MODULATION OF CYTOSOLIC PHOSPHOLIPASE A_2

BY

YAN JENNY JIANG

A thesis submitted to the Faculty of Graduate Studies
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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of Master of Science

To my family

Dad, Mom, Sisters and Brother

and

my husband Biao, my son Frank

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ABSTRACT

Phospholipase A_2 (PLA_2) is an ubiquitous enzyme found in mammalian cells. In the last two decades, several forms of PLA_2 have been identified, including a 14-kDa secretory PLA_2 ($sPLA_2$) and a 85-kDa cytosolic PLA_2 ($cPLA_2$). The $cPLA_2$ is a calcium-dependent phospholipase which catalyses the hydrolysis of glycerophospholipids at the *sn*-2 position. The enzyme has a high degree of specificity for the release of arachidonic acid (AA) which is a precursor for the biosynthesis of eicosanoids. The arachidonic acid and its metabolites possess diverse biological properties, many of which are related to vascular homeostasis. $cPLA_2$ activity is modulated by a number of factors, including $[Ca^{2+}]_i$, protein phosphorylation, and other forms of post-translational regulation. However, the mechanism for controlling its expression at the transcriptional level has not been well-defined.

Peroxisome proliferator activated receptors (PPARs) belong to the superfamily of nuclear hormone receptors that heterodimerize with the retinoid X receptor and regulate the transcription of several genes responsible for lipid metabolism and adipocyte differentiation. Three subtypes of PPAR have been found so far, including PPAR-α, -γ and -δ (or β). PPARα is predominantly expressed in tissues with high catabolic rates for fatty acids and peroxisomal metabolism. PPARγ plays a pivotal role in the control of metabolic function in the adipocyte. PPARδ is ubiquitously expressed, but the function is less known. In this study, we hypothesize that the catabolism of phospholipids by the $cPLA_2$ is regulated via the activation of PPAR pathways at the transcriptional level. The objective of our study is to elucidate the role of PPARs in the regulation of $cPLA_2$ in mammalian cells. Since cyclooxygenase-1/2 (COX-1 and -2) are key enzymes for conversion of AA to eicosanoids,

and $cPLA_2$ and COX-2 are functionally coupled in eicosanoids biosynthesis, we also examined the gene expression of COX-1 and COX-2.

Using the human preadipocyte SW872 cell line as a model, the modulation of arachidonic acid release by PPAR activators was examined. Clofibrate, a PPARα activator, caused an enhancement of arachidonate release in SW cells. This increase was the result of an elevated $cPLA_2$ activity which was caused by an increase in enzyme protein. The presence of PPAR-α and -γ in preadipocytes was confirmed by the presence of the respective mRNAs for these two proteins. Clofibrate (10-200μM) caused increases in the mRNAs encoding $cPLA_2$ and cyclooxygenase (COX-2) in a dose dependent manner. However, at higher dosage (400 μM), clofibrate treatment resulted in the attenuation of $cPLA_2$ mRNA and protein expression. Colorimetric assay using WST-1, a cell proliferation reagent, confirmed that a high dose of clofibrate impaired the viability of preadipocytes. Our results suggest that the up-regulation of $cPLA_2$ gene expression is caused by an increase in its rate of transcription. As predicted, clofibrate has no effect on COX-1 gene expression. Taken together, these results suggest that the PPARα activator clofibrate induced the gene transcription of $cPLA_2$, which led to an increase in $cPLA_2$ protein. The resultant increase in enzyme activity caused the enhancement of A-23187-induced arachidonate release in SW872 cells.

The modification of $cPLA_2$ activity by lyso-PC at the post-translational level was investigated using rat heart myoblastic H9c2 cells as a model. The incubation of H9c2 cells with lyso-PC resulted in an enhanced release of arachidonate in both a time- and dose-dependent fashion. Lyso-PC species containing palmitoyl ($C_{16:0}$) or stearoyl ($C_{18:0}$) groups evoked the highest amount of arachidonate release, while other lysophospholipid species

were relatively ineffective. The presence of phospholipase A_2 inhibitors attenuated the lyso-PC enhanced arachidonate release. Lyso-PC caused the translocation of phospholipase A_2 from the cytosol to the membrane fraction and induced an increase in Ca^{2+} flux from the medium into the cells. Nimodipine, a specific Ca^{2+}-channel blocker, partially attenuated the lyso-PC-induced rise in intracellular Ca^{2+}. Concurrent with Ca^{2+} influx, lyso-PC caused an enhancement of protein kinase C activity. The lyso-PC induced arachidonate release was attenuated when cells were pre-incubated with specific protein kinase C and mitogen activated protein kinase kinase inhibitors. These results indicate that the lyso-PC induced an increase in the level of intracellular calcium and stimulation of protein kinase C, which lead to the activation of $cPLA_2$ and the enhancement of arachidonate release in H9c2 cells.

In conclusion, we have shown that the action of $cPLA_2$ is regulated both at the transcriptional and post-translational levels. Using the SW872 cell line as a model, the long term regulation of $cPLA_2$ at the transcriptional level by clofibrate was demonstrated. The precise mechanism for the regulation is unclear. In addition, lyso-PC activated $cPLA_2$ via post-translational modification of the enzyme. Lyso-PC caused an elevation of the intracellular calcium concentration and the activation of PKC, which stimulated $cPLA_2$ via the activation of the MAPK and resulted in an enhanced release of arachidonate.

LIST OF ABBREVIATIONS

AA	arachidonic acid
$AACOCF_3$	arachidonyltrifluoromethylketone
ADD-1	adipocyte differentiation and determination factor 1
ADD-water	autoclaved, double-distilled water
AMP	adenosine monophosphate
AP-1	activation protein-1
aP2	adipocyte fatty acid binding protein P2
ATCC	American type culture collection
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BIS-I	bisindolylmaleimide-I
BSA	bovine serum albumin
C/EBP	CCAATT enhancer binding protein
CaLB	Ca^{2+}-dependent lipid-binding
cAMP	cyclic AMP
cDNA	complementary deoxyribonucleic acid
CLO	clofibrate (clofibric acid)
CoA	coenzyme A
COX	cyclooxygenase
COX-1	cyclooxygenase 1

COX-2	cyclooxygenase 2
$cPLA_2$	cytosolic phospholipidase A_2
DBD	DNA-binding domain
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulfoxide
dNTP	deoxynucleotide
dpm	disintegrations per minute
DR	direct repeat
DR-1(2)	direct repeat spaced by one (two) nucleotide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glyco-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
ERE	estrogen-response element
FABP	fatty acid binding protein
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GAS	interferon-gamma activation site
GR	glucocorticoid receptor
GRE	glucocorticoid response element

HDL	high density lipoprotein
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid
HODE	hydroxyoctadecadienoic acid
hPPARα	human proxisome proliferator-activated receptor alpha
$hPPAR\alpha_{tr}$	human proxisome proliferator-activated receptor alpha truncated (protein)
$hPPAR\alpha_{wt}$	human proxisome proliferator-activated receptor alpha wild type
HRE	hormone response element
γ-IRE	interferon-γ response element
$iPLA_2$	Ca^{2+}-independent phospholipase A_2
LBD	ligand-binding domain
LDL	low density lipoprotein
LPL	lipoprotein lipase
LPS	lipopolysacharide
lyso-PC	lysophosphatidylcholine
M-MEV	Moloney murine leukemia virus
MAPK	mitogen-activated protein kinase
MBP	membrane binding domain
mPPARα	mouse proxisome proliferator-activated receptor alpha
mRNA	messenger ribonucleic acid
MSH	mitochondrial succinate dehydrogenase
NF-κB	nuclear factor-κB
NIM	nimodipine

NSAIDS	non-steriodal anti-inflammatory drugs
OCT	octamer transcription factor
pBPB	*Para*-bromophenacyl bromide
PBS	phosphate buffered saline
PEA3	polyomavirus enhancer A-binding protein-3
PGH	prostaglandin endoperoxide
PGH_2	prostaglandin H_2
PGJ_2	prostaglandin J_2
PIP_3	phosphatidylinositol 1,4,5-triphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PMA	phorbol myristate acetate
PPAR	peroxisome proliferator-activated receptor
PPRE	proxisome proliferator response element
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
RXR	retinoid X receptor
SDS/PAGE	sodium dodecylsulfate/ polyacrylamide gel electrophoresis
$sPLA_2$	secretory phospholipase A_2
SREBP-1	sterol regulatory element binding protein 1

TBS	Tris-buffered saline
TG	triacylglycerol
Tris	tris (hydroxymethyl) aminomethane
TLC	thin layer chromatography
TX	thromboxane
TXA_2	thromboxane A_2
WST-1	4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate
$[Ca^{2+}]_i$	intracellular calcium concentration

I INTRODUCTION AND LITERATURE REVIEW

1. The catabolism of phospholipids by phospholipase A_2

1.1 Catabolism of phospholipids in mammalian cells

All cells contain phospholipids which provide the essential milieu of cellular membranes and act as barriers for the entry of compounds into cells (Vance, 1991). Within the phospholipid bilayer second messenger precursors are stored and these messengers are released after a cell has received an appropriate signal. These second messengers include eicosanoids, diacylglycerols and phosphatidylinositol 1,4,5-triphosphate (PIP_3) (Nishizuka, 1992; Berridge, 1993). In this study we will focus on the enzyme that hydrolyzes phospholipids to release arachidonic acid, an eicosanoid precursor (Fig 1).

Phospholipases A_2 (EC 3.1.1.4) are a group of enzymes that catalyze the hydrolysis of phospholipids at the *sn*-2 position, to yield a lysophospholipid and free fatty acid (Vance, 1991). One of the important free fatty acids released is arachidonic acid, a 20-carbon fatty acid with *cis* double bonds at carbon 5,8,11, and 14. Once released, the arachidonic acid may be converted into eicosanoids via three major pathways (Fig 2). The cyclooxygenase (COX) pathway leads to the synthesis of prostaglandins and thromboxanes. The first enzyme in this pathway is known as prostaglandin endoperoxide synthase (PGH synthase), also called COX (Needleman *et al.*, 1986; Smith *et al.*, 1991). PGH synthase exists in an already active form in the endoplasmic reticulum. This enzyme exhibits two different catalytic activities: a cyclooxygenase (*bis*-oxygenase) which catalyzes the formation of PGG_2 from arachidonate and a peroxidase (or hydroperoxidase) which facilitates the reduction of PGG_2 to PGH_2

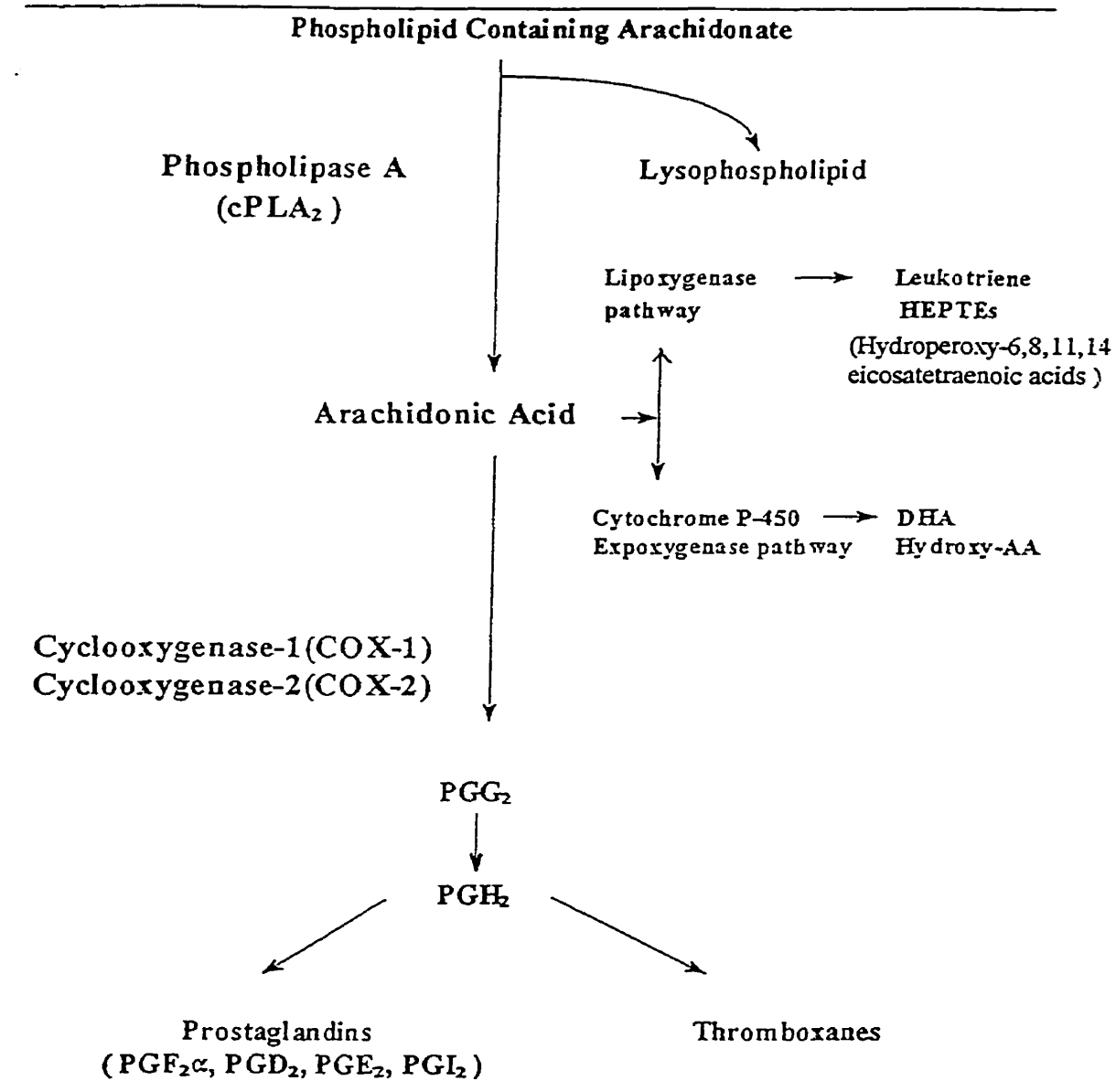


Fig 1. The catabolism of phospholipids

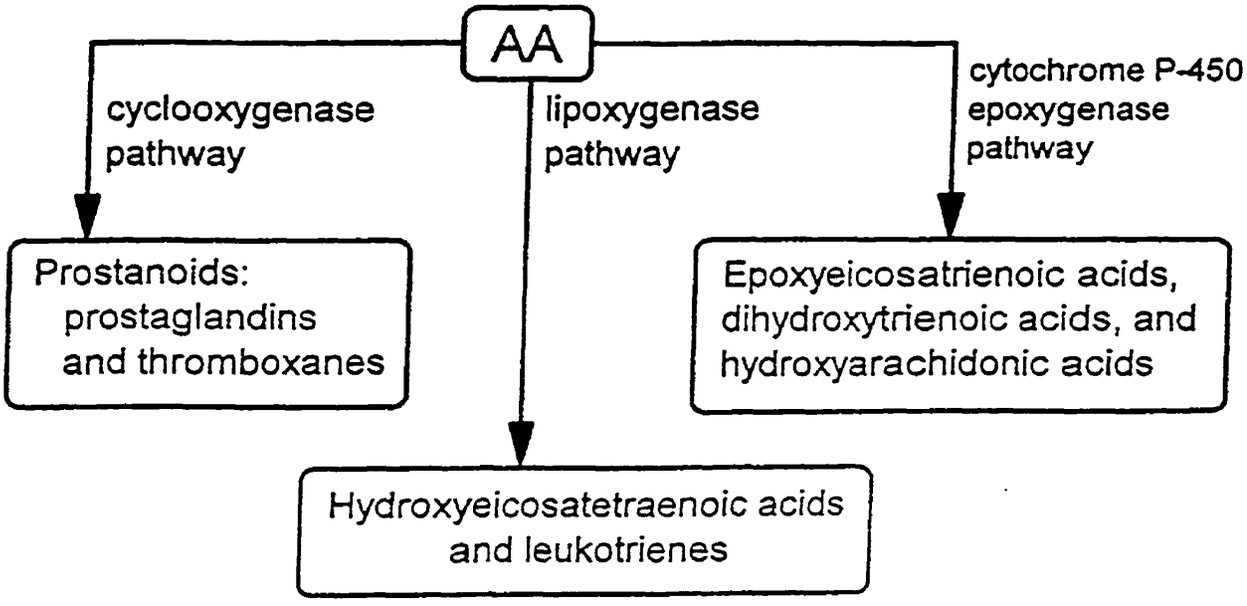


Fig 2. Pathways for the metabolism of arachidonic acid (AA)

(Smith and Marnett, 1991; DeWitt, 1991). Lipoxygenase catalyzes the first step in the synthesis of the hydroxy-eicosatetraenoic acids and the leukotrienes. The cytochrome P450 epoxygenase catalyzes the first step in the synthesis of the epoxyeicosatrienoic acids.

In contrast to hormones which have broad systemic effects despite being released from a single site in the body, prostaglandins are synthesized in a broad range of tissue types and serve as autocrine or paracrine mediators to signal changes within the immediate environment. Two classes of prostaglandin receptors exist to transduce signals upon binding of ligand, the G-coupled cytoplasmic receptor class and the nuclear PPAR receptor class (Forman *et al.*, 1996).

1.2 Classification of Phospholipase A_2

Phospholipase A_2 (PLA_2) plays a central role in diverse cellular processes including phospholipid digestion and metabolism, host defense, and signal transduction (Dennis *et al.*, 1991). Phospholipase activity was first studied in the pancreatic juice and cobra venom in the early 1900s (Waite, 1991), since the enzymes from these sources were abundant, easily purified, and stable to a variety of manipulations. Further study showed that they are 13-15 kDa molecules which are extensively cross-linked by disulfide bonds, making them relatively rigid (Dennis, 1994). Subsequently, the phospholipases A_2 have been shown to be a diverse class of enzymes with regard to function, localization, regulation, mechanism, sequence and structure (Dennis, 1994). PLA_2s have been divided into four main groups according to their structure and function. Group I, II, and III are extracellular enzymes, with a relatively high disulfide bond content, low molecular mass, and require Ca^{2+} for catalysis. The group IV

PLA_2 is distinctly different from the other groups since it is a high molecular weight intracellular enzyme that is highly specific for arachidonic acid. This enzyme has been identified in the cytosol of a variety of cells, including the human monocytic cell line U937 (Clark *et al.*, 1990; Kramer *et al.*, 1991), rat kidney (Gronich *et al.*, 1990), and mouse spleen (Wijkander and Sundler, 1991). Its general presence in the cytosolic fraction has led to the nomenclature of the cytosolic phospholipase A_2 ($cPLA_2$). The enzyme has been purified, sequenced and cloned from variety of sources (Clark *et al.*, 1991; Sharp *et al.*, 1991), and it has a molecular mass of 85-110 kDa. This enzyme can be activated by mitogen-activated protein kinase (MAPK) phosphorylation, which facilitates its translocation from the cytosol to the nuclear envelope, a process which requires a micromolar concentration of Ca^{2+} for membrane association and full activity (Clark *et al.*, 1995).

A newly identified secreted PLA_2 in human (Dufton and Hider, 1983), rat (Davidson and Dennis, 1990) and murine derived P388D1 macrophages (Wong and Dennis, 1990) does not clearly align with either Group I or Group II enzymes and lacks the unique disulfide bond of both. This enzyme is also distinguished from the Group III in that it has lower molecular mass. It has been assigned as Group V PLA_2.

A Ca^{2+}-independent PLA_2 ($iPLA_2$) has been identified. It has a molecular mass of 85 kDa and contains a lipase consensus sequence and eight ankyrin repeats. Because this $iPLA_2$ possesses a unique sequence that differs from the Groups I-V PLA_2, it is listed as a Group VI enzyme (Tang *et al.*, 1997). Another 40 kDa calcium-independent PLA_2 has been isolated from the myocardium. This enzyme has a high preference for the hydrolysis of the arachidonoyl group in plasmenylcholine and plasmenylethanolamine. It is regulated by

Ca^{2+}/calmodulin inhibition (Hazen *et al.*, 1990).

There are other Ca^{2+}-dependent and -independent intracellular PLA_2 activities from various sources. For example, a Ca^{2+}-dependent, 30-kDa dimeric PLA_2 was purified from sheep platelets (Loeb and Gross, 1986). A Ca^{2+}-independent, phosphatidylserine-specific PLA_2 was purified from rat brain (Nakaoka *et al.*, 1993). These enzymes have sequences which are homologous to a brain protein that belongs to the family of 14-3-3 proteins (Zupan *et al.*, 1992).

1.3 The role of $cPLA_2$

$cPLA_2$ mRNA is ubiquitously expressed in all mammalian tissues. It is abundantly expressed in the heart, spleen, lung and kidney. $cPLA_2$ can be activated by a wide variety of stimuli, including tumor necrosis factor (Hoeck *et al.*, 1993), ATP (Lin *et al.*, 1992), lipopolysachariде (LPS) (Rodewald *et al.*, 1994), mitogens (Chepenik *et al.*, 1994) and endothelin (Barnett *et al.*, 1994). The central role of $cPLA_2$ is to provide arachidonic acid as the precursor for prostaglandin and leukotriene biosynthesis. In addition, it has been shown that arachidonic acid is important not only as a precursor of the eicosanoids, but also as a second messenger (Jayadev *et al.*, 1994; Kolesnick and Golde, 1994). $cPLA_2$ also plays an important role in platelet activation (Smith *et al.*, 1991). Furthermore, there is evidence that arachidonic acid and its metabolites, and possibly $cPLA_2$ itself, are in some way involved in the regulation of cell proliferation (Chepenik *et al.*, 1994; Virdee *et al.*, 1994).

1.4 Lyso-PC and its role in modulation of $cPLA_2$

The hydrolysis of phosphatidylcholine by the action of phospholipase A_2 results in the production of lysophosphatidylcholine (lyso-PC) and a fatty acid. Due to its amphipathic property, lyso-PC is cytolytic at high concentrations and its intracellular levels are therefore under rigid control (Weltzien, 1979). The majority of lyso-PC formed in the tissue is rapidly metabolized or reacylated under normal physiological conditions (Giffin *et al.*, 1988; Choy and Arthur, 1989; Choy *et al.*, 1997). In ischemic myocardium, lyso-PC accumulates (Sobel *et al.*, 1978; Man *et a.*, 1983; Datorre *et al.*, 1991) and leads to electrophysiological and mechanical dysfunction of the heart (Hoque *et al.*, 1997; Woodley *et al.*, 1991). However, the work of Shaikh and Downar (1981) indicated that the elevated level of lyso-PC observed by Sobel and his co-workers might be formed during the extraction of lipids from tissues (Shaikh and Downar, 1981). Subsequently, a relationship between high lyso-PC concentrations and calcium overloading in cardiac tissues has been postulated (Ver Donck *et al.*, 1992; Yu *et al.*, 1998; Golfman *et al.*, 1998), but the mechanism for this phenomenon has been subjected to much debate (Yu *et al.*, 1995; Golfman *et al.*, 1998; Ahumada *et al.*, 1979; Watanabe and Besch, 1974). In plasma, the concentration of lyso-PC is normally low (Phillips, 1957) but high amounts of lyso-PC are found in atherogenic lipoproteins such as the oxidatively modified low-density lipoprotein and ß-very-low-density lipoprotein (Portman and Alexander, 1969; Steinberg *et al.*, 1989). In addition, the accumulation of lyso-PC in atherosclerotic and inflammatory lesions of vascular vessels has been reported (Witztum and Steinberg, 1991). Furthermore, lyso-PC induces the expression of mononuclear leukocyte adhesion molecules (Kume *et al.*, 1992). It also induces the gene expression of potent smooth muscle growth factors in monocytes and in cultured human

endothelial cells (Kume and Gimbrone, 1994), modulates smooth muscle contractility (Satio *et al.*, 1988) and acts as a chemotactic factor for human T lymphocytes (McMurray *et al.*, 1993) and monocytes (Quinn *et al.*, 1988).

It is clear that lyso-PC produced in the plasma is an important signal molecule that impairs endothelium-dependent relaxation of blood vessels (Witztum and Steinberg, 1991). In addition to lyso-PC, free fatty acids are produced from the hydrolysis of phosphatidylcholine by phospholipase A_2. The release of arachidonic acid from phospholipids is regarded as an important step for the biosynthesis of eicosanoids (Dennis, 1987). In most mammalian tissues including the cardiac tissue, arachidonate is converted to prostacyclin, a potent vasodilator which would attenuate the impairment of endothelium-dependent relaxation of blood vessels produced by lyso-PC. We have shown recently that lyso-PC stimulates the release of arachidonate in human endothelial cells. Lyso-PC caused the elevation of cellular Ca^{2+} and the activation of PKC, which stimulated cytosolic PLA_2 in an indirect manner and resulted in an enhanced release of arachidonate (Wong *et al.*, 1998).

1.5 The $cPLA_2$ gene

The human $cPLA_2$ gene has been mapped to chromosome 1q25 and is encoded by a 3.4-kb mRNA. While the complete genomic DNA for $cPLA_2$ has not yet been sequenced, there seems to be at least 7 introns in this gene, covering longer than 50 kb (Morii *et al.*, 1994). The gene also contains a microsatellite series of CA repeats which may be useful in linking polymorphisms in this region with $cPLA_2$-related diseases (Tay *et al.*, 1994). The cDNA of $cPLA_2$ comprised a total of 2880 nucleotides, including about 200 nucleotides for

the 5'-untranslated region and about 500 nucleotides for the 3'-untranslated region (Miyashita *et al.*, 1995; Morii *et al.*, 1994). The promoter region of $cPLA_2$ gene contains no TATA or CCAAT boxes. However, unlike the typical TATA-less housekeeping genes which contain SP-1 binding sites, these binding sites are absent in the $cPLA_2$ promoter (Morii *et al.*, 1994). Although the majority of TATA-less genes are house-keeping genes, studies have demonstrated that many of these TATA-less genes are highly and variously regulated at the level of transcription (Azizkhan *et al.*, 1993). In addition, there are consensus sites for NF-κB, NF-IL-6, AP-1 (activator protein-1), AP-2, PEA3 (polyomavirus enhancer A-binding protein-3), OCT (octamer transcription factor), C/EBP (CCAATT enhancer binding protein), GAS (gamma-interferon-activating-site), γ-IRE (γ-interferon response element) and GRE (glucocorticoid response elements). The roles of these elements in $cPLA_2$ regulation remain to be defined. Recently, study has shown that TNFα activates both $cPLA_2$ and COX-2 via NF-κB pathway in astrocytoma cells (Hernandez *et al.*, 1999).

1.6 The protein structure of $cPLA_2$

The human $cPLA_2$ cDNA encodes a 749 amino acid protein with a predicted molecular mass of 85.2 kDa, which migrates as a 100 ~110 kDa protein on SDS-PAGE (Sharp *et al.*, 1991). As shown in Fig 3, the deduced $cPLA_2$ protein sequence has several interesting structural features. Firstly, the $cPLA_2$ sequence contains a 68 amino acid stretch in the N-terminal region termed the CaLB (Ca^{2+}-dependent lipid-binding) domain that shows sequence homology with the C2 region of protein kinase C. This domain was found to be responsible for the Ca^{2+}-dependent binding of $cPLA_2$ to membranes or phospholipid

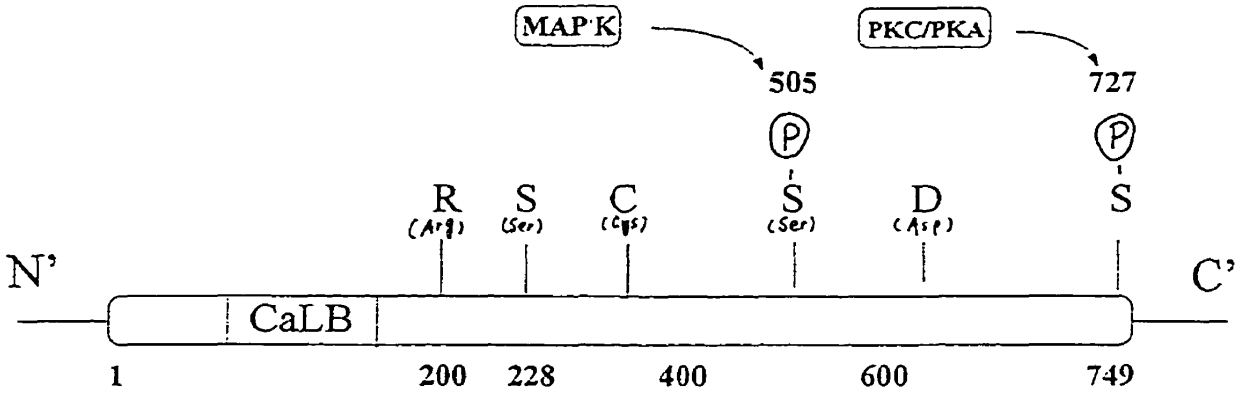


Fig 3. Functional domains of protein structure of $cPLA_2$ (human). CaLB: Ca^{2+}-dependent lipid binding domain. Two conserved phosphorylation sites: Ser-505 recognized by MAPK and Ser-727 recognized by PKA/PKC. Several essential residues: Ser-228, Asp-549, Arg-200.

substrates (Nalefski *et al.*, 1994). Secondly, several essential amino acid residues have been identified that may be important for the catalytic mechanism of $cPLA_2$. The $cPLA_2$ sequence containing the segment Gly-Leu-Ser228-Gly-Ser resembles the amino acid motif of Gly-X-Ser-X-Gly that is present in many serine esterases and neutral lipases. Replacing the central serine-228 with alanine by site-directed mutagenesis yielded a catalytically inactive $cPLA_2$, suggesting that the Ser-228 is required for the catalytic function of $cPLA_2$ (Sharp *et al.*, 1994). Following mutation of Asp-549 to alanine, the enzymatic activity of $cPLA_2$ was abolished, suggesting that Asp-549 is also required for the catalytic function (Pickard *et al.*, 1996). Thirdly, the $cPLA_2$ sequence contains consensus phosphorylation sites for both serine/threonine and tyrosine protein kinases (Sharp *et al.*, 1991). Among them, the Ser-505 and Ser-727 are conserved in $cPLA_2$ from other species. Ser-505 resides within the sequence Pro-Leu-Ser-Pro and is typically recognized by proline-directed kinases, such as MAP kinase (Cobb and Goldsmith, 1995). Ser-727 is flanked by arginines, which is a typical site preferred by basotrophic kinases, such as protein kinase C and protein kinase A. Finally, the $cPLA_2$ sequence contains a domain towards the C-terminus modestly enriched in proline that may be responsible for the reduced electrophoretic mobility of $cPLA_2$ on sodium dodecylsulfate (SDS) polyacrylamide gels (Kramer and Sharp, 1997).

1.7 The regulation of $cPLA_2$

The $cPLA_2$ is tightly coupled to signal transduction. The product of $cPLA_2$, arachidonic acid (AA), not only functions as a precursor of ecoisanoids but also as a second messenger (Clark *et al.*, 1995). For example, AA produced in response to TNFα stimulates

sphingomyelin hydrolysis, leading to the generation of the second messenger ceramide (Jayadev *et al.*, 1994). In this regard, $cPLA_2$ is believed to be of vital importance in cellular homeostasis, and the regulation of expression and function of $cPLA_2$ is of interest for the understanding of normal cellular homeostasis and inflammatory processes (Irvine, 1982; Axelrod, 1990; Bonventre, 1992).

The $cPLA_2$ is regulated by multiple mechanisms. At the post-translational level, this enzyme is subjected to complex modulations including Ca^{2+}-dependent translocation to the nuclear membrane and phosphorylation at serine 505 by MAPK (Clark *et al.*, 1995). In a prolonged response, the expression levels of $cPLA_2$ can be regulated by a number of cytokines and growth factors at the transcriptional level (Wu *et al.*, 1994; Maxwell *et al.*, 1993). Other modes of regulation at the transcriptional level have yet to be explored.

The $cPLA_2$ is the only known PLA_2 which is regulated by a receptor-mediated process (Kramer and Sharp, 1997). The best understood process regulating $cPLA_2$ is the post-translational regulatory mechanism responsible for rapid activation of $cPLA_2$ in response to a variety of physiological stimuli, including the calcium-dependent translocation to the membrane fraction via the CaLB domain, and protein phosphorylation (Clark *et al.*, 1991; Winitz *et al.*, 1994) (Fig 4). The $cPLA_2$ activity is enhanced by phosphorylation, a process mediated by mitogen activated protein kinase, as well as by the indirect activation of protein kinase C and G-protein coupled receptors (Fig 4) (Cobb and Goldsmith, 1995; Nemenoff *et al.*, 1993; Clark *et al.*, 1995).

The $cPLA_2$ mRNA level may also be subjected to modest regulation in some cell lines by mediators such as IFN-γ (Wu *et al.*, 1994), macrophage colony stimulating factor

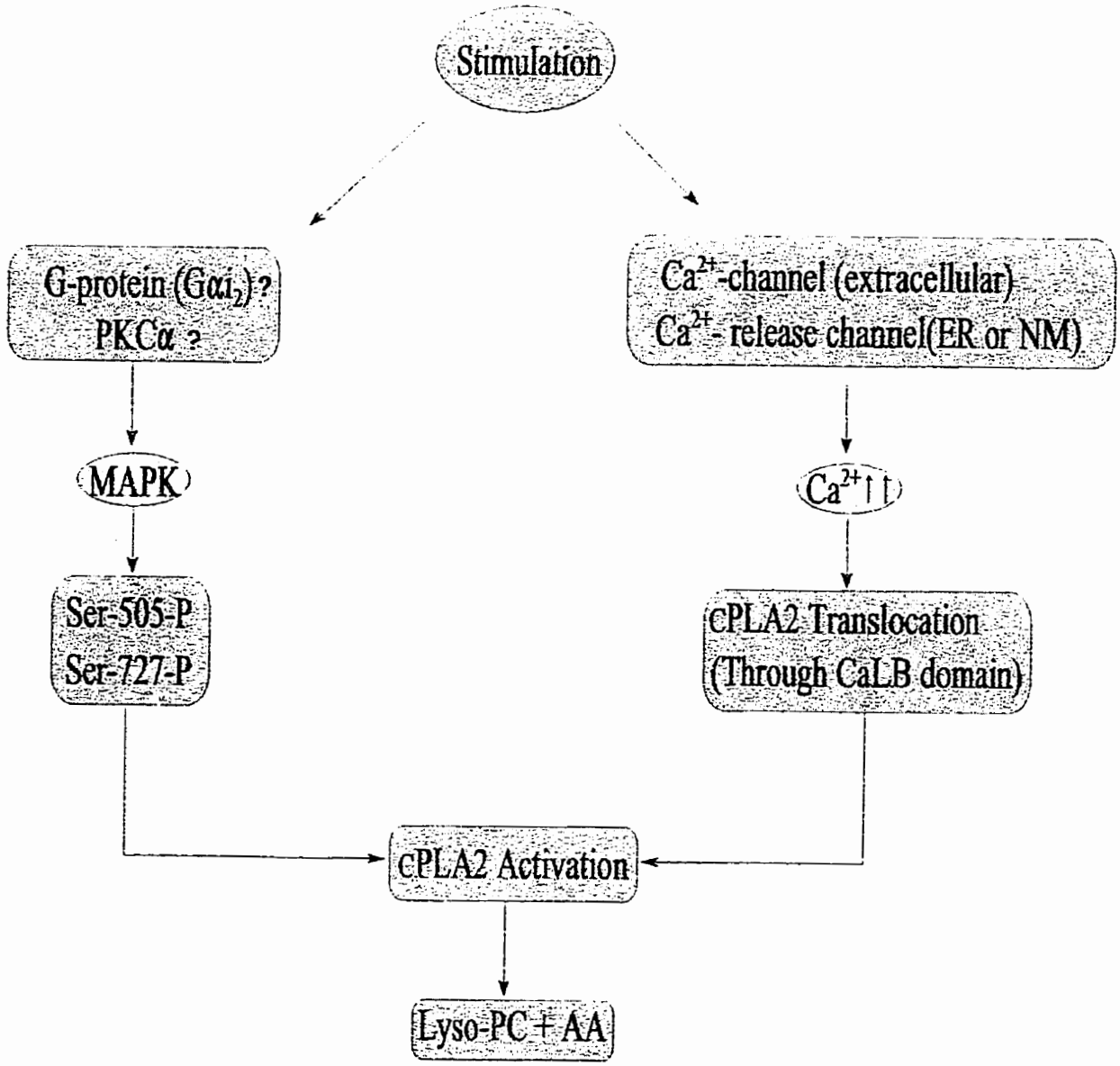


Fig 4. $cPLA_2$ Regulation at Post-transcriptional Level

(Nakamura *et al.*, 1992), TNFα (Hoeck *et al.*, 1993; Hernandez *et al.*, 1999), epidermal growth factor (Maxwell *et al.*, 1993), and glucocorticoid (Newton *et al.*, 1997). The existence of both GAS (Interferon-gamma Activation Site) and γ-IRE (Interferon-γ Response Element) consensus elements in the 5'-flanking region of the $cPLA_2$ gene have been identified (Wu *et al.*, 1994). There is evidence to show that the GAS and γ-IRE elements may be critical for the induction of the $cPLA_2$ gene by IFN-γ. In addition, the promoter region of $cPLA_2$ contains several inducible elements, including AP-1 binding sites and a potential NF-κB binding site, which may account for the transcriptional control of $cPLA_2$ (Tay *et al.*, 1994). The fact that $cPLA_2$ can be regulated by mechanisms affecting its long-term activity supports the idea that $cPLA_2$ could participate in processes such as the development of nervous system (Yoshihara *et al.*, 1992) or differentiation of premonocytic U937 cells (Burke *et al.*, 1999).

1.8 The nuclear receptors

Nuclear hormone receptors comprise of a large superfamily of ligand-modulated transcription factors that mediate response to steroids, retinoids, thyroid hormones and vitamin D_3. These receptors play key roles in development, cell differentiation, and organ physiology (Beato *et al.*1995). Unlike water-soluble peptide hormones and growth factors, which bind to cell surface receptors and activate a cascade of second messengers, the fat-soluble steroid hormones can enter the cell by simple or facilitated diffusion and transduce their signals to the genome via intracellular receptors (Evans, 1988; Mangelsdorf *et al.*, 1995). After binding to its receptor, the hormone-receptor complex translocates from the

cytoplasm to the nucleus, induces an allosteric change that enables the complex to bind to high affinity sites in the chromatin, and modulate transcription (Kurokawa *et al.*, 1994; Forman *et al.*, 1995a). Nuclear receptors can also bind to DNA in the absence of a ligand (Mangelsdorf *et al.*, 1995).

There are more than 150 members of nuclear receptor superfamily, spanning a large diversity of animal species from worms to insects to human (Mangelsdorf *et al.*, 1995). Using low stringency hybridization screening as well as genetic and molecular cloning techniques, numerous members of the family for which there are no apparent ligands have been identified. These are so-called "orphan" receptors (Fig 5).

The superfamily of nuclear receptor is often divided into the steroid receptor family and the thyroid/retinoid/vitamin D (or nonsteroid) receptor family. Each type of receptor constitutes a subfamily (eg., PPAR subfamily). Receptor subtypes are the products of individual genes (eg., PPARα), and receptor isoforms are the products of alternate splicing, promoter usage, or both (eg., PPARγ1, PPARγ2) (Mangelsdorf *et al.*, 1995).

The nuclear receptor superfamily can be broadly grouped into four classes based on their dimerization and DNA binding properties (Fig 5). Class I receptors include the steroid hormone receptors which function as ligand induced homodimers and bind to DNA half-sites organized as inverted repeats. Class II receptors heterodimerize with the retinoid X-activated receptor (RXR) and characteristically bind to direct repeats. Class III receptors bind primarily to direct repeats as homodimers. Class IV receptors typically bind to extended core sites as monomers (Mangelsdorf *et al.*, 1995; Stunnenberg, 1993).

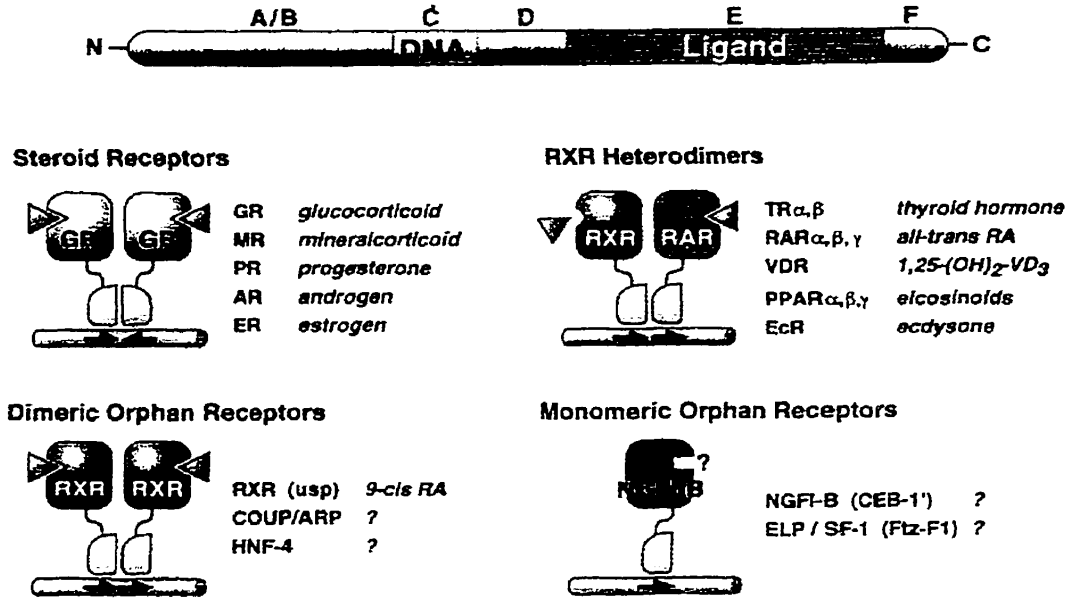


Fig 5. Nuclear receptors sharing common structure/function domains. Top figure depictes the modular structure of the nuclear receptor. (Adapted from Mangelsdorf *et al*, 1995)

1.9 The modular structure of nuclear receptors

A typical nuclear receptor contains several functional domains (Fig 5, top part): a variable N-terminal region (A/B), a conserved DNA-binding domain (C), a variable hinge region (D), a conserved ligand-binding domain (E) and a variable C-terminal region (F). The nuclear receptors are characterized by a central DNA-binding domain (DBD), which targets receptor to specific DNA sequences known as hormone response elements. The DBD is the composed of two highly conserved zinc fingers that set nuclear receptors apart from other DNA-binding proteins (Klug and Schwabe, 1995). The C-terminal half of the receptor encompasses the ligand-binding domain (LBD), which possesses the essential property of hormone recognition and ensures both specificity and selectivity of the physiologic response. In other words, the LBD can be thought of as a molecular switch that, upon ligand binding, shifts the receptor to a transcriptionally active state (Mangelsdorf *et al.*, 1995; Slack, 1999a; 1999b). The functions of other domains are summarized in elsewhere (Tenbaum *et al.*, 1997).

1.10 Peroxisome proliferator-activated receptors (PPARs)

The DNA binding domain is highly conserved among all members of the nuclear receptor superfamily and permits the cloning of many new members of this family. In 1987, a peroxisome proliferator-binding protein in rat liver was identified and characterized (Lalwani *et al.* 1987). Subsequently, the first peroxisome proliferator-activated receptor (PPAR) was cloned from mouse liver (Isseman and Green, 1990), followed by other PPAR homologs in several species (Dreyer *et al.*, 1992; Tontonoz *et al.*, 1994a; Amri *et al.*, 1995).

With the discovery of many ligands of the PPARs, these groups of receptors is no longer considered as orphan receptors.

To date, three different types of PPARs, α, γ, and δ have been identified in vertebrates. They are encoded from separate genes, have distinct tissue distribution and carry out different function. Mammalian PPARα is predominantly expressed in liver, heart, kidney, and tissues with high catabolic rates for fatty acids and peroxisome metabolism, such as the brown adipose tissue (Isseman and Green, 1990; Braissant *et al.*, 1996). PPARα activation mediates pleiotropic effects such as stimulation of lipid oxidation, alteration in lipoprotein metabolism and inhibition of vascular inflammation (Schoojans *et al.*, 1996a; 1996b; Staels *et al.*, 1998). PPARα is also present in endothelial and smooth muscle cells (Delerive *et al.*, 1999; Marx *et al.*, 1998), monocytes and monocyte-derived macrophages (Chinetti *et al.*, 1998; Ricote *et al.*, 1998), and in keratinocytes (Hanley *et al.*, 1998). The dysfunction of PPARα may be associated with hyperlipoproteinemia (Staels *et al.*, 1998; Delerive *et al.*, 1999). PPARγ has been implicated as a mediator of adipocyte differentiation and provides a mechanism for thiazolidinedione drugs to exert their *in vivo* insulin sensitization (Berger *et al.*, 1999). It is proposed that PPARγ acts with C/EBPβ (CCAATT enhancer binding protein) and provides the initial trigger for adipogenic development (Fajas *et al.*, 1998). However, it is unclear whether PPARγ is the only PPAR that plays a role in the induction of adipocyte differentiation. Transcriptional activation of PPARγ also initiates a positive feedback loop that enhances the expression of the oxidized-LDL receptor CD36, which is involved in a metacrine regulatory circuit whereby the oxidized-LDL may contribute to foam cell formation during atherogenesis (Nagy *et al.*, 1998; Tontonoz *et al.*,

1998). The altered activity of PPARγ may be associated with the pathogenesis of obesity and non-insulin-dependent diabetes mellitus, two risk factors of atherosclerosis (Schoojans *et al.*, 1996a).

PPARδ is abundantly and ubiquitously expressed, but the function is less known. Recently, it has been shown to be associated with the inhibition of human colorectal tumorigenesis mediated by non-steroidal anti-inflammatory drugs (NSAIDS) (He *et al.*, 1999).

1.11 Natural and synthetic activators of PPARα and PPAR γ

All PPARs are, albeit to different extents, activated by fatty acids and their derivatives. With the exception of short-chain fatty acids ($<C_{10}$), a broad range of naturally occurring fatty acids are capable of activating different PPARs (Gottlicher *et al.*, 1992; Keller *et al.*, 1993). Substituted fatty acids, such as sulphur-substituted fatty acid analogues, are more potent PPAR activators (Demoz *et al.*, 1994). Several exogenous substances, such as lipid-lowering drugs (fibrates), phthalate ester plasticizers, and herbicides also activate PPARs (Isseman and Green, 1990; Dreyer *et al.*, 1992). Among them, fibrates bind to and activate PPARα, with low affinity but high specificity (Forman *et al.*, 1997). WY-14643 (pirinixic acid; 4-chloro-6-(2,3-xylidino)-2-pyrimidinyl thioacetic acid) is a another specific synthetic PPARα ligand (Table 1). GW2331 (2-(4-[2-(3-[2,4-difluorophenyl]-1-heptylurieido)ethylphenoxy)-2-methylbutyric acid, a synthetic fibrate, has been found function as a high affinity ligand for both PPARα and PPARγ (Kliewer *et al.*, 1997).

Antidiabetic agents, such as thiazolidinedione, bind selectively with high affinity and

Table 1. Activators of PPARs and RXR

Receptor	Natural Activator	Synthetic Activator
PPARα	Oleic acid	Fibrates
	Linoleic acid	WY 14,463 (pirinixic acid; 4-chloro-6-(2,3-xylidino)-2-pyrimidinyl thioacetic acid)
	Arachidonic acid	ETYA (5,8,11,14-eicosatetruynoic acid)
		GW2331 (2-(4-[2-(3-[2,4-Difluorophenyl]-1-hept-2-enylureido)ethyl]phenoxy)2-methylbutyric acid
PPARγ	9-HODE (9-hydroxyoctadecadinoic acid)	15-D PGJ_2 (15-deoxy$^{\Delta 12,14}$ prostaglandin J_2)
	13-HODE (13-hydroxyoctadecadinoic acid)	TZD (thiazolidinedione)
	15-HETE (15-OH metabolite of arachidonic acid)	Ciglitizone
	Arachidonic acid	GW2331 (2-(4-[2-(3-[2,4-Difluorophenyl]-1-hept-2-enylureido)ethyl]phenoxy)2-methylbutyric acid
RXR	9-cis-RA (9-cis- retinoic acid)	LG268
	All-trans-RA (all-trans retinoic acid)	

potently activate the PPARγ subtype, suggesting that they are synthetic ligands for PPARγ (Lehmann *et al.*, 1995). Arachidonic acid metabolites, such as the prostaglandin J_2 group are the natural ligands for PPARγ (Yu *et al.*, 1995) (Table 1). Hypolipidemic fibrates, such as cipofibrate, clofibrate and gemfibrozil activated PPARα maximally at 300 μM in CV-1 cells. At the same concentration, they exhibited only weak activity on PPARγ. However, at 1mM, all three drugs displayed significant activity on PPARγ (Forman *et al.*, 1997).

1.12 Clofibrate: a hypolipidemic drug

Clofibrate (ethyl-p-chlorophenoxyisobutyrate) is a synthetic lipid-lowering agent, which belongs to the fibrate superfamily (Fig 6). It has a molecular mass of 214.6 kDa. As the first generation of lipid-lowering drugs, clofibrate has been approved for use in the United States for the last three decades. Treatment with clofibrate results in a substantial decrease in plasma triglyceride-rich lipoproteins and an increase in high-density lipoprotein (HDL) cholesterol concentration (Davignon, 1994; Sirtori and Franceschini, 1988). It also induces a reduction of the small dense LDL, resulting in better resistance of LDL to oxidation (Erkelens *et al.*, 1981). These alterations are correlated with decreased apoB, apoC-III, and apoE levels in triglyceride-rich lipoproteins and increased apoA-I and apoA-II in HDL (Fruchart *et al.*, 1987). The fibrate effect on human apo A-I is mediated by the transcription factor PPARα, which interacts with a positive PPAR-response element (PPRE) in its promoter region (Staels and Auwerx, 1998).

In one placebo-controlled study, the mortality rate was increased among patients who were receiving clofibrate, as a result of diseases of the biliary tract and cancer (The

Committee of Principle Investigators, 1978). Another follow-up study reported in 1982 showed that during the whole period of the study, (the mean observation was 13.2 years), there were 70 (11%) more deaths in the clofibrate-treated group (The Committee of Principle Investigators, 1980; 1984). Combined with other adverse effects such as erectile dysfunction in men and myositis in patients with impaired renal function (Schonfeld, 1994), clofibrate is not considered as the drug of choice in some cases. The second and third generation of fibrates, such as fenofibrate and gemfibrozil , are more powerful in their lipid-lowering action and have less adverse effects. Therefore, fenofibrate and gemfibrozil have been used as drugs of choice for the treatment of hyperlipidemia (Fig 6).

1.13 Activation of PPAR and cross-talk with other signaling pathways

After binding to its ligand, the activated PPARs heterodimerize with another nuclear receptor, the retinoid X receptor (RXR), and initiate downstream gene transcription by recognizing and binding to a specific peroxisome proliferator response element (PPRE). PPRE is a direct repeat (DR) of the nuclear receptor hexameric AGGTCA DNA core recognition motif separated by one or two nucleotides (DR1 and DR2) (Fig 7).

Some receptors can also be activated by ligand-independent mechanisms (Denner *et al.*, 1990; Power *et al.*, 1991), suggesting that the intracellular and membrane signaling pathways are not completely separated and that cross-talk between these signaling pathways may have important implications. Similar mechanisms occur in PPARs (Juge-Aubry *et al.*, 1999; Gelman *et al.*, 1999). It has been shown that PPREs embody structures that have potential for multiple cross-talk, and PPARs can interact with other hormone receptors in

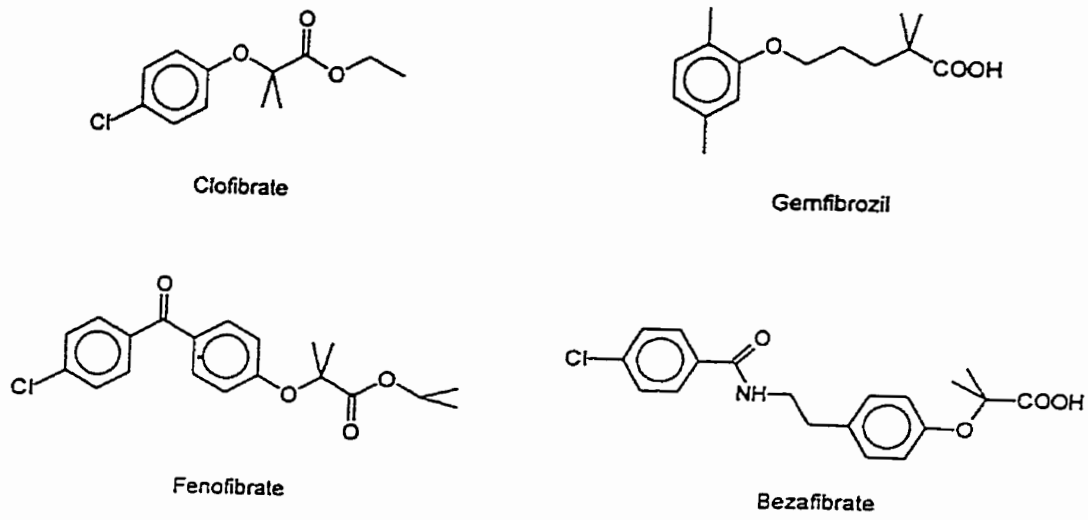


Fig 6. Structures of widely used fibrates

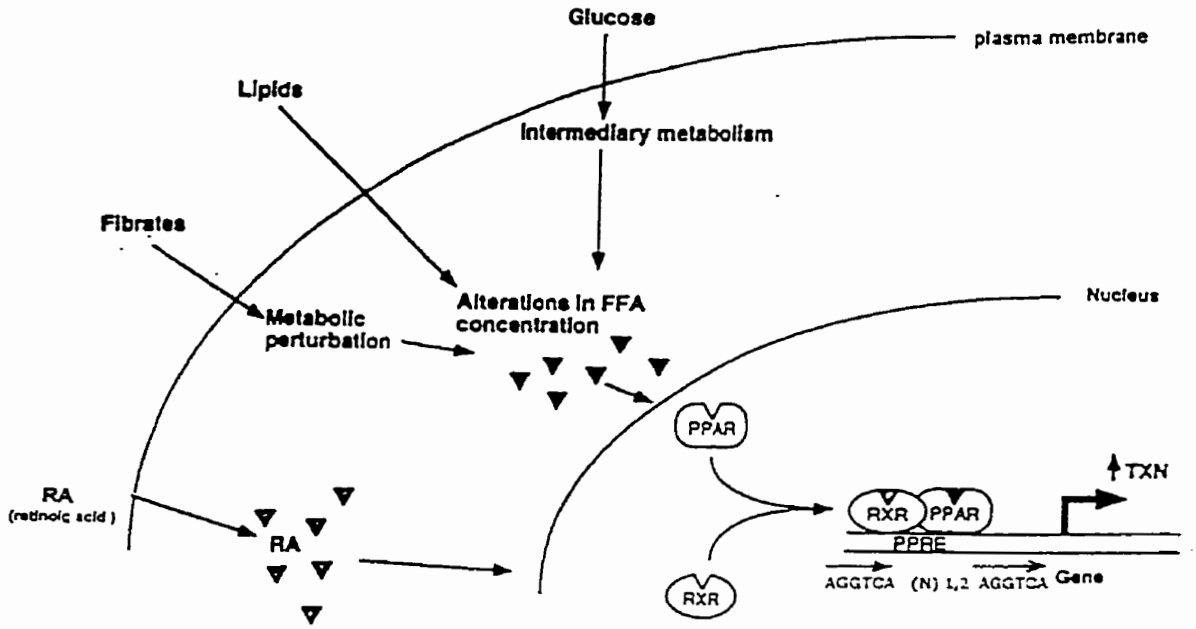


Fig 7. Nuclear receptor PPAR-RXR signaling pathway

addition to RXR. For example, the regulation of the PPARα gene by glucocorticoids has been linked to a diurnal rhythmicity in PPARα expression and its induction in response to stress (Lemberger *et al.*, 1996). This reaction is mediated through the interaction between PPAR and the glucocorticoid receptor (GR). Some PPARs also act as co-regulatory factors for the thyroid receptor in thyroid hormone signaling (Bogazzi *et al.*, 1994). Furthermore, PPARs have been implicated in estrogen signaling pathways in the regulation of cardiac and hepatic lipid metabolism (Djouadi *et al.*, 1998). In PPARα null mice (PPARα -/- mice), the inhibition of cellular fatty acid flux was shown to cause massive hepatic and cardiac lipid accumulation, hypoglycemia and death in 100% of males, but only in 25% of females. Interestingly, the metabolic phenotype of male PPARα-/- mice was rescued by a 2-week pretreatment with beta-estradiol. Six out of eight estradiol-treated PPARα-/- mice survived without developing hypoglycemia and exhibited reduced hepatic and myocardial lipid accumulation (Djouadi *et al.*, 1998).

1.14 Target genes and functional implication of PPAR

PPAR play an important role in lipid metabolism and homeostasis. The activation of PPAR has been shown to be involved in multiple cellular processes, such as alteration in lipoprotein metabolism and preadipocyte differentiation. For example, the activation of PPARα by fibrates regulates both extracellular and intracelluar lipid metabolism. Fibrates up-regulate the transcription of lipoprotein lipase (LPL) as well as down-regulate apoC-III, therefore resulting in an increase in the extracellular hydrolysis of triglyceride-rich lipoprotein (Schoonjans *et al.*, 1996a; Staels *et al.*, 1995). Fibrates exert an intracellular

hypolipidemic action by concomitant increases in the up-take and metabolism of the released fatty acids, through activation of PPARα. Various cytosolic proteins, such as the liver fatty acid-binding protein (FABP) and the adipose tissue-specific adipocyte fatty acid binding protein P2 (aP2) are induced upon administration of fibrates (Isseman *et al.*, 1992; Tontonoz *et al.*, 1994a). These proteins facilitate the cellular fatty acid uptake. Fibrates also regulate the genes encoding the acyl-CoA oxidase (Dreyer *et al.*, 1992) and microsomal cytochrome P450 fatty acid ω-hydroxylase (Muerhoff *et al.*, 1992), the rate-limiting enzymes in the peroxisomal β-oxidation pathway and in the microsomal ω-oxidation pathway, respectively. Another extra-peroxisomal gene regulated by fibrates is acyl-CoA synthetase, which is localized in peroxisomes, mitochondria, and microsomes (Schoojans *et al.*, 1995). Finally, fibrates reduce the activities of acetyl-CoA carboxylase (Maragoudakis and Hankin, 1971) and fatty acid synthase (Balke and Clarke, 1990), therefore inhibiting the *de novo* fatty acid synthesis. All of these actions exerted by fibrates are mediated through activation of PPARα, and PPREs have been identified in each of the genes for the enzymes at the promoter region.

PPARα was originally cloned from rodent liver (Isseman and Green, 1990). A diverse group of rodent non-genotoxic carcinogens, which are amphipathic carboxylates in nature, are collectively defined as proxisome proliferators (PPs), including hypolipidemic drugs (fibrates), plasticizers and herbicides. Although most of the PPARα functions are conserved, some important differences in response to PPs exist. For example, treatment of rodents with PPs results in hypolipidemia, peroxisome proliferation and liver hyperplasia leading to non-genotoxic hepatocarcinogenesis (Hertz and Bar-Tana, 1998). In contrast to rodents, the

hypolipidemic effect exerted by PPs (such as fibrates) in humans is never accompanied by peroxisome proliferation or by induction of hepatocarcinogenesis. Such different responses to fibrates among species may be linked to variations in the PPARα signalling pathways. Recently, James and co-workers identified a naturally occurring PPARα variant from human liver, which shares the ability of mouse PPARα (mPPARα) to bind to DNA but, unlike mPPARα, could not be activated by PPs (James *et al.*, 1998). Further studies showed that the mRNA of hPPARα variant, which lacks the entire exon 6 due to alternative splicing, is expressed in several human tissues and cells, representing between 20-50% of total PPARα mRNA (Gerviois *et al.*, 1999). This deletion leads to the introduction of a premature stop codon, resulting in the formation of a truncated PPARα protein (PPARαtr) lacking part of the hinge region and the entire ligand-binding domain. By contrast, PPARαtr mRNA could not be detected in rodent tissues. The truncated hPPARα protein ($hPPAR_{tr}$) produced by the splicing variant may act as a dominant negative regulator of the gene transcription mediated by the PPARα wild type ($PPAR\alpha_{wt}$) (Gerviois *et al.*, 1999). Thus, the protection of human beings from hepatocarcinonoma by PPs may arise due to the presence of the PPARα variant (Hertz and Bar-Tana, 1998).

Several highly specialized proteins are induced during adipocyte differentiation. Most of them are involved in lipid storage and metabolism, such as aP2 (Tonotonoz *et al.*, 1994a), acyl-CoA synthetase (Schoojans *et al.*, 1993), and LPL (Schoojans *et al.*, 1996b). *In vitro* studies show that in each of these enzymes, PPRE has been identified at the promoter region in the respective gene. Among them, the LPL gene is more efficiently activated by PPARγ than by PPARα. This is an another piece of evidence that is consistent with the specific role

for PPARγ in adipose tissue differentiation (Tonotonoz *et al.*, 1994a; 1994b; Schoojans *et al.*, 1996a). Moreover, LPL has the ability to increase the availability of fatty acids to the adipoctye. This increase in uptake of fatty acids is further enhanced by the increase in acyl-CoA synthetase activity mediated by PPAR. Fatty acids, which are potent activators of PPAR and provide the necessary building blocks for triglyceride accumulation, also cause adipocyte differentiation. This positive feedback loop, which involves the PPARγ, LPL, acyl-CoA synthetase, fatty acid transport protein, and perhaps other genes, promotes and maintains the mature adipocyte phenotype.

1.15 Structural, regulatory and functional difference of COX-1 and COX-2

Cycooxygenase (COX) (EC 1.14.99.1) is the enzyme which catalyzes the conversion of arachidonic acid to prostaglandin H_2 (PGH_2). PGH_2 is subsequently converted to a variety of eicosanoids that include PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2, and thromboxane (TX) A_2 (Fig 1 and Fig 2). Different tissues or cells produce different PGs, depending on the downstream enzymatic machinery present in a particular cell type. For example, endothelial cells primarily produce prostacyclin (PGI_2), which can induce the relaxation of vascular smooth muscle by increasing cyclic AMP (cAMP) levels (Moncada and Vane, 1979), whereas platelets mainly produce TXA_2 (Herschman, 1996).

COX is encoded by two related genes located on different chromosomes, *cox-1* and *cox-2*, which are differentially expressed and regulated (Smith, 1992). The *cox-1* gene is 22 kb in length, contains 11 exons (Kraemer *et al.*, 1992), and is constitutively and ubiquitously expressed. The *cox-2* gene is 8 kb in length, contains 10 exons (Kujubu and Herschman,

1992), and is expressed at high levels upon induction by growth factors, cytokines and extracellular stimuli associated with cell activation (Smith *et al.*, 1996). Many cell types related to inflammation, such as monocytes, endothelial cells and fibroblasts express the *cox-2* gene upon induction (Hla and Neilson, 1993). The cDNA of human *cox-2* encodes a polypeptide of 604 amino acids that is 61% identical to the previously isolated human COX-1 polypeptide (Hla *et al.*, 1992). COX-1 and -2 are integral membrane proteins. The interactions of the enzymes with the lipid bilayer involve an unique membrane binding domain (MBD), which was first recognized by analysis ofthe crystal structure of ovine COX-1 (Picot *et al.*, 1994). Both COX-1 and -2 are bound to the lumenal surface of the endoplasmic reticulum (ER) and contiguous *outer* membrane of the nuclear envelope (Otto and Smith, 1994). However, COX-2 is more concentrated on the nuclear envelop than COX-1 (Morita *et al.*, 1995).

Although some cells express both COX-1 and -2, the biological function of these two isoforms is different. COX-1 is mainly responsible for the biosynthesis of prostaglandins (PGs) involved in homeostatic regulation, which act as local "housekeeping" responses to circulating hormone. This action is mediated via G protein-linked receptors (Smith *et al.*, 1996). COX-2, on the other hand, probably has two roles. One major role of COX-2 is involved in co-localizing with COX-1 in the ER to augment the function of COX-1 (or to substitute for COX-1 in cells lacking this isozyme). Another role of COX-2 is to produce PGs that act through nucleoplasmic or nuclear membrane targets in association with cell differentiation and replication. The latter action is mediated through the nuclear receptor. For example, PGJ_2, a derivative of PGI, can activate PPARγ, which in turn triggers 3T3

murine preadipocyte differentiation (Forman *et al.*, 1995b).

1.16 Relationship between $cPLA_2$ and COX-2

The synthesis of prostaglandins is regulated by the AA release by $cPLA_2$ and subsequently, the conversion of AA to PGs by cyclooxygenase (COX). $cPLA_2$ and COX represent two crucial rate-limiting steps for the PG-biosynthetic pathway. Immunofluorescence and confocal microscope studies have shown that $cPLA_2$ translocates from the cytosol to the nuclear membrane and endoplasmic reticulum (ER) upon stimulation by Ca^{2+} (Schievella *et al.*, 1995). Using similar approaches, both COX isoforms were found to reside in the ER and nuclear envelope (Regier *et al.*, 1993). Evidence showed that a functional coupling exists between $cPLA_2$ and COX-2 in activated cells (Balsinde *et al.*, 1998; Murakami, 1999). The functional coupling of $cPLA_2$/COX-2 has been shown to be associated in both the immediate and delayed PG-biosynthetic responses (Murakami *et al.*, 1999). In addition, the human $cPLA_2$ gene has been mapped to chromosome 1q25, a region that also contains the COX-2 gene (Sharp and Kramer, 1997). Collectively, these data suggest that there may be coordinated regulation of $cPLA_2$ and COX-2.

II HYPOTHESIS AND OBJECTIVES OF THE STUDY

Arachidonate is an important precursor for eicosanoid biosynthesis. The release of arachidonate from membrane phospholipids is facilitated by the action of $cPLA_2$. In this study, we hypothesized that the action of $cPLA_2$ is regulated both at the transcriptional and post-translational levels.

The study in this thesis is divided into two parts. The first part was to investigate the modulation of $cPLA_2$ at the transcriptional level. Human preadipocyte SW 872 cells were used as a model in this study, and the regulation of $cPLA_2$ transcription was modulated by clofibrate. The involvement of PPARα was examined since the action of clofibrate has been shown to be mediated via the activation of this nuclear hormone receptor. The second part of the study was to elucidate the role and mechanism of lyso-PC in the modulation of $cPLA_2$. The H9c2 cells were selected as a model for this study because they were derived from the rat heart but retained many of the properties of the skeletal muscle. The results obtained from both studies are discussed together in the discussion section.

III. MATERIALS AND METHODS

1. Materials and Supplies

The following reagents were purchased from specific manufacturers and used according to instructions supplied, unless specifically annotated.

Sigma Chemical Company: (St. Louis, MO. U.S.A)	Dulbecco's Modified Eagle's medium (DMEM)
	Dulbecco's phosphate buffered saline (PBS)
	Bovine serum albumin (BSA, essential fatty acid free)
	Clofibrate
	9-cis retinoic acid
	Calcium ionophore A-23187
	Para-bromophenacyl bromide (pBPB)
Gibco Life Technology, Inc.: (Burlington, ON)	Fetal bovine serum (FBS)
	Medium 199
	TRIZOL Reagent
	Moloney murine leukemia virus reverse transciptase (M-MLV RT)
	Taq DNA polymerase
	Heat-inactivated newborn calf serum
	Trypsin

Amersham Pharmacia Biotech Inc.: (Quebec)	1-stearoyl-2-[1-^{14}C]-arachidonyl-*sn*-glycerol 3-phosphatidycholine (55 mCi/mmol)
	[5,6,8,9,11,12,14,15-^{3}H]-Arachidonic acid (209.6Ci/mmol)
	1-[1-^{14}C]palmitoyl-L-lyso-3-phosphatidyl-choline (55 mCi/mmol)
	Adenosine 5'-[γ-^{32}P]-triphosphate (3,000 Ci/mmol)
	Peroxidase labelled anti-rabbit antibody
	Enhanced chemiluminescence detection system (ECL)
Biomol Inc.: (Plymouth Meeting, PA. U.S.A)	Staurosporine
	H-89
	Arachidonyltrifluoromethylketone(AACOCF$_3$)
	PD98059
	Bisindolylmaleimide I
Calbiochem-Novabiochem Corp.: (La Jolla, CA. U.S.A)	Ro31-8220
Serdary Research Lab.: (London, Ontario, Canada)	Lipid standards
	Lysophospholipids
Promega Inc.: (Madison, WI, U.S.A)	Restriction enzymes
	Ribonuclease inhibitor (RNasin)
	Deoxynucleotide (dNTP)
Roche Inc: (Quebec)	Expand™ High Fidelity PCR System
	4-[3-(4-iodophenyl)-2-(4-

	nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1)
American Type Culture Collection: (ATCC)	Human preadipocyte cell line SW 872
	Human bladder cancer cell line T-24
	Rat heart myoblast (H9c2) cells
Difco Laboratories, Inc.:	LB agar and medium
Qiagen, Inc:	Plasmid Mini-preparation kit
	Plasmid Midi-preparation kit
Invitrogen Corp. : (Carlsbad, CA, USA)	One Shot™ TOP10 competent cells
	Zero Blunt™ TOPO™ PCR Cloning vector
Fisher Scientific:	Thin layer chromatographic plates (TLC)
	Chloroform, methanol, acetic acid (HPLC grade)
Cayman Chemical Co. : (Ann Arbor, MI, U.S.A)	5-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ_2,)
Bio-Rad Laboratories, Inc:	Hybond nitrocellulose membranes
	Pre-stained SDS-PAGE standard (Broad range)
Perkin-Elmer, Inc: (Foster City, CA, U.S.A)	BigDye Terminator Cycle Sequencing Ready Reaction Kit

2. Methods

2.1 Cell culture

Preadipocyte SW 872 cells, derived from a human liposarcoma, originally from the American Type Culture Collection, were kindly provided to us by Dr. Garry Shen (University of Manitoba). Cells were cultured in Dulbecco's modified Eagle medium /Ham's F12 nutrition mixture at a ratio of 3/1 (DEME/F12, 3:1), and further supplemented with 5 % heat-inactivated fetal bovine serum, 50 μg/ml gentamicin, 20 mM N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid (HEPES). Cells were grown in 35 mm or 60 mm dishes at 37 ^{0}C, in a water-saturated atmosphere with 5 % CO_2 and 95 % air. Cells were allowed to grow up to 30% confluence in a monolayer, and then incubated in a medium containing either clofibrate, 5-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ_2), 9-cis retinoid acid, or vehicle alone (< 0.05 % DMSO, < 0.05% ethanol) for the prescribed amount of time. The medium was changed every 48 hours until the end of the experiment. The human bladder cancer cell line T-24 was cultured in medium 199 with 10 % heat-inactivated fetal bovine serum. Rat heart myoblast (H9c2) cells were obtained from ATCC and cultured according to the company's guidelines. The cells were grown in flasks or culture dishes in Dulbecco's Modified Eagle's medium supplemented with 10% bovine serum albumin, 100 units/ml penicillin, 100 μg/ml streptomycin and 1.25 μg/ml fungizone. Cells of 85-90% confluency were used for all subsequent experiments.

2.2 Radiolabeling of cells

Preadipocytes were cultured in DMEM/F12 medium (3/1), and grown to 90 % confluency in 35 mm dishes. Cells were washed three times with HEPES-buffered saline (HBS) which contained 140 mM NaCl, 4 mM KCl, 5.5 mM glucose, 10 mM HEPES, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 0.025% (W/V) bovine serum albumin, with a final pH of 7.4. Cells were then incubated for 16 hours with 0.88-1.0 μCi/ml [5,6,8,9,11,12,14,15-^{3}H]-arachidonic acid (209.6 Ci/mmol) (Tran and Chan, 1988), in a DMEM/F12 mixture (3/1) containing 5 % fetal bovine serum.

H9c2 cells were radiolabelled (Wong *et al.*, 1998) in 35-mm culture dishes for 16-20 h with 1μCi/ml [^{3}H]arachidonate in Dulbecco's Modified Eagle's medium containing 10 % bovine serum albumin. The cells were washed 3 times with HEPES-buffered saline containing 140 mM NaCl, 4 mM KCl, 5.5 mM glucose, 10 mM HEPES, 1.5 mM $CaCl_2$, and 1.0 mM $MgCl_2$, pH 7.4 and 0.1 % (w/v) essentially fatty acid-free bovine serum albumin.

The binding of lysophospholipid to H9c2 cells was studied in the following manner: cells were cultured on 60 mm plates with Dulbecco's Modified Eagle's medium containing 100 nM [^{14}C]lysoPC (55 nCi/nmol) for 15 min. Subsequently, the medium was removed, and cells were incubated for another 15 min with the same medium containing 10 μM non-radioactive lysophospholipid (100 times excess) or 0.1% bovine serum albumin. After the second incubation, the cells were dislodged from the culture dish in HEPES-buffered saline. The labeled lysophospholipid content in each dish was determined by scintillation counting.

2.3 Determination of arachidonate release

Preadipocytes were cultured in DMEM/F12 medium as described above, until 30 %

confluent. Cells were then treated with clofibrate for 32 hours, and then incubated with clofibrate and 0.88 μCi/ml [^{3}H]-arachidonic acid for another 16 hours. At the end of the incubation, cells were rinsed three times with HEPES-buffered saline containing 0.025 % bovine serum albumin in order to remove any nonspecific binding of arachidonic acid to the cells (Wong *et al.*, 1998) prior to agonist stimulation. Cells were then challenged with 10 μM calcium ionophore A-23187 for 35 min. The labelled arachidonate released into the medium was determined by scintillation counting.

2.4 Determination of intracellular Ca^{2+}

H9c2 cells were plated on poly-D-lysine coated 35-mm glass cover slips 3-4 days prior to experimentation. $[Ca^{2+}]_i$ levels were determined using the Ca^{2+}-specific fluorescent probe Fura-2/AM. Cells were incubated for 40 min at 25°C followed by 10 min at 37°C in HEPES-buffered saline buffer containing 1.2 mM Ca^{2+}, 0.1% BSA and 2 μM Fura-2/AM. The cover slips containing Fura-2 loaded cells were placed in a PDMI-2 open perfusion micro-incubator (Medical Microsystems Corp., Greenvale, NY) set at 37°C. Cells were superfused at a rate of 2 ml/min with HEPES-buffered saline during baseline measurements. After 5 min, the flow was stopped and the buffer was removed and replaced with HEPES-buffered saline containing lyso-PC (25-150 μM). Cells were alternately excited at 340 and 380 nM, and emission was recorded at 510 nM with a video-based Universal Imaging System (EMPIX, Mississauga, ON). R_{max}/R_{min} ratios were converted to nanomolar $[Ca^{2+}]_i$ according to the method of Grynkiewicz *et al.* (1985). Images were acquired every 15 sec during baseline measurements and every 5 sec following lyso-PC additions by real time

averaging of 16 frames of each wavelength that included a background reference subtraction from each of the acquired images. Increases in $[Ca^{2+}]_i$ represent average cytosolic concentrations determined by subtracting $[Ca^{2+}]_i$ levels at 5, 10, and 15 min following lyso-PC applications from baseline $[Ca^{2+}]_i$. All cells in the visual field were monitored for time periods up to 30 min.

2.5 Determination of cell viability

Cell viability assays were performed with the reagent WST-1 according to the manufacturer's instruction. In this assay, the tetrazolium salt is used as a reagent for the colorimetric determination of viable cells. Tetrazolium is a hydrophobic molecule which can permeate through the biological membrane, and can be reduced by the mitochondrial succinate dehydrogenase (MSH) (Savard and Snyder, 1971; Scudiero *et al.*, 1988). When a sulfonated tetrazolium salt, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) is added to the cell, it is cleaved to produce a highly water-soluble formazan dye by the cellular mitochondrial succinate dehydrogenase (Fig 8) (Iwaki *et al.*, 1994). The colour development of this assay is dependent on the viability of the cells, and can be quantified by measuring the absorbance of the dye solution. In essence, preadipocytes were cultured in a 96-well plate and allowed to grow until 30 % confluence. Cells were treated with 50-1000μM clofibrate for 48 hours, and the WST-1 solution (10 μl) from a commercial kit was immediately added to each well containing 100 μl medium. After incubation for 2 hours at 37 ^{0}C, the formazan dye produced by metabolically active cells in each well was quantified by reading the plates with a Bio-Rad Model 3550 multi-well

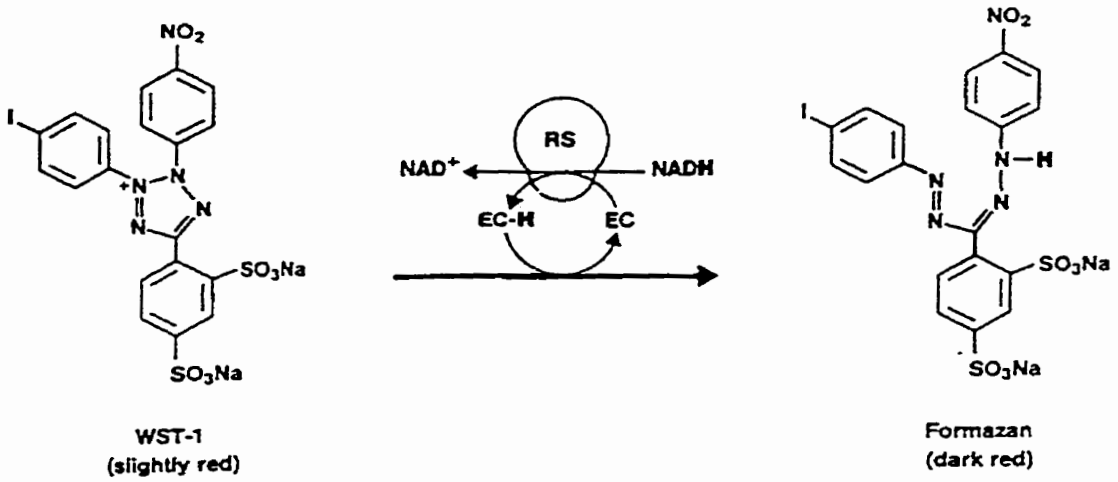


Fig 8. Cleavage of the tetrazolium salt WST-1 to formazan. (EC = electron coupling reagent. RS = mitochondrial succinate -tetrazolium-reductase system).

spectrophotometer (ELISA reader) at 450 nm, with a reference wavelength of 650 nm.

2.6 Morphological study on the conversion of preadipocyte to adipocyte

In order to detect morphological changes during the conversion of preadipocytes to adipocytes, SW cells were grown on a Chamber Slide until 20 % confluence. Cells were then treated with the indicated concentration of clofibrate for 4 days, with a change of medium every 48 hours. At the end of the incubation, the slide was washed twice with PBS at 4°C, air-dried for a few min, and then fixed with 10 % formalin for 3-5 min. The slide was stained with Giemsa for 30 sec. A photograph was taken with the objective magnification of 400 ×.

2.7 Determination of cellular protein

Preadipocytes were treated with clofibrate (10-500 μM) or DMSO vehicle (< 0.05 %) for 48 hours, and then washed three times with ice-cooled PBS and sedimented by centrifugation for 5 min at 600 × g. The cells were re-suspended in the lysis buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1 mM EGTA, 1% NP-40, 1 mM PMSF, 50 mM NaF and 10 % glycerol). Cells were lysed by sonication twice with Fisher Sonic Dismembrator, Model-300, at 35% power for 10 sec each time. The cell lysate was centrifuged at 600 × g for 5 min at 4 °C to remove cell debris and unbroken cells. The protein content in the supernatant was quantified using the bicinchoninic acid (BCA) reagent (Pierce, Rockford, IL, USA) (Smith *et al.*, 1985).

2.8 Electrophoresis and Western blot analyses

Samples containing 50 μg of protein were subjected to sodium dodecylsulfate/ 7.5 % polyacrylamide gel electrophoresis (SDS/PAGE), at constant 150 V for 1 hour. In a control experiment, the standard curve showed that the amount of protein used was within the linear range of the enhanced chemiluminescence detection system (ECL). $cPLA_2$ protein standard (15-20 *n*g) was used as a reference. Protein fractions in the gel were transferred to the hybond nitrocellulose membrane (Bio-Rad), using the Semi-Dry Transfer Cell (Bio-Rad). The membrane was incubated with the polyclonal anti-$cPLA_2$ antibody (1:1500-2000 dilution) dissolved in TBS containing 0.1 % Tween-20 (TTBS) and 2 % skim milk for 2-3 hours at room temperature. Both the $cPLA_2$ polyclonal antibody and the $cPLA_2$ protein standard were generous gifts from the Genetics Institute, Boston, MA, USA. Subsequently, the membrane was washed 4 times with TTBS and then incubated with the secondary antibody (peroxidase labelled anti-rabbit secondary antibody, 1:3000 dilution) for 30 min at room temperature. Protein bands in the membrane were visualized by ECL. In order to avoid saturation of the X-ray film, the exposure time was optimized. The relative intensities of bands were analyzed by scanning the film, and subsequently determined by Scion Image software (NIH Image modified for Windows by Scion Corp).

2.9 Determination of phospholipase A_2 activity

After clofibrate (10-500 μM) treatment, SW cells were washed twice with ice-cold PBS and re-suspended in the lysis buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA,10 μM leupetin, 10 μM aprotinin, 20 mM NaF, 10 mM Na_2HPO_4 and 1 mM dithiothreitol (DTT). The suspension was sonicated twice for 10 sec each and the mixture

was sedimented at 600 × g for 5 min to remove cell debris and unbroken cells. The supernatant was designated as the cell homogenate. This supernatant was further subjected to centrifugation at 100,000 × g for 60 min, and the cytosolic fraction was obtained from the supernatant whereas the membrane fraction was obtained from the pellets. Phospholipase A_2 activity in the subcellular fractions was determined by the hydrolysis of 1-stearoyl-2-[1-^{14}C]-arachidonyl-*sn*-glycerol-3-phosphocholine to yield the radiolabelled arachidonate. To prepare the substrate for the assay, the organic solvent was removed and the phospholipid was suspended in the assay buffer containing 0.5 % DMSO. A homogenous mixture was obtained by vigorous shaking and sonication. The assay mixture contained 50 μl (10 μg protein) of the cell homogenate, 50 μl of the assay buffer (50 mM Tris-HCl, pH 8.0, 3.0 mM $CaCl_2$), and 0.9 nmol of 1-stearoyl-2-[1-^{14}C]-arachidonyl-*sn*-glycerol-3-phosphocholine (100,000 dpm/assay), in a final volume of 100 μl. Oleic acid was added as a fatty acid carrier. The mixture was incubated for 30 min at 37^{0}C, and the reaction was terminated by the addition of 1.5 ml of chloroform/methanol (2/1, by volume). Water (0.5 ml) was added to the reaction mixtures to cause phase separation, and the lower phase (organic phase containing the labelled arachidonic acid) was collected. The upper phase was re-extracted with 1 ml chloroform, and the resultant lower phase was combined with the first extraction. The fatty acid released in the lower phase was separated by thin layer chromatography (TLC) in a solvent system consisting hexane/diethyl ether/acetic acid (70/30/1, by volume). The fatty acid fraction was visualized by exposure of the plate to iodine vapor, and the radioactivity of the arachidonic acid released was determined by liquid scintillation counting. Similar approaches were employed to determine the $cPLA_2$ activity after treating the T-24

cells with described concentrations of clofibrate for 48 hours.

2.10 Determination of PKC activity

Cells were sonicated in buffer B (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 10 mM EGTA, 0.25 M sucrose, 0.3% ß-mercaptoethanol, 10 μM benzamidine, 1 mM PMSF, 10 μg/ml leupeptin. and 10 μg/ml aprotinin) and were centrifuged at 1,500 x g for 10 min. The supernatants were subjected to ultracentrifugation at 100,000 *g* for 1 h to obtain soluble and membrane fractions. Approximately 15-30 μg of protein from these fractions were used to determine PKC activity using a PKC assay kit (Amersham), which is based on the incorporation of ^{32}P from [γ-^{32}P] ATP into a PKC-specific substrate peptide.

2.11 Isolation of total RNA

The total cellular RNA was extracted using the TRIZOL Reagent according to the manufacturer's instruction. Briefly, cells were washed three times with ice-cold PBS, and 1 ml of TRIZOL Reagent was added into each 100-mm dish. The content of each dish was collected and incubated at room temperature for 10 min, following which 200 μl chloroform was added. After mixing, the sample was centrifuged at 10,000 × g for 15 min at 4 ^{0}C. The upper phase was transferred to a new sterile tube, and mixed with 500 μl ice-cold isopropanol. The solution was allowed to sit at room temperature for 10 min. The RNA pellet was obtained by centrifugation at 10,000 × g for 10 min at 4 ^{0}C. The RNA pellet was stored at -20 ^{0}C in 75 % ethanol for future use, or re-suspended in an appropriate volume of autoclaved, double-distilled (ADD)-water after washing with 75 % ethanol. In a control

study, both the ADD-water and the diethyl pyrocarbonate (DEPC)-treated water were used to compare the RNA integrity in the preparation and analysis of RNA. There was no significant difference in RNA degradation between the DEPC-treated water and the ADD-water. Similar results were obtained in another study (Huang *et al.*, 1995). Therefore, the ADD-water was used in subsequent experiments.

The purity and the yield of isolated RNA were determined by monitoring absorbance at 260 nm and 280 nm. The integrity of the RNA was confirmed by performing denaturing agarose gel electrophoresis on the isolated RNA sample.

2.12 PCR primers and RT-PCR

The cDNA for $cPLA_2$, COX-1, COX-2, PPARα or PPARγ was amplified with a pair of specific primers. Using the known gene sequence of each protein, the primers were designed using the Mac Vector program (Sequence Analysis Software, Version 6.0.1. Oxford Molecular Group). Each primer was synthesized by Gibco Life Technology, Inc. The sequence of primers and the length of predicted PCR products were listed in Table 2.

The first strand cDNA from 1 μg total RNA was synthesized by employing 150 U of Moloney murine leukemia virus (M-MLV) reverse transcriptase, 25 pmol of random hexamer primer, 20 U of ribonuclease inhibitor, 1mM dithiothreitol (DTT), and 10 pmol each of the four deoxynucleotides (dNTPs), in a total volume of 15 μl (Leygue *et al.*, 1996). The reaction was incubated at 37^0 C for 1 hour, and terminated by boiling at 95 ^{0}C for 5 min. An aliquot of 1.0 ~1.2 μl of the resultant cDNA preparation was used directly for each amplification reaction.

Table 2. PCR Primer Design

NAME	SEQUENCE	START SITE	PRODUCT LENGTH (bp)
GAPDH(U)	5'-ACC CAC TCC TCC ACC TTTG-3'		176
GAPDH(L)	5'-CTC TTG TGC TCT TGC TGG G-3'		
$cPLA_2$ (U)	5'-AAA GAA CAC TAT AGG GAG AG-3'	609'- 628'	502
$cPLA_2$ (L)	5'-AAA GAG GTA AAG GGC ATT GT-3'	1072' - 1091'	
COX-1(U)	5'- TGC CCA GCT CCT GGC CCG CCG CTT-3'	174' - 198'	306
COX-1(L)	5'- GTG CAT CAA CAC AGG CGC CTC TTC-3'	456' - 480'	
COX-2(U)	5'- TTC AAA TGA GAT TGT GGG AAA ATT GCT-3'	2613'- 2640'	307
COX-2(L)	5'- AGA TCA TCT CTG CCT GAG TAT CTT-3'	3746'- 3770'	
PPAR-α (U)	5'- AAG TGC CTT TCT GTC GGG ATG-3'	601'- 622'	433
PPAR-α (L)	5'- CGT TCA GGT CCA AGT TTG CG-3'	1014'- 1034'	
PPAR-γ (U)	5'- CAG AAA TGC CTT GCA GTG GG-3'	276'-286'	289
PPAR-γ (L)	5'- GGG GGT GAT GTG TTT GAA CTT G-3'	581'- 602'	

Polymerase chain reaction (PCR) was performed in 20 μl reaction mixtures containing 8 pmol of each primer, 8 pmol of each dNTP and 0.4U Taq DNA polymerase. The mixture was overlaid with 30 μl mineral oil to prevent evaporation. The reaction mixture was incubated in a Perkin-Elmer DNA Thermal Cycler under conditions listed in Table 3.

The amplified RT-PCR product was analyzed by 1.2-1.8 % agarose gels electrophoresis in TAE buffer (40 mM Tris acetate, 2 mM sodium EDTA), and visualized by staining with 0.5μg/ml ethidium bromide. The GAPDH (glyceraldehyde 3-phosphate dehydrohenase) was used as an internal control.

2.13 mRNA stability assay

Actinomycin D (Fig 9) is an antibiotic extracted from *streptomyces* species mixture. (purchased from Sigma. Catalog number: A1410). It inhibits the proliferation of cells in a nonspecific way by forming a stable complex with double-stranded DNA, thus inhibiting the DNA- primed RNA synthesis. To test the stability of $cPLA_2$ mRNA, preadipocytes were grown to 30 % confluence, and then incubated in the presence or absence of clofibrate. Cells were grown in clofibrate for 48 hours, and then challenged with 5 μg/ml actinomycin D for up to 24 hours. The $cPLA_2$ mRNA level at each time point was measured by RT-PCR.

2.14 Sequence analysis of cDNA

The identity of each PCR product generated from RT-PCR using *Taq* DNA polymerase was verified and confirmed by direct DNA sequencing. The gel fraction containing the target DNA, including COX-1, COX-2, $cPLA_2$, PPARα or PPARγ, was

Table 3. PCR Condition

NAME	Cycle	Denaturation	Annealing	Extension
$cPLA_2$	28-30	94°C, 1 min	55°C, 2 min	72°C, 1 min
COX-1/2	25-28	94°C, 1 min	60°C, 1 min	72°C, 1 min
PPARα	25-30	94°C, 30 sec	57-60°C, 30 sec	72°C, 30 sec
PPARγ	25-30	94°C, 30 sec	58-60°C, 30 sec	72°C, 30 sec
GAPDH	25	94°C, 30 sec	55°C, 30 sec	72°C, 30 sec

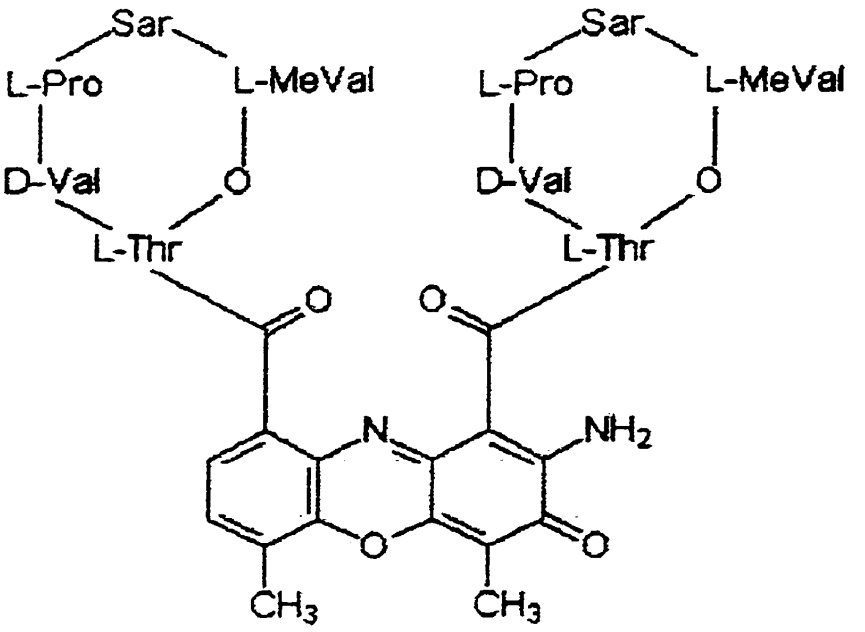


Fig. 9 Structure of Actinomycin D

excised and purified using standard methods. In brief, the agarose block with DNA fractions were cut under UV light, each fraction was placed in a Spin-X centrifuge tube equipped with a filter (0.22 μm cellulose acetate in 2.0 ml tube) and 100 μl H_2O was added. The solution containing the DNA fraction was obtained by centrifugation at 12,000 rpm for 5 min at 4 ^{0}C. The DNA in each fraction was extracted with phenol/chloroform, and the DNA in the aqueous fraction (100 μl) was allowed to precipitate in 10 μl of 3 M sodium acetate (pH 5.5) mixed with cold 100 % ethanol (250 μl). The DNA was pelleted by centrifugation at 12,000 rpm for 15 min (4 ^{0}C), followed by washing with ice-cold 75 % ethanol. The DNA was re-suspended in H_2O, and an aliquot was submitted for sequencing analysis. The sequencing was performed using the Perkin-Elmer Applied Biosystems ABI 310 Genetic Analyzer and the BigDye Terminator Cycle Sequencing Ready Reaction Kit at the Institute of Cell Biology, University of Manitoba, Winnipeg, Canada.

The nucleotide sequences obtained were used for homologous search by the Basic Local Alignment Search Tool (BLAST). Using a heuristic algorithm, BLAST can seek local as opposed to global alignments and is therefore able to detect relationships among sequences that share only isolated regions of similarity.

2.15 Statistical analysis

The data were analyzed with a two-tailed independent Student's t test, or where appropriate, a one-way analysis of variance (ANOVA), followed by Duncan's new multiple range post hoc test to detect individual differences. In all cases, the level of significance was defined as $P < 0.05$. Results are presented as the mean ± standard deviation or standard error.

IV EXPERIMENTAL RESULTS

Part I Modulation of $cPLA_2$ by Clofibrate in SW Cells

4.1.1 The effect of clofibrate on A-23187-induced arachidonic acid release in SW cells

Clofibrate is a synthetic chemical which belongs to the fibrate family. It was the first generation of triglyceride-lowering drugs to be recommended for treatment of patients with hyperlipidemia. In order to examine the ability of clofibrate to activate cytosolic phospholipase A_2 ($cPLA_2$), the effect of clofibrate on the release of arachidonic acid in human preadipocyte SW 872 cells was studied. SW cells were pre-incubated in DMEM/F12 (3/1; by volume) medium containing 5 % FBS and 50-200 μM clofibrate for 32 hours, prior to incubation in the presence of [^{3}H]-arachidonate. A prolonged period of arachidonate labeling may result in arachidonic acid break down, or chain elongation of arachidonate into adrenate (Rosenthal and Hill 1986). The presence of adrenate has been shown to resist the agonist-induced release of arachidonate from the phospholipids (Rosenthal and Hill, 1986). Hence, the labeling of SW cells with [^{3}H]-arachidonate (0.88 μCi/ml) was confined to the last 16 hours of the clofibrate treatment. About 33 % of the arachidonic acid was taken up by the cells. In a control study, the presence of clofibrate did not significantly alter the total cellular uptake of radiolabeled arachidonate in SW cells (Table 4). When these cells were stimulated with calcium ionophore A-23187 (10μM) (Street *et al.*, 1993; Dennis, 1997), arachidonate release was significantly enhanced in the clofibrate-treated cells (Table 5). No morphological change in the shape or size of cells was apparent during the course of treatment. A 34 % increase in arachidonate release was observed when 50 μM

Table 4. The effect of clofibrate on total cellular uptake of [^{3}H] labeled-arachidonic acid in human preadipocyte cells

Monolayer cells were cultured in regular media to 25 % confluence in 35 mm dishes, and then treated with 0, 50 or 200μM clofibrate for 36 hours. Cells were then incubated with 0.88 μCi/ml [^{3}H]-arachidonic acid for 16 hours in media containing the indicated concentrations of clofibrate. The culture media were collected and cells were washed three times with HEPES-buffered saline. The buffer was combined with the culture media and an aliquot of solution was taken for determination of radioactivity by scintillation counting. Results are expressed as mean ± standard deviation of three separate experiments.

Clofibrate (μM)	Radioactivity remaining in medium (dpm $\times 10^5$/ml)	Total cellular uptake (%)
0	13.04 ± 0.30	32.8
50	13.02 ± 0.17	31.9
200	12.80 ± 0.30	34.0

Table 5. The effects of clofibrtae on A-23187-induced arachidonic acid release in preadipocytes.

Cells were treated with indicated concentrations of clofibrate for 32 hours, and then cultured with the medium containing 0.88 μCi/ml [^{3}H]arachidonic acid and same amounts of clofibrate for another 16 hours. Cells were rinsed and challenged with 10 μM Calcium ionophore A-23187 for 35 min. Arachidonate release into the medium was determined. Results are expressed mean ± standard error of the mean from 3 independent experiments, each carried out in triplicate. * $P < 0.05$ and ** $P < 0.01$ when compared with control.

Clofibrate Treatment (μM)	Arachidonate Release (dpm/dish × 10^{-4})
0	7.22 ± 0.392
50 μM	9.64 ± 0.941*
200 μM	10.8 ± 0.413**

of clofibrate was added, whereas a 50 % increase was obtained when 200 μM of clofibrate was used. In the absence of the agonist, there was a small but insignificant increase in the release of arachidonic acid from cells treated with a high concentration (200μM) of clofibrate (data not shown). Our results indicate that clofibrate enhances the A-23187-induced arachidonic acid release in human preadipocytes.

4.1.2 The effect of clofibrate on cPLA$_2$ activity

The enhancement of arachidonic acid release may result from the activation of the cytosolic or secretory phosphoslipase A_2. In mammalian cells, the major pathway for the release of arachidonate is catalyzed by the hydrolytic action of cPLA$_2$ (Wong *et al.*, 1998, and references therein), and the contribution of the secretory phosphoslipase A_2 to this process is very limited (Golfman *et al.*, 1999). Since clofibrate caused the increase of A-23187-induced arachidonic acid release in SW cells, it would be of interest to demonstrate that the increase was associated with an enhancement of cPLA$_2$ activity. In this study, SW cells were incubated with increasing concentrations of clofibrate for 48 hours. Subsequently, cells were lysed and the protein content in each sample was determined. As shown in Fig 10, cPLA$_2$ activity was increased in the whole cell homogenates with increasing concentration of clofibrate. The activity of the secretory phospholipase A_2 was severely attenuated in the assay system by the presence of 1 mM of dithiothreitol (DTT). The presence of DTT would reduce the disulfide bonds, which would render the secretory phospholipase A_2 inactivate. When cPLA$_2$ activities were examined in both the cytosolic and membrane fractions, we did not observe a translocation of the enzyme from the cytosol to the membrane upon clofibrate

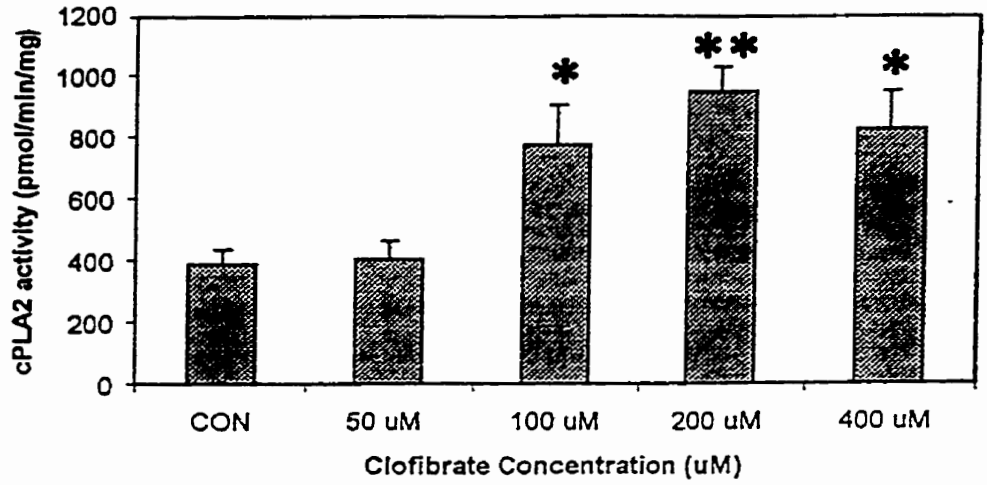


Fig 10. The effect of clofibrate on cPLA$_2$ activity in human preadipocyte. SW cells were treated with increasing concentrations of clofibrate in DMEM/F12 (3:1) medium containing 5 % FBS. Cells were lysed and cPLA$_2$ activity was assayed in the total homogenate. Results are expressed as means ± standard deviation from three separate experiments. * $P < 0.05$. ** $P < 0.01$.

stimulation (data not shown). The maximum effect of clofibrate on $cPLA_2$ activity was near 200 μM. The $cPLA_2$ activity was attenuated from the maximal value when cells were incubated with 400 μM clofibrate. The biphasic effect of clofibrate on $cPLA_2$ activity in SW cells was also observed in the human bladder cancer cells (Table 6).

4.1.3 The effect of clofibrate on the protein level of $cPLA_2$

The enhancement of the $cPLA_2$ activity by the clofibrate might be related to an increase in its protein level. Hence, the protein level of $cPLA_2$ in SW cells was determined by Western blot analyses using polyclonal anti-$cPLA_2$ antibodies. As shown in Fig 11, incubation of cells with increasing concentrations of clofibrate (up to 200 μM) resulted in progressive increases in $cPLA_2$ protein levels. This indicates that the increase in $cPLA_2$ activity by clofibrate stimulation may be caused by an increase in enzyme protein level. Similar to the enzyme activity study, the enzyme protein level was attenuated from the maximal value when cells were incubated with 500 μM clofibrate.

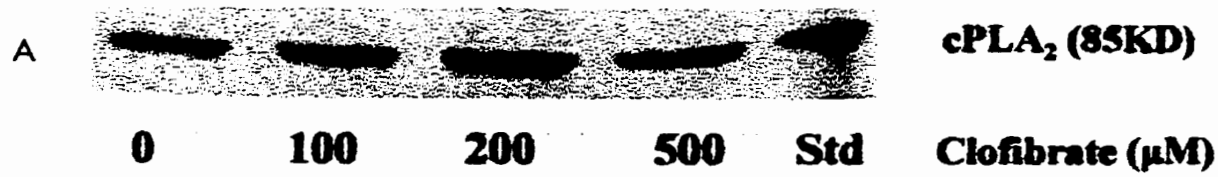
4.1.4 Study on the integrity of RNA after extraction

We have shown in the previous section that clofibrate caused an enhancement in arachidonate release which was mediated via an increase in $cPLA_2$ protein. In this study, the mechanism for the observed increase in $cPLA_2$ protein was examined. The possible increase in gene transcription was studied by determining the mRNA level of the enzyme after clofibrate stimulation. Initially, a control experiment was performed to examine the integrity of the RNA extracted from the cells under various conditions. SW cells were incubated in

Table 6. Effects of clofibrate on $cPLA_2$ activity in human bladder cancer cells T-24

Cells were incubated with indicated concentrations of clofibrate for 48 hours. Cells were then lysed, and $cPLA_2$ activity in whole cell homogenate was measured. Results are expressed as mean ± standard deviation of three separate experiments. * $P < 0.05$ when compared with control.

Clofibrate treatment (μM)	$cPLA_2$ activity (Pmol/min/mg protein)
0	623.3 ± 34.5
100	818.3 ± 62.3 *
200	847.7 ± 1.4 *
400	709.0 ± 48.9



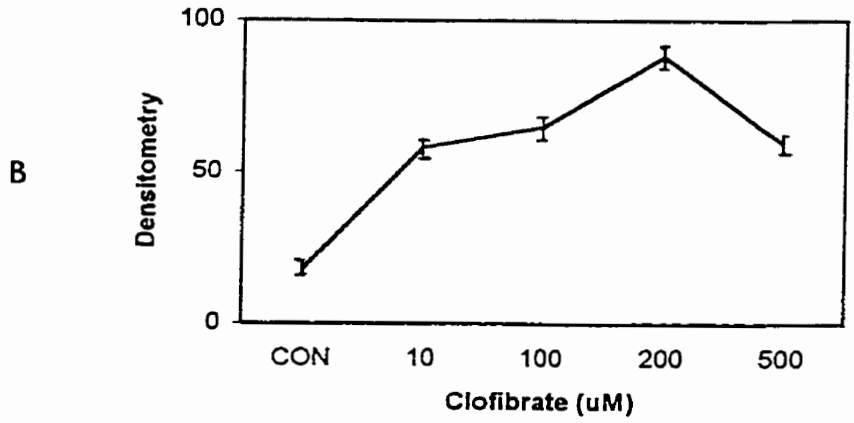


Fig 11. The effect of clofibrate on the protein level of $cPLA_2$. SW cells were incubated for 48h in DMEM/F12 (3:1) medium containing different concentrations of clofibrate. Western blot analyses of $cPLA_2$ protein were performed using a rabbit polyclonal $cPLA_2$ antibody (1: 2000 dilution). (A) A representative blot is depicted. Std: $cPLA_2$ standard. (B) Densitometry plot of A.

a medium containing the DMSO vehicle, 1 μM prostaglandin J_2 (PGJ_2) or 200 μM clofibrate for 48 hours. After incubation, the total cellular RNA was extracted, and resolved by formaldehyde-agarose gel electrophoresis. As depicted in Fig 12, the 28S ribosomal RNA (rRNA) was appropriately 2-fold higher that of the 18S rRNA (Johnson *et al.*, 1977). Our results indicate that there was no substantial degradation in the total RNA sample, suggesting that the integrity of the RNA was maintained after extraction. In addition, the integrity of mRNA was not affected by the presence of DMSO, prostaglandin J_2 or clofibrate. Therefore, the protocol for the total RNA extraction was used in subsequent experiments.

4.1.5 Up-regulation of $cPLA_2$ mRNA by clofibrate

In this study, the role of clofibrate in the regulation of gene expression of $cPLA_2$ was examined. Pre-confluent SW cells were cultured in the DMEM/F12 medium and then incubated in the same medium containing clofibrate (200μM), prostaglandin J_2 (PGJ_2, 1μM, 3μM) or the vehicle (DMSO, < 0.05 %) for 48 hours. The mRNAs for $cPLA_2$ and GAPDH were amplified by RT-PCR. The PCR products were analyzed by agarose gel electrophoresis as described in *Materials and Methods* (Fig 13A). The relative intensity of each band in the agarose gel was quantified by scanning, and then normalized by the intensity of GAPDH. The results were depicted in Fig 13B. Treatment of the cells with clofibrate caused a 1.8-fold increase of the $cPLA_2$ mRNA level, whereas treatment with 1-3 μM of PGJ_2 did not elicit any significant effect. When cells were treated with 5 μM of PGJ_2 under the same conditions, only 50% of the cells survived after the incubated period. In another set of experiments, the effect of various concentrations of clofibrate (10-1000 μM) on $cPLA_2$ mRNA levels were

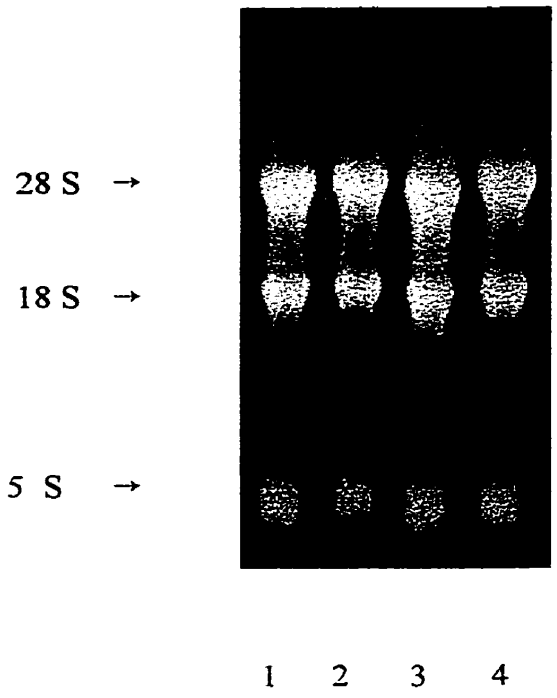


Fig 12. Analysis of RNA by formaldehyde-agarose gel. The total RNA was extracted from the SW cells after treatment with vehicle control (DMSO), 1μM PGJ_2 or 200μM clofibrate for 48 h. Each lane contained 20 μg RNA sample. The fractions were resolved by electrophoresis on 1% formaldehyde-agarose gel, at 120 constant voltage for 1.5 ~2 h. Lane 1 and 3, vehicle control; lane 2, PGJ_2 treatment; lane 4, clofibrate treatment. The three major bands indicate the 28S, 18S and 5 S subunits of the total RNA.

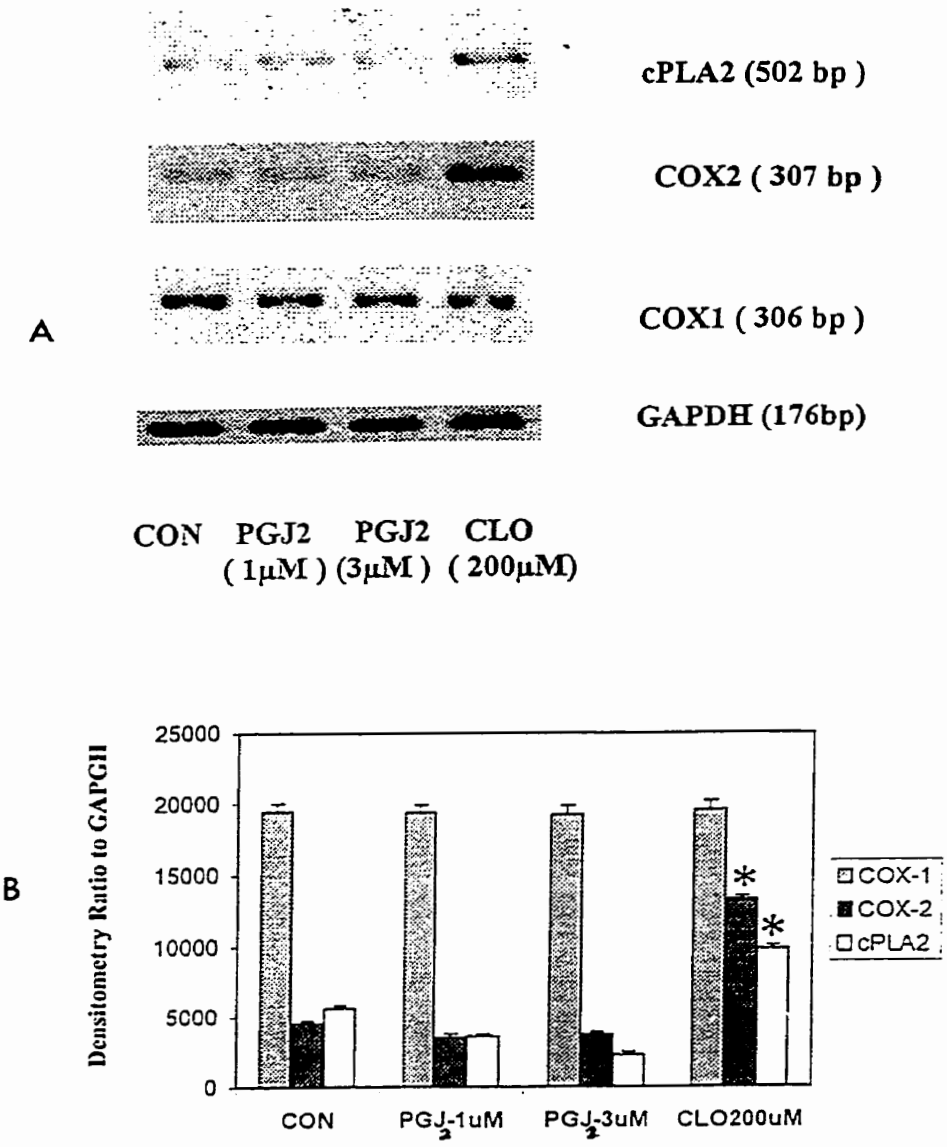


Fig 13. Effects of PPAR ligands on gene expression of $cPLA_2$ and COX-2 in human preadipocytes. Pre-confluent SW cells were cultured for 48 h in DMEM/F12 medium containing either PPARα ligand clofibrate (CLO, 200 μM) or PPARγ ligand PGJ_2 (1-5 μM). The total RNA was extracted and RT-PCR analyses were performed using primer sets specific for $cPLA_2$, COX-1, COX-2 and GAPDH. (A) A typical photograph is depicted. (B) Densitometry plot of A. Results represent the mean ± standard deviation from three independent experiments. * $P < 0.05$

investigated. As depicted in Fig 14, mRNA levels of $cPLA_2$ were significantly increased by 10 μM clofibrate, and the maximal effect was achieved at 200 μM clofibrate. The level of $cPLA_2$ mRNA was attenuated from the maximal value when the cells were incubated with 500 μM or 1000 μM clofibrate. It should be noted that the RT-PCR method is only semi-quantitative, and it would provide us with indications on changes in the mRNA level.

4.1.6 The role of clofibrate on $cPLA_2$ mRNA stability

The accumulation of a specific mRNA can be regulated by either an increase in its rate of synthesis, a decrease in its rate of degradation, or a combination of these two process (Ross, 1996). Actinomycin D, which is extracted from a mixture of *Streptomyces* species, is an anti-neoplastic antibiotic that can inhibit DNA-paired RNA synthesis by forming a stable complex with double-stranded DNA via deoxyguanosine residues. Hence, mRNA stability assays were conducted using actinomycin D as an inhibitor for RNA synthesis in SW cells. Cells were incubated with the vehicle control (DMSO), or clofibrate (50 μM, 200 μM) for 48 hours, and challenged with 5 μg/ml actinomycin D. After the challenge, the total RNA was isolated and the RT-PCR was conducted to determine mRNA levels for $cPLA_2$ and GAPDH/COX-1. The data obtained are shown in Fig 15A, and based on these data, a regression line (Fig 15B) was determined. Due to technical limitations, the photograph shown in Fig 15 has some smear and non-specific bands on the gel. In this studies, we did not observe the degradation of both GAPDH and COX-1 mRNA. Although the reasons are not known, we postulated that: (1) GAPDH and COX-1 mRNAs are relatively stable and have long half-lives in SW cells; (2) They are present in great abundance in SW cells and our

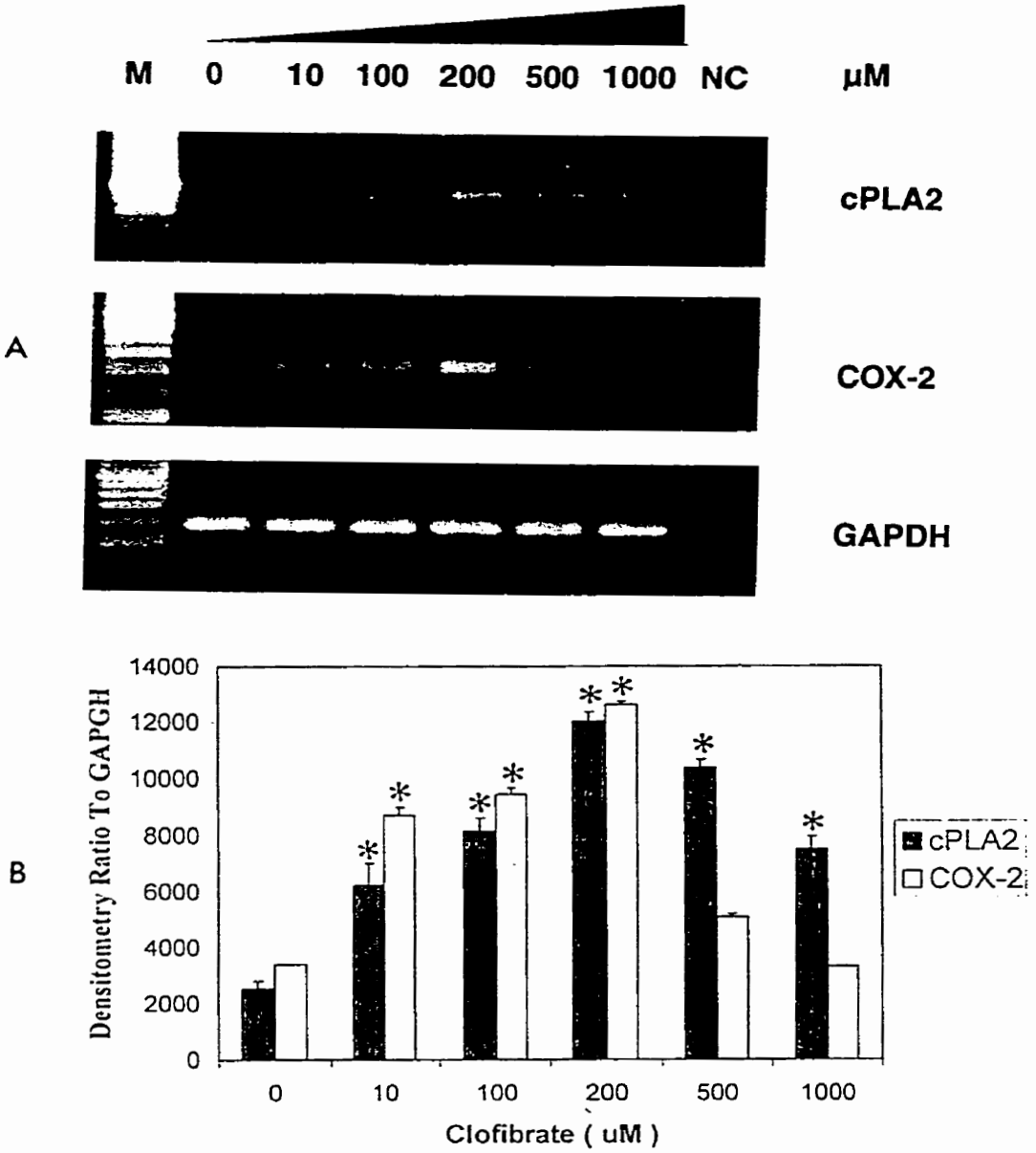


Fig 14. Effects of clofibrate concentration on $cPLA_2$ and COX-2 mRNA levels. Pre-confluent SW cells were incubated for 48h in DMEM/F12 medium containing 5% FBS and increasing concentrations of clofibrate. The total RNA sample was isolated and RT-PCR analyses were performed. (A) A typical photograph is depicted. (B) Densitometry plot of A. Results represents mean ± standard deviation of three separate experiments. M: molecular marker. * $P < 0.05$. ** $P < 0.01$. NC: negative control.

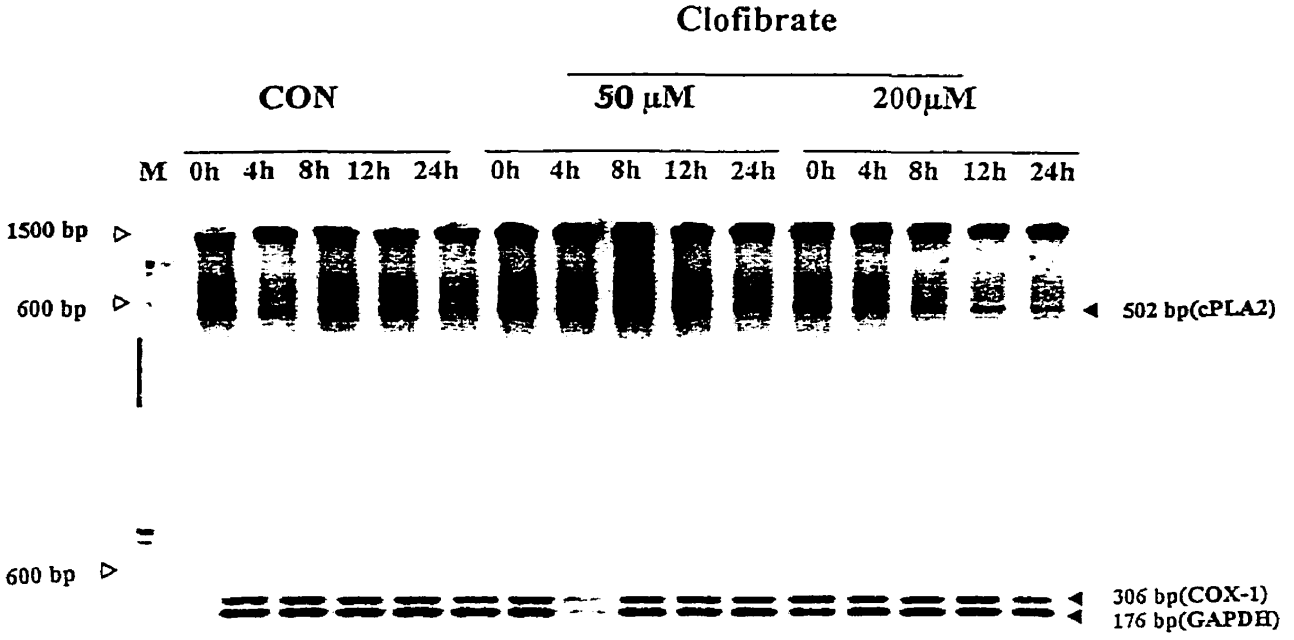


Fig 15A. Effects of clofibrate on $cPLA_2$ mRNA stability. SW cells were incubated with either vehicle (DMSO) or clofibrate (50 μM, 200 μM) for 48 h, and then challenged with 5 μg/ml actinomycin D. The RNA was isolated and mRNA levels of $cPLA_2$ and GAPDH/COX-1 were determined by RT-PCR and gel analysis. M: molecular marker.

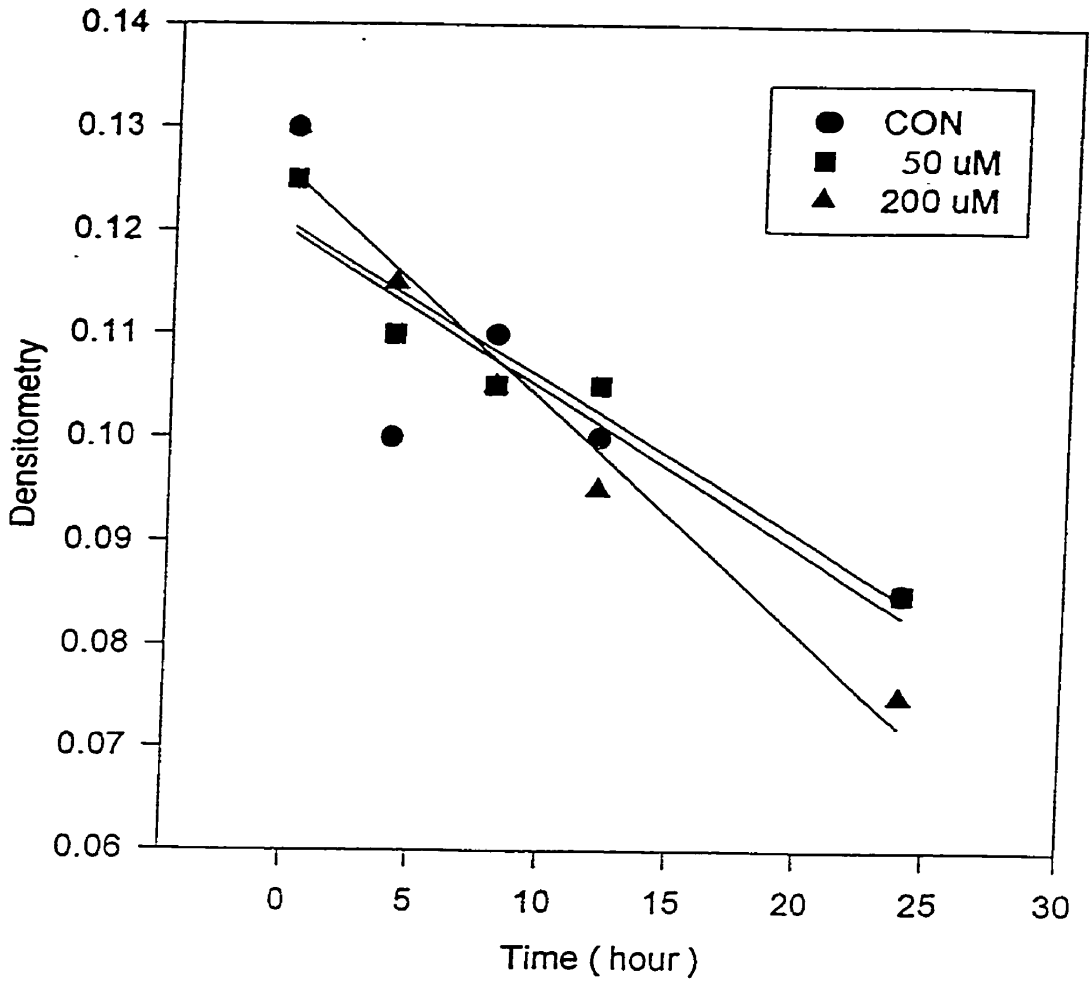


Fig 15B. Effects of clofibrate on cPLA$_2$ mRNA degradation. The bands on the agarose gel depicted in Fig. 15A were scanned and the relative intensities of densitometry were calculated. Each point in the figure is the mean of 3-4 experiments.

technique was not sensitive enough to measure their changes. Therefore, it appears that the data shown in Fig 15 would not enable us to draw any solid conclusion. However, they suggest that there was no apparent changes in mRNA degradation after clofibrate treatment.

4.1.7 Expression of PPARα and -γ in cultured SW cells

The action of fibrates on the regulation of transcription of lipid metabolic enzymes is mediated via the peroxisome proliferator-activated receptors. Specifically, the interaction between clofibrate and PPARα has been shown to regulate the transcription of a variety of lipid metabolic enzymes including acyl-CoA oxidase, lipoprotein lipase, and HMG-CoA synthetase. PPARα is found in almost all mammalian tissues including the liver, kidney, heart, endothelial cells and adipose tissues. In order to show that PPARα is a participant in the control of transcription of $cPLA_2$ in SW cells, our initial approach was to demonstrate the presence of PPARα in these cells. SW cells were grown in DEME/F12 medium containing 5% fetal bovine serum, and harvested at 90% confluency. The total RNA was isolated, and RT-PCR was performed using *Taq* DNA polymerase and specific primers for PPAR-α, -γ and GAPDH as depicted in the *Materials and Methods*. As shown in Fig 16, mRNA for both PPARα and PPAR-γ were present in SW cells.

Although the human PPARα (hPPARα) genomic structure has not been published, heterogeneity and polymorphism of this receptor have been detected (Tugwood *et al.*, 1996). There is considerable variation in the hPPARα cDNAs obtained from different individuals, both at the gross structural level (lack of a coding exon) and of a more subtle nature (single base changes leading to amino acid substitutions). At least two hPPARα cDNA have been

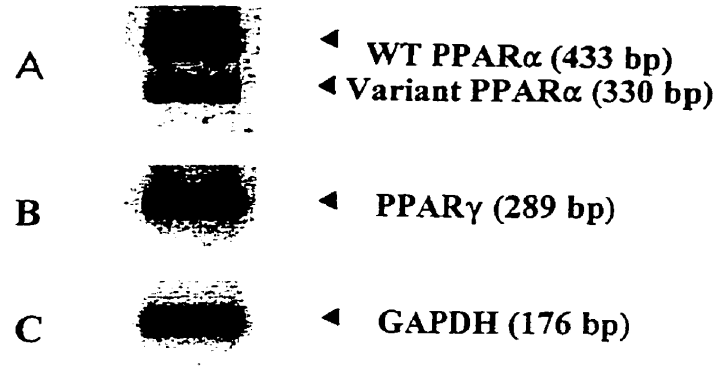


Fig 16. Expression of mRNA for PPARα and -γ in cultured SW cells. The total RNA was extracted from cultured SW cells and analyzed by RT-PCR. (A) Expression of PPARα mRNA in SW with a wild-type 433-bp fragment and a variant 330-bp fragment. (B) PPARγ mRNA with a 289-bp fragment. (C) GAPDH mRNA with a 176-bp fragment as a internal control.

characterized and designated as hPPARα6/29 and 8/14 due to alternative splicing. The hPPARα6/29 clone is identified as a wild type. The novel hPPARα8/14 clone lacks 203 base pairs of the coding sequence and results in a truncated protein with 170 amino acids of the normal PPARα sequence. In this study, a wild-type 433 bp fragment and a variant 330 bp fragment of PPARα mRNAs were both detected in SW cells. The function of the PPARα variant in preadipocytes is unknown. In liver cells, the PPARα variant functions as a dominant negative regulator of the PPARα wild type signaling pathway. The presence of the PPARα variant may protect human being from tumorigenesis by proxisome proliferators (Gerviois *et al.*, 1999).

4.1.8 The effect of clofibrate on gene expression of COX-1 and COX-2

COX-1 and -2 are integral membrane proteins. COX-1 is mainly responsible for the biosynthesis of prostaglandins involved in homeostatic or "house-keeping" regulation. COX-2 produces prostaglandins that act through nucleoplasmic or nuclear membrane targets in association with cell differentiation and replication through the nuclear receptor. Fibrates have been shown to regulate the gene expression of COX-2 via its interaction with PPAR (Fruchart *et al.*, 1999). Evidence has shown that $cPLA_2$ is functionally coupled with COX-2 to involve in both the immediate and delayed PG-biosynthetic responses (Balsinde *et al.*, 1998; Murakami, 1999). Furthermore, COX-2 and $cPLA_2$ may be coordinated regulated (Sharp and Kramer 1997). Hence, the effect of PPARα activator (clofibrate) on the COX gene expression in SW cells was also examined. Cells were incubated in the presence of either vehicle (DMSO, < 0.05 %) or 200 μM clofibrate for 48 hours, and mRNA levels of

COX-1, COX-2 and GAPDH were determined by RT-PCR. As predicted, clofibrate exhibited an effect on COX-2 similar to that observed in $cPLA_2$. Specifically, clofibrate elicited a 2.0 ~ 3.8 fold increase in COX-2 mRNA level when compared with the control (Fig 13A and Fig 14). The effect of clofibrate on COX-2 was found to be concentration dependent, and the maximum effect was obtained at 200μM (Fig 14). The enhancement of the COX-2 mRNA was attenuated from the maximal level when 500 μM of clofibrate was used. Taken together, these results demonstrate that clofibrate has the ability to up-regulate mRNA levels of both COX-2 and $cPLA_2$, suggesting that there is a coordinated regulation of both enzymes upon stimulation in SW cells.

No change in COX-1 mRNA level was observed in SW cells treated with clofibrate (Fig 13). This is not surprising since COX-2 can be induced by many inflammatory factors and subsequently producing prostaglandins in response to a wide spectrum of environmental insults and internal stimuli. COX-1, however, is mainly responsible for the biosynthesis of prostaglandins involved in homeostatic regulation (Dubois *et al.*, 1998). Our result is consistent with the postulation that COX-1 functions as a house-keeping enzyme whereas COX-2 is an inducible enzyme subjected to stimuli.

4.1.9 Sequencing of PCR products

The PCR method is extremely sensitive. It can detect a single DNA molecule in a sample. Trace amounts of RNA can be analyzed in the same way by RT-PCR, in which the RNA is first transcribed into DNA with reverse transcriptase. Hence, the identity of the PCR product has to be confirmed. In order to verify the sequence of the PCR product of interest

in this study, the double-stranded DNA produced from RT-PCR was subjected to sequencing analysis. All the DNA fragments, including $cPLA_2$, COX-1, COX-2, PPARα or PPARγ obtained by RT-PCR was separated by agarose gel electrophoresis. Each target band from the gel was excised, and the DNA was extracted and purified from the gel by phenol/chloroform extraction. The purified DNA fragment obtained was sequenced by a sequencing facility. The results obtained from the sequencing were compared with published sequences for $cPLA_2$, COX-1, COX-2, PPARγ and PPARα variant. The amplified cDNA fragments from SW cells were found to have a high degree of identity (>95%) with the published sequences (see Table 7 and related references). Despite several attempts, it was not possible to sequence the DNA sample obtained from the mRNA for the PPARα wild type (433 bp). One reason for the inability to sequence is the difficulty in separating the PPAR wild type from the variant fragments on the agarose gel. Since the double-stranded DNA sequencing technique is based on PCR amplification, any trace DNA contamination would interfere with sequencing of the target gene.

4.1.10 The effect of clofibrate on cell viability

Clofibrate has been shown to induce DNA synthesis (Uno *et al.*, 1999). However, when SW cells were incubated with a high concentration of clofibrate (>400 μM), levels of $cPLA_2$ activity, mRNA and protein were attenuated from the maximal value. A similar result was observed for the COX-2 mRNA level. Hence, the toxic effect of clofibrate on cell viability at higher concentration was investigated. WST-1, a reagent for determining cellular viability via the mitochondrial succinate dehydrogenase activity, was used for this study.

Table 7. Published Sequences and Homology

Name	Gene Bank Accession NO	Identity (%)	Reference
$cPLA_2$	M68874	97%	Sharp JD et al. 1991
	M72393	97%	Clark JD et al. 1991
COX-2	NM 000963.1	95%	Jones DA et al. 1993
	M90100	95%	Hla Tet al. 1992
COX-1	M59979	100%	Funk CD et al. 1991
	S36219	100%	Diaz A et al. 1992
	S36271	100%	Diaz A et al. 1992
	S78200	100%	Takahashi et al. 1992
PPARα (330 bp variant)	S74349	98%	Mukherjee et al. 1994
	Z79997	98%	Whiteley M. 1998 *
	Y07619	98%	Tugwood et al. 1996
PPARγ	U79012 (HSU63415)	99%	Mukherjee et al. 1997
	U63415 (HSU 79012)	99%	Elbrecht et al. 1996
	X90563 (HSPPARGAM)	99%	Lambe KG and Tugwood JD. 1996

* This sequence was submitted to GeneBank directly.

As depicted in Fig 17, there was no significant difference in the mitochondrial dehydrogenase activity when cells were incubated with up to 200 μM clofibrate. The proliferation of cells was not affect by clofibrate under the same experimental condition (data not shown). When cells were treated with 500 μM or higher concentrations of clofibrate (Fig 17), the viability of SW cells was significantly affected. The failure to induce higher levels of $cPLA_2$ activity, mRNA and enzyme protein, therefore, could be caused by the toxic effect of clofibrate at high concentrations.

4.1.11 Morphological study on the conversion of preadipocyte to adipocyte

The differentiation of preadipocytes into adipocytes is regulated by at least two families of transcription factors: the PPARs and the C/EBPs (Pessin and Bell, 1992; Cao *et al.*, 1991). Of the known members of PPAR family, ligand-induced PPARγ activation appears to be primarily involved in adipogenesis (Schoonjans *et al.*, 1996a; Fajas *et al.*, 1998). However, PPARα ligands, such as bezafibrate, were also found to induce the conversion of preadipocytes to adipocytes (Brandes *et al.* 1986; Brandes *et al.* 1987).

In order to determine whether clofibrate has a role in mediating the differentiation of preadipocytes into adpocytes, cells were grown on chamber slides (Nalge Nunc Inc. USA) in DMEM/F12 medium for 12 hours, and incubated with the same medium containing clofibrate for 4 days. At the end of incubation, the cells on the slides were fixed and stained with Giemsa dye. The vacuoles in the cytoplasm represent the neutral lipids (oil droplets) which were removed during fixation (see the arrows in Fig 18 A and B). The presence of oil droplets in these cells was confirmed by the red oil "O" dye staining.

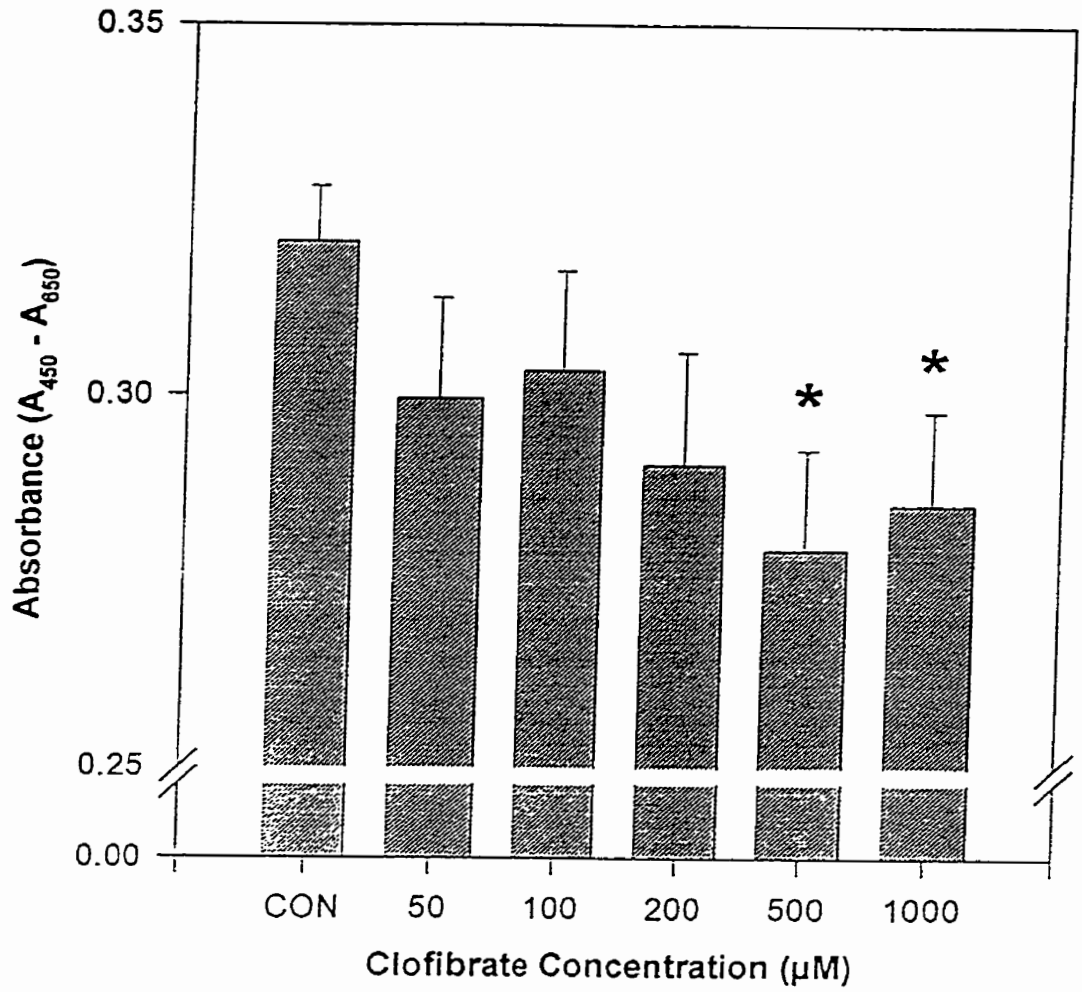


Fig 17. The effect of clofibrate on cell viability in SW cells. Cells were cultured with 96-well plates in a medium containing different concentrations of clofibrate (50μM-1000μM) for 48 hours. At end of the incubation, the cell proliferation reagent WST-1 was added into each well, and the mixture was incubated for another 2 h at 37 °C. Mitochondrial succinate dehydrogenase activity in each well was quantified by measuring the absorbance at 450 nm. For each experiment, 10 wells were used for each group of study. Results are expressed as the means ± standard deviation from three separate experiments. A non-continuous Y axis is used to highlight the differences between experimental groups. * $P < 0.05$.

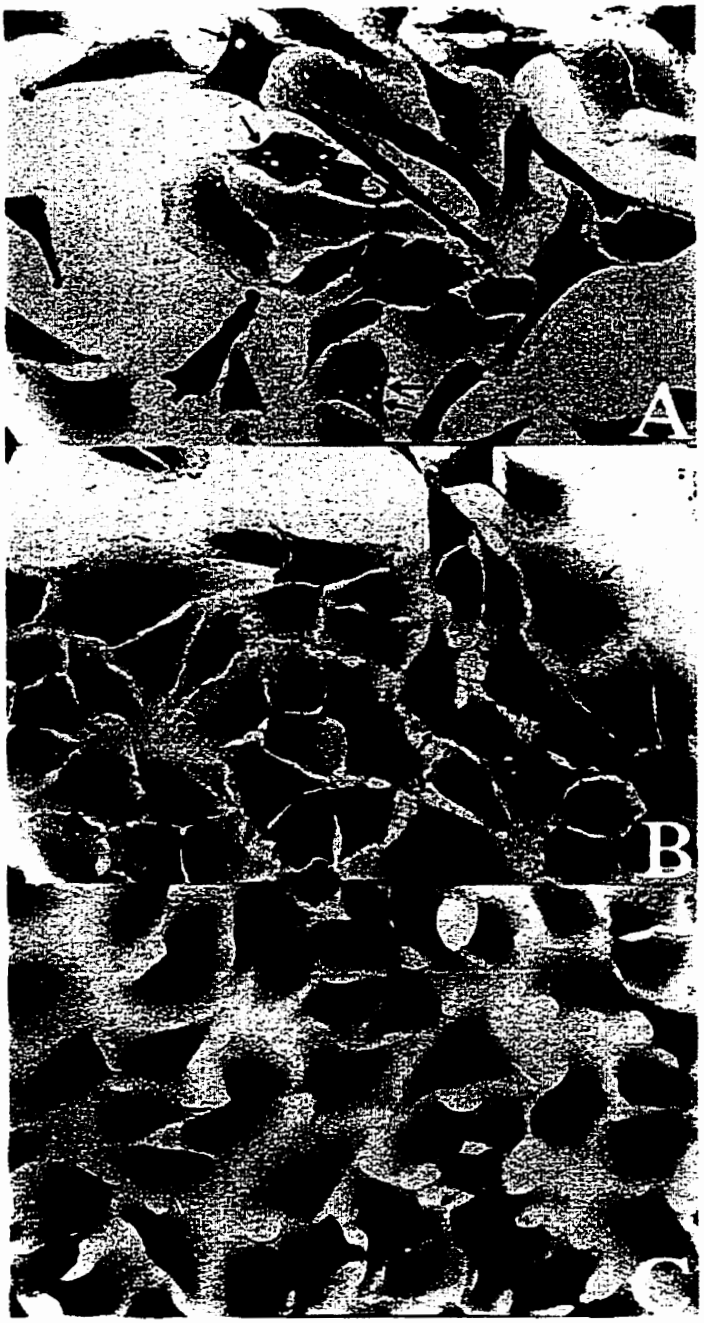


Fig 18. Morphological study of conversion of preadipocytes to adipocytes. SW cells on slides were treated for 4 days with (A) 200μM clofibrate (B) 50μM clofibrate (C) vehicle. Slides were fixed in 10 % formalin buffer and stained with Giemsa. The cytoplasmic vacuoles in A and B correspond to neutral lipids, and were indicated by arrows. (The objective magnification: 400 ×).

As shown in Fig 18, cells treated with clofibrate contained more oil droplets when compared with vehicle-treated cells. At higher clofibrate concentration, more oil droplets appeared in the cytoplasm. The presence of a high amount of oil droplets in the cytoplasm is indicative of the conversion of preadipocytes to adipocytes. Although the action of clofibrate has been shown to be mediated via the activation of PPARα, it is not clear if other factors may be involved in this process. It is possible that cross-talk between PPARα and PPARγ pathways may play a role in the cellular transformation process.

Part II Modulation of $cPLA_2$ by lyso-PC in H9c2 cells

Preamble

The study on the "Modulation of $cPLA_2$ by lyso-PC in H9c2 cells" was initiated by Dr. Leonard S. Golfman in Dr. Choy's lab. Subsequently, I was invited to join the study. My contribution to this study include: (1) participation in the design of experiments; (2) participation in the actual experimental works except for the calcium determination; (3) participation in the analysis of data; (4) participation in the writing of the manuscript. This study has been published in 1999 (Golfman *et al.*, *J.Lipid. Res.* **40**: 1818-26. See reference for authorships).

4.2.1 Modulation of arachidonate release

The effect of lyso-PC on arachidonate release in H9c2 cells was examined. Cells were pre-labelled with [^{3}H]arachidonate and then incubated with HEPES-buffered saline containing 0.1% bovine serum albumin and 0 or 150 μM lyso-PC for various time periods (Fig 19A). Lyso-PC elicited a time-dependent enhancement of arachidonate release, which reached a maximum at 15 min of incubation. The enhancement of arachidonate release was slightly diminished after the first 15 min. The presence of 100 μM lyso-PC resulted in optimal arachidonate release (Fig 19B) irrespective of the bovine serum albumin concentration (0.025-0.1 % w/v) (4-16 μM). Therefore, 100 μM lyso-PC in a medium containing 0.1% bovine serum albumin was used in all subsequent studies. Cell viability was confirmed by trypan blue exclusion that showed minimal dye infiltration under the conditions

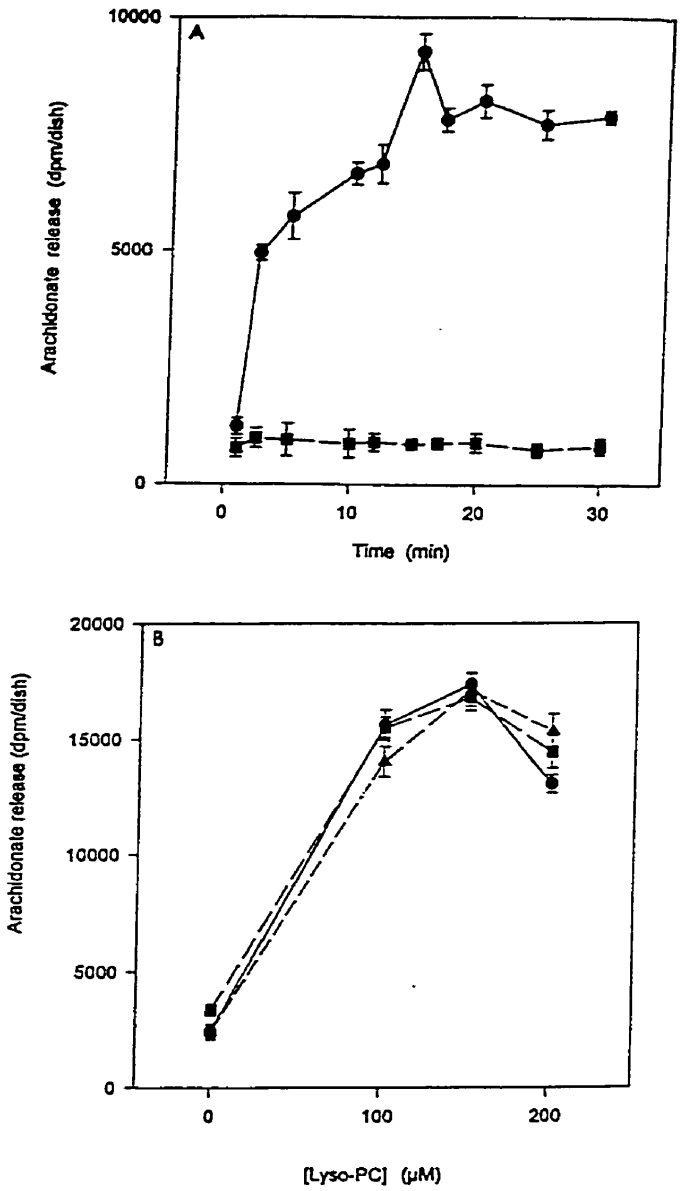


Figure 19. Effect of lyso-PC on arachidonate release in H9c2 cells. Cells were prelabelled with 1 μCi/ml of [^{3}H]arachidonate in Dulbecco's Modified Eagle's medium containing 1% newborn calf serum for 16-20h. Cells were washed 3× with HEPES-buffered saline containing 0.1% bovine serum albumin (w/v) prior to challenge. A, cells were challenged with 0 μM (■) or 100 μM (▲) lyso-PC in HEPES-buffered saline containing 0.1% bovine serum albumin for the time period as indicated. B, cells were challenged for 15 min with lyso-PC in HEPES-buffered saline containing 0.025% (▲), 0.05% (■), or 0.10% (●) bovine serum albumin. Arachidonate release into the medium was determined. Values represent means ± standard error of the mean from 4 separate experiments.

described above.

4.2.2 Binding study of lyso-PC to H9c2 cells

Lyso-PC is an amphiphilic molecule which can be incorporated into lipid membranes. Thus, we performed binding studies to determine the nature of the association of lyso-PC with the H9c2 cells. These cells were labeled with 1-[1-^{14}C] palmitoyl-lyso-PC (100 nM, 55 nCi/nmol) for 15 min, and a considerable amount (45 ± 7%) of radioactivity was found to be associated with cells after the incubation. When lipids were extracted from these cells and analyzed by thin-layer chromatography, the majority of the radioactivity (>90%) in the lipid extract was found in the lyso-PC fraction. Subsequently, these cells were incubated for another 15 min in the control medium (without lyso-PC) or medium containing 10 μM non-radioactive lyso-PC. The majority of the radioactivity (70-76%) associated with the cells was not removed by either treatment. However, more than two-thirds (67-74%) of the radioactivity was removed from these cells by incubation with medium containing 0.1% albumin. Taken together, our data indicate that the binding of lyso-PC to cells was non-specific and was not significantly metabolized within 15 min of incubation.

4.2.3 Acyl specificity of lyso-PC

Since egg lysolecithin contains mainly saturated species of lyso-PC, the ability of myristoyl- ($C_{14:0}$), palmitoyl ($C_{16:0}$)-, stearoyl ($C_{18:0}$)- lyso-PC as well as several unsaturated lyso-PC species (i.e. $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$) to stimulate arachidonate release were investigated. As depicted in Fig 20, lyso-PC containing palmitoyl and stearoyl chains caused

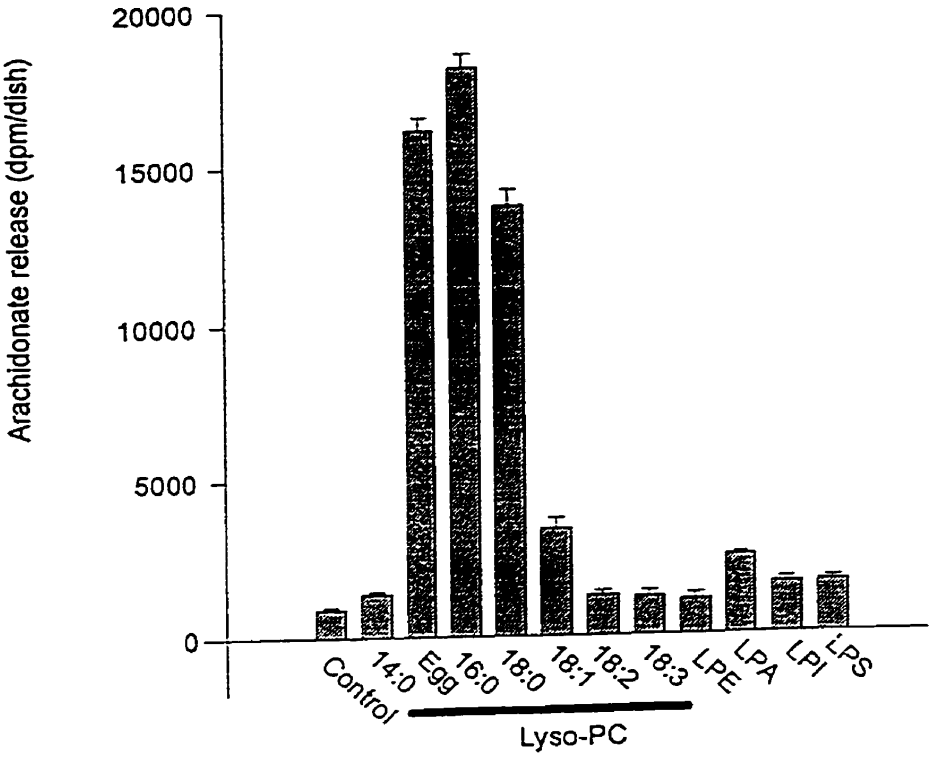


Figure 20. Effect of various lysolipids on arachidonate release in H9c2 cells. Cells were prelabelled with [^{3}H]arachidonate and challenged for 15 min with 0 μM (Control) or 100 μM of the indicated lysolipid. Myristoyl-lyso-PC (14:0); lysolecithin from egg (egg); palmitoyl-lyso-PC (16:0); stearoyl-lyso-PC (18:0); oleoyl-lyso-PC (18:1); linoleoyl-lyso-PC (18:2); linolenoyl-lyso-PC (18:3); lysophosphatidylethanolamine (LPE); lysophosphatidic acid (LPA); lysophosphatidyl-inositol (LPI); lysophosphatidylserine (LPS). Arachidonate release into the medium was determined. Values represent means ± standard error of the mean from 3 experiments.

the highest amount of arachidonate release by these cells. The specificity of other lysophospholipid for the stimulation of arachidonate release in H9c2 cells was also examined. Cells were incubated with 100 μM of lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol, or lysophosphatidate under identical conditions and the arachidonic acid released was determined. As indicated in Fig 20, lysophospholipids with head groups other than choline were minimally effective in the stimulation of arachidonate release. Based on these results, lyso-PC containing palmitoyl ($C_{16:0}$) chain was used in subsequent experiments.

4.2.4 Activation of $cPLA_2$ by lyso-PC

In order to determine whether the enhanced release of arachidonate is mediated via phospholipase A_2, the effect of lyso-PC on arachidonate release in H9c2 cell was studied in the presence of arachidonoyl trifluoromethyl ketone ($AACOCF_3$), a specific inhibitor of $cPLA_2$ (Clark *et al.*, 1995; Dennis, 1997). As shown in Table 8, cells pre-incubated with $AACOCF_3$ at concentrations of 0.5, 5.0 and 25 uM significantly reduced arachidonate release when compared to control cells. Our results indicate that $cPLA_2$ is a major phospholipase involved in lyso-PC-induced arachidonate release in the H9c2 cells.

To determine the mechanism of enzyme activation by lyso-PC, the enzyme activities in the cystosolic and membrane fractions were assayed. Direct addition of lyso-PC to the *in vitro* assay of phospholipase A_2 activity did not cause any significant changes in enzyme activity (data not shown). When enzyme activity was assayed in subcellular fractions of H9c2 cells incubated with lyso-PC, enzyme activity was decreased by 40% in cytosolic

Table 8. The effect of arachidonoyl trifluoromethyl ketone ($AACOCF_3$) on arachidonate release in H9c2 cells

Cells were prelabelled with [^{3}H] arachidonate and incubated with the indicated concentrations of $AACOCF_3$ for 2 minutes prior to challenge with 120 μM lyso-PC for 15 min. The release of arachidonate into the medium was determined. Results are expressed as mean ± standard error of the mean from 5 separate experiments. *P <0.05.

Treatment	Arachidonate release dpm/dish ($\times 10^{-2}$)	Inhibition %
Control	10 ± 1.1	
Lyso-PC	132 ± 2.3	
$AACOCF_3$ + Lyso-PC		
0.5 μM	75 ± 2.2*	43
5 μM	69 ± 1.9*	48
25 μM	54 ± 2.4*	59

fractions and increased by 47 % in membrane fractions (Table 9). These results are consistent with lyso-PC induced translocation of the enzyme from the cytosol to the membrane where it is in its most active form (Clark *et al.*, 1995).

4.2.5 Involvement of PKC and MAPK in arachidonate release induced by lyso-PC

Enzyme phosphorylation has been shown to play a major role in the regulation of phospholipase A_2 activity in a number of cell types (Clark *et al.*, 1995; Qui and Leslie, 1994). We therefore examined the involvement of protein kinase A and/or C as potential modulators of phospholipase A_2 activity. Cells were pre-treated with the protein kinase inhibitors staurosporine (Tamaoki *et al.*, 1986), Ro31-8220 (Nixon *et al.*, 1992), bisindolylmaleimide-I (BIS-I) (Gekeler *et al.*, 1996) and H89 (Chijiwa *et al.*, 1990) prior to the lyso-PC challenge. Ro31-8220, BIS-I and staurosporine inhibited significantly lyso-PC induced arachidonate release from 47 to 86% (Table 10). In contrast, the arachidonate release was not significantly attenuated by H89 even at concentrations 20-times its Ki value. Thus, on the basis of pharmacological inhibition, it appeared that protein kinase C was involved in the activation of phospholipase A_2 activity that resulted in the enhancement of arachidonate release. Furthermore, cells incubated with lyso-PC for 5 min resulted in a 84% increase in protein kinase C activity in the membrane fraction (Table 11). It is clear, however, that lyso-PC was a less potent modulator of protein kinase C activity than was phorbol 12-myristate 13-acetate.

It has been shown that the phosphorylation of $cPLA_2$ via the activation of protein kinase C is mediated downstream by the mitogen-activated protein kinase (MAPK) (Qui and

Table 9. The effect of lyso-PC on $cPLA_2$ activity in H9c2 cells.

Cells were treated with or without 100 μM lyso-PC in HEPES-buffered saline containing 0.1% bovine serum albumin. Cells were lysed and phospholipase A_2 activity was assayed in the cytosolic and membrane fractions. Results are expressed as mean ± standard error of the mean from four separate experiments. *P < 0.05.

Treatment	$cPLA_2$ activity (pmol/min/mg)	
	Cytosol	Membrane
Control	8.2 ± 0.1	1.2 ± 0.1
Lyso-PC (100 μM)	4.9 ± 0.2*	1.8 ± 0.1*

Table 10. The effect of protein kinase C and protein kinase A inhibitors on lyso-PC-induced arachidonate release in H9c2 cells.

Cells were prelabelled with [^{3}H] arachidonate and then treated with indicated concentrations of staurosporine, Ro31-8220, or H-89 for 15 min or BIS (bisindolylmaleimide I) for 30 minutes prior to challenge with 100 μM lyso-PC for an additional 15 min. Arachidonate release into the medium was determined. Results are expressed as mean ± standard error of the mean from 5 separate experiments. * $P < 0.05$.

Treatment	Arachidonate release dpm/dish ($x10^{-2}$)	Inhibition %
Control	7.3 ± 0.6	
Lyso-PC	130 ± 2.2	
Staurosporine + lyso-PC		
0.1μM	49 ± 1.9*	62
1.0 μM	36 ± 2.0*	72
Ro31-8220 + lyso-PC		
5 μM	51 ± 2.0*	61
10 μM	18 ± 2.0*	86
BIS + lyso-PC		
1.0 μM	69 ± 2.5*	47
10.0 μM	30 ± 2.2*	77
H-89 + lyso-PC		
0.5 μM	112 ± 3.0	14
1.0 μM	109 ± 2.0	17

Table 11. Effects of lyso-PC and phorbol 12-myristate 13-acetate (PMA) on protein kinase C activity in H9c2 cells.

Cells were challenged with 100 μM lyso-PC or 200 nM phorbol 12-myristate 13-acetate for 5 min. Protein kinase C activity was determined in the cell lysate. Results are expressed as mean ● standard error of the mean from 4 separate experiments. * $P < 0.05$.

Treatment	Protein kinase C activity (pmol/min/mg)	% increase
Control	130 ± 10	
Lyso-PC	239 ± 8*	84
PMA	412 ± 17*	214

Leslie, 1994; Lin *et al.*, 1993). Hence, the involvement of the MAPK pathway in lyso-PC induced arachidonate release was tested using PD098059, an inhibitor of the MAPK/extracellular regulated kinase kinase (Alessi *et al.*, 1995). Cells were preincubated with 10, 30 and 50 uM concentrations of PD098059 for 30 min prior to challenge with lyso-PC (Table 12). PD098059 significantly inhibited lyso-PC induced increases in the arachidonate release by 51% at 10 uM, 55% at 30 uM, and 69% at 50 uM (Table 12).

4.2.6 Involvement of Ca^{2+} in arachidonic acid release induced by lyso-PC

Another mode of PLA_2 activation occurs by increased levels of intracellular calcium $[Ca^{2+}]_i$. An elevation of $[Ca^{2+}]_i$ induces enzyme translocation and increases enzyme activation (Nalefski *et al.*, 1994). Hence, cells were challenged with varying levels of lyso-PC (50 to 150 μM) in the presence of 0-1.5 mM Ca^{2+}. As shown in Fig 21, arachidonate release induced by each level of lyso-PC was progressively suppressed at the lower Ca^{2+} concentrations. When extracellular Ca^{2+} was removed from the challenge buffer (calcium-free-buffer also contained 1.0 mM EDTA and 1.0 mM EGTA), arachidonate release was completely abolished (data not shown). Thus, the lyso-PC-induced arachidonate release was dependent on Ca^{2+} from the extracellular media. In determining the mechanism(s) by which lyso-PC increased $[Ca^{2+}]_i$ cells were treated with varying concentrations of lyso-PC (0-150 μM) in the presence of 1.2 mM Ca^{2+} in HEPES buffer. As shown in Fig 22, the absence of lyso-PC did not cause any change in $[Ca^{2+}]_i$. A dose dependent increase in $[Ca^{2+}]_i$ was observed at 25, 50 and 100 μM lyso-PC. A progressive decrease in the time to achieve maximum $[Ca^{2+}]_i$ was also dose related. At 150 μM lyso-PC, a dramatic increase in $[Ca^{2+}]_i$

Table 12. The effect of PD098059 on lyso-PC-induced arachidonate release in H9c2 cells.

Cells were prelabelled with [^{3}H]arachidonate and then treated with the indicated concentrations of PD098059 for 30 min prior to challenge with 100 μM lyso-PC for 15 min. Arachidonate release into the medium was then determined. Results are expressed as mean ± standard error of the mean from 5 experiments. * $P < 0.05$.

Treatment	Arachidonate release dpm/dish ($\times 10^{-2}$)	% inhibition
Control	7.4 ± 0.8	
Lyso-PC	131 ± 1.9	
PD98059		
10 μM PD98059 + lyso-PC	64 ± 3.2*	51
30 μM PD98059 + lyso-PC	59 ● 2.8*	55
50 μM PD98059 + lyso-PC	41 ± 2.3*	69

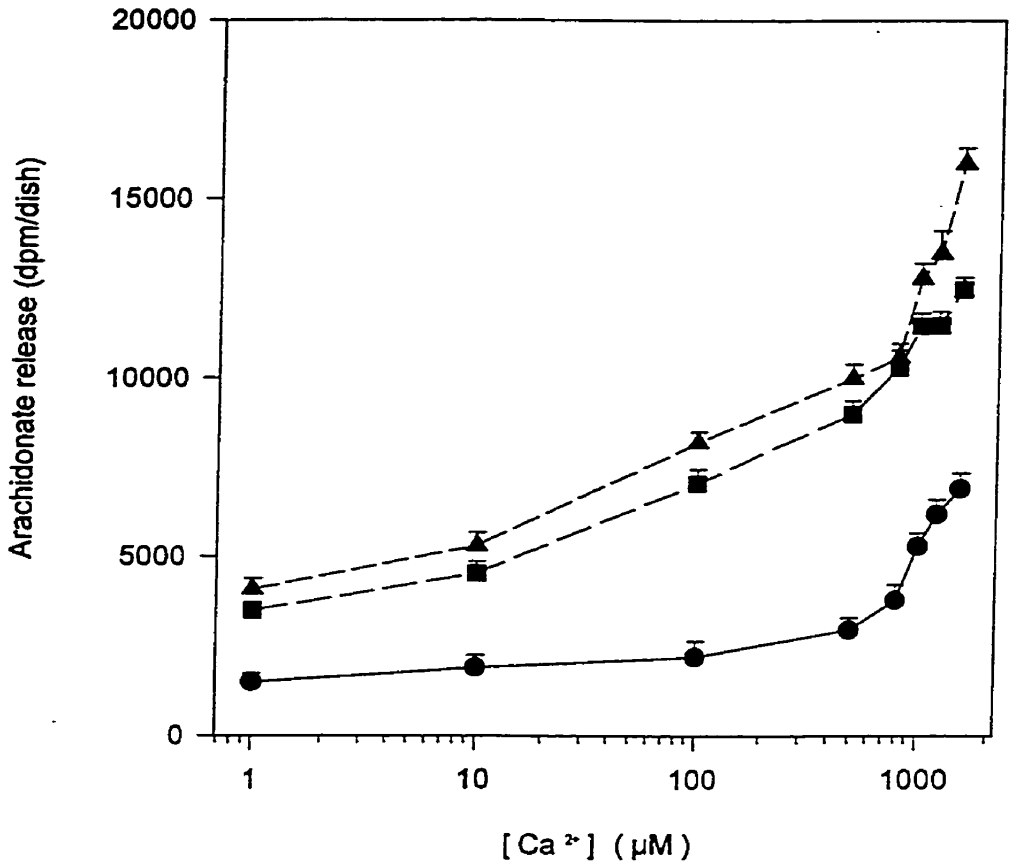


Fig 21. The effect of extracellular Ca^{2+} on lyso-PC-induced arachidonate release in H9c2 cells. Cells were prelabelled with [^{3}H]arachidonate and challenged for 15 min with 50 μM (●), 100 (■) and 150 μM (▲) LPC at various Ca^{2+} concentrations. Arachidonate release into the medium was determined. Values represent means ± standard error of the mean from 5 separate experiments.

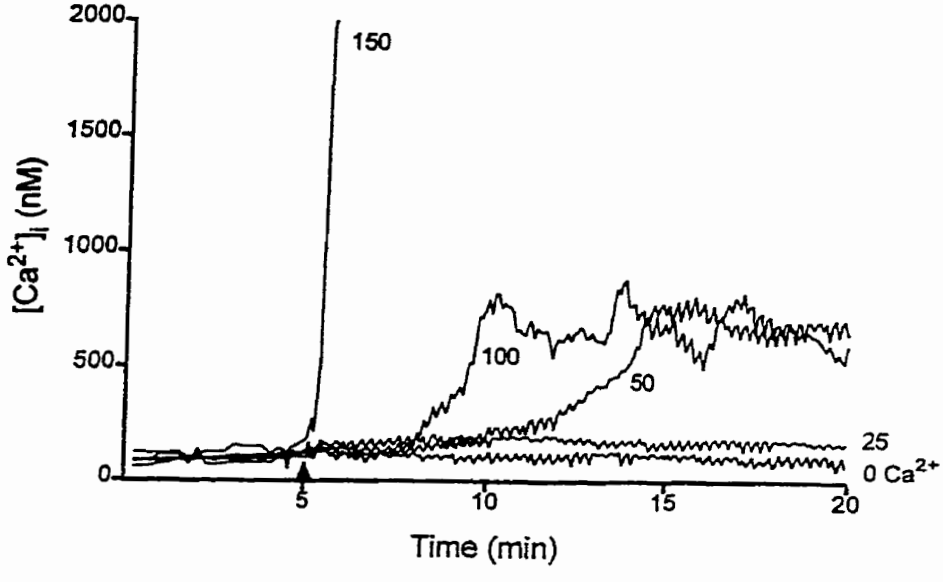


Fig 22. The effect of lyso-PC on intracellular calcium concentration $[Ca^{2+}]_i$ in H9c2 cells. Numbers next to traces indicate micromolar (μM) concentrations of lyso-PC in HEPES-buffered saline containing 0.1% bovine serum albumin. "0 Ca^{2+}" represents the absence of extracellular calcium. Arrow indicates the time of lyso-PC application. $[Ca^{2+}]_i$ was monitored using Fura-2 as described under "MATERIALS AND METHODS".

was followed by quenching of Fura 2-fluorescene, and a significant number of these cells stained trypan blue positive, suggesting that some of the cells became leaky. The increased levels of $[Ca^{2+}]_i$ achieved at various doses of lyso-PC and at different time points subsequent to lyso-PC challenge are depicted in Table 13.

The involvement of L-type Ca^{2+} channel in the lyso-PC induced elevation of $[Ca^{2+}]_i$ was investigated. Cells were preincubated with nimodipine for 5 min prior to incubation with lyso-PC. As shown in Fig 23, nimodipine in the presence of lyso-PC did not significantly influence the time required to achieve peak $[Ca^{2+}]_i$, however, the amplitude of peak $[Ca^{2+}]_i$ was significantly decreased. In order to investigate the potential effect of nimodipine on attenuation of lyso-PC-induced $[Ca^{2+}]_i$ levels and lyso-PC induced arachidonate release, cells were stimulated with 50, 100 and 150 μM lyso-PC in the presence and absence of nimodipine (10 μM), and arachidonic acid release was determined. Table 14 indicates that at all three lyso-PC concentrations, nimodipine caused significant declines in arachidonate release 2.5 and 10 min subsequent to lyso-PC challenge.

Table 13. The effect of lyso-PC concentration on intracellular calcium concentration $[Ca^{2+}]_i$ in H9c2 cells.

Cells were incubated with different concentrations of lyso-PC for the time indicated, and $[Ca^{2+}]_i$ was determined. Numbers in parentheses indicate cells that maintained flourescent signal during the time periods indicated. L.O.F. indicates loss of fluorescence.

Lyso-PC incubation (min)	Increases of $[Ca^{2+}]_i$ above baseline (nM) Lyso-PC concentration			
	25 μM	50 μM	100 μM	150 μM
5	100 ± 5 (46)	216 ± 20 (28)	990 ± 7 (26)	1781 ± 93 (34)
10	109 ± 5 (46)	496 ± 47 (27)	1174 ± 111(18)	L.O.F.
15	64 ± 5 (46)	575 ± 41 (27)	877 ± 216 (10)	L.O.F.

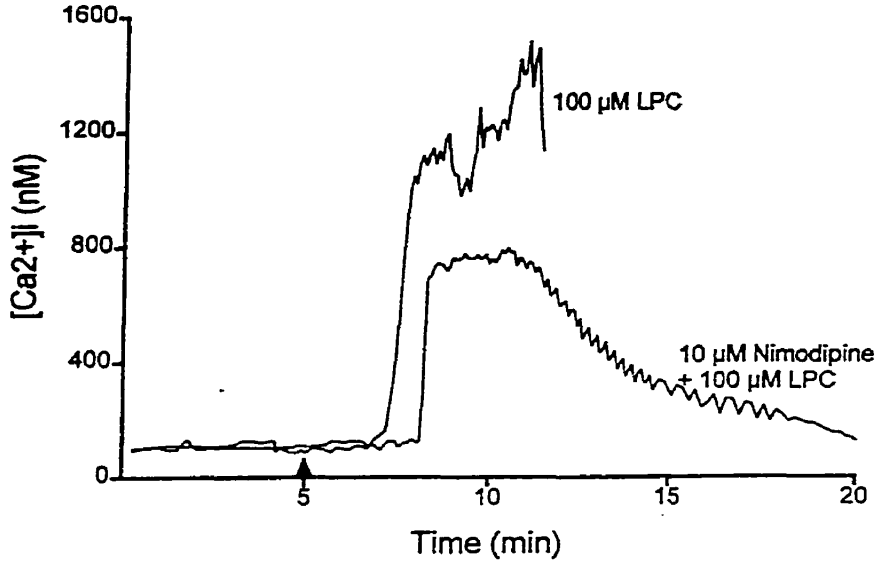


Fig 23. The effect of nimodipine (NIM) on the lyso-PC induced elevation of $[Ca^{2+}]_i$ in H9c2 cells. Cells were incubated with lyso-PC (100 µM) in the presence or absence of (10 µM) NIM. The presence of NIM attenuated $[Ca^{2+}]_i$ elevation induced by lyso-PC. NIM caused a delay in obtaining maximum $[Ca^{2+}]_i$ (from 3.3 ± 0.3 min to 4.09 ± 0.5 min; P>0.05), and the maximum value of $[Ca^{2+}]_i$ was also reduced. Arrow indicates the point of lyso-PC application.

Table 14. Influence of nimodipine (NIM) on lyso-PC-induced arachidonate release in H9c2 cells.

Cells were prelabelled with [^{3}H]arachidonate and then treated with lyso-PC in the presence (+) or absence (-) of nimodipine (NIM) for the indicated period of time. Arachidonate release into the medium was determined. Results are expressed as mean ± standard error of the mean from 6 separate experiments. *P<0.05, +NIM vs. -NIM under the same incubation conditions.

	Time (min)	
	2.5	10
Lyso-PC (μM)	Arachidonate release (dpm/dish $\times 10^{-2}$)	
50 (-NIM)	11 ± 1.0	91 ± 2.5
50 (+NIM)	6.9 ± 0.7*	67 ± 3.1*
100 (-NIM)	41 ± 1.9	143 ± 1.3
100 (+NIM)	22 ± 3.5*	121 ± 6.9*
150 (-NIM)	85 ± 4.2	168 ± 5.0
150 (+NIM)	51 ± 3.7*	132 ± 5.1*

V DISCUSSION

5.1 The metabolism of arachidonate

Arachidonic acid (AA) is an important precursor for ecoisanoid synthesis. Eicosanoids, which include prostaglandins, leukotrines and thromboxanes, have been implicated in biological processes as diverse as signal transduction (Habenicht *et al.*, 1986; Kiesel *et al.*, 1991), the regulation of cell growth, and the maintenance of vascular integrity (Janssen-Timmen *et al.*, 1994, and references therein). Under normal physiological conditions, the vast majority of the arachidonic acid is stored in the esterified form in phospholipids. The arachidonic acid is released from the phospholipid by the action of PLA_2. The other product of the PLA_2 reaction, lysophospholipid, can be converted into platelet-activating factor, which is a potent mediator of inflammation (Hanahan, 1986). Since the release of free arachidonic acid is the key step in eicosanoid biosynthesis, the enzyme for its hydrolysis from the phospholipid is under rigid control. Among the different types of PLA_2 found in the mammalian cell, $cPLA_2$, is the intracellular enzyme which directs the release of arachidonic acid under stimulation (Kramer, 1994). The activation of $cPLA_2$ can be regulated by many mechanisms at both the transcriptional and post-translational levels. It has been shown that the enzyme is regulated at the transcriptional level by cytokines and growth factors (Nakamura *et al.*, 1992; Maxwell *et al.*, 1993). The transcriptional regulation of this enzyme is touted as a prolonged process for the activation of the enzyme during tissue development and differentiation (Hoeck *et al.*, 1993; Wu *et al.*, 1994). Alternatively, the post-translational regulation would provide the cell with a rapid process for the activation

of $cPLA_2$. Several mechanisms for the post-translational regulation of the enzyme have been identified, including Ca^{2+}-dependent translocation to membrane and phosphorylation-dependent activation mediated by MAPK and PKC (Lin *et al.*, 1993). An additional mechanism involved in $cPLA_2$ activation is mediated through G-protein (s), which regulates the MAP kinase cascade (Lin *et al.*, 1992; Winitz *et al.*, 1994). Recently, we have demonstrated that lyso-PC is a potent activator of the enzyme activity in human endothelial cells (Wong *et al.*, 1998). In this study, lyso-PC caused the elevation of intracellular calcium ion concentration and the activation of PKC, which stimulated $cPLA_2$ via the activation of the MAPK and resulted in an enhanced release of arachidonate.

5.2 The modulation of $cPLA_2$ at the transcription level

Clofibrate is a classical peroxisome proliferator and PPARα activator (Schoojans *et al.*, 1996a). It has been shown to activate the peroxisome proliferator response element in the cultured murine 3T3 preadipocytes (Fajas *et al.*, 1999). In the present study, the release of arachidonic acid was increased in SW cells incubated in the presence of clofibrate in a dose dependent manner (50-200 μM). Accordingly, the clofibrate induced increase in $cPLA_2$ activity was also found to be dose-dependent, with the maximum effect at 200 μM. The protein level of this enzyme was also increased under the same conditions. The regulation of $cPLA_2$ by clofibrate was identified at the transcriptional level, where the mRNA for $cPLA_2$ was markedly increased by clofibrate. This increase was caused by the enhanced gene transcription of $cPLA_2$, since the $cPLA_2$ mRNA stability was not affected by clofibrate treatment.

The nuclear hormone receptor PPARα is a member of the thyroid/vitamin D/retinoid superfamily of transcription factors that require heterodimerization with the RXR for optimal DNA binding (Manglsdorf and Evans, 1995). Whereas effects by PPARα activators on activation of $cPLA_2$ have not been previously reported, the ligand/activator of other hormone receptors in this superfamily, such as the analog or precursor of vitamin D, have been shown to activate $cPLA_2$ or COX-2 (Pirianov *et al.*, 1999; Kanekura *et al.*, 1998). In this study, we postulated that the PPAR pathway is involved in the activation of $cPLA_2$. In order to establish the involvement of PPAR in this process, our initial approach was to demonstrate the presence of PPARα in SW cells. However, at the time the study was embarked, an antibody to PPARα was not commercially available. In lieu of a direct determination of this receptor in the SW cells, the ability of the cell to express the mRNA encoding PPARα was demonstrated. It is clear from our study that both PPARα and γ were expressed in SW cells. This is not surprising since this cell line was derived from the human liposarcoma and contained specific gene sets of lipogenesis and lipolysis upon stimulation. Using the RT-PCR approach, we were able to detect not only the wild-type 433-bp fragment, but also a naturally occurring variant of PPARα mRNA consisting of a 330-bp fragment. These two fragments correspond to the full length PPARα receptor and truncated PPARα receptor, respectively (Gerviois *et al.*, 1999). The function of this PPARα variant which presented in preadipocytes remains unclear.

PPARα is abundant in liver and kidney where it plays a role in the regulation of fatty acid metabolism (reviewed in Schoojans *et al.*, 1996a). It is activated by molecules such as long-chain free fatty acids and hypolipidemic drugs (Keller *et al.*, 1993; Yu *et al.*, 1995).

Fibrates, including clofibrate, are ligands of PPARα (Forman *et al.*, 1997). Moreover, the lipid lowering effect of clofibrate has been shown to be mediated via the activation of PPARα. In this study, the up-regulation of $cPLA_2$ mRNA by clofibrate was suggested at the transcription level. However, the precise mechanism of the induction of gene transcription of $cPLA_2$ by clofibrate remains unclear. We postulate that the transcriptional regulation of clofibrate on $cPLA_2$ in the SW cells is mediated via the activation PPARα. The activated PPARα may interact with a positive PPAR-response element (PPRE) in the promoter region of the target gene. The sequence of PPRE for PPARs is highly conserved, and attempts were made to match the sequence of the PPRE with the sequence of the $cPLA_2$ at the promoter region. Although we failed to obtain a sequence alignment with a typical conserved PPRE at the promoter region of $cPLA_2$ gene, which would be a direct repeat of AGGTCA (or TGACCT), a half consensus sequence was found at the other region of DNA sequence encoding the $cPLA_2$ (the consensus sequence TGACCT was at 3032'-3037' of human $cPLA_2$ gene. GenBank accession number: U11239). Whether this sequence plays a role in $cPLA_2$ gene expression by clofibrate is unknown. Further investigation is needed to confirm the role of this sequence.

Phospholipase A_2 (PLA_2) and cyclooxygenase (COX) are two key enzymes in the prostaglandin synthesis pathway. Whereas clofibrate had no effect on gene expression of COX-1, it induced 3.5-fold increase in the mRNA level of COX-2. A functional coupling and differential regulation of cytosolic phospholipase A_2 and COX-2 in inflammation has been shown (Scott *et al.*, 1999). For example, both $cPLA_2$ and COX-2 mRNAs were induced by pre-inflammatory cytokines but were suppressed by glucocorticoids in human epithelial

cells (Newton *et al.*, 1997). We have shown in this study that both $cPLA_2$ and COX-2 mRNA were induced by clofibrate, suggesting that these two enzymes are regulated in a coordinated fashion during eicosanioid biosynthesis.

SW cells exposed for 4 days to clofibrate were found to be converted to fat-loaded adipocytes. This differentiation was not triggered by cell confluence in the presence of fetal bovine serum, since confluent cells in the control group did not show the accumulation of lipid droplet in the cytoplasm. Similar studies in 3T3-L1 preadipocytes also showed that fibrates induced adipose conversion through activation of PPARα (Brandes *et al.*, 1995). It should be noted that cells exposed to clofibrate for 2 days should no gross signs of transformation into adipocytes.

The pivotal role of PPARγ in adipogenesis has been well documented (Kliewer *et al.*, 1995; Wu *et al.*, 1998). Activated PPARγ induces cells to exit from the cell cycle and triggers the expression of adipocyte-specific genes, resulting in an increased delivery of energy to the cells (Tontonoz *et al.*, 1994a; 1994b; Schoonjans *et al.*, 1996a). Furthermore, a gene mutation of PPARγ has been discovered which leads to a receptor that cannot be inactivated. This mutation, while probably rare, is associated with extreme obesity (Freake, 1999). Adipocyte differentiation is also coordinately regulated by several other transcription factors, such as C/EBP beta, C/EBP delta and adipocyte differentiation and determination factor 1/ sterol regulatory element binding protein 1 (ADD-1/SREBP-1) (Fajas *et al.*, 1998).

In light of the above, it is somehow surprising that the PPARγ ligand (PGJ_2) had no effect on the SW cell conversion (data not shown). This lack of effects does not exclude the role of PPARγ in preadipocyte differentiation. On the contrary, the PPARα activator

(clofibrate) caused the induction of preadipocyte differentiation. In SW cells, it is possible that the activation of PPARα dramatically changed the concentrations of free fatty acid inside the cell. This change may trigger the activation of PPARγ and C/EBPα, whose expression coincide with the later stages of differentiation. In turn, activated C/EBPα cooperates with PPARγ in inducing additional target genes and sustaining a high level of PPARγ in the mature adipocyte as part of a feed-forward loop (Schoonjans *et al.*, 1996a). Furthermore, clofibrate at 300 μM has been found to exhibit weak activity on PPARγ (Forman *et al.*, 1997). On the other hand, the observation that both PPARα and PPARγ were co-expressed in the same cell also support the idea for the existence of cross-talk between these two signaling pathways which may contribute to cell differentiation.

5.3 The modulation of $cPLA_2$ at the post-translational level

For the investigation on the modulation of $cPLA_2$ at the post-translational level, H9c2 cells were used in this study. This cell line is derived from quiescent cardiac myoblastic cells, and it retains many morphological and structural properties similar to the cardiac muscle myoblast (Kimes and Brandt, 1976). Alternatively, the biochemical and electrophysiological properties of these cells resemble those found in skeletal muscle cells. Using the H9c2 cells as a model, we observed that lyso-PC induced both a time- and dose-dependent increase in the release of arachidonate. The concentration of lyso-PC used in this study was similar to its plasma concentrations (0.13-0.15 mM) (Phillips, 1957; Okita *et al.*, 1997), but the presence of serum proteins may attenuate the ability of lyso-PC to enhance arachidonate release. The effect of lyso-PC was most pronounced in lyso-PC species

containing long and saturated acyl chains. Although the rationale for the effectiveness of lyso-PC with acyl chains containing $C_{16:0}$ or $C_{18:0}$ remains unclear, the ability of these lyso-PC species containing aliphatic chains of 16-18 carbon atoms to produce optimal lytic activity has been documented (Weltzien, 1979). Lyso-PC appears to induce arachidonate release by the elevation of $[Ca^{2+}]_i$ and activation of protein kinase C . We postulate that both regulatory factors acted in concert to fully activate the cytosolic phosholipase A_2, leading to the enhanced release of arachidonate in H9c2 cells.

In cardiac and other mammalian cells, the content of unesterified arachidonate is very low, and the majority of the acyl group is incorporated into the membrane phospholipid pool (Dennis, 1987). The esterified and free arachidonate levels are controlled by a fine-tuned deacylation-reacylation cycle of phopholipids in which phospholipase A_2 plays a dominant role (Dennis, 1987). Although there are several types of phospholipase A_2, the cytosolic form is the predominant form of the enzyme for the intracellular release of arachidonate. It has a molecular mass of 85-110 kDa, requires Ca^{2+} in the micromolar range for translocation of the enzyme to its target (phospholipid-containing membrane) and in contrast to the secretary form of the enzyme, possesses a high specificity for the arachidonoyl residues at the *sn*-2 position of the phospholipid molecule (Clark *et al.*, 1995; Sharp *et al.*, 1991; Dennis, 1997). This form is activated by phosphorylation followed by the translocation of the phosphorylated enzyme to the membrane in a calcium-dependent manner (Clark *et al.*, 1995; Lin *et al.*, 1993). Using $AACOCF_3$, a specific inhibitor of the c-form of phospholipase A_2, we have shown that the increase in arachidonate release was mediated by the enhancement of $cPLA_2$. In agreement with our earlier study (Wong *et al.*, 1998), we were unable to

demonstrate that the loss of enzyme activity in the soluble fraction was quantitatively recovered in the membrane fraction. Based on previous findings from our group and by others, the failure to recover all the enzyme activity after translocation was probably caused by interference of the enzyme assay with the membrane fraction (Wong *et al.*, 1998; Channon and Leslie, 1990). This phenomenon also occurs when other enzymes are translocated to the membrane fraction (Channon and Leslie, 1990). Factors attributing to this loss of activity include the reduced accessibility of exogenous radioactive substrate to the enzyme and the dilution effect on the exogenous radioactive substrate.

The resting $[Ca^{2+}]_i$ in quiescent cardiomyocytes is about 100 nM (Xu *et al.*, 1997) and values obtained for resting H9c2 cells (85-110 nM) are in agreement with this value. Lyso-PC caused a 2- to 17-fold rise in $[Ca^{2+}]_i$ levels (~200 nM to >1.7μM), consistent with the elevation level of $[Ca^{2-}]_i$ shown to cause the association of $cPLA_2$ with membranes and also correlates with the level of calcium required to stimulate the catalytic activity of the enzyme *in vitro* using phospholipid vesicles as substrates (Clark *et al.*, 1995; Nalefski *et al.*, 1994; Channon and Leslie, 1990). Inhibition of lyso-PC-induced increases of $[Ca^{2+}]_i$ by nimodipine suggests the involvement of L-type Ca^{2+} channels.

Although $cPLA_2$ is a substrate for protein kinase C *in vitro*, the direct phosphorylation of $cPLA_2$ by protein kinase C does not result in enhanced phospholipase activity, nor is there any evidence that the enzyme is phosphorylated *in vivo* by protein kinase C (Leslie, 1997; Lin *et al.*, 1993). However, protein kinase C has been shown to phosphorylate and activate Raf-1 (Seger and Krebs, 1995), which in turn activates MAP-kinase through a protein kinase cascade. Indeed, the direct phosphorylation and activation of $cPLA_2$ by MAP-kinase has been

demonstrated (Lin *et al.*, 1993; Nemenoff *et al.*, 1993). Our study indicates that the MAP kinase kinase is involved in the lyso-PC induced activation of $cPLA_2$ for the enhancement of arachidonate release, and suggests the involvement of MAP-kinase in this process.

5.4 Conclusion

Arachidonate is an important precursor for eicosanoid biosynthesis. The release of arachidonate from cellular phospholipids is facilitated by the hydrolytic action of $cPLA_2$. In this study, we have shown that the action of $cPLA_2$ is regulated, both at the transcriptional and post-translational levels. Using the SW 872 cell as a model, the long term regulation of $cPLA_2$ at the transcriptional level by clofibrate was demonstrated. The precise mechanism for the regulation is unclear. Alternatively, lyso-PC activated PLA_2 via the post-translational modification of the enzyme. Lyso-PC caused an increase in the level of intracellular calcium, and stimulated protein kinase C, which activated the $cPLA_2$ via the stimulation of the MAPK and resulted in an enhanced release of arachidonate.

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