

**ARACHIDONIC ACID AS A POTENTIAL INTRACELLULAR
REGULATOR OF AROMATASE ACTIVITY IN
HUMAN TERM TROPHOBLAST**

by

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ABSTRACT

The potential role of arachidonic acid as an intracellular regulator of aromatase activity of human term trophoblast was investigated in short term (3 h) incubations. Melittin, a known activator of phospholipase A_2 , suppressed the aromatase activity of physically dissociated trophoblast cells in a dose-dependent manner. Indomethacin ($10^{-4}M$), a cyclooxygenase inhibitor of arachidonic acid metabolism, suppressed aromatase activity and potentiated the inhibitory effect of melittin ($P < 0.05$). Nordihydroguaiaretic acid (NDGA) alone, a lipoxygenase inhibitor, also decreased aromatase activity but did not affect the inhibitory effect of melittin. The addition of exogenous arachidonic acid ($10^{-4}M$) to trophoblast cells decreased aromatase activity in a dose-dependent manner ($P < 0.01$). Arachidonic acid metabolites such as prostaglandin $F_{2\alpha}$ ($10^{-6}M$) and leukotriene B_4 ($10^{-6}M$) had no effect on aromatase activity. The presence of hypoxanthine ($10^{-3}M$) and xanthine oxidase (10 mU/ml), also attenuated aromatase activity in trophoblast cells ($P < 0.01$), presumably via an elevation of intracellular free arachidonic acid concentration. Interestingly, melittin suppressed aromatase activity during short term (3 h) incubation but was ineffective on trophoblast cells from 24 h and 48 h incubations.

The role of cAMP in the action of aromatase activity was investigated. Cyclic AMP had no effect on 17β -estradiol production in human trophoblast cells during short term (3 h) incubation. But cAMP enhanced 17β -estradiol production during 24 h and 48 h incubations. These results suggested that aromatase activity of freshly

obtained term trophoblast cells was at or near maximal capacity or cAMP may not have been able to exert its steroidogenic action on human term trophoblast cells rapidly enough to be detected during a 3 h incubation. Cyclic AMP stimulated aromatase activity on human term trophoblast cells during 24 h and 48 h incubation, suggesting that cAMP achieves its actions by increasing protein and mRNA synthesis.

Effects of arachidonic acid, cAMP, hCG and 25-OH-cholesterol on progesterone production were investigated. None of them had effects on progesterone production during a 3 h incubation. These observations suggest that fresh term trophoblast cells may perform at or near optimal capacity on progesterone production.

The results supported the potential intracellular regulatory role of arachidonic acid on aromatase activity in human term placenta.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ACTH	Adrenocorticotrophic hormone
ATP	Adenosine trisphosphate
cGMP	cyclic guanosine-3',5'-monophosphate
CL	Corpus luteum
DHA	Dehydroepiandrosterone
DHAS	Dehydroepiandrosterone sulfate
DG	Diacylglycerol
E ₁	Estrone
E ₂	Estradiol
E ₃	Estriol
EDTA	Ethylenediamine-tetraacetic acid
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin releasing hormone
IGF-I	Insulin growth factor I
Indo	Indomethacin
InsP ₃	Inositol 1,4,5-trisphosphate
LDL	Low density lipoprotein
LH	Luteinizing hormone
LTB ₄	Leukotriene B ₄
M199	Medium 199
NDGA	Nordihydroguaiaretic acid
P ₄	Progesterone
P-450 _{acc}	(pigment)-450 side chain cleavage enzymes
P-450 _{arom}	(pigment)-450 aromatase
PKC	Protein kinase C
PLA ₂	Phospholipase A ₂
PLC	Phospholipase C
PGF _{2α}	Prostaglandin F _{2α}
Ptd-Ins	Phosphatidylinositol
Ptd-InsP ₂	Phosphatidylinositol-4,5-bisphosphate
RIA	Radioimmunoassay
TRH	Thyrotropin-releasing hormone
3β-HSD	3β-hydroxysteroid dehydrogenase
8-br-cAMP	8-bromo-cyclic adenosine monophosphate

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1.0 LITERATURE REVIEW

1.1 The human placenta

The human placenta is a transient organ that mediates physiological exchange between the mother and the developing fetus. The human placenta is genetically programmed to function for 9 months. The placenta provides a barrier between maternal and fetal compartments and is a site of hormonal production and metabolism. The barrier function of the placenta is similar to membranes active in physiological exchange, such as the pulmonary alveolar lining and the nephron of the kidney. The endocrine function mimics certain functions of the adult pituitary, ovary and hypothalamus.

1.1.1 Development, anatomy and histology of the placenta

1.1.1.1 Development of the placenta

The ovulated human ovum is fertilized at the outer third of the fallopian tube and transported to the uterus by ciliary action of tubal cilia approximately 4 days after ovulation. As the fertilized ovum passes through the fallopian tube, it undergoes cellular cleavage and forms a solid mass of cells called the morula. The morula continues dividing to form the blastocyst which floats free in the

intrauterine fluid, from which it receives nutrients, for about 3 days.

The human blastocyst, surrounded by the primitive trophoblast cells, consists of the blastocyst cavity and inner cell mass. The inner cell mass differentiates into the fetus. The primitive trophoblast underlying the inner cell mass penetrates the decidua (pregnant endometrium) when the trophoblast cells contact the uterine epithelium. The trophoblast is the precursor of the fetal placenta.

The penetrating embryo is totally covered by uterine epithelium by 11 days. Microscopic examination of the 11-day embryo reveals that rapid growth and differentiation of the trophoblast has occurred around the entire circumference. The trophoblast has differentiated into two cell layers: an inner layer of cytotrophoblast composed of individual cells and an outer layer of syncytiotrophoblast without discrete cell boundaries. The syncytiotrophoblast is formed by fusion of cytotrophoblast which are mitotically active (Richart, 1961). The syncytium now possesses spaces called lacunae that contain maternal blood. Lacunae communicate with each other and with maternal sinusoids and veins. These vascular connections allow the placental protein hormone, human chorionic gonadotropin (hCG), to enter maternal circulation at this time. This is evidence of a functional maternal vascular connection. Proliferation of the cellular trophoblasts forms the tip-like villi which become covered with syncytiotrophoblast. The villi enlarge and branch and progressively assume the form of the fully developed human placenta.

1.1.1.2 Anatomy and histology of the placenta

A full term placenta is discoid in shape with a diameter of 15-25 cm. It is approximately 3 cm thick and has a weight of 500-600 gm. It consists of a fetal part formed by the chorion and a maternal part formed by the decidua basalis. The fetal side is bordered by the chorionic plate, the maternal side by decidua basalis. Between the chorionic plate and decidua basalis are the intervillous spaces which are filled with maternal blood. After delivery, the maternal side of the placenta has 15-20 slightly bulging areas, the cotyledons, covered by a thin layer of decidua basalis. The fetal surface of the placenta is covered entirely by chorionic plate.

The chorionic villi are the structures of the placenta central to function. All placental functions are centered in these areas. By the beginning of the third week, primary villi are formed. Primary villi are trophoblastic cords coated with syncytiotrophoblast. During further development, mesodermal cells invade the primary villous core and the secondary villi develop. By the end of the third week, blood cells and vessels are differentiated in the mesodermal core and mature chorionic villi are formed. The mature chorionic villus consists of 6 layers; syncytiotrophoblast, cytotrophoblast, basement membrane, interstitial cell layer, capillary endothelium and basement membrane. As pregnancy advances the interstitial cell space is reduced and the villous vessels move from a central position to a peripheral position (Fox, 1979). The thickness of the syncytiotrophoblasts decreases from approximately 10 μm in early gestation to 1.7 μm in late gestation (Robertson *et al.*, 1975).

1.1.2 Functions of the placenta

The placenta has several functions; exchange of metabolic and gaseous products between maternal and fetal circulation, immunological protection of the fetus from the maternal immune system and hormone production.

1.1.2.1 Transfer functions of the placenta

The transport activities of the placenta are complex because numerous materials required for the synthesis of fetal tissues must be transferred and the waste products of fetal metabolism removed. The transfer of substances across the placenta occurs by several mechanisms including simple diffusion, facilitated diffusion and active transport.

Simple Diffusion: It is the most common and quantitatively significant transport mechanism. O_2 , CO_2 , steroids and fatty acids are transferred by this method. Free fatty acids passively diffuse across the placenta and fetal levels are lower than maternal levels (Elphick *et al.*, 1976).

Facilitated diffusion: Transfer via a membrane bound carrier, usually at rates greater than achieved by simple diffusion. Some carbohydrates and glucose are transported by facilitated diffusion, leading to rapid equilibrium with only a

small maternal-fetal gradient (Schneider *et al.* 1981; Hauguel *et al.*, 1983). A protein (molecular weight 52,000) believed to bind glucose and act as a carrier in facilitated glucose diffusion has been isolated from placental membrane fractions (Johnson *et al.* 1982).

Active transport: Transfer against a chemical gradient requires energy. Some amino acids and cations (Fe, Ca) are actively transported. Amino acid are actively transported across the placenta, making the concentrations in the fetal blood higher than in the maternal blood (Schneider *et al.*, 1979).

1.1.2.2 Immunologic functions of the placenta

The placenta prevents the fetus from attack by the maternal immune system. The mechanisms involved are poorly understood. The fetal trophoblast of the chorionic villi, which is in contact with maternal blood and immunocompetent cells in the intervillous spaces over a large surface area, has been shown to lack antigens of the major histocompatibility complex (MHC) (ie. those antigens important in allograft rejection reactions) (Sunderland *et al.*, 1981; Bulmer *et al.*, 1985). Placental progesterone (P_4) levels in humans are in the range of 2×10^3 ng/g wet tissue to 6×10^3 ng/g. It has been demonstrated that P_4 at these concentrations acts as an immunosuppressive agent in lymphocyte cultures stimulated by allogenic antigens (Mori *et al.*, 1977; Stites *et al.*, 1979). Estradiol-17 β (E_2) and testosterone

are also able to block [^3H]thymidine incorporation into human mixed lymphocyte cultures (Clemens *et al.*, 1979). In studies utilizing nonpregnant rats, an inverse relationship was demonstrated between thymic weight and circulating E_2 or testosterone. In the absence of gonadal steroids (as by castration) thymic weight increased while in the presence of elevated steroid levels thymic weight decreased (Chiodi *et al.*, 1976; Grossman *et al.*, 1979). Elevated E_2 most certainly causes the changes in thymic tissue structure observed in pregnancy.

The action of these sex steroid hormones during pregnancy may play an important role in the prevention of maternal-fetal allograft rejection and thus maintains pregnancy to term.

1.1.2.3 Endocrine function of the placenta

In 1905, Halban first suggested that the placenta was an endocrine organ (Halban, 1905). Since then, many investigators have contributed to a definition of the endocrine functions of the human placenta, including the formation of steroid, glyco-protein, and peptide hormones.

The syncytiotrophoblast is thought to be the placental source of human chorionic gonadotropin (hCG). Human CG is a gonadotropic glycoprotein hormone that maintains the corpus luteum (CL) of pregnancy by mimicking luteotropic actions of luteinizing hormones (LH) during the first few months of pregnancy. The syncytiotrophoblast is also believed to be the site of production of human placental

lactogen (hPL). Human PL is a protein hormone that is primarily lactogenic in action but also has detectable growth-promoting activity. In addition, P_4 and estrogens (predominantly estriol(E_3)) are produced by the placenta but placental formation of estrogens requires cooperation of the fetal adrenal cortex and liver.

1.1.2.3.1 Protein hormones of the placenta

The endocrine alterations that accompany human pregnancy increase the formation of sex steroid hormones and produce protein and polypeptide hormones such as hPL and hCG. The placenta also produces gonadotropin-releasing hormone (GnRH), human chorionic thyrotropin (hCT), corticotrophin-releasing factor (CRF), somatostatin and inhibin.

Human chorionic gonadotropin--Human CG is a glycoprotein with a molecular weight of 36,700. The polypeptide portion accounts for 70% of the molecular weight and the carbohydrate portion, which appears to be essential for bioactivity, accounts for approximately 30%. Human CG shares immunologic and biologic properties with LH (Hussa, 1980). Like LH, follicle-stimulating hormone (FSH) and TRH, hCG consists of two subunits, α and β . Human CG and its subunits have been localized to syncytiotrophoblasts by immunocytochemical methods (Dreskin *et al.*, 1970; De Ikonoff *et al.*, 1973). Human CG can be detected in maternal blood by the tenth day after ovulation (Lenton *et al.*, 1982). From day 10 onward, the hCG

concentration in pregnant women increases rapidly until week 8 of pregnancy. From weeks 8 to 12, hCG concentrations are fairly constant and then decrease until about week 18. After week 18 of pregnancy, the hCG concentration remains relatively constant for the remainder of pregnancy (Kletzky *et al.*, 1985).

The principal role of hCG is similar to that of LH, that is stimulation of steroidogenesis. Several lines of evidence suggest that hCG rescues the CL during the luteal phase of a fertile cycle by the action of extension of the life span of CL and increases the P_4 and $17\alpha\text{-OH-}P_4$ concentration in the maternal circulation (Caldwell *et al.*, 1980).

The effect of hCG in the CL is mediated by the action of adenyl cyclase, which stimulates the conversion of adenosine trisphosphate (ATP) to cyclic adenosine-3'-5'-monophosphate (cAMP). Subsequently, cAMP augments the conversion of cholesterol to pregnenolone and then increases P_4 production (Hamberger *et al.*, 1979; Dennefors *et al.*, 1982). However, the role of hCG in the regulation of human placental steroidogenesis is still controversial. Human CG has been reported to have no effect on the rate of conversion of either cholesterol or pregnenolone to P_4 (Macome *et al.*, 1972; Talwar GP. 1979; Paul *et al.*, 1981). The role of hCG in the regulation of estrogen synthesis in the human placenta is not clearly defined. It has been reported that hCG either stimulates estrogen production (Cedard *et al.*, 1970) or has no effect on estrogen synthesis (Laumas *et al.*, 1968).

Human placental lactogen--Human PL consists of a single polypeptide chain with

191 amino acid residues and two disulfide bonds. It has 96% sequence homology with human growth hormone and 67% homology with human prolactin (Bewley *et al.*, 1972; Cooke *et al.*, 1981). Immunochemical location studies have demonstrated that hPL is present in the syncytiotrophoblast by the second week after conception (Beck, 1970; Fujimoto *et al.*, 1986).

Human PL may stimulate basal insulin secretion (Nielsen, 1982). Human PL also has growth-promoting activity, as demonstrated by hPL-induced body weight gain (Kaplan *et al.*, 1964). In rats, the luteotropic activity of hPL is established (Zumpe *et al.*, 1974). However, several women are known to have maintained a normal duration pregnancy despite nearly undetectable hPL levels (Nielsen *et al.*, 1979, Sideri *et al.*, 1983).

Gonadotropin-releasing hormone--Human placental tissues have been shown to contain gonadotropin-releasing hormone-like activity (Khodr *et al.*, 1978b). It was reported that gonadotropin-releasing hormone-like factor (LRF) stimulates secretion of placental hCG, α -hCG, P_4 , E_2 , estrone (E_1) and E_3 release *in vitro* (Khodr *et al.*, 1978a).

1.1.2.3.2 Steroid hormones of the placenta

The placenta produces large amounts of P_4 , E_1 , E_2 and E_3 during pregnancy and distributes these steroid hormones to both maternal and fetal compartments (Tulchinsky *et al.*, 1975). Normal pregnancy depends upon placental steroid actions

on the maternal reproductive organs and metabolic system. In pregnancy, estrogen stimulates growth of the uterine muscle mass, which will eventually supply the contractile force needed to deliver the fetus. Progesterone may maintain the uterine stability during pregnancy.

1.2 Biosynthesis of sex steroid hormones by term placenta

1.2.1 Feto-placental unit

The biomolecular interactions between the fetus and mother during human pregnancy are orchestrated by means of a fetal-maternal communication system.

The human placenta lacks 17α -hydroxylase and $C_{17,20}$ lyase and is, therefore, unable to convert acetate into estrogen. The placenta is capable of converting C_{19} steroids into estrogen (Ryan, 1959; Siiteri *et al.*, 1963). It was suggested that the human fetal adrenal may provide precursors for estrogen biosynthesis (Frandsen *et al.*, 1961). It has also been shown that DHAS of maternal and fetal adrenal origin contributed about equally to placental E_1 and E_2 formation, while over 90% of the E_3 as synthesized from 16-hydroxy-DHAS of fetal origin (Siiteri *et al.*, 1963). (Fig.

1)

1.2.2 Progesterone synthesis and secretion in the placenta

Most of the low density lipoprotein (LDL) in fetal plasma arises by *de novo* synthesis in the fetal liver (Carr *et al.*, 1984). During pregnancy the placenta uses maternal plasma cholesterol, primarily in the form of LDL, as its precursor for the formation of placental steroids, especially P_4 , rather than synthesizing cholesterol from acetate (Hellig *et al.*, 1970) (Fig. 2).

Progesterone is one of the major hormones synthesized during pregnancy. During the first trimester, the CL is primarily responsible for synthesis of this hormone, whereas during the second and third trimesters the main site of P_4 synthesis is the placenta. Conversion of cholesterol to P_4 involves several steps. The conversion of cholesterol to pregnenolone in mitochondria is the first rate-limiting and hormonally regulated step in the synthesis of all steroid hormones (Golos *et al.*, 1987; Ringler *et al.*, 1989). Three enzymes are involved in this step; 20 α -hydroxylase, 22-hydroxylase and 20,22-lyase (Lieberman, *et al.*, 1984). Most pregnenolone is readily converted to P_4 the action of 3 β -hydroxysteroid dehydrogenase (3 β -HSD) and Δ^5 , Δ^4 -isomerase. Approximately, 90% of P_4 which is formed by the placenta appears in maternal circulation, where it is metabolized to pregnanediol. The other 10% of P_4 transported to the fetus as precursor for other steroids (Tseng *et al.*, 1978).

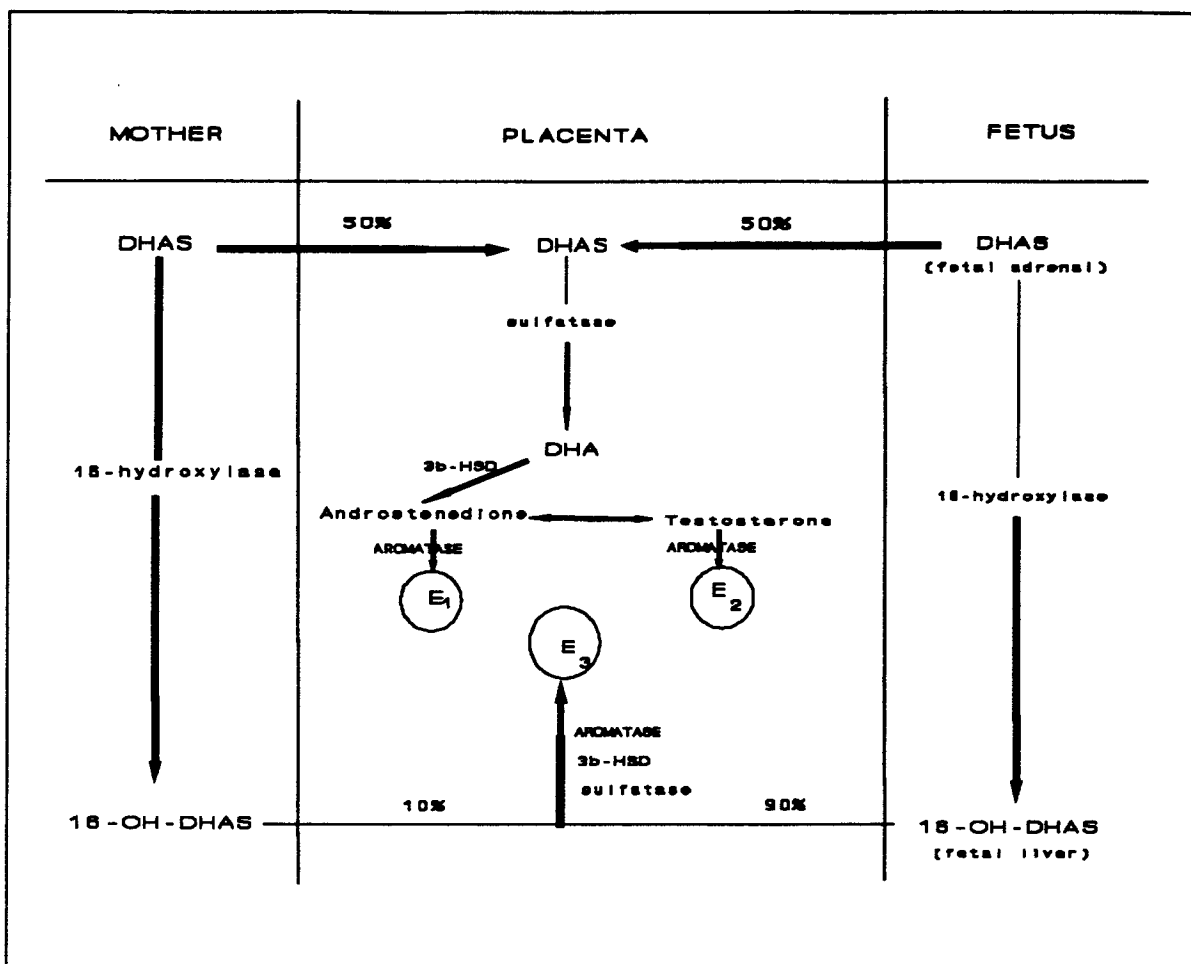


Fig. 1 Estrogen biosynthesis in the feto-placental unit.

Fetal DHA is synthesized and sulfatized in adrenal. Around 70% of DHAS produced by fetal adrenal is converted to 16 α -OH-DHAS in fetal liver and then transported to placenta for E_3 synthesis. Maternal and fetal compartment contributed the same amount of DHAS for the synthesis of E_1 and E_2 , while over 90% of the estriol was synthesized from 16 α -OH-DHAS obtained from the fetus. Estriol formation in the placenta is not from the conversion of E_1 , but directly from 16 α -OH-DHAS under a series of enzymatic action (sulfatase, 3 β -HSD, and $\Delta^{4,5}$ isomerase and aromatase).

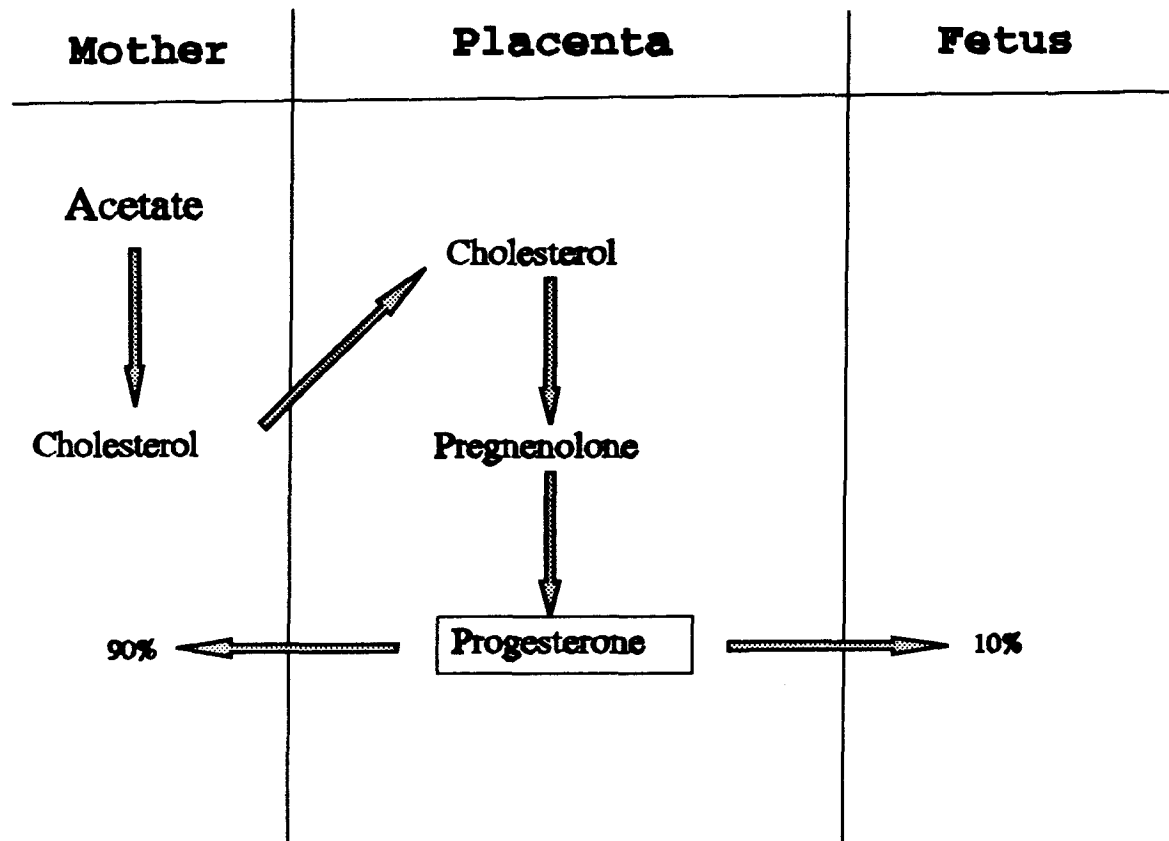


Fig. 2 The main sources of steroid hormone precursor.

Placenta uses maternal cholesterol, mainly LDL, as a primary source for the P_4 production. Ninety percent of P_4 production are transported to the maternal compartment and 10% to fetal compartment.

1.2.2.1 Role of LDL and cholesterol in steroid hormone synthesis

Cholesterol is the major substrate of steroid hormone biosynthesis in human placenta. The minimal capacity of the placenta to synthesize cholesterol is confirmed by the lack of conversion of acetate to cholesterol demonstrated by placental perfusion (Telegdy *et al.*, 1970). A large proportion of cholesterol comes from LDL (Brown *et al.*, 1979). A simplified model for the structure of LDL is shown in Fig. 3 (based on Brown *et al.*, 1991). LDL contains a nonpolar core in which many molecules of hydrophobic cholesteryl esters are packed to form an oil droplet. Hydrophobic cholesteryl esters account for most of the mass of the LDL particles. Uptake of lipoprotein from plasma is regulated by the serum concentration of lipoprotein and the lipoprotein receptor-dependent uptake system. In humans 70%-80% of LDL removed from the plasma each day by the LDL receptor pathway. Receptor-bound LDL enters the cells by receptor-mediated endocytosis and cholesterol is stored within cytoplasm in an esterified form.

Structure of Low Density Lipoprotein (LDL)

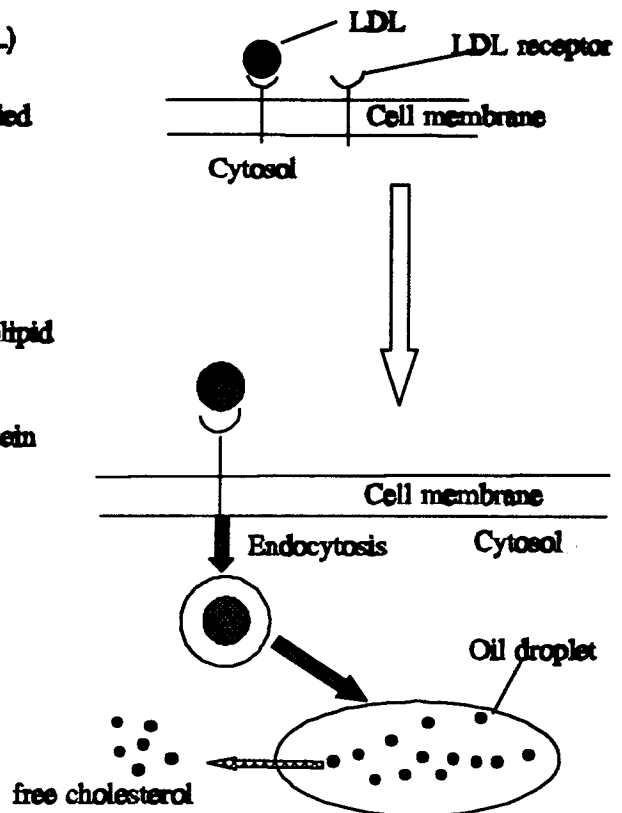
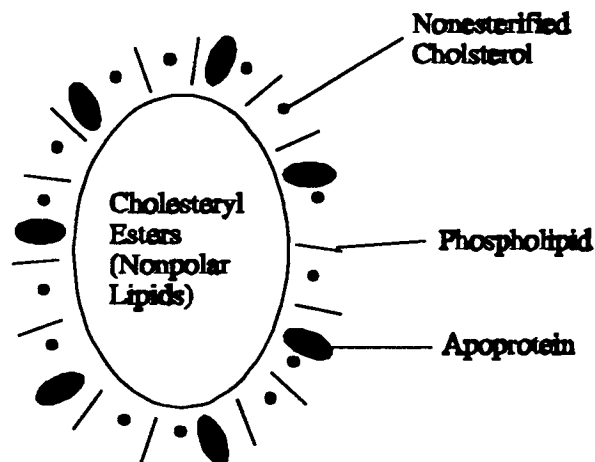


Fig. 3 A simple schematic structure of LDL and its transportation across the membrane.

The core of the spherical lipoprotein particle is consisted of two nonpolar lipids which are triglyceride and cholesteryl ester. The nonpolar core is surrounded with an outer coat composed chiefly of phospholipids. LDL carried by the plasma binds to the LDL receptors in the cell membrane and is taken up by the cell with endocytosis. LDL is digested by lysosomes within the cells and cholesteryl esters are hydrolyzed into cholesterol by cholesteryl esterase.

Upon stimulation of the target organs by tropic hormones such as LH, adrenocorticotrophic hormone (ACTH) or cAMP, a cholesterol esterase is activated, and the free cholesterol formed is transported into mitochondria, where a cytochrome P-450 side chain cleavage enzyme (P-450_{scc}) converts cholesterol to pregnenolone. Free cholesterol, which is insoluble in the aqueous cytosol, may be transported to mitochondria by a Sterol Carrier Protein 2 (SCP-2) (Noland *et al.*, 1980, Tanaka *et al.*, 1984). All mammalian sex steroid hormones are synthesized from cholesterol via pregnenolone through a series of reactions that occur in either the mitochondria or endoplasmic reticulum of sex steroid specific producing cells.

1.2.2.2 Cholesterol side chain cleavage (P-450_{scc}) enzyme and 3 β -HSD/ $\Delta^5 \rightarrow \Delta^4$ isomerase

Conversion of cholesterol to pregnenolone is the first rate-limiting and hormonally regulated step in the synthesis of steroid hormones (waterman *et al.* 1985; Golos *et al.* 1987; Voutilainen *et al.* 1987). It was suggested that three separate and distinct enzymes were involved: 20-hydroxylase, 22-hydroxylase and a 20,22-lyase (Lieberman *et al.* 1984). Carbon monoxide studies suggested that these steps were mediated by separate cytochrome P-450 enzyme. P-450 functions as a terminal electron receiver in the mitochondrial electron transport system. The electron transport process begins with the accepting electrons (e⁻) of adrenodoxin reductase from NADPH. These e⁻ are passed to adrenodoxin, an iron-sulfur protein

within mitochondria (Picado-Leonard *et al.* 1988). P-450_{scc} accepted the e⁻ from adrenodoxin and catalyzed the cleavage of cholesterol to pregnenolone.

Conversion from pregnenolone to P₄, it involves two enzymes: 3 β -HSD, and $\Delta^{5\rightarrow4}$ isomerase. The enzymatic reactions of these two enzymes are poorly understood. Some studies suggested the presence of two to three different isozymes of isomerase specific for the three steroidogenic pathways i.e mineralocorticoid, gluco-corticoidogenic and sex steroid (Gower et al. 1983).

1.2.3 Estrogen Synthesis and secretion in the placenta

The placenta becomes the primary source of estrogen after approximately week 9 of pregnancy (Oakey, 1970). Estrogens are formed in the placenta by maternal and fetal Δ^5 androgen precursors. The placenta is unable to produce these precursors *de novo*. Estrogen production rate increases continuously during pregnancy and reaches 100-120 mg/24h. E₃ comprises 60-70% of the total estrogens; 300-500 times greater than in the non-pregnant state.

1.2.3.1 Estradiol and estrone synthesis and secretion

The most important androgen precursor for E₁ and E₂ synthesis is DHAS. The maternal compartment contributes 40-45% of DHAS and DHA for the synthesis of E₁ and E₂ (Siiteri *et al.*, 1966). The fetal compartment and particularly the fetal

adrenals provide 60% of the DHAS. The first step in estrogen production is hydrolyzation of DHAS by steroid sulfatase followed by transformation of Δ^5 DHA to Δ^4 androstenedione by 3β -HSD and $\Delta^{5\rightarrow4}$ isomerase. Androstenedione is aromatized to E_1 by placental aromatase or is transformed to testosterone and then aromatized to E_2 .

1.2.3.2 Estriol synthesis and secretion in the placenta

Estriol is synthesized by the placenta primarily from fetally derived 16α -OH-DHAS and estriol diffuses to the maternal and fetal circulations (Siiteri *et al.*, 1966; Tulchinsky *et al.*, 1973). It is estimated that 90% of the 16α -OH-DHAS precursor for E_3 synthesis is derived from the fetus and 10% from the mother, which is much less than that for E_1 and E_2 (Madden *et al.*, 1978).

Fetal adrenals use maternal LDL as substrate to produce DHA and sulfatize DHA to DHAS. Approximately 70% of total fetal DHAS is transferred to the fetal liver where DHAS is hydroxylized to 16α -OH-DHAS. 16α -OH-DHAS is quantitatively the most important androgen circulating in the fetal compartment. 16α -OH-DHAS reaches the placenta from both maternal and fetal compartments but the maternal contribution is less than from the fetus (fetus 90%, mother 10%). In the placenta, E_3 is formed after desulfation and aromatization of 16α -OH-DHAS by placental sulfatase and placental aromatase respectively.

1.2.3.3 Steroid sulfatase and sulfotransferase

Steroid sulfates may be synthesized directly from cholesterol sulfate or may be formed by sulfation of steroids by cytosolic sulfotransferases (Miller *et al.*, 1981).

An important biochemical characteristic of the placenta is its capacity to hydrolyze steroid sulfates. Placental steroid sulfatase is a microsomal enzyme (Rose *et al.*, 1982). It was detected in syncytiotrophoblasts by immunohistochemistry and hybridization studies. Neither steroid sulfatase immunoreactivity nor steroid sulfatase mRNA were detected in cytotrophoblast (Salido *et al.*, 1990). In human placenta, P_4 is synthesized from cholesterol, whereas estrogens are produced mainly from maternal and fetal steroid sulfate precursors, DHAS and 16α -OH-DHAS. These Δ^5, C_{19} androgen precursors are desulfated by steroid sulfatase in the placenta before entering the pathway catalyzed by 3β -HSD, $\Delta^5 \rightarrow \Delta^4$ isomerase and aromatase to yield estrogens.

The fetal adrenal and liver convert estrogen and P_4 precursors, pregnenolone, 17α -OH-pregnenolone, DHA and 16α -OH-DHA, into sulfated forms mostly at the 3β position by sulfotransferase. The sulfated forms are transported to the placenta where the sulfated group is cleaved off by sulfatase to produce estrogens and P_4 .

1.2.3.4 Placental aromatase

The placenta is a major site of conversion of C_{19} androgen precursors to

estrogens because of the amount and the high rate of activity of aromatase (Doody *et al.*, 1989). The enzyme was first purified and its amino acid sequence characterized from human placenta (Chen *et al.*, 1986; Nakajin *et al.*, 1986). Major (P2a) and minor (P3) forms of human placental aromatase have been solubilized and identified by chromatography (Osawa *et al.*, 1987).

Most steroidogenic enzymes are members of the cytochrome P-450 groups of oxidases (Nebert *et al.*, 1987). Aromatization of C₁₈ estrogenic steroids from C₁₉ androgens is mediated by P450_{arom}, found in the endoplasmic reticulum. The system consists of an aromatase cytochrome P-450 and a flavoprotein, NADPH-cytochrome P-450 reductase (Thompson *et al.*, 1974). The aromatization process involves loss of the angular C-19 methyl group and stereospecific elimination of the 1-β and 2-β hydrogens from the androgens (Cole *et al.*, 1990). This process includes 3 enzymatic hydroxylations requiring 3 moles of O₂ and NADPH per mole of estrogen formed. Furthermore, these irreversible reactions require the participation of both cytochrome P-450 and NADPH cytochrome C-reductase enzymes (Seymour *et al.*, 1984) (Fig. 2).

1.2.3.5 Regulation of placental aromatase

Follicle-stimulating hormone (FSH) and cAMP have been shown to stimulate aromatase activity in ovarian tissues (Dorrington *et al.*, 1975, Erichson *et al.*, 1978, Gore-Langton *et al.*, 1981). Aromatase activity in human choriocarcinoma cells may

be stimulated by cAMP and theophylline (Bellino *et al.*, 1978). Insulin, insulin-like growth factor I (IGF-I) and insulin-like growth factor II (IGF-II) may be potent inhibitors of aromatase activity in human placental cytotrophoblasts (Nestler *et al.*, 1987 & 1990).

Estrogen biosynthesis sequence

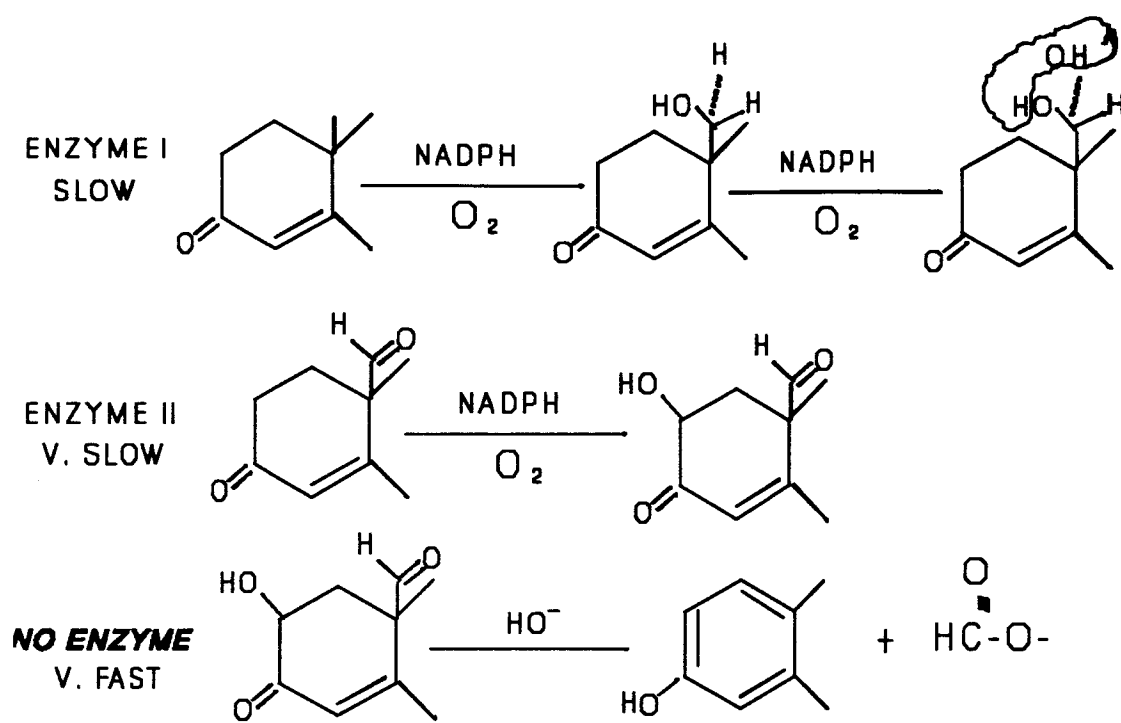


Fig. 4 A schematic representation of aromatization of androgen catalyzed by aromatase.

Aromatization process requires a sequence of several microsomal enzymes, referred to as aromatase. The process involves three enzymatic hydroxylations. Enzyme I catalyzes two hydroxylations. Enzyme II catalyzes one hydroxylation and is the slowest step of the three. The third step is no enzyme involved.

1.3 Signal transduction and regulation of hormone production:

Cells respond to their environment through cell surface receptors that bind specific ligands. These "signals" are transduced into cells and change cellular metabolism and function. Many extracellular ligands act by increasing the intracellular concentrations of second messengers such as cAMP, Ca^{2+} or the phosphoinositides. Receptors generally associate with a transmembrane signaling system with three separate components. The first messenger (ligand) is recognized by membrane-bound receptors specific to the ligand. The activated ligand-receptor complex triggers the activation of G-protein located inside the plasma membrane. The G-protein in turn changes the activity of effector elements, such as adenylate cyclase and PLC. The effector element for cAMP is adenylate cyclase, which is a transmembrane protein that converts intracellular ATP to cAMP. PLC and guanylate cyclase, respectively, are the effector elements of Ca^{2+} , DG and cGMP.

1.3.1 The second messenger: cAMP

Cyclic AMP, which is derived from ATP through the action of adenylate cyclase, plays an important role in the action of a number of hormones such as FSH, LH, angiotensin II, etc. Cyclic AMP binds to the regulatory subunits of a cAMP-dependent protein kinase and dissociates the catalytic subunits from the protein kinase. The active catalytic subunits catalyze the phosphorylation of proteins.

Cyclic AMP is an important intracellular mediator in reproductive endocrinology. Stimulation of P_4 secretion from placenta by $(Bu)_2cAMP$ and β -adrenergic agonists has been reported (Caritis *et al.*, 1983). Cyclic AMP also increases P_4 production by human chorion (Tonkowicz *et al.*, 1985). The regulation of P_4 secretion by 8-br-cAMP was proposed as a result of 8-br-cAMP-provoked increases in protein mRNA in placental cells (Nulsen *et al.*, 1989). There are also conflicting reports regarding cAMP. It had been shown that cAMP had no effect on the conversion of pregnenolone to P_4 of human placenta (Ferre *et al.*, 1975). It was also reported that 8-br-cAMP inhibited aromatase activity in cultured first trimester human placenta (Rodway *et al.*, 1990).

1.3.2 The second messengers: Ca^{2+} and DG

This second messenger system involves hormonal stimulation of Ptd-Ins hydrolysis. As soon as the extracellular signal (such as GnRH or angiotensin) binds with its specific receptor in the plasma membrane, a series of chain reactions occurs. The binding of ligand and receptor activates GTP-binding regulatory proteins (G-proteins) and then triggers the enzyme, phosphoinositide-specific PLC. PLC catalyzes plasma membrane PtdIns- P_2 breakdown and generates two products: IP_3 and DG.

IP_3 is water soluble and diffuses into cytoplasm where it triggers the release of sequestered Ca^{2+} from within the cell. The elevation of cytosolic Ca^{2+} enhances

the binding of Ca^{2+} with the calcium-binding protein calmodulin, which regulates activities of calcium-dependent protein kinase.

DG has two potential signaling roles. DG is confined to the plasma membrane where it activates the calcium-dependent PKC. DG can also be cleaved to release AA, which can be used as an intracellular regulator and/or continue to breakdown into its eicosanoid metabolites.

1.3.3 The second messenger: cGMP

Unlike cAMP, the versatile intracellular second messenger, cyclic guanosine-3',5'-monophosphate (cGMP), is a more localized messenger in only a few cell types, such as intestinal mucosa and vascular smooth muscle. Increased intracellular cGMP concentrations cause vascular smooth muscle relaxation through a kinase-mediated mechanism. This seems to involve phosphorylation of myosin light chain kinase.

Atrial natriuretic factor (ANF) and endothelial derived releasing factor (EDRF, probably nitric oxide) cause to guanylate cyclase activation. How ANF and EDRF regulate guanylate is unknown, but the system does not appear to use a G-protein.

1.3.4 Phospholipase C

The activation of PLC is most likely mediated by a GPT-binding protein (Cockcroft S. 1987; Spiegel AM. 1987). Recent studies to purify the enzyme have shown the existence of three immunologically distinct forms of PLC: type I, type II, type III (Suh et al. 1988). PLC-I was evenly distributed between the cytosol and the particulate fraction. PLC-II is exclusively cytosolic. It was reported recently that EGF-induced tyrosine phosphorylation of PLC-II, suggesting direct activation of effector by the receptor (Margolis et al. 1988).

The activation of receptors stimulates PLC which degrades membrane-bound PtdIns-4,5-bisphosphate to produce two second messengers: IP₃, which can liberate Ca²⁺ from intracellular stores and DG, which activates PKC (Berridge M.J., 1987, Nishizuka Y., 1984). The fatty acid in the sn-1 position of DG is hydrolyzed by DG lipase followed by hydrolytic cleavage of the fatty acid from the sn-2 position by mono-acylglycerol lipase (Okazaki *et al.*, 1981). This leads to the release of AA from the sn-2 position of 1,2-diacyl-sn-glycerols (Fig. 5).

Upon the liberation of intracellular unesterified fatty acid from above phospholipases, AA is rapidly oxidized in two separate pathways. One pathway is catalyzed by an enzyme complex referred to as prostaglandin synthase or cyclooxygenase and leads to the production of prostaglandins and thromboxanes. Another pathway of AA oxidation is catalyzed by lipoxygenase, an enzyme complex independent of the cyclooxygenase system, and leads to the formation of leukotriene B₄ leukotriene C₄, D₄, E₄. (Fig. 6)

1.3.5 Phospholipase A₂ and arachidonic acid

PLA₂:

PLA₂ enzyme is found in nearly all animal cell types examined. PLA₂ liberates AA from phospholipids in the membrane (Irvine, 1982). This enzyme may be present in the plasma membrane or the cytosol (Balsinde *et al.*, 1988). Most PLA₂ enzymes need Ca²⁺ ions for full activity and also can be regulated by G proteins linked to adrenergic or cholinergic receptors (Burch *et al.*, 1987; Felder *et al.*, 1990). In the presence of elevated cytosolic Ca²⁺ ions or phospholipase activators (e.g. melittin), phospholipase A₂ is activated and splits membrane phospholipids into lysophospholipid and AA (Fig. 7). PLA₂ activity is the major source of AA in the cell (Bell *et al.*, 1979).

Arachidonic acid:

Several lines of evidence suggest that AA and its metabolites play an important role as intracellular regulators in reproductive endocrinology. AA has been shown to stimulate P₄ production in rat ovarian cells (Wang and Leung 1988, 1989). It was demonstrated that GnRH-stimulated LH release is mediated by AA (and/or its metabolites) and protein kinase C in pituitary cells (Chang *et al.*, 1986). PKC activated by phorbol ester (TPA) was shown to stimulate AA mobilization and phospholipid hydrolysis in human uterine decidua cells (Schrey *et al.* 1987). They concluded that PKC activation by TPA leads to AA mobilization from decidua-cell phospholipid by a mechanism involving phospholipase A-mediated Ptd-Ins hydrolysis and phospholipase C-mediated phosphatidylcholine (Ptd-C) hydrolysis,

coupled with further hydrolysis of the DG product. It was reported that AA stimulated calcium and hPL release in isolated trophoblast cells (Zeitler *et al.* 1986). The stimulation of hPL by AA may be due, at least in part, to the effects of the fatty acid on cellular calcium mobilization. AA also stimulated the rapid appearance of inositol monophosphate in placental cells (Zeitler *et al.* 1985). The effect on AA was specific for hydrolysis of phosphoinositides and phosphatidylserine and did not involve other phospholipids. Arachidonate and unsaturated fatty acid have also been suggested that they might activate directly and regulate protein kinase C as equally important as DG in receptor function and cellular regulation in the neutrophil (McPhail *et al.*, 1984). In neutrophils, AA directly activates a GTP-binding protein and therefore may act as a role of second messenger in signal transduction (Abramson *et al.*, 1991). PGF_{2α} stimulates Ptd-Ins 4,5-bisphosphate hydrolysis and mobilizes intracellular Ca²⁺ in bovine luteal cells (Davis *et al.*, 1987). It was suggested that PGF_{2α} evokes its cellular responses in luteal cells by stimulating the phospholipase C-inositol 1,4,5-trisphosphate (InsP₃) and DG second messenger system. Recently, data had been reported suggesting that ovarian leukotriene B₄ (LTB₄), one of the lipoxygenase pathway products, may induce ovulation by mechanical events within the ovary that are required for rupture of mature Graafian follicles in the rabbit (Yoshimura *et al.* 1991).

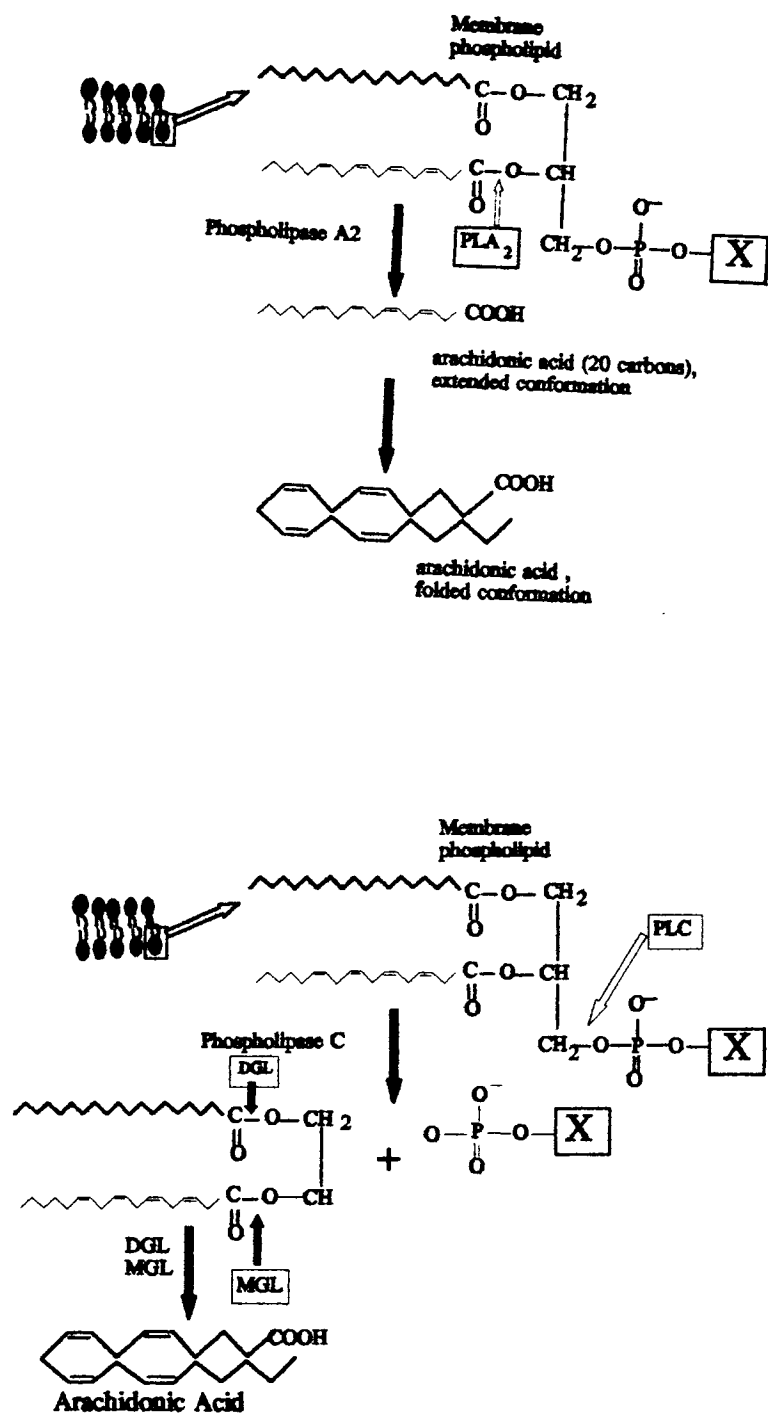


Fig. 5 Molecular basis of PLA₂ and PLC action. PLA₂ cleaves phospholipid on sn-2 position and yields AA and lysophospholipid (upper panel).

The lower panel shows PLC action on membrane phospho-lipids. The PLC splits the phospho-lipids into IP₃ and DG. DG may further break-down into AA.

DGL:diacylglycerol lipase.

MGL:monoacylglycerol lipase.

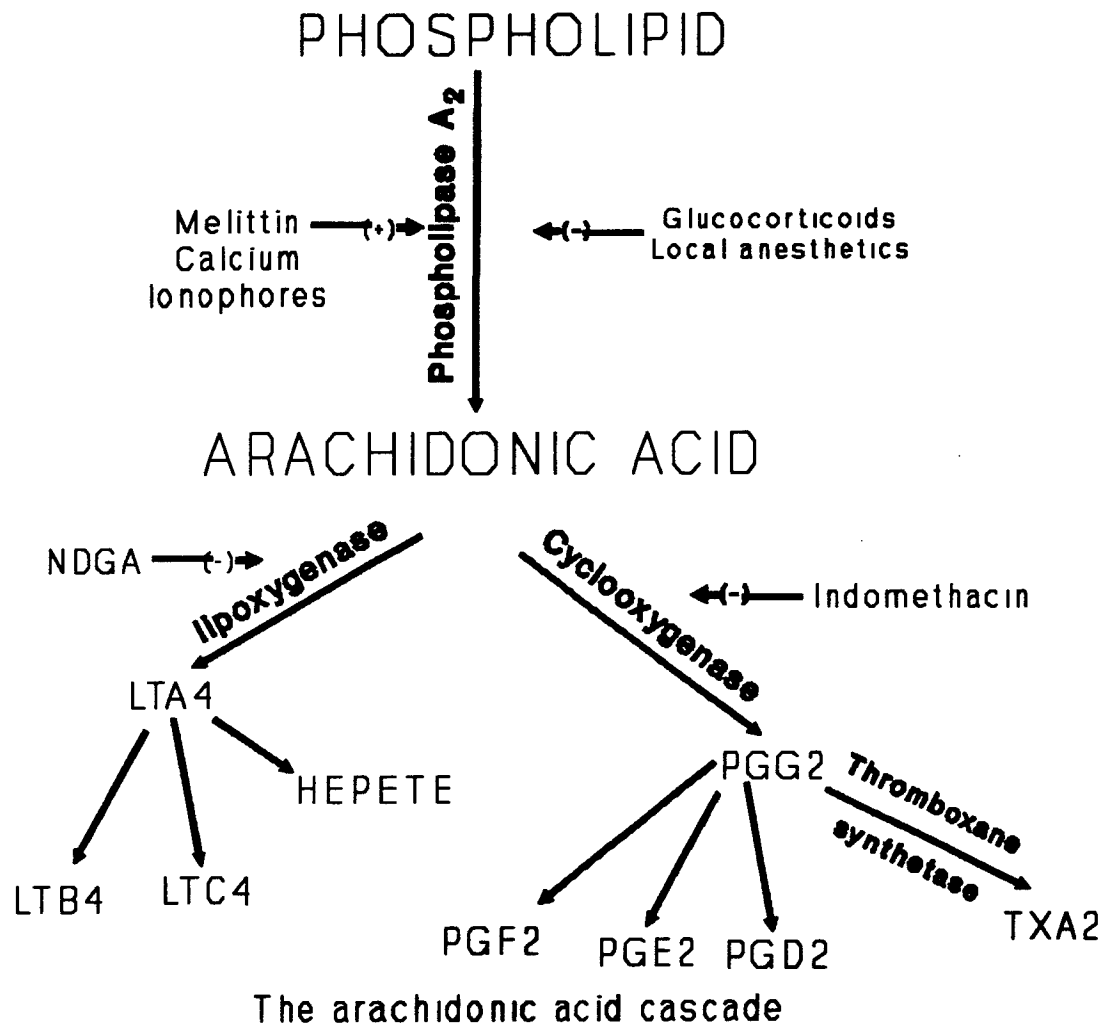


Fig. 6 The arachidonic acid cascade.

This is one pathway through which AA is generated. Release of AA is facilitated by the hydrolytic action of PLA₂ on membrane phospholipid. The liberation of AA is followed rapidly by the actions of cyclooxygenase and lipoxygenase, producing eicosanoid metabolites.

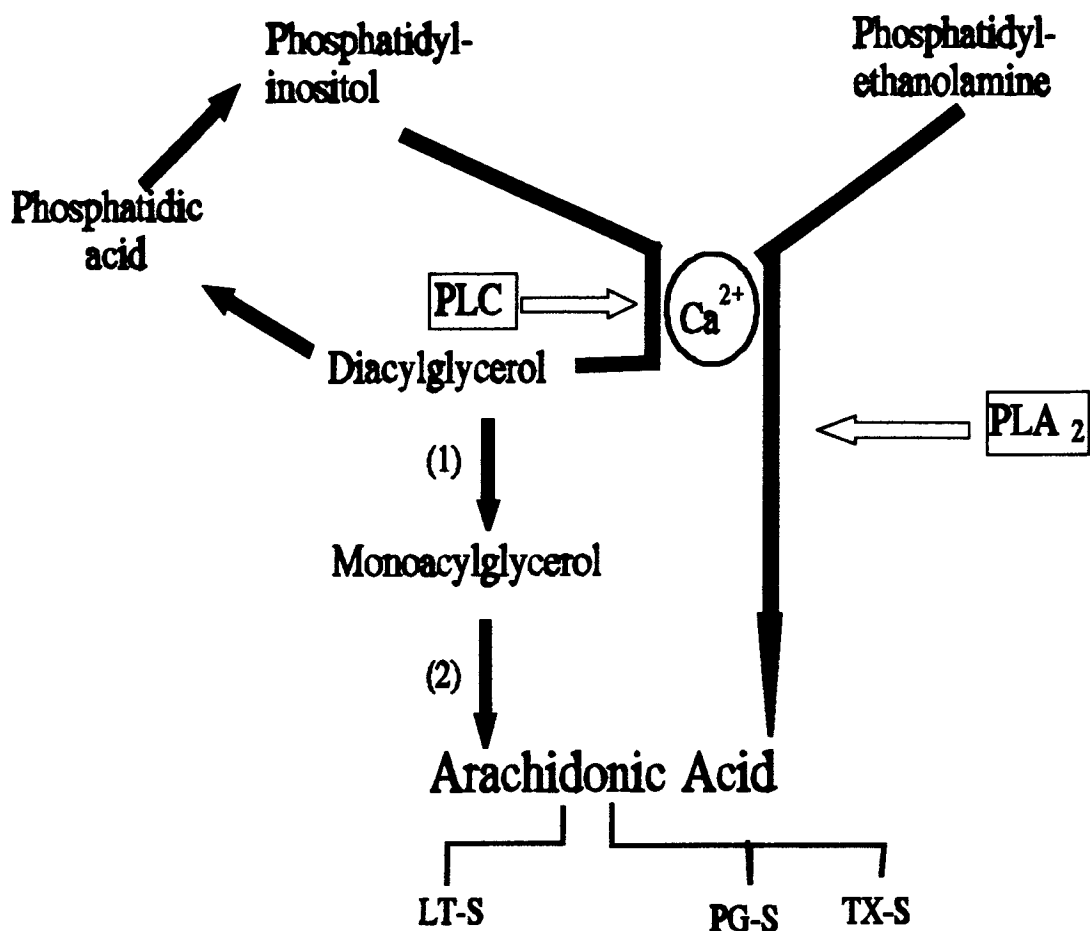


Fig. 7 Proposed pathways for the mobilization of AA from Ptd-Ins and Ptd-E in the cell.

Phospholipids are catalyzed by PLA₂ and PLC. DG produced from Ptd-I has at least two pathways: phosphorylation to phosphatidic acid and hydrolysis to monoacylglycerol (MG) by DG-lipase (1). MG is further degraded to AA and glycerol by MG-lipase (2). Mobilization of AA from phosphatidylethanolamine (Ptd-E) seems to involve a single reaction catalyzed by PLA₂.

AA also enhanced P_4 production in rat granulosa cells and AA may act as a stimulatory mediator of LH-releasing hormone action in the rat ovary (Wang & leung., 1988 & 1989).

1.4 Objectives

This study was intended to obtain information relative to understanding AA and cAMP effects on human term placenta. The study examined AA and cAMP regulation of aromatase activity and $P-450_{\alpha}$ in short term (3 h) human term placental cell cultures. Human term placental cells cultured for 3 to 4 days were used to examine differences in response of aromatase activity to AA and cAMP in short term *versus* long-term cell cultures.

2.0 MATERIALS AND METHODS

2.1 Cell preparation

2.1.1 Preparation of human term trophoblasts

General materials:

* Supplemented M199 (Gibco, Mississauga, Ontario):

1% (v/v) heat inactivated, defined fetal calf serum (FCS, Hyclone, Logan, UT).

Penicillin G sodium (100 U/ml, Gibco).

Streptomycin (100 µg/ml, Gibco).

Hepes (15 mM, Gibco).

NaHCO₃ (26 mM, Sigma, St. Louis, MO).

* Hank's balanced salt solution (HBSS, Gibco).

* Arachidonic acid (Sigma).

* Cyclic adenosine-3',5'-monophosphate (Sigma).

* Human chorionic gonadotropin (Sigma).

* Hypoxanthine (Sigma).

* Indomethacin (Sigma).

* Leukotriene B₄ (Sigma).

* Melittin (Sigma).

* Nordihydroguaiaretic acid (Sigma).

- * Prostaglandins $F_{2\alpha}$ (Sigma).
- * Scintiverse (Fisher, Vancouver, B.C).
- * $[1\beta,2\beta,^3H]$ -testosterone (40.4 mCi/mmol, Dupont, Boston, MA).
- * Xanthine oxidase (Sigma).

Methods--Normal full term placentae (36-42 weeks gestation) were obtained immediately after elective cesarean section. Four cotyledons dissected from underlying fibrous tissues were rinsed with supplemented M199 and dissociated through a stainless steel sieve (150 μ m screen, Sigma, St. Louis, MO). Aggregated cells were removed by filtration through 48 μ m nylon mesh (B&SH Thompson, Scarborough, ONT). Dissociated cells were centrifuged (1000g x 6 min), supernatant decanted and cells resuspended in M199. Hematocytes were removed by centrifuging (1700g x 20 min) the suspension on 40% (v/v) percoll (Sigma) in HBSS. Cell viability as determined by 0.05% trypan blue (Gibco) exclusion staining was consistently greater than 90%.

Cells were pipetted into borosilicate glass tubes (12 x 75 mm, Canlab, Burnaby, BC) at a density of 2×10^6 cells/ml M199. Each tube was treated with a mixture of $[1\beta,2\beta,^3H]$ -testosterone (30,000 cpm) and unlabelled testosterone (2×10^{-7} M). Tubes containing this testosterone mixture were then treated with additional factors such as indomethacin (1×10^{-4} M), melittin (1.5×10^{-6} M), NDGA (1×10^{-5} M), AA (1×10^{-5} M), $PGF_{2\alpha}$ (1×10^{-6} M), LTB_4 (1×10^{-6} M) or hypoxanthine (1 mM) plus xanthine oxidase (10 mU/ml). Cells were cultured in a shaking incubator (60 rpm,

5% CO₂, 37C) for 3 h. After 3 h incubation, 500 µl of a charcoal (BDH, Vancouver, BC, 20 mg/ml distilled water) and dextran (BDH, 2 mg/ml distilled water) mixture (4C) was added at 4C. The tubes were vortexed (30 cycles/sec) for 1 minute with a multiple-tube vortexer and let them stand for 15 minutes. The tubes were then centrifuged (2000g x 15 min) at 4C. The supernatant was decanted to scintillation vials (Packard, Canberra, Australia) and 3 ml of scintillation cocktail added. Radiation was counted (1 min/vial) using a beta-counter (LKB-Wallac C1217 RackBeta, Turku, Finland).

All experiments were performed in hexaplicate.

2.1.2 Preparation of porcine granulosa cells

General Materials--As outlined in section 2.1.1.1

Methods--Porcine ovaries were obtained from the abattoir in the morning. Pigs were electrically stunned (400 volts) and exsanguinated. Ovaries were immediately removed, submerged in 0.154 M saline in an insulated container and transported to the laboratory.

Disposable plastic syringes (5 ml) with 23 gauge needles were used to puncture and aspirate follicular fluid. The mixture of follicular fluid and granulosa cells was centrifuged (1000g x 6 min). The supernatant was decanted and the pellet resuspended in M199. The cells were pelleted and resuspended a second time.

Cells were counted using an Improved Neubauer Hemocytometer (Canlab) and a reverse phase microscope (10X, Nikon). Cell viability was determined using 0.05% trypan blue exclusion staining. Cell viability was approximately 30-40%.

Cells were pipetted into borosilicate glass tubes (12 x 75 mm) at a density of 1×10^6 cells/ml. Each tube received a mixture of [$1\beta,2\beta$, ^3H]testosterone (30,000 cpm) and unlabelled testosterone ($2 \times 10^{-7}\text{M}$) and subsequently treated with combinations of indomethacin ($1 \times 10^{-4}\text{M}$), melittin ($3 \times 10^{-7}\text{M}$) and NDGA ($1 \times 10^{-5}\text{M}$).

2.2 Enzyme assay: aromatase

Human term trophoblast cells (2×10^6 cells/ml) incubated with [$1\beta,2\beta$, ^3H]testosterone (30,000 cpm) and a corresponding amount of unlabelled testosterone to give a final concentration of 200 nM. The formation of total estrogen by the aromatase is equimolar to the tritiated water produced. Detection of tracer was with a liquid scintillation counter (LKB-Wallac C1217 RackBeta) detecting ^3H decay with 60% efficiency. Scintillation vials were counted for 60 sec each. Aromatase activity was measured by the recovery of tritiated water from the conversion of [$1\beta,2\beta$, ^3H]-testosterone to estrogen.

The following equations were use for calculation of conversion:

(200 nM was the testosterone concentration added in the media.)

$$\text{Rate of convert} = \frac{\text{the recovery rate of tritiated water} - \text{Background}}{\text{total count}}$$

$$\text{Estrogen} = \text{rate of convert} \times 200 \text{ nM}$$

2.3 Radioimmunoassay (RIA)

2.3.1 Estradiol RIA

Reagents

1. Buffer was 50 mM phosphate buffered saline (pH 7.4, 12 mM EDTA) with 0.1% w/v gelatin. All buffer reagents were from BDH.

Mix and stir until dissolved in 900 ml distilled water:

1.0 g gelatin, dissolved in 100 ml hot distilled water

9.0 g sodium chloride, NaCl

5.3 g monobasic sodium phosphate, NaH_2PO_4 crystalline.

8.8 g dibasic sodium phosphate, Na_2HPO_4 anhydrous.

0.1 g sodium azide, NaN_3

2. The standard (Sigma E1132, Sigma) was prepared and stored in polystyrene at -20°C. Standards were diluted in M199 media without serum.
3. The antiserum was rabbit #3, bled 12/10/82, raised against 1,3,5(10)-estratriene-3,17 β -diol-6-one-6-carboxymethyl-oxide:BSA conjugate (Steraloids,

Wilton, New Hampshire). The antiserum was used at a final dilution of 1:750000 and gave 71% binding of label (12.000 cpm).

4. The labelled hormone was 2,4,6,7,-³H-E₂ (Amersham, Oakville, Ontario) with a specific activity of approximately 114 Ci/mmol. Initial stock was 1 µCi/µl in toluene:ethanol (9:1 v:v). Solvents were evaporated and stock was diluted in buffer (200µl ≡ 15,000 cpm).
5. The charcoal suspension (0.25% w/v Norit A + 0.025% Dextran w/v, BDH) in buffer was mixed in glass for 30 min at 20C, stored at 4C and mixed for 30 min at 4C prior to use.
6. The extraction solvent was ethyl ether (Fisher). Extraction efficiency was approximately 90%.
7. The scintillation cocktail was Scintiverse (Fisher).

Procedure

Standards (range from 1pg-1ng/ml) and samples (diluted 5X) were assayed in quadruplicate. Extraction tubes were 16 x 150 mm borosilicate glass (Fisher); RIA tubes were 12 x 75 mm borosilicate glass (Fisher). Scintillation vials were polyethylene (Packard).

Day 1: Extraction Efficiency

100 µl tracer added to 3 scintillation vials and 3 extraction tubes.

0.5 ml M199 added.

M199 extracted, ether phase evaporated and 3 ml Scintiverse added.

Extraction

500 µl sample added to extraction tubes.

4 ml ethyl ether added to extraction tubes.

Extraction tubes vortexed twice for 30 s at a 1 h interval at 21C and incubated for an additional 1 h at 21C.

Extraction tubes cooled at -70C for 12 min.

Ether phase decanted to assay tubes and evaporated.

RIA

200 µl standard added to appropriate tubes.

200 µl buffer added to total counts assayed tubes (TCA) and non-specific binding tubes (NSB).

200 µl antiserum in buffer added to standards and samples.

Vortexed.

Incubated for 1 h at 20C.

200 µl tracer added to all tubes.

Vortexed.

Incubated for 24 h at 4C.

Day 2:

500 µl charcoal suspension added in less than 2 min to all but TCA tubes.

500 µl buffer added to TCA tubes.

Vortexed.

Tubes loaded in centrifuge precooled to 4°C.

Assay tubes centrifuged at 2000g for 15 min, beginning 10 min after addition of charcoal to first tube.

Supernatant decanted to scintillation vials.

3 ml Scintiverse added.

Vortexed and incubated for 1 h at 21°C.

Counted for 1 min per vial (LKB-Wallac).

2.3.2 Progesterone RIA

Reagents and procedure:

Tracer: [1,2-³H(N)]progesterone with a specific activity of approximately 115 Ci/mmol (Amersham).

Procedures: The P₄ concentration in the culture medium was determined by the method of Leung *et al.*, 1979, and antiserum was kindly provided by Dr. D.T. Armstrong of the University of Western Ontario (Leung and Armstrong, 1979).

Standards (range from 80pg-10ng/ml) were used triplicate and samples were assayed in duplicate. RIA tubes were 10 x 75 mm borosilicate glass (Fisher). Scintillation vials were polyethylene (Packard).

Day 1:

100 µl std added to appropriate tubes.

300 µl buffer added to TCA and NSB tubes.

100 µl antiserum in buffer added to stds and samples.

100 µl tracer added to all tubes.

Vortexed.

Incubated for 24 h at 4C.

Day 2:

500 µl charcoal suspension added in less than 2 min to all but TCA tubes.

Vortexed.

Tubes loaded in centrifuge precooled 4C.

Assay tubes centrifuged at 2000g for 15 min, beginning 10 min after addition of charcoal to first tube.

Supernatant decanted to scintillation vials.

3 ml Scintiverse added.

Incubated for 4 h at 4C.

Counted for 1 min per vial in beta-counter (LKB-Wallac).

2.3 Statistical analysis

Control and treatment groups were compared by one-way or multiple analysis of variance (ANOVA) where applicable. Briefly, a variance ratio (the variance of

between treatment groups/the variance of within treatment groups) of approximately 1 indicates no true treatment differences. A variance ratio much greater than 1 suggests a true treatment effect and was followed by Sheffe's multiple comparison test (Thomas, 1991).

3.0 RESULTS

Aromatase activity was measured by the recovery of tritiated water from the conversion of [$1\beta,2\beta$ - ^3H]-testosterone to estrogen.

3.1 Aromatase activity of human term trophoblasts:

3.1.1 Effects of melittin on aromatase activity (Fig. 8):

Human term trophoblasts dissociated from full term placenta were incubated for 3 h with tritiated testosterone (30,000 cpm, $2 \times 10^{-7}\text{M}$) and in the presence or absence of increasing melittin or exogenous AA. Following a 3 h incubation, there was a dose-dependent decrease in aromatase activity of the cells in the presence of melittin. Whereas melittin at the concentration of 10^{-7}M was ineffective, the aromatase activity was attenuated ($P < 0.01$) in the presence of 10^{-6}M and 10^{-5}M melittin (Fig. 8A). Treatment of the cells with 10^{-4}M AA also resulted in 80% suppression of aromatase activity (Fig. 8B).

3.1.2 Effects of a cyclooxygenase inhibitor, indomethacin, on aromatase activity (Fig. 9):

The possible involvement of the cyclooxygenase pathway in

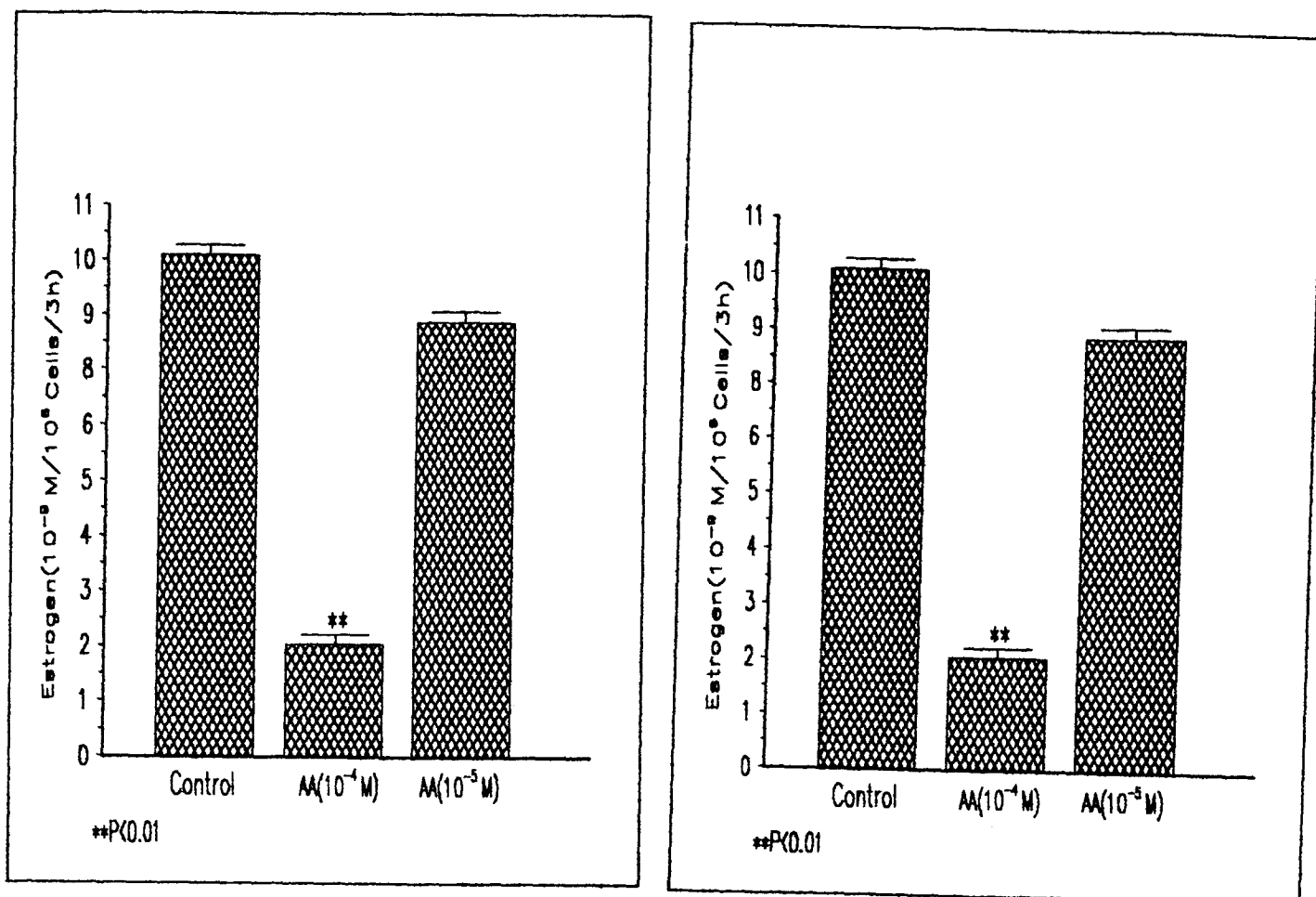


Fig. 8 Effects of melittin and AA on aromatase activity.

Melittin suppressed aromatase activity in a dose-dependent manner (10⁻⁴-10⁻⁶ M) during a 3 h incubation (P<0.01). Melittin (10⁻⁷ M) was ineffective. AA (10⁻⁴ M) also suppressed aromatase activity of trophoblast cells (P<0.01).

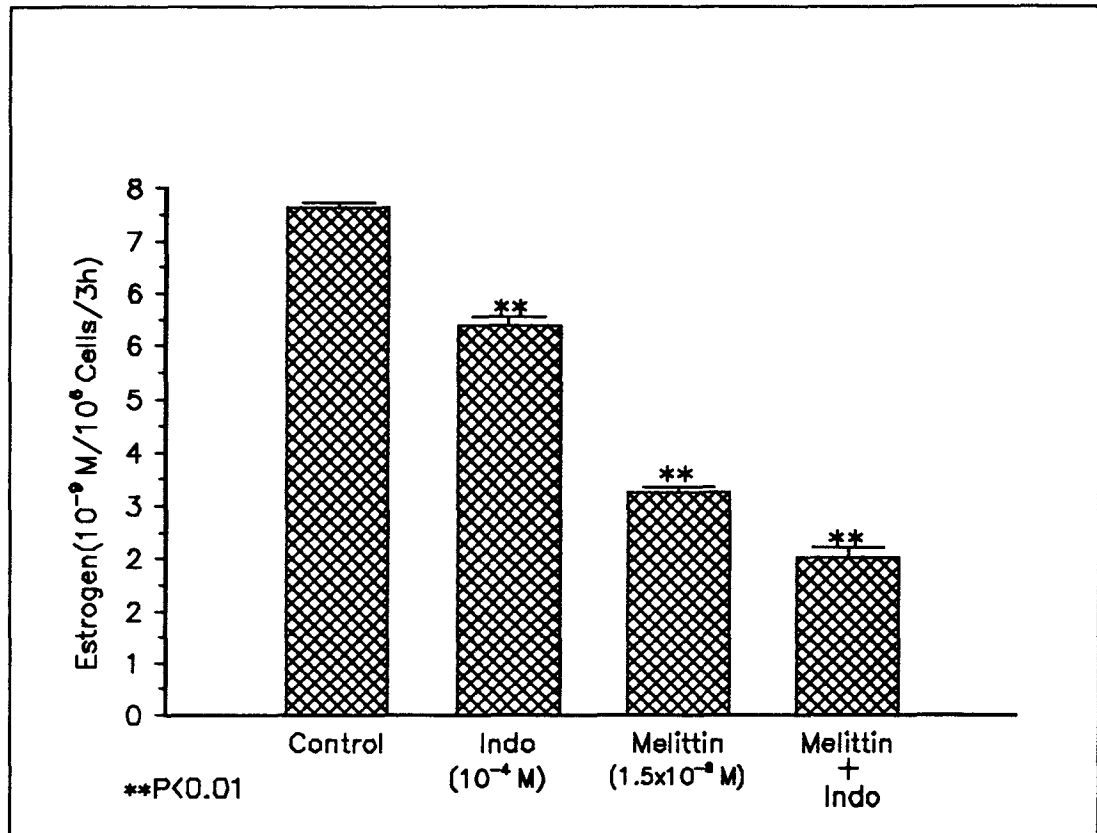


Fig. 9 Effects of a cyclooxygenase inhibitor, indomethacin.

A cyclooxygenase pathway inhibitor, indomethacin (10⁻⁴M), suppressed aromatase activity of human term trophoblast cells (P<0.01). Indomethacin + melittin further suppressed aromatase activity relative to melittin alone (P<0.05).

the inhibitory effect of melittin on aromatase activity was investigated. Human trophoblast cells were incubated in the presence or absence of melittin ($1.5 \times 10^{-6}\text{M}$), indomethacin (10^{-4}M) or melittin + indomethacin. The presence of this dose of indomethacin suppressed aromatase activity following a 3 h incubation period (Fig. 9), whereas lower concentrations had no effect (data not shown). As expected, melittin suppressed aromatase activity ($P < 0.01$) without affecting cell viability. The combined presence of melittin + indomethacin further attenuated aromatase activity in the cells (2.4 ± 0.2 vs 3.4 ± 0.1 nM; indomethacin + melittin vs melittin, $P < 0.05$).

3.1.3 Effects of a lipoxygenase inhibitor, NDGA, on aromatase activity (Fig. 10):

The effect of a lipoxygenase inhibitor, NDGA, on the action of melittin was investigated in parallel experiments. NDGA (10^{-5}M) suppressed aromatase activity following a 3 h incubation period (Fig. 10). Melittin ($1.5 \times 10^{-6}\text{M}$) decreased aromatase activity ($P < 0.01$). The combined treatment of cells with melittin + NDGA resulted in similar suppression of aromatase activity as in the melittin group alone (3.3 ± 0.3 nM vs 3.4 ± 0.1 nM).

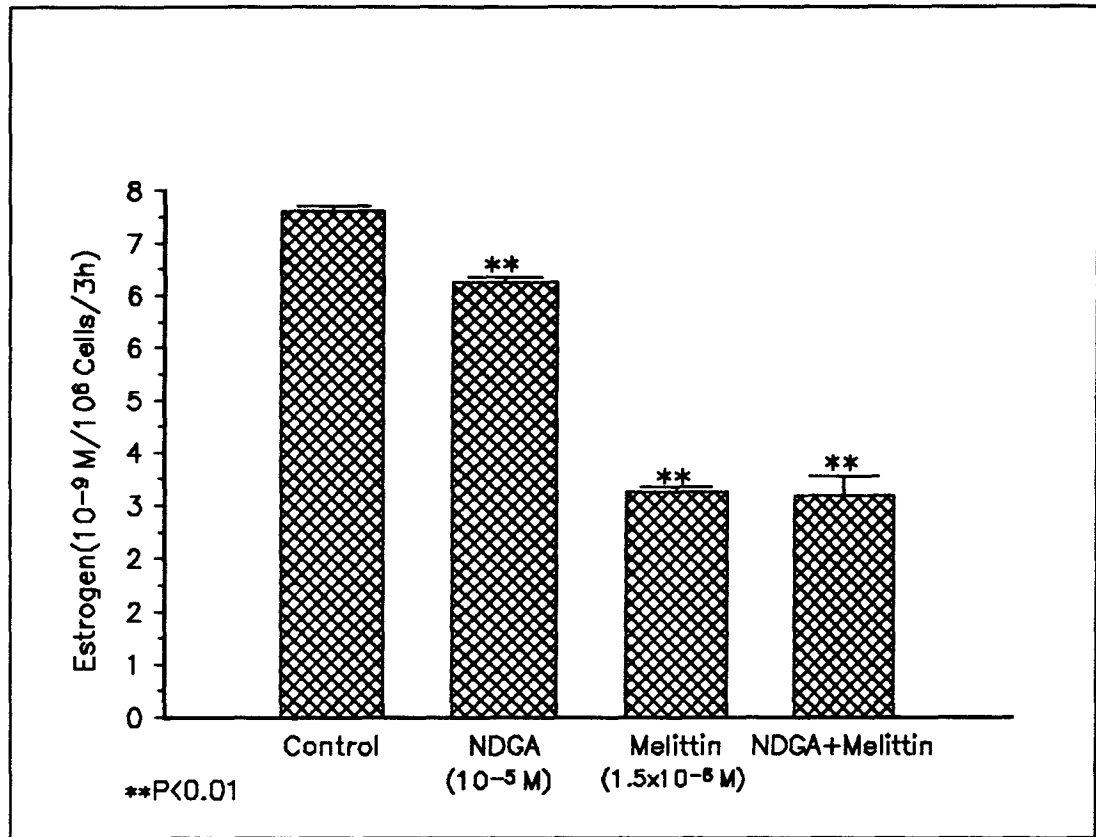


Fig. 10 Effects of a lipoxygenase inhibitor, NDGA, on aromatase activity.

NDGA (10⁻⁵M) suppressed the aromatase activity of trophoblast cells during a 3 h incubation (P<0.01). Melittin (1.5 x 10⁻⁶M) inhibited aromatase activity (P<0.01). Treatment of the cells with NDGA + melittin had an effect similar to melittin alone.

3.1.4 Effects of combined melittin, indomethacin and NDGA on aromatase activity (Fig. 11):

The effect melittin on aromatase activity of trophoblast cells was investigated in the presence or absence of NDGA and/or indomethacin. Melittin ($1.5 \times 10^{-6}\text{M}$) suppressed aromatase activity following a 3 h incubation (Fig. 11, $P < 0.01$). The effect of melittin was not affected by the presence of NDGA (10^{-5}M). As previously observed, indomethacin (10^{-4}M) + melittin suppressed aromatase activity further than melittin alone ($P < 0.01$). Combined treatment with melittin + NDGA + indomethacin suppressed aromatase activity further than NDGA + melittin ($P < 0.05$) but not further than melittin + indomethacin.

3.1.5 Effects of arachidonic acid with or without NDGA and indomethacin on aromatase activity (Fig. 12):

The presence of AA (10^{-4}M) decreased aromatase activity in trophoblast cells during a 3 h incubation period ($P < 0.01$). treatment with AA + NDGA (10^{-5}M) did not suppress aromatase activity further than AA alone (Fig. 12). Concomitant treatment with AA and indomethacin further suppressed aromatase

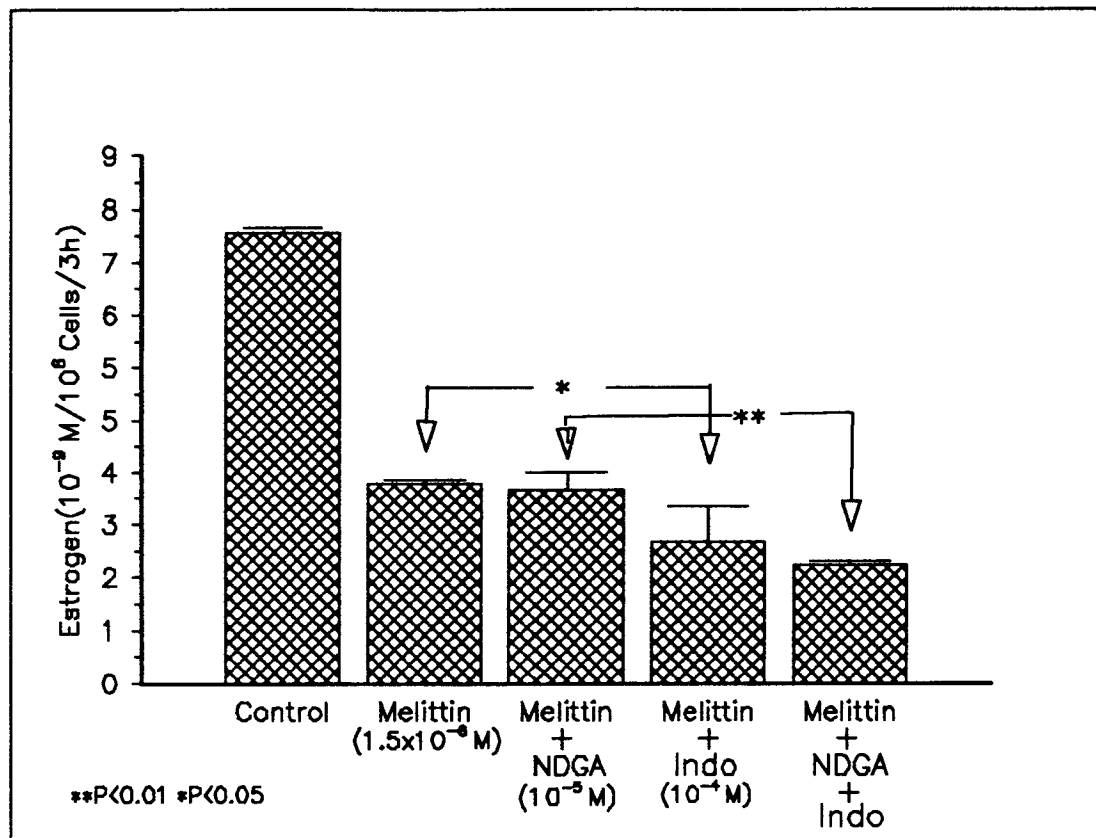


Fig. 11 Effects of melittin, indomethacin and NDGA on aromatase activity.

Trophoblast cells were treated with indomethacin and/or NDGA in the presence of melittin. Melittin suppressed aromatase activity ($P<0.01$). There was no difference in aromatase activity of the cells treated with melittin and those treated with melittin + NDGA. Melittin + indomethacin decreased aromatase activity further than melittin alone ($P<0.05$). The aromatase activity of cells treated with melittin + indomethacin did not differ from cells treated with melittin + indomethacin + NDGA.

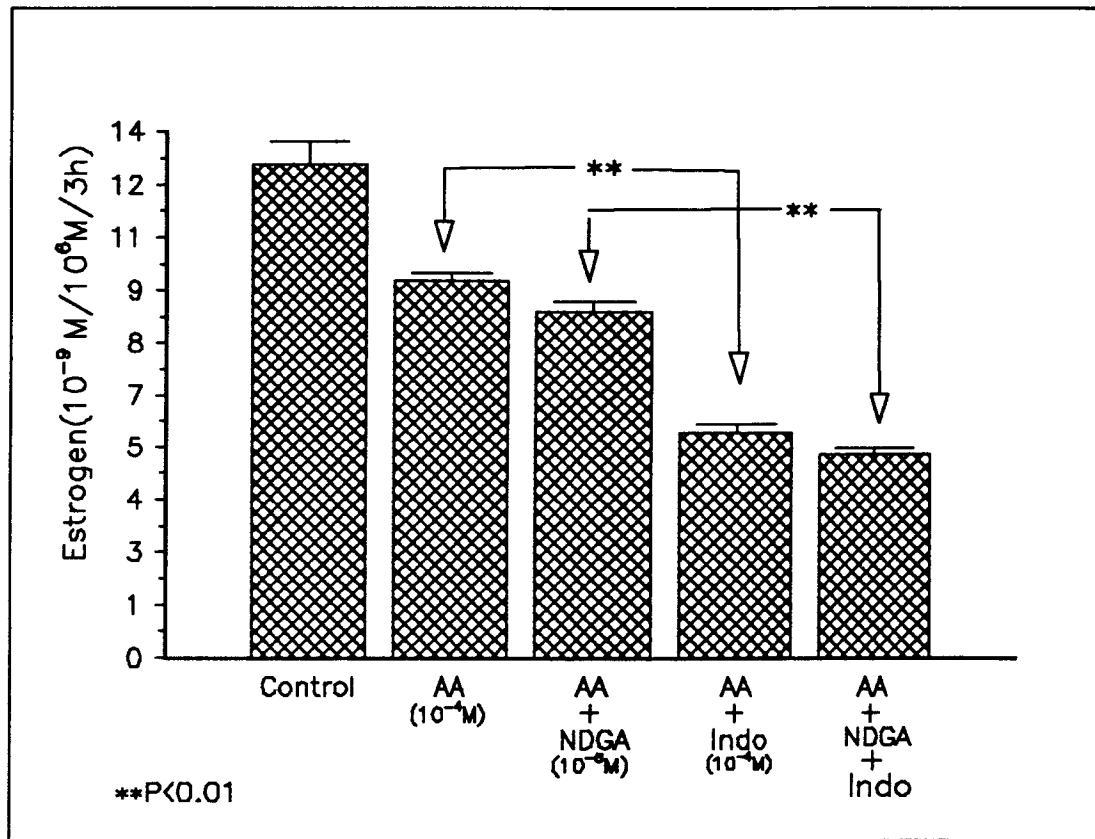


Fig. 12 Effects of arachidonic acid with or without NDGA and indomethacin on aromatase activity.

AA (10^{-4} M) inhibited aromatase activity during a 3 h incubation ($P < 0.01$). Treatment with AA and NDGA did not affect aromatase activity. AA + indomethacin suppressed aromatase activity further than AA alone ($P < 0.01$). Again, there was no difference between aromatase activity in cells treated with AA + indomethacin or AA + NDGA + indomethacin.

activity when compared with AA alone ($P < 0.01$). Finally, combined treatment with AA + indomethacin + NDGA did not suppress aromatase activity further than AA + indomethacin.

3.1.6 Effects of arachidonic acid metabolites, prostaglandin $F_{2\alpha}$ and leukotriene B_4 (Fig. 13):

The possible effects of two products of AA metabolism were investigated. Neither $PGF_{2\alpha}$ (a cyclooxygenase pathway metabolite) nor LTB_4 (lipoxygenase pathway metabolite) affected aromatase activity, whereas melittin suppressed aromatase activity in trophoblast cells (Fig. 13, $P < 0.01$).

3.1.7 Effects of hypoxanthine and xanthine oxidase on aromatase activity (Fig. 14):

Trophoblast cells were incubated with either melittin or hypoxanthine + xanthine oxidase. Both treatments suppressed aromatase activity in the trophoblast cells (Fig. 14, $P < 0.01$). Treatment of cells with hypoxanthine + xanthine oxidase resulted in 35% suppression of aromatase activity, which was less than that observed with $1.5 \times 10^{-6}M$ melittin. Increasing the concentrations of xanthine oxidase to 20 mU/ml did not further suppress aromatase activity (Fig. 14a).

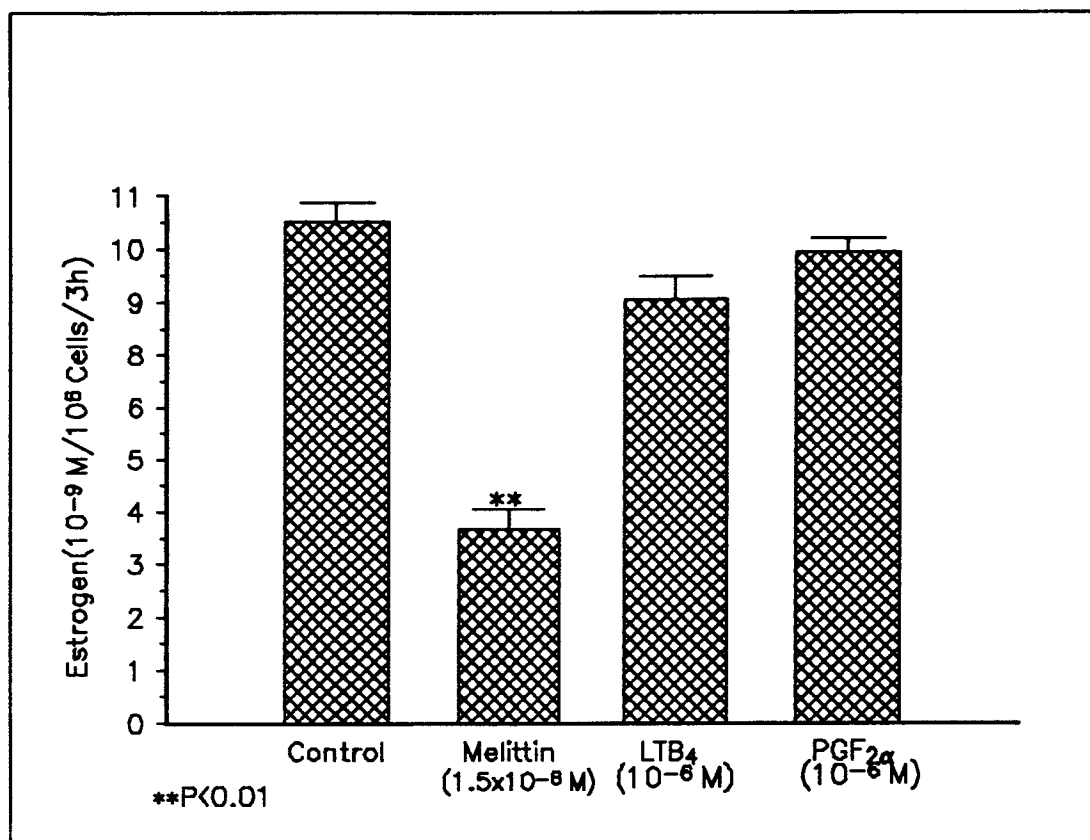


Fig. 13 Effects of arachidonic acid metabolites, PGF_{2α} and leukotriene B₄ on aromatase activity.

Trophoblast cells were treated with two AA metabolites, PGF_{2α} (a cyclooxygenase inhibitor) and LTB₄ (lipoxygenase metabolite). Neither affected aromatase activity of trophoblast cells during a 3 h incubation period. Melittin suppressed aromatase activity (P<0.01).

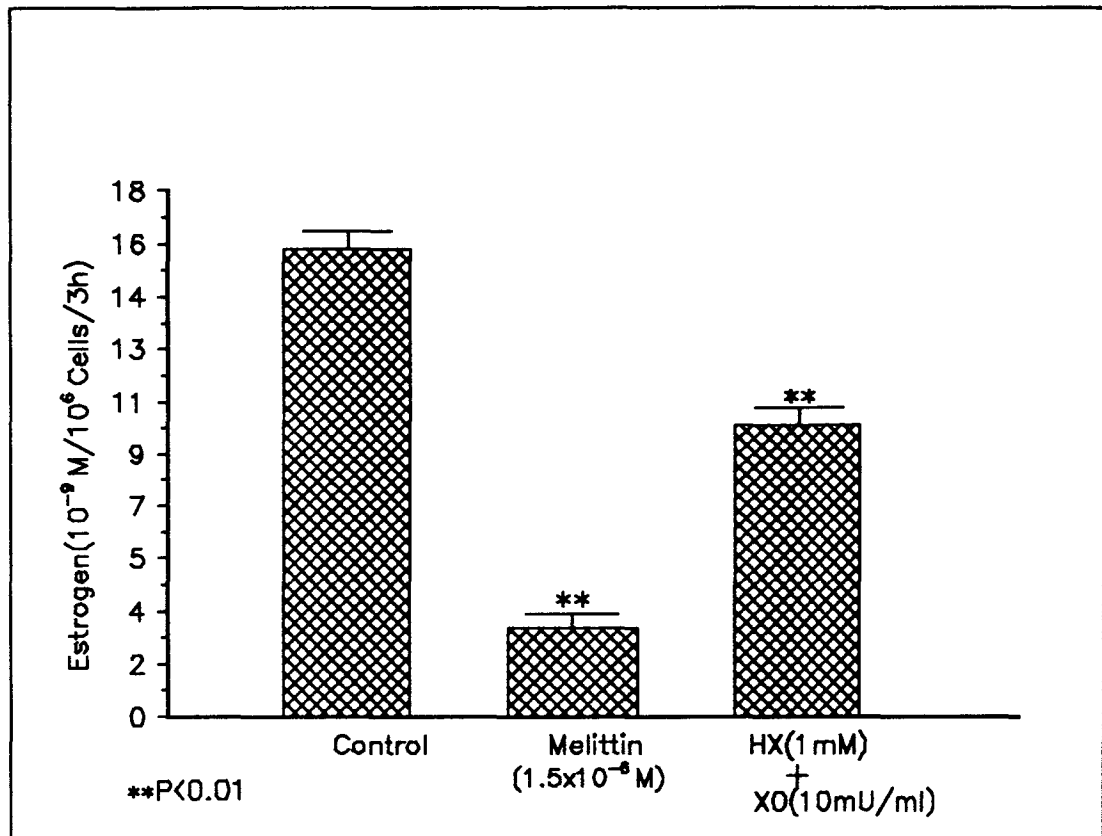


Fig. 14 Effects of hypoxanthine and xanthine oxidase on aromatase activity.

Hypoxanthine combined with xanthine oxidase had been shown to generate AA production. Hypoxanthine (1 mM) + xanthine oxidase (10 mM) suppressed aromatase activity of human term trophoblast cells (35% suppression). Melittin (1.5×10^{-6} M) suppressed aromatase activity more potently than hypoxanthine + xanthine oxidase.

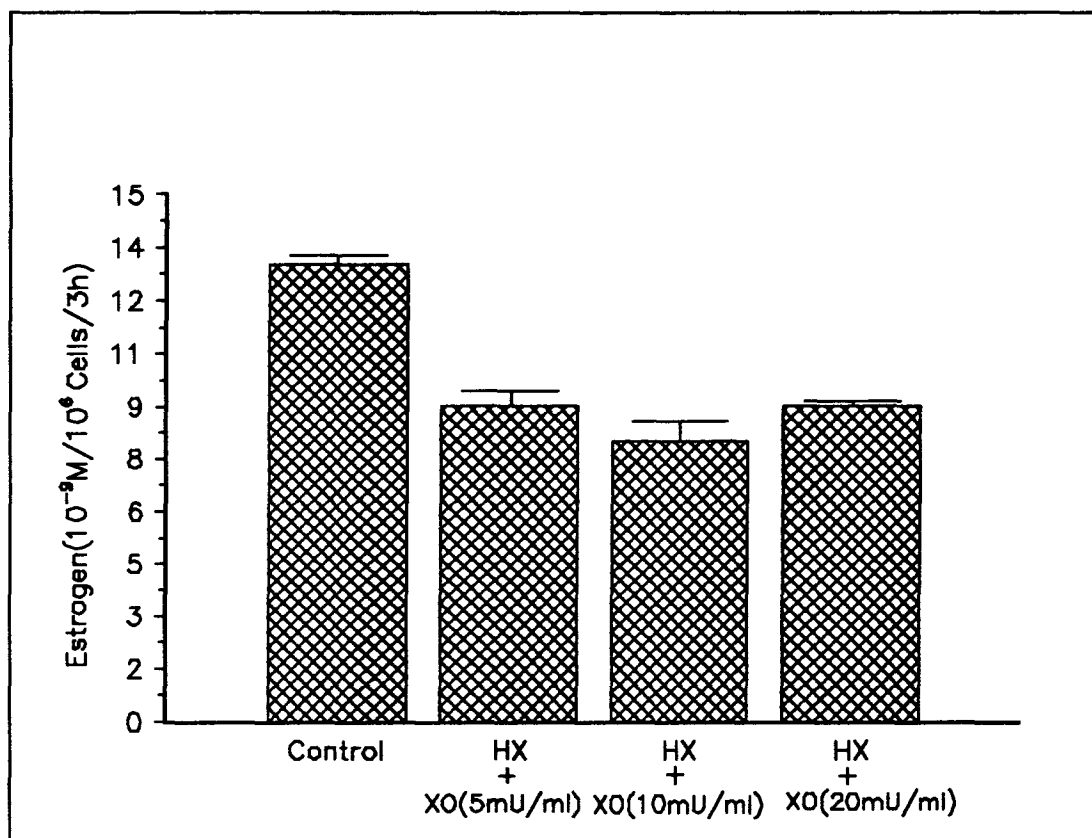


Fig. 14a Effects of different doses of xanthine oxidase and hypoxanthine on aromatase activity.

Xanthine oxidase (5 mU/ml to 20 mU/ml) + hypoxanthine (1 mM) had inhibitory effects on aromatase activity of trophoblast cells during a 3 h incubation period. Increasing xanthine oxidase concentrations did not further suppress aromatase activity.

3.1.8 Effects of hCG or 8-br-cAMP on aromatase activity (Fig. 15):

The presence of hCG (1 IU/ml) or 8-br-cAMP (2 mM) had no effect on trophoblast cells during the 3 h incubation period. Melittin ($1.5 \times 10^{-6}\text{M}$) and AA (10^{-4}M) decreased ($P < 0.01$) aromatase activity. Human CG and 8-br-cAMP did not affect aromatase activity (Fig. 15).

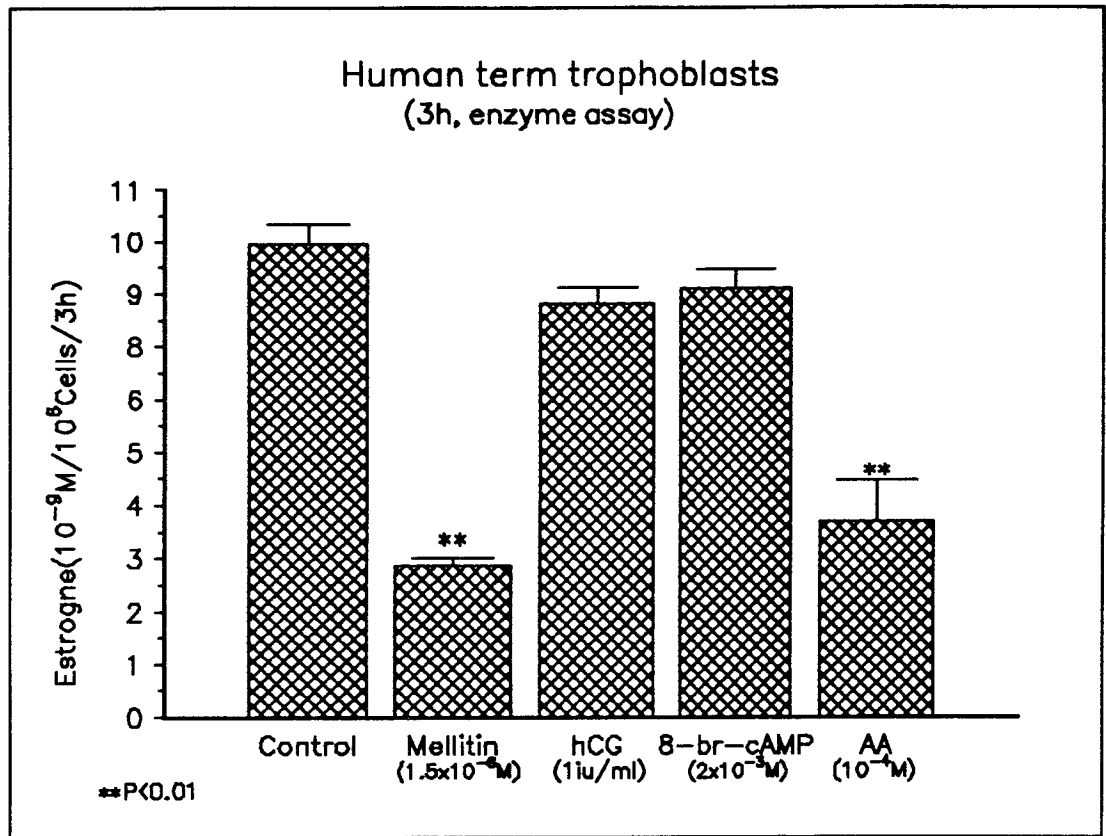


Fig. 15 Effects of hCG or 8-br-cAMP on aromatase activity.

Human CG (1 IU/ml) and 8-bromo-cAMP ($2 \times 10^{-3} \text{ M}$) had no effect on aromatase activity during a 3 h incubation period. Melittin and AA inhibited aromatase activity of trophoblast cells ($P<0.01$).

3.1.9 Comparison of RIA and enzyme assay determinations of aromatase activity (Fig. 16):

The RIA and enzyme assay provided similar data. Melittin suppressed aromatase activity as determined by both RIA and enzyme assay (Fig. 16). Human CG and 8-br-cAMP had no effect on aromatase activity in 3 h trophoblast incubation as determined by both methods. Data suggesting that AA inhibited aromatase activity ($P < 0.01$) was obtained using the enzyme assay but was not evaluated using RIA .

3.1.10 Effect of aromatase activity of melittin and cAMP after 24 h and 48 h incubation (Fig. 18):

Dispersed trophoblast cells were precultured for 48 h with 10% FCS in M199 on the first day and 1% FCS in M199 on the second day. During preculture these cells were exposed to melittin ($1.5 \times 10^{-6}M$) or cAMP ($2 \times 10^{-3}M$) in the presence of testosterone ($2 \times 10^{-7}M$). Media samples were collected after 24 h and 48 h incubation. During 24 h incubation, cAMP increased aromatase activity (Fig. 17). During 48 h incubation, cAMP enhanced aromatase activity (Fig. 18). Aromatase activity was higher in cells incubated for 48 h than for 24 h. Melittin had no effect on aromatase activity of cells cultured for either 24 or 48 h.

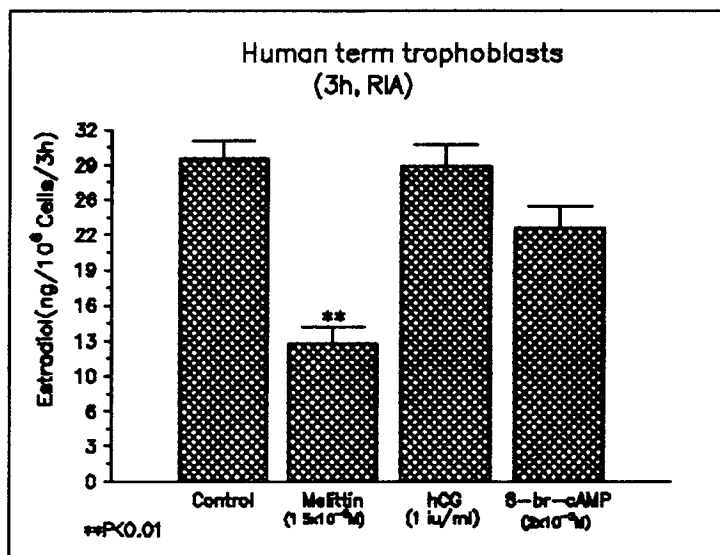
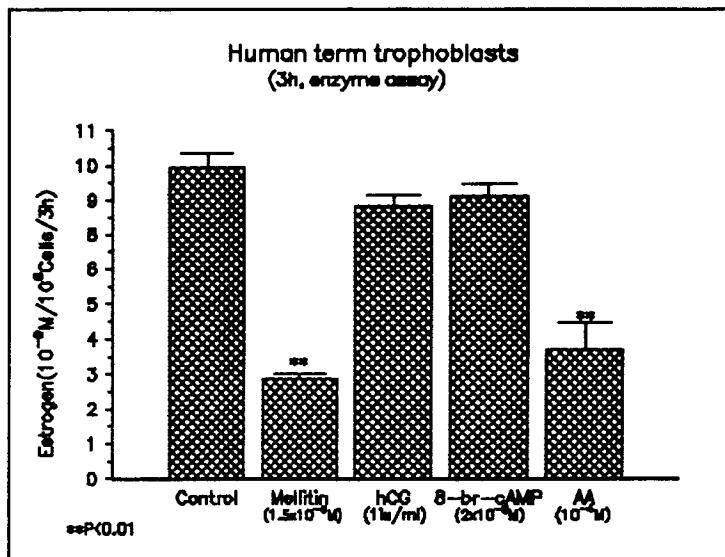


Fig. 16 The comparison between RIA and enzyme assay of aromatase activity. The upper panel shows aromatase activity as determined by enzyme assay. The lower panel shows E_2 values as determined by RIA. Both experiments used cells from the same placenta. AA and melittin attenuated aromatase activity. Neither 8-br-cAMP nor hCG affected aromatase activity of human term trophoblasts in short term incubation (3 h).

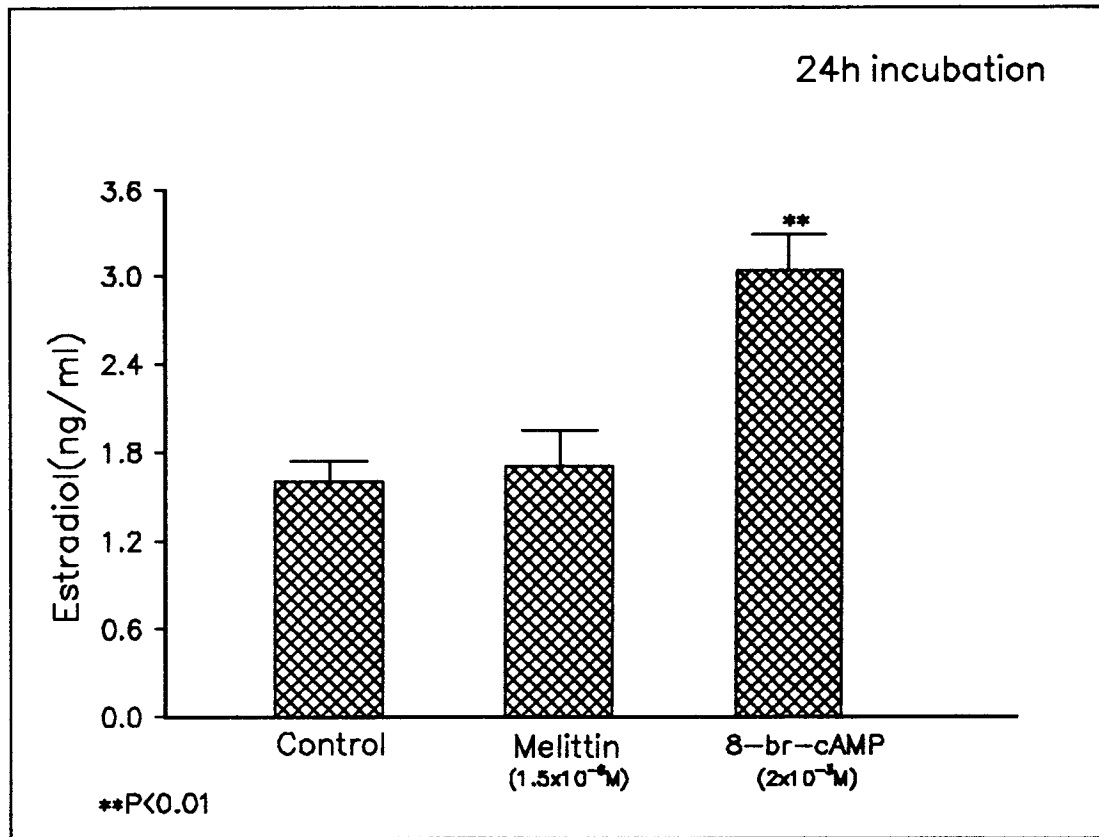


Fig. 17 Effects of melittin and cAMP after 24 h incubation on aromatase activity.

Dispersed trophoblast cells were precultured for 48 h and incubated with melittin (1.5×10^{-6} M) or cAMP (2×10^{-3} M) in the presence of testosterone (2×10^{-7} M) for 24 h. Melittin did not affect aromatase activity following 24 h incubation. cAMP increased aromatase activity ($P < 0.01$).

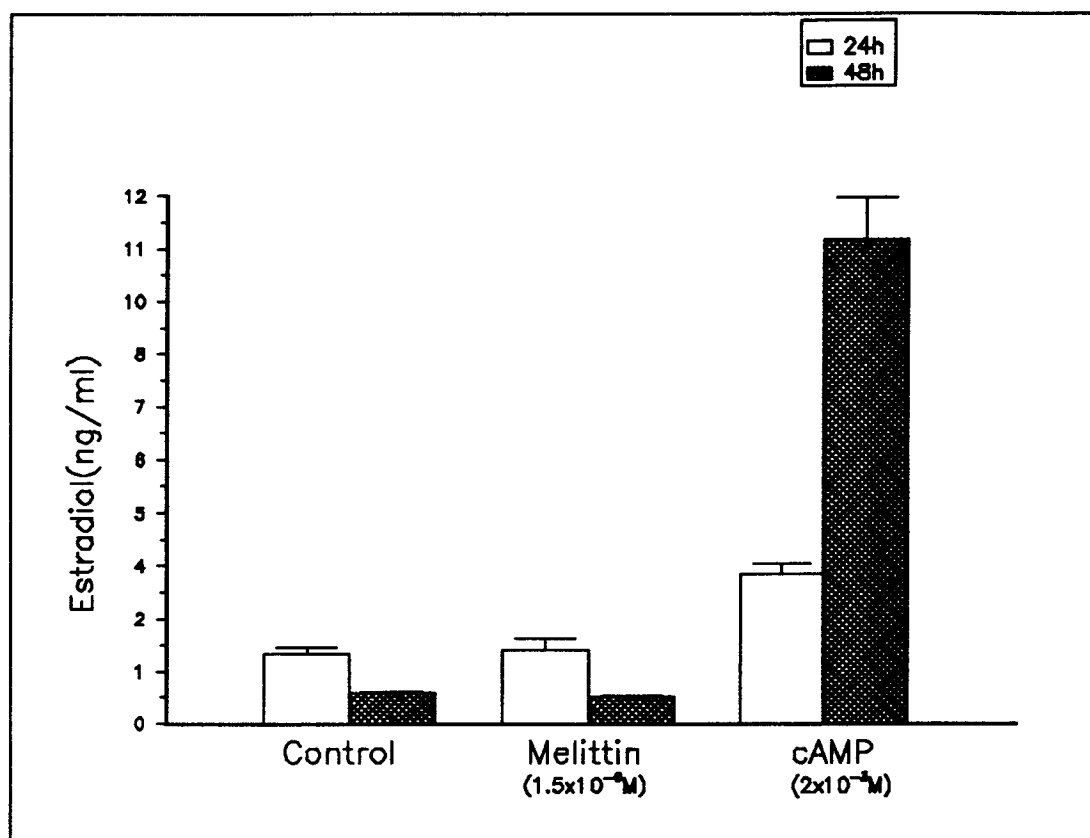


Fig. 18 Effect of melittin and cAMP after 24 h and 48 h incubation.

Cells were precultured for 48 h and incubated with melittin ($1.5 \times 10^{-6} \text{M}$) or cAMP ($2 \times 10^{-3} \text{M}$) in the presence of testosterone ($2 \times 10^{-7} \text{M}$) for 24 h and 48 h. Melittin (24 h & 48 h) did not affect aromatase activity. However, cAMP (24 h & 48 h) stimulated aromatase activity ($P < 0.01$).

3.2 Aromatase activity on porcine granulosa cells:

3.2.1 Effects of treatment with indomethacin and melittin on aromatase activity of porcine granulosa cells (Fig. 19):

Indomethacin and melittin were stimulatory to aromatase activity compared to controls (3.55 ± 0.27 pg E_2 /ml in the melittin treated cells, 4.37 ± 0.28 pg/ml in indomethacin treated cells and 2.12 ± 0.21 pg/ml in the control group, both $p < 0.01$). Indomethacin + melittin increased aromatase activity further than indomethacin or melittin alone (8.01 ± 0.07 pg E_2 /ml in indomethacin + melittin treated cells $p < 0.01$).

3.2.2 Dose-response effects of melittin and indomethacin on porcine granulosa cells (Fig. 20):

Indomethacin stimulated aromatase activity in a dose-dependent manner over the range of 10^{-6} to 10^{-4} M. The highest concentration of indomethacin doubled aromatase activity over control levels. Melittin (3×10^{-7} M) had no effect on aromatase activity. Combined indomethacin (10^{-6} to 10^{-4} M) and melittin treatment.

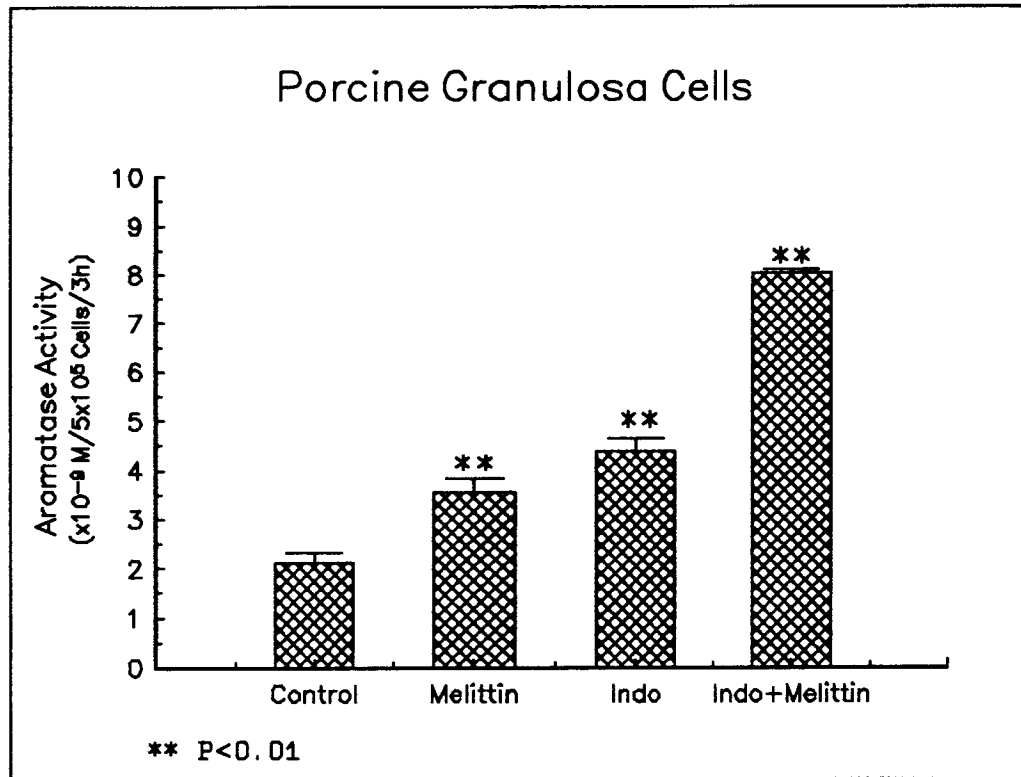


Fig. 19 Effects of indomethacin and melittin on aromatase activity of porcine granulosa cells.

Unlike their effects in human term trophoblast cells, indomethacin (10^{-4} M) and melittin (3×10^{-7} M) stimulated aromatase activity of porcine granulosa cells ($P<0.01$). Indomethacin + melittin further stimulated aromatase activity of porcine granulosa cells ($P<0.01$).

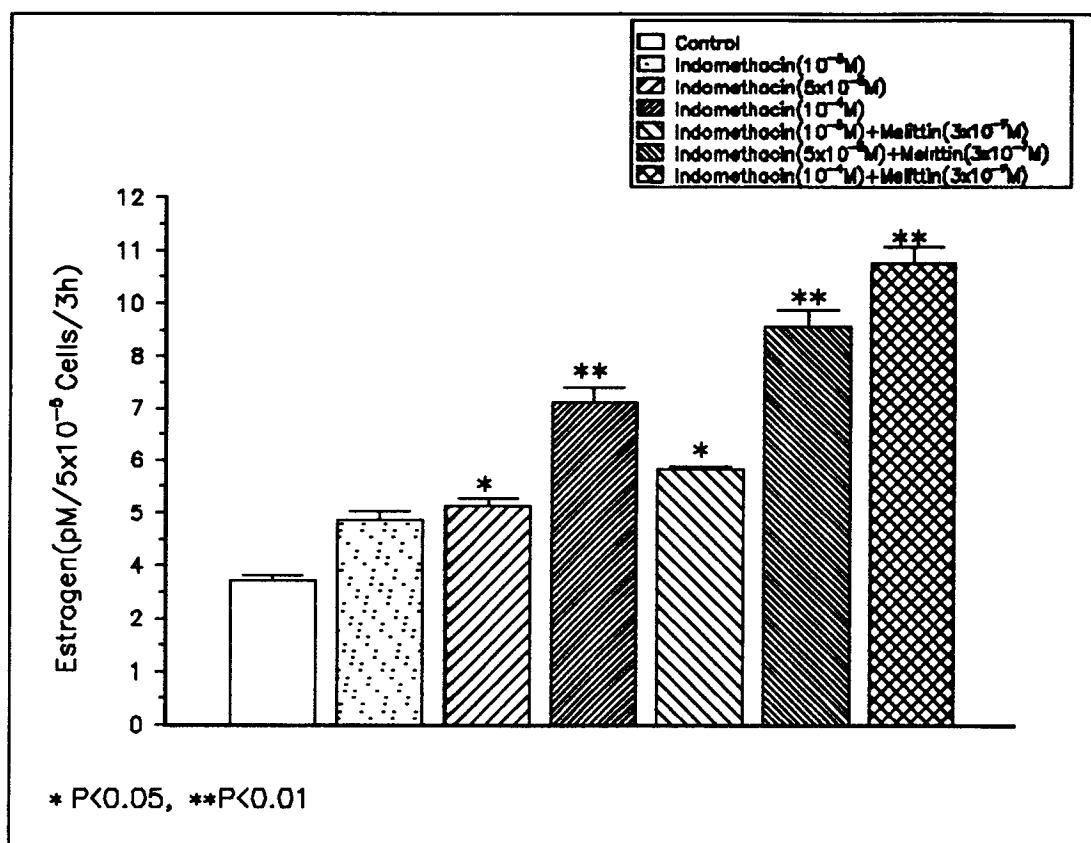


Fig. 20 Dose-response effects of melittin and indomethacin on porcine granulosa cells.

This figure illustrates the effect of increasing concentrations of indomethacin (10^{-5} to 10^{-4} M), in the presence or absence of melittin (3×10^{-7} M), on aromatase activity during 3 h incubation. Indomethacin increased aromatase activity in a dose-dependent manner and melittin enhanced the effect of indomethacin.

3.3 The modulation of Progesterone production of human term trophoblasts:

The P-450 cholesterol side-chain cleavage step is the rate-limiting step in steroidogenesis and is hormonally regulated. LH stimulates the adenyl cyclase system and increases cAMP production (Marsh, 1975). Activation of LH receptors is followed by cAMP formation and P₄ synthesis.

3.3.1 Effects of melittin, hCG, cAMP on P₄ production by human trophoblast cells (Fig. 21):

The possible involvement of melittin ($1.5 \times 10^{-6}\text{M}$), hCG (1 IU/ml) and cAMP ($2 \times 10^{-3}\text{M}$) in P₄ production were investigated using a 3 h incubation. None affected P₄ production in human term trophoblasts during short term incubation (Fig. 21).

3.3.2 Effects of melittin and 25-OH-cholesterol on P₄ production (Fig. 22):

The universal steroid hormone precursor, cholesterol, was tested for progestin production in human term trophoblasts during short term incubation. 25-OH-cholesterol (10^{-5}M), a water-soluble cholesterol, was not stimulatory to the P₄ production in this study. Melittin ($1.5 \times 10^{-6}\text{M}$) + 25-OH-cholesterol also failed to affect P₄ production during short term incubation of human term trophoblasts.

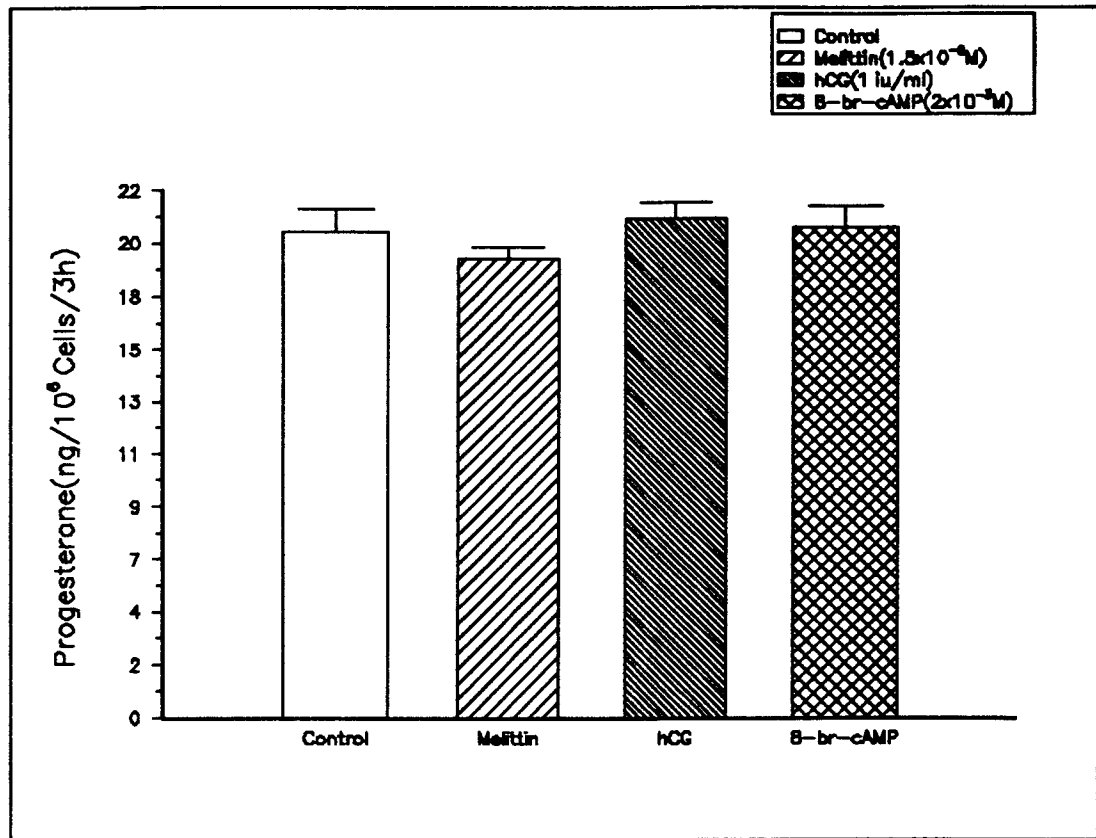


Fig. 21 Effects of melittin, hCG and cAMP on P₄ production in human term trophoblasts.

Human CG, AA and cAMP are known to enhance intracellular P₄ synthesis in the ovary. This study examined the effects of hCG, melittin and cAMP on P₄ production by human term trophoblasts. Human term trophoblasts were exposed to melittin (1.5 x 10⁻⁶M), hCG (1 IU/ml) and cAMP (2 x 10⁻³M) during 3 h incubations and P₄ production was examined. There were no differences between control and treatment values observed.

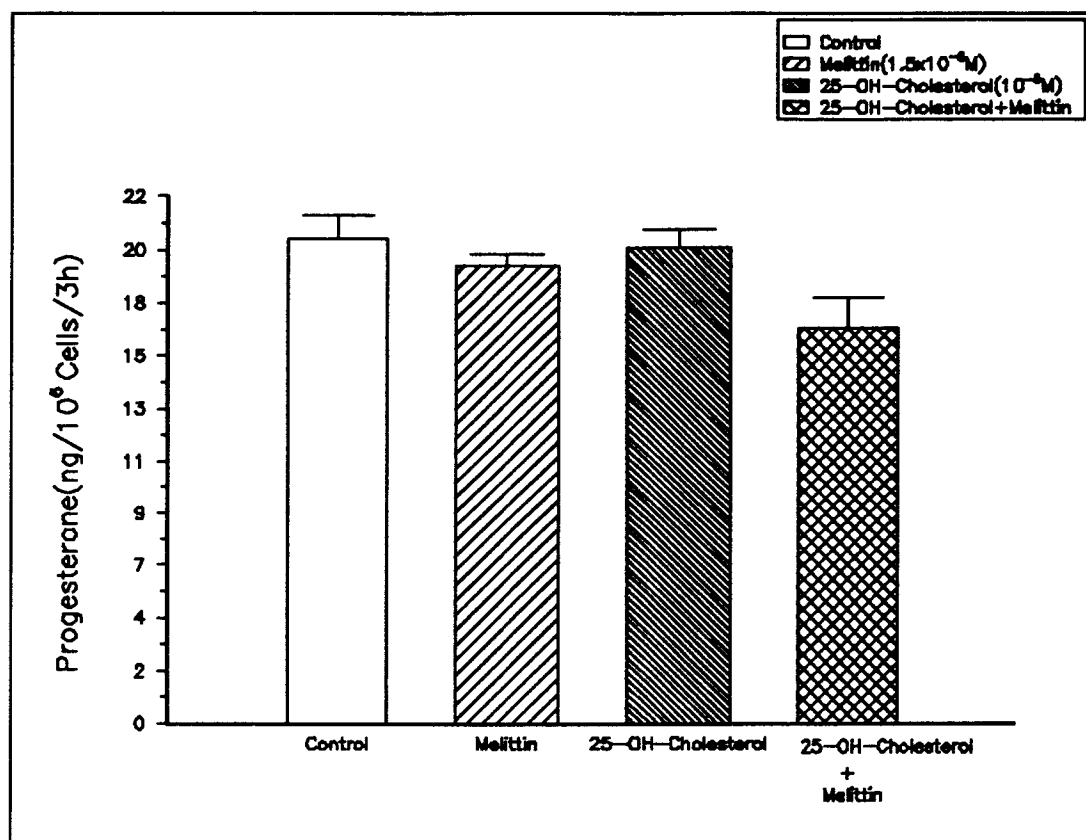


Fig. 22 Effects of melittin and 25-OH-cholesterol on P₄ production.

The steroid precursor, 25-OH-cholesterol, and melittin were administered to human term trophoblasts to measure P-450_{sec} function during the third trimester of pregnancy. The effects of melittin (1.5 x 10⁻⁶M) and 25-OH-cholesterol (10⁻⁵M) on P₄ production by human term trophoblasts was examined using a 3 h incubation. There were no differences between control and treatment values observed.

4.0 DISCUSSION

4.1 Effects of arachidonic acid on aromatase activity of human term trophoblasts:

The trophoblast, through complex endocrine mechanisms, plays an essential role in the initiation and maintenance of pregnancy. However, the mechanisms regulating hormonogenesis within this tissue appear to remain poorly understood. The role of intracellular regulators, including second messengers, enzyme function and the molecular events resulting in synthesis of sex steroids and peptide hormones require further elucidation for an improved understanding of the maintenance of pregnancy.

Results of this study suggested that melittin, an activator of PLA₂, inhibited aromatase activity in human term trophoblast cells *in vitro*. The effect of melittin was dose-dependent and could not be blocked by the concomitant presence of a cyclooxygenase inhibitor, indomethacin, or a lipoxygenase inhibitor, NDGA. Indeed, the presence of high concentrations of indomethacin or NDGA alone had a suppressive effect on aromatase activity. This may have resulted from an accumulation of intracellular AA due to the enzymatic blockade of AA metabolism. Treatment of trophoblast cells with exogenous AA also reduced aromatase activity. Again, the inhibitory effect of exogenous AA was not reversible by the concomitant

presence of indomethacin or NDGA. Thus, inhibition of aromatase activity by melittin could result from a direct action of intracellular unesterified AA on aromatase, rather than via conversion of AA to its metabolites of the cyclooxygenase or lipoxygenase pathways. Preliminary evidence has shown that neither $\text{PGF}_{2\alpha}$ nor LTB_4 could mimic the inhibitory action of melittin or AA on aromatase activity in trophoblast cells.

Melittin activates PLA_2 , a membrane associated, calcium-dependent enzyme which liberates AA from membrane phospholipids. There is increasing evidence that PLA_2 activation and AA liberation could be an important link in hormonogenesis in reproduction. As an example, it has been reported that GnRH-stimulated gonadotropin release from anterior pituitary cells is closely associated with the dynamic action of AA and PKC (Chang *et al.*, 1987). A similar situation may be found to occur in the placenta. One or more of the lipoxygenase metabolites of AA might be a component of a cascade of reactions initiated by interleukin-1. The cascade ultimately results in gonadotropin release (Spangelo *et al.*, 1991). AA itself may be an intracellular regulator of prolactin release from GH_3 cells (Kolesnick *et al.*, 1984). It has been demonstrated that AA stimulates P_4 production in rat ovarian cells (Wang *et al.*, 1988 & 1989). It has been reported that both melittin and AA stimulated hPL release but not hCG release from human trophoblast (Handwerger *et al.*, 1981; Zeitler *et al.*, 1983). There is preliminary evidence that AA stimulates aromatase activity in porcine granulosa cells (Nickerson *et al.*, 1990; Ledwitz-Rigby *et al.*, 1992). Together with the observation above, the present

finding of an inhibitory action of AA in human trophoblast cells suggests that effects of melittin or AA on aromatase could depend upon species and/or cell type under investigation.

The mechanism by which activation of PLA₂ suppresses aromatase activity in human trophoblast remains unknown. The present data suggest that AA, itself, rather than the cyclooxygenase or lipoxygenase metabolic products may mediate this action. The lack of effect of PGF_{2α} or LTB₄ supports this notion of a direct action of AA. Interestingly, treatment of the trophoblast cells with hypoxanthine and xanthine oxidase also suppressed aromatase activity. The combination of hypoxanthine and xanthine oxidase is known to generate intracellular hyperoxide anions as well as increased intracellular AA concentrations (Ikebuchi *et al.*, 1991). Although a possible direct effect of hyperoxide anions on aromatase activity cannot be excluded, these data are consistent with an inhibitory action of AA.

The majority of the cells used in this study are presumably cytotrophoblasts. Filtration to remove cellular clumps excluded syncytiotrophoblasts greater than 48 μm in diameter. The present data on aromatase activity are in agreement with those of Kliman *et al.*, (1986) and Nestler (1987), showing that freshly isolated cytotrophoblasts aromatized androgens to estrogens. However, recent immunohistochemical data have suggested that the cytochrome P-450 aromatase is localized in the syncytiotrophoblasts of first and second trimester placentae (Inkster *et al.*, 1989). It is difficult to explain the apparent discrepancy between these immunohistochemical and biochemical data. Nevertheless, the possibility exists

that there might be different forms of aromatase that are recognized by the antibodies in the immunohistochemical studies (Harada 1988).

The present results suggest that activation of PLA₂ and AA may have an inhibitory effect on aromatase activity in human trophoblast cells. Thus, in addition to cAMP (Bellino *et al.*, 1978; Hochberg *et al.*, 1982; Rodway *et al.*, 1990), AA could be another intracellular regulator of aromatase activity in human trophoblasts. The inhibition caused by melittin could result from a direct action of intracellular unesterified AA rather than via its conversion to metabolites of the cyclooxygenase or lipoxygenase pathways. The possible involvement of epoxygenated products of AA metabolism remains unknown.

Further, the physiological role of AA in the regulation of placental aromatase activity remains to be determined. Modulators of human placental hormonogenesis, such as IGF-I and insulin (Nestler *et al.*, 1987), have been reported to stimulate phosphatidylinositol turnover and release AA (Michell, 1975). IGF-1, insulin and IGF-II have been reported to decrease aromatase activity in human trophoblast (Hochberg *et al.*, 1983; Nestler, 1987 & 1990). The role of AA in the action of IGF-1, insulin or other regulators of placental hormonogenesis warrants further investigation. Interestingly, melittin suppressed aromatase activity during short term incubation but was ineffective on cells from 24 h and 48 h incubations.

4.2 Effects of cAMP on aromatase activity in human term trophoblasts:

Cyclic AMP has been shown to be a second messenger in the release of many hormones. Acute release (10 min) of hPL by cAMP was reported (Harman *et al.*, 1987). FSH and LH act via cAMP to exert their steroidogenic actions in the ovary. However, the steroidogenic effects of cAMP on several other tissues (like placenta) are still controversial. Data obtained by RIA and enzyme assay in this study found that cAMP had no effect on estrogen production in human trophoblasts during short term (3 h) incubation (Fig. 16). The results suggested that cAMP may not have been able to exert its steroidogenic action on human term trophoblasts rapidly enough to be detected during only a 3 h incubation. Alternately, aromatase activity of freshly obtained term trophoblasts was at or near maximal. The possibility of the latter explanation is less likely. It had been reported that aromatase specific activity of trophoblast cells increased 10- to 15-fold 24 h after plating (Lobo *et al.*, 1989). Cyclic AMP increased cytochrome P-450 mRNA within 24 h of the initiation of culture (Ringler *et al.*, 1989).

After 24 h and 48 h incubation with cAMP in this experiment, the aromatase activity of trophoblast cells was significantly increased ($P < 0.01$). Moreover, cells from 48 h incubation produced approximately 3 times more E_2 than cells from 24 h incubation (11 ng/ 10^6 cells/ml *vs* 3 ng/ 10^6 cells/ml). The results suggested that the first 24 h incubation with cAMP primed the trophoblast cells to produce more E_2 in the following 24 h. The mechanism behind this event may have involved early cAMP activation of the mRNA and protein synthetic machinery of the trophoblast. The results were in agreement with the report that cAMP-induced aromatase

specific activity was approximately 1.8-fold higher at 48 h than at 24 h of incubation (Lobo *et al.*, 1989).

Previous studies have demonstrated that 8-br-cAMP decreased basal production of estradiol in cultured term trophoblast cells (Benoit *et al.*, 1988; Rodway *et al.*, 1988). This discrepancy may be due to the different methods of cell preparation use in the previous *versus* the present study. Placental cells were physically dissociated (see "2.1.1 preparation of human term trophoblasts" for details) in this study but were enzymatically dissociated in the previous studies. It has been suggested that enzymatic dissociation has the inherent advantage of stripping trophoblast tissues while releasing few fibroblasts from the villous core (Truman *et al.*, 1989). Even small numbers of fibroblasts may be subject to overgrowth in placental culture (Truman *et al.*, 1989; Veger *et al.*, 1989). However, trypsin and DAase used in the enzymatic dissociation of cells is suspected to extract membrane bound receptor proteins from the cell surface, making the cells unresponsive to physiological ligands for the first several days in culture. The advantages of physical dissociation for freshly harvested tissues are no enzymatic interference of cell membrane receptors. The viabilities achieved are consistently between 95 and 100%. The disadvantage is that some large syncytiotrophoblast cells (>45 μm) may be excluded. It has been reported, for example, that aromatase regulation by cAMP is critically dependent upon the methods used to isolate and culture the trophoblast cells (Lobo *et al.*, 1989).

One other thing should be considered when comparing the short term (3 h)

incubation to the long term (24 h or 48 h) incubation. The majority of freshly prepared cells are trophoblasts. After 48 h of preculture, the cells are transformed to syncytiotrophoblasts (Kliman *et al.*, 1986). The different cell populations may respond differently to cAMP.

It was concluded that the effects of cAMP on aromatase activity of human trophoblast cells were not significant during short term incubation (3h). The stimulatory effects of cAMP on aromatase activity during 24 h and 48 h incubation could be due to the enhanced activity of mRNA and protein synthetic machinery of the trophoblast cells.

4.3 Short-term modulation of human placental progesterone production *in vitro*:

Cyclic AMP, hCG and melittin:

It is documented that acute stimulation of steroidogenesis in the gonads is LH-dependent and mediated by the activation of cAMP-dependent protein kinase A (PKA) (Dufau *et al.*, 1982). Cyclic AMP induced P_4 synthesis has been reported in term trophoblast cells (Tonkowicz *et al.*, 1985; Feinman *et al.*, 1986; Rodway *et al.*, 1988). Most of the experiments above involved incubation periods that were relatively long-term (24 h or 48 h). However, data presented in this study demonstrated that P_4 production is not stimulated by 8-br-cAMP, hCG or melittin during short term (3 h) incubation of human term trophoblasts (Fig. 21). Lack of

short term (3 h) modulation of *in vitro* ovine placental P_4 secretion by LH, 8-br-cAMP and 25-OH-cholesterol has also been observed (De La Llosa-hermier *et al.*, 1988). These results suggest that hCG, cAMP and possibly PLA_2 activation and increased free AA substrate are not implicated in the short-term regulation of P_4 synthesis by human term trophoblasts.

25-OH-cholesterol:

Cholesterol is the most important substrate for the production of P_4 by human term trophoblasts. The side chain cleavage enzymes are rate-limiting in the conversion of cholesterol to P_4 (Toaff *et al.*, 1982; Bagavandoss *et al.*, 1987). 25-OH-cholesterol is a water-soluble steroid which readily enters cells and is metabolized to pregnenolone in mitochondria (Toaff *et al.*, 1982; Lino *et al.*, 1985). 25-OH-cholesterol (2 $\mu\text{g/ml}$) was not observed to stimulate P_4 production during short term incubation (3 h) in human term trophoblasts in this study (Fig. 22). It was reported that LDL cholesterol had no effect on basal output of 17β -estradiol, P_4 or hCG (Haning, *et al.*, 1982). It was reported that 25-OH-cholesterol (2.5 $\mu\text{g}=10^{-5}\text{M}$) did not increase P_4 production over a 4 h incubation period of human chorion (Tonkowicz *et al.*, 1985). However, 25-OH-cholesterol (20 $\mu\text{g/ml}$) increased P_4 production in term trophoblast cells during a 3 h incubation (Kliman *et al.*, 1986). The concentration of 25-OH-cholesterol (20 $\mu\text{g/ml}=10^{-4}\text{M}$) is 10x higher than in this experiment (2 $\mu\text{g/ml}=10^{-5}\text{M}$). Increase incubation period (5 h) and/or dosage of 25-OH-cholesterol also increased P_4 production (Tonkowicz *et al.* 1985).

25-OH-cholesterol (10^{-5}M) did not affect P_4 production by trophoblast cells during a 3 h incubation in this study.

Melittin:

This study used melittin to examine the involvement of PLA_2 activation and cytochrome P450scc enzyme in P_4 production by trophoblast cells. Melittin had suppressive effects on aromatase activity (been discussed) above during short term incubation. In the process of P_4 production, several steps in the cholesterol SCC reaction, such as uptake of cholesterol by mitochondria, the intramitochondrial access of cholesterol to the SCC enzyme complexes, and the modulation of the mitochondrial cytochrome P-450 levels, are suspected to be under hormonal control (Leaven *et al.*, 1981). It was found that melittin had no effect on P_4 production by human term trophoblast cells during a 3 h incubation in this experiment. Melittin did increase P_4 production by rat granulosa cells during a 5 h culture period (Wang *et al.*, 1987). Melittin + 25-OH-cholesterol also had no effect on P_4 production in this study. This suggested that AA had no regulatory role in this regard. As mentioned above, full term placenta may make optimal use of its resources, performing at or near maximal capacity. Consequently, AA stimulated P_4 production in other tissues but was unable to further increase the near maximal rate of P_4 production by human term trophoblasts.

4.4 Effects of indomethacin and melittin on porcine granulosa cells

Contrasting the effects on human trophoblast cells, indomethacin or melittin stimulated aromatase activity of porcine granulosa cells. The stimulatory effect of indomethacin on aromatase activity seems more effective than melittin in porcine granulosa cells. This suggests that AA generated by melittin was rapidly hydrolyzed to further eicosanoid metabolite. Whereas AA generated by indomethacin with blockade of the cyclooxygenase pathway can maintain higher concentration to exert its stimulatory effect on aromatase activity. Indomethacin enhanced aromatase activity in a dose-dependent manner over a range of 10^{-6} M to 10^{-4} M. Indomethacin synergized with melittin to further stimulate aromatase activity. The synergistic effect result from the accumulation of AA via different pathways. One is from increasing AA production, yielded by the stimulatory action of melittin on PLA₂ and PLA₂ enhanced membrane phospholipid hydrolysis and subsequently release of AA. The other is from the blockade of AA metabolism by indomethacin. It was shown that PLA₂ + indomethacin had the same synergistic effect as melittin + indomethacin (Ledwitz-Rigby *et al.*, 1992). Granulosa cells from different size of follicles (large, medium, and small) had varying levels of aromatase activity responding to indomethacin treatment (Ledwitz-Rigby, *et al.*, 1992). Melittin and indomethacin stimulate aromatase activity of porcine granulosa cells by blocking the cyclooxygenase and lipoxygenase pathways of the AA cascade and then increase intracellular AA concentration. The regulatory action of AA on

aromatase is still not clearly understood. However, increased intracellular Ca^{2+} , PKC activation, and oxidation of AA via lipoxygenase (see 4.1) are all possible routes through which AA may mediate P_4 production by placental trophoblast cells. Observed differences in the effects of indomethacin and melittin on aromatase activity between porcine granulosa cells and human term trophoblast cells could be due to variation in cell types and/or species examined.

CONCLUSION

AA inhibition of aromatase activity of human term trophoblasts during short term (3 h) incubation is a direct action of AA rather than of its eicosanoid metabolites. AA had no attenuated effect on aromatase activity of human term trophoblasts after 24 h and 48 h incubation.

Human CG or 8-br-cAMP did not show any effect on aromatase activity during short term (3 h) incubation with both RIA and enzyme assay method. However, 8-br-cAMP was significantly stimulatory of aromatase activity in human trophoblasts after 24 h and 48 h incubation. Moreover, the estradiol production during 48 h incubations was 3 times more than that of 24 h incubation (11 ng/10⁶ cells/ml vs 3 ng/10⁶ cells/ml).

Human CG or 8-br-cAMP do not mediate short term regulation of progesterone production in human term trophoblasts. 25-OH-cholesterol had no stimulatory action on progesterone production during short term (3 h) incubation, suggesting that the rate of progesterone synthesis in human term trophoblasts is maximal. Melittin had no effect on progesterone production of human term trophoblasts during short term incubation period. It can be concluded that regulatory role is not implicated for arachidonic acid in the progesterone production of human term trophoblasts in short term (3 h) incubation.

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