NOVEL PARACRINE/AUTOCRINE ROLES OF PROSTAGLANDINS IN THE HUMAN OVARY

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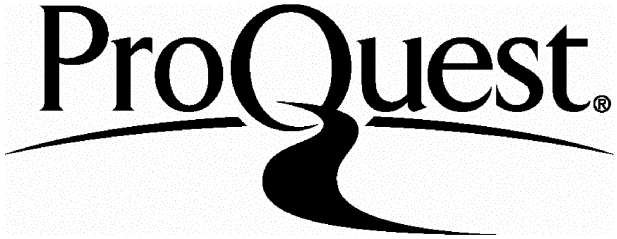

To my dearest parents:

Iris and John

ABSTRACT

Prostaglandin (PG)E_2 and $PGF_{2\alpha}$ exert important roles in ovulation and luteinisation. The studies reported herein have used human granulosa-lutein cells to investigate: (1) the relationship between basal PG and progesterone production; (2) participation of PGs in the steroidogenic response to high-density-lipoproteins (HDL); (3) the role for PGs in controlling ovarian cortisol metabolism by 11β-hydroxysteroid dehydrogenase (11β-HSD); (4) the role of EP_1 and EP_2 receptors in mediating ovarian responses to PGE_2. In culture, basal PG production decreased with a concomitant rise in progesterone synthesis. However, meclofenamic acid suppressed both PGE_2 and $PGF_{2\alpha}$ output without affecting progesterone production, and aminoglutethimide inhibited progesterone production without affecting either PG concentration. Both HDL and apolipoprotein A-I increased PGE_2, cAMP and progesterone concentrations. Each of these effects was abolished by co-treatment with meclofenamic acid. Meclofenamic acid, indomethacin and niflumic acid each suppressed ovarian cortisol oxidation, whereas co-treatment with PGE_2, PGD_2 and $PGF_{2\alpha}$ each increased 11β-HSD activities. Human follicular fluid was confirmed to contain intrinsic aqueous stimuli and hydrophobic inhibitors of $NADP^+$-dependent 11β-HSD activity that eluted from C18 mini-columns at 0-10% and 65-90% (v/v) methanol, respectively. PGE_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ each eluted at 20-50% (v/v) methanol. The ability of PGE_2 to stimulate progesterone production was inhibited by SC19220 and abolished by AH6809 (preferential EP_1 and EP_2 antagonists respectively). While SC19220 had no significant effect on the stimulation of cAMP accumulation and 11β-HSD activity by PGE_2, these effects were abolished by co-treatment with AH6809. In conclusion, while changes in the ouput of PGs and progesterone in luteinizing human granulosa cells appeared to occur independently of each other, PGs were implicated in the steroidogenic actions of HDL and apolipoprotein A-I. Locally synthesised PGs appear to maintain ovarian 11β-HSD activity but do not contribute to the hydrophilic stimuli of 11β-HSD activity present in follicular fluid. Both EP_1 and EP_2 receptors appear to participate in the stimulation of progesterone synthesis, cAMP accumulation and 11β-HSD activity by PGE_2.

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Chandras C., Jonas K.C., Thurston L.M., Abayasekara D.R.E. & Michael A.E. Paracrine modulation of human ovarian 11β-HSD activity by prostaglandins. *(in preparation)*

Thurston L.M., Chandras C. & Michael A.E. Candidate paracrine modulators of 11β-HSD activity in ovarian follicular fluid. *(in preparation)*

ABBREVIATIONS AND CONVENTIONS

Abbreviations used in this thesis:

aa	amino acids
AA	arachidonic acid
ABC1 transporter	ATP-binding cassette transporter 1
AC	adenylyl cyclase
ACAT	acyl-coenzyme A: cholesterol acyltransferase
ACTH	adrenocorticotrophic hormone
AcylCoA	Acyl coenzyme A
α_i	α-subunit of G_i
α_q	α-subunit of G_q
α_s	α-subunit of G_s
ADP	adenosine diphosphate
AG	aminoglutethimide
AME	apparent mineralocorticoid excess
AMH	anti-Mullerian hormone
AMP	adenosine 5'-monophosphate
ANOVA	analysis of variance
Apo	apolipoprotein
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor
βγ	G-protein βγ subunit
3β-HSD	3β-hydroxysteroid dehydrogenase
17β-HSD	17β-hydroxysteroid dehydrogenase
11β-HSD	11β-hydroxysteroid dehydrogenase
11β-HSD1	11β-hydroxysteroid dehydrogenase type 1
11β-HSD2	11β-hydroxysteroid dehydrogenase type 2
BSA	bovine serum albumen
Ca^{2+}	calcium ions

cAMP	cyclic adenosine-3', 5'-monophosphate
cDNA	complementary DNA
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
CG	chorionic gonadotrophin
CHO	Chinese Hamster Ovary
CL	corpus luteum [sing.] / corpora lutea [pl.]
CO_2	carbon dioxide
COX	cyclo-oxygenase
CRE	cAMP response element
CREB	cAMP response element binding protein
CYP19	cytochrome P450 aromatase
DAG	diacylglycerol
dbcAMP	dibutyryl-cAMP
DBD	DNA binding domain
11-DHC	11-dehydrocorticosterone
DHEA	dehydro-epiandrosterone
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DP	PGD_2 receptor
E_1	oestrone
E_2	oestradiol
EDTA	ethylene diaminotetra-acetic acid
EP	PGE_2 receptor
EP_1	PGE_2 receptor type 1
EP_2	PGE_2 receptor type 2
EP_3	PGE_2 receptor type 3
EP_4	PGE_2 receptor type 4
ER	endoplasmic reticulum
FAD	flavin adenine dinucleotide

FC	free cholesterol
FCS	foetal calf serum
FP	$PGF_{2\alpha}$ receptor
FSH	follicle-stimulating hormone
GCs	granulosa cells
GDP	guanosine diphosphate
GnRH	gonadotrophin releasing-hormone
GPI	glycerophosphatidylinositol
G-protein	GTP-binding protein
G_i	G-protein which mediates inhibition of AC
G_q	G-protein which mediates activation of PLC
G_s	G-protein which mediates stimulation of AC
GR	glucocorticoid receptor
GTP	guanosine triphosphate
H^+	proton
^3H	tritium
hCG	human chorionic gonadotrophin
HCl	hydrochloric acid
$HClO_4$	perchloric acid
HDL	high density lipoproteins
HBP	HDL binding protein
HETE	hydroxy-eicosa-tetraenoic acid
hMG	human menopausal gonadotrophin
HMG CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
HPETE	hydroperoxy-eicosa-tetraenoic acid
HRE	hormone response element
^{125}I	iodine-125 radioisotope
IBMX	iso-3'-butyl-5'-methyl xanthine
IDL	intermediate-density lipoproteins
IC_{50}	inhibitory concentration 50%
IgG	immunoglobulin

IGF	insulin-like growth factors
IL	interleukin
IP	PGI_2 receptor
IP_1	inositol monophosphate
IP_2	inositol bis-1,4-phosphate
IP_3	inositol triphosphate (unless otherwise stated this abbreviation refers to the biologically active 1,4,5 isomer)
IVF	*in vitro* fertilisation
IVF/ET	*in vitro* fertilisation-embryo transfer
K^+	potassium ions
K_d	dissociation constant
KH_2PO_4	potassium dihydrogen *ortho*-phosphate
K_m	Michaelis-Menten enzyme rate constant
KBr	potassium bromide
K_3PO_4	potassium phosphate
LBD	ligand binding domain
LCAT	lecithin-cholesterol acyltransferase
LDL	low density lipoproteins
LH	luteinizing hormone
LHr	luteinizing hormone/chorionic gonadotrophin receptor
LO	lipoxygenase
LPL	lipoprotein lipase
LTs	leukotrienes
MA	meclofenamic acid
Mg^{2+}	magnesium ions
MgCl	magnesium chloride
MIF	Mullerian inhibition factor
M_r	molecular weight
MR	mineralocorticoid receptor
mRNA	messenger RNA

n	number of repetitions of each experiment using cells from different patients on each occasion
Na^+	sodium ions
NaCl	sodium chloride
NAD^+	β-nicotinamide adenine dinucleotide
$NADP^+$	β-nicotinamide adenine dinucleotide phosphate
NADPH	reduced form of NADP
Na_2-EDTA	sodium ethylene diaminotetra-acetate
NaN_3	sodium azide
NaOH	sodium hydroxide
NSB	non-specific binding
O_2	oxygen
P	probability
P_4	progesterone
$P450_{aro}$	cytochrome P450: aromatase enzyme
$P450_{C11}$	cytochrome P450: 11β-hydroxylase
$P450_{C17}$	cytochrome P450: 17α-hydroxylase, C17,20-lyase enzyme
$P450_{C21}$	cytochrome P450: 21-hydroxylase enzyme
$P450_{SCC}$	cytochrome P450: cholesterol side-chain cleavage enzyme
PA	phosphatidic acid
PAS-gel buffer	phosphate azide saline – gelatin buffer
PBRs	benzodiazepine receptors
PBS	phosphate-buffered saline
PCA	perchloric acid
PDE	cyclic nucleotide phosphodiesterase
PEG	polyethylene glycol
PG	prostaglandin
PGDH	prostaglandin dehydrogenase
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$

PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGI_2	prostaglandin I_2
PGHS	prostaglandin H synthase
PGHS-1	prostaglandin H synthase-1
PGHS-2	prostaglandin H synthase-2
PI	phosphatidylinositol
PIP_1	phosphatidylinositol monophopshate
PIP_2	phosphatidylinositol bis-4,5-phosphate
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12-myristate 13-acetate
PR	progesterone receptor
PRL	prolactin
RCT	reverse cholesterol transport
RIA	radioimmunoassay
RIP	receptor interacting protein
RNA	ribonucleic acid
22ROHC	22R-hydroxycholesterol
rRNA	ribosomal RNA
SAP	steroidogenic activator peptide
SCP_2	sterol carrier protein 2
SD	standard deviation

SE	standard error
SR-BI	scavenger receptor class B, type I
SR-BII	scavenger receptor class B, type II
StAR	steroidogenic acute regulatory protein
TAF	transactivating factor
TAG	triacylglycerol
TBS	tris buffered saline
TDF	testis-determining factor
TLC	thin layer chromatography
TNF	tumor necrosis factor
TP	thromboxane A_2 receptor
Tris	Trizma base
TX	thromboxane
TXA_2	thromboxane A_2
VEGF	vascular endothelial growth factor
VLDL	very low density lipoproteins
v/v	volume per volume
w/v	weight per volume

UNITS AND MEASURES

Centrifugal Force:	***g***	=	gravity
Concentrations:	**IU**	=	international units
	mol	=	moles
	M	=	Molar (moles/L)
Mass:	**Da**	=	daltons
	g	=	grams
Radioactivity:	**cpm**	=	counts per minute
	dpm	=	disintegrations per minute
	Bq	=	Becquerel (1 nuclear disintegration per second)
		=	60 cpm
	Ci	=	curie (3.7×10^{10} Bq)
Temperature:	**°C**	=	degrees Celsius
Time:	**h**	=	hours
	min	=	minutes
Volume:	**L**	=	litres

Unit Prefixes:

giga	**(G)**	:	$\times 10^{9}$
mega	**(M)**	:	$\times 10^{6}$
kilo	**(K)**	:	$\times 10^{3}$
milli	**(m)**	:	$\times 10^{-3}$
micro	**(μ)**	:	$\times 10^{-6}$
nano	**(n)**	:	$\times 10^{-9}$
pico	**(p)**	:	$\times 10^{-12}$
femto	**(f)**	:	$\times 10^{-15}$

Chapter One

GENERAL INTRODUCTION

Chapter One

GENERAL INTRODUCTION

This thesis describes the paracrine and autocrine roles for prostaglandins in the control of steroidogenesis and cortisol metabolism by human luteinised granulosa cells, with particular reference to prostaglandins E_2 and $F_{2\alpha}$. The mechanisms by which these prostaglandins may exert their effects have also been investigated. In the general introduction, the nature of the human ovary is defined and the hormonal control of ovarian steroidogenesis is reviewed, with particular emphasis on luteal function. Cholesterol and plasma lipoproteins are described with particular reference to their significance in ovarian physiology. Prostaglandins are defined and their importance in ovarian function is discussed along with the receptors involved in their actions. Finally, the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) is outlined with particular reference to its role in ovarian function.

1.1 The Human Ovary

1.1.1 Formation of the Human Ovary

During the first forty or so days of embryo development the female and male gonads appear to be similar. The absence of a Y chromosome in an XX (or XO) embryo results in the lack of testis-determining factor (TDF) and hence the formation of the ovaries. The absence of anti Mullerian hormone (AMH) allows the Mullerian ducts to differentiate into the uterus and oviducts/Fallopian tubes. The absence of testosterone causes the Wolffian ducts to regress and facilitates development of the female external genitalia. The functional units of the ovary, the ovarian follicles, don't seem to develop until the second trimester of pregnancy and have by then reached a number of approximately six to seven million. The production of new oogonia ceases by the end of the second trimester of pregnancy and never resumes again. Towards the end of gestation the oogonia begin meiosis, at which time they are called primary oocytes. Oogenesis is arrested at prophase I of the first meiotic division (primary oocytes are still diploid) and follicles reach a resting state until puberty. At birth the ovaries contain

about two million oocytes, but this number is reduced to 300,000 – 400,000 by the time the girl enters puberty. Primary oocytes are contained within primordial follicles [Johnson & Everitt, 2000].

1.1.2 Structure of the Human Ovary

The two adult ovaries are located within the body cavity, suspended by means of ligaments from the pelvic girdle. Fimbriae of the Fallopian tubes partially cover each ovary. Oocytes that are released from the ovary at ovulation, are normally drawn into the Fallopian tubes by the action of the ciliated epithelial lining of the Fallopian tubes, and transported to the uterus. The ovary has a diameter of about 3cm and consists of stromal tissue, containing the primordial follicles, and glandular tissue, or so-called interstitial glands. Primordial follicles have a diameter of about 20μm and are comprised of flattened mesenchymal cells, condensed around a primordial germ cell [Johnson & Everitt, 2000].

1.1.3 The Ovarian Cycle, Follicular Development, Ovulation and Luteinisation

As previously mentioned (section 1.1.1), the human ovary contains a large number of follicles, each of which encloses an oocyte. From the onset of puberty through to menopause, folliculogenesis occurs in the ovary, whereby activated follicles leave the resting phase, and begin to develop. The ovary has a cyclic pattern, which is controlled by a series of interactions between the hypothalamus, the anterior pituitary gland and the ovaries. Each ovarian cycle can be resolved into a follicular phase, prior to ovulation and a post-ovulatory, luteal phase (Figure 1.1).

Gonadotrophin-releasing hormone (GnRH), a decapeptide synthesised by the neurosecretory cells of the hypothalamus, is secreted in a pulsatile manner [McCann, 1998]. Under its influence, and hence in the same pulsatile manner, the anterior pituitary secretes luteinizing hormone (LH) and follicle stimulating hormone (FSH) [Yen *et al*, 1977; Fink *et al*, 1979]. The variations of FSH and LH, in turn, cause the ovary to respond in a cyclical manner, with each cycle lasting approximately 28 days

(varying from 25-35 days), consisting of two phases [reviewed Macklon & Fauser, 1998].

The follicular phase (14 days) starts on day 1 which corresponds to the first day of menses. In primates, a number of primordial follicles start to grow under the influence of FSH and undergo a number of both structural and functional changes. A major part of follicular growth occurs in the primary oocyte, which increases in its diameter to its final size of 60-120μm. Early on during the oocyte growth, glycoproteins are secreted which condense around the oocyte to form a translucent acellular layer called the zona pellucida. The zona separates the oocyte from the surrounding granulosa cells (GCs), which at the same time proliferate to become several layers thick. Gap junctions are formed both between the zona and GCs as well as between adjacent GCs, thus providing an extensive network of intercellular communication. As the granulosa cells of the pre-antral follicle proliferate, a viscous fluid starts to appear between them, in the form of droplets. This follicular fluid is comprised partly of a serum transudate and partly of mucopolysaccharides. High concentrations of progesterone and oestradiol are produced by the granulosa, theca cells are also known to be present [Fitz *et al.*, 1982; Bergeron *et al*, 1988]. The drops of fluid coalesce to form a single follicular antrum. The follicular fluid reflects the hormonal microenvironment of the follicle.

In addition to oocyte growth and GC proliferation, the theca cells also undergo development and proliferation forming two distinct layers: an inner, highly vascular theca interna, surrounded by a fibrous capsule, the theca externa. In response to LH, the theca cells secrete high concentrations of androgens which are in turn taken up by GCs. At the same time the developing follicles synthesise large amounts of rRNA and mRNA, the latter being used to generate stores of proteins that are essential for later stages of oocyte maturation and for the first day or so of development of the fertilised ovum.

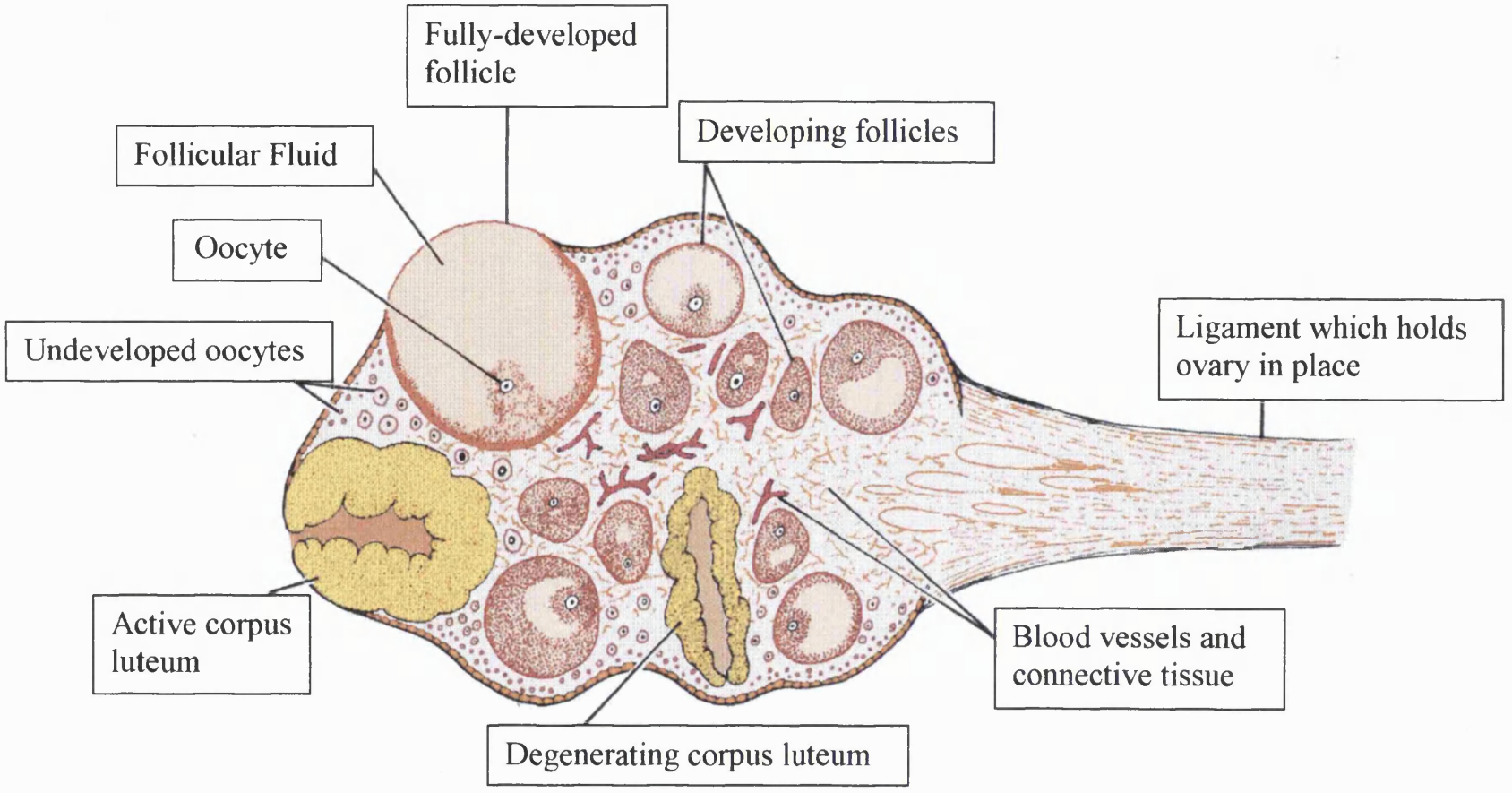

Figure 1.1 Schematic representation of the development of one follicle in the human ovary

Rises in progesterone concentrations prior to the mid-cycle gonadotrophin surge may also play a role in the release of LH. Studies in the macaque ovary using progesterone inhibitors have demonstrated that the LH surge can be blocked [Collins & Hodgen, 1986], signifying the importance of progesterone prior to the LH surge. It has also been reported that increases in serum progesterone concentrations prior to the LH surge have been measured in normal cycling women [Chang & Jaffe, 1978].

Even though a number of follicles start to develop at the beginning of each cycle only one (or occasionally two) is selected to achieve Graafian status, while the others undergo atresia. This may involve genetic and/or metabolic defects in the follicle [Moley & Schreiber, 1995]. Granulosa cells exhibit nuclear pyknosis and become free-floating in the follicular fluid. The oocyte undergoes pseudomaturation with condensation of the chromatin and the nuclear envelope becomes invaginated. The cells of the atretic follicle lyse and are phagocytosed by leucocytes and macrophages. It is still not clear how follicles are selected to become atretic or Graafian. At the end of the follicular phase in primates, usually only one of the follicles fully matures to form the Graafian follicle.

The LH surge triggers ovulation in the pre-ovulatory follicle. LH is thought to stimulate specific biochemical and physical changes in the follicle, by inducing the transient expression of genes essential for ovulation in the preovulatory granulosa cells [reviewed Richards *et al*, 1998; Tsafriri & Reich, 1999]. These may include induction of the progesterone receptor (PR) gene, and/or the induction of prostaglandin H synthase-2 (PGHS-2), (both of which are thought to induce the activation of matrix metalloproteinases), synthesis of growth factors, and recruitment of inflammatory cells [Petrovska *et al*, 1992; Buksovsky *et al*, 1995; Brannstrom & Friden, 1997].

The endocrine control of ovarian function by gonadotrophins is thought to be modulated by endocrine regulatory systems, involving a variety of molecules, including oestradiol, growth factors and inhibins [Gougeon *et al*, 1996]. Low oestradiol levels and inhibins are the main regulators of gonadotrophin secretion, with oestradiol exerting a negative

effect over FSH and LH production from the pituitary, mainly during the follicular phase [reviewed Gougeon *et al*, 1996; Chabbert Buffet *et al*, 1998]. In contrast, when the concentrations of oestradiol increase greatly, a positive effect on gonadotrophin production has been shown. This results in an increase in the responsiveness of cells to GnRH, a rise in the number of GnRH receptors and triggering of a GnRH surge from the hypothalamus [Sarkar *et al*, 1976; Fraser & Bouchard, 1994; Clarke *et al*, 1995] which translates into an LH surge from the anterior pituitary. Following the mid-cycle LH surge (which is oestrogen-dependent [reviewed Clarke *et al*, 1995; Mahesh & Brann, 1998]), ovulation occurs, resulting in the rupture of the follicle, and the expulsion of the oocyte from the ovary.

After ovulation, the cells constituting the remaining follicular tissue become highly vascularised and undergo structural and functional differentiation (luteinisation), giving rise to a new structure called the corpus luteum (CL) [reviewed by Channing *et al*, 1980; Duncan, 2000]. Activation of proteases within the follicle alters the integrity of the extracellular matrixes and allows capillaries from the surrounding stroma to proliferate and penetrate the basal lamina, thus providing the luteinized cells with plasma-borne substances. Luteinisation is the remodelling process of the follicle whereby the individual cells lose their previously defined structures, and both the theca and granulosa cells differentiate into the luteal phenotype, a process that is irreversible.

The follicular tissue becomes very rapidly, highly vascularised. This process is thought to be regulated by the paracrine release of angiogenic inducers such as basic fibroblast growth factor (bFGF) [Gospodarowicz *et al*, 1985; Gospodarowicz *et al*, 1986; Reynolds *et al*, 1998], vascular endothelial growth factor (VEGF), and angiotensin [Reynolds *et al*, 1998; Lebovic *et al*, 1999]. These factors induce endothelial migration, followed by the proliferation and formation of blood capillaries. Angiogenic factors have been shown to be present in human follicular fluid [Koos *et al*, 1989; Yamada & Gentry, 1995; Artini *et al*, 1998] and ovarian tissues [Gordon *et al*, 1996]. Predominant staining of such factors are dominant in theca cells of healthy follicles corresponding with luteinisation of the follicle, and in the highly vascularised CL [Ferrara *et al*, 1998; Fraser

et al, 1999]. Moreover, the CL number decreases in the presence of the angiogenic inhibitor TNP-470 [Fraser *et al*, 1999]. Thus the angiogenic factors are essential for vascularisation of the ruptured follicle, and the formation of the CL.

During the luteal phase, the CL actively secretes progesterone (the main steroid synthesised and secreted by the CL) and (in primates) oestradiol, under the influence of LH [Channing *et al*, 1980]. They together act to inhibit secretion of the pituitary gonadotrophins and hence the onset of another ovarian cycle. In response to progesterone secretion during the luteal phase, the epithelial cells of the endometrial glands begin to secrete nutrients in preparation to support the embryo in case fertilisation occurs. In the event of fertilisation of the released oocyte, the CL maintains the circulating progesterone concentrations required to sustain pregnancy until implantation occurs and the placenta is formed. In the absence of fertilisation, the endometrium sheds, leading to menstruation [De Ziegler *et al*, 1998], and FSH levels rise, resulting in the initiation of a new cycle [Le Nestour *et al*, 1993]. The CL regresses both structurally and functionally, with decreases in both progesterone and oestradiol production. The exact mechanisms for controlling the function and lifespan of the CL are still to be resolved [Knobil *et al.*, 1973; Auletta & Flint, 1988]. However, there are two classes of compounds that can either enhance or inhibit the life span of the CL, termed the luteotrophins or luteolysins respectively. Prostaglandins, and especially PGE_2 and $PGF_{2\alpha}$, are known to affect the lifespan of the CL in a number of species. However, their effects will be described in more detail in a later section (1.4.3).

1.1.4 The physiological role of the corpus luteum

Since the major secretory product of the corpus luteum is progesterone, the actions of this steroid must be defined in order to appreciate the physiological role of the CL in reproduction.

The principal actions of progesterone are to prepare the uterine endometrium for implantation to occur in the event of fertilisation of the released oocyte, and to stimulate the release of nutrients (*e.g.* sugars, glycoproteins and amino-acids) from the uterine

glands [Graham & Clarke, 1997]. In addition, progesterone is also capable of exerting a dual function in regulating gonadotrophin secretion. The high plasma concentrations of progesterone, such as are seen in the luteal phase (12.7-25.4nM in humans) can enhance the negative feedback effects of oestradiol on FSH and LH, and also block the positive feedback effect of oestradiol [Schaison & Cozinet, 1991; Leung *et al*, 1996]. Hence, ovulation and oestrus behaviour are inhibited during the luteal phase of the non-conception cycle and in pregnancy when copulation would represent a waste of time and energy in the biological sense that it would not result in any further offspring. The human CL also secretes large amounts of oestradiol and inhibin which are thought to act synergistically in suppressing LH release [Channing *et al*, 1980]. Thus the overall role of the CL is to provide the hormonal support required in early pregnancy and to inhibit ovarian cycles during gestation. In humans, the CL remains the major source of progesterone for the first three months of pregnancy until the placenta is established and able to produce adequate levels of progesterone to sustain the pregnancy.

1.1.5 Autonomous luteal function

At present, the precise mechanism(s), which initiates luteal regression is unknown. It has been hypothesised that progesterone is secreted from the CL for a fixed period dependent only upon conception. In keeping with this hypothesis, Rothchild (1981) suggested that progesterone inhibits the esterification and storage of intracellular cholesterol, resulting in increased steroid synthesis. However, studies on the macaque CL have shown that LH is required to maintain progesterone synthesis during the luteal phase [Fraser *et al*, 1987]. As the CL requires LH for maintenance, the progressive decrease in LH plasma concentrations during the luteal phase lead to the alternative hypothesis, that the CL regresses due to the decrease in LH [Baird *et al*, 1985]. Baird (1985) suggested that decreased LH or progesterone production would result in increased production and secretion of LH, due to decreased negative feedback mechanisms to the pituitary, resulting ultimately in increased LH support of the CL. However, it has also been postulated that the decreases in LH may increase the susceptibility of the CL to other luteolytic agents or mechanisms (section 1.6.4).

1.1.6 Structural and functional changes associated with luteolysis

Regression of the CL is characterised by functional changes including the loss of gonadotrophin receptors [Pang & Behrman, 1981], decrease in cAMP production [Thomas *et al*, 1978] and the activation of phospholipase C (PLC) (section 1.5.2) [Leung *et al*, 1986]. Such changes ultimately result in decreased progesterone synthesis and secretion. Structural changes include membrane breakdown, indicated by decreased membrane fluidity [Greenhalgh *et al*, 1990; Carlson *et al*, 1982; Riley & Behrman, 1991] with an increase in phospholipase A_2 (PLA_2) activity [Sawada & Carlson, 1991] and a loss of human chorionic gonadotrophin (hCG) binding [Gatzuli *et al*, 1991]. The activation of PLA_2 leads to the release of arachidonic acid, the precursor of all eicosanoids, including prostaglandins E_2 and $F_{2\alpha}$ which are known to affect luteal function. However, their precise effects will be discussed in a later section (1.6).

1.2 Ovarian Steroid Synthesis

1.2.1 Cholesterol uptake and synthesis

Cholesterol is the main precursor for steroid synthesis in all steroidogenic tissues (Figure 1.2). There are four sources of cholesterol:

i) free cholesterol

ii) cholesteryl esters (CE)

iii) *de novo* synthesis of cholesterol from acetate, the 2-carbon precursor, via a number of intermediate steps, of which the 3-hydroxy-3-methylglutaryl co-enzyme A reductase (HMG-CoA reductase: EC:1.1.1.34) is the rate-limiting enzyme

iv) plasma membrane free cholesterol.

Free cholesterol and cholesteryl esters are carried within plasma lipoproteins and are admitted into the cells by receptor-mediated endocytosis. In several species including humans, the major steroidogenic tissues (*i.e.* the adrenal glands, ovaries, testis and placenta) are known to acquire steroidogenic substrate by the uptake of cholesterol from lipoproteins.

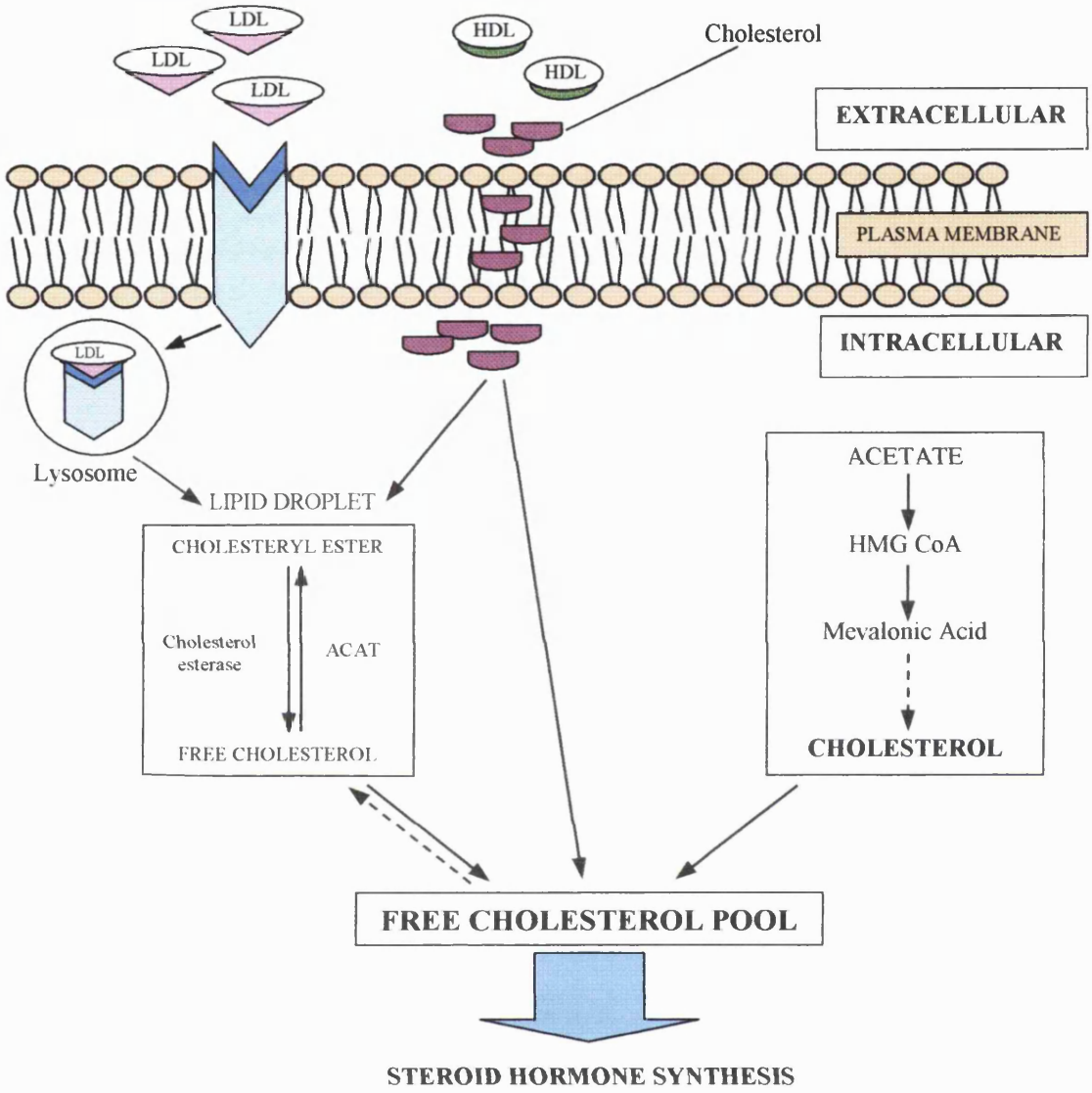

Figure 1.2 Schematic representation of cholesterol synthesis in a steroidogenic cell

Cholesteryl esters present in the core of the lipoprotein particle are released by hydrolysis and stored inside the cell as cholesteryl oleate in cytosolic lipid droplets. This storage is controlled by two opposing enzymes: acyl coenzyme A cholesterol acyltransferase (ACAT, EC:2.3.1.16), and cholesterol esterase (EC:3.1.1.13), the latter of which liberates free cholesterol from the lipid droplets according to the requirements of the cell [Flint & Armstrong, 1972].

Steroidogenic tissues may also synthesise cholesterol by *de novo* synthesis via HMG-CoA reductase. However, the activity of the enzyme is regulated by lipoprotein cholesterol such that *de novo* cholesterol synthesis is inhibited by lipoproteins at their physiological concentrations in luteal cells [Schuler *et al*, 1981; Savion *et al*, 1982; Pate & Codon, 1989]. In fact, cultured granulosa cells in the absence of serum or lipoproteins (the major sources of cholesterol for cell in culture) exhibited a significant decrease in progesterone production, suggesting that *de novo* synthesis of cholesterol was unable to support maximal steroidogenesis [Parinaud *et al*, 1987].

1.2.2 Intracellular cholesterol transport

Newly synthesised free cholesterol and cholesterol released from cytosolic lipid droplets has to be transported into the mitochondria for pregnenolone synthesis. Due to its insolubility in aqueous solutions (including plasma), it is suggested that free cholesterol is transported in association with a protein(s). Candidates for the cholesterol transport protein have included the 14kDa sterol carrier protein 2 (SCP_2), which is known to transfer cholesterol from lipid droplets to the mitochondria in adrenal cells [Vahouny *et al*, 1987], and the steroidogenic activator peptide (SAP), a 3.2kDa cytosolic protein [Pedersen & Brownie, 1987].

1.2.2.1 Steroidogenic acute regulatory (StAR) protein and peripheral benzodiazepine receptors (PBRs)

The recently discovered Steroidogenic Acute Regulatory (StAR) protein has been shown to play a critical role in facilitating cholesterol transport across the mitochondrial inter-membrane space in steroidogenic tissues [reviewed by Stocco, 1997; Cherradi *et al*, 1997; Arakane *et al*, 1998; Strauss III *et al*, 1999]. This short half-life protein is synthesised on the cytoplasmic ribosomes in response to hormone-induced generation of cyclic adenosine-3',5'-monophosphate (cAMP) [Kiriakidou *et al*, 1996] with subsequent activation of phosphorylation [Arakane *et al*, 1997] and gene transcription by the cAMP-dependent protein kinase A (PKA). A 37kDa preproprotein is hence formed, accompanied by chaperone proteins which prohibit folding of the precursor N-terminal

mitochondrial targeting sequence [Stocco & Clark, 1996; Stocco, 1997; Arakane *et al*, 1998]. This preproprotein becomes associated with the mitochondria, where the import and processing of the precursor protein takes place. It has been proposed that, as a result of the processing of this protein, a contact site between the two membranes is formed. At the same time, through a process that is still not understood, the C-terminus of the protein begins to interact with the outer mitochondrial membrane and result in cholesterol transfer to the inner mitochondrial membrane [Stocco & Clark, 1996; Kallen *et al*, 1998]. Cleavage of the precursor protein by the matrix processing protease gives rise to a 30kDa mature form of StAR which then becomes refolded and associated with both the inner mitochondrial membrane and the intermembrane space [Stocco, 2000].

In situ hybridisation studies revealed that the StAR gene is expressed in the theca cells of human preovulatory follicles but not in the granulosa cells. However, in the human CL, both the theca-lutein and granulosa-lutein cells express StAR [Kiriakidou *et al*, 1996]. Studies performed by Sugawara (1997) suggest that steroidogenic tissues that do not express the StAR gene, including the placenta, can synthesise pregnenolone. This finding suggests that there must be a StAR-independent mechanism for the movement of cholesterol to the P450 side chain cleavage ($P450_{SCC}$) enzyme. However, work performed by Watari (1997) described the steroidogenic properties of a protein, MLN64, which has homology to the C-terminal region of StAR. Its expression in COS-1 cells resulted in a two-fold increase in steroid production. This suggests that alternative pathways for the transport of cholesterol inside the mitochondria may exist in different steroidogenic tissues. Finally, recent findings have shown that the expression and transcription of StAR may be inhibited by environmental pollutants, which disrupt steroidogenesis and impair reproductive function [Walsh *et al*, 2000].

The peripheral benzodiazepine receptor (PBR) has also been suggested as a strong candidate for the transfer of cholesterol across the mitochondrial membranes. PBR expression has been shown to be localised at the outer mitochondrial membrane in steroidogenic tissues [Papadopoulos *et al*, 1998], and PBR agonists have been shown to

stimulate intramitochondrial transport and pregnenolone synthesis in isolated mitochondria [Amsterdam & Luh, 1991; Bar-Ami *et al*, 1991; Gavish, 1994].

1.2.3 Steroid Biosynthesis

The initial and rate-determining enzymatic step in the biosynthesis of all steroids is the conversion, within the mitochondria, of cholesterol (C_{27}), to the 21-carbon molecule pregnenolone [Belfiore *et al*, 1994] (Figure 1.3). This involves a hydroxylation at carbon positions 20 and 22, followed by cleavage of the cholesterol side-chain between these positions to yield pregnenolone and a 6-carbon fragment, isocaproic acid [Burstein & Gut, 1971; Voutilainen *et al*, 1986]. All of the above mentioned reactions are catalysed by a single enzyme, the cytochrome P450 cholesterol side-chain cleavage enzyme [($P450_{SCC}$), EC:1.14.15.6]. This enzyme is located on the inner face of the inner mitochondrial membrane [Simpson *et al*, 1979]. Thus, $P450_{SCC}$ functions as an oxidase by being the acceptor of electrons from NADPH, via an electron transfer system localised in the inner mitochondrial membrane. The electrons are transferred from the NADPH to the $P450_{SCC}$ via the FAD-containing flavoprotein, ferrodoxin reductase, and the non-haem iron-sulphur protein, ferrodoxin [Tuckey *et al*, 1997]. The *CYP11A* gene encoding the $P450_{SCC}$ enzyme has been cloned [Chung *et al*, 1986; Morohashi *et al*, 1987], and has been shown to be hormonally regulated in the human adrenal glands [DiBlasio *et al*, 1987; Sparkes *et al*, 1991] and ovarian tissues [Golos *et al*, 1987b]. Once pregnenolone has been formed, it leaves the mitochondrion, and passes to the smooth endoplasmic reticulum where the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD, EC: 1.1.1.145) is known to act, resulting in the formation of pregn-4-ene-3,20-dione (progesterone) [Lorence *et al*, 1990; Albrecht & Daels, 1997; Conley & Bird, 1997]. To date there are six cloned isoforms of 3β-HSD [Thomas *et al*, 1989; The *et al*, 1989; Lorence *et al*, 1990; Rheaume *et al*, 1991]. Even though progesterone is the main steroid product of the CL, both pregnenolone and progesterone may undergo 17α-hydroxylation in the smooth endoplasmic reticulum, followed by cleavage of the c17,20 carbon bond, thus giving rise to dehydroepiandrosterone (DHEA) and androstenedione respectively [Voutilainen *et al*, 1986; McAlister & Hornsby, 1988]. Both of these reactions are catalysed by the enzyme cytochrome $P450_{C17}$ ($P450_{C17}$) [Albrecht & Daels,

1997; Conley & Bird, 1997] which receives electrons from NADPH via the membrane bound flavoprotein, cytochrome P450 reductase [Nakajin *et al*, 1981].

Isoforms of 17β-hydroxysteroid dehydrogenase (17β-HSD, EC: 1.1.1.209) catalyse the interconversion of weak 17-ketosteroids (androstenedione & oestrone) to more potent 17β-hydroxysteroids (testosterone & oestradiol respectively) [Ghersevich *et al*, 1994].

1.2.4 The two cell-two gonadotrophin hypothesis

In the preovulatory follicle, steroidogenesis occurs in both the theca and the granulosa cells. However the two steroidogenic pathways occur independently in each cell type due to the presence of the membrana propria/granulosa which renders the granulosa cell layer avascular. Since the granulosa cells receive no cholesterol from the blood stream, they must synthesise oestradiol by the aromatisation of androgen substrates produced from the theca cell layer. Hence in accordance with the two-cell, two-gonadotrophin theory the theca cells under the influence of LH produce androgens, the latter of which undergo aromatisation in the granulosa cells under the influence of FSH [McNatty *et al*, 1979; Hillier *et al*, 1984; Hillier *et al*, 1994]. The enzyme 17β-HSD expressed in the granulosa cells, catalyses the reduction of oestrone to oestradiol. LH initially stimulates $P450_{C17}$ gene expression, and the subsequent stimulation of androgen formation in the theca cells via the steroidogenic pathway described above (section 1.2.3). The stimulation of androstenedione in response to both LH (and FSH) appears to be modulated by intra-ovarian factors and paracrine feedback from the granulosa cells, whereas FSH regulates oestrogen biosynthesis from androstenedione in granulosa cells. Oestrogen inhibits the enzymes involved in theca androgen production by negative feedback, whereas, intra-ovarian peptides such as inhibin and the insulin-like growth factors (IGF) stimulate theca enzyme activities [Hernandez *et al*, 1989; Hillier *et al*, 1991]. The factors regulating oestrogen synthesis in accordance with the two-cell, two-gonadotrophin model have been extensively reviewed [Hillier *et al*, 1994].

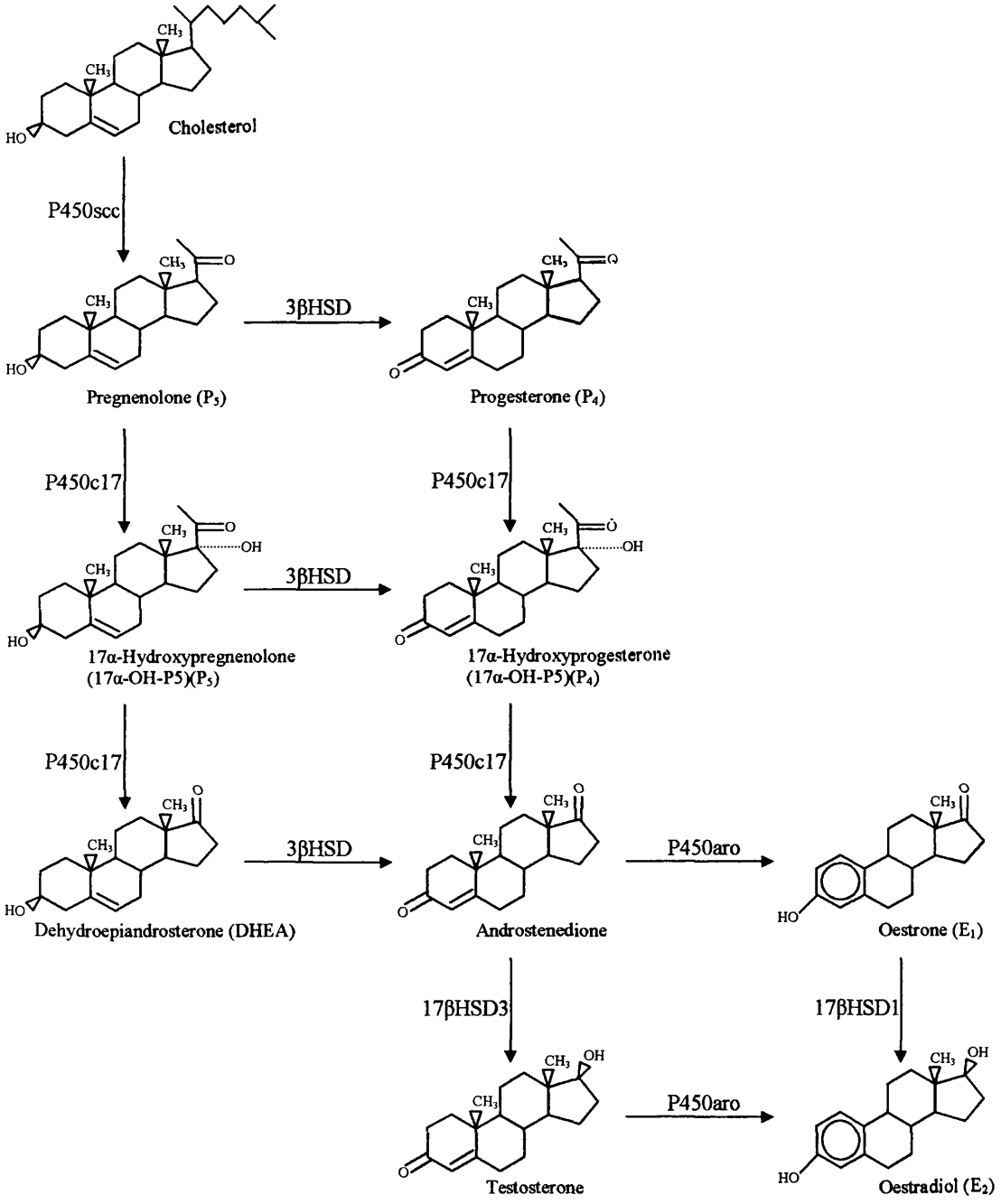

Figure 1.3 Principal pathways of human ovary steroidogenesis

During ovulation and luteinisation initiated by the LH surge, the cells of the ruptured follicle reorganise both structurally and functionally to form the CL. The transcription of human genes encoding the steroidogenic enzymes responsible for the synthesis of progesterone, hence $P450_{SCC}$ [Sparkes *et al*, 1991] and 3β-HSD [Lorence *et al*, 1990], is increased by LH.

1.3 Prostaglandin Biosynthesis

Prostaglandins (PGs) have a unique fatty acid skeleton called prostanoic acid (Figure 1.4). This compound, however, does not exist in nature.

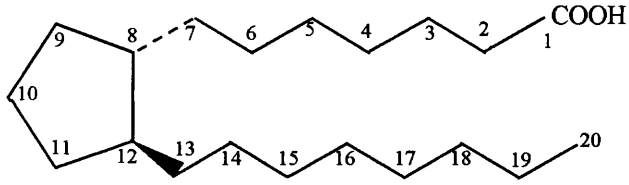

Figure 1.4 Generalised Prostaglandin Structure

The carbon skeleton of prostanoic acid, the prostaglandin parent compound

All naturally occurring prostaglandins have various oxygen-containing substituents in the molecule. The nature and position of these substituents determine the biological activity of the compound. The most important positions in the PG molecule are carbons 9, 11 and 15.

Prostaglandins are further subgrouped into three different series, the 1-, 2-, and 3-series, differing in their degree of unsaturation, and originating from three different polyunsaturated fatty acids. 8,11,14-eicosatrienoic acid is the precursor for the 1-series PGs, 5,8,11,14-eicosatetranoic acid (arachidonic acid) is the precursor for the 2-series and 5,8,11,14,17-eicosapentaenoic acid is the precursor for the 3-series of PGs.

The most important of the prostaglandin precursors is arachidonic acid (AA) which is stored in cell membranes esterified at the C2 position of membrane phospholipids. The

production of arachidonate metabolites is controlled by the rate of arachidonate release from these stores, through four alternative pathways (Figure 1.5):

i) PLA_2 pathway, hydrolyses acyl groups at the C2 of phospholipids (section 1.5.5)

ii) PLC pathway, specifically hydrolyses the phosphatidylinositol head group to yield 1,2-diacylglycerol, which is phosphorylated by diacylglycerol kinase to phosphatidic acid, a PLA_2 substrate (section 1.5.2)

iii) 1,2-diacylglycerol can also be hydrolysed directly to liberate AA by diacylglycerol lipase

iv) PLD pathway, generating phosphatidic acid (PA) which can then enter the PLA_2 pathway (section 1.5.4).

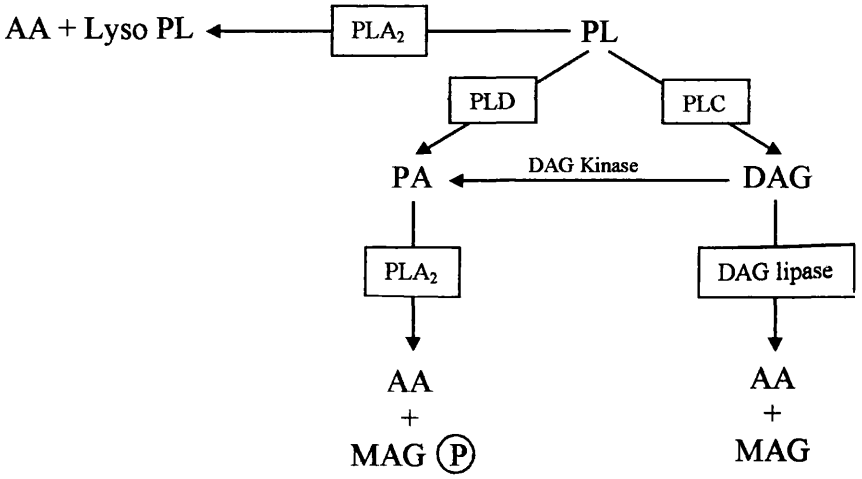

Figure 1.5 Arachidonic Acid Release
AA = arachidonic acid; Lyso PL = lyso phospholipid; PLA_2 = phospholiase A_2; PL = phospholipid; PLC = phospholipase C; PLD = phospholipase D; DAG = Diacyl glycerol; PA = phosphatidic acid; MAG (P) = monoacyl glycerol (phosphate)

There are two main pathways for arachidonate metabolism: the cyclooxygenase pathway (or "cyclic pathway") through which prostaglandins, thromboxanes and prostacyclins are formed (Figure 1.6), and the lipoxygenase pathway (or "linear pathway") through which leukotrienes and hydroperoxyeicosatetranoic acids (HPETEs) are derived (Figure 1.7).

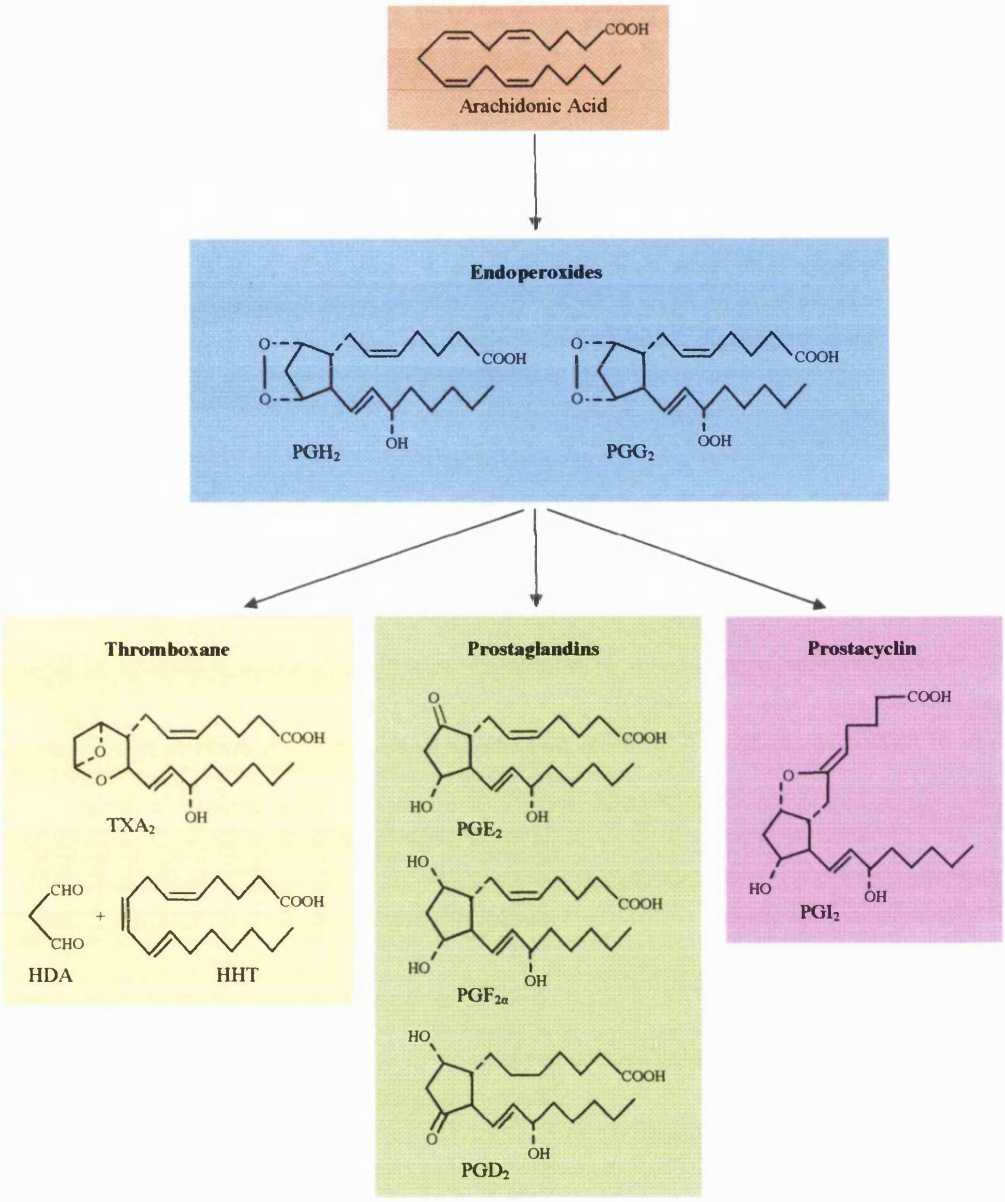

Figure 1.6 Schematic representation of the cyclo-oxygenase pathway

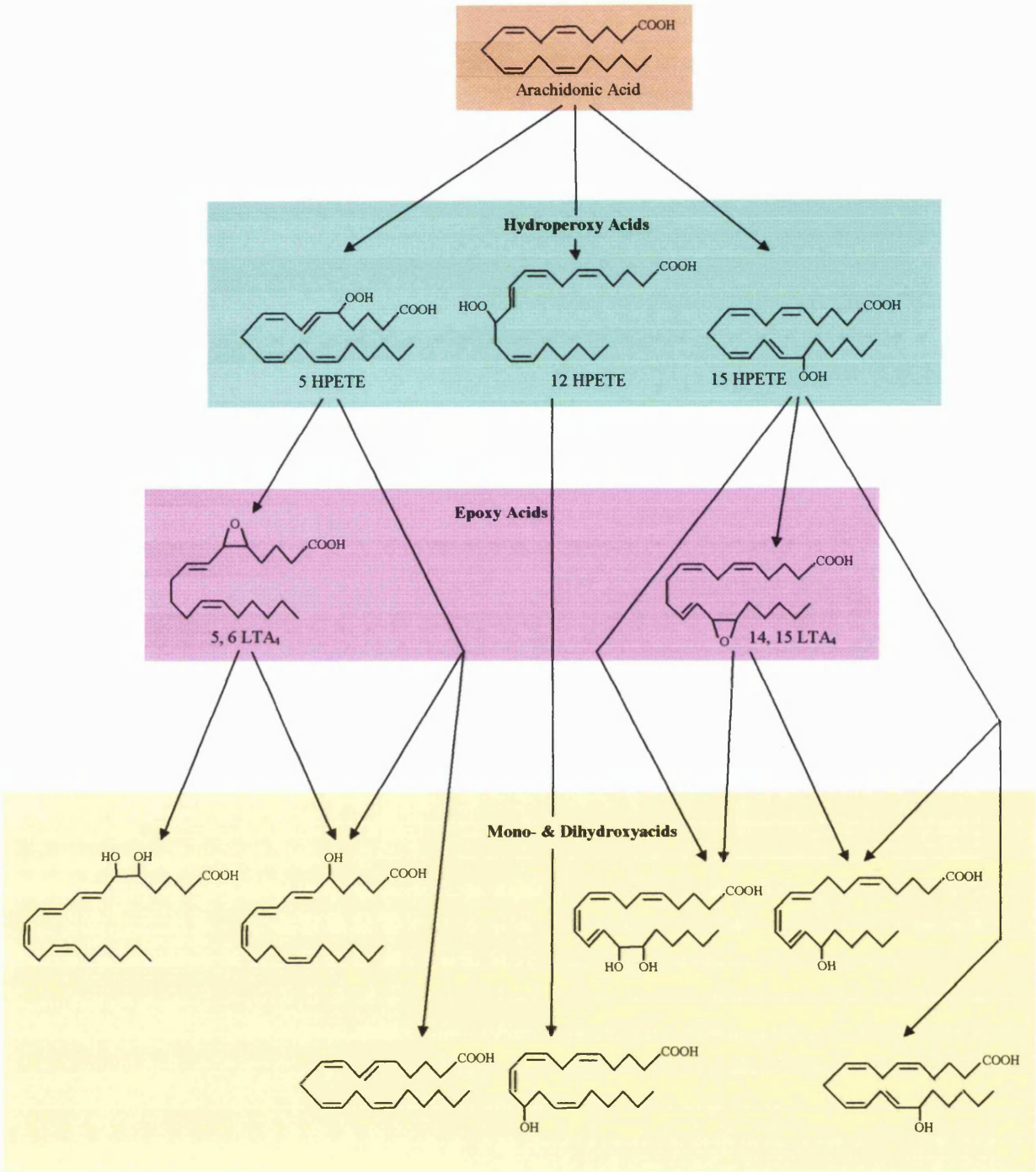

Figure 1.7 Schematic representation of the lipoxygenase pathway

1.3.1 The cyclo-oxygenase pathway of arachidonate metabolism

The first step on the cyclic pathway of arachidonic acid metabolism is catalysed by prostaglandin H_2 synthase [PGHS, EC: 1.14.99 formerly termed cyclo-oxygenase (COX)]. This haem-containing enzyme contains two catalytic activities; a cyclo-oxygenase activity and a peroxidase activity. The former catalyses the addition of two oxygen molecules to arachidonic acid, forming prostaglandin G_2 (PGG_2). The latter converts the hydroperoxy function of PGG_2 to a hydroxyl group, giving rise to prostaglandin H_2 (PGH_2). To date there are two identified isoforms of PGHS: PGHS-1 and PGHS-2. Both isoforms of the PGHS enzyme are homodimeric, haem containing, glycosylated, integral membrane proteins with two catalytic sites [reviewed by Smith *et al*, 1996]. They are anchored to one leaflet of the lipid bilayer through the hydrophobic surfaces of amphipathic helices rather than through transmembrane domains and are bound to the lumenal surface of the ER and the outer membrane of the nuclear envelope [Otto & Smith, 1994]. The reason for the existence of two PGHS isozymes in unknown, however, their expression differs which suggests that they serve different purposes inside the cell.

PGHS-1 is the constitutive isozyme whereas PGHS-2 is an inducible enzyme. PGHS-1 can be detected in most tissues although not within all cells of the tissue. PGHS-2 on the other hand is undetectable in most mammalian tissues. However, in those that do express PGHS-2 (*e.g.* fibroblasts [DeWitt & Meade, 1993], endothelial cells [Jones *et al*, 1993] & ovarian follicles [Sirois *et al*, 1992]), its expression can be induced rapidly in response to growth factors, tumour promoter hormones and cytokines. Furthermore, PGHS-2 mRNA and protein expression is inhibited by anti-inflammatory steroids (*e.g.* cortisol) [Kujubu & Herschman, 1992; Chofford *et al*, 1994].

PGHS-1 and -2 are encoded by separate genes located on different chromosomes. The gene for PGHS-1 is approximately 22kb and contains 11 exons [Kraemer *et al*, 1992], whereas the PGHS-2 gene is 8kb and contains 10 exons [Kujubu & Herschman, 1992]. Both PGHS-1 and -2 have similar cyclooxygenase K_m values for arachidonate ($\approx 5\mu M$) and O_2 ($\approx 5\mu M$) [Barnett *et al*, 1994; Laneuville *et al*, 1994; Lands *et al*, 1978].

Furthermore, the key residues that are involved in catalysis are conserved between the isozymes, and the crystal structures of the two isozymes are essentially superimposable. The cyclo-oxygenase active site lies on the opposite side of the haem at the end of a long narrow channel. Tyrosine 385, which lies near the top of the channel, just beneath the haem, has been shown to form a transient radical during the cyclo-oxygenase reaction. The peroxidase active site of PGHS-2 occurs in a shallow cleft that contains the Fe(III)-haem prosthetic group. The cleft exposes a large portion of the haem to solvent and is therefore thought to comprise the substrate binding site.

It has been hypothesised that PGHS-1 and -2 represent at least partially independent prostanoid biosynthetic systems [Smith *et al*, 1996]. PGHS-1 occurs as part of an ER prostanoid biosynthetic system, which forms prostanoids that act extracellularly as "local" hormones functioning through cell surface, G-protein coupled receptors to mediate acute "housekeeping" responses to circulating hormones. PGHS-2 on the other hand is thought to have two roles. The first one involves augmentation of the PGHS-1 function whereas the second one is thought to involve the sub-population of PGHS-2 present in the lumenal surface of the inner membrane of the nuclear envelope. The latter it thought to operate as part of a unique nuclear prostanoid biosynthetic system forming products that act on nuclear membrane targets in association with cell differentiation and replication [reviewed by Smith *et al*, 1996].

PGH_2 is the immediate precursor for all the series-2 PGs, prostacyclins and thromboxanes. The fate of the PGH_2 depends on the relative activities of the enzymes catalysing the specific interconversions (Figure 1.6).

1.3.2 The lipoxygenase pathway of arachidonate metabolism

The first reaction in the conversion of arachidonic acid to leukotrienes is its oxidation to form 5-hydroperoxyeicosatetraenoic acid (5-HPETE), a substance that, in itself, is not a physiological mediator [Peters-Golden, 1998]. 5-lipoxygenase (5-LO; EC: 1.13.11) contains an iron atom that must be in its Fe(III) state to be active. The 5-LO reaction is thought to proceed as follows:

i) the active site iron ion abstracts an electron from the central methylene group of the 5,8-pentadiene moiety of arachidonate and the resulting free radical loses a proton to an enzymatic base

ii) the free radical rearranges and adds an oxygen atom to form a hydroperoxide radical

iii) the hydroperoxide radical reacts with the active site iron, now in its Fe(II) form, to yield the hydroperoxide in its anionic form, which the enzyme then protonates to yield the hydroperoxide product, regenerating the active Fe(III) enzyme.

Once the 5-HPETEs is formed, several further reactions take place for the generation of the other HPETEs and leukotrienes according to cell requirements [Rouger *et al*, 1986; Ueda *et al*, 1986].

The actions of epoxygenase on AA generates epoxides, which are subsequently converted to hydroxy acids, although the involvement of these products in PLA_2-mediated intracellular signalling is not well understood [Voorhis *et al*, 1993].

1.3.3 Prostanoid Receptors

Extensive research has elicited several binding sites for prostanoids [reviewed by Coleman *et al*, 1994]. These are on the cell surface and have been classified as follows:

i) PGD_2 (DP) receptors

ii) PGE_2 (EP) receptors

iii) $PGF_{2\alpha}$ (FP) receptors

iv) PGI_2 (IP) receptors

v) TXA_2 (TP) receptors

The cDNAs encoding numerous types of prostanoid receptors have been cloned, and the structures of the encoded receptors have been elucidated [Narumiya *et al*, 1999]. Analysis of these sequences has revealed that all of the cloned receptors have seven hydrophobic segments, characteristic of transmembrane domains, indicating that they are G-protein-coupled, rhodopsin-type receptors. The overall homology among the

receptors in not high, and the amino acid identity is scattered over the entire sequences, showing that they are derived from different genes. However, these receptors appear to have ability to couple other prostanoid receptors than those reflecting their name. Following binding studies, receptor potencies are evaluated as the concentration at which that tested ligand displaces binding of the prefered ligand by 50%. For example the FP receptor can bind to several ligands with the following rank order of affinities: $PGF_{2\alpha}$>PGD_2>PGE_2 [adapted from Abramovitz *et al*, 2000]. Receptors that are relevant to this thesis will be described in more detail in section 1.6.

1.4 Lipoproteins and ovarian function

1.4.1 Lipoproteins

Lipoproteins have as their main function the transport of lipids inside the body. One of the most important lipids for all steroidogenic tissues is cholesterol, which, as mentioned above, is the essential precursor for steroid biosynthesis. Lipoproteins are globular complexes (Figure 1.8) and can be categorised into four major classes according to their size and function (Table 1.1).

Table 1.1 Major lipoproteins and their constituents
(Adapted from Mackness & Durrington, 1995)

LIPOPROTEIN CLASS*	DENSITY RANGE (g/L)	COMPOSITION (% by weight)				
		PROTEIN	TAGs	CHOLESTEROL		PHOSPHOLIPIDS
				FREE	ESTER	
Chylomicrons	<0.98	1-2	85-95	1-3	2-4	3-6
VLDL	0.98-1.006	6-10	50-65	4-8	16-22	15-20
LDL	1.006-1.063	18-22	4-8	6-8	45-50	18-24
HDL	1.063-1.21	45-55	2-7	3-5	15-20	26-32

Mature plasma lipoproteins have a hydrophobic core, composed of triacylglycerols (TAGs) and cholesteryl esters (CE). This core is surrounded by a monolayer consisting

of both phospholipids (mainly phosphatidylcholine) and cholesterol molecules, which are embedded amongst the phospholipids, with their polar hydroxyl groups pointing towards the outer surface. Part of the phospholipid tails consists of AA (a precursor for the synthesis of prostaglandins; section 1.3) and cholesterol (Fig 1.8). The outer surface of this complex is surrounded by apolipoproteins (Table 1.2), which generate a more hydrophilic surface hence increasing solubility of lipoproteins in aqueous solutions (*e.g.* plasma and follicular fluid).

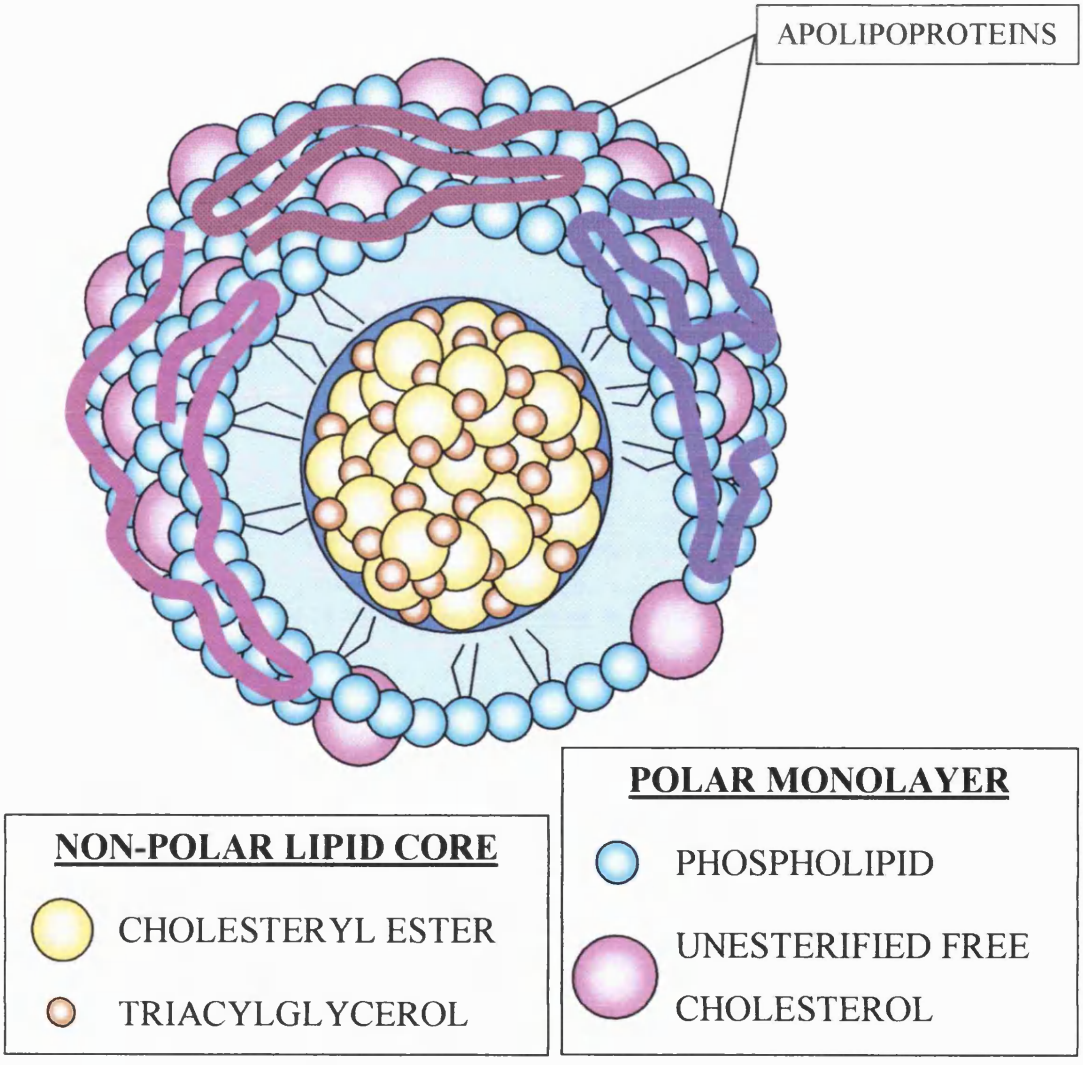

Figure 1.8 Schematic representation of a lipoprotein particle

Table 1.2 Properties and functions of the major apolipoproteins

(Adapted from Mackness & Durrington, 1995)

APOLIPOPROTEIN	PLASMA CONCENTRATION (approximate mg/100ml)	MOLECULAR WEIGHT (kDa) (approximate)	KNOWN FUNCTION
AI	6-160	28.5	Activation of LCAT
AII	20-55	17.4	Activation of hepatic lipase
AIII	?	22	Unknown
AIV	15	44.5	Lipid Transport (?)
B_{48}	0-2	264	Secretions of chylomicrons
B_{100}	60-160	550	Secretion of VLDL, receptor mediated LDL uptake
CI	3-11	6	Co-factor with LCAT (?)
CII	1-7	9	Activation of lipoprotein lipase
CIII	3-23	9	Inhibition of hepatic uptake of VLDL and chylomicrons
D	6-10	33	LCAT reaction (?)
E	2-6	34	Hepatic clearance of chylomicron remnants and IDL, cellular lipoprotein uptake
H	20	43-54	Triacylglycerol metabolism (?)
J	10	70	Lipid transport (?)
(a)	1-100	300-700	Inhibitor of fibrinolysis (?)

1.4.1.1 Lipoprotein Metabolism

The metabolism of plasma lipoproteins is summarised in Figure 1.9. Lipoproteins are in a dynamic state with continuous synthesis and degradation, accompanied by rapid exchange of lipid and protein components between particles. The major sites of synthesis are the intestine and liver. There are three enzymes throughout the different stages of the metabolic cycle which regulate the delivery, storage and mobilisation of lipoprotein-associated cholesterol, and these are the lipoprotein lipase (LPL, EC:3.1.1.34), lecithin: cholesterol acyltransferase (LCAT, EC:2.3.1.43) and ACAT.

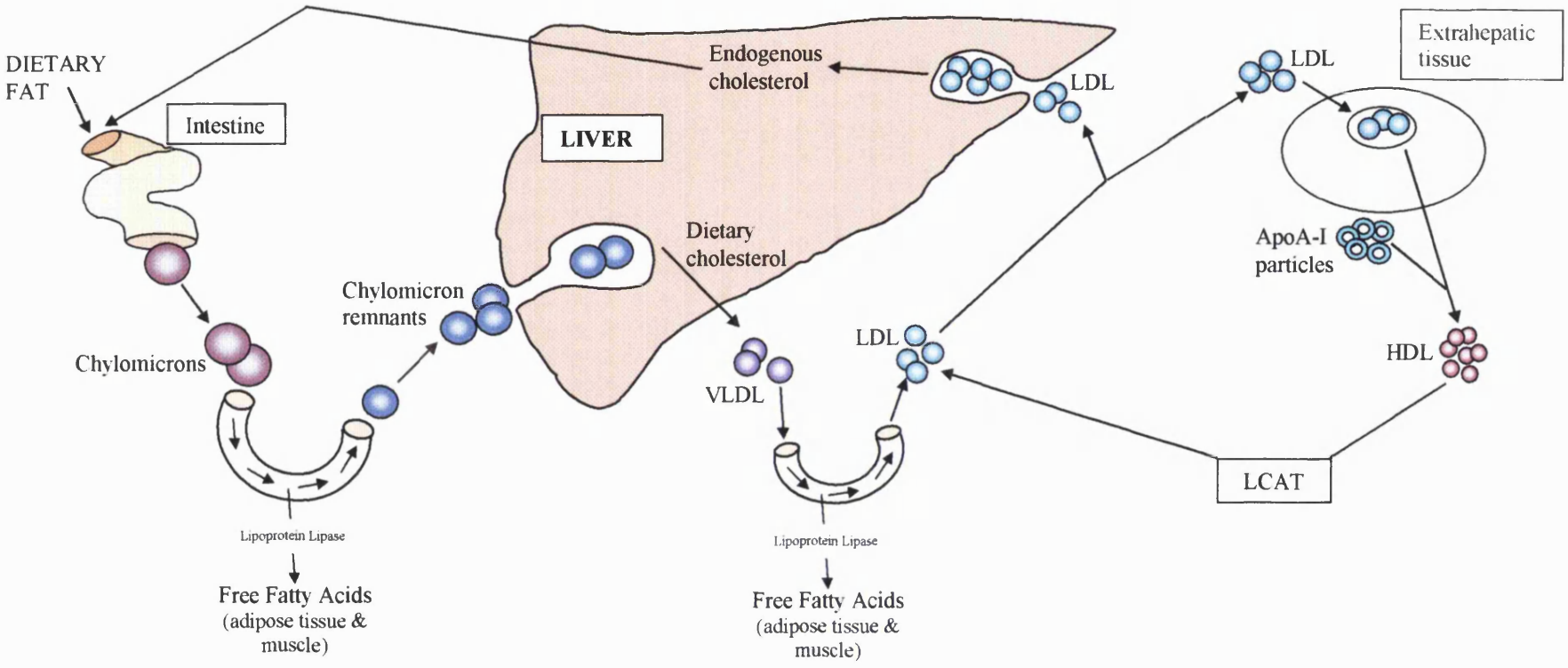

Figure 1.9 Schematic representation of lipoprotein metabolism

Chylomicrons and VLDL are the two major lipoproteins that carry TAG (Figure 1.9). Chylomicrons transport dietary fat from the small intestine, whereas VLDL transport endogenously synthesised TAG from the liver to peripheral tissues [Yang *et al*, 1995; Wiggins *et al*, 1996]. Chylomicron Apo-CII interacts with LPL which catalyses the hydrolysis of the TAG core into glycerol and fatty acids, which are then taken up by various tissues [Schaefer *et al*, 1978]. Following the loss of TAG, the chylomicron decreases in volume and at the same time the phospholipids, cholesterol and Apo-AI, AIV and CII are shed from the particle surface in the form of discs which are thought to be the precursors of HDL particles [Redgrave & Small, 1979]. VLDL are TAG-rich particles synthesised by hepatocytes and their metabolism is similar to that of chylomicrons.

Cholesterol rich LDL are formed by the action of LPL on VLDL particles, with the subsequent release of cholesterol, which is then transported to the peripheral tissues. LDL are considered to be the principal cholesterol provider for the peripheral tissues and contain a single Apo-B_{100} as the sole apolipoprotein on their surface [Eisenberg *et al*, 1973]. The distribution and delivery of cholesterol is mediated by specific binding of the LDL Apo-B_{100} to the receptors present at surface of hepatocytes and extrahepatic tissues.

LDL are taken up by cells via receptor-mediated endocytosis, which is regulated according to the cholesterol requirements of the cell (Figure 1.10) [Brown & Goldstein, 1984]. The "LDL receptor" is a five domain-glycoprotein comprised of 839 amino acids [Brown & Goldstein, 1986] and is specific for Apo-B_{100} lipoproteins [Brown & Goldstein, 1984; Brown & Goldstein, 1986]. The LDL-receptor complex migrates to a specialised area of endocytosis contained in clathrin-coated pits within the plasma membrane. This leads to invagination of the pits and the formation of endosomes [Brown & Goldstein, 1986]. The endosome fuses with a primary lysosome containing hydrolases and LDL is degraded to its component amino acids and CE, the latter of which are hydrolysed to yield fatty acids and free cholesterol. The cholesterol concentration is precisely regulated by three co-ordinated reactions:

i) HMG-CoA reductase is suppressed;

ii) synthesis, distribution and recycling of the Apo-B_{100} receptors on the cell surface is also suppressed;

iii) microsomal ACAT is activated [Brown & Goldstein, 1984], resulting in the esterification of excess free cholesterol which, due to its hydrophobic nature, forms CE storage droplets in the cytoplasm.

The uptake of lipoproteins serves a dual purpose in lipid homeostasis. Firstly, lipoprotein uptake mediates the delivery of cholesterol required for maintenance of cellular functions, and secondly, lipoprotein uptake regulates the concentration of cholesterol rich lipoproteins in the circulation. Hence, patients with hereditary hypercholesteraemia who lack the Apo-B_{100} receptor activity due to gene defects have increased circulating cholesterol-rich lipoproteins [Goldstein & Brown, 1989].

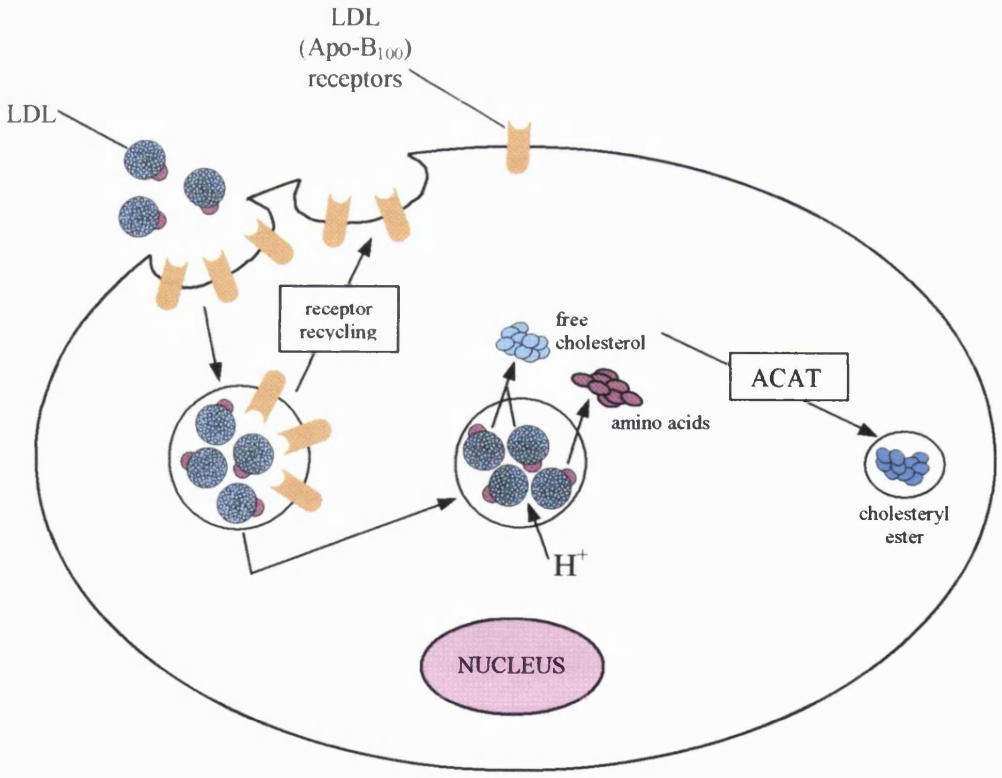

Figure 1.10 Schematic representation of the LDL receptor cycle

1.4.1.2 High Density Lipoproteins

HDL are initially formed as precursor lipoproteins from hepatic and intestinal tissues. The initial particle is a discoidal lipoprotein composed of phospholipids, apolipoproteins (usually Apo-AI and AII) and small amounts of free cholesterol. These particles are called "nascent" HDL particles. True HDL precursors are formed when free cholesterol becomes associated with the apolipoprotein-phospholipid complexes, giving rise to discoidal HDL. Mature HDL is assembled by acquiring more free cholesterol which is converted to CEs through the action of LCAT. In fact apolipoprotein-AI has been shown to serve as an effective activator of LCAT which, in turn, catalyses the esterification of cholesterol [Fielding & Moser, 1982].

HDL contain a host of apolipoproteins, but apolipoprotein A-I (Apo-AI) is believed to constitute 95% of all apolipoprotein composition of HDL particles [Eisenberg *et al*, 1972]. Human Apo-AI is a polypeptide of 243 amino acids [Brewer *et al*, 1978] and is known to be primarily synthesised in liver and intestine. Apo-AI is also expressed in the ovary [Ndikum-Moffor *et al*, 1997] and its plasma levels are hormonally regulated (*e.g.* oestradiol) [Harnish *et al*, 1998].

Follicular fluid has been shown to contain high levels of HDL with very low, or undetectable levels of LDL and VLDL [Simpson *et al*, 1980; Perret *et al*, 1985; Volpe *et al*, 1991; Jaspard *et al*, 1996; Jaspar *et al*, 1997]. The differences between the concentrations of the various lipoproteins in human follicular fluid and those in human plasma may be due to the difference in sizes and a sieving effect of plasma through the basement membrane.

1.4.1.3 High Density Lipoprotein receptors

Unlike LDL with its specific and unique receptors, HDL appears to have several modes of interaction with cells, some of which include the influx or efflux of cholesterol to or from the cells.

HDL binding proteins have been identified on the plasma membranes of many cell types including fibroblasts [Graham & Oram, 1987], hepatocytes [Bachorik *et al*, 1982], macrophages, adipocytes [Barbaras *et al*, 1990] and steroidogenic tissues [Chen *et al*, 1980]. Purification and cloning of these receptors has led to the identification of candidate HDL receptors, including the HDL binding protein (HBP) [Mcknight *et al*, 1992; Chiu *et al*, 1997], HDL binding protein$_2$ (HB_2) [Matsumoto *et al*, 1997], and the scavenger receptor, class B, type I (SR-BI) [Acton *et al*, 1996].

SR-BI is an ~82kDa, palmitoylated, cell surface glycoprotein that binds HDL and LDL with high affinity [Acton *et al*, 1994; Acton *et al*, 1996; Stangl *et al*, 1998] and was first identified by its homology to the CD-36 scavenger receptor [Calvo & Vega, 1993]. SR-BI is also known to mediate selective lipid uptake from HDL in ovarian cells from humans [Azhar *et al*, 1998a], and rats [Reaven *et al*, 1995; Reaven *et al*, 1998], and in human adrenocortical cells [Temel *et al*, 1997; Rigotti *et al*, 1997]. Expression of SR-BI has been identified at high levels in liver and in the major steroid-producing regions of the adrenal gland, ovary and testis of rodents [Landschulz *et al*, 1996; Rigotti *et al*, 1996; Li *et al*, 1998]. Furthermore, SR-BI is involved with selective lipid uptake which is regulated by trophic hormones via the cAMP second messenger pathway [Azhar *et al*, 1998b]. However, there is evidence to support the idea that after desensitisation of SR-BI occurs in the ovary, expression of the receptor loses its gonadotrophin responsiveness and its regulation becomes dependent upon other factors, such as the cellular cholesterol status of the ovary [Reaven *et al*, 1998]. Both the CD36 and BI scavenger receptors mediate HDL-CE uptake. However, SR-BI has been shown to facilitate cholesterol efflux to both HDL and phosphatidylcholine vesicles but not to Apo-AI in transfected CHO cells [Ji *et al*, 1997; Jian *et al*, 1998; De Llera-Moya *et al*, 1999]. Recent studies in macrophages have reported that SR-BI does not facilitate cholesterol efflux when intracellular cholesterol stores are increased [Chen *et al*, 2000].

SR-BII has also been identified, as a derivative of alternative splicing of the gene for SR-BI, and has also been characterised as being capable of mediating lipid transfer between cells and HDL [Webb *et al*, 1997; Webb *et al*, 1998]. About 30% of the total

expressed SR-BI gene has been estimated to generate SR-BII mRNA, which in turn mediates cholesterol transport with four times less efficiency than that of SR-BI [Webb *et al*, 1998]. The physiological relevance of this alternative sliced SR-BI receptor remains unclear.

Another protein reported of being involved in cholesterol transport is the ATP-binding cassette transporter 1 (ABC1). To date, the ABC1 gene has been cloned from mice [Luciani *et al*, 1994] and humans [Langmann *et al*, 1999]. Like other ABC proteins, the ABC1 protein contains two ATP-binding domains and 12 transmembrane domains, which are believed to form a channel-like structure and are essential for the transport function of ABC proteins [Decottignies & Goffeau, 1997; Riordan *et al*, 1989]. The ABC1 transporter has been shown to mediate cholesterol efflux from the cells to Apo-AI or HDL [Lawn *et al*, 1999; Langmann *et al*, 1999].

1.4.1.4 Reverse cholesterol transport

The term "reverse cholesterol transport" (RCT) is reserved for that part of the cholesterol flux that is described as net movement of free cholesterol molecules out of cells. This cholesterol subsequently binds to components of plasma and eventually is used by other tissues, predominantly the liver, where it is either recycled or excreted in bile [Pieters *et al*, 1994; Barter & Rye, 1996]. There are four steps involved in RCT:

i) the efflux of cholesterol from the cell membranes to HDL
ii) the esterification of cholesterol taken up by HDL, by LCAT
iii) the transfer of cholesteryl esters to other plasma lipoproteins via cholesterol ester transfer protein (CETP)
iv) the delivery of the cholesteryl esters to the liver

In the presence of lipoproteins, free cholesterol is distributed in endocytotic vesicles within the Golgi network and transient lipid domains in the plasma membrane which mediate the transfer of cholesterol to extracellular acceptors [reviewed by Fielding & Fielding, 1995; Fielding & Fielding, 1997]. This transfer of cholesterol is thought to be mediated by caveolae, which increase relative to the rate at which cholesterol is

delivered to the plasma membrane [Smart *et al*, 1996]. At present lipid-poor preβ-HDL particles, known to be present in human follicular fluid [Jaspard *et al*, 1996], are thought to be the primary acceptors of cholesterol in RCT [Barrans *et al*, 1996].

1.5 Second Messenger Pathways Involved in the Regulation of CL Function

The receptors that bind LH and FSH are coupled to G-proteins. These in turn are coupled to an effector, thus generating intracellular signals via second messengers. Receptors associated with G-proteins share a common tertiary structure, comprised of seven hydrophobic trans-membrane domains. The N-terminus of the receptor is located on the outside of the cell membrane, whereas the C-terminus is found in the cell cytoplasm [reviewed by Spiegel *et al*, 1992; Devivo & Iyengar, 1994]. G-proteins are hetero-trimers comprised of three subunits, defined as α, β, and γ. Within the α-subunit, a guanine nucleotide-binding region has been characterised. In the resting state the three subunits are associated as one complex, with guanidine diphosphate (GDP) occupying the specific region on the α-subunit. Following binding of a hormone/ligand to the receptor, the G-protein complex is activated resulting in the displacement of GDP by guanine triphosphate (GTP). This displacement results in the dissociation of the GTP-bound α-subunit from the βγ heterodimeric subunit, and the subsequent association of the α-GTP subunit to the effector, which in turn mediates the generation of a second messenger. The intrinsic GTPase activity of the α-subunit hydrolyses the GTP to GDP on the α-subunit which then reassociates with the βγ subunit returning the G-protein complex to its resting state [Devivo & Iyengar, 1994; Birnbaumer & Birnbaumer, 1995].

1.5.1 Cyclic adenosine-3',5'-monophosphate-dependent protein kinase A pathway

The cAMP stimulated protein kinase A (PKA; EC: 2.7.10) pathway is the major signalling system stimulated by LH in ovarian tissues [Cooke, 1999; Richards, 2001] (Figure 1.11). Cyclic AMP is generated by the integral membrane protein adenylyl cyclase (AC) which catalyses the hydrolysis of ATP into cAMP and pyrophosphate, and promotes the cyclation of the remaining phosphate group to the adenosine ring [Della-Fazia *et al*, 1997]. Once formed, cAMP exerts its actions by activating cytoplasmic

PKA [Lalli & Sassone-Corsi, 1994]. Cyclic AMP is subsequently hydrolysed by a cyclic nucleotide phosphodiesterase (PDE), which converts cAMP into its inert 5'AMP derivative. Several isozymes of cAMP phosphodiesterase exist, each with different affinities for cAMP [Strada *et al*, 1989].

PKA exists is a tetrameric protein, and consists of two catalytic (C) subunits and two regulatory (R) subunits [Nishizuka *et al*, 1986; Nishizuka *et al*, 1992]. To date, four types of PKA regulatory subunits and three PKA catalytic subunits have been described [Nishizuka *et al*, 1992] allowing the possible formation of 12 PKA holoenzymes. The R subunits function as a "pseudosubstrate" obscuring the active sites of the C subunits. Binding of one cAMP molecule to each of the R subunits results in their dissociation and migration to cellular compartments, where they phosphorylate cytoplasmic and nuclear substrates [reviewed by Tamai *et al*, 1997]. This results in the activation of transcriptional factors including the cAMP-response element binding (CREB) protein [Hagiwara *et al*, 1993; Delmas & Sassone-Corsi, 1994; Della -Fazia *et al*, 1997] and CCAAT/enhancer-binding protein (C/EBP) [Piontkewitz *et al*, 1993]. CREB interacts with the cAMP responsive element (CRE) found in the promoters of several cAMP-responsive genes, to promote transcription of the enzymes required for steroidogenesis [Simpson *et al*, 1990; Tamai *et al*, 1997]. To date there are two C/EBP genes (C/EBP-α and C/EBP-β) that are expressed both in the ovary and other tissues [Sirois & Richards, 1993]. Furthermore, C/EBP-β has been shown to regulate gonadotrophin action [Sterneck *et al*, 1997].

The activation of LH receptor is the major signalling pathway for the stimulation of ovarian progesterone synthesis in most species. LH and hCG exert stimulatory effects on progesterone synthesis by activating AC and increasing cAMP levels in luteal tissues of the human [Soto *et al*, 1986; Yong *et al*, 1992; Davis, 1994; Furger *et al*, 1996; Azhar *et al*, 1998a] and non-primates [*e.g.* Davis *et al*, 1986; Allen *et al*, 1988]. The involvement of cAMP in mediating LH and hCG action in the production of luteal progesterone production was demonstrated by stimulation of progesterone concentrations with dibutyryl cAMP, a cell permeable cAMP analogue, and the AC

activator, forskolin [Golos *et al*, 1986; Golos *et al*, 1987a; Reaven *et al*, 1995; Azhar *et al*, 1998a]. Other signalling pathways activated by LH include the phospholipases C (PLC) and D (PLD), and the protein kinase C (PKC) pathways. Since these non-cAMP pathways may also mediate the actions of LH (and other hormones) on luteal progesterone synthesis, these pathways shall be discussed briefly in sections 1.5.2, 1.5.3 & 1.5.4.

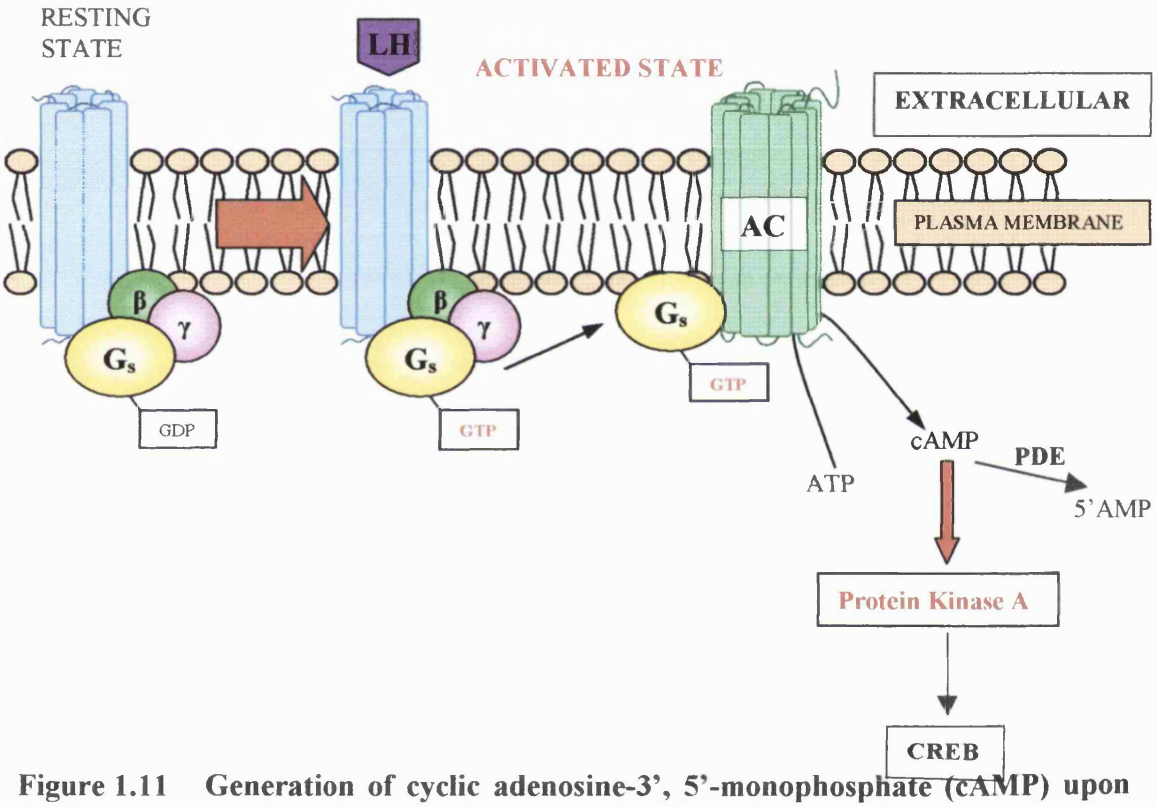

Figure 1.11 Generation of cyclic adenosine-3', 5'-monophosphate (cAMP) upon ligand binding and G-protein activation

1.5.2 Phospholipase C pathway

Activation of PLC (EC:3.1.4.3) results in the generation of multiple second messengers and activation of multiple signal transduction pathways [reviewed by Steele & Leung, 1993; Leung & Wang, 1989]. The inositol 1,4,5-trisphosphate/diacylglycerol second messenger system is an example of such a signal transduction pathway. Activation of a

specific cell surface receptor promotes rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate. The pivotal enzyme for this second messenger system is phosphoinositide-specific PLC which hydrolyzes the sn-3 phosphodiester bond of the phosphatidylinositides [phosphatidylinositol (PI), phosphatidylinositol-4-monophosphate (PIP_1) and phosphatidylinositol-4,5-bisphosphate (PIP_2)], to generate diacylglycerol (DAG) and inositol polyphosphates [inositol-1-monophosphate (IP_1), inositol-1,4-bisphosphate (IP_2) and inositol-1,4,5-trisphosphate (IP_3) respectively]. Purification, characterisation and cDNA cloning of PLC has revealed three PI-specific PLC isoenzymes (PLC-β, PLC-γ and PLC-δ) [reviewed by Rhee & Choi, 1986]. However, not all isozymes are phosphoinositide-specific [Crooke & Bennett, 1989].

The D enantiomer of inositol-1,4,5-P_3 binds to specific subcellular receptors resulting in the release of calcium (Ca^{2+}) ions from the intracellular calcisomes [Taylor *et al*, 1992] (Figure 1.12). Intracellular Ca^{2+} concentrations are also increased by the opening of integral membrane calcium channels [*e.g.* by inositol-1,3,4,5-tetrakis phosphate (IP_4)], and influx of Ca^{2+} ions into the cells [Berridge *et al*, 1987; Taylor *et al*, 1992]. The mobilisation of calcium stores is a signal for many cellular responses, including the activation of protein kinase C (PKC, EC:2.7.1.) and other calcium-dependent kinases. DAG stimulates the translocation of PKC from the cytosol to the membrane, and stimulates PKC activity by increasing its affinity for Ca^{2+} [Berridge *et al*, 1987; Leung & Wang, 1989]. Therefore, stimulation of phosphoinositide hydrolysis activates PKC first by increasing intracellular Ca^{2+} concentrations and secondly by increases in DAG levels. Hence both DAG and Ca^{2+} work in a co-operative way to stimulate PKC activity which subsequently phosphorylates and activates cellular proteins. DAG may also be hydrolysed by DAG lipase resulting in the generation of AA (section 1.3).

Binding of LH to receptors in the ovary has been shown to stimulate PLC activity [Leung & Wang, 1989]. Radiolabelled phospholipids, phosphatidic acid and PI have been incorporated into luteal cells following gonadotrophin stimulation [Leung *et al*, 1986], with LH-induced release of intracellular calcium [Davis *et al*, 1986; Wang *et al*,

1989]. The addition of calcium ionophores to granulosa cells mimics the effect of LH on steroidogenesis [Leung *et al*, 1988].

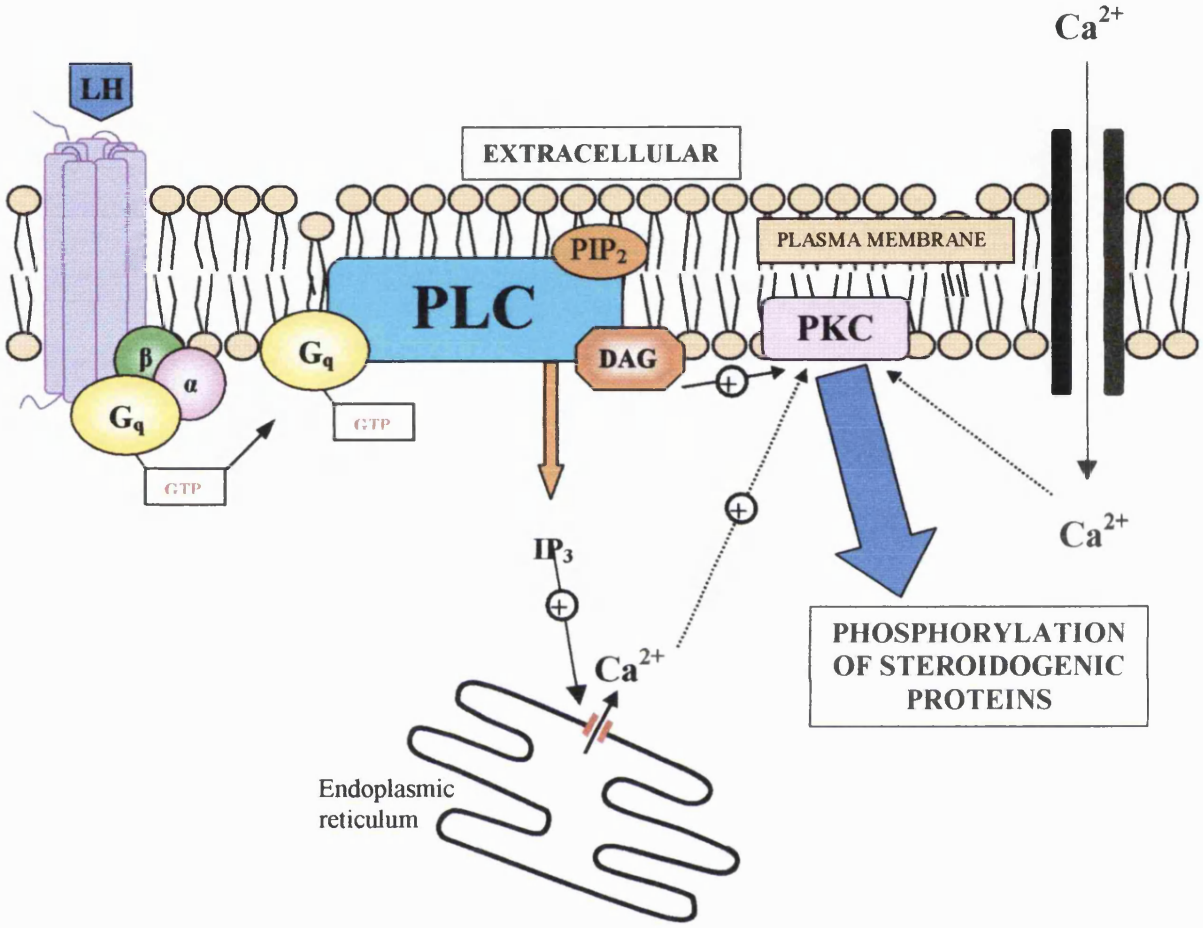

Figure 1.12 Schematic representation of the activated PLC and Protein Kinase C pathways

1.5.3 Calcium dependent-protein kinase C (PKC) pathway

There are at least eleven isoforms of PKC which vary in their requirement for phospholipids, DAG and Ca^{2+} for activation, their substrate specificity, and their end biological response [Nishizuka *et al*, 1986; Nishizuka *et al*, 1992; Liu & Simon, 1996; Breuiller-Fouche *et al*, 1998]. The conventional PKC isoforms (α, βI, βII and γ), are activated by DAG, which increases their affinity for intracellular Ca^{2+}. Both IP_3 and DAG are required for the physiological response of conventional PKC isoforms, which

are active only in the presence of Ca^{2+} and the co-factor phosphatidylserine. However, the novel PKC isoforms (δ, ε, η, θ and μ) are Ca^{2+}-independent [Nishkawa *et al*, 1995], and the activities of the atypical isoforms (λ, ξ and τ), are both DAG and Ca^{2+}-independent [Hussain & Abdel-Latif, 1996].

In its inactive state, PKC resides in the cytosol with its active site occupied by a pseudosubstrate region of the regulatory domain. Calcium ions bind the regulatory domain such that the enzyme translocates to the membrane where it interacts with phosphatidylserine and undergoes a conformational change, which exposes the active site to intracellular proteins [Huang *et al*, 1986]. The distribution of PKC between the cytosol and membrane is thus thought to be a measure of PKC activity [Kraft & Anderson, 1983; Nishizuka *et al*, 1986]. If PKC resides at the membrane for prolonged periods, then the enzyme becomes irreversibly down-regulated, due to activation of proteases which digest the enzyme protein [Huang *et al*, 1989].

PKC has been measured in the ovine CL [Belfiore *et al*, 1994], although its role in ovarian steroidogenesis is not fully understood. The DAG-sensitive isoforms of PKC can be activated by tumour-promoting phorbol esters, which show physical homology to DAG and exert their biological action by binding to DAG-sensitive regulatory region of PKC, increasing the enzyme affinity for Ca^{2+}, leading to enzyme activation. Phorbol esters, such as 4β-phorbol 12-myristate 13-acetate (4β-PMA), have been used acutely to stimulate PKC activity with subsequent inhibition of basal and LH-stimulated progesterone production in luteinized cells of the human [Jalkanen *et al*, 1987; Abayasekara *et al*, 1993a] pig [Wiltbank *et al*, 1989; Wheeler & Veldhuis, 1989], rat [Sender Baum & Rosenberg, 1987] and cow [Benhaim *et al*, 1987]. However, chronic exposure of human luteal cells to 4β-PMA has been shown to down-regulate PKC, with a 90% reduction in the enzyme activity [Abayasekara *et al*, 1993b], and a subsequent increase in basal progesterone synthesis. Low doses of 4β-PMA can enhance LH-stimulated cAMP accumulation and progesterone synthesis, suggesting that stimulants such as 4β-PMA, hCG, cAMP analogues and forskolin share a common signalling protein, stimulated by both PKA and PKC [Birnbaumer & Birnbaumer, 1995].

1.5.4 Phospholipase D pathway

Phospholipase D (PLD, EC: 3.1.4.4) is a phosphodiesterase that hydrolyses the terminal phosphodiester bond of membrane phospholipids, generating phosphatidic acid (PA) and the polar head group alcohol. The principal phospholipid hydrolysed by PLD is phosphatidylcholine, cleaved to generate PA and choline. However phosphatidylinositol and phosphatidylethanolamine may also be hydrolysed. The liberation of PA and its subsequent conversion to DAG implicates the PLD pathway in the activation of PKC. PA has also been shown to act as a calcium ionophore, and as well as activating PKC, may be a potential regulator of intracellular Ca^{2+} [Shukla & Halenda, 1991].

The involvement of PLD in the regulation of luteal steroidogenesis is unclear. However GnRH stimulates PLD activity in the ovary [Liscvovitch & Amsterdam, 1989; Amsterdam *et al*, 1994]. Moreover, PA mimics the effect of GnRH, and neither PLD nor GnRH antagonists differ in their stimulatory effects on progesterone synthesis by luteal cells.

1.5.5 Phospholipase A_2 pathway; Arachidonic acid metabolites

As previously described, AA is released by the action of PLA_2 or other lipases (section 1.3) and provides substrate for three subsequent eicosanoid pathways [Lapetine *et al*, 1989]:

i) Cyclooxygenase pathway (section 1.3.1)

ii) Lipoxygenase pathway (section 1.3.2)

iii) cytochrome P450 epoxygenase pathway

The oxygenation of AA by the prostaglandin H synthases results in the formation of thromboxane, prostacyclin and a variety of prostaglandins (1.3.1). Aspirin and indomethacin inhibit prostaglandin synthesis by acetylation of PGHS and are used extensively in studies of AA mediated intracellular signalling. The oxidation of AA yields hydroperoxy metabolites which are rapidly metabolised to hydroxy analogues,

like leukotrienes which are involved in intracellular signalling [Puustinen *et al*, 1987] (section 1.3.2).

In the ovary there is evidence suggesting increased PLA_2 activity in the signal transduction pathways involved with an accumulation of AA in rat granulosa cells [Minegishi & Leung, 1985] and luteal tissue [Watanabe *et al*, 1990; Nomura *et al*, 1994]. Moreover, the actions of GnRH in luteal cell cultures are mimicked by both AA and PLA_2 [Wang *et al*, 1989]. The action of $PGF_{2\alpha}$ is thought to occur via an accumulation of AA [Watanabe *et al*, 1990] which may be generated by either the PLC or PLA_2 pathways. Co-operative coupling of the two pathways to the $PGF_{2\alpha}$ receptor has been suggested by the accumulation of both AA and IP_3 in luteal cells [Watanabe *et al*, 1990].

The inhibition of luteal progesterone production using the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) suggests that the products of lipoxygenase-mediated AA metabolism stimulate luteal progesterone production in the human [Feldman *et al*, 1986], rat [Reich *et al*, 1983; Mikuni *et al*, 1998], and cow [Milvae *et al*, 1986].

1.6 Paracrine Control of Luteal Function by Prostaglandins

The production of progesterone in luteal tissue is controlled by different hormones, which can be broadly categorised into two groups, the luteotrophins and the luteolysins. Their role is to either stimulate or inhibit progesterone production respectively. In doing so they control both luteal steroidogenesis [Hillier & Wickings, 1985; Richardson *et al*, 1986] and the functional lifespan of the CL. The principal luteotrophin is LH and several studies have demonstrated that its luteotrophic action is required for normal luteal function in both primates and non-primate species [Simmons & Hansel, 1964; Fuller & Hansel, 1970; Van de Wiele *et al*, 1970; Moudgal *et al*, 1972; Hutchinson & Zeleznik, 1984; Hutchinson & Zeleznik, 1985]. While LH appears to be the main endocrine stimulus in primates, non-gonadotrophic luteotrophins include prolactin (PRL), oestradiol, growth factors and prostaglandin E_2 (PGE_2) [Richardson, 1986]. In the absence of PRL, LH is not sufficient to sustain the survival of the rat CL. However,

LH binding is up regulated in the pregnant rat CL by PRL, and PRL related hormones [Grinwich *et al*, 1976; Gibori *et al*, 1978], thus emphasising the requirement of PRL for luteal steroidogenesis and function.

A luteotrophic role for PGE_2 has been described in the human [Dennefors *et al*, 1982; Hahlin *et al*, 1988] and bovine [Shelton *et al*, 1990; Tsai *et al*, 1996; Sharif *et al*, 1998] CL. Moreover, luteotrophic actions of PGE_2 have been demonstrated in luteinized granulosa cells [McNatty *et al*, 1975; Veldius *et al*, 1987] and luteal cells of the rhesus macaque [Stouffer *et al*, 1979], rat [Thomas *et al*, 1978] and hamster [Yang *et al*, 1998].

1.6.1 The role of cAMP in PGE_2 action

The cellular mechanisms underlying the luteotrophic effect of PGE_2 have been investigated. A number of studies have shown that stimulation of progesterone production by PGE_2 is associated with a significant increase in cAMP accumulation. More specifically, *in vitro* studies have demonstrated an increase in both cAMP accumulation and progesterone production in response to PGE_2 both in the human [Hamberger *et al*, 1979; Dennefors *et al*, 1982; Hahlin *et al*, 1988] and non-human primate CL [Molskness *et al*, 1987; Webley *et al*, 1989]. This increase in cAMP accumulation is thought to reflect stimulation of AC (as opposed to decreased PDE activity) [Rojas *et al*, 1989; Davis *et al*, 1989; Michael *et al*, 1993a].

1.6.2 EP receptors

EP receptors mediate an impressive range of biological activities, including contraction and relaxation of smooth muscle, inhibition and enhancement of neurotransmitter release, inhibition of lipolysis, and inhibition of inflammatory mediator release [reviewed by Coleman *et al*, 1990]. EP receptors are expressed both in the rat and human endometrium [Kennedy *et al*, 1983; Hofmann *et al*, 1985; Adelantado *et al*, 1998] as well as the human myometrium during the menstrual cycle, pregnancy and labour [Giannopoulos *et al*, 1985; Senior *et al*, 1993; Adelantado *et al*, 1998]. Furthermore, recent studies have suggested that EP receptors in the rabbit CL decrease as the CL ages [Boiti *et al*, 2001]. The fact that there are PGE_2 binding sites in diverse

tissues implicated in opposite biological actions suggested the existence of more than one receptor subtype. To date there are four subtypes of EP receptors that have been cloned (EP_1, EP_2, EP_3 & EP_4) [Hirata *et al*, 1991; Sugimoto *et al*, 1992; Honda *et al*, 1993; Funk, *et al*, 1993], with multiple isoforms of the EP_3 receptor subtype [reviewed by Coleman *et al*, 1994]. All of the cloned EP receptors have been shown to be seven transmembrane domain, G-protein coupled receptors, linked to AC or PLC, IP_3 pathway [Probst *et al*, 1992]. These receptors have different affinities for PGE_2 and structural analogues. Hence, receptor subtype-specific agonists and antagonists can be used to delineate the role of particular EP receptor subtypes in a given cellular response [Coleman *et al*, 1994].

1.6.2.1 EP_1 receptors

The EP_1 receptor consists of 405 aa [Sugimoto *et al*, 1992]. EP_1 receptors have been localised in smooth muscle of guinea pig (trachea, gastrointestinal tract, uterus and bladder) where they mediate muscle contraction. They are also present in human myometrium and are thought to mediate myometrial contractility [Senior *et al*, 1991; Senior *et al*, 1993]. However, expression of EP_1 is generally low compared with other EP receptors. Their expression in COS cells has revealed that they have a high affinity for ^{3}[H]-PGE_2 (K_d=1nM) [Coleman *et al*, 1994]. The EP_1 receptors appear to mobilise Ca^{2+} concentrations from intracellular stores [Watanabe *et al*, 1993]. However, it has not yet been established whether IP_3 is involved in mediating EP_1 receptor responses.

1.6.2.2 EP_2 receptors

EP_2 receptors consist of 513 amino acids [Sugimoto *et al*, 1992]. They are quite widespread and the responses mediated are quite varied. These prostanoid receptors are widely distributed in smooth muscle, where they mediate relaxation. Recent studies have also demonstrated that EP_2 receptors are expressed in rat endometrium [Pappay *et al*, 2000] and baboon myometrium and cervix during labour [Smith *et al*, 1999]. Binding studies of recombinant human EP_2 receptors have demonstrated K_d values ranging from 2 to 11 nM [Ann *et al*, 1993]. Studies performed in rabbit kidney have

shown that cAMP is elevated in response to PGE_2 [Sonnenburg & Smith, 1988]. This implies that EP_2 receptors are coupled to AC presumably through G_s.

The EP_2 receptor has been extensively implicated with fertility and parturition. Female mice lacking the gene encoding the EP_2 receptor exhibit reduced fertility which is attributable to reduction in ovulation and predominantly attributable to severe failure of fertilisation [Kennedy *et al*, 1999]. The EP_2 receptor is implicated in the completion of fertilisation competent eggs [Hizaki *et al*, 1999]. Furthermore, EP_2 receptors are also important for the establishment of the microenvironment required for successful fertilisation of the released oocyte [Tilley *et al*, 1999]. Recent studies have confirmed previous observations by Tilley *et al*, (1999) and have suggested that *in vivo* PGE_2 acts through the EP_2 receptor on the cumulus granulosa cells, which in turn support the oocyte, the oviduct, and/or the spermatozoa for successful fertilisation [Elvin *et al*, 2000].

1.6.2.3 EP_3 receptors

The EP_3 receptors consist of 365 amino acids [Sugimoto *et al*, 1992], and are present in smooth muscle of the gastrointestinal tract and the uterus, where they mediate contraction [Goureau *et al*, 1992; Coleman *et al*, 1987]. Furthermore they have been localised to gastric mucosal cells [Reeves *et al*, 1988] and human platelets [Jones & Wilson, 1990], and are highly expressed in the uterus, kidney, stomach, spleen, lung and brain [Sugimoto *et al*, 1992].

Ligand-binding studies performed in the human myometrium have shown that EP_3 receptors have high affinity for their ligands (K_d<10nM) [Schillinger *et al*, 1979]. Human EP_3 receptors have been expressed in COS cells and have been found to have K_d values ranging from 0.3 to 6.6 nM [Adam *et al*, 1994].

To date there are four cloned EP_3 isoforms. Studies performed by Namba (1993) have shown that four EP_3 isoforms are expressed in bovine adrenal cells. These have been designated as EP_{3A}, EP_{3B}, EP_{3C} and EP_{3D} and are linked to different second messenger

pathways. More specifically, the EP_{3A} isoform is thought to be coupled to G_i, to induce inhibition of AC, whereas the EP_{3B} and EP_{3C} isoforms have been found to couple to G_s, to increase cAMP. Finally EP_{3D} receptors is coupled to G_q, in addition to G_i and G_s causing both stimulation and inhibition of cAMP and increasing PI turnover [Narumiya *et al*, 1999]. Namba *et al* (1993) demonstrated that in the bovine adrenal, EP_3 mRNA undergoes alternative splicing to produce the four mentioned EP_3 isoforms. However, the presence of EP_3 isoforms, has not only been demonstrated in the cow. Three EP_3 isoforms have been cloned in human, designated as hEP_3-I, hEP_3-II and hEP_3-III [Adam *et al*, 1994].

1.6.2.4 EP_4 receptors

The EP_4 receptors have only recently been identified [Coleman *et al*, 1994], hence very little is known about their characteristics and modes of action. Like all the other EP receptors, they are 7-transmembrane domain receptors, coupled to G-proteins. They have recently been cloned and are expressed in the rabbit kidney cortex [Breyer *et al*, 1996] and in baboon chorion [Smith *et al*, 2001]. These receptors act in the myometrium through AC and cAMP [Senior *et al*, 1993]. This can be further supported by recent findings in baboon, where EP_4 receptors coupled to AC mediating an effect which causes inhibition of receptor expression in chorion thus promoting parturition [Smith *et al*, 2001].

1.6.3 Luteolytic actions of $PGF_{2\alpha}$

In non-primates, the physiological luteolysin synthesised and secreted from the uterus is prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) [reviewed by Horton & Poyser, 1976; Auletta & Flint, 1988]. In support of this theory, the administration of $PGF_{2\alpha}$ antiserum in cycling ewes disrupts the luteolytic process [Scaramuzzi *et al*, 1973; Fairclough *et al*, 1981]. Moreover, administration of $PGF_{2\alpha}$ analogues induces luteolysis in the rat [Jordan *et al*, 1981] and sheep [Flint & Sheldrick, 1985]. However, in women and non-human primates the luteolytic mechanism is poorly understood. Both in women [Neill *et al*, 1969] and in the rhesus macaque [Beiling *et al*, 1970] hysterectomy failed to affect luteal function which suggests that if a luteolysin is responsible for the demise of the primate CL, such a

hormone cannot originate from the uterus. Instead it has been suggested that luteal regression in the primate requires a luteolytic agent originating from the ovary and possibly from the CL itself, acting in a paracrine/autocrine manner [Beiling *et al*, 1970; Rothchild, 1981; Auletta & Flint, 1988]. To date the identity of such an agent remains unclear. Since $PGF_{2\alpha}$ is synthesised in the primate ovary [Challis *et al*, 1976; Swanston *et al*, 1977; Patwardhan & Lanthier, 1985; Houmard & Ottobre, 1989] it is therefore regarded as the best candidate for this luteolytic effect [Auletta *et al*, 1984]. In support of this theory, administration of $PGF_{2\alpha}$ has been shown to induce transient [Wentz *et al*, 1973; Coudert *et al*, 1974] and permanent luteal regression [Auletta *et al*, 1984; Summers *et al*, 1985] in primates. In contrast to the luteolytic effects of $PGF_{2\alpha}$ in non-human primates, infusion of analogues of $PGF_{2\alpha}$, had no effect on circulating progesterone levels in women [LeMaire & Shapiro, 1972; Karim & Hillier, 1979; Hamberger *et al*, 1980]. However, rapid metabolism of $PGF_{2\alpha}$ to its inactive metabolites may be the reason for such observations. Hence, the luteolytic actions of $PGF_{2\alpha}$ may be one mechanism by which the CL functionally regresses.

1.6.3.1 The role of calcium/PKC in $PGF_{2\alpha}$ action

Even though the luteolytic effects of $PGF_{2\alpha}$ have been well documented [Auletta & Flint, 1988], its precise mode of action is still not completely understood. Even though, the cell surface receptor for $PGF_{2\alpha}$ has been identified (section 1.6.3.2) in the human ovary the second messenger signals utilised by $PGF_{2\alpha}$ are still unknown. $PGF_{2\alpha}$ activates PLC which generates IP_3 and transiently increases intracellular Ca^{2+} in cultured human granulosa cells [Davis *et al*, 1989; Currie *et al*, 1992]. $PGF_{2\alpha}$ can also induce cellular redistribution of PKC activity from the cytosol to the membrane in rat [Veldhuis, 1987] and pig [Musicki *et al*, 1990] luteal cells. Recent studies have demonstrated that the antigonadotrophic action of $PGF_{2\alpha}$ and cloprostenol are mediated via PKC [Abayasekara *et al*, 1993a; Abayasekara *et al*, 1993b] in human granulosa-lutein cells. A proposed mechanism of this antigonadotrophic action of $PGF_{2\alpha}$ is thought to be through an increase in intracellular Ca^{2+} which activates PKC and cAMP phosphodiesterase which in turn inhibit LH-stimulated AC activity and steroidogenesis in luteal cells [Michael & Webley, 1991; Michael & Cooke, 1994].

1.6.3.2 FP receptors

The cDNA for the FP receptor has been isolated from mouse ovary [Sugimoto *et al*, 1994]. This prostanoid receptor appears to be a seven transmembrane domain protein of 366 amino acids, homologous to other prostanoid receptors (*e.g.* mouse TP and EP_1 receptor). To date there are two FP receptor subtypes termed FP_A and FP_B [Fujino *et al*, 2000]. FP receptors are expressed in a variety of different tissues from a range of different species [reviewed by Narumiya *et al*, 1999]. One tissue in which they are particularly prevalent is the CL where they may mediate luteolysis [Behrman *et al*, 1985], however, FP receptors have also been identified in human GCs [Carrasco *et al*, 1997; Narko *et al*, 1997; Ristimaki *et al*, 1997]. Indeed, FP receptors appear to be present in the corpora lutea of all species [*e.g.* Sugimoto *et al*, 1994; Abramovitz *et al*, 1994], including the human and their mRNA levels have been shown to be dependent upon the luteal steroid output [Rao *et al*, 1979; Feng *et al*, 1996]. In some rodents and in humans, there are contractile FP receptors on the myometrium [Whalley & White, 1980; Senior *et al*, 1992]. Moreover, FP receptor protein expression increases in the rabbit CL, as the CL age increases [Boiti *et al*, 2001] and mRNA expression occurs exclusively in the large luteal cells of the ovine CL [Tsai *et al*, 1998].

Ca^{2+} mobilisation in conjunction with formation of IP_3 is associated with the FP receptor pathway [Raymond *et al*, 1983; Behrman *et al*, 1985]. This suggests that FP receptors can interact with a member of the G_q protein family to activate PLC. Moreover, the number of $PGF_{2\alpha}$ binding sites, is regulated by luteotrophic agents (*e.g.* gonadotrophins and cAMP analogues) or agents that inhibit luteal progesterone production [Ristimaki *et al*, 1997; Juengel *et al*, 1996].

1.7 Significance of ovarian cortisol metabolism

1.7.1 Synthesis and action of glucocorticoids

The adrenal cortex synthesises and secretes glucocorticoids (cortisol & corticosterone) and mineralocorticoids (aldosterone) in response to adrenocorticotrophic hormone (ACTH). The precursor for all glucocorticoids is pregnenolone which is oxidised at C3,

resulting in a ketone and the isomerisation of the double bond and the formation of progesterone (section 1.2.3). A series of hydroxylations at C17, C21 and then C11 give rise to cortisol (Figure 1.13).

Glucocorticoid availability and transport depends on corticosteroid-binding-globulins (CBG). These specific plasma transport glycoproteins, bind C21 steroid hormones (cortisol, corticosterone & progesterone) with high affinity and play an important role in transporting these steroids and disposing them to target tissues, altering their concentrations in blood and influencing their biological action [Hammond, 1990; Rosner, 1990]. It is generally accepted that CBG-bound plasma cortisol in humans has a restricted access to target cells [Siiteri *et al*, 1982]. CBG are mainly produced by the liver in all species examined [Hammond *et al*, 1991] and mRNA has been detected in the human ovary [Misao *et al*, 1999a], placenta [Misao *et al*, 1999b] and CL [Misao *et al*, 1997]. Furthermore CL CBG levels of synthesis are thought to be regulated by steroid hormone levels [Misao *et al*, 1999c].

The glucocorticoids perform a broad range of metabolic and other functions that include many aspects of homeostasis and the response to stress. More specifically glucocorticoids have the ability to increase gluconeogenesis by inducing the enzymes associated with this pathway. Furthermore they stimulate muscle breakdown and the turnover of adipose tissue. Glucocorticoids are also involved in immune responses and cell differentiation [Brook & Marshall, 1996].

Identification of glucocorticoid receptors in a range of ovarian and testicular cell types has given strong reason to believe that glucocorticoids can directly affect gonadal tissue and may modulate the processes of gametogenesis. Indeed recent studies have demonstrated that glucocorticoids can exert direct effects on gonadal steroidogenesis both *in vivo* and *in vitro* [Evain *et al*, 1976; Fitzpatrick & Richards, 1991; Michael *et al*, 1993c; Kowalski & Chatterton, 1992]. Association between hypercortisolaemia and infertility may include direct suppressive effects at the level of the ovary and testis.

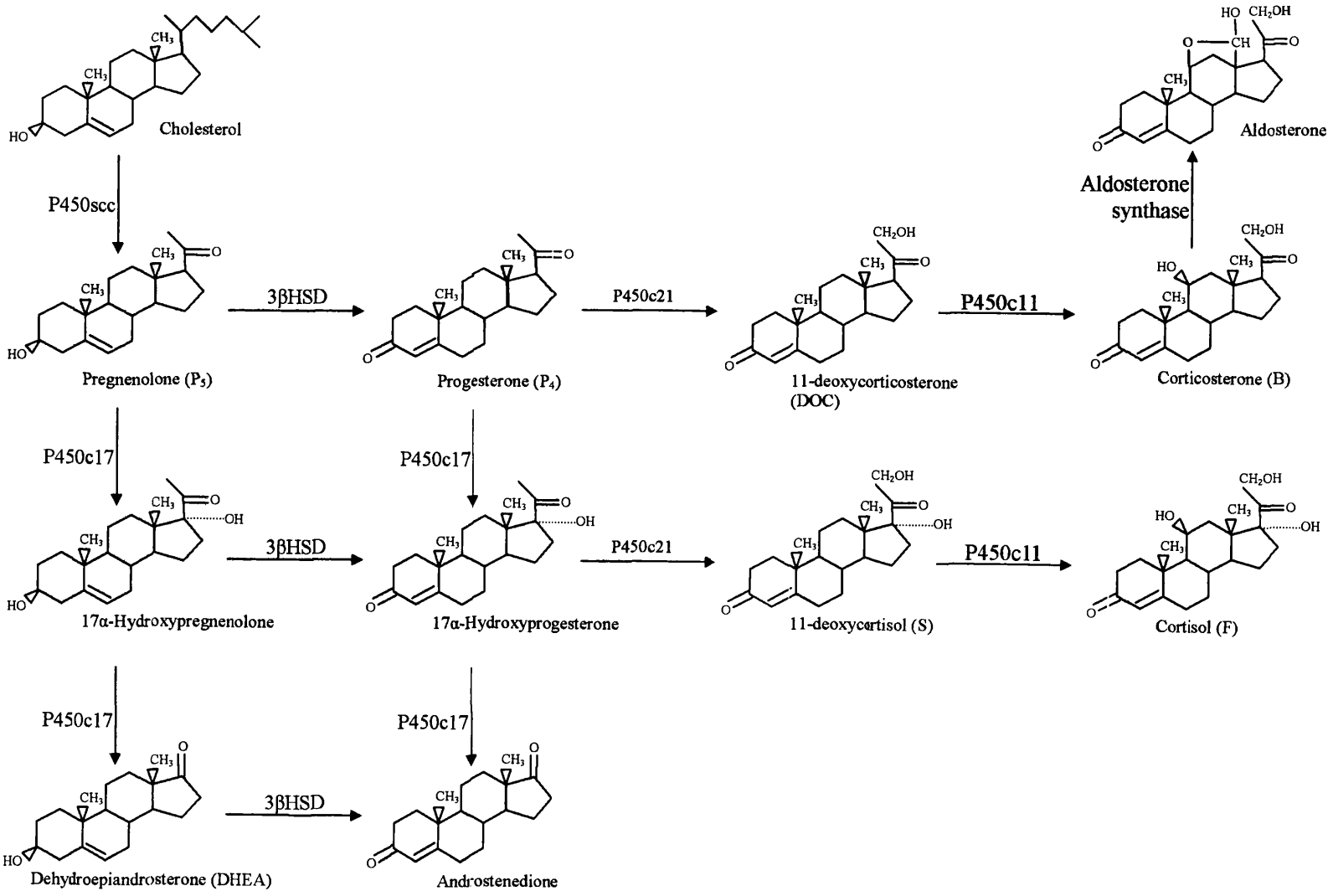

Figure 1.13 Principal pathways of corticosteroid biosynthesis

Glucocorticoids potentially affect gonadal function by acting in one or more of the following levels of the HPG axis:

i) the hypothalamus, to decrease the synthesis of GnRH [Fonda *et al*, 1984; Rosen *et al*, 1988].

ii) the anterior pituitary, to decrease the synthesis and release of LH and/or FSH [Sakakura *et al*, 1975; Brann *et al*, 1990]

iii) the ovary/testis, to modulate steroidogenesis and/or gametogenesis directly [Evain *et al*, 1976; Fitzpatrick & Richards, 1991; Michael *et al*, 1993c; Kowalski & Chatterton, 1992].

In granulosa cells, glucocorticoids may enhance FSH action during the follicular phase of the ovarian cycle but predominantly attenuate LH-stimulated steroidogenesis during the luteal phase. Furthermore, elevation of plasma cortisol concentrations following the administration of exogenous ACTH has no effects on the serum concentrations of oestradiol in the follicular phase but significantly suppresses progesterone concentrations in the luteal phase [Kowalski & Chatterton, 1992]. Very few studies have been performed regarding the effects of glucocorticoids on oocyte maturation. Those studies performed in fish have revealed a possible role for cortisol or corticosterone in promoting final maturation of the oocyte [Greeley *et al*, 1986; Patino & Thomas, 1990].

1.7.2 Corticosteroid receptors

Steroids are highly lipophilic compounds and can thus readily diffuse through the plasma membrane and enter most cells to access their intracellular receptors which are members of the nuclear-hormone-receptor superfamily [Evans, 1988; Funder *et al*, 1993] of ligand activated transcription factors. These receptors have four major domains:

i) ligand binding domain (LBD)

ii) dimerisation domain

iii) DNA binding domain (DBD)

iv) transactivating factor (TAF) domains

In the resting state, the receptors are located in the cytoplasm of the nucleus, associated with chaperone proteins. Once a steroid binds to the receptor, it undergoes a conformational change, resulting in the dissociation of some of the chaperone proteins. The activated ligand-receptor complex then undergoes "back to back" dimerisation which enables it to recognise and bind the response elements (via the zinc fingers of the DBD in each receptor) within the DNA [Bamberger *et al*, 1996] and regulate the DNA transcription rate.

There are 2 principal corticosteroid receptors, the "mineralocorticoid" (MR) and the glucocorticoid (GR) receptors. GR are expressed in virtually all tissues, but some tissues also express the higher affinity MR [Evans, 1988]. Both purified and recombinant MR bind cortisol, corticosterone and aldosterone with similar affinity *in vitro*. However, even though there is at least a 100-fold excess of "free" circulating glucocorticoid over mineralocorticoid, MR can selectively bind aldosterone *in vivo* [Edwards *et al*, 1988; Funder *et al*, 1988]. This specificity is conferred by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD). The enzyme rapidly oxidises, active cortisol, to its inert metabolite cortisone. By virtue of its 11-keto group, cortisone is unable to bind to MR, hence allowing aldosterone to bind and specifically regulate Na^+, K^+, H^+ and water balance.

In the rare genetic syndrome of apparent mineralocorticoid excess (type-I AME), congenital defects in 11β-HSD allow cortisol to illicitly bind and activate MR, causing antinatriuresis, hypokalaemia and hypervolemic hypertension [Stewart *et al*, 1988]. Similarly, if the renal metabolism of cortisol is inhibited by ingestion of such compounds as liquorice, glycyrrhetinic acid or carbonoxolone, the acquired form of AME (type-II AME) ensues with the same clinical symptoms. In both forms of AME, the patient presents with signs of hyperaldosteronism, despite suppressed plasma renin activity and normal/decreased plasma concentration of aldosterone [Stewart *et al*, 1987].

1.7.3 Corticosteroid metabolism by 11β-HSD

The tissue response to glucocorticoids is modulated not only by circulating concentrations of steroids and the cellular expression of their receptors, but also by the presence of 11β-HSD. Demonstration that glucocorticoids can exert direct effects on the functions of numerous unconventional target tissues including the gonads lead scientists to investigate the role of 11β-HSD (EC: 1.1.1.146), a microsomal enzyme belonging to the short-chain alcohol dehydrogenase (SCAD) superfamily, as an important determinant of glucocorticoid action [Krozowski *et al*, 1992]. 11β-HSD activity is of great importance in a number of tissues (including the brain [Seckl, 1997], liver [Whorwood *et al*, 1992], foetal [Stewart *et al*, 1994; Brown *et al*, 1996], placenta [Lopez-Bernal & Craft, 1981; Hofmann *et al*, 2001] and uterus [Waddell & Burton, 2000], as well as the gonads [Monder *et al*, 1994; Michael *et al*, 1993c]. This enzyme catalyses the interconversion of physiologically active glucocorticoids (cortisol & corticosterone) and their inert 11-keto metabolites [cortisone & 11-dehydrocorticosterone (11-DHC)] [Monder & White, 1993; Krozowski *et al*, 1999].

As previously mentioned, cortisol can exert direct effects on gonadal steroidogenesis both *in vivo* and *in vitro* [1.7.1]. Indeed studies performed by Michael *et al* (1994) demonstrated that human granulosa-lutein cells express both isoforms of 11β-HSD suggesting that the enzyme activities may play an important role in regulating glucocorticoid metabolism in the ovary.

1.7.3.1 Cloned isoforms of 11β-HSD

Biochemical and molecular studies have demonstrated that 11β-HSD exists in at least two distinct isoforms, type1 11β-HSD (11β-HSD1) and type2 11β-HSD (11β-HSD2) [Smith *et al*, 1997; Monder *et al*, 1993; Stewart *et al*, 1999; Albiston *et al*, 1994; Agarwal *et* al, 1994], of which the hepatic type1 isoform is best characterised. The two isoforms of the enzyme differ in their actions and co-factor requirements and their genes share only 14% sequence identity [Albiston *et al*, 1994].

11β-HSD1 is expressed in a wide range of tissues including the gonads [Agarwal *et al*, 1989; Krozowski *et al*, 1990; Whorwood *et al*, 1992]. Although the precise physiological role of 11β-HSD1 is currently unclear it has been suggested to play a role in maintaining expression of glucocorticoid-regulated genes [Jamieson *et al*, 1999]. This isozyme is bi-directional, preferentially utilises NADP(H) as a cofactor and has a low binding affinity for the active glucocorticoids cortisol and corticosterone (K_m for cortisol≈27μM; K_m for corticosterone≈2μM) [Lakshmi & Monder, 1988; Stewart & Mason, 1995]. Furthermore, there has been great controversy regarding the preferential direction of 11β-HSD1. Although 11β-HSD1 can catalyse $NADP^+$-dependent inactivation of glucocorticoids in cell homogenates, this enzyme appears to act predominantly as an 11-ketosteroid reductase (to generate cortisol from cortisone) in intact cells. This applies in primary cultures of cells from liver [Jamieson *et al*, 1995], adipose tissue [Bujalska *et al*, 1997], lung [Hundertmark *et al*, 1995], CNS [Rajan *et al*, 1996] and vascular smooth muscle cells [Brem *et al*, 1995] which suggests that reactivation of glucocorticoids occurs.

In contrast 11β-HSD2, acts predominantly as a high affinity 11β-dehydrogenase thus converting active cortisol (in human) and corticosterone (in rat) to their inert metabolites, cortisone and 11-DHC respectively [Albiston *et al*, 1994; Stewart *et al*, 1994; Brown *et al*, 1996]. This isoform shows an absolute requirement for NAD^+ as a cofactor [Rundle *et al*, 1989; Mercer & Krozowski, 1992] and its affinity for cortisol and corticosterone are far higher (K_m=40 and 26nM respectively) than those observed for 11β-HSD1. 11β-HSD2 is susceptible to inhibition not only by derivatives of glycyrrhetinic acid (*e.g.* carbenoxolone) but also by the end products of 11β-dehydrogenase action (*i.e.* cortisone and 11-dehydrocorticosterone) [Albiston *et al*, 1994]. 11β-HSD2 has recently been cloned and sequenced [Albiston *et al*, 1994; Agarwal *et al*, 1994] and is expressed in tissues other than the kidney, including the human ovary [Albiston *et al*, 1994]. Furthermore, 11β-HSD2 is also highly expressed in human placenta [Murphy *et al*, 1974] and in many foetal tissues [Stewart *et al*, 1994; Brown *et al*, 1996] suggesting that it may serve a role as a placenta "barrier"

[Benediktsson *et al*, 1997] in providing the foetus with a low glucocorticoid environment for most of pregnancy.

In human granulosa cells, both 11β-HSD isoforms are thought to be expressed which reversibly interconvert glucocorticoids [Michael *et al*, 1997]. Studies performed in the laboratories of SG Hillier have shown that in the preovulatory follicle, granulosa cells (GCs) mainly express 11β-HSD2 mRNA with little of no expression of 11β-HSD1. At the time of follicular rupture, in GCs, both the dehydrogenase and oxoreductase reactions seem to be catalysed by 11β-HSD1 [Thomas *et al*, 1998]. Following ovulation 11β-HSD2 mRNA expression is suppressed and at the same time 11β-HSD1 is greatly enhanced [Tetsuka *et al*, 1997 & others]. This suggests that in the follicle glucocorticoid oxidation should prevail whereas in the CL it would be expect for glucocorticoid reduction to predominate. In rat granulosa cells, LH is able to stimulate 11β-HSD1 mRNA expression directly, in the absence of theca cells [Hillier & Tetsuka, 1998].

1.7.3.2. *In vitro* fertilisation outcome and 11β-HSD

11β-HSD activity has been localised in the ovary, however its function has yet to be fully understood. In human granulosa-lutein cells (hGLC) both 11β-HSD activities exist and their relative activities may be a local determinant of intrafollicular glucocorticoid exposure [Albiston *et al*, 1994; Smith *et al*, 1997]. However, the 11β-dehydrogenase activity predominates and correlates negatively with success of *in vitro* fertilisation-embryo transfer (IVF-ET) [Michael *et al*, 1995]. They reported that in granulosa cells obtained from patients undergoing IVF-ET, 11β-HSD activities were detectable in 2/3 of the patients studied (137 patients in total) and none became pregnant. Of the remaining 1/3 of patients studied, ≈60% of them became pregnant coinciding with low 11β-HSD activity. It was therefore proposed that 11β-HSD activity in cultured GCs, can be used in patients to predict IVF outcome.

1.7.3.3. Prostaglandins and 11β-HSD

Measurements of free cortisol in human follicular fluid have demonstrated that after the onset of the LH surge, dramatic increases in free and total cortisol were detected

[Harlow *et al*, 1997]. It is hence suggested that differential regulation of 11β-HSD gene expression before follicular rupture may be the means by which the intrafollicular concentrations of cortisol increase before ovulation. High glucocorticoid concentrations inhibit most immunological responses and are well-known anti-inflammatory agents. It is thus likely that they participate in uterine events, such as menstruation, implantation, cervical softening and parturition, all of which are inflammatory situations [Kelly *et al*, 1996]. Prostaglandins are also known participants in inflammatory situations. However, unlike glucocorticoids they induce inflammation rather than suppress it. Since glucocorticoids and PGs have opposing actions, their effects may be inter-linked.

PGs play a key role in the initiation of labour by enhancing uterine contractility [Challis *et al*, 1997; O'Brien, 1995]. Furthermore, the activity of 11β-HSD1 is increased by both PGE_2 and $PGF_{2\alpha}$ in cultured chorionic trophoblast cells [Sun *et al*, 1997; Patel *et al*, 1999]. These cells are thought to utilise inactive cortisone to produce cortisol that may then act in an autocrine/paracrine fashion to decrease prostaglandin dehydrogenase (PGDH) mediated PG metabolism and increase PGHS activity. In Challis *et al* (2000) reference is made of 11β-HSD1 activity increasing in response to PGE_2 and $PGF_{2\alpha}$ and be dependent upon a transient increase in intracellular Ca^{2+}. Unlike 11β-HSD1, studies performed by Hardy *et al* (2001) demonstrated that Ca^{2+} can inhibit 11β-HSD2. Furthermore, Hardy *et al*, (1999) and co-workers have suggested that prostaglandins and leukotriene B_4 are potent inhibitors of 11β-HSD2 in human choriocarcinoma JEG-3 cells. This will thus mean that greater amounts of maternal cortisol will be crossing the placenta, into the foetal circulation at term. Excessive exposure of the foetus to glucocorticoids is known to reduce birth weight and interfere with foetal development [Seckl *et al*, 1997].

1.8. Project Justifications and Aims

Prostaglandins have been extensively implicated in the biochemistry of the human ovary. However, their involvement within several biochemical pathways is still unclear and requires further study. Although PGs are known to be involved in the functional lifespan of the CL, their mode of action is not completely understood and even though existence of PG receptors has been confirmed, the second messenger pathways via which they act still need to be enlightened. Finally cortisol metabolism, another very important aspect in reproductive endocrinology is also known to be affected by PGs in some cell types, but has not yet been investigated in the ovary.

Hence the main aims of this study were to:

i) investigate the relationship between the production of PGs and progesterone in luteinising human granulosa cells

ii) elucidate the role of PGs in the steroidogenic response of hGLC to HDL

iii) examine the possible role for PGs in the control of ovarian cortisol metabolism by 11β-HSD

iv) investigate which EP receptors mediate the effects of PGE_2 on steroidogenesis and cortisol metabolism in human granulosa-lutein cells.

Chapter Two

MATERIALS AND METHODS

Chapter Two

MATERIALS AND METHODS

2.1 Chemicals and Reagents

PGE_2 and $PGF_{2\alpha}$ antibodies were generously donated by Drs. N.L. Poyser and R.W. Kelly respectively (University of Edinburgh, UK). [^{125}I]-succinyl-cAMP was kindly prepared by Ros Foot (Royal Veterinary College, London, UK) as described in section 2.3.4. Apolipoprotein-AI (Apo-AI) was purchased from Athens Research & Technology (Athens, Georgia, USA). [1,2,6,7-^{3}H]-cortisol (60Ci/mmol), Dextran T500, octadecyl (C18) mini-columns, PD-10 columns, [1,2,6,7-^{3}H]-progesterone (86Ci/mmol), [5,6,8,11,12,14,15-^{3}H]-PGE_2 (164Ci/mmol), [5,6,8,9,11,12,14,15-^{3}H]-$PGF_{2\alpha}$ (219Ci/mmol), [5,8,9,11,12,14,15-^{3}H]-6-keto-$PGF_{1\alpha}$ (190Ci/mmol) and Na ^{125}I were obtained from Amersham Pharmacia Biotech UK Ltd (Little Chalfont, Bucks., UK). Charcoal, chloramine T, chloroform, di-sodium hydrogen orthophosphate 12-hydrate ($Na_2HPO_4.12H_2O$), ethanol, ethyl acetate, gelatin (powder), methanol, sodium di-hydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$), sodium metabisulphite, succinyl cAMP-Tyrosyl methyl ester (ScAMP-TME) were supplied by BDH (Poole, Dorset, UK). Cyclic AMP standard and anti-cyclic AMP antibody was obtained from Calbiochem Novabiochem (UK) Ltd, Beeston (Notts, UK). AH 6809, butaprost and SC 19220 were purchased from Alexis Biochemicals (Cayman Chemical, Notts, UK). 1:1(v/v) Dulbecco's modified Eagle's medium: Ham's F_{12} (DMEM: F_{12}) medium, Earles balanced salt solution (heat inactivated), foetal calf serum (FCS), L-glutamine, penicillin/streptomycin and Dulbecco's phosphate-buffered saline (PBS) were obtained from Life Technologies Ltd (Gibco BRL, Paisley, UK). TLC aluminium sheets (20×20cm, Silica gel 60) were supplied by Merck, Poole, Dorset, UK. Ultima-Gold scintillant was purchased from Packard Bioscience B.V. (Pangbourne, Berks, UK). Bicinchoninic Acid (BCA) protein assay reagent A and BCA protein assay reagent B were obtained from Pierce Science UK Ltd (Tattenhall, Cheshire, UK). All other chemicals and solvents were supplied by Sigma (Poole, Dorset, UK).

2.2 Collection and storage of follicular aspirates

2.2.1 Patient samples

Ovarian follicular aspirates containing granulosa cells were collected from women undergoing assisted conception at the Lister Hospital, Chelsea Bridge Road, London, UK. Collections were carried out in accordance with the Declaration of Helsinki and with approval from the local ethics committee.

Pituitary down-regulation was achieved by administration of a GnRH analogue (Suprecur: Shire Pharmaceuticals, Andover, Hants., UK; 500μg per day from day 2 of the cycle for 10-21 days). Administration of the GnRH analogue was then continued in conjunction with purified urinary human menopausal gonadotrophin (Menogon: Ferring Pharmaceuticals, Feltham, Middlesex, UK; 2-4 ampoules daily for 10-14 days) followed by a single intramuscular injection of hCG (Profasi: Serono, Welwyn Garden City, Herts., UK; 5,000-10,000IU) 36 hours prior to oocyte collection. Follicles were subsequently aspirated under local anaesthesia by the transvaginal route.

2.2.2 Isolation of human granulosa cells

Human granulosa cells were isolated from follicular aspirates as previously described [Webley *et al*, 1988]. Granulosa cells and contaminating blood cells from a single patient were precipitated from the supernatant follicular fluid and follicular flushing medium by centrifugation at 250*g* for 10 min at +4°C in a Beckman GS-6R centrifuge. The supernatant was discarded and the pelleted cells were resuspended in 10ml Dulbecco's modified PBS. The cell suspension was overlaid onto 10ml 60% (v/v) Percoll. The Percoll gradients were subjected to centrifugation at 1000*g* for 20 min at +4°C with no brake. This resulted in the sedimentation of any erythrocytes at the bottom of the tube, while the granulosa cells settled at the PBS-Percoll interface (equivalent density range: 1.04-1.07 g/ml). Granulosa cells were aspirated from the interface, resuspended in 10ml PBS and centrifuged at 250*g* for 10 min at +4°C. The supernatant was discarded and the pellet was resuspended in 10ml PBS. This washing step was repeated three times in total and in the final wash, all cells from the same patient were pooled together.

Following the final wash, the cell pellet was resuspended in 1 or 2ml PBS and a 10µl aliquot was mixed with 10µl 0.4% (w/v) Trypan Blue solution. Viable granulosa cells did not take up the Trypan blue stain and were therefore counted under the microscope (×200 magnification) using a haemocytometer. The cells were cultured in 1:1 (v/v) DMEM: F12 medium supplemented with 10% (v/v) foetal calf serum (FCS), penicillin (87,000 IU/L), streptomycin (87 mg/L) and 2mM L-glutamine. For secretion studies, cells were either seeded into sterile 24-well or 96-well cell culture plates at a density of 1×10^5 cells/ml culture medium, with a volume of 1ml or 250µl medium per well respectively. For 11β-HSD experiments, cells were seeded into sterile 24-well plates at a density of 5×10^4 cells/ml culture medium, with a volume of 1ml medium per well. Cells were subsequently incubated for 2 days at 37°C in a humidified atmosphere of 5% (v/v) CO_2 in air to allow cells attach to the wells of the cell culture plate.

At the end of the 2-day incubation the medium was aspirated from the wells and discarded. To remove any residual serum, wells were each rinsed with ~200µl warmed serum-free medium. This washing medium was discarded before addition of serum-free medium and treatments to a total volume of 1ml or 250µl medium per well depending on cell culture plates (as described above). Cells were subsequently incubated at 37°C in a humidified atmosphere of 5% (v/v) CO_2 in air for a period of 0-24h according to the experimental design.

2.3 Radioimmunoassays (RIAs)

2.3.1 Progesterone Radioimmunoassay (RIA)

Progesterone concentrations were determined by RIA as previously described [Pallikaros *et al*, 1995]. A progesterone standard curve was prepared from a 1µg/ml stock solution of progesterone in ethanol, stored at –20°C. A volume of 10µl (10ng) was removed from the progesterone standard. The ethanol was evaporated under nitrogen and the progesterone standard was resuspended in 1ml serum-free medium to give a top standard progesterone concentration of 10ng/ml (31.797nM). The top standard was double diluted to generate the following standards: 15.898, 7.949, 3.975, 1.987, 0.994, 0.497 and 0.248nM progesterone. Volumes of 100µl of each standard were aliquoted in triplicate and 100µl serum-free medium was aliquoted into the 0nM progesterone (B_0), total (T) and non-specific binding (NSB) tubes. The

unknown samples were diluted (by serial dilution) in serum-free medium into a total volume of 100µl. The unknown samples were diluted in serum-free medium at dilutions ranging between 1/10 to 1/400 (v/v) into a total volume of 100µl per tube.

Progesterone antiserum was diluted 1/100 (v/v) in phosphate azide saline-gelatin (PAS-gel) buffer [refer to Appendix I] and stored in 100µl aliquots at –20°C. Immediately prior to use, a 100µl aliquot was thawed and further diluted 1/100 (v/v) in PAS-gel buffer resulting in a working antibody dilution of 1/10,000 (v/v). A volume of 100µl of the diluted antibody was added into all sample and standard tubes excluding those for the determination of the total and NSB radioactivity, where 100µl PAS-gel buffer was added instead.

Stock [1,2,6,7 ^{3}H]-progesterone was diluted in PAS-gel buffer to give the required 10,000 cpm per 100µl. A volume of 100µl of the diluted [^{3}H]-progesterone was added into all assay tubes (including B_0, T and NSB tubes). Each tube was vortexed, covered in aluminium foil and incubated at +4°C overnight.

The next morning/day, a solution of 0.025% (w/v) dextran was first prepared in 0.01M phosphate buffer. After the dextran had dissolved, 0.25% (w/v) charcoal was added and mixed for 30min at +4°C. A volume of 500µl of the activated dextran-coated charcoal was added into each of the assay tubes, except the T tubes, which received 500µl 0.01M phosphate buffer. The tubes were vortexed and subjected to centrifugation for 10min at 1000*g* at +4°C with no brake, to precipitate the unbound progesterone. The supernatants were decanted into mini-scintillation vials and 2ml Ultima-Gold scintillant was added into each vial, which was then capped and vortexed thoroughly. Radioactivity (cpm) was measured on a Beckman Coulter LS 6500 Multipurpose Scintillation counter, with a count time of 1min per vial. The counts recorded were analysed in a DOS - RIA software program using the logit % B/B_0 whereby the data for the standard curve was linearised (see Appendix II for representative standard curve).

Intra- and inter-assay coefficients of variation were 8.9% (n=15) and 13.6% (n=40) respectively at 31% binding with a working assay range of 0.5-8.0nM progesterone.

2.3.2 PGE_2 Radioimmunoassay

PGE_2 concentrations were determined by RIA as previously described [Poyser *et al*, 1987]. A PGE_2 standard curve was prepared from a 1μg/ml stock solution of PGE_2 in ethanol, stored at –20°C. A volume of 10μl was removed from the PGE_2 standard, dried under nitrogen and resuspended in 1ml serum-free medium to give a top standard PGE_2 concentration of 10ng/ml (28.369nM). The top standard was double diluted to generate the following standards: 14.185, 7.092, 3.546, 1.773, 0.887, 0.443, 0.223, 0.111, 0.055 and 0.0277nM PGE_2. Volumes of 100μl of each standard were aliquoted in triplicate and 100μl serum-free medium was aliquoted into the 0nM PGE_2 (B_0), total (T) and non-specific binding (NSB) tubes. The unknown samples were either diluted 1/10 (v/v) in serum-free medium or were assayed neat, with a total volume of 100μl per tube.

PGE_2 rabbit antiserum was stored in 50μl aliquots at –20°C. A 50μl aliquot of antiserum was diluted 1/100 (v/v) in phosphate buffer [Appendix I] and stored in 500μl aliquots at –20°C. Immediately prior to assay, a 500μl aliquot of pre-diluted antibody was thawed and further diluted 1/30 (v/v) in phosphate buffer resulting in a working antibody dilution of 1/3,000 (v/v). A volume of 100μl of the diluted antibody was added into all sample and standard tubes excluding those for the determination of the T and NSB radioactivity, where 100μl of phosphate buffer were added instead.

Stock [5,6,8,11,12,14,15-^{3}H]-PGE_2 was diluted in phosphate buffer to give the required 10,000 cpm per 100μl. A volume of 100μl of the diluted [^{3}H]-PGE_2 was added into all standard and unknown sample tubes. Each tube was vortexed, covered in aluminium foil and incubated at +4°C overnight.

The next day, a solution of 0.1% (w/v) dextran was first prepared in phosphate buffer and after the dextran had dissolved, 1% (w/v) charcoal was added and mixed for 30min at +4°C. A volume of 200μl activated dextran-coated charcoal was added into each assay tube, excluding the totals, which instead received 200μl phosphate buffer. The tubes were vortexed and subjected to centrifugation for 15min at 800*g* at +4°C with no brake, to precipitate the unbound PGE_2. The supernatants were decanted into mini-scintillation vials and 2ml Ultima-Gold scinillant was added into each vial,

which was then capped and vortexed thoroughly. Radioactivity (cpm) was measured on a Beckman Coulter LS 6500 Multipurpose Scintillation counter, with a count time of 1min per vial. The counts recorded were analysed in a DOS - RIA software using the logit % B/B_0 whereby the data for the standard curve was linearised (see Appendix II for representative standard curve).

Intra- and inter-assay coefficients of variation were 9.6% (n=15) and 16.3% (n=28) respectively at 40% binding with a working range of 0.11-1.77nM PGE_2.

2.3.3 $PGF_{2\alpha}$ Radioimmunoassay

$PGF_{2\alpha}$ concentrations were determined by RIA as previously described [Kelly *et al*, 1986]. A $PGF_{2\alpha}$ standard curve was prepared from a 1µg/ml stock solution of $PGF_{2\alpha}$ in ethanol, stored at –20°C. A volume of 80µl was removed from the $PGF_{2\alpha}$ standard, dried under nitrogen and resuspended in 1ml serum-free medium to give a top standard $PGF_{2\alpha}$ concentration of 80ng/ml (225.67nM). The top standard was double diluted to generate the following standards: 112.835, 56.418, 28.209, 14.104, 7.052, 3.526, 1.763, 0.882, 0.441 and 0.22nM $PGF_{2\alpha}$. Volumes of 100µl of each standard were aliquoted in triplicate and 100µl serum-free medium was aliquoted into the 0nM $PGF_{2\alpha}$, T and NSB tubes. The unknown samples were either diluted 1/10 (v/v) in serum-free medium or were assayed neat, with a final volume of 100µl per tube.

$PGF_{2\alpha}$ antiserum was stored in 100µl aliquots at –20°C. A 100µl aliquot was diluted 1/100 (v/v) and stored in 50µl aliquots at –20°C. Immediately prior to assay a 50µl aliquot of pre-diluted antibody was thawed and diluted 1/20 (v/v) in assay buffer [Appendix I] resulting in a working antibody dilution of 1/2,000 (v/v). A volume of 100µl of the diluted antibody was added into all sample and standard tubes excluding those for the determination of the T and NSB radioactivity, where 100µl phosphate buffer were added instead.

Stock [5,6,8,9,11,12,14,15-^{3}H]-$PGF_{2\alpha}$ was diluted in phosphate buffer to give the required 10,000 cpm per 100µl. A volume of 100µl of the diluted [^{3}H]-$PGF_{2\alpha}$ was added into all standard and unknown sample tubes. Each tube was vortexed, covered in aluminium foil and incubated at +4°C overnight.

A solution of 0.1% (w/v) dextran was first prepared in phosphate buffer and after the dextran had dissolved, 1% (w/v) charcoal was added and mixed for 30min at +4°C. A volume of 200µl activated dextran-coated charcoal was added into each of the assay tubes, excluding the Ts, which instead received 200µl phosphate buffer. The tubes were vortexed and subjected to centrifugation for 15min at 800*g* at +4°C with no brake, to precipitate the unbound $PGF_{2\alpha}$. The supernatants were decanted into mini-scintillation vials and 2ml Ultima-Gold scinillant was added into each vial, which was then capped and vortexed thoroughly. Radioactivity (cpm) was measured on a Beckman Coulter LS 6500 Multipurpose Scintillation counter, with a count time of 1min per vial. The counts recorded were analysed in a DOS - RIA software using the logit % B/B_0 whereby the data for the standard curve was linearised (see Appendix II for representative standard curve).

Intra- and inter-assay coefficients of variation were 9.6% (n=15) and 14.4% (n=15) at 36.8% binding, with a working range of 0.9-14.1nM $PGF_{2\alpha}$.

2.3.4 Cyclic adenosine-3', 5'-monophosphate (cAMP) iodination and radioimmunoassay

2.3.4.1 Iodination of Succinyl-cAMP-Tyrosyl Methyl Ester (ScAMP-TME)

[^{125}I]-succinyl-cAMP was kindly prepared by Mrs. Ros Foot using the method of Brooker *et al* (1979). The following volumes were added to a glass tube containing a micromagnet; 20µl 0.5M potassium phosphate (KH_2PO_4, pH 7.0), 20µl ScAMP-TME (equivalent to 800ng in 5mM sodium acetate) and 10µl radiolabelled Na ^{125}I (1mCi). Chloramine T solution (1mg/ml) in 0.5M KH_2PO_4 (pH 7.0) was prepared fresh and 5µl were added to the above solution. The tube was then capped immediately and vortexed for 1min at room temperature. The reaction was stopped with the addition of 50µl 5mg/ml sodium metabisulphite (in ddH_2O). A volume of 800µl 0.1M sodium acetate, (pH4.75) was then added and the tube was again vortexed.

2.3.4.2 Rapid purification of iodinated cAMP

[^{125}I]-succinyl-cAMP was resolved by the method of Wilson *et al* (1988). A C18 mini-column was washed with 20ml methanol followed by 20ml ddH_2O. The reaction mixture (section 2.3.4.1) was loaded onto the column and eluted with 17.5:82.5 (v:v) propan-1-ol:0.1M sodium acetate and 0.5ml fractions were collected in borosilicate tubes. The radioactivity was determined in aliquots of each fraction, and fractions containing the highest radioactivity were pooled, adjusted to 50% (v/v) propan-1-ol by the addition of an appropriate volume of propan-1-ol and stored at –20°C.

2.3.4.3 cAMP Radioimmunoassay

Cyclic adenosine-3', 5'-monophosphate (cAMP) concentrations were determined by RIA as previously described [Steiner *et al*, 1972] with the acetylation step subsequently proposed by Harper & Brooker *et al* (1975). A cAMP standard curve was prepared from a 5µM stock cAMP solution, stored at –20°C. A volume of 10µl was diluted 1/250 (v/v) in 10mM sodium acetate solution (pH 4.75) [Appendix I] to give a cAMP concentration of 20nM. From the 20nM solution, 250µl were removed and diluted 1/4 (v/v) to a total volume of 1ml, giving rise to the 5nM top standard cAMP concentration. The top standard was then double diluted in 10mM sodium acetate solution to generate the following standards: 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.039nM. Volumes of 100µl of each standard were aliquoted in triplicate and 100µl 10mM sodium acetate solution were aliquoted (in triplicate) into the B_0, T and NSB tubes. The unknown samples were diluted 1/20 or 1/25 (v/v) in 10mM sodium acetate solution into a total volume of 100µl.

Acetylation mix was prepared (in glass) immediately prior to use by mixing triethylamine and acetic anhydride in a ratio of 2:1 (v:v). A volume of 10µl was added into each tube (excluding Ts), to increase assay sensitivity by improving the competition between sample and tracer. Both the preparation and addition of the acetylation mix was performed in a fume cupboard. Samples were allowed to stand for ~3h for the acetylation mix to evaporate.

Rabbit polyclonal anti-succinyl-cAMP antibody was first diluted 1/120 in 10mM sodium acetate and stored in 100µl aliquots at –20°C. Immediately prior to assay, a 100µl aliquot of pre-diluted antibody was thawed and diluted 1/50 (v/v) in 50mM sodium acetate with 0.3% (w/v) γ-globulins resulting in a working antibody dilution of 1/96,000 (v/v). A volume of 100µl of the diluted antibody was added into all assay tubes excluding those for the determination of the total and non-specific binding radioactivity, where 100µl 50mM sodium acetate solution containing 0.3% (w/v) γ-globulins were added instead.

[^{125}I]-succinyl-cAMP was diluted in 50mM sodium acetate solution with 0.3% (w/v) γ-globulins to give the required 10,000 cpm per 100µl. A volume of 100µl of the diluted [^{125}I]-cAMP was added into all assay tubes. Each tube was vortexed, covered in aluminium foil and incubated at +4°C overnight.

The next day, immediately prior to use, a stock solution of 30% (w/v) polyethylene glycol (PEG) in 50mM sodium acetate buffer (pH 6.2) was prepared [Appendix I]. In parallel, a 0.4% (w/v) γ-globulin solution in 50mM sodium acetate (pH 6.2) was prepared. Equal volumes of the two solutions were mixed, by addition of PEG to the γ-globulins, to make the total volume required to precipitate all of the assay tubes. Once the mixture became uniform, 0.05% (v/v) of Tween was added, to prevent the antibody-antigen complex from sticking to the tube. A volume of 1ml of the precipitant mix was added into each of the assay tubes, excluding the T tubes. The tubes were vortexed and subjected to centrifugation in an MSE Coolspin centrifuge, for 30min at 900*g* at +4°C with no brake, to precipitate the bound cAMP. The supernatants were carefully aspirated off using an automatic aspirator. The assay tubes (including T tubes) were then put on a Wallac wizard 1470 automatic gamma counter with a count time of 1min per tube. The counts recorded were automatically analysed using on-line radioimmunoassay software [see Appendix II for representative standard curve].

Intra- and inter-assay coefficients of variation were 4.7% (n=13) and 9.5% (n=30) at 48.9% binding with a working range of 0.2-1.3nM cAMP.

2.4 Preparation of HDL

2.4.1 Isolation of HDL from human plasma

HDL were isolated from plasma by sequential ultracentrifugation as previously described [Edelstein *et al*, 1986]. Isolation of the lipoproteins is based on their density, as shown below (Table 2.1).

Table 2.1 Lipoprotein groups and their densities

LIPOPROTEINS	(d) DENSITIES (g/ml)
VLDL	<1.006
IDL	1.006 – 1.019
LDL	1.019 – 1.063
HDL_2	1.063 – 1.125
HDL_3	1.125 – 1.21

Fresh blood was collected (~60ml) from healthy male human volunteers and every 20ml blood were mixed with 1ml 5.4% (w/v) anticoagulant ethylene-diamine-tetra-acetic acid (EDTA). Plasma with lipoproteins were separated in the supernatant from the precipitated blood cells by centrifugation at 800*g* for 20min at +4°C with no brake, in a Beckman GS-6R centrifuge.

16ml plasma (density 1.006g/ml) were transferred into a Beckman ultracentrifuge polycarbonate tube, together with 8ml 1.045 g/ml potassium bromide (KBr), to give a final density solution of 1.019g/ml at which the chylomicrons, VLDL and IDL will float. The tubes were capped, balanced to within 0.1g of each other using appropriate density solution (in this case 1.006g/ml) and centrifuged at 10,000*g* for 24h under vacuum in a Beckman-L8-70M ultracentrifuge (70Ti rotor), at +12°C.

The lipoproteins (chylomicrons, VLDL & IDL) formed a yellow layer at the top of the tube which was carefully removed (accounting for ~2ml of the total volume). A

further volume of 6ml of the supernatant was removed, to make a total volume of 8ml removed and 16ml of density 1.019g/ml left in the tube. The density was then increased to 1.065g/ml (at which LDL will float) by the addition of 8ml of 1.157g/ml density solution KBr into each tube. As before the tubes were capped, balanced using the 1.019 g/ml KBr density solution and mixed. The tubes were centrifuged at 10,000*g* for 24h under vacuum as above.

The LDL were removed from the top of the tube in a similar way as described previously (accounting for ~2ml of the total volume), followed by a further removal of 6ml of the supernatant (total volume of 8ml), leaving 16ml of 1.065g/ml density in the tube. The density was then increased to 1.21g/ml by the addition of 8ml of 1.478g/ml KBr density solution at which the HDL will float. The tubes were again capped, balanced using the density solution 1.065g/ml KBr as described before and centrifuged at 10,000*g* as above.

The HDL were removed from the top of the tube in a similar way as previously described. HDL was then stored at +4°C covered with aluminium foil for protection from the light, to prevent oxidation.

KBr concentrations were calculated using the following formula:

$$X_{(gKBr)} = [\text{Volume } (\text{density}_{final} - \text{density}_{initial})] / [1 - (0.312 \times \text{density}_{initial})].$$

Oxidation of the lipoproteins was assessed by measuring thiobarbituric acid reactive substances (TBARS) using a spectrophotometric method as previously described [Janero *et al*, 1990]. The HDL preparations used for the studies reported in this thesis had values of <0.6nmol TBARS/mg proteins, indicating that no significant oxidation had taken place.

2.4.2 HDL Desalting

The lower end of a PD-10 column was cut using a pair of scissors. The lid was then removed from the top and the solution was decanted. The column was then washed by the addition of 3ml PBS, allowing it to slowly pass through the column. A volume of 2-2.5 ml HDL (if the HDL sample was bigger a second column would be used) was pipetted at the top of the column and allowed to enter while PBS already

inside the column was being eluted and discarded. A volume of 3ml PBS was then added to elute the HDL from the PD-10 column. Desalted HDL was stored at +4°C, protected from the light.

2.4.3 Protein Assay

For determination of HDL protein concentrations (mg protein/ml), a bovine serum albumin (BSA) standard curve was prepared from a 2mg/ml stock solution of BSA in ddH_2O, stored at +4°C. A volume of 50µl was removed from the BSA standard and was diluted 1/2 (v/v) in ddH_2O to give the second standard of 1mg/ml. This standard was double diluted in ddH_2O to generate the following standards: 0.5, 0.25, 0.125, 0.063, 0.031mg/ml. Volumes of 10µl of each standard were aliquoted in triplicate in a 96-well cell culture plate. For the top standard of 2mg/ml, 10µl were pipetted out straight from the stock BSA bottle, in triplicate, while 10µl ddH_2O were aliquoted in triplicate for the "zero" standard. The HDL unknown samples were diluted 1/2 (v/v) and 1/5 (v/v) in PBS into a total volume of 10µl (in triplicate). A volume of 10µl PBS was also assayed in triplicate as an assay blank.

A total volume of 5ml was prepared from the two bicinchoninic acid (BCA) reagents (A and B) at a ratio of 49:1 (v:v) A:B. The reagents were mixed well and a volume of 100µl was added into each of the assay tubes. The samples were incubated at +37°C for 30min and the Optical Density (OD) was then read for 1min at a wavelength of 620nm, in a Dynex Technologies micro-plate reader. The data obtained were averaged and analysed in Microsoft Excel [see Appendix II for representative standard curve].

2.5 Apolipoprotein A-I preparation

Apolipoprotein A-I (ApoA-I) purchased from Athens (USA) was supplied in 10mM NH_4HCO_3 solution at a protein concentration of 1mg/ml. However, ApoA-I purchased from Sigma (UK) was lyophilised and needed processing before use. 1mg of ApoA-I protein was dissolved in 600µl 3M guanidine hydrochloride. A large tube-o-dialyzer (Chemicon) was emptied, rinsed with ddH_2O and then dried. The dissolved ApoA-I was then transferred into the dialysis tube and the lid was replaced. The dialysis tube was then inverted and placed on top of a 250ml conical flask filled with 10mM Tris-buffered saline (TBS) (pH 7.4) and left on a magnetic stirrer. Only

half of the lid was immersed into the TBS, so that the membrane was in contact with the TBS for dialysis to take place. Dialysis was performed over a 5-day period at +4°C with the TBS being changed regularly (1-2 times a day). The dialyzed ApoA-I was removed from the dialysis tube and a protein assay was performed as previously described (section 2.4.3) to determine the final protein concentration. This was necessary because the total volume increased during dialysis. When performing the protein assay for the ApoA-I sample, the dilutions of the samples were done in TBS (instead of PBS) and a triplicate of TBS alone served as the assay blank.

2.6 11β-HSD Assay

Human granulosa cells were isolated as previously described (section 2.2.2) and cultured at a density of 5×10^4 cells/ml culture medium, with a volume of 1ml medium per well in a 24 well cell culture plate. Cells were subsequently incubated for 1 or 2 days in serum-supplemented medium according to the experimental design, at 37°C in a humidified atmosphere of 5% (v/v) CO_2 in air.

At the end of the 1- or 2-day pre-incubation, the serum-supplemented medium was aspirated from the wells and discarded. To remove any residual serum, each well was rinsed with ~200µl warmed serum-free medium. This washing medium was discarded before addition of serum-free medium and treatments in a total volume of 1ml medium per well. Cells were subsequently incubated at 37°C in a humidified atmosphere of 5% (v/v) CO_2 in air for a period of 0-20h according to the experimental design. Following this pre-incubation, the medium was again aspirated from the wells and discarded. Fresh warmed serum-free medium containing treatments were added in a volume of 900µl medium per well. 100µl serum-free medium containing 0.5µCi [1,2,6,7-^{3}H]-cortisol were then added to each well and the cells were incubated for a further 4 hours.

At the end of the 4-hour incubation, the medium from each well was transferred into a pyrex screw cap glass tube containing 2ml chloroform. The tubes were then capped, vortexed and subjected to centrifugation at 1000*g* for 30min at +4°C. Following centrifugation, the aqueous phase was removed and discarded. The samples were then dried in a sample concentrator Techne Dri-Block DB-3 at +45°C

under nitrogen before being stored at +4°C for up to 14 days pending resolution of steroids by thin layer chromatography (TLC) (described below).

2.7 Cortisol cortisone resolution by thin layer chromatography

After evaporation to dryness, steroid residues were re-suspended in 25µl ethyl acetate containing 1mM cortisol and 1mM cortisone. The tubes were then vortexed and 20µl samples from each tube were transferred to individual lanes of a TLC plate. TLC plates were developed in TLC tanks containing 92:8 (v/v) chloroform:95% (v/v). The bands containing the labelled cortisol and cortisone were visualised by the absorption of ultraviolet (UV) light at 254nm. The radioactivity of the cortisol and cortisone regions were quantified by a Bioscan System 200 Radiochromatohramme Scanner (Lablogic, Sheffield, UK). The amount of cortisone (pmol) formed by oxidation of cortisol was calculated by multiplication of percentage conversion of ^{3}H-cortisol to ^{3}H-cortisone by the total amount of cortisol present at the outset of the assay (100pmol/tube).

2.8 C18 Chromatography

2.8.1 Follicular fluid fractionation

Before use each mini-column was washed with 10ml methanol and then rinsed with 10ml ddH_2O. Acellular human follicular fluid (1.5ml) was then loaded onto the mini-column, and the eluent was collected. The cartridges retain hydrophobic molecules, so elution of the different compounds in fractions occurs according to their hydrophobicity and affinity for the C18 mini-column. Increasing concentrations of methanol (0, 10, 20, 30, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 & 100% (v/v) methanol in ddH_2O) were added sequentially, in 1ml volumes. Each fraction was collected separately into borosilicate glass tubes.

2.8.2 Follicular fluid spiking

Follicular fluid samples from three individual patients was aliquoted in three 1.5ml aliquots. Each of the aliquots were spiked by the addition of either 1µCi [^{3}H]-PGE_2, [^{3}H]-$PGF_{2\alpha}$ or [^{3}H]-6-keto $PGF_{1\alpha}$. The spiked samples were each loaded onto a C18 mini-column for fractionation as previously described (section 2.8.1). Triplicate 100µl aliquots of each column fraction were transferred to scintillation vials containing 2ml Ultima-Gold scintillant and the amount of radioactivity was

measured on a Beckman Coulter LS 6500 Multipurpose Scintillation counter with a count time of 1min per tube.

2.9 Effects of human follicular fluid fractions on renal 11β-HSD activities

Kidneys from male Sprague-Dawley rats were dissected and stored at –20°C. Into 27ml hypotonic Tris-EDTA lysis buffer (Appendix I), 0.75g rat kidney were added and homogenised. Restoration of isotonicity was achieved by the addition of 3ml 1.5M potassium chloride (KCl) giving a renal tissue homogenate concentration of 25mg (net weight/ml). The homogenate was subsequently centrifuged at 1000*g* for 20 min at +4°C (to precipitate intact tissue and cells). From this supernatant, 100µl volumes were transferred into tubes together with 600µl PBS. Each tube then received 100µl fractions of follicular fluid (generated from C18 mini-column fractionation, section 2.8.1) or 100µl PBS (controls) before being pre-incubated for 30min at +37°C in a gyratory water-bath. Those follicular fluid fractions eluted at methanol concentrations >20% (v/v) were dried in a sample concentrator Techne Dri-Block DB-3 at 45°C under nitrogen and reconstituted in 1ml of 20% (v/v) methanol prior to use. To initiate the 11β-HSD assays, each tube received 100µl 4mM nicotinamide adenine dinucleotide phosphate ($NADP^+$) and 100µl PBS containing 0.5µCi [^{3}H]-cortisol. The tubes were further incubated for 1 hour at +37°C in a gyratory water-bath. After 1 hour, steroids were extracted as previously described (section 2.6) and ^{3}H-cortisone was resolved and quantified as described above (section 2.7).

2.10 Statistical Analysis

All experimental data are presented as the mean±SE of upto 5 independent experiments each performed using cells from a different patient. In some cases, there was great variation in the absolute concentrations of hormone/cAMP or enzyme activities measured between cells from different patients. As a result, data were standardised within each experiment, as a percentage of the defined basal control concentration/ enzyme activity before pooling data. However, the statistical analyses in such experiments were performed on the non-referenced (absolute) data.

All statistical evaluations were performed using GraphPad Prism2 software (San Diego, CA, USA). Since data conformed to Gaussian (normal distributions) statistical analyses were performed using parametric tests. In the case of analysing changes across one variable (*e.g.* concentration, time), parametric statistical analyses were performed using one-way analysis of variance (ANOVA) with repeated measures (for individual experiments), with Dunnett's *post hoc* analysis. When data were analysed across different treatments, when there were two variables within the same experimental design (*e.g.* concentration and treatment), parametric statistical analyses were performed using a combination of two-way ANOVA and Bonferroni's *multiple comparison* test.

Probability values of <0.05 were accepted as significant in all experiments.

Chapter Three

THE RELATIONSHIP BETWEEN PROSTAGLANDIN SYNTHESIS AND PROGESTERONE

Chapter Three

THE RELATIONSHIP BETWEEN PROSTAGLANDINS AND PROGESTERONE

3.1 INTRODUCTION

Following the preovulatory LH surge, induction of the expression of PGHS-2 confers on luteinizing granulosa cells the capacity to synthesise high concentrations of prostaglandins (PGs) [LeMaire *et al*, 1975; Espey *et al*, 1980; Ainsworth *et al*, 1984; Richards *et al*, 1994; Sirois *et al*, 1994; Narko *et al*, 1997]. These inflammatory mediators are known to play important regulatory roles in follicular rupture and ovulation of the mature oocyte [Grinwich *et al*, 1972; Munalulu *et al*, 1987]. However, the significance of these locally synthesised PGs in the paracrine/autocrine control of ovarian steroidogenesis is still not completely understood. A number of studies in a wide range of species have demonstrated that treatment of luteinizing cells with exogenous $PGF_{2\alpha}$ and its analogues inhibit luteal steroidogenesis whereas PGE_2 stimulates progesterone production [Richardson, 1986; Michael *et al*, 1993b; Michael *et al*, 1994; Olofsson & Leung *et al*, 1994]. Based on these documented effects, it is hypothesised that locally synthesised PGs may act in a paracrine/autocrine manner to determine the level of progesterone synthesis in luteinizing granulosa cells. Moreover, since progesterone has been inversely correlated with PG synthesis in the CL [Patek & Watson, 1976; Rothchild, 1981], it is also speculated that increasing output of progesterone from luteinizing granulosa cells may suppress $PGF_{2\alpha}$ and/or PGE_2 production from such cells. The main objective of the work described in this chapter was, therefore, to investigate the relationship between the production of both $PGF_{2\alpha}$ and PGE_2 and the output of progesterone from human granulosa cells as they luteinise *in vitro*.

3.2 EXPERIMENTAL PROTOCOLS

In experiments described in this chapter from section 3.3.2 onwards, cells were seeded into sterile 24-well cell culture plates at a density of 1×10^5 cells/ml serum-supplemented culture medium with a volume of 1ml medium per well and cultured for 2 days. At the end of the 2-day incubation, medium was discarded, cells were rinsed with warmed serum-free medium and then further incubated in serum-free

medium depending on the individual experimental protocol. The experimental design described in section 3.3.1 was somewhat different and is hence described in more detail within that section. However, cells were cultured and seeded into 24-well cell culture plates as described above. In all experiments, at the end of the incubation period the spent serum-free medium was transferred to micro test-tubes and stored frozen at –20°C pending PG and progesterone assays (section 2.3).

Unless otherwise stated, for the purpose of presentation and discussion, data were internally standardised. Such data are presented graphically as percentage values (mean±SE) (where the control $PGF_{2\alpha}$, PGE_2 or progesterone production for each experimental design was standardised to 100%). However, all statistical analyses were performed on non-referenced data.

3.3 RESULTS

3.3.1 Daily patterns of prostaglandin and progesterone production

To investigate the patterns of $PGF_{2\alpha}$, PGE_2 and progesterone production over the first three days in culture, granulosa-lutein cells were cultured for 0, 1 or 2 days in 10% (v/v) FCS-supplemented medium being switched to serum-free medium for a period of 24 hours. To remove any residual serum, wells were each rinsed with 200-300µl warmed serum-free medium and this washing medium was discarded before adding 1ml fresh serum-free medium to each well. At the end of the 24-hour incubation period, the spent serum-free medium was transferred to micro test-tubes and stored frozen at –20°C pending PG and progesterone assays (section 2.3).

Over the first three days in culture, the concentration of $PGF_{2\alpha}$ declined progressively by 36.4±7.0% (n=5; $P<0.05$) and 82.1±13.1% (n=5; $P<0.01$) on days 2 and 3 relative to the output on day 1 (Figure 3.1). PGE_2 concentrations followed a similar pattern over the same time frame but were only significantly decreased on the third day in culture by 75.0±7.5% (n=5; $P<0.05$) (Figure 3.2). Over the same culture period, the concentration of progesterone increased by 52.4±16.9% and 54.0±10.9% (n=5; $P<0.05$) on days 2 and 3 of culture respectively, relative to the progesterone concentrations on the first day (Figure 3.3).

3.3.2 Effects of meclofenamic acid on PG and progesterone production

3.3.2.1 Concentration-dependent effects of meclofenamic acid

As previously described, after incubation for 2 days, cells were rinsed with warmed serum-free medium and then incubated for a further 24 hours in serum-free medium containing 0, 0.01, 0.1, 1 or 10μM meclofenamic acid (MA). (These concentrations were selected based on an IC_{50} for the inhibition of PGHS by meclofenamic acid of 0.6μM [Boctor *et al*, 1986]). At the end of the 24 hour incubation period, the spent medium was collected and stored at -20°C pending assay of the PG and progesterone concentrations (section 2.3). Meclofenamic acid was prepared to a stock concentration of 100mM in dimethylsulphoxide (DMSO), the final concentration of which was adjusted in all wells to 0.1% (v/v).

Incubation for 24 hours with meclofenamic acid resulted in a concentration-dependent suppression of $PGF_{2\alpha}$ production which was significant at concentrations ≥ 10nM (Figure 3.4). At the maximum tested concentration of 10μM, meclofenamic acid suppressed $PGF_{2\alpha}$ production by 69.6±22.4% (n=5; $P<0.01$). At concentrations of 1μM and 10μM, meclofenamic acid also inhibited PGE_2 accumulation by 73.0±17.0% and 63.8±16.4% respectively (n=5; $P<0.05$; Figure 3.5). However, progesterone synthesis remained unaffected by meclofenamic acid at any of the tested concentrations (Figure 3.6).

3.3.2.2 Time-dependent effects of meclofenamic acid

In order to investigate the effects of meclofenamic acid over time, cells were cultured for 2 days (as previously described) and then further incubated in serum-free medium containing 10μM meclofenamic acid for 0, 1, 2, 4, 8 & 24 hours. At each time point the spent medium was collected and stored frozen at -20°C pending assay of the PG and progesterone concentrations (section 2.3).

Basal concentrations of $PGF_{2\alpha}$ increased in a time-dependent manner at 4, 8 and 24 hours by up to 109.1±28.5% relative to time zero (n=3; $P<0.01$; Figure 3.7). Basal PGE_2 production exhibited a similar pattern increasing in a time-dependent manner by 100.0±16.7% (n=5; $P<0.01$) (Figure 3.8). Treatment with 10μM MA prevented the progressive increases in $PGF_{2\alpha}$ and PGE_2 production. Progesterone concentrations increased in a time-dependent manner over the 24-hour incubation

period by upto 2.2±0.5-fold (n=4; $P<0.01$) relative to time zero (Figure 3.9). Incubation with 10μM MA had no effect on progesterone output at any of the time points.

3.3.3 Effects of aminoglutethimide on PG and progesterone production

3.3.3.1 Concentration-dependent effects of aminoglutethimide

At the end of the 2-day incubation, cells were rinsed with serum-free medium and then incubated for a further 24 hours in serum-free medium containing 0, 1, 10 or 100μM aminoglutethimide. (These concentrations were selected on the basis of previous reports demonstrating inhibition of adrenal and gonadal steroidogenesis at aminoglutethimide concentrations of 86-500μM [Wilroy *et al*, 1986; Touitou *et al*, 1973; Peluso & Pappalardo *et al*, 1999]). At the end of the 24-hour incubation period, medium was collected and stored at –20°C pending assay of PG and progesterone concentrations (section 2.3). A stock concentration of 100mM aminoglutethimide was prepared in chloroform. The final concentration of this organic solvent was adjusted in all wells to 0.1% (v/v).

Incubation for 24 hours with 100μM aminoglutethimide, as expected, suppressed progesterone production by 64.0±14.2% (n=5; $P<0.01$) with no significant effect at 1μM or 10μM AG (Figure 3.10). However, treatment with an effective concentration of this cytochrome P_{450} inhibitor had no effect on the production of either $PGF_{2\alpha}$ (Figure 3.11) or PGE_2 (Figure 3.12).

3.3.3.2 Time-dependent effects of aminoglutethimide

In order for the effects of aminoglutethimide to be examined over time, cells cultured for 2 days in serum-supplemented medium and then incubated in serum-free medium containing 100μM aminoglutethimide for 0, 1, 2, 4, 8 & 24 hours. At each time point the spent medium was collected and stored frozen at -20°C pending assay of the PG and progesterone concentrations (section 2.3).

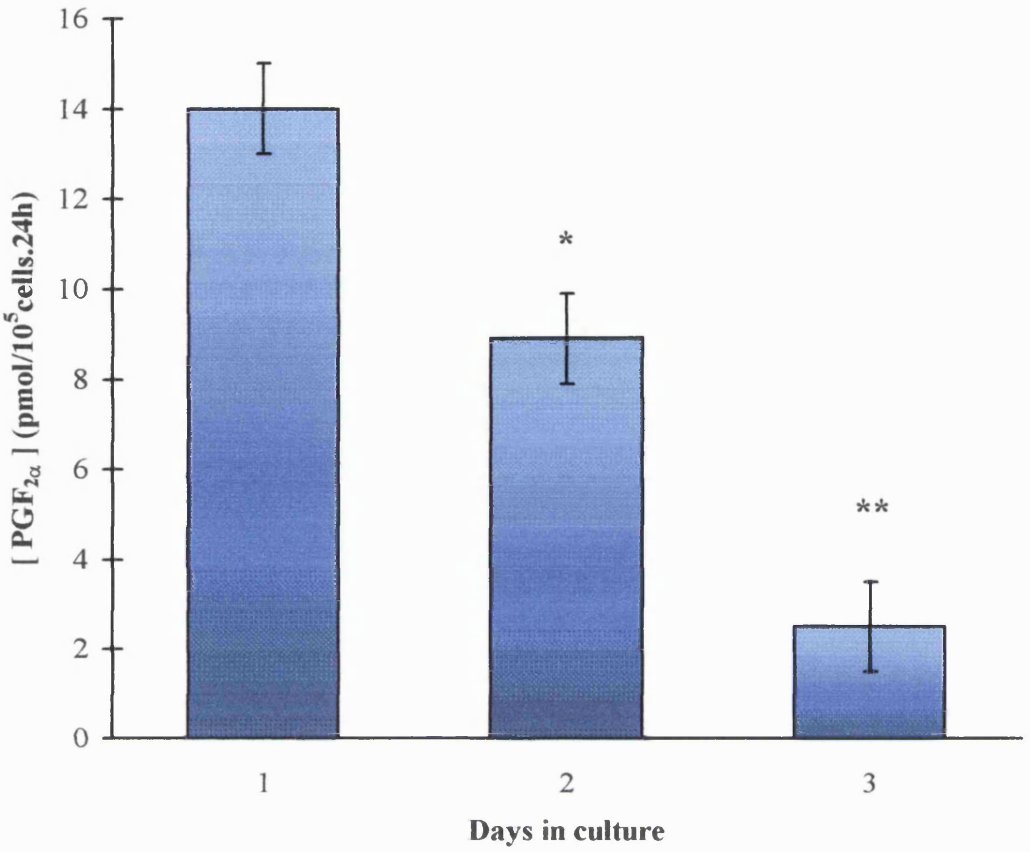

Figure 3.1 Daily pattern of $PGF_{2\alpha}$ production by human granulosa-lutein cells over a three-day period

$PGF_{2\alpha}$ concentrations in spent medium collected over 24 hour intervals during continuous culture of human granulosa-lutein cells. Values are the mean±SE for 5 independent experiments with triplicate determinations in each experiment. *$P<0.05$, **$P<0.01$ relative to the $PGF_{2\alpha}$ concentration on day 1 of culture. (One way ANOVA; Dunnett's *post hoc* test)

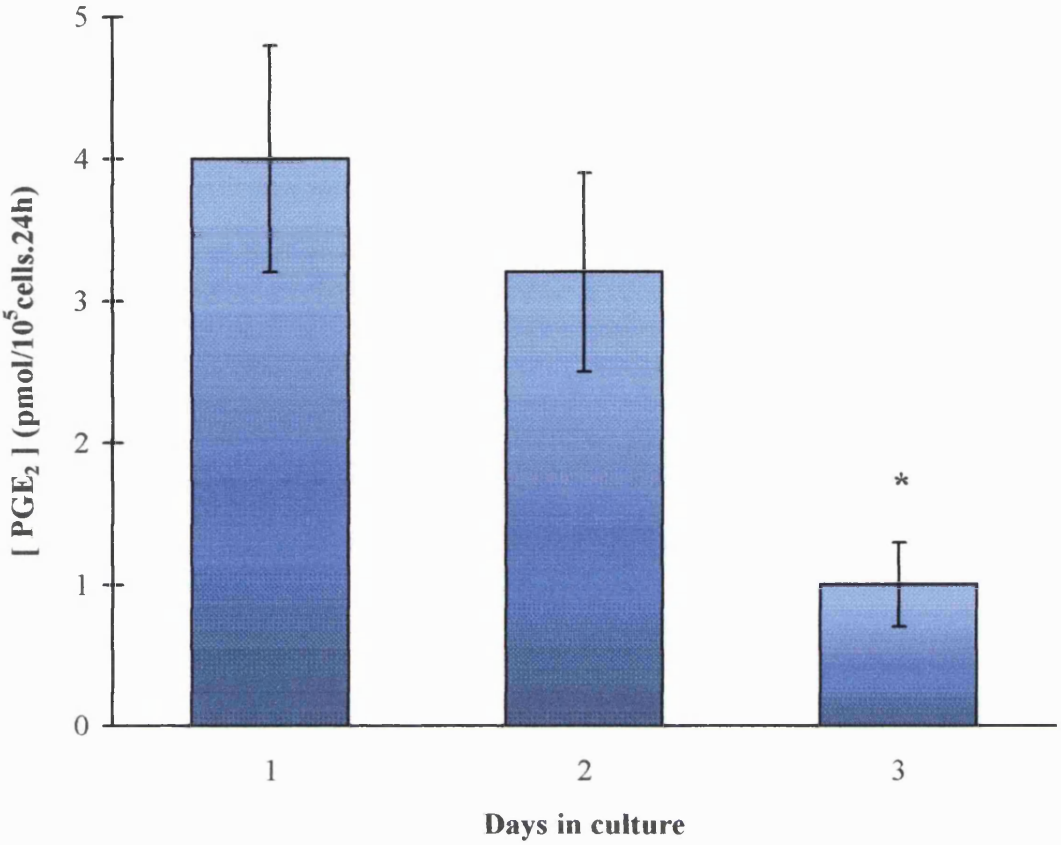

Figure 3.2 Daily pattern of PGE_2 production by human granulosa-lutein cells over a three-day period

PGE_2 concentrations in spent medium collected over 24 hour intervals during continuous culture of human granulosa-lutein cells. Values are the mean±SE for 5 independent experiments with triplicate determinations in each experiment. *$P<0.05$ relative to the PGE_2 concentration on day 1 of culture. (One way ANOVA; Dunnett's *post hoc* test)

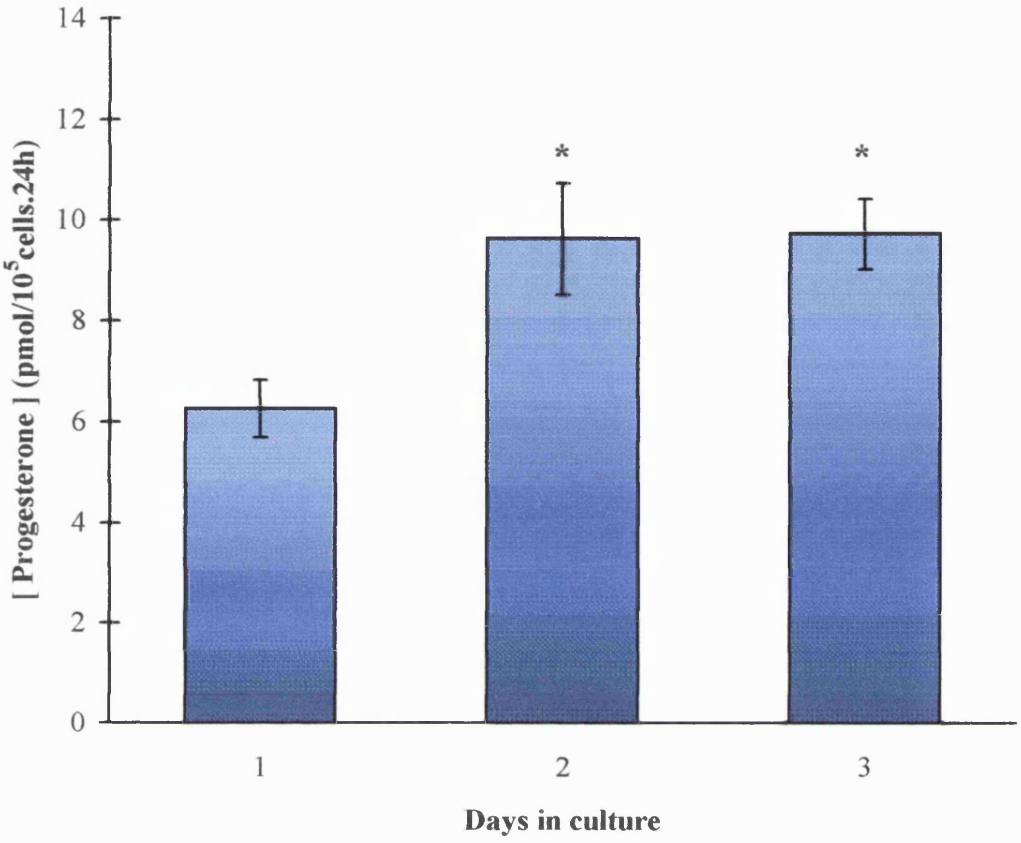

Figure 3.3 Daily pattern of progesterone production by human granulosa-lutein cells over a three-day period

Progesterone concentrations in spent medium collected over 24 hour intervals during continuous culture of human granulosa-lutein cells. Values are the mean±SE for 5 independent experiments with triplicate determinations in each experiment. *P<0.05 relative to the progesterone concentration on day 1 of culture. (One way ANOVA; Dunnett's *post hoc* test)

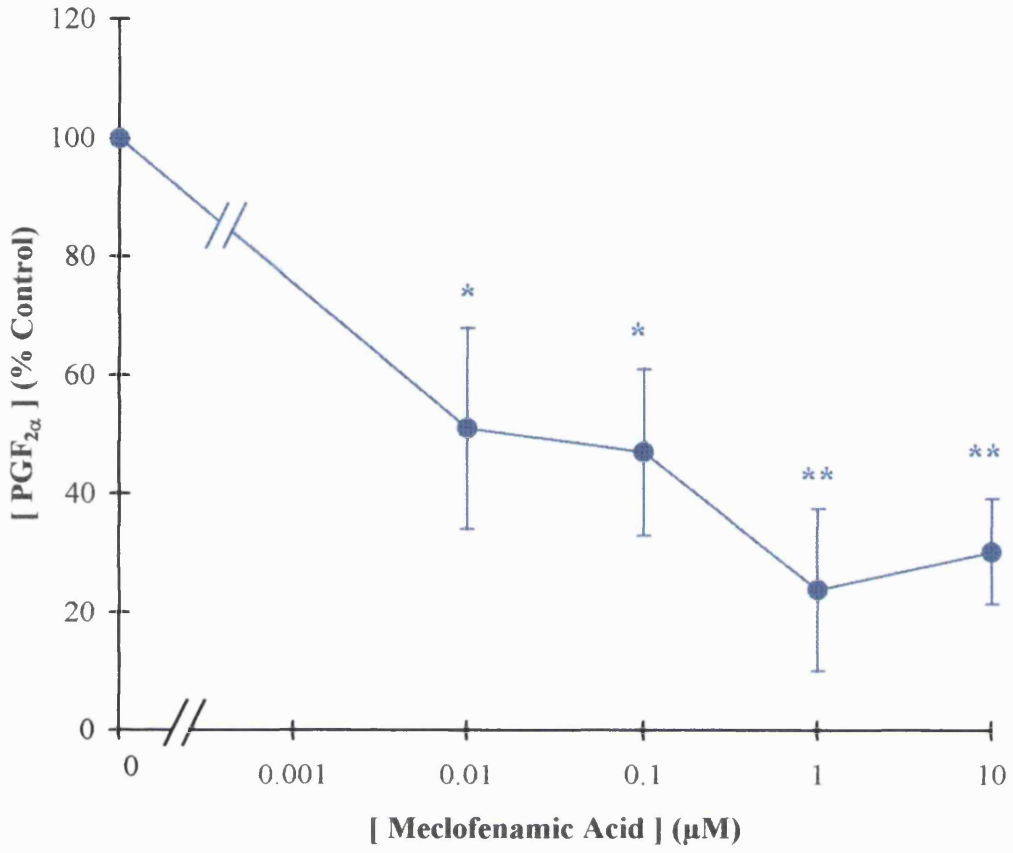

Figure 3.4 Concentration-dependent effects of meclofenamic acid on $PGF_{2\alpha}$ output by human granulosa-lutein cells

$PGF_{2\alpha}$ concentrations in spent medium collected after a 24-hour incubation in the presence of 0-10μM meclofenamic acid. Values are the mean±SE for 5 independent experiments with triplicate determinations in each experiment. $*P<0.05$, $**P<0.01$ relative to 0μM meclofenamic acid (One way ANOVA; Dunnett's *post hoc* test)

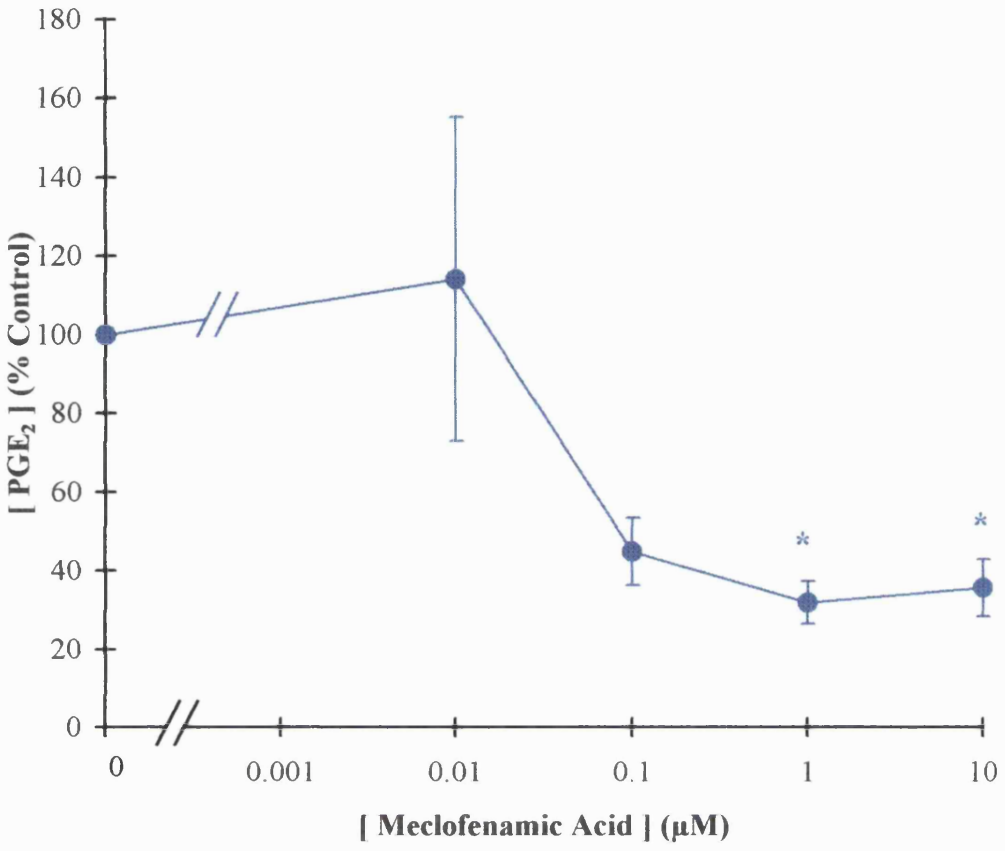

Figure 3.5 Concentration-dependent effects of meclofenamic acid on PGE_2 output by human granulosa-lutein cells

PGE_2 concentrations in spent medium collected after a 24-hour incubation in the presence of 0-10μM meclofenamic acid. Values are the mean±SE for 5 independent experiments with triplicate determinations in each experiment. *$P<0.05$ relative to the 0μM meclofenamic acid (One way ANOVA; Dunnett's *post hoc* test)

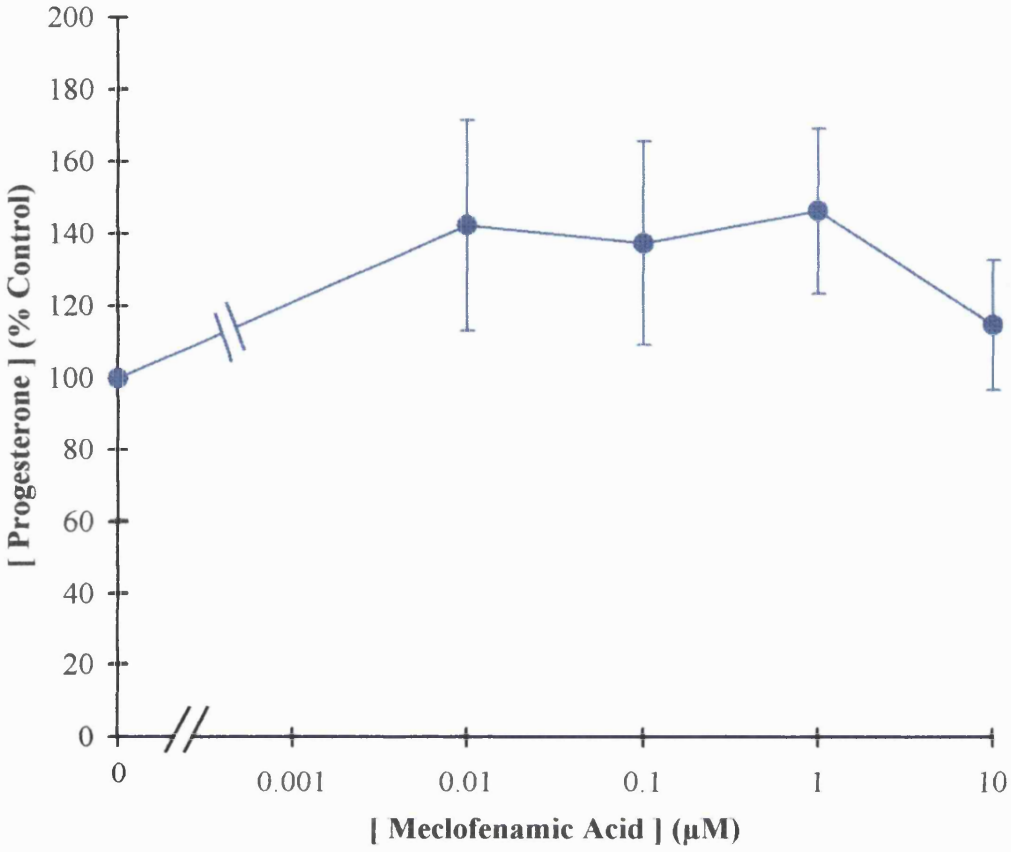

Figure 3.6 Concentration-dependent effects of meclofenamic acid on progesterone output by human granulosa-lutein cells

Progesterone concentrations in spent medium collected after a 24-hour incubation in the presence of 0-10µM meclofenamic acid. Values are the mean±SE for 5 independent experiments with triplicate determinations in each experiment. (One way ANOVA; $P>0.05$)

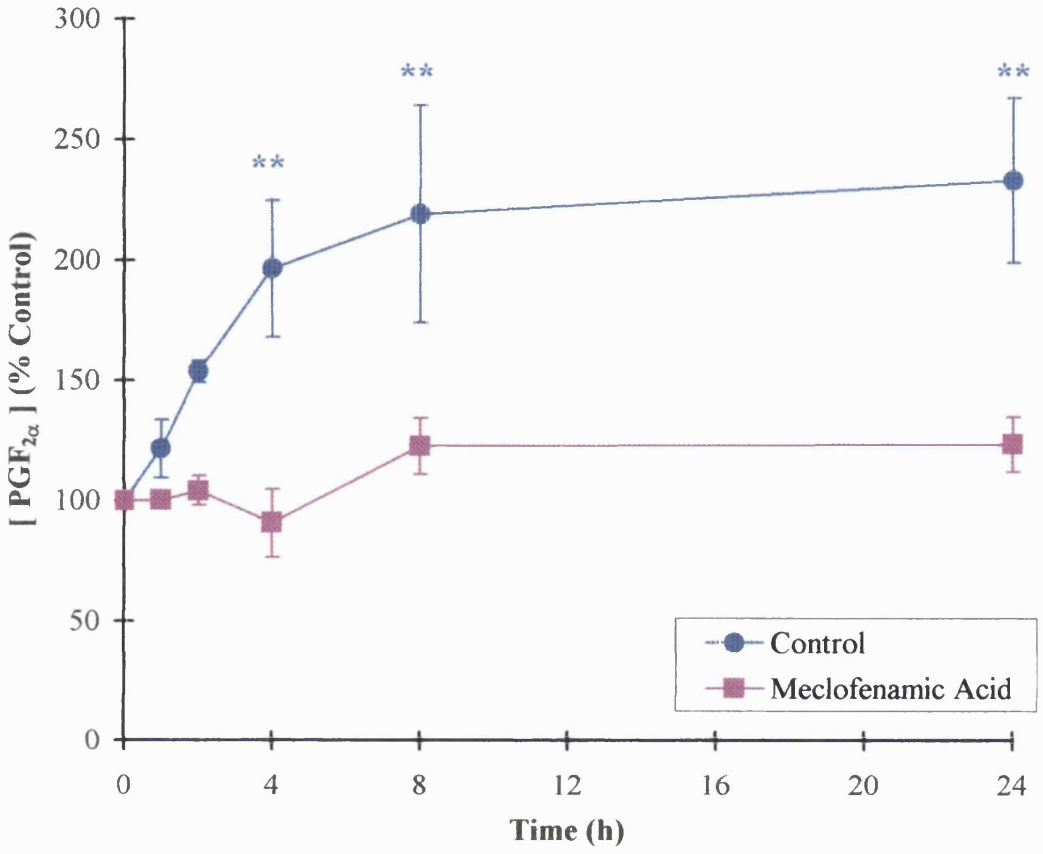

Figure 3.7 Time-dependent effects of meclofenamic acid on $PGF_{2\alpha}$ output by human granulosa-lutein cells

$PGF_{2\alpha}$ concentrations in spent medium, collected after incubation in the absence (-●-) or the presence (-■-) of 10μM meclofenamic acid for 0, 1, 2, 4, 8 or 24 hours. Values are the mean±SE for 3 independent experiments, with triplicate determinations in each experiment. $^{**}P<0.01$ relative to time zero in the absence or presence of meclofenamic acid as appropriate. (One way ANOVA; Dunnett's *post hoc* test)

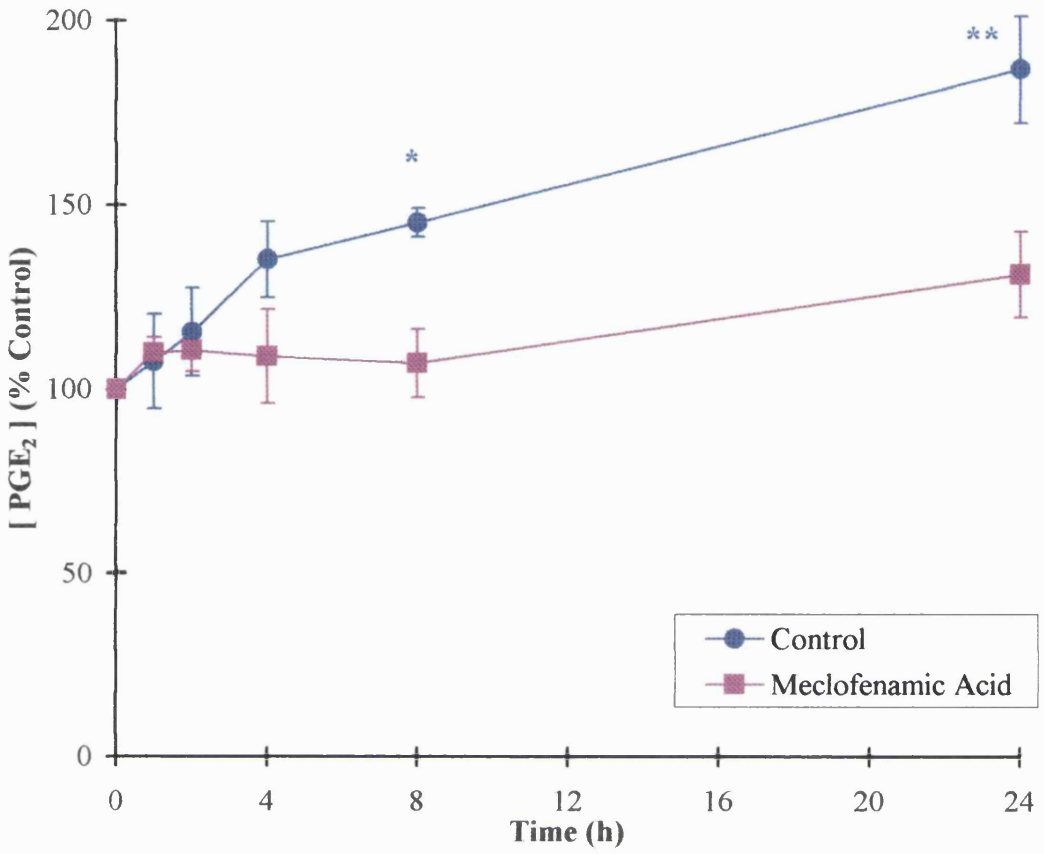

Figure 3.8 Time-dependent effects of meclofenamic acid on PGE_2 output by human granulosa-lutein cells

PGE_2 concentrations in spent medium, collected after incubation in the absence (—●—) or the presence (—■—) of 10μM meclofenamic acid for 0, 1, 2, 4, 8 or 24 hours. Values are the mean±SE for 3 independent experiments, with triplicate determinations in each experiment. *P<0.05, **P<0.01 relative to time zero in the absence or presence of meclofenamic acid as appropriate. (One way ANOVA; Dunnett's *post hoc* test)

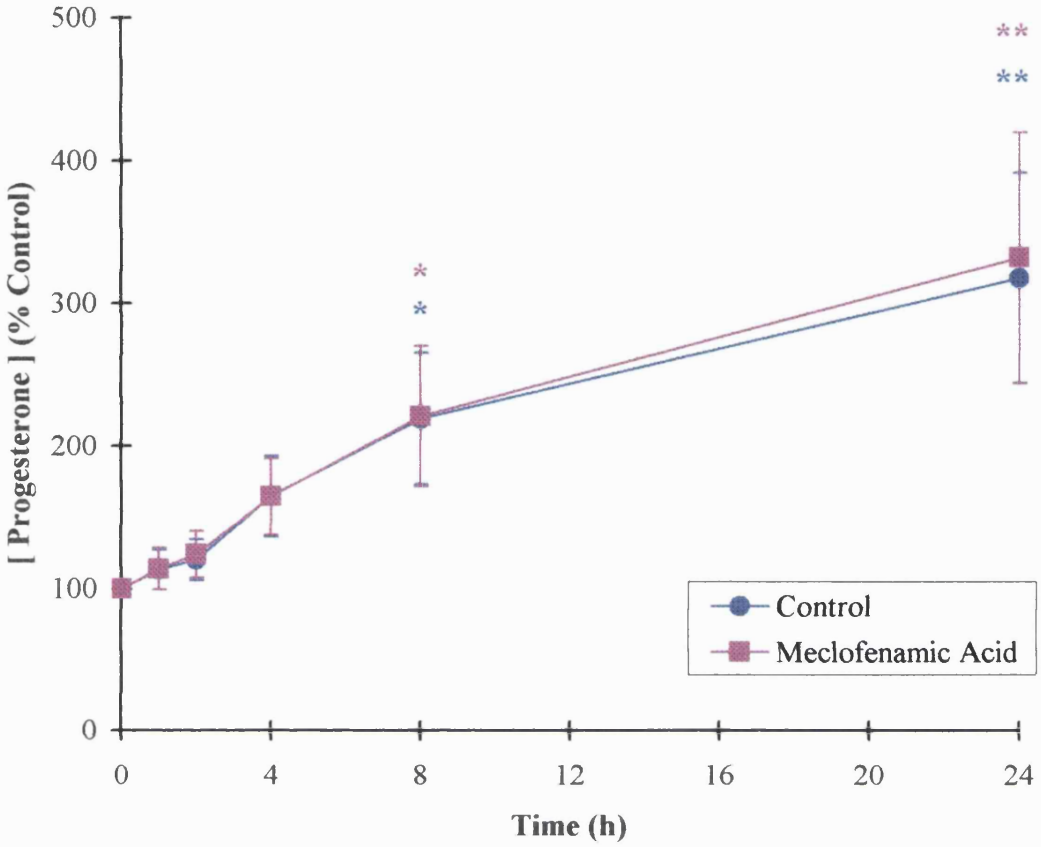

Figure 3.9 Time-dependent effects of meclofenamic acid on progesterone output by human granulosa-lutein cells

Progesterone concentrations in spent medium, collected after incubation in the absence (—●—) or the presence (—■—) of 10μM meclofenamic acid for 0, 1, 2, 4, 8 or 24 hours. Values are the mean±SE for 3 independent experiments, with triplicate determinations in each experiment. **P*<0.05, ***P*<0.01 relative to time zero in the absence or presence of meclofenamic acid as appropriate. (One way ANOVA; Dunnett's *post hoc* test)

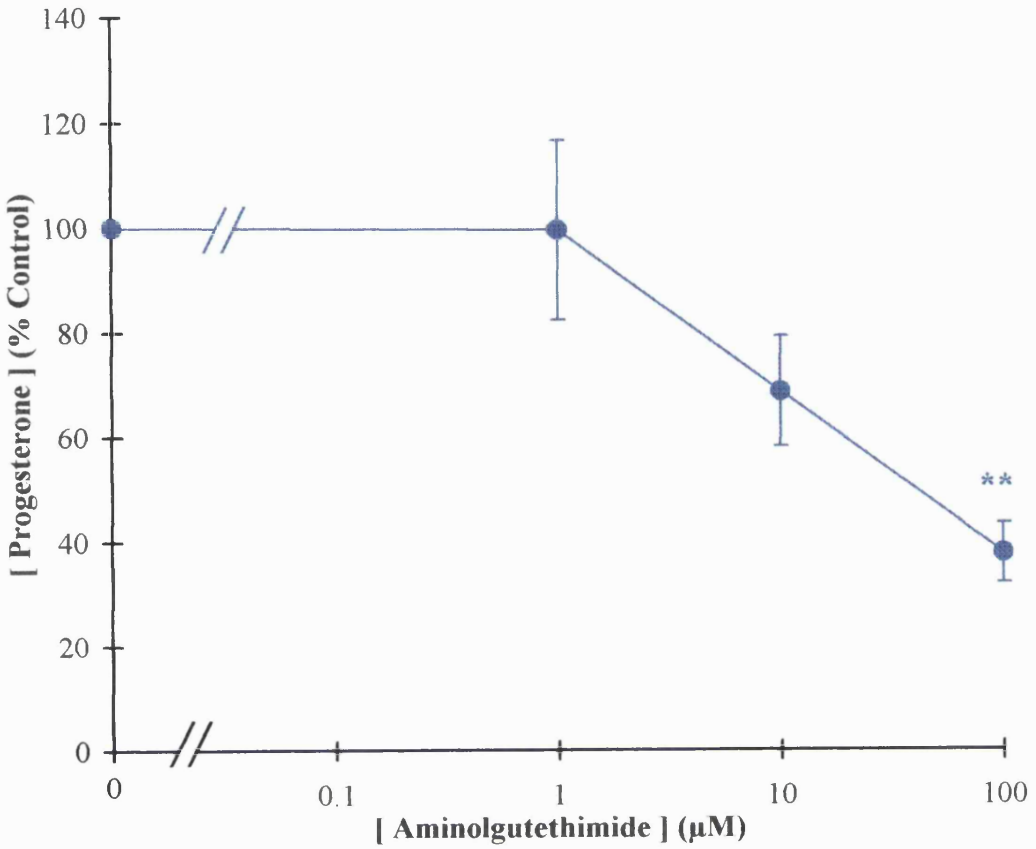

Figure 3.10 Concentration-dependent effects of aminoglutethimide on progesterone output by human granulosa-lutein cells

Progesterone concentrations in spent medium collected after a 24-hour incubation in the presence of 0-100μM aminoglutethimide. Values are the mean±SE for 5 independent experiments with triplicate determinations in each experiment. $^{**}P<0.01$ relative to 0μM aminoglutethimide (One way ANOVA; Dunnett's *post hoc* test)

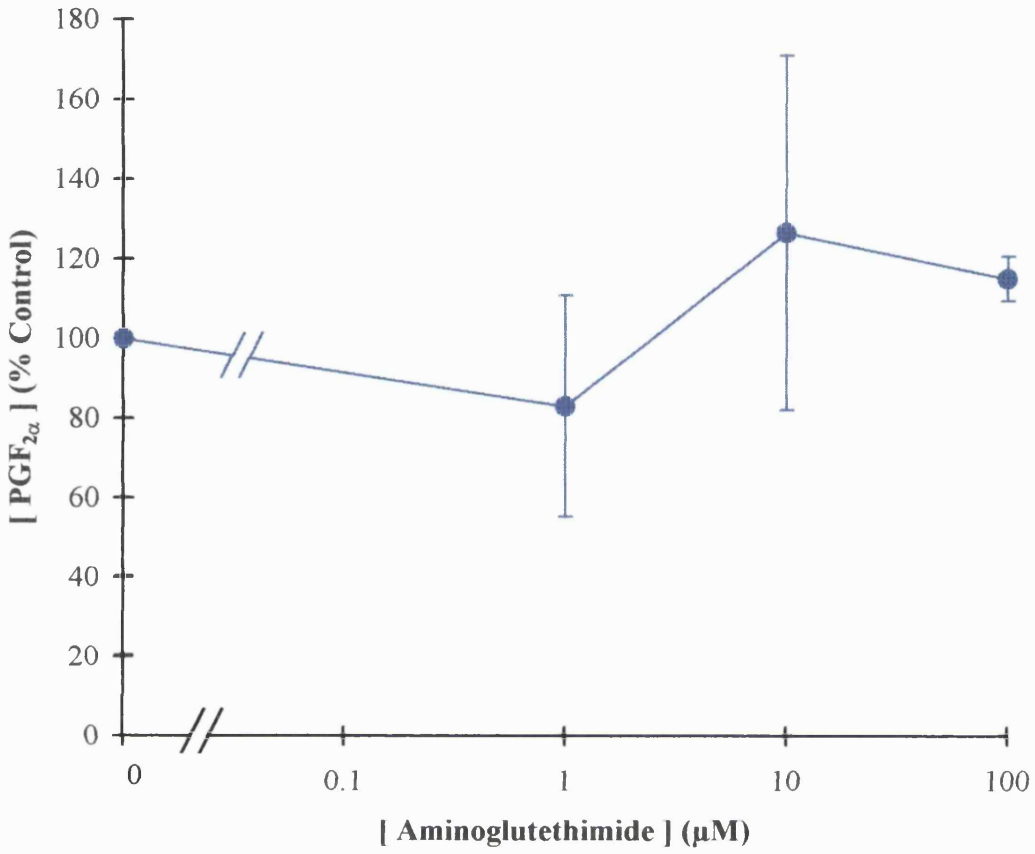

Figure 3.11 Concentration-dependent effects of aminoglutethimide on $PGF_{2\alpha}$ output by human granulosa-lutein cells

$PGF_{2\alpha}$ concentrations in spent medium collected after a 24-hour incubation in the presence of 0-100μM aminoglutethimide. Values are the mean±SE for 5 independent experiments with triplicate determinations in each experiment. (One way ANOVA; $P>0.05$)

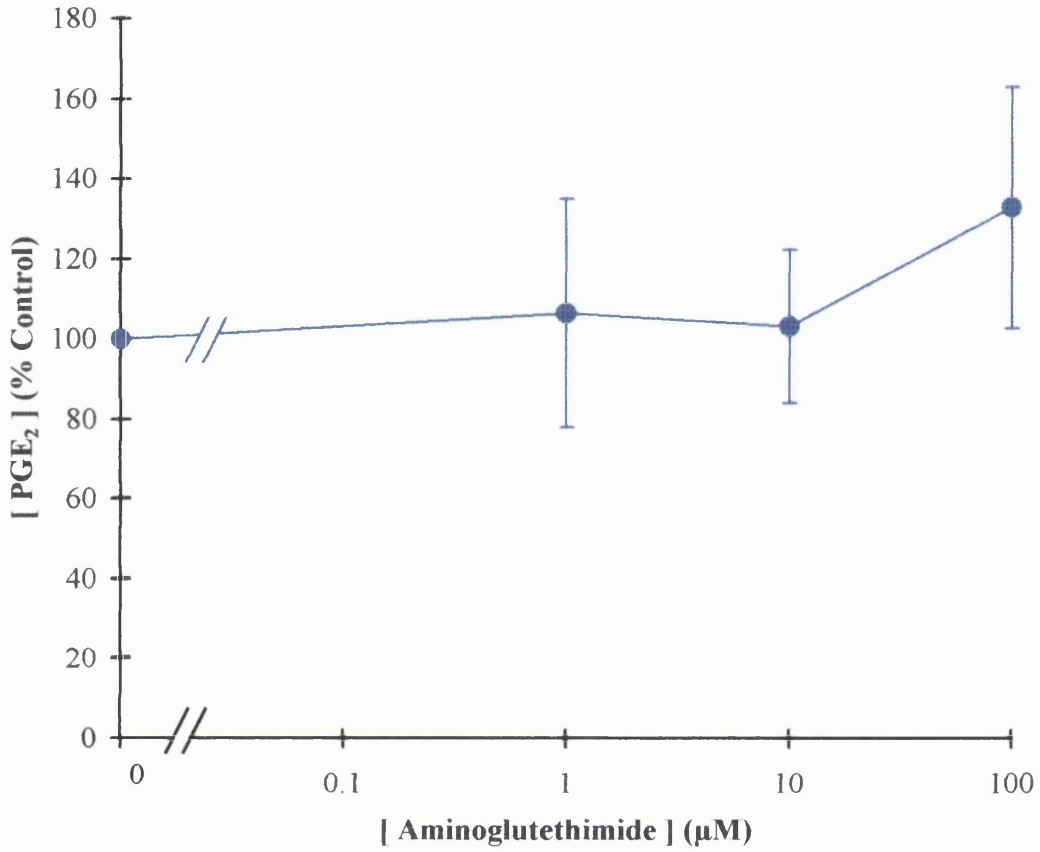

Figure 3.12 Concentration-dependent effects of aminoglutethimide on PGE_2 output by human granulosa-lutein cells

PGE_2 concentrations in spent medium collected after a 24-hour incubation in the presence of 0-100μM aminoglutethimide. Values are the mean±SE for 5 independent experiments with triplicate determinations in each experiment. (One way ANOVA; $P>0.05$)

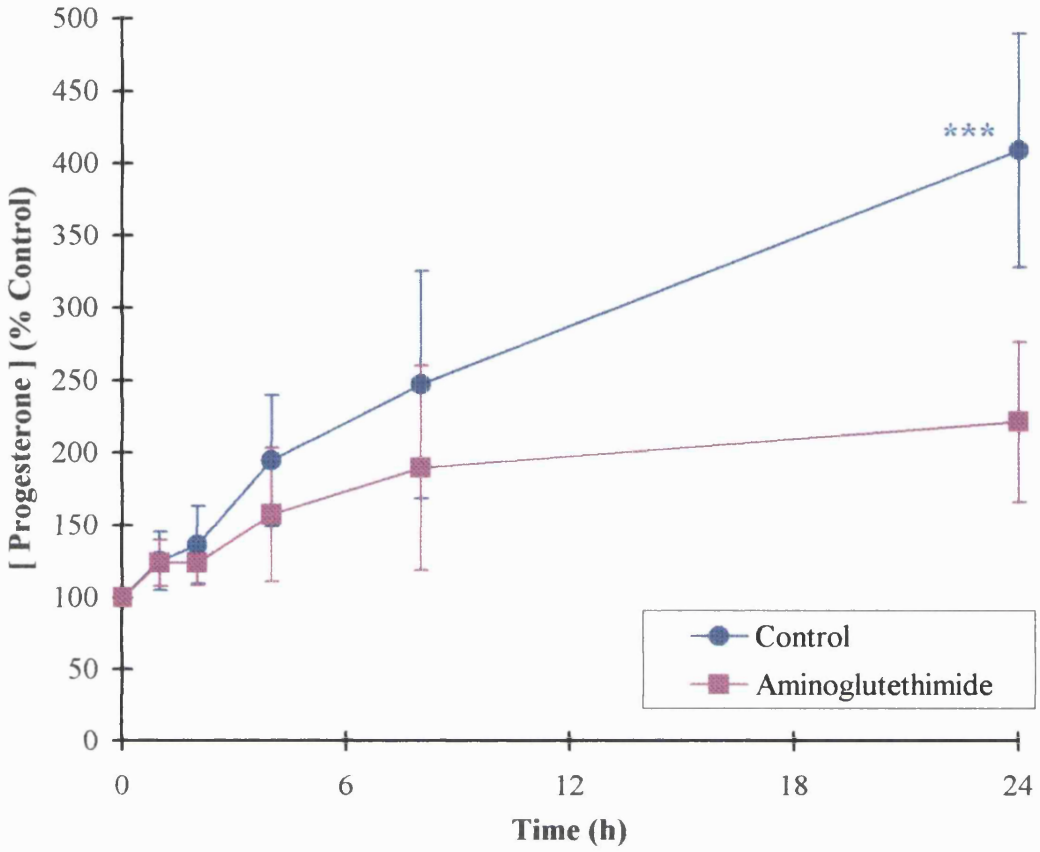

Figure 3.13 Time-dependent effects of aminoglutethimide on progesterone output by human granulosa-lutein cells

Progesterone concentrations in spent medium, collected after incubation in the absence (-●-) or the presence (-■-) of 100μM aminoglutethimide for 0, 1, 2, 4, 8 or 24 hours. Values are the mean±SE for 3 independent experiments, with triplicate determinations in each experiment. ***P<0.001 relative to time zero in the absence or presence of aminoglutethimide as appropriate. (One way ANOVA; Dunnett's *post hoc* test)

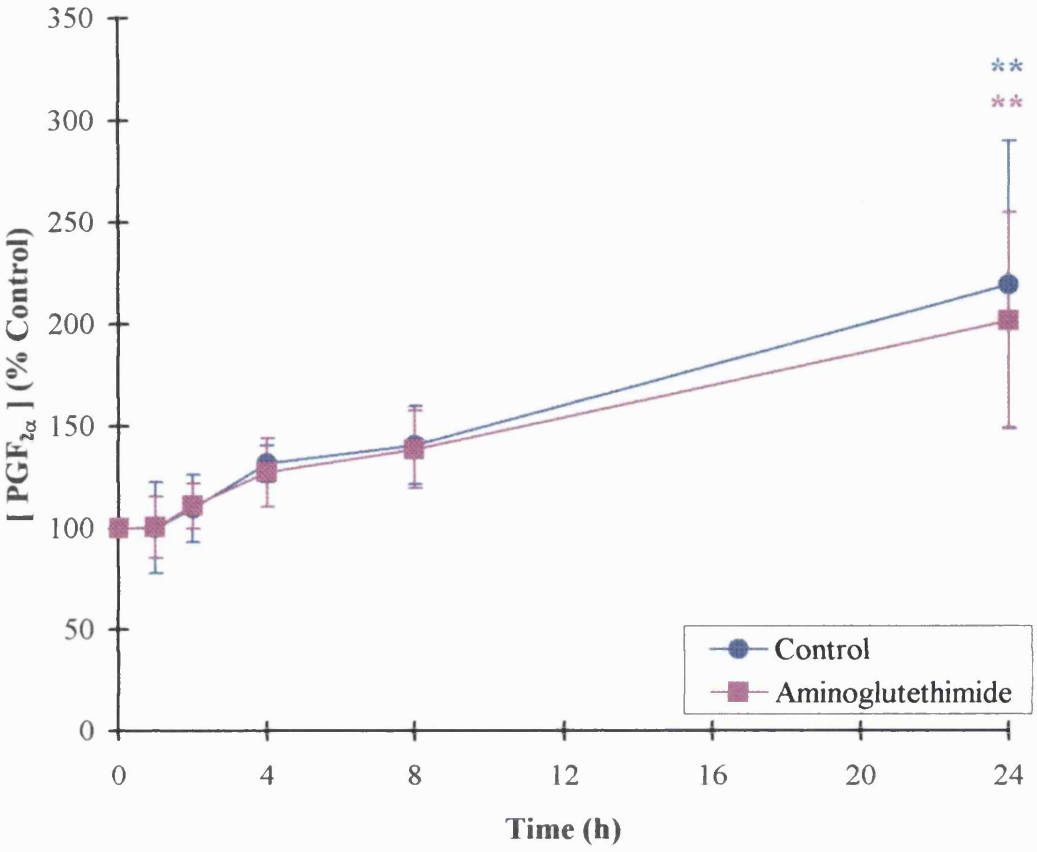

Figure 3.14 Time-dependent effects of aminoglutethimide on $PGF_{2\alpha}$ output by human granulosa-lutein cells

$PGF_{2\alpha}$ concentrations in spent medium, collected after incubation in the absence (—●—) or the presence (—■—) of 100μM aminoglutethimide for 0, 1, 2, 4, 8 or 24 hours. Values are the mean±SE for 3 independent experiments, with triplicate determinations in each experiment. $^{**}P<0.01$ relative to time zero in the absence or presence of aminoglutethimide as appropriate. (One way ANOVA; Dunnett's *post hoc* test)

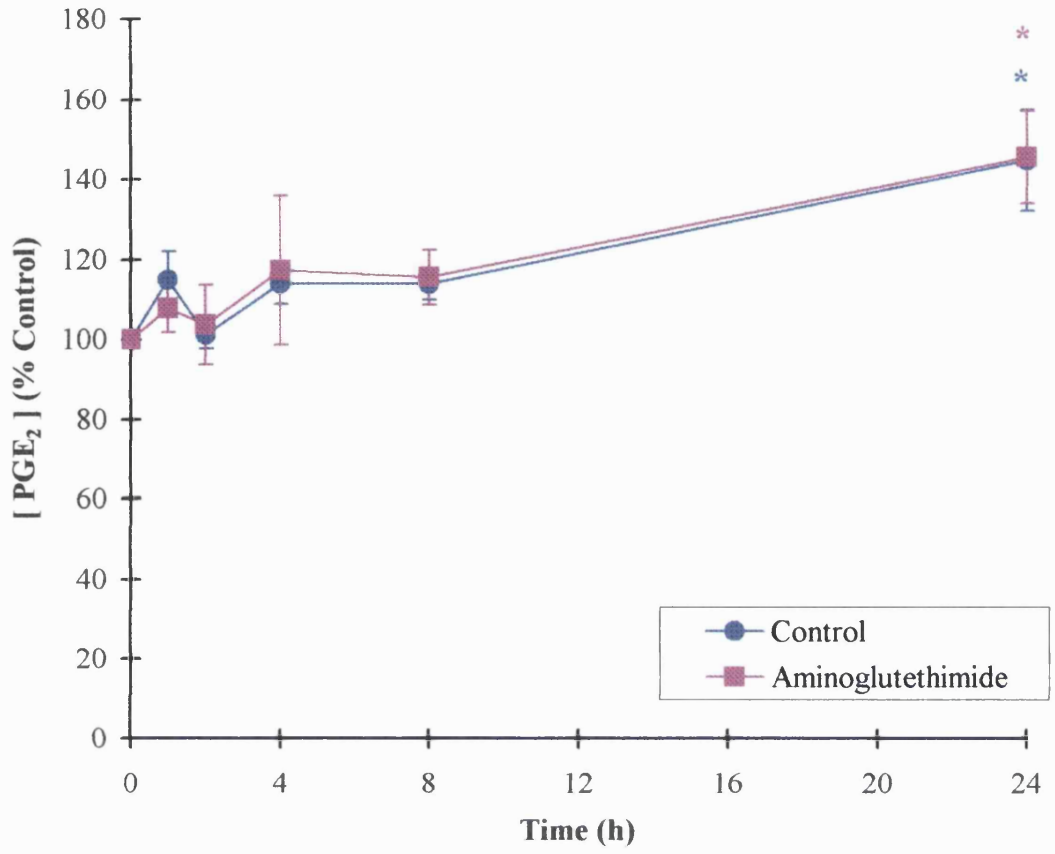

Figure 3.15 Time-dependent effects of aminoglutethimide on PGE_2 output by human granulosa-lutein cells

PGE_2 concentrations in spent medium, collected after incubation in the absence (—●—) or the presence (—■—) of 100μM aminoglutethimide for 0, 1, 2, 4, 8 or 24 hours. Values are the mean±SE for 3 independent experiments, with triplicate determinations in each experiment. *$P<0.05$ relative to time zero in the absence or presence of aminoglutethimide as appropriate. (One way ANOVA; Dunnett's *post hoc* test)

Basal concentrations of progesterone in the absence of aminoglutethimide increased in a time-dependent manner by up to 2.9±0.7-fold at 24 hours (n=3; $P<0.001$; Figure 3.13). However, incubation with 100μM aminoglutethimide abolished this time-dependent rise in progesterone output. Both $PGF_{2\alpha}$ and PGE_2 concentrations increased in a time-dependent manner for both untreated and treated cells. Treatment with an effective concentration of aminoglutethimide no effect on either $PGF_{2\alpha}$ (Figure 3.14) or PGE_2 (Figure 3.15) synthesis at any of the tested time points.

3.4 DISCUSSION

The data presented in this chapter suggest that there is a decline in the production of both $PGF_{2\alpha}$ and PGE_2 by human granulosa-lutein cells over the first 3 days of culture, which coincides with an increase in the output of progesterone through out the same period of time. This finding raises 3 possibilities.

1. Based on the documented ability of $PGF_{2\alpha}$ to inhibit ovarian progesterone synthesis [Richardson, 1986; Auletta & Flint, 1988; Michael *et al*, 1994; Olofsson & Leung, 1994], the decline in $PGF_{2\alpha}$ production may permit the rise of progesterone synthesis by the human granulosa cells.
2. The increase of progesterone output within the first 3 days in culture, may cause the inhibition of $PGF_{2\alpha}$ and PGE_2 production.
3. Finally, there may be no causal relationship between the pattern of production of PGs and progesterone in luteinizing cells.

In order for endogenous $PGF_{2\alpha}$ and PGE_2 to dictate the progesterone synthesis in luteinizing cells, the concentrations of these PGs need to be close to the respective K_d values for each of the FP and EP receptors respectively. On day 1 of culture, this was indeed the case with a mean $PGF_{2\alpha}$ concentration of 14.0±2.6nM (*i.e.* 14.0±2.6pmol/10^5 cells × 10^5 cells/ml) which approximates to the K_d of 13nM for [^{3}H]-$PGF_{2\alpha}$ binding to the human corpus luteum [Rao *et al*, 1977] and exceeds the K_d for the cloned human FP receptor of 8.3±2.5nM [Lake *et al*, 1994]. At present, there are four cloned subtypes of the EP receptor, named EP_1 to EP_4, with K_d values that range from 1nM for the human EP_1 receptor to 11nM for the human EP_2 receptor [Coleman *et al*, 1994] (section 1.6.2). According to these K_d values, the PGE_2 concentration of 4.0±0.8nM, as measured on day 1 of culture, should be capable of activating at least EP_1 and EP_3 receptor subtypes.

In order to examine whether the gradual decrease of $PGF_{2\alpha}$ production could account for the coincident increase in progesterone output, the concentration- and time-dependent effects of MA, a PGHS inhibitor, were examined. This anti-inflammatory agent resulted in the concentration-dependent decline of both $PGF_{2\alpha}$ and PGE_2 synthesis by up to 69.6±22.4% and 63.8±16.4% respectively at the highest tested meclofenamic acid concentration. Moreover, the effects of MA on PG production by human granulosa-lutein cells over time resulted in suppression of both $PGF_{2\alpha}$ and PGE_2 concentrations in the presence of this PGHS inhibitor. However, since MA did not significantly affect progesterone output either in a concentration- or in a time-dependent manner, this suggests that endogenously synthesised PGs do not dictate the steroidogenic output of human granulosa-lutein cells *in vitro*.

To establish whether the progressive rise in progesterone production could be the reason for the decrease in $PGF_{2\alpha}$ and PGE_2 concentrations, cells were incubated with a range of concentrations of AG. This cytochrome P_{450} inhibitor resulted in the decrease of progesterone production by 64.0±14.2% at the highest tested concentration used in this study, but had no effect on the output of either $PGF_{2\alpha}$ or PGE_2. As with MA, the effects of AG over time were also investigated. Treatment with an effective AG concentration over time resulted in suppression of progesterone output without affecting $PGF_{2\alpha}$ or PGE_2 production. This suggests that the decline in PG production in human granulosa-lutein cells is unlikely to be due to the increase in progesterone output.

In conclusion, inhibition of $PGF_{2\alpha}$ and PGE_2 output in human granulosa-lutein cells did not have an effect in the progesterone production, and *vice versa*, suppression of progesterone synthesis had no impact on the PG concentrations. This suggests that there is no causal relationship between the pattern of basal production of PGs and progesterone from luteinizing human granulosa cells *in vitro* and that the dynamic changes in their production occur independently of each other.

Chapter Four

ROLE OF PROSTAGLANDINS IN THE STEROIDOGENIC RESPONSE TO HIGH DENSITY LIPOPROTEINS (HDL)

Chapter Four

ROLE OF PROSTAGLANDINS IN THE STEROIDOGENIC RESPONSE TO HIGH DENSITY LIPOPROTEINS (HDL)

4.1 INTRODUCTION

Number of studies have investigated the HDL in the ovary. HDL_3 has shown an inability to support progesterone synthesis in human granulosa lutein cells [Tureck *et al*, 1982] and the human CL in organ culture has been shown to be unresponsive to HDL [Carr *et al*, 1982]. In contrast to these non-stimulatory effects, in human granulosa-lutein cells (hGLC), total HDL subfractions [Enk *et al*, 1987; Parinaud *et al*, 1987] and HDL_3 [Azhar *et al*, 1998a] have been shown to stimulate progesterone production. This suggests that HDL may provide cholesterol for steroid biosynthesis to human granulosa-lutein cells.

Since cholesterol is the substrate for steroidogenesis, granulosa cells require cholesterol in order to synthesise progesterone. However, the mechanism involved in the uptake of cholesterol from lipoproteins, and its fate once cholesterol is inside the cell, is still not completely understood in this cell type. One of the suggested mechanisms for HDL action is through the selective uptake of cholesterol from HDL particles. This has been shown to occur through SR-BI in ovarian cells from humans [Azhar *et al*, 1998b] and rats [Reaven *et al*, 1995; Reaven *et al*, 1998], and in human adrenocortical cells [Temel *et al*, 1997; Rigotti *et al*, 1997]. Furthermore, SR-BI has also been shown to act as a mediator of cellular cholesterol efflux in transfected CHO cells [Ji *et al*, 1997; Jian *et al*, 1998; De Llera-Moya *et al*, 1999]. SR-BII could also participate in cholesterol transport since it has been shown to be capable of mediating lipid transfer between cells and HDL [Webb *et al*, 1997; Webb *et al*, 1998]. Finally, the ABC-I transporter may be another mediator of cholesterol transport since it has been reported to be involved with cholesterol efflux from the cells.

Another possible cholesterol-independent mechanism for the HDL particle to stimulate progesterone production may be through the arachidonic acid residing in the phospholipid monolayer of HDL. Arachidonic acid is a known precursor for the

synthesis of eicosanoids including PGE_2 which in turn is able to stimulate progesterone production [Richardson *et al*, 1986; Michael *et al*, 1993a; Michael *et al*, 1994; Olofsson *et al*, 1994]. Furthermore, preliminary data indicate that HDL can also stimulate the production of PGE_2 in human granulosa-lutein cells [Ragoobir, 1999]. Moreover, Cockerill *et al* (1999) have shown that HDL has the ability to stimulate the expression of PGHS-2. Since PGE_2 is known to be luteotrophic, as previously mentioned, these observations raise the possibility that the steroidogenic response to HDL may be mediated by prostaglandins (PGs) such as PGE_2.

In any experiment involving plasma HDL, net delivery of cholesterol will be a potential contributing factor. Hence, to circumvent the confounding issues of net cholesterol (and arachidonic acid) delivery, all experiments were repeated using apolipoprotein-AI (Apo-AI), the major apolipoprotein of the HDL particle.

The main objective of the work described in this chapter was to investigate the role of PGs in the steroidogenic response of human granulosa lutein cells to HDL and Apo-AI.

4.2 EXPERIMENTAL PROTOCOLS

In all experiments described in this chapter, cells were seeded into sterile 96-well culture plates at a density of 1×10^5 cells/ml culture medium with a volume of 250µl medium per well. All experiments were stopped by addition of 10µl 3M perchloric acid per well, followed by storage at –20°C pending assay. Immediately prior to assays, 20µl 2.16M tripotassium orthophosphate were added into each well.

For the purpose of presentation and discussion all data were internally standardised. Those data are presented graphically as percentage values (mean±SE) (where the control PGE_2, cAMP or progesterone concentrations for each experimental design was standardised to 100%), however, all statistical analyses were performed on non-referenced data.

4.3 RESULTS

4.3.1 Effects of HDL on PGE_2, cAMP and progesterone production

4.3.1.1 Concentration-dependent effects of HDL

Cells were cultured for two days in serum-supplemented medium to allow the cells to attach to the plate. On the third day of culture, cells were rinsed with warmed serum-free medium and then incubated for a further 24 hours in serum-free medium containing HDL at concentrations of 0, 1, 3, 10, 30 and 100 μg protein/ml in the presence of 0.5mM isobutyl-methyl xanthine (IBMX). At the end of the 24 hour incubation period, 10μl 3M perchloric acid solution were added to each well and the spent medium was collected and stored at -20°C pending assay of the PGE_2, cAMP and progesterone concentrations by specific RIAs (section 2.3).

Consistent with previous observations [Ragoobir, 1999], incubation for 24 hours with HDL resulted in a concentration-dependent increase of progesterone production which became significant only at the highest tested HDL concentration of 100μg protein/ml (Figure 4.1.); progesterone production increased by 5.7±2.3-fold relative to the untreated cells (n=4; $P<0.05$).

Treatment with HDL over a 24-hour period resulted in a similar concentration-dependent increase of PGE_2 production which was significant only at an HDL concentration of 100μg/ml (Figure 4.2). At this maximum tested concentration, HDL stimulated PGE_2 production by 14.4±4.8-fold relative to the control cells (n=4; $P<0.001$).

Incubation for 24 hours with HDL appeared to have a similar but less marked effect on cAMP accumulation. A significant increase was obtained only at the highest tested concentration of 100μg/ml (Figure 4.3). The cAMP concentration increased by 42.3±9.2% relative to the cells that had not been treated with HDL (n=4; $P<0.01$).

4.3.1.2 Time-dependent effects of HDL

Cells were incubated for two days in serum supplemented medium and on the third day of culture, were rinsed with warmed serum-free medium and then incubated in serum-free medium containing HDL (100μg/ml) for 0, 1, 2, 4, 8 & 24 hours (in the presence of 0.5mM IBMX). At each time point, selected wells were terminated by

the addition of 10µl perchloric acid and concentrations of progesterone, cAMP and PGE_2 were assessed as discussed above.

The basal concentrations of progesterone did not change at any of the tested time points (Figure 4.4). In the presence of HDL (100µg/ml), at time points 1, 2, 4 and 8 hours progesterone concentrations did not differ from control, however there was a significant increase in the progesterone production at 24 hours. At this time point, the progesterone output increased to 13.3±33.7-fold (n=4; $P<0.01$).

Basal concentrations of PGE_2 did not increase at any of the tested time points. However, incubation with HDL over the same time frame resulted in an increase of PGE_2 production which became significant only after treatment for 24 hours (n=4; $P<0.001$) (Figure 4.5).

A similar pattern was noted for the cAMP concentrations (Figure 4.6). Basal cAMP production did not increase over the 24-hour period whereas incubation with HDL (100µg/ml) caused the production of cAMP to increase to 47.1±6.5% at 24 hours (n=4; $P<0.01$).

4.3.2 Concentration-dependent effects of Apolipoprotein-AI on progesterone, PGE_2 and cAMP production

As mentioned before (Section 1.4.1.2), Apo-AI is one of the major protein components of the HDL particle. It comprises approximately 70% of the protein moiety of HDL. Hence the ability of Apo-AI to stimulate PGE_2, cAMP and progesterone production by human granulosa cells was examined.

Cells were cultured for two days in serum-supplemented medium to allow the cells to attach to the plate. On the third day of culture, cells were rinsed with warmed serum-free medium and then incubated for a further 24 hours in serum-free medium containing HDL or Apo-AI at concentrations of 0, 1, 3, 10, 30 and 100 µg/ml (in the presence of 0.5mM IBMX). Apo-AI was purchased from two sources; Sigma (UK) and Athens (USA). At the end of the 24 hour incubation period the spent medium was collected and stored at -20°C pending assay of the progesterone, PGE_2 and cAMP concentrations as mentioned above. HDL, Apo-AI Sigma (UK) and Apo-AI

Athens (USA) concentrations were prepared in phosphate-buffered saline (PBS), Tris-buffered saline (TBS) and 10mM ammonium bicarbonate solution respectively, the final concentrations of which were adjusted in all wells to 10% (v/v) respectively.

Cells incubated with HDL exhibited a concentration-dependent increase in progesterone production which became significant only at the highest tested concentration of 100μg/ml. At this concentration of HDL, progesterone production increased by 113.7±8.7% (n=3; $P<0.01$) (Figure 4.7) similar to the responses observed in earlier experiments (Figure 4.1). In cells treated with Apo-AI from Sigma (UK), production of progesterone only increased significantly at the highest tested concentration of Apo-AI (44.7±7.5% increase; n=3; $P<0.01$). However, the progesterone output by cells treated with Apo-AI purchased from Athens, USA, was not affected at any of the tested concentrations of Apo-AI.

Incubation for 24 hours with increasing concentrations of HDL resulted in a similar stimulation of PGE_2 production, as previously described; HDL stimulated a concentration-dependent increase in PGE_2 accumulation that was only significant at the highest tested concentration of 100μg/ml (1.7±0.3-fold increase n=4; $P<0.01$) (Figure 4.8). Incubation over the same period of time with range of concentrations of Apo-AI, purchased from Sigma (UK), resulted in a similar PGE_2 response. Significance was exhibited only at the highest tested concentration of 100μg Apo-AI protein/ml (1.5±0.3-fold increase; n=4; $P<0.01$). However, treatment with the Apo-AI purchased from Athens (USA), did not stimulate PGE_2 production in human GCs over the 24-hour incubation at any of the tested concentrations.

As previously shown cAMP concentrations increased following the 24-hour incubation with a range of concentrations of HDL. The rise in cAMP accumulation was only significant at the highest tested concentration of HDL (100μg/ml) (70.0±26.4% increase; n=4 $P<0.05$) (Figure 4.9). Cells that were incubated with increasing concentrations of Apo-AI form Sigma (UK), exhibited increased accumulation of cAMP in a similar way as that described for HDL. The production of cAMP increased by 47.1±17.0% relative to the respective control at the maximum tested Apo-AI concentration of 100μg/ml (n=4; $P<0.01$). In contrast, incubation

with Apo-AI Athens, USA, did not affect cAMP production by the cells at any of the tested concentrations.

4.3.3 Effects of ammonium bicarbonate on PGE_2, cAMP and progesterone production

Apolipoprotein-AI (Apo-AI) purchased from Athens (USA), was commercially prepared at a concentration of 1mM in a 10mM solution of ammonium bicarbonate (NH_4HCO_3). In order to reach Apo-AI concentrations as high as 100μg protein/ml, as required for the design of the above experiments, the final concentration of NH_4HCO_3 in the well had to reach concentrations as high as 1mM. Such a high concentration might have directly affected the production of PGE_2, cAMP and progesterone by human granulosa cells. As a consequence, the effects of NH_4HCO_3 on PGE_2, cAMP and progesterone production by human GCs had to first be assessed. Cells were cultured for two days in serum-supplemented medium to allow the cells to attach to the plate. On the third day of culture, cells were rinsed with warmed serum-free medium and then incubated for a further 24 hours in serum-free medium containing 0, 0.01, 0.1, 1, 10, 100, 1000 and 10,000μM NH_4HCO_3, in the presence of 0.5mM IBMX. At the end of the 24-hour incubation period, the spent medium was collected and stored at -20°C pending assay of the PGE_2, cAMP and progesterone concentrations as mentioned above.

Incubation for 24 hours with NH_4HCO_3 resulted in a concentration-dependent decrease in the production of PGE_2. This effect was maximal at a NH_4HCO_3 concentration of 1mM; at this concentration, NH_4HCO_3 suppressed PGE_2 concentration by 50.4±5.8% (n=5; $P<0.01$) (Table 4.1). However, incubation for 24-hours with a range of concentrations of NH_4HCO_3 did not have any effect on the production of either cAMP or progesterone by human granulosa cells.

Table 4.1 **Concentration-dependent effects of ammonium bicarbonate on the accumulation of PGE_2, cAMP and progesterone**
PGE_2, cAMP and progesterone concentrations in spent medium collected after a 24-hour incubation in the presence of a range of concentrations of NH_4HCO_3, ranging from 0 to 10,000μM. Values are the mean±SE for 5 independent experiments with quadruplicate determinations in each experiment. *$P<0.05$, **$P<0.01$ relative to the respective control (One way ANOVA; Dunnett's *post hoc* test)

[NH_4HCO_3] (μM)	[PGE_2] (pmol/10^5cells.24h)	[cAMP] (pmol/10^5cells.24h)	[Progesterone] (pmol/10^5cells.24h)
0	4.4±1.0	5.7±0.5	1042.7±159.0
0.01	3.4±1.0	5.4±0.6	958.1±136.4
0.1	2.5±0.8 **	5.2±0.4	858.9±124.3
1	3.1±1.0	5.1±0.2	849.2±163.7
10	2.4±0.9 **	5.1±0.6	854.8±224.5
100	2.6±0.7 *	5.1±0.4	944.0±208.5
1,000	2.0±0.3 **	5.7±0.5	875.5±125.1
10,000	3.0±0.6	5.2±0.4	964.8±119.4

4.3.4 Effects of Meclofenamic acid on PGE_2, cAMP and progesterone production in response to HDL

In order to examine the possible involvement of PGs in the steroidogenic and cAMP responses to HDL and Apo-AI, cells were co-treated with meclofenamic acid (MA). Cells were cultured for two days in serum-supplemented medium to allow the cells to attach to the plate. On the third day of culture, cells were rinsed with warmed serum-free medium and then incubated for a further 24 hours in serum-free medium containing HDL (100μg protein/ml) or Apo-AI, Sigma, UK (100μg protein/ml) each in the presence and absence of 10μM MA. At the end of the 24 hour incubation the spent medium was collected and stored at -20°C pending RIAs for measurement of PGE_2, cAMP and progesterone concentrations as previously described. Since data obtained from experiments described in section 4.2.2 demonstrated that PBS and TBS alone did not affect the concentrations of PGE_2, cAMP or progesterone, the control cells for HDL (0μg protein/ml) and Apo-AI Sigma (UK) (0μg protein/ml) acquired PBS, the HDL vehicle. The final concentration of PBS was adjusted in all wells to 10% (v/v).

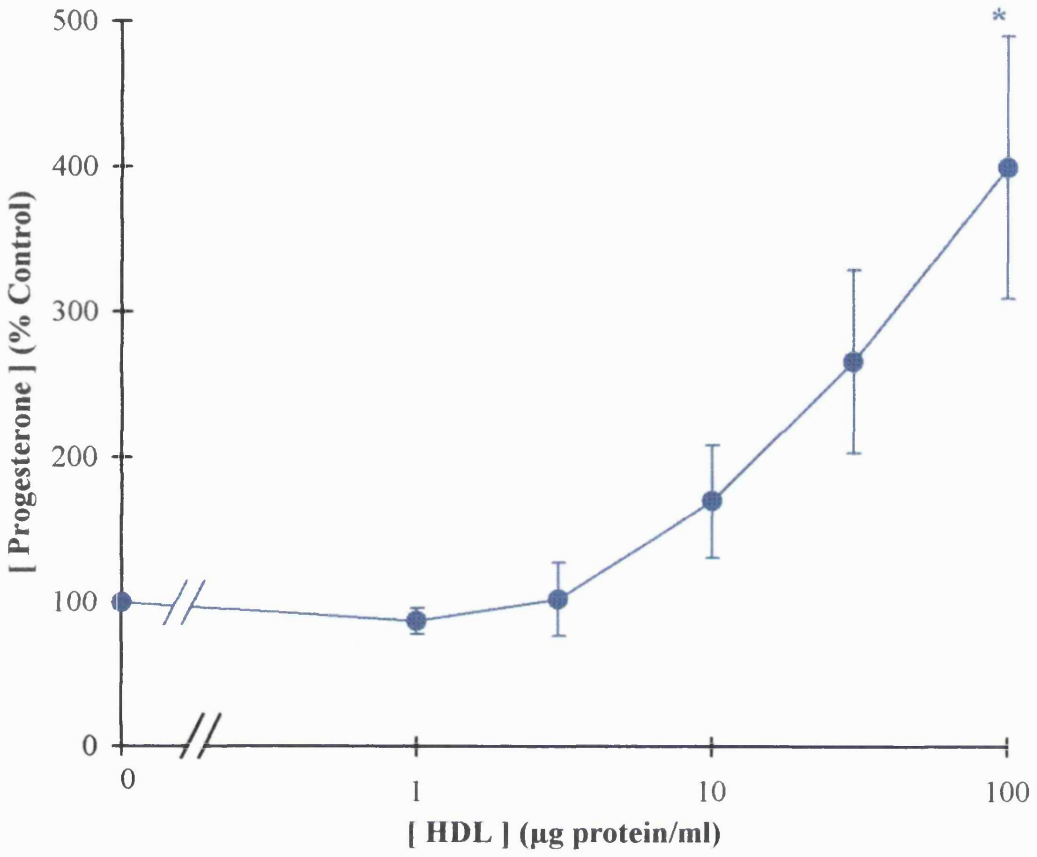

Figure 4.1 Concentration-dependent effects of HDL on progesterone production by human granulosa-lutein cells

Progesterone concentrations in spent medium collected after a 24-hour incubation in the presence of a range of concentrations of HDL, ranging from 0 to 100µg protein/ml. Values are the mean±SE for 4 independent experiments with quadruplicate determinations in each experiment. *$P<0.05$ relative to 0µg/ml protein (One way ANOVA; Dunnett's *post hoc* test)

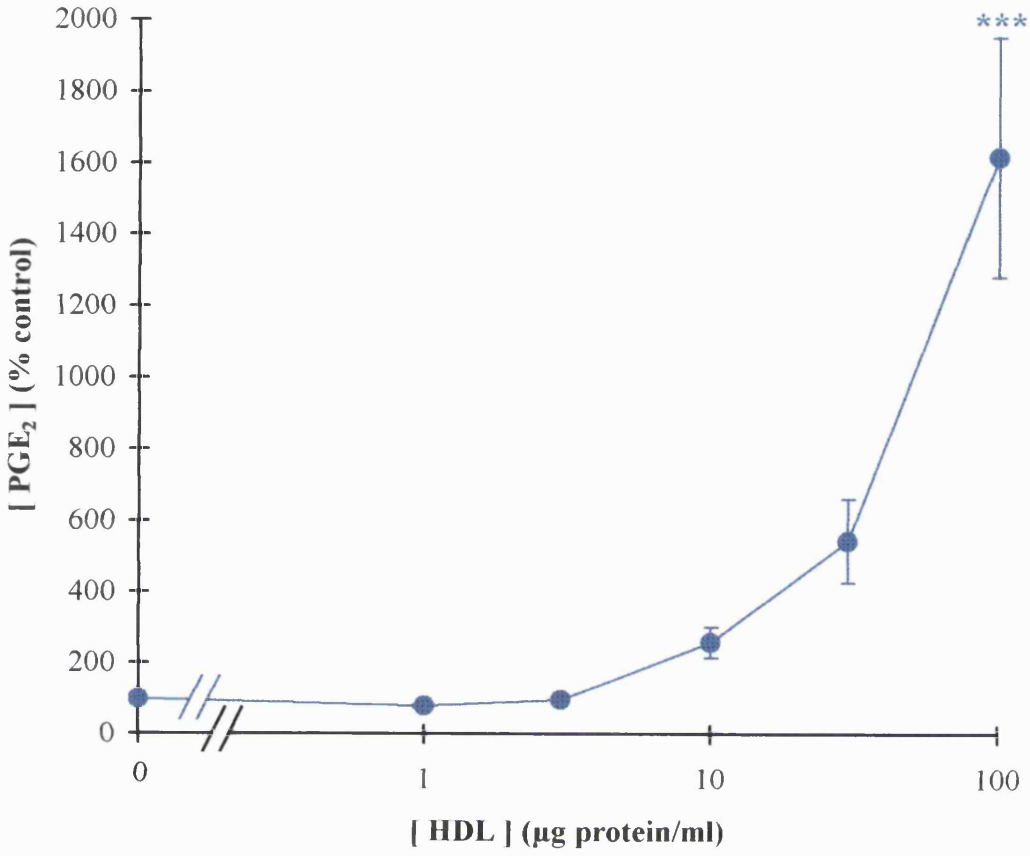

Figure 4.2 Concentration-dependent effects of HDL on production of PGs in human granulosa-lutein cells

PGE_2 concentrations in spent medium collected after a 24-hour incubation in the presence of a range of concentrations of HDL, ranging from 0 to 100µg protein/ml. Values are the mean±SE for 4 independent experiments with quadruplicate determinations in each experiment. ***$P<0.001$ relative to 0µg/ml protein (One way ANOVA; Dunnett's *post hoc* test)

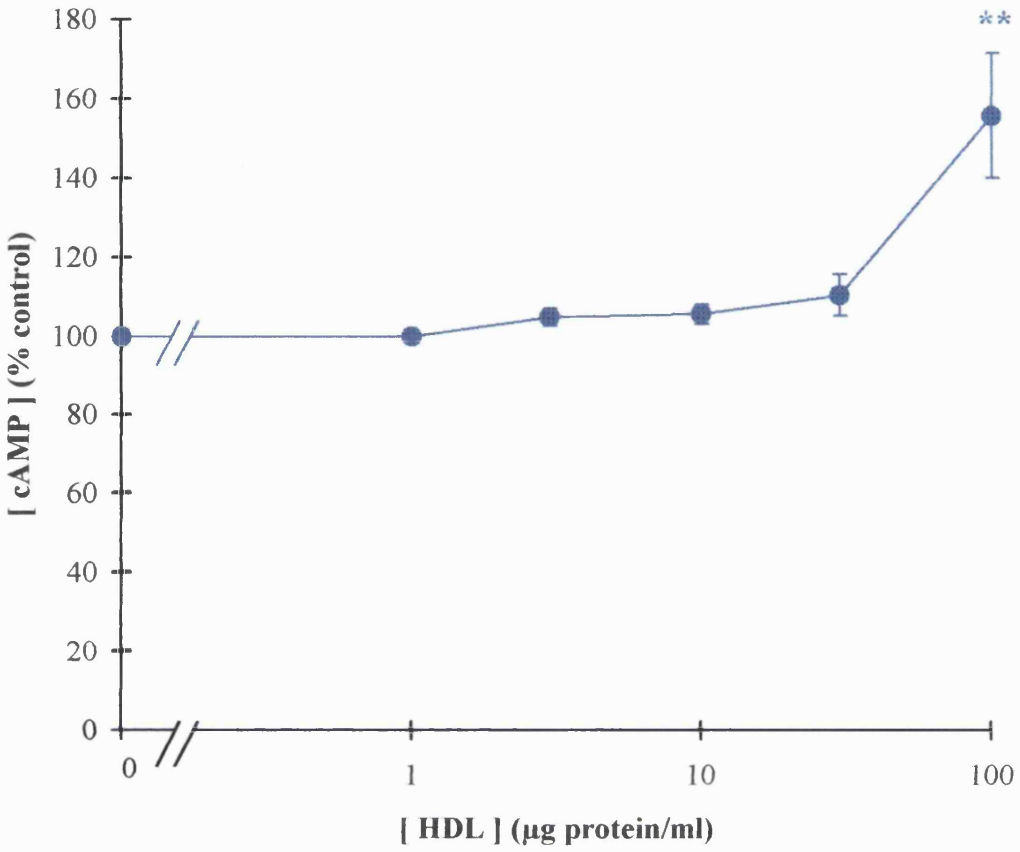

Figure 4.3 Concentration-dependent effects of HDL on the accumulation of cAMP in human granulosa-lutein cells

cAMP concentrations after a 24-hour incubation in the presence of a range of concentrations of HDL, ranging from 0 to 100µg protein/ml. Values are the mean±SE for 4 independent experiments with quadruplicate determinations in each experiment. $^{**}P<0.01$ relative to 0µg/ml protein (One way ANOVA; Dunnett's *post hoc* test)

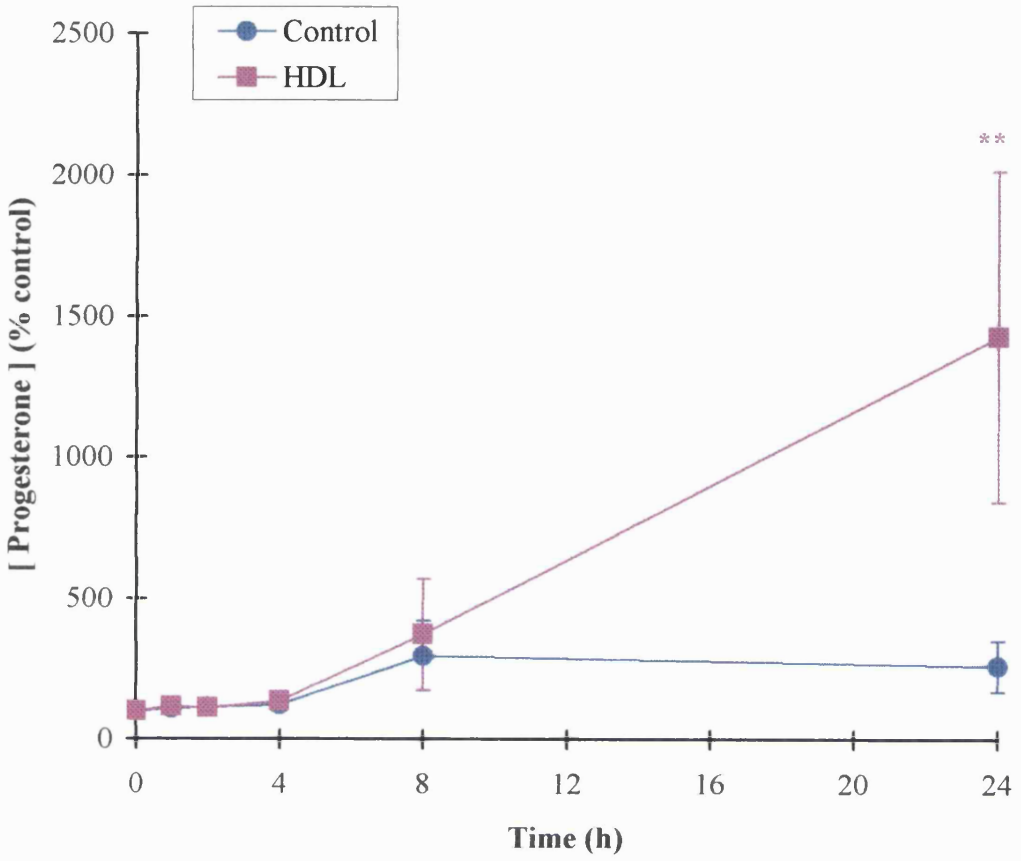

Figure 4.4 Time-dependent effects of HDL on progesterone production by human granulosa-lutein cells

Progesterone concentrations in spent medium, collected after incubation in the absence (-●-) or the presence (-■-) of HDL (100μg protein/ml) for 0, 1, 2, 4, 8 or 24 hours. Values are the mean±SE for 4 independent experiments, with quadruplicate determinations in each experiment. $^{**}P<0.01$ relative to control (cells treated with HDL at time point zero). (One way ANOVA; Dunnett's *post hoc* test)

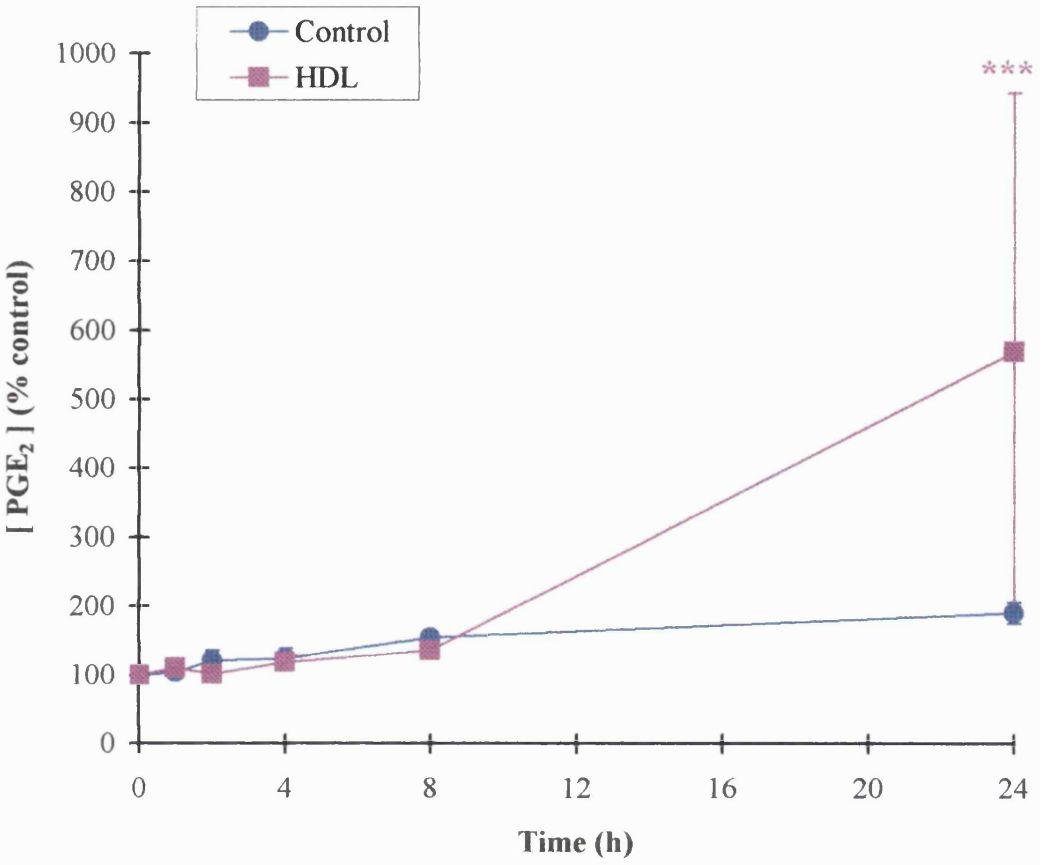

Figure 4.5 Time-dependent effects of HDL on PG production by human granulosa-lutein cells

PGE_2 concentrations in spent medium, collected after incubation in the absence (-●-) or the presence (-■-) of HDL (100μg protein/ml) for 0, 1, 2, 4, 8 or 24 hours. Values are the mean±SE for 4 independent experiments, with quadruplicate determinations in each experiment. ***$P<0.001$ relative to control (cells treated with HDL at time point zero). (One way ANOVA; Dunnett's *post hoc* test)

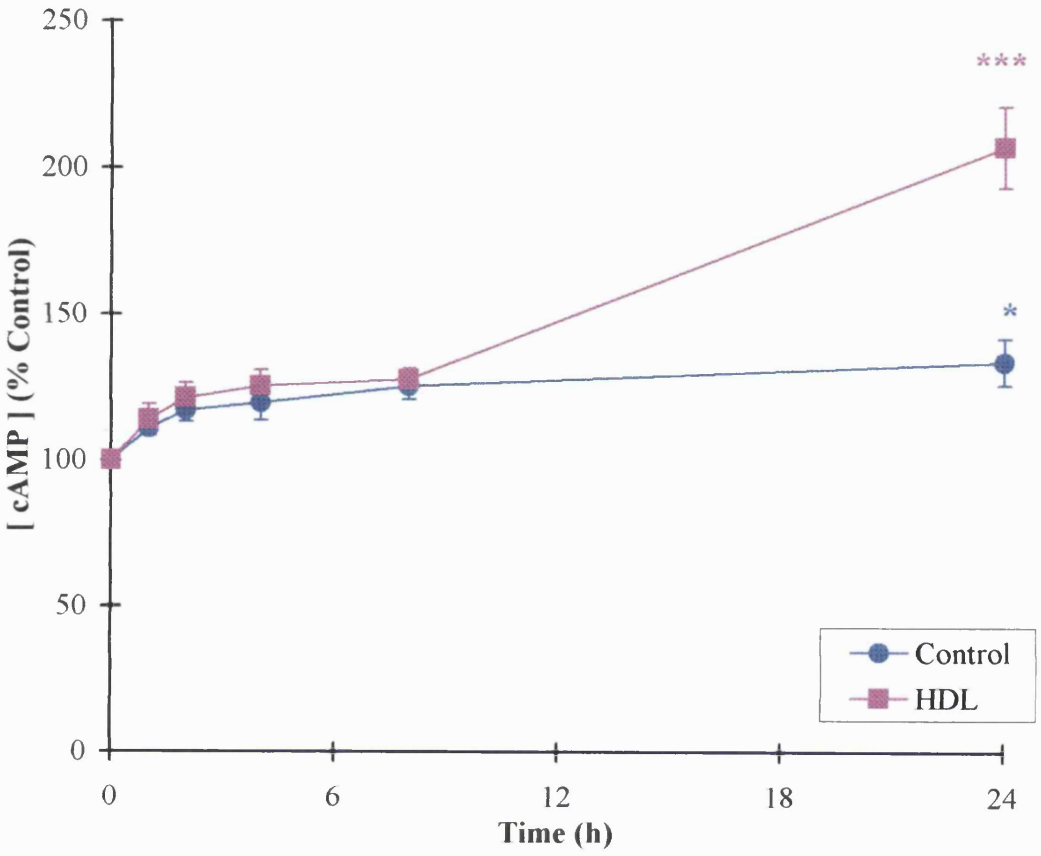

Figure 4.6 Time-dependent effects of HDL on the accumulation of cAMP in human granulosa-lutein cells

cAMP concentrations after incubation in the absence (—●—) or the presence (—■—) of HDL (100μg protein/ml) for 0, 1, 2, 4, 8 or 24 hours. Values are the mean±SE for 4 independent experiments, with quadruplicate determinations in each experiment. $^{**}P<0.01$ relative to control (cells treated with HDL at time point zero); (One way ANOVA; Dunnett's *post hoc* test)

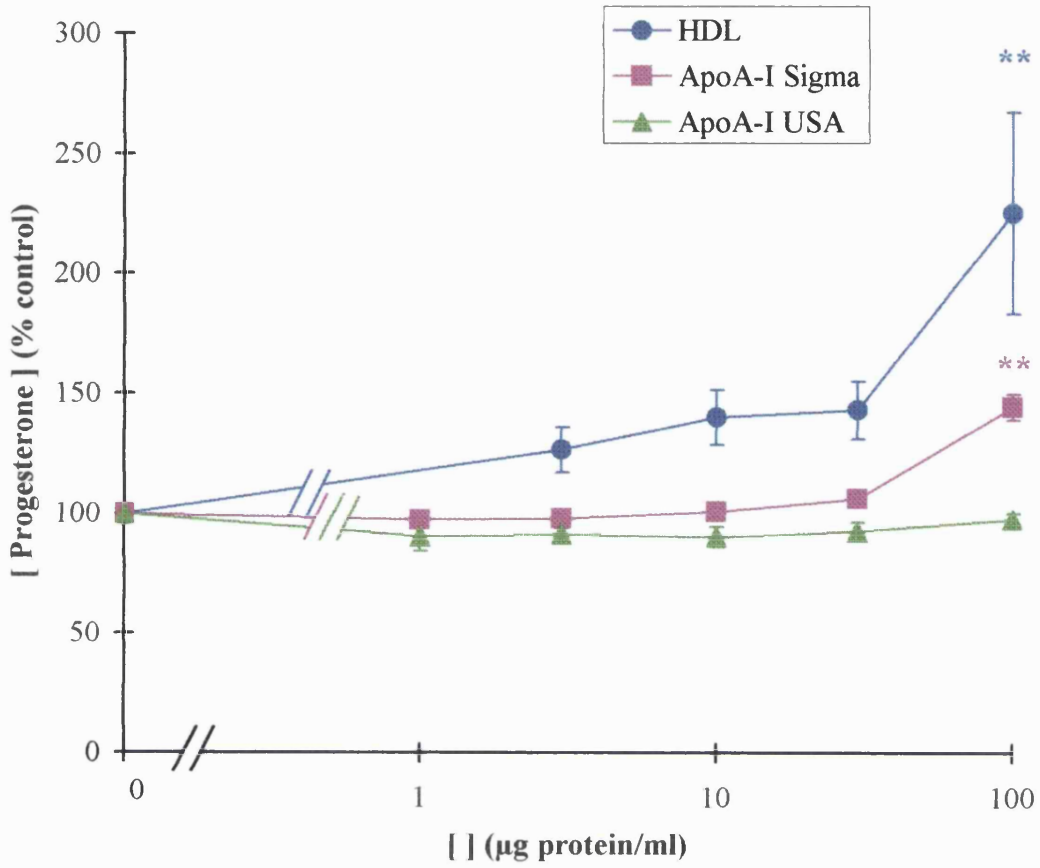

Figure 4.7 Concentration-dependent effects of HDL and Apo-AI on progesterone production by human granulosa-lutein cells

Progesterone concentrations in spent medium collected after a 24-hour incubation in the presence of a range of concentrations of HDL (—●—), Apo-AI Sigma (—■—), or Apo-AI Athens (—▲—) ranging from 0 to 100µg protein/ml. Values are the mean±SE for 4 independent experiments with quadruplicate determinations in each experiment. $^{**}P<0.01$ in the absence of HDL or Apo-AI Sigma as appropriate. (One way ANOVA; Dunnett's *post hoc* test)

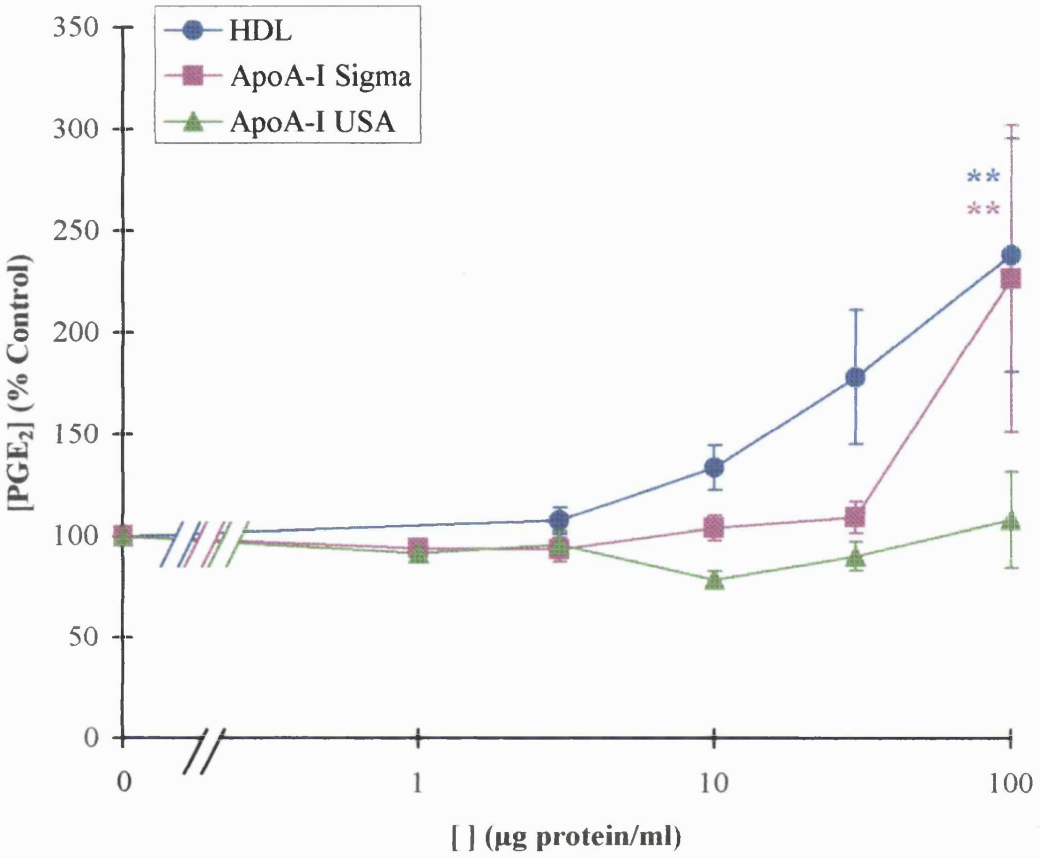

Figure 4.8 Concentration-dependent effects of HDL and Apo-AI on production of PGs by human granulosa-lutein cells

PGE_2 concentrations in spent medium collected after a 24-hour incubation in the presence of a range of concentrations of HDL (—●—), Apo-AI Sigma (—■—), or Apo-AI Athens (—▲—) ranging from 0 to 100µg protein/ml. Values are the mean±SE for 4 independent experiments with quadruplicate determinations in each experiment. $^{**}P<0.01$ in the absence of HDL or Apo-AI Sigma as appropriate. (One way ANOVA; Dunnett's *post hoc* test)

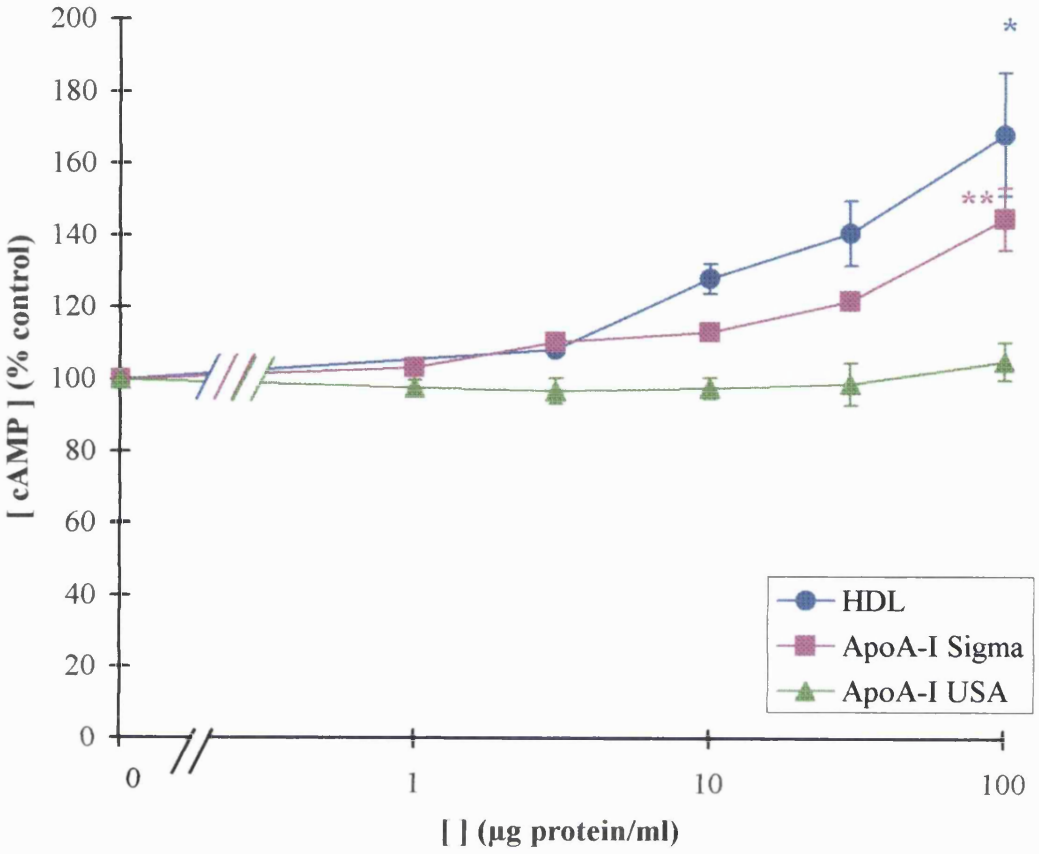

Figure 4.9 Concentration-dependent effects of HDL and Apo-AI on the accumulation of cAMP in human granulosa-lutein cells

cAMP concentrations after a 24-hour incubation in the presence of a range of concentrations of HDL (—●—), Apo-AI Sigma (—■—), or Apo-AI Athens (—▲—) ranging from 0 to 100µg protein/ml. Values are the mean±SE for 4 independent experiments with quadruplicate determinations in each experiment. *P<0.05, **P<0.01 in the absence of HDL or Apo-AI Sigma as appropriate. (One way ANOVA; Dunnett's *post hoc* test)

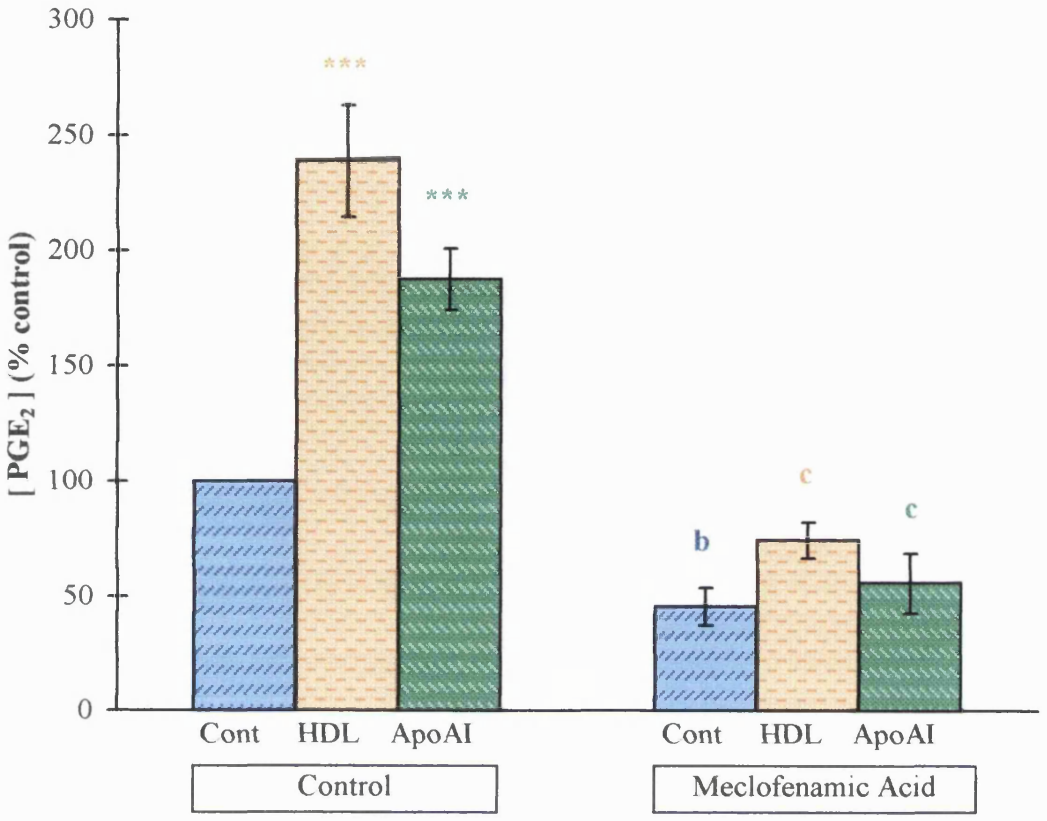

Figure 4.10 Effects of meclofenamic acid on PG production by human granulosa-lutein cells treated with HDL and Apo-AI, Sigma

PGE_2 accumulation after incubation for 24h without (▨) or with HDL (100μg protein/ml) (□) or Apo-AI Sigma (100μg protein/ml) (▩). Incubations were performed in the absence or presence of MA. Values are the mean±SE for 4 independent experiments with quadruplicate determinations in each experiment. ***$P<0.001$ in the absence of HDL; $^{b}P<0.01$, $^{c}P<0.001$ relative to the cells treated with HDL or Apo-AI alone as appropriate. (One way ANOVA; Bonferonni's *post hoc* test).

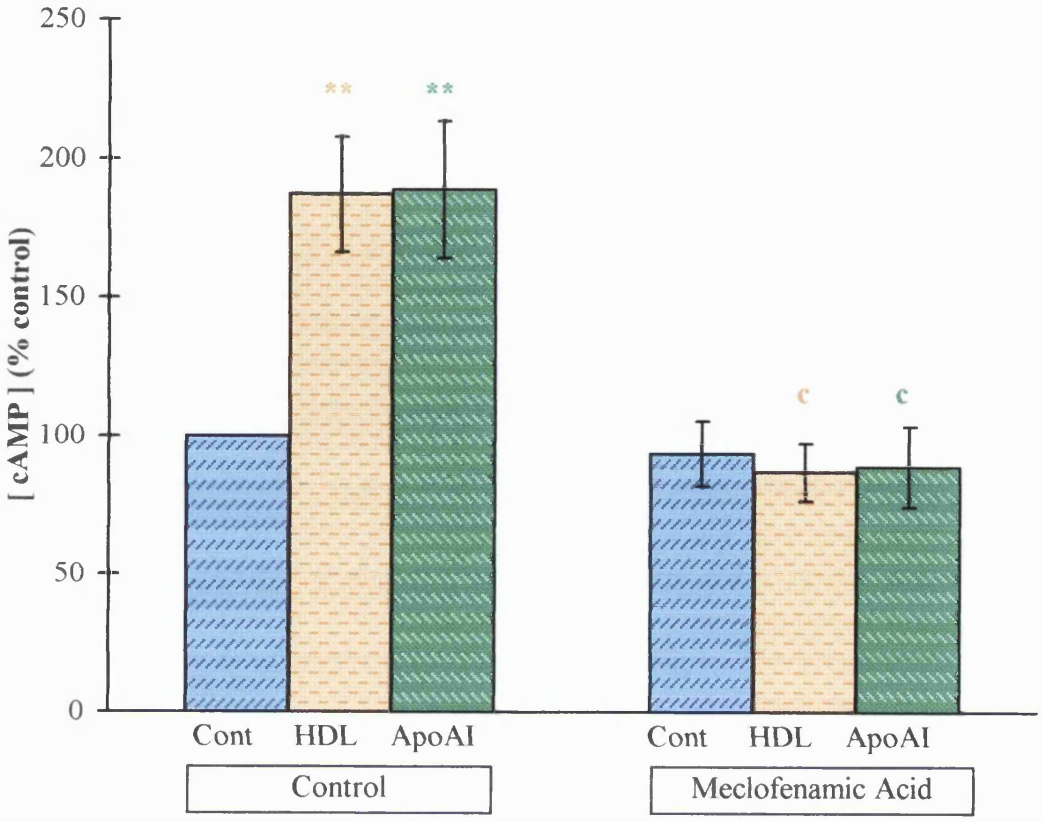

Figure 4.11 Effects of meclofenamic acid on cAMP accumulation in human granulosa-lutein cells treated with HDL and Apo-AI, Sigma

Granulosa lutein cells were incubated for 24h without (▨) or with HDL (100µg protein/ml) (□) or Apo-AI Sigma (100µg protein/ml) (▩). Incubations were performed in the absence or presence of MA. Values are the mean±SE for 4 independent experiments with quadruplicate determinations in each experiment. $^{**}P<0.01$ in the absence of HDL; $^{c}P<0.001$ relative to the cells treated with HDL or Apo-AI alone as appropriate (One way ANOVA; Bonferonni's *post hoc* test).

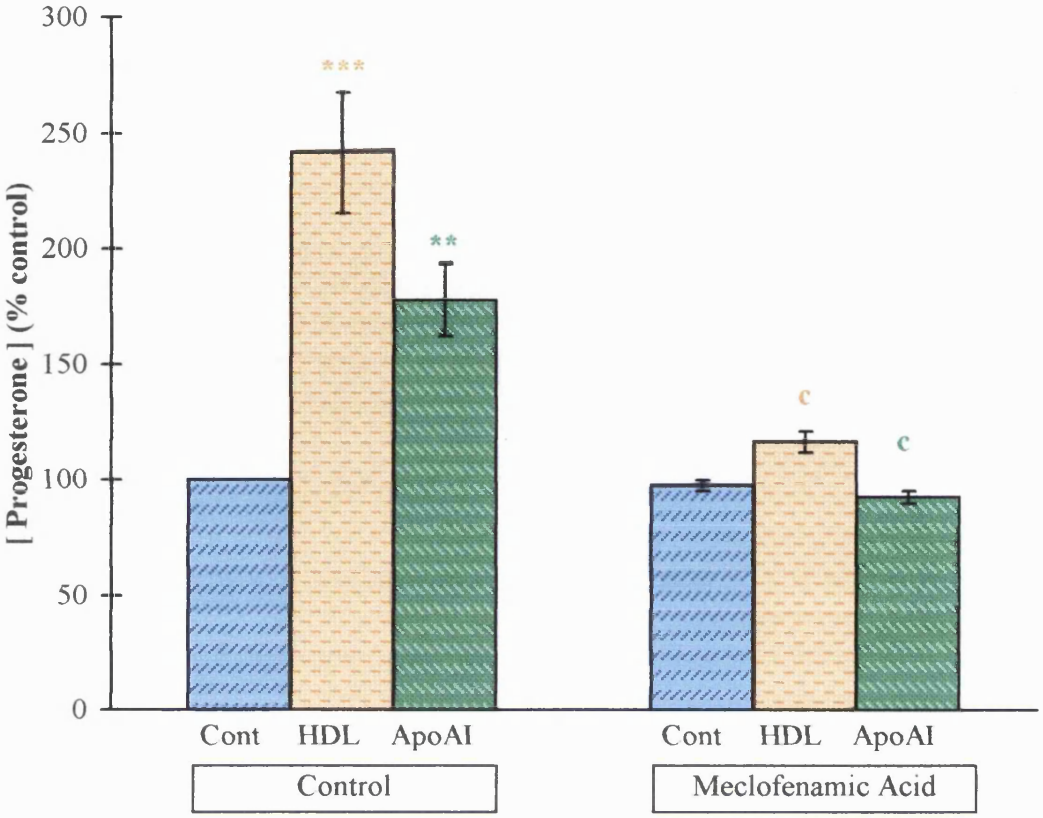

Figure 4.12 Effects of meclofenamic acid on progesterone production by human granulosa-lutein cells treated with HDL and Apo-AI, Sigma

Granulosa lutein cells were incubated for 24h without (▨) or with HDL (100µg protein/ml) (□) or Apo-AI Sigma (100µg protein/ml) (▩). Incubations were performed in the absence or presence of MA. Values are the mean±SE for 4 independent experiments with quadruplicate determinations in each experiment. $^{**}P<0.01$, $^{***}P<0.001$ in the absence of HDL; $^{c}P<0.001$ relative to the cells treated with HDL or Apo-AI alone as appropriate. (One way ANOVA; Bonferonni's *post hoc* test).

Consistent with previous observations, treatment with HDL and Apo-AI significantly stimulated PGE_2 production by 136.8±6.1% (n=4; $P<0.001$) and 89.5±7.5 (n=4; $P<0.001$) respectively (Figure 4.10). Co-treatment with MA abolished the PGE_2 responses to both HDL and Apo-AI.

Treatment with either HDL or Apo-AI (100μg/ml), resulted in a significant increase in cAMP concentrations by 79.3±8.4% ($P<0.001$) and 80.0±8.9% ($P<0.001$) respectively (Figure 4.12). Co-treatment with MA resulted in the abolition of both the HDL and Apo-AI responses.

Incubation for 24 hours in the presence of 100μg/ml HDL or Apo-AI stimulated the progesterone synthesis by 135.1±25.1% ($P<0.001$) and 74.8±15.0% ($P<0.01$) respectively (Figure 4.11). Co-treatment with MA (10μM) completely abolished the progesterone responses to both HDL and Apo-AI.

Similar results were obtained when indomethacin was used in place of MA for experimental design (data not shown).

4.4 DISCUSSION

The data presented in this chapter suggest that PGs may mediate, at least in part the steroidogenic response to HDL, and that Apo-AI may participate in the stimulation of PGE_2, cAMP and progesterone by HDL.

In order to investigate the effects of HDL on the production of PGE_2, cAMP and progesterone by hGLC, the concentration- and time-dependent effects of this lipoprotein were examined. HDL was able to stimulate both concentration- and time-dependent increases in the accumulation of PGE_2, cAMP and progesterone. At the highest tested concentration of HDL, the PGE_2 production was increased to 12.3±4.1nM which is above the K_d values for all four cloned EP receptors (range from 1nM to 11nM; [Coleman *et al*, 1994]). The concentrations of PGE_2 produced in response to HDL hence appeared to be at a level capable of activating all four EP receptor subtypes. However, in response to HDL the concentrations of PGE_2, cAMP and progesterone all increased at the same time points, thus questioning the hypothesis that PGE_2 may be mediating the steroidogenic response to HDL. One

would expect for PGE_2 concentrations to increase at an earlier time point than that at which the cAMP and progesterone concentrations increase. However, the interval between the last two time points is relatively long (16h) such that elevation of the PGE_2 concentration may indeed have occurred prior to the elevation of cAMP and progesterone without being detected.

In order to exclude the net delivery of cholesterol to cells from HDL, the effects of recombinant lipid-poor Apo-AI were examined. A parallel concentration curve was set up between HDL and two commercial Apo-AI preparations, purchased form either Sigma (UK) or Athens (USA). Both HDL and Apo-AI from Sigma (UK) stimulated concentration-dependent increases in PGE_2, cAMP and progesterone production. However, the progesterone concentrations generated in response to Apo-AI Sigma (UK) appeared to be lower than those produced in response to HDL, even though cells from the same patients were used for both experiments. This suggests that HDL may have alternative ways of stimulating progesterone production. One possible method employed for the steroidogenic action of HDL would be the provision of cholesterol to the cells, either through selective lipid uptake, through a receptor (*e.g.* SR-BI) or direct diffusion of cholesterol from the HDL particle to the cell membrane. Another possible mechanism utilised by the cells could be through the apolipoproteins surrounding the HDL particle, such as Apo-AI, Apo-AII or Apo-E. Another possible mechanism for the steroidogenic action of HDL may be through the arachidonic acid existing as part of the phospholipids which form the HDL monolayer. Arachidonic acid is a known precursor for eicosanoids including PGE_2 which in turn is able to stimulate progesterone production.

Finally it should be considered that the lipid poor Apo-AI used for treatment in the above mentioned experiments may actually not remain the same throughout the course of the 24-hour incubation. Lipid poor Apo-AI can stimulate cholesterol efflux and thus acquire cholesterol [Phillips *et al*, 1998]. Indeed a mechanism has been proposed involving lipidation of lipid-poor Apo-AI and the circulation of cholesterol. Chen *et al* (2000), suggested that the ABC1 transporter, may efflux cholesterol to Apo-AI which then becomes nascent HDL. Cholesterol from this newly formed nascent HDL particles can then enter back into the cell via the SR-BI. This proposed mechanism is though to be important for the mobilisation and recycling of

cholesterol inside the cell. From these observations lipidation of lipid-poor Apo-AI in the experiments performed herein should be considered. However, as previously mentioned this may only be the step of a mechanism acquired by the cells to mobilise cholesterol.

Unlike the responses generated by HDL and Apo-AI Sigma (UK), treatment with increasing concentrations of Apo-AI Athens (USA) did not stimulate the production of PGE_2, cAMP or progesterone. However Apo-AI Athens was purchased in a 10mM solution of NH_4HCO_3 the possible direct effects of which were assessed. NH_4HCO_3 inhibited basal PGE_2 production without affecting the basal accumulation of cAMP or progesterone. The latter set of results suggest that NH_4HCO_3 inhibits PGE_2 production and hence suggests that the increase in PGE_2 elicited by Apo-AI Sigma may be required to stimulate both progesterone output and cAMP accumulation. Consequently further experiments were undertaken to examine the possible implication of PGE_2 in the steroidogenic response to HDL.

Cells were hence cultured with HDL or Apo-AI, Sigma (UK), both in the absence and presence of MA, a PGHS inhibitor. Both HDL and Apo-AI stimulated the concentrations of PGE_2, cAMP and progesterone. Co-treatment with MA abolished the effects of HDL and of Apo-AI on both cAMP accumulation and on progesterone synthesis. This strongly suggests that the steroidogenic and cAMP responses to HDL occur at least in part through the generation of PGs and/or thromboxanes. In view of the known action of PGE_2 as a luteotrophin, acting via cAMP, PGE_2 is a strong candidate as a mediator of HDL and Apo-AI action.

From the data presented in this chapter, it can be concluded that PGs may mediate the steroidogenic response to HDL and that Apo-AI participates in the steroidogenic response to HDL.

Chapter Five

ROLE OF PROSTAGLANDINS IN THE PARACRINE/AUTOCRINE CONTROL OF OVARIAN 11β-HSD ACTIVITIES

Chapter Five

ROLE OF PROSTAGLANDINS IN THE PARACRINE/AUTOCRINE CONTROL OF OVARIAN 11β-HSD ACTIVITIES

5.1 INTRODUCTION

Glucocorticoids have been shown to exert direct effects on gonadal function and glucocorticoid receptors have been identified in a range of ovarian tissues [Evain *et al*, 1976; Fitzpatrick & Richards, 1991; Michael *et al*, 1993c; Kowalski & Chatterton, 1992]. Furthermore, glucocorticoids are thought to be involved with oocyte maturation [Greeley *et al*, 1986; Patino & Thomas, 1990], as well as inflammatory responses such as menstruation, implantation, cervical softening and parturition [Kelly *et al*, 1996]. The effects of glucocorticoids in potential target cells are determined not only by their local concentrations and the presence of receptors but also by the degree of their conversion to inactive metabolites. As previously mentioned (section 1.7.3), the enzyme 11β-HSD converts active cortisol to its inactive metabolite, cortisone. 11β-HSD activity has been measured in human granulosa-lutein cells [Michael *et al*, 1997].

Concentrations of prostaglandins, known participants in inflammatory responses, have been shown to be suppressed by glucocorticoids in the uterus [Pakrasi *et al*, 1983], and play a key role in the initiation of labour [Challis *et al*, 1994; O'Brien *et al*, 1995]. Furthermore, high concentrations of PGs are known to be secreted following the LH surge [Sirois *et al*, 1994; Narko *et al*, 1997; LeMaire *et al*, 1975; Espey et al, 1980; Richards, 1994; Ainsworth et al, 1984]. Recent studies have demonstrated a direct dynamic interaction between PGs and glucocorticoid concentrations which appears to be of great importance in the regulation of birth [Challis *et al*, 2000]. Furthermore, Challis *et al* (2000) reports increases in 11β-HSD1 activity in response to PGE_2 and $PGF_{2\alpha}$ which is thought to be dependent upon a transient increase in intracellular Ca^{2+}. However, studies performed by Hardy *et al* (1999) and co-workers in human choriocarcinoma JEG-3 cells have suggested that PGs and leukotriene B_4 are potent inhibitors of 11β-HSD2. Furthermore, Hardy *et al* (2001) demonstrated that Ca^{2+} inhibits 11β-HSD2 activity and that this mechanism underlies the inhibition of 11β-HSD2 activity by $PGF_{2\alpha}$.

PG availability is controlled by the enzyme prostaglandin dehydrogenase (PGDH) which is responsible for the initial oxidation of PGs to their biologically inactive metabolites [Okazaki *et al*, 1981]. Moreover, both PGDH expression and activity are suppressed by glucocorticoids [Patel *et al*, 1999] which results in a paradoxical increase in active PG concentrations (especially PGE_2 and $PGF_{2\alpha}$). However, in the same way that glucocorticoids may affect PG levels, PGs have also been demonstrated to affect 11β-HSD1 activity [Challis *et al*, 2000]. Consequently, a relationship between PGDH and 11β-HSD activities emerges. This may rely on the high degree of sequence homology between cloned PGDH and 11β-HSD1 [Baker, 1991]; the two enzymes share more than 90% homology at their active sites [Baker, 1994]. In view of the molecular similarities between 11β-HSD and PGDH, it is important to clarify the relationship between prostaglandin-PGDH and cortisol-11β-HSD systems in the ovary. Specifically the primary objective of this chapter was to examine the paracrine/autocrine effects of PGs on the activity of the enzyme 11β-HSD in hGLCs.

Ongoing research in the laboratory of A.E.Michael has demonstrated that human follicular fluid contains endogenous modulators of 11β-HSD activity which can be resolved by C18 column chromatography. Hence, this chapter also compared the retention properties of these endogenous ovarian 11β-HSD modulators to those of ovarian PGs.

5.2 EXPERIMENTAL PROTOCOLS

In all experiments described in this chapter, cells were seeded into sterile 24-well culture plates at a density of 5×10^4 cells/ml with a volume of 1ml medium per well. Cells were initially cultured for two days in serum-supplemented medium to allow cells to attach to the plate and luteinise. 11β-HSD activities were assessed, in the presence and absence of treatments. At the end of each experiment, the spent medium was transferred into pyrex screw cap glass tubes containing 2ml chloroform for steroid extraction. After aspirating the aqueous supernatant, the organic extracts were evaporated to dryness under nitrogen at + 40°C, reconstituted in 25μl ethyl acetate and were then resolved by TLC (section 2.7). 11β-HSD activities were quantified as the net oxidation of cortisol to cortisone/5×10^4cells.4h.

In all experiments described in this chapter, results were internally standardised. All data are presented graphically as percentage values (mean±SE) (where the control enzyme activity for each experimental design was standardised to 100%). Data presented in sections 5.3.5.2 and 5.3.5.3 were also internally standardised and are presented as percentage values (mean±SE). However, the value standardised to 100% was the total PG concentration per column. In all experiments statistical analyses were performed on non-referenced data.

5.3 RESULTS

5.3.1 Effects of PGHS inhibitors on 11β-HSD activity

5.3.1.1 Concentration-dependent effects of indomethacin

Cells were plated in a 6×2 factorial design and cultured for two days as previously described (section 5.2). On the third day of culture, cells for experimental design A (Figure 5.1) were rinsed with warmed serum-free medium and then incubated for a further 20 hours in serum-free medium. After the 20-hour incubation, cells were treated for a further 4 hours in serum-free medium containing 0, 0.01, 0.1, 1, 10 and 100μM indomethacin, each in the presence of 100nM ^{3}H-cortisol. Cells for experimental design B (Figure 5.1) were rinsed with warmed serum-free medium before being incubated for a further 20 hours in serum-free medium containing 0, 0.01, 0.1, 1, 10 and 100μM indomethacin. After the 20-hour incubation, cells were treated for a further 4 hours in fresh serum-free medium containing 0, 0.01, 0.1, 1, 10 and 100μM indomethacin, in the presence of 100nM ^{3}H-cortisol. Hence, irrespective of whether cells were incubated with indomethacin for 4 or 24 hours, the enzyme assay was performed over the same 4 hour period. Indomethacin was prepared to a stock concentration of 100mM in DMSO, the final concentration of which was adjusted in all wells to 0.1% (v/v).

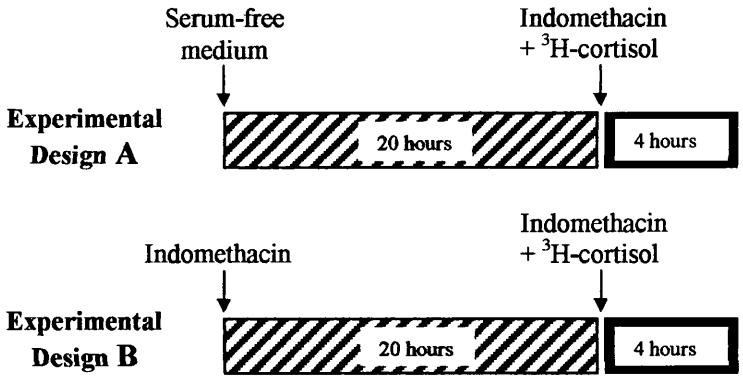

Figure 5.1 Diagrammatic representation of experimental designs for assessing concentration-dependent effects of indomethacin both acutely and chronically

Over a 4h period, indomethacin inhibited 11β-HSD activity in a concentration-dependent manner. At indomethacin concentrations of 0.01μM and 1μM, 11β-HSD activity was decreased by 47.2±12.4% and 44.4±15.5% respectively (n=4; $P<0.05$) (Figure 5.2). However, at concentrations of 0.1, 10 and 100μM, the acute effects of indomethacin on net oxidation of cortisol were insignificant.

Treatment with indomethacin for 24 hours resulted in a more consistent, concentration-dependent inhibition of the 11β-HSD activity at indomethacin concentrations ≥ 1μM. The conversion of cortisol to cortisone was suppressed by up to 50.8±5.8% (n=4; $P<0.01$) at the highest tested indomethacin concentration of 100μM.

Due to the erratic effects of indomethacin at 4 hours, the experimental design was repeated with two other, structurally different PGHS inhibitors: meclofenamic acid (MA) (section 5.3.1.2) and niflumic acid (NA) (section 5.3.1.3).

5.3.1.2. Concentration-dependent effects of MA

A similar experimental design to that described above (section 5.3.1.1) was followed, but using MA in place of indomethacin. MA was prepared to a stock concentration of 100mM in dimethylsulphoxide (DMSO), the final concentration of which was adjusted in all wells to 0.1% (v/v).

Incubation for 4 hours with MA, resulted in a concentration-dependent suppression of 11β-HSD activity (Figure 5.3). Even at the lowest tested concentration of 0.01μM MA, the conversion of cortisol to cortisone was inhibited by 50.0±2.5% (n=4; $P<0.01$). Inhibition of the enzyme activity was maximal (80.0±30.0% inhibition; n=4; $P<0.01$) at the highest tested concentration of 100μM MA.

Similarly, incubation for 24 hours with MA resulted in a concentration-dependent inhibition of cortisol oxidation at concentrations ≥ 1μM. At the highest tested concentration of MA (100μM), the net 11β-dehydrogenase activity was suppressed by 69.2±17.3% (n=4; $P<0.01$).

5.3.1.3 Concentration-dependent effects of NA

The experimental design described above (section 5.3.1.1) was repeated using NA in place of indomethacin. NA was prepared to a stock concentration of 100mM in DMSO, the final concentration of which was adjusted in all wells to 0.1% (v/v).

Incubation with NA for 4 hours resulted in a concentration-dependent suppression of the enzyme activity (Figure 5.4). Even at the lowest tested concentration of 0.01μM NA, cortisol oxidation was inhibited by 34.6±8.1% (n=4; $P<0.01$). At the highest tested concentration of 100μM NA, the conversion of cortisol to cortisone was decreased by 44.2±9.1% (n=4; $P<0.01$).

Cells treated with NA for 24 hours exhibited similar responses. 11β-HSD activity was suppressed by NA in a concentration-dependent manner by up to 61.2±16.1% at 100μM NA (n=4; $P<0.01$).

5.3.2 Comparative effects of PGE_2, PGD_2 and $PGF_{2\alpha}$ on 11β-HSD activity

Following the two-day incubation in serum-supplemented medium, cells were rinsed with warmed serum-free medium and then incubated for a further 4 hours in serum-free medium, containing 1μM MA plus PGE_2, $PGF_{2\alpha}$, or PGD_2, each at concentrations of either 30 or 3000nM, in the presence of 100nM ^{3}H-cortisol. Assays were terminated and 11β-HSD activities were quantified as described in sections 2.6 & 2.7. The prostaglandins were each prepared to a stock concentration

of 3mM in ethanol (EtOH), the final concentration of which was adjusted in all wells to 0.1% (v/v).

Incubation for 4 hours with 1μM MA resulted, as above, in a decrease in the oxidative activity of 11β-HSD by 51.9±12.5% (n=3; $P<0.05$) (Figure 5.5). Cortisol oxidation after co-treatment with $PGF_{2\alpha}$ and PGD_2 both at 30 and 3000nM did not differ from that in control cells not treated with MA. However, the effects of PGE_2 on 11β-HSD activities in MA-treated cells were concentration-dependent. Although 11β-HSD activities after co-treatment with 30nM PGE_2 did not differ from control cells, this was not the case after incubation with 3000nM PGE_2. At this concentration, cortisol oxidation remained inhibited by 46.0±21.0% (n=3; $P<0.05$) relative to untreated control cells. Moreover, the enzyme activities were the same in cells treated with MA+3000nM PGE_2 as in cells treated with MA alone.

Identical results were obtained when indomethacin (1μM) was used in place of MA for this experimental design (*data not shown*).

5.3.2.1 Concentration-dependent effects of PGE_2 on 11β-HSD activity

In view of the differing actions of PGE_2 at 30 and 3000nM, the concentration-dependent effects of PGE_2 on 11β-HSD activity were further investigated. Cells were cultured for two days in serum-supplemented medium to allow the cells to attach to the plate. On the third day of culture, cells were rinsed with warmed serum-free medium and then incubated for a further 4 hours in serum-free medium containing 0, 10, 30, 100, 300, 1000 and 3000nM PGE_2, each in the presence of 1μM MA and 100nM ^{3}H-cortisol. Assays were terminated and 11β-HSD activities were quantified as described in sections 2.6 & 2.7. MA and PGE_2 were prepared and diluted as previously described in sections 5.3.1.2 and 5.3.2.

Incubation with a range of PGE_2 concentrations for 4 hours in the presence of MA resulted in a biphasic effect on the net dehydrogenase activity of 11β-HSD (Figure 5.6). At the three lower tested concentrations of 10, 30 and 100nM PGE_2, the 11β-HSD activity was increased by 37.5±4.1%, 27.5±1.1% and 37.5±0.7% respectively (n=3; $P<0.01$). However, at the three higher tested concentrations of 300, 1000 and

3000nM PGE_2, the oxidative activity of the enzyme did not differ significantly from that in cells treated with MA alone.

5.3.3 Concentration-dependent effects of AG on 11β-HSD activity

Since PGE_2 can stimulate progesterone production in human granulosa-lutein cells (section 1.6) and progesterone is known to inhibit 11β-HSD activity [Ricketts *et al*, 1998; Burton *et al*, 1999], the biphasic actions of PGE_2 on 11β-HSD activity may be as a result of increased progesterone production stimulated at the higher concentrations of PGE_2. The experimental design described above was therefore repeated in the presence and absence of a steroidogenic inhibitor to re-examine the effects of PGE_2 on 11β-HSD activity in the absence of progesterone (section 5.2.4). However, before using AG in such experiments, possible direct effects of AG on 11β-HSD activities were first examined.

Cells were cultured for two days in serum-supplemented medium to allow the cells to attach to the plate. On the third day of culture, cells were rinsed with warmed serum-free medium and then incubated for a further 4 hours in serum-free medium containing 0, 0.01, 0.1, 1, 10 and 100μM AG in the presence of 100nM ^{3}H-cortisol. Assays were terminated and 11β-HSD activities were quantified as described in sections 2.6 & 2.7. AG was prepared to a stock concentration of 100mM in chloroform, the final concentration of which was adjusted in all wells to 0.1% (v/v).

Incubation for 4 hours with AG resulted in a concentration-dependent increase in 11β-HSD activity (Figure 5.7). While aminoglutethimide concentrations $\leq$ 10μM had no significant effect on cortisol oxidation, at the maximum tested concentration of 100μM AG, the 11β-HSD activity was increased by 41.5±3.6% (n=5; $P<0.01$).

5.3.4 Concentration-dependent effects of PGE_2 in the absence and presence of AG

Cells were cultured for two days in serum-supplemented medium to allow the cells to attach to the plate. On the third day of culture, cells were rinsed with warmed serum-free medium and then incubated for a further 4 hours in serum-free medium, containing an abridged concentration curve of PGE_2 (0, 10, 100 & 1000nM PGE_2), in the absence and presence of 100μM AG. All cells were incubated over this 4-hour period in the presence of 1μM MA and 100nM ^{3}H-cortisol. Cells were incubated for

30min with MA and AG prior to addition of PGE_2 and ^3H-cortisol for the initiation of the 4-hour assay incubation. Assays were terminated and 11β-HSD activities were quantified as described in sections 2.6 & 2.7. PGE_2, MA and AG were prepared and diluted as previously described in sections 5.3.2, 5.3.1.2 and 5.3.3, respectively.

In the absence of AG, incubation for 4 hours with PGE_2, resulted in stimulation of 11β-HSD activity by 47.7±6.5% and 43.1±6.5% at PGE_2 concentrations of 10 and 100nM respectively (n=3; $P<0.01$) (Figure 5.8). However, at a PGE_2 concentration of 1000nM, 11β-HSD activities did not differ from those measured in the presence of MA alone.

Co-treatment with AG increased 11β-HSD activities at all tested concentrations of PGE_2. Moreover, in the presence of AG, at PGE_2 concentrations of 100 and 1000nM, 11β-HSD activities increased significantly with a maximum stimulation (42.5±3.1%; n=3; $P<0.01$) at 1000nM PGE_2.

5.3.5 Potential effects of intra-follicular PGs on 11β-HSD activity

5.3.5.1 Effects of follicular fluid fractions eluted from C-18 mini-column on $NADP^+$-dependent 11β-HSD activity

Follicular fluids from three patients were loaded onto C18 mini-columns as previously described (section 2.8) and the eluted fractions were collected and tested for effects on $NADP^+$-dependent oxidation of cortisol in a rat kidney homogenate (section 2.9).

Human follicular fluid was confirmed to contain intrinsic aqueous stimuli and hydrophobic inhibitors of the enzyme 11β-HSD activity that could be eluted from a C18 mini-column at methanol concentrations of 0-10% (v/v) and 70-85% (v/v) respectively (Figure 5.9). However, follicular fluid fractions eluted at 20-65% and 90-100% methanol had no significant effect on the $NADP^+$-dependent oxidation of cortisol to cortisone.

5.3.5.2 C-18 mini-column elution profiles for ^3H-PGs

Follicular fluids from the same three patients used for the experiment described in section 5.3.5.1 were aliquoted in three 1.5ml aliquots. Each of the aliquots were

spiked by the addition of 1μCi [^{3}H]-PGE_2, [^{3}H]-$PGF_{2\alpha}$ or [^{3}H]-6-keto $PGF_{1\alpha}$. The spiked follicular fluids were each loaded on a separate C18 mini-column for fractionation as previously described (section 2.8.1). The amount of ^{3}H-PGE_2, ^{3}H-$PGF_{2\alpha}$ and ^{3}H-6-keto-$PGF_{1\alpha}$ eluted in each fraction was measured on a Beckman Coulter LS 6500 Multipurpose Scintillation counter (section 2.8.2).

All three PGs were eluted from the C18 mini-column at methanol concentrations between 20 and 45% (v/v) (Figure 5.10). Maximum elution for all three PGs was at either 30% or 40% (v/v) methanol.

5.3.5.3 C-18 mini-column elution profiles for intra-follicular PGs

500μl from each of the follicular fractions from section 5.2.5.1 were evaporated to dryness in a sample concentrator Techne Dri-Block DB-3, at 45°C, under nitrogen, and each reconstituted in 500μl distilled water. The samples were subjected in duplicate to RIAs for PGE_2 and $PGF_{2\alpha}$ (sections 2.3.2 and 2.3.3 respectively).

PGE_2 and $PGF_{2\alpha}$ concentrations were undetectable in those follicular fluid fractions eluted at <20% or >50% methanol (Figure 5.11). The concentrations of both PGs were maximum (0.3±0.0nM and 5.1±2.0nM) in the 40% (v/v) methanol fraction. PGE_2 and $PGF_{2\alpha}$ concentrations in the same follicular fluid samples prior to fractionation were 1.3±0.2 and 27.3±1.2nM, respectively.

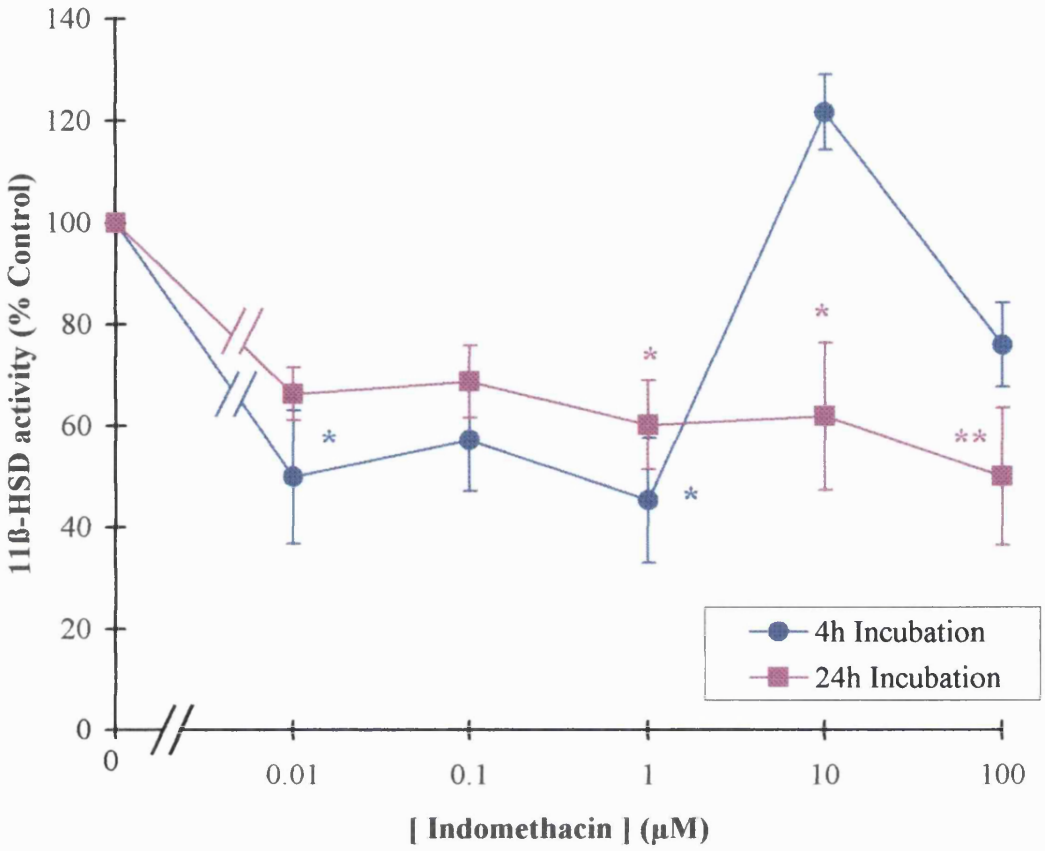

Figure 5.2 Concentration-dependent effects of indomethacin on 11β-HSD activity in human granulosa-lutein cells

11β-HSD activities, measured over 4h, following treatment of hGLC with 0-100µM indomethacin for 4h (—●—) or 24h (—■—). Values are the mean±SE for 4 independent experiments with triplicate determinations in each experiment. **P*<0.05, ***P*<0.01 relative to 0µM indomethacin at 4 or 24 hours as appropriate (One way ANOVA; Dunnett's *post hoc* test)

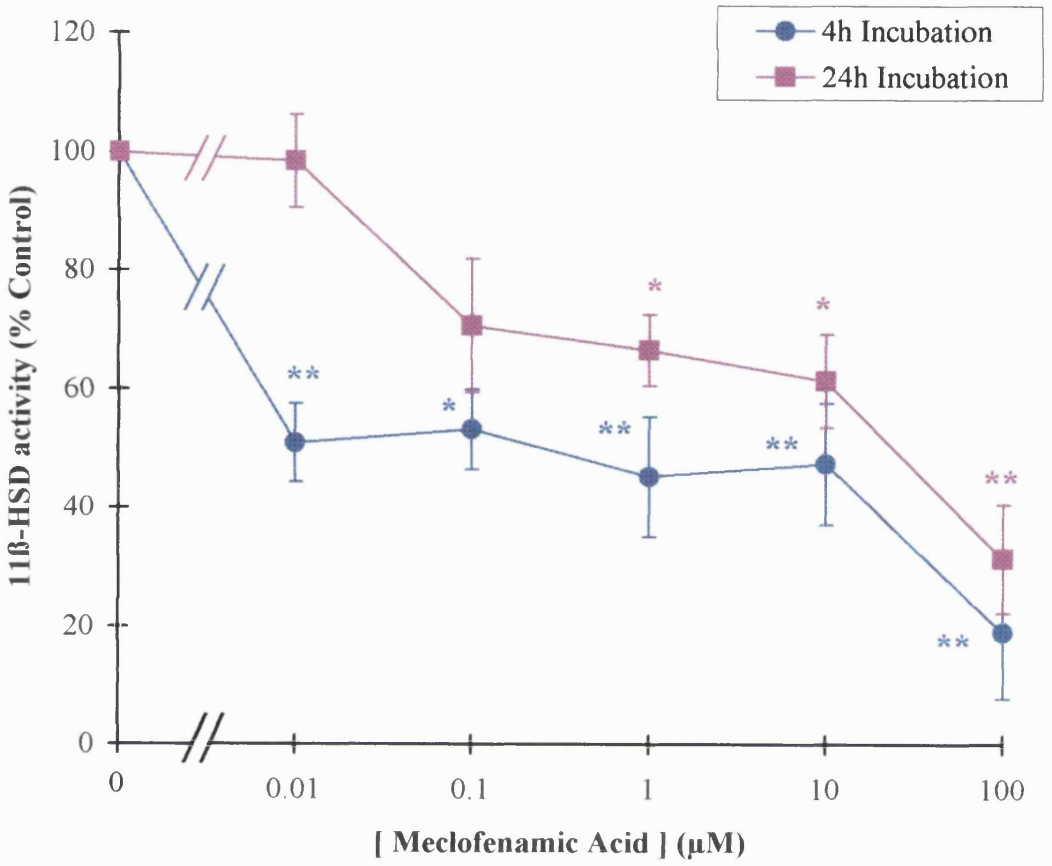

Figure 5.3 Concentration-dependent effects of meclofenamic acid on 11β-HSD activity in human granulosa-lutein cells

11β-HSD activities, measured over 4h, following treatment of hGLC with 0-100µM meclofenamic acid for 4h (—●—) or 24-hour (—■—). Values are the mean±SE for 4 independent experiments with triplicate determinations in each experiment. **P*<0.05, ***P*<0.01 relative to 0µM meclofenamic acid at 4 or 24 hours as appropriate (One way ANOVA; Dunnett's *post hoc* test)

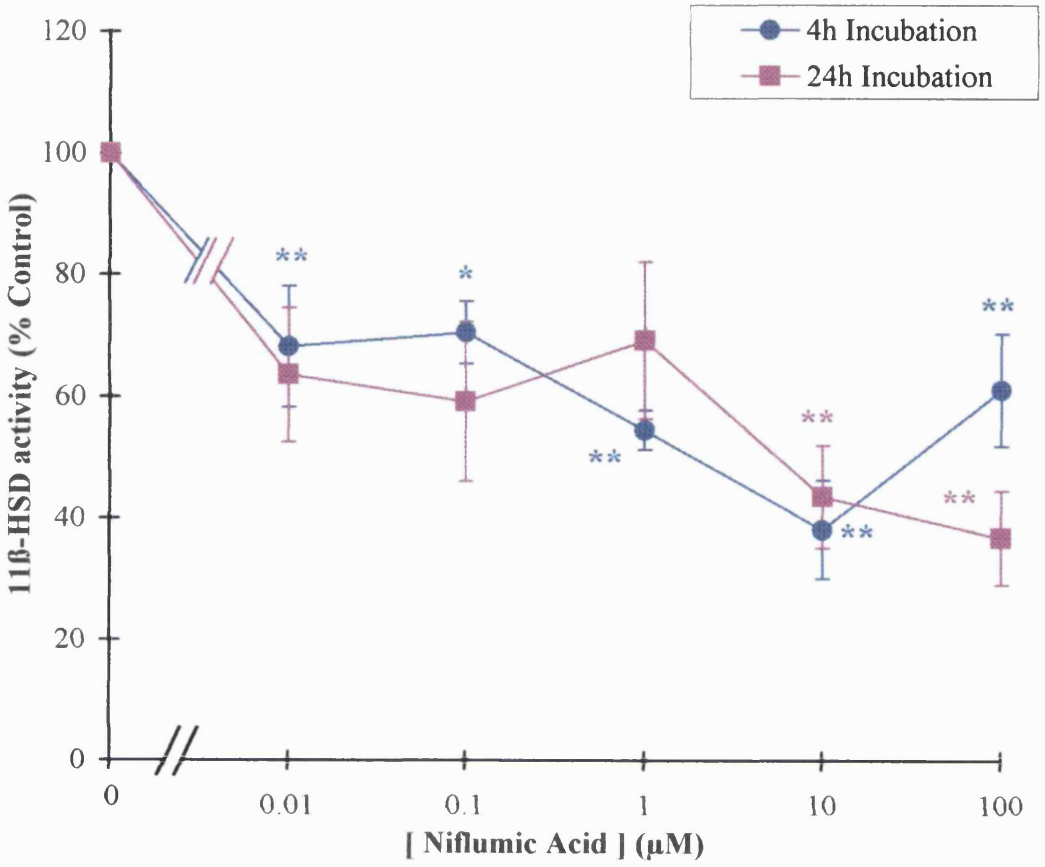

Figure 5.4 Concentration-dependent effects of niflumic acid on 11β-HSD activity in human granulosa-lutein cells

11β-HSD activities, measured over 4h, following treatment of hGLC with 0-100µM niflumic acid for 4h (—●—) or 24h (—■—). Values are the mean±SE for 4 independent experiments with triplicate determinations in each experiment. *P<0.05, **P<0.01 relative to 0µM niflumic acid at 4 or 24 hours as appropriate (One way ANOVA; Dunnett's *post hoc* test)

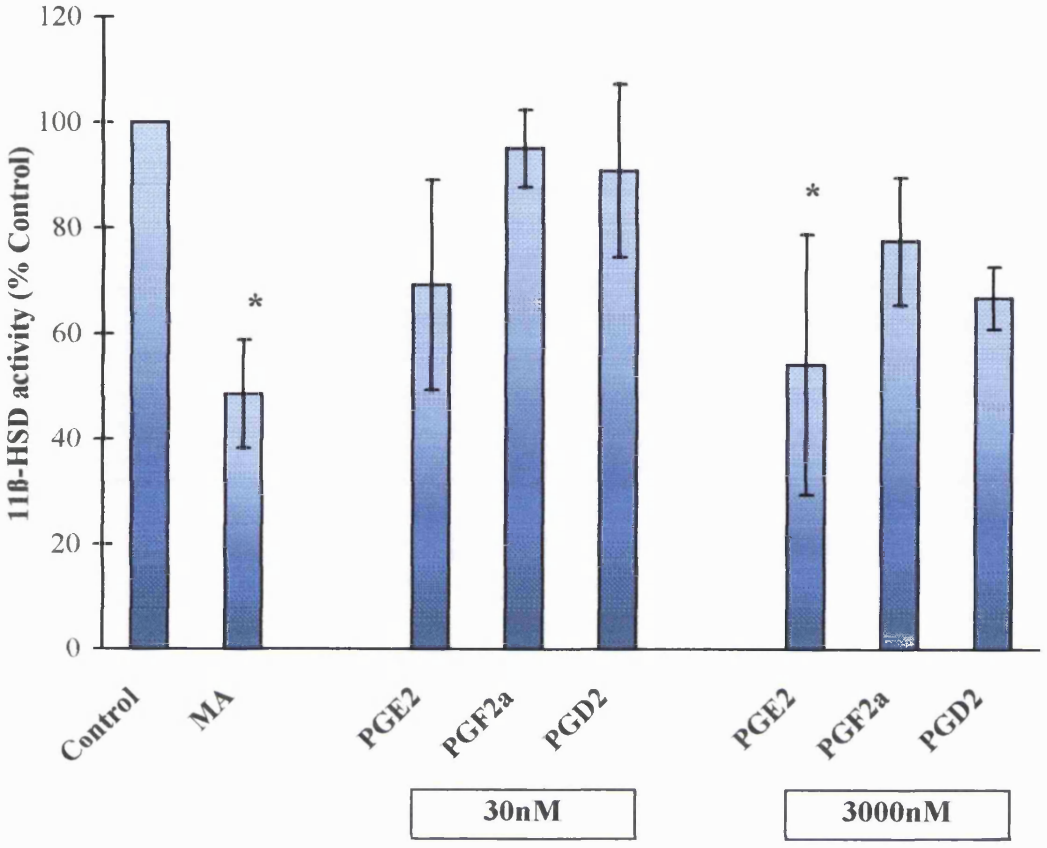

Figure 5.5 Acute effects of selected prostaglandins on 11β-HSD activity in human granulosa-lutein cells

11β-HSD activities, measured over 4h, following treatment of hGLC with 1μM meclofenamic acid ± PGE_2, $PGF_{2\alpha}$ or PGD_2 at concentrations of either 30nM or 3000nM. Values are the mean±SE for 4 (for PGE_2) or 3 (for PGD_2 and $PGF_{2\alpha}$) independent experiments with triplicate determinations in each experiment. *$P<0.05$ relative to the control which corresponds to cells not treated with meclofenamic acid (One way ANOVA; Dunnett's *post hoc* test).

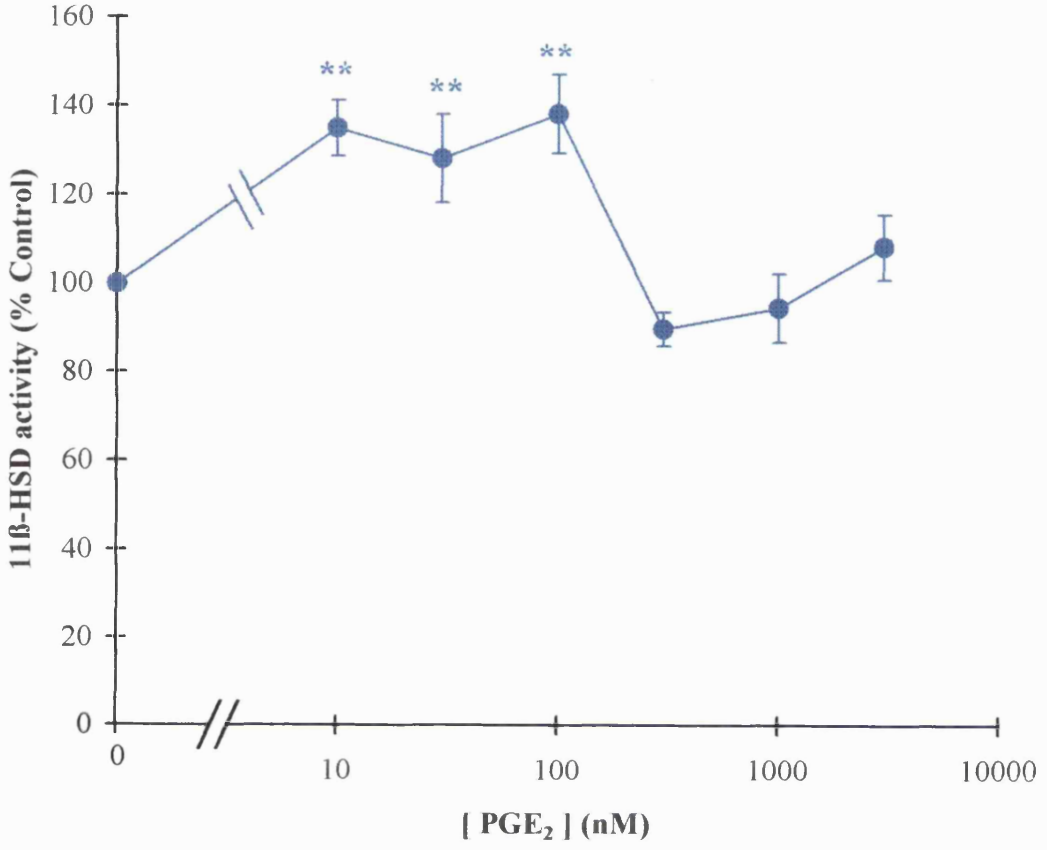

Figure 5.6 Concentration-dependent effects of PGE_2 on 11β-HSD activity in human granulosa-lutein cells

11β-HSD activities, measured over 4h, following treatment of hGLC with 0-3000nM PGE_2 in the presence of 1μM meclofenamic acid. Values are the mean±SE for 3 independent experiments with triplicate determinations in each experiment. $^{**}P<0.01$ relative to 0nM PGE_2 (One way ANOVA; Dunnett's *post hoc* test)

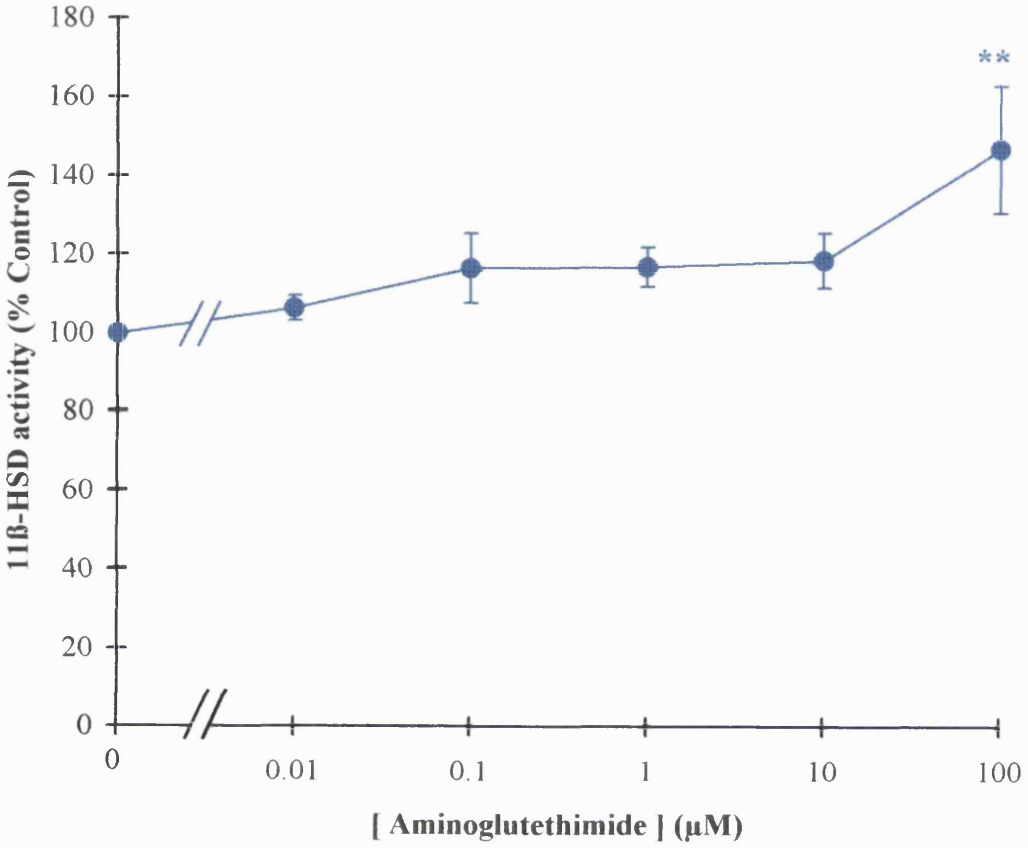

Figure 5.7 Concentration-dependent effects of aminoglutethimide on 11β-HSD activity in human granulosa-lutein cells

11β-HSD activities, measured over 4h, following treatment of hGLC with 0-100µM aminoglutethimide. Values are the mean±SE for 5 independent experiments with triplicate determinations in each experiment. $^{**}P<0.01$ relative to 0µM aminoglutethimide (One way ANOVA; Dunnett's *post hoc* test)

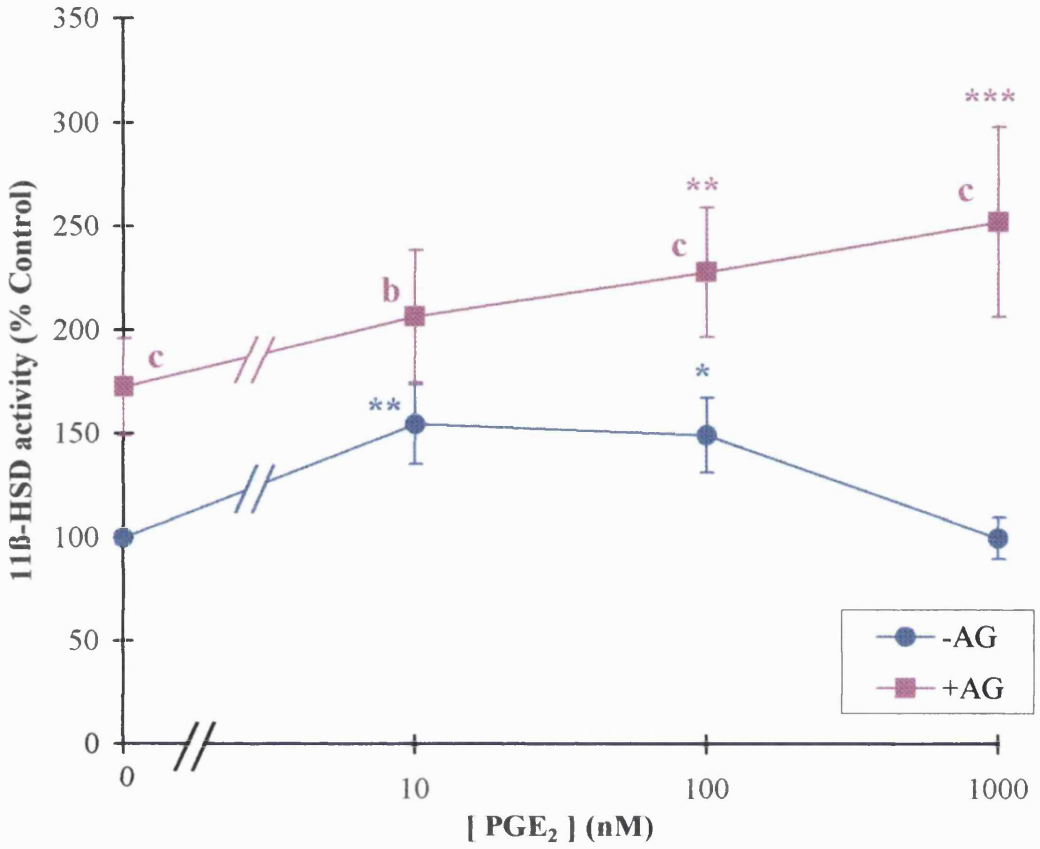

Figure 5.8 Concentration-dependent effects of PGE_2 on 11β-HSD activity both in the absence and presence of aminoglutethimide

11β-HSD activities, measured over 4h, following treatment of hGLC with 0-1000nM PGE_2 in the presence of 1μM meclofenamic acid. Parallel abridged PGE_2 concentration curves were performed either in the absence (-●-), or presence (-■-) of 100μM aminoglutethimide (AG). Values are the mean±SE for 3 independent experiments with triplicate determinations in each experiment. *P<0.05, **P<0.01, ***P<0.001 relative to 0nM PGE_2 in absence or presence of aminoglutethimide as appropriate. ^{b}P<0.01, ^{c}P<0.001 relative to cells treated with the equivalent PGE_2 concentration in the absence of AG (One way ANOVA; Bonferonni's multiple comparison test)

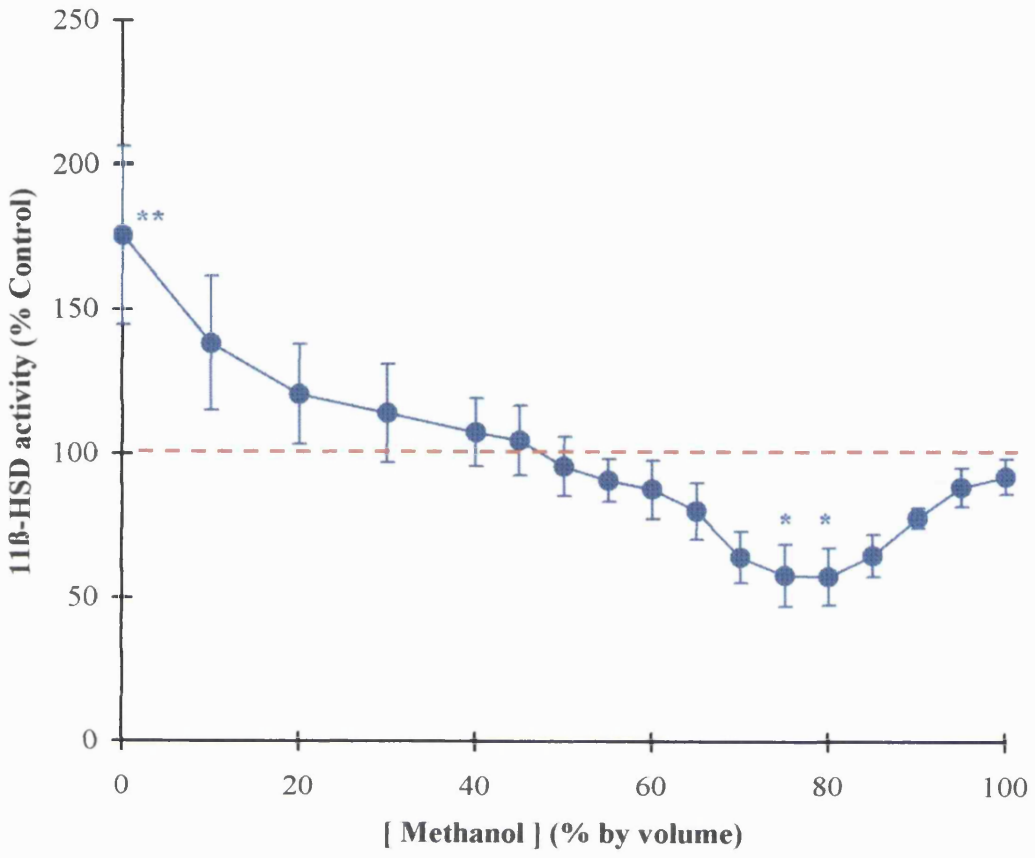

Figure 5.9 Effects of follicular fluid fractions eluted from C18 mini-column on NADP$^+$-dependent 11β-HSD in a rat kidney homogenate

11β-HSD activities measured over 1h, following treatment of rat kidney homogenate with follicular fluid fractions eluted from a C18 mini-column with increasing methanol concentrations. Values are the mean±SE for 3 independent experiments with triplicate determinations in each experiment expressed as percentage of 11β-HSD activities in the absence of follicular fluid. This control enzyme activity (represented by the red broken line) was equal to 6.9±0.3 pmol cortisone/1h. $^*P<0.05$, $^{**}P<0.01$ relative to enzyme activities in the absence of follicular fluid treatment (One way ANOVA; Dunnett's *post hoc* test).

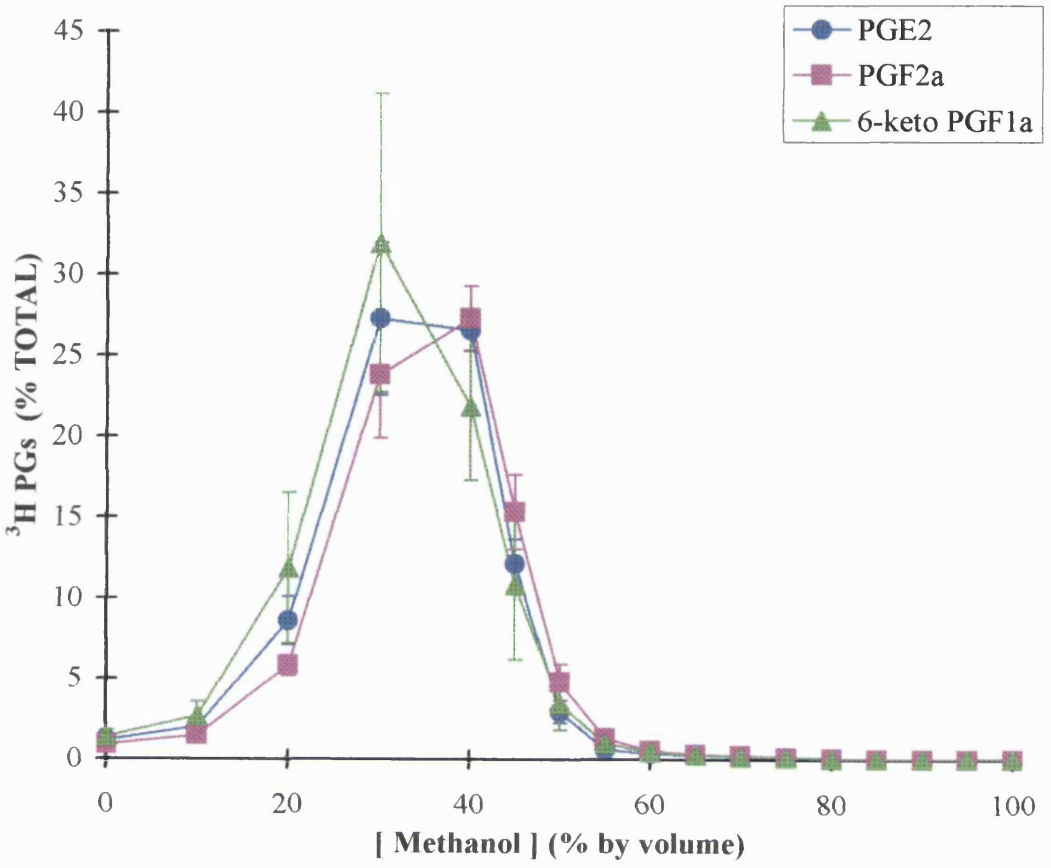

Figure 5.10 Elution of selected ^{3}H-PGs from a C18 mini-column

^{3}H-PGE$_2$, ^{3}H-PGF$_{2\alpha}$, ^{3}H-6-keto-PGF$_{1\alpha}$ from spiked samples of human follicular fluid eluted from a C18 mini-column and eluted with increasing concentrations of methanol. Values are the mean±SE for 3 independent experiments with triplicate determinations in each experiment. The results are expressed as percentage of the total eluted radioactivity per mini-column.

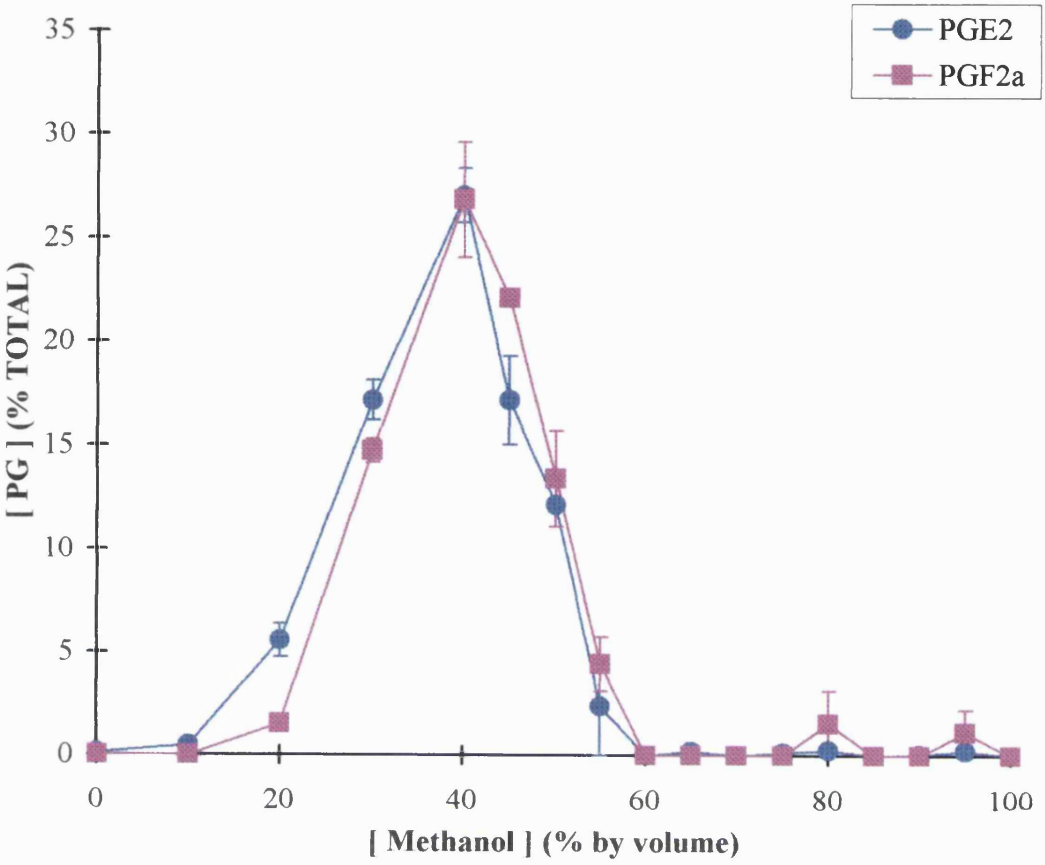

Figure 5.11 Elution of intra-follicular PGE_2 and $PGF_{2\alpha}$ from a C18 mini-column

Elution of intra-follicular PGE_2 and $PGF_{2\alpha}$ from a C18 mini-column with increasing concentrations of methanol. Intra-follicular PGE_2 and $PGF_{2\alpha}$ concentrations were determined by RIA. Values are the mean±SE for 3 independent experiments with duplicate determinations in each experiment expressed as percentage of total PGE_2 or $PGF_{2\alpha}$ concentration eluted from each mini-column respectively. Maximum PGE_2 and $PGF_{2\alpha}$ concentrations were 0.3±0.0 and 5.1±2.0nM respectively.

5.4 DISCUSSION

The data presented in this chapter demonstrate that three structurally dissimilar PGHS inhibitors, (indomethacin, MA and NA) can each inhibit 11β-HSD activity in human granulosa-lutein cells and that enzyme activity can be attenuated by co-treatment with PGE_2, $PGF_{2\alpha}$ and PGD_2. However, endogenous intra-follicular PGs seem to have no effect on type-1 11β-HSD activity in a rat kidney homogenate.

In order to establish whether PGs and thromboxanes may exert paracrine/autocrine effects on ovarian 11β-HSD activity, the concentration- and time-dependent effects of indomethacin, MA and NA on cortisol oxidation were examined. Both MA and NA suppressed 11β-HSD activity both at 4 and 24 hours, even though their effects were more evident at 4h. However, the results obtained after treatment for 4 hours with indomethacin appeared to be a bit erratic. This may reflect the limited specificity of indomethacin action. This PGHS inhibitor, apart from its potential to suppress the cyclo-oxygenase pathway, is known to inhibit the lipoxygenase pathway [Randall *et al*, 1980]. Furthermore, the fact that the effects of MA and NA are more evident at 4 hours suggest that over the 24-hour period, other mechanisms inside the cells may be activated and hence affect 11β-HSD activity. It is possible that beyond 4h, PGHS inhibitors exert some other biochemical action that increases 11β-HSD activity relative to that measured after 4 hours of treatment at the same concentration of PGHS inhibitor. Chronic absence of PGs and/or thromboxanes may also lead to an increase in the expression of 11β-HSD which would oppose the decrease in enzyme activity.

Co-treatment with PGs negated the effect of MA on 11β-HSD activity suggesting that the 3 tested PGs may be important for maintaining cortisol oxidation in human granulosa-lutein cells. Co-treatment with 30nM PGE_2, PGD_2 and $PGF_{2\alpha}$ attenuated the inhibition of 11β-HSD activity by MA. When cells were co-treated with 3000nM of PGD_2 and $PGF_{2\alpha}$, 11β-HSD activity was only partially restored. At this concentration, PGE_2 was unable to alter the inhibited 11β-HSD activity which remained significantly decreased relative to the untreated control cells.

As described above, all 3 tested PGs, under defined conditions, were capable of increasing 11β-HSD activity in human granulosa-lutein cells. However, as

considered previously (section 1.3.3) although the name of each PG subclass reflects the ligand for which they have the highest affinity, PGs have the ability to bind to classes of PG receptor other than their definitive receptor. Table 5.1 defines the K_i values for each potential ligand at each receptor (sub)class, where Ki is the concentration at which that prostaglandin displaces binding of the appropriate ^3H-ligand by 50% (hence K_i reflects K_a, the association constant of a specified ligand for the corresponding prostaglandin receptor).

Table 5.1 Competition for radioligand binding on prostaglandin recombinant receptors (adapted from Abramovitz *et al*, 2000)

	[PGE_2] K_i (nM)	[PGD_2] K_i (nM)	[$PGF_{2\alpha}$] K_i (nM)
EP_1	9.1±1.5	5820±1801	547±104
EP_2	4.9±0.5	2973±100	964±64
EP_{3III}	0.3±0.3	421±60	38±6
EP_4	0.8±0.1	1483±189	288±27
DP	307±106	1.7±0.3	861±139
FP	119±12	6.7±0.5	3.2±0.3

From the data presented in this table it is obvious that at PGE_2 concentrations above 1000nM, it becomes very difficult to establish through which receptor each prostaglandin is acting. At the higher tested concentration of 3000nM, both PGE_2 and $PGF_{2\alpha}$ are capable of binding DP receptors, all 4 cloned EP receptor subtypes and FP receptors. Likewise PGD_2 is also capable of binding all receptors (with the possible exception of the EP_1 receptor). At 30nM, PGE_2 would not have been able to bind significantly with any other than its own designated receptors. However, even at 30nM, PGD_2 can bind to both DP and FP receptors while $PGF_{2\alpha}$ can bind FP and EP_{3III} receptors.

Although at 30nM, PGE_2 is likely to work through the EP receptors, from the data obtained it is not possible to specify which of the four EP receptor classes may be involved in mediating the actions of PGE_2 on cortisol metabolism (K_i range 0.3-9.1nM) (Table 5.1). Most EP receptor pathways are linked to increases in cAMP accumulation, which would be expected to stimulate 11β-HSD activity [Sun *et al*, 1998; Patel *et al*, 1999; Barker *et al*, 2001]. Even at high concentrations where PGE_2 can activate the DP receptors the effect on 11β-HSD may still be mediated through cAMP.

$PGF_{2\alpha}$ is likely to be stimulating 11β-HSD activity through the FP receptor-Ca^{2+} signalling pathway. This observation is consistent with the Ca^{2+}-mediated increase in placental cortisol-cortisone interconversion in response to PGE_2 and $PGF_{2\alpha}$ as previously described by Challis *et al* (2000). However, the current findings contrast with the observation that $PGF_{2\alpha}$ can inhibit placental 11β-HSD2 activity, apparently via the elevation of intracellular Ca^{2+} [Hardy *et al*, 2001]. Hence these findings reported herein are consistent with reports that human granulosa-lutein cells express predominantly if not exclusively type-I 11β-HSD [Tetsuka *et al*, 1997]. While these observations can be explained by $PGF_{2\alpha}$ acting via the FP-Ca^{2+} pathway, the possibility that $PGF_{2\alpha}$ can also act via the EP_{3III} receptor cannot be excluded.

PGD_2 is likely to be stimulating 11β-HSD activity either through the DP or FP receptors. The DP receptor pathway is linked to increases in cAMP accumulation which, as previously mentioned, can stimulate 11β-HSD activity. Even at 30nM PGD_2 can activate both the DP and FP receptors. This activation could result in a positive effect through the FP receptors, as described above.

Although PGE_2 at a concentration of 30nM appears to mediate its actions on 11β-HSD activity through its own receptors, from the data obtained it is impossible to accurately assess which of the four EP receptor classes are implicated in this response. More specific receptor antagonists for each of the four EP receptor classes need to become available in order to further pursue such investigations.

In view of the fact that 3000nM PGE_2 had less of an effect on 11β-HSD activity than 30nM PGE_2, the concentration-dependent effects of PGE_2 on the enzyme activity were further examined. A 4-hour incubation with a range of PGE_2 concentrations resulted in a biphasic effect on conversion of cortisol to cortisone. At concentrations <100nM, PGE_2 is more likely to be mediating its effects on 11β-HSD solely through its own receptors which result in a stimulation of cortisol oxidation. At concentrations >100nM PGE_2, other receptors may become implicated (*e.g.* FP and/or DP receptors) in mediating the actions of PGE_2. At these concentrations of PGE_2, 11β-HSD activity did not differ from that in the control cells. This observation suggests that the stimulation of 11β-HSD activity at low concentrations

of PGE_2 is antagonised at the high concentrations of PGE_2. PGE_2 is known to exert trophic actions on both primate and non-primate ovarian tissues, stimulating progesterone biosynthesis [reviewed by Richardson, 1986; Michael *et al*, 1994; Olofsson *et al*, 1994]. Furthermore progesterone is known to inhibit 11β-HSD activity [Ricketts *et al*, 1998; Burton *et al*, 1999]. Hence, at higher PGE_2 concentrations, an increase in progesterone production may offset the stimulation of 11β-HSD activity by PGE_2 in cells co-treated with MA. In order to test this hypothesis, the concentration-dependent effects of PGE_2 were compared in the absence and the presence of aminoglutethimide, a known inhibitor of steroid synthesis (section 3.3.3). Incubation with an abridged range of PGE_2 concentrations for 4 hours in the presence of AG resulted in a concentration-dependent increase in the activity of the enzyme 11β-HSD by all tested concentrations of PGE_2, supporting the hypothesis that progesterone antagonises the stimulation of 11β-HSD activity by PGE_2. At low concentrations (<30nM), PGE_2 can only activate the EP receptors but no stimulation of 11β-HSD activity is observed at these PGE_2 concentrations. At concentrations of 100 and 1000nM PGE_2, 11β-HSD activity increased significantly suggesting that PGE_2 may be mediating its effects on cortisol oxidation partly through other receptors than its own (*e.g.* FP and/or DP receptors).

Ongoing research in our laboratory has shown that human follicular fluid contains intrinsic aqueous stimuli and hydrophobic inhibitors of $NADP^+$-dependent 11β-HSD activity and that these components can be eluted from a C18 mini-column at methanol concentrations of 0-10% (v/v) and 65-90% (v/v) respectively. It was therefore suggested that PGE_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ (the stable metabolite of prostacyclin) should be tested to see whether they may be contained within the follicular fractions that are either stimulatory or inhibitory to $NADP^+$-dependent 11β-HSD activity. Two independent lines of data indicated that PGE_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ in follicular fluid elute from a C18 mini-column at 20-50% (v/v) methanol. Hence these PGs probably do not contribute to the modulation of 11β-HSD activity by follicular fluid. According to the data obtained from the human granulosa-lutein cell-experiments, it would be expected that the fractions of follicular fluid eluted at 20-50% methanol would stimulate 11β-HSD activity since they contain PGE_2 and $PGF_{2\alpha}$. There are several possible explanations as to why this did not happen:

1. The intra-follicular concentrations of PGE_2 and $PGF_{2\alpha}$ measured by RIA were 1.3±0.2 and 27.3±1.2nM respectively. Results obtained from hGLC experiments demonstrated that the 11β-HSD activity was increased when cells were co-treated with 30nM PGE_2, $PGF_{2\alpha}$ and PGD_2. Hence at concentrations <30nM, prostaglandins would probably be too low to increase 11β-HSD activity in the rat kidney homogenate. Furthermore, the PGE_2 and $PGF_{2\alpha}$ concentrations measured in follicular fluid prior to fractionation were higher than those measured by RIA in the follicular fluid fractions (1.1±0.0 and 18.2±7.9nM for PGE_2 and $PGF_{2\alpha}$ respectively). This suggests that PG concentrations may have been slightly underestimated when measured in follicular fractions, probably due to reconstitution of the samples which was performed in ddH_2O (since methanol, even at low concentrations, appeared to interfere with the RIA). Prostaglandins are not very soluble in aqueous solutions, which makes it more difficult for the dried samples to be fully reconstituted in ddH_2O.
2. Since follicular fluid fractions are heterogeneous there may be other modulators contained within the 20-50% methanol fractions that may obscure the effects of PGs. Hence stimulation of 11β-HSD activity may occur which may be simultaneously suppressed by another compound present in the same follicular fluid fractions.
3. The time of incubation for the rat kidney bioassay may be too short. Incubation with follicular fluid fractions was only performed for 1 hour which may not be enough time for PGs contained within these follicular fluid fractions to increase 11β-HSD activity. When testing the effects of PGs on granulosa cells, the incubation was conducted over a period of 4h.
4. $NADP^+$-dependent 11β-HSD isoforms in rat kidney homogenate may be less sensitive to stimulation by PGs than the major 11β-HSD isoform(s) of human granulosa-lutein cells.
5. The signal transduction pathways via which PGs stimulate 11β-HSD activity in human granulosa-lutein cells may be compromised in the rat kidney homogenate. The rat kidney bioassay experiment is performed on lysed kidney cells, whereas studies in human granulosa-lutein cells involve intact isolated cells. As a result, components of the cell membrane or structures usually found inside the cell that may be required for PGs to stimulate 11β-HSD activity in human granulosa-lutein cells, may not be functional in the rat kidney homogenate.

Studies performed by L.M. Thurston have demonstrated that modulators on 11β-HSD in bovine follicular fluid have comparable biophysical properties to those in human follicular fluid. In both cases, the endogenous ovarian enzyme stimuli elute at 0-10% (v/v) methanol and 11β-HSD inhibitors elute at 55-85% methanol. However, whereas the inhibitory fractions of porcine follicular fluid have similar properties, to those in human and cow, the biophysical properties of the enzyme stimuli in porcine follicular fluid are somewhat different. The enzyme stimuli do not elute in the aqueous fractions, but elute at 20-40% (v/v) methanol. Hence, unlike human and bovine follicular fluids, the paracrine stimuli of 11β-HSD from porcine follicular fluid coelute with PGs. Whereas the lack of effect of PGs in human follicular fluid may be due to low PG concentrations, it maybe that in porcine follicular fluid PG concentrations are high enough to stimulate 11β-HSD activity.

In conclusion, the data presented in this chapter suggest that locally produced PGs are important for maintaining ovarian 11β-HSD activity. However, they do not seem to contribute to the hydrophilic stimuli or hydrophobic inhibitors of 11β-HSD activity as resolved from human follicular fluid.

Chapter Six

ROLE OF EP_1 AND EP_2 RECEPTOR SUBTYPES IN THE OVARIAN ACTIONS OF PGE_2

Chapter Six

ROLE OF EP_1 AND EP_2 RECEPTOR SUBTYPES IN THE OVARIAN ACTIONS OF PGE_2

6.1 INTRODUCTION

As reviewed in Chapter 1, the actions of PGE_2 have been shown to be mediated through EP receptors (section 1.6.2). To date there are four subtypes of EP receptors that have been cloned (EP_1, EP_2, EP_3 & EP_4) [Hirata *et al*, 1991; Sugimoto *et al*, 1992; Honda *et al*, 1993; Watabe *et al*, 1993, Funk, *et al*, 1993], with multiple isoforms of the EP_3 receptor subtype [reviewed by Coleman *et al*, 1994]. While EP_1 and EP_3 receptors mediate increases in the intracellular Ca^{2+} concentrations, EP_2 EP_3 and EP_4 can each increase cAMP accumulation. Since EP receptors have different affinities for synthetic PGE_2 analogues, receptor subtype-specific agonists and antagonists can be used to delineate the role of particular EP receptor subtypes in a given cellular response [Coleman *et al*, 1994].

In chapters 3, 4 and 5, it has been suggested that PGE_2 plays an important role in the control of both steroidogenesis and 11β-HSD activity. Consequently, the main objective of the work described in this chapter was to investigate which EP receptors mediate the effects of PGE_2 on steroidogenesis and cortisol metabolism in human granulosa-lutein cells. These studies involved the use of EP receptor antagonists which preferentially inhibit binding of PGE_2 to EP_1 and EP_2 receptors (SC19220 and AH6809, respectively) and an EP_2 receptor agonist, butaprost.

6.2 EXPERIMENTAL PROTOCOLS

For the experiments described in sections 6.3.1 and 6.3.2, cells were seeded into sterile 96-well cell culture plates at a density of 1×10^5 cells/ml culture medium with a volume of 250µl medium per well. All experiments were stopped by the addition of 10µl 3M perchloric acid per well, followed by storage at –20°C pending assay. Prior to each RIA, 20µl 2.16M potassium phosphate were added into each well.

For the experiments described in sections 6.3.3, 6.3.4 and 6.3.5, cells were seeded into sterile 24-well cell culture plates at a density of 5×10^4 cells/ml culture medium

with a volume of 1ml medium per well. 11β-HSD activities were quantified as described in sections 2.6 & 2.7.

In all experiments described in this chapter, results were internally standardised. All data are presented graphically as percentage values (mean±SE) where the control, progesterone or cAMP concentrations, or 11β-HSD activity for each experimental design, was standardised to 100%. However, all statistical analyses were performed on non-referenced data.

6.3 RESULTS

6.3.1 Concentration-dependent effects of the EP receptor antagonists SC19220 and AH6809 on basal progesterone production

Before use, both EP receptor antagonists were tested for possible direct effects on basal progesterone output from human granulosa-lutein cells. Cells were cultured for two days in serum-supplemented medium to allow the cells to attach to the plate. On the third day of culture, cells were rinsed with warmed serum-supplemented medium and then incubated for a further 24 hours in serum-supplemented medium containing 0, 0.01, 0.1, 1 and 10μM SC19220 or AH6809, both in presence of MA (1μM). SC19220 and AH6809 were prepared to a stock concentration of 10mM in DMSO, the final concentration of which was adjusted in all wells to 0.1% (v/v). At the end of the 24-hour incubation period, the spent medium was collected and stored at -20°C pending assay of the progesterone concentrations by specific RIAs (section 2.3).

Under conditions in which intrinsic PG synthesis had been suppressed with 1μM MA, incubation for 24 hours with the EP receptor antagonists SC19220 or AH6809 had no significant effect on basal progesterone concentrations (Figure 6.1).

6.3.2 Concentration-dependent effects of PGE_2 on progesterone production in the absence and presence of SC19220 and AH6809

Cells were cultured for two days in serum-supplemented medium to allow the cells to attach to the plate. On the third day of culture, cells were rinsed with warmed serum-supplemented medium and then incubated for a further 24 hours in serum-supplemented medium containing 1μM MA and a range of PGE_2 concentrations (0-

3000nM) both in the absence and presence of either 10μM SC19220 or 10μM AH6809 in a 3×8 factorial design. Since cAMP concentrations were going to be measured by RIA, treatments were performed in the presence of 0.5mM IBMX. At the end of the 24 hour incubation period, the spent medium was collected and stored at -20°C pending assay of the cAMP and progesterone concentrations by specific RIAs (section 2.3).

Treatment with PGE_2 for 24 hours resulted in concentration-dependent increases in progesterone accumulation (Figure 6.2). However, co-treatment with SC19220 attenuated the responses to PGE_2 at concentrations of 1000 and 3000nM and co-treatment with AH6809 completely abolished the ability of PGE_2 to stimulate progesterone output. At PGE_2 concentrations $\geq$ 30nM, progesterone production increased progressively by up to 90.5±5.5% ($P<0.01$; n=3) at the highest tested PGE_2 concentration of 3000nM. Co-treatment with SC19220 (10μM) attenuated the progesterone response to PGE_2 by 55.9±4.1% ($P<0.001$) and 35.0±3.3% ($P<0.001$) at PGE_2 concentrations of 1000 and 3000nM respectively, relative to the cells treated with PGE_2 alone.

Incubation for 24 hours with PGE_2 resulted in concentration-dependent increases in cAMP accumulation which were significant at concentrations of 1000 and 3000nM (Figure 6.3). Responses were either enhanced or decreased by co-treatment with SC19220 or AH6809 respectively. At the highest tested concentration of 3000nM, PGE_2 stimulated cAMP production by 17.7±6.8-fold (n=3; $P<0.01$) in the absence of EP receptor antagonists. In cells co-treated with SC19220, the highest tested PGE_2 concentration of 3000nM stimulated a 40.3±27.8-fold increase in cAMP accumulation (n=3; $P<0.01$) which was significantly higher than that in cells treated with PGE_2 alone. However, co-treatment with AH6809 (10μM) significantly decreased the ability of PGE_2 to elevate cAMP accumulation. Hence, in cells co-treated with AH6809, 3000nM PGE_2 was only able to increase cAMP by 4.1±1.2-fold (n=3; $P<0.05$).

6.3.3 Concentration-dependent effects of the EP receptor antagonists SC19220 and AH6809 on basal 11β-HSD activity

Following the two-day incubation in serum-supplemented medium cells were rinsed with warmed serum-free medium and further incubated for 4 hours in serum-free medium containing 0, 0.01, 0.1, 1 and 10μM SC19220 or AH6809, both in the presence of MA (1μM). 11β-HSD activities were quantified as previously described (sections 2.6 & 2.7).

Under conditions in which intrinsic PG synthesis had been suppressed with 1μM MA, incubation with a range of concentrations of SC19220 and AH6809, had no significant effect on basal 11β-HSD activities (Figure 6.4).

6.3.4 Concentration-dependent effects of PGE_2 on 11β-HSD activity in absence and presence of SC19220 and AH6809

In this 3×4 factorial design, cells were rinsed with warmed serum-free medium and then incubated for a further 4 hours in serum-free medium containing an abridged range of concentrations of PGE_2 (0, 10, 100 and 1000nM) each in the absence or presence of 10μM SC19220 or AH6809. The experiments testing the concentration-dependent effects of PGE_2 were performed in the presence of 1μM MA for suppression of intrinsic PG synthesis. In view of the potential confounding effects of progesterone on 11β-HSD activities incubations were performed in the presence of 100μM AG. 11β-HSD activities were subsequently quantified as previously described (sections 2.6 & 2.7).

Consistent with previous observations (section 5.2.4) PGE_2 significantly increased 11β-HSD activity in a concentration-dependent manner; net oxidation of cortisol to cortisone was increased by 42.5±3.1% at the highest tested PGE_2 concentration of 1000nM ($P<0.001$; n=3) (Figure 6.5). Incubation in the presence of SC19220 (10μM) resulted in an increase in the 11β-HSD activity at each of the tested concentrations of PGE_2. At the highest tested PGE_2 concentration of 1000nM, co-treatment with SC19220 increased the 11β-HSD activity by 30.2±0.2%($P<0.05$; n=3) relative to the cells treated with PGE_2 in the absence of EP receptor antagonist. In contrast, co-treatment with AH6809 completely abolished the 11β-HSD response to all tested concentrations of PGE_2.

6.3.5 Concentration-dependent effects of butaprost on 11β-HSD activity in the absence and presence of SC19220 and AH6809

Following the two-day incubation in serum-supplemented medium cells were rinsed with warmed serum-free medium to remove any residual serum and further incubated for 4 hours in serum-free medium containing an abridged range of butaprost concentrations (0, 10, 100 and 1000nM) each in the absence or presence of 10μM SC19220 or 10μM AH6809. Butaprost was prepared to a stock concentration of 1mM in DMSO, the final concentration of which was adjusted in all wells to 0.1% (v/v). These experiments were performed in the presence of 1μM MA for suppression of intrinsic PG synthesis and 100μM aminoglutethimide for suppression of potential confounding effects of progesterone. 11β-HSD activities were quantified as previously described (sections 2.6 & 2.7).

Incubation for 4 hours with butaprost resulted in a concentration-dependent increase of 11β-HSD activity at butaprost concentrations of 100 and 1000nM (Figure 6.6). At the higher tested concentration of 1000nM, butaprost increased net cortisol oxidation by 40.0±3.0% ($P<0.01$; n=3). Co-treatment with SC19220 (10μM) resulted in a further increase in the 11β-HSD activity at each tested concentration of butaprost; at the highest tested concentration of 1000nM butaprost, co-treatment with SC19220 increased cortisol oxidation by 30.5±0.6% (n=3; $P<0.05$) relative to cells treated with butaprost alone. However, co-treatment with AH6809 completely abolished the effects of butaprost on 11β-HSD activity.

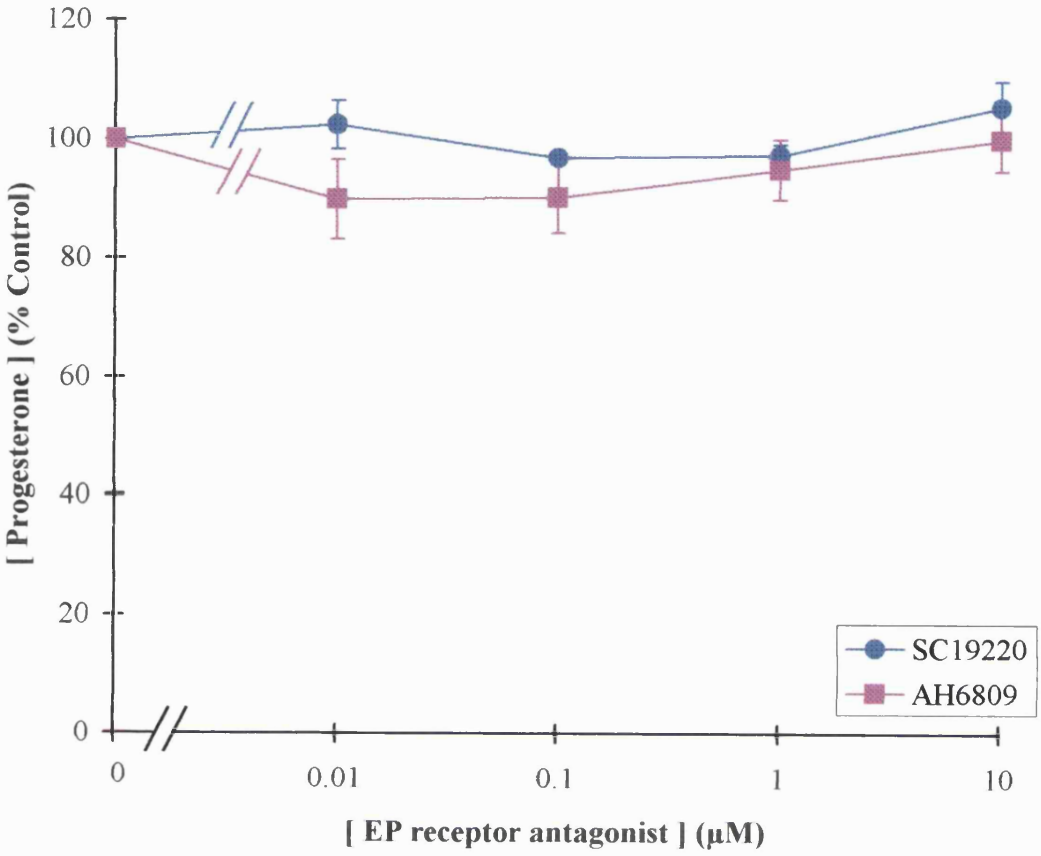

Figure 6.1 Concentration-dependent effects of SC19220 and AH6809 on progesterone production by human granulosa-lutein cells

Progesterone concentrations in spent medium collected after a 24-hour incubation in the presence of 0-10µM SC19220 (-●-) or AH6809 (-■-). Values are the mean±SE for 3 independent experiments with quadruplicate determinations in each experiment. (One way ANOVA; $P>0.05$)

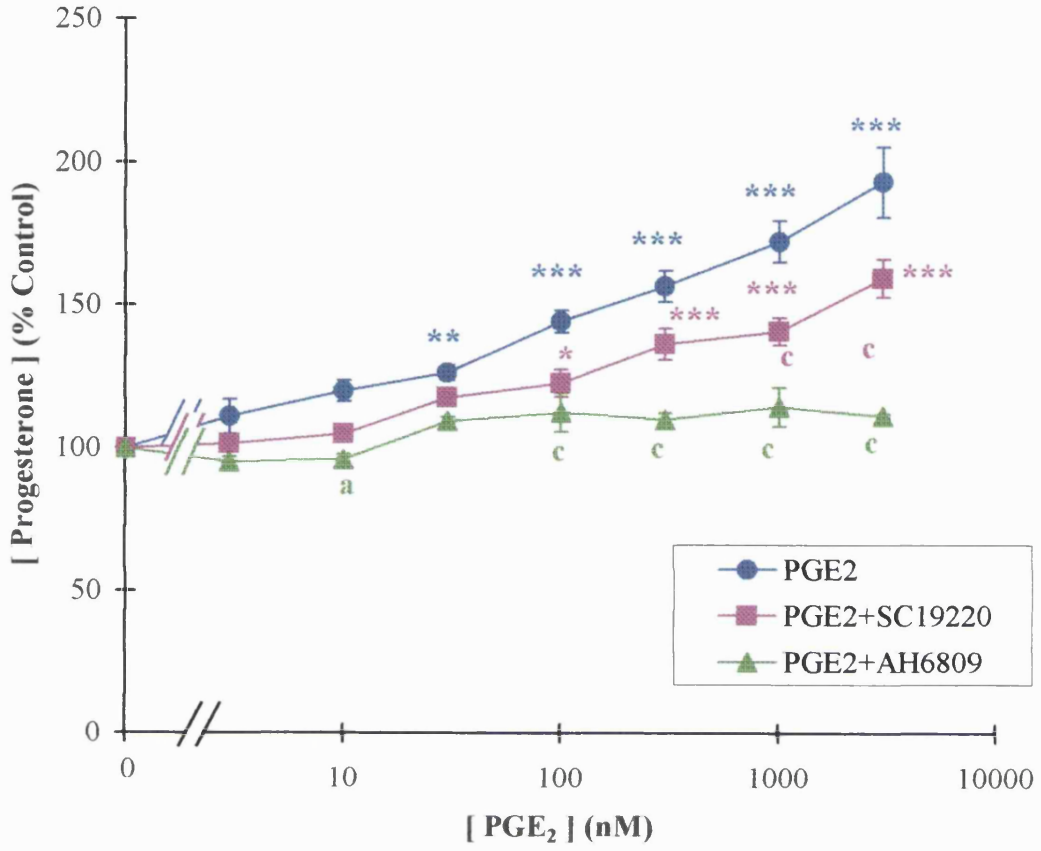

Figure 6.2 Effects of SC19220 and AH6809 on the progesterone responses to PGE_2 by human granulosa-lutein cells

Progesterone concentrations in spent medium collected after a 24-hour incubation in the presence of 0-3000nM PGE_2, either in the absence (—●—) or the presence of 10μM SC19220 (—■—) or 10μM AH6809 (—▲—). Values are the mean±SE for 3 independent experiments with quadruplicate determinations in each experiment. *P<0.05, **P<0.01, ***P<0.001 relative to 0nM PGE_2 in the absence or presence of the respective EP receptor antagonist. ^{a}P<0.05, ^{c}P<0.001 relative to cells treated with the equivalent PGE_2 concentration in the absence of EP receptor antagonist (One way ANOVA; Bonferonni's *post hoc* test)

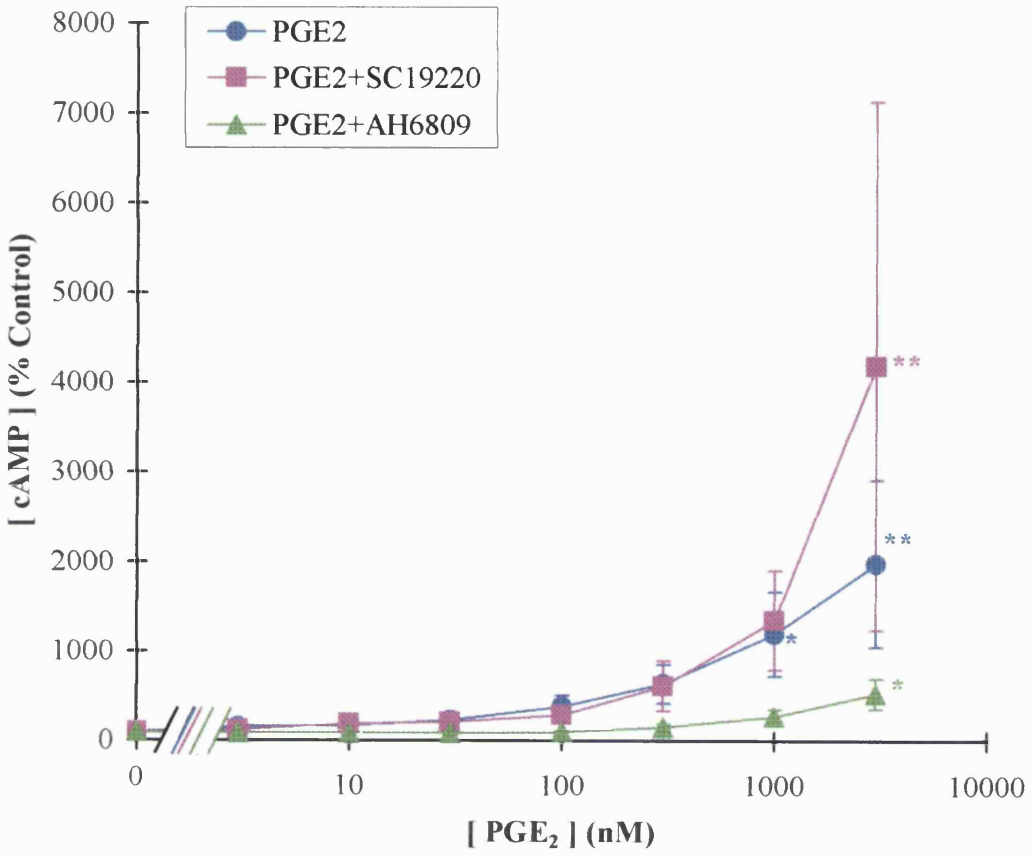

Figure 6.3 Effects of SC19220 and AH6809 on the cAMP responses to PGE_2 by human granulosa-lutein cells

Concentrations of cAMP after a 24-hour incubation in the presence of 0-3000nM PGE_2, either in the absence (—●—) or the presence of 10μM SC19220 (—■—) or 10μM AH6809 (—▲—). Values are the mean±SE for 3 independent experiments with quadruplicate determinations in each experiment. *P<0.05, **P<0.01 relative to 0nM PGE_2 in the absence or presence of the respective EP receptor antagonist (One way ANOVA; Dunnett's *post hoc* test)

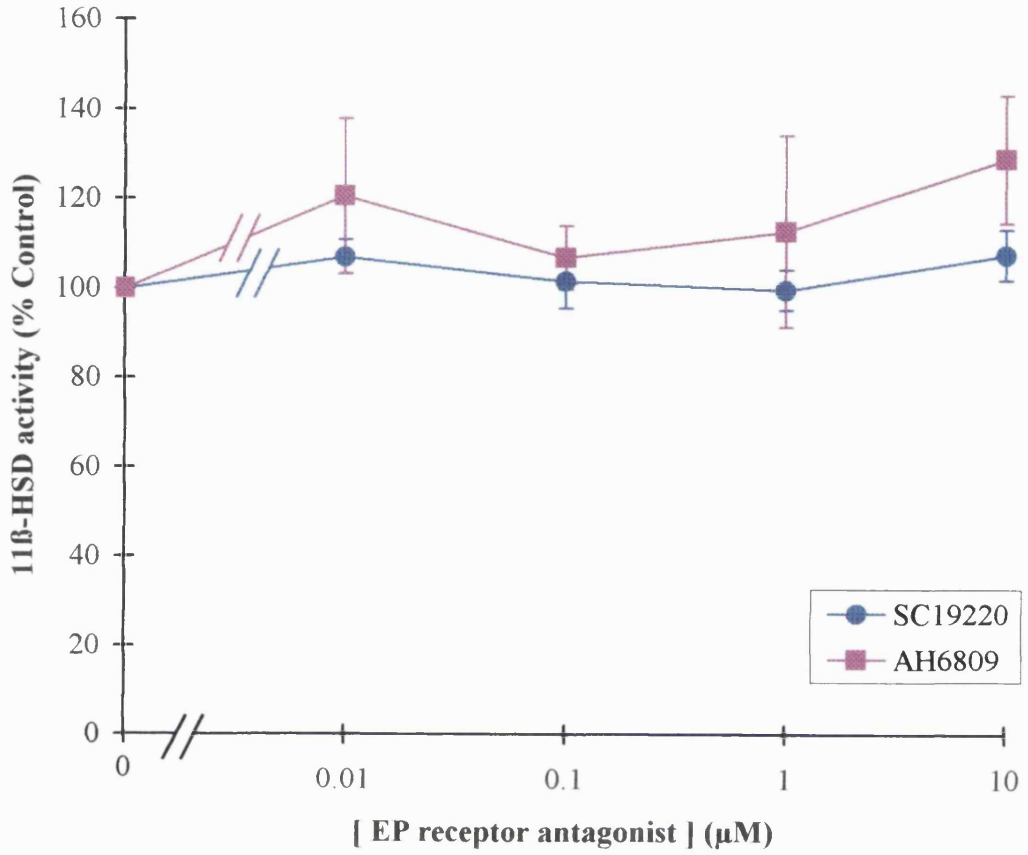

Figure 6.4 Concentration-dependent effects of SC19220 and AH6809 on 11β-HSD activity in human granulosa-lutein cells

11β-HSD activities following treatment of hGLC with 0-10µM of either SC19220 (-●-) or AH6809 (-■-) in the presence of 1µM MA. Values are the mean±SE for 4 independent experiments with triplicate determinations in each experiment. (One way ANOVA; $P>0.05$)

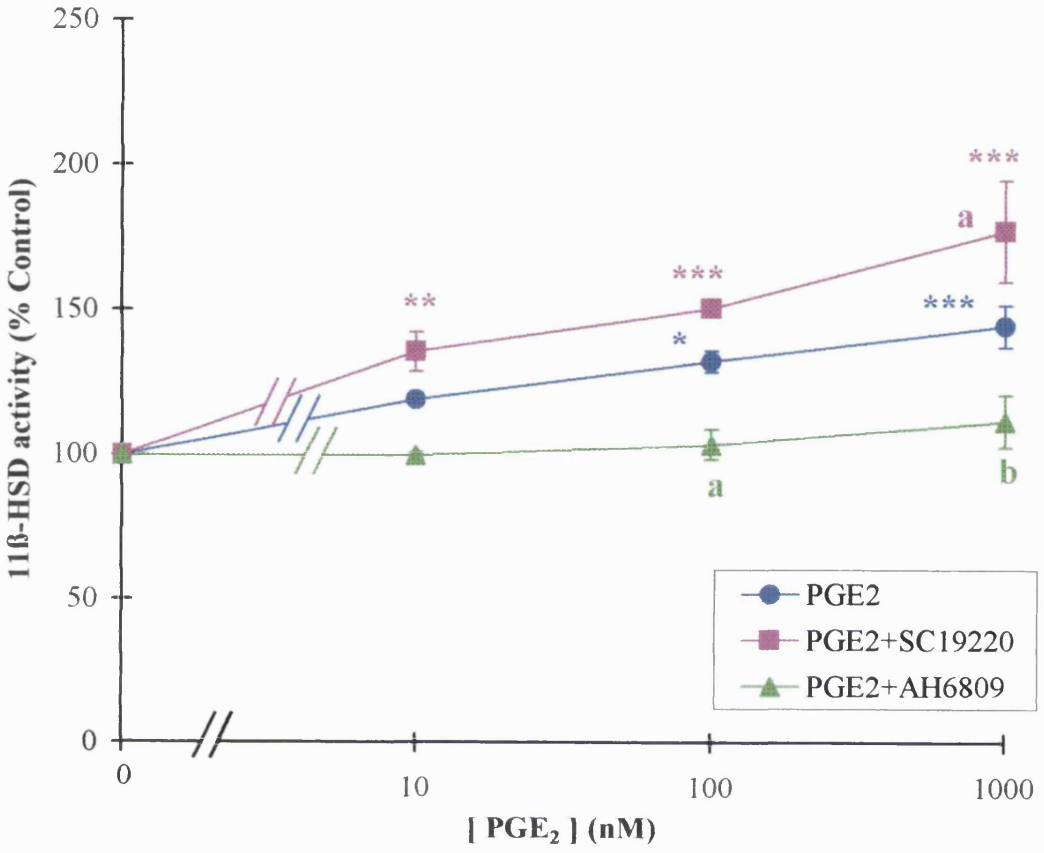

Figure 6.5 Effects of SC19220 and AH6809 on 11β-HSD activities in response to PGE_2 in human granulosa-lutein cells

11β-HSD activities following treatment of hGLC with 0-1000nM PGE_2 either in the absence (●) or the presence of 10μM SC19220 (■) or 10μM AH6809 (▲). Values are the mean±SE for 3 independent experiments with triplicate determinations in each experiment. **P*<0.05, ***P*<0.01, ****P*<0.001 relative to 0nM PGE_2 in absence or presence of the respective EP receptor antagonist. [a]*P*<0.05, [b]*P*<0.01 relative to cells treated with the equivalent PGE_2 concentration in the absence of EP receptor antagonist (One way ANOVA; Bonferonni's *post hoc* test)

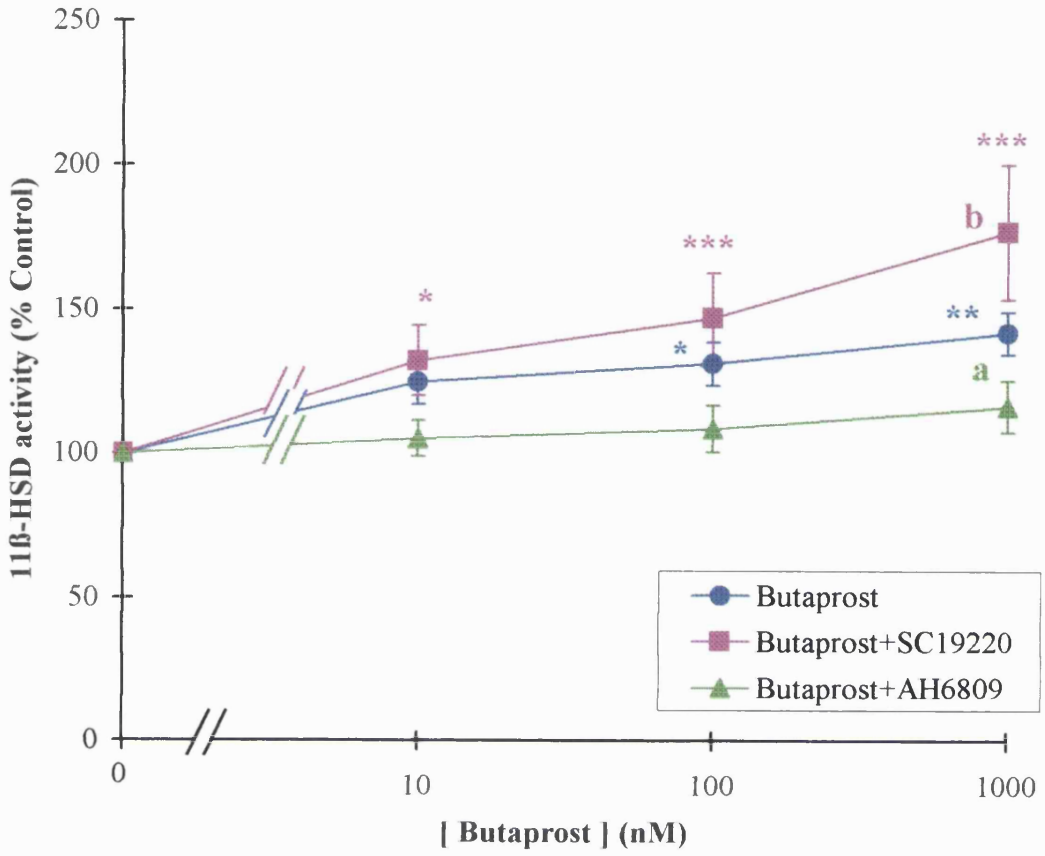

Figure 6.6 Effects of SC19220 and AH6809 on 11β-HSD activities in response to butaprost in human granulosa-lutein cells

11β-HSD activities following treatment of hGLC with 0-1000nM butaprost either in the absence (—●—) or the presence of 10μM SC19220 (—■—) or 10μM AH6809 (—▲—). Values are the mean±SE for 3 independent experiments with triplicate determinations in each experiment. **P*<0.05, ***P*<0.01, ****P*<0.001 relative to 0nM PGE_2 in the absence or presence of the respective EP receptor antagonist. [a]*P*<0.05, [b]*P*<0.01 relative to cells treated with the equivalent butaprost concentration in the absence of EP receptor antagonist (One way ANOVA; Bonferonni's *post hoc* test)

6.4 DISCUSSION

Experiments described in this chapter involved the use of commercially available PG receptor agonists/antagonists, each of which has limited receptor specificity. Therefore, before interpreting the experimental data obtained with these agents, the disadvantages of receptor pharmacology approaches need to be reviewed. Although the available receptor agonists and antagonists are useful for investigating new receptors and signal transduction pathways, high specificity is not always achieved and prohibits precise interpretation of the data. For example both SC19220 and AH6809, employed in the current studies, have weak affinities for the receptors to which they bind [Ito *et al*, 1990; Abramovitz *et al*, 2000]. Moreover, AH6809, apart from being a mixed EP_1/EP_2 receptor antagonist, has also been shown to block DP and EP_{3III} receptors [Abramovitz *et al*, 2000]. In some cases, the use of receptor selective agonists and antagonists in various combinations allows tentative conclusions to be reached. In the current studies, this approach has been taken in comparing the effects of AH6809 (a mixed EP_1/EP_2 receptor antagonist) and SC19220 which appears to act solely at the EP_1 receptor [Coleman *et al*, 1994]. The interpretations offered in this discussion are based on the assumptions that:

1. if both AH6809 and SC19220 inhibit a given response to PGE_2, the EP_1 receptor is likely to participate in the relevant response
2. if AH6809 inhibits a given response, but SC19220 does not, that action of PGE_2 must be mediated through a receptor that is sensitive to inhibition by AH6809 but not by SC19220, such as the EP_2 or DP receptor.

Consistent with previous observations, PGE_2 stimulated concentration-dependent increases in the production of progesterone. Co-treatment with AH6809 prevented the steroidogenic response to PGE_2 at all tested concentrations, suggesting that an AH6809-sensitive receptor (*e.g.* EP_1, EP_2 and/or DP receptor(s)) is implicated in this steroidogenic response. Co-treatment with SC19220 also suppressed the responsiveness of the cells to PGE_2 but only by ~50%, suggesting that the EP_1 receptor is partly implicated in stimulating progesterone production in response to PGE_2. According to Table 5.1, at concentrations <10nM, PGE_2 should be able to activate all four EP receptor subtypes, and at concentrations >30nM, activation of each of the cloned EP receptors should be maximal. However, in the studies reported herein, 10nM PGE_2 was unable to stimulate progesterone production, and

the progesterone concentrations were increased in a concentration-dependent manner at PGE_2 concentrations >30nM. These observations suggest that the cloned EP receptors may not mediate the steroidogenic response to PGE_2, but that PGE_2 may stimulate progesterone synthesis via other PG receptors (*e.g.* FP &/or DP receptors; Table 5.1). Since FP receptors mediate inhibition (rather than stimulation) of progesterone production in human granulosa-lutein cells [Lopez-Bernal, 2001], it seems highly unlikely that the stimulation of steroidogenesis by PGE_2 could reflect cross-reaction with the FP receptor. However, the DP receptor remains a strong candidate for mediating the steroidogenic action of PGE_2.

Cyclic AMP accumulation increased when granulosa cells were treated with PGE_2. Co-treatment with AH6809 suppressed, but did not completely abolish, the cAMP response to PGE_2, suggesting that an AH6809-sensitive receptor may be required for stimulation of cAMP accumulation. SC19220 on the other hand did not suppress the responsiveness of the cells to PGE_2; if anything, it enhanced the increase of cAMP in response to PGE_2. These observations raise 2 possibilities. Firstly, inhibition of EP_1 receptor binding by SC19220 may have facilitated increased PGE_2-induced activation of other receptors which are capable of stimulating cAMP accumulation (*e.g.* EP_2, EP_4 and/or DP receptors). Secondly, co-stimulation of the EP_1 receptor would be expected to elevate the intracellular Ca^{2+} concentration [Narumiya *et al*, 1999] which, via PKC, can inhibit the stimulation of cAMP production [Abayasekara *et al*, 1993a; Abayasekara *et al*, 1993b]. Therefore, blocking the EP_1 receptor pathway would decrease Ca^{2+}-dependent effects on cAMP accumulation. Based on these observations, a receptor which is sensitive to AH6809 but insensitive to SC19220 (*e.g.* EP_2 and/or DP receptor) is implicated in the ability of PGE_2 to stimulate cAMP accumulation. At 30nM PGE_2, when all EP receptors should be saturated (Table 5.1), cAMP accumulation was not elevated in response to PGE_2. This observation implies that the EP receptors may not participate in the cAMP response of the cells to PGE_2. However, cAMP accumulation increased at PGE_2 concentrations of 1000 and 3000nM. At such high concentrations, the increase in cAMP accumulation is likely to be mediated through a non-EP receptor. At a concentration of 3000nM PGE_2, co-treatment with AH6809 did not completely prevent 3000nM PGE_2 from increasing cAMP accumulation, indicating that the residual effect on cAMP must be mediated through an AH6809-insensitive receptor.

As regards effects of PGE_2 on ovarian cortisol-cortisone interconversion, 11β-HSD activities in human granulosa-lutein cells were stimulated at PGE_2 concentrations of 100 and 1000nM PGE_2. Co-treatment with AH6809 prohibited PGE_2 from stimulating cortisol oxidation implicating an AH6809-sensitive receptor (*e.g.* EP_1, EP_2 and/or DP receptor) in mediating the effects of PGE_2. When granulosa cells were co-treated with SC19220, 11β-HSD activities increased in response to PGE_2, which suggests that the increase in 11β-HSD activity in response to PGE_2 is unlikely to be mediated through the EP_1 receptor. These observations raise 3 possibilities:

1. Inhibition of binding of PGE_2 to the EP_1 receptor pathway may have facilitated increased activation of other receptors and signalling pathways which may have a positive effect on 11β-HSD activity
2. As previously mentioned the EP_1 receptor pathway modulates intracellular Ca^{2+} concentrations [Narumiya *et al*, 1999]. If, in common with the 11β-HSD2 isoform expressed in placenta, the major 11β-HSD isoform in human granulosa-lutein cells is sensitive to Ca^{2+}-mediated inhibition by PGs [Hardy *et al*, 2001], then activation of the EP_1 receptor pathway may inhibit cortisol oxidation such that suppression of the EP_1 receptor pathway would result in a higher 11β-HSD activity. However, luteinised granulosa cells are thought to express only 11β-HSD1 [Tetsuka *et al*, 1997] which appears to be positively regulated by PG-induced increases in the intracellular Ca^{2+} concentration [Challis *et al*, 2000].
3. If it is assumed that PGE_2 is increasing cortisol oxidation by a cAMP pathway (activated via the EP_2, EP_4 or DP receptor), then simultaneous elevation of the intracellular Ca^{2+} concentration via the EP_1 receptor might be expected to compromise the cAMP-stimulation of 11β-HSD activity such that blockade of the EP_1 receptor could potentiate the 11β-HSD response to PGE_2.

Finally a combination of the above mentioned events may be taking place and the 11β-HSD activity measured may be the result of all of the above actions.

As previously mentioned incubation for 4 hours in the presence of AH6809 prevented PGE_2 from stimulating 11β-HSD activity. In conjunction with the data obtained from co-treatment with SC19220 it is implied that PGE_2 actions on cortisol oxidation may be mediated by an AH6809-sensitive receptor that is not blocked by

SC19220 (*e.g.* EP_2 and/or DP receptor). The EP_2 receptor is coupled to G_s known to activate AC and hence the production of cAMP [Narumiya *et al*, 1999]. Furthermore cAMP has also been shown to stimulate 11β-HSD activity in the placenta [Sun *et al*, 1998; Patel *et al*, 1999] and in human granulosa-lutein cells [Barker *et al*, 2001]. When granulosa cells were treated with butaprost, an EP_2 receptor-selective agonist, 11β-HSD activity increased at 100 and 1000nM butaprost. Such observations strongly implicate the EP_2 receptors in mediating the stimulation of cortisol oxidation in response to PGE_2. Co-treatment with AH6809 prevented butaprost from stimulating 11β-HSD activities, whereas SC19220 potentiated the response to butaprost. Since butaprost is a preferential EP_2 agonist, one would have not expected SC19220 to have any effect on the stimulation of cortisol oxidation by butaprost. The most probable explanation for these observations would be that butaprost is not a specific EP_2 agonist and/or that SC19220 is not acting solely as an EP_1 antagonist.

As described above, there are inevitable disadvantages when using receptor agonists and antagonists. Limitations on receptor specificity should be taken into consideration when interpreting results from such studies. For example, AH6809 is currently thought to block the actions of EP_1, EP_2 and DP receptors [Abramovitz *et al*, 2000]. However, at a concentration of 10μM, as used in the current studies, one cannot exclude the possibility that AH6809 might also block other prostanoid receptors. At lower concentrations, AH6809 may exhibit more selective properties for those receptors for which it has the highest affinity. Although there are no reports that SC19220 can block receptors other than the EP_1 receptor, this possibility cannot be excluded. Hence repetition of the experiments previously described using lower concentrations of SC19220 and AH6809 might generate more conclusive results.

The design of subtype specific EP receptor antagonists is necessary for definitive investigation of the signalling pathways employed by PGE_2 to stimulate progesterone production, cAMP accumulation and 11β-HSD activity in human granulosa-lutein cells. More specifically, selective agonists and antagonists for the DP, EP_2, EP_4 and FP receptors would be required in order to examine which of the above mentioned receptors may be mediating PGE_2 actions. Such specific compounds are not currently available and so further investigations await their development.

The data reported in this chapter indicate that the ovarian actions of PGE_2 are likely to be mediated via those EP and/or DP receptors coupled to the AC-cAMP signalling pathway.

Chapter Seven

GENERAL DISCUSSION

Chapter Seven

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The overall objective of this thesis was to examine the involvement of locally synthesised PGs in the paracrine/autocrine control of steroidogenesis and cortisol metabolism in human granulosa-lutein cells by investigating:

i) the relationship between the basal production of endogenous PGs and progesterone
ii) the role of PGs in the steroidogenic response to HDL
iii) the possible role for PGs in the regulation of ovarian cortisol metabolism
iv) which EP receptors mediate the effects of PGE_2 on steroidogenesis and cortisol metabolism

In this study the dynamic changes in the production of PGs and progesterone were shown to occur independently of each other, PGs were implicated in the steroidogenic responses to HDL and Apo-AI, and locally synthesised PGs were found to be important for maintaining ovarian 11β-HSD activity. Obtained data implicate certain classes of prostanoid receptors (EP_2 and DP receptors) in the above mentioned responses.

With regard to the first aim, data presented in this thesis have shown that there is no causal relationship between the pattern of endogenous production of either of the two prostaglandins measured (PGE_2 and $PGF_{2\alpha}$) and that of progesterone (Chapter 3). Although endogenous prostaglandins did not seem to affect basal progesterone production, exogenously administered PGE_2 was capable of stimulating steroidogenesis. This luteotrophic role of PGE_2 has been described previously in the human CL [Dennefors *et al*, 1982] and in human granulosa-lutein cells [McNatty *et al*, 1975; Veldius *et al*, 1987].

PGs were also shown to mediate, at least in part, the steroidogenic response to HDL (Chapter 4). HDL can stimulate the production of both PGE_2 and progesterone in human granulosa cells [Azhar *et al*, 1998a; Ragoobir, 1999]. Indeed in the studies reported herein, treatment with HDL increased PGE_2, cAMP and progesterone concentrations. Co-treatment with meclofenamic acid suppressed all three of these

responses to HDL, suggesting that luteotrophic PGs, such as PGE_2, might mediate the steroidogenic action of HDL (Figure 7.1). As for HDL, the responses to Apo-AI were also inhibited by meclofenamic acid. These findings suggest that Apo-AI may be one of the ways that HDL may be acting to exert its steroidogenic action. Since the EP_1 and EP_2 receptors have been implicated in the steroidogenic action of PGE_2, (Chapter 6), their role in mediating the stimulation of cAMP accumulation and progesterone synthesis by HDL and Apo-AI should be investigated. However, such studies should use specific antagonists for the EP_1, EP_2, EP_4 and DP receptors which have yet to be developed.

Another role for prostaglandins in the human ovary appears to be maintaining 11β-HSD activity (Chapter 5); treatment of human granulosa cells with PGHS inhibitors resulted in suppression of 11β-HSD activity and co-treatment with exogenous PGE_2, $PGF_{2\alpha}$ or PGD_2 all attenuated this inhibition. While PGs where implicated in paracrine stimulation of 11β-HSD activity in human granulosa-lutein cells, the concentrations of PGs in follicular fluid were too low to regulate cortisol oxidation. Hence those fractions of follicular fluid that contained PGs had no significant effects on renal $NADP^+$-dependent (type-I) 11β-HSD activity. Even though, in the human and bovine follicular fluids, PGs do not appear to be implicated in the stimulation of 11β-HSD activity, PGs might contribute to the stimulation of cortisol oxidation by fractions of porcine follicular fluid. Such observations would therefore be worth further investigation.

Overall, the data reported herein indicate that the EP_1 receptor plays a relatively minor role (if any) in the paracrine/autocrine actions of PGE_2 in the human ovary. The EP_1 receptor mediates the elevation of Ca^{2+} concentrations which is thought to be mainly dependent upon availability of extracellular Ca^{2+} [Coleman *et al*, 1994]. Mobilisation of intracellular Ca^{2+} has also been demonstrated [Watabe *et al*, 1993]. However, this increase in Ca^{2+} concentrations from intracellular stores appears to be via an IP_3-independent mechanism. Increases in Ca^{2+} concentrations linked to activation of PKC and other calcium/calmodulin-dependent protein kinases can inhibit the generation of cAMP [Abayasekara *et al*, 1993a; Abayasekara *et al*, 1993b] and increase cAMP breakdown via PDE activity [Michael *et al*, 1991]. Ca^{2+} can also affect the cAMP response through a direct effect on AC [Will-Shahab *et al*, 1985;

Wei *et al*, 1996]. Hence, co-activation of the EP_1 receptor signalling pathway might be expected to compromise the ability of PGE_2 to increase cAMP concentrations via the EP_2, EP_4 and possibly DP receptors. Co-treatment with SC19220, a selective EP_1 antagonist, decreased the progesterone response to PGE_2, suggesting that the EP_1-Ca^{2+} pathway is important for a full steroidogenic response. However, co-treatment with SC19220 appeared to have no effect on the ability of PGE_2 to stimulate cAMP accumulation and 11β-HSD activity. If anything, SC19220 enhanced these responses to PGE_2. Hence, the EP_1-Ca^{2+} pathway appears not to be required for PGE_2-induced stimulation of cAMP accumulation or cortisol metabolism, and simultaneous activation of this pathway may exert an antagonistic effect on the AC-cAMP-PKA signalling pathway (Figure 7.2 and Figure 7.3). Ca^{2+} is known to inhibit LH-stimulated AC activity and steroidogenesis [Knecht *et al*, 1985] such that activation of a Ca^{2+} signalling pathway(s) could result in suppression of cAMP accumulation and steroidogenesis. Results described herein are in accordance with these well-documented effects. Firstly, SC19220 enhanced the cAMP accumulation in response to PGE_2, which suggests that blocking the EP_1-Ca^{2+} pathway results in a further elevation of cAMP accumulation. Secondly, inhibition of the EP_1-Ca^{2+} pathway by SC19220 also increased the ability of PGE_2 to stimulate 11β-HSD activity, again suggesting that simultaneous activation of this pathway is antagonistic. Finally another possible explanation for the observed effects of SC19220 to both cAMP accumulation and cortisol oxidation may be that blockage of the EP_1 receptor renders PGE_2 more accessible for binding to other receptors.

Most of the data reported herein implicate AC-coupled PG receptors, such as EP_2 and DP receptors, in the paracrine/autocrine actions of PGE_2 in the human ovary. The EP_2 receptor is coupled to G_s and AC which mediate increases in cAMP concentrations [Narumiya *et al*, 1999]. Increases in intracellular cAMP concentrations have been linked in a number of studies to the stimulation of gonadal steroidogenesis [reviewed by Cooke, 1999]. Therefore, inhibition of the EP_2-cAMP pathway would suppress the stimulation of the steroidogenic pathway by PGE_2. In accordance with these predictions, co-treatment of cells with AH6809, an EP_2 antagonist, prevented PGE_2 from increasing progesterone concentrations, cAMP accumulation and 11β-HSD activity. Moreover, butaprost, an EP_2 agonist, mimicked the stimulation of 11β-HSD activity by PGE_2, further implicating the EP_2 receptor in

this response. However, as previously discussed, the concentrations of PGE_2 required to elevate progesterone production, cAMP accumulation and cortisol oxidation were all higher than those expected to activate EP_2 receptors (Table 5.1). Furthermore, the expected concentrations of PGE_2 at which saturation of the EP receptors would be achieved was estimated at ~30nM PGE_2 (Table 5.1) and yet the progesterone, cAMP and 11β-HSD responses were not maximal at this low concentration of PGE_2. Indeed, all three of these ovarian responses continued to increase in a concentration-dependent manner at PGE_2 concentrations >100nM. These observations strongly implicate other prostanoid receptors which PGE_2 might activate at such high concentrations (*e.g.* DP receptors). Moreover, both progesterone production and 11β-HSD activity increased at lower PGE_2 concentrations than those required for elevation of cAMP accumulation. Similar observations have previously been reported whereby low LH concentrations stimulated steroidogenesis without increasing cAMP accumulation [Cooke, 1999]. Two possible explanations have been proposed to elucidate such observations which could also account for those reported above. Firstly that the available RIAs employed to measure cAMP accumulation are not sensitive enough to detect such changes in cAMP concentrations. Secondly, that the increases in cAMP accumulation are compartmentalised inside the cell, resulting in local increases of cAMP concentrations which are high enough to stimulate steroidogenesis (or 11β-HSD activity) but would not significantly alter the overall cAMP concentrations inside the cells.

Unlike EP_2 receptors, the EP_1 receptors do not appear to be required for stimulation of 11β-HSD activity in response to PGE_2 and, if anything, activation of the EP_1-Ca^{2+} pathway may antagonise the positive effects mediated through the EP_2 receptor pathway (see above). At PGE_2 concentrations >30nM, this eicosanoid may be acting to increase 11β-HSD activity not only through EP receptors, but alternatively through DP (and possibly FP) receptors (Table 5.1). While activation of DP receptors could increase net cortisol oxidation via increased cAMP accumulation, activation of FP receptors would only be expected to increase 11β-HSD activity if this ovarian enzyme activity is positively regulated by Ca^{2+} (as for 11β-HSD1 activity in the placenta) [Challis *et al*, 2000]. In order to further investigate which of the EP receptor subtypes and DP receptors mediate the effects of PGE_2 in human

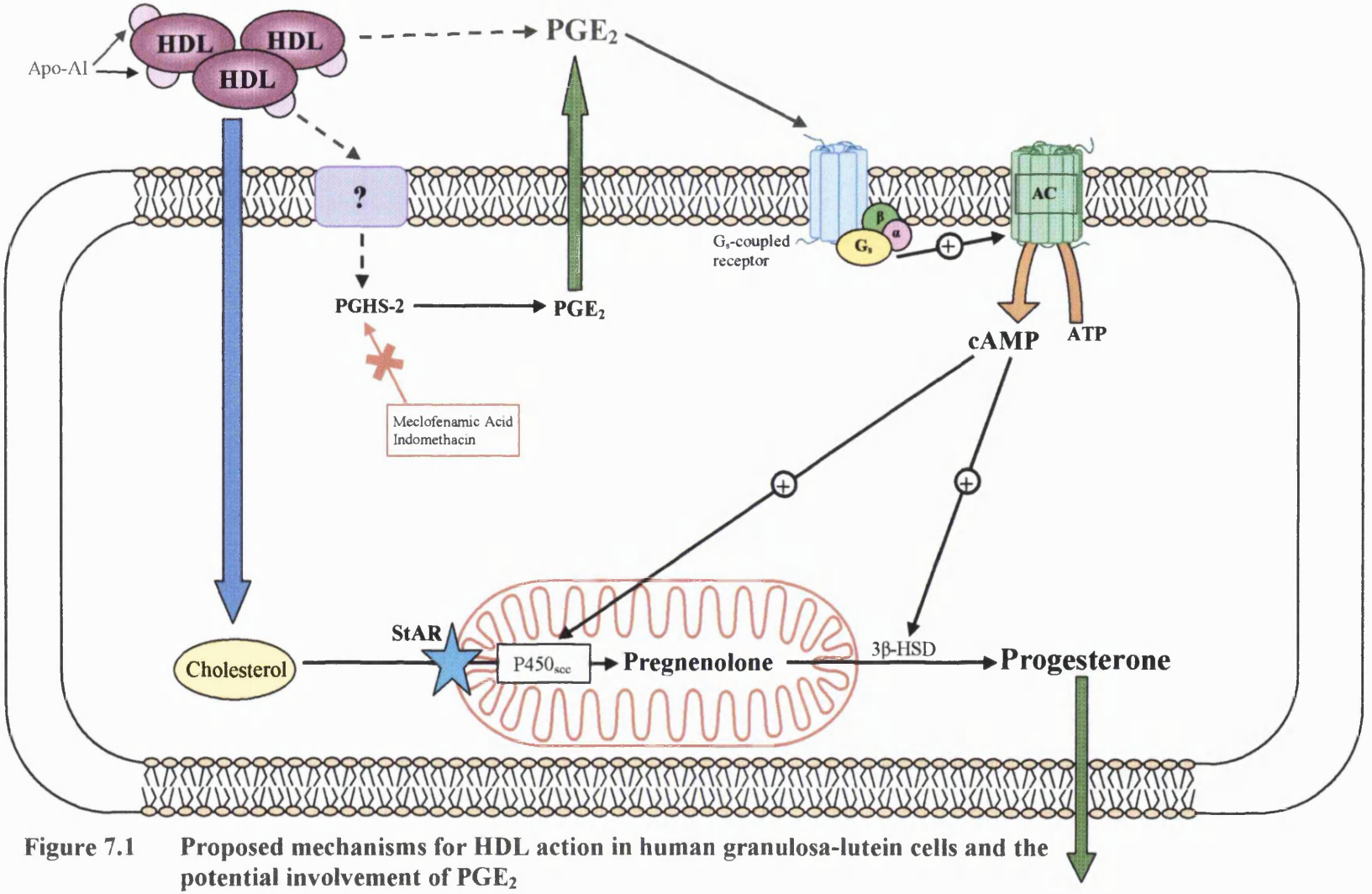

Figure 7.1 **Proposed mechanisms for HDL action in human granulosa-lutein cells and the potential involvement of PGE_2**

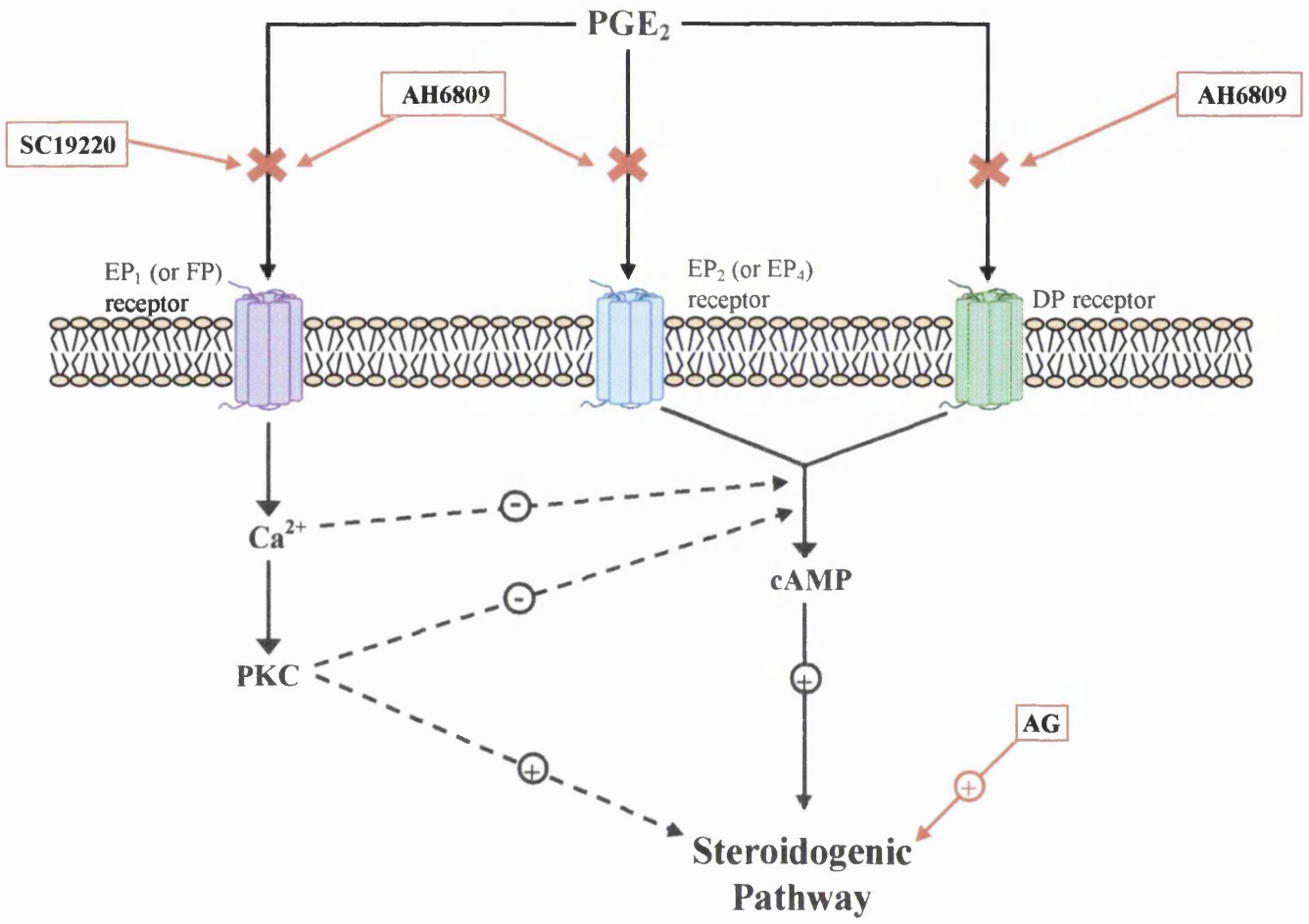

Figure 7.2 Hypothesised mechanisms by which PGE_2 may increase steroidogenesis

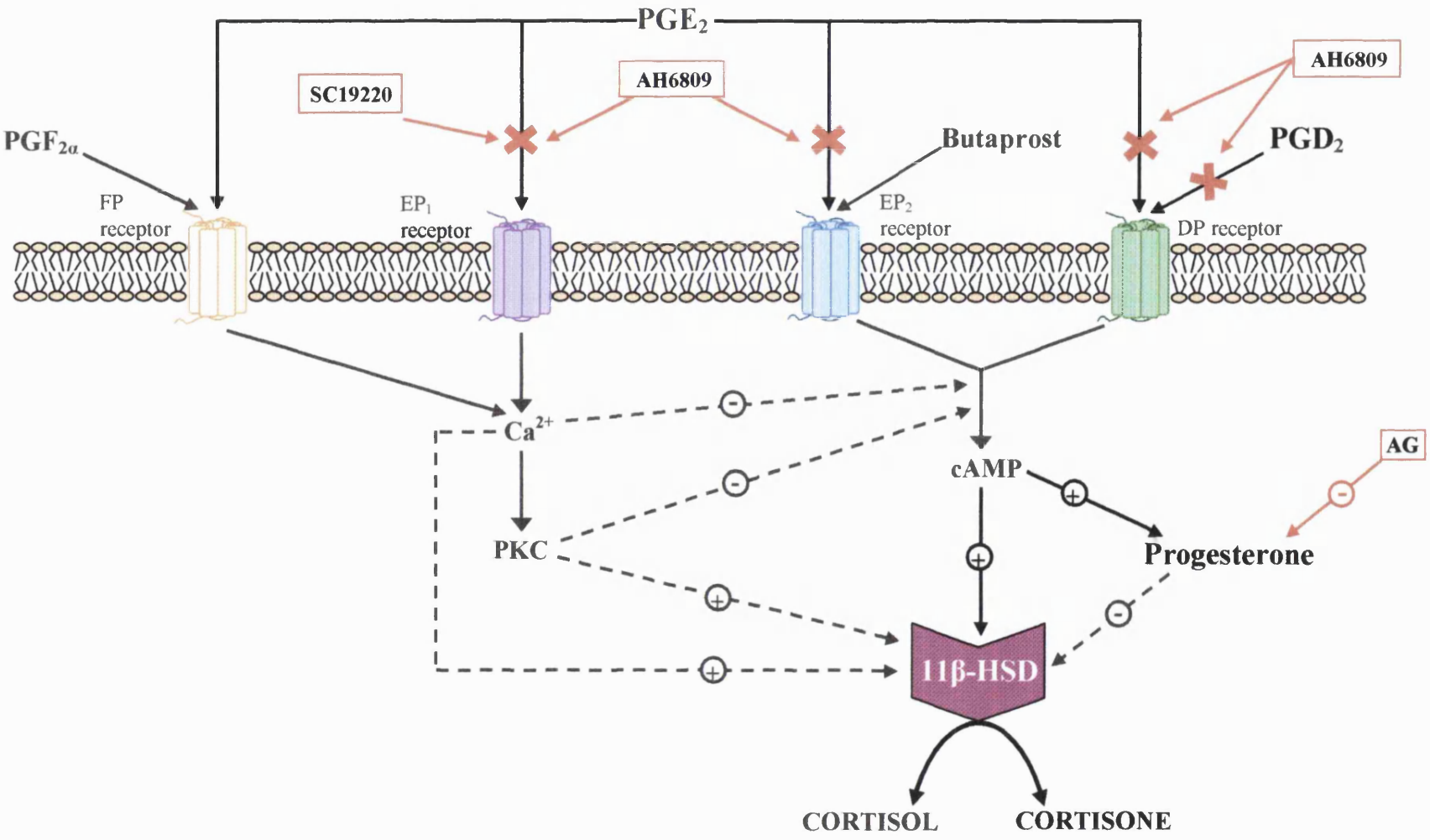

Figure 7.3 **Hypothesised mechanisms by which PGs may increase 11β-HSD activity**

granulosa-lutein cells, selective antagonists are required for each of the EP receptor subtypes and DP receptors. In the interim use of a combination of the available agonists and antagonists will enlighten this field of research.

Irrespective of whether PGE_2 elevates intracellular cAMP accumulation via EP_2 receptors, DP receptors or an alternative AC-coupled prostanoid receptor, the fact remains that the ability of PGE_2 to increase progesterone synthesis and cortisol metabolism appears to involve increased cAMP production (Figure 7.3). As reviewed previously, elevation of cAMP concentrations can acutely regulate 11β-HSD activity in both the placenta [Sun *et al*, 1998; Patel *et al*, 1999] and in human granulosa-lutein cells [Barker *et al*, 2001]. This second messenger can either have a direct effect on 11β-HSD or it could exert effects through protein kinases (*i.e.* PKA). In the latter event, changes in second messenger concentrations may be relayed to 11β-HSD activity via downstream protein kinases (*e.g.* tyrosine-kinase and mitogen-activated protein (MAP) kinases) that have consensus phosphorylation sequences in the cytosolic C-terminus of the cloned 11β-HSD proteins [Michael, Pers.Comm.]. Future experiments should examine the roles for specific protein kinases in the control of ovarian 11β-HSD activity through the use of specific protein kinase agonists and inhibitors.

In conclusion, this thesis has presented data regarding novel paracrine/autocrine roles for PGs in mediating the stimulation of steroidogenesis by HDL (and Apo-AI) and in the regulation of ovarian 11β-HSD activity. Knowing which receptors mediate these responses will await the development of more specific agonists and antagonists.

THE END

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APPENDIX I

Recipes for solutions and buffers

A. Percoll solution

Reagent/Solution	Volume per 15 ml
Percoll	9 ml
PBS	5 ml
1.5M NaCl (filter sterilised)	1 ml

B. Phosphate buffer for progesterone RIA

Reagent	Mass per litre
$NaH_2PO_4.2H_2O$	0.6g
$Na_2HPO_4.12H_2O$	2.2g

C. PAS-Gelatin buffer pH 7.0

Reagent	Mass per litre
$NaH_2PO_4.2H_2O$	0.6g
$Na_2HPO_4.12H_2O$	2.2g
NaCl	9.0g
NaN_3	1.0g
Gelatin	1.0g

D. Phosphate buffer for PGE_2 and $PGF_{2\alpha}$ RIA

Reagent	Mass per litre
$NaH_2PO_4.2H_2O$	0.6g
$Na_2HPO_4.12H_2O$	2.2g
NaCl	8.2g
BSA	1.0g
NaN_3	1.0g

E. 10mM Sodium acetate Buffer (pH 4.5)

Reagent	Mass per litre
Sodium acetate	0.82g

F. 50mM Sodium acetate Buffer (pH 4.5 or pH 6.2)

Reagent	Mass per litre
Sodium acetate	4.1g

G. Stock (×10) Tris buffered saline solution (pH 7.4)

Reagent	Mass per litre
Trizma Base	24.22g
NaCl	87.66g

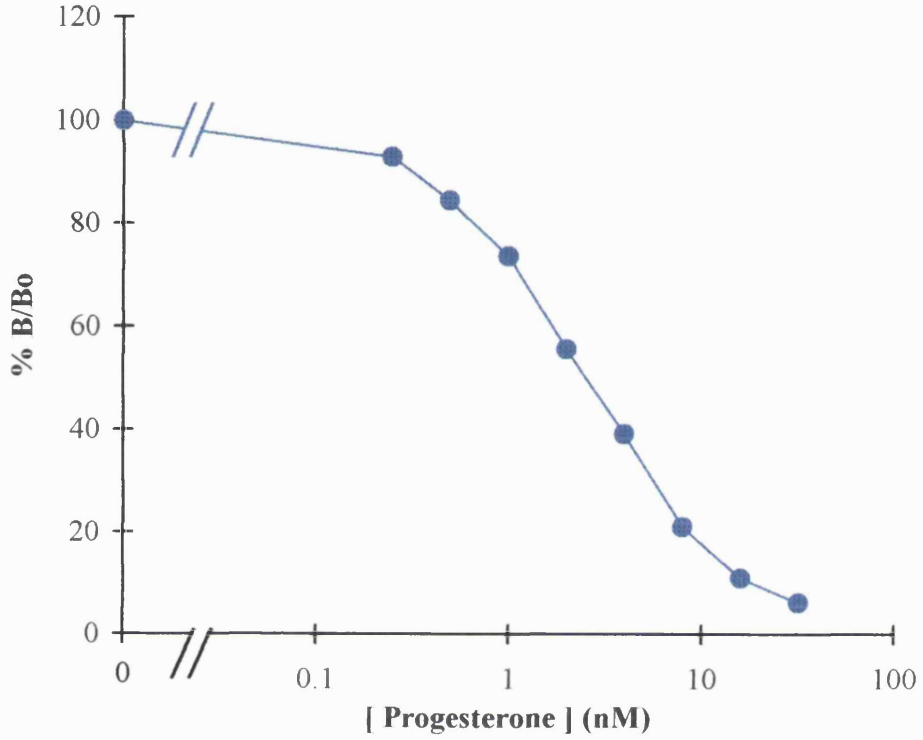
For use, a 1/10 dilution was performed in ddH_2O.

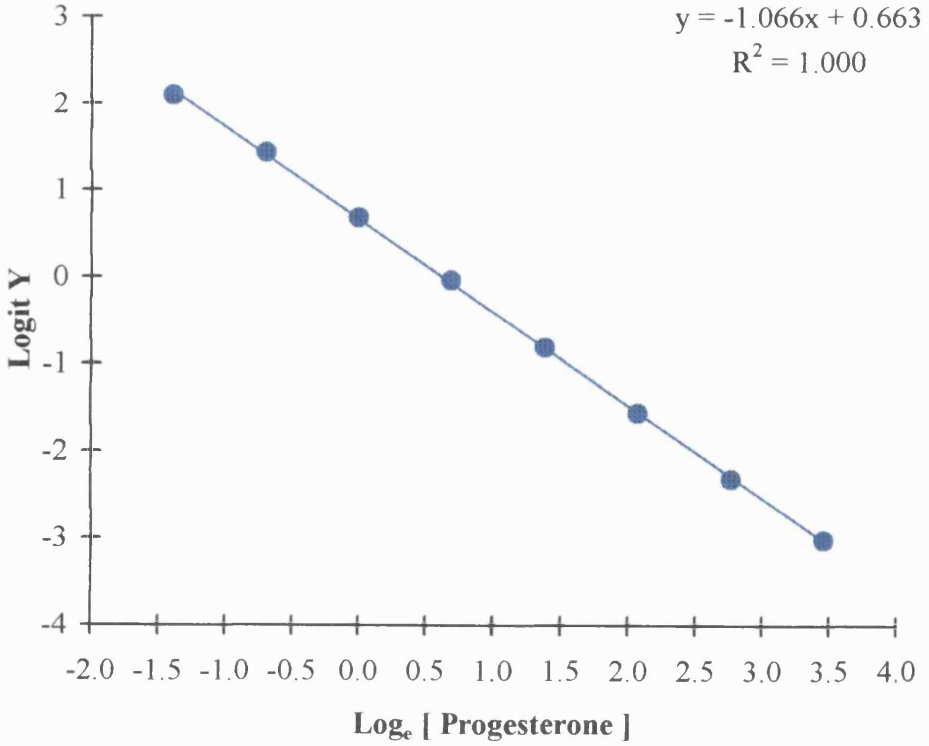
H. Lysis buffer (pH 7.4)

Solution	Mass per litre
Trizma Base	0.6g
Magnesium Chloride	0.3g
EDTA	0.6g

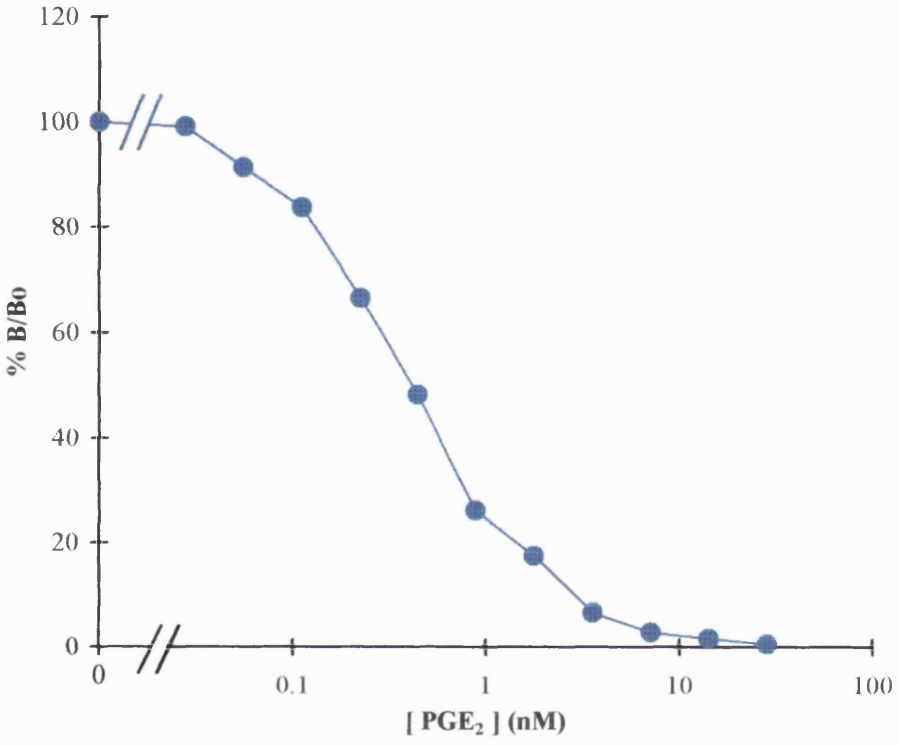
APPENDIX II

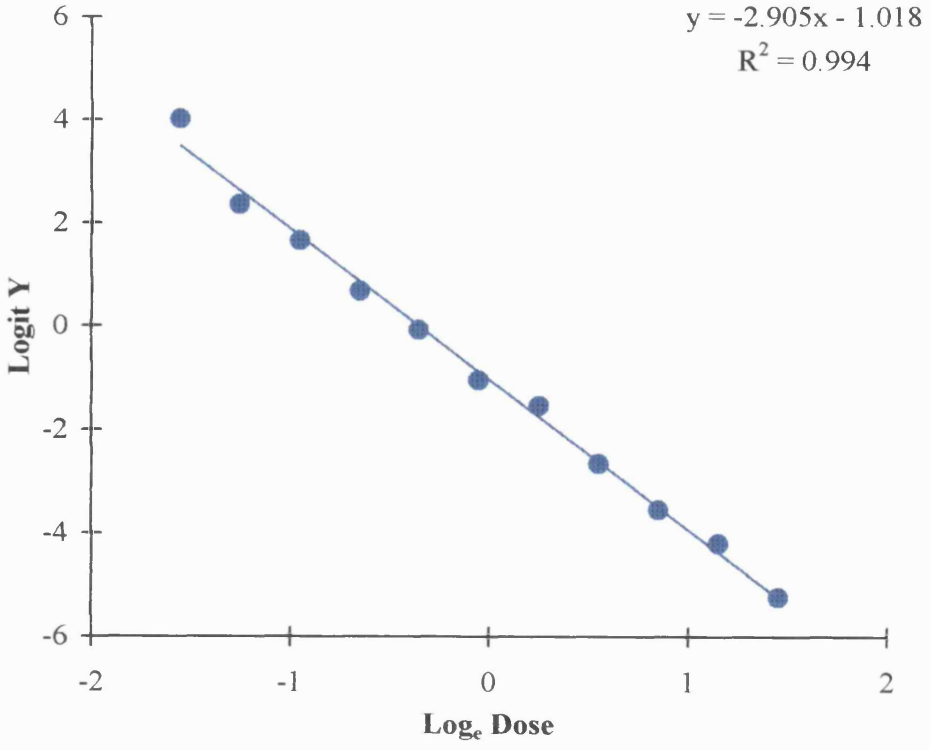
Standard Curve for a progesterone radioimmunoassay



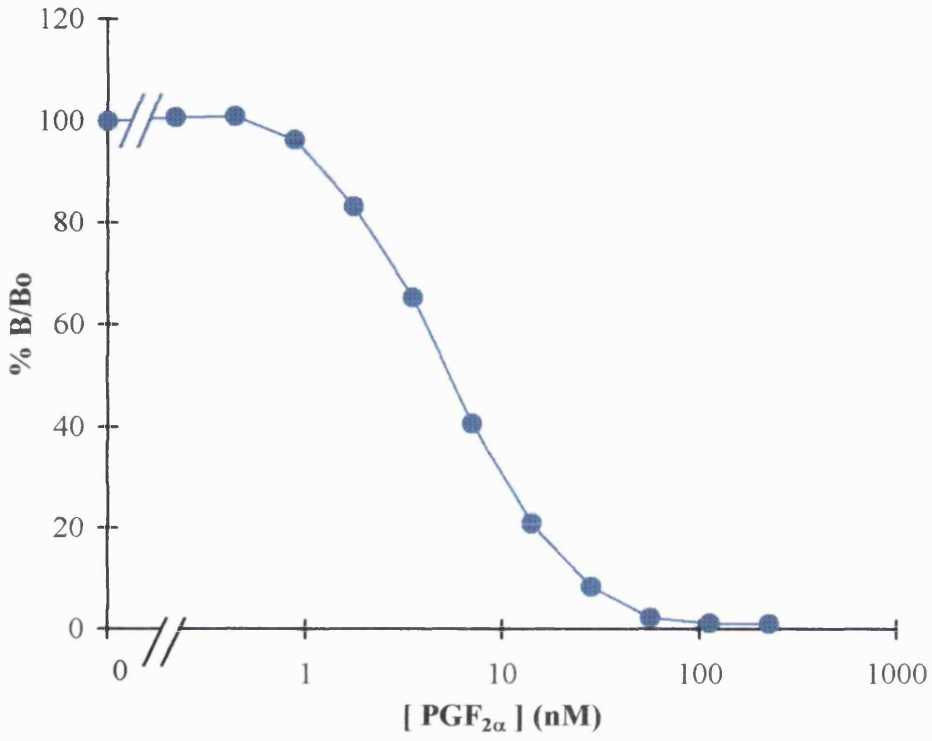


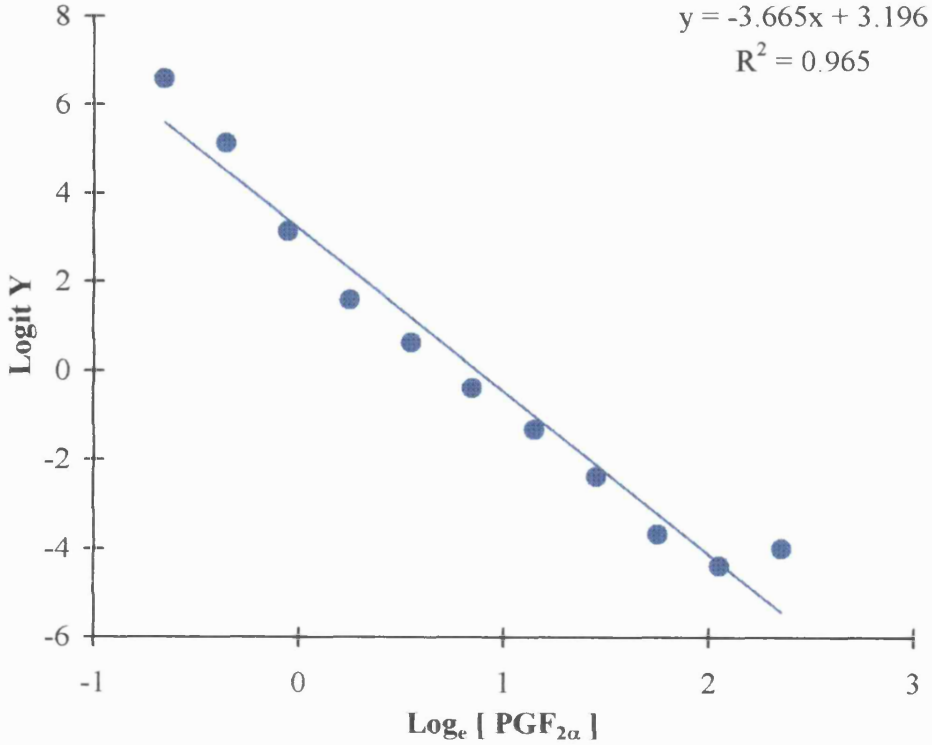
Standard Curve for a prostaglandin E_2 radioimmunoassay



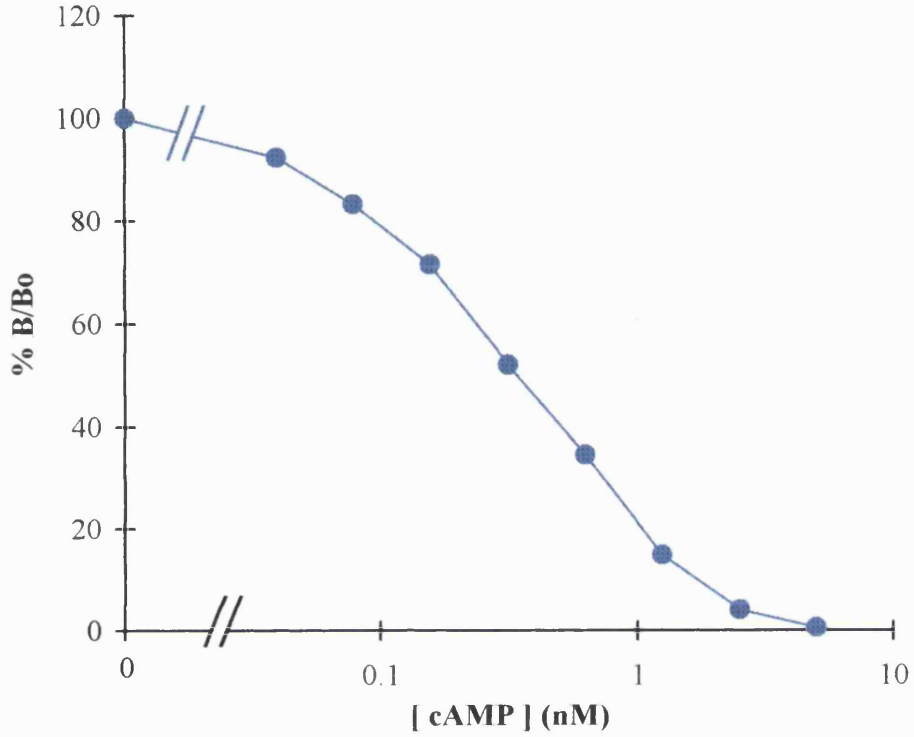


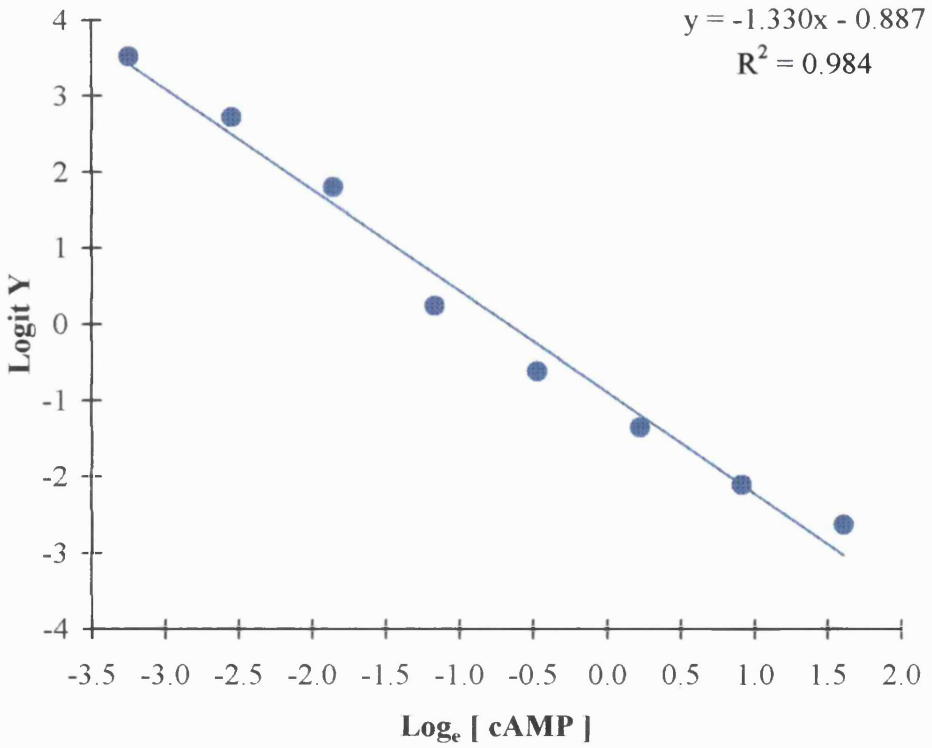
Standard Curve for a prostaglandin $F_{2\alpha}$ radioimmunoassay





Standard Curve for a cAMP radioimmunoassay





BSA protein assay