PHD

An investigation of platelet-activating factor metabolism during normal and pre-eclamptic pregnancies

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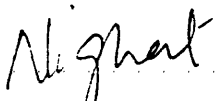
Award date:
1993

Awarding institution:
University of Bath

AN INVESTIGATION OF PLATELET-ACTIVATING FACTOR METABOLISM DURING NORMAL AND PRE-ECLAMPTIC PREGNANCIES.

Submitted by Nighat Murad Khan
for the degree of Ph.D
of the University of
Bath
1992



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This work is dedicated to Myra and Murad. Myra, for being a wonderful daughter, who trod along her milestones delightfully and Murad for his patience.

ACKNOWLEDGEMENTS:

First of all, I would like to thank Dr K I Williams for his supervision. I am indebted to Prof John Westwick, who inspite of his busy schedule found time for extremely useful discussions regarding my results and his encouragements during my stay in Bath.
I wish to thank Dr C. Page and C.O'Neill for provision of references which I could never have dug out.
I also would like to thank Dr D. Bamford for being extremely helpful in recovery of precious amniotic fluid and other samples.

Finally, I wish to thank Prof Amin Suria for his encouragement and support for my Ph.D and for the critical review of my write-up and Dr S.A.Saeed for his supervision.

This research was supported by a Faculty Development Award from the Aga Khan Foundation.

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ABBREVIATIONS:-

ALKYL-ACYL-GPC: ACYL-ALKYL-GLYCEROPHOSPHOCHOLINE.
ADP: ADENOSINE DIPHOSPHATE.
AA: ARACHIDONIC ACID.
BPB: BROMOPHENACYL BROMIDE.
CPZ: CHLORPROMAZINE
DFP: DIISOFLUOROPHOSPHATE
INDO: INDOMETHACIN.
NEM: N-ETHYLMALEIMIDE.
NDGA: NORDIHYDROGUAIARETIC ACID.
PAF: PLATELET-ACTIVATING FACTOR.
PMA: PHORBOL 12-MYRISTATE 13-ACETATE.
PDB: PHORBOL 12,13 DIBUTYRATE.
PMSF: PHENYLMETHYLSULPHONYLFLUORIDE.
PPZ: PIPERAZINE.
TMX: TAMOXIFEN.

ABSTRACT:

Changes in PAF acetylhydrolase activity with respect to gestational age were estimated in normotensive and pre-eclamptic maternal plasma, amniotic fluid and intrauterine tissues. The enzyme activity was 52 nmol/min/ml, 53 nmol/min/ml, 33 nmol/min/ml, 32nmol/min/ml, 49nmol/min/ml in nonpregnant females, pregnant females in their first trimester, 2nd trimester, 3rd trimester, during full term normal spontaneous labour and 48 hrs postpartum respectively.

There was no significant gestational age-related changes in the enzyme activity in amniotic fluid obtained during 16-17 weeks, at term, but not in labour, and during full term normal labour.

PAF acetylhydrolase activity was highest in the chorion > amnion > decidua vera > placenta. Amongst the cellular fractions the activity was highest in, cytosol > mitochodria > microsomes.

The amnion cytosol PAF acetylhydrolase activity was characterized physico-chemically. The enzyme had optimal pH of 7-8 and the activity was destroyed by acidic pH. The enzyme was inhibited by 20 %TCA, Protease (DFP, PMSF. PAF acetylhydrolase is a non-dialyzable and molecular size appeared to be < 50,000.

Maternal steroid hormones modulated the enzyme activity in amnion cells in culture. After 48 hrs of incubation, 17-ß-oestradiol inhibited the enzyme in a concentration dependent manner ($p<0.01$), whereas progesterone stimulated the enzyme activity

($p<0.05$). Treatment of amnion cells with oxytocin (10mU/ml), caused a significant loss in enzyme activity ($p<0.001$). Prostaglandins E_2 and $F_{2\alpha}$ stimulated the PAF acetylhydrolase activity ($p<0.05$). Modulation of PAF acetylhydrolase activity by hormones, prostanoids and oxytocin, suggests that these factors interact in paracrine manner during pregnancy and parturition.

PAF, is a hypotensive lipid. Its role in pregnancy-induced hypertension was investigated. PAF acetylhydrolase activity in maternal plasma, obtained at various stages of gestation and during

labour, however was not significicantly different from that in normotensives. The activity was significantly higher in cytosols of amnion and chorion, obtained from pre-eclamptics, as compared to that obtained from the normotensive pregnancies, suggesting the local derangements in PAF metabolism, rather than maternal circulation, during pre-eclampsia, which could lead to compromised blood flow to growing fetus and caused intrauterine growth retardation.

Exogenous ^{14}C PAF(0.18nM) was taken up and metabolized by amnion cells in culture. WEB-2086 inhibited the uptake significantly ($p<0.01$) in a dose-dependent manner. Uptake was also inhibited by Indomethacin (100nM $p<0.05$), Nordihydroguaiaretic acid (100 nM, $p<0.02$). PAF uptake was also regulated by changes in extracellular calcium ($p<0.01$), Na+/K+ ATPase inhibition ($p<0.05$). Merthiolate and N-ethylmaleimide (100uM), also caused a significant reduction in total uptake of PAF by amnion cells ($p<0.01$).

The uptake was also regulated by maternal steroid hormones and dexamethasone, the former increasing it ($p<0.01$) and the latter inhibiting it ($p<0.05$).

Phorbol 12-13 dibutyrate and Phorbol 12 myristate 13-acetate-treatment caused a time- and dose-dependent decrease in PAF uptake ($p<0.001$).

These results suggest that amnion cells take up exogenous PAF actively, via a receptor-mediated mechanism.

SUMMARY:

1. PAF plays an important role in events related to foetal lung maturation, as seen by reduction in PAF acetylhydrolase enzyme activity in maternal plasma.

2. PAF acetylhydrolase in human amnion cytosol resembles the plasma acetylhydrolase in physico-chemical characteristics and is modulated by maternal hormones, prostanoids and oxytocin.

3. PAF released from foetal tissues is actively taken up by the amnion cells. The uptake being partly receptor mediated and in part by other membrane associated mechanism (s).

4. The receptor-mediated PAF uptake and hence its actions are down regulated by the phorbol esters in human amnion cells.

5. Maternal sex hormones play a crucial role in PAF metabolism. Oestradiol, by inhibition of acetylhydrolase and acylase enzymes, potentiate effects of PAF in amnion.

6. Pre-eclampsia is a multi-system disorder. PAF metabolism disturbance alone does not play a role in its pathogenesis.

BACKGROUND:

Recently, there has been a surge of literature documenting, the role of platelet-activating factor (PAF) in various pathological as well as physiological processes (Koltai et al, 1991). PAF is now reported to have numerous effects in various events related to reproductive biology (Johnston, 1989; O'Neill, 1989).

The following paragraphs will give an account of basic biochemistry and basic concepts regarding PAF metabolism, an outline of effects of PAF at various cells, organs and followed by discussion on the role of platelet-activating factor in reproduction.

INTRODUCTION AND BIOCHEMISTRY:

Platelet-activating factor, is an ether phospholipid associated inflammation, vasodilation and is produced by many cells (Braquet et al, 1987). Its chemical structure has been established as 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (Blank et al, 1979).

PAF BIOSYNTHESIS.

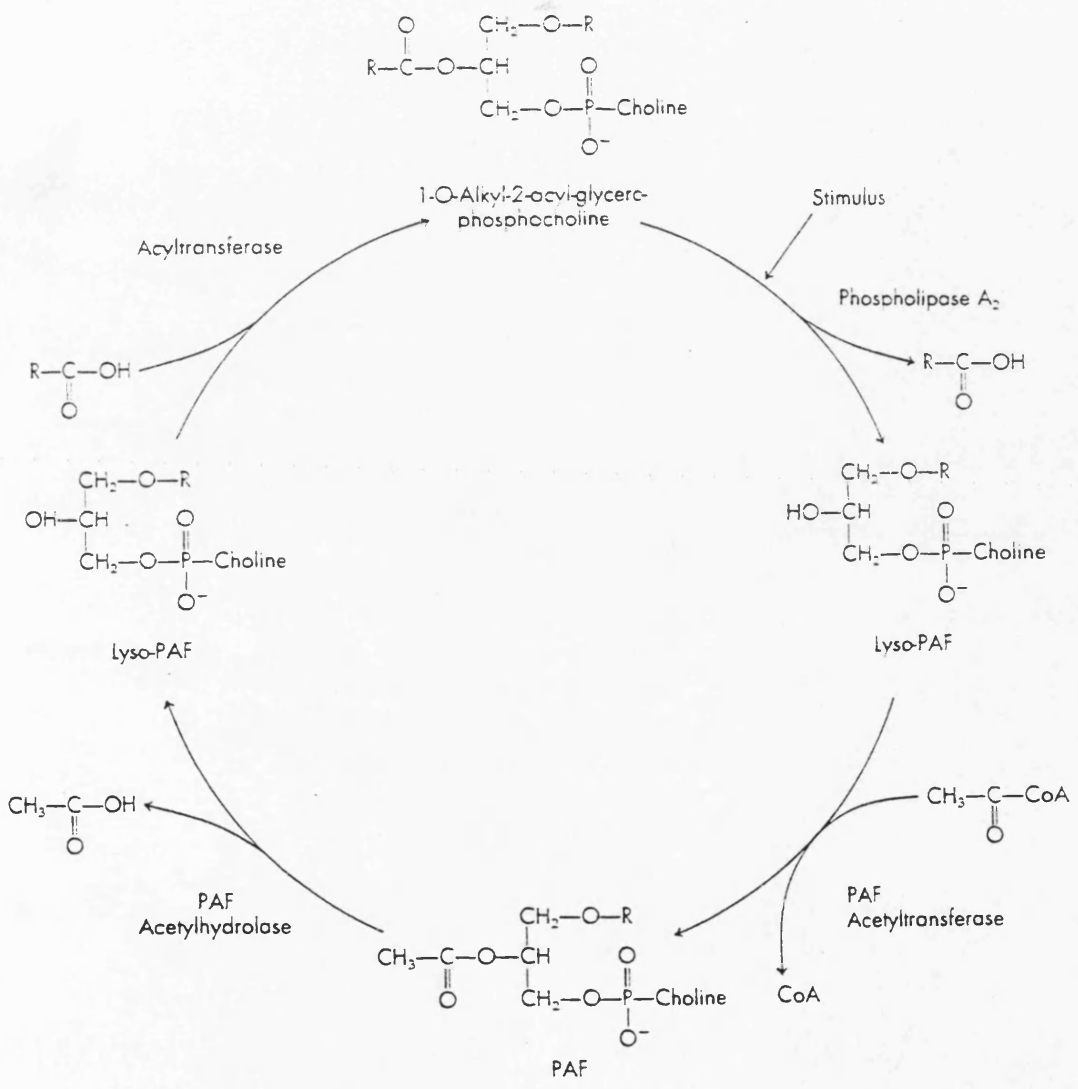
PAF is synthesized from membrane phospholipids, specifically the ether-linked phospholipids which are enriched with arachidonic acid (Snyder, 1990). Two distinct pathways are involved and are active under different circumstances. The first, called the remodelling pathway (Snyder et al 1986), involves the hydrolysis of membranes phospholipids by a phospholipase A_2, releasing lyso-PAF, which is acetylated to PAF by the rate limiting coenzyme A-acetyltransferase (Fig:i). This pathway is stimulated by the agents like calcium ionophore, thrombin, bradykinin and angiotensin, as well as inflammatory cells. The common outcome of different stimuli is an increase in intracellular calcium (Vandongen, 1991). Phospholipase A_2 action is also accompanied by release of arachidonic acid, a precursor of cyclo-oxgenase as well as lipoxygenase products (Snyder, 1990).

Fig: (i).

Biosynthetic pathway of platelet-activating factor (PAF), showing inter-relationship with arachidonic acid pathway. GPC, glycerophosphocholine.

(From: Vandongen, R: (1991). Platelet-activating factor and the circulation. J. Hypertension. 9: 771).

Platelet-Activating Factor



The second pathway, termed the de novo pathway (Snyder, 1986), is independent of phospholipase A_2 and PAF is generated from a precursor, 1-O-alkyl-2-acetyl-sn-glycerol via a specific choline phosphotransferase. This pathway is believed to be responsible for the production of PAF under basal physiological conditions and may be the primary source of renal PAF (Woodard, 1987).

INACTIVATION OF PAF.

The inactivation of PAF occurs in two steps (Chilton et al, 1983). Initially, the acetyl group at 2(R) position of PAF is removed by the highly active enzyme PAF acetyl hydrolase (E.C 3.1.1.3.4), to form lyso-PAF. This enzyme is present both in intracellular as well as extracellular compartments. Its intracellular form is found in the cytosolic fraction of various cells and tissues (Blank et al., 1981), whereas the extracellularform is recoverable from plasma (Blank et al., 1983; Farr et al.,1983).The properties of the plasma enzyme are similar to those of the cytosolic enzyme except that the former is resistant to the actions of proteases (Blank et al., 1983) and is sensitive to serine hydrolase inhibitors, PMSF and DFP. It is reported that the activity of the plasma enzyme is closely associated with the LDL fraction of plasma. Stafforini et al (1987), have purified, to near homogeneity the plasma enzyme from the LDL fraction and have suggested that the biologically active acetyl hydrolase is associated with the LDL fraction, although significant activity is also present in the HDL fraction. The enzyme activity could be transferred from HDL to LDL fractions and vice versa. It was proposed that intracellular acetyl hydrolase may undergo modification, such as glycosylation, to facilitate its secretion into the vascular compartment (Snyder et al., 1983). In contrast to PLA2, acetylhydrolase cleaves only the short chain fatty acids esterified at the 2(R) position of phospholipids and is calcium independent.There is positive correlation between the cells sensitivity to PAF effects and their capacity to hydrolyse PAF.

Stafforini et al (1987) studied the ability of PAF acetyl hydrolase to interact with 1-acyl rather than with 1-alkyl phospholipids by testing the effect of 1-acyl-2-acetyl-glycerophosphocholine on activity of the purified enzyme. The compound proved to be a competitive inhibitor of the enzyme

with a Ki of 27.5uM. Thus, the recognition of the substrate by the enzyme is not dependent on the type of bond, i.e., ester or ether, at the sn-1 position. The enzyme did not demonstrate a preference for the L-isomer of PAF since, 3-O-alkyl-2-acetyl-sn- glycero- phosphocholine competitively inhibited the hydrolysis of PAF with a Ki of 17uM. The Ki is likely to be the Km for the hydrolysis of the stereoisomer as it actually acted as a substrate for the enzyme. In contrast to the weak specificity at the sn-1 position, the acetylhydrolase did show a strong specificity for the residue at the sn-2 position. PAF acetyl hydrolase did not recognize phospholipids with a long chain fatty acid esterified at the sn-2 position since egg phosphatidylcholine and other 1,2 diacylglycerophospholipids (pentanoyl, hexanoyl, and octanoyl) did not significantly inhibit PAF hydrolysis. These results demonstrated that the acetyl hydrolase is distinct from other previously described phospholipase A_2 enzymes, which utilize long chain diacylphospholipids as substrates and show that there is specificity for the length of the acyl chain esterifying the sn-2 position. To define this specificity, Stafforini and her coworkers (1987), synthesized, 1-O-^{3}[H] alkyl-2-acyl-glycerophospholipids containing fatty acids of various chain lengths and tested as substrates of PAF acetyl hydrolase. The relative velocities of hydrolysis of the homolog was C2 >> C3 = C4 > C6, demonstrating that the purified enzyme has a pronounced preference for an acetyl residue at the sn-2 position. Moreover, the enzyme has an absolute requirement for an ester linkage at the sn-2 position of the glycerol backbone, since 1-O-alkyl-2-O-ethyl-sn-glycero phosphocholine, at concentrations up to 40uM, did not inhibit the hydrolysis of its ester analog, PAF. Stafforini et al (1987), examined the ability of the enzyme to hydrolyse compounds containing acetyl groups, but where the PAF glycerol backbone was either absent or highly modified. acetylsalicylic acid (up to 200 uM) inhibited the hydrolysis of 20uM PAF by only 10%, phorbol 12-myristate 13-acetate at the concentrations of 200uM was without effect as an inhibitor, and up to 200uM 4-methylumbelliferyl acetate (a substrate for esterase D, or sialic acid-specific O-acetylesterase (Varki et al, 1986) also was not inhibitory. In addition, [^{3}H]acetylcholine was not a substrate for the purified enzyme. These results indicate that the acetyl hydrolase is not a non-specific acetyl esterase. Whatever the route of formation of PAF, lyso-PAF is cytotoxic having lytic and detergent properties (Weltzein, 1986), its elimination occurs by an acylation system which introduces a long chain fatty

acid into 2(R) position of lyso-PAF, the resulting alkyl-acyl GPC then becomes an integral part of the cell membrane. Exogenous lyso-PAF is principally converted to alkyl-acyl GPC (Chilton et al,1983). Arachidonic acid is one of the major fatty acids incorporated into lyso PAF. The re-acylation reaction is inhibited by calcium ions, leading to accumulation of lyso-PAF (Touqui et al, 1987). The other possible fates of lyso-PAF include, oxidative cleavage of the alkyl bond either before or after the removal of polar head (Lee et al, 1981), removal of the base and phosphate group by lysophospholipase D and phosphohydrolase (Wykle et al, 1980).

SYSTEMIC EFFECTS OF PAF:

PAF is the most active, low molecular weight platelet-activating agent, exhibiting a wide range of activities of which some e.g., bronchoconstriction, depend on the presence of platelets, whereas other effects, including hypotensive effect, occur independently (Snyder, 1985).

The pharmacological effects of PAF is enlisted below:

a). PAF and inflammation (Ney and Schror, 1989).

b). PAF and anaphylaxis (Braquet at al, 1987).

c). PAF and hypotension (Lagente et al, 1988; Vandongen, 1991).

d). PAF and thrombosis, platelet aggregation (Braquet et al, 1987).

e). PAF and bronchoconstriction and asthma (Page et al, 1984).

f) PAF and gastrointestinal injury (Dembinski et al, 1989).

g) PAF and myocardial negative inotropic effects (Robertson et al, 1988).

The list of PAF effects in various pathophysiological phenomena is by no means complete. There are number of recent reviews (Koltai et al, 1991, Snyder, 1990, Braquet et al, 1987) describing the role played by PAF at various sites in body.

BIOCHEMICAL MODE OF ACTION OF PAF.

There is an abundance evidence that PAF elicits its functions through multiple signalling pathways (Shukla et al,1992). PAF stimulates rapid phospholipid turn over via phospholipase C, D, A_2, which give rise to inositol trisphosphate (IP_3) plusdiglyceride, phosphatidic acid and prostaglandins, respectively (Shukla et al, 1991). In given system, one or more of these phospholipase-mediated pathways may operate. Furthermore, PAF causes Ca^{2+} mobilization (both intracellular Ca^{2+} release and influx). Intracellular Ca^{2+} release is mediated by IP_3, whereas the influx could be via a receptor-mediated channel (Avdonin et al, 1991) or by some other unknown mechanism. In a number of systems, PAF activates phosphorylation of protein kinase C (PKC) (O'Flaherty, et al, 1990), tyrosine kinase (Dhar et al, 1990) and Ca^{2+}-calmodulin-dependent kinase.In cells pre-treated with PAF, any subsequent addition of PAF exhibited a homologous desensitization of PAF signalling responses (phosphoinositide turnover, Ca^{2+} mobilization, protein kinase activation) (Hwang, 1990; Mazer et al., 1991; O'Flaherty et al., 1990). In this regard, it is of interest to note that internalization of PAF binding sites and translocation of PKC from cytosol to membranes (Mazer et al, 1991), may be relevant as its regulatory mechanism (s). However, the mechanism for the desensitization remains to be elucidated.

PLATELET-ACTIVATING FACTOR AND REPRODUCTION:

There is an array of literature regarding the effects of PAF on various processes related to reproductive biology. Hence the role of PAF in reproduction is discussed under various headings.

PAF AND NON-PREGNANT FUNCTIONS:

Recently high affinity receptors have been demonstrated for platelet-activating factor in endometrium (Kudolo & Harper, 1990). Lyso-PAF has been shown to displace PAF from endometrium cells (Harper et al, 1989).

PAF is also recently reported to modify prostaglandins release from primary cultures of endometrial cells of human origin (Salamansen and Findlay, 1990) as well as of ovine endometrium (Salamansen et al, 1991).

Similar interactions have been reported between PAF and prostaglandins in hormone-treated human luteal phase endometrium in vitro. PAF levels in the uterus were elevated by progesterone and PGE2 (Alecozay et al, 1990; 1991; Harper et al, 1989).

Nakayama et al (1991), studied the effects of 17-ß-oestradiol on PAF and prostaglandin $F_{2\alpha}$ levels in rat uterus. Oophorectomy resulted in the decrease in the PAF levels to 1/3rd of that in natural estrus. The levels were recovered by subcutaneous administration of 17-ß-oestradiol. These workers observed that levels of uterine phospholipids which are rich in arachidonic acid were decreased by oestradiol treatment. Arachidonate-PC was depleted more than arachidonate-PE. The amount of $PGF_{2\alpha}$ in oophorectomized uterine tissue was 10-times that of PAF, but like PAF, it also increased on oestradiol treatment.These data suggest a correlation between the formation of PAF and $PGF_{2\alpha}$ and oestradiol could be regulating the physiological formation of PAF and PGs in non-pregnant uterus.

PAF AND OVULATORY PROCESSES:

Follicle rupture during ovulation is associated with inflammation-like changes (Harper et al, 1989). Ginkgolide B, a PAF antagonist, inhibited follicle rupture, hormone-stimulated increase of ovarian collagenolysis and vascular permeability in rats whose ovulation was stimulated by human chorionic gonadotrophin (Abisogun et al, 1989). Simultaneous PAF administration reversed this effect. Espey et al (1989) have shown that at 2-hr sampling intervals during an ovulatory process in gonadotrophin-primed Wister rats, the ovaries contained lower PAF levels than normal. The ovarian lipid extract contained PAF inhibitors, that co-migrated with PAF whose levels were also decreased in response to gonadotrophins.Kikukawa et al (1991), have recently compared the ovulation processes with inflammatory reaction. PAF is known to be an important mediator of inflammation and has numerous interactions with cytokines and other agonits (Koltai et al, 1991). Kikikawa et al, (1991) evaluated the potential role of PAF in pregnant mare serum globulin (PMSG) and HCG induced ovulation in super ovulated ICR mice (9-12 weeks old). They observed that CV 3988, a specific PAF-receptor antagonist, blocked ovulation in a dose dependent manner and reduced ovulatory efficiency at a dose > 500ug. The ovulatory efficiency decreased by CV 3988 was reversed by PAF. However the in vitro fertilization rate of follicular oocytes treated with CV 988 was not different from that of untreated ova.

PAF AND SPERMATOZOAL FUNCTIONS:

PAF has been shown to be produced by human (Kuzan et al, 1990; Minhas et al, 1990) as well as rabbit spermatozoa (Kumar et al, 1988). Kuzan et al (1990), have demonstrated that exogenous PAF increased the in vitro fertilization rate of mouse oocytes by mouse epididymal spermatozoa and PAF receptor antagonist WEB-2086 blocked the fertilization of these oocytes. This would suggest existence of PAF receptor in spermatozoa.C-16 PAF has greater potential of causing vasoconstriction and mediation if inflammation that C-18 analogue (Surles et al, 1985). Sanwick et al (1992), have shown that human spermatozoa synthesized C-16 platelet-activating factor but not

C-18 PAF. Angle and her coworkers (1991), recently delineated the physiological role of PAF in the development and function of human gametes and that PAF regulates a wide spectrum of events involved in normal gamete physiology. Incubation of human spermatozoa with PAF resulted in an increase in the acrosome response (7-fold over the controls) and enhanced penetration of hamster oocytes (a two-fold increase in the percent of eggs penetrated and in the number of spermatozoa bound per egg as compared to controls), and an approximately 40% increase in the percent of hyperactivated spermatozoa. However PAF failed to enhance the total percent of motile spermatozoa or improve the overall motion parameters for the entire population of motile cells from either normal donors or subfertile patients.

PAF AND FERTILIZATION AND IMPLANTATION:

Mouse embryos have been shown to produce PAF, similar to authentic PAF (Collier et al., 1988; O'Neill et al., 1989a,b). SRI 63-441, SRI 63-412, WEB-2086 and ginkgolide B reduced PAF release from the embryos challenged by phospholipase A_2. Ryan et al (1989), found that in vitro production of PAF could influence embryonic metabolism, as indicated by increased production of CO_2 from the carbon-1 position of lactate. Exogenous PAF stimulated glucose and lactate metabolism by embryos (Ryan et al, 1990 a). Ryan et al (1990b), observed that supplementation of culture medium with PAF increased the proportion of 2-cell embryos which developed through to expand to blastocyst stage after 72 hr in culture. The specificity of PAF action was confirmed by lack of any such effect by lyso-PAF and SRI-63-441 significantly decreased the amount of CO2 generated (Ryan et al, 1990 b). There was no significant difference in control versus PAF-treated embryos which resorbed, nor there was any effect on fetal weight or crown rump length, however, PAF-treated embryos had slightly heavier placentae. Collier et al (1990), reported that 53% of media samples in which single human embryos were cultured for 24 hrs had PAF levels > than corresponding control media.They assigned it as embryo-derived PAF and corresponding embryos termed as PAF-positive. The medium from the PAF-positive embryos transferred to patients who achieved an ongoing pregnancy had PAF levels significantly higher that media of PAF-positive

embryos transferred to patients who failed to become pregnant.Adno et al (1990), have also shown that mouse embryos produce an embryo-derived PAF (EDPAF) during their developmental stages. Treatment of specific PAF antagonist significantly decreased EDPAF, without affecting circulating platelet concentrations.PAF injected into left uterine horn induced a dose-dependent decidua like reaction in pseudopregnant rats (Acker et al, 1989). Ginkgolide B inhibited the effect of PAF on decidua-like reaction, indicating the specific receptor sites. These receptor sites were characterized in purified endometrium from pregnant rabbits and relative potencies of PAF and its antagonists in displacing [^{3}H]PAF were lyso-PAF> CV 3988 > PAF > U66985 > AO2405 > ginkgolide B> U66982 (Kudolo and Harper, 1989). Kudolo and Harper (1991), described the presence of unique PAF receptor subtypes in the uterus of pregnant rabbits, using specific antagonists and PAF analogues and emphasized the importance of species specificity of receptor binding.However, Milligan and Finn (1990), showed failure of PAF antagonists to inhibit implantation in mice.

SOURCES OF PAF IN AMNIOTIC FLUID:

Billah and his coworkers (1983) reported the presence of PAF in amniotic fluid in women undergoing full term normal labour. This finding was confirmed by Nishihara et al (1984), using GC-MS. The source of this PAF in amniotic fluid is uncertain, but likely tissues sources include the fetal lung, the fetal kidney, and the amniotic membrane. Billah et al (1984), have previously detected PAF in the urine of newborn humans, and they postulated that the fetal kidney may release PAF into the amniotic fluid via fetal urine. The human fetal kidney has considerable acetyltransferase activity and is capable of producing PAF. The activity of a second biosynthetic enzyme, 1-alkyl-2-acetyl-glycerol: cytidine-5'-diphosphate choline-phosphotransferase, has also been found in the adult rat kidney (Snyder, 1985). Hoffman et al (1986), however could not detect any significant changes in PAF acetyltransferase or acetylhydrolase activities in rabbit fetal kidneys. Billah et al (1985), had reported that PAF biosynthesis occurs in human amnion tissue which was stimulated in vitro by the calcium ionophore A23187. However, they failed to demonstrate, the release of PAF in these experiments. They postulated that the amnion is not a likely source of PAF in amniotic fluid. Other workers had also shown that although PAF, is synthesized by neutrophils (Lynch and Henson, 1986) and endothelial cells (Lewis et al, 1988), only a fraction of the total PAF is released, the remainder being bound to the cell. PAF present in amniotic fluid is associated with the lamellar body fraction (35,000 x g pellet) of amniotic fluid (Hoffman et al, 1986). It was therefore suggested that fetal lung could be the possible source of PAF present in amniotic fluid, as a secretory product of type II pneumocytes.

Hoffman et al (1986; Johnstn and Maki, 1989), were able to show that the concentrations of PAF lipid precursors lyso-PAF and alkyl-acyl glycerophosphocholine were sufficient to support synthesis of PAF. They also demonstrated that the activity of PAF acetyltransferase increased as lung maturation progressed and that the concentration of PAF was elevated during this period of development. Furthermore there was a reciprocal relationship between the levels of glycogen and

PAF in the lung explants. In contrast to that in the liver (Shukla et al, 1983), glycogen breakdown in lung tissue should result in increased pyruvate formation since this tissue contains a relatively low activity of glucose-6-phosphate (Sorokin et al, 1959). The increased glycolytic rate could provide the glycerol backbone for phospholipid biosynthesis as well as elevating the levels of acetyl Coenzyme A, which in, turn, is utilized for fatty acid biosynthesis. Ultimately, the glycerol and fatty acid moieties derived from glycogen result in the increased rate of surfactant biosynthesis that occurs during fetal lung development. Increases in acetylcoenzyme A concentration may also serve to further increase PAF biosynthesis, thus creating a cyclic process to perpetuate glycogenolysis. PAF synthesized in the type II pneumocyte cells could then be secreted in association with surfactant.

FATE OF PAF IN AMNIOTIC FLUID:

PAF in amniotic fluid, before the onset of labour could be inactivated by hydrolysis by acetylhydrolase present in amniotic fluid or could be taken up by the amnion cells. PAF acetylhydrolase activity in amniotic fluid has to decrease significantly before the onset of labour to allow greater PAF levels locally. Ban et al (1986), however could not observe any significant difference in acetylhydrolase activity in amniotic fluid, obtained from women in full term normal labour and those at term, but not in labour.

PAF uptake has been studied in various cells like, human umbilical vein endothelial cells (HUVEC) (Blank et al, 1986), rabbit platelets (Malone et al, 1985), rabbit neutrophils (Chilton et al, 1983), rabbit alveolar macrophages (Robinson et al, 1985), rat lung type II epithelial cells (Kumar et al, 1987),fibroblasts from rat lungs (Kumar et al, 1987) and rabbit kidney medulla (Kawasaki and Snyder, 1987). Little is known about thePAF uptake from amniotic fluid by fetal membranes. Amnion cells are capable of metabolising exogenous PAF (Ban et al, 1986). However, it is not known whether PAF uptake could occur in amnion cells. PAF receptor has been reported and isolated in a variety of cells, most notably in cell involved in inflammation, neutrophils (Valone, 1988a), murine macrophages (Valone, 1988b) platelets (Kloprogge et al, 1984), smooth muscle cells (Hwang et al, 1983) and more recently in endometrium (Harper and Kudolo, 1989), human

myometrium (Zhu et al, 1992). The involvement of PAF receptor in fetal membranes has not been studied to date. The existence and implications of PAF receptor(s) in this part of intrauterine tissue needs to be elucidated.

ROLE OF PAF IN PARTURITION.

The presence of PAF in amniotic fluid of women at full term and in spontaneous labour as compared to those at term, but not in labour (Billah et al, 1983; Nishihara et al, 1984; Hoffman et al, 1990; Silver et al, 1992), suggests that this autacoid has an important role to play in events of parturition.Previously, Ban et al (1986), were able to demonstrate, the enzymes necessary for PAF metabolism in fetal membranes and decidua vera. The activities of enzyme Lyso-PAF:acetylCoA transferase and acetylhydrolase in intrauterine tissues were present in microsomal and cytosolic fractions predominantly respectively. Although 0.1 uM Ca++ caused activation of acetylhydrolase enzyme, these workers, could not detect any gestational age related change in activities of acetyltransferase as well as acetylhydrolase in intrauterine tissues. However, the highest acetylhydrolase activity was found in order, chorion> amnion> decidua vera> placenta.Billah et al (1985), observed that amount of PAF in amnion tissueobtained either during second trimester or at term (before labour) were similar. However, after labour the amount of PAF in amnion increased to 2.5 times that of before labour, without any discernible changes in lyso-PAF or alkyl-acyl-GPC. Increased PAF in amnion tissue could be because of increased PAF biosynthesis and/or increased uptake of PAF from amniotic fluid.Increased PAF biosynthesis was demonstrated in amnion tissue disks in vitro (Billah et al, 1985) by calcium ionophore A23187, in presence of Ca^{2+}, but PAF was released into the incubation medium. The stimulation of PAF biosynthesis by A23187 and calcium was not affected by the addition of indomethacin.

elease of prostaglandins, PGE_2 and $PGF_{2\alpha}$ by the fetal membranes may herald the onset of labour (Dray et al, 1979; Chelsey et al, 1984). In same experiments, Billah et al (1985), reported stimulation of PGE_2 biosynthesis by exogenous PAF, as well as by calcium ionophore A23187, suggesting PAF may be exerting a stimulatory effect on PGE_2 production via changes in intracellular

calcium.Recently, similar observations have been made by Morris et al (1992). PAF stimulated PGE_2 and its metabolites by three-foldby intact amnion and chorion-decidua after 24 hrs of incubation. PAF had greater effect on PGE_2 production by the intact membranes obtained after the onset of labour, such that PGE_2 production was increased by 100-fold.It had been reported earlier on by Montrucchio et al (1986) that PAF evoked myometrial contractions in two different patterns. In spontaneously active myometrial strips, both PAF and oxytocin enhanced the amplitude of contractions in guinea-pig myometrium. In quiescent myometrial strips, PAF induced contractions were characterized by a prompt development of tension, a plateau and a final relaxation. Interestingly the PAF-induced contractions was followed by rhythmic activity. Nishihara and coworkers (1984) demonstrated that PAF was a potent agonist of myometrial contractions in the rat. Yasuda et al (1986) reported presence of PAF in rat uterus. Zhu et al (1992), have recently demonstrated that PAF affects contraction in strips of human myometrium at a concentration as low as 0.1nM/L. PAF receptor has been characterized in human myometrium. PAF caused increase in intracellular Ca^{2+} concentration of isolated myometrial smooth muscle cells in culture and increase in phosphorylation of the 20 kd light chain of myosin in a concentration-dependent manner. The presence of PAF receptor(s) (Zhu et al, 1992) and related molecular changes, provide further support for the importance of PAF in initiation as well as maintenance of parturition.

PAF ACETYLHYDROLASE ACTIVITY IN PREGNANCY:

Maki et al (1988) reported that PAF acetylhydrolase activity, decreases to 10% of the non-pregnant level in the maternal plasma during the last half of pregnancy in rabbits. After delivery, the PAF acetylhydrolase activity returned to pre-pregnancy levels. A decrease in PAF acetylhydrolase activity has also been demonstrated in the maternal plasma of the human (Johnston, 1989). However as PAF is being synthesized in human fetal lung type II cells, an increasingly high concentration of PAF reaching maternal circulation and hence intrauterine compartments, has to be prevented. The correlation between the role of PAF in fetal lung maturation (Hoffman et al, 1986) and changes in acetylhydrolase activity needs to be established.

PAF AND MATERNAL AND HORMONES:

PAF metabolism in rat uterus (Nakayama et al, 1991), and PAFacetylhydrolase activity has now been shown to be modulated by various hormones in rat plasma (Yasuda and Johnston, 1992). PAF acetylhydrolase activity in rat plasma, was decreased by the administration of 17 α-ethynylestradiol, and increased by dexamethasone treatment. Moreover, PAF acetylhydrolase was lowered by treatment of estrone, 17-ß-estradiol, estriol in female rat plasma, estriol being the most potent. The administration of medroxyprogesterone to female rats resulted in 2-fold increase in plasma acetylhydrolase and the activity remained elevated for several weeks. The effects of estrogens and medroxyprogesterone in male rat plasma were much less significant. Similarly, the juvenile male or female rats responded to hormone-treatment much less significantly as compared to the adult female rats. The effects of dexamethasone on juvenile rats male or females, were similar to those of adult rats.Maternal hormones increase in maternal circulation with increasing gestational age (Heap and Flint, 1990). Human fetal membranes possess, the ability to metabolize maternal steroid hormones (Mitchell et al, 1982; 1984; 1987) and constitute an integral part of feto-placental unit and are also known to stimulate prostaglandin biosynthesis in fetal membranes (Challis et al, 1987; Chibbar and Mitchell, 1988; Schatz et al, 1985). The effects of maternal steroid hormones on PAF acetylhydrolase activity in intrauterine tissues, especially the amnion tissue needs to be studied.

PAF AND OXYTOCIN:

Oxytocin is a neuropeptide, released from posterior pituitary around parturition, to serve important role in initiation and maintenance of uterine contractions. PAF and oxytocin have been reported to synergies in myometrium (Tetta et al, 1986). In resting myometrial strips pre-treated with oxytocin PAF caused rhythmic contractions characterized by a prompt development of tension, followed by a short plateau, finally leading to relaxation. The effects of PAF but not oxytocin, on myometrial contractions were abolished by indomethacin completely and partially by lipoxygenase inhibitor, FPL 55712 (Montruccio et al, 1986). Little is known about the effects of oxytocin on PAF metabolism in the intrauterine tissues. Modulation of PAF metabolism in fetal membranes, by hormones and other factors can have important implications in the onset of labour. The local interactions of PAF, $PGF_{2\alpha}$ and oxytocin which causes contraction of myometrium (Zhu et al, 1992) and progesterone, known to inhibitory effect on gravid myometrium would play a crucial role.

PAF AND PROSTAGLANDINS:

At several instances, local mediators are known to regulate each other's metabolism. PAF and arachidonic acid metabolism are
important autacoids, documented to have various interactions at numerous sites in the body (Peplow and Mikhailidis, 1990). PAF is known to stimulate prostaglandin E_2 production in amnion tissue (Billah et al, 1985). Although lipoxygenase metabolites have known to cause stimulation of PAF biosynthesis by human neutrophils (Billah et al, 1985), however, there is paucity of literature on the role of prostaglandins on PAF metabolism and as mentioned earlier, indomethacin treatment abolished PAF-induced effects on myometrium.

PAF AND COMPLICATED PREGNANCIES:

a. PAF AND PRE-TERM LABOUR:

Although PAF is detectable in amniotic fluid of women with uncomplicated pregnancies at term and in labour, very little PAF is detectable in amniotic fluid of women at term, but not in labour (Billah et al, 1983). Hoffman et al (1990) determined PAF in amniotic fluid from group patients with complicated pregnancies. There was a 20-fold greater concentration of PAF in amniotic fluid obtained from patients incurring "preterm labour" with an average gestational age of 32 weeks as compared with those at term but not in labour group. Moreover, PAF concentrations were also significantly higher in amniotic fluid in group of patients with an average gestational age of 31.5 weeks who had premature rupture of membranes as compared to those at term, but not in labour.The presence of PAF in amniotic fluid of human pregnancy appears to correlate with normal and pathological labour.Similar observations were made by Silver et al (1992). These workers found a 12-fold increase in PAF levels in amniotic fluid of women in preterm labour, in whom, tocolysis failed. Patients with premature rupture of membrane had similar raised levels of PAF as compared to those with full term normal delivery.Patients with preterm labour with positive amniotic fluid gram stain had the highest PAF levels associated with very low levelsof acetylhydrolase activity in amniotic fluid (Silver et al, 1992). These studies clearly suggest that raised PAF production may be associated with preterm labour.

b. PAF AND PREGNANCY-INDUCED HYPERTENSION.

DEFINETION AND INCIDENCE OF PIH.

Pregnancy-induced hypertension (PIH) is defined as the development of a diastolic blood pressure (phase IV) greater than 90 mm Hg after twenty weeks gestation in a primigravida with no prior hypertension or renal disease. Blood pressure returns to normal post partum. This diagnosis is most secure if these features are accompanied by an early (booking) diastolic pressure less than 90 mm Hg and a subsequent rise of more than 25mm Hg from this level (Redman, 1988). Pregnancy-induced hypertension is peculiar to primate pregnancy and it includes pre-eclampsia and eclampsia. This condition usually occurs in primigravida, however its occurrence in multigravida is well documented (Villar and Sibai, 1988). Pregnancy-induced hypertension complicates up to 15% of all pregnancies. Maternal mortality rates of 0 to 14 % have been attributed to pre-eclampsia, as have perinatal mortality rates of 10 to 28%.Many hypotheses regarding the aetiology and pathophysiology of pre-eclampsia have been proposed, but none as yet has been confirmed (Brown, 1989).

PAF AND CONTROL OF BLOOD PRESSURE:

PAF possesses potent hypotensive activity (Magusi et al.,1982) and is known to be identical to "antihypertensive polar renomedullary lipid" (Smith et al.,1981). These facts suggest that PAF may play an important role in the pathophysiology of hypertension. PAF has been detected by bioassay in the arterial blood of unclipped one-kidney, one clipped hypertensive rats, with high levels in rats exhibiting early fall in blood pressure (McGowan et al, 1988). Furthermore, increased plasma levels of lyso-PAF have been reported within 24 hrs of unclipping the one kidney, one clipped rat (McGowan et al, 1986). However, treatment of rats with PAF inhibitor CV 3988 and WEB-2086, prevented fall in blood pressure only incompletely (Magusi et al, 1985; Cotter et al, 1990). Magusi et al (1982), and other workers (Blank et al, 1983) have reported higher activities of PAF

acetylhydrolase inhypertensive rats. Recently, Satoh et al, (1989) studied the plasma acetyl hydrolase activity in patients with essential hypertension, the levels were significantly elevated in patients as compared with that of controls. There was also a significant difference in the activity associated with LDL between the patients and controls, but there was no difference in the distribution.Since PAF plays an important role in various events related to reproduction and pregnancy, both normal as well complicated, the role of this hypotensive lipid needs to be explored in pregnancy-induced hypertension.

AIMS AND OBJECTIVES:

Although an abundance of literature is available on PAF metabolism in various cells there is still little known about the regulation of PAF metabolism during pregnancy. It is believed that regulation of PAF metabolism could occur by regulation of its activation. (Snyder, 1989).PAF acetylhydrolase activity has been described to change during rabbit pregnancy (Maki et al, 1988) human pregnancy (Johnston and Miyaura, 1990), little information is available on gestational age related changes in human maternal plasma and amniotic fluid and their correlation with molecular events in fetal lung maturation.

This project will be carried out to investigate the PAF acetylhydrolase enzyme activity in maternal plasma obtained at various intervals of gestation and human amniotic fluid obtained from early pregnancy, at term and at term but in spontaneous labour in normal pregnancy as well pregnancy complicated by pregnancy-induced hypertension.

PAF inactivation in human intrauterine tissues and any change with respect to onset of labour would be investigated. Moreover, plasma acetylhydrolase is well characterised enzyme (Stafforini et al, 1987). However little information is available on physico-chemical characteristics of the tissue enzyme. There is paucity of work reported on various pregnancy-associated factors modulating the PAF inactivation in fetal membranes. Human amnion cells have been extensively used as a model for study of prostaglandin metabolism as well as PAF metabolism (Billah et al, 1985). There is no evidence of fate of PAF in amniotic fluid. The epithelial cells of amnion could take up the PAF present in amniotic fluid. In these experiments, the uptake and subsequent disposition of [14-C]PAF by human amnion cells in culture would be studied. It will also be tried to elucidate the mechanism (s) underlying PAF uptake by amnion cells.

MATERIALS AND METHODS.

REAGENTS AND CHEMICALS.

The following reagents and chemical were purchased from Sigma Chem. Co. St Louis, Missouri, U.S.A. Arachidonic acid, ADP, Albumin, Aspirin, Collagen, Collagenase, Calcium ionophore A23187, Cellulose, CHAPS, Dexamethasone, Diastase, Epinephrine, Fibrinogen, Folin Ciocalcteo reagent, Ferric chloride, glutathione, Goat serum, Gentamycin, Haemoglobin, Heparin, HEPES, Indomethacin, Lyso PAF, Merthiolate, N-ethylmaleimide, Newborn calf serum, Oxytocin, Oestradiol, Progesterone, Papain, Phorbol esters, Phospholipids, Prostaglandins, PMSF, TLCK, Trypan blue, Thrombin, Trypsin.

BUFFERS AND SOLVENT SYSTEMS:

a) PHOSPHATE BUFFERED SALINE.

Stock solution.

i). 0.2 M monobasic sodium phosphate.

ii). 0.2 M dibasic sodium phosphate.

190 ml of (i) and 810 ml of (ii) = 1000 ml (pH 7.4).

b) Calcium-free Krebs solution consisted of.

NaCl =0.690 g

KCl = 0.035 g

$NaHCO_3$ = 0.210 g

KH_2PO_4 = 0.016 g

$MgSO_4$ = 0.029 g

GLUCOSE = 0.200 G

WATER = 100 ML.

c) Tris HCl buffer (pH 7.5) contained

TRIS = 15 mM

NaCl =125 mM

$MgCl_2$ =0.5 mM

KCl = 5.0 mM

GLUCOSE = 5.5 mM

EDTA =4 mM

pH maintained by HCl to 7.5.

RADIOCHEMICAL.

PLATELET ACTIVATING FACTOR ([^{14C}]methyl).

{1-O-hexadecyl-2-acetyl-sn-glycero-3-phospho-[N-methyl-C]14choline}

Source = Amersham.

Specific activity = 55 mCi/mmol : Radioactive Conc = 20 uCi/ml

5 ul was taken and 995 ul of 0.1% bovine serum albumin, gave 1.0uCi PAF (1.8nmol /ml).

SOLVENT SYSTEMS USED FOR SEPARATION OF:

PHOSPHOLIPIDS (Touchstone et al, 1979).

CHLOROFORM : ETHANOL: WATER : TRIETHYLAMINE.

(60: 68: 16: 70 v/v).

PLATELET-ACTIVATING FACTOR AND OTHER PHOSPHATIDYLCHOLINES (Billah et al, 1983).

CHLOROFORM: METHANOL : ACETIC ACID : WATER.

(50: 30: 8: 4 v/v).

The TLC tanks were equilibrated prior to use.

SAMPLE COLLECTION:

a).BLOOD.

Blood was obtained from healthy subjects and pregnant females at antenatal clinics and the labour rooms at the Aga Khan University Hospital Karachi, and Royal United Hospital, Bath. Blood was drawn with butterfly needles No. 21 mounted on a polypropylenesyringe and without applying a tourniquet, in order to avoid vascular stasis (Slater and Benedetto, 1987). Blood samples (9 volumes) were transferred to siliconized tubes containing sodium citrate (1 volume) (3.8 %) as an anticoagulant in polystyrene test tubes. Heparinized tubes were avoided, because heparin interferes with platelet aggregation.

The subjects were divided into the following groups.

i). Normal healthy individuals, who had not taken any medication for the past two weeks prior to sample collection (this group included healthy males and non-pregnant, normotensive females (N=15).

ii). Normotensive pregnant females (15-20 in first, second and third trimester and 20 in labour, preferably primigravidas).

iii). Patients with pregnancy-induced hypertension (gestational age range 20 weeks onwards 10-12 in each trimester, 15 in labour, primigravidas).

Criteria for pregnancy-induced hypertension were:.

Maternal age : (19-38 years).

Blood pressure: Blood pressure 140/90 mm Hg on three subsequent readings in women who had been normotensive in first trimester or early booking. Diastolic blood pressure was taken at Korotoff IV stage.

Proteinuria: Significant proteinuria (> 0.3 g/24 hrs).

Gestational age : 20 weeks onwards.

Excessive weight gain / oedema .

b) PLACENTAE AND FETAL MEMBRANES:

Placentae along with intact fetal membranes were collected from: 1. Women undergoing full term normal spontaneous labour

2. Those not in labour but undergoing elective caesarean section for indications other than pregnancy- related disease.

The placental tissues were transported immediately at 4°C to the laboratory. Placental cotyledons were chopped and blood was removed by thorough washing with 0.9% ice-cold saline. Placental vessels were perfused with 0.9% saline to wash any blood products. Fetal membranes were manually separated and were washed with ice-cold normal saline and were homogenized at 4°C using a polytron homogenizer for 20 sec at 4°C. The homogenates were centrifuged for 600 x g at 4°C for

10 minutes. The supernatants were centrifuged again at 12000 x g at 4°C for 30 min to obtain the mitochondrial fraction in the pellets. The supernatant was again centrifuged at 105,000 x g at 4°C for 45 min. The pellet, thus obtained, contained microsomal fractions and supernatant mainly comprised of cytosolic fraction.

c) AMNIOTIC FLUID:

Amniotic fluid was collected transvaginally and /or transuterinally from:

1. Women undergoing diagnostic amniocentesis (Transuterinally)(16-17 weeks of gestation, n=5).
2. Women in full term normal labour admitted to labour room before the rupture of membranes and whom artificial rupture of membranes was carried out (Transvaginally, n=6).
3. Women undergoing elective caesarian section for indications other than pregnancy-related complication (Transuterinally, n=5).

Care was taken to maintain aseptic conditions while samples were being collected. All the samples (blood as well as amniotic fluid), obtained from the Royal United Hospital, Bath were subjected to freeze-drying for transportation. There was no loss in PAF acetylhydrolase activity on freeze-drying (Stafforini et al, 1987) and as tested the effects of freeze-drying previously on few samples before transportation.

AMNION CELL LINE PREPARATION:

Cell culture was carried out under sterile conditions. All glassware was sterilized by autoclaving at 120° C for 20 minutes, heat stable media and solution were autoclaved for 10 min while heat-labile media and solutions were sterilized by membrane filteration through a 0.22um millipore filter.The amnion cell line was prepared by modified method of Bennet et al (1987). In brief, human fetal membranes and placentae were obtained from labour room and aseptically transported to the laboratory. Amnion was manually separated from chorio-decidua and transferred to Tris-HCl, pH 7.7 at 25°C . The tissue was chopped into 2" pieces and incubated with 0.2% collagenase in

Tris-HCl pH 7.7 in Erlenmeyer flasks at 37° C in the shaking water bath for an hour with, vigorous shaking every 15 mins. The cell suspension was decanted and centrifuged for 5 min at 600 x g . The pellet was washed with Dulbecco's medium with 20 mM L-glutamate and sodium bicarbonate and the cells were plated in plastic 10 cm diameter plates in a laminar flow cabinet and incubated in a humidified atmosphere with 95% air and 5% CO_2 at 37° C and grown in monolayer. Media and media additives were checked for infection both before and during culture by adding samples to Brain Heart Infusion and Sabouraud liquid media. The epithelial cells were identified on their microscopic characteristics (Schwartz et al, 1977). The amnion cells were grown in monolayer culture in Dulbecco's modification of Eagle's medium supplemented with 10% new born calf serum. When amnion cells reached confluence as determined by light microscopy, they were harvested and split 1:3 for continous culture (Fig.ii). Each preparation was subcultured three times, after that, new cell line was prepared and subcultured. More than three subcultures were avoided, as epithelial cells characteristics would change during prolonged culture and various subcultures (Casey et al, 1984). These cells in culture provide a useful model for metabolic studies in the cells, as such cells in culture closely resemble the in vivo conditions (Schwartz et al, 1977 a, b).

[^{14}C] PAF UPTAKE AND INCORPORATION IN MEMBRANE LIPIDS:

Amnion cells grown in monolayer, and reaching the confluence, were harvested after 2-3 subcultures. The harvested cells were suspended in fresh culture media (Dulbecco's modified medium supplemented by heat inactivated newborn calf serum) and incubated with 0.18nm PAF (0.1uCi) at various time intervals (0, 2, 5, 15, 30, 60 min and later on 0, 0.5, 1, 2, 4, 6, 8, 12, 24,36 hours) at 37°C in the incubator with humidified 95% air supplemented with 5% CO_2.Incubations were stopped by pouring off the incubation media and washing the cells twice with ice-cold phosphate buffered saline (pH 7.4). The cells were scraped with rubber police man and re-suspended in phospahte buffer saline (pH 7.4) and lipid-extraction was carried out by chloroform: methanol (2:1) (Bligh and Dyer, 1959). After vortex mixing for 2 min the samples were centrifuged at 600xg for 10 min at 4°C. The organic layer was separated and subsequently treated depending on the

experiments.For the estimation of total radioactivity incorporated in the amnion cells, the extracted lipids were added on to scintillation vials and radioactivity was estimated by gamma counting by liquid scintillation counting (Robison et al, 1988). For the separation and analyses of radiolabel products of PAF, the extracted lipids were dried under nitrogen flow. The concentrated lipids were reconstituted in 2-5 ul of chloroform and spotted onto Silica gel G thin layer chromatograph plates and developed in a solvent system containing Chloroform: Methanol: Acetic acid: Water (50: 30: 8:4), (Billah et al, 1983) or by the method of Touchstone et al (1979). The separated radioactive lipid products were quantified and analyzed by the use of Berthold TLC linear analyzer, coupled to chromatography data system (Saeed et al, 1988).

INCUBATION WITH DRUGS

Various drugs and their vehicles were added along with the radioactive PAF to the media. WEB-2086 (0.2-20 nanomolar), NDGA (0.1-100 nanomolar), Indomethacin (0.1-100 nanomolar) were added to Dulbecco's modified medium with 5% heat-inactivated newborn calf serum and incubated with [14-C]PAF (0.1u Ci) with amnion cells for upto 60 minutes at 37°C in an incubator. The incubations were stopped by pouring off the drug-containing incubation medium and washing the cells with twice with ice-cold phosphate buffer saline (pH 7.4). The total lipids were extracted by modified method of Bligh and Dyer (1959) and divided into two equal portions. To the first half, scintillation fluid was added and total radioactivity was estimated by the method of Robison et al (1988). The results were expressed as total radioactivity cpm. The second half of samples were dried and concentrated under at stream of nitrogen. These samples were reconstituted in 5 ul of chloroform, applied on to Silica gel G TLC plates and developed in a solvent system containing chloroform: methanol: acetic acid: water (50: 30: 8:4) (Ban et al, 1986). The radioactive zones were identified and quantified by using Berthold TLC linear scanner, coupled with a chromatography data system (Saeed et al, 1988).Merthiolate (0.1-100 micromolar), N-ethylmaleimide (0.1-100 micromolar), Phorbol esters, Phorbol, Phorbol 12-myristate 13-acetate, Phorbol 12,13-dibutyrate (0.1-10 micromolar), PKC inhibitors Tamoxifen (0.1 micromolar), Piperazine (0.1 micromolar) and

Chlorpromazine (1 micromolar) were incubated with the amnion cells along with [^{14}C]PAF (0.18 nM, 0.1 uCi) for 0-60 min at 37°C in an incubator. The incubations were terminated as described above and total radioactivity was determined by the method of Robison et al (1988).

INCUBATIONS WITH HORMONES:

Various hormones like corticosteroids and ovarian sex homones, exert their effects via receptors and their effect would appear after the stimulation of receptors at effector sites (Dong et al, 1988). The effects of progesterone and 17-ß-estradiol (0.01 micromolar to 10 micromolar in 0.01% DMSO) on PAF uptake by amnion cells in culture, and subsequent metabolism were determined by incubation of hormones with [14 PAF] (0.1uCi/ml) with amnion cells for upto 48 hours in culture at 37°C. After 48 hours, the incubations were stopped, cells labelled with PAF were washed thrice with ice-cold phosphate buffer saline (pH 7.4) and lipid extraction was carried out by modified method of Bligh and Dyer (1959). The extracted lipids were divided into two equal portions. To the first half, scintillation fluid was added and total radioactivity was determined by the method of (Robison et al, 1988). The results were expressed as total radioactivity present in cells (cpm). The second half of samples were dried and and concentrated under a stream of nitrogen. The dried samples were reconstituted in 5ul of chloroform, applied on to the Silica gel G thin layer chromatography plates and developed in a solvent system containing, Chloroform: Methanol: Acetic acid: Water (50:30:8:4), (Ban et al, 1986) The separated radioactive products were identified and quantified by using Berthold TLC linear analyzer coupled with chromatography data system (Saeed et al, 1988).
Dexamethasone (2.5-250 microgram/ml in 0.01% ethanol, 2.5-250 ug/ml was incubated with the amnion cells under similar incubation conditions for 48 hours and estimating the total uptake of PAF and its metabolism.

CHROMATOGRAPHIC ANALYSIS OF TOTAL MEMBRANE PHOSPHOLIPIDS:

The dried samples along with standard phospholipids were reconstituted in chloroform and applied to silica gel G plates and developed in a solvent system containing, chloroform: ethanol: triethylamine: water to a height of 17 cm.The radiolabeled phospholipids were analysed by a TLC linear analyser coupled with chromatographic data system as mentioned above and/or the plates were exposed to iodine vapours and non radioactive standards as well as samples were visualised by spraying with 10% molybidic acid and drying the plates in an oven at 80-100°C until the phospholipids appeared blue.

CHROMATOGRAPHIC ANALYSIS OF PHOSPHATIDYLCHOLINES:

For the separation of PAF, lysoPAF, and alkyl acyl GPC, the samples were spotted onto the silica gel G TLC plates and developed in a solvent system containing :
CHLOROFORM: METHANOL: ACETIC ACID: WATER. (50:30:8:4) to a distance of 17 cm. The separated phosphatidylcholine were analysed by a TLC linear analyser coupled with the chromatographic data system.

PLATELET AGGREGATION.

Platelet counts were estimated in the platelet rich plasma by standard procedures. A small quantity of PRP was sucked to mark 0.5 of a haemocytometer pipette and diluted with ammonium oxalate (1 % v/v in water). After gentle mixing, the distal fraction was discarded and the tip of the pipette was laid on both chambers of a Burker chamber. After waiting for 20 min, to allow platelets to settle, platelet counting was carried out.Platelet aggregation was studied by adding aliquots of 0.45 ml of platelet-rich plasma (PRP) to the aggregometer cuvettes of Dual Channel Chronolog Lumi Aggregometer (model 400, Chronolog, Chicago, U.S.A) at 37°C and stirring by magnetic bar (Saeed et al, 1991). After 1 minute the various aggregating agents (arachidonic acid, 1.73 mM, PAF 80 nM, Collagen 0.638 uM, and ADP 2.17 uM) were added and aggregation was monitored for an average of 4 minutes (3-5 min) and recorded as a change in light transmission on a strip chart recorder (Khan and Saeed, 1992).The effects of amnion cytosol on platelet aggregation induced by various agents was tested. This was carried out by incubating amnion cytosol (0-0.3mg) with various aggregating agents (PAF, Collagen, ADP and arachidonic acid) at 37° C for 0-60 min. After each time point the aggregating agent along with the amnion cytosol was added to the PRP and aggregation was recorded as above. These methods were particularly employed in the experiments carried out on the characterization of PAF acetylhydrolase. PAF (80 nM) was incubated with 0.1mg of amnion cytosol and this cytosol-treated PAF was subsequently tested for its effects on platelet aggregation. Any loss of PAF activity was quantified as percentage loss of PAF-induced aggregation as compared with the untreated PAF (80 nM).The effects of changes in pH, temperature, various enzyme inhibitors [20% Tricholoracetic acid, Phenylmethylsulfonylflouride, (PMSF), Diisoflourophosphate (DFP), organic solvents, protease) were determined by subjecting the amnion cytosol to above treatments and incubating unlabelled PAF (80nM) with cytosol. Any loss in cytosolic activity was observed by its capacity to cause PAF inactivation and decrease in PAF-induced aggregation.The molecular size of PAF acetylhydrolase was determined by:

1. Dialysis of amnion cytosol:

5-10 ml of amnion cytosol was added into cellophane dialysis membrane against 0.9% NaCl for 48

hours at 4°C. The control enzyme was also kept under similar experimental conditions. After 48 hrs, the enzyme activity was estimated in control and dialysed cytosol by incubating with unlabelled PAF (80nM) for 30 min at 37° C. Any loss in activity was recorded by determining PAF-induced aggregation.

2. Amnion cytosol was subjected to amicon cell ultrafilteration under pressure of 7psi, using XM-10, XM-30, YM-100 membranes. The enzyme activity was determined in filterates and retentants, by the platelet aggregation method.

3. Amnion cytosol was filtered through, Sephadex G-100, Sephadex G-50 column chromatography at 4° C. Various fractions were collected and enzyme activity was estimated in the collected fractions by the PAF-induced platelet-aggregation method.

PLATELET ACTIVATING FACTOR ACETYL HYDROLASE ENZYME ASSAY:

PAF acetylhydrolase enzyme assay was carried out by method of Blank et al (1981). In brief, the enzyme was assayed by incubation of either cytosolic fraction, plasma or amniotic fluid (0.1 to 0.4 ml of protein) with (N-methyl-^{14}C) [1-O-Hexadecyl-2-acetyl- sn- glycero-3- phospho-[N-methyl-^{14}C] choline (Platelet activating factor) (0.1u Ci, specific activity 55.0 mCi/ mmol) in Tris-HCl (30 mM, pH 7.4) at 37°C for 20 min in a shaking water bath. The blanks were boiled and inactivated cytosols or plasma or amniotic fluid. Each incubation was carried out in duplicate and each experiment was atleast thrice, keeping the incubation conditions similar, each time.The reaction was stopped by adding 0.4 ml of 1 M citric acid. The lipids were extracted by a modified method of Bligh and Dyer (1959). In short, lipids were extracted by addition of chloroform : methanol (2:1 v/v) and dried under nitrogen. The dried samples were reconstituted in 50 ul of chloroform and applied onto the silica gel G (TLC) plates separated by one-dimensional thin layer chromatography and developed to a distance of 17 cm in a solvent system containing :Chloroform : Methanol : Acetic acid : Water (50 : 30 : 8 :4 v/v) to distance of 17 cm. The radiolabeled zones were located and quantified as described above (Saeed et al, 1988).The effects of 17-ß-estradiol and progesterone (0.1-10 micromolar in 0.01% dimethylsulphoxide (DMSO) was tested by incubation

of steroid hormones with the amnion cytosol for 30 min and carrying out the enzyme assay as above. The effects of various prostaglandins PGE_2, $PGF_{2\alpha}$ and prostacyclin (0.1-10ug/ml in 0.025% ethanol) and oxytocin (0.1-10 mU/ml, along with TLCK 50 ug/ml (as a protease inhibitor) on PAF acetylhydrolase was also carried out as above. The incubations carried out in absence of drugs and in presence of drug vehicles only served as controls.The effects of maternal steroid hormones, prostanoids and oxytocin was also estimated in amnion cells in culture. In short, the above agents along with their vehicle controls were incubated with amnion cells in culture at 37°C in a humidified air with 5% CO_2. After 24-48 hrs of incubation, the amnion cells were harvested and cytosol was prepared by centrifugation of homogenised cells at 105,000 x g at 4°C for 45 min. The cytosol, thus obtained was used for the enzyme assay by using [^{14}C]PAF (0.18 nM) by the method of Blank et al (1981).

PROTEIN ESTIMATION:

Protein estimation was carried out by using the method of Lowry et al (1951), using bovine serum albumin as standard.

The following stock solutions were prepared and stored at 4°C before use .

a). Solution A : 1% w/v $CuSO_4.5H_2O$

b). Solution B : 2% w/v Sodium potassium tartrate.

c). Bovine serum albumin : 200ug /ml in 0.1 M NaOH

The following solutions were prepared fresh before use .

d). Solution C : 2% w/v Na_2CO_3 in 0.1 M NaOH

e). Solution D : 1 ml of each solution A and B mixed with 100 ml solution C .

f). Folin-Ciocalteau reagent (Sigma, USA) : This was made 1 N by dilution with distilled water .

The samples were diluted with 1N NaOH and distilled water to bring the protein concentration within the assay range 50-200 ug /ml in 0.1 M NaOH. The bovine serum albumin (BSA) solution was diluted where appropriate with 0.1 M NaOH, to produce the series of dilutions containing 50-200 ug BSA/ml.To 1 ml of sample or standard, 4 ml of solution D was added. After 10 min at room temperature, 0.5 ml of Folin-Ciocalteau reagent was added and the tubes mixed immediately.After the samples were left in this condition for at least 30 min at room temperature, the absorbance at 680 nm was measured using a DU 7 spectrophotometer. Sample protein estimation was carried out by reference to the standard curve.

STATISTICAL ANALYSIS:

The results of the majority of experiment are described as mean ± s.d, with N=3-5 on average for assay procedures. Differences in the treatment between two groups was detected by Student's t-test and differences in the treatments between various groups were detected by Dunnet s'test. Gestational-age related changes were analysed by drawing the correlation line by simple regression analysis. Statistical analyses were carried out by a computer programme STATGRAPHICS for descriptive statistics, regression analysis and test of hypotheses Student's t-test.

For graphics, STATGRAPHICS and SIGMAPLOT were used.

Fig: ii

The confluent human amion cells in monolayer culture. The 2" x2" pieces of freshly delivered amnion membrane was incubated with 0.01% collagenase in phosphate buffer saline, pH 7.4 at 37°C for 60 min in shaking water bath, with vigorous shaking every 15 min (Bennet et al, 1987). The dissociated cells were washed twice with Dulbecco's medium and the cells were culture in Dulbecco's medium supplemented with 10% new born calf serum in a humidified air with 5% CO_2. After 27-96 hours the cells reached confluence and the morphology verified by microscopic characteristics.

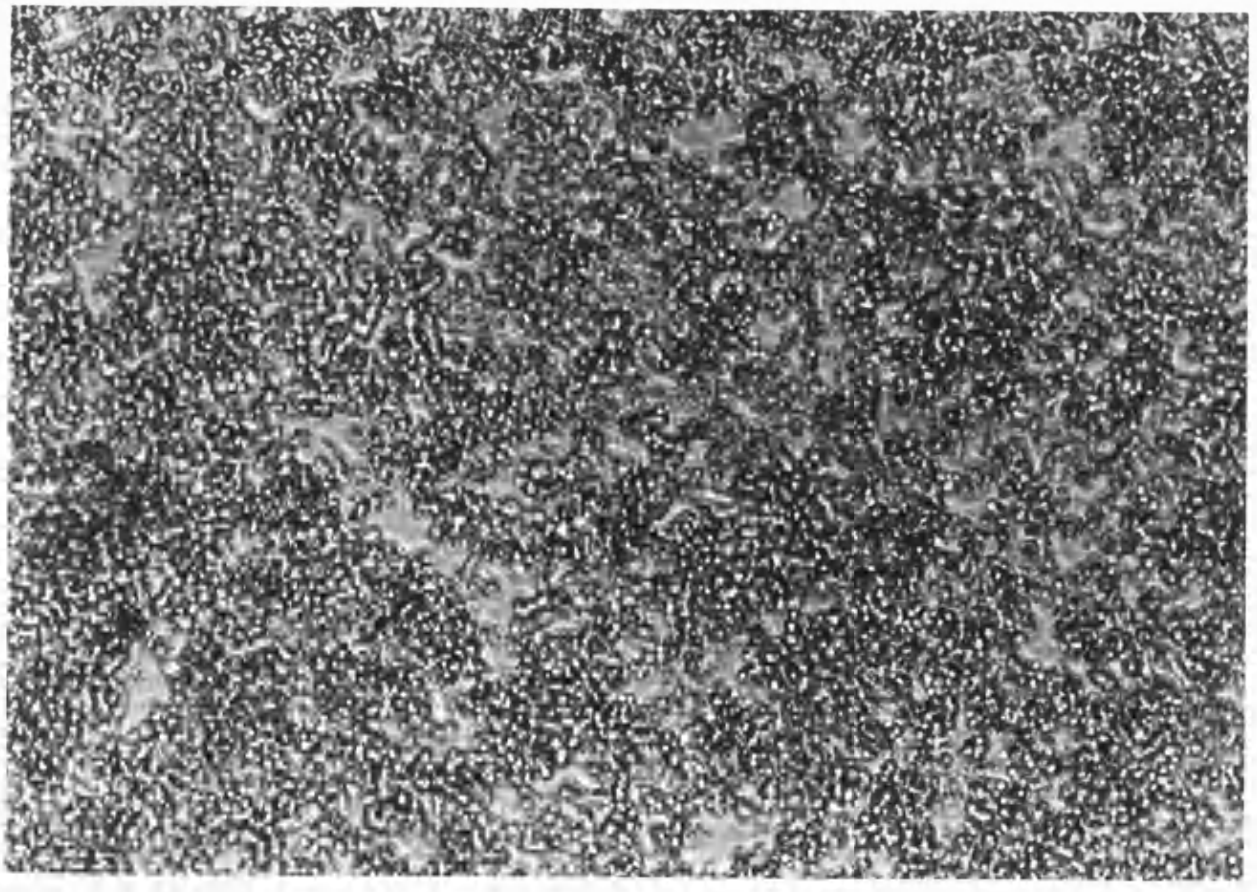


Fig:iii

Human platelet aggregation recorded by Lumiaggregometer (A). 0.45 ml of platelet rich plasma (PRP), is added to the cuvette and stirred by magnetic bar (B).

Aggregating agent is added and any change in light transmission is recorded in strip chart.

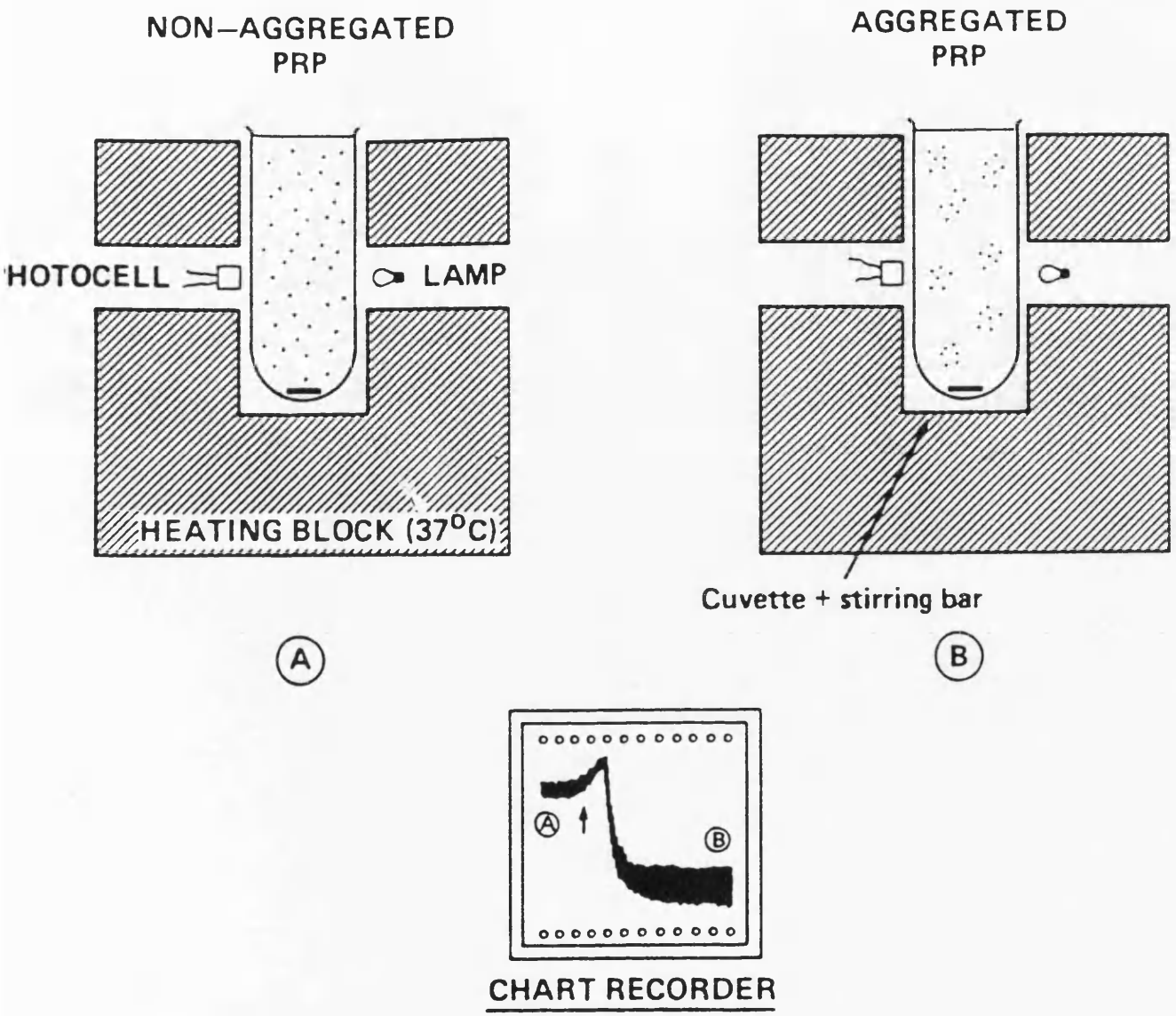
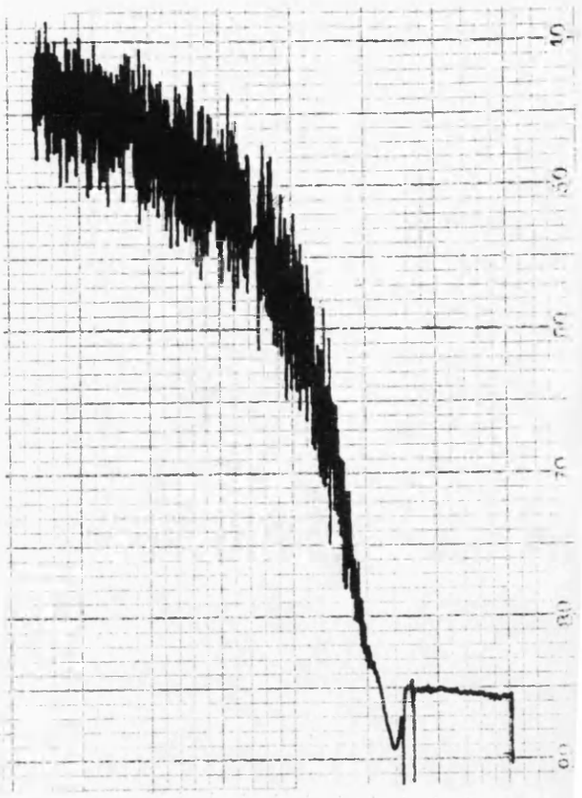
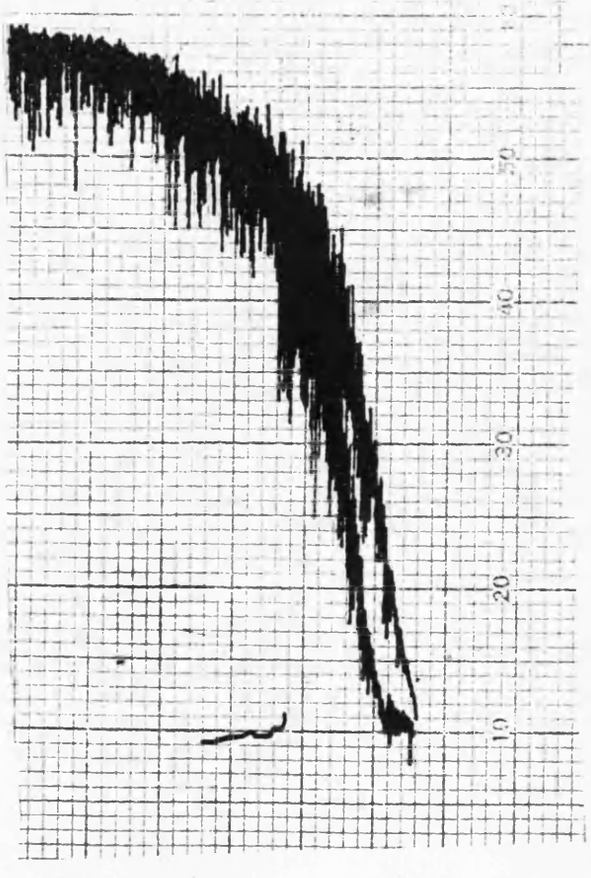

NON–AGGREGATED
PRP
AGGREGATED
PRP
HOTOCELL
LAMP
HEATING BLOCK (37°C)
Cuvette + stirring bar
A
B
A
B
CHART RECORDER

Fig: iv.

Typical tracings of human platelet aggregation induced by various aggregating agents as recorded by a Dual Channel Chronolog Lumi aggregometer (Model 400, Chronolog, Chicago, U.S.A)(Khan & Saeed,1992) .



(a) Arachidonic acid (100ug/ml of PRP).



(b) ADP (3.5uM).

(c) PAF.(80nm)

(d) Collagen (0.5 ug/ml).

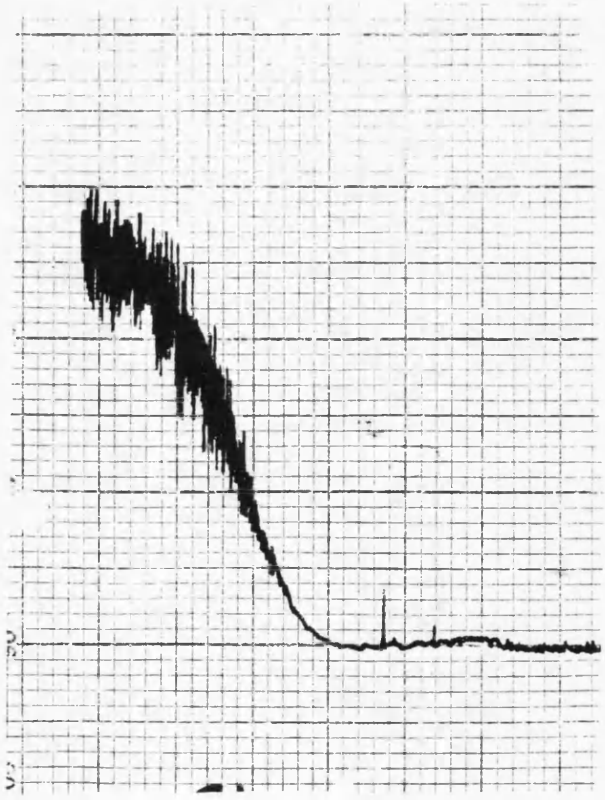


Fig: v.

Radiochromatogram of PAF and its metabolite, Lyso-PAF after incubation of radiolabel PAF with PAF acetylhydrolase at 37°C for 20 min (Blank et al,1981). The lipids were extracted and separated by TLC and developed in solvent system, containing, choroform, methanol, acetic acid, water. The radioactive zones were located and quantified by a Berthold TLC linear analyzer coupled with a chromatography data system. Lyso-PAF being more polar, moves ahead of PAF. The tracing show a typical concentration-dependent Lyso-PAF conversion.

Fig: v.

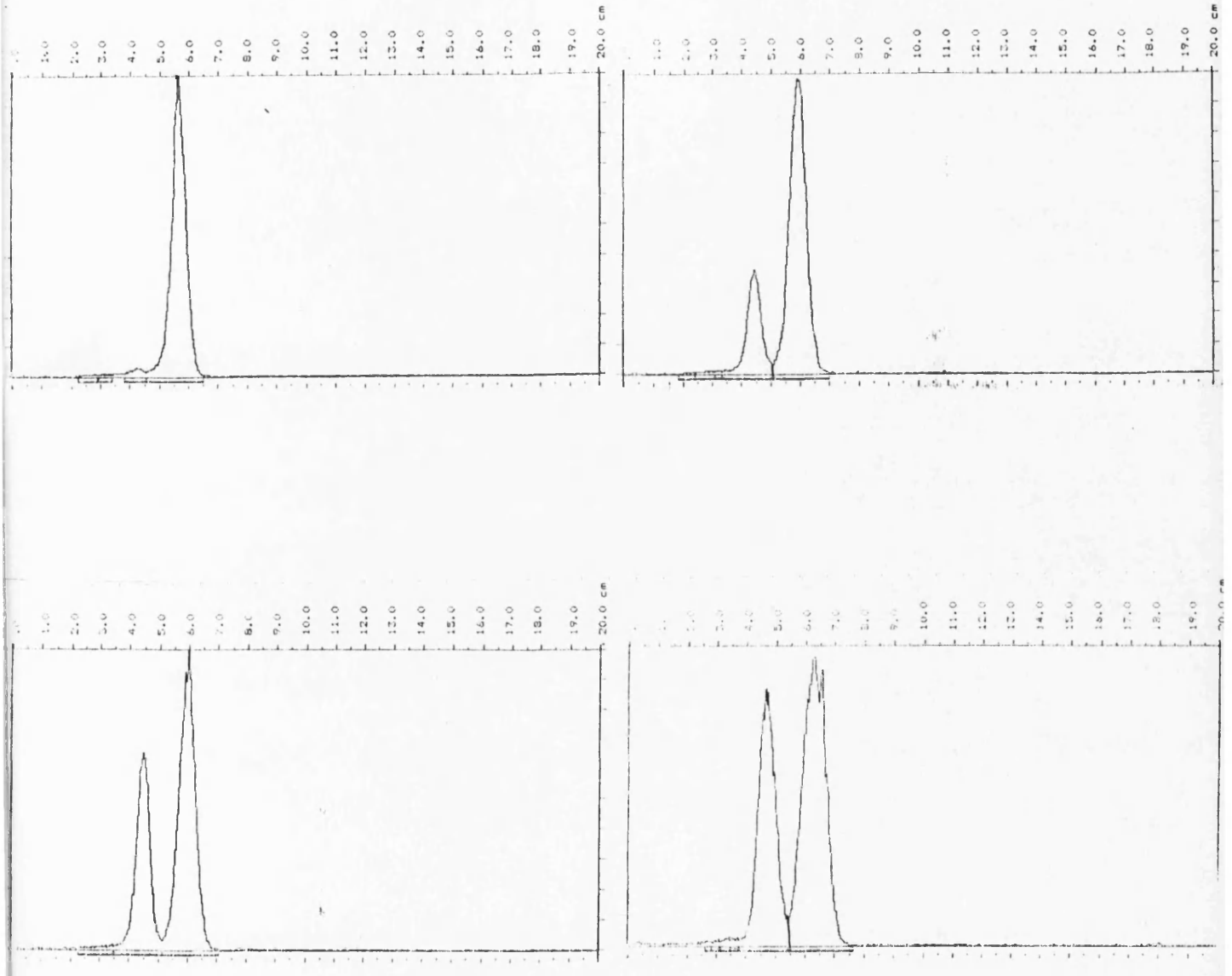
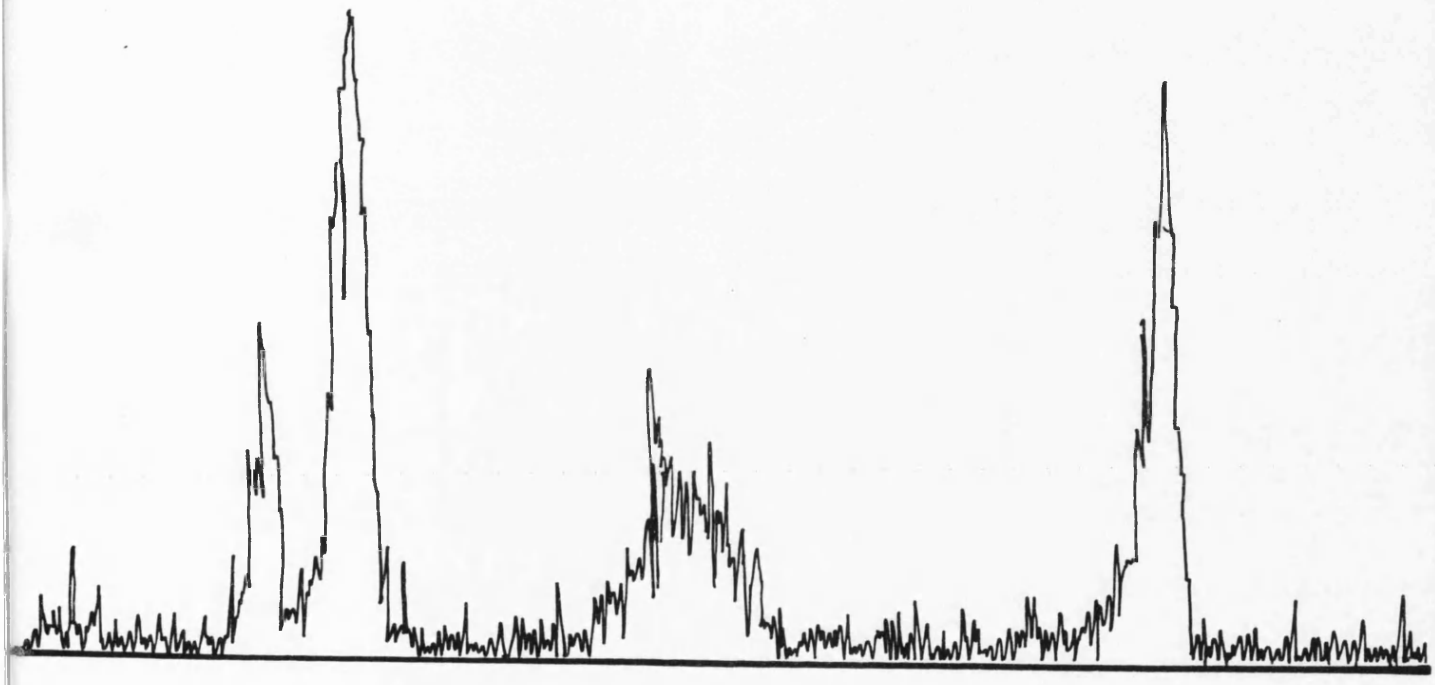


Fig: vi.

Radiochromatogram of PAF and its metabolites, Lyso-PAF, AAGPC etc. Tha amnion cells were incubated with 0.18nM [1-^{14}C] PAF in 0.01% bovine serum albumin. After 60 min, the incubation was terminated and the lipids were extracted and separated by TLC and developed in solvent system, containing, choroform, methanol, acetic acid, water. The radioactive zones were located and quantified by a Berthold TLC linear analyzer coupled with a chromatography data system. Lyso-PAF being more polar, moves ahead of PAF, AAGPC trails after PAF. The effects of various drugs on PAF metabolism was estimated by incubation of PAF in presence/absence of drugs/hormones.



RESULTS.

Recently, there has been a surge of interest in the role of PAF in physiological processes related to pregnancy. It has been shown that the regulation of PAF biosynthesis could occur via inactivation and conversion into the precursors such as alkyl-acyl-GPC. However there is still not sufficient information to explain PAF metabolism and its regulation during pregnancy and parturition. Therefore, PAF metabolism intracellularly as well as extracellularly, in normal and preeclamptic pregnancies was investigated in this study.

The results of the experiments carried out in this project will be described in four sections. The first section describes the occurrence of PAF acetylhydrolase enzyme in maternal plasma, amniotic fluid and human intrauterine tissues. Gestational-age related changes in the specific activity of acetylhydrolase enzyme are also reported.

The second section contains studies on the physico-chemical properties of the cytosolic acetylhydrolase enzyme.

This section also highlights the hormonal as well as humoral pregnancy-related factors modulating catabolism of PAF. The third section deals with the fate of exogenous radiolabelled PAF in human amnion cells in culture and also the mechanism underlying the uptake, binding and disposition of exogenously added PAF by the amnion cells.

As PAF is described as a hypotensive agent, its metabolism in pre-eclampsia will be described in the final section.

Section I.

PAF acetylhydrolase activity was determined in maternal plasma (n=15-20 in each trimester), with respect to gestational age and during labour. The enzyme assay was carried out as described in method section, by the method of Blank et al (1981). The results showed that the enzyme specific activity was highest in first trimester (53 nmol/min/ml of plasma) (Fig: 1). The enzyme activity

began to decline (32nmol/min/ml), significantly, by 22-24 weeks of gestation and remained unchanged during the latter half of pregnancy. The specific activity did not change during active labour (33 nmol/min/ml). The differences in activities were determined by Dunnet's test, $p< 0.01$. The correlation coeffient was 0.83 by simple regression analysis. The values for normotensive males, nonpregnant females and 48-72 hrs postpartum are 51.2nmol/min/ml, 52.5 nmol/min/ml and 49.8nmol/min/ml respectively (Table:1).

PAF acetylhydrolase activity was then determined in human amniotic fluid, obtained from women in full term normal labour (Fig: 2). The activity was linear with amniotic fluid protein for up to 300 ug and when incubated for 30 min. Blank values (incubation without enzyme or with boiled enzyme) were < 0.05 % of the total radioactivity added and the recovery was greater than 89% in the incubation blanks.

The enzyme activity was determined, in liquor amnii, obtained from

i) early gestation (16-17 weeks), ii) at term but not in labour (elective caesarean section), and iii) during full term spontaneous labour (Fig: 3). The specific activity was significantly lower than in plasma samples taken at similar periods. No statistically significant difference was found in the enzyme activities at various stages of pregnancy and labour, as determined by Student's t-test.

The enzyme involved in PAF metabolism has been described in fetal membranes, decidua vera and in amniotic fluid during labour.(Ban et al, 1983). The next set of experiments were carried out to assay PAF acetylhydrolase activity in human intrauterine tissues.

PAF acetylhydrolase was assayed in various cellular fractions, crude homogenate, mitochondrial fraction, microsomal fraction and cytosolic fractions of chorion, amnion, decidua vera and placental cotyledons, obtained from women in full term normal labour(Table:2). The results showed that the enzyme activity was highest in the cytosolic fractions (105,000 x g) of foetal membranes and decidua vera. However, chorion had the highest specific activity > amnion > decidua vera. Placental cotyledons, after thorough washing to remove blood contamination, had negligible activity.There was no statistically significant difference between samples obtained from active labour and those not in labour (Fig:4).

The importance of amnion tissue in prostaglandin biosynthesis during pregnancy is well documented (Casey and MacDonald, 1984). Moreover, plasma acetylhydrolase enzyme is well studied and characterised (Stafforini et al, 1987) as compared to cytosolic enzyme. Therefore, further work on acetylhydrolase was carried out on amnion cytosol.

Section II.

A comparison of the physico-chemical properties of the plasma and cytosolic enzymes was carried by determination of various physico-chemical characteristics of cytosolic enzyme and then comparing the results with plasma enzyme properties reported in the literature (Stafforini et al, 1987). The enzyme activity was estimated by two methods:

1. The cytosolic enzyme was incubated with 80 nmol of cold PAF. The cytosol-treated PAF was used to induce human platelet aggregation, as compared with the untreated PAF.

PAF (80 nmol) incubated with amnion cytosolic protein at various time points (0-30 min), showed that PAF was completely degraded after 30 minutes incubation (Fig: 5) at 37°C as estimated by the cytosol-treated PAF-induced aggregation as compared to the untreated PAF-induced aggregation. No effect of cytosol treatment was observed with arachidonic acid, collagen, ADP-induced aggregation (Fig: 6).

2. Enzyme assay by the method of Blank et al (1981), using 1-O-hexadecyl-2-acetyl-sn-glycerophosphocholine [^{14}C]-methyl (1.8 nmol/ml, 0.1 uCi/ml) as substrate. The concentration-dependent effect of cytosol was obtained (Fig: 7).

The effects of temperature on enzyme activity were then determined by carrying out incubations at 4, 56, 37, 80 and 100°C. The loss of activity was 20%, 28%, 85% and 100% at 4, 56, 80 and 100°C respectively (Fig: 8).

The plasma acetylhydrolase is an acid-labile enzyme. The effects of changes in pH on cytosolic activity were tested by carrying out incubations at pH 7.4, 3.0, 6.8, 10.0. The losses in the activity were 80%, 38% and 42% (Fig: 9). The results confirm earlier findings (Blank et al,1981) that the cytosolic enzyme is also acid-labile as is the plasma acetylhydrolase.

Plasma acetylhydrolase enzyme is < 43,000 dalton and is hypothesised to be glycosylated form of cytosol or intracellular enzyme. There was no significant loss in the enzyme activity after dialysis, indicating the cytosolic enzyme to be a non-dialysable protein (Fig: 10).

Molecular size of the enzyme was also determined by using amicon ultrafiltration under pressure of 7 psi, using XM-10, XM-30 and YM-100 membranes and sephadex G-100, and G-50 column chromatography. The collected fractions were used to detect the PAF acetylhydrolase enzyme activity.

The results showed that the enzyme activity was present in YM-100 retentent. (Table :3).

Blank et al (1983), had reported that the plasma enzyme is resistant to the effects of proteases and is resistant to serine hydrolase inhibitors. Based on these reports experiments were carried out to study the effects of various inhibitors on the enzyme activity.

The enzyme activity was determined in presence of Trichloroacetic acid (10%), protease, Difluoro isophosphate (DFP), and PMSF and various organic solvents (Fig: 11-12).

The results showed that enzyme activity was inhibited by trichloroacetic acid (80%), organic solvents (90%), protease (75)%, DFP (45%) and PMSF (52%).

The cytosolic enzyme was stable through several cycles of freezing and thawing, as is the plasma enzyme (Stafforini et al, 1987). There was no significant loss of activity on freeze-drying the cytosol (Table: 4).

Both the cyclooxygenase and lipoxygenase products are closely associated with various functions of PAF. The role of cyclooxgenase enzyme products on PAF metabolism has not well studied. Therefore, in the next series of experiments, the effects of various prostanoids on PAF acetylhydrolase activity and hence PAF metabolism in human amnion tissue was studied.

Amnion cytosol (0.2 mg) was incubated with various doses (0-10ug) of Prostaglandins E_2, F_2 and Prostacyclin and the enzyme activity was assayed using [^{14}C] PAF as described in the methods

section (Chapter II.11.c).

The results showed that PGE_2 caused a significant dose-dependent stimulation of acetylhydrolase (>30% ($p<0.05$ between control and PgE_2). The effects of $PGF_{2\alpha}$ were apparent at high dose only, whereas prostacyclin failed to show any effect (Fig: 13).

The effects of oxytocin on PAF acetylhydrolase enzyme were then investigated. The enzyme assay was carried out as described in the methods (Chapter II 11.c). The effects of oxytocin were studied in broken cell model, as well as in intact cells in culture. Oxytocin (0.1 to 10 mU) caused a marked reduction in the specific activity of acetylhydrolase, both in cytosolic incubation as well as in the intact cells in culture ($p< 0.001$, between control and 10mU oxytocin-treatment). However, the effect was pronounced in the intact cells (>60% inhibition) as compared with cytosol (25% inhibition) (Fig: 14, 15).

Moreover, ovarian steroid hormones have been reported to have important effects on prostanoids in human endometrium and endometrial epithelial cells (Schatz et al, 1985) and human fetal membranes (Challis et al, 1987). Levels of maternal steroid hormones vary with gestational age (Heap and Flint, 1990) and towards the end of gestation, a nocturnal surge of oestradiol forward is found in pregnant baboons. This may induce nocturnal uterine contractions by oxytocin release and/ or increase in uterine oxytocin receptors (Wilson et al, 1991). Recently Nakayama et al (1991), described the effects of oestradiol on PAF metabolism in oophorectomised rats.

The effects of oestradiol and progesterone (10^{-8} to 10^{-5} M) on PAF acetylhydrolase were studied using two models, amnion cytosolic preparation and amnion cells in culture. When the cytosol was incubated with steroids at 37°C for 20 min, 17-ß-oestradiol caused an inhibition of acetylhydrolase activity (13% less than that of control $p< 0.01$), the effects of progesterone were only significant at 10uM concentration, as compared with the control incubation ($p<0.05$). When the amnion cells were cultured in Dulbecco's medium with or without addition of above doses of 17-ß-oestradiol and progesterone

for 48-72 hours, the effects of oestradiol on acetylhydrolase were much more prominent (Fig: 16-18). These results show that oestradiol caused over 45% concentration-dependent inhibition of acetylhydrolase as compared with the control ($p<0.001$).

Since these experiments revealed that acetylhydrolase in amniotic fluid remains fairly constant throughout gestation, therefore PAF secreted in amniotic fluid has to be disposed of by the mechanisms other than enzyme hydrolysis. One such mechanism could be uptake and storage by the amnion cells. The next experiments were carried out to investigate the uptake and distribution of ether-phospholipid by human amnion cells and also to elucidate the mechanism(s) underlying this.

Section III.

Amnion cells in culture provide a useful model for the study of prostaglandin and related lipid metabolites (Schwartz et al, 1977 a,b). The cell line was established to study the uptake and disposition of exogenous radiolabel PAF by amnion cells. The cells were prepared by obtaining foetal membranes from women in full term normal labour. Amnion membrane was manually separated and amnion cells were cultured in monolayer as described in the Method Section. The amnion cells (Fig: ii) reached confluence on sixth day as observed under light microscopy. The confluent cells were subsequently used for 2-3 subcultures for further experiments.Human amnion cells grown in monolayer in Dulbecco's medium supplemented by 10% new born calf serum were incubated with 0.18nmol [^{14}C] PAF (0.1uCi in 0.01% bovine serum albumin) at different time points (0, 1, 3, 5, 10, 30, 60 min) at 37°C. The results showed that amnion cells took up the radiolabel linearly. (Fig: 19). Subsequently, the amnion cells were incubated for longer times, 0, 30 min, 1, 2, 4, 8, 20, 36 hrs. The total radioactivity present in amnion cells revealed uptake of radiolabel ether

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The estimation of radioactivity present in the culture medium at corresponding time-points showed

that there was a reciprocal decline in the radiolabel in the medium, and at 20 hrs. The [^{14} C] PAF may have been secreted from the cells (Fig: 21).

PAF in the cellular phospholipids is not stored as such. Unlike arachidonic acid PAF is hydrolysed by acetylhydrolase into lyso-PAF, which is acylated at position 2 with a fatty acid (usually arachidonic acid) and stored in cell membrane phospholipids,

phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol etc. The distribution of [^{14}C]PAF in the cellular phospholipids, as determined by the method of Touchstone et al (1979), showed that PAF is distributed in the phosphatidylcholine, predominantly, as alkylacyl phosphatidylcholine, diacylphosphatidylcholine (Fig:22).

In the next set of experiments the fate of bound [^{14}C]PAF

in the amnion cells was estimated. Total lipid extraction was carried out and dried under nitrogen stream. The reconstituted samples were applied on Silica gel G plates and various choline containing lipids separated by TLC (see methods).

The standards of 1-O-alkyl-2-acetyl-glycerophosphocholine, 1-O-alkyl-2-lyso-glycerophosphocholine, and alkylacylglycerophosphocholine were also applied and developed in solvent system containing, Chloroform: Methanol: Acetic acid: Water. The distribution of radioactivity in the products that comigrated with the corresponding standards was calculated. The results showed that within 5 minutes, almost the entire radioactivity was bound to amnion cells and was demonstrable as 1-O-alkyl-2-acetyl-glycerophosphocholine (PAF) alone. The corresponding time points contained radioactivity as PAF alone (Fig:23). No product was secreted into the medium by the cells.

At later time points (15-60 min), it was possible to identify the metabolites of PAF in cellular lipids. Initially, about 12% of total radioactivity was detected in the product that comigrated

with 1-alky-2-lyso-GPC and by 60 min, the radioactivity was distributed amongst, 1-alkyl-2-acetyl-GPC (41%), 1-alkyl-2-lyso-GPC (11%), 1-alkyl-2-acyl-GPC (33%) and Diacyl-GPC (9%).

At the corresponding time points, PAF was the predominant radioactive lipid. Lyso-PAF were detectable in the medium after 30 min (7%) and by 60 min, lyso-PAF increased to 16% and alkyl-acyl-GPC (10% of total radioactivity) was secreted into the culture medium (Fig: 24).

The amnion cells with incorporated PAF were washed twice with ice-cold phosphate buffer and re-suspended in Dulbecco's medium. These cells were then treated with 5.0 units/ml of snake venom phospholipase A_2 enzyme. The enzyme treated cells were subjected to lipid-extraction and the remaining radioactivity was estimated. The enzyme-treatment resulted in almost complete loss of radiolabel from the cells into the medium (Fig: 25). The amnion cell were labelled with [^{14}C] PAF in presence of Bromophenacyl bromide an inhibitor of phospholipase A_2, and subsequently treated with phospholipase A_2. This resulted in loss of phospholipase A_2 enzyme effects. BpB-treatment alone increased PAF uptake as compared to the untreated cells ($p<0.05$). The predominant product in BPB-treated cells was however alkyl-acetyl-GPC (73%), whereas alkyl-acyl-GPC was the major radioactive product in the culture medium (Fig: 26a,b).

The next series of experiments were carried out to elucidate the mechanism underlying uptake of ether phospholipid from the medium into the cells.

Incubations were carried out in control and calcium-free

buffer and that containing calcium ionophore A23187. The results showed that for the uptake of ether phospholipid extracellular calcium may be required, as PAF decreased in absence of extracellular calcium (Fig: 27 $p< 0.01$). Moreover, A23187 significantly increased the uptake, specially at longer incubations (30-60 min $p< 0.01$).

Merthiolate is a metabolic inhibitor and a topical anti-infective, known to inhibit SH-group enzyme reactions. It has been reported to decrease the incorporation of arachidonic acid into the cellular lipids by inhibiting the transacylase reactions. N-ethylmaleimide a is general metabolic inhibitor. In the next experiments, the effects of both drugs on PAF uptake was studied.

Amnion cells were incubated with (1.8nmol, 0.1uCi/mmol) [^{14}C]PAF in the presence and absence

of metabolic inhibitors, Merthiolate and N-ethylmaleimide (0.1-100 uM). Both compounds caused a significant reduction in radiolabel incorporation as compared to controls. Merthiolate inhibited the uptake in a dose-dependent manner (Fig 28, $p < 0.01$). Similarly N-ethylmaleimide caused 50% reduction in PAF binding to the cells at 100 micromolar concentration (Fig: 29, $p < 0.01$). PAF receptor has been described and characterised in various tissues, however no data is available on the presence and characterisation of PAF receptor(s) in human foetal membrane. PAF receptor involvement in PAF uptake mechanisms was studied by using the PAF receptor antagonist WEB-2086. Amnion cells were incubated in the presence and absence of PAF-receptor blocker, WEB-2086 (0.02 to 20.0 nM) and the radiolabel incorporation was estimated. WEB-2086 treatment reduced PAF uptake in a concentration-dependent manner (Fig: 30, $p < 0.001$ 20.0nM-treated cells as compared to controls).

These results suggest that receptor-mediated PAF binding is functional in human amnion cells. The estimation of various metabolites of PAF in WEB-treated cells showed that there was loss of alkyl-acyl-GPC peak in WEB-2086-treated cells as compared to untreated cells. The alkyl-acyl-GPC peak was however detectable in the culture medium (Fig: 31 a,b).

As seen by previous experiments in this study, prostaglandin E_2 stimulates PAF acetylhydrolase (Fig:15) that could lead to its acylation and storage.

Next set of experiments were designed to study the regulation of PAF uptake and storage in the cell membrane by cyclooxygenase and lipoxygenase products. Amnion cells were treated with cyclooxyge-nase inhibitor, Indomethacin (0.1 to 100 nM) and lipoxygenase inhibitor, Nordihydroguaretic acid (0.1 to 100nM). Treatment with Indomethacin failed to affect the total uptake of [^{14}C]PAF by amnion cells except at 100 nM dose (Fig: 32, $p<0.05$). NDGA-treatment lead to a non-dose related inhibition of radiolabel uptake (Fig:33 $p<0.03$). However, the lipoxygenase inhibitor treatment diminished of alkyl-acyl-GPC peak both in the cells and culture medium (Fig: 34).

To exclude the possibility of involvement of any energy requiring mechanism in the PAF binding, we treated the cells with various concentrations (0.1nM, 1.0nM and 10nM) of Ouabain, an inhibitor of Na^+/K^+-ATPase. The results showed that inhibition of Na^+/K^+ pump lead to a dose-dependent reduction in PAF incorporation in to the amnion cells (Fig: 36 $p < 0.001$), revealing the existence of ATPase-dependent process(s) in binding of ether phospholipids. Since, earlier experiments revealed that the maternal steroid hormones had markedly affected PAF metabolism, therefore the effects of steroids on [^{14}C]PAF uptake and distribution kinetics in the amnion cells was investigated. Treatment of amnion cells with 17-ß-oestradiol (10^{-8} to 10^{-5} M), led to an increase in radiolabel incorporation by amnion cells (Fig: 37). Treatment of cells with oestrogen led to inhibition of acetylhydrolase as well as a reduction in the activity of reacylation enzymes, leading to accumulation of PAF in the cells in a concentration-dependent manner (Fig 38 & 39). Treatment of cells with progesterone (10^{-8} to 10^{-5} M), also led to an increase in radiolabel uptake which was much more pronounced than that of oestrogen (Fig: 40). However, there was also stimulation of PAF inactivation, as seen by increased levels of lyso-PAF and acylated GPCs, particularly the latter. This means, progesterone stimulates the inactivation and storage of the ether phospholipid, effects opposite to those of oestrogen (Fig 41). These acylated glycerophosphocholines were secreted into the medium only at lower doses (10^{-7} and 10^{-8} M), as compared with higher concentrations (Fig: 42).

The next set of experiments were to study the effects of treatment of the amnion cells with the combination of various concentrations of oestradiol and progesterone. The results showed that oestradiol was the dominant hormone in the combination as seen by its effects on acetylhydrolase as well as the acylation enzymes (Fig: 43). Of these two hormones, oestrogen causes potentiation of PAF levels intracellularly (Fig: 44, 45).

Corticosteroids are known to play an important role in the processes related to pregnancy like human foetal lung maturation and parturition in different species. The effects of Dexamethasone on PAF metabolism in human amnion cells were therefore investigated. Incubation of amnion cells with different concentrations of dexamethasone (2.5ug/ml, 25ug/ml and 250ug/ml) led to inhibition of radiolabelled PAF binding in a dose-dependent manner (Fig: 46).

The corticosteroid-treatment lead to significant increase in the acylated-GPC both in the cells as well in the culture medium (Fig: 47 and 48). These acylated phospholipids can be used for the synthesis of disaturated phosphatidyl choline (lecithin), known to be the lung surfactant.

It is now well documented that PAF binding to its receptor leads to activation of phosphatidylinositol breakdown leading to formation of diacylglycerol (DAG) and in turn leading to an increase in intracellular calcium, involving the PKC.

In the subsequent experiments, the role of PKC activation in PAF disposition by the amnion cells was studied. Three phorbol esters Phorbol, Phorbol 12-myristate 13-acetate and Phorbol 12,13-dibutyrate in doses of 10^{-7} to 10^{-5}M were used. When the [^{14}C] PAF binding experiments were carried in the phorbol ester-treated cells (i.e. after PKC activation) such treatement led to a significant reduction in PAF binding to the amnion cells in both dose-dependent and time-dependent manner. The effects being marked at higher doses and at later time points. (Fig 49 a, b, c).

The effects were pronounced with Phorbol 12-myristate 13-acetate and Phorbol 12,13-dibutyrate as compared to the Phorbol alone.

These effects were not reversed or were partially reversed by PKC inhibitors, like Tamoxifen, Piperazine and Chlorpromazine respectively (Fig: 50). Treatment of cells with a combination of Oestradiol and Phorbol esters, did not lead to any significant change in the effects of phorbol esters on PAF binding by amnion cells (Fig: 51).

Section IV.

This set of experiments was carried out to investigate the role of PAF in pregnancy-induced hypertension. PAF acetylhyrolase activity was estimated in maternal plasma obtained from the women diagnosed to be pre-eclamptic according to the criteria described in the Methods. There was no statistically significant difference (Student's t-test) in acetylhydrolase activity between normotensive and pre-eclamptic plasma, during the last trimester, at term or in labour (Fig: 52). However the specific activity of PAF acetylhydrolase in fetal membranes of pre-eclamptic pregnancies was significantly increased, as compared to normal pregnancies (Fig: 53).

Fig: 1.

Specific activity of PAF acetylhydrolase in maternal plasma in normotensive women obtained at various stages of gestation and from those in full term spontaneous labour, before any oxytocic intervention. The enzyme activity was assayed by incubation of ^{14}C PAF (0.18 nM/ml, 0.1uCi/ml) with 0.1ml/ml of plasma for 30 min at 37°C by the method of Blank et al (1981). Boiled enzymes served as blanks (only 0.45% of control enzyme activity). The incubation were stopped by 1 M citric acid and lipids were extracted and separated by one-dimensional thin layer chromatography. The separated radioactive products were identified and quantified by the use of Berthold TLC analyzer (Saeed et al, 1988). The specific activity was calculated by estimated nanomoles of lyso-PAF formed/min/ml of plasma.
The values are Mean ± S.D (nmol/min/ml of plasma). n=15-20 in each group.
$p<0.01$ (first vs 2nd & 3rd trimester,& labour).
Not significant (2nd, 3rd vs labour).

Gestational age–related changes in PAF acetylhydrolase activity in maternal plasma.

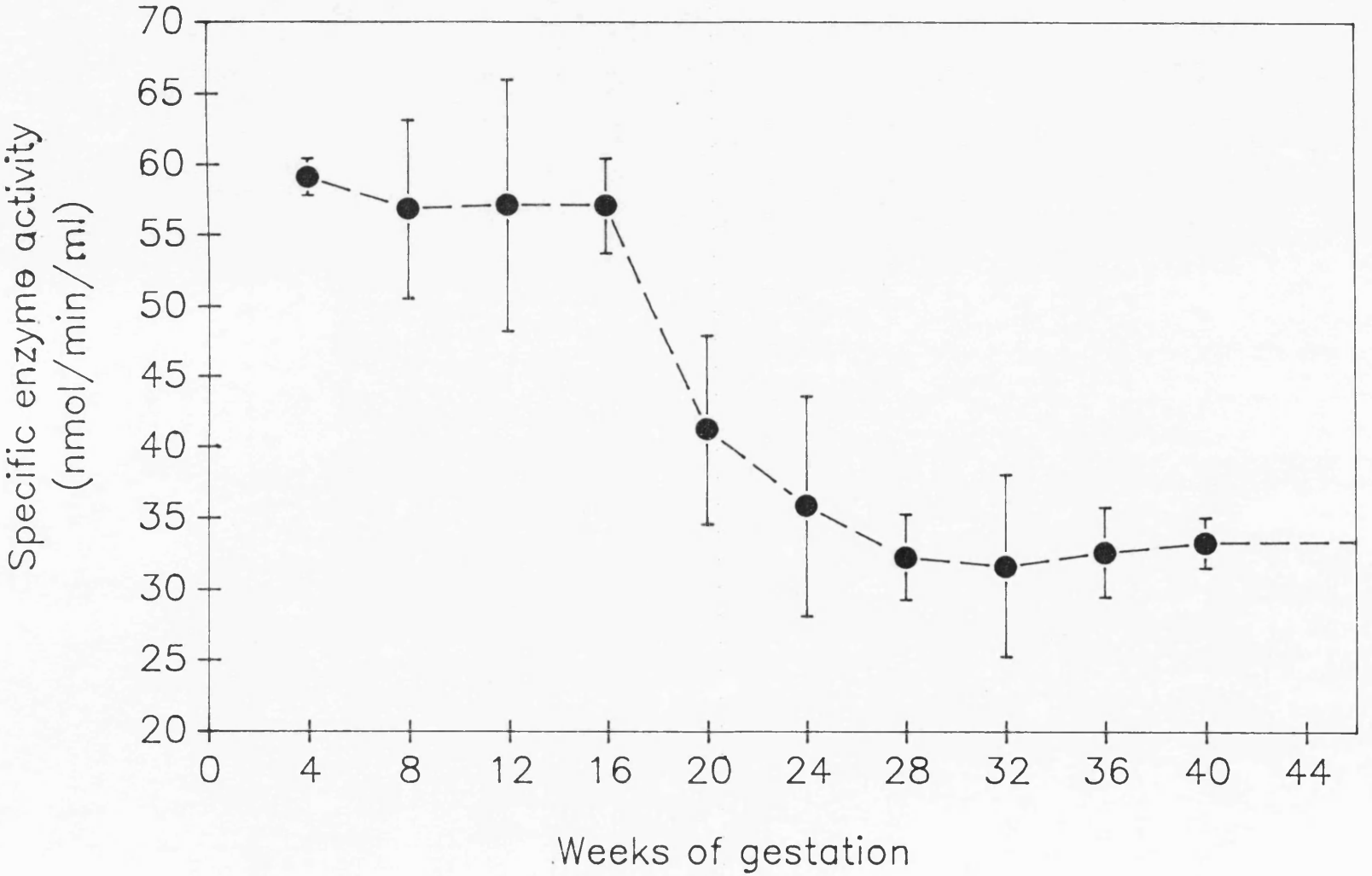


Fig:1

Table: 1.

Comparison of specific activities of PAF acetylhydrolase activity in plasma obtained from (a): controls (normotensive nonpregnant females (23-34 yrs n=7) and normotensive males (18-33 yrs n=5), (b): normotensive pregnant females at 1st, 2nd, 3rd trimesters n=15-20 in each trimester), (c): normotensive females in full term spontaneous labour (n=15), (d) 48 hrs after full term spontaneous vaginal delivery of normotensive females (n=12).
PAF acetylhydrolase activity was assayed as described in Fig:1.
Results are Mean ± S.D.

Normotensive males males	Normotensive females (non-preg)	Normotensive females (preg)	Normotensive postpartum (48hrs)
45 (3.5)	48 (5.2)	33 (3.7)	47 (6)

Fig:2.

The specific activity of PAF acetylhydrolase in human amniotic fluid obtained transvaginally, from women in full term spontaneous labour. The enzyme activity was assayed by incubation of ^{14}C PAF (0.18 nM/ml, 0.1uCi/ml) with 0.1ml/ml of amniotic fluid for 30 min at 37°C by the method of Blank et al (1981). Boiled enzymes served as blanks (only 0.45% of control enzyme activity). The incubation were stopped by 1 M citric acid and lipids were extracted and separated by one-dimensional thin layer chromatography. The separated radioactive products were identified and quantified by the use of Berthold TLC analyzer (Saeed et al, 1988). The specific activity was calculated by estimated nanomoles of lyso-PAF formed/min/ml of amniotic fluid.
The values are Mean ± S.D ,n=5.

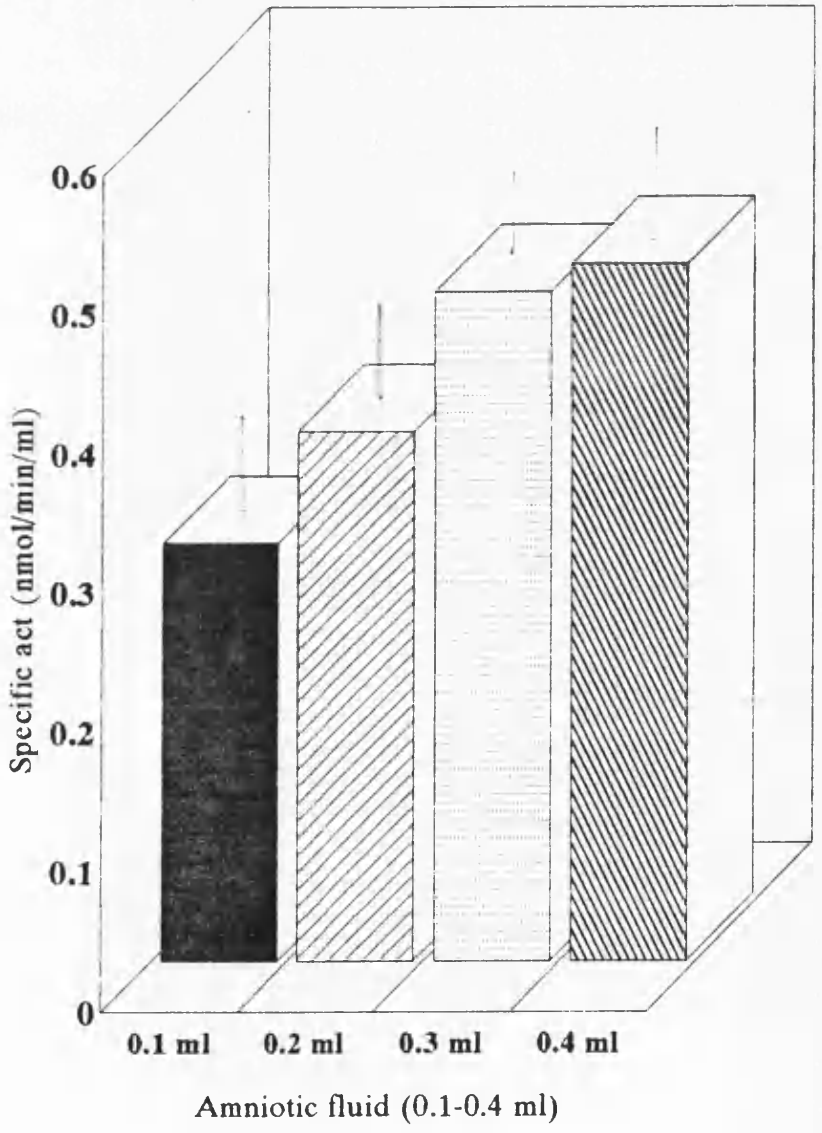


Fig:3.
The comparison of PAF acetylhydrolase in (a): amniotic fluid obtained from early gestation (16-17 weeks) during amniocentesis (n=5) (transuterinally).
(b): amniotic fluid obtained from at term, not in labour and undergoing elective caesarian section (n=5) (transuterinally).
(c): amniotic fluid obtained from women in full term sponatneous active labour (n=6) (transvaginally).
The enzyme activity was assayed by incubation of ^{14}C PAF (0.18 nM/ml, 0.1uCi/ml) with 0.1ml/ml of amniotic fluid for 30 min at 37°C by the method of Blank et al (1981). Boiled enzymes served as blanks (only 0.45% of control enzyme activity). The incubation were stopped by 1 M citric acid and lipids were extracted and separated by one-dimensional thin layer chromatography. The separated radioactive products were identified and quantified by the use of Berthold TLC analyzer (Saeed et al, 1988). The specific activity was calculated by estimated nanomoles of lyso-PAF formed/min/ml of
amniotic fluid.
The results are Mean ± S.D.

Fig:3

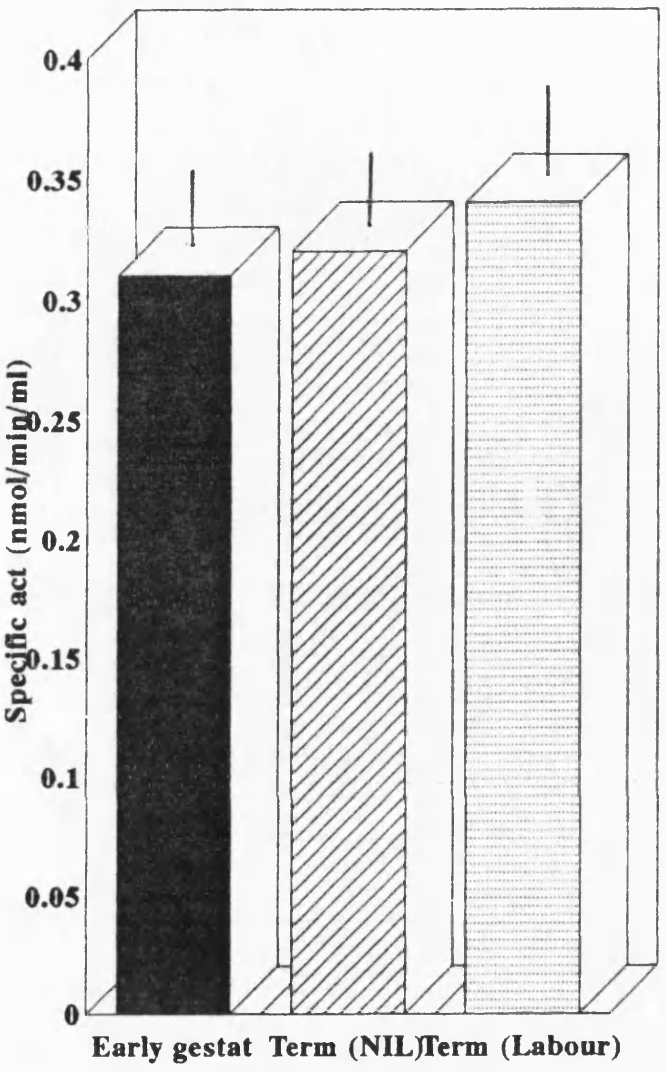

0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Specific act (nmol/min/ml)
Early gestat
Term (NIL)
Term (Labour)

Table: 2.

PAF acetylhydrolase enzyme activity in various cellular fractions (crude homogenate, mitochondria, microsomal fraction and cytosol) of chorion, amnion, deidua vera and placental cotyledons of the placentae obtained from women in full term normal spontaneuos vaginal delivery.

	Crude homogenate	Mitochondria	Microsomes	Cytosol
Chorion	7	3	0.9	9
Amnion	4	2	0.8	5
Decidua Vera	0.43	0.02	0.05	1
Placenta	0.01	0.001	0.002	0.02

Fig:4.
PAF acetylhydrolase activity (nmol/min/mg of protein) in amnion, chorion, decidua vera and placental cytosols obtained from normotensive women after full term spontaneous vaginal delivery and those undergoing elective caesarean section at term. The enzyme activity was assayed by incubation of ^{14}C PAF (0.18 nM/ml, 0.1uCi/ml) with 0.1mg/ml of cytosols for 30 min at 37°C by the method of Blank et al (1981). Boiled enzymes served as blanks (only 0.45% of control enzyme activity). The incubation were stopped by 1 M citric acid and lipids were extracted and separated by one-dimensional thin layer chromatography. The separated radioactive products were identified and quantified by the use of Berthold TLC analyzer (Saeed et al, 1988). The specific activity was calculated by estimated nanomoles of lyso-PAF formed/min/mg of protein.
The results are Mean ± S.D, n=7.

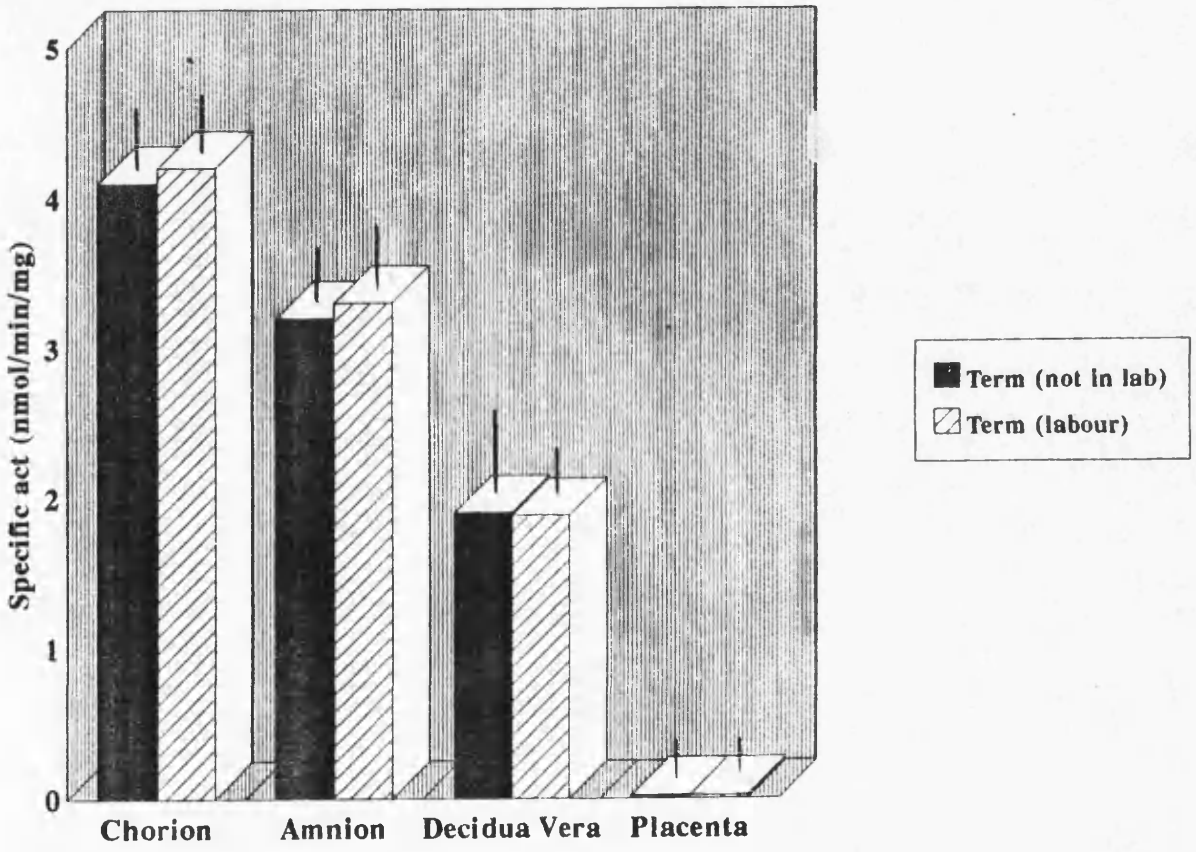


Fig: 5.

Effects of pre-incubation of PAF acetylhydrolase activity human amnion cytosol with PAF (80nm) at 0, 2, 5, 7, 10, 15, 20, 30, 60 min at 37°C. The enzyme activity was estimated by an assay of human platelet aggregation (Khan & Saeed, 1992), induced by control untreated-PAF (80nM) and cytosol-treated PAF (80nM). The enzyme activity was determined by percentage decrease in cytosol treated-PAF-induced aggregation.
The results are Mean ± S.D, n=6.

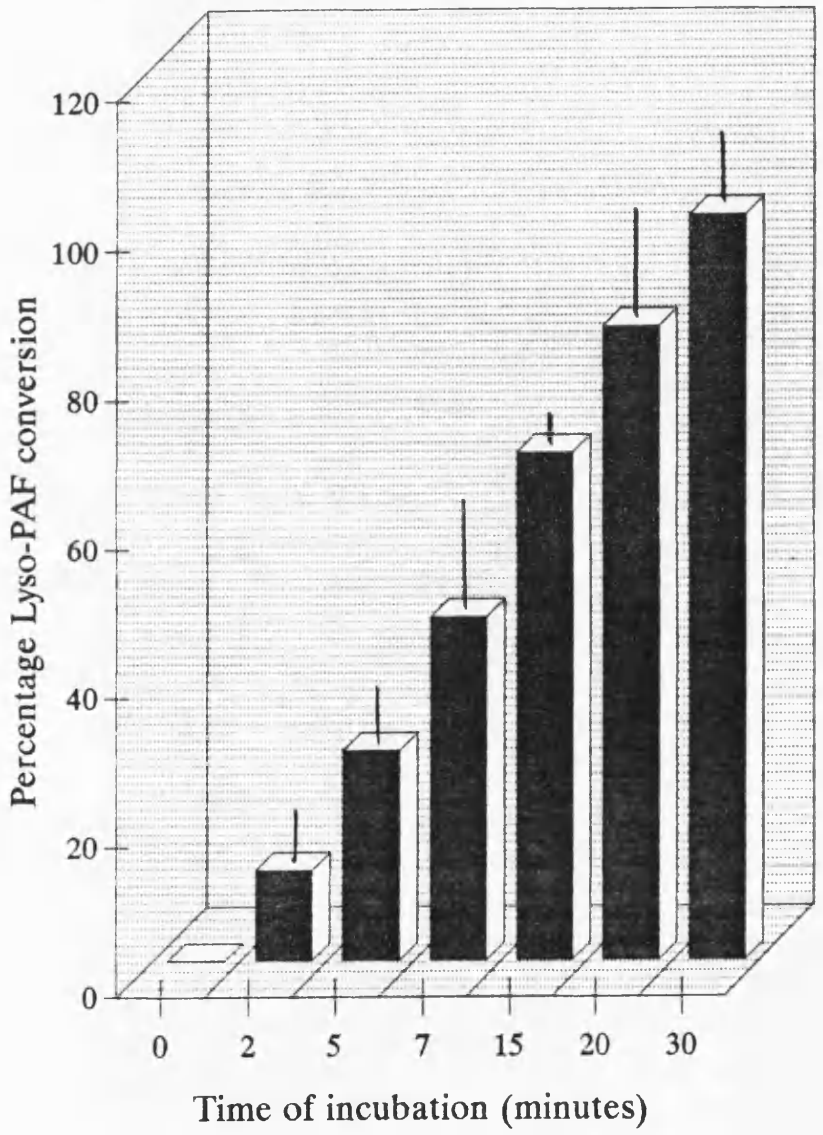


Fig: 6.
Effects of human cytosol (0.1mg) on (a) arachidonic acid (AA),
(b) ADP, (c) PAF and (d) collagen-induced human platelet aggregation.
(i) aggregating agent alone
(ii) cytosol alone
(iii) aggregating agent + cytosol.

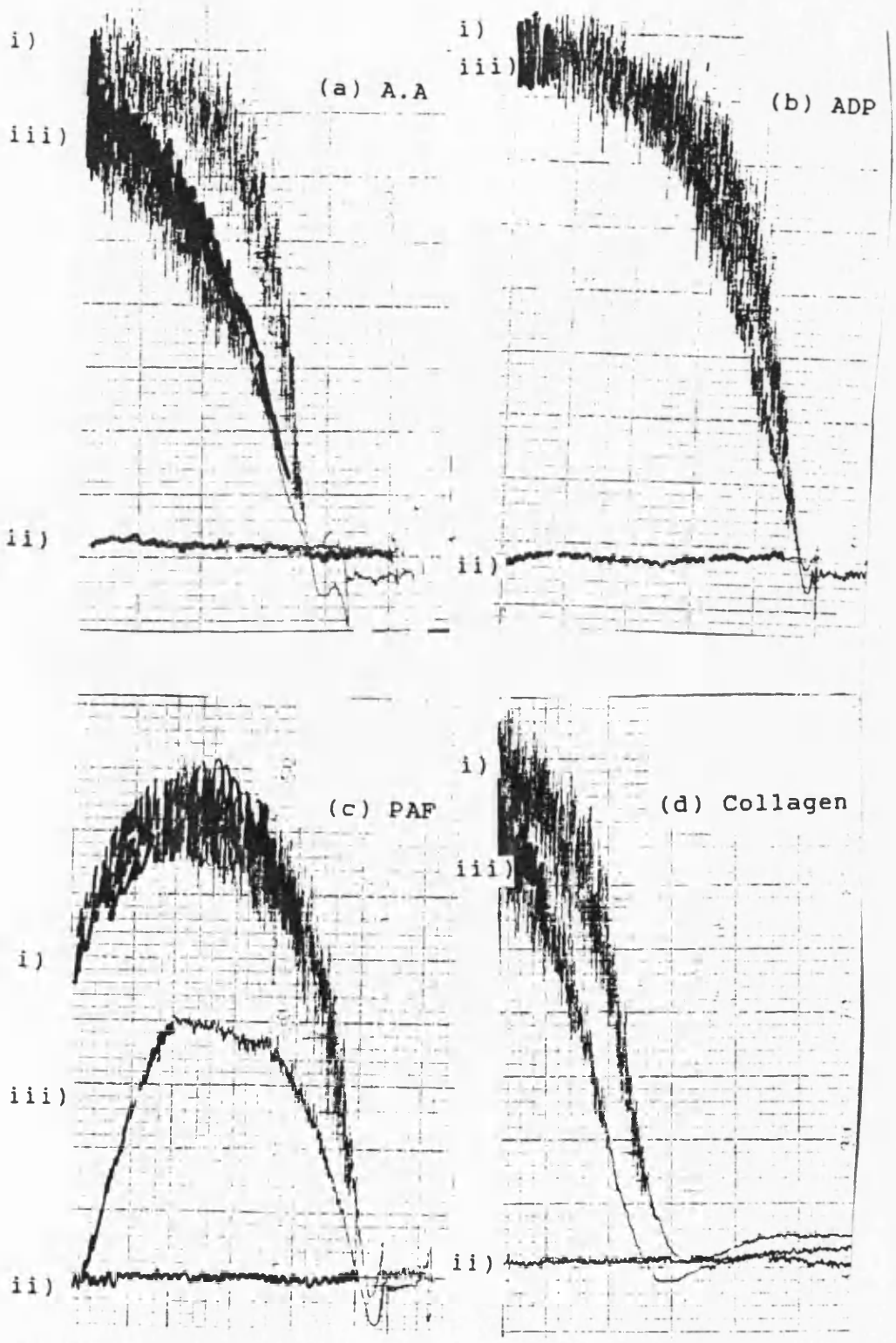


Fig: 7:
The activity of PAF acetylhydrolase in human amnion cytosol. The enzyme activity was assayed by incubation of ^{14}C PAF (0.18 nM/ml, 0.1uCi/ml) with 0-0.3 mg/ml of cytosols for 30 min at 37°C by the method of Blank et al (1981). Boiled enzymes served as blanks (only 0.45% of control enzyme activity). The incubation were stopped by 1 M citric acid and lipids were extracted and separated by one-dimensional thin layer chromatography. The separated radioactive products were identified and quantified by the use of Berthold TLC analyzer (Saeed et al, 1988). The enzyme activity is described as percentage lyso-PAF production.
Values are Mean S.D, n=5.

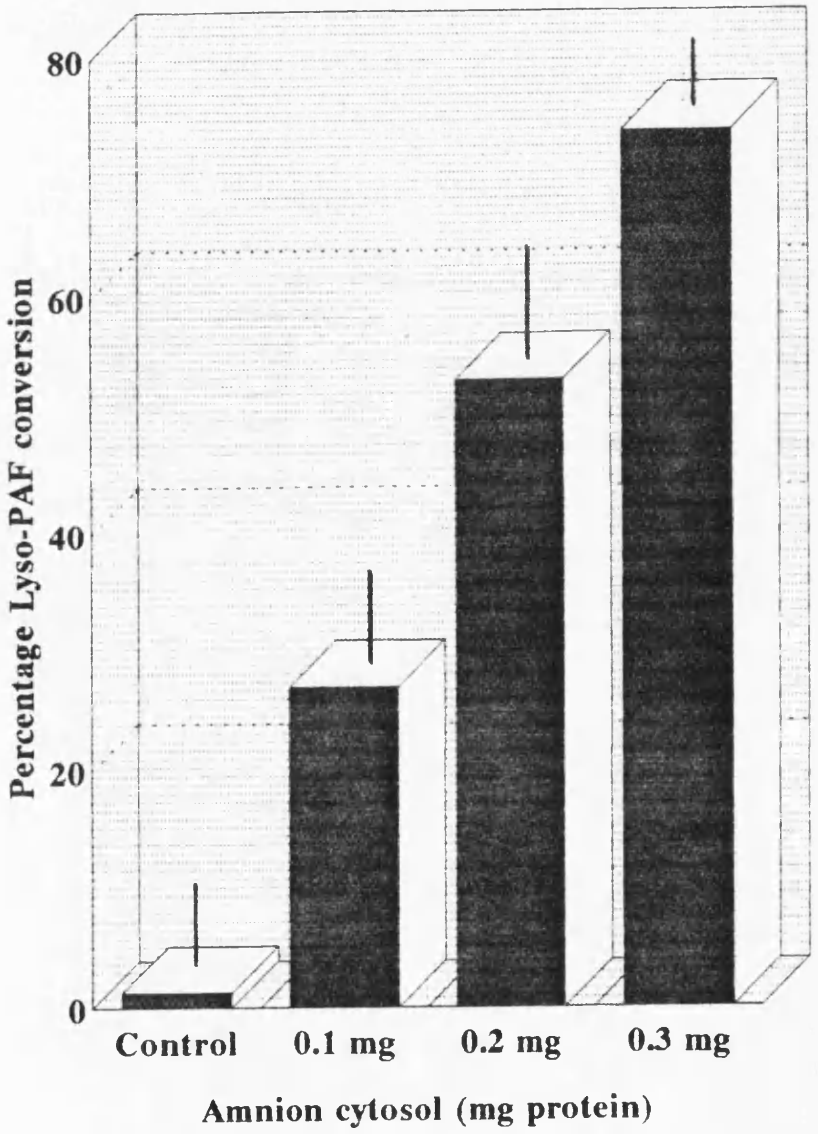


Fig:8.
Effects of temperature on cytosolic activity. The cytosols were heated at 4°, 37°, 56°, 80° and 100°C for 5 min. The enzyme activity was estimated by an assay of human platelet aggregation (Khan & Saeed, 1992), induced by control untreated-PAF (80nM) and cytosol-treated PAF (80nM). The effect of change in temperature on cytsol activity was noted by its effect on PAF-induced aggregation.

Results are Mean ± S.D, n=4.

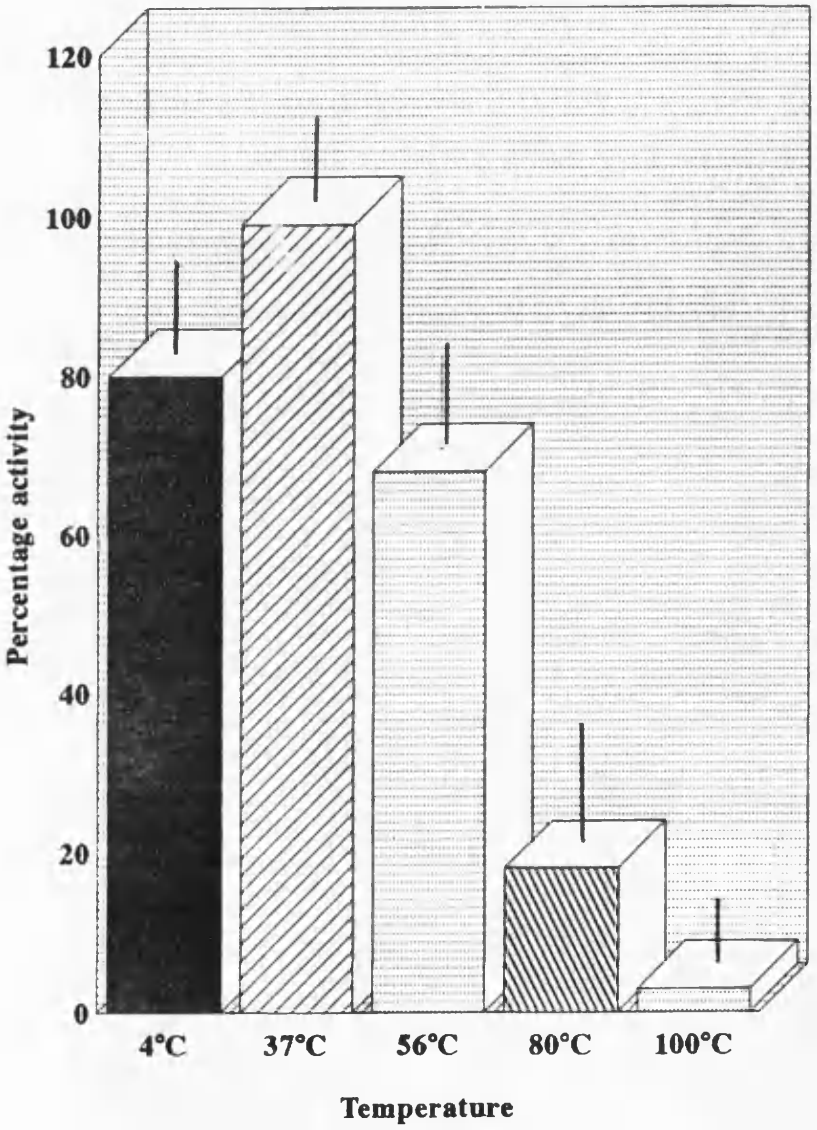


Fig:9.

Effects of changes in pH on amnion cytosol acetylhydrolase activity as measured by PAF (80nM)-induced aggregation. The cytosol was incubated at pH, 7.4, 3, 6.8, 10.0 for 20 min and PAF (80nM) was incubated with these cytosols for 30 min at 37°C. The effects of pH-treated cytosols was compared with that of pH 7.4 treated-cytosol on PAF-induced aggregation, (Khan & Saeed, 1992).
$p<0.001$: pH 7.4 vs pH 3.0.
$p<0.01$: pH 7.4 vs pH 6.8 & 10.0
The results are mean ± s.d, n=3.

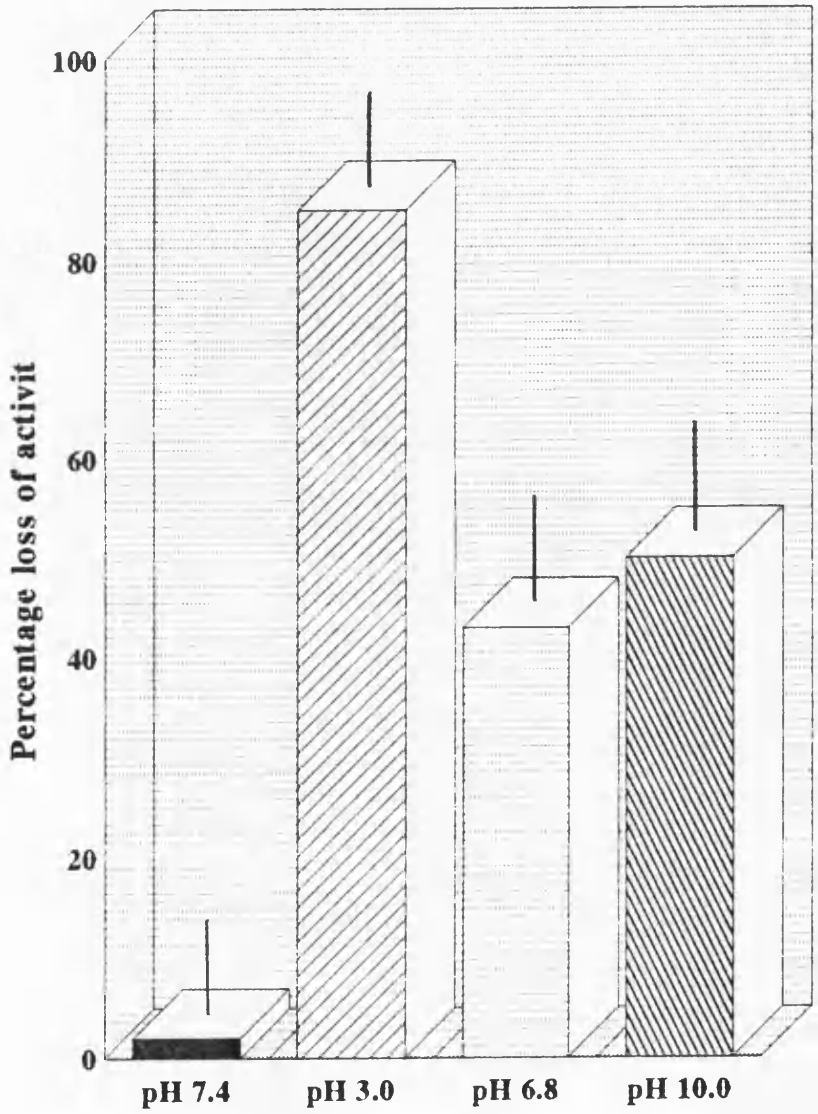


Fig:10.

Effects of overnight dialysis on PAF acetylhydrolase activity in human amnion cytosol. Dialysis of cytosol was carried out against 0.9% saline at 4°C for 48 hrs, using cellophane membrane. The effect of dialysis on amnion cytosolic enzyme was estimated by incubation of dialysed and undialysed (control) cytosol with PAF (80nM) for 30 min at 37°C and subsequently assaying human platelet aggregation induced by dialysed cytosol-treated PAF and undialysed cytosol-treated.
Results are Mean ± S.D, n=3.

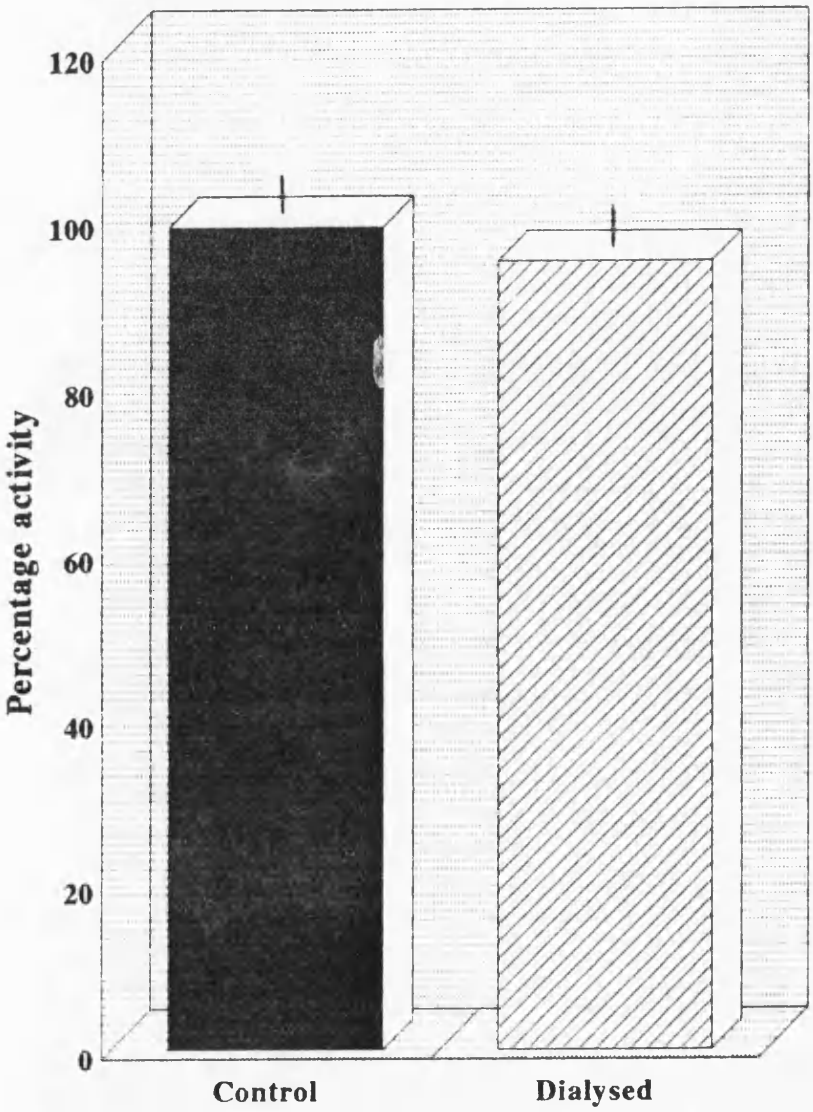


Table:3.
(a):
PAF acetylhydrolase activity in amnion cytosol after amicon cell ultrafiltration at 4°C. The enzyme activity in filterates and retentants by an assay of human platelet aggregation (Khan and Saeed, 1992). Filtered as well as corresponding retentant cytosol were incubated with PAF (80nM) at 37°C for 30 min. Platelet-aggregation was recorded in untreated PAF and that incubated with various cytosols. The results are described as Mean ± S.D, N=3.
(b):
PAF acetylhydrolase activity in various fractions of amnion cytosol after Sephadex-G 50, 100 column chromatography. The enzyme activity was estimated by an assay of human platelet aggregation, as described above (a). The results are Mean ± S.D.

	Control	XM-10	XM-30	YM-100
Filterate	100%	2%	20%	78 (8.5)
Retentant		99%	75%	30 (6.4)

Values are percentage mean aggregation (s.d)

	Control	Sephadex-G 50	Sephadex-G 100
	100		
Fraction		58 (7.7)	85 (12)
(0-10)			
Fraction		49 (8.30	23 (6.4)
(11-15)			

Fig:11.

Effects of organic solvents on amnion cytosolic acetylhydrolase activity. Amnion cytosol was incubated with various solvents for 10 min at 37°C. The enzyme activity was estimated by an assay of human platelet-aggregation induced by PAF (80nM). PAF was incubated with untreated and organic solvent-treated cytosol for 30 min at 37°C and platelet aggregation recorded (Khan & Saeed, 1992).

Results are Mean ± S.D, n=5.
$p < 0.001$: control (untreated cytosol) vs organic solvent-treated.

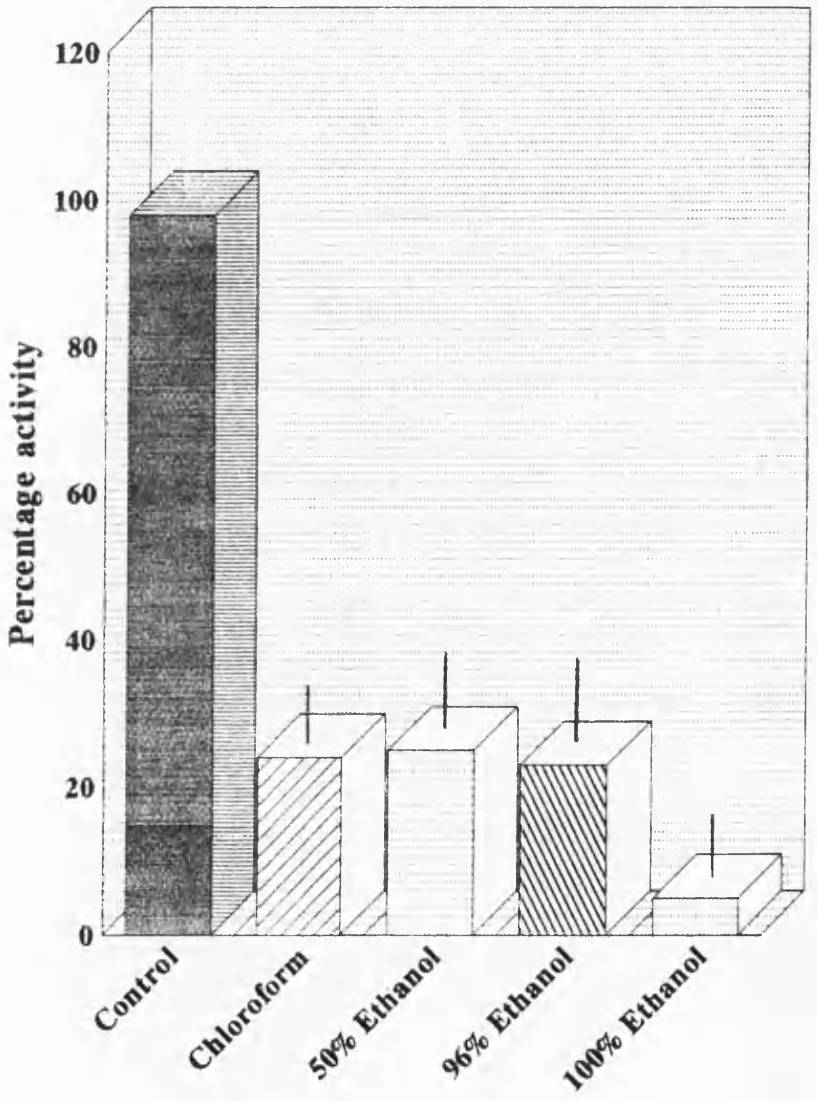


Fig: 12.
Effects of various inhibitors on PAF-acetylhydrolase activity in human amnion cytosol. Amnion cytosol was incubated with various inhibitors for 30 min at 37°C. The enzyme activity was estimated by an assay of human platelet-aggregation induced by PAF (80nM). PAF was incubated with untreated and inhibitor-treated cytosol for 30 min at 37°C and platelet aggregation recorded (Khan & Saeed, 1992). The results are mean ± s.d, n=3.
TCA: 20 %Trichloroacetic acid.Protease: 20 microunit/ml.
PMSF: Phenylmethylsulphonylfluoride (5nM).DFP: Diisofluorophosphate (2nM). $p < 0.01$. control vs inhibitor-treated activity.

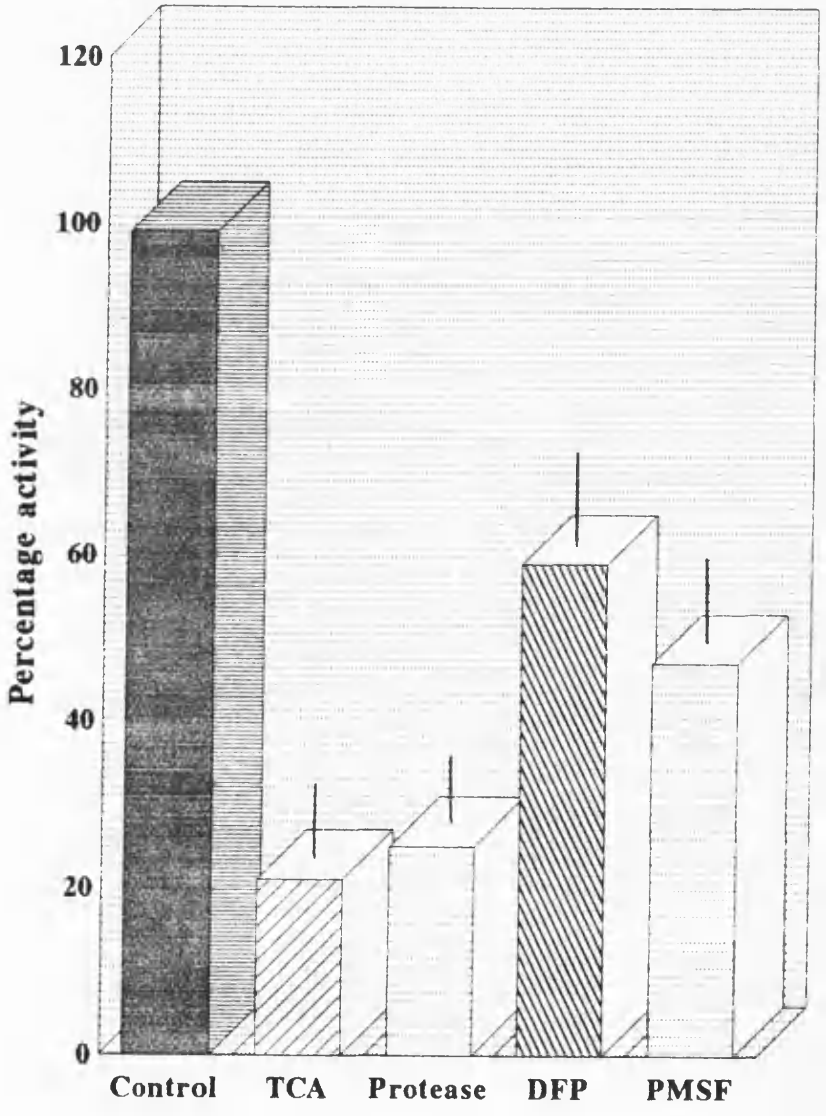


Table:4.

Effect of freezing and thawing of human amnion cytosolic protein on PAF acetylhydrolase enzyme activity. The enzyme activity was estimated as described in the methods.

Control	**98 (5.9)**
Freeze-thawing	**96 (7.4)**

Fig: 13.

Effects of various doses of Prostaglandin E_2 Prostaglandin $F_{2\alpha}$ and Prostacyclin on PAF acetyl hydrolase activity in human amnion cytosol. The enzyme activity was assayed by incubation of ^{14}C PAF (0.18 nM/ml, 0.1uCi/ml) with 0.1 mg/ml of cytosols in presence and absence of various doses (0-10 micrograms in 0.01% ethanol) of Prostaglandins for 30 min at 37°C by the method of Blank et al (1981). Boiled enzymes served as blanks (only 0.45% of control enzyme activity).
Values are Mean ± S.D, n=3.

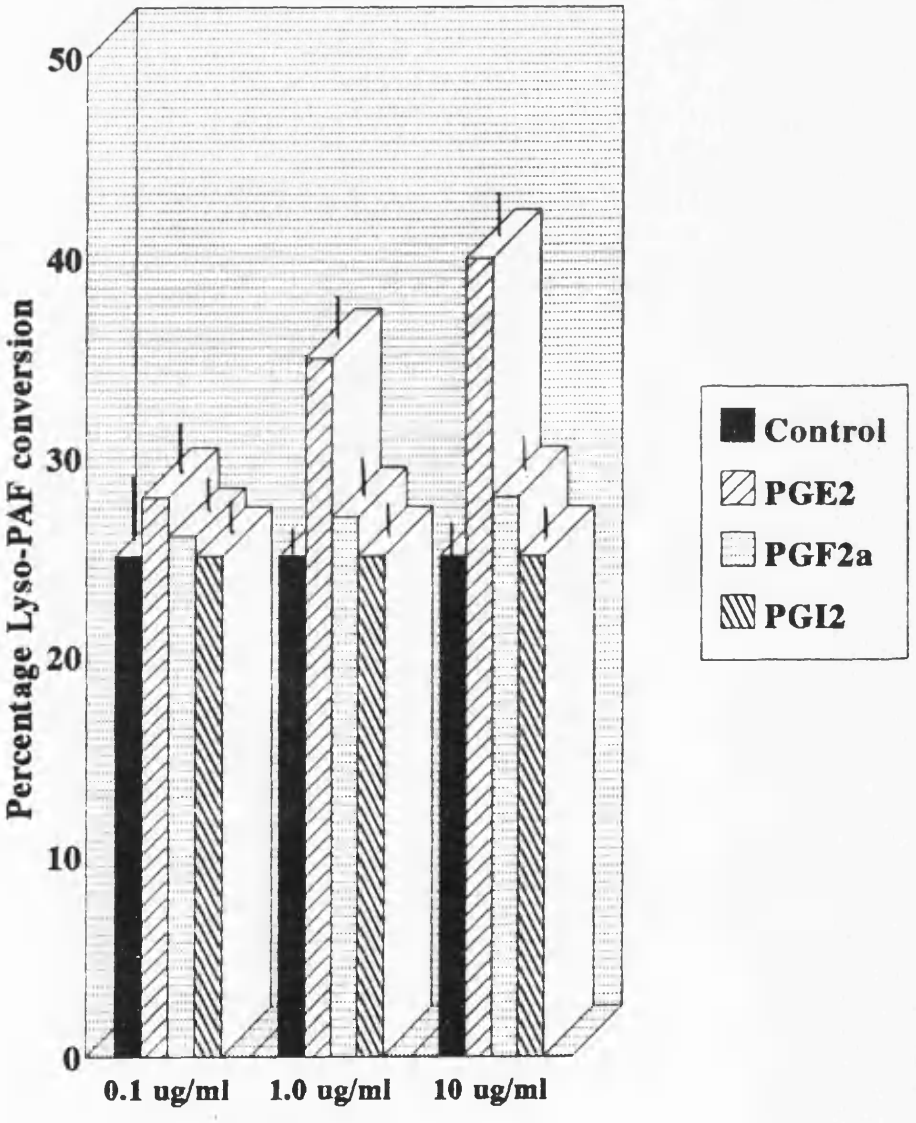


Fig:14.
The dose-related effects of oxytocin (0-10 microgram) on PAF acetylhydrolase activity in human amnion cytosol. The enzyme activity was assayed by incubation of ^{14}C PAF (0.18 nM/ml, 0.1uCi/ml) with 0.1 mg/ml of cytosol in presence and absence of various doses (0-10 mU in 0.9% saline) of oxytocin for 30 min at 37°C by the method of Blank et al (1981). Boiled enzymes served as blanks (only 0.45% of control enzyme activity). The enzyme activity is described as percentage lyso-PAF production.
Values are Mean±S.D, n=3

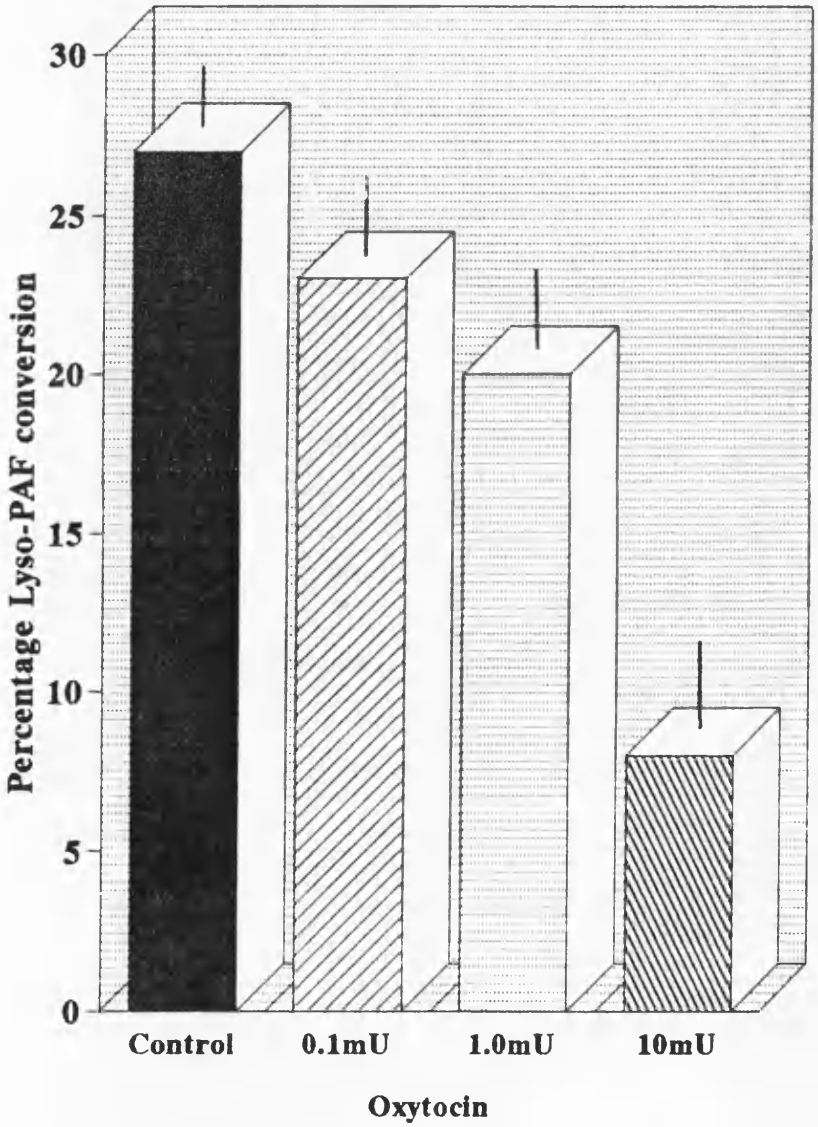


Fig:15.
The effects of treatment of human amnion cells in monolayer culture with various doses of oxytocin on PAF acetylhydrolase. The amnion cells were incubated with oxytocin (0-10 mU in 0.9% saline) with TLCK (50ug/plate) as general protease inhibitor in monolayer culture grown in humidified air (95%) with 5% CO^2 at 37°C for 48 hrs. The cytosols obtained from treated and untreated cells was used for the enzyme assay. The enzyme activity was assayed by incubation of ^{14}C PAF (0.18 nM/ml, 0.1uCi/ml) with 0.1 mg/ml of cytosol for 30 min at 37°C by the method of Blank et al (1981). Boiled enzymes served as blanks (only 0.45% of control enzyme activity). The enzyme activity is described as percentage lyso-PAF production. The values are Means ±S.D of three separate experiments.

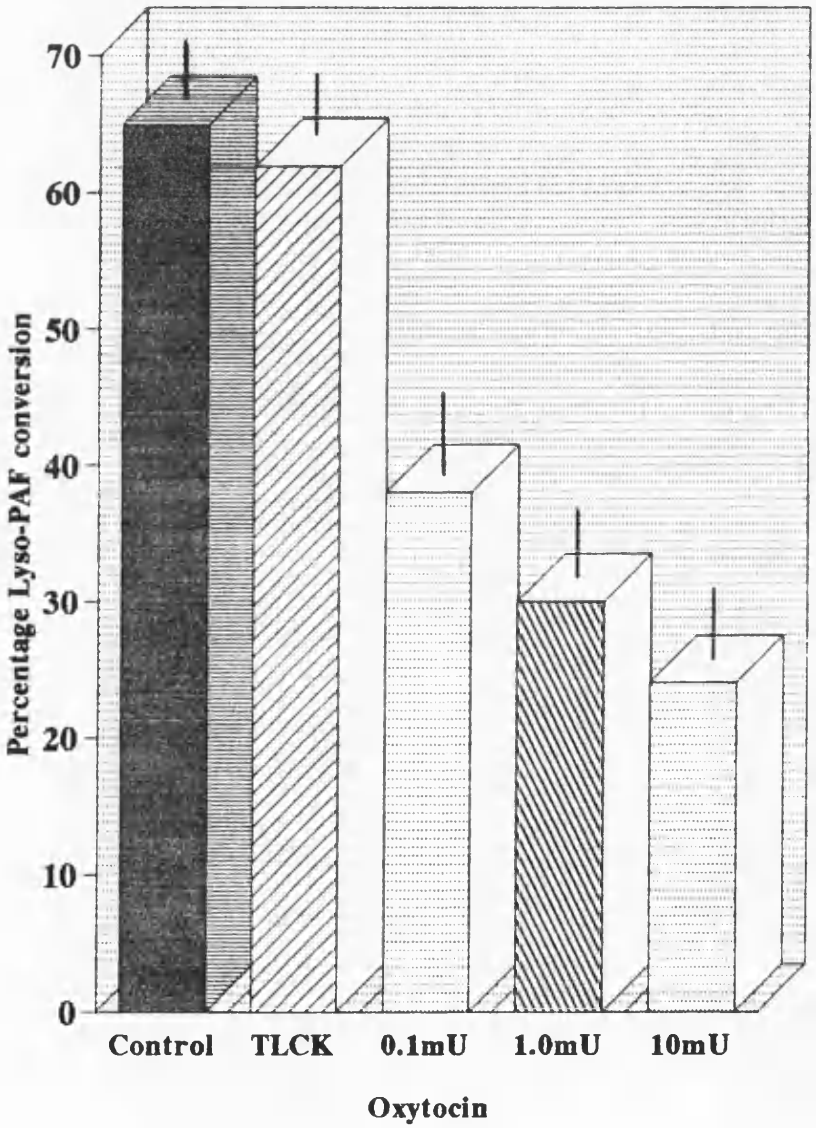


Fig: 16
The effects of various doses of 17-ß-Oestradiol on PAF acetylhydrolase in human amnion cells. The amnion cells were incubated with oestradiol (0-10 mM in 0.01 DMSO) in monolayer culture grown in humidified air (95%) with 5% CO_2 at 37°C for 48 hrs. The cytosols obtained from treated and untreated cells was used for the enzyme assay. The enzyme activity was assayed by incubation of ^{14}C PAF (0.18 nM/ml, 0.1uCi/ml) with 0.1 mg/ml of cytosol for 30 min at 37°C by the method of Blank et al (1981). The enzyme activity is described as percentage lyso-PAF production. The values are Means± S.D, n=3.

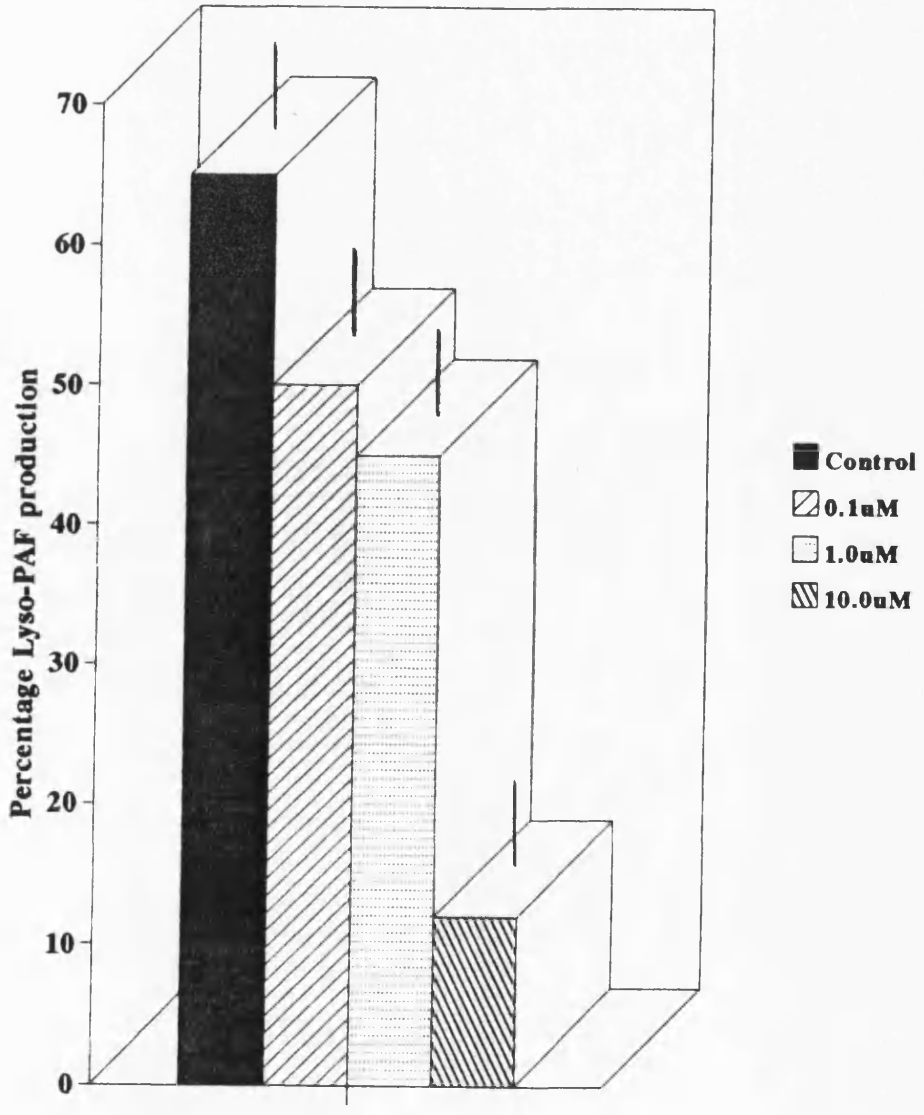


Fig:17.

The effects of 17-ß-Oestradiol on PAF acetylhydrolase in human amnion cytosol. The enzyme activity was assayed by incubation of ^{14}C PAF (0.18 nM/ml, 0.1uCi/ml) with 0.1 mg/ml of cytosol in presence and absence of various doses of oestradiol (0-10 mM in 0.05% DMSO) for 30 min at 37°C by the method of Blank et al (1981). The enzyme activity is described as percentage lyso-PAF production.
Values are Mean ± S.D, n=3.

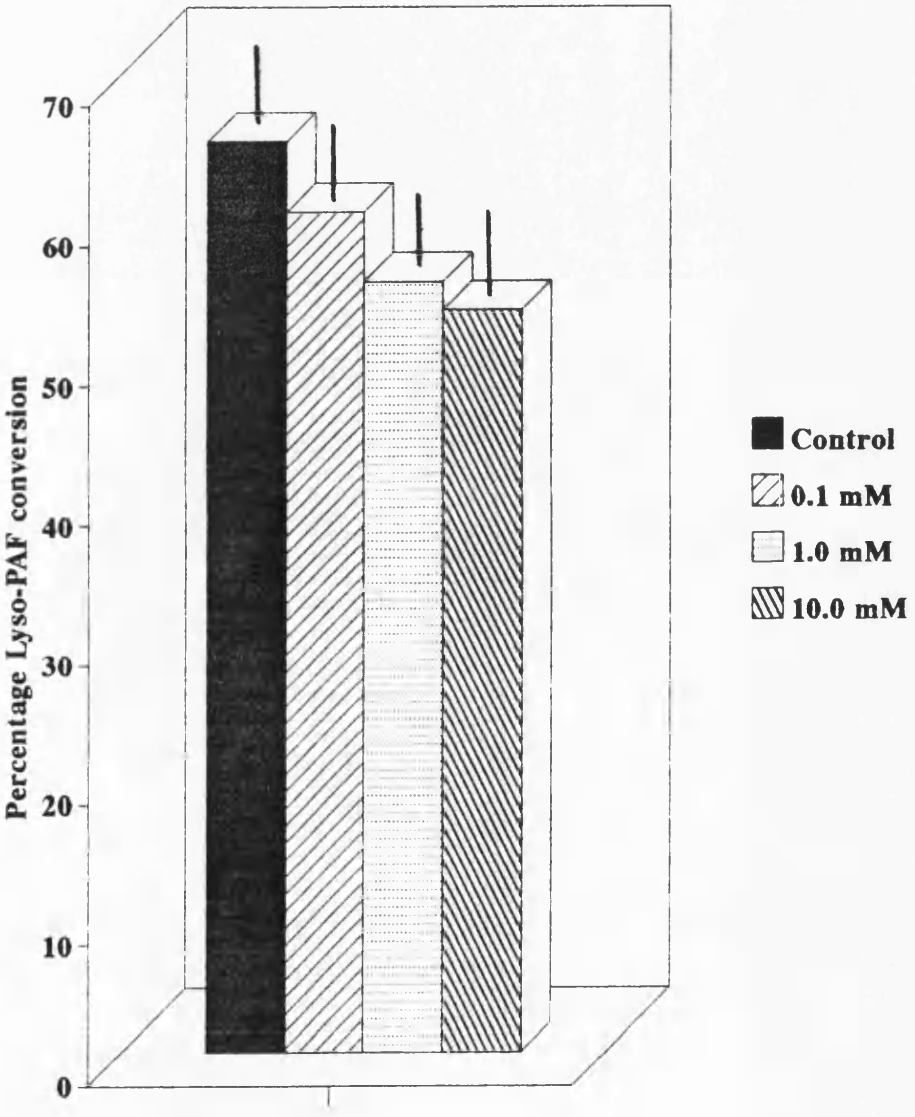


Fig:18.
The effects of various doses of Progesterone on PAF acetylhydrolase in human amnion cells. The amnion cells were incubated with Progesterone (0-10 mM in 0.01 DMSO) in monolayer culture grown in humidified air (95%) with 5% CO^2 at 37°C for 48 hrs. The cytosols obtained from treated and untreated cells was used for the enzyme assay. The enzyme activity was assayed by incubation of ^{14}C PAF (0.18 nM/ml, 0.1uCi/ml) with 0.1 mg/ml of cytosol for 30 min at 37°C by the method of Blank et al (1981). The enzyme activity is described as percentage lyso-PAF production. The values are Means ± S.D, n=3.

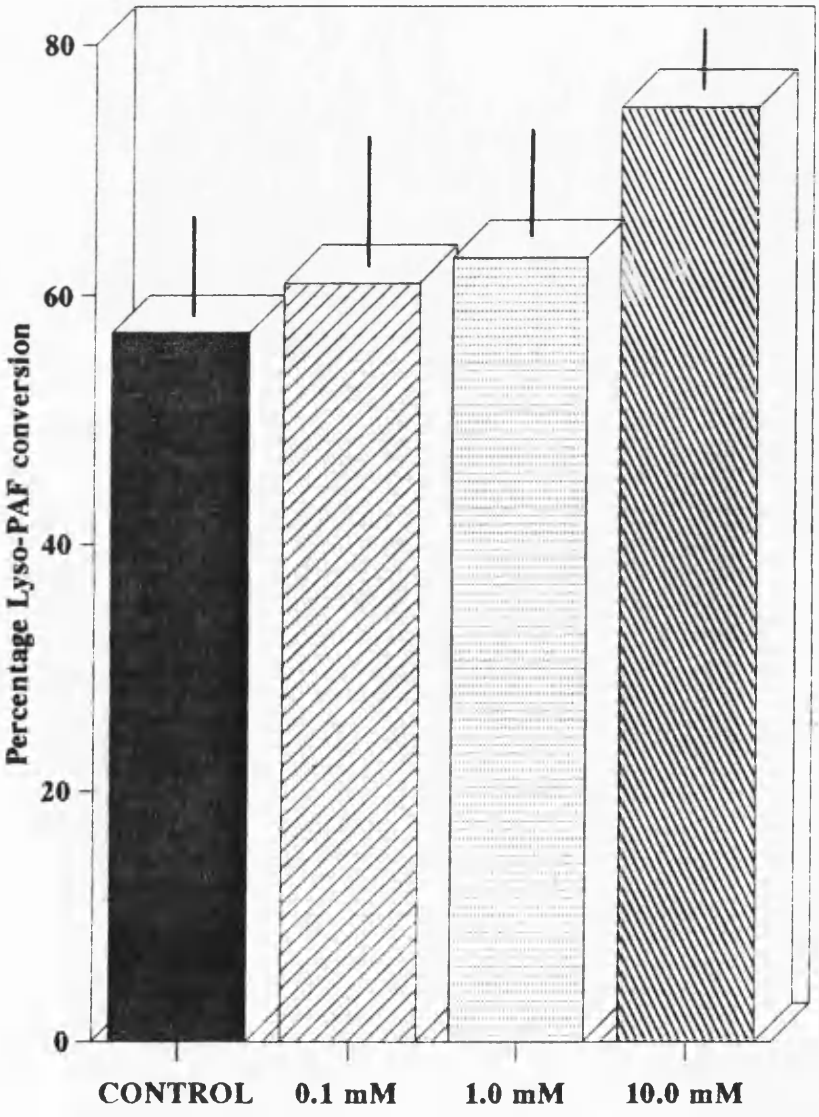
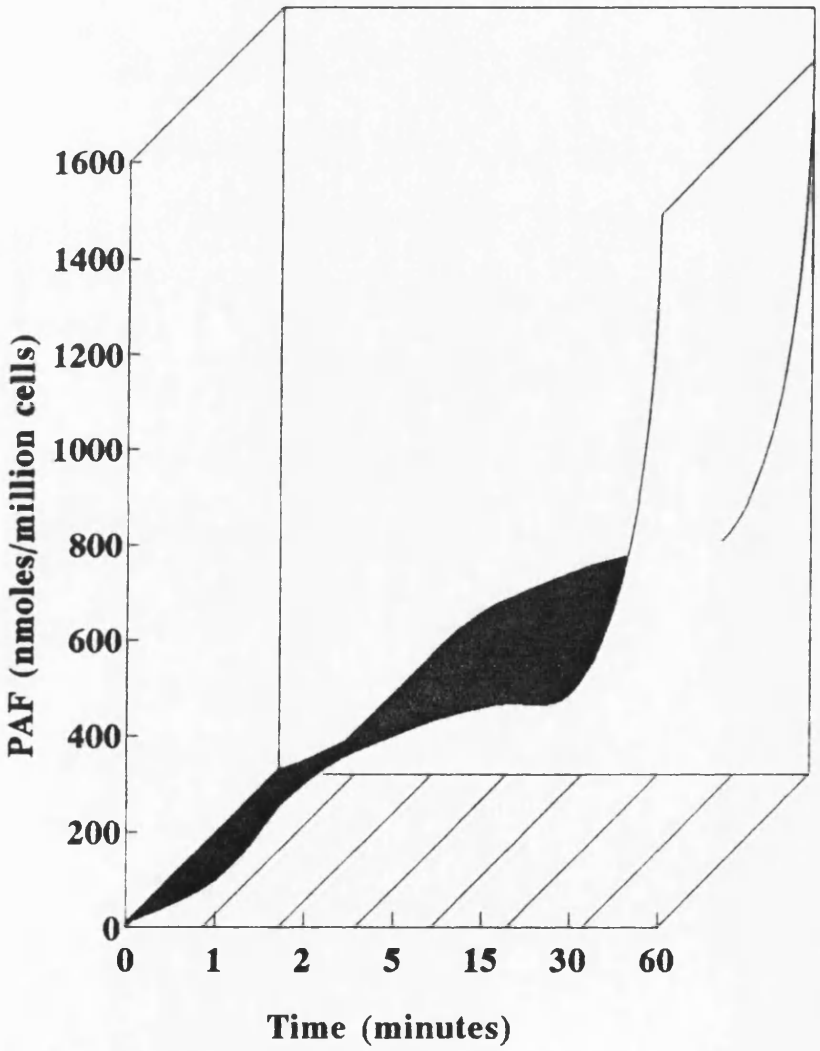
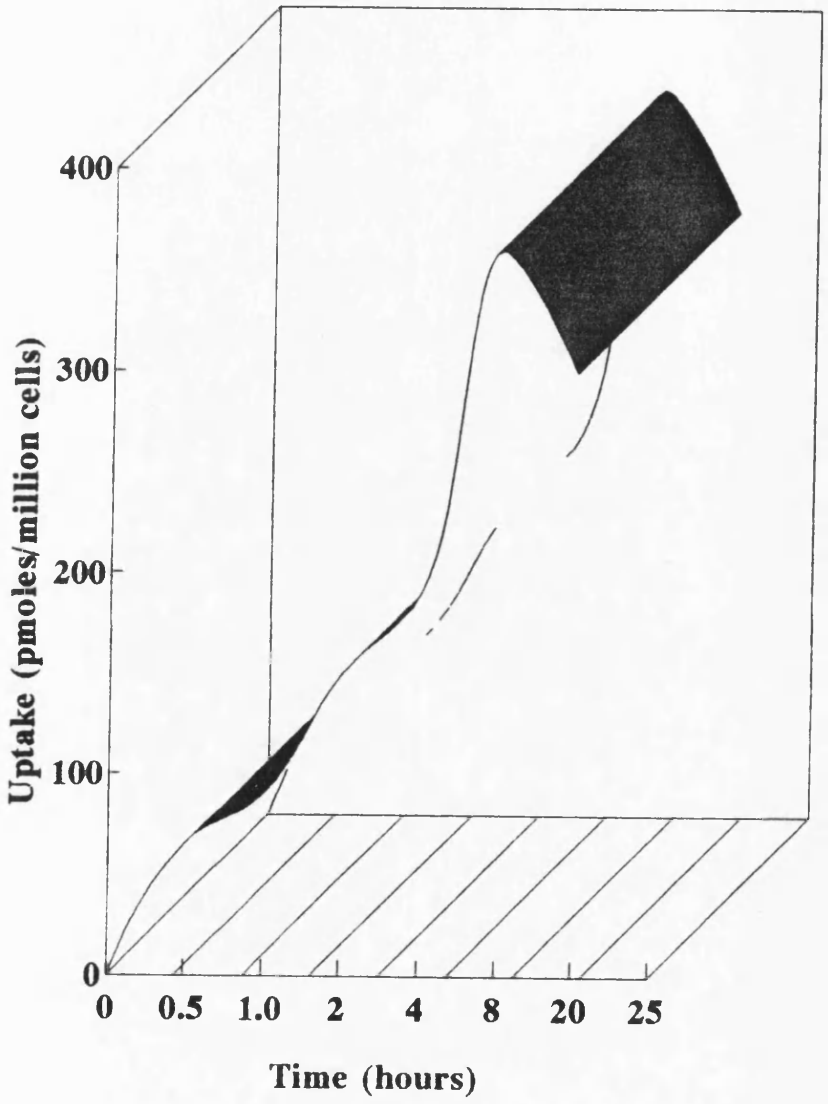


Fig:19.
The time-course profile of ^{14}C PAF uptake by human amnion cells in culture. 0.18 nM PAF (0.1uCi) in 0.01% bovine serum albumin was incubated with amnion cells for 0, 1, 2, 5, 15, 30, 60 min. After each time-point, the cells were washed with phosphate buffer saline and treated with Chloroform : Methanol (2:1). The extracted radioactivity was measured by liquid scintillation counting after addition of Aquasol and Tissue solubilizer (Amersham).



Values are mean of three separate experiments

Fig:20.
The longer time-course (0-20 hours) profile of uptake of C^{14} PAF uptake by human amnion cells in culture. 0.18 nM PAF in 0.01% bovine serum albumin was incubated with amnion cells for 0, 30min, 1, 2, 5, 8, 20 hrs. After each time-point, the cells were washed with phosphate buffer saline and treated with Chloroform : Methanol (2:1). The extracted radioactivity was measured by liquid scintillation counting after addition of Aquasol and Tissue solubilizer (Amersham).



Values are mean of 3 experiments

Fig:21.
Radioactivity present in Dulbecco's culture medium at corresponding time-points after uptake of C^{14}-PAF by human amnion cells in culture. At each time-point, the medium was treated with Chloroform:Methanol (2:1). The extracted radioactivity was measured by liquid scintillation counting. Values are Mean ± S. D. n=3.

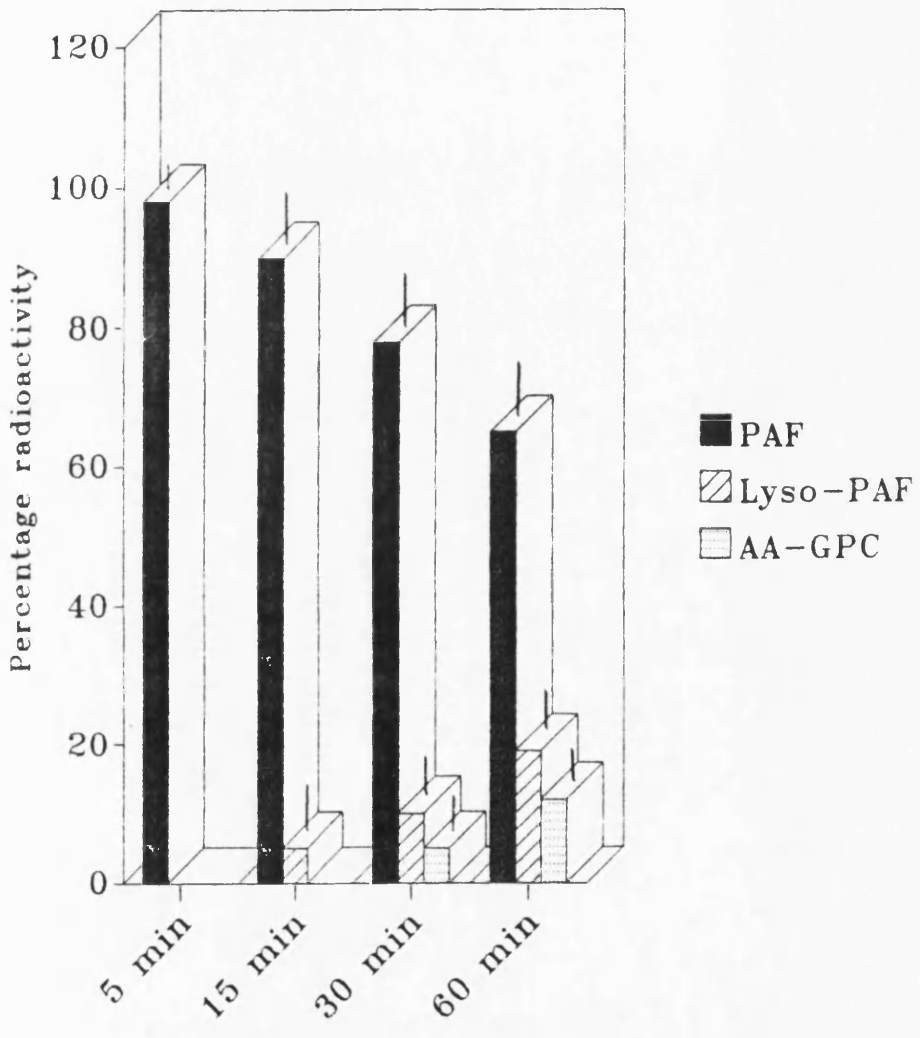


Fig:22.
Radiochromatogram showing the separation by thin layer chromatography of various phospholipids containing (0.18nM)[C^{14}]PAF in human amnion cells at various time-points by the method of Touchstone et al (1979).

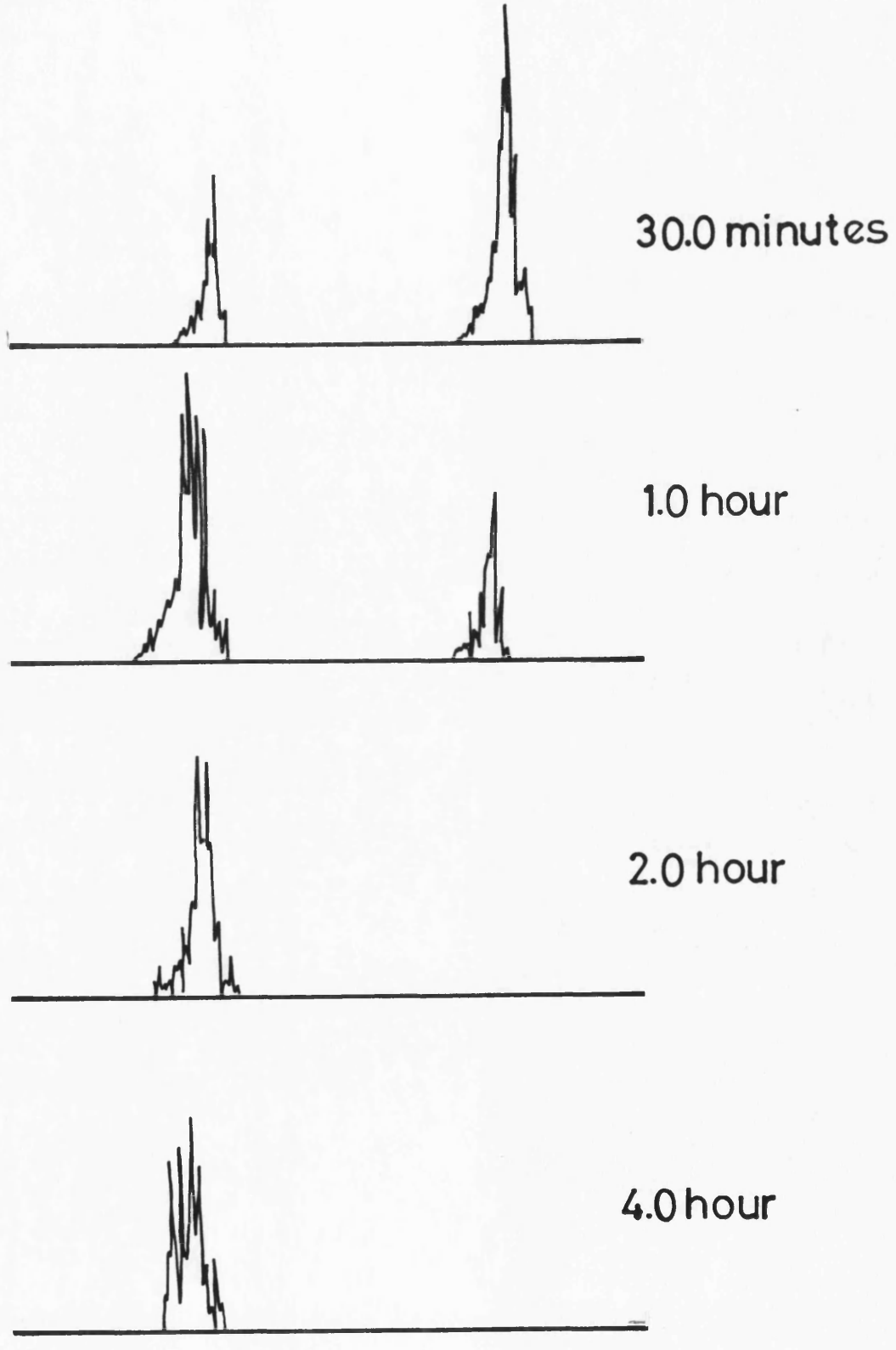


Fig:23.

Distribution of radioactivity in choline-containing phospholipids correponding in human amnion cells in culture at various time intervals. After each time points, the incubation were stopped by washing the cells with phosphate buffer saline and lipids were extracted and separated by one-dimensional thin layer chromatography in solvent system for choline-phospholipids (Ban et al, 1986). The separated radioactive products were identified and quantified by the use of Berthold TLC analyzer (Saeed et al, 1988).

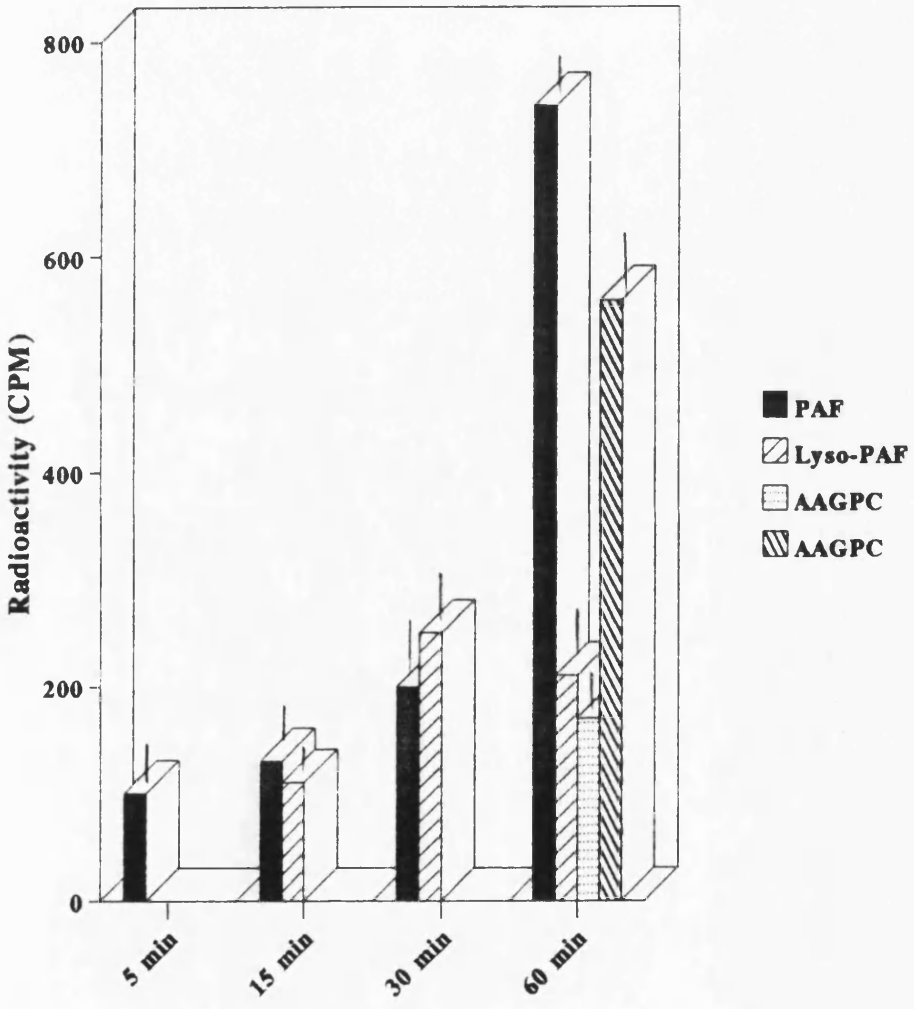


Fig: 24.
Distribution of radioactivity in choline-containing phospholipids correponding culture medium at various time intervals.
After each time points, the incubation were stopped and lipids from the culture medium, were extracted and separated by one-dimensional thin layer chromatography in solvent system for choline-phospholipids (Ban et al, 1986). The separated radioactive products were identified and quantified by the use of Berthold TLC analyzer (Saeed et al, 1988).

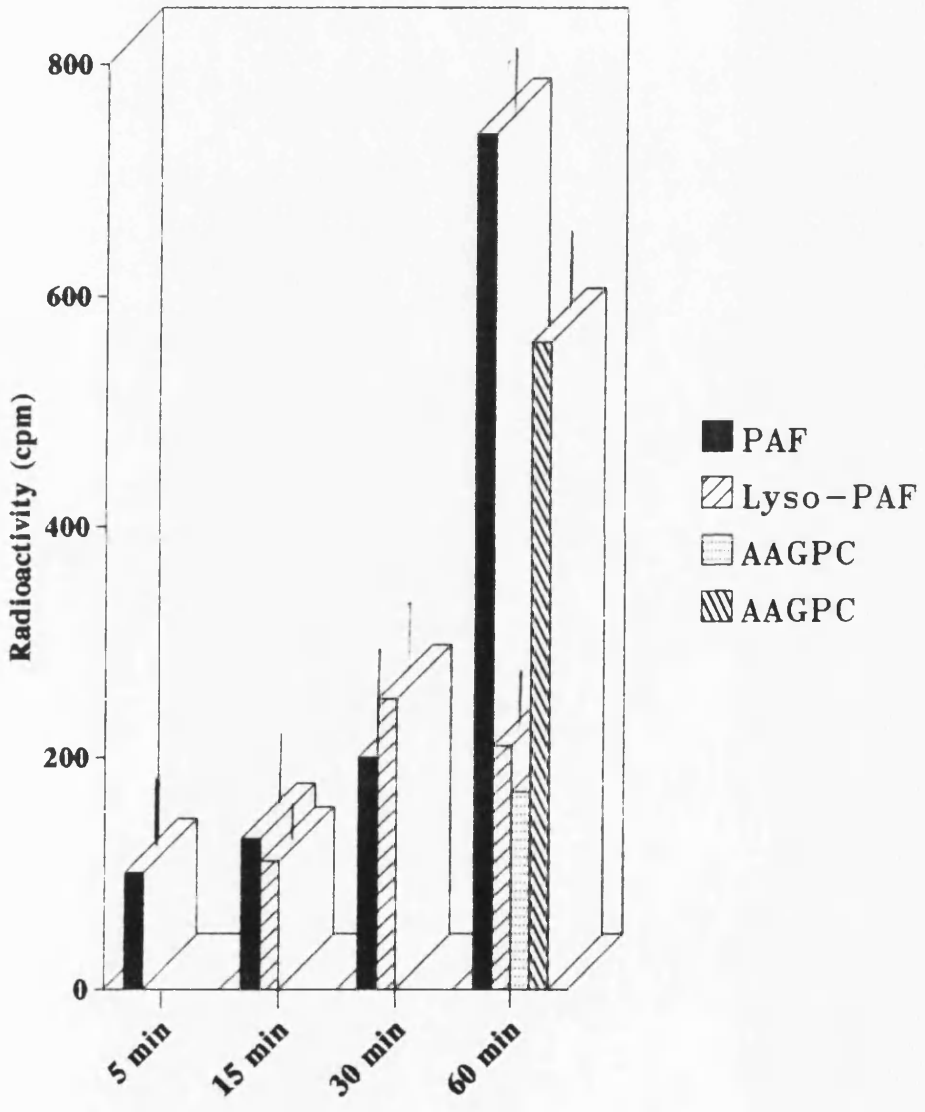


Fig:25.
Effects of 5.0 mUnits Phospholipase A_2-treatment on incorporated ^{14}C-PAF in human amnion cells in culture. The amnion cells were incubated with 1.8nM ^{14}C-PAF, washed with ice-cold phosphate buffer saline and treated with phospholipase A_2 for 20 min at 37°C (Robison et al, 1988). After incubation the cells were washed with phosphate buffer saline and treated with Chloroform : Methanol (2:1). The extracted radioactivity was measured by liquid scintillation counting after addition of Aquasol and Tissue solubilizer (Amersham).

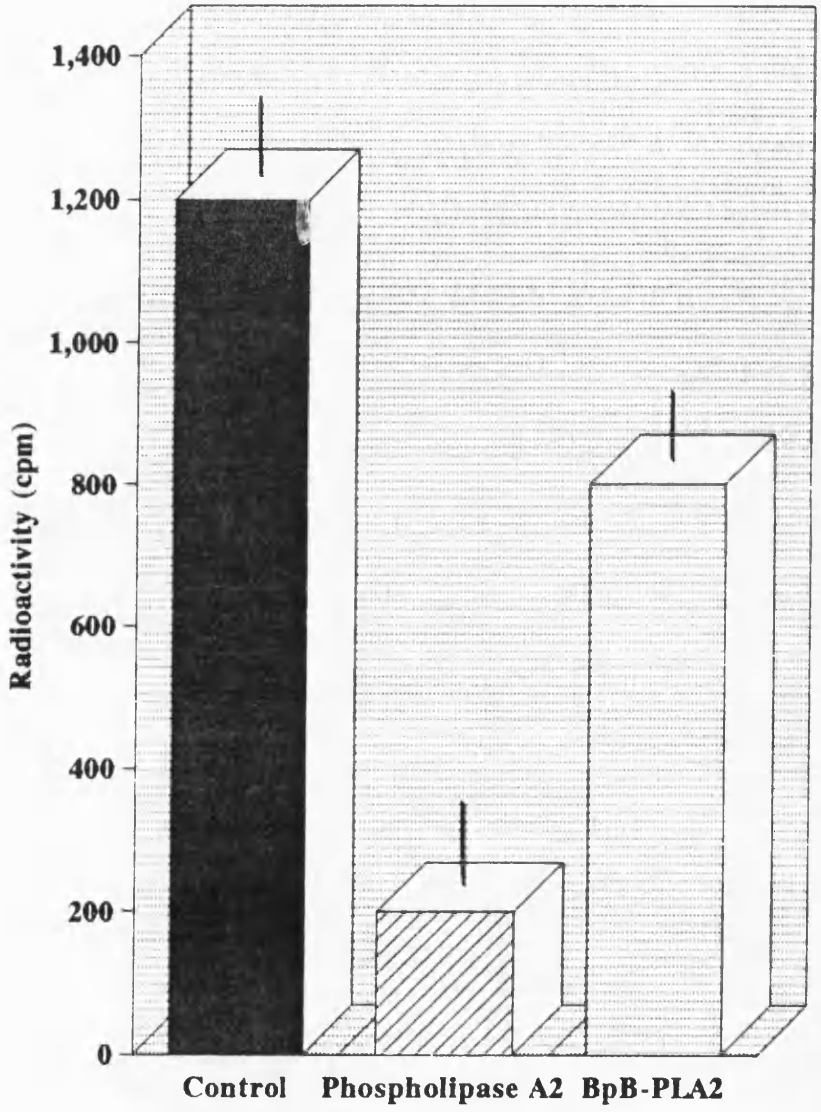


Fig: 26.
Effects of Bromophenacyl bromide on PAF metabolism in human amnion cells in culture. The amnion cells were incubated ^{14}C PAF (0.18 nM/plate, 0.1uCi in 0.01 % bovine serum albumin in presence and absence of BpB (0.5 mM) for 60 min at 37°C in humidified air (95%) with 5% CO_2 in an incubator. After 60 min, the incubation was stopped and cells treated with phospholipase A_2 for 20 min at 37°C. The cells were washed with phosphate buffer saline and lipids were extracted by the method of Bligh and Dyer (1959), by choloroform:methanol (2:1). The lipids were separated by one-dimensional thin layer chromatography in solvent system for choline-phospholipids (Ban et al, 1986). The separated radioactive products were identified and quantified by the use of Berthold TLC analyzer, coupled with chromatography data system (Saeed et al, 1988).
The results are representative of three separate experiments.

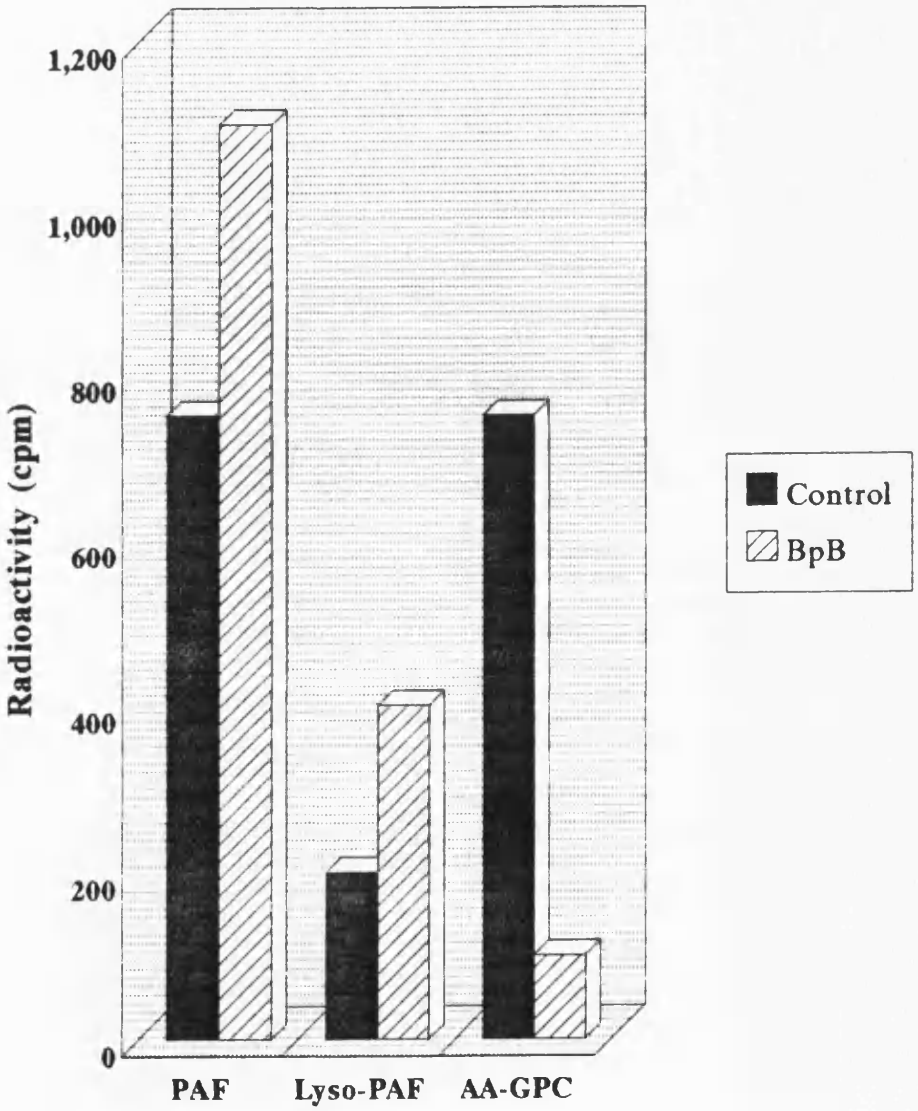


Fig: 27.

Effects of changes in extracellular calcium (1uM) on ^{14}C PAF uptake by human amnion cells in culture at various time points. The amnion cells were incubated ^{14}C PAF (0.18 nM/plate, 0.1uCi in 0.01 % bovine serum albumin in :(a) presence of Kreb's solution (with 1uM calcium) as control, (b) Kreb's calcium-free solution, (c) Kreb's solution with 1uM calcium and calcium ionophore A23187 (5mM) for 10, 30, 60 min at 37°C in humidified air (95%) with 5% CO_2 in an incubator. After 10, 30, 60 min, the incubation was stopped and the cells were washed with phosphate buffer saline and lipids were extracted by the method of Bligh and Dyer (1959), by choloroform:methanol (2:1). The extracted radioactivity was measured by liquid scintillation counting after addition of Aquasol and Tissue solubilizer (Amersham).
The results are representative of three experiments.
$p < 0.01$ (control vs calcium-free).
$p < 0.001$ (calcium-free vs ionophore-treated).

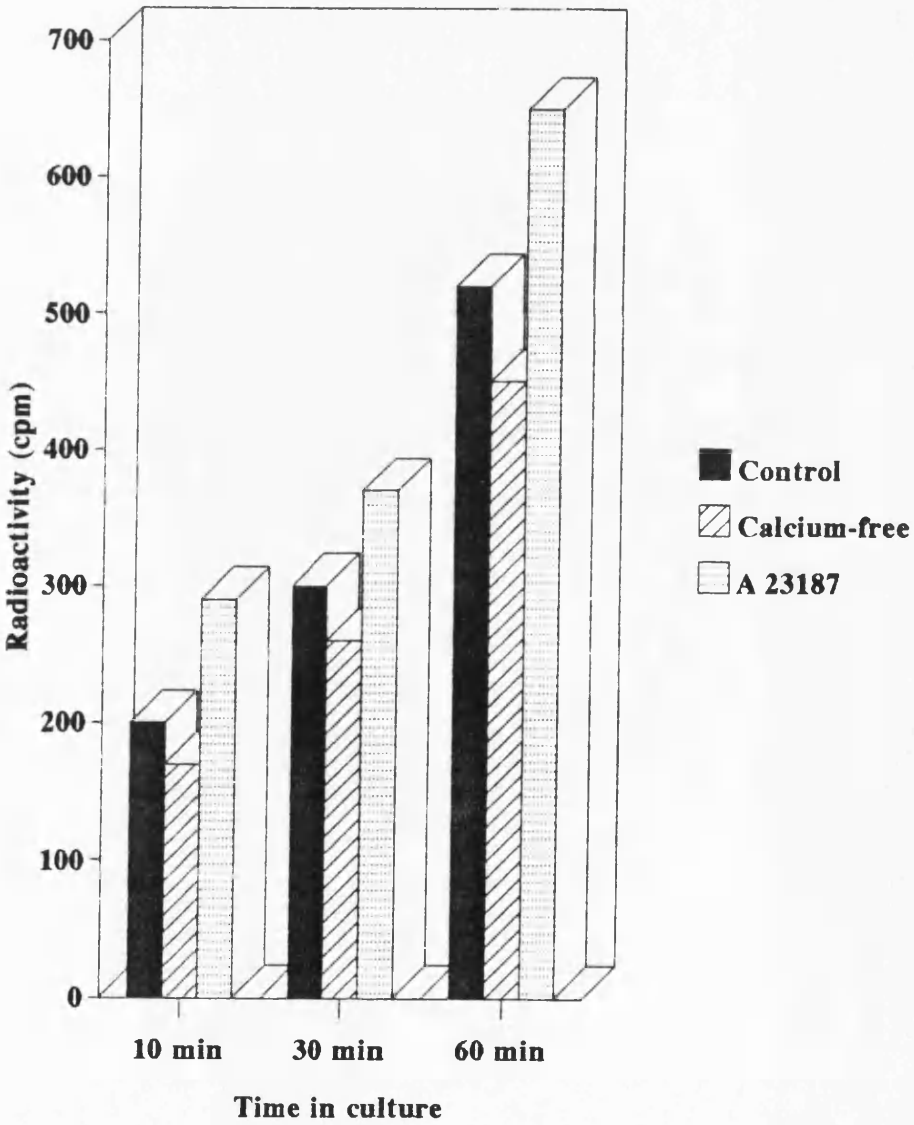


Fig:28.
Effects of Merthiolate on ^{14}C PAF uptake by human amnion cells in culture. The amnion cells were incubated ^{14}C PAF (0.18 nM/plate, 0.1uCi in 0.01 % bovine serum albumin in presence and absence of Merthiolate (0–100 uM in 0.9% saline) for 60 min at 37°C in humidified air (95%) with 5% CO_2 in an incubator. After 60 min, the incubation was stopped and the cells were washed with phosphate buffer saline and lipids were extracted by the method of Bligh and Dyer (1959), by choloroform:methanol (2:1). The extracted radioactivity was measured by liquid scintillation counting after addition of Aquasol and Tissue solubilizer (Amersham).

Results are Mean ± S.D, n=3.
p<0.05: control vs 0.1, 1.0, 10uM merthiolate.
p<0.01: control vs 100uM merthiolate.

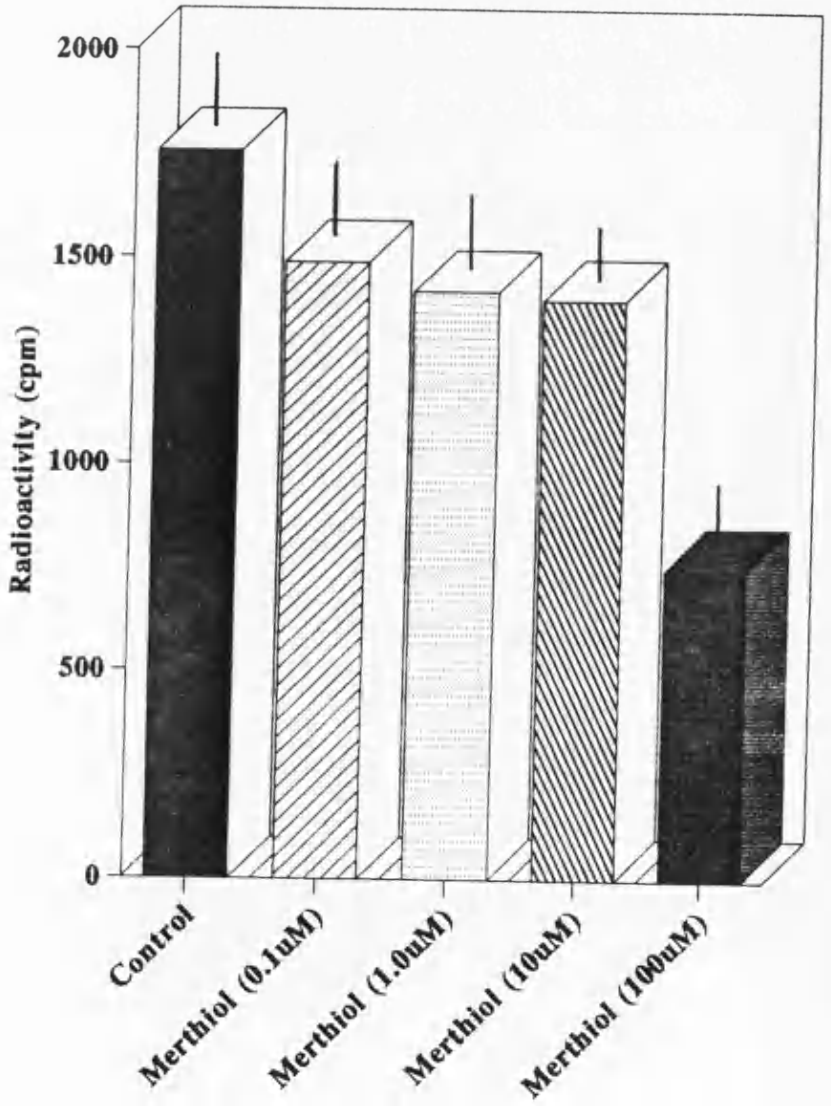


Fig:29.
Effects of N-ethylmaleimide on ^{14}C PAF uptake by human amnion cells in culture. The amnion cells were incubated ^{14}C PAF (0.18 nM/plate, 0.1uCi in 0.01 % bovine serum albumin in presence and absence of N-ethylmaleimide (0-100 uM in 0.9% saline) for 60 min at 37°C in humidified air (95%) with 5% CO_2 in an incubator. After 60 min, the incubation was stopped and the cells were washed with phosphate buffer saline and lipids were extracted by the method of Bligh and Dyer (1959), by choloroform:methanol (2:1). The extracted radioactivity was measured by liquid scintillation counting after addition of Aquasol and Tissue solubilizer (Amersham).

Results are Mean ± S.D, n=3.
p<0.05: control vs 0.1, 1.0, 10uM N-ethylmaleimide.
p<0.01: control vs 100uM N-ethylmaleimide.

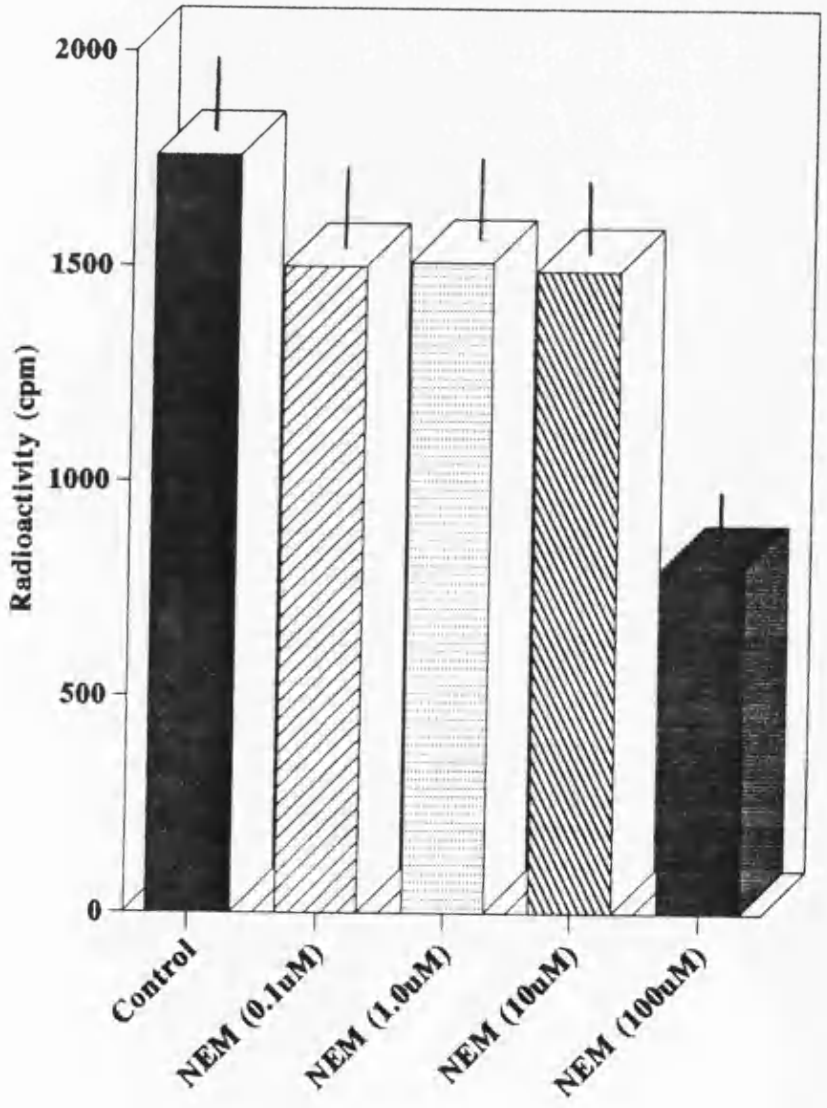


Fig:30.
Effects of WEB-2086 on ^{14}C PAF uptake by human amnion cells in culture. The amnion cells were incubated ^{14}C PAF (0.18 nM/plate, 0.1uCi in 0.01 % bovine serum albumin in presence and absence of WEB-2086 (0-20nM in 0.9% saline) for 60 min at 37°C in humidified air (95%) with 5% CO_2 in an incubator. After 60 min, the incubation was stopped and the cells were washed with phosphate buffer saline and lipids were extracted by the method of Bligh and Dyer (1959), by choloroform:methanol (2:1). The extracted radioactivity was measured by liquid scintillation counting.

Results are Mean ± S.D, n=3.
$p<0.05$: control vs 0.02, 0.2 nM WEB-2086.
$p<0.01$: control vs 2.0, 20 nM WEB-2086.

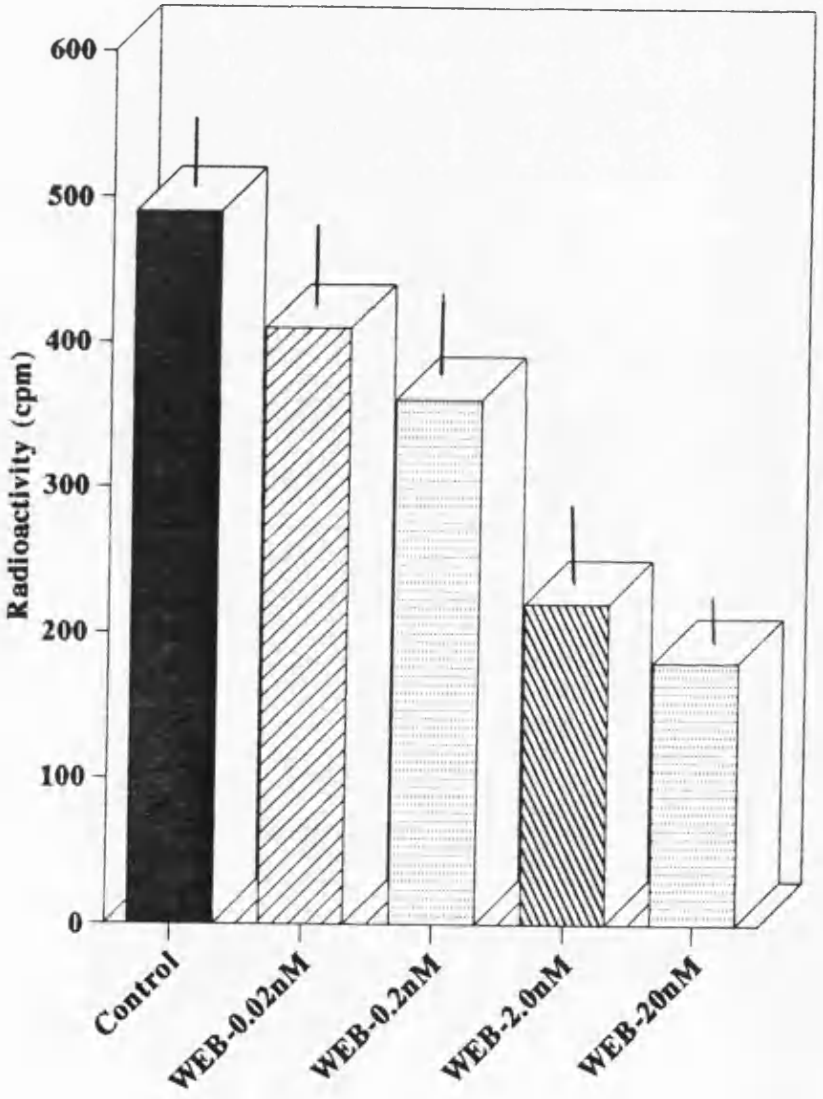
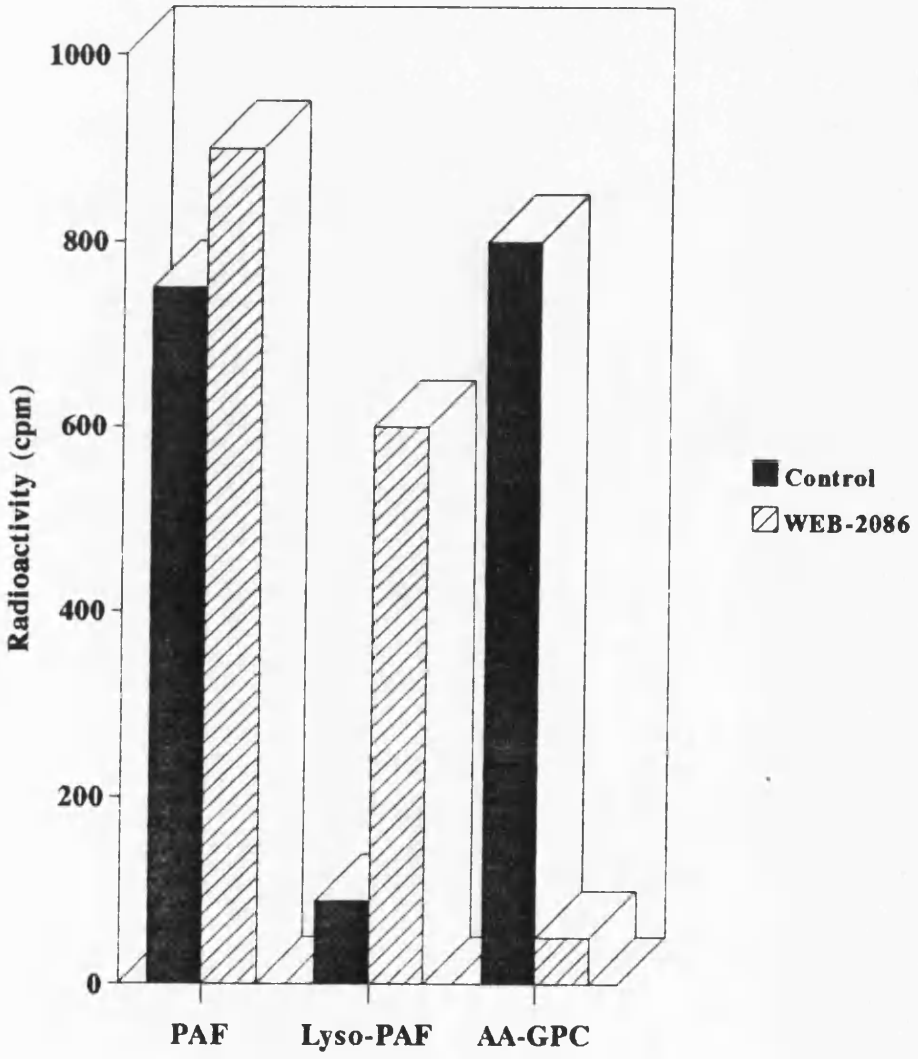


Fig:31 (a,b).

Effects of WEB-2086 on PAF metabolism in human amnion cells and in culture medium. The cells were incubated with ^{14}C PAF (0.18nM, 0.1uCi) and WEB-2086 (20nM in 0.9% NaCl) for 60 min in humidified air (95%) with CO_2 at 37°C, the lipids were extracted from the cells and culture medium by chloroform:methanol 2:1. The lipids were separated by TLC and the radiolabel products were identified and quantified using Berthold TLC analyzer as described in the Methods (Saeed et al, 1988). The results are representative of separate experiments.
a: cells.
b: culture medium.

(a): amnion cells



(b):culture medium

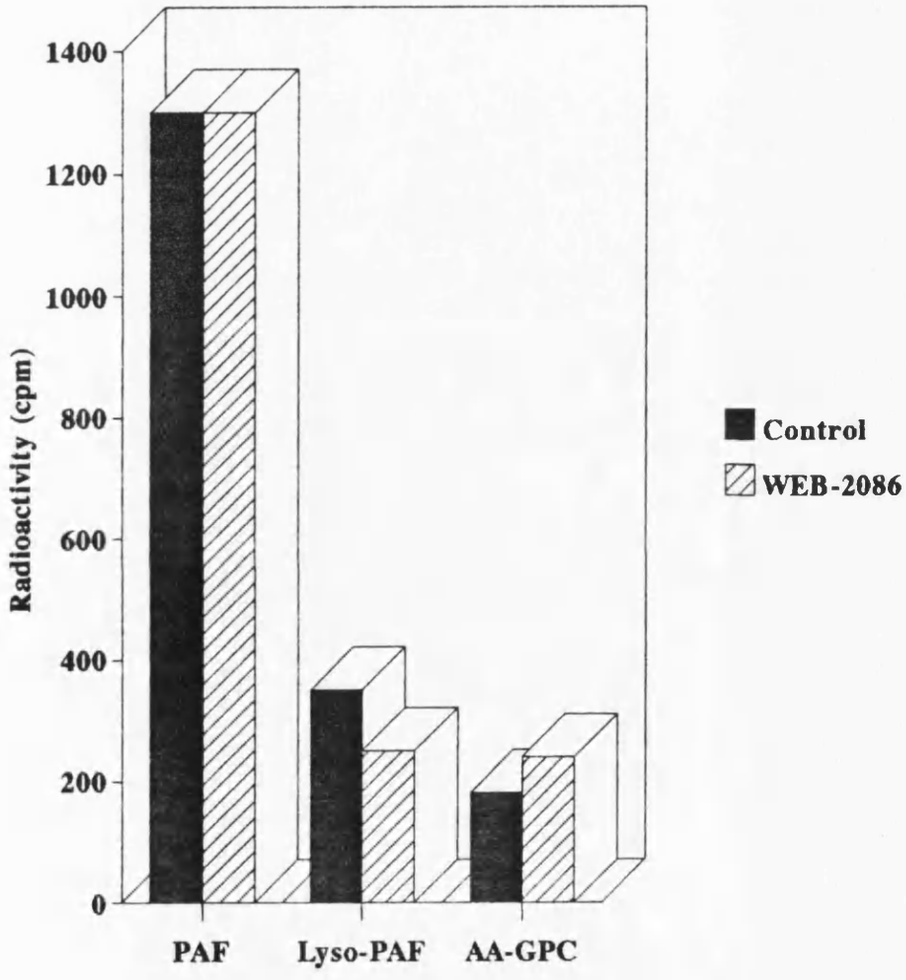


Fig:32.
Effects of Indomethacin on ^{14}C PAF uptake by human amnion cells in culture. The amnion cells were incubated ^{14}C PAF (0.18 nM/plate, 0.1uCi in 0.01 % bovine serum albumin in presence and absence of Indomethacin (0-100nM in 0.5% Na_2CO_3) for 60 min at 37°C in humidified air (95%) with 5% CO_2 in an incubator. After 60 min, the incubation was stopped and the cells were washed with phosphate buffer saline and lipids were extracted by the method of Bligh and Dyer (1959), by choloroform:methanol (2:1). The extracted radioactivity was measured by liquid scintillation counting.
Results are Mean ± S.D, n=3.
$p < 0.05$: control vs 100nM.

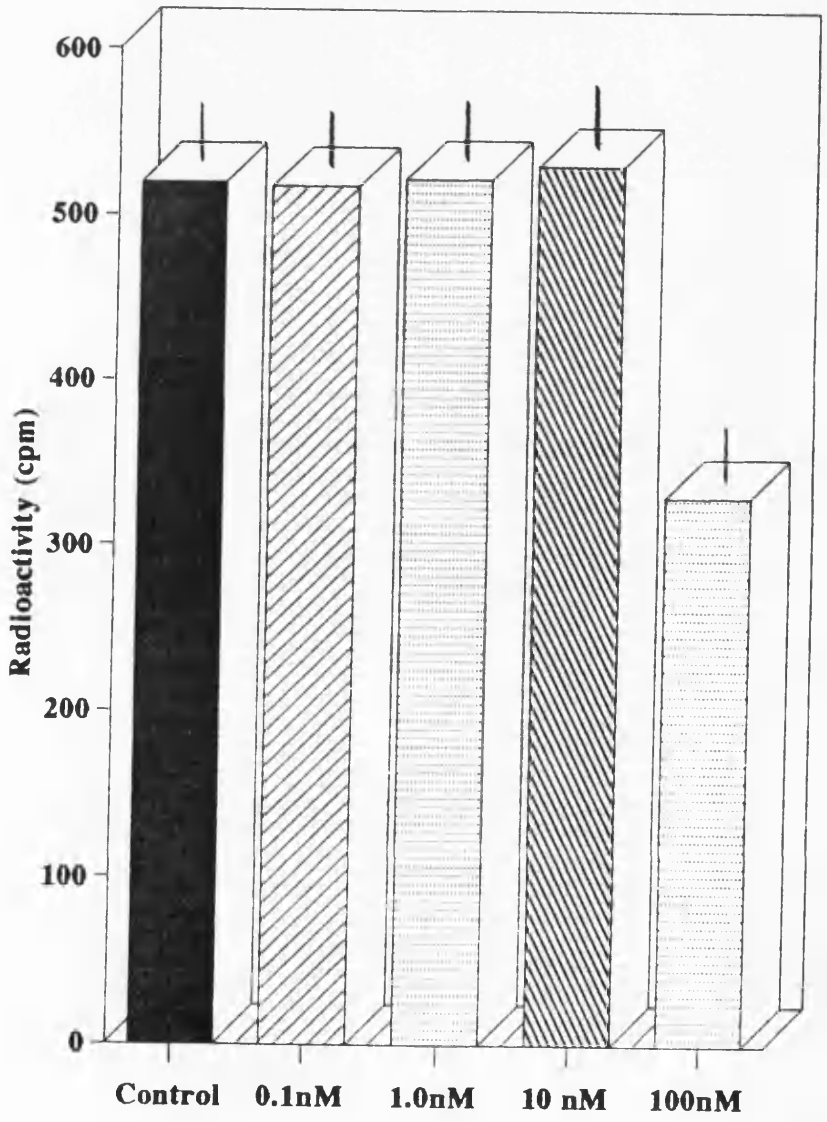


Fig:33.
Effects of Nordihydroguaiaretic acid (NDGA) on ^{14}C PAF uptake by human amnion cells in culture. The amnion cells were incubated ^{14}C PAF (0.18 nM/plate, 0.1uCi in 0.01 % bovine serum albumin in presence and absence of NDGA (0-100nM) for 60 min at 37°C in humidified air (95%) with 5% CO_2 in an incubator. After 60 min, the incubation was stopped and the cells were washed with phosphate buffer saline and lipids were extracted by the method of Bligh and Dyer (1959), by choloroform:methanol (2:1). The extracted radioactivity was measured by liquid scintillation counting.
Results are Mean ± S.D, n=3.
p< 0.05: control vs 0.1,1,10,100nM NDGA.

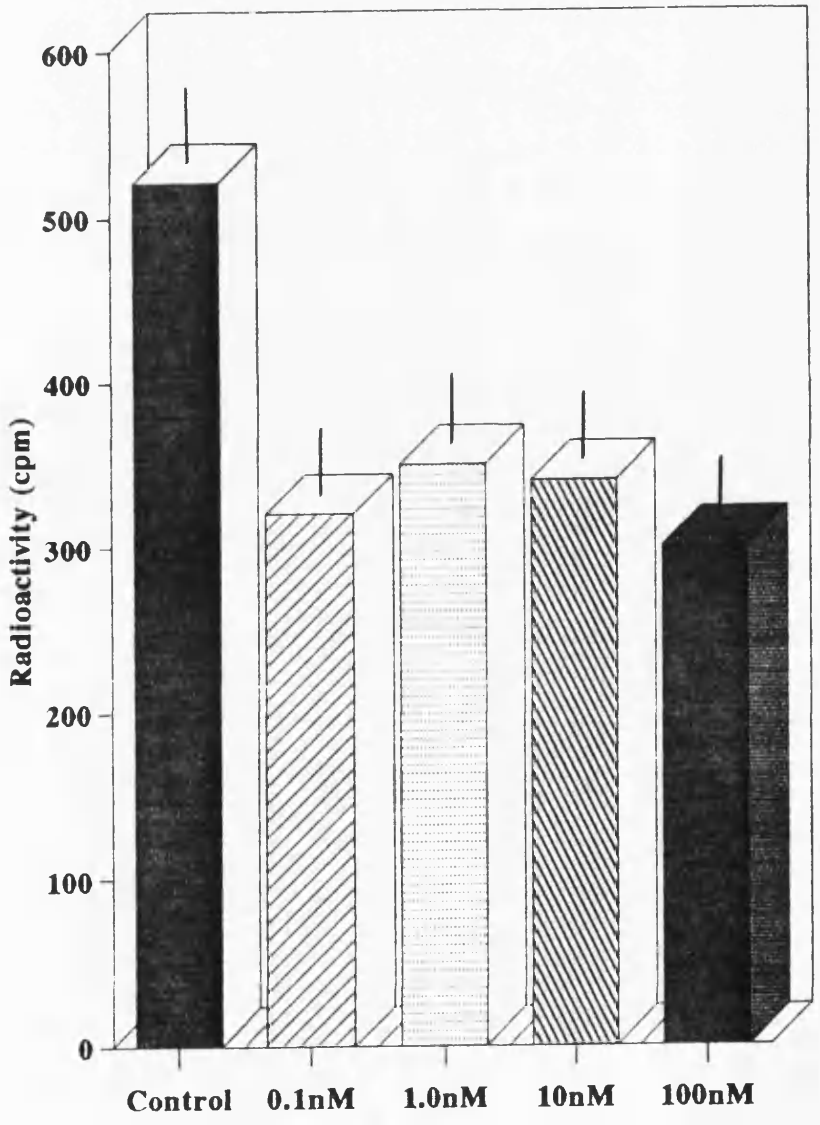


Fig:34 (a,b).

Effects of Nordihyroguaiaretic acid on PAF metabolism in human amnion cells and in culture medium. The cells were incubated with ^{14}C PAF (0.18nM, 0.1uCi) and NDGA (100nM) for 60 min in humidified air (95%) with CO_2 at 37°C, the lipids were extracted from the cells and culture medium by chloroform:methanol 2:1. The lipids were separated by TLC and the radiolabel products were identified and quantified using Berthold TLC analyzer as described in the Methods (Saeed et al, 1988). The results are representative of separate experiments.
a: cells.
b: culture medium.

(a): cells

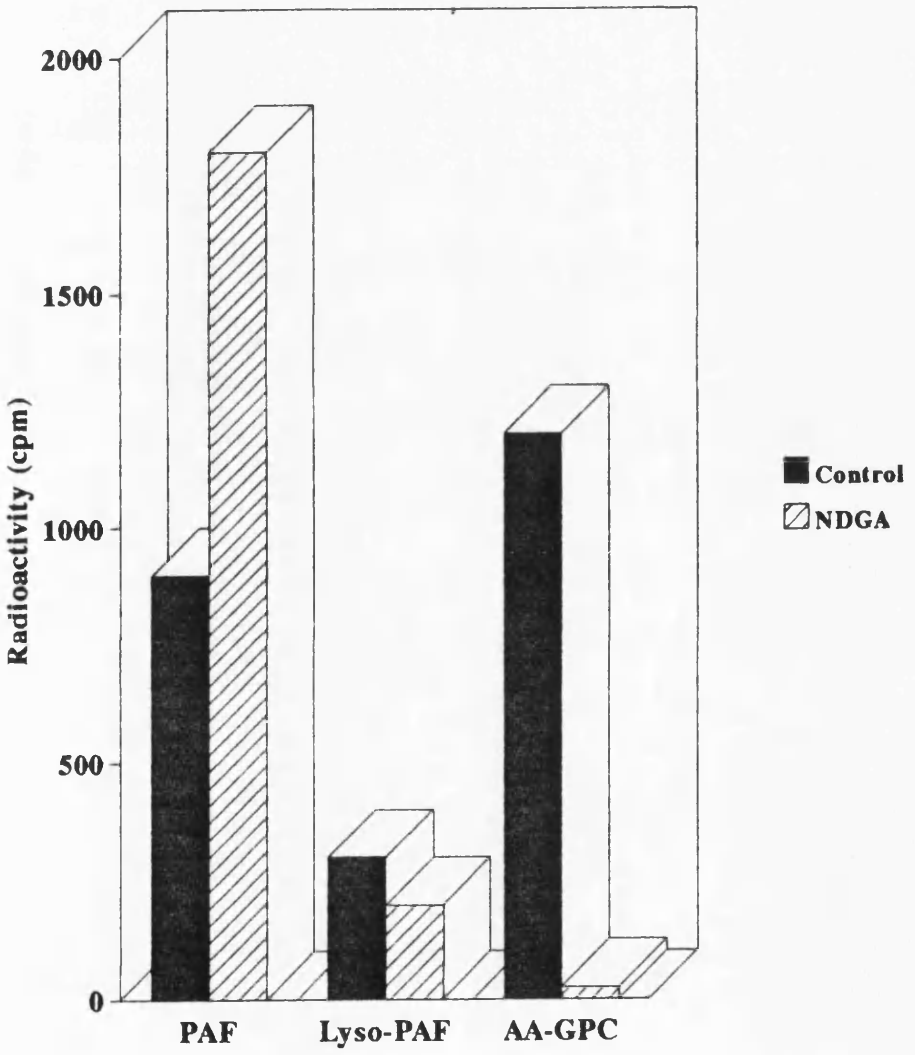


Fig:34

(b): culture medium

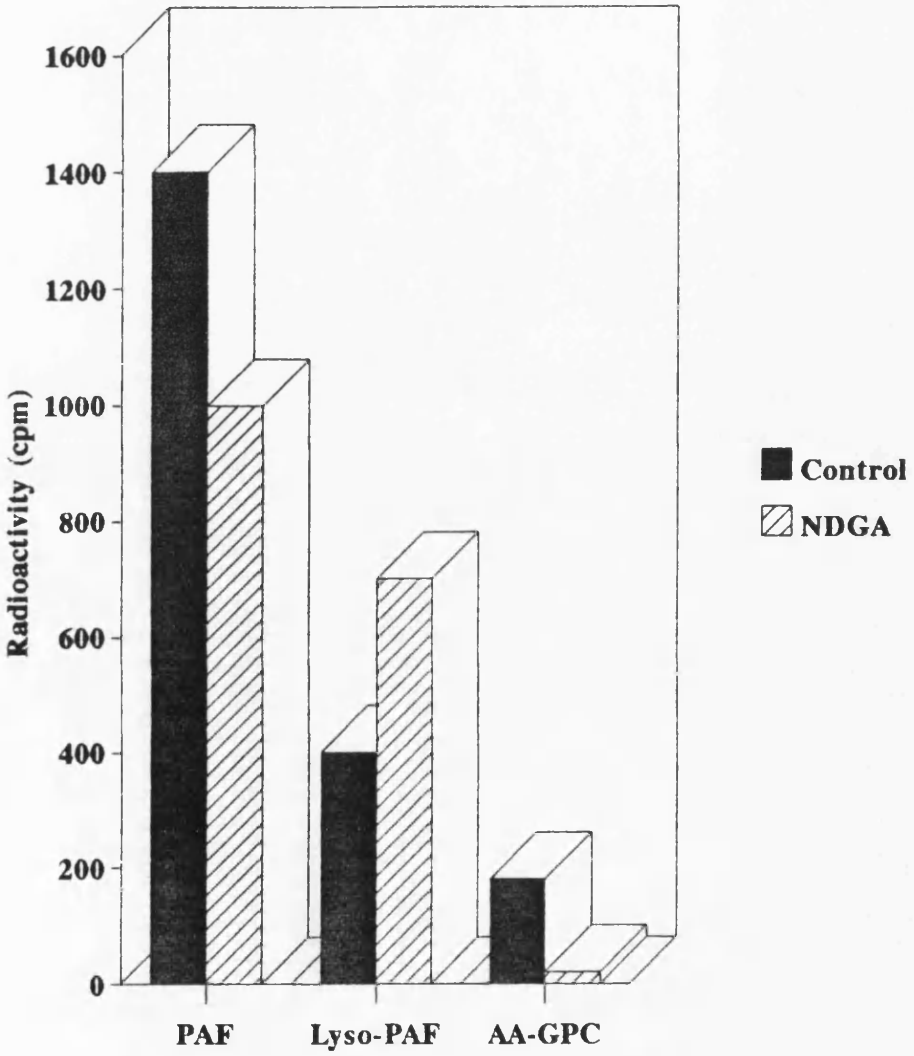
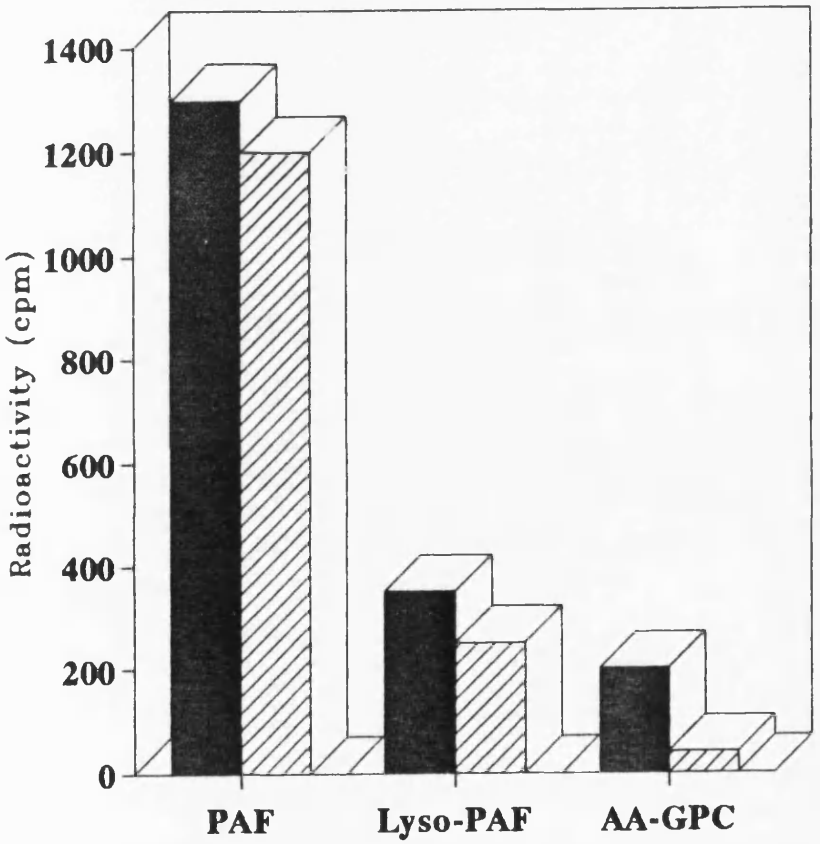


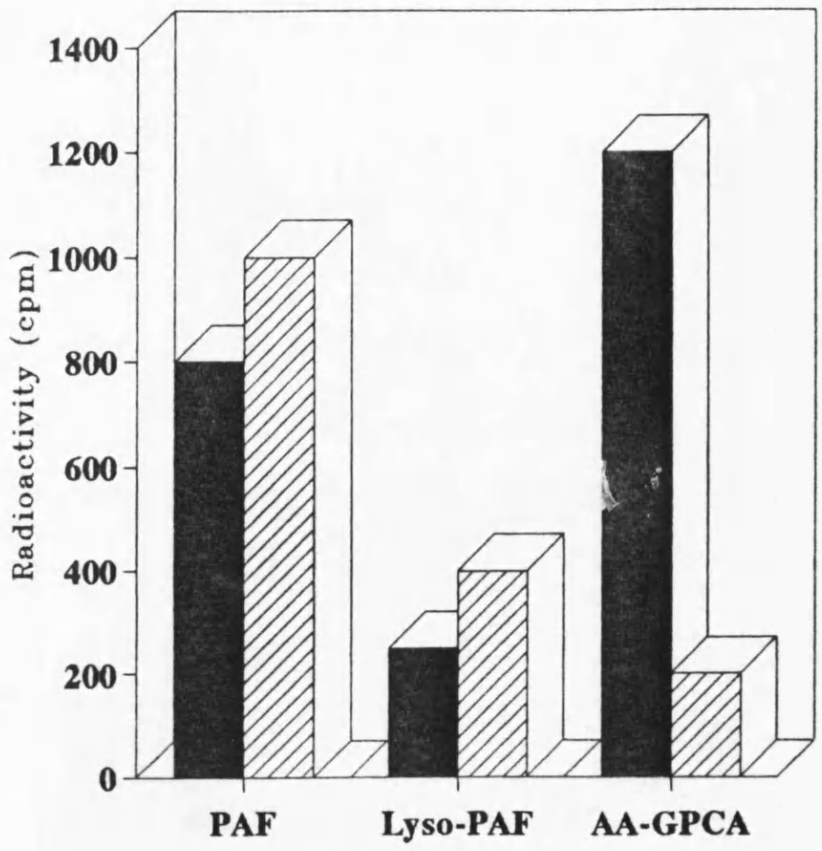
Fig:35.

Effects of Indomethacin on PAF metabolism in human amnion cells and in culture medium. The cells were incubated with ^{14}C PAF (0.18nM, 0.1uCi) and Indomethacin (100nM in 0.5% Na_2CO_3) for 60 min in humidified air (95%) with CO_2 at 37°C, the lipids were extracted from the cells and culture medium by chloroform:methanol 2:1. The lipids were separated by TLC and the radiolabel products were identified and quantified using Berthold TLC analyzer as described in the Methods (Saeed et al, 1988). The results are representative of separate experiments.
a: cells.
b: culture medium.



ntrol: Solid bars
domethacin (100nM): Shaded bars
): cells

Fig: 35



Control: Solid bars
Indomethacin: Shaded bars
(b):culture medium

Fig:36.
Effects of Ouabain on ^{14}C PAF uptake by human amnion cells in culture. The amnion cells were incubated ^{14}C PAF (0.18 nM/plate, 0.1uCi in 0.01 % bovine serum albumin in presence and absence of Ouabain (0-10 uM) for 60 min at 37°C in humidified air (95%) with 5% CO_2 in an incubator. After 60 min, the incubation was stopped and the cells were washed with phosphate buffer saline and lipids were extracted by the method of Bligh and Dyer (1959), by choloroform:methanol (2:1). The extracted radioactivity was measured by liquid scintillation counting.
Results are Mean ± S.D, n=3.
p< 0.01: control vs 1,10 uM Ouabain.

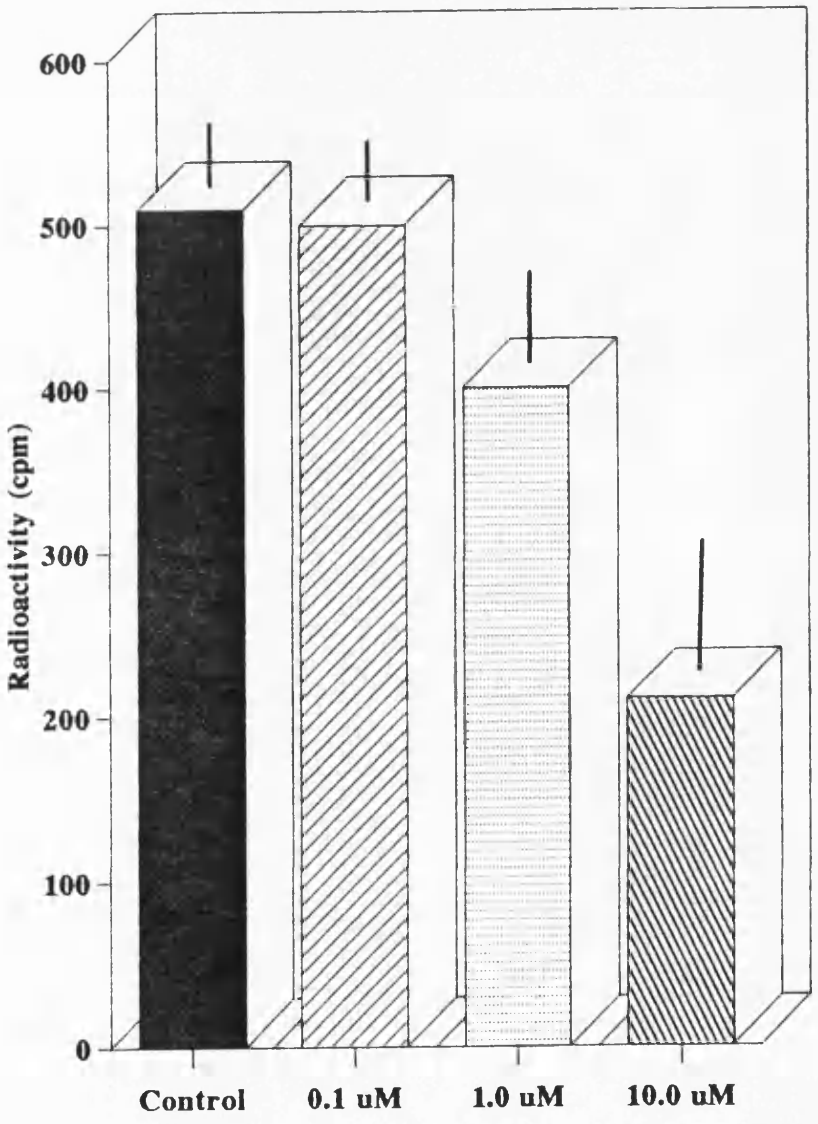


Fig:37.
Effects of Oestradiol on ^{14}C PAF uptake by human amnion cells in culture. The amnion cells were incubated ^{14}C PAF (0.18 nM/plate, 0.1uCi in 0.01 % bovine serum albumin in presence and absence of Oestradiol (0-10 uM in 0.05% DMSO) for 48 hrs at 37°C in humidified air (95%) with 5% CO_2 in an incubator. After 48 hrs, the incubation was stopped and the cells were washed with phosphate buffer saline and lipids were extracted by the method of Bligh and Dyer (1959), by choloroform:methanol (2:1). The extracted radioactivity was measured by liquid scintillation counting.
Results are Mean ± S.D, n=3.
p< 0.05: control vs 10 uM.

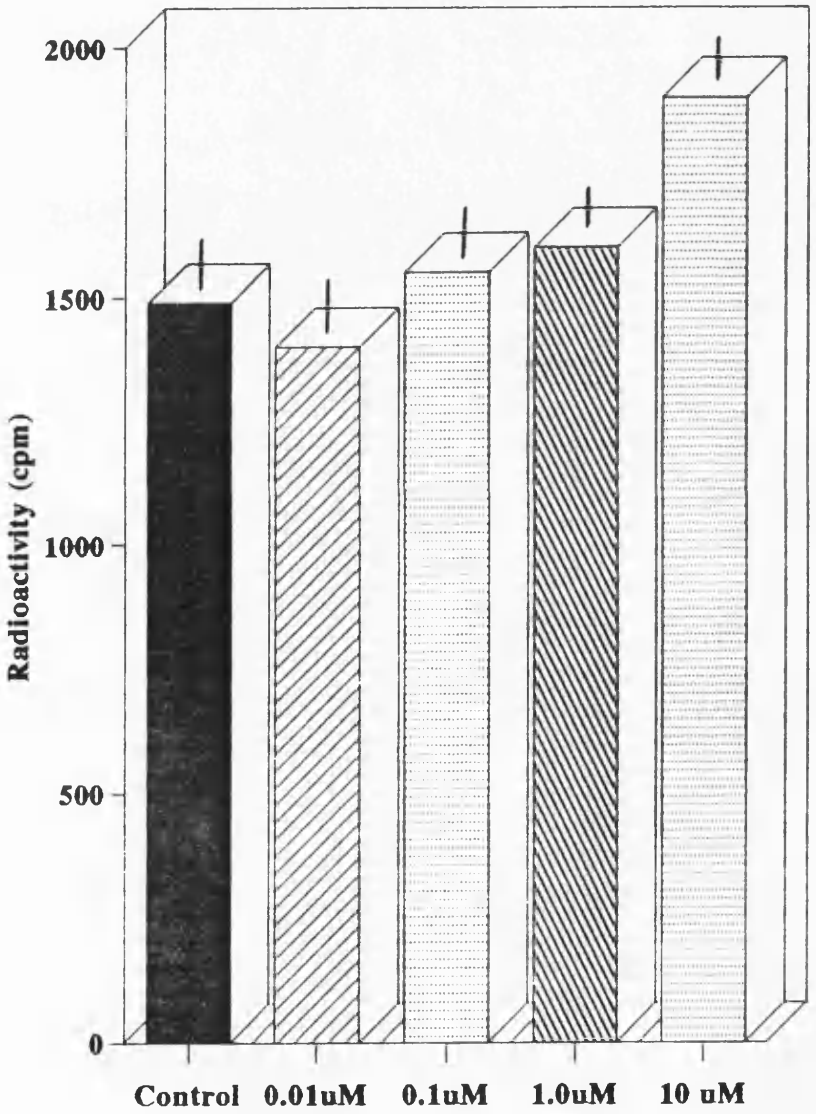


Fig:38.
Distribution of radioactivity in Lyso-PAF(1-O-Hexadecyl-2-lyso-sn-Glycerophosphocholine), PAF (1-O-Hexadecyl-2-acetyl-sn-Glycerophosphocholine),andPhosphatidylcholine(Hexadecyl-2-acyl-sn-Glycerophosphocholine) in human amnion cells. After treatment of C^{14} PAF (1-O-Hexadecyl-2-acetyl-sn-Glycerophosphocholine) incubated cells with various concentrations of Oestradiol (0.1,1,10 uM in 0.05% DMSO) for 48 hrs, the lipids were extracted and radioactive products were separated by TLC and analysed by Berthold TLC linear analyzer with chromatographic data system. The values are representative of three separate experiments.

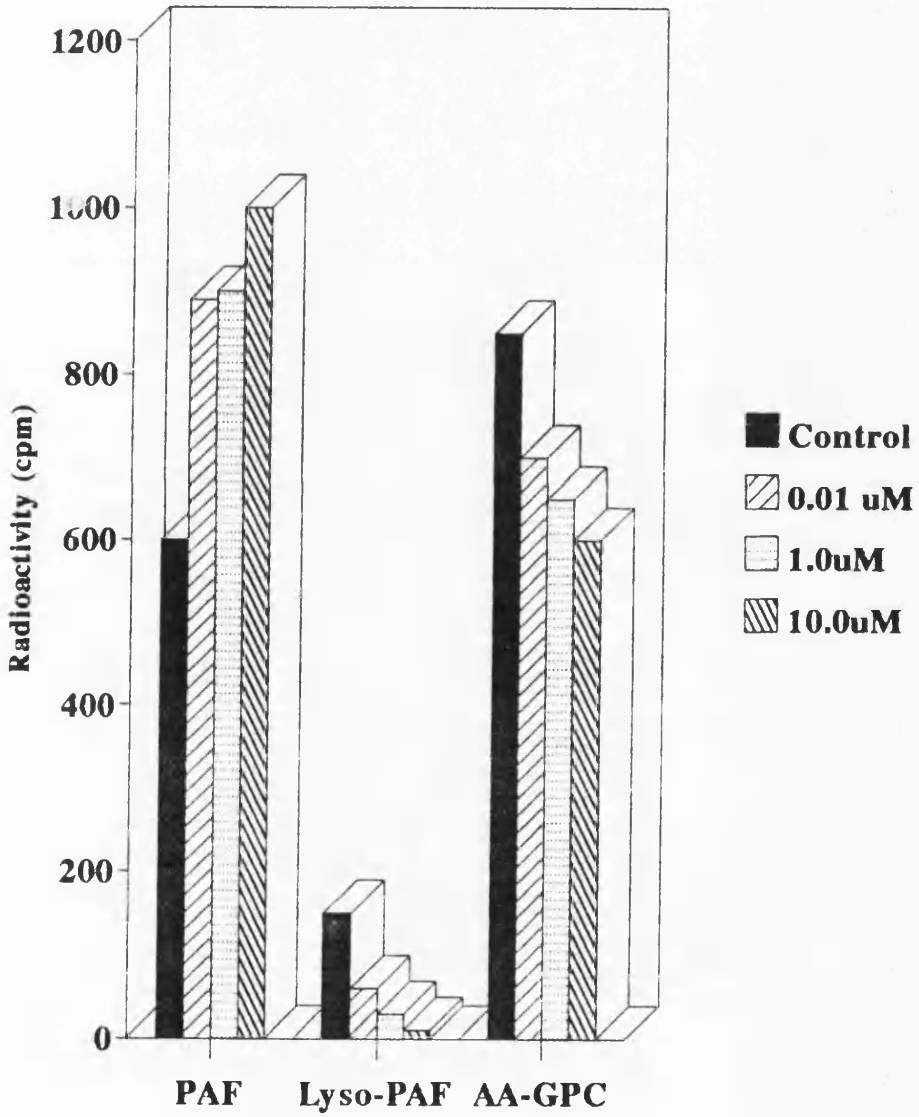


Fig:39.
Distribution of radioactivity in Lyso-PAF(1-O-Hexadecyl-2-lyso-sn-Glycerophosphocholine), PAF (1-O-Hexadecyl-2-acetyl-sn-Glycerophosphocholine),andPhosphatidylcholine(Hexadecyl-2-acyl-sn-Glycerophosphocholine) in corresponding culture medium of oestradiol treated-cells. After treatment of C^{14} PAF (1-O-Hexadecyl-2-acetyl-sn-Glycerophosphocholine) incubated cells with various concentrations of Oestradiol for 48 hrs, the lipids were extracted and radioactive products were separated by TLC and analysed by Berthold TLC linear analyzer with chromatographic data system.
The values are representative of three separate experiments.

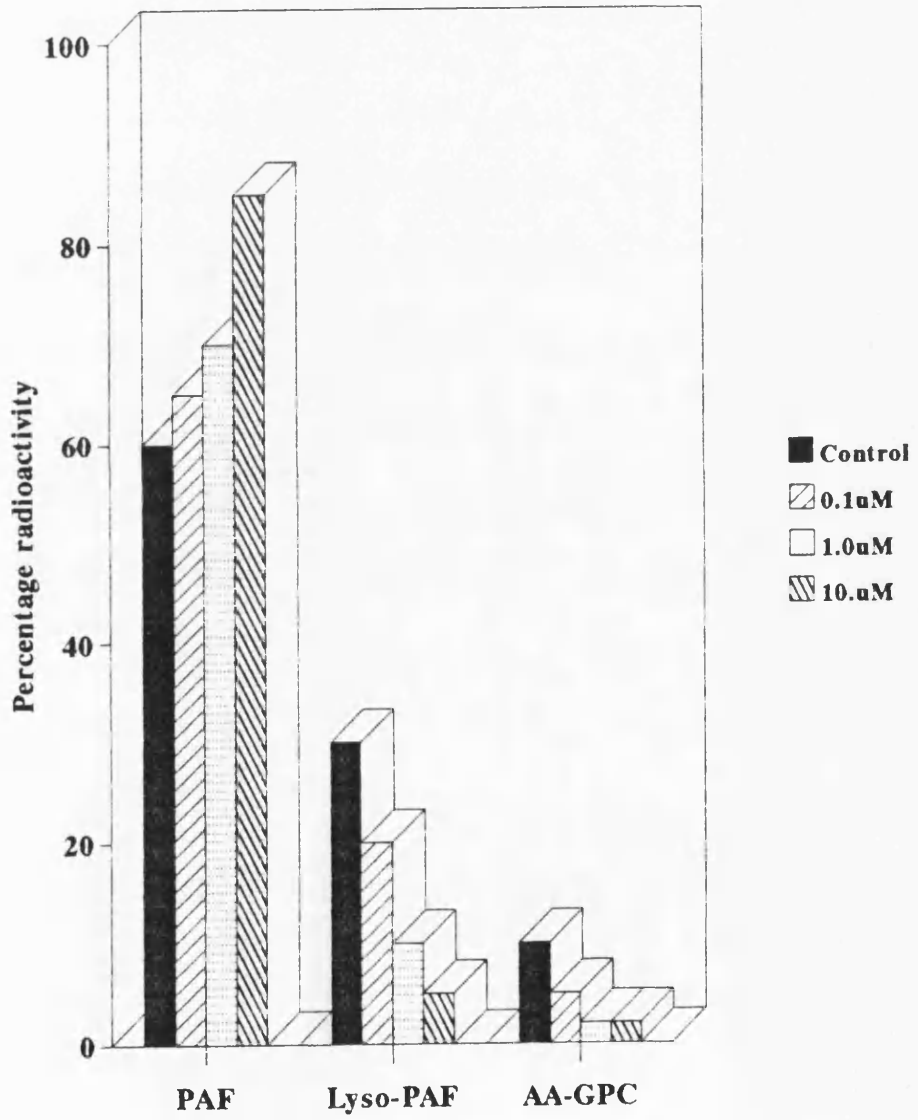


Fig:40.
Effects of Progesterone on ^{14}C PAF uptake by human amnion cells in culture. The amnion cells were incubated ^{14}C PAF (0.18 nM/plate, 0.1uCi in 0.01 % bovine serum albumin in presence and absence of Progesterone (0-10 uM in 0.05% DMSO) for 48 hrs at 37°C in humidified air (95%) with 5% CO_2 in an incubator. After 48 hrs, the incubation was stopped and the cells were washed with phosphate buffer saline and lipids were extracted by the method of Bligh and Dyer (1959), by choloroform:methanol (2:1). The extracted radioactivity was measured by liquid scintillation counting.
Results are Mean ± S.D, n=3.
$p < 0.05$: control vs 10 uM.

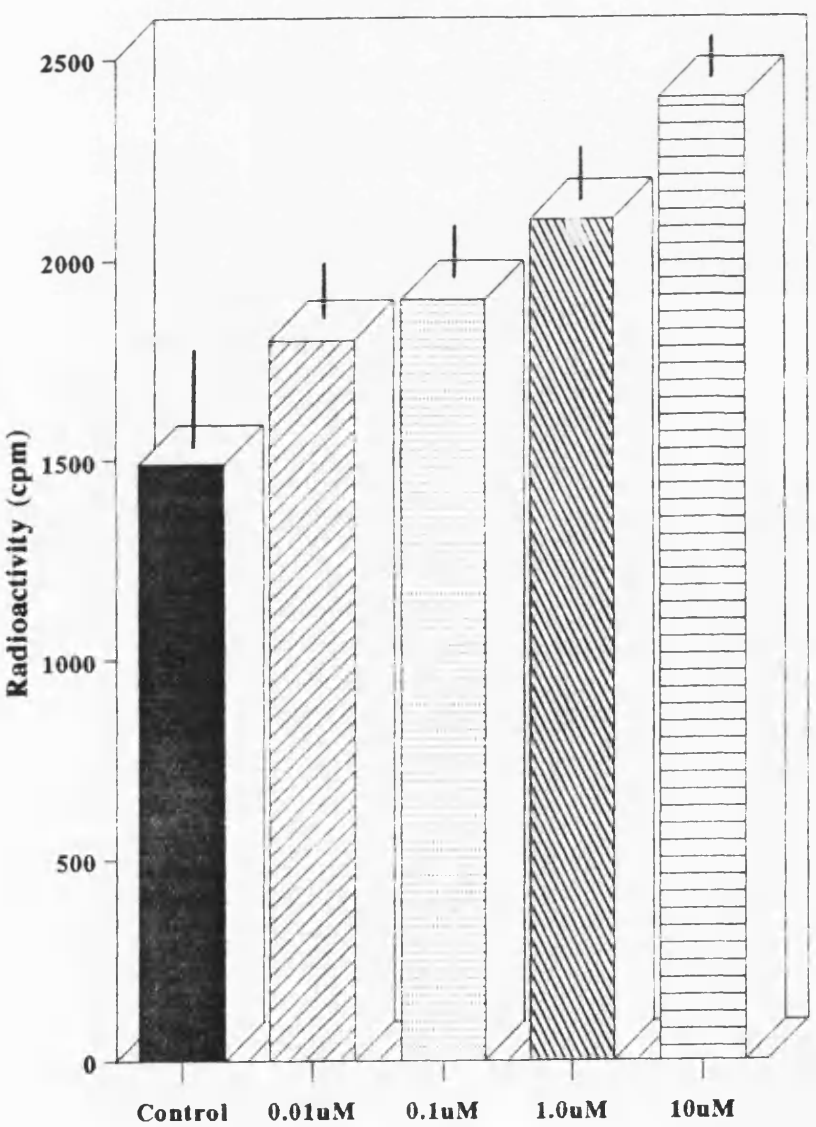


Fig:41.
Distribution of radioactivity in Lyso-PAF(1-O-Hexadecyl-2-lyso-sn-Glycerophosphocholine), PAF (1-O-Hexadecyl-2-acetyl-sn-Glycerophosphocholine),andPhosphatidylcholine(Hexadecyl-2-acyl-sn-Glycerophosphocholine) in human amnion cells. After treatment of C^{14} PAF (1-O-Hexadecyl-2-acetyl-sn-Glycerophosphocholine) incubated cells with various concentrations of Progesterone (0.1,1,10 uM in 0.05% DMSO) for 48 hrs, the lipids were extracted and radioactive products were separated by TLC and analysed by Berthold TLC linear analyzer with chromatographic data system. The values are representative of three separate experiments.

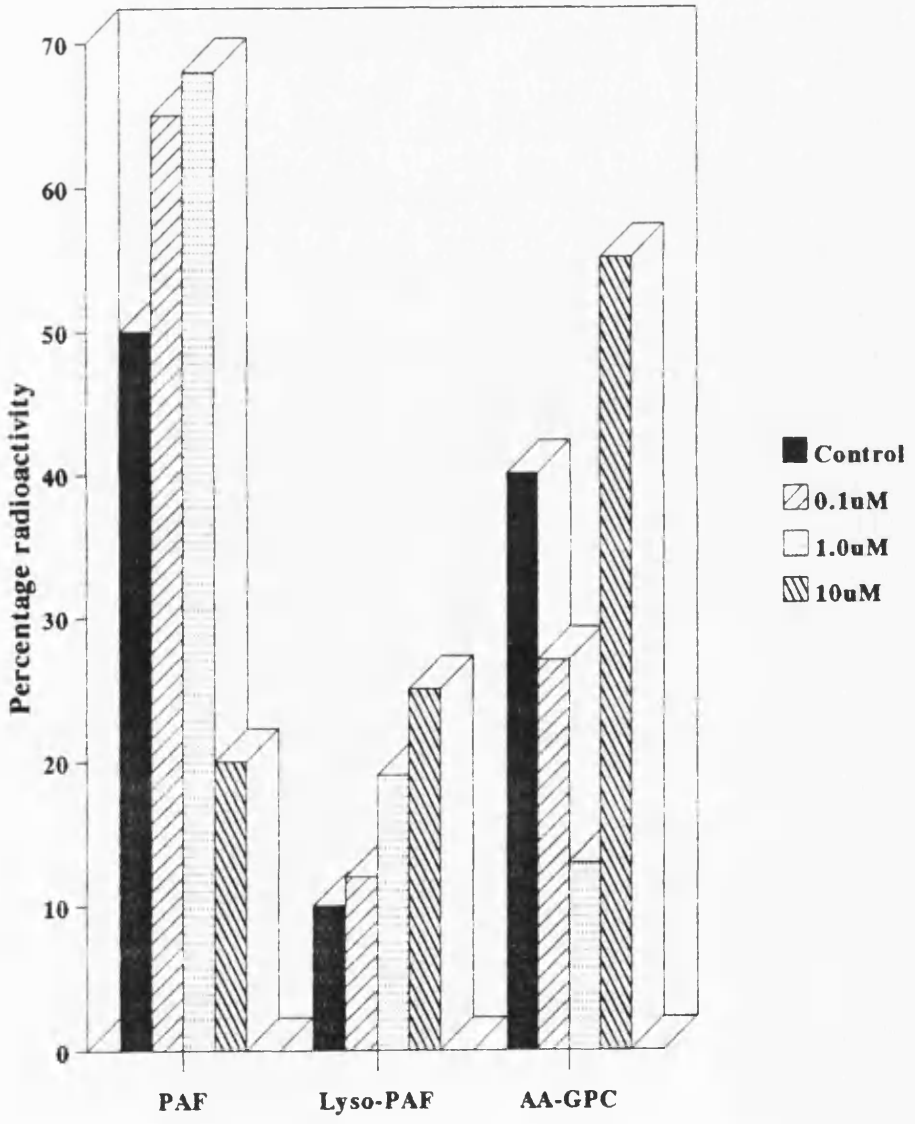


Fig:42.
Distribution of radioactivity in Lyso-PAF(1-O-Hexadecyl-2-lyso-sn-Glycerophosphocholine), PAF (1-O-Hexadecyl-2-acetyl-sn-Glycerophosphocholine),andPhosphatidylcholine(Hexadecyl-2-acyl-sn-Glycerophosphocholine) in corresponding culture medium of Progesterone treated-cells. After treatment of C^{14} PAF (1-O-Hexadecyl-2-acetyl-sn-Glycerophosphocholine) incubated cells with various concentrations of Progesterone for 48 hrs, the lipids were extracted and radioactive products were separated by TLC and analysed by Berthold TLC linear analyzer with chromatographic data system.
The values are representative of three separate experiments.

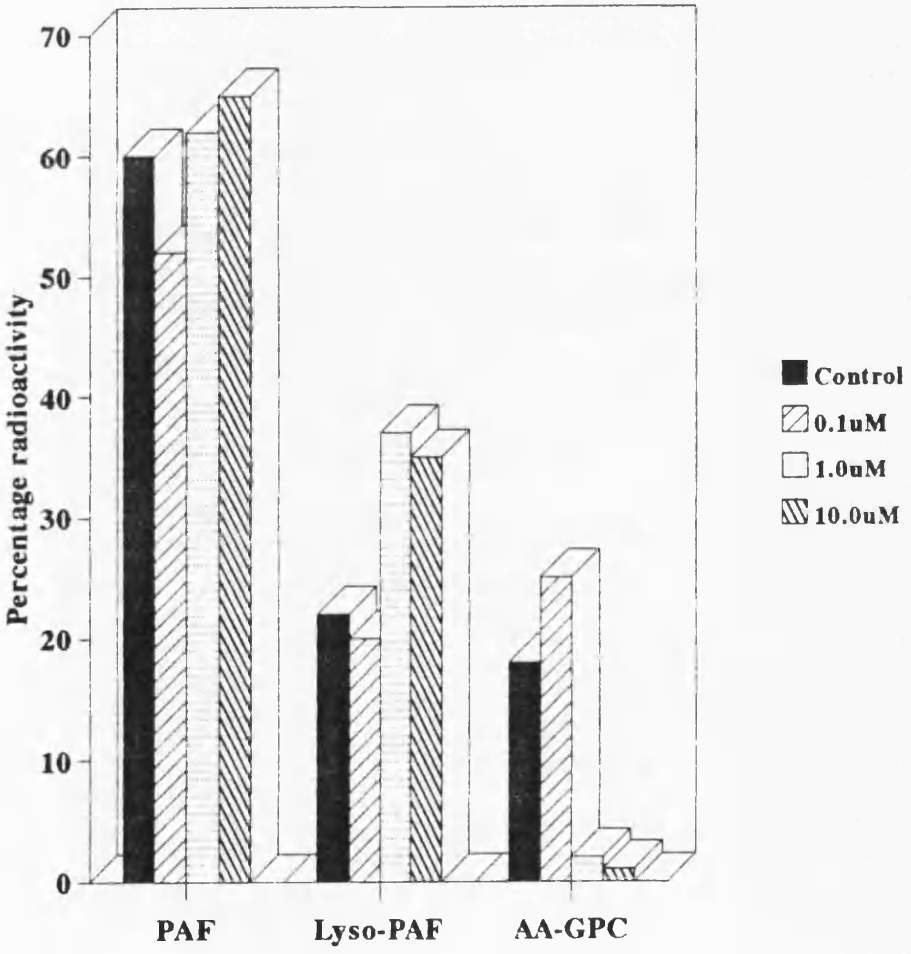


Fig:43.
Effects of Oestradiol and Progesterone on ^{14}C PAF uptake by human amnion cells in culture. The amnion cells were incubated ^{14}C PAF (0.18 nM/plate, 0.1uCi in 0.01 % bovine serum albumin in presence and absence of Oestradiol and Progesterone (0-10 uM in 0.05% DMSO) for 48 hrs at 37°C in humidified air (95%) with 5% CO_2 in an incubator. After 48 hrs, the incubation was stopped and the cells were washed with phosphate buffer saline and lipids were extracted by the method of Bligh and Dyer (1959), by choloroform:methanol (2:1). The extracted radioactivity was measured by liquid scintillation counting.
Results are Mean ± S.D, n=3.
$p < 0.05$: control vs 10 uM.

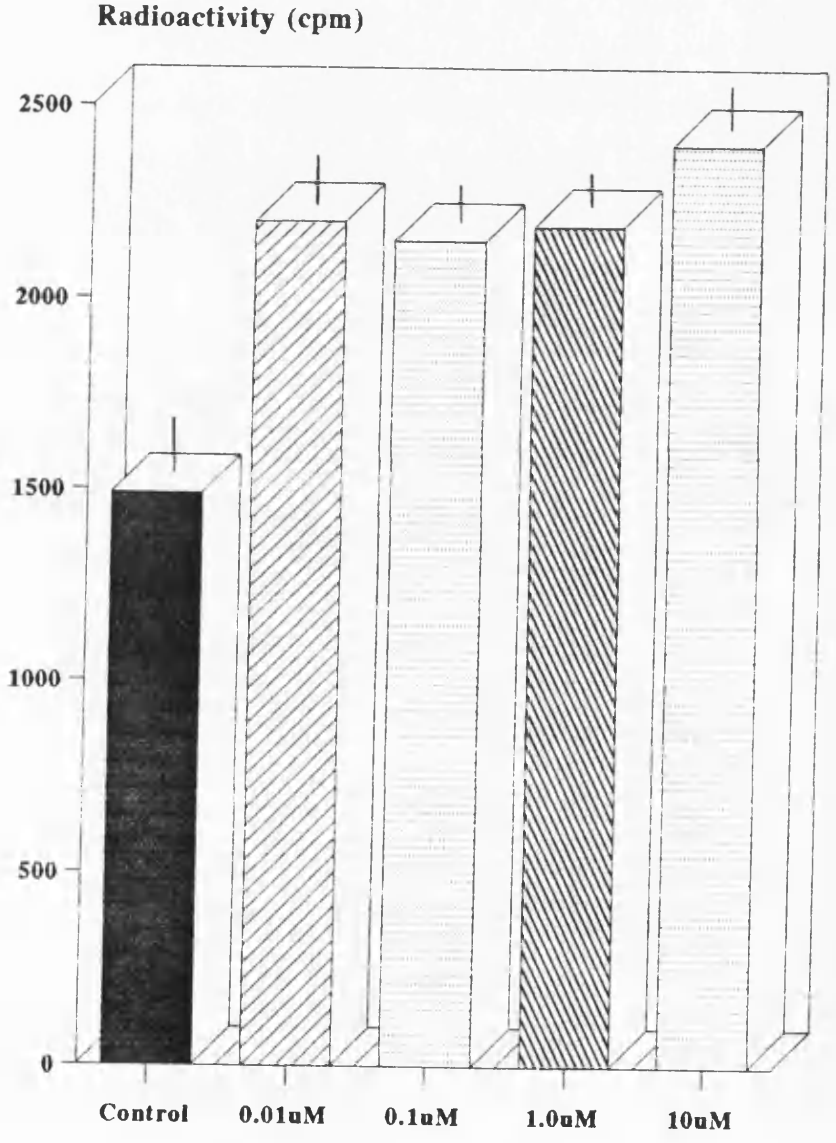


Fig:44.
Distribution of radioactivity in Lyso-PAF(1-O-Hexadecyl-2-lyso-sn-Glycerophosphocholine), PAF (1-O-Hexadecyl-2-acetyl-sn-Glycerophosphocholine),andPhosphatidylcholine(Hexadecyl-2-acyl-sn-Glycerophosphocholine) in human amnion cells. After treatment of C^{14} PAF (1-O-Hexadecyl-2-acetyl-sn-Glycerophosphocholine) incubated cells with various concentrations of Oestradiol and Progesterone (0.1,1,10 uM in 0.05% DMSO) for 48 hrs, the lipids were extracted and radioactive products were separated by TLC and analysed by Berthold TLC linear analyzer with chromatographic data system.
The values are representative of three separate experiments.

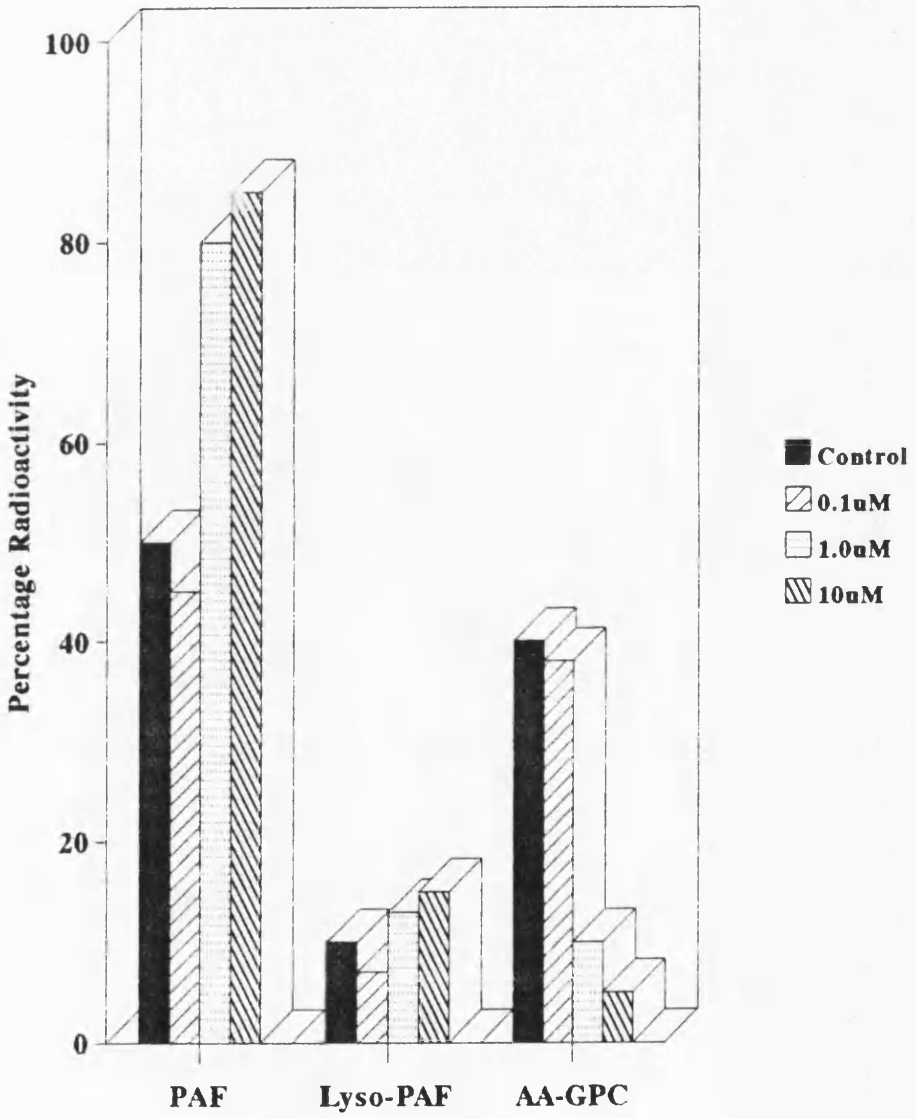


Fig:45.
Distribution of radioactivity in Lyso-PAF(1-O-Hexadecyl-2-lyso-sn-Glycerophosphocholine), PAF (1-O-Hexadecyl-2-acetyl-sn-Glycerophosphocholine),andPhosphatidylcholine(Hexadecyl-2-acyl-sn-Glycerophosphocholine) in corresponding culture medium of Oestradiol and Progesterone treated-cells. After treatment of C^{14} PAF (1-O-Hexadecyl-2-acetyl-sn-Glycerophosphocholine) incubated cells with various concentrations of Oestradiol and Progesterone for 48 hrs, the lipids were extracted and radioactive products were separated by TLC and analysed by Berthold TLC linear analyzer with chromatographic data system.
The values are representative of three separate experiments.

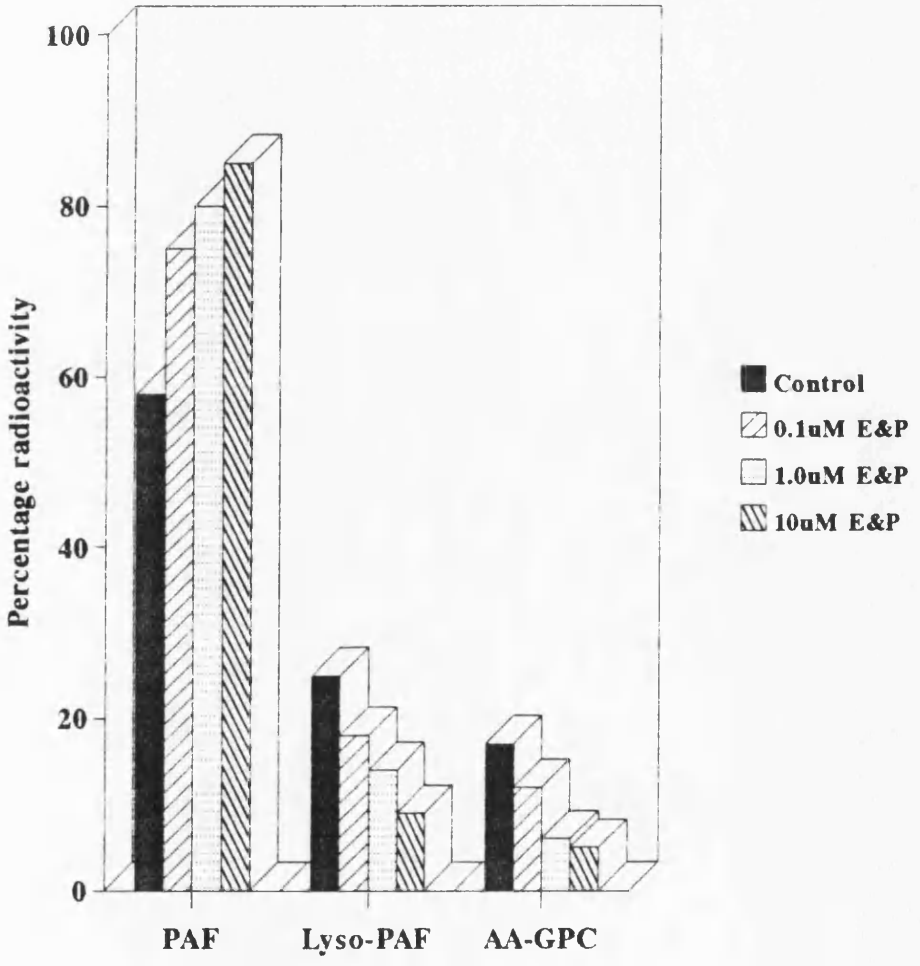


Fig:46.
Effects of Dexamethasone on ^{14}C PAF uptake by human amnion cells in culture. The amnion cells were incubated ^{14}C PAF (0.18 nM/plate, 0.1uCi in 0.01 % bovine serum albumin in presence and absence of Dexamethasone (2.5,25, 250ug/ml in 0.05% DMSO) for 48 hrs at 37°C in humidified air (95%) with 5% CO_2 in an incubator. After 48hrs the incubation was stopped and the cells were washed with phosphate buffer saline and lipids were extracted by the method of Bligh and Dyer (1959), by choloroform:methanol (2:1). The extracted radioactivity was measured by liquid scintillation counting.
Results are Mean ± S.D, n=3.
p< 0.05: control vs 10 uM.

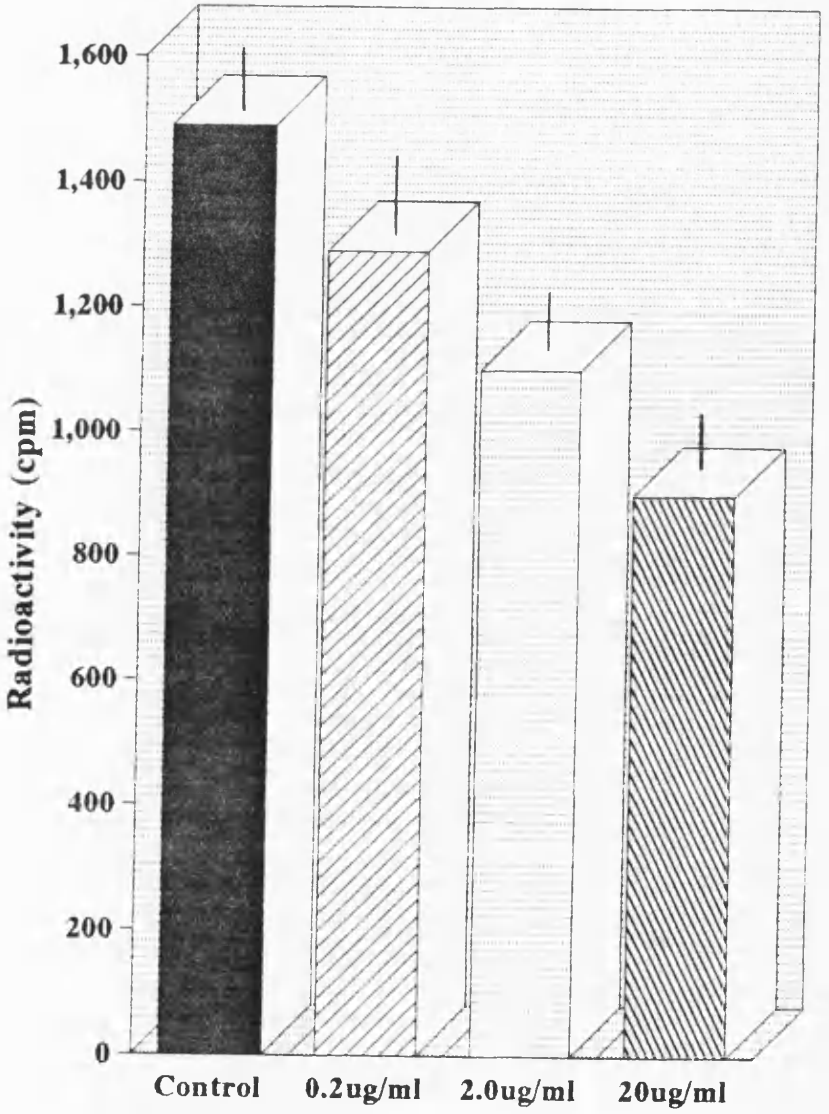


Fig:47.
Distribution of radioactivity in Lyso-PAF(1-O-Hexadecyl-2-lyso-sn-Glycerophosphocholine), PAF (1-O-Hexadecyl-2-acetyl-sn-Glycerophosphocholine),andPhosphatidylcholine(Hexadecyl-2-acyl-sn-Glycerophosphocholine) in human amnion cells. After treatment of C^{14} PAF (1-O-Hexadecyl-2-acetyl-sn-Glycerophosphocholine) incubated cells with various concentrations of Dexamethasone (2.5,25,250 ug/ml in 0.05% DMSO) for 48 hrs, the lipids were extracted and radioactive products were separated by TLC and analysed by Berthold TLC linear analyzer with chromatographic data system.
The values are representative of three separate experiments.

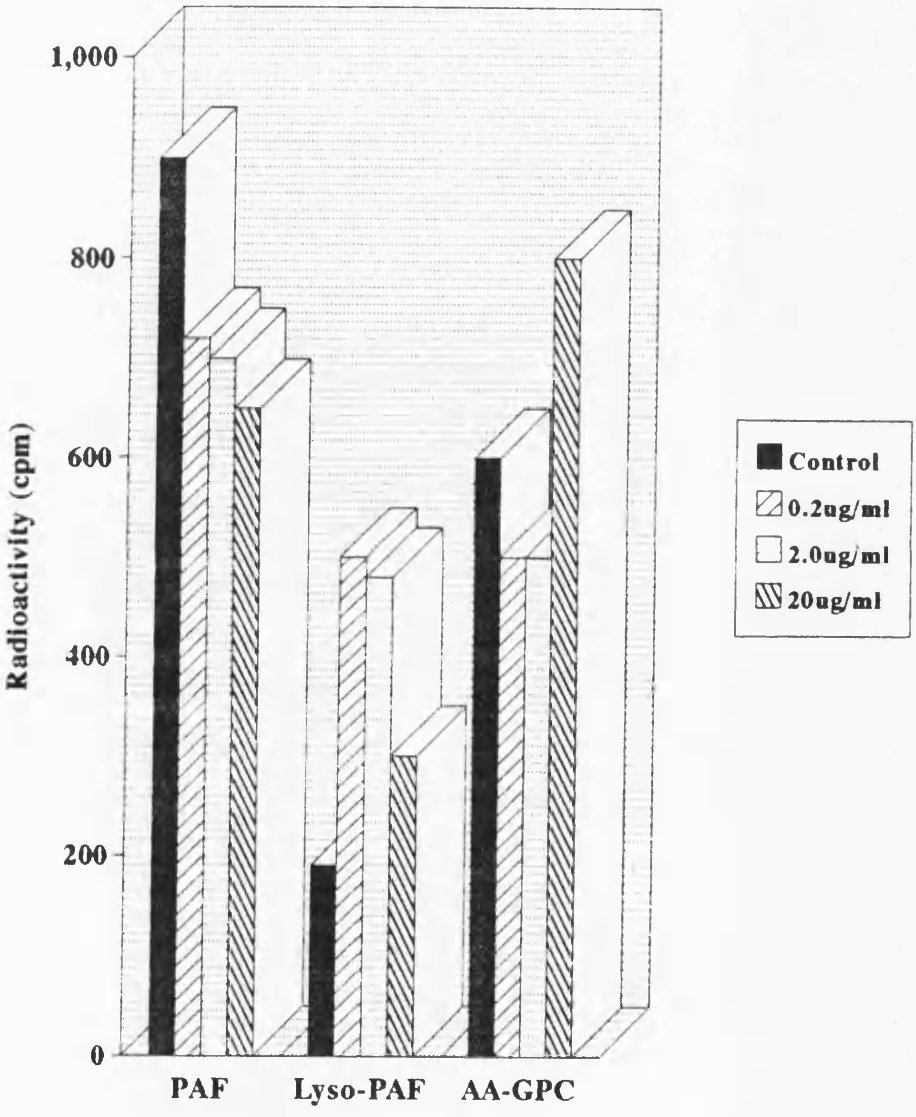


Fig:48.
Distribution of radioactivity in Lyso-PAF(1-O-Hexadecyl-2-lyso-sn-Glycerophosphocholine), PAF (1-O-Hexadecyl-2-acetyl-sn-Glycerophosphocholine),andPhosphatidylcholine(Hexadecyl-2-acyl-sn-Glycerophosphocholine) in corresponding culture medium of Dexamethasone treated-cells. After treatment of C^{14} PAF (1-O-Hexadecyl-2-acetyl-sn-Glycerophosphocholine) incubated cells with various concentrations of Dexamethasone for 48 hrs, the lipids were extracted and radioactive products were separated by TLC and analysed by Berthold TLC linear analyzer with chromatography data system.
The values are representative of three separate experiments.

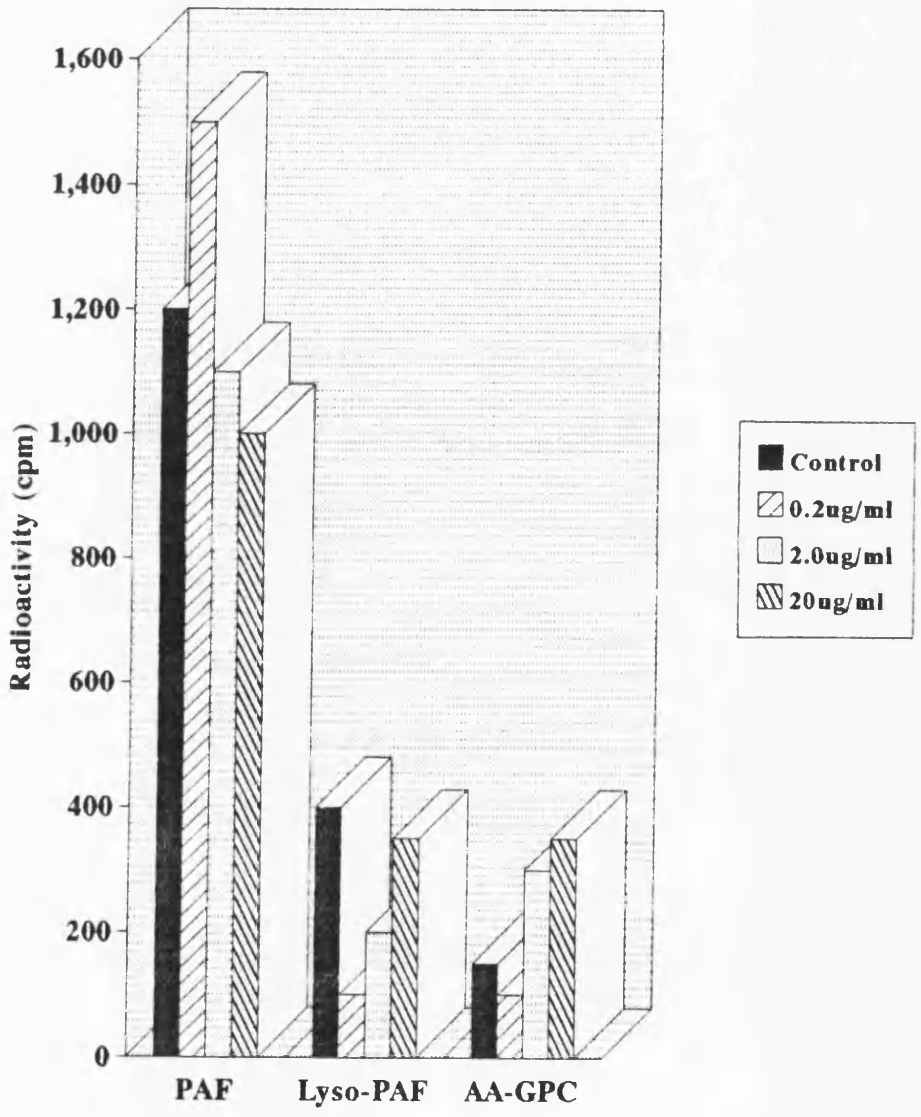


Fig:49.

The effects of treatment of Phorbol esters on [C^{14}] PAF uptake by human amnion cells in culture at various time-points (10, 30, 60 min). The amnion cells were incubated ^{14}C PAF (0.18 nM/plate, 0.1uCi in 0.01 % bovine serum albumin at 37°C in humidified air (95%) with 5% CO_2 in an incubator in presence and absence of Phorbol esters (10^{-7}-10^{-5} M in 0.01% ethanol). After 10, 30, 60 min, the incubation was stopped and the cells were washed with phosphate buffer saline and lipids were extracted by the method of Bligh and Dyer (1959), by choloroform:methanol (2:1). The extracted radioactivity was measured by liquid scintillation counting.
(a): With Phorbol alone.
(b): With Phorbol 12-myristate 13-acetate.
(c): With Phorbol 12,13-dibutyrate.

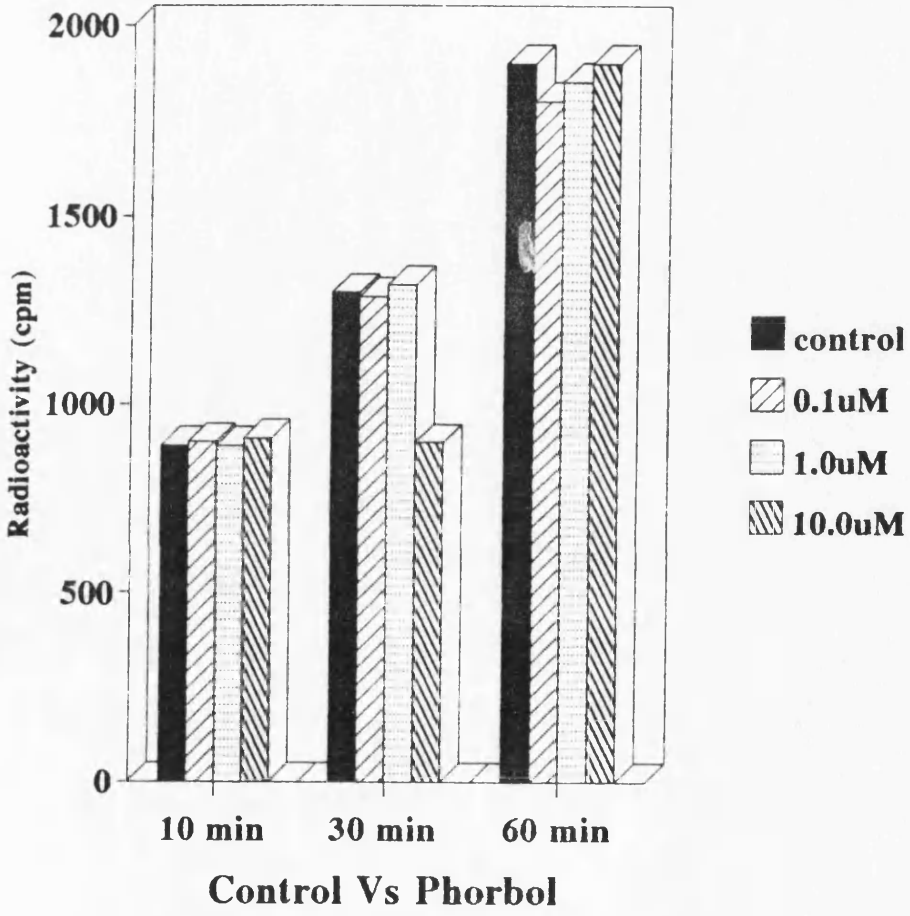


Fig: 49.

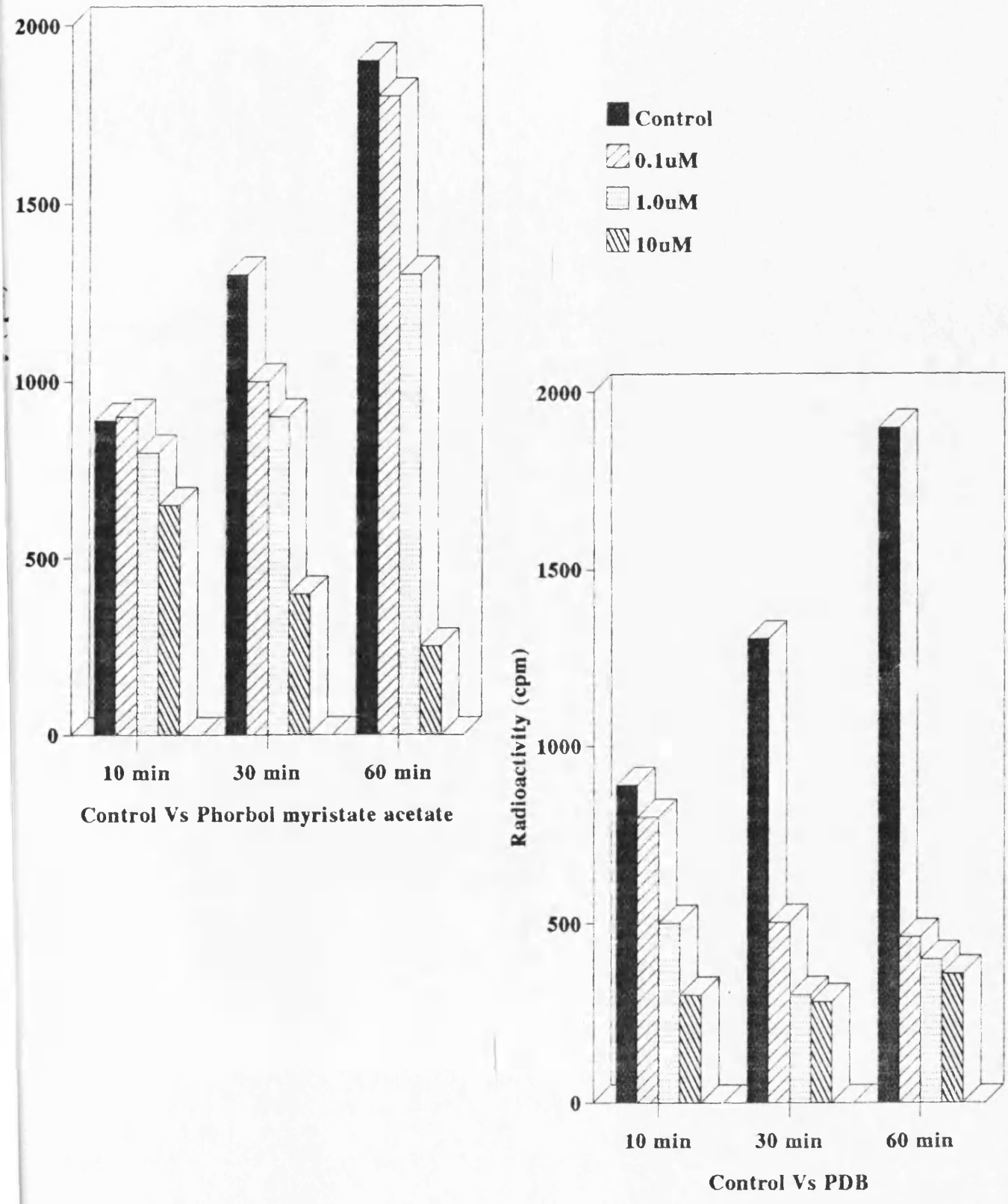
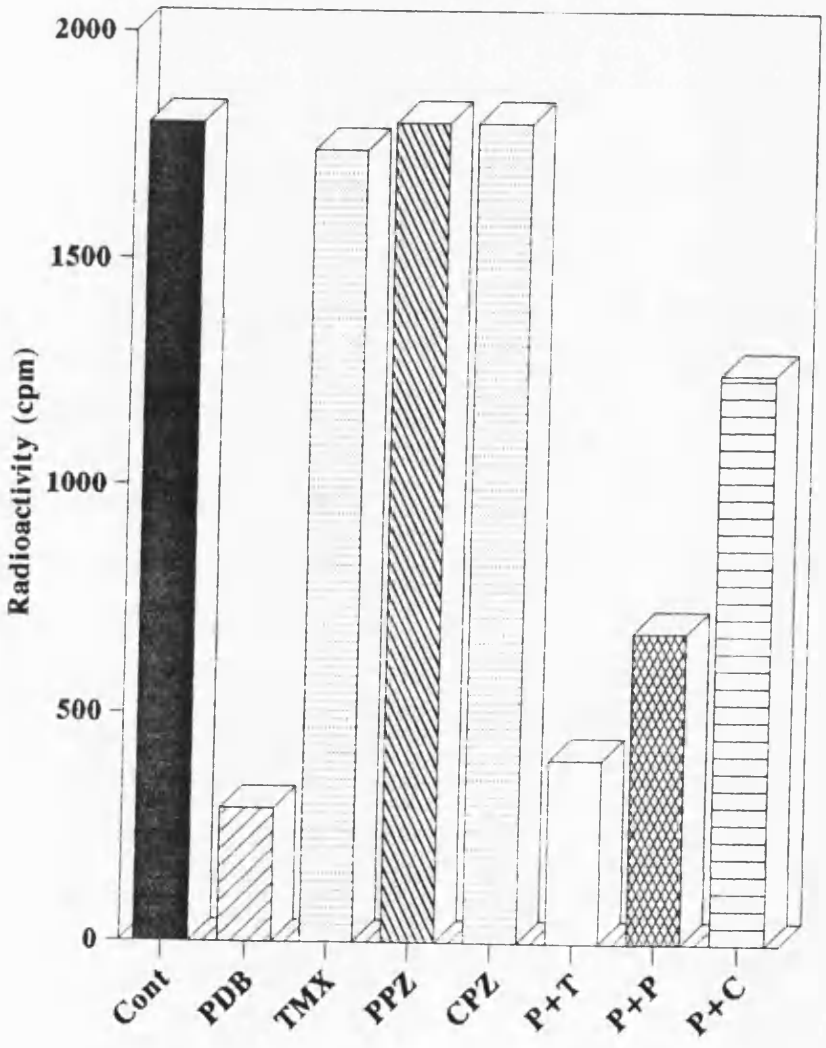

2000
1500
1000
500
0
Control
0.1uM
1.0uM
10uM
Radioactivity (cpm)
10 min
30 min
60 min
Control Vs Phorbol myristate acetate
Control Vs PDB

Fig:50.
Effects of various PKC inhibitors on Phorbol 12,13 Dibutyrate (PDB) (10^{-5}M) or Phorbol 12 myristate acetate-treated amnion cells in culture for 60 min at 37°C in monolayer culture. The cells were prelabelled with ^{14}C PAF (0.18nM) and treated with (a) PDB with or without PKC inhibitor (b) PMA with or without PKC inhibitor. After 60 min, the incubation was stopped and the cells were washed with phosphate buffer saline and lipids were extracted by the method of Bligh and Dyer (1959), by choloroform:methanol (2:1). The extracted radioactivity was measured by liquid scintillation counting.
The results are representative of three separate incubations.
Tamoxifen (TMX): 10^{-7}M
Piperazine (PPZ): 10^{-6}M
Chlorpromazine (CPZ): 10^{-6}M



Phorbol dibutyrate (PDB,10uM), Piperazine (PPZ,1uM),Chorpromazine (CPZ,1uM). Tamoxifen. (TMX,0.1uM).

Fig: 51.
Effects of 17-ß-oestradiol on ^{14}C PAF uptake in Phorbol 12 Myristate-13 Acetate (PMA) and Phorbol 12,13 Dibutyrate (PDB)-treated human amnion cells in culture at 37°C. After 60 min, the incubation was stopped and the cells were washed with phosphate buffer saline and lipids were extracted by the method of Bligh and Dyer (1959), by choloroform:methanol (2:1). The extracted radioactivity was measured by liquid scintillation counting.
The results are representative of three separate incubations.
Oestradiol: 10^{-6}M
PMA: 10^{-5}M
PDB:10^{-5}M

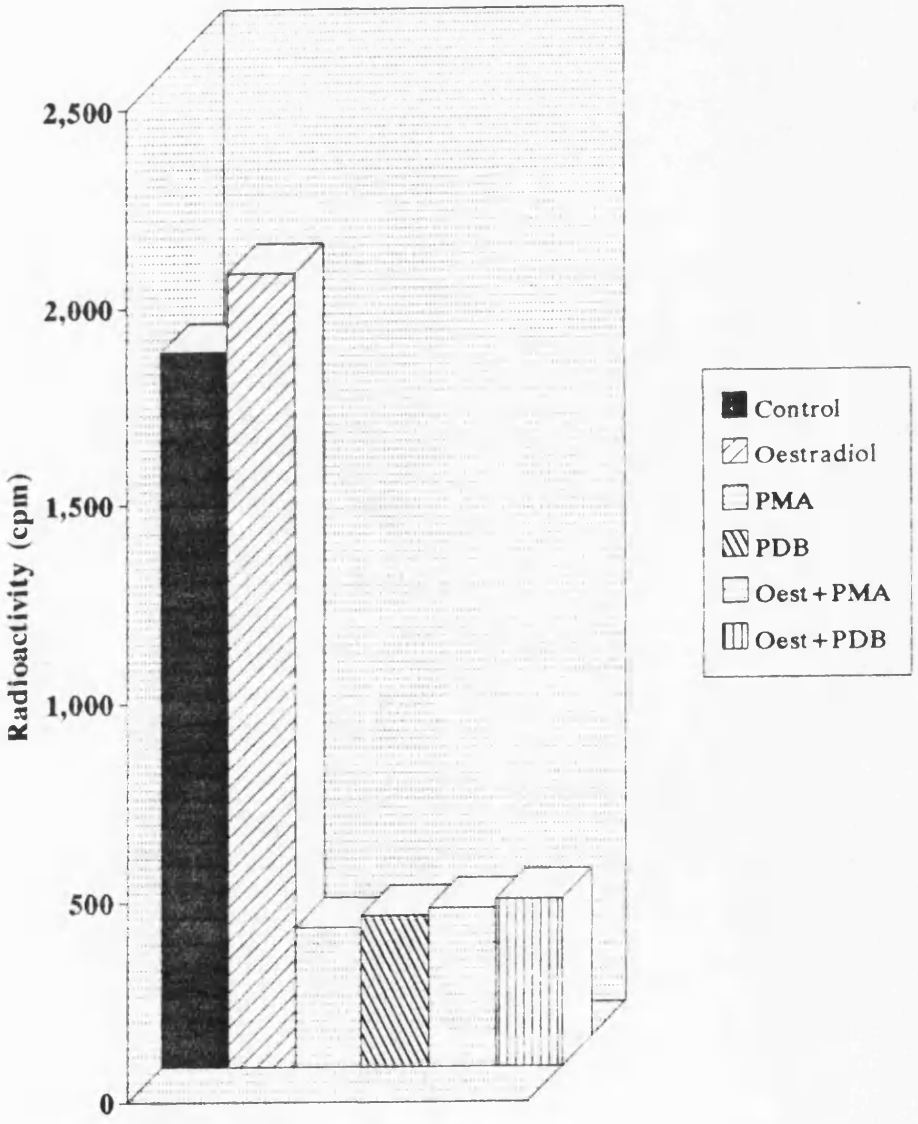


Fig:52.

Comparison of specific activities of PAF acetylhydrolase in maternal plasma from normotensive vs pre-eclamptic pregnancies during third trimester and active labour.
Plasma was obtained from : (a).Normotensive women attending attending antenatal clinics and those admitted in labour room with full term spontaneous normal labour (n=20 in each group) and (b). Women diagnosed to be pre-eclamptics according to the criteria in the methods, attending antenatal clinics or admitted to the labour room with spontaneous labour or those selected for induction of labour, blood was withdrawn prior to induction of labour (n=15).
PAF acetylhydrolase activity was estimated by the method of Blank et al (1981).
The values are Mean ± S. D.

$p < 0.1$ (between 3rd trimester of normal and pre-eclamptic pregnancies).
NS (not significant, between labour samples of normal vs pre-eclamptic pregnancies.

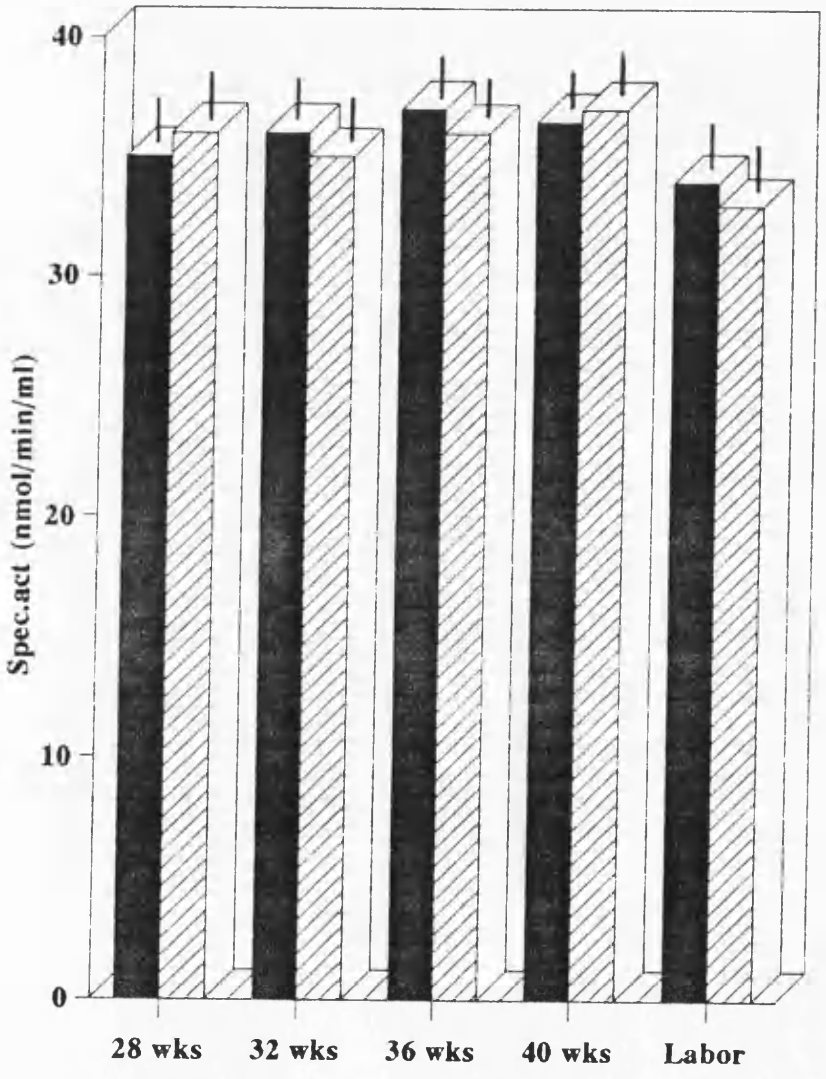
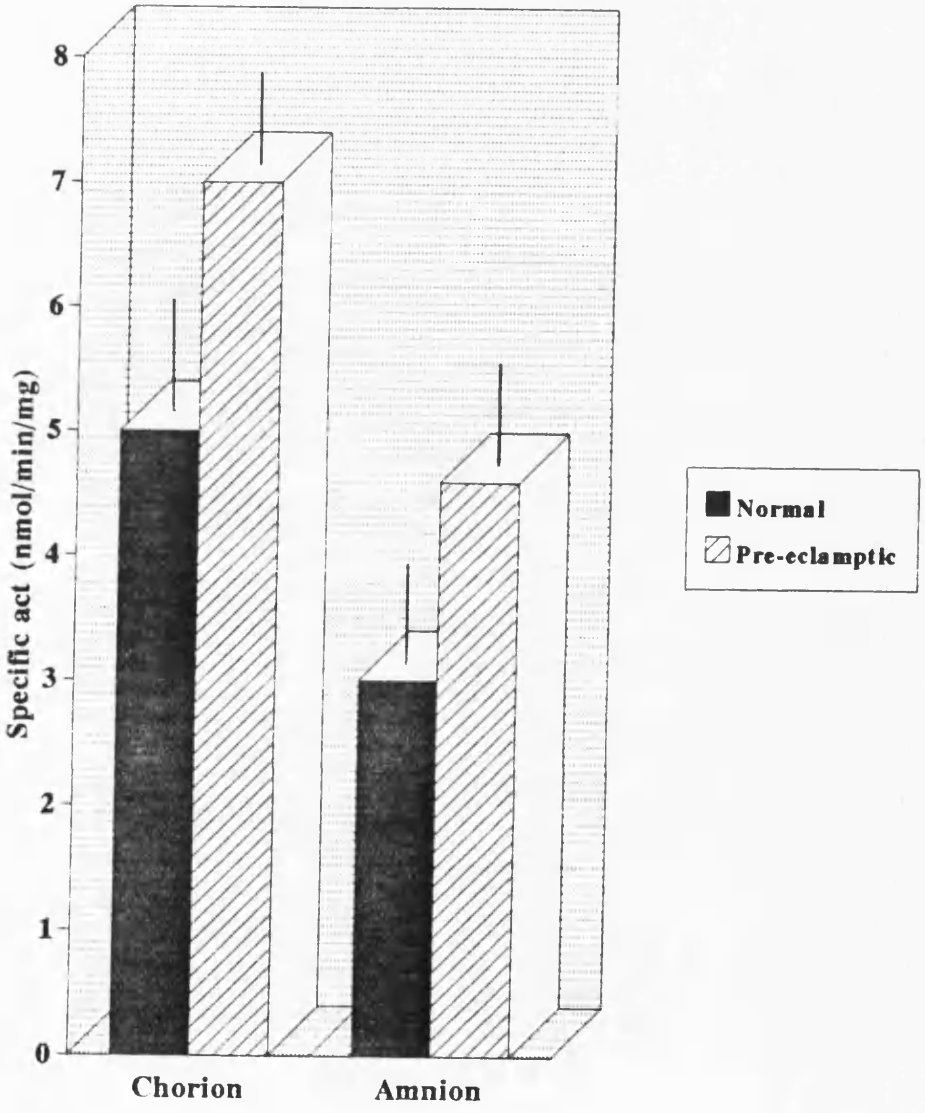


Fig: 53.

Comparison of specific activities of PAF acetylhydrolase in cytosols of amnion, chorion obtained from (a): Normotensive women in full term spontaneous vaginally delivery (n=5). (b) Pre-eclamptic women admitted to labour romm at 36-38 weeks with spontaneous or induced labour (n=5). Cytosols were prepared as described in the methods and PAF acetylhydrolase activity was assayed by the use of [14-C]PAF (0.18 nM) as substrate (Blank et al, 1981).
$p < 0.01$: (between normotensive and preeclamptic tissues).
The results are described as Mean ± S.D.

The values are Mean ± S. D. (n=5)



DISCUSSION :-

SECTION:1

It has been postulated that the regulation of PAF metabolism occurs via inactivation and conversion into precursors like alkyl-acyl-GPC (Braquet et al, 1987). The primary route for intracellular inactivation of PAF is catabolism by a cytosolic acetylhydrolase. Maki et al (1988), had reported earlier on that the plasma acetyl hydrolase activity in rabbit maternal plasma decreased with gestational age and was minimal near term and returned to pre-pregnancy levels during post partum period. Similar changes have been described in human pregnancy (Johnston, 1989).

The results of experiments carried out human maternal plasma showed that plasma PAF acetyl hydrolase enzyme activity, was highest during first 22 weeks of gestation. Subsequently, the activity declined and remained unchanged during labour, only to return the pre-pregnancy levels at 48-72 hr after delivery (Table:1).

Since PAF is now documented to effects on PGE2 metabolism fetal membranes, i.e., amnion (Billah et al., 1985; Morris et al, 1992), contraction of myometrium (Tetta et al, 1986), the functions mostly required at term, rather than early gestation. It appears that during early gestational period, high activity of acetylhydrolase is required to check the PAF levels in maternal circulation.

Moreover, the changes in plasma acetyl hydrolase seem to closely coincide with the molecular events related to fetal lung maturation, as PAF in amniotic fluid is associated with the lamellar body fraction a secretory product of lungs (Hoffman et al, 1986). It has been also shown by Kumar et al (1985), that glycerol ether occur in the phosphatidylcholine fractions of the surfactant from dog lungs.

Human fetal lung development begins at eight weeks of gestation with the appearance of lung buds. By 20-22 weeks the primitive saccules are lined with precursors of cytoplasmic glycogen. These cells progressively differentiate to contain the intracellular surfactant granules, the lamellar bodies by 22-24 weeks.

Foetal lung maturation is assessed clinically by Lecithin (Dipalmitoyl phosphatidyl choline) :

Sphingomyelin ratio in amniotic fluid. The role of PAF in fetal lung maturation is substantiated by results reported in this study that PAF acetylhydrolase activity in normotensive maternal plasma was elevated during early gestation The specific activity of the enzyme reduced at 20-22 weeks of gestation, seems to be coinciding with the events in foetal lung. However, unlike rabbit maternal plasma (Maki et al, 1988), the activity remained constant till term. We failed to observe any significant reduction in the acetylhyrolase activity during spontaneous labour.

The reduction in maternal plasma acetylhydrolase could occur under the influence of maternal hormones, particularly the estrogens, which rise in maternal plasma by mid-gestation (Heap and Flint 1990; Yasuda and Johnston, 1992)). These findings were substantiated in subsequent results in this project.

PAF acetylhydrolase activity, however did not change significantly, in amniotic fluid samples obtained during amniocentesis at 17 weeks, from those at term, but not in labour and amniotic fluid obtained from women in full term spontaneous labour, without any drug intervention. This suggests that, perhaps, hydrolysis by acetylhydrolase amniotic fluid is not the primary route of disposition of PAF in amniotic fluid. Some other mechanisms could be responsible for keeping low or undetectable levels of PAF during pregnancy.

SECTION II:

There are several differences between plasma and tissue enzymes as studied by treatment with various proteases. Snyder and his coworkers (1985) suggested that the differences were due to various degrees of glycosylation of plasma enzyme. Similarly Wardlow et al, (1981) reported that human serum acetyl hydrolase

was sensitive to trypsin and to pronase and resistant to papain.

Stafforini et al (1987), suggested that the biologically active acetylhydrolase was associated with LDL fraction. The enzyme activity could also be transferred from LDL to HDL fractions and vice versa. Our data, suggests that the cytosolic acetylhydrolase appeared to have similar physico-chemical

properties to the plasma enzyme. The amnion cytosol enzyme appears to be an acid-labile enzyme also. The optimal pH was 7-8 and any further change in pH caused a significant loss in enzyme activity. The optimal temperature for PAF acetylhydrolase in amnion was 37 C. There was a reduction in activity on heat-treatment at >60 C. The enzyme is inhibited by various known inhibitors of plasma acetylhydrolase, such as, PMSF (5nM), DFP (2nM), 20% trichloroacetic acid and is protease-sensitive. Various organic solvent-treatment caused almost complete loss of activity. PAF acetylhydrolase enzyme is non-dialysable, as observed by cellophane membrane dialysis for 48 hrs. Sephadex-G column separation and amicon cell ultrafiltration studies suggest that molecular weight of tissue acetylhydrolase is < 50,000. We are not able to describe the accurate molecular size at this point. However, plasma acetylhydrolase enzymes is reported be around 42700 daltons (Maki et al, 1988). Cytosolic enzyme also appears to be of close range.

FACTORS MODULATING ENZYME ACTIVITY;

Recently, it has been shown by Yasuda and Johnston (1992) that PAF acetylhydrolase is decreased in rat plasma by administration of estrogens, while dexamethasone and progestins caused stimulation of enzyme activity. Out data showed that 17-ß-oestradiol inhibited the PAF acetyl hydrolase activity in human amnion cells in a concentration-dependent manner (Fig: 16). Progesterone, on the other hand stimulated the acetylhydrolase enzyme. It is of particular importance to mention that the effects of steroids were time-dependent. Shorter incubations for 30 minutes were devoid of any effect. Some effect was observed at 60 min incubation. However, when the incubations were carried out for up to 48 hrs, using intact cells in culture, the effect was much more pronounced. The predominant effect of oestradiol was inhibition of acetyl hydrolase in amnion cells in culture.
These effects of oestradiol on PAF metabolism are in consistency with to those recently reported by Nakayama et al, (1991) and Yasuda and Johnston (1992). Nakayama had reported that oophorectomy resulted in decrease in uterine PAF levels to 1/3rd of those seen in natural estrus.

The levels were recovered by subcutaneous administration of 17-ß oestradiol. In their work, the level of uterine phospholipids, rich in arachidonic acid were significantly decreased by oestradiol treatment and the arachidonate-PC was depleted more than arachidonate-PE. Schatz and Gurpide (1983) and Olson et al, (1983) have shown that oestradiol stimulates PGE2 output from the decidual cells. Chorion and decidua have high activities of 3-ß-hydroxysteroid dehydrogenase, thereby converting pregnenolone to progesterone (Mitchell et al 1982). In addition, these tissues can convert oestrone sulphate to oestrone and to oestradiol (Mitchell et al, 1984). The concentration of oestrone increases in amniotic fluid in late gestation. Therefore, the potential exists for the foetal membranes and decidua to synthesize the steroid hormones and thereby modulate the local prostaglandin and PAF production.

Ramesha and Pickett (1986) have reported that stimulated cells depleted of arachidonic acid produced little PAF; production could be restored by pre-labelling the cells with arachidonate.

It is also known that arachidonic acid and the products of the lipoxygenase pathway stimulate the production of PAF (Ramesha and Pickett,1986; Billah et al, 1985; Saito et al, 1985; McIntyre et al, 1986). These reports suggest that PAF inactivation could be regulated by eicosanoids. Our results demonstrate that PGE2 significantly stimulated the acetylhydrolase activity in amnion. PGF2a also increased PAF breakdown, however the effect was demonstrable only at significantly higher doses than those of PGE2. Prostacyclin was devoid of any effects.

It is likely that the inter-actions of prostaglandins, PAF and maternal steroid hormones could lead to important consequences.

Oxytocin and PAF synergies in myometrium (Tetta et al, 1986).

These results showed that oxytocin inhibited the inactivation of PAF in a dose-dependent manner, by inhibiting the acetylhydrolase enzyme (Fig: 14,15). PAF is also reported to evoke a contractile response of myometrial strips obtained from the human pregnant uterus. Moreover, PAF receptor has been isolated in human myometrium recently (Zhu et al, 1992). It seems logical to hypothesize that PAF and oxytocin could be augment each other's effects on myometrium during labour via two

mechanisms: (a) PAF-induced release of PGs which potentiate the effects of oxytocin on the myometrium (b) Oxytocin mediated inhibition of PAF breakdown.

SECTION III:

Billah et al (1983), reported that PAF is undetectable in human amniotic fluid obtained before labour, but is present in the majority of samples obtained after labour. This finding was later confirmed by Nishihara et al, (1986). We have previously observed that PAF acetylhydrolase activity in human amniotic fluid does not change significantly, with progression of gestation.

It seems that PAF, secreted from fetal lung or fetal kidney, is only partly inactivated by an acetylhydrolase present in amniotic fluid. The levels of PAF could be regulated by its being taken up by the amnion membrane cells from amniotic fluid where it could meet different fates and consequently affect various biochemical events related to arachidonic acid metabolism.

^{14}C-PAF was taken up by the amnion cells in culture linearly with time. The analysis of radioactive zones revealed that PAF was the predominant peak during first 20-30 min, Lyso-PAF appeared after that, constituting 15% of the radioactivity. By 60 min, alkyl-acyl-GPC also appeared. This pattern of PAF metabolism in amnion cells is somewhat different from that described in other tissues e.g. platelets (Malone et al, 1985; Chilton et al, 1983), rabbit neutrophils (Chilton et al, 1983), macrophages (Robinson et al, 1985) and human umbilical vein endothelial cells (Blank et al, 1986) where lyso-PAF appeared much earlier.

It could be that these tissues probably need to inactivate and store PAF in the form of precursors. In amnion cells however, delayed inactivation means the lipid mediator is involved in a variety of biochemical events related to intracellular calcium mobilization, as well as stimulation of PGE_2 production at the onset of labour. At later time points the lyso-PAF was acylated to form alkyl-acyl-GPC, which constituted 35% of radiolabel. Analysis of radiolabel in the media at corresponding time points revealed that lyso-PAF and later on alkyl-acyl-GPC were secreted from the cells.

The distribution of the exogenous PAF in various membrane phospholipids, showed that the radioactivity was detectable in phosphatidylcholine and in a product with an Rf value similar to that of phosphatidylethanolamine (20%). However phosphatidylcholine(alkyl-acylGPC or diacyl GPC) remained the principle phospholipid, in which PAF was stored.

Phospholipase A_2 treatment of pre-labelled cells, resulted in loss of 95% of the radiolabel, suggesting that the loss of acyl group at sn-2 position of 1-O-alkyl-2-acyl-GPC or diacyl-GPC results in the release of lyso-PAF and hence PAF, along acyl group which predominantly is arachidonic acid. Bromophenacyl bromide reversed the phospholipase effects.

The structural requirement for the biological responses mediated by PAF are highly specific, suggesting that its functions may be mediated through a receptor. There is a good correlation between its biological actions either in vitro or in vivo and its

affinity for the receptor on isolated platelet membranes (Hwang et al, 1986). Our data corroborated the findings that very low concentration are necessary to trigger biological effects. Recently a battery of PAF receptor antagonists are under laboratory and clinical investigation, e.g, WEB 2086 (Apafant, Boeringer Ingelheim), WEB 2170, BN 50726, BN 50730, BN 52021 (Ipsen-Beaufor, France), RP 55778, RP 59227 (Rhone-Poulenc, France) and Y-24180 (Yoshimoto, Japan) (Koltai et al, 1991).

The presence of PAF-receptors in amnion cells is reported

using WEB 2086, a potent PAF receptor antagonist. We observed that although the antagonist reduced the uptake of PAF by amnion cells in a dose-dependent manner, however did not completely abolish it suggesting other mechanisms could be operating besides, the receptor-mediated uptake.

This work substantiates the existing information regarding PAF uptake by various cells (1991, TIPS; receptor supplement), which suggests that PAF uptake is in part receptor-mediated, and in part by other membrane uptake mechanisms, in various tissue.

WEB-treated cells metabolised PAF into lyso-PAF, but there was loss of GPC, from the cellular lipids, as compared to controls.

However, GPC peak was detectable in culture medium of WEB-treated cells. This could well mean that, the WEB-treated cells may be synthesizing GPC, but not retaining it, instead GPC was released into the medium. The significance of this observation at this point is not clear. Indomethacin and lipoxygenase inhibitor NDGA also caused non-dose-dependent inhibition of uptake of PAF. The profile of distribution of radioactivity showed that indomethacin and NDGA-treatment caused loss of GPC from amnion cells. This GPC loss was different from WEB-2086 effects, as it was both from the cells as well as in culture media. It is likely that inhibition of cyclo-oxygenase and lipoxygenase enzymes, in turn be regulating the uptake and metabolism of PAF. We had earlier observed that PGE2 stimulates PAF breakdown by increasing PAF acetylhydrolase activity in amnion cells and it appears that blocking PGE2 effects by Indomethacin had led to reduction in PAF hydrolysis and reduced formation of lyso-PAF and GPC in amnion cells. Gilfillan et al (1985), had reported that archidonic acid stimulated the release of phosphatidylcholine from type II pneumonocytes and NDGA reduced this stimulatory effect, whereas, Indomethacin had no effects.

Merthiolate and N-ethylmaleimide are known to possess inhibitory activity for sulfhydryl group of acyl-CoA-alkyl-sn-glycerophosphocholine transferase enzyme (Herman et al, 1986).

Treatment of cells with Merthiolate and N-ethylmaleimide led to significant decrease in radiolabel uptake of PAF and incorporation into membrane lipids, suggesting the possibility that the re-acylation of lyso-PAF also modulates the uptake of this lipid mediator. It appears from results that the uptake and metabolic fate of alkyl-acetyl-GPC (PAF) into alkyl-lyso-GPC and alkyl-acyl-GPC, is affected by acylation processes, which, in turn affects the incorporation of arachidonic acid. This is in accord with the above observations that cyclo-oxygenase and lipoxygenase blocked PAF uptake.

Incubations with cellular Na+/K+ ATPase inhibitor Ouabain, suggest that there is an involvement of active energy in uptake process. It is not clear at this moment, at what site this ATP-related energy is required. Most likely the energy is involved in receptor-mediated processes. Calcium plays a crucial role in various cellular reactions. PAF and calcium are documented to stimulate PGE2

production by amnion cells (Billah et al, 1986). We observed that calcium ionophore A23187 in presence if extracellular calcium, stimulated PAF uptake. It is possible that calcium-induced stimulation of PAF uptake could be that of receptor-mediated. Furthermore, we observed an actual decrease in uptake in absence of extracellular calcium.

ROLE OF PROTEIN KINASE C:

The tumour promoting phorbol esters are currently used as probes to study the involvement of protein kinase C (PKC) in regulating a variety of cell functions (Ashendel, 1985). These agents are extremely potent activators of PKC and in this respect mimic diacylglycerol (DAG), which is the endogenous signal for PKC stimulation (Nishizuka, 1984; 1986). DAG is formed rapidly but transiently as a result of receptor mediated breakdown of membrane phosphatidylinositol, a process that yields IP_3, the intracellular messenger responsible for the mobilization of cytosolic Ca^{++} (Berridge, 1984: Berridge and Irvine, 1984). Okazaki et al, (1984) have shown that amnion tissue contains a Ca^{++}-activated phospholipid-dependent PKC.

Lytton and Mitchell (1988) reported that Phorbol 12-myristate 13-acetate (PMA) and Phorbol 12,13-dibutyrate (10^{-8}-10^{-6} M) induced a concentration-dependent stimulation (10.8-fold and 5.9 fold respectively) of PGE_2 by amnion cells, and 4-_-Phorbol 12,13-didecanoate had little or no effect.

The data from this study showed that the incubation of amnion cells with various concentrations of Phorbol, Phorbol 12-myristate 13-acetate and Phorbol 12,13-dibutyrate (10^{-7}-10^{-5} M) resulted in a significant time-dependent reduction of PAF uptake at all concentrations by Phorbol 12 myristate 13 acetate and Phorbol 12,13 dibutyrate. However, Phorbol itself was devoid of any activity. Phorbol is not considerd to be a potent PKC activator.

The inhibition was particularly significant at later time points at 60 min. Subsequently treatment was carried out with Tamoxifen, Piperazine and Chorpromazine, prior to phorbol ester treatment. These

carried out with Tamoxifen, Piperazine and Chorpromazine, prior to phorbol ester treatment. These agents are known to inhibit PKC. Tamoxifen was ineffective in reversing the effects of phorbol myristate acetate or dibutyrate. Piperazine could only partly reverse, whereas chlorpromazine was the most effective. On their own, none of these PKC inhibitors had any effects on PAF uptake.

Recently, several reports have appeared in the literature that implicate PKC-activation in the down-regulation of receptors that are coupled to Inositol Phosphate metabolism, in a number of tissues and clonal cell lines. These studies show that pre-incubation with phorbol esters, results in a reduced accumulation of intracellular inositol phosphate in response to a subsequent agonist challenge (Labarca et al, 1984; Orelana et al, 1985; Vicentini et al, 1985; Monaco and Mufson, 1986).

The results of this investigation have shown that uptake of PAF in dispersed human amnion cells, is in part receptor-mediated internalization, as WEB 2086 treatment leads to dose-related reduction in PAF uptake. Moreover, PAF incorporation is partly mediated by some other unknown membrane uptake mechanism (s), which could be regulated by transacylation of arachidonic acid.

The receptor-mediated PAF binding is perhaps regulated by PKC, as PKC activation by phorbol esters for longer incubation periods leads to down regulation of PAF receptors in amnion tissue. These results are consistent with those reported by Pearce et al, (1988), Murphy and Welk (1989; 1990) and Brock et al, (1987).

The results of this study and those of various investigators are contrary to what has been reported by Lytton and Mitchell (1988).

EFFECTS OF STEROID HORMONES ON PAF UPTAKE AND METABOLISM:

It is well documented that increasing levels of oestrogens with the progress of gestation are associated with functions like increased protein synthesis, such as those of oxytocin receptors, increased conduction (gap-junction) in individual myometrium cells (Heap and Flint, 1990). The

present results demonstrate that role of oestradiol in elevating the of PAF levels intracellularly. This elevation was attained by :a):increased PAF uptake by oestradiol-treated amnion cells, b):by inhibition of PAF inactivation i.e., reduction in acetylhydrolase enzyme activity, c): by reducing GPC levels in cellular lipids. The concomitant decrease in acylated-GPC could be due to direct inhibition of Co-A dependent transacylase, or could be a natural sequelae of decreased substrate availability for transacylation. This further lends credence to the role of oestrogen in modulation of PAF metabolism.

Progesterone treatment caused a significant increase in uptake. However, progesterone caused a stimulation of PAF acetylhydrolase, but the effect was not as pronounced as that of oestrogen. There are number of instances in the body, where the effects of steroid hormones can be complementary and/or antagonistic to each other. During menstrual cycle, the effects of progesterone are seen in oestrogen-primed endometrium (Clark et al, 1970) and their effects on breast tissue (Jaffe et al, 1986). The inhibitory effect of progesterone on pregnant myometrium and increased synthesis of oxytocin receptors by oestradiol are also well documented. The combination of oestrogen and progesterone increased total uptake of PAF by amnion cells, greater than oestradiol and progesterone alone.

However, the effect of combination of oestradiol and progesterone on PAF metabolism had different results. The combination was accompanied by predominance of oestradiol effects on PAF metabolism.

Glucocorticoids decrease prostaglandin biosynthesis by inhibiting phospholipase A_2 in various tissues (Casey et al, 1985).

However, the results in reproductive organs are contradictory.

Casey et al (1985) found that during parturition in uterine tissue in vivo high amounts of PG occur that are associated with high cortisol values in serum. No inhibition of PG production by was observed after Dexamethasone in endometrial tissue in vitro. However, in myometrial tissue PI_2 synthesis was markedly reduced by dexamethasone. These results were interpreted as an inversion

of prostaglandin balance in uterine tissue, finally inducing labour. Yasuda and Johnston (1992), have observed a stimulatory effects of dexamethsone on PAF acetylhydrolase activity in adult, as well as juvenile rat plasma.

No work update has been done to investigate the role of corticosteroids on PAF metabolism in human fetal membranes. The experiments of incubation with amnion cells with ^{14}C PAF in the presence of Dexamethasone, revealed that, dexamethasone reduced PAF incorporation in a dose-related manner (Fig: 52).

Dexamethasone treatment however promoted acylation of lyso-PAF, leading to increased radiolabel in the alkyl-acyl-GPC, which in turn could be due to increased hydrolysis of PAF into Lyso-PAF by acetylhydrolase. These findings are in consistency with those of Yasuda and Johnston (1992). This effect of dexamethasone on phospholipid metabolism can explain the therapeutic rationale of giving dexamethasone to premature babies, to stimulate fetal lung maturity and to prevent fetal respiratory distress syndrome, which occurs because of a deficiency of lung surfactant like dipalmitoyl glycerophosphocholine (lecithin). By stimulating acetylhydrolase and subsequent, acylation of lyso-PAF, dexamethasone may in turn be increasing the diacylation of glycerophosphocholine in type II pneumonocytes. These effects of dexamethasone on phospholipid metabolism needs to be extrapolated to type II pneumonocytes.

These data on PAF metabolism in human amnion cells and possible modulation by various humoral and hormonal factors have given impetus to the role of PAF in physiological processes related to reproductive biology.

SECTION IV:

ROLE OF PAF IN PRE-ECLAMPSIA:

PAF is a hypotensive agent, and the regulation of its metabolism is thought to occur by its inactivation. PAF acetylhydrolase activity has been reported to be high in hypertensive rats (Blank

in patients with essential hypertension.

Pregnancy-induced hypertension is evident after 22-24 weeks of gestation. Johnston et al, (1989) reported that plasma acetylhydrolase activity remains elevated in pre-eclamptic pregnancies, in a limited number of samples. Our results failed to confirm any such finding. PAF acetylhydrolase activity in plasma was not significantly higher in pre-eclamptic pregnancies than in normotensive pregnancies (Fig: 59).

However, specific activity of the enzyme was significantly raised in chorioamnion cytosolic fractions obtained from pre-eclamptic patients, as compared to the normotensive. We suggest that since PAF is rapidly taken up by the amnion cells, where it could be playing crucial roles in initiation of parturition, as well as regulating local homeostasis, like vasodilation of placental vasculature and hence ensuring uninterrupted blood flow to the growing fetus. Increased PAF acetylhydolase activity in fetal membranes as compared to those from normotensive placentae, suggests that, increased catabolism of hypotensive lipid, is carried out locally, which could be disrupting the placental blood flow.

Thus the results of the present study have shown that during pregnancy PAF catabolism in maternal plasma showed gestational-age related reduction, only to facilitate the events related to foetal lung maturation.

Amnion cytosolic PAF acetylhydrolase seemed characteristically to be the same as its plasma counterpart. PAF metabolism is modulated by sex steroids and other factors present during pregnancy.

PAF receptor is present in human amnion and is regulated by PKC.

PAF is also taken up by the amnion cells by the mechanism(s) regulated by acylation of phospholipids and dependent on energy provided by ATP breakdown.

PAF acetylhydrolase did not significantly differ in pre-eclampsia as compared to the normotensive pregnancies, suggesting this is not only mechanism functional in pathogenesis of pregnancy-induced hypertension.

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