% of women suffering from the condition at some point in their reproductive life time (Brosens JJ, 2000; Dawood, 1985).

Primary dysmenorrhoea is an extremely common condition, typically occurring in adolescence and in the absence of any recognisable pathology. Secondary dysmenorrhoea is recognised as a condition associated with other pelvic pathologies such as endometriosis, pelvic inflammatory disease and uterine fibroids. It is much less common than primary dysmenorrhoea and the role of prostaglandins is less well documented.

Primary dysmenorrhoea is associated with uterine hypercontractility. During contractions, uterine blood flow decreases which leads to relative uterine ischaemia. Good correlation has been found between uterine blood flow and pain (Brosens JJ, 2000; Dawood, 1985; Fraser, 1992). However, pain has been documented in the absence of uterine contractility and it is recognised that prostaglandins have an important alternate role in the aetiology. Prostaglandins have a known role in inflammatory nocosomial pathways and may have a direct effect in stimulating nerve pain pathways (Bley *et al.*, 1998). Excessive synthesis and secretion of prostaglandins by the endometrium have been detected in menstrual fluid taken form women with primary dysmenorrhoea (Lundstrom *et al.*, 1976; Pickles *et al.*, 1965). A clear demonstration of increased PGE_2 and $PGF_{2\alpha}$ levels in menstrual fluid of women with dysmenorrhoea compared to controls shows that this increase was most prominent in the first two days of menstruation and that there is a greater proportional increase in $PGF_{2\alpha}$ levels (Lumsden *et al.*, 1983). Furthermore, infusion of $PGF_{2\alpha}$ into the uterine cavity produces an increase in uterine contractility and dysmenorrhoea like pain (Lundstrom, 1977). The role of prostaglandins in dysmenorrhoea is additionally confirmed by the effective use of COX enzyme inhibitors (Lundstrom, 1978; Rees, 1989) including selective COX-2 enzyme inhibitors (Daniels *et al.*, 2002). In summary, the vasoconstrictive properties of $PGF_{2\alpha}$ together with the ability to cause myometrial contractions could contribute to dysmenorrhoea and in addition the increase availability of PGE_2 could have additional direct pain inducing properties (Bley *et al.*, 1998).

Endometriosis

Endometriosis occurs when tissue histologically similar to the endometrium is found in extrauterine sites with tissue implants most often found over visceral and peritoneal surfaces (Farquhar, 2007). The extent of disease is highly variable and endometriosis can be associated with pelvic pain and infertility. Although not a malignant disorder, endometriotic tissue exhibits all the hallmarks of tumorigenic tissue, namely proliferation, cellular invasion and angiogenesis. According to Sampson's classical theory of retrograde menstruation; peritoneal adhesion of endometrial tissue and outgrowth are essential in the pathogenesis of endometriosis. However, retrograde menstruation is a common occurrence with viable endometrial cells found in 76-90% of women (Bartosik *et al.*, 1986), a much higher incidence than endometriosis (2-3%). Other factors must therefore determine the susceptibility of women to develop endometriosis. This susceptibility is likely to be a result of changes to both the peritoneal environment and to the characteristics of the menstrual tissue.

The aetiology of endometriosis is multifactorial and requires a number of peritoneal substances, in particular angiogenic factors, to facilitate implantation in ectopic sites (Oral *et al.*, 1996). The peritoneal fluid contains a variety of free-floating cells including macrophages, natural killer (NK) cells and lymphocytes. These cells all have immune mediating roles and can promote cellular growth and viability through the secretion of growth factors and cytokines. Macrophages are the most abundant cell type in the peritoneal fluid and have their highest endometrial expression in menstrual tissue. Once activated, they can release products including prostaglandins (Gazvani *et al.*, 2002). It has been demonstrated that peritoneal macrophages from women with endometriosis release more PGE_2 and $PGF_{2\alpha}$ compared to those without endometriosis (Karck *et al.*, 1996). Further evidence implicating prostaglandins in the aetiology of endometriosis arises from histological analysis of endometrial tissue. A higher degree of immunostaining for COX-2, the rate-limiting enzyme in prostaglandin production, is found in endometrial glandular epithelium in women suffering from endometriosis compared to control endometrium (Ota *et al.*, 2001). The increased prostaglandin synthesis and expression (in particular

PGE_2 and $PGF_{2\alpha}$) may play a role in the regulation of cytokines and various growth factors within this disorder.

Although the exact mechanism whereby prostanoids exert their function in endometriosis is unclear, PGE_2 is known to be a potent inducer of aromatase activity in endometrial stromal cells (Noble *et al.*, 1997). Aromatase gives rise to the synthesis of oestrogen, which in turn will feedback positively on further prostaglandin synthesis. Endometriosis is classically described as an oestrogen dependent disease, but the lack of ER expression in pre- menstrual and menstrual tissue (Brenner *et al.*, 2002; Critchley *et al* 2001a) suggests that initiation of the disease is independent of oestrogen. However once implantation has occurred, prostaglandins provide a mechanism for stimulating oestrogen production to sustain the endometriotic lesions.

COX enzymes, PGE_2 and $PGF_{2\alpha}$ and their receptors have been shown to promote the transcription of angiogenic factors such as VEGF and the angiopoietins (Tsujii *et al.*, 1998, Sales *et al.*, 2002) The peritoneal fluid of women with endometriosis displays greater angiogenic activity than women without endometriosis (Gazvani and Templeton, 2002). Angiogenic factors may be important for creating a microvascularised peritoneal environment that would allow for establishment of endometriotic lesions. In this regard, it is interesting that VEGF and its receptor KDR are dramatically and specifically up regulated in the superficial stromal cells of the endometrium in response to the decline of progesterone (Brenner *et al.*, 2002). It is postulated that VEGF has a unique role in stimulating matrix metalloproteinases (MMP) expression within the endometrium. Therefore, the rich content of MMPs, VEGF and inflammatory prostaglandins found simultaneously in menstrual fragments could facilitate attachment and angiogenesis of endometriotic lesions within the pelvis (Brenner *et al.*, 2002).

Heavy Menstrual Bleeding (Menorrhagia)

Treatment of menorrhagia using non steroidal anti-inflammatory drugs (NSAIDs) which are inhibitors of COX enzymes have repeatedly been shown to reduce menstrual blood loss (Anderson *et al.*, 1976; Cameron *et al.*, 1990; Makarainen and Ylikorkala, 1986; Reid *et al.*, 2005), thus implicating disturbances of prostaglandin pathways in the aetiology of heavy menstrual bleeding.

As stated in section 1.4.2, the majority of cases of heavy menstrual bleeding occur in the absence of any pathology, a condition known as dysfunctional uterine bleeding (DUB). There is a large body of evidence linking local mediators, in particular prostaglandins, to ovulatory DUB and this is discussed below. However, the role of prostaglandins in heavy menstrual blood loss secondary to pathologies such as fibroids is unclear. There is evidence that the use of NSAIDs is less effective in treating women with menorrhagia when fibroids exist (Makarainen and Ylikorkala, 1986).

Investigations into the aetiology of ovulatory DUB have not been able to discover any differences in circulating steroid hormone levels in women with heavy menstrual blood loss compared to women with normal menstruation (Eldred *et al.*, 1994). Additionally, there are no specific histological differences within the endometrium of women with heavy menstrual blood loss (Rees *et al.*, 1984). Of note, no differences in expression of oestrogen and progesterone receptors have been discovered (Critchley *et al.*, 1994). However, substantial evidence does exist implicating local mediators, in particular disturbances of arachidonic acid metabolism and disturbances of angiogenic processes. Elevated levels of PGE_2 have been found in endometrium of women complaining of heavy periods (Willman *et al.*, 1976). Further experiments confirmed an elevation of total prostaglandins with an apparent shift in synthesis in favour of PGE_2 over $PGF_2\alpha$ in the endometrium of women with DUB (Smith, S. K. *et al.*, 1981a). Additionally, an increase in PGE_2 binding sites has been reported in uterine tissue associated with menorrhagia (Adelantado *et al.*, 1988), although the specific receptors mediating this pathology remain to be elucidated. Mefanamic acid, a COX inhibitor and member of the Fenmate family of drugs, is routinely used as a first line treatment for menorrhagia. A dual mode of action has been documented for this family of drugs; as well as inhibiting prostaglandin synthesis they also inhibit binding of PGE_2 to its receptor (Rees *et al.*, 1988). PGE_2 has vasodilatory properties and it is interesting to note that prostacyclin and nitric oxide synthesis, vasodilatory substances, are also both elevated in uterine tissue of women with heavy menstrual blood loss (Smith, S. K. *et al.*, 1981b; Zervou *et al.*, 1999). It appears that local mediators with vasodilatory effects on the endometrial vasculature are implicated in menorrhagia.

As mentioned prostaglandin and PGE_2 signalling has been shown to promote angiogenesis via autocrine-paracrine mechanisms. Endothelial cells in the endometrium of women with heavy periods have been shown to proliferate more as compared to controls (Kooy *et al.*, 1996). In addition the spiral arterioles in menorrhagic endometrium have a less established covering of vascular smooth muscle cells (Abberton *et al.*, 1996; Abberton *et al.*, 1999). Lack of cellular support to the spiral arterioles prevents sufficient vasoconstriction of these blood vessels, hence potentially increasing menstrual blood loss. However, hysteroscopic appearance of endometrial surface vessels appears to be very similar between women with DUB compared to normal endometrium (Hickey *et al.*, 2000). The hypothesis that heavy menstrual bleeding arises as a result of poor vascular maturation is supported by the finding of decreased angiopoetin-1 mRNA expression in the endometrium of women with heavy MBL (Hewett *et al.*, 2002). Immunohistochemical studies have additionally demonstrated an increase in expression of Ang-2 and it is receptor Tie-2 in menorrhagic endometrium (Blumenthal *et al.*, 2002). Since there is evidence that Ang-1 and Ang-2 are regulated by PGE_2 (Sales *et al.*, 2002), immature vessels in turn could be more susceptible to the vasodilatory actions of prostaglandins such as PGE_2, leading to heavier menstruation, and this may be enhanced via the autocrine/paracrine actions of PGE_2 via one or multiple EP receptors and their intracellular signalling pathways.

Further investigation into the diversity of signalling events triggered by one or multiple prostanoid receptors is needed to define the roles of prostanoid receptor signalling pathways in reproductive function and pathology.

Selective Progesterone Receptor Modulators (SPRMs)

Early studies into the effects of the selective progesterone receptor modulators (SPRMs; see chapter 6) have provided hope for advancements in the treatments of abnormal menstruation, endometriosis associated pelvic pain and also fibroid disease. Phase II trials have shown that asoprisnil, a SPRM, produces a dose dependent amenorrhoea, reduces endometriosis associated pain and shrinks the volume of fibroid uteri (Chwalisz *et al.*, 2005). The mechanism of action for this family of compounds is complex, exerting a tissue-selective agonist, antagonist or partial agonist/antagonist effect on the progesterone receptor. Interestingly, evidence

demonstrates that asoprisnil causes the formation of unusual, thick-walled arterioles in the endometrium when compared to progesterone agonists or antagonists. Overall, early trials suggest that asoprisnil induces its therapeutic benefits such as suppression of menstruation via an endometrial specific vascular effect. The molecular mechanisms that lead to these vascular changes have yet to be elucidated and may well involve changes in local mediators such as prostaglandins and hence subsequent alterations in angiogenic factors and altered vessel formation. The effects of asoprisnil upon the prostanoid signalling are explored in chapter 6.

Chapter 2

Hypothesis and Aims

Hypothesis

The events of normal menstruation involve complex interactions between the endocrine system, locally produced inflammatory mediators and angiogenic growth factors (Critchley *et al.*, 2001b). The causes of menstrual disturbances and benign pathologies of the endometrium are poorly understood (reviewed in section 1.4.3). It is recognised that they involve disturbances in local cellular and molecular signalling events that subsequently lead to defective vascular function within the endometrium.

This thesis is based on the hypothesis that menstrual dysfunction, including heavy menstrual blood loss is due to (a) up-regulated expression/synthesis of cyclooxygenase enzymes and prostaglandin receptors, and (b) initiation of enhanced intracellular signalling pathways in response to prostaglandins.

Aims

1) The first aim of this thesis is to outline the expression of the cyclooxygenase enzymes in human endometrium and to examine the expression of these enzymes in women with both normal (≤80ml) and heavy (>80ml) menstrual blood loss.

2) The second aim of this thesis is to determine the pattern of expression of various components of the COX-prostaglandin signalling pathways present in the endometrium of women with both normal and heavy menstrual blood loss.

3) The third aim of this thesis is to examine the functional capabilities of the PGE_2 signalling pathways within endometrium taken from women with normal and heavy menstrual blood loss.

The above aims centre on work utilising endometrium collected from women who have consented to both an endometrial biopsy and also measurement of their menstrual blood loss. However, whilst exploring the role of prostaglandins within human endometrium, access to well characterised human tissue from consenting patients is too limited to explore molecular signalling pathways in detail.

4) The fourth aim of this thesis is to therefore explore intracellular signalling pathways using an endometrial epithelial cell line (Ishikawa) and human endometrial tissue. The intracellular signalling transduction pathways activated following PGI_2-IP ligand-receptor interaction have been chosen as the target of this more detailed signalling investigation.

5) The fifth and final aim of this thesis is to explore the effect of the selective progesterone receptor modulator, asoprisnil, upon the endometrial expression of COX enzymes and the various component genes involved in prostanoid signalling.

Asoprisnil is a selective progesterone receptor modulator (SPRM), which has undergone early phase I and II trials, demonstrating favourable safety and tolerability. It induces cessation of menstruation, reduces fibroid volume and reduces non-menstrual pain and dysmenorrhoea in women with endometriosis (Chwalisz *et al.*, 2005). Overall, early results suggest that menstrual suppression by asoprisnil in humans is primarily due to a local endometrial-specific vascular effect. The exact mechanisms of action are however poorly understood.

Chapter 3

General Methods

This chapter provides information for procedures used widely for this thesis. Methodologies more specific to individual chapters are contained therein.

3.1 Human Endometrial Tissue Collection and Characterisation

Human endometrial tissue samples were obtained from patients attending the Royal Infirmary of Edinburgh gynaecology department. Local Lothian Regional Ethical Committee (LREC) approval was obtained and all patients provided written informed consent (contained within Appendices).

3.1.1 Endometrial Specimens

All women participating in the study were aged between 18 to 50 years old and had regular menstrual cycles lasting between 25 and 35 days. Patients had been referred to the outpatient department by their general practitioner with a complaint of heavy menstrual bleeding. A patient history was taken to exclude the use of any hormonal preparations for the proceeding three months and subjects with endometrial pathology or unexplained vaginal bleeding were excluded. Abnormal gynaecological examination at initial presentation led to anatomical survey of the pelvis by ultrasound investigation. Abnormalities such as uterine fibroids ($\geq$ 3cm) excluded use of endometrial tissue from experimentation. Samples were obtained in the out patient setting using a Pipelle endometrial sampler. Endometrial samples were additionally obtained at the time of minor procedures in theatre by curettage of the endometrium. In addition, full thickness endometrial biopsies (figure 3.1) had previously been collected from women undergoing hysterectomy for benign gynaecological indications. These samples therefore include the surface epithelium and endometrial–myometrial junction, thus upper functional and deeper basal zones.

Endometrium was collected from women during all stages of the menstrual cycle. Following collection, the specimens were immediately divided as follows:

1) A proportion of endometrium was fixed in 4% NBF overnight before being embedded in paraffin wax the following day.
2) A proportion was transferred into RNA Later (Ambion Inc, Huntingdon, UK) to prevent RNA degradation before early transfer to storage at –80°C.

3) Finally, if quantities of endometrial tissue allowed, endometrium was incubated overnight in RPMI 1640 culture medium for experimentation the following day.

Histological dating was carried out according to the criteria of Noyes *et. al.* (Noyes, 1950) to confirm the stage of the menstrual cycle. All endometrial samples were consistent for; patient's reported last menstrual period (LMP); histological stage and; circulating oestradiol and progesterone concentrations in venous blood obtained at the time of tissue collection. This is a robust method for characterizing endometrial samples. Detailed gene microarray studies support this method for characterizing endometrial samples with consistency across these three parameters (Critchley, H. O. *et al.*, 2006; Talbi *et al.*, 2006).

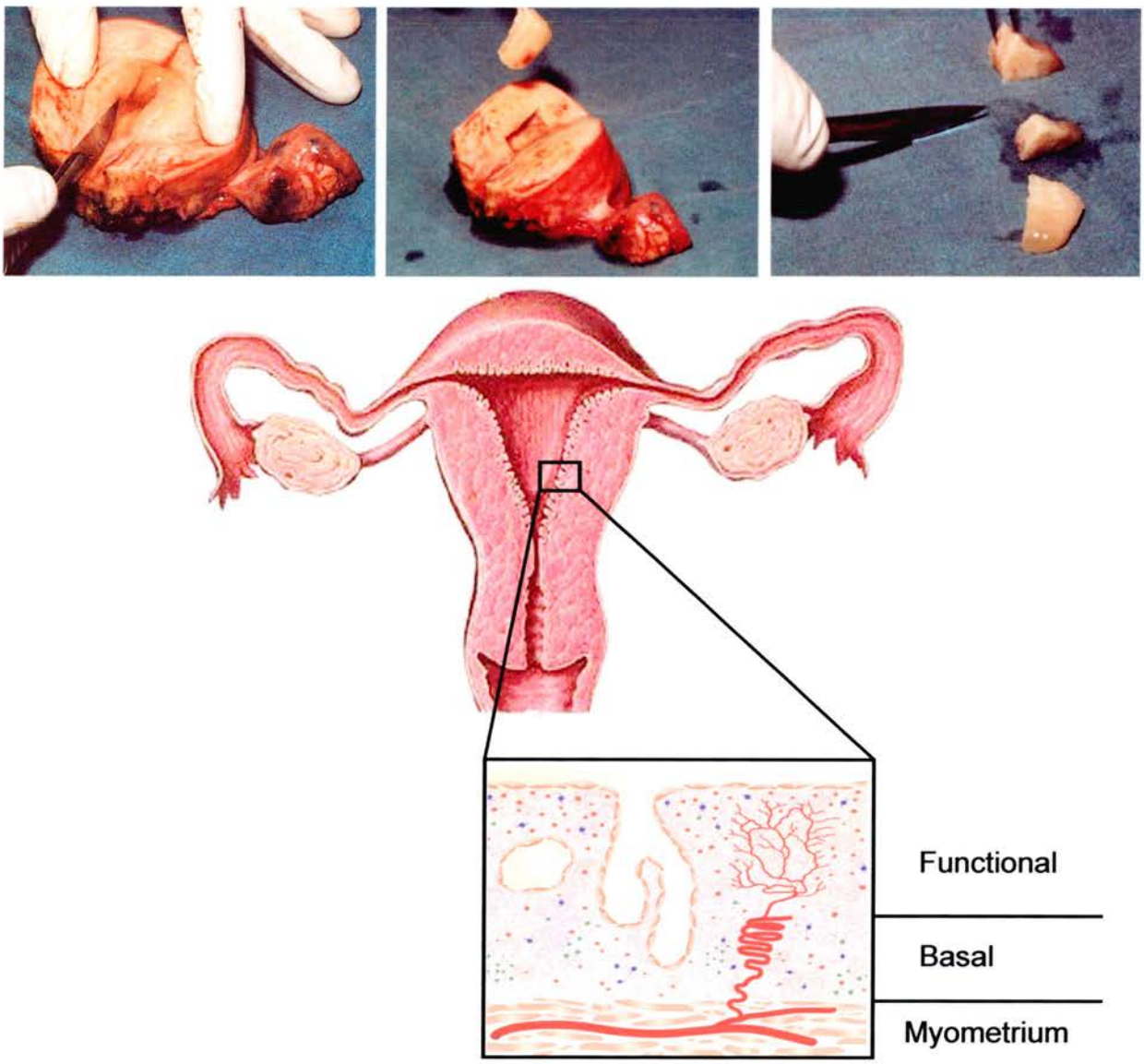
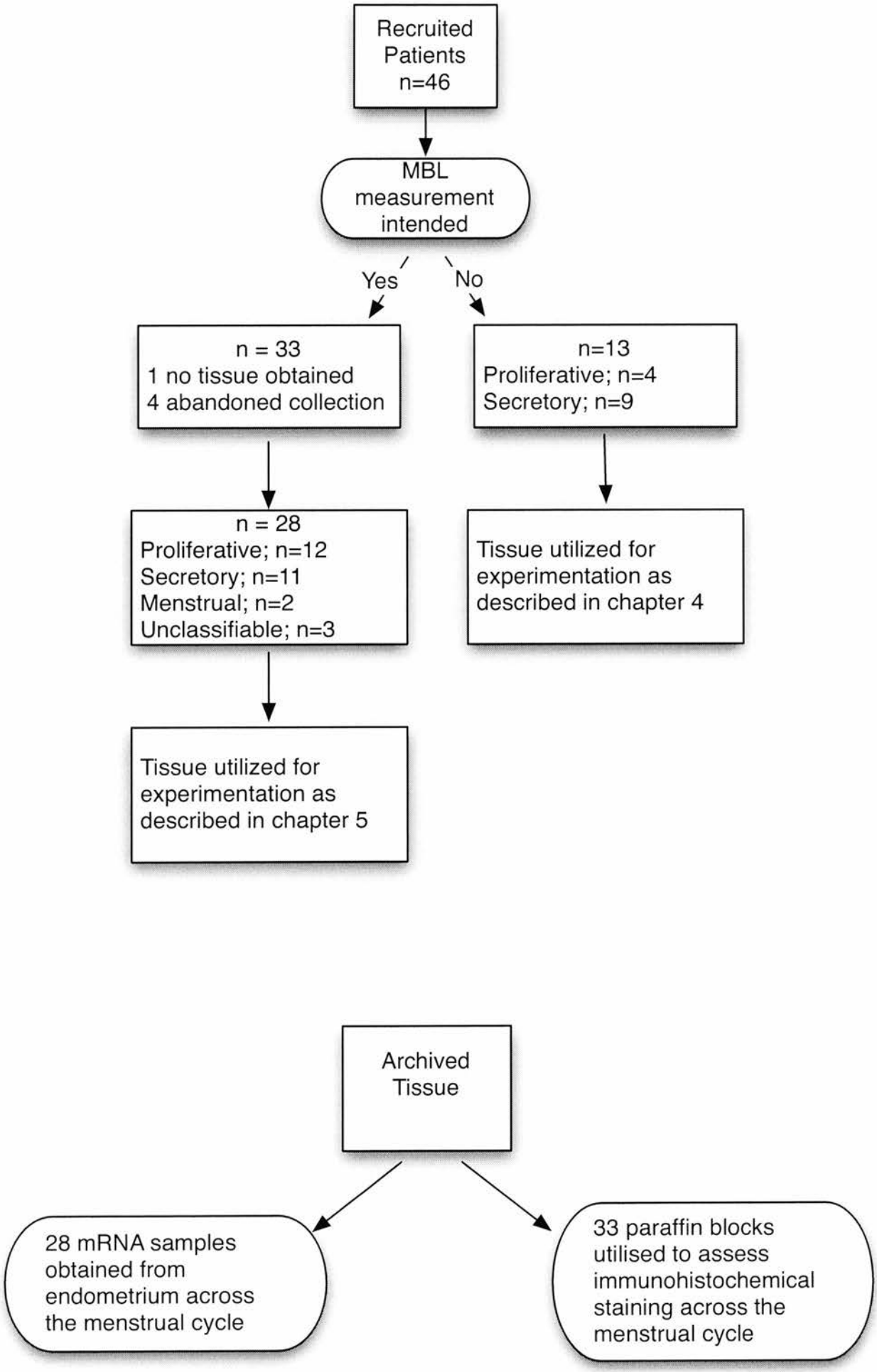


Figure 3.1

Images illustrate the bisection of the uterus enabling identification of the uterine cavity. A biopsy incorporating the endometrium and a portion of the myometrium is removed. The sample is then divided into separate portions for subsequent processing as detailed in 3.1.1. The diagram illustrates the layer architecture for the full thickness endometrial biopsy.


Recruited Patients n=46
MBL measurement intended
Yes
No
n = 33
1 no tissue obtained
4 abandoned collection
n=13
Proliferative; n=4
Secretory; n=9
n = 28
Proliferative; n=12
Secretory; n=11
Menstrual; n=2
Unclassifiable; n=3
Tissue utilized for experimentation as described in chapter 4
Tissue utilized for experimentation as described in chapter 5
Archived Tissue
28 mRNA samples obtained from endometrium across the menstrual cycle
33 paraffin blocks utilised to assess immunohistochemical staining across the menstrual cycle

3.1.2 Measuring Menstrual Blood Loss

Measurement of Menstrual Blood Loss

To further investigate and understand the endometrial signalling mechanisms that may cause aberrant heavy menstrual bleeding, this study set out to objectively measure menstrual blood loss (MBL) in order to allow grouping of associated endometrial samples based on this valuable clinical characteristic. Measurement of menstrual blood loss was based on a modified alkaline-haematin method as described by Hallberg *et al* (Hallberg, Nilsson, 1964). This method involves extraction of the haem from used sanitary towels using 5% sodium hydroxide. Although expensive and labour intensive, it is an objective, reproducible technique for measuring blood loss during menses. For the purposes of research regarding menstrual blood loss, this technique is recognised as the "gold standard" for objective measurement of MBL.

All women were given packages containing a standard choice for sanitary protection (tampons or pads) and detailed oral and written instructions on how to collect all sanitary wear during one period. Care was taken to minimise blood loss that could not be recovered for measurement. After menstruation, packages of sanitary wear were recovered and stored in refrigeration ($4^{o}C$).

For measurement of menstrual blood loss, pads and tampons were added to a known volume of 5% sodium hydroxide (between 2 to 4 litres, approximately 100ml / sanitary product) within a bucket with fitted lid. The contents were then left for 48 hours to allow conversion of haemoglobin to haem.

During the same time period a stored sample of the patient's venous blood was used to create a 1 in 200 dilution of blood with 5% sodium hydroxide. An aliquot was stored alongside the menstrual blood collection.

After 48 hours, tampons and pads were thoroughly mixed within the sodium hydroxide. An aliquot was removed from the bucket and filtered through hardened filter paper (Whartman no. 54, Maidstone, UK). A known volume was then carefully diluted by a measured addition of further sodium hydroxide, to create a close colour match with the control venous blood solution. Theoretical total volume of sodium hydroxide added was therefore possible to calculate by multiplying the added volume by dilution factor. The optical density (OD) of menstrual blood loss solution and venous blood sample were then measured using spectrophotometry at 546nm (A_{546}).

Menstrual blood loss was then calculated as a quantity of patients own venous blood using the following equation (van Eijkeren *et al.*, 1986);

$$MBL = \frac{(OD \text{ of Menstrual Blood Solution} \times \text{Total Volume of added NaOH})}{(OD \text{ of Venous Blood} \times 200)}$$

All patients had provided informed consent for collection of an endometrial suction biopsy during the month proceeding or immediately following collection of menstrual loss.

3.2 Immunohistochemistry (IHC).

Standard immunohistochemical techniques were performed to localise a number of cellular proteins within the human endometrium. All protocols were optimised to determine the best conditions for maximal immunostaining with minimal background staining. The protocols used for individual primary antibodies are summarised in section 3.2.4

3.2.1 Theory of Immunohistochemical Staining Methods

Theory of Antibodies

The antibody is the pivotal reagent to all IHC techniques. Antibodies belong to a group of proteins termed immunoglobulins, which comprise of five major classes; IgA, IgM, IgD, IgE and IgG. All primary antibodies used within this research were of the IgG class and were polyclonal in nature.

The structure of the immunoglobulin molecule is depicted in figure 3.2. The variable domain is specific to the target protein and is responsible for antigen binding. Polyclonal antibodies are produced by a variety of cells within an animal species. As such, they are immunochemically dissimilar and may react against various epitopes on the antigens against which they have been raised (figure 3.3).

Basic Immunohistochemistry

Antibodies vary with regard to their affinity for their target antigen and their non-specific binding. Basic IHC methods are optimised in order to obtain the highest quality, specific immunostaining pattern possible.

In order to visualise the areas of antibody-antigen binding, IHC methods utilise enzyme-substrate reactions to convert colourless chromagens into coloured end products. Horse-radish peroxidase and alkaline phosphatase are two enzymes used in this work, the theory of which is explained in the staining methods section on the following pages.

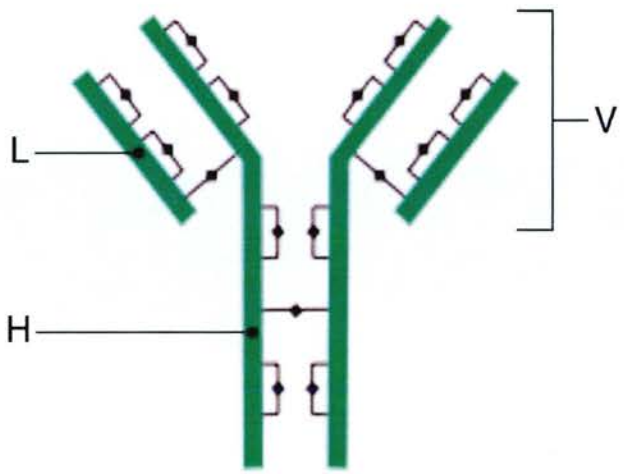


Figure 3.2

Immunoglobulin molecule; comprising two identical heavy (H) chains and two identical light (L) chains. Inter- and intra-chain disulfide bonds contribute to structural stability. Variable domain (V) is responsible for antigen binding.

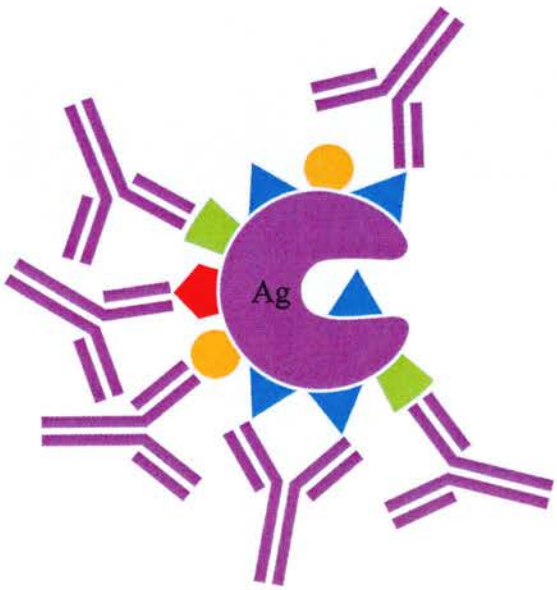


Figure 3.3

Polyclonal antibodies raised against an antigen (Ag) may react against a variety of epitopes upon such antigen.

Fixation

The use of a fixative prevents tissue autolysis by inactivating lysosomal enzymes and inhibiting bacterial growth. Furthermore, stabilisation of the tissue is obtained to reduce degradation during the rigorous process of IHC staining. All endometrial tissue samples in this study were fixed overnight in Neutral Buffered Formalin (NBF) solution, a well-tolerated fixative with good tissue penetration. Some shrinkage and distortion of tissue architecture is recognised but in general good results are obtained. Overnight tissue incubation within NBF was consistent, as varying fixative times can alter antibody penetration of the tissue during IHC staining. Formaldehyde fixes by reacting primarily with basic amino acids to form cross-linking "methylene bridges". This leads to relatively low permeability to macromolecules and a structural preservation of intracytoplasmic protein structures. Once fixed, endometrial tissue blocks were then embedded in paraffin wax for preservation. It is important to beware of variation in IHC staining that can be attributed to differences in tissue fixation e.g. type of tissue, time in fixative and tissue size.

Antigen Retrieval (AR)

Fixation may unfortunately result in the loss of immunoreactivity by many antigens. The use of an antigen retrieval step is designed to "improve" the immunoreactivity of formalin-fixed antigens.

The principal of AR relies on the application of heat to the tissue sections in an aqueous medium. Within this study, all tissue slides were pressure cooked for five minutes in 0.01M sodium citrate buffer which has a pH of 6. Cooling then occurred over twenty minutes before resumption of IHC staining protocols. The exact mechanism of AR is not completely understood. In some way a degree of fixative cross-bridges are reversed therefore allowing antigenicity whilst the remaining cross-links (methylene bridges) are unaltered thereby preventing complete heat denaturation.

Staining Methods

All IHC performed used an indirect staining method, whereby an enzyme labelled secondary antibody directed against the primary antibody (now the antigen)

is applied (figure 3.4). This allows for amplification of the primary antibody-antigen complex thereby enhancing the target signal. To visualize the enzyme labelled secondary antibody, a substrate-chromagen solution is applied to allow ultimate visualization of the target antigen.

Further amplification of the signal can be obtained by use of the high affinity ($k = 10^{-15}M$) that avidin has for biotin. Avidin has four binding sites for biotin, therefore the use of a biotinylated secondary antibody allows for a subsequent incubation with pre-formed avidin-biotin complexes. Because the affinity of avidin for biotin is over one million times higher than that of an antibody for most antigens, the binding can be essentially thought of as irreversible. If these complexes are attached to an enzyme-substrate system, visualization of the now amplified antibody-antigen complex can be obtained. This tertiary signal amplification increases the sensitivity of staining techniques, which helps to reduce unwanted background staining. Visualisation is concluded with the substrate solution.

Tyramide Amplification

An alternate method of signal amplification involves the phenolic substrate, tyramide, which is catalysed by peroxidase to from insoluble biotinylated phenols. Extreme signal amplification can be obtained by using a biotinylated labelled secondary antibody, followed by a streptavidin-biotin-peroxidase complex. The peroxidase enzyme will act upon a tertiary tyramide substrate to cause biotin deposition, which in turn acts with the streptavidin-enzyme to form additional complexes that can be detected.

Controls

Validation of all IHC protocols was carried out by the inclusion of control tissue slides. To assure the primary antibodies were working specific to their target, the inclusions of positive and negative controls were required. For negative controls, the use of a blocking peptide was used where available. Affinity absorbtion of the primary antibody with a blocking peptide was carried out 24 hours prior to commencing the IHC protocol. This preparation renders the primary antibody "pre-absorbed" thereby preventing specific complex formation with the tissue target antigen, leading to reduced staining. When the blocking peptide was not

commercially available, an immunoglobulin fraction from the same species as the primary antibody was used at a matching protein concentration for the negative control. Identical methodology was used on control experimental tissue sections.

For a positive control, a tissues sample, which is known to express the protein of interest, was also included in the experiment. This inclusion is required to ensure that the immunostaining achieved is within the expected cellular compartments.

Background immunostaining

All additional steps within the protocols were included to reduce background staining which can occur via a variety of causes. Specifically, the following steps were carried out:

- Prior to application of the primary antibody, separate incubation of the tissue with a blocking serum was performed in order to prevent non-specific binding of the primary antibody to tissue as a result of hydrophobic interactions.
- Endogenous peroxidase activity was quenched by the incubation of the sections in 3% hydrogen peroxidase (H_2O_2) for ten minutes. This is required in order to prevent non-specific activity of a peroxidase labelled secondary antibody.
- Endogenous (strept) avidin binding activity is a problem for all biotin-based techniques and is primarily due to endogenous biotin. This activity is suppressed by sequential 15-minute incubations of the sections first with an avidin solution followed by a biotin solution, prior to incubation with the primary antibody.

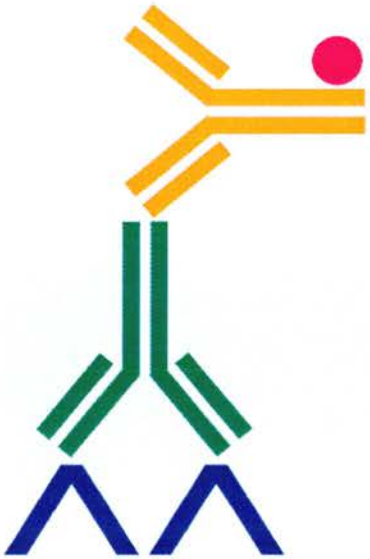


Figure 3.4

Indirect staining method: Enzyme labelled secondary antibody binds with primary antibody bound to tissue target antigen.

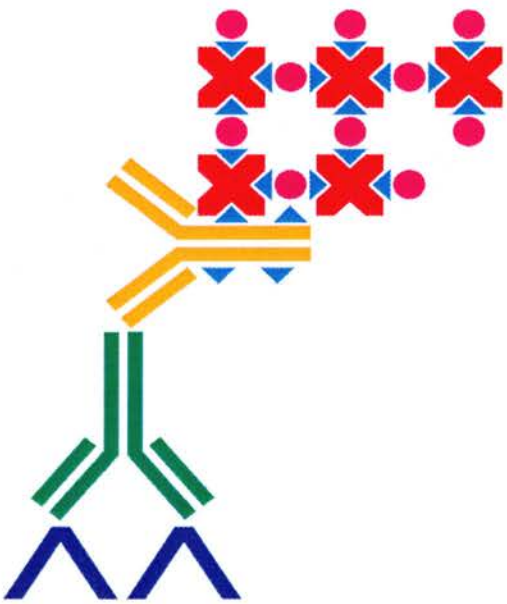


Figure 3.5

Streptavidin-biotin complexes react with biotinylated secondary antibody

3.2.2 General Immunohistochemistry Protocol

Immunohistochemical staining techniques were optimised for each individual protein of interest. Below is a general account of staining techniques; more detailed methods are included in sections 3.2.4 to 3.2.6 and within the specific methods sections of each data chapter.

Tissue sections embedded in paraffin wax were cut to a width of 5 microns and mounted on superfrost glass slides (BDH, Merck House, Poole). Slides were then dewaxed by placement in histoclear for two five-minute episodes. Sections were then rehydrated through graded alcohols, 100% ethanol (2 x 2 min), 95% ethanol (2 min) and 70% ethanol (2 min) before receiving two five-minute washes in distilled water. Thereafter washes were performed in 0.01M phosphate buffered saline (PBS).

After two washes in PBS, antigen retrieval was then carried out to unmask antigenic sites that may be hidden due to tissue fixation (section 3.2.1). Antigen retrieval was performed by pressure-cooking in 0.01M sodium citrate for 5 minutes, followed by a 20 minute cooling period. Sections were then washed twice in PBS for five minutes each time.

To reduce background staining, it was necessary to quench endogenous peroxidase activity by submerging tissue sections in 3% H_2O_2 in distilled water for 10 minutes followed by two further five minute washes in PBS. Next, endogenous avidin / biotin activity was blocked with a 15 minute incubation within an avidin solution (Vector Laboratories, Peterborough, UK) followed by a biotin solution with a two minute PBS wash separating incubations. Finally, non-specific binding was further reduced by 20 min incubation with non-immune serum, of the same species as the secondary antibody, in a humidified chamber at room temperature. Primary antibody incubation overnight then occurred at 4°C. During the optimization experiments for a specific immunohistochemical staining, various concentrations of antibody were utilised. The time and temperature for the incubation period could also be altered in order to achieve optimal immunostaining i.e. one hour incubation at 37°C instead of overnight incubation at 4°C.

All single antigen staining techniques used a standard Avidin-Biotin Peroxidase immunohistochemical protocol, requiring the use of a biotinylated

secondary antibody (an antibody with biotin covalently linked to its structure; figure 3.5).

Excess primary antibody was washed with PBS containing 0.01% Tween 20 (PBST) for two five minute washes. A biotinylated secondary antibody (raised against the specific species of the primary antibody) is then applied, diluted in serum as a concurrent non-immune block for 60 minutes. During this incubation time, a solution of preformed avidin-biotin-peroxidase enzyme complex (ABC Elite, Vectastain, Peterborough, UK) was prepared. The ABC-Elite system can be employed to improve antigen detection. The avidin–biotinylated peroxidase complexes are small and highly active, allowing greater tissue diffusion and binding to the biotinylated secondary antibody. As the most sensitive peroxidase-based detection system, providing five to ten times more sensitivity than regular ABC methods, this was most often used. This greater sensitivity allowed for better detection of biotinylated targets and hence less detection of non-specific background antigens.

After excess secondary antibody was removed with two five minute PBST washes, incubation with the ABC Elite tertiary detection solution was performed for a further 60 minutes. The peroxidase substrate, diaminobenzidine tetra hydrochloride (DAB, DAKO Corp, Ca, USA) was then employed as the chromagen. The peroxidase enzyme leads to oxidation of the DAB substrate, resulting in a stable enzyme-substrate complex and a brown precipitation, allowing visualization of the sites of positive immunoreactivity.

Sections were washed in distilled water before counter staining with Harris' Haematoxylin and then subsequently dehydrated in ascending grades of alcohol. Final incubation in xylene was performed prior to mounting in Pertex.

3.2.3 Scoring and analysis of immunoreactivity

The immunostaining intensity of epitopes in all tissue sections was assessed in a semi-quantitative manner on a 4-point scale: 0 = no immunostaining, 1= mild immunostaining, 2 = moderate immunostaining, 3 = intense immunostaining. All tissue sections were scored blind by two independent observers. This scoring system has been previously validated in a subset of tissue sections in which immunoreactivity was measured with a computerised image analysis system, a strong correlation between quantitative data derived from the image analysis and subjective scores by a trained observer was obtained (regression coefficient of 0.963 (Wang *et al.*, 1998)).

Due to the discontinuous nature of this semi-quantitative data, analysis was performed using a non-parametric method, the Kruskall-Wallis test, followed by Dunn's post-hoc multiple comparison test. Results with a p value <0.05 were considered to be significant.

3.2.4 Table of Immunohistochemical Protocols

Antigen	Primary Antibody			Secondary Antibody			Antigen retrieval	Tertiary amplification	Negative Control
	Species	**Conc.**	**Incubation**	**Species**	**Conc.**	**Incubation**			
COX-1*	Goat	1:500	120 min room temp	Rabbit – biotinylated	1:500	60 min machine temp	0.01 M sodium citrate	ABC HRP	PPA
COX-2*	Goat	1:500	120 min room temp	Rabbit – biotinylated	1:500	60 min machine temp	0.01 M sodium citrate	ABC HRP	PPA
EP2	Rabbit	1:400	4^0C overnight	Goat – biotinylated	1:200	60 min room temp	0.01 M sodium citrate	ABC Elite	Rabbit IgG
EP4	Rabbit	1:400	4^0C overnight	Goat – biotinylated	1:200	60 min room temp	0.01 M sodium citrate	ABC Elite	Rabbit IgG
IP	Goat	1:30	4^0C overnight	Horse - biotinylated	1:200	60 min room temp	0.01 M sodium citrate	ABC Elite	PPA
CD31 (dual staining with IP)	Mouse (monoclonal)	1:20	4^0C overnight	Rabbit – biotinylated	1:500	60 min room temp	0.01 M sodium citrate	ABC Elite	1) Goat Anti IP 2) Mouse anti CD31
EGFR (dual staining with IP)	Mouse	1:25	4^0C overnight	Rabbit – biotinylated	1:500	60 min room temp	0.01 M sodium citrate	ABC Elite	1) Goat Anti IP 2) Mouse anti EGFR

* Use of Bond automated IHC machine for these proteins (see section 3.2.5)

3.2.5 Automated immunohistochemistry for localisation of cyclooxygenases 1 (COX-1) and 2 (COX-2)

The immunostaining protocols for COX-1 and COX-2 followed the general immunohistochemical protocol outlined in section 3.2.2. However the timings and concentrations of reagents used were markedly different. This deviation from previous staining methodology was due to the utilisation of a Bond™ Automated immunohistochemistry system. This system was able to deliver a more efficient staining method with an improved quality and reproducibility of immunostaining.

Slide identification details were pre-programmed into the machine and slide labels applied. Slides were the dewaxed and underwent antigen retrieval as per normal protocol (section 3.2.2) before being mounted on the automated machine. The Bond™ system facilitates tissue staining by applying a series of pre-optimised reagents to tissue sections under tight control of conditions including, accurate timings for each stage of the staining procedure (step times) and slide temperatures. Cross contamination of reagents is carefully avoided.

The machine is fully automated and could offer batch processing if required. Sections were automatically counterstained and washed. Slides were then removed from the system and dehydrated in ethanol and xylene prior to being mounted in Pertex.

3.2.6 Immunofluorescent confocal laser microscopy

Immunohistochemical co-localisation of the site of expression of prostacyclin receptor (IP) with epidermal growth factor receptor (EGFR) or the endothelial cell marker CD31 was performed in human endometrium by dual immunofluorescence immunohistochemistry and confocal laser microscopy (see chapter 4).

Human endometrial sections (5μm) were dewaxed, rehydrated and washed as previously described in section 3.2.2. The immunohistochemical methodology was repeated for antigen retrieval by pressure cooking, quenching of hydrogen peroxidase activity and blocking of endogenous streptavidin activity. Non-specific binding was further reduced by 20 minute incubation with 5% non-immune rabbit serum diluted in PBS before overnight incubation at 4^0C with the polyclonal mouse anti-EGFR primary antibody (NCL-EGFR-384, Nova-Castra, Newcastle-upon-Tyne, UK) at a dilution of 1 in 25. For co-localization of the IP receptor with CD31, a monoclonal mouse anti CD31 antibody (Dako Cytomation, CA, USA) was used at a dilution of 1 in 20. Control sections were incubated with polyclonal goat anti-IP primary antibody at a dilution of 1 in 100 in order to demonstrate specificity of the secondary antibody for the mouse primary antibodies. The following day, sections were washed with PBS tween and incubated with a 1 in 500 dilution of biotinylated rabbit anti-mouse IgG for one hour. A further one hour incubation with the fluorochrome streptavidin 488 Alexifluor (Molecular Probes Inc. Cambridge Bioscience, Cambridge, UK) diluted at 1 in 200 in PBS was performed. Next, sections were incubated for 20 minutes in a PBS solution containing biotin in order to enhance fluorescent signal before re-blocking with 5% non-immune rabbit serum. Incubation with the goat anti-IP antibody (Santa Cruz Inc.) at a 1 in 100 dilution at 4^oC overnight was then performed. A second control slide was incubated with mouse anti-EGFR or mouse anti-CD31 primary antibody. Incubation with a 1 in 200 dilution of rabbit anti-goat peroxidase (Vector Laboratories, Peterborough, UK) secondary antibody was performed for 30 minutes. Tertiary detection was performed with an 8 minute incubation with Tyramide Cy3 solution (PerkinElmer Life Sciences, Boston, USA) at a 1 in 50 dilution as per manufacture's instructions. Slides were counterstained with To Pro (Molecular probes Inc.), at a 1 in 2000 dilution for 2 minutes and then mounted in permafluor.

3.3 Real Time Quantitative Reverse Transcription Polymerase Chain Reaction (Q-RT PCR).

3.3.1 General Theory

Transcription is the process in which the DNA sequence of a gene acts as a template for mRNA synthesis. The enzyme driven reverse-transcription (RT) reaction is used to generate complementary DNA (cDNA) from mRNA. The polymerase chain reaction (PCR) is used to amplify specifically defined regions of a particular gene. Combining RT and PCR (RT-PCR) has revolutionised the study of gene expression providing a highly sensitive and rapid method for mRNA analysis.

Once synthesised cDNA is used as a template for cycles of amplification of specific target sequences. Following denaturing of secondary structures by heating, short oligonucleotides (primers) are added and allowed to anneal to the target cDNA strands by cooling of the mixture. Primers are designed to be complementary to the 5' ends of the sequence chosen for amplification, ideally they will result in a product containing a spliced intron-exon boundary, thereby ruling out any possibility of genomic DNA contamination exerting an effect. A thermostable polymerase enzyme is then employed to extend the sequence complementary to the cDNA. The template and nascent strand are then separated by heating, allowing them to be used as template stands for the next cycle of amplification. Subsequent cycles allow the exponential generation of DNA fragments of a size defined by the added primers.

Real-time quantitative PCR analysis involves the simultaneous amplification and detection of target complimentary DNA (cDNA), such that quantification is performed once per cycle. The system used in this thesis is based on the taqman assay developed by PerkinElmer-Applied Biosystems (Ca, USA). This system relies on the same principles as normal PCR, rounds of annealing, polymerisation and denaturation (figure 3.7). However, this more sensitive technique employs an additional short oligonucleotide designed to anneal to sequences within a region to be amplified. This additional oligonucleotide, known as the hybridisation probe, is chemically altered such that it cannot initiate polymerisation and contains a fluorescent reporter dye at its 5' end and a quencher dye at its 3' end. Due to the proximity of the quencher dye, fluorescence from the reporter dye is undetectable from an intact probe. Detection

only becomes possible due to the 5' nuclease activity of DNA polymerase, which will hydrolyse the hybridisation probe only when it is bound to the target DNA sequence. Because the hybridisation probe anneals within the region bound by the forward and reverse amplification primers it will encounter the DNA polymerase. Once hydrolysed the reporter dye loses its proximity to the quencher and can now be detected. It is therefore possible to follow the accumulation of PCR products by monitoring the increase in fluorescence of the reporter dye (see section 3.3.5).

Figure 3.6. Summary of processes involved in real time RT-PCR.

Practical Aspects of PCR

Theoretically, the PCR reaction should occur at an exponential rate, as it is a cyclical process incorporating the product of one cycle in the pool of templates for the next cycle. However, in practice the exponential increase is not unlimited and occurs only relatively early on because one or more factors become limiting, resulting in a reduced rate that eventually tails off to give the so-called "plateau effect". For example, products may accumulate such that template re-association competes with primer annealing and extension, or the molar ratio between polymerase and template is no longer correct. Small differences in efficiency of the RT and PCR reactions, inevitable in such complex reactions, can result in large apparent differences in expression in similar samples following product amplification. Thus, it is necessary to carefully optimise each PCR reaction, such that the plateau phase is not reached and incorporated into quantification data.

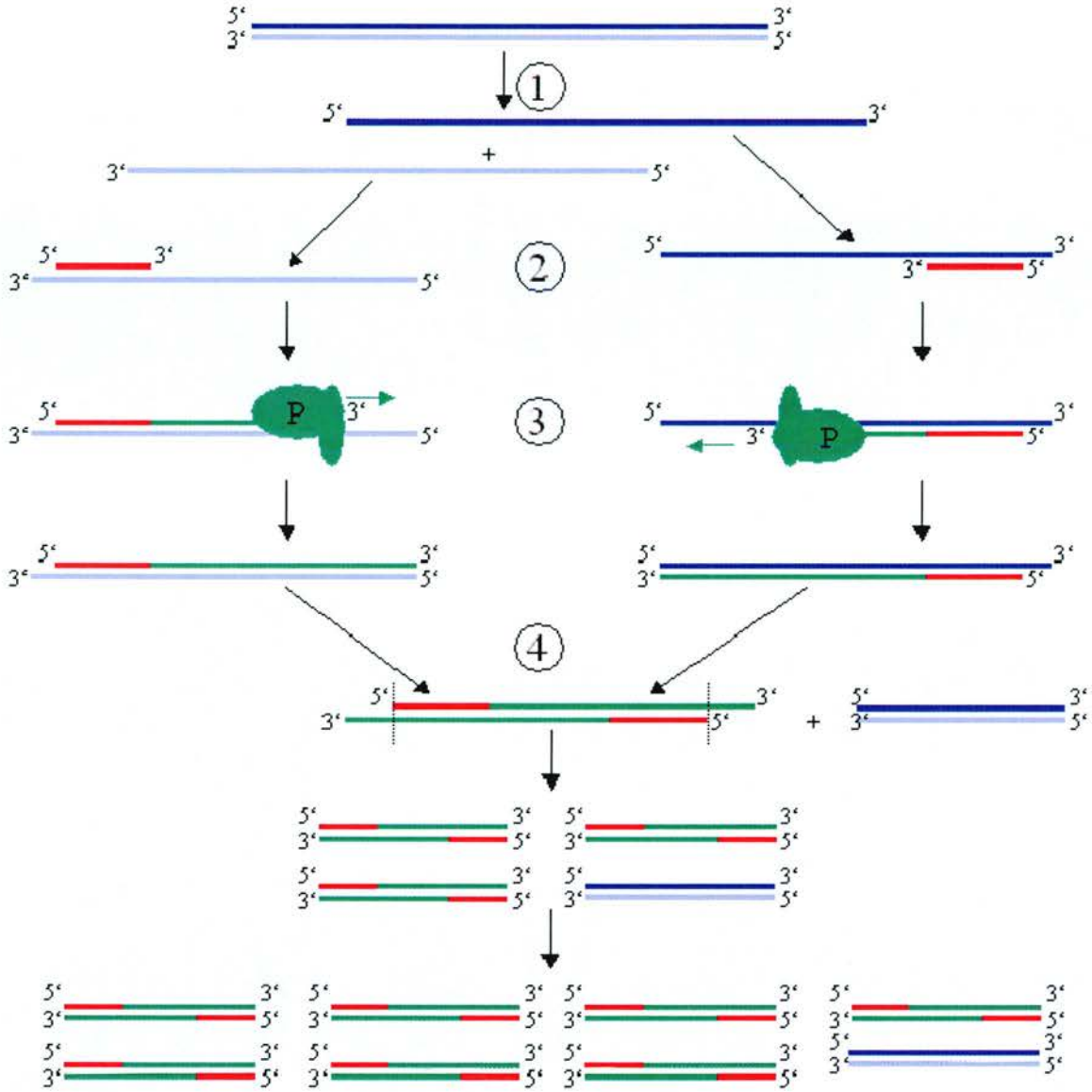


Figure 3.7 Schematic drawing of the PCR cycle.

(1) Denaturing at 96°C. (2) Annealing at 68°C. (3) Elongation at 72°C (P=Polymerase). (4) The first cycle is complete. The two resulting DNA strands make up the template DNA for the next cycle, thus doubling the amount of DNA duplicated for each new cycle.

3.3.2 RNA Extraction

All tissue or cell lysate handling was conducted to minimise the possibility of RNAse contamination. All instruments and disposables used in the procedure were autoclaved or certified as RNAse free.

Tissue RNA Extraction

Upon collection, endometrial tissue was immediately transferred to a solution of RNA Later (Ambion, Huntington, UK) to aid RNA stability before processing could occur at the laboratory. On arrival at the laboratory, tissue was transferred to storage at –80°C.

RNA was extracted using RNAeasy midispin columns (Qiagen, Crawley, UK) as per manufacturer's protocols, in combination with on-column DNase-I treatment (Qiagen).

In summary, 100-250mg samples of endometrium were homogenized, in a RNA- stabilizing buffer medium (provided by Quiagen), using a hand held rotator blade homogenizer. This solution was then centrifuged prior to passing the supernatant through a RNA extracting column pre-prepared by Quiagen. The column is designed to efficiently bind RNA out of solution and maintain its stability at room temperature. A wash solution was then passed through the column prior to a 15 minute incubation with DNase I to remove contaminating genomic DNA. After a further wash the RNA is then eluted from the column in a known volume of RNase treated sterile water.

Ishikawa Cell Extraction

For this study, total RNA was isolated from Ishikawa cells using a single-step extraction reagent, Total RNA Isolating Reagent (TRIR, Abgene, Surrey, UK). This method of RNA extraction is based on the one-step acid guanidinium method first described by Chomczynski and Sacchi (Chomczynski *et al.*, 1987). The procedure was carried out according to a modified protocol provided with the reagent. Briefly, TRIR is a combination of guanidine salts and urea, which act as denaturing agents. Cells were lysed in 1ml of TRIR and allowed to stand at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes. Addition of

0.2ml chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase and can be precipitated by the addition of isopropanol, washed with ethanol and solubilised in DEPC treated water.

3.3.3 Assessing the Quality and Quantity of total RNA extracted.

The concentration of the RNA extracted from Ishikawa cells was determined by measuring the optical density (OD) at 260nm (A260nm) using an automated spectrophotometer (Genequant), with 1 A260nm unit being an equivalent of 40ng of RNA in 1mL dH2O.

A 1:10 dilution of RNA was made in nuclease free water. A ratio of the optical density at a wavelength of 260nm to a wavelength of 280nm (260:280) was additionally calculated. If the ratio was less than 1.6, the sample was considered not to have sufficient purity for use in further work.

For total RNA extracted from endometrial tissue, in addition to the above method of assessment, aliquots (1μl) of purified RNA were removed for quantification and quality assessment using the Agilent 2100 Bioanalyser system in combination with RNA6000nano chips (Agilent Technologies, Cheshire, UK). Only RNA that displayed intact 18S and 28S peaks was reverse transcribed (RT) to cDNA for real-time PCR analysis. This quality-control step was included for each experimental run to avoid generation of false-negative results due to RNA degradation before and during extraction steps, and also as a quantification method to ensure equal amounts of RNA were transcribed in each RT reaction.

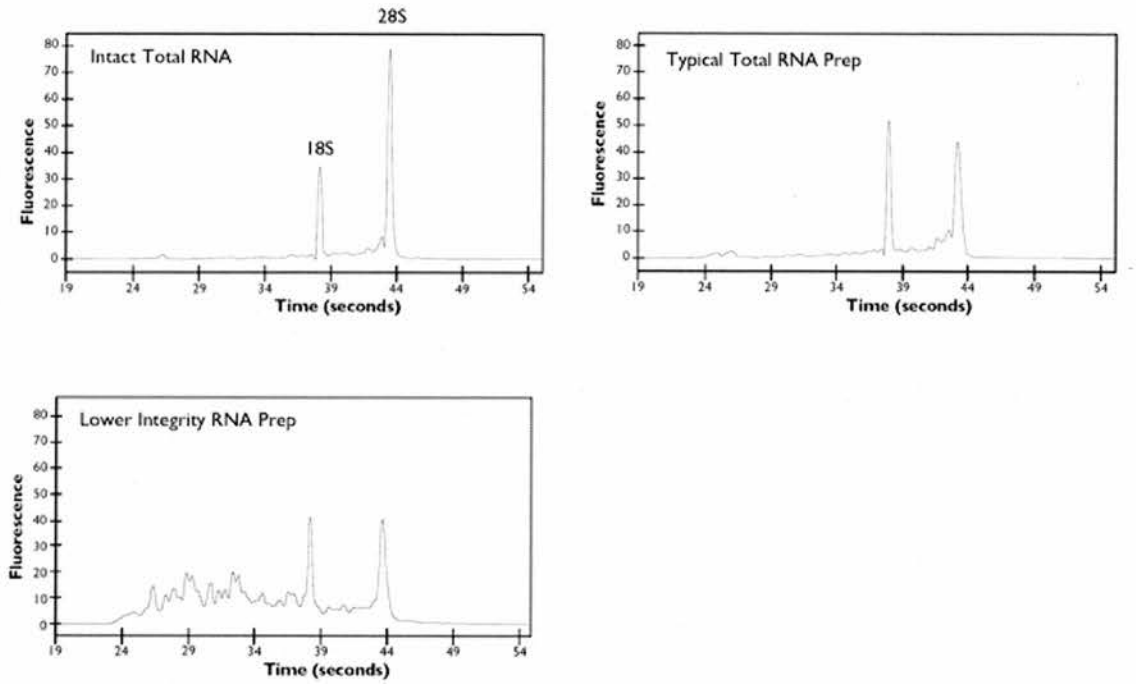


Figure 3.8 Bioanalyzer Images of Total RNA Preparations.

These electropherograms (from the Agilent 2100 bioanalyzer) show RNA samples with decreasing integrity. The trace labelled "Intact Total RNA" represents the ideal for a bioanalyzer trace of total RNA. Notice how the ribosomal RNA peaks are at a ratio of about 2 (28S:18S) in this sample; this represents ultrahigh quality RNA in terms of integrity. Most RNA samples will more closely resemble the right trace where there are nearly equal amounts of 28S and 18S rRNA. The lower trace shows a degraded RNA preparation with rRNA peaks that are lower than in the other two traces and where lower molecular weight degradation products become apparent.

3.3.4 Synthesis of Complementary DNA - Reverse Transcription and Polymerase Chain Reaction (RT-PCR).

The reverse transcription polymerase chain reaction (RT-PCR) is a sensitive method for detecting low abundance mRNA. RNA is unable to form a template for PCR; therefore the initial step in an RT-PCR reaction is the reverse transcription in order to form complementary DNA (cDNA). Exponential amplification of this cDNA is then achieved in a PCR reaction. These steps are carried out individually.

Reverse transcription was carried out in a 20μl volume of reaction solution. 400ng of RNA was diluted in nuclease free H_2O to a volume of 7.7μl. 12.3 μl of master mix solution containing; $MgCl_2$ (5.5mM), deoxy (d)-NTPs (0.5 mM each), random hexamers (2.5 μM), ribonuclease inhibitor (0.4 U/μl) and multiscribe reverse transcriptase (1.25U/μl) were then added to the 400ng of RNA, (all solutions supplied from Applied Biosystems, Warrington, UK). After mixing by brief centrifugation, samples were reverse transcribed on a thermal cycler. Reverse transcription involved incubation for 90 min at 25°C, 45 min at 48°C and 5min at 95°C for one cycle. Thereafter, cDNA samples were stored at –20°C. A tube with no reverse transcriptase was included to control for any DNA contamination.

Several techniques based on RT-PCR technology are available, including detection of mRNA by conventional (non-quantitative PCR), semi-quantitative PCR or 'real-time' quantitative PCR. The method used in this project was "real time" quantitation.

3.3.5 Real Time Quantitative RT-PCR

The Taqman assay (Perkin-Elmer – Applied Biosystems, CA, USA) is a real time RT-PCR assay. It requires the use of the ABI Prism 7700 - a combined thermal cycler/detector, which utilises sequential laser stimulation of its 96 reaction wells to detect fluorescence between 500nm and 660nm. Detected fluorescence is then analysed to quantify the amount of target gene of interest in the samples being analysed.

To successfully detect the target gene within cDNA, two specific oligonucleotide primers are designed to flank the endpoints of the gene or amplicon. This allows for specificity of amplification, which is additionally improved by the incorporation of an oligonucleotide probe that hybridizes to the amplicon during the annealing/extension phase of the PCR. To avoid amplification of contaminant DNA, the primers should be designed to span an intron. If this is not possible, the RNA to be quantified should be treated with RNAse-free DNAse to minimise the potential for genomic DNA contamination of the cDNA.

The assay utilises the 5'-3' nuclease activity of the DNA polymerase, Taq polymerase, to hydrolyse the hybridization probe that is bound to the amplicon. The probe used for Taqman is a 5'labelled FAM (6-carboxyfluorescene; a fluorophore), 3'labelled TAMRA (6-carboxytetramethylrhodamine; a quencher) FRET (Fluorescene Resonance Energy Transfer) probe. In FRET probes, the fluorophore donor transfers its energy to the quencher, suppressing fluorescence due to its close proximity. If the probe is correctly bound to an area within the target amplicon during the denaturing step of the PCR, subsequent action by the Taq polymerase, extends from the primers until it reaches and displaces the 5' end of the probe. Continued movement of the polymerase enzyme results in cleavage of the probe, separating the FAM reporter and TAMRA quencher dyes, allowing fluorescence to be produced and hence measurement of PCR product (figure 3.10).

Real-time PCR probe validation and calibration

The Taqman system incorporates its own design program, Primer Express, to facilitate adherence to the design specifications required for Taqman oligonucleotides. This ensures successful hybridization and amplification of template

cDNA during the procedure. An endogenous reference is also required within the experimental well to ensure there are similar amounts of starting material between experimental samples. A multiplexing strategy was utilised to allow relative quantitation of cDNAs between samples. Multiplexing is the use of more than one pair of primers within the same well. In real time quantitation, one primer pair amplifies the target and the other amplifies the endogenous reference within the same experimental well. The endogenous reference used in these experiments was ribosomal RNA (rRNA). rRNA constitutes 85-95% of cellular RNA and levels are unlikely to vary under conditions that might effect expression of mRNA (Barbu *et al.*, 1989). It has also been shown to be more reliable than normalisation with genes such as β-actin or GAPDH in a comparison study of human malignant cell lines (Zhong *et al.*, 1999). PE Applied Biosystems supplied VIC-labelled ribosomal 18S housekeeping primers and probe, which were multiplexed in reactions with the primers and probes for the mRNAs of interest. VIC is an alternative fluorescent probe that is detected at a different wavelength from FAM hence allowing discrimination between the two different labelled probes.

Prior to using the designed primers and probes for real time RT-PCR, the linearity of the response was determined. Serial dilutions of a cDNA standard (containing the target gene of interest) up to a 1/32 dilution were amplified with the reaction mixture containing target gene and endogenous 18s rRNA primers and probes.

It is vital to ensure a linear relationship between template concentration and threshold cycle value for both target gene and endogenous housekeeping gene. The threshold cycle value is the cycle number at which logarithmic PCR plots cross a calculated threshold line (Ct value) with Ct values decreasing as template concentration increases. The difference between the values for the target amplicon and the housekeeping 18s is called the delta Ct. The log of the total RNA (concentration prior to cDNA conversion) was plotted against the delta Ct and a best fit line applied. The gradient of this line should be < 0.1, see figure 3.9.

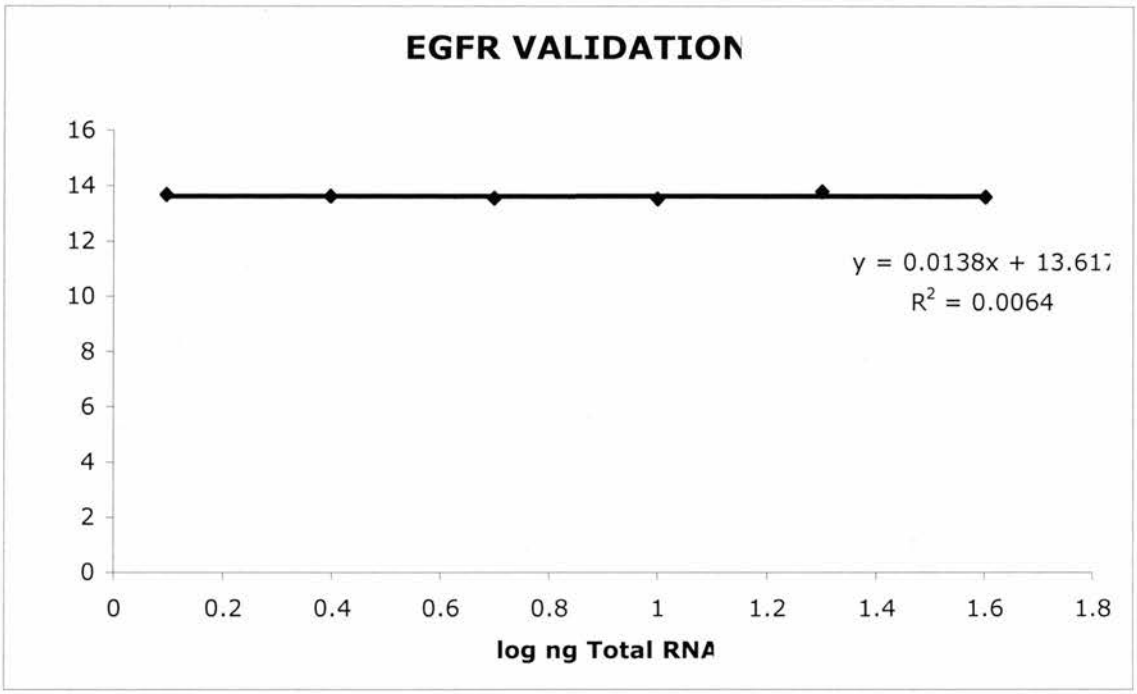


Figure 3.9 Validation of EGFR primers and probes.

Representative plot for Taqman primers and probe validation. Validation was performed for each set of primers and probes used in this thesis. The mean delta Ct is represented on the y-axis and the log concentration of total RNA is represented on the x-axis. The equation for the gradient of the slope is shown and is <0.1

Taqman Protocol

The expression of target enzymes in both endometrium from across the menstrual cycle and Ishikawa cells were investigated by quantitative RT-PCR. Total RNA was extracted, quantified and reverse transcribed to cDNA according to methods previously described (sections 3.3.2-3.3.4).

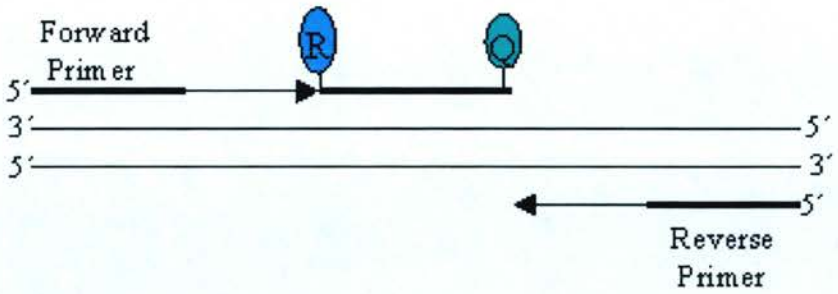
To measure cDNA expression, a reaction mix was prepared containing Taqman buffer (5.5 mM $MgCl_2$, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 400 µM dUTP), ribosomal 18S forward and reverse primers and probe (all at 50 nM), forward and reverse primers for the target gene / amplicon (300 nM), specific probe (100 nM), AmpErase UNG (0.01U/µl) and AmpliTaq Gold DNA Polymerase (0.025 U/µl; PE Biosystems).

After mixing, 48 µl was aliquoted into separate tubes and 2 µl (40 ng) of cDNA added and mixed before placing duplicate 24 µl samples into a PCR plate. To

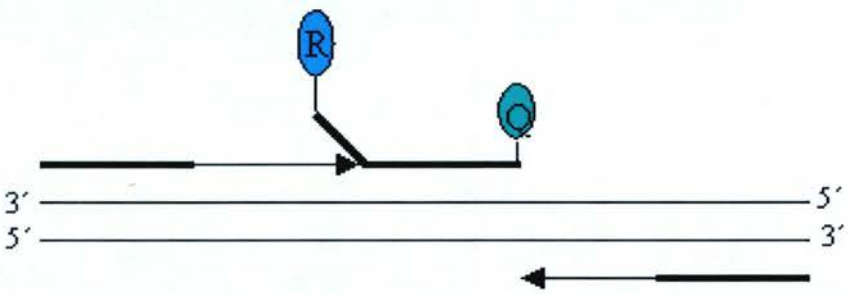
assess for genomic DNA contamination, two negative controls were added to the plate in duplicate; a no template (containing water) control and a no reverse transcriptase (containing RNA) control. Wells were sealed with optical caps and the PCR reaction run on the ABI prism 7700 using standard conditions.

All primers and probes were designed using the PRIMER express program (Applied Biosystems, Warrington, UK) and their sequences can be found in chapters 4 and 5.

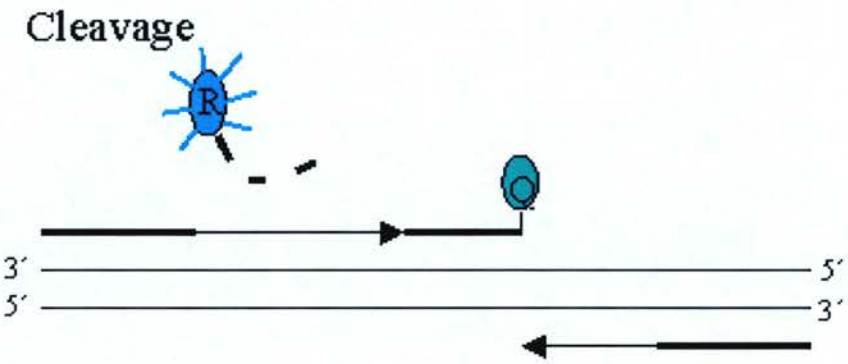
Polymerization



Strand Displacement



Cleavage



Polymerization Completed

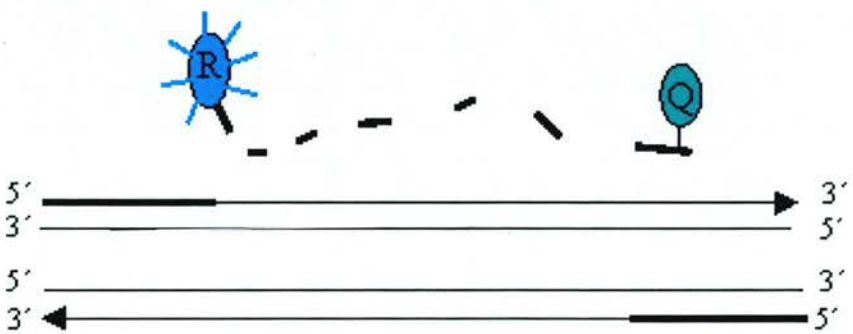


Figure 3.10 Diagrammatic representation of RT-PCR

Polymerisation

Two fluorescent dyes, a reporter (R) and a quencher (Q), are attached to the probes used with the TaqMan PCR Reagent Kit. The probe specifically anneals to the target gene between forward and reverse primer sites. The 3' end of the probe is blocked, so it is not extended during the PCR reaction.

Strand Displacement

When both dyes are attached to the probe, reporter dye emission is quenched due to fluorescence energy transfer from the reporter dye to the quencher dye. During each extension cycle, the probe is displaced at the 5' end by the DNA polymerase.

Cleavage

Taq DNA polymerase then cleaves the reporter dye from the probe via its 5'-3' exonuclease.

Polymerisation Complete

Once separated from the quencher, the reporter dye emits its characteristic fluorescence which can then be measured by the 7700 detector. The amount of fluorescence measured is proportional to the amount of PCR product made.

3.3.6 Taqman Analysis and Statistics

Data were processed using Sequence Detector version 1.6.3 (Applied Biosystems, Warrington, UK) according to manufacturer's instructions. Expression of target genes were normalised to RNA loading for each sample using 18S ribosomal RNA as an internal standard. All results were expressed relative to a control standard (cDNA obtained from a single sample of endometrial tissue and included in all experiments).

Analysed data was imported into a spreadsheet and analysed using the formula

$2^{-\Delta\Delta CT}$. Ct is the cycle number at which the PCR signal crosses a designated threshold. The ΔCT is the difference between the Ct value for the target gene and the internal normalisation control (18S). ΔΔCT is the difference between the ΔCT and the control standard mentioned above.

Data were subjected to statistical analysis with analysis of variance (ANOVA) and Least Significance Difference (LSD) post testing (Fishers PLSD tests; Statview 4.0; Abacus Concepts Inc., Piscataway, NJ, USA) and statistical significance accepted when $p<0.05$.

3.4 Cell Culture and Protein Signalling

3.4.1 Ishikawa Cell culture and cell experimentation

The Ishikawa Cell

The cyclical changes undergone by the human endometrium are orchestrated by the circulating sex steroids, progesterone and oestrogen. The regulation of the progesterone and oestrogen receptors is tightly controlled and their expression has been shown to vary throughout the menstrual cycle as discussed in the section 1.2.5. It is difficult to study protein signalling in the normal human endometrium because of the limited availability of suitable model systems, which contain functional oestrogen and progesterone receptors. Ishikawa cells are derived from a well-differentiated endometrial carcinoma cell line that expresses both oestrogen receptors (ER) and progesterone receptors (PR). (Hata *et al.*, 1992; Lessey *et al.*, 1996).

Cell Culture

Human Ishikawa endometrial adenocarcinoma cells (European Collection of Cell Culture, Centre for Applied Microbiology, Wiltshire, UK) were routinely cultured under sterile conditions in culture flasks with a surface area of 170 cm^2 (Corning Science Products, UK). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) nutrient mixture F-12 with glutamax-1 and pyridoxine (Life Technologies, Paisley, UK), supplemented with 10% fetal calf serum (FCS), and 1% antibiotics (stock 500IU/ml penicillin and 500ug/ml streptomycin, PAA Laboratories Ltd, Middlesex, UK) at 37°C and 5% CO_2 (v/v). Cells grew as a monolayer on the bottom of the flask and were observed using inverted light microscopy (Olympus CK40) to determine confluence. At about 80% confluency, cells were passaged. For passage of cells, growth medium was removed by pipette and cells washed twice with PBS before 2 ml of trypsin-EDTA (0.1% trypsin and 0.04% EDTA in PBS) was added to the culture flask. The trypsin was allowed to flow over the monolayer of cells at 37°C for 2 minutes. Flasks were then tapped to allow cells to loosen from the flask and 10 ml of DMEM was added to re-suspend cells and inhibit trypsinization. 1 ml of re-suspended cells was added to a new culture flask and then diluted 1 part to 30 in DMEM containing FCS.

For experimentation, Ishikawa cells were seeded out to sterile petri dishes (number of cells calculated according to dish size) and grown to the required confluence; 60 to 80% for all experimentation except transient transfection (section 3.4.3). The culture medium was then replaced with serum free DMEM and 8.4μM Indomethacin (a dual COX-enzyme inhibitor used to inhibit endogenous prostanoid biosynthesis) for overnight incubation in order to achieve cell synchronization prior to experimentation. Details of specific experimentation can be found in chapters 4 and 5.

3.4.2 Cell Lysis and Protein Concentration

Protein extraction

After experimental stimulation of Ishikawa cells, two washes with cold PBS were carried out to stop further cellular signalling. Cells were then lysed by addition of protein lysis buffer (1% Triton X-100, 150mM NaCl, 10mM Tris/HCl pH7.4, 1mM EDTA, 5mM EGTA, 0.1% SDS containing 2mM PMSF, 1mM Na_3VO_4 and 5μg/ml aprotinin). Proteins were extracted by allowing experimental dishes to sit on ice for 10 minutes prior to scraping of cells using a plate scraper and collection by sterile pipette. The collected suspension of cells in lysis buffer was centrifuged at 14 000 rpm for 15 minutes at 4°C to cause insoluble material to pellet. The clarified lysate was then transferred to a new eppendorf tube for protein quantification prior to immunoprecipitation or SDS-PAGE.

For endometrial tissue experiments, following stimulation, tissue was snap frozen in dry ice and stored at –20°C for subsequent protein extraction. Protein was later harvested by homogenization of tissue (kept on ice to limit protein degradation) in protein lysis buffer (as described above). The collected suspension of tissue homogenate in lysis buffer was centrifuged at 14 000 rpm for 15 minutes at 4°C to cause insoluble material to pellet. The clarified lysate was then transferred to a new eppendorf tube for protein quantification prior to immunoprecipitation or SDS-PAGE.

Protein Quantification

Proteins were quantified according to the BIO-RAD DC colorimetric protein microassay (Biorad, UK) as per manufacturer's instructions. This is a method based upon the Method of Lowry in which colour development results from the reaction between protein and an alkaline copper tartrate solution and the subsequent reduction of Folin reagent. This reaction results in the formation of several reduced species all with a characteristic blue colour, the absorbance of which can be measured between 650 and 750nm.

Samples were diluted in distilled water to a ratio of 1:30. A range of bovine serum albumin (BSA, supplied with the assay) protein standards (0 - 200 μg/ml) was additionally made up in the same distilled water to achieve an OD_{595} response from 0.1 to 1.0 OD units. For the assay, 25μl of each standard and sample (Ishikawa cell or tissue homogenate) were dispensed in duplicate into individual wells of a 96-well plate. To each aliquot 25μl of Reagent A (alkaline copper tartrate solution) was added followed by 100μl of Reagent B (dilute Folin reagent). The reactions were allowed to proceed for 5 minutes at room temperature before the OD 690nm was recorded using a spectrophotometric plate reader (Multiskan Biochromatic Eliza Plate-Reader, Labsystems, UK).

A standard curve (x-axis; μg of protein against y-axis; OD690nm) was produced using the AssayZap computer software program (Biosoft) and used to determine the average protein concentration of each sample.

3.4.3 Transient Transfections and Immmunoprecipitation

Cell signalling to ERK1/2 (Chapter 4) was determined using a dominant negative (DN) mutant isoform targeted against epidermal growth factor receptor (EGFR).

Ishikawa cells were seeded to a density of $5x10^5$ per well in 6cm dishes and then transfected with a c-myc-tagged ERK1/2 cDNA construct together with either empty vector cDNA (pcDNA3; Invitrogen, de Schelp, The Netherlands) or DN-EGFR cDNA using Superfect (Qiagen, Crawley, UK) as per the manufacturer's protocol (The DN-EGFR and c-myc tagged ERK constructs were a kind gift from Professor Zvi Naor, Department of Biochemistry, Tel-Aviv University, Israel). Optimal concentrations of cDNA for transfection had been determined by titration. The transfection efficiency of the Ishikawa cell line was determined by transfection with a pcDNA6/V5/His/lacZ cDNA construct (Invitrogen) and β-Galactosidase assay. Transfection efficiency as reported previously for this cell line using standard procedures according to the manufacturer protocol is 45 ± 5 percent (Sales *et al.*, 2005). The following day, cells were starved by overnight incubation in serum free medium containing 3μg/ml indomethacin and then underwent experimentation.

Cells were then lysed and protein quantified as described in section 3.4.2. The tagged ERK1/2 allowed immunoprecipitation from whole cell lysate. For immunoprecipitation, equal amounts of protein were incubated with specific c-myc antibody pre-conjugated to protein A Sepharose overnight at 4°C with gentle rotation. Beads were washed extensively with lysis buffer and immune complexes eluted and solubilised in Laemmli buffer (125 mM Tris-HCl pH6.8, 4% SDS, 5% 2-mercaptoethanol, 20% glycerol and 0.05% bromophenol blue), boiled for 5 minutes and subjected to Western blot analysis.

3.4.4 Theory of protein analysis

Proteins examined in this thesis were derived from Ishikawa cells or from normal human endometrium.

Most of the methods used in protein purification can be divided into four distinct but interrelated groups depending upon protein characteristics:

Methods based on surface features of proteins

Surface features include charge distribution and surface distribution of hydrophobic amino acid side chains amongst others. Most methods exploiting these surface properties depend mainly on protein solubility in various solvents. Also included in this category is the highly specific technique of immunoaffininty, in which an antibody directed against an epitope on the protein surface is used to pull out the desired protein from a mixture. This method is utilised in immunoprecipitation described in section 3.4.3.

Methods based on whole structure: Protein size and shape

Proteins range in size from 5000 to many million Daltons (Da) and the chief methods of exploiting size and shape of a protein is gel-filtration chromatography and preparative gel electrophoresis (section 3.4.5).

Methods based on net charge

Separation methods exploiting the overall charge of a protein are not utilised in this thesis, however protein charge would in part determine travelled distance in preparative gel electrophoresis.

Methods based on bioproperties (affinity)

This method exploits the strong affinity of a protein for its ligand thereby separating a desired protein from a mix. Although not specifically utilised in this thesis, the immunoaffinity of a protein for a specific antibody is a closely related method of isolation used in immunoprecipitation (section 3.4.3)

3.4.5 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting

Western blot analysis can detect one protein in a mixture of any number of proteins while giving you information about the size of the protein. The proteins must be separated by size using SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane to provide a protein imprint, which can be detected using standard immunological techniques. A negative charge is created on the side of the gel and the positive charge on the side of the membrane to drive the negatively charged proteins over to the positive charged nitrocellulose membrane.

SDS-PAGE

SDS-PAGE was performed using pre-cast 4%-20% Tris-Glycerine gels (Novex, Invitrogen, UK). Gels were set up in the gel running tank with running buffer (25 mM Tris-HCL, 0.2M glycine, 0.1% SDS). An equal amount of protein (40μg) was resuspended in a total volume of 25μl sample loading buffer (125mM Tris-HCL pH 6.8, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol and 0.05%bromophenol blue), boiled for 5 minutes at 95°C to denature the protein content and then loaded into separate wells of the gel. 15μl of Seeblue (Novex) pre-stained molecular weight markers were loaded into a separate well. Gels were run at 4mA constant current for about 90 minutes prior to immunoblotting.

Western Blotting

Following electrophoresis, the gel was transferred to a protein-free tray and equilibrated with transfer buffer (25 mM Tris/HCL, 0.192M glycine, 20% methanol) for 5 minutes. Whatman No.3 filter paper and polyvinylidene difluoride membrane (PVDF, Millipore, Watford, UK) were cut to the gel dimensions and equilibrated in the transfer buffer. The PVDF membrane was pre-soaked in methanol for 1 minute followed by a rinse in distilled water prior to equilibration in transfer buffer. The blot was assembled by overlaying three pieces of Whatman paper with the PVDF membrane followed by the gel and finally three further pieces of Whatman paper. Care was taken to remove any air bubbles prior to protein transfer to the membrane

for 1 hour 45 minutes at 14V constant voltage using a semi-dry blotter (BioRad, UK). Following transfer, membranes were incubated with the relevant primary antibody at 4°C overnight. After washing with TBS-Tween, membranes were subsequently incubated for one hour with the relevant alkaline phosphatase conjugated secondary antibody.

Immunoreactive proteins were visualised by the ECF chemiluminescence system according to manufacture's instructions. Specific proteins were revealed and quantified by and normalized to total protein expression using STORM 860 Phosphorimager (Molecular Dynamics, Amersham Biosciences, UK). The molecular weight of the proteins was identified relative to the mobility of the pre-stained markers on SDS-PAGE.

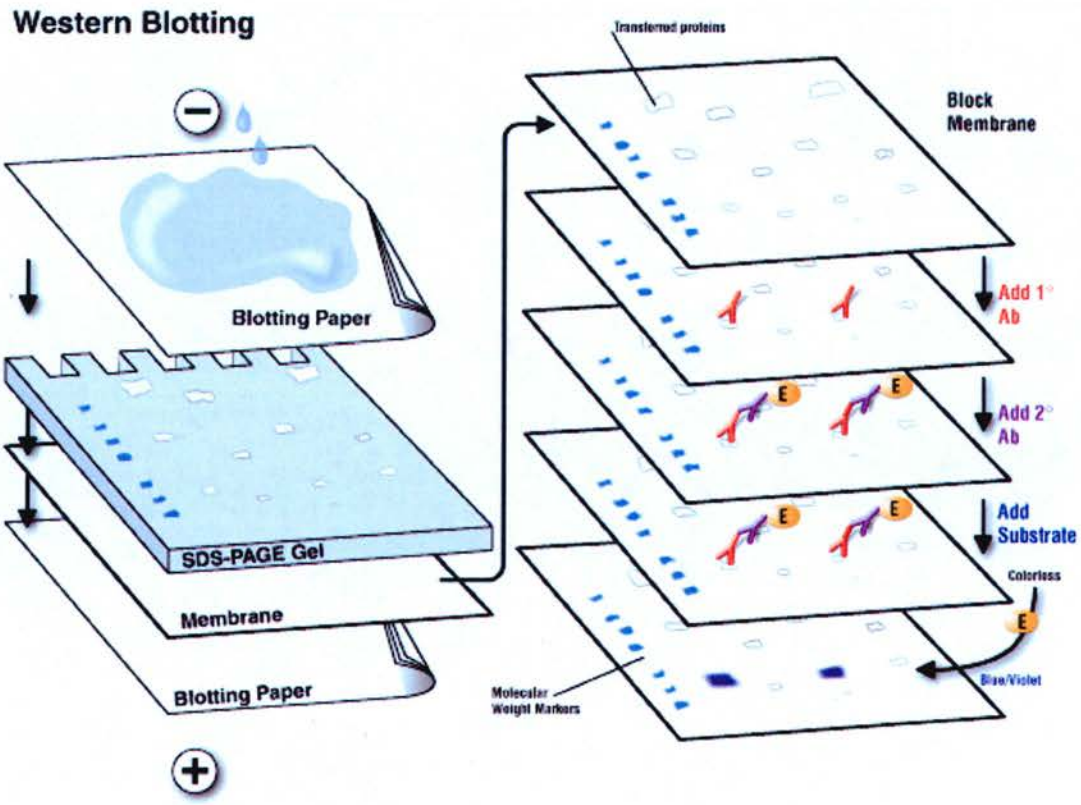


Figure 3.11 Diagrammatic representation of the Western Blotting process

3.5 Cyclic AMP Assay

PGE_2 signalling was assessed in endometrial biopsies obtained from proliferative and secretory phases of the menstrual cycle. Tissue was minced finely with scissors and incubated overnight at 37°C in a humidified 5% CO_2 incubator in 2 ml RPMI medium (Sigma, Poole, UK) containing, 2 mmol/l L-glutamine, 100 IU penicillin and 100□g streptomycin and 3μg/ml indomethacin (Sigma, Poole, UK). Following overnight treatment, approximately 1/3 of the tissue was removed and stored at –20°C. The remaining tissue was incubated in the same medium containing 1mM 1-methyl-3-isobutylxanthine (Sigma, Poole, UK) at 37°C for 30 minutes. It was then divided in two and treated with control medium or 100nM PGE_2 for 10 minutes at 37°C. Tissue was then lysed in 0.1M HCl and frozen until assayed.

Cyclic AMP concentration was measured by ELISA (Biomol, Affiniti, Exeter, UK) according to manufacturer's instructions and normalised to protein concentration determined by a modification of the method of Lowry (section 3.4.2). The ELISA was performed using a 96 well microtitre plate provided in the assay kit. The wells of the plate were pre-coated with goat anti-rabbit IgG. cAMP standards (100μl of each), ranging form 200 pmol/ml up to 0.78pmol/ml, were added to the plate to produce a standard curve. A 100μl volume of sample was then added to the plate in duplicate. 50μl of alkaline phosphatase-cAMP conjugate and 50μl of polyclonal rabbit anti-cAMP antibody (both provided in kit) were added to each well of the plate.

The plate was then incubated at room temperature for two hours on a plate shaker at ~500rpm. Thereafter, the wells were aspirated and washed three times with the provided wash buffer (TBST; 50mM Tris-HCL, 150mM NaCl, 0.5% Tween20, pH 7.4; containing sodium azide).

The assay was developed by the addition of 200μl/well of p-nitrophenyl phosphate. The colour reaction was allowed to develop for twenty minutes before being stopped with the addition of 200ul/well of provided "stop solution" (trisodium phosphate in water). Colour reaction was measured at 405nm by spectrophotomery. The concentration of cAMP per sample was extrapolated from the standard curve using the Assay Zap software (Biosoft, UK).

Chapter 4

Prostacyclin receptor signalling in the Ishikawa cell

4.1 Introduction

Prostacyclin (PGI_2) was discovered in 1976 during the generation of unstable eicosanoids in blood vessels (Bunting *et al.*, 1976) and is best known for its anticoagulant effect on platelets and vasodilatory effect on the vascular endothelium, where expression is found in abundance (Smyth *et al.*, 2002). Like many eicosanoids, PGI_2 is produced via the prostaglandin signalling pathways, which are described in detail in section 1.3.1. Briefly, arachidonic acid (AA) is released from plasma membrane phospholipids and is cyclized, oxygenated and reduced to the unstable intermediary prostaglandin H_2 (PGH_2) by COX enzymes (Marnett, 1992; Narumiya *et al.*, 2001). The action of the COX enzymes is the rate limiting and committing step of prostaglandin signalling. The PGH_2 intermediary serves as a substrate for terminal prostaglandin synthase enzymes, such as prostacyclin synthase (PGIS), which completes the synthesis of prostacyclin (figure 4.1). PGI_2 has a mode of action via coupling to the heptahelical G-protein coupled prostacyclin receptor (IP receptor) (Battersby *et al.*, 2004a; Narumiya *et al.*, 1999). The IP receptor is known to mediate a cAMP rise and has been termed a "relaxant" receptor (Narumiya and FitzGerald, 2001) with routine activation of the IP receptor activating adenylate cyclase via the Gs subunit in a dose dependent manner.

Temporal expression of prostaglandins such as PGE_2 and $PGF_{2\alpha}$ and their receptors (EP2/EP4 and FP receptors) has been demonstrated in the human endometrium and has been shown to vary with the phase of the menstrual cycle (Milne and Jabbour, 2003; Milne *et al.*, 2001). Recent data implicate a role for PGE_2 and $PGF_{2\alpha}$ in proliferation of glandular epithelial cells via diverse signalling pathways (Jabbour *et al.*, 2003; Milne and Jabbour, 2003). In contrast, little is known of the expression pattern and function of IP receptor in human endometrium, although PGIS and IP receptor expression have been demonstrated in pregnant and non-pregnant myometrium (Chegini *et al.*, 1989; Giannoulias *et al.*, 2002; Moonen *et al.*, 1986) and prostacyclin is known to act as a smooth muscle relaxant (Dyal *et al.*, 1988; Lumsden *et al.*, 1986a).

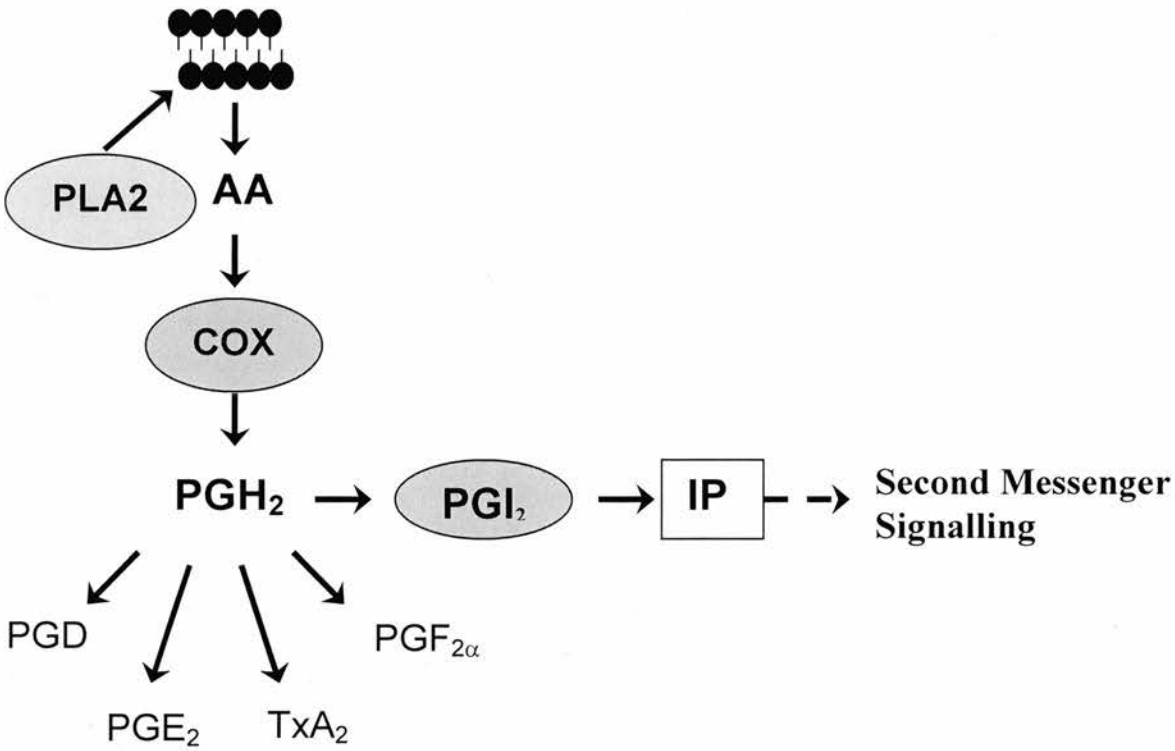


Figure 4.1 Prostacyclin signalling pathway

Arachidonic acid (AA) is released from plasma membrane via the action of phospholipase A2 (PLA2). AA is acted upon by cyclooxygenase enzymes (COX) to form the prostaglandin intermediary PGH_2. Terminal synthase enzymes then convert this intermediary to the respective prostanoid. Prostacyclin (PGI_2) acts upon its cognate receptor (IP) to initiate second messenger signalling.

Knockout studies disrupting the IP gene in mice have demonstrated thrombotic tendencies and decreased inflammatory responses (Murata *et al.*, 1997; Ueno *et al.*, 2000). The cyclical regeneration and repair undergone by the human endometrium during the menstrual cycle (section 1.4.1) necessarily involves physiological processes involving clotting and inflammation. Production of uterine prostacyclin may be involved in myometrial smooth muscle relaxation, vasodilatation and also prevention of clot formation. These physiological actions are all involved in the process of menstruation and a role for PGI_2 in menstruation and menstrual disturbances can be hypothesised. Indeed, endometrium collected from women with heavy menstrual blood loss (MBL) has a greater capability of enhancing myometrial prostacyclin production compared to endometrium collected from women with normal MBL (Smith, S. K. *et al.*, 1981b). Previous studies have also demonstrated an increase in expression of PGIS and IP receptor mRNA during the menstrual compared with proliferative and secretory stages of the cycle (Battersby *et al.*, 2004a).

The remodelling of the human endometrium required during the menstrual cycle requires tight control of angiogenic growth factors to coordinate the growth of new vessel formation. Previous studies have reported that COX enzymes, in particular COX-2 (Gately *et al.*, 2004), and prostaglandins such as PGE_2 (Sales *et al.*, 2004a) and $PGF_{2\alpha}$ (Sales *et al.*, 2005) can modulate the expression of target angiogenic genes within the human endometrium. The angiopoietins (Ang-1 and Ang-2) are a family of growth factors that act as ligands for the largely endothelial restricted Tie-2 receptor tyrosine kinase which is essential for vascular development. Investigation into the expression of the angiopoietins 1 and 2 in human endometrium showed minor variations in expression of Ang-1 mRNA at different stages of the cycle and a significant up regulation of expression of Ang-2 mRNA during the secretory stage of the cycle (Hirchenhain *et al.*, 2003). Ang-1 binds and causes autophosphorylation of Tie-2, promoting endothelial cell migration, sprouting and survival. In contrast, Ang-2 binds to Tie-2 without eliciting a signal. The effect of this natural antagonism is to cause vessel destabilization with vascular regression in the absence of additional vascular endothelial growth factor (VEGF) or indeed neo-vascularization in its presence (Smith, O. P. *et al.*, 2005).

The aim of this chapter is to examine the prostacyclin receptor within the human endometrium. Whilst exploring the role of prostaglandins within human endometrium, access to well characterised human tissue from consenting patients is too limited to explore molecular signalling pathways in detail. Using an endometrial epithelial cell line (Ishikawa) and human endometrial tissue, the intracellular signalling transduction pathways activated following PGI-IP ligand-receptor interaction is investigated.

4.2 Methods

Patients and tissue collection

Ethical approval was obtained from Lothian Research Ethics Committee and written informed consent was obtained from all subjects before tissue collection. (The patient information sheet and ethical approval are included in Appendices.)

Endometrial biopsies were collected at different stages of the menstrual cycle with an endometrial suction curette (Pipelle, Laboratoire CCD, Paris, France) from women attending the gynaecological outpatient setting. In addition, full thickness endometrial biopsies (section 3.1.1) at all stages of the menstrual cycle, were collected from women undergoing hysterectomy for benign gynaecological indications. All subjects were aged between 18 to 50 and reported regular menstrual cycles (cycle length, 21-35 days) with no unscheduled, non-menstrual bleeding. No woman had received hormonal preparation in the 3 months preceding biopsy collection. Patients were clinically examined and clinical pelvic abnormalities, such as an enlarged uterus, were further investigated by pelvic ultrasound imaging. Patients with known uterine pathology such as fibroid disease and endometriosis were excluded from the study.

Immediately after collection, tissue was divided, transferred into RNA Later (Ambion Inc, Huntingdon, UK) and stored at –70°C (for RNA extraction), fixed in neutral buffered formalin and wax embedded (for immunohistochemical analysis) or placed in RPMI 1640 medium (containing 2 mM L-glutamine, 100 U penicillin and 100 μg/ml streptomycin) and transported to the laboratory for in vitro culture.

All biopsies were dated according to stated last menstrual period (LMP) and dating was confirmed by histological assessment according to criteria of Noyes and co-worker (Noyes, 1950). Furthermore, circulating oestradiol and progesterone serum levels were measured at the time of biopsy collection and were consistent for both LMP and histological assignment of menstrual cycle stage. This is a robust method for characterizing endometrial samples. Detailed gene microarray studies support this method for characterizing endometrial samples with consistency across these three parameters (Critchley *et al.*, 2006; Talbi *et al.*, 2006). Inconsistencies between measured circulating hormone levels, stated day of last menstrual period and/or histological assessment led to exclusion of three tissue biopsies from the study.

Tissue Culture

Tissue samples were finely minced using sterile forceps and scissors before overnight incubation in serum free RPMI medium (as described above) and 3μg/ml indomethacin (a dual COX enzyme inhibitor to inhibit endogenous prostanoid production). The next day, tissue was pre-treated with a specific chemical inhibitor of EGFR kinase (AG1478, 100nM) for 1 hour prior to stimulation with 100nM Iloprost for time period stated in figure legend. Following stimulation, tissue was either snap frozen in dry ice and stored at -20^0C for subsequent protein extraction or stored at -70^0C for RNA extraction. Protein was harvested by homogenization of tissue in protein lysis buffer. Protein content was determined using a protein assay kit (Bio-Rad, Hemel Hempstead, UK) as described in section 3.4.2.

Cell culture

Ishikawa human endometrial epithelial cells (European Collection of Cell Culture, Wiltshire, UK) were maintained as described in section 3.4.1. The cells were grown on monolayer in 6cm dishes to 60-80% confluence after which the culture medium was replaced with serum free DMEM containing 3μg/ml indomethacin for overnight incubation. The next day cells were pre-treated with specific inhibitors of either EGFR kinase (AG1478, 100nM) or mitogen-activated protein kinase kinase (MEK; PD98059, 50 μM) for 1 hour prior to stimulation with 100nM Iloprost or v/v 100% ethanol as a vehicle control for the time period specified in the figure legend. Following stimulation with Iloprost, proteins were harvested and extracted and the protein content in the supernatant fraction was determined using a protein assay kit (Bio-Rad) (see section 3.4.2).

Taqman quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The expression of IP receptor across the menstrual cycle and the effects of Iloprost on pro-angiogenic gene expression in Ishikawa cells or endometrial tissue were investigated by Taqman quantitative RT-PCR analysis. Total RNA was extracted from endometrial biopsies using an RNeasy Midi Kit (Qiagen Ltd, Sussex, UK) according to the manufacturer's instructions (section 3.3.2). Samples were treated for DNA contamination by DNA digestion during RNA purification.

Following extraction, total RNA was eluted in 150µl of nuclease free water and stored at -80^0C. RNA was extracted from Ishikawa cells using Total RNA Isolating Reagent (TRIR, Abgene, Surrey) as described in section 3.3.2. Extracted RNA was again eluted in nuclease free water and stored at -80^0C. Quality and concentration of RNA was assessed using the Agilent 2100 Bioanalyser system in combination with RNA6000nano chips (Agilent Technologies, Cheshire, UK). Only RNA that displayed intact 18S and 28S peaks was reverse transcribed to cDNA for real-time PCR analysis. Additionally, the concentration of the RNA extracted was confirmed by measuring the optical density (OD) at 260nm (A260nm) as described in section 3.3.3. Once extracted and quantified, RNA samples were reverse transcribed (section 3.3.4) and subjected to real time quantitative PCR using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Warrington, UK) as previously described in section 3.3.5. All primers and probe were designed using the PRIMER express program (Applied Biosystems, Warrington, UK; Table 4.1)

Data were analysed and processed using Sequence Detector version 1.6.3 (Applied Biosystems, Warrington, UK) according to manufacturer's instructions. Expression of IP receptor and pro-angiogenic genes was normalised to RNA loading for each sample using the 18s RNA as an internal standard. Results were expressed relative to an internal positive control of pooled human endometrial cDNA, which was included in all reactions. Fold increase was determined by dividing the relative expression in Iloprost-treated cells/tissues by the relative expression of the vehicle control.

Transient transfections and immunoprecipitation

To confirm the role of EGFR in Iloprost-mediated ERK1/2 phosphorylation, we used a dominant negative (DN) mutant EGFR. Ishikawa cells were seeded to a density of $5x10^5$ per well in 6cm dishes and then transfected with a c-myc-tagged ERK1/2 cDNA construct together with either empty vector cDNA (pcDNA3; Invitrogen, de Schelp, The Netherlands) or DN-EGFR cDNA using Superfect (Qiagen, Crawley, UK) as per the manufacturer's protocol described in section 3.4.3. The DN-EGFR and c-myc tagged ERK constructs were a kind gift from Professor Zvi Naor, Department of Biochemistry, Tel-Aviv University, Israel. Optimal concentrations of cDNA for transfection were determined by titration. The

transfection efficiency of the Ishikawa cell line had previously been investigated by transfection with a pcDNA6/V5/His/lacZ cDNA construct (Invitrogen) and utilizing a β-Galactosidase assay. Transfection efficiency as reported previously for this cell line using standard procedures according to the manufacturer protocol is 45 ± 5 percent (Sales *et al.*, 2005). The following day, cells were starved by overnight incubation in serum free medium containing 3μg/ml indomethacin and then treated with 100nM Iloprost or vehicle for 10 minutes. Cells were then lysed and protein quantified as described above. The tagged ERK1/2 was immunoprecipitated from whole cell lysate. For immunoprecipitation, equal amounts of protein were incubated with specific c-myc antibody pre-conjugated to protein A Sepharose overnight at 4°C with gentle rotation. Beads were washed extensively with lysis buffer and immune complexes eluted and solubilised in Laemmli buffer (125 mM Tris-HCl pH6.8, 4% SDS, 5% 2-mercaptoethanol, 20% glycerol and 0.05% bromophenol blue), boiled for 5 minutes and subjected to Western blot analysis.

Western blot analysis

Western blot analysis was conducted to investigate ERK1/2 expression in Ishikawa cells and human endometrial tissue. A total of 50 μg of protein from whole cell lysate was resuspended in 20 μl of Laemmli buffer. Proteins were resolved on 4-20% Tris-Glycine gels (NOVEX, Invitrogen), transferred onto polyvinylidene difluoride membrane (PVDF, Millipore, Watford, UK) and subjected to immunoblot analysis as described in section 3.4.5. Blots were incubated with anti-phospho-p42/p44 ERK (9101, Cell signalling technologies/New England Biolabs, Herts, UK) and alkaline-phosphatase conjugated secondary antibodies (Sigma). Immunoblots were stripped and reprobed with antibody recognizing total ERK (sc-93, Santa Cruz Biotechnology/Autogen-Bioclear, Wiltshire, UK). Immunoreactive proteins were visualized by the ECF chemiluminescence system according to the manufacturers instructions (Amersham Biosciences, Amersham, UK). Proteins were revealed and quantified by Phosphorimager analysis using a Typhoon 9400 Phosphorimager (Molecular Dynamics, Amersham Biosciences, UK). Relative density in immunoblots was calculated by dividing the value obtained from the phosphorylated immunoblots by the value obtained from the total immunoblots in the same experiment and expressed as fold increase above vehicle control.

Immunohistochemistry

To investigate the expression of the prostacyclin receptor (IP) in human endometrium, endometrial sections (5μm) from across the menstrual cycle were dewaxed in xylene and rehydrated using decreasing grades of ethanol followed by water. All washes were carried out in 0.01M phosphate buffered saline (PBS; Sigma-Aldrich Ltd, Ayrshire, UK). Antigen retrieval was performed by pressure-cooking in 0.01M sodium citrate, pH 6.0, for 5 minutes, followed by a 20 minute cooling down period. Thereafter, slides were sequentially incubated with 3% hydrogen peroxide (H_2O_2, VWR Inc. Poole, UK) in distilled water for 10 minutes (to quench endogenous peroxidase activity) followed by a 15 minute incubation with avidin and biotin solutions (Vector Laboratories, Peterborough, UK) to block endogenous steptavidin activity. Non-specific binding was further reduced by 20 minutes incubation with non-immune horse serum (Vector Laboratories, Peterborough, UK) in a humidified chamber at room temperature before overnight incubation with the primary antibody at 4^0C. For localization of IP receptor, slides were incubated with a goat polyclonal antibody raised against a peptide mapping near the carboxy terminus of IP receptor of human origin (sc-20436, Santa Cruz Biotechnology Inc. CA, USA) at a 1:30 dilution in normal horse serum. Pre-absorption of the antibody with a specific blocking peptide (Santa Cruz Biotechnology Inc.) was used as the negative control in addition to a control goat IgG antibody at matched protein concentration to the IP antibody. Following a wash in PBS with 0.01% Tween 20, the slides were incubated in biotinylated horse anti-goat secondary antibody (Vector Laboratories, Peterborough, UK) in normal horse serum at a 1:200 dilution for 60 minutes at room temperature. Tertiary detection was carried out using an avidin-biotin peroxidase complex (Vectastain Elite, Vector Laboratories) for 60 minutes at room temperature and visualization carried out with the substrate and chromagen 3,3'-diaminobenzidine (Dako Cytomation, CA, USA). Sections were counterstained with haematoxin, dehydrated in xylene and mounted.

Scoring and analysis of immunoreactivity

The immunostaining intensity of the IP receptor epitope in all tissue sections was assessed in a semi-quantitative manner on a 4-point scale: 0 = no immunostaining, 1= mild immunostaining, 2 = moderate immunostaining, 3 = intense immunostaining. All tissue sections were scored blind by two observers. This scoring system has been previously validated in a subset of tissue sections in which immunoreactivity was measured with a computerised image analysis system, a strong correlation between quantitative data derived from the image analysis and subjective scores by a trained observer was obtained (Wang *et al.*, 1998).

Immunofluorescent confocal laser microscopy

Co-localisation of the site of expression of prostacyclin receptor (IP) with epidermal growth factor receptor (EGFR) or the endothelial cell marker CD31 was performed in human endometrium by dual immunofluorescence immunohistochemistry and confocal laser microscopy. Human endometrial sections (5μm) were dewaxed, rehydrated and washed as described above. The immunohistochemical methodology was repeated for antigen retrieval by pressure cooking, quenching of hydrogen peroxidase activity and blocking of endogenous streptavidin activity. Non-specific binding was further reduced by 20 minute incubation with 5% non-immune rabbit serum diluted in PBS before overnight incubation at 4^0C with the polyclonal mouse anti-EGFR primary antibody (NCL-EGFR-384, Nova-Castra, Newcastle-upon-Tyne, UK) at a dilution of 1 in 25. For co-localization of the IP receptor with CD31, a monoclonal mouse anti CD31 antibody (Dako Cytomation, CA, USA) was used at a dilution of 1 in 20. Control sections were incubated with polyclonal goat anti-IP primary antibody at a dilution of 1 in 100 in order to demonstrate specificity of the secondary antibody for the mouse primary antibodies. The following day, sections were washed with PBS tween and incubated with a 1 in 500 dilution of biotinylated rabbit anti-mouse IgG for one hour. A further one-hour incubation with the fluorochrome streptavidin 488 Alexifluor (Molecular Probes Inc. Cambridge Bioscience, Cambridge, UK) diluted at 1 in 200 in PBS was performed. Next, sections were incubated for 20 minutes in a PBS solution containing biotin in order to enhance fluorescent signal before re-blocking with 5% non-immune rabbit serum. Incubation with the goat anti-IP antibody (Santa Cruz Inc.) at a 1 in 100

dilution at 4^0C overnight was then performed. A second control slide was incubated with mouse anti-EGFR or mouse anti-CD31 primary antibody. Incubation with a 1 in 200 dilution of rabbit anti-goat peroxidase (Vector Laboratories, Peterborough, UK) secondary antibody was performed for 30 minutes. Tertiary detection was performed with an 8-minute incubation with Tyramide Cy3 solution (PerkinElmer Life Sciences, Boston, USA) at a 1 in 50 dilution as per manufacture's instructions. Slides were counterstained with To Pro (Molecular probes Inc.), at a 1 in 2000 dilution for 2 minutes and then mounted in permafluor.

Statistics

Unless otherwise stated and where appropriate, data were subjected to statistical analysis with ANOVA and Fishers PLSD tests (Statview 4.0; Abacus Concepts Inc., Piscataway, NJ, USA) and statistical significance accepted when $p<0.05$. Semi-quantitative scoring results for immunohistochemical staining were analysed by a non-parametric method, the Kruskall-Wallis test, followed by Dunn's post-hoc multiple comparison test.

4.3 Results

Prostacyclin receptor (IP) mRNA and protein expression within human endometrium

The IP receptor mRNA expression in human endometrium across the menstrual cycle was determined by Taqman quantitative RT-PCR analysis (figure 4.2). IP receptor mRNA was significantly upregulated during the menstrual stage of the cycle when compared to all other stages in the cycle ($p<0.05$).

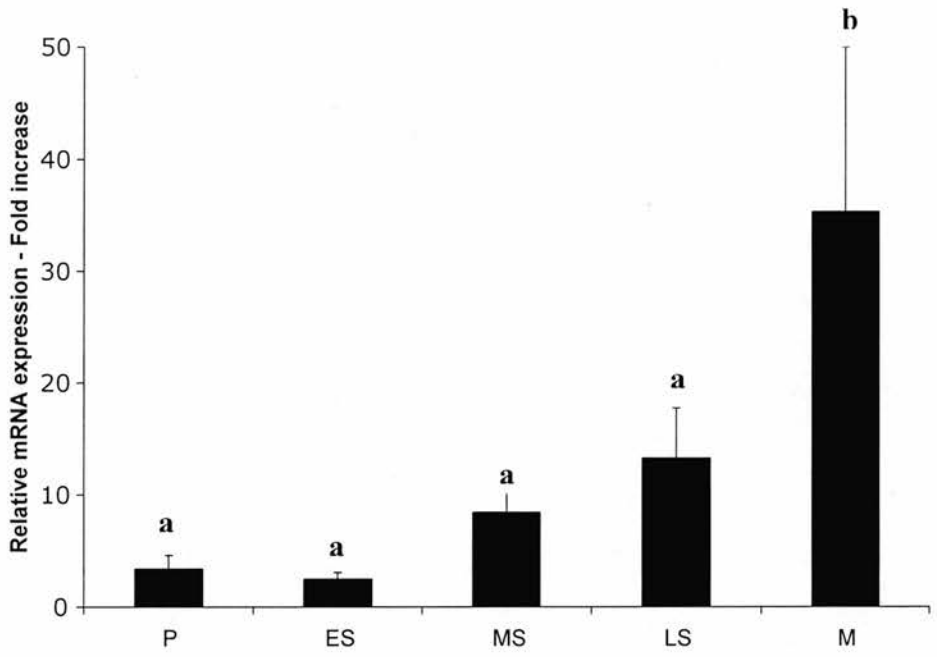


Figure 4.2

Relative mRNA expression of the IP receptor in the human endometrium across the menstrual cycle as determined by real-time quantitative RT-PCR. Results are expressed as the mean ± S.E.M. of relative mRNA expression levels. ***b*** *is significantly elevated from* ***a*** *($p<0.05$). Tissue sample numbers are; n=7 proliferative (P); n=6 early secretory (ES); n=5 mid secretory (MS); n=6 late secretory (LS); n=4 menstrual (M).*

This rise in RNA expression was found to precede the expression of the IP receptor protein as detected by immunohistochemistry. The temporal spatial expression of the IP receptor was examined in human endometrium across the

menstrual cycle (figure 4.3). Subjective scoring of the IP receptor immunoreactivity showed a significant variation of temporal–spatial expression across the menstrual cycle within the glandular epithelium of the functional layer of human endometrium (figure 4.4). IP receptor immunostaining was greatest during the proliferative (figure 4.3 i-iii) and early secretory (figure 4.3 iv) phases within the glandular compartment of the functional layer and was observed to decrease during the late secretory stages (figure 4.3 vi).

Figure 4.3 Immunohistochemical localization of the IP receptor within the human endometrium across the menstrual cycle.

*Variation in temporal/spatial localization of the IP receptor is demonstrated. Glandular epithelial immunostaining (**g**) was present in both basal (**b**) and functional (**f**) layers. Some stromal immunostaining was demonstrated in the functional layer only. Prominent immunostaining of vessel endothelium (**v**) is seen throughout full thickness of sections within endometrial and muscularis layers. Representative sections from; (i) early proliferative; (ii) mid proliferative; (iii) late proliferative; (iv) early secretory; (v) mid secretory (vi) late secretory, (vii) menstrual (insert shows control staining with primary antibody after specific peptide pre-absorbtion) and (viii) muscularis layer are shown.*

(Scale bar =10μm)

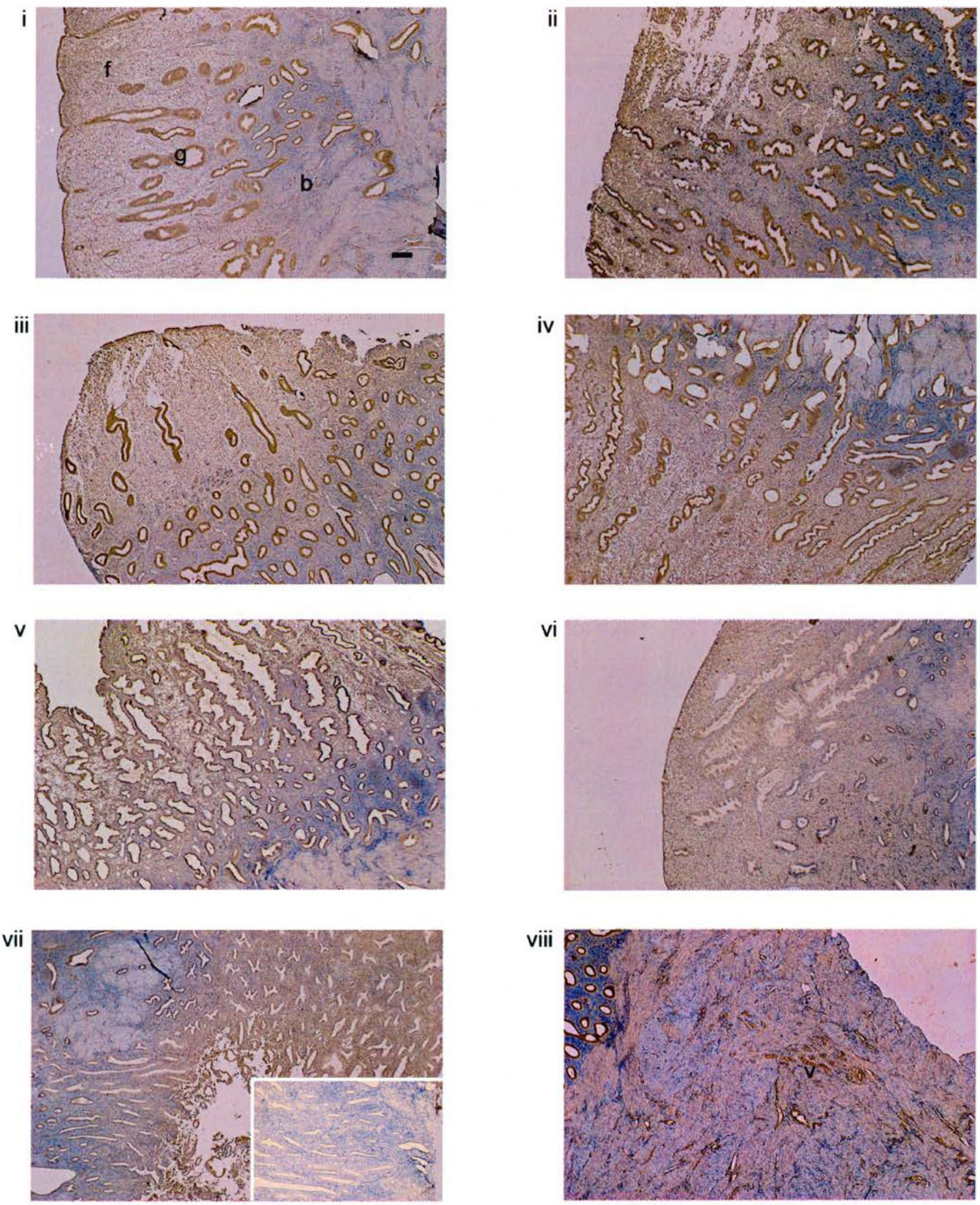


Figure 4.3

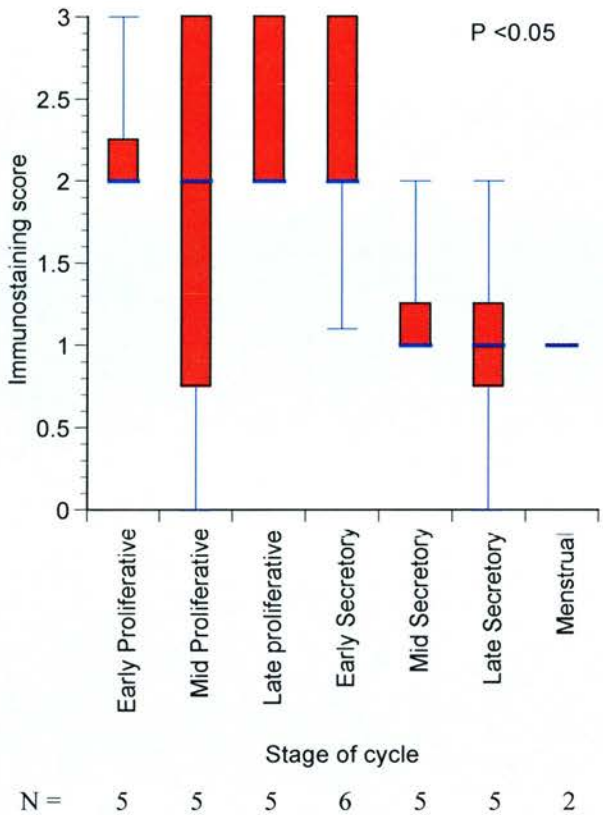


Figure 4.4

Box-plot demonstrating results of subjective scoring of IP receptor immunostaining within the glandular epithelium of the functional layer of human endometrium. Statistical analysis using the non-parametric ANOVA Kruskal-Wallis test indicated that variation of immunostaining intensity was significantly different across the menstrual cycle ($p<0.05$). The data are presented as box-and-whisker plots: box represents the 25^{th} and 75^{th} percentiles and the heavy bar represents the median. The whiskers are the 10^{th} and 90^{th} percentiles. Tissue sample numbers for each stage of the cycle are presented.

High magnification views (figure 4.5A) of a representative endometrial section show the plasma membrane localisation of the IP receptor, with some cytoplasmic immunoreactivity also present. Minimal basal layer stromal immunostaining was observed in all tissue sections across the menstrual cycle in comparison to the functional stroma.

In addition, IP receptor immunoreactivity appeared prominent in vessel endothelium throughout the full thickness of endometrial tissue sections, involving vessels within the endometrial and muscularis layers (figure 4.3 viii). To establish this immunostaining as being vascular in location, dual confocal immunofluorescence immunohistochemistry was used to co-localize the IP receptor with the endothelial cell marker, CD 31 (figure 4.5B). IP receptor (red) was observed to co-localise (yellow) with CD31 (green) in the blood vessels of all tissue sections investigated indicating that IP receptor was present in the vascular compartment.

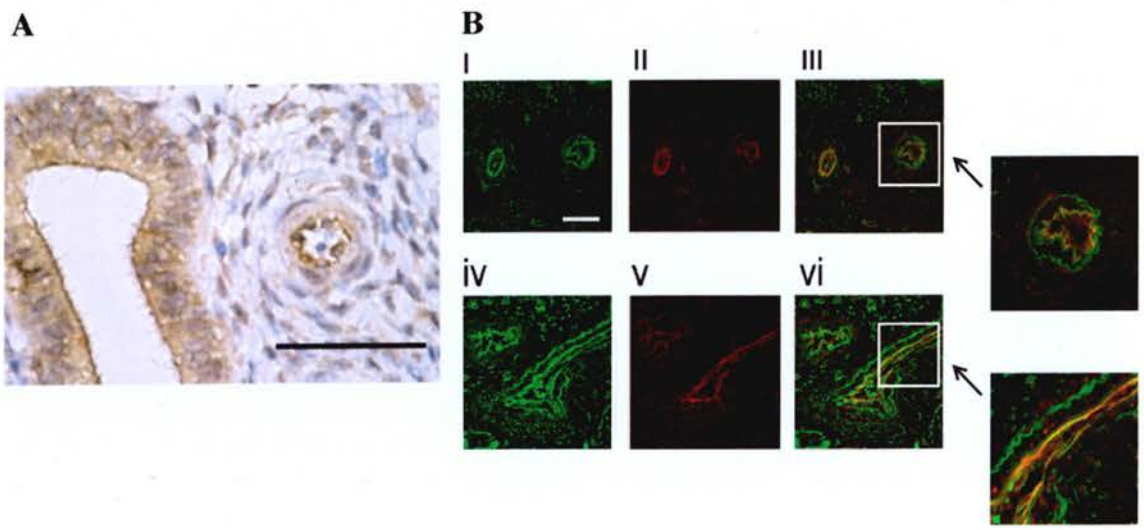


Figure 4.5

(A) High magnification (x100) view of immunohistochemical staining for IP receptor within epithelial gland and blood vessel of the functional layer of early secretory endometrium (Scale bar =10μm).

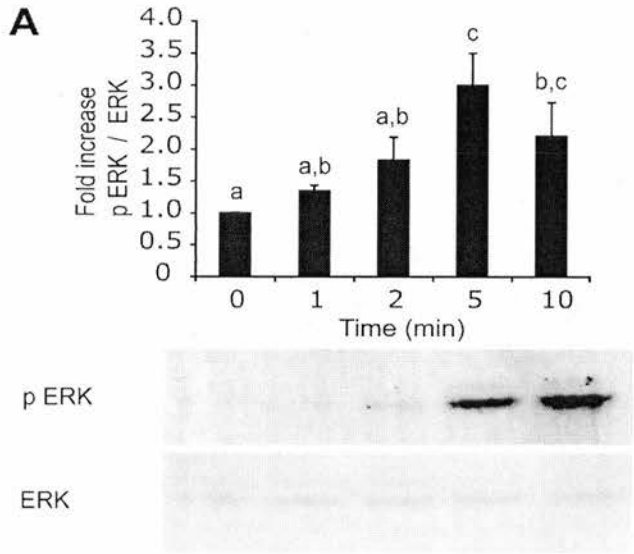
(B) Endothelial staining is confirmed by confocal immunofluorescent colocalization (merged; yellow) of the site of expression of IP receptor (red; figures ii and v) with the endothelial cell marker, CD31 (green; figures i and iv) in early secretory endometrium. Colocalization of IP receptor with CD31 (yellow; figures iii and vi) is demonstrated in vascular endothelium. (Scale bar =10μm)

Iloprost activation of the prostacyclin receptor in the Ishikawa Cell

To investigate the role of the IP receptor in endometrial epithelial cells, the Ishikawa endometrial epithelial cell line was used. This cell line has previously been shown to express functional IP receptors in our laboratory.

Treatment of Ishikawa cells with 100nM Iloprost elicited a significant time-dependent increase in phosphorylation of the ERK1/2 pathway with maximal phosphorylation detected at 5 minutes (figure 4.6A; $p<0.05$). Previous studies in the laboratory have demonstrated that prostanoid (including PGE_2 and $PGF_{2\alpha}$) signalling to downstream MAPK pathways involves transactivation of the EGFR (Sales *et al.*, 2005; Sales *et al.*, 2004a; Sales *et al.*, 2004b). To investigate the potential involvement of the EGFR in transducing the PGI_2-IP receptor signal to ERK1/2, the selective EGFR tyrosine kinase inhibitor, AG1478, was used. Pre-incubation of cells for 1 hour with the EGFR kinase inhibitor (AG1478, 100nM) or the inhibitor of MAPK Kinase (MEK; PD98059, 50μM) abolished the phosphorylation of ERK1/2 in response to a 5 minute 100nM Iloprost treatment (figure 4.6B). No significant alteration in basal levels of ERK phosphorylation was observed in cells treated with chemical inhibitor on its own.

In order to further confirm a role for the EGFR in mediating the signalling of the IP receptor to ERK1/2, Ishikawa cells were co-transfected with a c-myc-tagged ERK cDNA construct with either a dominant negative mutant cDNA isoform of the epidermal growth factor receptor (DN-EGFR) or empty vector cDNA (pcDNA3). Ishikawa cells were then treated with either vehicle or 100nM Iloprost for 5 minutes. The tagged ERK was immunoprecipitated with anti-c-myc antibody and ERK activity of the tagged construct was determined by Western blotting as described in section 3.4.5. Iloprost treatment of Ishikawa cells resulted in a significant phosphorylation of ERK1/2 in cells transfected with the empty vector (figure 4.6C, lane 2). This elevation in ERK1/2 phosphorylation by Iloprost was abolished by co-transfection of cells with the DN-EGFR cDNA (figure 4.6C, lane 3).


A
Fold increase p ERK / ERK
4.0
3.5
3.0
2.5
2.0
1.5
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a
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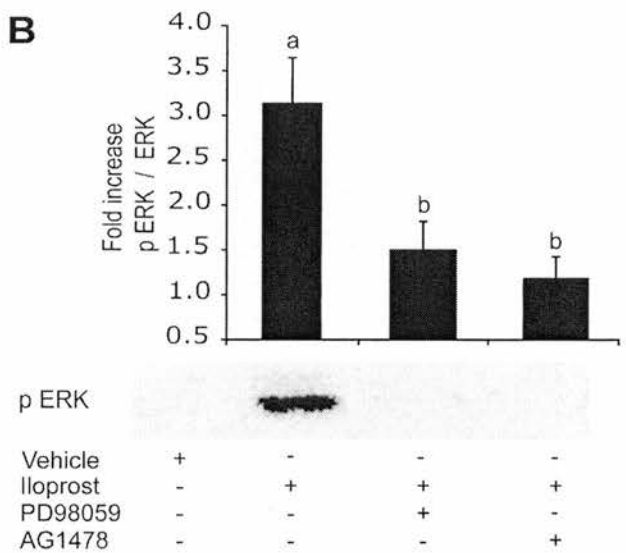
10
Time (min)
p ERK
ERK


B
Fold increase p ERK / ERK
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
a
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b
p ERK
Vehicle + - - -
Iloprost - + + +
PD98059 - - + -
AG1478 - - - +

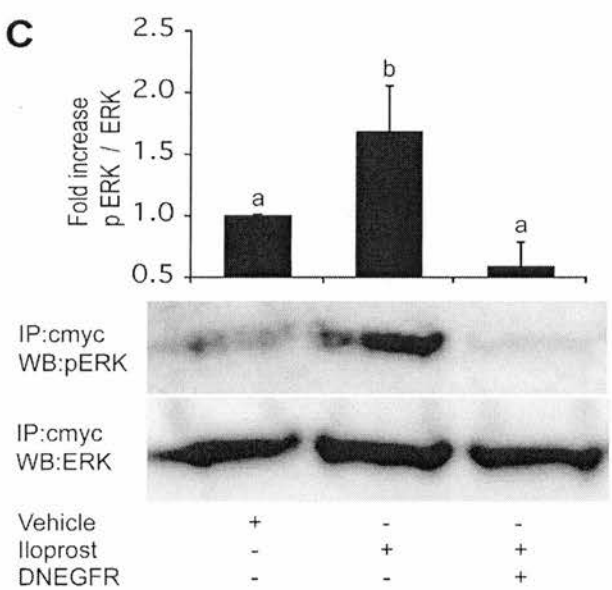

C
Fold increase p ERK / ERK
2.5
2.0
1.5
1.0
0.5
a
b
a
IP:cmyc
WB:pERK
IP:cmyc
WB:ERK
Vehicle + - -
Iloprost - + +
DNEGFR - - +

Figure 4.6 ERK1/2 phosphorylation following treatment of Ishikawa cells with 100nM Iloprost.

*(**A**) Western blot analysis of time course for ERK1/2 phosphorylation. Representative blot demonstrates phosphorylated ERK1/2 (upper panel). Total ERK1/2 was identified by re-probing the same blot with antibody directed against total ERK protein (lower panel). Graph shows semi-quantitative analysis of n=4 experiments as described in the methods. Superscripts indicate significant differences ($p<0.05$).*

*(**B**) Western blot analysis of the effects of chemical inhibitors on ERK1/2 phosphorylation. PD98059 is a MEK inhibitor (inhibitor of ERK phosphorylation), AG1478 is an inhibitor of EGFR tyrosine kinase. For each, a representative blot is shown. Graph shows semi-quantitative analysis of n=3 experiments as described in the methods. **b** is significantly different from **a** ($p<0.05$).*

*(**C**) Ishikawa cells were co-transfected with a c-myc-tagged ERK cDNA construct, together with either dominant negative cDNA isoform targeted against the EGFR (lane 3) or empty vector pcDNA (lanes 1 and 2) and subsequently stimulated with vehicle (lane 1) or 100nM Iloprost (lanes 2 and 3) for 10 minutes. The tagged ERK construct was immunoprecipitated (IP) and ERK phosphorylation of the tagged construct determined by Western blot analysis (WB). Graph represents semi-quantitative analysis of n=4 experiments as described in the methods. (- denotes absence of agent, + denotes presence of agent; **b** is significantly different from **a** ($p<0.05$).*

PGI_2-IP receptor signalling in Ishikawa cells promotes the expression of pro-angiogenic genes

Recent studies have shown that COX enzymes and prostaglandins, such as PGE_2 and $PGF_{2\alpha}$ can promote angiogenesis by up-regulating the expression of pro-angiogenic genes (Sales *et al.*, 2005; Sales *et al.*, 2004a; Seno *et al.*, 2002; Sonoshita *et al.*, 2001; Tsujii *et al.*, 1998) and down regulating the expression of anti-angiogenic genes (Perchick *et al.*, 2003). Since the IP receptor is present in the epithelial and vascular compartment of the human endometrium, the potential role of IP receptor signalling on the expression of the pro-angiogenic genes, bFGF, Ang-1 and Ang-2, was investigated. These genes have been shown to be regulated by prostanoids in other model systems (Sales *et al.*, 2002; Tsujii *et al.*, 1998). Iloprost stimulation of Ishikawa cells caused a significant fold increase in the mRNA expression of the pro-angiogenic factors; bFGF, Ang-1 and Ang-2 at 24 hours compared to earlier time points (figure 4.7A). Co-treatment of the cells with the EGFR kinase inhibitor (AG1478, 100nM) significantly reduced the IP receptor induced mRNA expression of all target genes (figure 4.7B; $p<0.05$).

Prostacyclin receptor signalling in Human Endometrium

To correlate these findings using the Ishikawa cell line to IP receptor signalling in human endometrium *in situ,* human endometrial biopsy explants were used. In order to investigate whether IP signalling in the human endometrium involves cross talk with EGFR, co-localisation of IP receptor with EGFR by confocal immunofluorescence microscopy was examined. Dual immunofluorescence immunohistochemistry (figure 4.8) confirmed co-localization (merged; yellow) of the IP receptor (red, 4.8A and 4.8D) with the epidermal growth factor receptor (green, 4.8B and 4.8E). Co-localisation was most evident within the glandular epithelial compartments in both the basal (A, B and C) and functional (D, E and F) layers of the endometrium, with minimal stromal cell co-localisation.

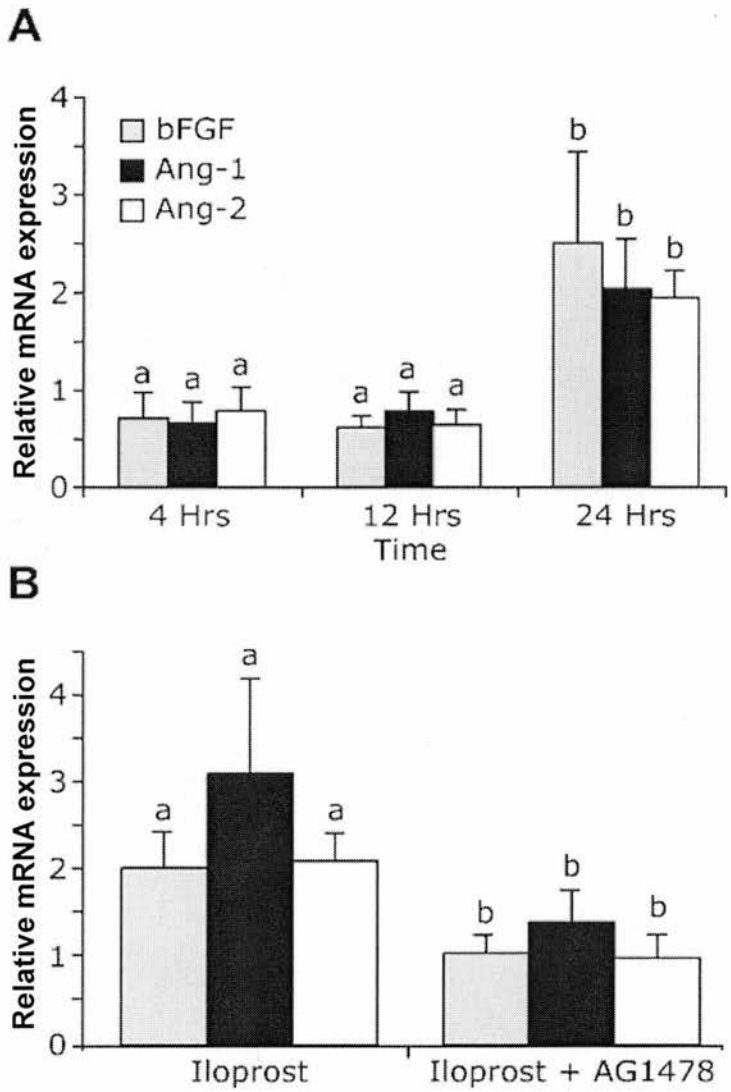


Figure 4.7

(A) Time course demonstrating basic fibroblast growth factor (bFGF), angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) gene expression in Ishikawa cells in response to 100nM Iloprost stimulation. Results are expressed as the mean ± S.E.M. (n=4 experiments). ***b*** *is significantly different from* ***a*** *($p<0.05$).*

(B) mRNA expression of bFGF, Ang-1 and Ang-2 in Ishikawa cells following stimulation with 100nM Iloprost for 24 hours in the absence or presence of EGFR tyrosine kinase inhibitor (AG1478, 100nM) as determined by real-time quantitative RT-PCR. Results are expressed as the mean ± S.E.M. (n=3 experiments). b is significantly different from a ($p<0.05$).

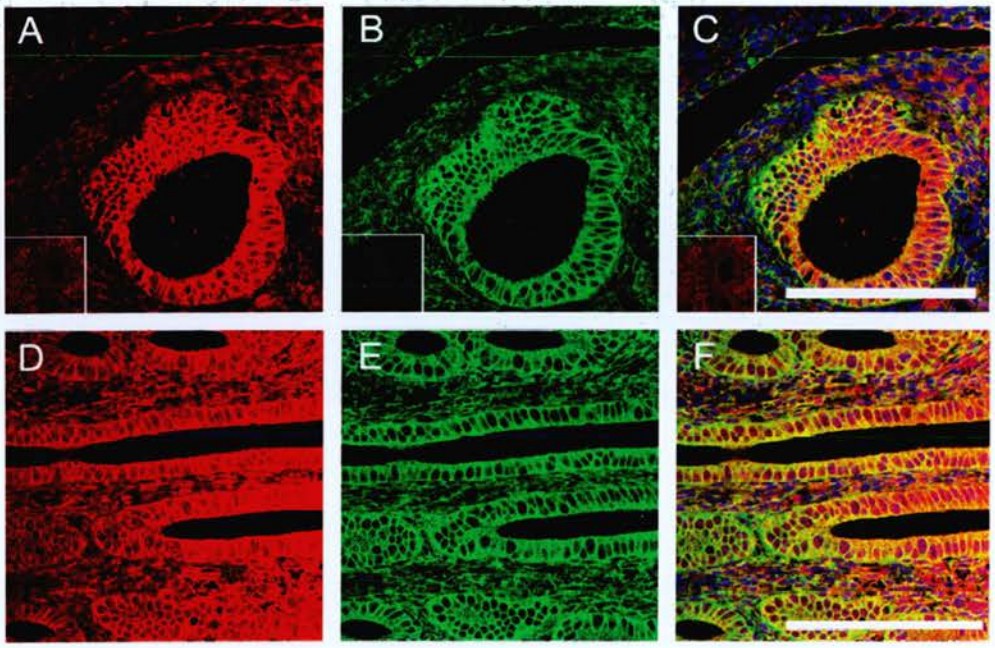


Figure 4.8

Confocal immunofluorescent localization of the site of expression of IP receptor (red; A and D) with EGFR (green; B and E) and co-localization of IP with EGFR (merged; yellow; C and F). Expression is demonstrated in the epithelial cells of the basal (A, B and C) and functional (D, E and F) human proliferative endometrium. Inserts are shown for negative control sections as described in the methods. (Scale bar =10μm).

Subsequently, we assessed the effect of PGI_2-IP receptor signalling on ERK1/2 phosphorylation and pro-angiogenic gene expression in human endometrial tissue. Treatment of human endometrial tissue explants with 100nM Iloprost (figure 4.9) caused a significant phosphorylation of ERK1/2 after 10 minutes. Pre-incubation of the tissue with 100nM AG1478 for one hour abolished the Iloprost-induced phosphorylation of ERK1/2.

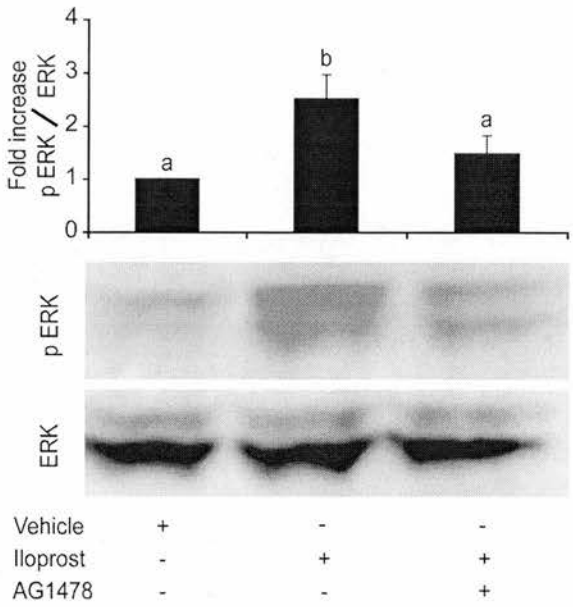


Figure 4.9

Western blot analysis of ERK1/2 phosphorylation following stimulation of human endometrial tissue explants with 100 nM Iloprost for 10 minutes in the absence or presence of AG1478. Graph shows semi-quantitative analysis of n=3 experiments. ***b*** *is significantly different from* ***a*** *($p<0.05$); - denotes absence of agent, + denotes presence of agent.*

As observed in Ishikawa cells, treatment of human endometrial tissue with 100nM Iloprost for 24 hours resulted in a significant increase in bFGF mRNA expression compared with vehicle control ($p<0.05$). Pre-incubation of the tissue with 100nM AG1478 for one hour abolished the Iloprost-induced elevation of bFGF. (figure 4.10). A similar trend in expression of the two angiopoietin genes, Ang-1 and Ang-2, was observed in response to treatment with 100nM Iloprost. Pre-incubation of endometrial tissue with 100 nM AG1478 reduced the Iloprost-mediated elevation of Ang-1 and Ang-2 expression, however the reduction was not statistically significant (figure 4.10).

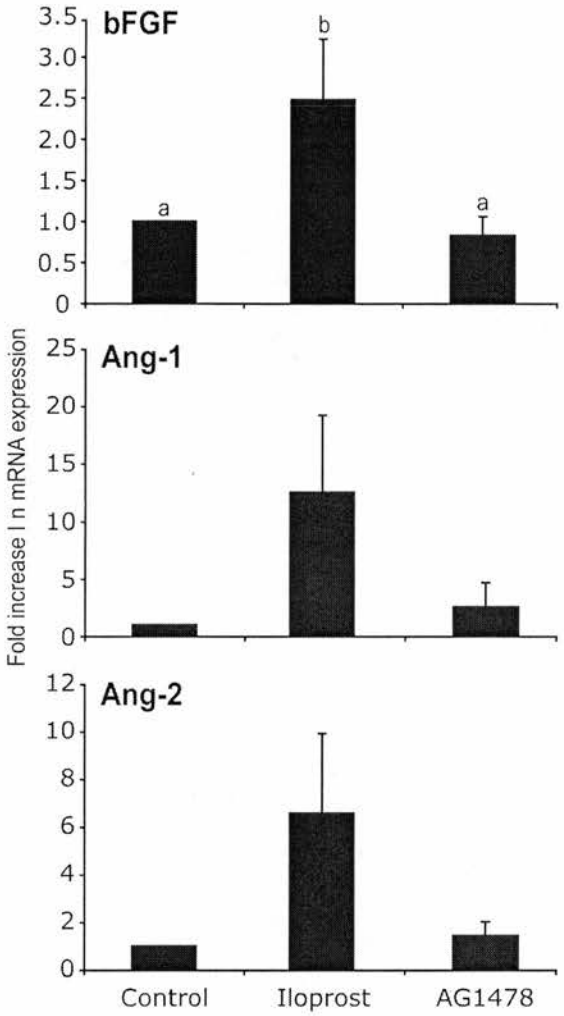


Figure 4.10

mRNA expression of bFGF (top panel), Ang-1 (middle panel) and Ang-2 (lower panel) within normal human endometrial tissue following stimulation with 100nM Iloprost for 24 hours in the presence or absence of AG1478, as determined by quantitative RT-PCR. Results are expressed as the mean ± S.E.M. (n=4 experiments). ***b*** *is significantly different from* ***a*** *($p<0.05$).*

4.4 Discussion

During the reproductive years, in the absence of pregnancy, the human endometrium undergoes a series of cyclical changes culminating in the process of menstruation. This process of physiological injury and repair requires continuous remodelling of the superficial layers of the endometrium together with a concurrent control of vessel remodelling and formation. Studies of angiogenesis in the endometrium have confirmed continuous cycles of angiogenic activity with a number of peaks of activity demonstrated throughout the menstrual cycle (Smith, S. K., 2001). The role of COX enzymes, prostanoids and prostanoid receptors in the reproductive tract has been well documented (Jabbour and Sales, 2004; Sales *et al.*, 2003; Smith, S. K. *et al.*, 1981a; Smith, S. K. *et al.*, 1981b) and the role for prostaglandins in the endometrium reviewed in section 1.4.2 and 1.4.3. Menstrual problems, such as painful periods (dysmenorrhoea), endometriosis and heavy menstrual blood loss (menorrhagia), are linked to prostaglandin signalling. Indeed first line treatment for such complaints involves the use of non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit COX, the rate-limiting enzyme in prostanoid production (Anderson *et al.*, 1976; Cameron *et al.*, 1990; Reid and Virtanen-Kari, 2005).

The data presented in this chapter demonstrate the expression and localisation of the IP receptor in epithelial, endothelial and stromal cells of the human endometrium across the menstrual cycle. IP receptor mRNA is dramatically elevated in human endometrium during the menstrual phase of the cycle and precedes the glandular expression of IP receptor protein, which is highest in proliferative phase of the menstrual cycle. Activation of prostanoid receptors such as EP and FP receptors results in initiation of numerous effector signalling pathways, including the MAPK pathway (Milne and Jabbour, 2003; Sales *et al.*, 2004a; Sales *et al.*, 2004b). The MAPK pathway is a key signalling mechanism that regulates many cellular functions such as growth, differentiation and transformation (Lewis *et al.*, 1998; Naor *et al.*, 2000). The data presented herein also demonstrates that activation of the IP receptor induces a rapid increase in p42 ERK phosphorylation in Ishikawa cells and p42/p44 ERK phosphorylation in endometrial tissue. Interestingly the differential phosphorylation of ERK between Ishikawa cells and endometrial biopsy tissue may

be due the presence of other cells types (stromal and endothelial) within the biopsy tissue compared with the homogeneity of the Ishikawa cells line. This phosphorylation of ERK in Ishikawa cells and endometrial tissues is inhibited with the specific chemical inhibitor of EGFR kinase or by transfection of Ishikawa cells with a dominant negative mutant isoform of the EGFR. Moreover the expression of the EGFR co-localised with IP receptor in the glandular epithelial compartment of the human endometrium. Thus, as observed with prostanoid receptor signalling to downstream ERK1/2 in other model systems (Buchanan *et al.*, 2003; Pai *et al.*, 2003; Pai *et al.*, 2002; Sales *et al.*, 2005; Sales *et al.*, 2004a), in the present study, the EGFR is in close proximity with the IP receptor. EGFR transactivation is required for the PGI_2-IP induced activation of the ERK1/2 signalling pathway within human endometrium. Whether the EGFR is held in a complex of protein-protein interactions with the IP receptor in the glandular epithelial compartment, or whether transactivation of the EGFR is mediated by intermediary scaffold adapter molecules to facilitate ERK1/2 signalling, remains to be determined.

These data also demonstrate that IP receptor activation leads to an increase in expression of several pro-angiogenic factors, including bFGF, Ang-1 and Ang-2 in both the Ishikawa cell and normal human endometrium. bFGF is a potent growth factor, which is known to promote growth and proliferation of numerous cell types by activation of membrane FGF receptor tyrosine kinases. Moreover, bFGF is also known to have potent pro-angiogenic effects in several model systems (Polnaszek *et al.*, 2003; Tsujii *et al.*, 1998), and has been implicated in promoting tumour angiogenesis (Bossard *et al.*, 2004; Kwabi-Addo *et al.*, 2004) and angiogenesis in proliferative lesions of endometriosis (Mihalich *et al.*, 2003). The angiopoietins are a family of growth factors that act as ligands for the largely endothelial restricted Tie-2 receptor tyrosine kinase which is essential for vascular development. Ang-1 is a Tie-2 receptor agonist, which is required for recruitment of perivascular cells leading to the formation and stabilisation of capillaries, vessel maturation and endothelial cell survival (Jabbour *et al.*, 2005). Ang-1 and other angiogenic factors such as VEGF and bFGF may act synergistically to increase vascular sprouting and branching (Jabbour *et al.*, 2005). In addition Ang-1/Tie-2 interaction enhances the mitogenic effect of angiogenic factors such as VEGF on endothelial cell growth (Jabbour *et al.*, 2005). By contrast, Ang-2 is a natural Tie-2 receptor antagonist, destabilising cell contacts

and thus allowing access to angiogenic factors such as VEGF. The process of angiogenesis is thus a fine balance between the expressions of numerous pro-angiogenic factors, all of which may be present concurrently in the cell to regulate vascularization in response to PGI_2-IP receptor interaction.

Furthermore, this chapter demonstrates that the action of PGI_2, via the IP receptor, on target angiogenic gene expression is dependent upon the presence of the EGFR. It is also possible that this up-regulation of angiogenic gene expression is mediated by ERK1/2 phosphorylation. This mechanism of target gene regulation in reproductive cells and tissues via prostanoid-receptor interaction is in agreement with previous studies. Transactivation of the EGFR and ERK1/2 phosphorylation, leading to increased expression of angiogenic genes including vascular endothelial growth factor (VEGF) has previously been shown for PGE_2-EP2 receptor (Sales *et al.*, 2004a) and $PGF_{2\alpha}$ -FP receptor interaction (Sales *et al.*, 2005), suggesting that EGFR transactivation is a central theme for the promotion of vascular function in the human endometrium by prostanoids. The present study focuses on the role of PGI_2-IP receptor signalling to angiogenic genes in epithelial cells, however it is possible that PGI_2-IP receptor signalling in stromal and endothelial cells may act in a synergistic manner to favour angiogenesis in the endometrium, since the parallel studies on whole tissue endometrial biopsy explants are in agreement with the data derived from the Ishikawa cell line.

The precise role of PGI_2 in human endometrium remains to be fully explored, however, PGI_2 has been implicated in menstruation (Baird *et al.*, 1996) and menstrual disturbances, where levels are elevated in endometrial pathologies such as heavy menstrual blood loss (menorrhagia; (Smith, S. K. *et al.*, 1981b). It is thus possible that vascular disturbances in the endometrium of women with menstrual pathologies such as heavy menstrual blood loss may be exacerbated by the elevation of pro-angiogenic factors, such as bFGF, Ang-1 and Ang-2 brought about by enhanced PGI_2-IP receptor signalling. In other studies, an aberration of expression levels of angiogenic growth factors have been demonstrated in endometrium taken from women with heavy menstrual bleeding, such that a decrease in expression of Ang-1 mRNA (Hewett *et al.*, 2002) and an increase of Ang-2 protein (Blumenthal *et al.*, 2002) are reported in endometrium collected from women with heavy menstrual

blood loss compared to control endometrium. These alterations of angiopoietin expression are coincident with an increase in expression of the bFGF receptor (FGF-R1) in the endometrium of women with excessive menstrual blood loss compared to control endometrium (Sangha *et al.*, 1997).

Taken together, these results demonstrate a potential role for PGI_2-IP receptor signalling in human endometrial glandular epithelial cells in regulating the expression of several pro-angiogenic factors, which could influence angiogenesis by acting on adjacent endothelial cells in an autocrine/paracrine manner, and have confirmed these observations using whole tissue biopsy explants from human endometrium. Moreover, these studies have demonstrated a role for IP-EGFR crosstalk in promoting angiogenic factor expression in the endometrium. Blockade of EGFR signalling with an orally active EGFR tyrosine kinase inhibitor has been used successfully in inhibiting angiogenesis in nude mice (Baker *et al.*, 2002). Additionally, in a mouse model of colorectal cancer, studies have demonstrated that a combinatorial approach using a non-selective COX-enzyme inhibitor in combination with an inhibitor of EGFR kinase is of greater therapeutic benefit than either compound on their own (Torrance *et al.*, 2000). These observations of EGFR inhibition and the data described in the present study suggest that targeted inhibition of EGFR function with small molecule chemical inhibitors alone or in combination with a COX enzyme inhibitor may modulate angiogenic activity in the endometrium with possible benefits for menstrual complaints that are associated with aberrant expression and signalling of prostanoids and altered angiogenesis or vascular function (Guidi *et al.*, 1996; Hickey *et al.*, 2000; Kooy *et al.*, 1996; Nap *et al.*, 2004).

Table 4.1 Sequences of primers and probes used for Taqman RT-PCR analysis.

Ang-1	Forward	5'-CTT GTG GCC CCT CCA ATC TA-3'
	Reverse	5'-TAG TGC CAC TTT ATC CCA TTC AGT T-3'
	Probe	5'-TGG TTT TGT CCC GCA GTA TAG AAC ATT CCA T-3'
Ang-2	Forward	5'-GCC GCT CGA ATA CGA TGA CT-3'
	Reverse	5'-ATT AGC CAC TTG CAG CCT CTG CA-3'
	Probe	5'-TTC TCC AGC ACT TGC AGC CTC TGC A-3'
FGF	Forward	5'-CCG ACG GCC GCG TTG AC-3'
	Reverse	5'-GAC ACA ACT CCT CTC TCT T-3'
	Probe	5'-AGA AGA GCG ACC CTC ACA-3'
Prostacyclin receptor (IP)	Forward	5'-GCC CTC CCC CTC TAC CAA-3'
	Reverse	5'-TTT TCC AAT AAC TGT GGT TTT TGT G-3'
	Probe	5'-CCA AGA GCC AGC CCC CTT TCT GC-3'
18S	Forward	5'-CGT CTA CCA CAT CCA AGG AA-3'
	Reverse	5'-GCT GGA ATT ACG GGG GCT-3'
	Probe	5'-TCG TGG CAC CAG ACT TGC CCT C-3'

Chapter 5

Endometrial prostaglandin receptor expression in women with normal and heavy menstrual blood loss.

5.1 Introduction

Initial studies of menstrual fluid identified vasoactive substances with the capabilities of inducing contractions in strips of ileal muscle (Pickles, 1957). These were subsequently identified as the prostaglandins F2α ($PGF_{2\alpha}$) and E2 (PGE_2), the two most abundant prostaglandins found in the endometrium and menstrual fluid (Lumsden *et al.*, 1983). Evidence has since continued to mount, supporting a role for prostaglandins in menstruation. This evidence is reviewed in section 1.4.

The prostaglandin synthesis pathway is described in detail in section 1.3.1. Briefly, the cyclooxygenase (COX) enzymes generate PGH2 from arachidonic acid. There are two main isoforms of the COX enzyme, COX-1 and COX-2, which catalyse the rate-limiting step in prostanoid synthesis. COX-1 is traditionally thought of as a constitutively expressed enzyme in many tissues and generates prostaglandins for normal physiological function. Recent studies have however shown an up regulation of COX-1 expression in various carcinomas (Hwang *et al.*, 1998; Kirschenbaum *et al.*, 2000; Sales *et al.*, 2002). COX-2 is rapidly induced in cells in response to varied stimuli (Vane *et al.*, 1998) such as growth factors, oncogenes and carcinogens. A role has been ascertained for COX-2 in rheumatic diseases, inflammation and tumorigenesis amongst other pathologies (Morita, 2002).

Once synthesised, PGH_2 acts as an intermediary for a specific terminal prostaglandin synthase enzymes. Synthesised prostaglandins mediate their actions via seven-transmembrane G-protein coupled receptors (GPCRs). PGE_2 can couple to four subtypes of GPCRs, which have been pharmacologically classified as EP1, EP2, EP3 and EP4 (Coleman *et al.*, 1994). These receptors are often co-expressed together in the same cell and utilize alternate and in some cases opposing intracellular signalling pathways (Ashby, 1998). EP2 and EP4 receptors, previously demonstrated in human endometrium (Milne et al. 2001), are coupled to Gαs and adenylyl cyclase, resulting in increased formation of cyclic AMP (cAMP). PDE4 is a member of the phosphodiesterase family of enzymes that has been previously demonstrated in human endometrium (Bartsch *et al.*, 2004) and is specific for hydrolysis of cAMP (Sanz *et al.*, 2005). The level of accumulation of the second messenger, cAMP, in response to prostaglandin signalling may therefore, in part, be dependent upon the level of PDE4 activity. The PGE_2 pathway has previously been implicated in the

problem of heavy MBL (Smith, S. K. *et al.*, 1981a; Willman *et al.*, 1976) although specific signalling mechanisms have yet to be elucidated.

Previous investigations into the aetiology of heavy menstrual blood loss (reviewed in section 1.4.2 and 1.4.3) have not been able to discover any differences in circulating steroid hormone levels (Eldred and Thomas, 1994) or any specific histological differences within the endometrium (Rees *et al.*, 1984) of women with heavy MBL compared to women with normal MBL. Additionally, there is no difference in endometrial expression of oestrogen and progesterone receptors (Critchley *et al.*, 1994). However, evidence does exist implicating local mediators, in particular prostaglandins (Sales *et al.*, 2003). Increased levels of total prostaglandins have been found in endometrium taken from women with heavy MBL (Smith, S. K. *et al.*, 1981a). Furthermore, treatment using inhibitors of COX enzymes have repeatedly been shown to reduce menstrual blood loss (Bonnar *et al.*, 1996; Cameron *et al.*, 1990; Coulter *et al.*, 1995) implicating disturbances of prostaglandin pathways in the aetiology of heavy menstrual bleeding although the mechanisms underlying the cause of heavy blood loss remain to be elucidated.

The aim of this chapter is to firstly, outline the expression of the cyclooxygenase enzymes in human endometrium; secondly, determine the pattern of expression of the various components of the COX-prostaglandin signalling pathways present in the endometrium of women with both normal ($\leq$80ml) and heavy MBL (>80ml); and thirdly, to examine the functional capabilities of the PGE_2 signalling pathways within endometrium taken from women with normal and heavy menstrual blood loss. These aims are based upon the hypothesis that disturbances of local mediator signalling, including prostaglandin signalling, are implicated in the aetiology of heavy menstrual blood loss.

5.2 Methods

Tissue Collection and Measurement of Menstrual Blood Loss

Patients complaining of heavy menstruation were recruited from the gynaecological outpatient setting. Ethical approval was obtained from Lothian Research Ethics Committee and written informed consent was obtained from all subjects before tissue collection. (The patient information sheet and ethical approval are included in Appendices.) All subjects were aged 18 to 50 years of age (range 22 to 49; mean 40 years of age). All subjects reported regular menstrual cycles (cycle length, 21-35 days) with no unscheduled, non menstrual, bleeding. No woman had received hormonal preparation in the 3 months preceding biopsy collection. Patients were clinically examined and clinical pelvic abnormalities, such as an enlarged uterus, were further investigated by pelvic ultrasound imaging. Patients with known uterine pathology such as fibroid disease and endometriosis were excluded from the study.

Endometrial biopsies (n=26) were collected for research purposes at different stages of the menstrual cycle with an endometrial suction curette (Pipelle, Laboratoire CCD, Paris, France) from women with measured MBL. No suction curette biopsies were taken during the menstrual phase of the cycle due to technical difficulties in obtaining sufficient quantities of endometrium suitable for RNA analysis.

Table 5.1 Summary of endometrial biopsies collected from women with measured menstrual blood loss.

Stage of Menstrual Cycle	Number of Biopsies	
	Menstrual blood loss ≤80ml	Menstrual blood loss >80ml
Proliferative	7	5
Secretory	6	5
Unclassifiable / Excluded	1	2

Immediately after collection, tissue was divided, transferred into RNA Later (Ambion Inc, Huntingdon, UK) and stored at –70°C (for RNA extraction); fixed in neutral buffered formalin for wax embedding (for immunohistochemical analysis and

histological dating); and placed in RPMI 1640 medium (containing 2 mM L-glutamine, 100 U penicillin and 100 μg/ml streptomycin) and transported to the laboratory for in vitro culture.

In addition to consenting to provide an endometrial biopsy, patients agreed to undergo measurement of menstrual blood loss (MBL) over one menstruation. Measurement was based on a modified alkaline-haematin method as previously described (Hallberg, Nilsson, 1964); see section 3.1.2). Briefly, used sanitary products were added to a measured volume of 5% sodium hydroxide (between 2 to 4 litres). The contents were then left for 48 hours to allow conversion of haemoglobin to haematin. During this same time period a 1 in 200 dilution of the patient's venous blood in 5% sodium hydroxide was made and additionally stored. After 48 hours, an aliquot of sodium hydroxide was removed from the volume soaking the sanitary products and filtered through hardened filter paper (Whartman No 54, Maidstone, UK). The optical density (OD) of menstrual blood loss solution and venous blood sample were then measured using spectrophotometry at 546nm (A546).

MBL was then calculated as a quantity of patient's own venous blood using the following equation (van Eijkeren *et al.*, 1986);

$$\text{MBL} = \frac{(\text{OD of Menstrual Blood Solution} \times \text{Total Volume of added NaOH})}{(\text{OD of Venous Blood} \times 200)}$$

Validation work for this method of measuring menstrual blood loss has previously been carried out by a collaborating research group based in Glasgow (data not shown, see acknowledgements). The range of measured MBL was between 10ml and 567ml with a median MBL of 42ml for the normal group (MBL <80ml) and a median of 183ml for the heavy group (MBL >80ml). The quantification of menstrual blood loss was not normally distributed (figure 5.1). All patients provided informed consent for collection of an endometrial biopsy during the month proceeding or immediately following collection of their menstrual loss.

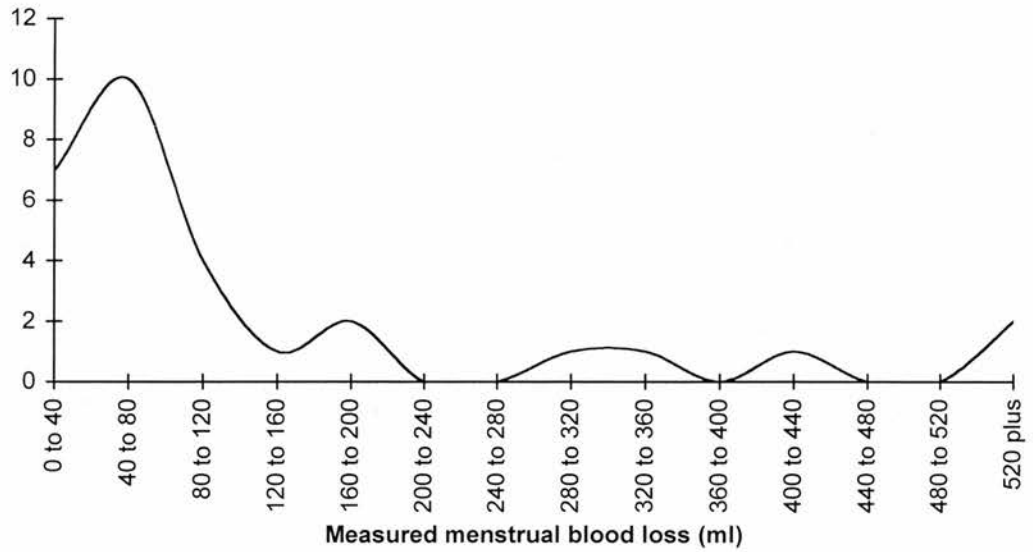


Figure 5.1 Distribution of measured menstrual blood loss plotted against patient numbers

Graph demonstrates the range of measured menstrual blood loss plotted against the patient numbers for each level of menstrual bleeding. Twenty-nine subjects provided a menstrual collection for measurement with a range of menstrual blood loss between 10ml and 567ml.

In addition, full thickness endometrial biopsies at all stages of the menstrual cycle (n=33), were previously collected from women undergoing hysterectomy for benign gynaecological indications. These tissue sections were fixed in neutral buffered formalin and wax embedded. Immunohistochemical analysis of the temporal and spatial expression of the proteins involved in prostaglandin signalling (COX-1, COX-2, FP, EP2, EP4 and IP) was then performed upon the tissue sections. Analysis of expression within full thickness endometrial sections, allowed confirmation of immunohistochemical staining techniques by comparison with previously published work. In addition, carrying out immunohistochemistry on full thickness endometrial tissue sections could provide a detailed immunostaining pattern for comparison with the endometrial suction biopsies taken from women with measured MBL.

All biopsies were dated according to stated last menstrual period (LMP) and dating was confirmed by histological assessment according to criteria of Noyes and co-worker (Noyes, 1950). Furthermore, circulating oestradiol and progesterone serum

levels were measured at the time of biopsy collection and were consistent for both LMP and histological assignment of menstrual cycle stage. This is a robust method for characterizing endometrial samples. Detailed gene microarray studies support this method for characterizing endometrial samples with consistency across these three parameters (Critchley *et al.*, 2006; Talbi *et al.*, 2006).

Inconsistencies between measured circulating hormone levels, stated day of last menstrual period and/or histological assessment led to exclusion of three tissue biopsies from the study.

Taqman quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The expression of COX enzymes (COX 1&2) across the menstrual cycle and expression of prostanoid receptors in endometrium characterised by MBL were investigated by quantitative RT-PCR. Total RNA was extracted from endometrial biopsies using the commercially available product RNeasy Midi Kit (Qiagen Ltd, Sussex, UK) according to the manufacturer's instructions (see section 3.3.2). Each tissue sample was able to provide sufficient quantities of RNA for complete analysis of target genes. Samples were treated for DNA contamination by DNA digestion during RNA purification. Following extraction, total RNA was eluted in 150µl of nuclease free water and stored at -80^0C. Quality of RNA was assessed using the Agilent 2100 Bioanalyser system in combination with RNA6000nano chips (Agilent Technologies, Cheshire, UK). Only RNA that displayed intact 18S and 28S peaks was reverse transcribed to cDNA for real-time PCR analysis. Once extracted and quantified, RNA samples were reverse transcribed as previously described in section 3.3.4. Thereafter, cDNA samples were stored at -20^0C. A tube with no reverse transcriptase was included to control for any DNA contamination.

Real time quantitative PCR was performed using an ABI 7700 Sequence Detection System (Applied Biosystems, Warrington, UK) as previously described (see section 3.3.5) using duplicate samples. A no template control (containing water) was included. The inclusion of ribosomal 18S primers and probes was used to act as a housekeeping gene for each reaction mix. cDNA obtained from a single sample of endometrial tissue was included as a duplicate in all experiments to act as a relative standard against which all expression levels are compared. All primers and probes

were designed using the PRIMER express program (Applied Biosystems, Warrington, UK) and their sequences can be found in table 5.2.

Data were analysed and processed using Sequence Detector version 1.6.3 (Applied Biosystems, Warrington, UK) according to manufacturer's instructions. Expression of target genes were normalised to RNA loading for each sample using 18S ribosomal RNA as an internal standard. All results were expressed relative to the standard cDNA obtained from a single sample of endometrial tissue and included in all experiments.

Table 5. 2 Sequences of primers and probes used for Taqman RT-PCR analysis.

COX-1	Forward	5'-TGT TCG GTG TCC AGT TCC AAT A-3'
	Reverse	5'-ACC TTG AAG GAG TCA GGC ATG AG-3'
	Probe	5'-CGC AAC CGC ATT GCC ATG GAG T-3'
COX-2	Forward	5'-CCT TCC TCC TGT GCC TGA TG-3'
	Reverse	5'-ACA ATC TCA TTT GAA TCA GGA AGC T-3'
	Probe	5'-TGC CCG ACT CCC TTG GGT GTC A-3'
PGES(MT)	Forward	5'-CCT CAT CAG CAA GCG ACT-3'
	Reverse	5'-CCA CTT GTC AGC AGC CTC A-3'
	Probe	5'-AGC AGG CAC CGC CTC CAG-3'
EP1	Forward	5'-AGA TGG TGG GCC AGC TTG T-3'
	Reverse	5'-GCC ACC AAC ACC AGC ATT G-3'
	Probe	5'-CAG CAG ATG CAC GAC ACC ACC ATG-3'
EP2	Forward	5'-GAC CGC TTA CCT GCA GCT GTA C-3'
	Reverse	5'-TGA AGT TGC AGG CGA GCA-3'
	Probe	5'-CCA CCC TGC TGC TGC TTC TCA TTG TCT-3'
EP3	Forward	5'-GAC GGC CAT TCA GCT TAT GG-3'
	Reverse	5'-TTG AAG ATC ATT TTC AAC ATC ATT ATC A-3'
	Probe	5'-CTG TCG GTC TGC TGG TCT CCG CTC-3'
EP4	Forward	5'-ACG CCG CCT ACT CCT ACA TG-3'
	Reverse	5'-AGA GGA CGG TGG CGA GAA T-3'
	Probe	5'-ACG CGG GCT TCA GCT CCT TCC T-3'
PGFS	Forward	5'-GGG ATC TCA ACG AGA CAA ACG-3'
	Reverse	5'-AAA GGA CTG GGT CCT CCA AGA-3'
	Probe	5'-TGG ACC CGA ACT CCC CGG TG-3'
FP	Forward	5'-GCA GCT GCG CTT CTT TCA A-3'
	Reverse	5'-CAC TGT CAT GAA GAT TAC TGA AAA AAA TAC-3'
	Probe	5'-CAC AAC CTG CCA GAC GGA AAA CCG-3'
TBXS	Forward	5'-CAG GTG TTG GTT GAG AAC TTC AGT A-3'
	Reverse	5'-TGT CGG CTA CCG ACT TGA ACT-3'
	Probe	5'-CAA ACC CGA CGC CAT TCT GTT GGT AAA G-3'
TBXR	Forward	5'-TGG TGG TGG CCA GCG T-3'
	Reverse	5'-CGG GTT TCG CAG CAC TGT-3'
	Probe	5'-TGC CCC TTC TGG TCT TCA TCG CCC-3'
PGIS	Forward	5'-ACG CAG ATG TGG AGA TCC CT-3'
	Reverse	5'-GTC GTG TTC CGG CTG CA-3'
	Probe	3'-CCT CAG CAG GTA CGG CTT CGG TCT G-5'
IP	Forward	5'-GCC CTC CCC CTC TAC CAA-3'
	Reverse	5'-TTT TCC AAT AAC TGT GGT TTT TGT G-3'
	Probe	5'-CCA AGA GCC AGC CCC CTT TCT GC-3'
PDE4B	Forward	5'-CCT TCA GTA GCA CCG GAA TCA-3'
	Reverse	5'-CAA ACA AAC ACA CAG GCA TGT AGT T-3'
	Probe	5'-AGC CTG CAG CCG CTC CAG CC-3'
18S	Forward	5'-CGT CTA CCA CAT CCA AGG AA-3'
	Reverse	5'-GCT GGA ATT ACG GGG GCT-3'
	Probe	5'-TCG TGG CAC CAG ACT TGC CCT C-3'

Immunohistochemical analysis of cyclooxygenase 1, cyclooxygenase 2 and prostanoid receptors in human endometrium

To investigate the expression of the COX 1 and 2 enzymes and prostanoid receptors in human endometrium, endometrial sections (5μm) were dewaxed in xylene and rehydrated using decreasing grades of ethanol. General immunohistochemical protocols were carried out as described in section 3.2.2 after preliminary optimization experiments had been performed to determine the best protocol environment and antibody concentrations (see table within section 3.2.4 and section 3.2.5 for experimental details). Sections were counterstained with haematoxin, dehydrated in xylene and mounted.

Scoring and analysis of immunoreactivity

The immunostaining intensity of target epitopes in all tissue sections were assessed in a semi-quantitative manner on a 4-point scale: 0 = no immunostaining, 1= mild immunostaining, 2 = moderate immunostaining, 3 = intense immunostaining. All tissue sections were scored blind by two observers. This scoring system has been previously validated in a subset of tissue sections in which immunoreactivity was measured with a computerised image analysis system, a strong correlation between quantitative data derived from the image analysis and subjective scores by a trained observer was obtained (Wang *et al.*, 1998).

Whole tissue cAMP Assay

Endometrial biopsies from proliferative and secretory phases of the menstrual cycle were minced finely with scissors and incubated overnight in RPMI medium containing 3μg/ml indomethacin (an inhibitor of COX-1 and COX-2 enzymes). Following overnight treatment, approximately 1/3 of the tissue was removed and stored at -20^0C. The remaining tissue was incubated in the same medium containing 1mM 1-methyl-3-isobutylxanthine (IBMX, Sigma, Poole, UK) for 30 minutes. (IBMX inhibits the action of phosphodiesterases and prevents the rapid degradation of cAMP.) It was then divided into two portions and treated with control medium or 100nM PGE_2 for 10 minutes. Tissue was then lysed in 0.1M HCl and frozen until assayed. Cyclic AMP concentration was measured by ELISA (Biomol, Affiniti, Exeter, UK) in accordance with manufacturer's instructions and normalised to protein

concentration determined by protein assay according to manufactures instructions (Bio-Rad, Hemel Hempstead, UK, see section 3.4.2 for more detailed methodology).

Statistical Analysis

Where data did not consistently fulfil the assumptions necessary for using analysis of variance and t-test, the non-parametric Mann-Whitney test was used (Graphpad InStat3) and statistical significance accepted when $p<0.05$.

Semi-quantitative scoring results (non-continuous data) for immunohistochemical staining were analysed by a non-parametric method, the Kruskall-Wallis test, followed by Dunn's post-hoc multiple comparison test.

Normally distributed data such as the cyclic AMP assay data were subjected to statistical analysis with ANOVA and Fishers PLSD tests (Statview 4.0; Abacus Concepts Inc., Piscataway, NJ, USA) and statistical significance accepted when $p<0.05$.

5.3 Results

Cyclooxygenase enzymes (COX)

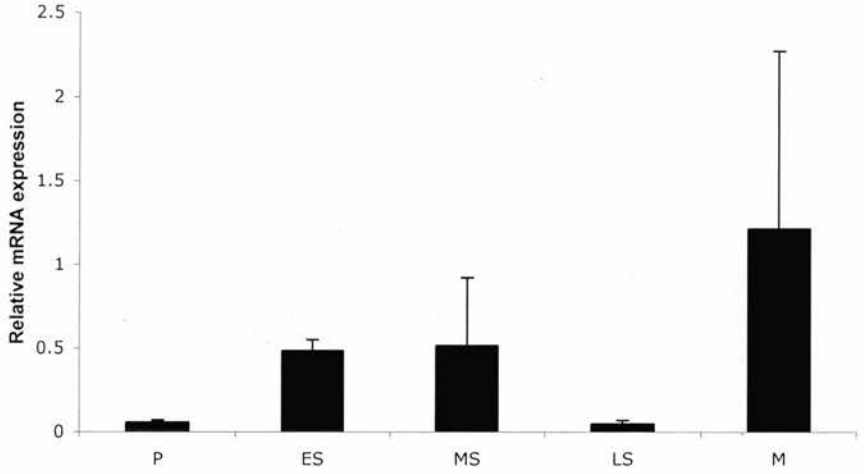
COX mRNA and protein expression within the human endometrium across the menstrual cycle

The expression of COX1 and 2 mRNA in human endometrium was determined by Taqman quantitative RT-PCR analysis (figure 5.2). No significant differences in expression were discovered for COX-1. COX-2 mRNA was shown to be significantly upregulated during the menstrual stage of the cycle when compared to proliferative, mid-secretory and late secretory endometrium ($p<0.05$). A significant increase of COX-2 mRNA expression was also noted in early secretory endometrium (figure 5.2).

Standard immunohistochemical techniques successfully demonstrated spatio-temporal expression of COX-1 and -2 proteins within the endometrium (figure 5.3A and 5.4A). COX-1 immunostaining is demonstrated within glandular epithelial compartment of both basal and functional layers. Some stromal immunostaining for COX-1 was demonstrated in the functional layer only. Vascular endothelial, perivascular cell and strong white blood cell immunostaining is also noted for COX-1. COX-2 immunostaining is also found within the glandular epithelial compartment of both basal and functional layers. The stromal compartment demonstrates immunostaining within the functional layer only. Very little vascular endothelium or perivascular cell immunostaining is noted for COX-2. Subjective scoring of both COX isoform's immunostaining demonstrates a significant variation of temporal spatial expression across the menstrual cycle within normal endometrium (figure 5.3B and 5.4B). There is a significant variation in protein expression levels within the stroma of the function layer of the endometrium for COX-1 across the menstrual cycle. Variation of temporal spatial COX-2 protein expression is significant within the glandular and stromal compartments of the functional layer of the endometrium and also within the glandular compartment of the basal layer.

Similar localisation of COX-1 and COX-2 is noted within the endometrium, however additional COX-1 staining is prominently seen within white blood cells of the endometrium (figure 5.3A).

COX 1



COX 2

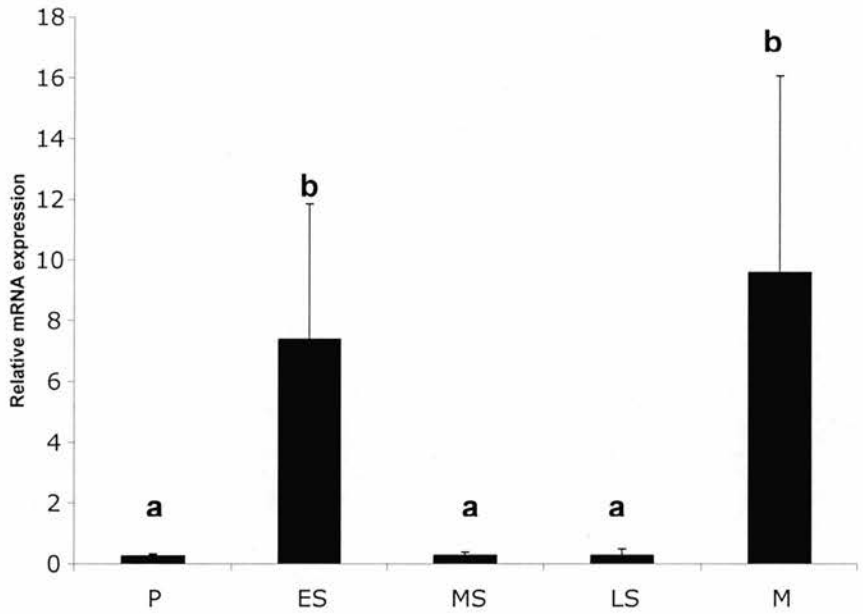


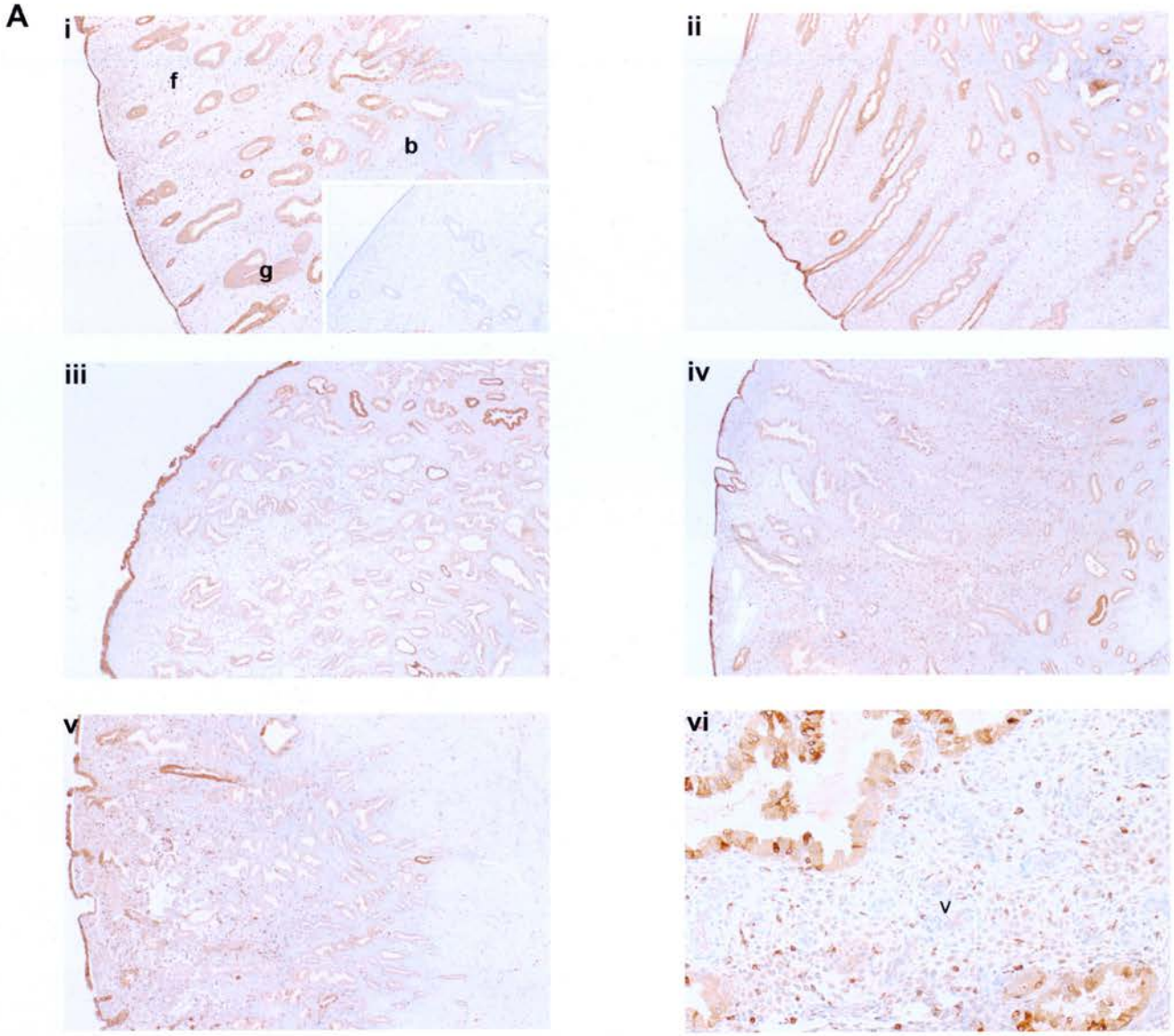
Figure 5.2

Relative mRNA expression of COX 1 and 2 in the human endometrium across the menstrual cycle as determined by real-time quantitative RT-PCR. Results are expressed as the mean ± S.E.M. of relative mRNA expression levels. Note the magnitude of change for COX-2 is greater than COX-1, hence the difference of scales for y-axis. b is significantly elevated from a ($p<0.05$). Tissue sample numbers are; n=7 proliferative (P); n=5 early secretory (ES); n=5 mid secretory (MS); n=6 late secretory (LS); n=4 menstrual (M).

Figure 5.3 Cyclooxygenase 1 Immunohistochemistry

*(A) Immunohistochemical localization of COX-1 within the human endometrium across the menstrual cycle. Variation in temporal/spatial localization of COX-1 is demonstrated. Glandular epithelial immunostaining (**g**) was present in both basal (**b**) and functional (**f**) layers. Some stromal immunostaining was demonstrated in the functional layer only. Representative sections from; (i) proliferative (insert shows control staining with primary antibody after specific peptide pre-absorbtion); (ii) early secretory; (iii) mid secretory; (iv) late secretory; (v) menstrual are shown. (vi) High magnification view of the functional layer of late secretory endometrium demonstrating immunohistochemical staining for COX-1 within epithelial gland and spiral arterial blood vessel endothelial and perivascular cells (**v**). Note the strong immunostaining of white blood cells distributed around the spiral arteriole vessel (**v**).*

(B) Box-plots demonstrating results of subjective scoring of COX-1 immunostaining within the cellular compartments of human endometrium. Statistical analysis using the non-parametric ANOVA Kruskal-Wallis test indicated that variation of immunostaining intensity was significantly different across the menstrual cycle ($p<0.05$) for COX-1 within the stroma of the functional layer. The data are presented as box-and-whisker plots: box represents the 25^{th} and 75^{th} percentiles and the heavy bar represents the median. The whiskers are the 10^{th} and 90^{th} percentiles. P, proliferative (n=16); ES, early secretory (n=6); MS, mid secretory (n=5); LS, late secretory (n=5); M, menstrual (n=2).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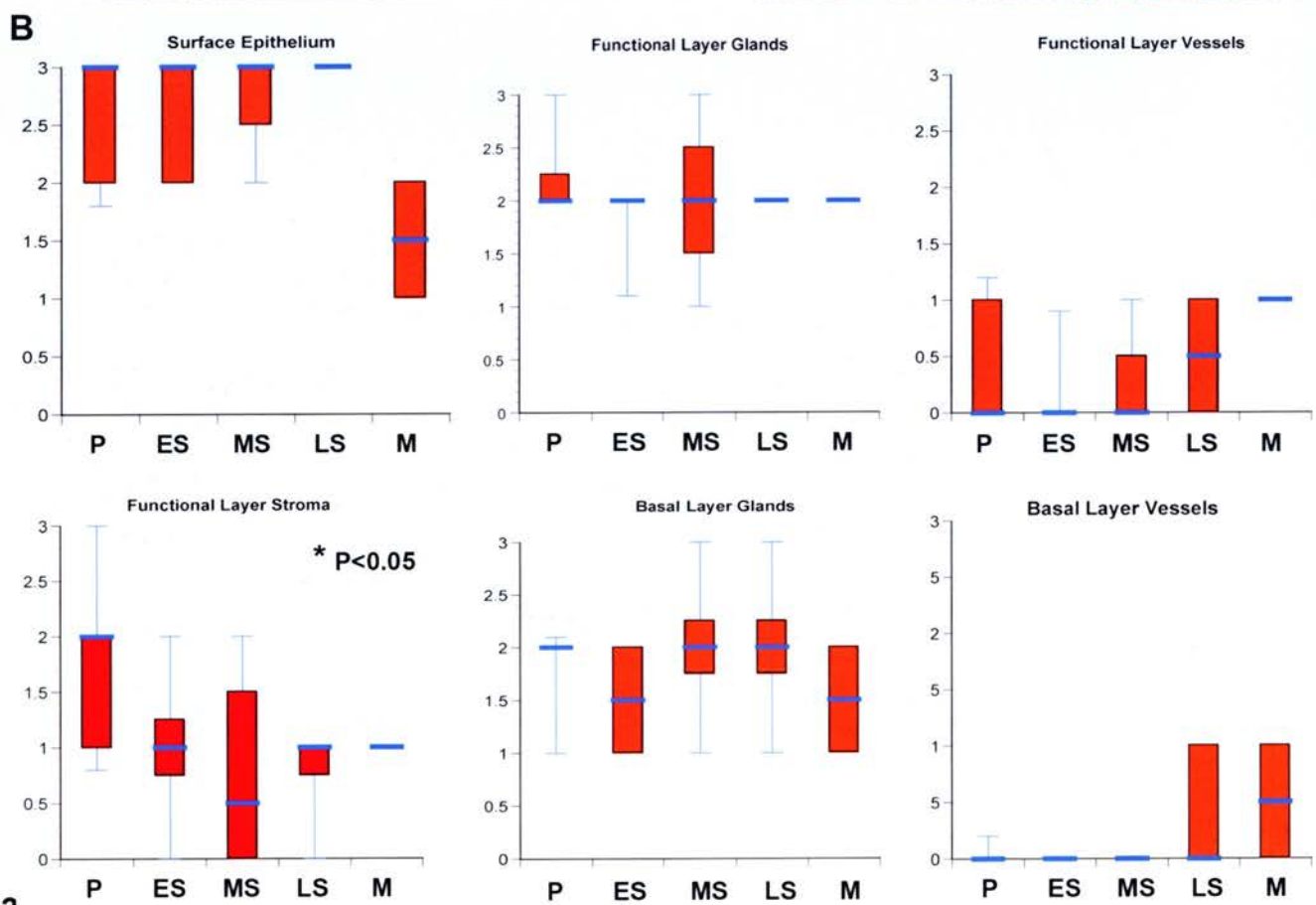
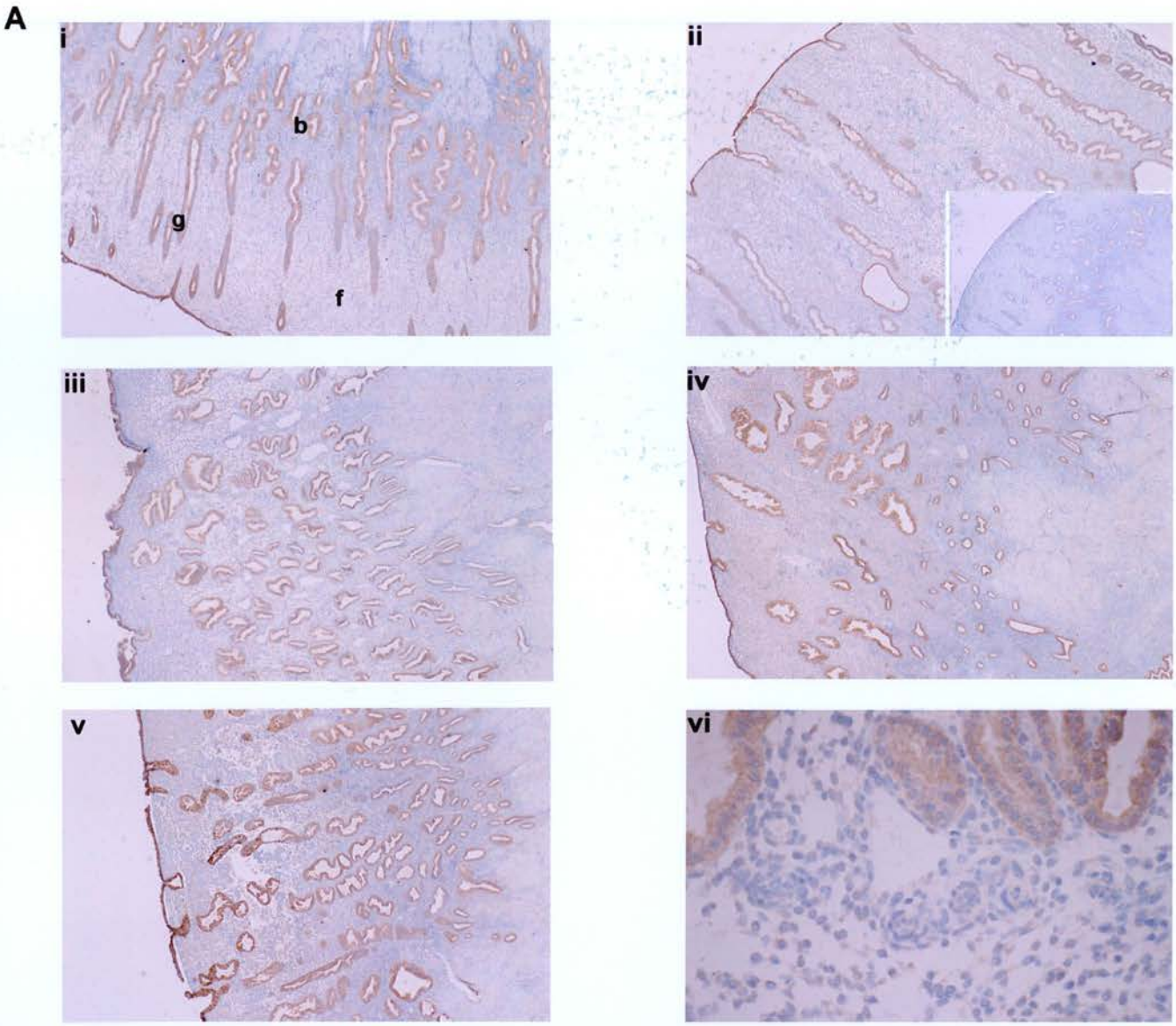

B
Surface Epithelium
Functional Layer Glands
Functional Layer Vessels
Functional Layer Stroma
* P<0.05
Basal Layer Glands
Basal Layer Vessels
P
ES
MS
LS
M

Fig. 5.3

Figure 5.4 Cyclooxygenase 2 Immunohistochemistry

*(A) Immunohistochemical localization of COX-2 within the human endometrium across the menstrual cycle. Variation in temporal/spatial localization of COX-2 is demonstrated. Glandular epithelial immunostaining (**g**) was present in both basal (**b**) and functional (**f**) layers. Some stromal immunostaining was demonstrated in the functional layer only. No vessel staining was detected in the vessel endothelium within the basal layer. Representative sections from; (i) proliferative; (ii) early secretory (insert shows control staining with primary antibody after specific peptide pre-absorbtion); (iii) mid secretory; (iv) late secretory; (v) menstrual are shown. (vi) High magnification view of immunohistochemical staining for COX-2 within the functional layer of late secretory endometrium. Note the reduced immunostaining within vessel endothelium and perivascular cells compared to figure 5.3 vi. There is also an absence of immunostaining for white blood cells.*

(B) Box-plots demonstrating results of subjective scoring of COX-2 immunostaining within the cellular compartments of human endometrium. Statistical analysis using the non-parametric ANOVA Kruskal-Wallis test indicated that variation of immunostaining intensity was significantly different across the menstrual cycle ($p<0.05$) for COX-2 within the epithelial glands and stroma of the functional layer and also within the epithelial glands of the basal layer. The data are presented as box-and-whisker plots: box represents the 25^{th} and 75^{th} percentiles and the heavy bar represents the median. The whiskers are the 10^{th} and 90^{th} percentiles. P, proliferative (n=16); ES, early secretory (n=6); MS, mid secretory (n=5); LS, late secretory (n=5); M, menstrual (n=2).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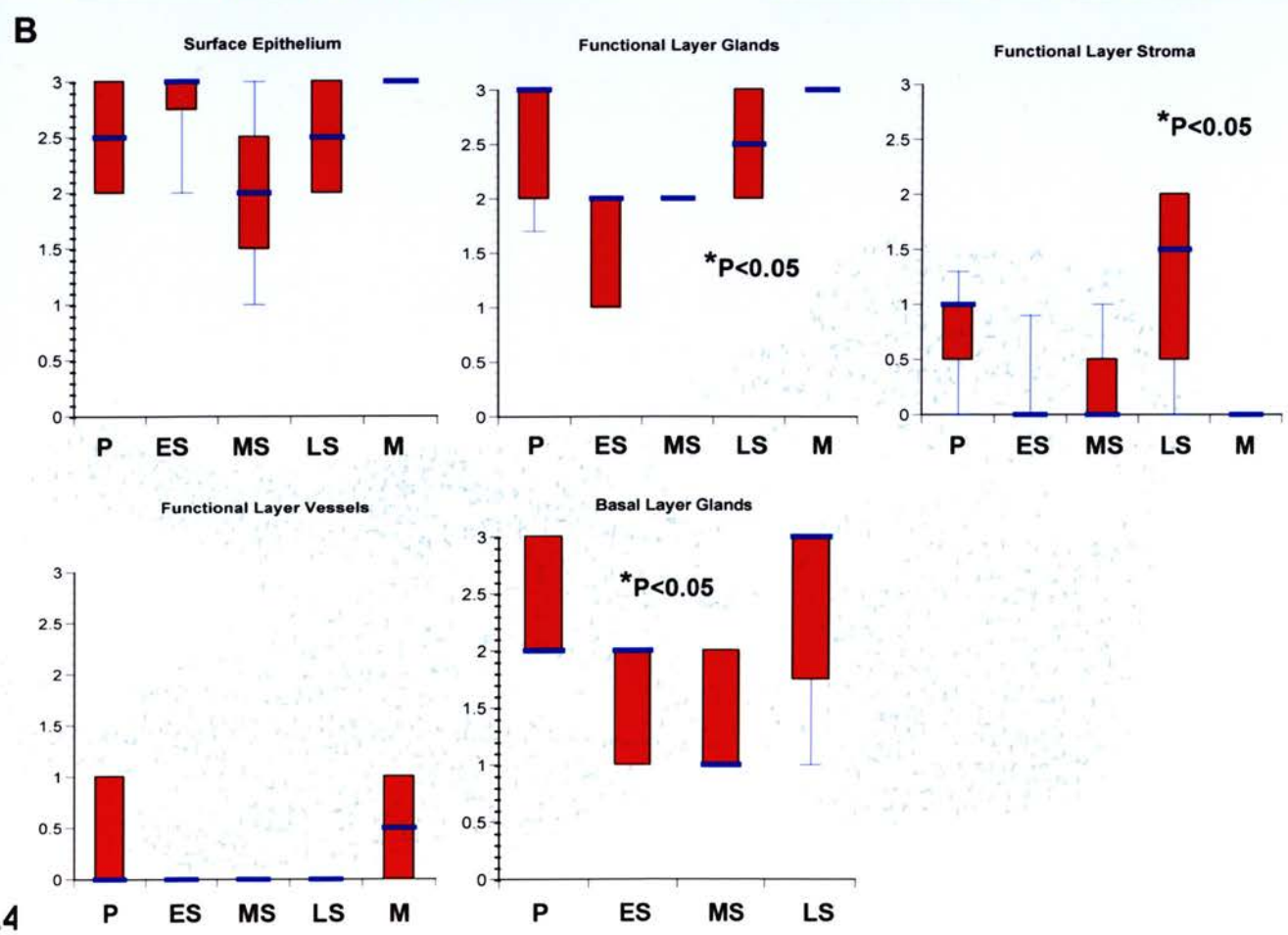

B
Surface Epithelium
Functional Layer Glands
*P<0.05
Functional Layer Stroma
*P<0.05
Functional Layer Vessels
Basal Layer Glands
*P<0.05
P
ES
MS
LS
M

Cyclooxygenase mRNA and protein expression within endometrium obtained from women with measured menstrual blood loss.

Quantitative RT-PCR analysis

Analysis of mRNA expression for a variety of genes associated with prostanoid signalling within the endometrium by quantitative RT-PCR was performed. Analysis of COX-1 and COX-2 mRNA expression demonstrated a significant increase in secretory endometrium of women with MBL in excess of 80ml compared to endometrium from women with MBL less than 80ml (figure 5.5).

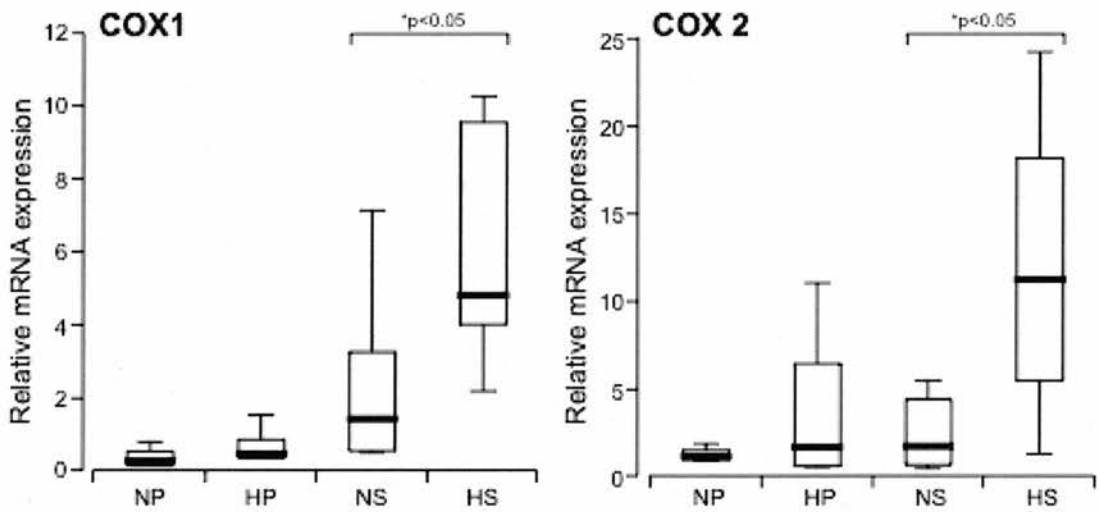


Figure 5.5

*Box-plot demonstrating relative cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) mRNA expression in proliferative and secretory endometrium of women with normal and heavy measured menstrual blood loss (normal MBL ≤80ml, heavy MBL >80ml). The data are presented as box-and-whisker plots: box represents the 25th and 75th percentiles and the heavy bar represents the median. The whiskers are the 10th and 90th percentiles. NP, normal proliferative (n=7); HP, heavy proliferative (n=5); NS, normal secretory (n=6); HS, heavy secretory (n=5). * indicates significant difference (p<0.05)*

Immunohistochemical Analysis

Immunohistochemical staining was performed upon the suction curette endometrial biopsies obtained from women with measured menstrual blood loss for the inducible form of the cyclooxygenase enzyme-COX2. Immunostaining intensities were analysed and subjectively scored using a semi quantitative scoring system (section 3.2.3). No differences in protein expression were detected in the different endometrial cellular compartments between women with normal and heavy MBL. An explanation for the lack of detection of differences in protein expression to support mRNA data is proposed in this chapter's discussion.

Prostanoid receptor and synthase expression within endometrium obtained from women with measured menstrual blood loss

Quantitative RT-PCR analysis

Analysis of synthase enzymes and respective prostanoid receptors downstream of COX action within prostaglandin signalling was performed. There were no significant differences in mRNA expression detected for the prostanoid synthase enzymes - prostacyclin, thromboxane, PGE_2 and $PGF_{2\alpha}$ synthases.

No significant differences in endometrial expression were detected for prostacyclin or thromboxane receptors. PGE_2 has four known receptors for its ligand action. No significant differences in expression were detected between the two groups of endometrial samples for EP2, EP3 and EP4. The mRNA expression of the PGE_2 receptor, EP1, was significantly increased in secretory endometrium of women with heavy MBL compared with normal MBL. Finally, mRNA expression of the receptor for $PGF_{2\alpha}$ (FP) was significantly reduced in proliferative endometrium of women with heavy MBL compared to normal MBL (figure 5.6).

Immunohistochemical analysis

In addition to immunohistochemistry for COX2, staining was performed upon the suction curette biopsies collected from women with measured MBL for PGE_2 receptors EP2 and EP4 together with the IP receptor (chapter 4). Using a semi quantitative scoring system to measure scoring intensity, no differences were noted

between endometrial groups classified according to menstrual blood loss. An explanation for the lack of detection of differences in protein expression to support mRNA data is proposed in this chapter's discussion.

Functional PGE_2 receptor signalling in endometrium of women with measured MBL

The PGE_2 pathway has previously been implicated in the problem of heavy MBL (Smith, S. K. *et al.*, 1981a; Willman *et al.*, 1976). The PGE_2 receptors, EP2 and EP4, are known to activate the cAMP / protein kinase A pathway within the human endometrium (Milne *et al.*, 2001; Regan, 2003). To investigate potential differences in PGE_2 receptor function between endometrial samples of women with measured MBL, we therefore looked at cAMP production as an endpoint for receptor function.

Cyclic AMP production in response to treatment with 100nM PGE_2 was higher in endometrial tissue explants collected from women with heavy MBL compared to endometrium from women with normal MBL (figure 5.7A).

As described, analysis of prostanoid receptor mRNA expression did not detect any significant differences for EP2 or EP4 in endometrium of women with measured MBL. Therefore, in order to investigate whether effects at the post-receptor level could explain this enhanced functionality of the EP receptor, we investigated the expression of phosphodiesterase isotype 4B (PDE4B) in the same endometrial samples. PDE4 is a member of the phosphodiesterase family of enzymes that has been previously demonstrated in human endometrium (Bartsch *et al.*, 2004) and is specific for hydrolysis of cAMP (Sanz *et al.*, 2005). Quantitative RT-PCR analysis demonstrated a significant reduction in expression of PDE4B in secretory endometrium from women with heavy MBL compared to normal MBL (figure 5.7B).

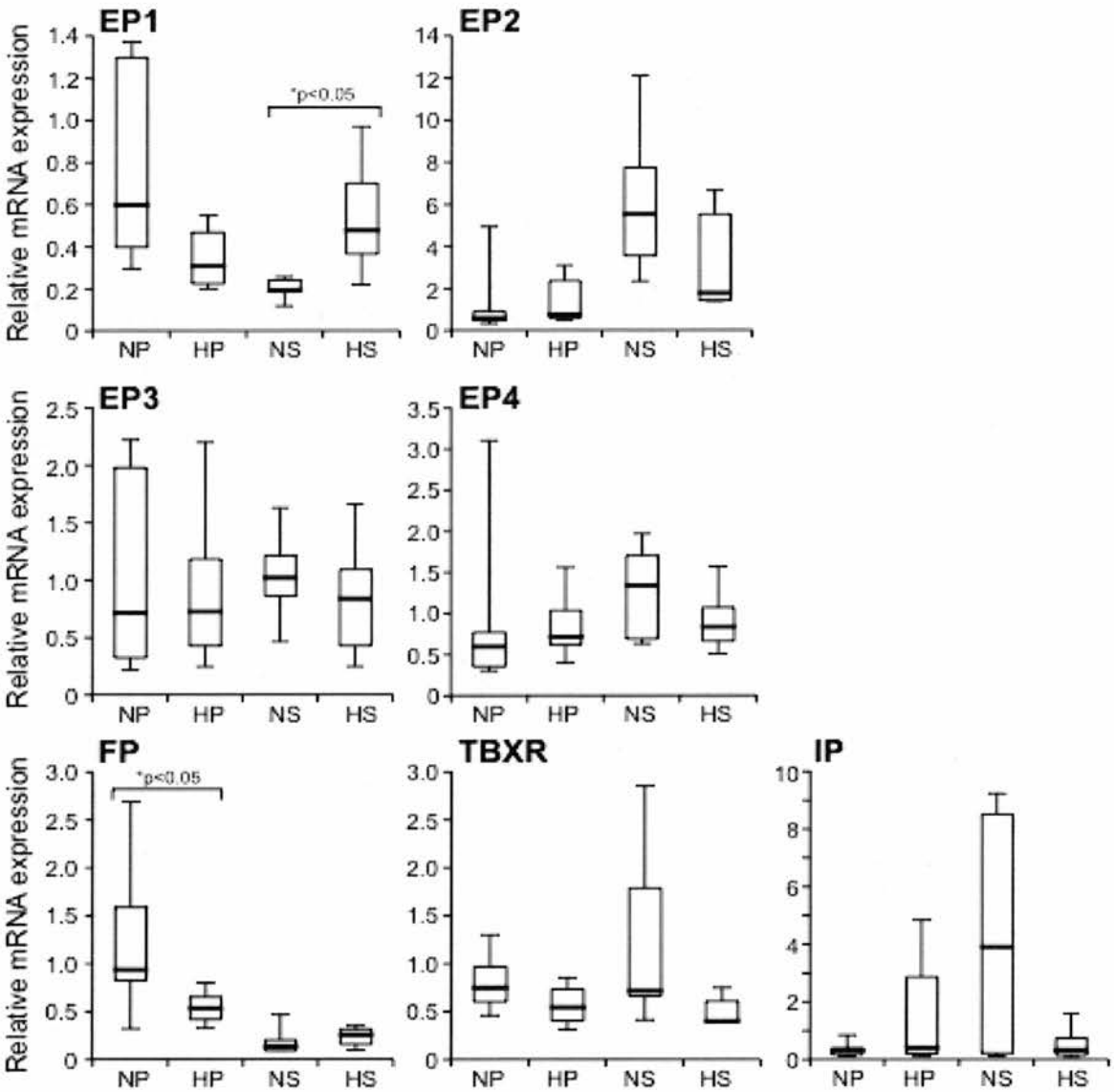


Figure 5.6

*Composite figure of box-plot graphs demonstrating relative mRNA expression for prostanoid receptors in proliferative and secretory endometrium of women with normal and heavy measured menstrual blood loss (normal MBL ≤80ml, heavy MBL >80ml). The data are presented as box-and-whisker plots: box represents the 25th and 75th percentiles and the heavy bar represents the median. The whiskers are the 10th and 90th percentiles. NP, normal proliferative (n=7); HP, heavy proliferative (n=5); NS, normal secretory (n=6); HS, heavy secretory (n=5). EP1 to EP4 are the receptors for prostaglandin E2; FP is the receptor for prostaglandin F2α; TBXR is the receptor for thromboxane; IP is the receptor for prostacyclin. * indicates significant difference (p<0.05).*

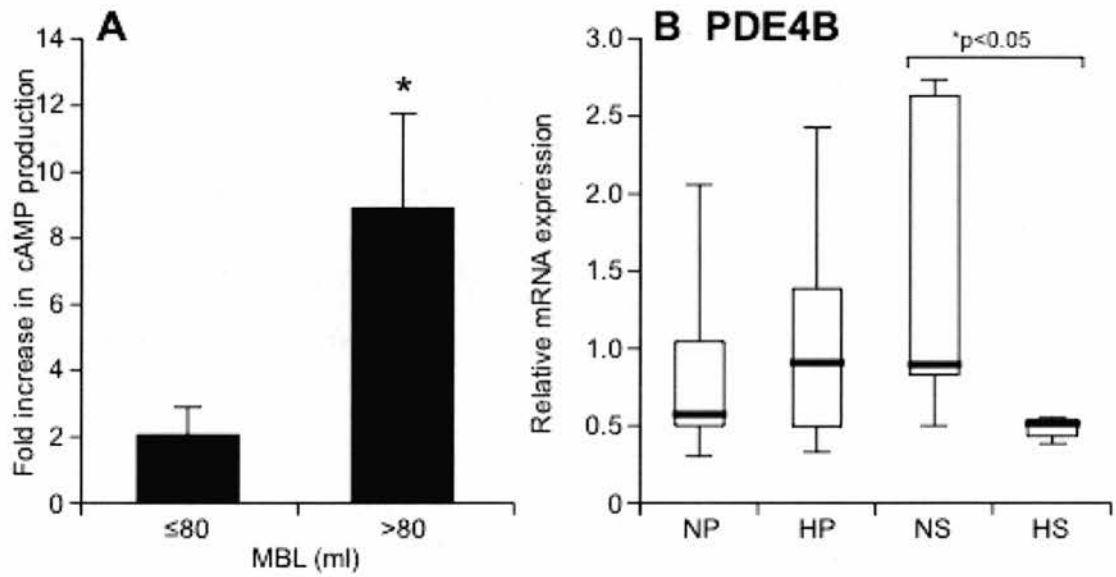


Figure 5.7

*A) Cyclic AMP (cAMP) production by endometrial tissue explants in response to a 10 minute stimulation with 100nM PGE_2 or vehicle. Graph shows average fold increase in cAMP production by endometrium from women with normal and heavy menstrual blood loss (normal MBL ≤80ml, heavy MBL >80ml). * indicates significant elevated cAMP production ($p<0.05$).*

B) Phophodiesterase 4B (PDE4B) mRNA expression in endometrium from women with normal and heavy menstrual blood loss (normal MBL ≤80ml, heavy MBL >80ml) as determined by real time quantitative RT-PCR. The data are presented as box-and-whisker plots: box represents the 25^{th} and 75^{th} percentiles and the heavy bar represents the median. The whiskers are the 10^{th} and 90^{th} percentiles. NP, normal proliferative (n=7); HP, heavy proliferative (n=5); NS, normal secretory (n=6); HS, heavy secretory (n=5). indicates significant difference ($p<0.05$).*

5.4 Discussion

This study demonstrates a significant variation in temporal and spatial protein expression for COX-1 in the endometrial stroma of the functional layer. Otherwise, very little change in expression of either mRNA or protein is noted for COX-1 in the endometrial compartments across the menstrual cycle. However, COX-2 demonstrates marked changes in mRNA expression across the menstrual cycle with co-ordinating differences in protein expression noted, particularly within the endometrial functional layer. These observations are consistent with current thinking that, in the normal physiological state, cells responsible for prostaglandin production constitutively express COX-1 and that COX-2 is the inducible form of the enzyme. However, it is important to note that previous studies have demonstrated an up regulation of COX-1 in various carcinomas (Sales *et al.*, 2002). This present study also demonstrates significantly elevated levels in mRNA expression of both COX-1 and COX-2 in endometrium obtained from women with measured heavy MBL, thereby adding heavy menstrual blood loss to the list of gynaecological complaints demonstrating an alteration in expression of both COX enzymes. Clinically, these data suggest that it is important to use a general inhibitor of both COX-1 and COX-2 enzyme action for treatment of the complaint of heavy menstruation. Mefenamic acid, a COX inhibitor and member of the Fenamate family of drugs, is routinely used as a first line treatment for heavy menstrual bleeding (Reid and Virtanen-Kari, 2005). As well as inhibiting prostaglandin synthesis, fenamates have been shown to inhibit binding of PGE_2 to its receptors (Rees *et al.*, 1988). In keeping with this dual mode of action, the PGE_2 pathway has previously been implicated in the problem of heavy menstrual blood loss. Elevated levels of PGE_2 have been found in endometrium of women complaining of heavy periods (Willman *et al.*, 1976) and further experiments confirmed an apparent shift in synthesis in favour of PGE_2 over $PGF_{2\alpha}$ in the endometrium of women with heavy MBL (Smith, S. K. *et al.*, 1981a).

These current data demonstrate an enhanced PGE_2-EP induced cAMP production in endometrium obtained from women with heavy MBL, however the expression of mRNA for EP2 and EP4 receptors, which are known to couple to cAMP production, does not display any significant correlation with measured MBL in the present studies. Previous published data have demonstrated an increase in

PGE_2 binding sites in uterine tissue associated with the complaint of heavy MBL (Adelantado *et al.*, 1988) although specific receptor subtypes were not identified. Indeed, it is plausible that EP2 and EP4 receptor numbers could be regulated at the post-transcriptional level resulting in increased PGE_2 receptor binding sites. However, in this study, no differences in expression of protein levels for EP2 and EP4 were detected using immunohistochemical techniques. Analysis of protein expression would have been best performed using western blot techniques, however not enough tissue quantity was available to perform these experiments.

$PGF_{2\alpha}$ and PGE_2 are two prominent prostaglandins found in human endometrium (Lumsden *et al.*, 1986b). In the endometrium, $PGF_{2\alpha}$ receptor (FP) and PGE_2 receptors EP2 and EP4 are responsible for their respective ligand action. Whilst there was a reduction in expression of FP mRNA in the endometrium taken from women with heavy MBL, no changes in the expression of EP2 and EP4 mRNA were found. Therefore, a shift of endometrial prostaglandin signalling in favour of the PGE_2 pathway over the $PGF_{2\alpha}$ pathway may exist in the endometrium of women with heavy MBL. Previous work has shown a decrease in the $PGF_{2\alpha}$ / PGE_2 ratio in the endometrium of women with measured heavy MBL compared to women with normal MBL (Smith, S. K. *et al.*, 1981a).

Immunohistochemistry confirmed the cellular localization of expression for COX-2 and the prostanoid receptors. These immunostaining patterns are consistent with previous published studies (Battersby *et al.*, 2004b; Jones *et al.*, 1997; Milne *et al.*, 2001). Using a semi quantitative analysis of staining intensity, no differences in protein expression could be detected in endometrium from women with heavy menstrual blood loss compared to normal MBL. It is expected that protein expression is temporally and spatially regulated within human endometrium (Battersby *et al.*, 2004a; Nayak *et al.*, 2000). However, in order to demonstrate this variation, analysis of full thickness endometrial biopsies, collected at the time of hysterectomy, is preferable. Our endometrial samples were collected by suction curette from, predominantly, the superficial layers of the endometrium. Unfortunately, this mechanism of tissue collection only samples a small portion of the endometrium and tissue architecture is disrupted during the collection process. As patients were not undergoing a hysterectomy, we did not have the benefit of access to full thickness

endometrial biopsies to detect the subtle differences of protein expression expected to exist.

Although it would have been preferable to have quantified protein expression using immunohistochemical staining techniques we feel it is more important that we were able to demonstrate a significant difference in the functional endpoint of the PGE_2 signalling pathway between endometrium taken from women with both normal and heavy menstrual blood loss (figure 5.7A).

The COX2-PGE_2-EP pathway has previously been shown to influence angiogenic factors such as vascular endothelial growth factor (VEGF) through a mechanism mediated by cAMP (Sonoshita *et al.*, 2001). Therefore, the enhanced ability for cAMP production by endometrium taken from women with heavy menstrual blood loss may have important effects on the expression of angiogenic factors. Altered endothelial function as a result of disturbances to angiogenic factors has previously been implicated in heavy menstrual blood loss (Kooy *et al.*, 1996).

Thromboxane and prostacyclin are implicated in platelet function and vascular haemostasis (Grosser *et al.*, 2006) and there is strong expression of their respective receptors in the endometrial vascular compartment (*Chapter 4, Figure 4.4*; Battersby, unpublished data). However, no differences in expression of the genes involved in their signalling pathways downstream of COX enzymes were detected between the two groups of endometrium from women with heavy and normal MBL.

Phosphodiesterases (PDE) are a large family of enzymes that are responsible for the hydrolysis of cyclic nucleotides (Sanz *et al.*, 2005). PDE4B is an isoenzyme that is found in the human endometrium (Bartsch *et al.*, 2001; Bartsch *et al.*, 2004) and shows specificity for hydrolysis of cAMP. We have shown that in addition to an enhanced ability to produce cAMP in response to PGE_2, endometrium taken from women with heavy MBL expresses a significantly reduced level of PDE4B isoform mRNA.

In summary, increased expression of the rate limiting COX enzymes in endometrium from women with heavy MBL will lead to an increase in prostaglandin production and signalling. In addition, enhanced functionality of the EP receptors coupled to cAMP production could in part be explained by a reduction in PDE4B expression within endometrium of women with heavy MBL. These data therefore present a novel mechanism of endometrial prostaglandin signalling that may lead to

the complaint of heavy menstruation. Enhanced COX-PGE_2 signalling and reduced PDE4B expression in endometrium from women with heavy MBL presents us with new therapeutic opportunities in the treatment of heavy menstrual bleeding.

Chapter 6

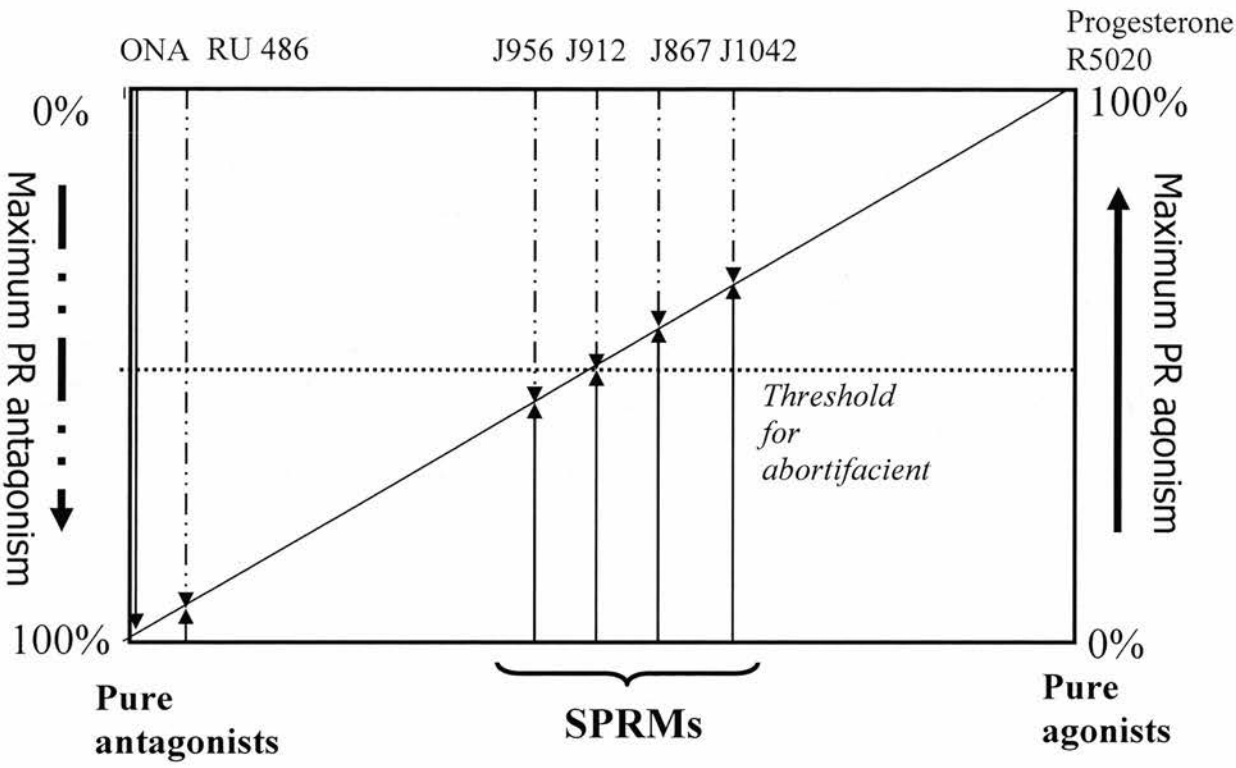
The effect of the Selective Progesterone Receptor Modulators (SPRM; asoprisnil) upon prostaglandin signalling pathways within human endometrium.

6.1 Introduction

The following chapter includes work supported by TAP Pharmaceuticals Products Inc., Lake Forest, IL 60045, USA.

Progesterone plays a crucial role in controlling various reproductive functions. The isolation of progesterone in 1934 (Allen WM, 1934) led to the search for synthetic, orally active progestins (PR agonists) that have found broad applications in fertility control and hormone therapy. Since the discovery of mifepristone (Baulieu, 1989), several progesterone antagonists (PAs) became available for potential clinical use. More recently, selective progesterone receptor modulators (SPRMs) have been synthesised and biologically characterised.

SPRMs represent a new class of PR ligands that exert clinically relevant tissue-selective progesterone agonist, antagonist, partial, or mixed agonist/antagonist effects on various progesterone target tissues in an in vivo situation depending on the biological action studied (Chwalisz *et al.*, 2005)). Asoprisnil (J867) is a novel steroidal compound that belongs to the class of 11β-benzaldoxime-substituted estratrienes (Chwalisz *et al.*, 2005). Asoprisnil demonstrates a high degree of receptor and tissue selectivity, with high-binding affinity for PR, moderate affinity for glucocorticoid receptor (GR), low affinity for androgen receptor (AR), and no binding affinity for oestrogen or mineralocorticoid receptors (DeManno *et al.*, 2003). Primate studies first provided evidence that the use of SPRM may induce amenorrhoea by directly targeting the endometrium and have direct endometrial antiproliferative effects at all times in the menstrual cycle (Chwalisz *et al.*, 2006). Similar effects have since been confirmed in women (Chwalisz *et al.*, 2004). In addition to inducing amenorrhoea, asoprisnil has been shown to shrink fibroid volumes, improve symptoms associated with fibroids and reduce endometriosis-associated pain (Chwalisz *et al.*, 2007; Chwalisz *et al.*, 2005; DeManno *et al.*, 2003). SPRMs are ranked according to their progesterone agonist / antagonist activity (figure 6.1). Asoprinil (J867) has been shown to have a pronounced progesterone agonist activity with endometrial antiproliferative effects and has an absence of abortifact or labour inducing activity (Chwalisz *et al.*, 2005). As such, it is currently undergoing drug trials to assess its suitability as a treatment for fibroid associated problems, including heavy menstrual bleeding.



Schubert et al. *Semin Reprod Med* 2005 **23**: 58-73

Figure 6.1

Diagram represents spectrum of progesterone agonist and antagonist activity. Asoprisnil (J867) has predominantly progesterone agonist activity with some antagonist activity. (ONA, onapristone; SPRMs, selective progesterone receptor modulators; RU486, mifepristone; adapted from (Schubert et al. Semin Reprod Med 2005 ***23****: 58-73)).*

Although the mechanism of asoprisnil-induced amenorrhea is still not completely understood, the current evidence suggests that asoprisnil may have a direct or indirect inhibitory effect on endometrial spiral arteries.

Interestingly, asoprisnil-induced morphological changes in spiral arteries, characterized by thickening of the wall, clearly differ from those commonly observed in women using long-acting progestins or levonorgestrel-containing intrauterine systems. These treatments are associated with the formation of "thin-walled" microvessels that are very fragile, and frequently lead to breakthrough bleeding (Smith, O.P. and Critchley, 2005; Williams *et al.*, 2007).

Disturbances of prostaglandin receptor signalling have previously been implicated in vascular function (Jabbour *et al.*, 2006). This chapter sets out to explore the effect of asoprisnil upon the expression of cyclooxygenase enzymes and components of prostanoid signalling within the endometrium obtained from women who underwent surgical hysterectomy for treatment of a fibroid uterus.

6.2 Methods

Study design

This was a Phase 2 multi-centre randomized double-blind placebo-controlled study of asoprisnil administered for 12 weeks. Dose selection was based upon data from Phase I and Phase II studies (Williams *et al.*, 2007; Chwalisz *et al.*, 2007) which showed asoprisnil at doses of 5 mg, 10 mg, and 25 mg to be safe in subjects treated for 12-weeks. Asoprisnil 10 mg and 25 mg doses and the 12-week duration of treatment were chosen because they were effective for treatment of excessive bleeding, in reducing leiomyoma and uterine volumes, and in decreasing mass effect symptoms, such as pelvic pressure and bloating, over a 3-month treatment period.

At baseline, screening procedures (performed within 45 days of study commencement) included pelvic and breast examination, complete physical examination, Pap test [ThinPrep® Pap Test, Cytyc Corp], ECG, transvaginal ultrasound (TVU), endometrial biopsy [Unimar Pipelle® Endometrial Suction Curette, Medscand], contraception counselling and clinical laboratory investigations - chemistry, haematology, urinalysis, endocrine panel, lipid profile, coagulation screen and serum and urine pregnancy tests. After successful enrolment based on inclusion and exclusion criteria, women were sequentially assigned subject numbers in ascending numerical order that encoded the assignment of the woman, via a randomization schedule, to one of the three treatment arms of the study. Patients were randomized to one of three parallel dose groups in a 1:1:1 ratio to receive daily doses of asoprisnil 10 mg, 25 mg or placebo. Patients and all study personnel were blinded to treatment groups. Asoprisnil or placebo tablets were supplied in blister cards of identical appearance, supplied to the site packaged in sealed kits. Drug was self-administered as an oral dose taken once daily. Treatment was initiated no later than the fifth day of the subject's menstrual cycle, and continued for 12 weeks, when subjects were to undergo hysterectomy within 24 hours of the final dose of drug. The patients returned all used and unused study medication blister cards at eight-week and final visits so that verification of medication compliance could be monitored.

Patients studied

The study group was composed of patients with symptomatic leiomyomata from four centres (Edinburgh, Southampton, Glasgow, Liverpool) in good general health with a menstrual cycle between 17 and 42 days, and symptoms related to overall fibroid size, pressure and/or heavy uterine bleeding, who were scheduled for hysterectomy. Each patient had at least one intramural non-pedunculated, submucosal or subserosal fibroid with a diameter of at least 2 cm or multiple small fibroids with uterine volume $\geq$ 200 cm^3 on ultrasonography. Other inclusion criteria included age over 18 years; negative pregnancy test; a washout period of 4 months for hormonal therapies; serum FSH of <30mIU/mL at commencement; agreement to use double barrier method of contraception (condom/diaphragm/sponge plus spermicide) throughout the study until hysterectomy, unless surgically sterile by bilateral tubal ligation or vasectomy of partner; and normal Papanicolaou (Pap) test. Patients were not permitted to enter the study without a normal endometrial biopsy report based on an adequate specimen taken within 3 months of commencement. Screening data were collected in the clinic by study nurses or gynaecologists. All patients voluntarily signed a full Informed Consent Form. The study was performed according to the ethical principles of the Declaration of Helsinki (1989 revision) and the protocol approved by the Institutional Review Board (Multicentre Research Ethics Committee).

Hysterectomy and processing of uterine samples

The following description of tissue sample processing and histological assessment is provided by Dr Alistair Williams, consultant pathologist supervising tissue processing.

After removal of the uterus, the unfixed specimen was placed on ice immediately and taken without delay to the local pathology laboratory, where the study pathologist or deputy was on hand to open and sample the specimen. The specimen was oriented and a probe inserted through the external os of the cervix to define the position of the cavity. The uterus was opened using a long-bladed knife along the plane of the probe. Endometrial tissue samples were collected from the fundus, the body and the isthmus of the uterus and placed in RNA Later (Ambion Inc, Huntingdon, UK) and stored at -70^0C (for RNA extraction). The remaining opened

specimen was then placed in an adequate volume of 10% buffered formaldehyde and allowed to fix overnight. For large specimens, parallel parasagittal slices 2 cm in thickness were made to permit adequate fixation overnight. The following day, the pathologist sampled the specimen for routine diagnostic reporting, and took additional blocks for study assessment. Endometrial samples were taken from areas away from leiomyomata wherever possible. All specimens were processed by routine methods to paraffin wax, and 3μm haematoxylin-eosin sections were prepared by microtomy and mounted on glass slides. Histological sections of endometrium were considered unsuitable for assessment if a leiomyoma was present within 10 mm of the endometrium.

During Phase I studies with asoprisnil, it became clear that SPRMs including asoprisnil induce unique changes in the endometrium that cannot be assessed by currently used criteria of endometrial dating (i.e. the Noyes criteria) (Noyes *et al.*, 1950), because of differential effects on glands and stroma. As a result, TAP Pharmaceutical Products Inc, Lake Forest, IL, Diagnostic Cytology Laboratories, Indianapolis, IN and a group of expert gynaecological pathologists developed a list of diagnostic criteria that allows for classification of changes induced by SPRMs ("Dictionary of Endometrial Biopsy Diagnoses for Clinical Trials with SPRMs" - data on file). This system supplements the conventional descriptive criteria of the normal menstrual cycle as described by Noyes (Noyes *et al.*, 1950). Endometrium, without pathology, is usually defined histologically as being proliferative, secretory, menstrual, inactive or unsatisfactory for classification. The majority of asoprisnil-treated subjects showed endometrial morphology that was consistently recognisable as showing a "non-physiologic secretory effect". This appearance is unfamiliar to histopathologists, as it is not seen in normal cycling endometrium or in any described condition, and is believed to reflect the partial agonist/antagonist effect of asoprisnil described in animal studies (DeManno *et al.*, 2003). The histological features defining the effect are endometrial glands showing tortuosity similar to the architecture of the secretory phase, but with a rarity of secretory activity or cytoplasmic vacuolation. Glands frequently showed cystic dilatation however, and although nuclear stratification was present, there was a paucity of mitotic activity. Appearances of cystic glandular dilatation are frequently associated in the minds of

histopathologists with simple hyperplasia of endometrium, but the non-physiologic secretory effect differed in that glandular crowding was absent, cystic change was often focal involving one or two glands in a field of non-dilated glands, and mitotic activity was very sparse or absent. Gland dilatation is a feature commonly seen in the inactive, non-secretory endometrium of the perimenopausal and postmenopausal woman, and is not considered indicative of glandular secretory activity. It may in fact represent glands in which the opening to the surface has become occluded, perhaps as a consequence of the lack of secretory activity.

With asoprisnil, the endometrial stroma showed increasing compactness without decidual change, but the most characteristic stromal effect involved vessels. Aggregations of thin-walled vessels were seen in endometrial stroma more frequently in asoprisnil-treated patients than in placebo-treated patients. Additionally, vessels with thickened muscularised walls were seen with greater frequency in asoprisnil patients than in controls.

Taqman quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The endometrial expression of COX enzymes (COX 1&2) and the expression of genes involved in prostanoid signalling were investigated by quantitative RT-PCR. Total RNA was extracted from endometrial biopsies using Trizol reagent (Invitrogen Life technologies, Paisley, UK) followed by the commercially available product RNeasy Midi Kit (Qiagen Ltd, Sussex, UK) according to the manufacturer's instructions (see section 3.3.2). Each tissue sample was able to provide sufficient quantities of RNA for complete analysis of target genes. Samples were treated for DNA contamination by DNA digestion during RNA purification. Following extraction, total RNA was eluted in 150µl of nuclease free water and stored at -80^0C. Quality of RNA was assessed using the Agilent 2100 Bioanalyser system in combination with RNA6000nano chips (Agilent Technologies, Cheshire, UK). Only RNA that displayed intact 18S and 28S peaks was reverse transcribed to cDNA for real-time PCR analysis. Once extracted and quantified, RNA samples were reverse transcribed as previously described in section 3.3.4. Thereafter, cDNA samples were

stored at -20^0C. A tube with no reverse transcriptase was included to control for any DNA contamination.

Real time quantitative PCR was performed using an ABI 7700 Sequence Detection System (Applied Biosystems, Warrington, UK) as previously described (see section 3.3.5) using duplicate samples. A no template control (containing water) was included. The inclusion of ribosomal 18S primers and probes was used to act as a housekeeping gene for each reaction mix. cDNA obtained from a single sample of endometrial tissue was included as a duplicate in all experiments to act as a relative standard against which all expression levels are compared. All primers and probes were designed using the PRIMER express program (Applied Biosystems, Warrington, UK) and their sequences can be found in table 5.2, chapter 5.

The PCR data is analysed by calculating the threshold cycle or C_T for each sample. This is the cycle number (range 0-40) at which the accumulation of PCR product shows exponential growth and crosses a threshold. The higher the initial product sequence of interest, the lower the C_T value, as the product accumulates earlier in the PCR run. This value is then related to the amount of total cDNA loaded by comparing it to the C_T value for the housekeeping gene, 18S. The difference between the two (the $\hat{}C_T$) is then related to the value for the standard cDNA, included in all experiments, giving a $\hat{}\hat{}C_T$ value for each sample. Finally the formula $2^{-\hat{}\hat{}CT}$ is applied. This formula means that the final results will then be comparative to the standard sample and will be normalised for differences in initial cDNA loading. The standard sample always has a figure of 1 and a sample with a final result of 10 has ten times more sequence of interest than the standard sample. If the sequence of interest is not detected after 40 cycles, the CT value is treated as right-censored at 40, and the delta CT values is treated as right-censored at 40-threshold cycle for 18s.

Immunohistochemical analysis of cyclooxygenase 1 and cyclooxygenase 2

To investigate the expression of the COX 1 and COX 2, endometrial sections (5μm) were dewaxed in xylene and rehydrated using decreasing grades of ethanol. The use of the Bond automated IHC machine was carried out as described in section 3.2.5 after preliminary optimization experiments had been performed to determine the best protocol environment and antibody concentrations (see table within section 3.2.4). Sections were counterstained with haematoxin, dehydrated in xylene and mounted.

Semi-quantitative analysis

Immunohistochemical staining was semi-quantitatively assessed using the histoscore method by two separate observers ranking the intensity of immunostaining on a scale of 0 to 3 (0=no staining to 3=intense staining). The amount of tissue in each slide, with specific immunostaining intensity, was estimated as a percentage in graduations of 10%. The final histoscore is an integer between 0 and 300.

Once the slides had been independently scored the values were tabulated and an overall score for each section determined following consultation between the two observers.

Data analysis and statistical methods

The SAS System 8.2 is used by TAP appointed statisticians to perform the statistical analysis. All subjects with evaluable data for a specific imunohistochemical variable are included for analysis of that variable. Based on morphological assessment by consultant pathologist, a placebo subject was classified into proliferative phase or secretory phase by a pre-agreed histological algorithm. There were only 2 samples obtained which were placebo-treated and allocated a proliferative categorisation. Meaningful comparison of asoprisnil treatment endometrium with proliferative endometrium is therefore difficult and differences should be interpreted with caution. Wilcoxon rank sum tests were performed to compare each asoprisnil group with the placebo subjects in the secretory phase. Hochberg's multiple comparison procedure was used to control the familywise Type 1 error rate at the level of 0.05 for each variable.

6.3 Results

Quantitative RT-PCR analysis

Results of mRNA expression for COX-1, COX-2 and various prostanoid signalling genes is summarised in table 6.1 and figure 6.2

Data showed a trend for down regulation of COX-1 mRNA expression in the asoprisnil treated group but statistical significance was not reached. In contrast, the trend in expression of COX-2 is up regulated in the asoprisnil treated groups, approaching significance for the 10mg Asoprisnil group when compared against expression in placebo/secretory tissue.

There is a significant up-regulation of prostaglandin dehydrogenase (PGDH) mRNA in both asoprisnil groups when compared with the placebo/secretory treatment groups. When the prostaglandin E_2 and F_2 series receptors are examined, a dramatic upregulation of FP expression is noted in both asoprisnil treatment groups when compared against the placebo/secretory treatment groups. No differences are noted in the expression of EP4. A significant decrease of mRNA expression is noted for the EP2 receptor in the asoprisnil 10mg group when compared with placebo/secretory endometrium.

Immunohistochemistry Analysis for Cyclooxygenase enzymes

The box plots of figure 6.3 and 6.4 represent the results of the histoscoring for each tissue compartment; surface epithelium, endometrial glands, endometrial stroma and endothelium. There is a significant increase in COX-1 in the glands for both asoprisnil doses when compared against placebo/secretory treatment groups. This effect alters in the surface epithelium where a reduction in expression for COX-1 is noted in the high dose asoprisnil treatment group.

No significant difference for COX-2 expression is detected when analysing immunohistochemical scoring intensity by this method.

Variable	Treatment Group	N	Median	Ratio	P-Value
COX-1	Placebo/Proliferative	2	17.2	-	-
	Placebo/Secretory	8	15.5	1	-
	Asoprisnil 10mg	12	17.3	0.27	0.09
	Asoprisnil 25mg	10	17.3	0.28	0.07
COX-2	Placebo/Proliferative	2	17.0	-	-
	Placebo/Secretory	8	16.9	1	-
	Asoprisnil 10mg	12	14.8	4.2	0.048
	Asoprisnil 25mg	10	15.2	3.2	0.10
PGDH	Placebo/Proliferative	2	18.6	-	-
	Placebo/Secretory	8	20.2	1	-
	Asoprisnil 10mg	12	18.4	3.3	**0.004**
	Asoprisnil 25mg	10	19.0	2.2	**0.02**
EP2	Placebo/Proliferative	2	22.3	-	-
	Placebo/Secretory	8	18.5	1	-
	Asoprisnil 10mg	12	20.6	0.24	**0.004**
	Asoprisnil 25mg	10	19.7	0.43	0.08
EP4	Placebo/Proliferative	2	21.4	-	-
	Placebo/Secretory	8	18.3	1.0	-
	Asoprisnil 10mg	12	18.2	1.09	0.99
	Asoprisnil 25mg	10	17.8	1.41	0.48
FP	Placebo/Proliferative	2	27.7	-	-
	Placebo/Secretory	8	26.8	1.0	-
	Asoprisnil 10mg	12	22.5	18.4	**0.004**
	Asoprisnil 25mg	10	21.6	35.8	**0.001**

Table 6.1 Q-RT-PCR Results for prostanoid signalling pathway mediators

The median represents the difference between the threshold cycles for the target and the reference 18s included in the reaction mix. P values are for each asoprisnil group compared with the placebo/ secretory group using Wilcoxon's rank sum test. The ratio is taken as the amount of target normalised to the reference and relative to the placebo/secretory subjects calculated as; $2^{\wedge(-[\text{median (asoprisnil)} - \text{median (placebo/secretory)}])}$.

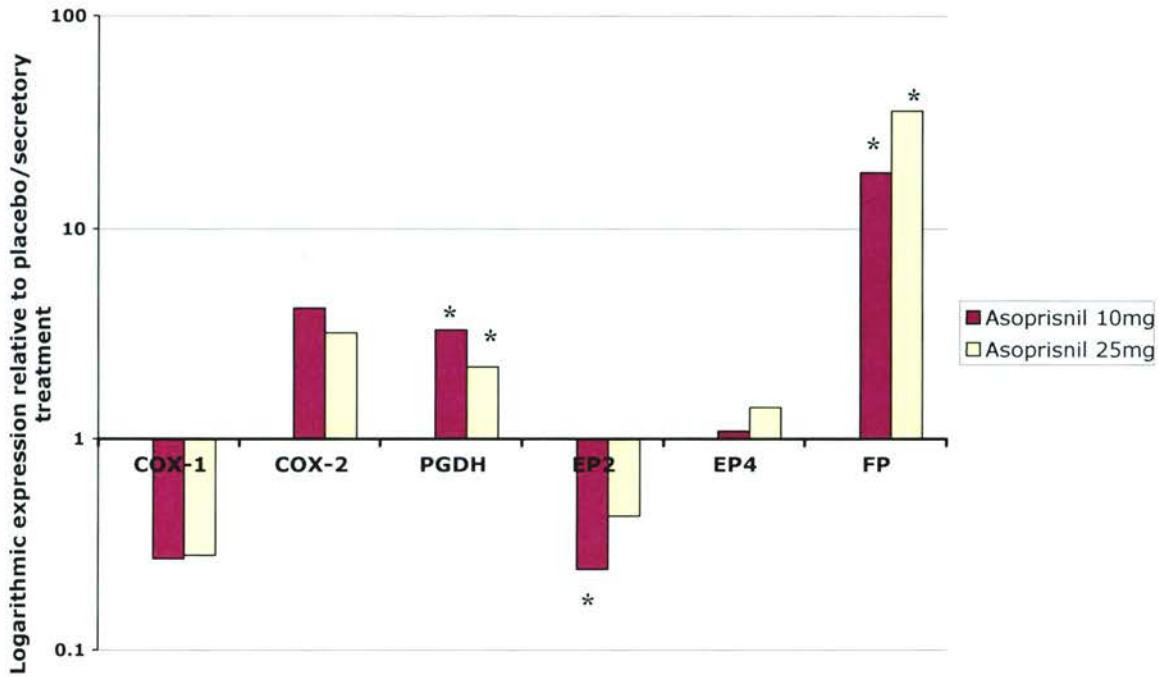


Figure 6.2 Asoprisnil effects on prostanoid signalling gene expression

*Graph demonstrates mRNA expression as determined by quantitative RT-PCR analysis. Expression is relative to the placebo/secretory treatment endometrium, which is represented by the x-axis on this logarithmic graph. * represents significant difference in expression from base line ($p<0.05$; COX, cyclooxygenase; PGDH, prostaglandin dehydrogenase; EP, prostaglandin E_2 series receptor; FP, prostaglandin F_2 receptor.)*

Figure 6.3

Box-plot demonstrating results of subjective scoring of COX-1 immunostaining within the human endometrium. Statistical analysis using the non-parametric Hochberg's multiple comparison procedure indicated that immunostaining intensity was significantly increased within the glandular epithelium for both asoprisnil doses and a decrease in immunostaining within the surface epithelial cell for 25mg asoprisnil treated group ($p<0.05$). The data are presented as box-and-whisker plots: box represents the 25^{th} and 75^{th} percentiles and the heavy bar represents the median. The whiskers are the 10^{th} and 90^{th} percentiles. There were only 2 samples obtained which were placebo-treated and allocated a proliferative categorisation. Meaningful comparison of asoprisnil treatment endometrium with proliferative endometrium is therefore difficult and differences should be interpreted with caution.

Figure 6.4

Box-plot demonstrating results of subjective scoring of COX-2 immunostaining within the human endometrium. Statistical analysis using Hochberg's multiple comparison procedure did not indicated any significant differences in immunostaining intensity within different tissue compartments. The data are presented as box-and-whisker plots: box represents the 25^{th} and 75^{th} percentiles and the heavy bar represents the median. The whiskers are the 10^{th} and 90^{th} percentiles. There were only 2 samples obtained which were placebo-treated and allocated a proliferative categorisation. Meaningful comparison of asoprisnil treatment endometrium with proliferative endometrium is therefore difficult and differences should be interpreted with caution.

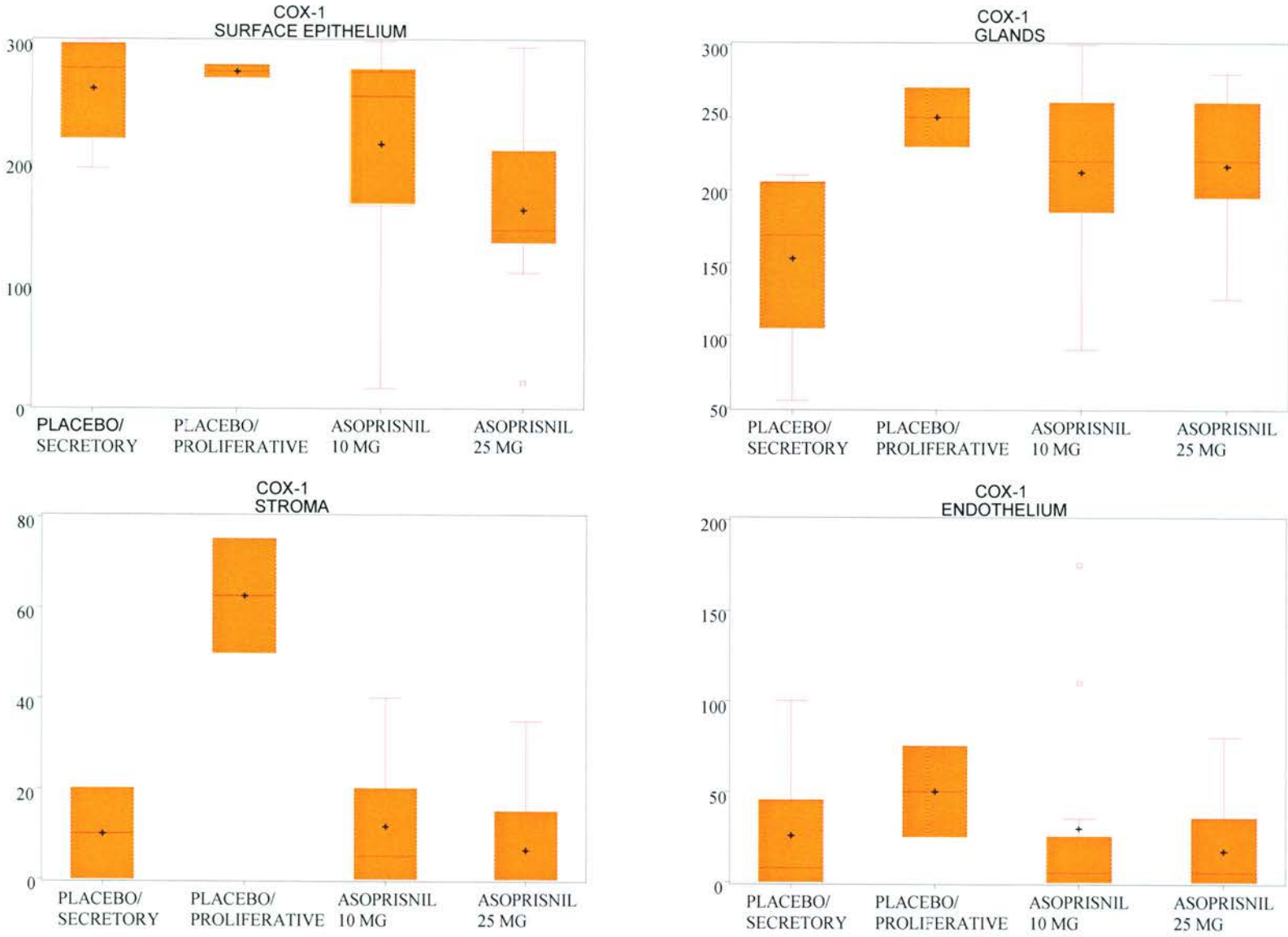


Figure 6.3 Box plot representation of Cyclooxygenase-1 immunostaining within various endometrial tissue compartments

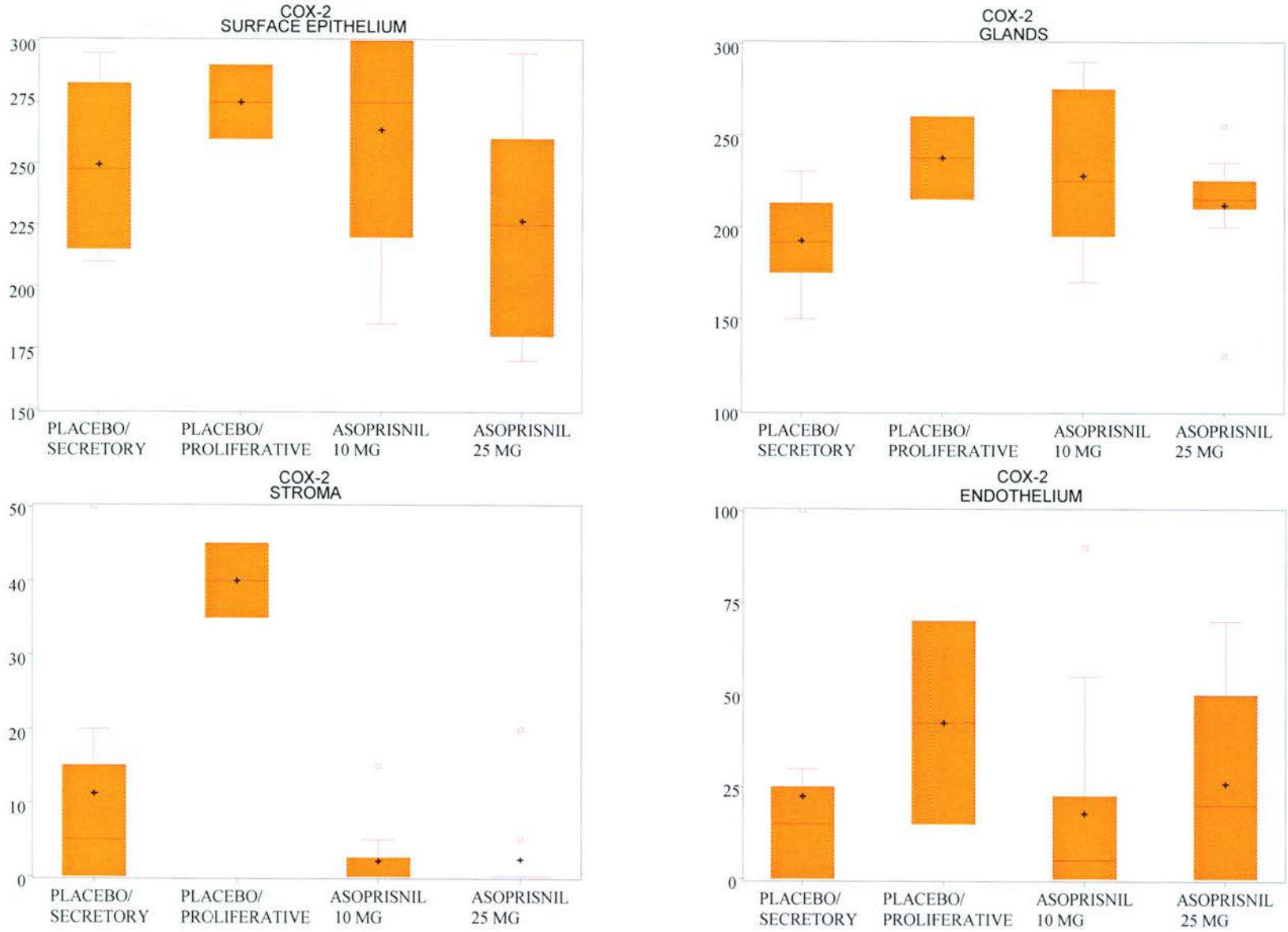


Figure 6.4 ***Box plot representation of*** *Cyclooxygenase-2* ***immunostaining within various endometrial tissue compartments***

6.4 Discussion

Many genes are regulated by the progesterone receptor (section 1.2.5). Progesterone is the natural ligand for this receptor and can influence many biological functions by gene regulation. Progesterone withdrawal is a key physiological event complicit in the mechanism of menstruation (section 1.4.1) and is known to influence the expression of genes at many levels of prostanoid signalling. Both *in vitro* and *in vivo* studies indicate that PR-A and PR-B can profoundly effect the biological response to progesterone and synthetic progestins (Chwalisz *et al.*, 2005). Progestins are widely used for treating many gynaecological disorders but they are often associated with side effects such as depression, bloating and break through bleeding thereby interfering with drug compliance (Smith, O.P. and Critchley, 2005). The effectiveness and side effects of progestins are dependent upon the type of progestin used, the dose of drug and indeed the mode of delivery e.g oral, subdermal, intramuscular. Progesterone antagonists such as mifepristone (RU486) also show potential for use in many studies of gynaecological disorders, however they are principally therapeutically used for their abortifact properties (Baird *et al.*, 2003). Selective progesterone receptor modulators (SPRMs) are able to provide the benefits of both progesterone agonist and antagonist actions whilst avoiding many of their drawbacks (Chwalisz *et al.*, 2005).

Asoprisnil produced a trend to upregulation of COX-2 mRNA in endometrium as compared with placebo/secretory endometrium. Previous studies have demonstrated an up regulation of COX-2 in the late secretory stage of the menstrual cycle in response to physiological or pharmacological progesterone withdrawal (Hapangama *et al.*, 2002; Jones *et al.*, 1997; Ota *et al.*, 2001). This suggests that with regard to COX-2 transcription, asoprisnil is demonstrating a progesterone antagonist action. The picture for COX-1 mRNA expression is for a trend in down- regulation although again, significance is not reached. The results of immunohistochemical staining for the COX enzymes provide more information. As for COX-2 mRNA expression, there is a similar trend for an increase in expression for COX-2 within the glandular compartment of the asoprisnil treated endometrium but a downtrend noted in the endometrial stroma. This difference of expression within endometrial tissue compartments may explain why true significance is not

obtained after Hochberg's multiple comparison. Differences in effect upon protein expression within tissue compartments is also seen for COX-1 immunostaining where a significant upregulation found in glandular cells is opposed by a down regulation within the surface epithelium. This makes meaningful analysis of mRNA expression difficult, as isolation of the various cellular compartments for mRNA analysis would be technically challenging and not possible within the boundaries of this study.

The mode of action of SPRMs upon COX transcription is therefore complex. Asoprisnil's ligand action upon the PR may be dependent upon the tissue compartment of action, local concentrations of drug and ratio of PR-A and PR-B isotypes. Additionally PR ligand binding induces a conformational change to the receptor altering the interaction with coregulators of transcriptional activity (Chwalisz *et al.*, 2005). The effect of a SPRM upon transcription of a particular gene is therefore difficult to predict.

In the endometrium, prostaglandins are not stored but are immediately synthesized and released and metabolized to inactive metabolites by PG 15 dehydrogenase (PGDH) enzyme (Casey *et al.*, 1980). Previous work has demonstrated a down-regulation of expression of endometrial PGDH in response to pharmacological progesterone withdrawal (Hapangama *et al.*, 2002). Asoprisnil significantly upregulated endometrial PGDH expression as compared to placebo/secretory endometrium thereby demonstrating a progesterone agonist action in contrast to its effects upon COX-2 mRNA expression.

$PGF_{2\alpha}$ and PGE_2 are two prominent prostaglandins found in human endometrium (Lumsden *et al.*, 1986b). In the endometrium, $PGF_{2\alpha}$ receptor (FP) and PGE_2 receptors EP2 and EP4 are responsible for their respective ligand action. The effects of asoprisnil upon endometrial mRNA expression for these receptors are examined. There is a significant upregulation of FP mRNA endometrial expression in women taking both doses of asoprisnil. EP2 mRNA expression is however down regulated in endometrium obtained from women taking 10mg asoprisnil compared to placebo/secretory treatment subjects.

The effects of asoprisnil upon prostaglandin signalling within human endometrium are therefore varied and complex. A reduction in menstrual blood loss has been demonstrated as a possible clinical benefit of asoprisnil and the mechanisms

of action for this effect have been postulated to involve changes to endometrial vasculature (Chwalisz *et al.*, 2004; DeManno *et al.*, 2003; Chwalisz *et al.*, 2007). Prostaglandins have additionally been implicated in disorders of menstruation (see section 1.4.3). Chapter 5 explores the role of these prostaglandin signalling in the aetiology of heavy menstrual bleeding and concludes that a shift in prostanoid signalling in favour of the PGE_2 pathway over $PGF_{2\alpha}$ may have a role in this pathology, thereby supporting earlier studies (Smith, S. K. *et al.*, 1981a). In contrast, the use of the SPRM asoprisnil, which leads to a cessation of menstrual bleeding, may cause a shift in signalling in favour of $PGF_{2\alpha}$ over PGE_2 pathways.

Whether asoprisnil associated changes in prostaglandin signalling within the endometrium participate in vascular and cellular changes that lead to a reduction of menstrual blood loss has however not yet been proven. The exact mechanism of action of the SPRM, asoprisnil, is currently being researched. Clinical studies with asoprisnil demonstrated that this SPRM effectively controls leiomyoma-associated symptoms, including heavy uterine bleeding and pressure-related symptoms, and reduces leiomyoma volume. Furthermore, studies with this SPRM showed its efficacy in controlling pain symptoms associated with endometriosis. Further studies, in a larger study population, are needed to determine the optimal treatment regimens with asoprisnil or other SPRMs regarding both safety and efficacy outcomes.

Chapter 7

Discussions

7.1 General Discussions

It has been postulated that the increasing incidence in menstrual disorders are due to changes in life style in the second half of the twentieth century. The development of the contraceptive pill, reduction in family size and /or the incidence of lactational amenorrhoea has meant that today a woman would experience a much greater number of menstrual cycles (approximately 400) compared to her ancestors (30-40 cycles).

Twenty years ago, 60% of women referred to secondary care with a complaint of heavy menstrual bleeding had a hysterectomy within 5 years. In 2005, 50% of these women had a hysterectomy within 2 years (Reid and Virtanen-Kari, 2005). Disorders of the menstrual cycle place a considerable burden on general practice and specialist health service resources and are the most common indication for hysterectomy (Vessey *et al.*, 1992). It is this health burden, which drives intense research into the exact mechanisms of the menstrual process despite the gross physiological aspects being well established. It has become clear that the cellular mechanisms involved in the aetiology of abnormal uterine bleeding are complex and involve a number of different molecular systems.

Menstrual dysfunction encompasses benign pathologies such as heavy menstrual blood loss (menorrhagia), painful periods (dysmenorrhoea), endometriosis and disorders of menstrual timing such as; frequent periods, infrequent or scanty periods (oligomenorrhoea), and amenorrhea (absent menstrual periods). Disorders of timing such as oligomenorrhoea and amenorrhoea are closely associated with disturbances in the hypothalamic-pituitary-ovarian axis and morphological changes within the uterus. These changes are not precipitated by local disturbances within the endometrial environment and therefore fall out with the remit of this thesis, which discusses prostaglandin signalling in the endometrium.

The molecular mechanism of menstruation in response to progesterone withdrawal is a complex cascade of events that have yet to be fully elucidated. It in part involves the production of prostaglandins that are able to induce vasoconstriction leading to a reduced blood flow to the endometrium. Subsequently there is increased expression of a range of locally acting mediators including cytokines, angiogenic factors, protease enzymes and further prostaglandins. The cumulative endpoint of

these changes in local mediators, together with an influx of migratory leukocytes is the process of menstruation (Brenner *et al.*, 2002; Critchley *et al.*, 2001b; Jabbour *et al.*, 2005) section 1.4; figure 1.5).

Evidence has since continued to mount, supporting a role for local disturbances of prostaglandin synthesis, secretion and metabolism in several pathologies of the endometrium. Endometrial pathologies, in which prostaglandins appear to have an aetiological role, are those in which the clinical symptoms of pain or bleeding disturbances are prominent. A role for COX enzymes and prostaglandins has been ascertained in reproductive tract pathology, including painful menstruation (dysmenorrhoea), heavy menstrual bleeding (menorrhagia) and endometriosis (Lundstrom *et al.*, 1976; Morita, 2002; Ota *et al.*, 2001; Smith, S. K. *et al.*, 1981a&b). Indeed first line treatment for such complaints involves the use of non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit COX, the rate-limiting enzyme in prostanoid production (Cameron *et al.*, 1990; Reid and Virtanen-Kari, 2005). Non-selective NSAID use has, however, been associated with gastrointestinal toxicity, ulceration, platelet dysfunction and kidney damage in at least 1% of users (Fosslien, 1998). In addition, well-publicised and unforeseen cardiovascular problems have arisen with selective COX-2 inhibitors (Krumholz *et al.*, 2007) that dramatically highlight the need for a further understanding of these enzymes, metabolites and their receptors.

It has traditionally been thought that in the normal physiological state, cells responsible for prostaglandin production, constitutively express COX-1 and are induced to express COX-2 in response to external stimulation. This present study demonstrates significantly elevated levels in mRNA expression of both COX-1 and COX-2 in endometrium obtained from women with measured heavy MBL (chapter 5, figure 5.5), thereby adding heavy menstrual blood loss to the list of gynaecological complaints demonstrating an alteration in expression of both COX enzymes. Clinically, these data suggest that it is important to use a general inhibitor of both COX-1 and COX-2 enzyme action for treatment of the complaint of heavy menstruation. Increased expression of the rate limiting COX enzymes in endometrium from women with heavy menstrual blood loss will lead to an increase in prostaglandin production and signalling. Of the two main prostaglandins in the

endometrium, PGE_2 and $PGF_2\alpha$, a shift in balance of signalling towards the PGE_2 pathway has previously been suggested (Smith, S. K. *et al.*, 1981a). A reduction in $PGF_2\alpha$ receptor mRNA in this study supports this finding. Furthermore, enhanced functionality of the PGE_2 receptors coupled to cAMP production has also been demonstrated in endometrium from women with heavy MBL as compared to normal (chapter 5, figure 5.7). The effect of this increased cAMP production was not studied. However, prostanoid coupled cAMP signalling has previously been linked to angiogenic influences (Sales *et al.*, 2004a) in the Ishikawa endometrial epithelial cell line.

Emerging evidence supports a role for COX enzymes, prostaglandins and prostaglandin receptor signalling pathways in a multitude of phenotypic changes within reproductive tissues including the promotion of angiogenesis and vascular function (Sales *et al.*, 2005; Tsujii *et al.*, 1998). The remodelling of the human endometrium required during the menstrual cycle requires tight control of angiogenic growth factors to coordinate the growth of new vessel formation.

By investigating the molecular signalling mechanism within both human endometrium and an endometrial epithelial cell line, this thesis demonstrates a potential role for PGI_2-IP receptor signalling in human endometrial glandular epithelial cells in regulating the expression of several pro-angiogenic factors. Such prostanoid signalling could influence angiogenesis by acting on adjacent endothelial cells in an autocrine/paracrine manner. Moreover, these studies have demonstrated a role for IP-EGFR crosstalk in promoting angiogenic factor expression in the endometrium. Prostanoid receptor crosstalk with EGFR, linked to angiogenic alterations, has previously been demonstrated for both PGE_2 and $PGF_{2\alpha}$ prostaglandin signalling within endometrial epithelial cell lines (Sales *et al.*, 2005; Sales *et al.*, 2004a).

A comparative study, which investigated endometrial tissue from women with heavy and normal blood loss, has demonstrated increased endothelial cell proliferation in women with heavy menstrual blood loss (Kooy *et al.*, 1996). Additionally, the proliferation and differentiation pattern of the vascular smooth muscle cells around spiral arterioles of the endometrium of women with heavy menstrual bleeding is significantly reduced compared to that of women with normal

blood loss (Abberton *et al.*, 1996; Abberton *et al.*, 1999). These data implicate disturbances of endometrial angiogenesis in the endometrial pathology leading to heavy menstrual blood loss. Whether these angiogenic alterations effect vascular development, composition and structural integrity or permeability is more difficult to ascertain. Heavy MBL has previously been reviewed as a defect of vasoconstriction, leading to reduced control of blood volume loss (Livingstone and Fraser, 2002). Ultimately, vascular permeability and leakage at the time of menstruation will be determined by a milieu of pro and anti-angiogenic factors. The demonstrated over-expression of COX enzymes in heavy MBL and the established role of COX enzymes in regulation of angiogenic factors would suggest a disturbance in the balance of these factors in heavy compared with normal menstruation. This is further exacerbated by the effect of COX enzymes upon decreasing anti-angiogenic factors. A reduction in expression of cathepsin D (an anti-angiogenic factor) mRNA and protein has previously been demonstrated in Ishikawa endometrial epithelial cells over-expressing COX-2 (Perchick *et al.*, 2003).

Early studies into the effects of the selective progesterone receptor modulators (SPRMs; see section 6) have provided hope for advancements in the treatments of abnormal menstruation, endometriosis associated pelvic pain and also fibroid disease. Phase II trials have shown that asoprisnil, a SPRM, produces a dose dependent amenorrhoea, reduces endometriosis associated pain and shrinks the volume of fibroid uteri (Chwalisz et al., 2005). The mechanism of action for this family of compounds is complex, exerting a tissue-selective agonist, antagonist or partial agonist/antagonist effect on the progesterone receptor. Interestingly, evidence demonstrates that asoprisnil causes the formation of unusual, thick-walled arterioles in the endometrium when compared to progesterone agonist or antagonists. Overall, early trials suggest that asoprisnil induces its therapeutic benefits such as suppression of menstruation via an endometrial specific vascular effect. The exact mechanisms of action are however poorly understood. The effect of asoprisnil upon prostanoid signalling has been examined within this thesis. The effects of asoprisnil upon expression of genes involved in prostanoid signalling are complex with alterations both in keeping with progesterone agonist and antagonist actions. Whether prostanoids are indirectly involved in the action of asoprisnil upon endometrial vasculature has not yet been ascertained.

Studies are advancing to elucidate the mechanism of action for this compound but research in to endometrial function is generally hampered by the absence of good animal models.

This thesis is based on the hypothesis that menstrual dysfunction, including heavy menstrual blood loss is due to (a) up-regulated expression/synthesis of cyclooxygenase enzymes and prostaglandin receptors, and (b) initiation of enhanced intracellular signalling pathways in response to prostaglandins. By identifying the prostanoids responsible for these effects and the specific receptors/signalling pathways that are associated with their function, we hope to establish information that may result in the development of novel therapeutic targets for menstrual pathology. Although data published within this thesis further implicate the COX enzymes in heavy MBL and also demonstrate an enhanced functional capability of endometrium in response to stimulation with PGE_2, specific receptor differences in endometrium taken form women with heavy as compared to normal MBL, have been more difficult to demonstrate. It would appear sensible to suggest that defects of prostaglandin signalling within endometrial pathologies are not attributable to a defective expression of just one receptor. Instead, that signalling defects are the end result of subtle alterations at any and probably many stages of prostanoid signalling. The concept of multi-gene activation patterns is now being explored to explain these complex molecular differences that lead to menstrual pathologies, principally with the use of gene microarray technologies.

Figure 7.1 Summary of investigations into prostaglandin signalling in the endometrium

Diagram highlights a postulated role of prostaglandin signalling in human endometrium with particular respect to the pathology of heavy menstrual bleeding.

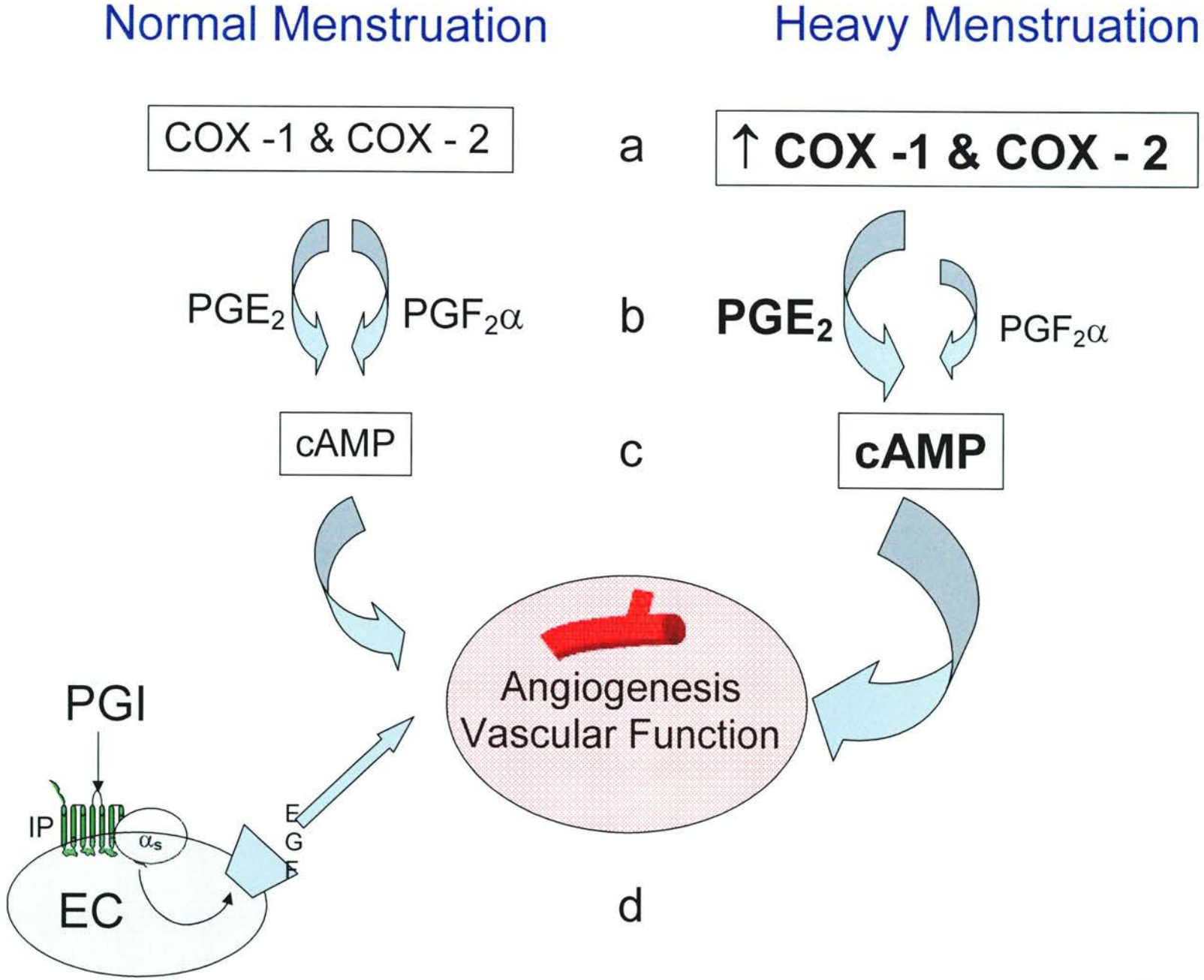
a) This study demonstrates significantly elevated levels in mRNA expression of both COX-1 and COX-2 in endometrium obtained from women with measured heavy menstrual bleeding (MBL), potentially providing a greater synthesis of prostaglandins.

b) Increased expression of the rate limiting COX enzymes in endometrium from women with heavy menstrual blood loss will lead to an increase in prostaglandin production and signalling. Of the two main prostaglandins in the endometrium, PGE_2 and $PGF_2\alpha$, a shift in balance of signalling towards the PGE_2 pathway has previously been suggested to have a role in heavy MBL. A reduction in $PGF_2\alpha$ receptor mRNA in this study supports this finding.

c) Furthermore, enhanced functionality of the PGE_2 receptors coupled to cAMP production has also been demonstrated in endometrium from women with heavy MBL as compared to normal. Prostanoid coupled cAMP signalling has previously been linked to angiogenic influences and disturbances in vessel structure and endothelial cell proliferation has been demonstrated in endometrium from women with heavy MBL compared to normal MBL.

d) Finally, this thesis demonstrates a potential role for PGI_2-IP receptor signalling in human endometrial glandular epithelial cells in regulating the expression of several pro-angiogenic factors. Moreover, these studies have demonstrated a role for IP-EGFR crosstalk in promoting angiogenic factor expression in the endometrium.

Abbreviations: COX, cyclooxygenase; cAMP, cyclic AMP; EC, endometrial epithelial cell; IP, prostacyclin receptor; EGFR, epidermal growth factor receptor


Normal Menstruation
Heavy Menstruation
COX -1 & COX - 2
a
↑ COX -1 & COX - 2
PGE2
PGF2α
b
PGE2
PGF2α
cAMP
c
cAMP
Angiogenesis
Vascular Function
PGI
IP
αs
EC
EP
d

7.2 For the future

This thesis has outlined the close relationship between prostaglandin signalling and angiogenic alterations. The signalling pathways linking prostanoid receptor activation and angiogenic endpoint appear to necessitate a "cross communication" with the epidermal growth factor receptor (EGFR). Blockade of EGFR signalling with an orally active EGFR tyrosine kinase inhibitor has been used successfully in inhibiting angiogenesis in nude mice (Baker *et al.*, 2002). Targeted inhibition of EGFR function with small molecule chemical inhibitors alone or in combination with a COX enzyme inhibitor may modulate angiogenic activity in the endometrium with possible benefits for menstrual complaints that are associated with aberrant expression and signalling of prostanoids and altered angiogenesis or vascular function.

The challenge in the future is to unravel the signalling pathways that precipitate menstrual disorders. In order to accomplish this, the exact nature of the molecules, the cross-talk that may exist between them and their role in signalling needs to be established. It is important to accurately assess whether these pathways function independently, or converge to amplify specific signals that are crucial for the progression of the studied pathology. Only with this knowledge will we be able to apply novel therapeutic intervention strategies.

Our knowledge of the factors regulating menstrual function/dysfunction has historically relied on candidate gene approaches with a requirement for knowledge of the gene structure and sequence. This approach has been applied to investigate the variation in expression of the candidate genes in the human endometrium across the menstrual cycle. It is also important to bear in mind that menstrual disorders may result from multiple aetiologies with different aetiologies occasionally found to be responsible for the same clinical complaint.

The advent of global mining of genes, using approaches such as genomics and proteomics, will greatly enhance our knowledge on the aberrations in gene expression in menstrual disorders. They offer a powerful approach of exploring the global transcriptional and signalling effects of drugs such as SPRMs upon the endometrium, thereby offering insight into their mode of action. Previous research has applied genomic array technology within human endometrial function (Giudice, 2004; Kao *et*

al., 2003; Kao *et al.*, 2002; Talbi *et al.*, 2006). These global approaches can give a great insight into the various genetic and molecular pathways when applied in context of specific endometrial pathologies. The application of such genomic approaches to menstrual disorders will greatly advance our knowledge of the library of genes that may be altered in these complaints. Moreover, they will outline a molecular "signature" that is the characteristic of the aetiology behind each of the menstrual complaints being investigated. With the advancement of these signatures, with time it may become possible to "tailor" the therapeutic option most appropriate to the individual patient from the variety of options available to the physician.

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Publications and Presentations

Original Articles

- Milling Smith OP, Jabbour HN, Critchley HOD (2007) "Cyclooxygenase enzyme expression and E series prostaglandin receptor signalling are enhanced in heavy menstruation" *Human Reproduction 22(5): 1450-6*

- Milling Smith OP, Battersby S, Sales K, Critchley HOD, Jabbour HN (2006) "Prostacyclin receptor up-regulates the expression of angiogenic genes in human endometrium via cross talk with EGF receptor and the ERK1/2 pathway" *Endocrinology* 147(4): 1697-1705

Reviews

- Jabbour HN, Sales K, Milling Smith OP, Battersby S, Wright S (2006) "Prostaglandin receptors are mediators of vascular function in endometrial pathologies" *Molecular and Cellular Endocrinology 27;252(1-2): 191-200*

- Milling Smith OP, Critchley HOD (2005) "Progesterone only contraception and endometrial break through bleeding." *Angiogenesis* 8(2):117-26

Published Abstracts

- O Milling Smith, S Battersby, HOD Critchley, HN Jabbour (2004) "Prostacyclin Receptor (IP) activated ERK 1/2 signalling via the EGF receptor in endometrial epithelial cells." *Proceedings of The Endocrine Society 86th Annual Scientific Meeting*, 466-467

Presentations

- Human Reproductive Science Unit (2005) - Invited Seminar; "Prostanoid signalling in the human endometrium"

- 14th Simpson Symposium (2005) – Poster; "EP receptor signalling is elevated in endometrium from women with excessive menstrual blood loss"

- Munro Kerr Society for the Study of Reproduction (2005) – Free Communication; "Menstruation, menorrhagia and prostaglandins"

- The Endocrine Society 86th Annual Meeting (2004) - Poster; "Prostacyclin Receptor (IP) activated ERK 1/2 signalling via the EGF receptor in endometrial epithelial cells."

- Munro Kerr Society for the Study of Reproduction (2004) –Free communication; "Prostacyclin signalling within an endometrial epithelial cell line"

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Cyclooxygenase enzyme expression and E series prostaglandin receptor signalling are enhanced in heavy menstruation

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BACKGROUND: Although the mechanisms underlying the causes of heavy menstrual blood loss (MBL) remain to be elucidated, prostaglandins have been previously implicated. This study was initiated to elucidate a pattern of expression of the various components of the cyclooxygenase (COX)–prostaglandin signalling pathways present in the endometrium of women with normal and heavy MBLs. METHODS: Endometrial biopsies were collected at different stages of the menstrual cycle from women who underwent measurement of MBL. Tissue was divided for either examination of gene expression by quantitative RT–PCR analysis or *in vitro* culture experimentation. RESULTS: Analysis of gene expression demonstrated a significant elevation in expression of COX-1 and COX-2 mRNA in endometrium obtained from women with heavy MBL when compared with endometrium obtained from women with normal MBL. Tissue culture with PGE_2 stimulation caused a significantly elevated production of cyclic AMP (cAMP) by endometrium of women with heavy MBL when compared with normal MBL. Expression of phosphodiesterase 4B, an enzyme involved in cAMP breakdown, was reduced in these same endometrial samples obtained from women with heavy MBL. CONCLUSIONS: These data identify the E series prostaglandin receptors and their signalling pathways as potential therapeutic targets in the treatment of heavy menstruation.

Key words: cyclooxygenase/heavy menstruation/menorrhagia/prostaglandin receptors

Introduction

Initial studies of menstrual fluid identified vasoactive substances with the capabilities of inducing contractions in strips of ileal muscle (Pickles, 1957). These were subsequently identified as prostaglandins $F_{2\alpha}$ ($PGF_{2\alpha}$) and E_2 (PGE_2), the two most abundant prostaglandins found in the endometrium and menstrual fluid (Lumsden *et al.*, 1983). Evidence has since continued to mount, supporting a role for prostaglandins in menstruation (Baird *et al.*, 1996).

In the prostaglandin synthesis pathway, the cyclooxygenase (COX) enzymes generate PGH_2 from arachidonic acid. There are two main isoforms of the COX enzyme: COX-1 and COX-2. COX-1 is constitutively expressed in many tissues and generates prostaglandins for normal physiological function, whereas COX-2 is rapidly induced in cells in response to varied stimuli (Vane *et al.*, 1998). Once synthesized, PGH_2 acts as an intermediary for a specific terminal prostaglandin synthase enzyme. Synthesized prostaglandins mediate their actions via seven-transmembrane G-protein coupled receptors (GPCRs). PGE_2 can couple to four subtypes of GPCRs, which have been pharmacologically classified as EP1, EP2, EP3 and EP4 (Coleman *et al.*, 1994). These receptors are often co-expressed together in the same cell and, utilize alternate and, in some cases, opposing intracellular signalling pathways (Ashby, 1998). EP2 and EP4 receptors, previously demonstrated in human endometrium (Milne *et al.*, 2001), are coupled to G proteins (Gαs) and adenylyl cyclase, resulting in the increased formation of cyclic AMP (cAMP). PDE4 is a member of the phosphodiesterase family of enzymes, which has been previously demonstrated in human endometrium (Bartsch *et al.*, 2004), and is specific for the hydrolysis of cAMP (Sanz *et al.*, 2005). The level of accumulation of the second messenger, cAMP, in response to prostaglandin signalling may therefore be dependent on the level of PDE4 activity. The PGE_2 pathway has previously been implicated in the problem of heavy menstrual blood loss (MBL) (Willman *et al.*, 1976; Smith *et al.*, 1981; Adelantado *et al.*, 1988), although specific signalling mechanisms have yet to be elucidated. Additional

prostaglandins include prostacyclin and thromboxane, which act upon their respective receptors (IP and TBXR). Prostacyclin and thromboxane are best known for their effects on the vascular endothelium, where their synthesis is well noted (Ullrich *et al.*, 2001). Menstruation is an active process whereby the upper two-thirds of the endometrium, the functional layer, are shed and regenerated on a cyclical basis. Menstrual problems account for much of the morbidity that occurs in women of reproductive age. Thirty percentage of women consider their menstruation to be excessive, rising to 50% in perimenopausal women (Prentice, 1999, 2000). Management typically involves invasive surgery. In 2002–3, over 13 000 surgical procedures (hysterectomy and endometrial ablation) were performed in UK for complaints of heavy bleeding (Reid and Mukri, 2005). An estimated 3.5 million workdays are lost annually (Weeks *et al.*, 2000).

The objective definition of heavy MBL is often based on the measurement of menstrual haemoglobin content. This method of measurement was first described in Scandinavian studies, which demonstrated the mean MBL to be 40 ml. Regular MBL in excess of 63 ml was associated with iron deficiency anaemia (Hallberg, 1964; Hallberg *et al.*, 1966). The 90th centile for measured blood loss was 80 ml and this has traditionally been accepted as the upper limit of normal in clinical evaluation of MBL.

Previous investigations into the aetiology of heavy MBL have not been able to discover any differences in circulating steroid hormone levels (Eldred and Thomas, 1994) or any specific histological differences within the endometrium (Rees *et al.*, 1984) of women with heavy MBL when compared with women with normal MBL. Additionally, there is no difference in endometrial expression of estrogen and progesterone receptors (Critchley *et al.*, 1994). However, evidence does exist implicating local mediators, in particular, prostaglandins (Sales and Jabbour, 2003). Increased levels of total prostaglandins have been found in endometrium taken from women with heavy MBL (Smith *et al.*, 1981). Furthermore, treatment using inhibitors of COX enzymes has repeatedly been shown to reduce MBL (Cameron *et al.*, 1990; Coulter *et al.*, 1995; Bonnar and Sheppard, 1996), implicating disturbances of prostaglandin pathways in the aetiology of excessive menstrual bleeding, although the mechanisms underlying the cause of heavy blood loss remain to be elucidated.

This study was initiated to determine a pattern of expression of the various components of the COX–prostaglandin signalling pathways present in the endometrium of women with normal and heavy MBLs (>80 ml). This is based on the hypothesis that disturbances of local mediator signalling, including prostaglandin signalling, are implicated in the aetiology of heavy MBL.

Materials and methods

Tissue collection and measurement of MBL

Patients complaining of heavy menstruation were recruited from the gynaecological outpatient setting. Ethical approval was obtained from the Lothian Research Ethics Committee and written informed consent was obtained from all subjects before tissue collection. All subjects were aged 18–50 (range 22–49; mean 40 years of age). All subjects reported regular menstrual cycles (cycle length 21–35 days), with no unscheduled, non-menstrual bleeding. No woman had received hormonal preparation in the 3 months preceding biopsy collection. Patients were clinically examined and clinical pelvic abnormalities, such as an enlarged uterus, were further investigated by pelvic ultrasound imaging. Patients with known uterine pathology such as fibroid disease and endometriosis were excluded from the study.

Endometrial biopsies ($n = 26$) were collected for research purposes at different stages of the menstrual cycle with an endometrial suction curette (Pipelle, Laboratoire CCD, Paris, France). No biopsies were taken during the menstrual phase of the cycle because of technical difficulties in obtaining sufficient quantities of endometrium suitable for RNA analysis. Biopsies were dated according to stated last menstrual period (LMP) and dating was confirmed by histological assessment according to criteria of Noyes *et al.* (1950). Furthermore, circulating estradiol and progesterone serum levels were measured at the time of biopsy collection and were consistent for both LMP and histological assessment of the menstrual cycle stage. This is a robust method for characterizing endometrial samples. Detailed gene microarray studies support this method for characterizing endometrial samples with consistency across these three parameters (Critchley *et al.*, 2006; Talbi *et al.*, 2006).

Inconsistencies between measured circulating hormone levels, stated day of LMP and/or histological assessment, led to exclusion of three tissue biopsies from the study. Table I provides a summary of the biopsies ($n = 23$) used within this work.

Immediately after collection, tissue was divided, transferred into RNA Later (Ambion Inc., Huntingdon, UK) and stored at −70°C (for RNA extraction), fixed in neutral-buffered formalin for wax embedding (for histological dating) and placed in Rosewell Park Memorial Institute (RPMI) 1640 medium (containing 2 mM L-glutamine, 100 U penicillin and 100 μg ml^{-1} streptomycin) and transported to the laboratory for *in vitro* culture.

In addition to consenting to provide an endometrial biopsy, patients agreed to undergo measurement of MBL over one menstruation. Measurement was based on a modified alkaline–haematin method as previously described (Hallberg, 1964). Briefly, used sanitary products were added to a measured volume of 5% sodium hydroxide (between 2 and 4 l). The contents were then left for 48 h to allow conversion of haemoglobin to haematin. During this same time period,

Table I. Summary of collected endometrial biopsies

Stage of menstrual cycle	Number of biopsies	
	Menstrual blood loss (MBL) ≤80 ml	MBL >80 ml
Proliferative	7	5
Secretory	6	5

a 1 in 200 dilution of the patient's venous blood in 5% sodium hydroxide was made and additionally stored. After 48 h, an aliquot of sodium hydroxide was removed from the volume soaking the sanitary products and filtered through hardened filter paper (Whartman No. 54, Maidstone, UK). The optical density (OD) of MBL solution and venous blood sample were then measured using spectrophotometry at 546 nm (A_{546}).

MBL was then calculated as a quantity of patient's own venous blood using the following equation (van Eijkeren *et al.*, 1986):

$$\text{MBL} = \frac{(\text{OD of menstrual blood solution} \times \text{total volume of added NaOH})}{(\text{OD of venous blood} \times 200)}$$

Validation work for this method of measuring MBL has previously been carried out (data not shown).

The range of measured MBL was between 10 and 567 ml with a median MBL of 42 ml for the normal group (MBL >80 ml) and a median MBL of 183 ml for the heavy group (MBL >80 ml). All patients provided informed consent for collection of an endometrial biopsy during the month proceeding or immediately following collection of their menstrual loss.

Whole tissue cAMP assay

Endometrial biopsies from proliferative and secretory phases of the menstrual cycle were minced finely with scissors and incubated overnight in RPMI medium containing 3 $\mu g\ ml^{-1}$ indomethacin (an inhibitor of COX-1 and COX-2 enzymes). Following overnight treatment, approximately one-third of the tissue was removed and stored at $-20°C$. The remaining tissue was incubated in the same medium containing 1 mM 1-methyl-3-isobutylxanthine (IBMX, Sigma, Poole, UK) for 30 min. (IBMX inhibits the action of phosphodiesterases and prevents the rapid degradation of cAMP.) It was then divided into two portions and treated with control medium or 100 nM PGE_2 for 10 min. Tissue was then lysed in 0.1 M HCl and frozen until assayed. Cyclic AMP concentration was measured by enzyme-linked immunosorbent assay (Biomol, Affiniti, Exeter, UK) in accordance with the manufacturer's instructions and normalized to protein concentration determined by protein assay according to the manufacturer's instructions (Bio-Rad, Hemel Hempstead, UK).

Taqman quantitative reverse transcriptase–polymerase chain reaction

The expression of COX enzymes across the menstrual cycle and the expression of prostanoid receptors in endometrium characterized by MBL were investigated by quantitative RT–PCR. Total RNA was extracted from endometrial biopsies using the commercially available product RNeasy Midi Kit (Qiagen Ltd, Sussex, UK), according to the manufacturer's instructions. Each tissue sample was able to provide sufficient quantities of RNA for the complete analysis of our target genes. Samples were treated for DNA contamination by DNA digestion during RNA purification. Following extraction, total RNA was eluted in 150 μl of nuclease-free water and stored at $-80°C$. Quality of RNA was assessed using the Agilent 2100 Bioanalyser system in combination with RNA6000nano chips (Agilent Technologies, Cheshire, UK). Only RNA that displayed intact 18S and 28S peaks was reverse transcribed to cDNA for real-time PCR analysis.

Once extracted and quantified, RNA samples were reverse transcribed as previously described (Milne *et al.*, 2001; Sales *et al.*, 2004). Thereafter, cDNA samples were stored at $-20°C$. A tube with no reverse transcriptase was included to control for any DNA contamination.

Real-time quantitative PCR was performed using an ABI 7700 Sequence Detection System (Applied Biosystems, Warrington, UK) as previously described (Milne *et al.*, 2001; Sales *et al.*, 2004) using duplicate samples. A no-template control (containing water) was included. The inclusion of ribosomal 18S primers and probes was used to act as a housekeeping gene for each reaction mix. All primers and probes were designed using the PRIMER express programme (Applied Biosystems) and their sequences can be found in Table II.

Data were analysed and processed using Sequence Detector version 1.6.3 (Applied Biosystems) according to manufacturer's instructions. Expression of target genes was normalized to RNA loading for each sample using 18S ribosomal RNA as an internal standard. All results were expressed relative to a control standard (cDNA obtained from a single sample of endometrial tissue and included in all experiments).

Statistical analysis

The quantitative RT–PCR data did not consistently fulfil the assumptions necessary for using analysis of variance (ANOVA) and *t*-test; therefore, the non-parametric Mann–Whitney test was used (Graphpad InStat3) and statistical significance accepted when $P < 0.05$. Cyclic AMP assay data were subjected to statistical analysis with ANOVA and Fishers PLSD tests (Statview 4.0; Abacus Concepts Inc., Piscataway, NJ, USA) and statistical significance accepted when $P < 0.05$.

Results

Quantitative RT–PCR analysis of genes involved with prostaglandin signalling

Analysis of mRNA expression for a variety of genes associated with prostanoid signalling within the endometrium by quantitative RT–PCR was performed. Analysis of COX-1 and COX-2 mRNA expressions demonstrated a significant increase in secretory endometrium of women with MBL in excess of 80 ml when compared with endometrium of women with MBL <80 ml (Figure 1).

No significant differences in endometrial expression were detected for prostacyclin or thromboxane receptors. Regarding the receptors for PGE_2, no significant differences in expression were detected between the two groups of endometrial samples for EP2, EP3 and EP4. The mRNA expression of EP1 was significantly increased in secretory endometrium of women with heavy MBL when compared with normal MBL. Finally, mRNA expression of the receptor for $PGF_{2\alpha}$ (FP) was significantly reduced in proliferative endometrium of women with heavy MBL when compared with normal MBL (Figure 2).

Table II. Sequences of primers and probes used for Taqman RT-PCR analysis

Cyclooxygenase-1 (COX-1)	Forward	5′-TGT TCG GTG TCC AGT TCC AAT A-3′
	Reverse	5′-ACC TTG AAG GAG TCA GGC ATG AG-3′
	Probe	5′-CGC AAC CGC ATT GCC ATG GAG T-3′
Cyclooxygenase-2 (COX-2)	Forward	5′-CCT TCC TCC TGT GCC TGA TG-3′
	Reverse	5′-ACA ATC TCA TTT GAA TCA GGA AGC T-3′
	Probe	5′-TGC CCG ACT CCC TTG GGT GTC A-3′
Prostaglandin E_2 receptor type 1 (EP1)	Forward	5′-AGA TGG TGG GCC AGC TTG T-3′
	Reverse	5′-GCC ACC AAC ACC AGC ATT G-3′
	Probe	5′-CAG CAG ATG CAC GAC ACC ACC ATG-3′
Prostaglandin E_2 receptor type 2 (EP2)	Forward	5′-GAC CGC TTA CCT GCA GCT GTA C-3′
	Reverse	5′-TGA AGT TGC AGG CGA GCA-3′
	Probe	5′-CCA CCC TGC TGC TGC TTC TCA TTG TCT-3′
Prostaglandin E_2 receptor type 3 (EP3)	Forward	5′-GAC GGC CAT TCA GCT TAT GG-3′
	Reverse	5′-TTG AAG ATC ATT TTC AAC ATC ATT ATC A-3′
	Probe	5′-CTG TCG GTC TGC TGG TCT CCG CTC-3′
Prostaglandin E_2 receptor type 4 (EP4)	Forward	5′-ACG CCG CCT ACT CCT ACA TG-3′
	Reverse	5′-AGA GGA CGG TGG CGA GAA T-3′
	Probe	5′-ACG CGG GCT TCA GCT CCT TCC T-3′
Prostaglandin $F_{2\alpha}$ receptor (FP)	Forward	5′-GCA GCT GCG CTT CTT TCA A-3′
	Reverse	5′-CAC TGT CAT GAA GAT TAC TGA AAA AAA TAC-3′
	Probe	5′-CAC AAC CTG CCA GAC GGA AAA CCG-3′
Thromboxane receptor (TBXR)	Forward	5′-TGG TGG TGG CCA GCG T-3′
	Reverse	5′-CGG GTT TCG CAG CAC TGT-3′
	Probe	5′-TGC CCC TTC TGG TCT TCA TCG CCC-3′
Prostacyclin receptor (IP)	Forward	5′-GCC CTC CCC CTC TAC CAA-3′
	Reverse	5′-TTT TCC AAT AAC TGT GGT TTT TGT G-3′
	Probe	5′-CCA AGA GCC AGC CCC CTT TCT GC-3′
Phosphodiesterase 4B	Forward	5′-CCT TCA GTA GCA CCG GAA TCA-3′
	Reverse	5′-CAA ACA AAC ACA CAG GCA TGT AGT T-3′
	Probe	5′-AGC CTG CAG CCG CTC CAG CC-3′
18S	Forward	5′-CGT CTA CCA CAT CCA AGG AA-3′
	Reverse	5′-GCT GGA ATT ACG GGG GCT-3′
	Probe	5′-TCG TGG CAC CAG ACT TGC CCT C-3′

Functional PGE_2 receptor signalling in endometrium of women with measured MBL

The PGE_2 receptors, EP2 and EP4, are known to activate the cAMP/protein kinase A pathway within the human endometrium (Milne *et al.*, 2001; Regan, 2003). To investigate the potential differences in PGE_2 receptor function between endometrial samples of women with measured MBL, we therefore looked at cAMP production as an end-point for receptor function.

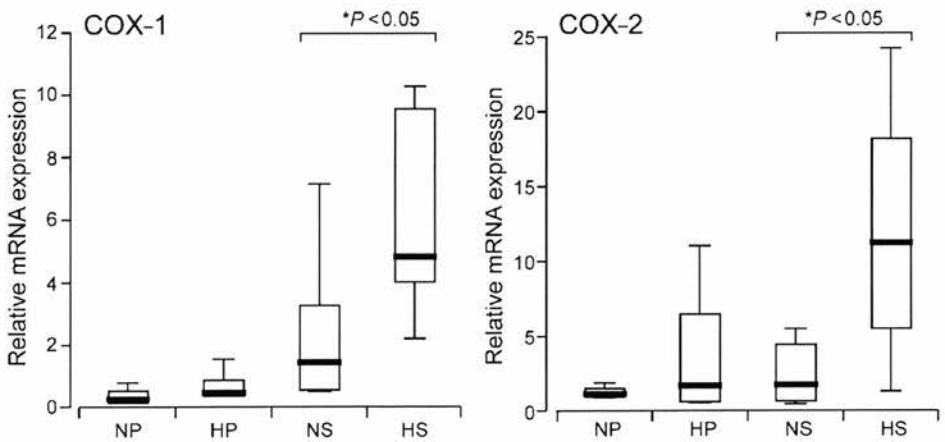

Figure 1. Box plot demonstrating relative cyclooxygenase-1 (COX-1) and COX-2 mRNA expression in proliferative and secretory endometrium of women with normal and heavy measured menstrual blood losses (MBLs) (normal MBL $\leq$80 ml and heavy MBL >80 ml). The data are presented as box-and-whisker plots: box represents the 25th and 75th percentiles and the heavy bar represents the median. The whiskers are the 10th and 90th percentiles. NP, normal proliferative ($n = 7$); HP, heavy proliferative ($n = 5$); NS, normal secretory ($n = 6$); HS, heavy secretory ($n = 5$). Asterisk indicates significant difference ($P < 0.05$).

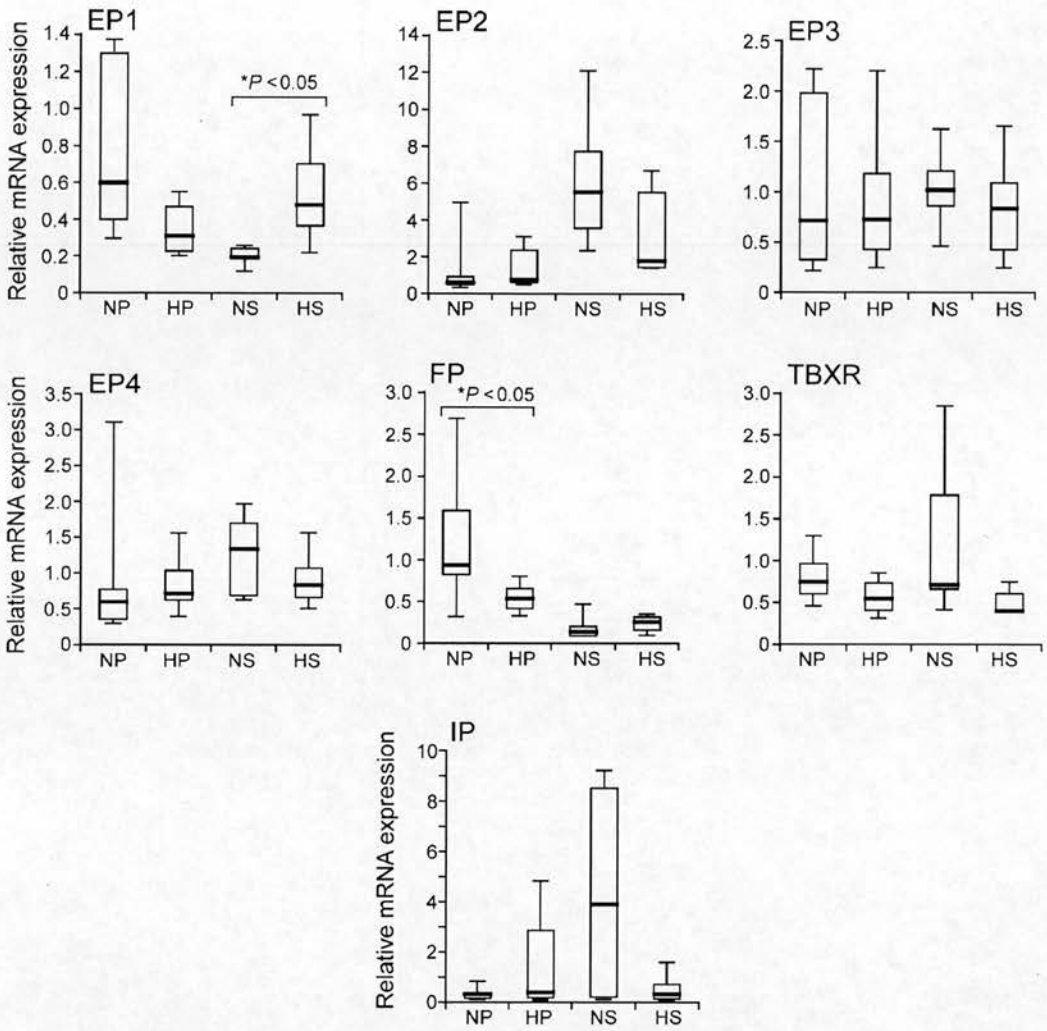

Figure 2. Composite figure of box plot graphs demonstrating relative mRNA expression for prostanoid receptors in proliferative and secretory endometrium of women with normal and heavy measured menstrual blood losses (MBLs) (normal MBL $\leq$80 ml and heavy MBL >80 ml). The data are presented as box-and-whisker plots: box represents the 25th and 75th percentiles and the heavy bar represents the median. The whiskers are the 10th and 90th percentiles. NP, normal proliferative ($n = 7$); HP, heavy proliferative ($n = 5$); NS, normal secretory ($n = 6$); HS, heavy secretory ($n = 5$). EP1 to EP4 are the receptors for prostaglandin E_2; FP is the receptor for prostaglandin $F_{2\alpha}$; TBXR is the receptor for thromboxane and IP is the receptor for prostacyclin. Asterisk indicates significant difference ($P < 0.05$).

Cyclic AMP production in response to treatment with 100 nM PGE_2 was higher in endometrial tissue explants collected from women with heavy MBL when compared with endometrium of women with normal MBL (Figure 3A).

As described earlier, the analysis of prostanoid receptor mRNA expression did not detect any significant differences for EP2 or EP4 in endometrium of women with measured MBL. Therefore, in order to investigate whether effects at the post-receptor level could explain this enhanced functionality of the EP receptor, we investigated the expression of phosphodiesterase isotype 4B (PDE4B) in the same endometrial samples. Quantitative RT–PCR analysis demonstrated a significant reduction in the expression of PDE4B in secretory endometrium of women with heavy MBL, compared with normal MBL (Figure 3B).

Discussion

This study demonstrates significantly elevated levels in mRNA expression of both COX-1 and COX-2 enzymes in endometrium obtained from women with measured heavy MBL. These data suggest that it is important to use a general inhibitor of both COX-1 and COX-2 enzyme action in treating the complaint of heavy menstruation. Mefenamic acid, a COX inhibitor and a member of the Fenamate family of drugs, is routinely used as a first-line treatment for menorrhagia. As well as inhibiting prostaglandin synthesis, it has been shown to inhibit binding of PGE_2 to its receptors (Rees *et al.*, 1988). In keeping with this dual mode of action, the PGE_2 pathway has previously been implicated in the problem of heavy MBL. Elevated levels of PGE_2 have been found in the endometrium of women complaining of heavy periods

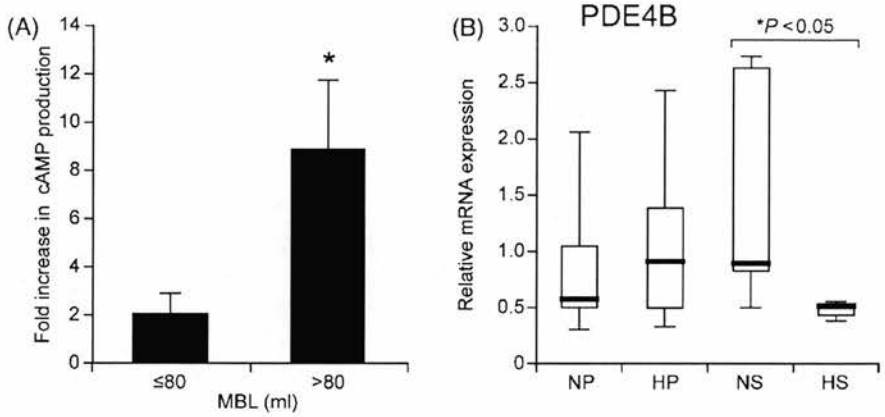

Figure 3. (**A**) Cyclic AMP (cAMP) production by endometrial tissue explants in response to a 10 min stimulation with 100 nM PGE_2 or vehicle. Graph shows average fold increase in cAMP production by endometrium of women with normal and heavy menstrual blood losses (MBLs) (normal MBL ≤80 ml and heavy MBL >80 ml). Asterisk indicates significant elevated cAMP production ($P < 0.05$). (**B**) Phosphodiesterase 4B (PDE4B) mRNA expression in endometrium of women with normal and heavy MBLs (normal MBL ≤80 ml and heavy MBL >80 ml) as determined by real-time quantitative RT–PCR. The data are presented as box-and-whisker plots: box represents the 25th and 75th percentiles and the heavy bar represents the median. The whiskers are the 10th and 90th percentiles. NP, normal proliferative ($n = 7$); HP, heavy proliferative ($n = 5$); NS, normal secretory ($n = 6$); HS, heavy secretory ($n = 5$). Asterisk indicates significant difference ($P < 0.05$).

(Willman *et al.*, 1976) and further experiments confirmed an apparent shift in synthesis in favour of PGE_2 over $PGF_{2\alpha}$ in the endometrium of women with heavy MBL (Smith *et al.*, 1981).

Additionally, our data demonstrate an enhanced PGE_2–EP-induced cAMP production in the endometrium obtained from women with heavy MBL; however, the expression of EP2 and EP4 receptors, which are known to couple to cAMP production, does not display any significant correlation with measured MBL in our studies. Previous data have demonstrated an increase in PGE_2 binding sites in uterine tissue associated with the complaint of heavy MBL (Adelantado *et al.*, 1988), although specific receptor subtypes were not identified. Indeed, it is plausible that EP2 and EP4 receptor numbers could be regulated at the post-transcriptional level, resulting in increased PGE_2 receptor binding sites. $PGF_{2\alpha}$ and PGE_2 are two prominent prostaglandins found in human endometrium (Lumsden *et al.*, 1986). In the endometrium, $PGF_{2\alpha}$ receptor (FP) and PGE_2 receptors, EP2 and EP4, are responsible for their respective ligand action. Although there was a reduction in the expression of FP in the endometrium taken from women with heavy MBL, no changes in the expression of EP2 and EP4 were found. Therefore, a shift of endometrial prostaglandin signalling in favour of the PGE_2 pathway over the $PGF_{2\alpha}$ pathway may exist in the endometrium of women with heavy MBL. Previous work has shown a decrease in the $PGF_{2\alpha}/PGE_2$ ratio in the endometrium of women with measured heavy MBL when compared with women with normal MBL (Smith *et al.*, 1981).

The COX-2–PGE_2–EP pathway has previously been shown to influence angiogenic factors such as vascular endothelial growth factor through a mechanism mediated by cAMP (Sonoshita *et al.*, 2001). Therefore, the enhanced ability for cAMP production by endometrium taken from women with heavy MBL may have important effects on the expression of angiogenic factors. Altered endothelial function as a result of disturbances to angiogenic factors has previously been implicated in excessive MBL (Kooy *et al.*, 1996).

Thromboxane and prostacyclin are implicated in platelet function and vascular haemostasis (Grosser *et al.*, 2006) and there is a strong expression of their respective receptors in the endometrial vascular compartment (Milling Smith *et al.*, 2006; Battersby, unpublished data). However, no differences in the expression of the genes involved in their signalling pathways downstream of COX enzymes were detected between the two groups of the endometrium of women with heavy and normal MBLs.

PDEs are a large family of enzymes that are responsible for the hydrolysis of cyclic nucleotides (Sanz *et al.*, 2005). PDE4B is an isoenzyme that is found in the human endometrium (Bartsch *et al.* 2001, 2004) and shows specificity for hydrolysis of cAMP. We have shown that in addition to an enhanced ability to produce cAMP in response to PGE_2, endometrium taken from women with heavy MBL expresses a significantly reduced level of PDE4B isoform mRNA.

In summary, increased expression of the rate-limiting COX enzymes in the endometrium of women with heavy MBL will lead to an increase in prostaglandin production and signalling. In addition, enhanced functionality of the EP receptors coupled to cAMP production could in part be explained by a reduction in PDE4B expression within endometrium of women with heavy MBL.

Our data, therefore, present a novel mechanism of endometrial prostaglandin signalling that may lead to the complaint of heavy menstruation. Enhanced COX–PGE_2 signalling and reduced PDE4B expression in the endometrium of women with heavy MBL present us with new therapeutic opportunities in the treatment of heavy menstrual bleeding.

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Prostacyclin Receptor Up-Regulates the Expression of Angiogenic Genes in Human Endometrium via Cross Talk with Epidermal Growth Factor Receptor and the Extracellular Signaling Receptor Kinase 1/2 Pathway

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Prostacyclin (PGI) is a member of the prostanoid family of lipid mediators that mediates its effects through a seven-transmembrane G protein-coupled receptor (IP receptor). Recent studies have ascertained a role for prostanoid-receptor signaling in angiogenesis. In this study we examined the temporal-spatial expression of the IP receptor within normal human endometrium and additionally explored the signaling pathways mediating the role of IP receptor in activation of target angiogenic genes. Quantitative RT-PCR analysis demonstrated the highest endometrial expression of the IP receptor during the menstrual phase compared with all other stages of the menstrual cycle. Immunohistochemical analysis localized the site of IP receptor expression to the glandular epithelial compartment with stromal and perivascular cell immunoreactivity. Expression of the immunoreactive IP receptor protein was greatest during the proliferative and early secretory phases of the menstrual cycle. To explore the role of the IP receptor in glandular epithelial cells, we used the Ishikawa endometrial epithelial cell line. Stimulation of Ishikawa cells and human endometrial biopsy explants with 100 nM iloprost (a PGI analog) rapidly activated ERK1/2 signaling and induced the expression of proangiogenic genes, basic fibroblast growth factor, angiopoietin-1, and angiopoietin-2, in an epidermal growth factor receptor (EGFR)-dependent manner. Furthermore, EGFR colocalized with IP receptor in the glandular epithelial compartment. These data suggest that PGI-IP interaction within glandular epithelial cells can promote the expression of proangiogenic genes in human endometrium via cross talk with the EGFR. (*Endocrinology* 147: 1697–1705, 2006)

ARACHIDONIC ACID (AA) is released from plasma membrane phospholipids and is cyclized, oxygenated, and reduced to the unstable intermediary prostaglandin (PG), prostaglandin H_2 (PGH_2) by cyclooxygenase (COX) enzymes (1, 2). This intermediary serves as a substrate for terminal PG synthase enzymes, such as prostacyclin (PGI) synthase (PGIS), which completes the synthesis of PGI. PGI is a member of the PG family and has a mode of action via coupling to the heptahelical G protein-coupled PGI receptor (IP receptor) (3, 4). PGI is best known for its effect on the vascular endothelium, where its expression is found in abundance (5).

The IP receptor is known to mediate a cAMP rise and has been termed a relaxant receptor (1), with routine activation of the IP receptor activating adenylate cyclase via the G_s subunit in a dose-dependent manner. Knockout studies disrupting the IP gene in mice have demonstrated thrombotic tendencies and decreased inflammatory responses (6, 7). The cyclical regeneration and repair undergone by the human endometrium during the menstrual cycle necessarily involves physiological processes involving clotting and inflammation. Uterine PGI production may be involved in myometrial smooth muscle relaxation, vasodilation, and prevention of clot formation. These physiological actions are all involved in the process of menstruation, and a role for PGI in menstruation and menstrual disturbances is likely. Indeed, endometrium collected from women with excessive menstrual blood loss has a greater capability of enhancing myometrial PGI production than endometrium collected from women with normal menstrual blood loss (8). Previous studies have also demonstrated an increase in the expression of PGIS and IP receptor mRNA during the menstrual stage compared with the proliferative and secretory stages of the cycle (4).

The cyclical remodeling of the human endometrium requires tight control of angiogenic growth factors to coordinate the growth of new vessel formation. Previous studies have demonstrated that COX enzymes, in particular COX-2 (9), and PGs such as PGE_2 (10) and $PGF_{2\alpha}$ (11) can modulate the expression of target angiogenic genes within the human endometrium.

This study was designed to investigate the expression of the IP receptor and its role within the human endometrium. Using an endometrial epithelial cell line (Ishikawa) and human endometrial tissue, we investigated the intracellular

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Abbreviations: AA, Arachidonic acid; Ang-1, angiopoetin-1; bFGF, basic fibroblast growth factor; COX, cyclooxygenase; DN, dominant negative; EGFR, epidermal growth factor receptor; IP receptor, G protein-coupled PGI receptor; MEK, MAPK kinase; PG, prostaglandin; PGI, prostacyclin; PGIS, PGI synthase; VEGF, vascular endothelial growth factor.

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signaling transduction pathways activated after PGI-IP ligand-receptor interaction. We found that the IP receptor is spatio-temporally regulated within the glandular epithelial compartment of human endometrium and is colocalized with the epidermal growth factor receptor (EGFR) in human endometrial glandular epithelial cells. Investigation of IP receptor signal transduction pathways using Ishikawa cells and human endometrial tissue showed rapid activation of the ERK1/2 signaling pathway in an EGFR-dependent manner. Moreover, activation of the IP receptor was shown to promote changes in the expression of proangiogenic genes, basic fibroblast growth factor (bFGF), angiopoietin-1 (Ang-1), and Ang-2.

Materials and Methods

Patients and tissue collection

Endometrial biopsies were collected at different stages of the menstrual cycle with an endometrial suction curette (Pipelle, Laboratoire CCD, Paris, France) from women attending the gynecological outpatient setting. In addition, full-thickness endometrial biopsies at all stages of the menstrual cycle were collected from women undergoing hysterectomy for benign gynecological indications. Immediately after collection, tissue was divided, transferred into RNA Later (Ambion, Inc., Huntingdon, UK), stored at −70 C (for RNA extraction), fixed in neutral buffered formalin, wax embedded (for immunohistochemical analysis) or placed in RPMI 1640 medium (containing 2 mM L-glutamine, 100 U penicillin, and 100 μg/ml streptomycin), and transported to the laboratory for *in vitro* culture. All subjects were 18–50 yr of age and reported regular menstrual cycles (cycle length, 21–35 d). No woman had received hormonal preparation in the 3 months preceding biopsy collection. Biopsies were dated according to stated last menstrual period and were confirmed by histological assessment according to criteria of Noyes and co-workers (12). Furthermore, circulating estradiol and progesterone serum levels were measured at the time of biopsy collection and were consistent for both last menstrual period and histological assignment of menstrual cycle stage. Ethical approval was obtained from Lothian research ethics committee, and written informed consent was obtained from all subjects before tissue collection.

Tissue culture

Tissue samples were finely minced using sterile forceps and scissors before overnight incubation in serum-free RPMI medium (as described above) and 3 μg/ml indomethacin (a dual COX enzyme inhibitor to inhibit endogenous prostanoid production). The next day, tissue was pretreated with a specific chemical inhibitor of EGFR kinase (AG1478; 100 nM) for 1 h before stimulation with 100 nM iloprost for the time period stated in the figure legends. After stimulation, tissue was either snap-frozen in dry ice and stored at −20 C for subsequent protein extraction or stored at −70 C for RNA extraction. Protein was harvested by homogenization of tissue in protein lysis buffer. Protein content was determined using a protein assay kit (Bio-Rad Laboratories, Inc., Hemel Hempstead, UK).

Cell culture

Ishikawa human endometrial epithelial cells (European Collection of Cell Culture, Wiltshire, UK) were maintained as previously described (10, 13). The cells were grown on monolayer in 6-cm dishes to 60–80% confluence, after which the culture medium was replaced with serum-free DMEM containing 3 μg/ml indomethacin for overnight incubation. The next day, cells were pretreated with specific inhibitors of EGFR kinase (AG1478; 100 nM) or MAPK kinase (MEK; PD98059; 50 μM) for 1 h before stimulation with 100 nM iloprost or 100% (vol/vol) ethanol as a vehicle control for the time period specified in the figure legends. After stimulation with iloprost, proteins were harvested and extracted as described previously (10), and the protein content in the supernatant fraction was determined using a protein assay kit (Bio-Rad Laboratories, Inc.).

TaqMan quantitative RT-PCR

The expression of IP receptor across the menstrual cycle and the effects of iloprost on proangiogenic gene expression in Ishikawa cells or endometrial tissue were investigated by TaqMan quantitative RT-PCR analysis. Total RNA was extracted from endometrial biopsies using an RNeasy Midi Kit (Qiagen, Sussex, UK) according to the manufacturer's instructions. Samples were treated for DNA contamination by DNA digestion during RNA purification. RNA was extracted from Ishikawa cells as described previously (10, 13). Once extracted and quantified, RNA samples were reverse transcribed and subjected to real-time quantitative PCR using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Warrington, UK) as previously described (10, 13). All primers and probes were designed using the PRIMER express program (Applied Biosystems; Table 1); IP primers and probes have been previously described (4).

Data were analyzed and processed using Sequence Detector version 1.6.3 (Applied Biosystems) according to the manufacturer's instructions. The expression of IP receptor and proangiogenic genes was normalized to RNA loading for each sample using 18S RNA as an internal standard. Results were expressed relative to an internal positive standard of pooled human endometrial cDNA, which was included in all reactions. The fold increase was determined by dividing the relative expression in PGI-treated cells/tissues by the relative expression of the vehicle control.

Transient transfections and immunoprecipitation

To confirm the role of EGFR in iloprost-mediated ERK1/2 phosphorylation, we used a dominant negative (DN) mutant EGFR. Ishikawa cells were seeded to a density of 5×10^5/well in 6-cm dishes, then transfected with a c-Myc-tagged ERK1/2 cDNA construct together with either empty vector cDNA (pcDNA3; Invitrogen Life Technologies, Inc., de Schelp, The Netherlands) or DN-EGFR cDNA using Superfect (Qiagen, Crawley, UK) according to the manufacturer's protocol (DN-EGFR and c-Myc tagged ERK constructs were gifts from Dr. Zvi Naor, Tel-Aviv University, Tel-Aviv, Israel). Optimal concentrations of cDNA for transfection were determined by titration, and the transfection efficiency of the Ishikawa cell line was determined by transfection with a pcDNA6/V5/His/lacZ cDNA construct (Invitrogen Life Technologies, Inc.) and a β-galactosidase assay. Transfection efficiency, as reported previously for this cell line using standard procedures according to the manufacturer protocol, is 45 ± 5% (11). The following day, cells were starved by overnight incubation in serum-free medium containing 3 μg/ml indomethacin, then treated with 100 nM iloprost or vehicle for 10 min. Cells were lysed, and protein was quantified as described above. The tagged ERK1/2 was immunoprecipitated from whole-cell lysate. For immunoprecipitation, equal amounts of protein were incubated with specific c-Myc antibody preconjugated to protein A Sepharose overnight at 4 C with gentle rotation. Beads were washed extensively with lysis buffer, and immune complexes were eluted and solubilized in Laemmli buffer [125 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate, 5% 2-mercap-

TABLE 1. Sequences of primers and probes used for TaqMan RT-PCR analysis

	Sequence (5′–3′)
Ang-1	
Forward	CTT GTG GCC CCT CCA ATC TA
Reverse	TAG TGC CAC TTT ATC CCA TTC AGT T
Probe	TGG TTT TGT CCC GCA GTA TAG AAC ATT CCA T
Ang-2	
Forward	GCC GCT CGA ATA CGA TGA CT
Reverse	ATT AGC CAC TTG CAG CCT CTG CA
Probe	TTC TCC AGC ACT TGC AGC CTC TGC A
FGF	
Forward	CCG ACG GCC GCG TTG AC
Reverse	GAC ACA ACT CCT CTC TCT T
Probe	AGA AGA GCG ACC CTC ACA
18S	
Forward	CGT CTA CCA CAT CCA AGG AA
Reverse	GCT GGA ATT ACG GGG GCT
Probe	TCG TGG CAC CAG ACT TGC CCT C

toethanol, 20% glycerol, and 0.05% bromophenol blue], boiled for 5 min, and subjected to Western blot analysis.

Western blot analysis

Western blot analysis was conducted to investigate ERK1/2 expression in Ishikawa cells and human endometrial tissue. A total of 50 μg protein from whole-cell lysate was resuspended in 20 μl Laemmli buffer. Proteins were resolved on 4–20% Tris-glycine gels (NOVEX, Invitrogen Life Technologies, Inc.), transferred onto a polyvinylidene difluoride membrane (Millipore Corp., Watford, UK), and subjected to immunoblot analysis as previously described (14). Blots were incubated with anti-phospho-p42/p44 ERK (9101, Cell Signaling Technologies/New England Biolabs, Hertfordshire, UK) and alkaline-phosphatase conjugated secondary antibodies (Sigma-Aldrich Corp., Ayrshire, UK). Immunoblots were stripped and reprobed with antibody recognizing total ERK (sc-93, Santa Cruz Biotechnology/Autogen-Bioclear, Wiltshire, UK). Immunoreactive proteins were visualized by the enhanced chemiluminescence system according to the manufacturer's instructions (Amersham Biosciences, Little Chalfont, UK). Proteins were revealed and quantified by PhosphorImager analysis using a Typhoon 9400 PhosphorImager (Molecular Dynamics, part of Amersham Biosciences). Relative density in immunoblots was calculated by dividing the value obtained from the phosphorylated immunoblots by the value obtained from the total immunoblots in the same experiment and was expressed as the fold increase above the vehicle control value.

Immunohistochemistry

To investigate the expression of the IP receptor in human endometrium, endometrial sections (5 μm) from across the menstrual cycle were dewaxed in xylene and rehydrated using decreasing grades of ethanol, followed by water. All washes were carried out in 0.01 M PBS (Sigma-Aldrich Corp.). Antigen retrieval was performed by pressure-cooking in 0.01 M sodium citrate (pH 6.0) for 5 min. Thereafter, slides were sequentially incubated with 3% hydrogen peroxide (VWR, Inc., Poole, UK) in distilled water for 10 min (to quench endogenous peroxidase activity), followed by a 15-min incubation with avidin and biotin solutions (Vector Laboratories, Inc., Peterborough, UK) to block endogenous streptavidin activity. Nonspecific binding was further reduced by 20-min incubation with nonimmune horse serum (Vector Laboratories, Inc.) in a humidified chamber at room temperature before overnight incubation with the primary antibody at 4 C. For localization of IP receptor, slides were incubated with a goat polyclonal antibody raised against a peptide mapping near the carboxyl terminus of IP receptor of human origin (sc-20436, Santa Cruz Biotechnology, Inc.) at a 1:30 dilution in normal horse serum. Preabsorption of the antibody with a specific blocking peptide (Santa Cruz Biotechnology, Inc.) was used as the negative control in addition to a control goat IgG antibody at a matched protein concentration to the IP antibody. After a wash in PBS with 0.01% Tween 20, the slides were incubated in biotinylated horse antigoat secondary antibody (Vector Laboratories, Inc.) in normal horse serum at a 1:200 dilution for 60 min at room temperature. Tertiary detection was carried out using an avidin-biotin-peroxidase complex (Vectastain Elite, Vector Laboratories, Inc.) for 60 min at room temperature, and visualization was carried out with the substrate and chromagen 3,3′-diaminobenzidine (DakoCytomation, Carpinteria, CA). Sections were counterstained with hematoxylin, dehydrated in xylene, and mounted.

Scoring and analysis of immunoreactivity

The immunostaining intensity of the IP receptor epitope in all tissue sections was assessed in a semiquantitative manner on a 4-point scale: 0, no immunostaining; 1, mild immunostaining; 2, moderate immunostaining; and 3, intense immunostaining. All tissue sections were scored blind by two observers. This scoring system has been previously validated in a subset of tissue sections in which immunoreactivity was measured with a computerized image analysis system; a strong correlation between quantitative data derived from the image analysis and subjective scores determined by a trained observer was obtained (15).

Immunofluorescent confocal laser microscopy

Colocalization of the site of expression of the IP receptor with EGFR or the endothelial cell marker CD31 was performed in human endometrium by dual-immunofluorescence immunohistochemistry and confocal laser microscopy. Human endometrial sections (5 μm) were dewaxed, rehydrated, and washed as described above. The immunohistochemical methodology was repeated for antigen retrieval by pressure cooking, quenching of hydrogen peroxidase activity, and blocking of endogenous streptavidin activity. Nonspecific binding was further reduced by 20-min incubation with 5% nonimmune rabbit serum diluted in PBS before overnight incubation at 4 C with the polyclonal mouse anti-EGFR primary antibody (NCL-EGFR-384; Nova-Castra, Newcastle-upon-Tyne, UK) at a dilution of 1:25. For colocalization of the IP receptor with CD31, a monoclonal mouse anti-CD31 antibody (DakoCytomation) was used at a dilution of 1:20. Control sections were incubated with polyclonal goat anti-IP primary antibody at a dilution of 1:100 to demonstrate the specificity of the secondary antibody for the mouse primary antibodies. The following day, sections were washed with PBS Tween 20 and incubated with a 1:500 dilution of biotinylated rabbit antimouse IgG for 1 h. An additional 1-h incubation with the fluorochrome streptavidin AlexiFluor 488 (Molecular Probes, Inc., Cambridge Bioscience, Cambridge, UK) diluted at 1:200 in PBS was performed. Next, sections were incubated for 20 min in a PBS solution containing biotin to enhance fluorescent signal before reblocking with 5% nonimmune rabbit serum. Incubation with the goat anti-IP antibody (Santa Cruz Biotechnology, Inc.) at a 1:100 dilution at 4 C overnight was then performed. A second control slide was incubated with mouse anti-EGFR or mouse anti-CD31 primary antibody. Incubation with a 1:200 dilution of rabbit antigoat peroxidase (Vector Laboratories, Inc.) secondary antibody was performed for 30 min. Tertiary detection was performed with an 8-min incubation with tyramide Cy3 solution (PerkinElmer Life Sciences, Boston, MA) at a 1:50 dilution according to the manufacturer's instructions. Slides were counterstained with To Pro (Molecular Probes, Inc.) at a 1:2000 dilution for 2 min, then mounted in Permafluor (Immunotech-Coulter, Buckinghamshire, UK).

Statistics

Unless otherwise stated and where appropriate, data were subjected to statistical analysis with ANOVA and Fisher's protected least significant difference tests (StatView 4.0; Abacus Concepts, Inc., Piscataway, NJ), and statistical significance was accepted at $P < 0.05$. Semiquantitative scoring results for immunohistochemical staining were analyzed by a nonparametric method, the Kruskal-Wallis test, followed by Dunn's *post hoc* multiple comparison test.

Results

IP receptor mRNA and protein expression within human endometrium

IP receptor mRNA expression in human endometrium across the menstrual cycle was determined by TaqMan quantitative RT-PCR analysis (Fig. 1). IP receptor mRNA was significantly up-regulated during the menstrual stage of the cycle compared with all other stages in the cycle ($P < 0.05$).

This rise in RNA expression was found to precede the expression of the IP receptor protein, as detected by immunohistochemistry. The temporal spatial expression of the IP receptor was examined in human endometrium across the menstrual cycle (Fig. 2A). Subjective scoring of IP receptor immunoreactivity showed a significant variation of temporal-spatial expression across the menstrual cycle within the glandular epithelium of the functional layer of human endometrium (Fig. 3; $P < 0.05$). IP receptor immunostaining was greatest during the proliferative (Fig. 2Ai) and early secretory (Fig. 2Aii) phases within the glandular compartment of the functional layer and was observed to decrease during the late secretory stages (Fig. 2Aiii). High magnification views (Fig. 2B) of a representative endometrial section

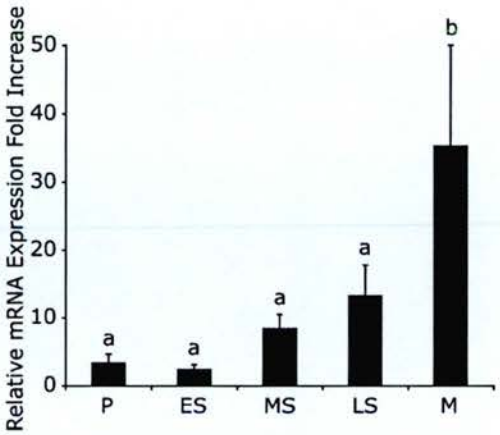

FIG. 1. Relative mRNA expression of the IP receptor in the human endometrium across the menstrual cycle as determined by real-time quantitative RT-PCR. Results are expressed as the mean ± SEM relative mRNA expression levels. b is significantly elevated from a ($P < 0.05$). Tissue sample numbers are: n = 7 proliferative, n = 6 early secretory, n = 5 midsecretory, n = 6 late secretory, and n = 4 menstrual.

show the plasma membrane localization of the IP receptor, with some cytoplasmic immunoreactivity also present. Minimal basal layer stromal immunostaining was observed in all tissue sections across the menstrual cycle compared with the functional stroma.

In addition, IP receptor immunoreactivity appeared prominent in vessel endothelium throughout the full thickness of endometrial tissue sections, involving vessels within the endometrial and muscularis layers. To establish this immunostaining as being vascular in location, dual-confocal immunofluorescence immunohistochemistry was used to colocalize the IP receptor with the endothelial cell marker, CD 31 (Fig. 2C). IP receptor (*red*) was observed to colocalize (*yellow*) with CD31 (*green*) in the blood vessels of all tissue sections investigated, indicating that IP receptor was present in the vascular compartment.

Iloprost activation of the IP receptor in Ishikawa cells

Treatment of Ishikawa cells with 100 nM iloprost elicited a significant time-dependent increase in phosphorylation of the ERK1/2 pathway, with maximal phosphorylation detected at 5 min (Fig. 4A; $P < 0.05$). Previous studies in our laboratory have demonstrated that prostanoid (including PGE_2 and $PGF_{2\alpha}$) signaling to downstream MAPK pathways involves transactivation of the EGFR (10, 11, 13). To investigate the potential involvement of the EGFR in transducing the PGI-IP receptor signal to ERK1/2, we used the selective EGFR tyrosine kinase inhibitor, AG1478. Preincubation of cells for 1 h with the EGFR kinase inhibitor (AG1478; 100 nM) or inhibitor of MEK (PD98059; 50 μM) abolished the phosphorylation of ERK1/2 in response to a 5-min 100-nM iloprost treatment (Fig. 4B). No significant alteration in basal levels of ERK phosphorylation was observed in cells treated with chemical inhibitor alone (data not shown).

To confirm a role for the EGFR in mediating the signaling

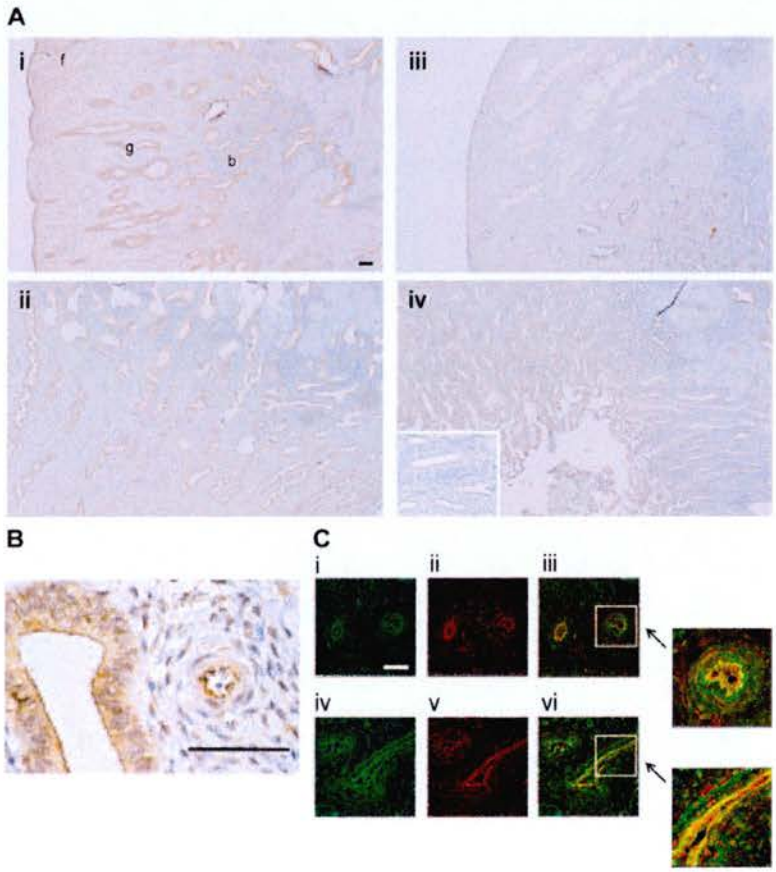

FIG. 2. A, Immunohistochemical localization of the IP receptor within the human endometrium across the menstrual cycle. Variation in temporal/spatial localization of the IP receptor is demonstrated. Glandular epithelial immunostaining (g) was present in both basal (b) and functional (f) layers. Some stromal immunostaining was demonstrated in the functional layer only. Representative sections from i) proliferative, ii) early secretory, iii) late secretory, and iv) menstrual (*inset* shows control staining with primary antibody after specific peptide preabsorption) stages are shown. *Scale bar*, 10 μm. B, High magnification (×100) view of immunohistochemical staining for IP receptor within epithelial gland and blood vessel of the functional layer of early secretory endometrium. *Scale bar*, 10 μm. C, Endothelial staining is confirmed by confocal immunofluorescent colocalization (merged; *yellow*) of the site of expression of IP receptor (*red*; ii and v) with the endothelial cell marker, CD31 (*green*; i and iv) in early secretory endometrium. Colocalization of IP receptor with CD31 (*yellow*; iii and vi) is demonstrated in vascular endothelium. *Scale bar*, 10 μm.

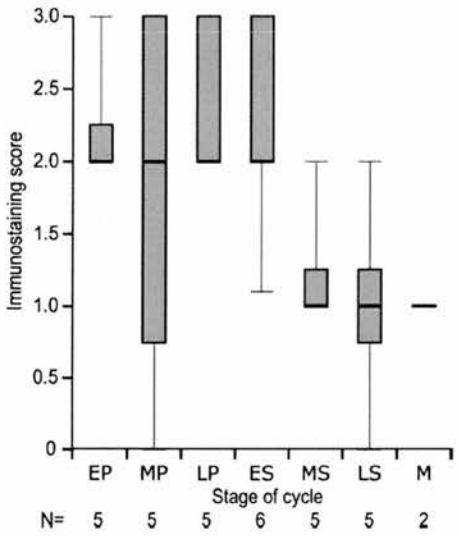

FIG. 3. Box plot demonstrating results of subjective scoring of IP receptor immunostaining within the glandular epithelium of the functional layer of human endometrium. Statistical analysis using the nonparametric ANOVA Kruskal-Wallis test indicated that variation in immunostaining intensity was significantly different across the menstrual cycle ($P < 0.05$). The data are presented as box and whisker plots; the *box* represents the 25th and 75th percentiles, and the *heavy bar* represents the median. The *whiskers* are the 10th and 90th percentiles. Tissue sample numbers for each stage of the cycle are presented.

of the IP receptor to ERK1/2, we cotransfected Ishikawa cells with a c-Myc-tagged ERK cDNA construct with either a DN mutant cDNA isoform of the epidermal growth factor receptor (DN-EGFR) or empty vector cDNA (pcDNA3). Ishikawa cells were then treated with either vehicle or 100 nM iloprost for 5 min. The tagged ERK was immunoprecipitated with anti-c-Myc antibody, and the ERK activity of the tagged construct was determined by Western blotting as described in *Materials and Methods*. Iloprost treatment of Ishikawa cells resulted in a significant phosphorylation of ERK1/2 in cells transfected with the empty vector (Fig. 4C, lane 2). This elevation in ERK1/2 phosphorylation by iloprost was abolished by cotransfection of cells with the DN-EGFR cDNA (Fig. 4C, lane 3).

PGI-IP receptor signaling in Ishikawa cells promotes the expression of proangiogenic genes

Iloprost stimulation of Ishikawa cells caused a significant fold increase in mRNA expression of the proangiogenic genes, bFGF, Ang-1, and Ang-2, at 24 h compared with earlier time points (Fig. 5A). Cotreatment of the cells with the EGFR kinase inhibitor (AG1478; 100 nM) significantly reduced IP receptor-induced mRNA expression of all target genes (Fig. 5B; $P < 0.05$).

IP receptor signaling in human endometrium

To correlate our findings using the Ishikawa cell line to IP receptor signaling in human endometrium *in situ*, we used human endometrial biopsy explants. Initially we investigated whether IP signaling in the human endometrium involves cross talk with EGFR and examined the colocalization of IP receptor with EGFR by confocal immunofluorescence microscopy. Dual-immunofluorescence immunohistochemistry (Fig. 6) confirmed colocalization (merged; *yellow*) of the IP receptor (Fig. 6, A and D, *red*) with the EGFR (Fig. 6, B and E, *green*). Colocalization was most evident within the glandular epithelial compartments in both basal (Fig. 6, A–C) and functional (Fig. 6, D–F) layers of the endometrium, with minimal stromal cell colocalization.

Subsequently, we assessed the effect of PGI-IP receptor signaling on ERK1/2 phosphorylation and proangiogenic gene expression in human endometrial tissue. Treatment of human endometrial tissue explants with 100 nM iloprost (Fig. 7A) caused a significant phosphorylation of ERK1/2 after 10 min. Preincubation of the tissue with 100 nM AG1478 for 1 h abolished the iloprost-induced phosphorylation of ERK1/2.

As observed in Ishikawa cells, treatment of human endometrial tissue with 100 nM iloprost for 24 h resulted in a significant increase in bFGF mRNA expression compared with vehicle control ($P < 0.05$). Preincubation of the tissue with 100 nM AG1478 for 1 h abolished the iloprost-induced elevation of bFGF (Fig. 7B). A similar trend in expression of the two angiopoietin genes, Ang-1 and Ang-2, was observed in response to treatment with 100 nM iloprost. Preincubation of endometrial tissue with 100 nM AG1478 reduced the iloprost-mediated elevation of Ang-1 and Ang-2 expression; however, the reduction was not statistically significant (Fig. 7B).

Discussion

During the reproductive years, in the absence of pregnancy, the human endometrium undergoes a series of cyclical changes, culminating in the process of menstruation. This process of physiological injury and repair requires continuous remodeling of the superficial layers of the endometrium together with a concurrent control of vessel remodeling and formation. Studies of angiogenesis in the endometrium have confirmed continuous cycles of angiogenic activity, with a number of peaks of activity demonstrated throughout the menstrual cycle (reviewed in Ref. 16). The roles of COX enzymes, prostanoids, and prostanoid receptors in the reproductive tract have been well documented (8, 14, 17, 18). In particular, menstrual problems, such as excessive blood loss, are linked to PG signaling. Indeed, first-line treatment for such complaints involves the use of nonsteroidal antiinflammatory drugs, which inhibit COX, the rate-limiting enzyme in prostanoid production (19, 20).

The data presented in this manuscript demonstrate the expression and localization of the IP receptor in epithelial, endothelial, and stromal cells of the human endometrium across the menstrual cycle. IP receptor mRNA is dramatically elevated in human endometrium during the menstrual phase of the cycle and precedes the glandular expression of IP receptor protein, which is highest in the proliferative phase of the menstrual cycle. The IP receptor is a G_s-coupled heptahelical transmembrane receptor that has been shown to activate the protein kinase A pathway, resulting in the production of cAMP (4). Activation of prostanoid receptors, such as E- and F-series prostanoid receptors, results in initiation of numerous effector signaling pathways, including

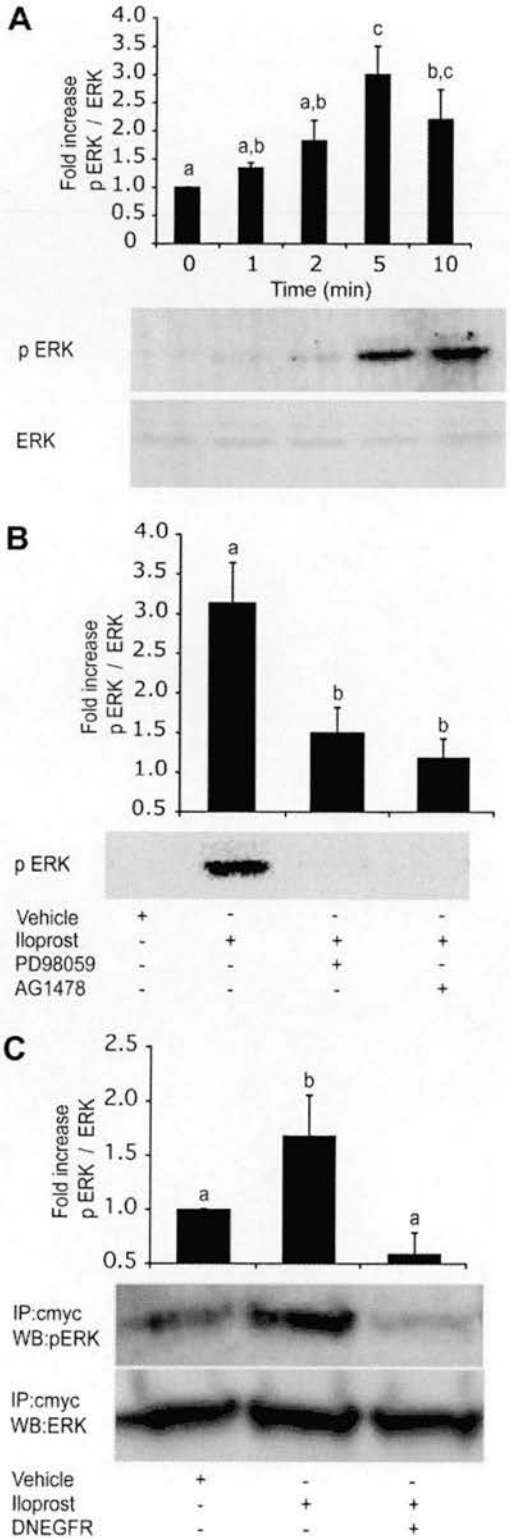

FIG. 4. ERK1/2 phosphorylation after treatment of Ishikawa cells with 100 nM iloprost. A, Western blot analysis of time course for ERK1/2 phosphorylation. A representative blot demonstrates phosphorylated ERK1/2 (*upper panel*). Total ERK1/2 was identified by reprobing the same blot with antibody directed against total ERK protein (*lower panel*). Graph shows semiquantitative analysis of four experiments, as described in *Materials and Methods*. *Superscripts* indicate significant differences ($P < 0.05$). B, Western blot analysis of the effects of chemical inhibitors on ERK1/2 phosphorylation. PD98059 is a MEK inhibitor (inhibitor of ERK phosphorylation); AG1478 is an inhibitor of EGFR tyrosine kinase. For each, a representative blot is shown. The graph shows semiquantitative analysis of three experiments as described in *Materials and Methods*. b is significantly different from a ($P < 0.05$). C, Ishikawa cells were cotransfected with a c-Myc-tagged ERK cDNA construct together with either a DN cDNA isoform targeted against the EGFR (lane 3) or empty vector pcDNA (lanes 1 and 2) and subsequently stimulated with vehicle (lane 1) or 100 nM iloprost (lanes 2 and 3) for 10 min. The tagged ERK construct was immunoprecipitated (IP) and ERK phosphorylation of the tagged construct determined by Western blot analysis (WB). The graph represents semiquantitative analysis of four experiments, as described in *Materials and Methods*. −, Absence of agent; +, presence of agent. b is significantly different from a ($P < 0.05$).

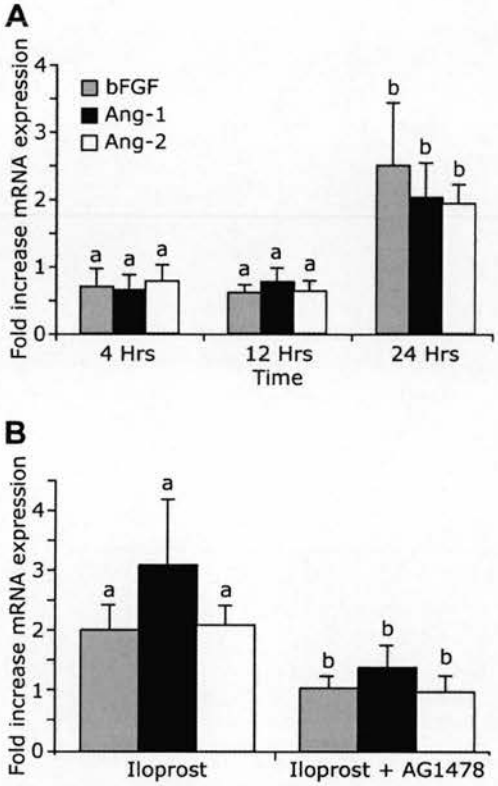

FIG. 5. (A) Time course demonstrating bFGF, Ang-1 and Ang-2 gene expression in Ishikawa cells in response to 100 nM Iloprost stimulation. Results are expressed as the mean + SEM (n = 4 experiments). b is significantly different from a ($P < 0.05$). B, mRNA expression of bFGF, Ang-1 and Ang-2 in Ishikawa cells following stimulation with 100 nM Iloprost for 24 h in the absence or presence of EGFR tyrosine kinase inhibitor (AG1478, 100 nM) as determined by real-time quantitative RT-PCR. Results are expressed as the mean + SEM (n = 3 experiments). b is significantly different from a ($P < 0.05$).

the MAPK pathway (10, 13, 21). The MAPK pathway is a key signaling mechanism that regulates many cellular functions, such as growth, differentiation, and transformation (22, 23). The data presented in this report also demonstrate that activation of the IP receptor induces a rapid increase in p42 ERK phosphorylation in Ishikawa cells and of p42/p44 ERK phosphorylation in endometrial tissue. Interestingly, the differential phosphorylation of ERK between Ishikawa cells and

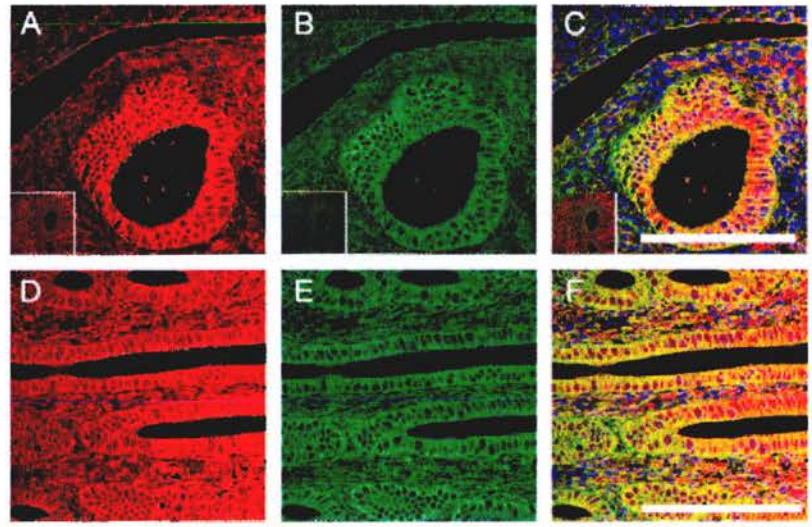

FIG. 6. Confocal immunofluorescent localization of the site of expression of IP receptor (*red*; A and D) with EGFR (*green*; B and E) and colocalization of IP with EGFR (merged; *yellow*; C and F). Expression is demonstrated in the epithelial cells of the basal (A–C) and functional (D–F) human proliferative endometrium. Inserts are shown for negative control sections as described in the methods. (*Scale bar*, 10 μm.)

endometrial biopsy tissue may be due to the presence of other cells types (stromal and endothelial) within the biopsy tissue compared with the homogeneity of the Ishikawa cell line. Alternatively, Ishikawa cells originate from an endometrial carcinoma and may respond differently from normal endometrial epithelial cells. This phosphorylation of ERK in Ishikawa cells and endometrial tissues is inhibited with the specific chemical inhibitor of EGFR kinase or by transfection of Ishikawa cells with a DN mutant isoform of the EGFR. Moreover the expression of EGFR colocalized with IP receptor in the glandular epithelial compartment of the human endometrium. Thus, as observed with prostanoid receptor signaling to downstream ERK1/2 in other model systems (10, 11, 24–26), in the present study, EGFR is in close proximity with the IP receptor, and EGFR transactivation is required for the PGI-IP-induced activation of the ERK1/2 signaling pathway within human endometrium. Whether the EGFR is held in a complex of protein-protein interactions with the IP receptor in the glandular epithelial compartment, or transactivation of the EGFR is mediated by intermediary scaffold adapter molecules to facilitate ERK1/2 signaling remains to be determined.

Our data also demonstrate that IP receptor activation by PGI leads to an increase in mRNA expression of several proangiogenic genes, including bFGF, Ang-1, and Ang-2, in both Ishikawa cells and normal human endometrium. bFGF is potent growth factor, which is known to promote the growth and proliferation of numerous cell types by activation of membrane FGF receptor tyrosine kinases. Moreover, bFGF is known to have potent proangiogenic effects in several model systems (27, 28) and has been implicated in promoting tumor angiogenesis (29, 30) and angiogenesis in proliferative lesions of endometriosis (31). The angiopoietins are a family of growth factors that act as ligands for the largely endothelial-restricted Tie-2 receptor tyrosine kinase, which is essential for vascular development. Ang-1 is a Tie-2 receptor agonist that is required for recruitment of perivascular cells, leading to the formation and stabilization of capillaries, vessel maturation, and endothelial cell survival. Ang-1 and other angiogenic factors, such as vascular endothelial growth factor (VEGF) and bFGF, may act synergistically to increase vascular sprouting and branching. In addition, the Ang-1/Tie-2 interaction enhances the mitogenic effect of angiogenic factors, such as VEGF, on endothelial cell growth (reviewed in Ref. 32). By contrast, Ang-2 is a natural Tie-2 receptor antagonist, destabilizing cell contacts and thus allowing access to angiogenic factors, such as VEGF. The process of angiogenesis is thus a fine balance among the expressions of numerous proangiogenic factors, all of which may be present concurrently in the cell to regulate vascularization in response to PGI-IP receptor interaction.

Furthermore, in the present study we have demonstrated that the actions of PGI, via the IP receptor, on target angiogenic gene expression are dependent upon the presence of the EGFR. It is also possible that this up-regulation of expression is mediated by ERK1/2 phosphorylation. This mechanism of target gene regulation in reproductive cells and tissues via prostanoid-receptor interaction is in agreement with previous studies. Transactivation of EGFR and ERK1/2 phosphorylation, leading to increased expression of angiogenic genes, including VEGF, have previously been shown for PGE_2-E-series prostanoid receptor 2 (10) and $PGF_{2\alpha}$-F-series prostanoid receptor interaction (11), suggesting that EGFR transactivation is a central theme for the promotion of vascular function in the human endometrium by prostanoids. In the present study we have focused on the role of PGI-IP receptor signaling to angiogenic genes using the Ishikawa endometrial epithelial cell line as a model system; however, it is possible that PGI-IP receptor signaling in stromal and endothelial cells may act in a synergistic manner with epithelial cells in the endometrium to favor angiogenesis, because our parallel studies of whole tissue endometrial biopsy explants are in agreement with our data derived from the Ishikawa cell line.

The precise role of PGI in human endometrium remains to be fully explored; however, PGI has been implicated in menstruation (33) and menstrual disturbances, where levels are elevated in endometrial pathologies such as menorrhagia (excessive menstrual blood loss) (8). It is thus possible that vascular disturbances in the endometrium of women with

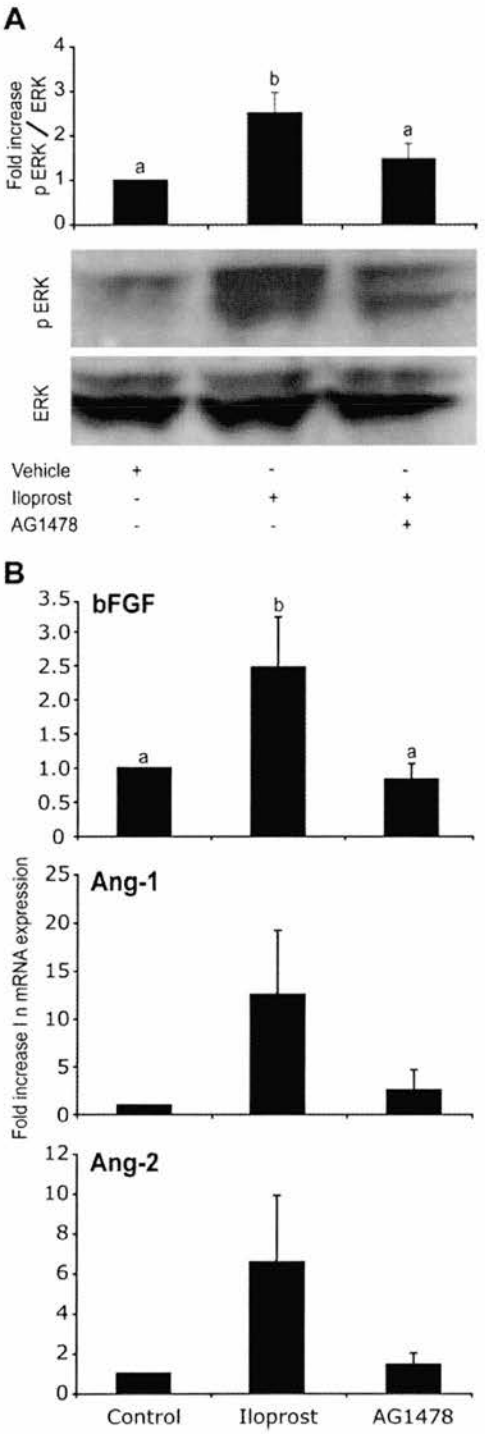

FIG. 7. ERK1/2 phosphorylation after treatment of endometrial tissue explants with 100 nM iloprost. A, Western blot analysis of ERK1/2 phosphorylation after stimulation with 100 nM iloprost for 10 min in the absence or presence of AG1478. The graph shows semiquantitative analysis of three experiments determined as described in *Materials and Methods*. b is significantly different from a ($P < 0.05$). −, Absence of agent; +, presence of agent. B, mRNA expression of bFGF (*top panel*), Ang-1 (*middle panel*), and Ang-2 (*lower panel*) within normal human endometrial tissue after stimulation with 100 nM iloprost for 24 h in the presence or absence of AG1478, as determined by quantitative RT-PCR. Results are expressed as the mean ± SEM (n = 4 experiments). b is significantly different from a ($P < 0.05$).

menstrual pathologies such as menorrhagia may be exacerbated by the elevation of proangiogenic genes, such as bFGF, Ang-1, and Ang-2, brought about by enhanced PGI-IP receptor signaling. In other studies, an aberration of expression levels of angiogenic growth factors has been demonstrated in endometrium from women with menorrhagia, such that a decrease in the expression of Ang-1 mRNA (34) and an increase in Ang-2 protein (35) were reported in endometrium collected from women with heavy menstrual blood loss compared with control endometrium. These alterations in Ang expression are coincident with an increase in the expression of bFGF receptor in the endometrium of women with excessive menstrual blood loss compared with control endometrium (36).

Taken together, we have demonstrated a potential role for PGI-IP receptor signaling in the Ishikawa endometrial epithelial cell line and whole human endometrial biopsy explants in regulating the mRNA expression of several proangiogenic genes. These genes can influence angiogenesis by acting on adjacent endothelial cells in an autocrine/paracrine manner. Moreover, these studies have demonstrated a role for PGI-EGFR cross talk in promoting angiogenic gene expression in the endometrium. Blockade of EGFR signaling with an orally active EGFR tyrosine kinase inhibitor has been used successfully in inhibiting angiogenesis in nude mice (37). Additionally, in a mouse model of colorectal cancer, studies have demonstrated that a combinatorial approach using a nonselective COX enzyme inhibitor in combination with an inhibitor of EGFR kinase is of greater therapeutic benefit than either compound alone (38). These observations of EGFR inhibition and ours reported in the present study suggest that targeted inhibition of EGFR function with small molecule chemical inhibitors alone or in combination with a COX enzyme inhibitor may modulate angiogenic activity in the endometrium, with possible benefits for menstrual pathologies that are associated with aberrant expression and signaling of prostanoids and altered angiogenesis or vascular function (39–42).

Acknowledgments

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Prostaglandin receptors are mediators of vascular function in endometrial pathologies

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Abstract

Prostaglandins are bioactive lipids produced from arachidonic acid by cyclooxygenase enzymes and specific terminal prostanoid synthase enzymes. Following biosynthesis, prostaglandins exert an autocrine/paracrine function by coupling to specific prostanoid G protein-coupled receptors to activate intracellular signaling and gene transcription. For many years prostaglandins have been recognised as key molecules in reproductive biology by regulating ovulation, endometrial physiology and proliferation of endometrial glands and menstruation. More recently a role for COX enzymes and prostaglandins has been ascertained in reproductive tract pathology, including dysmenorrhea, endometriosis, menorrhagia and cancer. Emerging evidence supports a role for COX enzymes, prostaglandins and prostaglandin receptor signaling pathways in a multitude of phenotypic changes in reproductive tissues including the promotion of angiogenesis and vascular function. Here we provide an overview of some of the findings from these studies with specific emphasis on the role of cyclooxygenase enzymes, prostaglandins and their receptors in benign and neoplastic pathologies of the human endometrium.

Keywords: Prostaglandins; Prostaglandin receptors; Endometrium; Angiogenesis

1. Introduction

The uterus plays a crucial role in survival of the species in all viviparous animals. Implantation of the fertilized egg is a critical event common to all species. Humans and old-world primates differ from most other animals in that they shed a significant proportion of their endometrium if pregnancy is not established at the opportune time. Human endometrium is thus a dynamic tissue which, in order to prepare for implantation, undergoes well-defined cycles of proliferation, differentiation and degradation (menstruation) in response to the prevailing endocrine and paracrine environment.

It is essential to have a detailed knowledge of the local mechanisms regulating local endometrial events involved in implantation and menstruation if we are to understand the mechanisms responsible for aberrations in endometrial function. This ever expanding knowledge base has the potential to contribute to far reaching applications in the medical management of benign and neoplastic pathologies of the endometrium such as unacceptable heavy menstrual bleeding, painful periods, endometriosis and cancer.

Cyclooxygenase (COX) enzymes and prostaglandins are known as important regulators of reproductive function and more recent data has implicated a role for these molecules in various pathologies including those of the endometrium. In this short review we will focus on the role of prostaglandins and their receptors in pathologies of the endometrium with special reference to their potential effects on angiogenic and vascular function.

2. Cyclooxygenase enzymes, prostaglandins and their receptors

Prostaglandins, thromboxanes and leukotrienes, collectively referred to as eicosanoids, are the COX and lipooxygenase (LOX) metabolites of arachidonic acid (AA). Following activation of phospholipase A2 (PLA2), AA is released from plasma membrane phospholipids or dietary fats and is cyclized, oxygenated and reduced to the intermediary prostaglandin H_2 (PGH_2) by COX enzymes. This intermediate serves as the substrate for terminal prostanoid synthase enzymes. These

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are named according to the prostaglandin they produce such that prostaglandin D_2 is synthesised by prostaglandin-D-synthase (PGDS), prostaglandin E_2 (PGE_2) by prostaglandin-E-synthase (PGES), prostaglandin $F_{2\alpha}$ by prostaglandin-F-synthase (PGFS), prostacyclin by prostaglandin-I-synthase (PGIS) and thromboxane (TXA_2) by thromboxane synthase (TXS) (Narumiya et al., 1999).

Three isoforms of COX enzyme (COX-1, COX-2 and COX-3) have been reported to catalyze the committed step in prostanoid (prostaglandins and thromboxanes) biosynthesis (Morita, 2002; Chandrasekharan et al., 2002). COX-1 is long thought of as a constitutive enzyme involved in performing normal physiological functions, but has now been shown by us and others to be up-regulated in various carcinomas (Sales et al., 2002a; Hwang et al., 1998; Kirschenbaum et al., 2000; Maldve et al., 2000) and to play a central role in tumorigenesis (Narko et al., 1997; Chulada et al., 2000; Kitamura et al., 2002). COX-2 is an immediate early gene that is rapidly induced by growth factors, oncogenes, carcinogens and tumor-promoting phorbol esters and a role has been ascertained for COX-2 in rheumatic disease, inflammation and tumorigenesis (Morita, 2002).

The roles played by COX enzymes in reproductive biology have been demonstrated using COX-deficient mice. Studies in COX-1-deficient mice have shown that the gestation period is prolonged and parturition is reduced coincident with a reduction in the number of viable offspring. Interestingly, conception and fetal development are unaltered suggesting that prostanoids produced by COX-1 are not critical for ovulation, fertilization or implantation, but are essential for bringing on normal labour at term. This is confirmed by observations between wild type mice giving natural birth and COX-1 deficient females having their young delivered by cesarean section, where there are no noticeable differences in the number or size of the offspring delivered (Langenbach et al., 1995; Gross et al., 1998).

By contrast, ablation of the COX-2 gene in mice results in multiple reproductive failures, including ovulation, fertilization, implantation and decidualization confirming that prostaglandins produced by COX-2 play a crucial role in these processes (Langenbach et al., 1999a,b; Dinchuk et al., 1995; Lim et al., 1997). Prior to ovulation, pituitary gonadotropins trigger the expression of COX-2 and synthesis of PGE_2 to promote follicle expansion and ovulation. In COX-2 deficient mice, absence of PGE_2 in preovulatory follicles disrupts follicle expansion and results in anovulation. Under these conditions administration of exogenous PGE_2 has been shown to rescue ovulation (Davis et al., 1999). Thus, although both COX isoforms essentially catalyze the same reaction, in the reproductive tract there are clear differences in the prostanoid profile and functions of the two COX enzymes.

Following biosynthesis, prostanoids are rapidly transported out of the cell by means of a prostaglandin transporter (PGT) and act in an autocrine/paracrine manner on their cognate heptahelical transmembrane G-protein-coupled receptors (GPCRs) in the vicinity of their sites of production. PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 exert their biological function through interactions with DP, EP, FP, IP and TP GPCRs respectively. There are four subtypes of EP receptors, termed EP1, EP2, EP3 and EP4, which are encoded by four separate genes (Narumiya et al., 1999). In addition, there are several splice variants of the EP3, FP and TP receptors, which differ only in their C-terminal tails. Generally, prostanoid receptor isoforms exhibit similar ligand binding but differ in their signaling pathways, their sensitivity to agonist-induced desensitisation, and their tendency toward constitutive activity. Phylogenetic analyses indicate that receptors sharing a common signal pathway have higher sequence homology than receptors sharing a common prostanoid as their preferential ligand. Among the different receptors, the IP, DP, EP2 and EP4 receptors elevate intracellular cAMP accumulation via $G_{\alpha s}$ and have been termed "relaxant" receptors since they induce smooth muscle relaxation. TP, FP and EP1 receptors induce calcium mobilisation via $G_{\alpha q}$ and constitute a "contractile" receptor group since they cause smooth muscle contractions. The remaining receptor, EP3, is generally associated with a decline in cAMP levels and has been termed the "inhibitory" receptor and usually stimulates smooth muscle contraction, however depending on the splice variant and cell type, the EP3 receptor can also elevate intracellular cAMP and mobilise calcium (Narumiya et al., 1999).

The specific roles played by prostanoids, especially PGE_2 and $PGF_{2\alpha}$ in modulating reproductive physiology, have been demonstrated using mice deficient for each of the prostanoid receptors (Narumiya et al., 1999). The most startling observations have been derived from the EP2 and FP receptor knockouts. Recent studies have shown that the EP2 and FP receptors are indispensible in female reproduction (Kennedy et al., 1999; Hizaki et al., 1999; Tilley et al., 1999; Narumiya and FitzGerald, 2001). Loss of EP2 receptor function in murine model systems by gene ablation, results in impaired ovulation and dramatic reduction in litter size (Tilley et al., 1999; Kennedy et al., 1999; Hizaki et al., 1999), whereas ablation of the FP receptor in mice results in loss of parturition (Narumiya and FitzGerald, 2001).

3. Vascular function in the endometrium: the role of COX enzymes, prostanoids and prostanoid receptors

The human endometrium is a dynamic tissue, which in the absence of pregnancy, undergoes well-defined episodes of tissue proliferation, differentiation and breakdown on a cyclical basis. This cyclical nature of tissue breakdown and remodelling is the menstrual cycle (Critchley et al., 2001b). The menstrual cycle is clinically described according to its regularity and length of bleeding (Chiazze et al., 1968; Treloar et al., 1967). The mean menstrual cycle length during a woman's reproductive years is between 28 and 32 days with the duration of menses lasting 4–5 days.

Menstruation is an active process whereby the upper two thirds of the endometrium (functional layer) is shed and regenerated on a repetitive basis, once every cycle in response to withdrawal of the circulating ovarian steroids. After menstruation, the functional layer is replenished by the sequential exposure to estradiol and progesterone (Critchley et al., 2001b; Lockwood et al., 2004), which are known to modulate the expression of a diverse array of effector molecules, including cytokines and

prostanoids, which in turn modulate vascular function in the endometrium to facilitate tissue regeneration.

In addition to replenishment of the superficial epithelium, the vascular compartment of the endometrium comprising the spiral arteries and arterioles also needs to be reconstructed following menstruation. Hence, new endothelial cells are encouraged to sprout and recruit pericytes to form capillaries and smooth muscle cells to form larger vessels (Smith, 1998; Gargett and Rogers, 2001). This process involves degradation of the extracellular matrix, endothelial cell proliferation and migration and organisation of the endothelial cells into capillary networks. Several angiogenic factors have been identified to-date and are believed to be involved in angiogenesis of the human endometrium (Gargett and Rogers, 2001). Of the known angiogenic factors, vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), is a major specific stimulator of endothelial cell proliferation and vascular permeability in the endometrium. VEGF acts through two tyrosine kinase receptors, VEGFR-1 (flt-1) and VEGFR-2 (KDR), the latter being considered the more important in regulation of angiogenesis.

Organisation of blood vessel formation, stabilisation, maintenance and regression also involves other factors. These include the angiopoietins, Ang-1 and Ang-2, which stabilise and destabilise blood vessels respectively to facilitate angiogenesis (Maisonpierre et al., 1997). Although the precise mechanism of action of the angiopoietins remains to be clarified, it is proposed that Ang-2, acting through its tyrosine kinase receptor Tie-2, enhances the action of VEGF by reducing endothelial contact with the extracellular matrix, and hence with adjacent endothelial cell interactions. In contrast, Ang-1 acting as a competitive antagonist via Tie-2 enhances the stability of the newly formed blood vessels by recruiting pericytes (Maisonpierre et al., 1997). Several other factors including basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF), endocrine gland VEGF (EG-VEGF) and their respective receptors (Battersby et al., 2004; Weston and Rogers, 2000) are also known to stimulate angiogenesis. Thus, the remodelling of the endometrial vasculature is a complex and multifactorial process, under the influence of multiple effector molecules.

Towards the end of the menstrual cycle and concomitant with the withdrawal of progesterone and sloughing of the functional layer of the endometrium, COX-2 expression increases in the endometrial glandular epithelium and lasts through the proliferative phase of the cycle (Jones et al., 1997; Uotila et al., 2002; Critchley et al., 1999). This is accompanied by an elevation in the expression of EP2, EP4 and FP receptors, which reach their peak during the mid-late proliferative phase of the menstrual cycle, coincident with an elevation in the expression of the prostaglandin transporter (PGT) (Kang et al., 2005). Once COX-2 expression and prostanoid biosynthesis is induced, a positive feedback system is set in motion to regulate the COX-PG biosynthetic signaling pathway and target genes involved in vascular function (Fig. 1). Auto-regulation of COX-2 expression has been reported in several in vitro studies (Tjandrawinata et al., 1997; Tjandrawinata and Hughes-Fulford, 1997; Maldve et al., 2000; Fujino and Regan, 2003). Following up-regulation of COX-2

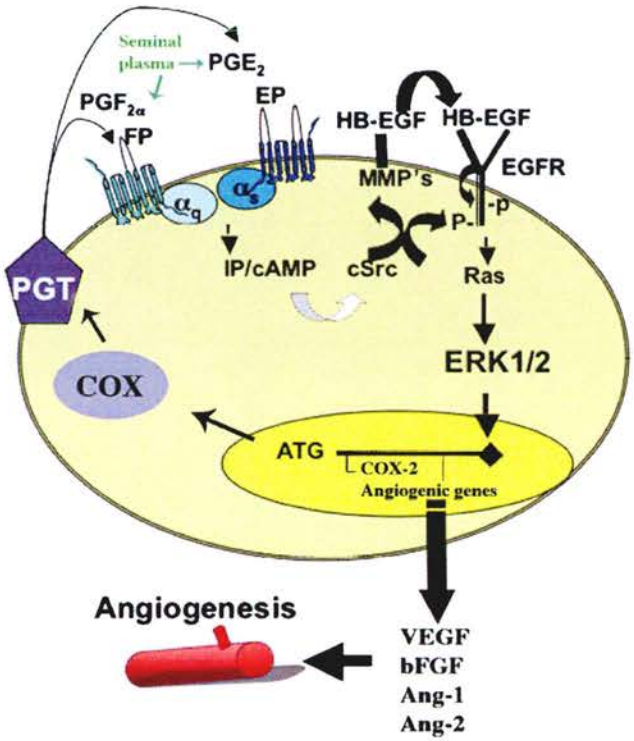

Fig. 1. Autocrine/paracrine regulation of prostanoid receptor signaling and the downstream effects on biological function. In this cartoon, prostaglandins (produced either intracellularly via the COX enzyme biosynthetic pathway or present in seminal plasma) activate specific prostanoid receptors (such as EP or FP), initiating second messenger production (inositol 1,4,5-trisphosphate, IP; cyclic adenosine 3′,5′-monophosphate, cAMP). Activation of the second messenger systems can initiate signaling via the small G-protein Ras-extracellular signal-regulated kinase (ERK1/2) pathway to initiate target gene transcription. Recent data suggest that target gene transcription can occur via prostanoid-receptor-mediated transactivation of receptor tyrosine kinases (RTKs) such as the epidermal growth factor receptor (EGFR), either via the release of a heparin-bound (HB)-EGF-like molecule to directly activate the RTK or by intracellular mechanisms involving non-receptor tyrosine kinases (such as c-SRC). The activation of target genes such as COX-2 can auto-regulate tumorigenesis by establishing a positive feedback loop to promote prostanoid-receptor signaling and can also in turn promote angiogenesis (by modulating vascular function and vascular tone) by elevating the expression of pro-angiogenic genes such as VEGF, bFGF, Ang-1 and Ang-2.

expression, the subsequent elevated prostanoid biosynthesis and signaling can promote the expression of pro-angiogenic factors, such as VEGF, bFGF, PDGF, Ang-1 and Ang-2 (Fig. 1) (Sales et al., 2002a; Tsujii et al., 1998) or down-regulate the expression of anti-angiogenic genes such as cathepsin-D (Perchick and Jabbour, 2003).

These angiogenic factors have been shown to act in a paracrine manner on endothelial cells to enhance endothelial cell proliferation and promote their rearrangement into tubular structures (Tsujii et al., 1998). These data suggest that endometrial angiogenesis may be under direct control of prostaglandins, produced by COX enzymes in the endometrium. Recently the role of specific prostaglandin receptors in modulating angiogenesis has been described. In endometrial epithelial cells (Sales et al., 2004a) and murine model systems (Seno et al., 2002; Sonoshita et al., 2001), elevated PGE_2-EP2 receptor interaction promotes

the expression of pro-angiogenic genes such as VEGF. Similarly, elevated $PGF_{2\alpha}$-FP receptor interaction in endometrial epithelial cells can also elevate the expression of VEGF in a similar manner to that of PGE_2 via the EP2 receptor to promote angiogenesis in the endometrium via the activation of convergent intracellular signaling effector molecules and signal transduction cascades, such as the ERK1/2 cascade (Sales et al., 2005).

4. COX enzymes and prostaglandins in benign endometrial pathologies

Problems of menstruation in which prostaglandins appear to have an etiological role, are those in which the clinical symptoms of pain or bleeding disturbances are prominent. The common disorders described here are those of pain associated with menstruation (dysmenorrhoea), the troublesome pathology of endometriosis and excessive menstrual blood loss (menorrhagia).

4.1. Dysmenorrhoea

Dysmenorrhoea describes painful periods and is a significant public health burden with up to 50% of women suffering from the condition at some point in their reproductive lifetime (Brosens et al., 2000; Dawood, 1985).

Primary dysmenorrhoea is an extremely common condition, typically occurring in adolescence and in the absence of any recognisable pathology. Secondary dysmenorrhoea is recognised as a condition associated with other pelvic pathologies such as endometriosis, pelvic inflammatory disease and uterine fibroids. It is much less common than primary dysmenorrhoea and the role of prostaglandins is less well documented.

Primary dysmenorrhoea is associated with uterine hypercontractility. During contractions, uterine blood flow decreases which leads to relative uterine ischaemia. Good correlation has been found between uterine blood flow and pain (Brosens et al., 2000; Dawood, 1985; Fraser, 1992). However, pain has been documented in the absence of uterine contractility and it is recognised that prostaglandins have an important alternate role in the etiology. Prostaglandins have a known role in inflammatory nocosomial pathways and may have a direct effect in stimulating nerve pain pathways (Bley et al., 1998). Excessive synthesis and secretion of prostaglandins by the endometrium have been detected in menstrual fluid taken form women with primary dysmenorrhoea (Lundstrom et al., 1976; Pickles et al., 1965). A clear demonstration of increased PGE_2 and $PGF_{2\alpha}$ levels in menstrual fluid of women with dysmenorrhoea compared to controls, shows that this increase was most prominent in the first 2 days of menstruation and that there is a greater proportional increase in $PGF_{2\alpha}$ levels (Lumsden et al., 1983). Furthermore, infusion of $PGF_{2\alpha}$ into the uterine cavity produces an increase in uterine contractility and dysmenorrhoea like pain (Lundstrom, 1977), presumably via interaction with the FP receptor and activation of intracellular signaling. The role of prostaglandins in dysmenorrhoea is additionally confirmed by the effective use of COX enzyme inhibitors (Lundstrom, 1978; Rees and Lopez Bernal, 1989) including selective COX-2 enzyme inhibitors (Daniels et al., 2002). In summary, the vasoconstrictive properties of $PGF_{2\alpha}$ together with the ability to cause myometrial contractions could contribute to dysmenorrhoea and in addition the increased availability of PGE_2 could have additional direct pain inducing properties (Bley et al., 1998).

4.2. Endometriosis

Endometriosis occurs when tissue, histologically similar to the endometrium, is found in extrauterine sites with tissue implants most often found over visceral and peritoneal surfaces. The extent of disease is highly variable and endometriosis can be associated with pelvic pain and infertility. Although not a malignant disorder, endometriotic tissue exhibits all the hallmarks of tumorigenic tissue, namely proliferation, cellular invasion and angiogenesis. According to Sampson's classical theory; retrograde menstruation, peritoneal adhesion of endometrial tissue and outgrowth are essential in the pathogenesis of endometriosis. However, retrograde menstruation is a common occurrence with viable endometrial cells found in 76–90% of women (Bartosik et al., 1986), a much higher incidence than endometriosis (2–3%). Other factors must therefore determine the susceptibility of women to develop endometriosis. This susceptibility is likely to be a result of changes to both the peritoneal environment and to the characteristics of the menstrual tissue.

The etiology of endometriosis is multifactorial and requires a number of peritoneal substances, in particular angiogenic factors, to facilitate implantation in ectopic sites (Oral et al., 1996). The peritoneal fluid contains a variety of free-floating cells including macrophages, natural killer (NK) cells and lymphocytes. These cells all have immune mediating roles and can promote cellular growth and viability through the secretion of growth factors and cytokines. Macrophages are the most abundant cell type in the peritoneal fluid and have their highest endometrial expression in menstrual tissue. Once activated, they can release products including prostaglandins (Gazvani and Templeton, 2002). It has been demonstrated that peritoneal macrophages from women with endometriosis release more PGE_2 and $PGF_{2\alpha}$ compared to those without endometriosis (Karck et al., 1996). Further evidence implicating prostaglandins in the etiology of endometriosis arises from histological analysis of endometrial tissue. A higher degree of immunostaining for COX-2, the rate-limiting enzyme in prostaglandin production, is found in endometrial glandular epithelium in women suffering from endometriosis compared to control endometrium (Ota et al., 2001). It is therefore thought that the increased PGE_2 and PGF_2 biosynthesis may play a role in the regulation of this disorder.

Although the exact mechanisms whereby prostanoids exert their function in endometriosis is unclear, PGE_2 is known to be a potent inducer of aromatase activity in endometrial stromal cells (Noble et al., 1997). Aromatase gives rise to the synthesis of estrogen, which in turn will feedback positively on COX enzyme expression and further prostaglandin synthesis. Endometriosis is classically described as an estrogen dependent disease, but the lack of ER expression in pre-menstrual and menstrual tissue (Critchley et al., 2001a) suggests that initi-

ation of the disease is independent of oestrogen. However, once implantation has occurred, prostaglandins provide a mechanism for stimulating estrogen production to sustain the endometriotic lesions.

COX enzymes, PGE_2, $PGF_{2\alpha}$ and their receptors have been shown to promote the transcription of angiogenic factors such as VEGF and angiopoietins (Fig. 1). The peritoneal fluid of women with endometriosis displays greater angiogenic activity than women without endometriosis (Gazvani and Templeton, 2002). Angiogenic factors may be important for creating a microvascularised peritoneal environment that would allow for establishment of endometriotic lesions and this may be regulated in part by the COX-PG biosynthetic pathway (Sales et al., 2002a, 2004a, 2005). VEGF and its receptor KDR are dramatically and specifically up regulated in the superficial stromal cells of the endometrium in response to the decline of progesterone (Brenner et al., 2002). It is postulated that VEGF has a unique role in stimulating matrix metalloproteinase (MMP) expression within the endometrium. Therefore, the rich content of MMPs, VEGF and inflammatory prostaglandins found simultaneously in menstrual fragments could facilitate attachment and angiogenesis of endometriotic lesions (Brenner et al., 2002), and this may be facilitated in an autocrine/paracrine manner by activation of specific prostanoid receptors and their diverse downstream signaling pathways.

4.3. Menorrhagia

Menorrhagia is the clinical definition describing excessive menstrual blood loss. Unacceptable heavy menstrual blood loss affects 10–30% of women of reproductive age and up to 50% of perimenopausal women (Prentice, 1999, 2000). In the United Kingdom, 5% of women will seek help for this symptom annually (Vessey et al., 1992) and the risk of hysterectomy performed for menstrual disorders is 20% (Coulter et al., 1988).

Treatment using inhibitors of COX enzymes have repeatedly been shown to reduce menstrual blood loss (Anderson et al., 1976; Makarainen and Ylikorkala, 1986; Cameron et al., 1990) implicating disturbances of prostaglandin pathways in the etiology of menorrhagia.

In research studies, the objective definition of menorrhagia is often based on measurement of menstrual hemoglobin content. Scandinavian studies demonstrated that the mean menstrual blood loss was around 40 ml and that regular menstrual blood loss in excess of 63 ml was associated with iron deficiency anemia (Hallberg et al., 1966). The 90th centile for measured blood loss was 80 ml and this was taken as the upper limit of normal (Hallberg et al., 1966). However, in the clinical setting only 40–60% of women with perceived heavy menstrual blood loss have an objective measurement of greater than 80 ml (Fraser et al., 1981; Chimbira et al., 1980).

Dysfunctional uterine bleeding (DUB) describes excessive menstrual blood loss in the absence of any pathology. This condition accounts for the majority of cases of menorrhagia and is ovulatory in 80% of cases (Cameron, 1989; Livingstone and Fraser, 2002). Other causes of menorrhagia are pelvic pathologies such as fibroids and systemic disease including blood clotting disorders such as Von Willebrands disease (Makarainen and Ylikorkala, 1986; Kouides, 2001). Bleeding disorders represent a small proportion of women presenting with menorrhagia. Reduced clotting is a known feature of menstruation (Livingstone and Fraser, 2002) however the exact mechanisms that lead to excessive menstrual blood loss for many systemic diseases are poorly understood. Pelvic pathologies such as fibroids are common, affecting between 20 and 25% of women and it is reported that around a third of women with fibroids complain of excessive menstrual blood loss (Buttram and Reiter, 1981). There is a large body of evidence linking local mediators, in particular prostaglandins, to ovulatory DUB and this is discussed below. However, the role of prostaglandins in excessive menstrual blood loss secondary to pathologies such as fibroids is unclear. There is evidence that the use of NSAIDs is less effective in treating women with menorrhagia when fibroids exist (Makarainen and Ylikorkala, 1986).

Investigations into the etiology of ovulatory DUB have not been able to discover any differences in circulating steroid hormone levels in women with excessive menstrual blood loss compared to women with normal menstruation (Eldred and Thomas, 1994). Additionally, there are no specific histological differences within the endometrium of women with excessive menstrual blood loss (Rees et al., 1984a,b). Of note, no differences in expression of estrogen and progesterone receptors have been discovered (Critchley et al., 1994). However, substantial evidence does exist implicating local mediators, in particular disturbances of arachidonic acid metabolism and disturbances of angiogenic processes. Elevated levels of PGE_2 have been found in endometrium of women complaining of heavy periods (Willman et al., 1976). Further experiments confirmed an elevation of total prostaglandins with an apparent shift in synthesis in favour of PGE_2 over $PGF_{2\alpha}$ in the endometrium of women with DUB (Smith et al., 1981). Additionally, an increase in PGE_2 binding sites has been reported in uterine tissue associated with menorrhagia (Adelantado et al., 1988), although the specific prostanoid receptors mediating this pathology remain to be elucidated. Mefenamic acid, a COX inhibitor and member of the Fenamate family of drugs, is routinely used as a first line of treatment for menorrhagia. A dual mode of action has been documented for this family of drugs; as well as inhibiting prostaglandin synthesis they also inhibit binding of PGE_2 to its receptors (Rees et al., 1988). It is thus plausible that one or multiple EP receptors may mediate the role of PGE_2 in this disorder. PGE_2 has vasodilatory properties and it is interesting to note that prostacyclin (PGI_2) and nitric oxide synthesis, vasodilatory substances, are also both elevated in uterine tissue of women with excessive menstrual blood loss (Smith et al., 1981; Zervou et al., 1999). It appears that local mediators with vasodilatory effects on the endometrial vasculature are implicated in menorrhagia.

As mentioned prostaglandins and PGE_2 signaling have been shown to promote angiogenesis via autocrine–paracrine mechanisms (Fig. 1). Endothelial cells in the endometrium of women with heavy periods have been shown to proliferate more as compared to controls (Kooy et al., 1996). In addition the spiral arterioles in menorrhagic endometrium have a less established

covering of vascular smooth muscle cells (VSMC) (Abberton et al., 1996, 1999). Lack of cellular support to the spiral arterioles prevents sufficient vasoconstriction of these blood vessels, hence potentially increasing menstrual blood loss. However, hysteroscopic appearance of endometrial surface vessels appears to be very similar between women with DUB compared to normal endometrium (Hickey et al., 2000). The hypothesis that menorrhagia arises as a result of poor vascular maturation is supported by the finding of decreased Ang-1 mRNA expression in the endometrium of women with menorrhagia (Hewett et al., 2002). Immunohistochemical studies have additionally demonstrated an increase in expression of Ang-2 and it is receptor Tie-2 in menorrhagic endometrium (Blumenthal et al., 2002). These disturbances of angiopoietin expression with reduced Ang-1:Ang-2 ratio would be in favour of vessel destabilisation. Since there is evidence that Ang-1 and Ang-2 are regulated by PGE_2 (Sales et al., 2002a), immature vessels in turn could be more susceptible to the vasodilatory actions of prostaglandins such as PGE_2, leading to heavier menstruation, and this may be enhanced via the autocrine/paracrine actions of PGE_2 via one or multiple EP receptors and their intracellular signaling pathways.

5. COX enzymes and prostaglandins in neoplastic pathology of the endometrium

Adenocarcinoma of the endometrium is one of the most frequently diagnosed malignancies of the female genital tract in the Western world, ranking fourth in incidence among invasive tumors in women, following breast, lung, and colon cancers. Incidence rates of the disease are described as 10–25 women per 100,000 with a clear geographic variation between European (United Kingdom, France and Spain) and North American (United States of America and Canada) countries, with a greater incidence recorded in North America (Parazzini et al., 1991). Adenocarcinoma of the endometrium is typically a disease of postmenopausal women with approximately 85% of the patients being over 50 years of age. The etiology of endometrial adenocarcinoma is poorly understood, however, menopause after age 52, nulliparity, obesity, diabetes and the administration of unopposed estrogen and tamoxifen are the main factors that are known to predispose women to this type of cancer, making the disease a significant factor in women's health in an aging population. Owing to symptoms such as abnormal vaginal bleeding, the disease is often diagnosed early, and this is reflected in the 86% 5-year survival rate (Ellenson and Wu, 2004). Endometrial adenocarcinomas arise from several cell types with adenocarcinoma arising from the glandular epithelium being the most common type, accounting for 80–90% of all uterine tumors (Gordon and Ireland, 1994; Mant and Vessey, 1994; Wingo et al., 1995).

Over the last 10 years, numerous studies using gene-disruption and gene overexpression systems in cell lines and laboratory animals have provided conclusive evidence to support a role for COX enzymes, prostanoids and prostanoid receptors in pathology (Chulada et al., 2000), including cancer of the endometrium (Sales and Jabbour, 2003a,b). Recent studies have indicated a correlation between up-regulated COX enzyme expression and incidence of uterine carcinomas. In all studies reported thus far, expression of COX-1 appears to be unaltered in endometrial carcinomas (Tong et al., 2000; Uotila et al., 2002; Sales and Jabbour, 2003a), indicating a possible housekeeping function. In other reproductive tract pathologies such as ovarian and cervical carcinomas, COX-1 expression is elevated above normal indicating a divergence of function in these tissues compared with that observed in the endometrium (Sales et al., 2002a; Dore et al., 1998). By contrast, COX-2 expression is up-regulated in neoplastic epithelial and endothelial cells of endometrial carcinomas (Tong et al., 2000; Jabbour et al., 2001; Ferrandina et al., 2002; Uotila et al., 2002). These findings have lead to the suggestion that COX enzyme inhibitors may be of potential benefit as therapeutic regimens for endometrial carcinomas.

Non-selective NSAID use has, however, been associated with gastrointestinal toxicity, ulceration, platelet dysfunction and kidney damage in at least 1% of users (Fosslien, 1998; Smith et al., 2000). Even highly selective NSAID derivatives, which showed enormous potential as a therapy for pathologies highly expressing COX-2 have recently been shown to pose a potential risk of myocardial infarction and sudden cardiac death in certain individuals (Graham et al., 2005). More recently it has been proposed that small molecule antagonists of prostaglandin receptor function may be more effective strategies against the effects of prostanoids in pathologies highly expressing COX enzymes. These ideas stem from the observations that expression and signaling of prostanoid receptors, such as EP2, EP4, FP and PPARδ are up-regulated in uterine carcinomas coincident with the elevation of COX-2 expression and synthesis and secretion of PGE_2 and $PGF_{2\alpha}$ (Tong et al., 2000; Jabbour et al., 2001; Sales et al., 2001; Ryu et al., 2000; Kulkarni et al., 2001). Indeed elevated PGE_2-EP_2 and $PGF_{2\alpha}$-FP receptor signaling has been shown to promote endometrial tumor cell proliferation and induce the expression of potent pro-angiogenic factors, like VEGF. This is mediated via convergent pathways by inducing the transactivation of growth factor receptors, such as the epidermal growth factor receptor (EGFR) in a predominantly ligand-independent manner leading to the activation of mitogenic signaling to ERK1/2 (Fig. 1) (Sales et al., 2004a,b, 2005).

In animal model systems, blockade of EGFR signaling with an orally active EGFR tyrosine kinase inhibitor has been used successfully in carcinomas of nude mice to inhibit tumor angiogenesis, by reducing VEGF expression (Kedar et al., 2002). Similarly the use of orally active inhibitors of the Ras-ERK cascade have proved efficacious in treatment of metastatic melanoma (Collisson et al., 2003). More, recently a combinatorial approach using a non-selective COX-enzyme inhibitor in combination with an inhibitor of EGFR kinase has been shown to reduce polyp formation in $APC^{\Delta 716}$ mice more effectively than either compound on their own (Torrance et al., 2000). In light of these findings and the fact that different prostanoids can activate convergent signaling pathways to perform a common function in promoting endometrial tumorigenesis, targeted disruption of specific common signaling pathways

such as ERK1/2 or EGFR may also be efficacious intervention strategies.

6. Modulation of endometrial pathologies by seminal plasma

There is much emerging evidence to suggest that the introduction of semen into the female reproductive tract during coitus orchestrates molecular and cellular changes to facilitate conception. Seminal plasma is known to contain estrogen, progesterone, cytokines and growth factors (Robertson, 2005), all of which have the capacity to modulate cellular function via their actions on their cognate receptors. Indeed, seminal plasma has been shown to influence the expression of genes in endometrial epithelial and stromal cells, where expression of a number of pro-inflammatory mediators such as IL-1β, IL-8 and VEGF is increased following treatment with seminal plasma (Gutsche et al., 2003).

In addition, it is well established that prostaglandins are present in high concentrations in the semen of primate species including man. Seminal plasma concentrations of prostaglandins are 10,000-fold greater than that detected at the site of inflammation and PGE_2 is one of the major types detected (Templeton et al., 1978).

This has prompted the suggestion that seminal plasma prostaglandins may exacerbate endometrial pathologies by activating intracellular signaling via the elevated prostanoid receptors (Fig. 1).

Recently, seminal plasma and PGE_2 have been shown to promote COX-2 and EP receptor expression and signaling in cervical adenocarcinoma cells (Sales et al., 2002b). In addition to contact with cervical tissue where semen is deposited during intercourse, seminal plasma is transported into the uterine cavity in humans (Kunz et al., 1997). Research using labelled albumin macrospheres deposited at the external cervical os demonstrates that the spheres can travel into the uterine cavity within minutes of deposition. The proportion of spheres entering the uterine cavity increases with increased intensity of uterine contractions during the menstrual cycle (Kunz et al., 1997). Similarly, in vivo studies show that the constituents of seminal plasma, bound to the post-acrosomal region of sperm, are carried together with the sperm into the uterus and higher tract, served by the peristaltic contractions observed in the reproductive tract, during the menstrual cycle (Chu et al., 1996; Kunz and Leyendecker, 2002). Therefore, in sexually active women, in addition to regulation by endogenous prostaglandins produced as a consequence of up-regulated COX enzyme expression, reproductive tract pathologies with elevated prostaglandin receptors may also be modulated by prostaglandins in seminal plasma (Fig. 1).

This highlights further the advantages of utilising combinatorial therapeutic approaches of COX inhibitors with receptor antagonists or signaling inhibitors in sexually active women with pathologies of the reproductive tract that are associated with elevated prostanoid receptor expression. The effectiveness of these combinatorial approaches as means of therapy will need to be extensively investigated.

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Review

Progestogen only contraception and endometrial break through bleeding

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Abstract

Progestogen only contraceptives (POC) provide a safe and effective method of fertility regulation. Unfortunately, they are commonly associated with the problem of endometrial break through bleeding (BTB), often leading to discontinuation of use. An increase in endometrial vascular fragility has been demonstrated as an important mechanism that contributes to BTB but our understanding of the interaction between exogenous steroid use and endometrial vasculature remains incomplete. This review sets out to describe a number of commonly used POC, their effects on endometrial morphology and possible molecular and cellular mechanisms that may lead to unscheduled bleeding.

Abbreviations: Ang – angiopoietin; BM – basement membrane; BTB – breakthrough bleeding; ER – oestrogen receptor; HEEC – human endometrial endothelial cells; HESC – human endometrial stromal cells; HIF-1α – hypoxia inducible factor-1α; IL-1β – interleukin-1β; LNG – levonorgestrel; LNG-IUS – levonorgestrel intrauterine system; MMP – matrix metalloproteinases; MPA – medroxyprogesterone acetate; PD-ECGF – platelet derived endothelial cell growth factor; POC – progestogen only contraceptives; PR – progesterone receptor; TF – tissue factor; TGF-β1 – transforming growth factor β1; TIMP – tissue inhibitor of matrix metalloproteinases; TP – thymidine phosphorylase; VEGF – vascular endothelial growth factor; VSMC – vascular smooth muscle cells

Introduction

The search for improved forms of contraception will always remain of importance. The world's population figures, around 6057 million in the year 2000, are due to rise to 9322 million by the year 2050 [1]. Virtually all this increase in population will occur in developing countries, leading to significant social and health implications. The 'perfect' contraceptive should combine safety and efficacy with convenience of use and ideally be able to offer additional health benefits. Progestogen only contraceptives (POCs) have a worldwide usage and are able to provide safe and effective contraception. Unfortunately the unwanted side effect of endometrial break through bleeding (BTB) is a universal problem for all modalities of POC and this is the commonest cause for discontinuation of contraceptive use [2, 3].

The exact mechanism of progestogen induced BTB has yet to be explained, with much evidence pointing towards superficial blood vessel fragility. In addition to enhanced blood vessel fragility, local changes in endometrial steroid response, structural integrity, tissue perfusion and local angiogenic factors are likely to contribute to the mechanism of progestogen induced BTB.

As yet there is no established long-term intervention available to manage BTB and thus a greater understanding of the mechanisms involved is required.

Progesterone and oestrogen are naturally occurring steroid hormones derived through steroidogenesis from cholesterol. In the normal menstruating woman, they are responsible for orchestrating the morphological characteristics in the endometrium required for its functional endpoints – implantation or menstruation with associated regeneration and repair.

Progestogens are often defined as any synthetic compound that can sustain human secretory endometrium. Small molecular changes to the structure of progesterone have led to dramatic changes in pharmacological properties such as absorbance, half-life, tissue availability and side effects. Hence, a variety of synthetic progestogens are used in contraceptive formulations (Table 1). The gonanes and estranes [4] are structural classifications for derivatives of 19-nortestosterone. All estranes require conversion into norethindrone for

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biological activity. Gonanes include the common contraceptive progestogen levonorgestrel as well as newer third generation agents developed to reduce drug androgenicity. A third group of progestogens, pregnanes, are derived from 17 α-hydroxy progesterone and include cyproterone acetate and medroxyprogesterone acetate (MPA), commonly used in a depot contraceptive.

A recently developed progestogen called drospironone, is not derived from testosterone or progesterone but from spironolactone, a mineralocorticoid receptor antagonist.

POCs prevent pregnancy via a number of mechanisms depending upon the contraceptive type [5]. These include suppression of ovulation, suppression of normal luteal activity, production of hostile cervical mucous and changes to endometrial morphology. There are a wide variety of POCs available (summarised in Table 2), all of which deliver a variety of progestogens, administered via different routes in different doses.

All POCs lead to problematic break through bleeding, the type of contraceptive use influences the amount and character of bleeding with many women reporting an improvement with time [5]. BTB can occur in up to 55% of women after 3 months' use of the levonorgestrel intra-uterine releasing system (LNG-IUS) [6] and over 50% of women using Norplant have been reported to request removal within the first year of use on account of menstrual disturbances [7].

Effects of progesterone and progestogens on endometrial morphology

The sequential exposure of the endometrium to oestrogen and progesterone leads to a series of classical morphological changes [8]. Exogenous steroids may not always lead to similar histological changes within the endometrium, as the response of this target organ is dependent upon the dose of steroid, the mode of delivery and duration of therapy. To understand the mechanisms of BTB, an understanding of normal endometrial morphology is required. The following description of morphological changes is summarised in Table 3.

Endometrial steroid receptors

The two layers of the endometrium are the superficial functional layer, which is shed during menstruation and the basal layer from which the endometrium has always been considered to regenerate. Three phases of endometrial development are recognised: a preovulatory *proliferative* phase, a postovulatory *secretory* phase and a *menstrual* phase. Oestrogen is responsible for the endometrial proliferative changes. Exposure to progesterone in the second half of the cycle results in secretory differentiation. The decline in circulating levels of steroid hormones, oestrogen and progesterone, in the absence of pregnancy, are responsible for the onset of menstruation. Overall progesterone exerts an anti-oestrogenic effect with inhibition of endometrial growth and induction of glandular differentiation. These effects require the progesterone receptor to be present which paradoxically only exists in an oestrogen primed endometrium. The expression of endometrial sex steroid receptors varies both temporally and spatially within the endometrium (reviewed by Critchley et al. [9]). Both oestrogen (ER) and progesterone (PR) receptors are upregulated in stromal and glandular tissue during the proliferative phase of the menstrual cycle. During the secretory phase, ER and PR are down regulated in the glands with ER expression also being suppressed in the stroma [10].

Steroid receptors are expressed as various subtypes. The ER has two structurally related subtypes, commonly known as ER-α and ER-β. The endometrium demonstrates differing levels of ER subtype expression within all cellular compartments [11]. ERα and ERβ mRNAs are present in glandular epithelial cells, stromal cells and smooth muscle of the uterine wall at every stage of the menstrual cycle with higher levels of ERα expression than ERβ. ERα, however, does show the greater decline within the superficial layer of the endometrium during the secretory stage [11]. A similar decline in ERα protein expression has also been reported within a study that demonstrated only ERβ expression within the endometrial endothelial cells of the vasculature. Both ER subtypes, however, were expressed in the perivascular cells [9].

Table 1. Classification of progestogenic compounds.

	First generation	Second generation	Third generation
Estranes	Norethindrone (Norethisterone) Norethindrone acetate Ethinodiol diacetate		
Gonanes	Norgestrel	Levonorgestrel	Desogestrel Norgestimate Gestodene
Pregnanes	Medroxyprogesterone acetate Cyproterone acetate Dydrogesterone		
Others	Drospironone		

Table 2. Common progestogen contraceptives.

Route of administration	Contraceptive	Type of progestogen	Dose
Oral (mini pills)	Second generation	Levonorgestrel	25 μg–75 μg/d
		Norethindrone	35 μg/d
	Third generation	Desogestrel	75 μg/d
Intra muscular injection	Depot provera	Depot MPA (DMPA)	150 mg/3 months
Sub dermal implants	Norplant	Levonorgestrel	40–50 μg/d
	Implanon	3-keto desogestrel (Etonogestrel)	67 μg/d
Intrauterine delivery system	Mirena (LNG-IUS)	Levonorgestrel	20 μg/d

Table 3. Summary of progestogen effects on endometrial morphology.

Endometrial type	Oestrogen receptor (ER)	Progesterone receptor (PR)	Vascular morphology	Vascular density	Structural integrity
Proliferative					
Glands	Increased	Increased	No change	No change	No change
Stroma	Increased	Increased			
Secretory					
Glands	Decreased	Decreased	No change	No change	No change
Stroma	Decreased	Persists (PRA predominant)			
Levonorgestrel intrauterine system					
Short term	Decreased	Decreased	Dilated thin walled	Increased	Decreased
Long term		Decreased (with increased PRA:PRB)			
Subdermal LNG (Norplant)		Increased protein Decreased mRNA	Dilated thin walled Fragile	Increased	Decreased
Medroxyprogesterone acetate		Decreased	Dilated thin walled	Decreased with high dose	
Northisterone				Decreased with high dose	
Etonogestrel (Implanon)				No change	Decreased

Empty cells indicate unknown findings.

The PR also exists as two subtypes PRA and PRB. These structurally related gene products are expressed from a single gene as a result of transcription from two alternative promoters [12]. The two isoforms can display distinct transactivational properties that are specific to cell type and ligand. For the few genes regulated by both receptors, PRB appears to function as a stronger activation of transcription [13]. PRA and PRB are differentially regulated with spatio-temporal variations in expression. Both subtypes are demonstrated within the glandular and stromal compartments during the proliferative phase, with both receptor types dramatically declining in the glandular cells during the secretory stage. However, PRA remains the predominant progesterone receptor within the stroma during the secretory phase and would appear to mediate progesterone effects on endometrium during this phase of the cycle [14].

Progestogen effects on steroid receptor expression

As POC are associated with breakthrough bleeding, an imbalance of either sex steroid concentration or receptor expression may contribute to the mechanisms responsible for aberrant endometrial bleeding.

The short term administration of synthetic progestogens decreases the expression of the progesterone receptor in endometrial stromal and epithelial cells [15]. However, not all POC have the same effect on steroid receptor expression as this phenomenon is the result of differences of dose, duration and mode of administration.

The use of the 20 μg LNG-IUS leads to an initial down regulation in both ER and PR expression [16, 17]. The study by Critchley et al. [17] also found that during 6–12 months exposure to intrauterine LNG, there was an increase in the subtype A receptor (PRA) relative to PRB.

Interestingly, although a decrease in PR is noted with initial progestogen use, there is no difference in expression between women experiencing BTB compared to those with no BTB. It is therefore suggested that local factors rather than systemic hormones are finally responsible for the control of endometrial bleeding. However, progestogen induced decidualisation varies from one region to another and it is possible that exogenous sex steroids may have an effect in areas where decidualisation is weak, leading to BTB [16].

Paradoxically, the use of subdermal levonorgestrel (Norplant) has been reported to raise [18] or lead to a persistence [19] of PR expression in the endometrium,

although the functionality of these receptors has not been examined. Although PR protein expression may remain elevated, studies investigating mRNA levels of PR have shown a decline within the glands and stroma with Norplant use. However, amenorrhoeic women did demonstrate more PR mRNA in the stroma than women with BTB [20]. It is likely that the differential PR expression in response to exposure to LNG is a result of local endometrial progestogen concentrations. The local endometrial LNG concentrations in women using a LNG-IUS are 1000-fold greater than serum concentrations or LNG levels among subdermal LNG (Norplant) users [21].

In summary, functional responses to progestogen use include alterations in sex steroid receptor expression, with a general trend towards down regulation in glandular and stromal cells. This decrease in expression will impact upon a cascade of downstream events as a consequence of altered ligand receptor binding. However, the data are not yet available to demonstrate effects of progestogens on endometrial endothelial cells. This absence of data along with the lack of evidence linking progestogen induced receptor level changes with clinical BTB, suggests that the mechanism of BTB may be locally mediated.

Endometrial vasculature

The mechanism of normal menstruation has been shown in classical studies to principally involve the constriction of endometrial spiral arterioles [22].

Break through bleeding is usually lighter in character and shorter in duration than menstruation. The pattern and character of BTB suggest that it likely occurs from a different vascular source as compared with normal menstrual bleeding.

Important hysteroscopic investigations of women complaining of BTB with both low and high dose progestogens have shown that bleeding occurs focally in the endometrium and originates from superficial, dilated vessels [23]. These vessels were also prone to contact bleeding during hysteroscopy. Vascular fragility, as examined during hysteroscopy, was also increased in Norplant users when compared to control women with normal menstrual cycles [24].

Progestogen use has also been shown to cause dilated venules in histological studies [25]. It is interesting that large, thin walled vessels have also been observed in the superficial endometrial stroma of women using the LNG-IUS [26]. It may be that such vessel abnormalities do not persist with prolonged LNG-IUS use as a result of ensuing LNG induced endometrial atrophy. If these dilated veins are a source of bleeding, this would correlate with the clinical picture of improved BTB with long term LNG-IUS use.

Despite the observations of abnormal vessel development as a result of long term progestogen only contraceptive use, the exact mechanism that causes aberrant angiogenesis and vascular fragility is as yet unknown.

Understanding changes in endometrial vessel morphology with exogenous progestogen use is essential to understanding mechanisms of BTB.

Normal endometrial vasculature

Blood supplies the uterus via the uterine arteries. In the myometrium, arcuate arteries then form, which branch at 90° towards the endometrium to form radial arteries. These radial arteries then branch to form basal arteries and spiral arterioles. The basal arteries supply the basal layer of the endometrium and the larger spiral arterioles supply the functional layer of the endometrium [27]. The endometrial microvasculature of capillaries and venous plexuses are formed downstream of the spiral arterioles. The spiral arterioles are under the control of steroid hormones [28], with both growth and differentiation mediated by presence of ER and PR of the vascular smooth muscle cells. Direct endothelial steroid receptor expression has been difficult to prove with recent data demonstrating human endometrium endothelial cells do not express ERα or PR but do express ERβ [9]. The mechanism for control of the microvasculature is poorly understood. Endometrial microvessels consist only of endothelial cells linked by tight junctions and are supported by their basal lamina. This specialised form of extracellular matrix contributes more than support, aiding vessel growth, differentiation and permeability [27].

Progestogen effects on endometrial blood vessels

Vessel density

During the normal menstrual cycle, vessel density does not substantially change [25, 29]. The effects of progestogens on the endometrial vasculature seem to be dose and type dependent. A low dose LNG implant system leads to an increase in microvessel density [29] from as early as after 3 weeks of use, with an association between higher vascular density in atrophic endometrium and bleeding patterns detected [30].

In contrast, in women exposed to higher doses of norethisterone or medroxyprogesterone acetate (MPA), a decrease in vessel density is seen [25]. Although differing in their effects, these results do indicate that exogenous steroid administration may cause a disruption in normal vessel formation. This variability of vascular structure in LNG exposed endometrium compared to normal endometrium has also been shown *in vitro*, in 3-dimensional culture systems [31]. These studies demonstrated uncannulated clumps of endothelial cells continuous with vessel-like structures. Dilated, thin walled vessels were also found within the functional layers of Norplant users.

Implanon is a subdermal implant, which delivers a progestogen, etonogestrel (3-keto-desogestrel). Investigations into endometrial effects of Implanon have not shown any change in endothelial density [32].

Structural integrity

During the first month of Implanon use, analysis of endometrial biopsies, showed that there was a significant reduction in the number of vessels surrounded by the basement membrane components; laminin, collagen IV and heparan sulphate proteoglycan [33]. Rogers et al. [34] have also reported a reduction in vascular smooth muscle α-actin in the endometrium of women using subdermal LNG (Norplant).

There is therefore considerable evidence that the integrity and support of endometrial small vessels is altered by POC, in particular implants, such as Norplant. Subdermal exogenous progestogens are thus likely to make small vessels more fragile and prone to break through bleeding. In addition to the loss of structural support, basement membrane components also have local effects on vessel formation and permeability. Basement membrane abnormalities could therefore lead to an increased incidence of endothelial break down. However, in a study by Hickey et al. [33], no correlation was found between bleeding episodes and basement membrane components. In addition, not all studies have demonstrated this reduction of basement membrane components with subdermal LNG use [35]. The large variability in immunohistochemical findings within biopsies of subjects make interpretation of these results difficult [36]. Assuming that a decrease in BM integrity does contribute to the mechanism of BTB, it is yet to be determined whether there is a decrease in BM components production or an increase in local proteolytic activity.

A breakdown in vascular structure only partially contributes to our current understanding about mechanisms of break through bleeding and a disruption of the endometrial epithelium is also required for bleeding to be clinically evident. This would explain the hysteroscopic findings of subepithelial bleeds seen as petechiae and ecchymoses in women when vaginal bleeding has not been noted [24]. Cytokeratin deposition within the epithelium is significantly reduced in Norplant users [37]. This change in epithelial structural component could allow for break down and therefore bleeding reaching the endometrial cavity.

Local mediators

Progestogen use has been shown to cause superficial vascular dilatation, new vessel formation, to increase microvessel density and to increase vascular fragility. These changes suggest that exogenous progestogen administration interferes with normal angiogenesis. Direct progestogenic effects on endometrial blood vessels are difficult to prove and increasing evidence is accumulating to show that changes in local mediators could contribute to the changes. The following description of progestogenic effects upon angiogenic factors and MMP activity is briefly summarised in Table 4.

Angiogenesis in the endometrium

This subject area has been addressed in more detail in this issue by Girling & Rogers, and thus only brief mention is made herein. A number of reviews have highlighted the need for continuing angiogenesis during the various stages of remodelling within the endometrium. Repair of the endometrium begins on the second day of menstruation, with general increases of proliferation indices noted from day five. After ovulation, under the influence of progesterone, stromal cells undergo differentiation and dense coiling of the spiral arterioles is noted. The above changes of endometrial structure and function require a concomitant development of vasculature (reviewed by Smith [38]). There are varying reports on endothelial cell proliferation during the menstrual cycle. Small peaks in

Table 4. Progestogen effects on local mediators of endometrial function.

Progestogen type	Vascular endothelial growth factor (VEGF)	Other angiogenic factors	Matrix metalloproteinases
In vitro progestogens	Increased in Ishikawa cell with MPA, levonorgestrel and norethisterone Decreased in endometrial fibroblasts but increased in epithelial cells with MPA		
Progesterone	Suppressed in baboon endometrial glandular and stromal cells Increased in human breast tissue		General suppression of MMP activity
LNG-IUS	Increased, especially in macrophages	Increased tissue factor levels	Increased mast cell MMP-1 expression
Sub dermal LNG	Declined in glandular cells	Increased tissue factor levels	Increased stromal MMP-1 and MMP-3.
Etonogestrel (Implanon)	Decreased endometrial glandular expression		
Depot medroxyprogesterone acetate (DMPA)			Decreased TIMP in all endometrial compartments Menstrual-like expression of stromal MMPs

MPA – Medroxyprogesterone acetate.

proliferation have been noted in the mid to late proliferative stage and later again in the secretory stage of the cycle [39]. A third peak in proliferation has also been shown during the menstrual stage [40]. Within the endometrium, most changes in proliferative activity occur in the superficial functional layer rather than the basal layer and new vessels are deemed to develop from intersusceptive mechanisms rather than sprouting [41]. Development of these new vessels is closely reliant on supporting mesenchymal cells such as vascular smooth muscle cells (VSMC). If no connection to VSMC has been established then vascular endothelial growth factor (VEGF) is required to prevent endothelial cell apoptosis [38]. This mechanism of increased endothelial cell stability by association with VSMC may involve other growth factors termed angiopoietins (Ang-1 and Ang-2). Ang-1 is expressed by VSMC and via binding to its receptor, tie-2, a stable endothelial–mesenchyme structure is formed. Interestingly, Ang-2 also binds to the same tie-2 receptor but without signal and therefore reduces endothelial stability [42].

VEGF is a growth factor responsible for angiogenesis and promotes permeability and dilatation in vessels. It is therefore a prime candidate for a role mediating the progestogen induced changes in angiogenesis. The VEGF family of genes has 6 members; A, B, C, D, E and placental growth factor. Three receptors have been noted, VEGFR 1, 2 and 3. The human endometrium is capable of synthesising all members of the VEGF A family which contains five splice variants. Interestingly, the VEGF A gene has two regions that are homologous to oestrogen response elements (ERE) and numerous studies demonstrate an increase of VEGF production in response to oestrogen [43, 44]. Hypoxia has also been shown to increase VEGF production [45]. It is postulated that hypoxia inducible factor-1α (HIF-1α) leads to an induction of VEGF gene transcription. In addition hypoxic conditions may lead to structural changes in the transcript, which promote stability of the mRNA [45]. The role of hypoxia in the endometrium is discussed later.

The exact mechanisms controlling angiogenesis in the endometrium have yet to be elucidated but angiogenic activity is a consequence of the balance between angiogenic and angiostatic factors. The effect of POC upon these factors is therefore of great interest.

Progestogen effects on angiogenic factors

The use of the Ishikawa cell line as an endometrial model has demonstrated that the steroid hormone oestrogen leads to an increase in VEGF protein and mRNA production. Various progestogens were shown to increase VEGF expression as well, MPA producing a stronger rise than norethindrone and levonorgestrel [44]. In primary cultures of epithelial and stromal cells isolated from human endometrium, oestrogen was also shown to induce VEGF expression [46, 47]; however, the reports with regard to the effects of progestogens are conflicting. MPA alone was shown to decrease VEGF production by stromal cells, but increase production by epithelial cells [48]. When applied together, MPA augmented the 17β-oestradiol response of VEGF expression in human endometrial epithelial cells and inhibited the 17β-oestradiol-induced VEGF expression in the stromal cells [48]. In contrast, studies in ovariectomised baboons have demonstrated that the addition of physiological doses of progesterone suppresses an oestradiol induced rise in VEGF [43]. Other *in vivo* studies using a mouse sponge assay to measure angiogenesis, demonstrated an increase with low dose LNG but not with higher doses of the same progestogen. The same study also showed an increase in angiogenesis with the use of MPA, norethisterone and nomegestrol [49].

The effect of exogenous steroids on VEGF levels is varied. Difference in findings may be due to cell type, progestogen formulation, dose and route of administration and even *in vivo*/*in vitro* laboratory techniques. It is therefore important to look at POC use and their effects on angiogenic factors within the human endometrium *in vivo*.

The LNG-IUS has been shown to cause an increase in VEGF levels in the endometrium at 3 and 6 months time points [50]. Importantly, this study demonstrated a positive correlation between changes in VEGF levels and clinically reported irregular bleeding, a correlation rarely shown in other studies. Studies investigating progestogen effects on tissues other than endometrium have shown that within breast cancer cells, progesterone and progestogens cause an increase in VEGF expression, which is mediated via the progesterone receptor [51]. However, the finding that LNG-IUS leads to a decrease in progesterone receptor levels [17], suggests that changes in steroid receptor expression alone is not fully responsible for progestogen induced changes in angiogenic factors within the endometrium.

Local paracrine mechanisms of angiogenic control are likely to involve a variety of cytokines. For example, IL-1β has been shown to cause a rise in VEGF in cultured human endometrial cells [52]. Additionally, endometrial biopsies taken from LNG-IUS users have shown that much of the increase in VEGF immunostaining is demonstrated in macrophage cells [32]. This migratory cell population could be a source of cytokine production leading to aberrant angiogenesis. A review on endometrial angiogenesis by Gargett and Rogers [41] documents changes of VEGF levels in the endometrial glands and stroma but is unable to correlate these changes with endothelial cell proliferation. It concludes that neutrophils, marginalised and adherent to the endothelium, release focal VEGF. It has been proposed that the neutrophils may be a source of angiogenic factor that are responsible for superficial microvessel development.

Implanon is a long term progestogen only subdermal implant that delivers the gestogen etonogestrel for contraception. In contrast to LNG-IUS, it has been

shown to decrease glandular VEGF expression [32]. This study demonstrated a correlation between endothelial cell density and stromal VEGF levels although no correlation with BTB was shown. Changes in glandular VEGF levels could therefore be less biologically important compared to stromal levels. This could explain why no change in endothelial cell density was demonstrated with Implanon despite a decline in VEGF expression within endometrial glands.

Platelet-derived endothelial cell growth factor (PD-ECGF), also known to be the intracellular enzyme thymidine phosphorylase (TP) [53], is a key player in endometrial angiogenesis. Its expression is spatio-temporally regulated within the human endometrium. Expression is mainly stromal during the proliferative phase of the menstrual cycle, changing to strongly glandular during the secretory stage [54]. Work carried out to elucidate steroid hormone control of this enzyme in a normal endometrial epithelial (NEE) cell culture, demonstrated that progesterone will only lead to a dramatic increase in PD-ECGF/TP expression when co-cultured with TGF-β1 [54].

Thrombin and hypoxia

Other factors acting as angiogenic and angiostatic factors are also influenced by progestogen use. The role of angiopoietins in angiogenesis is influenced with POC use and also linked to thrombin formation and tissue hypoxia. Break through bleeding will cause thrombin formation within the endometrium. Thrombin has been shown to decrease Ang-1 protein and mRNA expressed in HESC but only minimally decrease Ang-2, expressed within HEEC [55]. The outcome of this change in angiopoietin levels is a decrease in endothelial cell stability with an enhancement of further aberrant angiogenesis.

Tissue factor (TF) is an initiator of haemostasis, and levels of the protein are raised with LNG-IUS use, therefore aiding thrombin formation [56]. In contrast, levels are initially decreased with LNG subdermal system but levels rebound to a persistent elevated level after one year of use [57]. These elevated TF levels are significant in endometrial biopsies taken from bleeding sites identified during hysteroscopy. In addition to aiding haemostasis and thrombin formation, TF can act as an angiogenic factor with prolonged expression as seen with exogenous progestogen use.

Thrombin elevates levels of the pro-inflammatory and angiogenic factor, cytokine IL-8, levels of which are usually decreased with decidualisation. Hypoxia has also been demonstrated to cause an increase in IL-8 [56]. This is of importance as studies investigating uterine blood flow with Norplant use have shown a significant decrease in endometrial perfusion [58]. Therefore the use of LNG-IUS and LNG implant systems may lead to aberrant angiogenesis as a result of increasing TF levels and increased IL-8 levels secondary to both hypoxia and enhanced thrombin formation.

Hypoxia and reperfusion secondary to decreased endometrial blood flow with progestogen use may contribute to BTB by other mechanisms.

The link between HIF-1α and increased VEGF production has already received mention. It has also been shown that hypoxia, via free radical production, leads to an inhibition of Ang-1, leaving unopposed the expression of Ang-2 [59]. POC induced bleeding can therefore occur as a result of vessel damage secondary to hypoxia induced free radical changes in angiogenic factors.

In summary, progestogen effects on angiogenesis and angiogenic factors are varied but evident. Differences in findings could belie differences in dose, mode of administration and progestogen type as well as methods (*in vivo* or *in vitro*) used for investigation. It is interesting that much of the evidence showing increases in angiogenic factors, in particular VEGF, is in response to administration of synthetic progestogens whereas the use of progesterone does not lead to changes in VEGF expression. Progesterone has been shown to increase the angiogenic inhibitor thrombospondin-1 (TSP-1) whereas synthetic progestogens have little effect [60]. This discrepancy between progestogens could be one reason why aberrant angiogenesis is not normally seen in response to progesterone during the physiological menstrual cycle.

PD-ECGF/TP is upregulated in response to progesterone but only in combination with TGF-β1. This required combination may also explain the differences in angiogenesis between POC use and raised progesterone levels during the menstrual cycle.

Matrix metalloproteinases

Tissue repair and remodelling is a necessary process in the female reproductive tract. Menstruation is preceded by an increase in proteolytic substances including matrix metalloproteinases (MMPs), which are able to effect tissue breakdown. The trigger for this change in local tissue characteristics is thought to be the decline in steroid progesterone levels and also involve interactions between a migratory leukocyte population and the endometrial stroma [61, 62]. With the exception of membrane bound forms (MT)-MMP, MMPs are secreted as latent proenzymes and require activation by extracellular proteases [61]. The activity of MMPs is proportional to the balance of active enzyme to natural inhibitors, which are termed tissue inhibitors of metalloproteinases (TIMP).

Progesterone contraception and matrix metalloproteinases

Progesterone has been shown to have a regulatory effect on MMP activity (reviewed in [61]). Progesterone seems to suppress MMP activation in human endometrial cell cultures, its withdrawal then leading to an increase in MMP:TIMP ratio [63].

Although progesterone has been shown to influence MMP activity, the focal nature of MMP staining within the endometrium suggests there are additional local factors influencing activity as well as endocrine control.

Studies have identified leukocytes within the endometrium, the numbers of which rise perimenstrually [64]. High dose progestogens have also been shown to cause an increase in leukocyte numbers in the endometrium [65]. It is likely that endometrial leukocytes produce a range of cytokines and proteases, which are responsible for regulating MMP function. *In vitro* studies have shown that 'activation' of MMP requires co-culture of both endometrial fibroblasts and neutrophils [66] In addition, activated mast cells have also been shown to have the potential to stimulate MMP production by endometrial stromal cells [67]. The necessary involvement of these migratory cell populations could explain the focal nature of MMP expression and action.

The use of subdermal LNG implants (Norplant) and DMPA have both been shown to cause similar expression of MMP and TIMP to that seen in breaking down perimenstrual tissue [68]. MMP-1 expression was significantly higher in Norplant users compared with DMPA exposed endometrium or menstrual controls. In addition, activated mast cells, as detected by the presence of extracellular MC tryptase, predominated in the endometrium of Norplant users and were also seen with DMPA use in similar quantities to menstrual controls [68]. Unfortunately, within these findings there was no correlation between MMP immunostaining and reported number of bleeding days. However, endometrium exposed to the LNG-IUS has demonstrated a significant increase of mast cells containing MMP-1 in women experiencing BTB compared to those with no reported bleeding [69].

Further studies have shown an increase in MMP-3 in directed endometrial biopsies taken at the start of bleeding episodes with Norplant use. Tissue biopsied from non-bleeding endometrium did not display this finding [70]. MMP-3 is an important enzyme in the context of BTB as it is responsible for proteolytic activation of MMP-1 and/or MMP-9, both of which have been shown to be present in menstrual biopsies [71]. The number of MMP-9 positive cells was significantly increased in Norplant exposed endometrium that displayed a shedding morphology and in biopsies collected at the time of menses [72] i.e. endometrium associated with break down and bleeding.

Progestogens can also effect TIMPs thereby increasing MMP activity. The use of DMPA leads to a decrease in TIMP 1, 2 and 3 in endometrium. However, no correlation with bleeding patterns was noted [73].

In summary, POCs cause a perimenstrual pattern of expression of MMPs and their inhibitors. The variation in findings between progestogen type and between studies may in part be explained by sampling differences since the focal nature of MMP expression making consistent sampling difficult. This is supported by studies by Galant et al. [74] which demonstrated focal expression and activity of MMPs, associated with a focal tissue breakdown during dysfunctional bleeding episodes.

Conclusion

Progesterone only contraceptives are commonly associated with unscheduled endometrial bleeding.

Increased vascular fragility, as demonstrated by hysteroscopy, appears to be a key component of unscheduled bleeding. However, the mechanisms that lead to vessel fragility have yet to be determined. The majority of studies conducted to address the aetiology of BTB have been descriptive in nature. Interpretation of the data in the context of functional consequences within the steroid exposed endometrium is difficult. Thus far the correlation between clinical endpoint; i.e. bleeding days, and changes in local gene or protein expression have been disappointing, with no lead factors being identified. Changes in steroid receptor expression may cause downstream effects, altering vascular morphology, vessel density and structural integrity in the endometrium. These vascular changes are in turn likely to occur secondarily to alterations in endometrial local mediators such as angiogenic factors and MMPs.

The main implication of BTB is discontinuation of use, further reducing women's contraceptive options. A greater understanding of POC induced changes in the endometrium is thus necessary before effective management strategies for BTB can be achieved.

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EP receptor signalling is elevated in endometrium from women with excessive menstrual blood loss

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Introduction

Menstruation is an active process whereby the upper two thirds of the endometrium (functional layer) is shed and regenerated on a repetitive basis. Heavy menstrual blood loss (HMBL) is one of the most common gynaecological complaints, affecting 10-30% of otherwise healthy women. Treatment using inhibitors of COX enzymes have repeatedly been shown to reduce menstrual blood loss implicating disturbances of prostaglandin pathways in the aetiology of HMBL. Prostaglandin E2 has repeatedly been implicated in the aetiology of excessive menstruation (1-3) although the specific prostanoid receptors mediating this pathology remain to be elucidated.

This study was initiated to elucidate a pattern of expression of the various components of the COX-prostaglandin signalling pathways present in the endometrium of women with normal and heavy menstrual blood loss (>80ml).

Methods

Patients were recruited from the gynaecological outpatient clinic with local institutional ethical approval. Subjects were aged between 18 to 50 years and reported regular menstrual cycles (21-35 days). No women had received exogenous hormonal preparations in the preceding 3 months.Subjects collected all used sanitary products for one menstrual period and measurement of menstrual blood loss (MBL) was performed by a modified alkaline haematin method (4). Endometrial biopsies were collected by suction curette (Pipelle) at different stages of the menstrual cycle.

Patients, phase of menstrual cycle and results of measured MBL.

	Normal MBL (<80ml)	Heavy MBL (>80ml)
Patient Numbers	n =17	n =12
Proliferative phase	n = 7	n = 5
Secretory phase	n = 6	n = 5
Mean MBL (ml)	42	255
Median MBL (ml)	45	183
Range MBL (ml)	10 - 69	82 - 567

Results

Table 1. *Relative expression of mRNA (Q-RT-PCR) in endometrium obtained from women with MBL > 80ml compared with endometrium obtained from women with MBL < 80ml. *Indicates significant difference in endometrium from women with heavy MBL compared to women with normal MBL (p<0.05).*

Prostaglandin Pathway Gene	mRNA Expression
COX-1	Increased *
COX-2	Increased *
Prostacyclin Synthase	Decreased *
Prostacyclin Receptor (IP)	Decreased *
Thromboxane Synthase	Decreased *
Thromboxane Receptor (TP)	Decreased *
$PGF_{2\alpha}$ Synthase	No Change
$PGF_{2\alpha}$ Receptor (FP)	Decreased *
PGE_2 Synthase	No Change
PGE_2 Receptor (EP2)	No Change
PGE_2 Receptor (EP4)	No Change

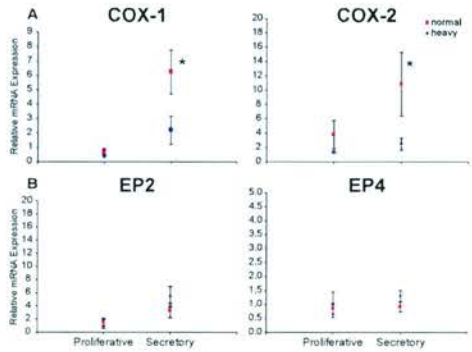

Figure 1. *Relative mRNA expression within endometrium of women with measured MBL* ***A.*** *Graphs demonstrate significant elevated expression of COX mRNA in secretory endometrium from women with heavy MBL.* indicates significant difference (p<0.05)* ***B.*** *Graph demonstrates mRNA expression of EP2 and EP4 receptors within endometrium of women with measured MBL. No significant differences of expression were noted.*

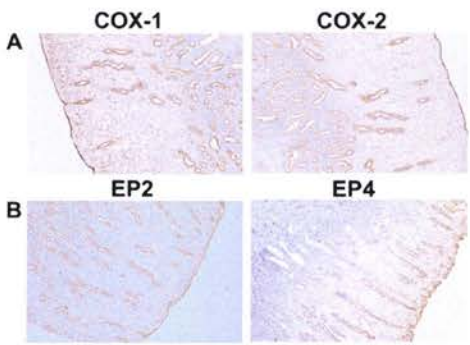

Figure 2. A. *Immunohistochemical localisation of COX-1 and COX-2 in mid-proliferative endometrium. Strong surface epithelial and glandular staining is noted for both COX enzymes. Stromal immnostaing is noted in the functional layer but absent in the basal layer. COX-1 immunostaining shows marked localisation to white blood cells infiltrated within the endometrium.*

B. *Immunohistochemical localisation of EP2 and EP4 in mid-proliferative endometrium, Similar immunostaining patterns are seen for both receptors with greatest immuno staining intensity seen in the surface epithelium and functional glands.*

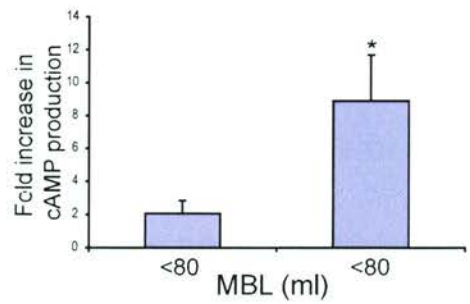

Figure 3. *cAMP production in response to a 10 minute stimulation with 100nM PGE_2 or vehicle. Graph shows the average fold increase of cAMP production by endometrium collected from women with heavy MBL and normal MBL. *Endometrium from women with excessive MBL showed significantly elevated cAMP production (p<0.05).*

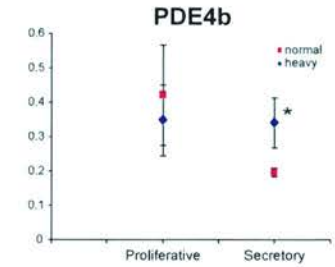

Figure 4. *Graph demonstrating phosphodiesterase 4b (PDE4b) mRNA expression in endometrium from women with normal and HMBL as determined by real time quantitative RT-PCR. Results are expressed as the mean ± S.E.M. of relative mRNA expression levels * indicates significant reduction in expression within endometrium from women with HMBL (p<0.05).*

Discussion

To our knowledge, this is the first study demonstrating significantly higher levels in expression of COX enzyme mRNA in endometrium obtained from women with measured excessive MBL. Furthermore differences in endometrial prostanoid synthase and receptor mRNA expression were demonstrated indicating that dysregulation of prostaglandin signalling may play a role in the aetiology of aberrant menstrual function in disorders such as excessive menstrual bleeding. Prostaglandin E_2 receptor did not demonstrate the reduced levels of expression seen for other prostanoids in endometrium from women with heavy MBL as compared to normal MBL. An elevated signalling response to PGE_2 ligand in endometrium taken from the same women with excessive MBL further implicates the PGE_2 signalling pathway in the aetiology of heavy bleeding. This elevated production of the second messanger, cAMP, could in part be explained by a reduced breakdown by phoshodiesterase enzymes in the post receptor setting.

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P3-9

Prostacyclin receptor (IP) activated ERK 1/2 signalling via the EGF receptor in endometrial epithelial cells

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INTRODUCTION

The prostaglandin family of lipid mediators are involved in vascular changes and have been implicated in the mechanism of menstrual function and dysfunction.

Prostacyclin (PGI) is a member of the prostaglandin family and has a mode of action via coupling to the heptahelical G-protein coupled prostacyclin receptor (IP receptor). Previous studies in our laboratory have demonstrated an increase in expression of IP receptor in epithelial and endothelial cells of the endometrium during the menstrual phase (Battersby et al. 2004). In addition, it has been demonstrated that endometrium from women with excessive menstrual blood loss is more effective at enhancing production of prostacyclin by myometrium compared to endometrium from women with normal blood loss (Smith et al. 1981). These data implicate a role for prostacyclin and IP receptor in menstruation. This study was initiated to investigate the intracellular signal transduction pathways mediating the role of the IP receptor in endometrial epithelial (Ishikawa) cells.

METHODS

Tissue Collection

Endometrial biopsies were collected with a suction curette (Pipelle) from women with regular menstrual cycles(25-35 days). No woman had received hormonal preparations in the three months preceding biopsy collection. Ethical approval was obtained from Lothian Research Ethics Committee and written informed consent obtained from all patients prior to tissue collection.

Cell Culture

Human Ishikawa endometrial adenocarcinoma cells were maintained in Dulbecco's modified Eagle's medium nutrient mixture F-12 with glutamax-1 and pyridoxine, supplemented with 10% FCS, and 1% antibiotics (stock 500IU/ml penicillin and 500ug/ml streptomycin) at 37°C and 5% CO_2 (v/v).

Cyclic AMP Assay

cAMP concentration in Ishikawa cells in response to 100nM Iloprost, a PGI analogue, was quantified by ELISA using a cAMP kit and normalised to protein concentration.

Western Blotting

Western Blot analysis was carried out on 50 μg of protein run on a 4-20% SDS-polyacrylmide gel. The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Watford, UK) and subject to immunoblot analysis. Membranes were blocked for 1 hour at 25°C in 4% Bovine Serum Albumin and then incubated with specific antibodies. Proteins were revealed and quantified by phosphoimager analysis. Fold increase in phosphorylation was determined by dividing the protein expression of the phosphorylated protein by the relative expression obtained from total protein immunoblots.

RESULTS

IP receptor signalling in Human Endometrium

We initially set out to examine the role of the IP receptor in the human endometrium. Endometrial tissue was incubated overnight in RPMI medium containing 3μg/ml indomethacin and subsequently incubated in the presence of 100nM Iloprost. A significant phosphorylation of ERK1/2 in response to an Iloprost (PGI analogue) challenge was detected after treatment for 10 minutes. Pre-incubation of tissue with 100nM AG1478, an EGFR kinase inhibitor for one hour, prior to co-treatment with 100nM Iloprost abolished the phosphorylation of ERK1/2 (Fig 1).

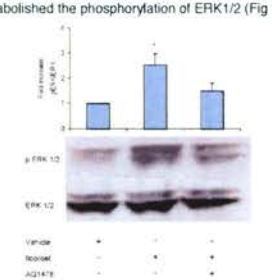

Figure 1

Activation of ERK1/2 in normal human endometrial tissue in response to 100nM Iloprost (PGI analogue). Phosphorylation is significantly reduced by preincubation with EGFR inhibitor. Graph shows semi-quantitative analysis of n=3 experiments.

Iloprost activation of Ishikawa Cells leads to cAMP accumulation and activation of Extracellular Regulated Kinase 1/2 (ERK1/2)

We investigated cAMP activation in Ishikawa cells in response to 100nM Iloprost. A significant time dependent increase in accumulation of cAMP was observed at 5, 10 and 15 minutes following Iloprost challenge ($p<0.05$) (Fig. 2).

Treatment of Ishikawa cells with 100nM Iloprost also resulted in a significant phosphorylation of ERK1/2 with maximum phosphorylation detected at 5 minutes (Fig 3).

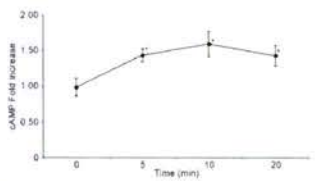

Figure 2

Fold increase in cAMP production by the Ishikawa cell line in response to a 100nM Iloprost challenge. *Fold change is significantly increased at each time point measured ($p<0.05$).

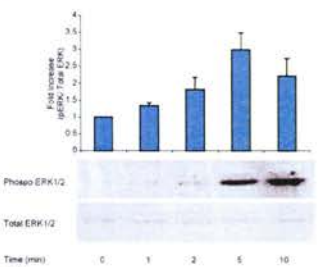

Figure 3

Western blot showing the effect of 100nM Iloprost on phosphorylation of ERK1/2 in the Ishikawa cell. Graph shows semi-quantitative analysis of n=4 experiments.

ERK 1/2 activation is dependent on cAMP and Epidermal Growth Factor Receptor (EGFR) activation.

Further experiments investigating the activation of the ERK1/2 signalling pathways in Ishikawa cells in response to Iloprost were carried out in the absence or presence of various chemical inhibitors to the EGFR and ERK1/2 signalling pathways. Treatment of Ishikawa cells with 100nM Iloprost for 5 minutes led to a significant increase in ERK1/2 phosphorylation.

Pre-incubation of cells for 1 hour with the EGFR kinase inhibitor AG1478 (100nM), Protein Kinase A inhibitor (H89, 10μM) and inhibitor of MAPK Kinase (PD98059, 50μM) abolished the phosphorylation of ERK1/2 (Fig 4).

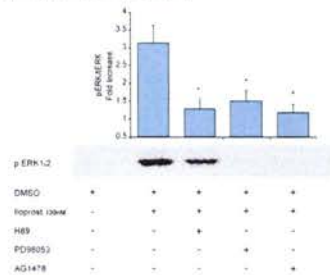

Figure 4

Western blot depicting the effects of chemical inhibitors on phosphorylation of ERK1/2 signalling protein. H89 is a cAMP/Protein Kinase A inhibitor, PD98059 is a MEK inhibitor (inhibitor of ERK phosphorylation), AG1478 is an inhibitor of EGFR. *indicates significant recuction of phosphorylation ($p<0.05$).

Finally, in order to determine whether the cAMP pathway could cross talk with the ERK 1/2 pathway, we treated Ishikawa cells with dibutyryl cAMP (a cell permeable cAMP analogue). Treatment of Ishikawa cells with 5mM dibutyryl cAMP for 10 minutes significantly phosphorylated ERK1/2. Co treatment of cells with 100nM AG1478 inhibited the dibutyryl cAMP mediated ERK1/2 phosphorylation, demonstrating that PGI- mediated cAMP accumulation could activate ERK1/2 signalling in Ishikawa cells via the EGFR (Fig 5).

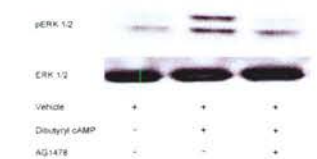

Figure 5

Representative Western Blot demonstrating ERK1/2 phosphorylation in response to dibutyryl cAMP. Phosphorylation is significantly reduced in the presence of EGFR inhibitor.

CONCLUSION

Taken together, our data demonstrate that PGI can activate ERK1/2 MAPK signalling via a cAMP dependent mechanism. This cAMP dependent activation of ERK 1/2 appears to require transactivation of the Epidermal Growth Factor Receptor (As summarised schematically in Fig 6). The downstream phenotypic effect of ERK1/2 activation by PGI-IP receptor interaction remains to be elucidated.

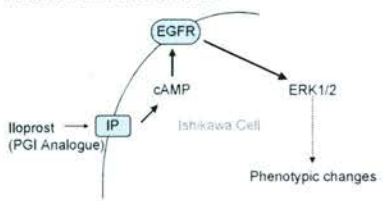

Figure 6

Cartoon demonstrating hypothesised IP intracellular signalling leading to activation of the ERK1/2 pathway.

References

1) Battersby S, Critchley HOD, Brum-Fernandes and Jabbour HN 2004. Temporal expression and signalling of prostacyclin receptor in the human endometrium across the menstrual cycle. Reproduction 127, 79-86

2) Smith SK, Abel MH, Kelly RW, Baird DT. 1981. The Lancet 1(8219): 522-4

Acknowledgements

Financial support for study provided by University of Edinburgh College of Medicine and Veterinary Medicine fellowship grant and the Medical Research Council.

Appendices

- Lothian Research Ethics Committee Certificate of ethical opinion

- Patient Information Sheet

- Instructions for menstrual collection

- Consent Form

- Optimisation procedures for immunohistochemical protocols

Lothian NHS Board

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Chancellor's Building, 49 Little France
Edinburgh, EH16 4SB

Date 31 March 2004
Your Ref
Our Ref LREC/2003/6/47

Enquiries to Joyce Clearie
Extension 89022
Direct Line 0131 536 9022
Email joyce.clearie@lhb.scot.nhs.uk
US IRB No.: IRB00001462

Dear Prof Critchley,

THE INVESTIGATION OF CELLULAR SIGNALLING WITHIN ENDOMETRIUM FROM WOMEN COMPLAINING OF MENSTRUAL DYSFUNCTION.

Thank you for submitting the above research proposal for ethical review. The Paediatrics/Reproductive Medicine Research Ethics Committee of the Lothian Research Ethics Committee has reviewed this proposed research and has given it a favourable ethical opinion. An official Certificate of Ethical Opinion outlining the conditions of this opinion is enclosed together with a list of members present at the meeting. Please note that the LREC reference number LREC/2003/6/47 **must** be quoted on all correspondence. Correspondence received without the LREC reference number will be returned.

Under the terms of the Scottish Executive Health Department Research Governance Framework for Health and Community Care this opinion has been notified to the Research & Development Office of the relevant NHS Trust(s) where the research is intended to take place. It is the NHS Trust(s) from whom you must obtain management approval before any work on the proposed research can proceed.

Details of the Lothian Research Ethics Committee and its documentation can be found on http://www.nhslothian.scot.nhs.uk/nhs_lothian/about_lothian_health/lrec/index.html

Yours sincerely

JOYCE CLEARIE
Committee Administrator

LOTHIAN RESEARCH ETHICS COMMITTEE

CERTIFICATE OF ETHICAL OPINION

LREC Reference Number: LREC/2003/6/47
Title: The Investigation of Cellular Signalling Within Endometrium from women Complaining of Menstrual Dysfunction.
Researcher: Prof Hilary O D Critchley

The Paediatrics/Reproductive Medicine Research Ethics Committee of the Lothian Research Ethics Committee (the Committee) reviewed this proposed research and is of the opinion that it is ethical and appropriate to be carried out in the Lothian Area. This opinion encompasses all aspects of the application including the Patient/Subject Information Sheet and all other accompanying documentation provided.

The LREC application form, protocol, subject information sheet, information on compensation arrangements, payments to researchers and the provision of expenses to subjects (where appropriate) were reviewed and approved and the members of the Committee present at the meeting are shown on the attached *Membership List*.

This opinion is issued subject to the following conditions and is invalid if they are not followed:

- You must obtain appropriate management approval from the relevant NHS Trust(s) before starting the proposed research. It is the NHS Trust(s) that ultimately decide whether or not this research should go ahead taking account of the advice of the Local Research Ethics Committee.
- You must notify the Sub-Committee and the relevant NHS Trust(s), in advance, of any significant proposed deviation from the original protocol or application form and obtain approval for any such amendments using the *Amendment Approval Request Form*.
- You must submit reports to the Sub-Committee and the NHS Trust(s) once the study is underway if there are any unusual or unexpected results which raise questions about the safety of the research.
- You must report annually on successes, or difficulties, in recruiting subjects in order to provide useful feedback on perceptions of the study among patients and volunteers using the *Progress Report Form*.
- Where the study is terminated prematurely you must report within fifteen days indicating the reasons for early termination.
- You must submit a final report within three months of the completion of the study using the *Progress Report Form*.

Peter Reith
Secretary
Lothian Research Ethics Committee

02 April 2004

Joyce Clearie
Administrator
Paediatrics/Reproductive Medicine
Research Ethics Committee

LOTHIAN RESEARCH ETHICS COMMITTEE

Members of the Paediatrics/Reproductive Medicine Committee present at the Meeting held on 29 October 2003

Dr Christine West (Medical Member) (Chairman)
Mr W Taylor (Lay Member)
Dr Hamish Wallace (Medical Member)
Dr David Semple (Medical member)
Mr Jonathon Spence (Expert)
Mrs Margaret Butcher (Lay Member)
Mr A S J Farrow (Lay Member)
Mrs J McCallum (Lay Member)
Ms Sinead McNally (Expert)
Ms Aileen Crosbie (Expert)
Mrs C R Milligan

Patient information sheet

LREC//2002/6/8

The role of prostaglandins in menstrual dysfunction

We would like to seek your help with the above study in which we are investigating the mechanisms regulating normal menstruation. It is particularly important that we seek the help of women like yourself who have been referred to our clinic with heavy periods. The information obtained from these studies will help us develop treatments for problems with heavy and troublesome menstruation.

We are asking for your help in several different ways: -

1. We would like to collect the sanitary protection from your next (or a future period) in order that we can measure in the laboratory the actual amount of blood lost during your period. Although this may sound an unusual request, helping us in this way will enable us to conduct crucial research. We will make every effort to ensure that the collection process is as easy and discreet as possible for you and we can provide airtight, sealable bags and all the packaging required. Furthermore, we would supply your sanitary protection (pads and / or tampons) in order that the sanitary products from which we measure the blood loss are the same for each patient participating in our study. We will also arrange uplifting of this collection by one of our team of research nurses, who are employed specifically to assist us with medical research projects.

2. The second request, if you agree to participate in the study is that you permit us to collect a sample of the lining of the womb (biopsy). The sample will be collected with a slim plastic sampler. The tissue thereafter would be used anonymously and processed in the laboratory in several different ways to allow us to investigate the function of the womb lining. Occasionally, patients experience a mild period pain type feeling following collection of the sample. I would like to emphasise that this is a routine procedure conducted in the outpatient clinic for women referred with menstrual bleeding problems.

3. It is also important that we seek your permission for the sample you have given to be stored anonymously at the Centre for Reproductive Biology, University of Edinburgh for possible use in future projects.

4. In addition, we would like to collect a small sample of blood for the measurement of hormone levels.

Your confidentiality will be strictly maintained throughout the study. Although we would like you to participate in the study if you agree to volunteer you are under no obligation or commitment and may withdraw at any time. Withdrawal would in no way affect your future treatment or management.

Should you wish to contact an independent adviser concerning this study please feel free to contact Dr C P West, Centre for Reproductive Health, Royal Infirmary of Edinburgh at Little France, 51 Little France Crescent, Edinburgh EH16 4SA.

For further information about the study, please contact: - **Professor Hilary O D Critchley** at the above address or telephone **0131 242 6441**

Date: 11th March, 2002

INSTRUCTIONS FOR MENSTRUAL COLLECTION

Thank you for agreeing to collect your used products.

We will provide you with the necessary items required for the collection including sanitary products. Please contact me for additional bags etc.

Here is a reminder of what we would like you to do:

1. Every time you change your sanitary protection please:
   put the used product(s) immediately into a storage bag.
   It does not matter how many pads /tampons you put in each bag
   i. Tie the neck of the bag.
   ii. As soon as possible, put the bagged product into the inner plastic bag, inside the outer courier bag.

2. If you use tampons, and you wish to bathe or to swim, please remove and collect the tampon you are wearing, just before you get into the water. If you want to wear another tampon while you are bathing, that is fine, but remove and discard it once out of the water. Then use a new tampon, which you will later collect in the normal way.

3. Please try to collect as carefully as you can, so as the measurement is as accurate as possible. Please note and record if you loose clots to the toilet, or fail to collect any pads etc. We would also like you to complete a simple menstrual chart throughout your period. The timing of product changes etc. may be completed on this.

4. Please enclose the piece of paper with your name, date of birth, hospital number and the date your period started, inside the courier bag.

5. When you are sure your period has finished, please telephone me, to arrange the hand-over of your collection. The completed menstrual chart should be handed in at the same time as the courier bag.

6. It may be necessary for you to have a sample of blood taken at the time you return your collection. This is for part of the laboratory process.

'What if I have to go out?'

This is very likely and should not be a problem. Please just take spare products and storage bags with you. When you need to change you can bag and tie your used product, to take home, for placing in the collection later.

'What if I need to change and I have no bags with me?'

In an emergency any plastic bag will do.

'What if I can't get collection in immediately?'

The test is not affected by time, therefore if for example holidays intervene, just bring the collection in at your convenience.

If you have any concerns please contact Oliver Milling Smith via one of the numbers below. Thank you again.

Radio Page -Tel: 07659547016 and leave your phone number followed by hash key;

or via Professor Critchley's secretary on: 0131 242 2512

Study Number: LREC/ 2003/6/147
Patient Identification Number for this trial:

CONSENT FORM

Department of Obstetrics and Gynaecology

Study Title **The investigation of cellular signalling within the endometrium.**

Name of Researcher:

Professor H O D Critchley
New Royal Infirmary of Edinburgh
Simpson Centre for Reproductive Health
51 Little France Crescent
Edinburgh EH16 4SA

Tel: 0131 242 2670
Or : 0131 242 6441

1. I confirm that I have read and understand the information sheet dated 3rd Oct. 2003 for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected.

3. I agree to a letter being sent to my General Practioner about my participation in this study.

4. I agree to the provision of any clinically significant information to my General Practioner.

5 I understand that my medical notes may be looked at by the researchers involved in the study or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.

6. I agree that the sample I have given and the information gathered from me may be stored by Professor Hilary O D Critchley at the Centre for Reproductive Biology, University of Edinburgh for possible use in future projects, as described in the attached information sheet. I understand that some of these projects may be carried out by researchers other than Professor Critchley's laboratory who ran the first project.

7. I understand that this is non-therapeutic research from which I cannot expect to derive any benefit.

8. I agree to take part in the above study.

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Optimising protocols for immunohistochemical staining

The purpose of immunohistochemical experimentation is to maximise specific protein staining with minimal background staining. Staining for each individual protein examined had to be optimised, often over the course of two to three experiments.

Before running the initial optimising experiments, literature searches were performed to look for any previous examples of immunohistochemistry for the target protein. The manufacture's recommendation for concentrations to use for individual antibodies was also recognised. During all optimisation experiments negative controls were included using species-specific IgG at a matched concentration or a peptide pre-absorbed antibody if available.

1) Optimising immunohistochemistry for the PGE_2 receptors EP2 and EP4:

Separate experimental staining runs were carried out for each of the EP receptors. However conditions utilised for each were identical and therefore described together. Previous immunostaining for these receptors had been performed on a variety of tissues within the laboratory (unpublished data). A concentration range for the primary antibody was therefore decided upon. Slides were divided to those for antigen retrieval by pressure-cooking and those not for antigen retrieval. Within these two groups, two concentrations of primary antibody (1:400 and 1:800) were also chosen. This optimisation run therefore had four subsets of slides. Slides are clearly labelled at the outset to avoid confusion during experimentation. The same tissue blocks were utilised to provide positive and negative slides for experimentation to allow for accurate comparison of staining results. The general immunohistochemical protocol was then followed as detailed in section 3.2.2.

This optimisation experiment demonstrated that the use of pressure-cooking for antigen retrieval enhanced the clarity of staining and also that staining was optimal with a primary antibody concentration of 1 in 400. The lower primary antibody staining produced staining that was too faint for accurate assessment. A full immunohistochemical run was then run with the best-assessed conditions.

2) Optimising immunohistochemistry for the PGI receptor IP:

The commercially obtained antibody for the IP had not previously been utilised within the laboratory. The first optimisation run was therefore run too try and assess a concentration to utilise this antibody at. 1:10, 1:50, 1:100, 1:200, 1:400, 1:800 concentrations of primary antibody were therefore prepared. Full section endometrial slides were utilised, as myometrial vasculature was known to express the prostacyclin receptor, thereby incorporating a control for the antibody's effectiveness. The general immunohistochemical protocol was then followed as detailed in section 3.2.2. Concentrations of primary antibody below 1:50 produced immunohistochemical staining that was too faint for assessment. A concentration of 1:10 produced staining that was too strong and had an increase of faint background staining. A second optimisation experiment was therefore run to try concentrations of 1:20, 1:30 and 1:40. The use of antigen retrieval with a primary antibody concentration of 1:30 produced the optimal immunostaining and these conditions were hence used for a full immunohistochemical run.

3) Optimising immunohistochemistry for COX-1 and COX-2:

Separate experimental staining runs were carried out for each of the COX enzymes. However conditions utilised for each were identical. Previous immunohistochemical staining had been carried out upon these proteins using the general immunohistochemical protocol detailed in section 3.2.2. However, within the work carried out for this thesis, the staining for these proteins was carried out utilising Bond Automated immunohistochemistry system. This system was able to deliver a efficient staining method with an improved quality and reproducibility of immunostaining. An optimisation run was therefore required to detect the best concentration for the primary antibody and also to ensure the systems specific reagents allowed for optimal staining. (The tertiary detection used with this system was ABC Horseradish peroxidase rather than ABC Elite used in the general immunohistochemical protocol). Clear and precise immunostaining was achieved with both primary antibodies at a concentration of 1 in 500.