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XIX CICLO DI DOTTORATO

TESI DI DOTTORATO IN IMMUNOLOGIA

"ACTIVATORY PROPERTIES OF LYSOPHOSPHATIDIC ACID (LPA) ON HUMAN THP-1 MONOCYTES"

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1. INTRODUCTION

1.1. Lysophosphatidic acid (LPA) structure, synthesis, transport and degradation

Besides lysophospholipids (LPLs) are minor structural components among the lipids which make up the cell membrane, they constitute, however, a group of important mediators and signalling molecules (Moolenaar, 1999). Among these lipids, the smallest and structurally simplest is the lysophosphatidic acid (LPA). Giving its unique biochemical and functional properties what makes LPA different from other lysophospholipids is that it does not have a head group attached to its phosphate moiety, such as choline or ethanolamine. It consists, in fact, of a glycerol backbone with a hydroxyl group at the sn-2 (or sn-1) position, a free phosphate group at the sn-3 position, and a long saturated or unsaturated fatty acid chain at the sn-1 (or sn-2) position (Fig. 1).

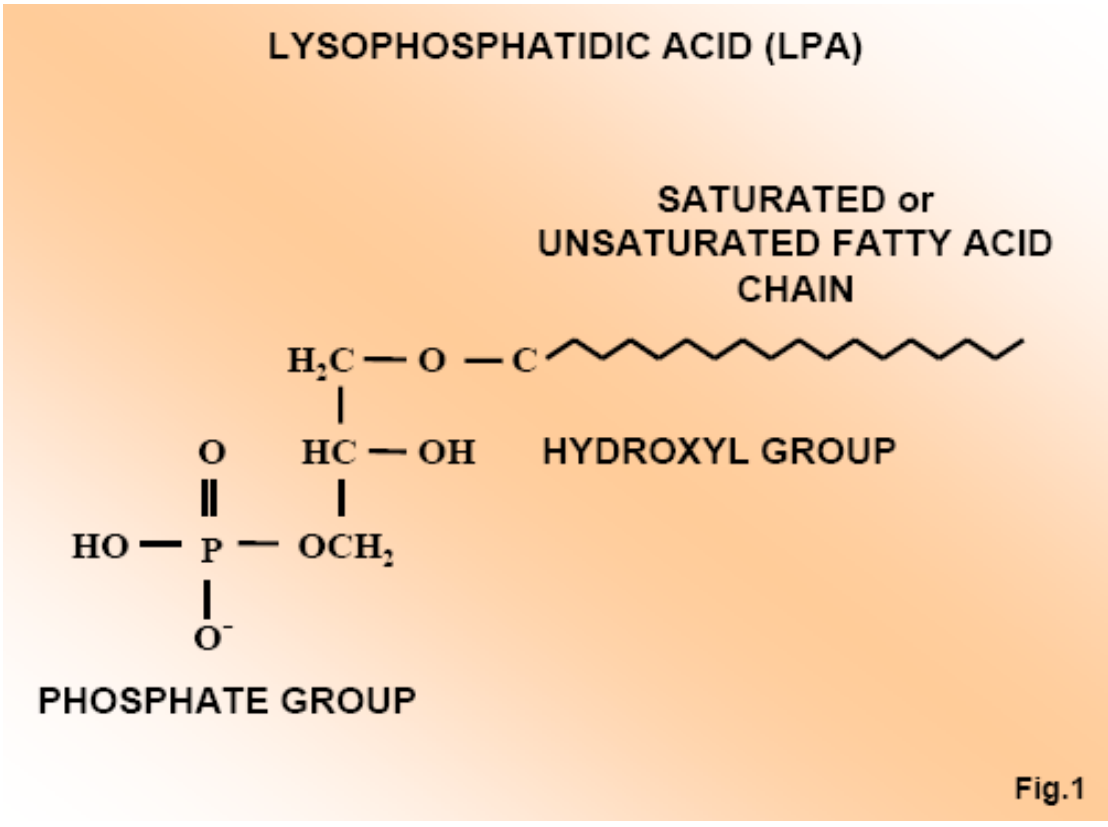


Fig.1

Moreover, unlike other phospholipids, it is also water soluble (Anliker and Chun, 2004a). In spite of such structural simplicity, LPA, identified for the first time in 1957 as the Darmstoff's active ingredient (smooth muscle-stimulating substance) (Vogt, 1957) and already known to be an important intermediate product in intracellular lipid metabolism, is nowadays considered one of the most attractive phospholipidic mediator, which is able to modulate a broad variety of biological processes depending on target cell type (Moolenaar et al., 1997).

The serum is the best characterized source of LPA, which is present at low concentrations (1-5 μM) depending on the equilibrium between its production, degradation, and clearance (Baker et al., 2000). Moreover, it circulates bound to albumin due to its high-affinity sites for long-chain fatty acids (Thumser et al., 1994). This lipid, however, is an ubiquitous molecule and its physiological production and degradation are tightly regulated, with protein-bound LPA levels ranging from 100 to 200 nM or lower concentrations (Aoki et al., 2002). Moreover, LPA has been found in plasma in physiopathological situations and significant amounts (~ μM) have been also detected in other biological fluids such as saliva (Sugiura et al., 2002), aqueous humor (Liliom et al., 1998), seminal (Hama et al., 2002) and follicular fluids (Tokumura et al., 1999), and malignant effusions from several cancers, including ovarian cancer ascites fluid (Xu et al., 1995).

LPA in serum, plasma and other biological fluids is a mixture of various fatty acids. Most of them are, in fact, long chain saturated (18:0, 16:0) or unsaturated (16:1, 18:1, 18:2, 20:4) fatty acids linked to the glycerol backbone by acyl- or alkyl-group (Xiao et al., 2000; Baker et al., 2001). Interestingly,

these LPA species exhibit differential biological activities; for example, it has been demonstrated that LPA carrying an unsaturated fatty acid is more biologically active than LPA carrying a saturated fatty acid (Yoshida et al., 2003). Moreover, the diversity in LPA composition may reflect the existence of multiple synthetic pathways for this lipid.

LPA, in fact, can be produced both inside and outside the cell by means of different enzymes. Whereas some of these enzymes, which appear to be critical for the extracellular LPA production and metabolism, act on the extracellular side of the cell leading to a direct release of the phospholipid, other enzymes act intracellularly but their effective contribution to the extracellular production of LPA remains unclear (Pagès et al., 2001). Indeed, it is not clear if intracellular LPA is exported from the cell to participate in intercellular signalling. To this regard, emerging evidences indicate that bioactive LPA is generated extracellularly rather than inside the cell with subsequent secretion or release (Sano et al., 2002).

As mentioned above, LPA has been recognized for decades as a key intermediate in the early steps of the synthetic pathway for neutral lipids, phospholipids (PLs) and triacylglycerol (TG) in almost every cell type (Bishop and Bell, 1988). In this pathway, the glycerol 3-phosphate acyltransferase (G3PAT), located in both the endoplasmic reticulum and the mitochondria, catalyzes the formation of LPA by acylation of glycerol 3-phosphate (G3P). LPA is then rapidly acylated in mithocondria by the monoacylglycerolphosphate acyltransferase (MGAT or LPA acyltransferase) to form phosphatidic acid (PA), the precursor of all glycerolipids (Haldar and Vancura, 1992). An alternative pathway for LPA production is the reduction of

dihydroxy acetone phosphate (acyl DHAP) occurring in the peroxysomes; this pathway contributes to LPA formation in pancreatic islets exposed to high glucose concentrations (Dunlop and Larkins, 1985). Finally, LPA may also be synthesized within mithocondria and microsomes by the monoacylglycerol kinase (MAGK)-directed phosphorylation of monoacylglycerol (MAG), an important precursor of phosphatidylinositol synthesis (Simpson et al., 1991).

Intracellular LPA synthesis can also occur from PA and it is depending on the sequential actions of phospholipase D (PLD) and phospholipase A (PLA). In mammalian cells, two PLD isozymes, called PLD_1 and PLD_2, have been identified and characterized, even though their specific roles in LPA production remain obscure (Aoki, 2004).

PLD catalyzes the hydrolysis of diacyl phospholipids (mainly phosphatidylcoline, PC) resulting in PA generation on the inner leaflet of the plasma membrane (Exton, 2002; Cummings et al., 2002). Once produced, PA is subsequently converted to LPA by PLA-catalyzed deacylation: in this context, both PLA_1 and PLA_2 isozymes could be involved in LPA production. However, since most of LPA species comprise saturated fatty acids, they are likely to derive from PLA_2.

Recently, the possible involvement of PLA_1 in the production of some LPA species has been proposed as well. This hypothesis is supported by the identification of two membrane-associated PA-selective PLA_1s, called mPA-$PLA_1\alpha$ (Sonoda et al., 2002) and mPA-$PLA_1\beta$ (Hiramatsu et al., 2003). Particularly, mPA-$PLA_1\alpha$, which is highly expressed in human platelets, has been recently proposed to be involved in the production of LPA with unsaturated fatty acid (Sonoda et al., 2002).

In an alternative LPA-producing pathway, the diacylglycerol kinase (DAGK) generates PA through diacylglycerol (DAG) phosphorylation, which, in turn, can be converted to LPA by PLA_2 (Mauco et al., 1978). Although many DAGKs isozymes have been identified (Topham and Prescott, 2003), it remains to be determined which isozymes are effectively involved in LPA production under physiological conditions. Moreover, since no evidences of the release of the so-formed LPA into the extracellular fluid can be found, LPA generated through these pathways is not considered to be involved in extracellular LPA signalling.

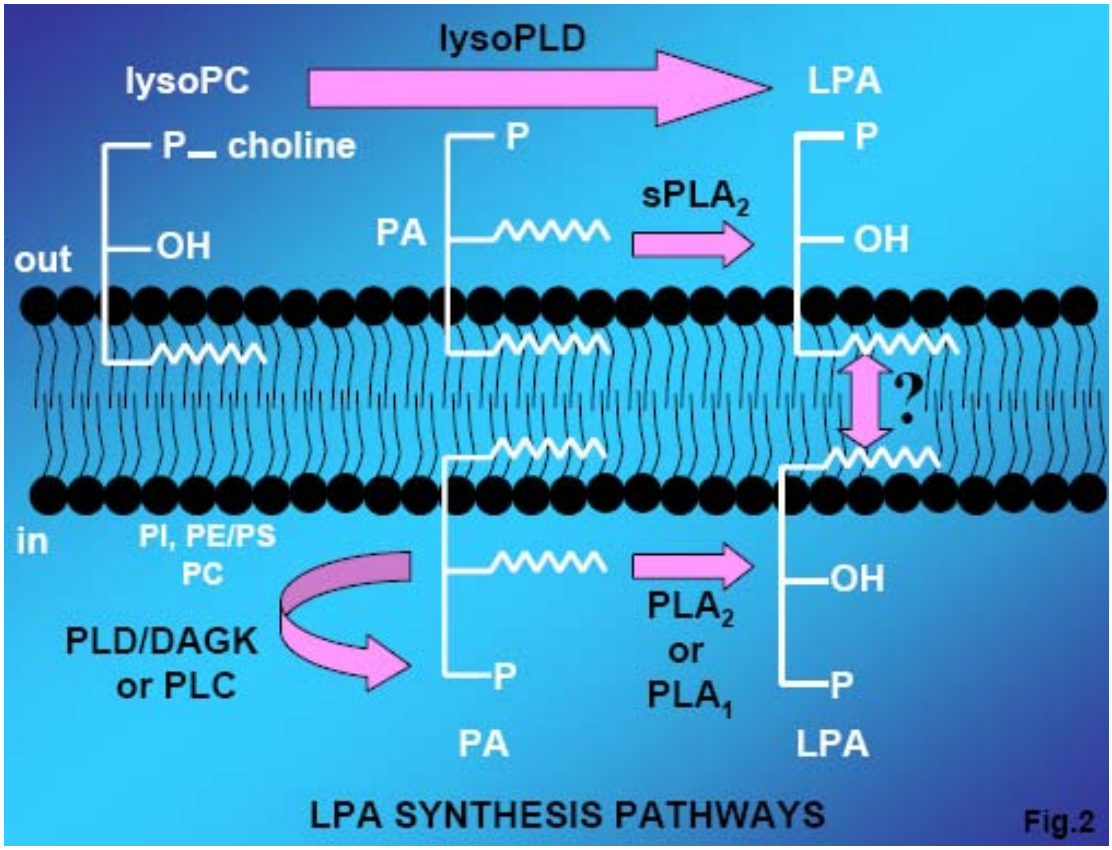
Extracellularly, LPA is produced as a result of cell activation which is triggered by several stimuli and agonists (Shen et al., 1998; Xie et al., 2002). Cellular activation occurring during blood clotting, wound healing, or inflammation increases local LPA production and activity. Thus, whereas cellular LPA production is tightly regulated, lipid production in biological fluids may also be unregulated (Aoki, 2004).

In biological fluids such as the serum, LPA is mainly generated during clotting and, although activated platelets appear to be the major source of the lipid, their full contribution to serum LPA production remains to be determined. Approximately, it can be extimated that half of serum LPA is synthesized through a platelet-dependent pathway, while the other half is produced by a platelet-independent pathway (Aoki et al., 2002). Particularly, serum LPA can be produced through at least three different pathways. In the first pathway, activated platelets produce and release a large amount of LPLs. LPLs, which are generated from membrane phospholipids (Yokoyama et al., 1995) and mainly by phosphatidylserine-specific PLA_1 (PS-PLA_1) (Sato et al., 1997) and

type IIA secretory PLA_2 ($sPLA_2$-IIA) enzymes, are subsequently converted to LPA by a constitutively present plasma enzyme, called lysophospholipase D (lysoPLD) (Sano et al., 2002). PS-PLA_1 specifically hydrolyzes PS and produces lysophosphatidylserine (LPS) with unsaturated fatty acids, which may explain the accumulation of unsaturated LPA in serum (Sato et al., 1997), while $sPLA_2$-IIA prefers amino phospholipids such as phosphatidylethanolamine (PE) and PS (Murakami and Kudo, 2002). Since these phospholipids are predominantly located in the inner leaflet of the cell membrane, they may not be accessible to PS-PLA_1 and $sPLA_2$-IIA, which are extracellular enzymes, unless the phospholipid asymmetry is disrupted. Such a phenomenon is known to occur in activated platelets. This synthetic route is, in fact, generally observed at the site of injury where blood coagulation occurs; it explains the fact that the remarkable increase in serum LPA appears to depend on platelets, although activated platelets alone were found to produce only a small part (~ 10%) of the serum LPA (Aoki, 2004). In the second pathway, LPA can be produced through the hydrolysis of PA by $sPLA_2$-IIA or mPA-$PLA_1\alpha$, which may explain LPA production in isolated platelets upon their activation. The amount of LPA generated in this route makes minor contributions to serum LPA formation; however, it rapidly forms LPA and may have a role in pathophysiological states such as wound healing and inflammation (Tigyi and Parrill, 2003).

In the third pathway, a part of extracellular LPA is produced through a cell-free system; particularly, LPA was shown to be converted from lysophosphatidylcholine (LPC), which is present at high concentrations in the plasma (Tokumura et al., 1986), by the sequential actions of lecithin-

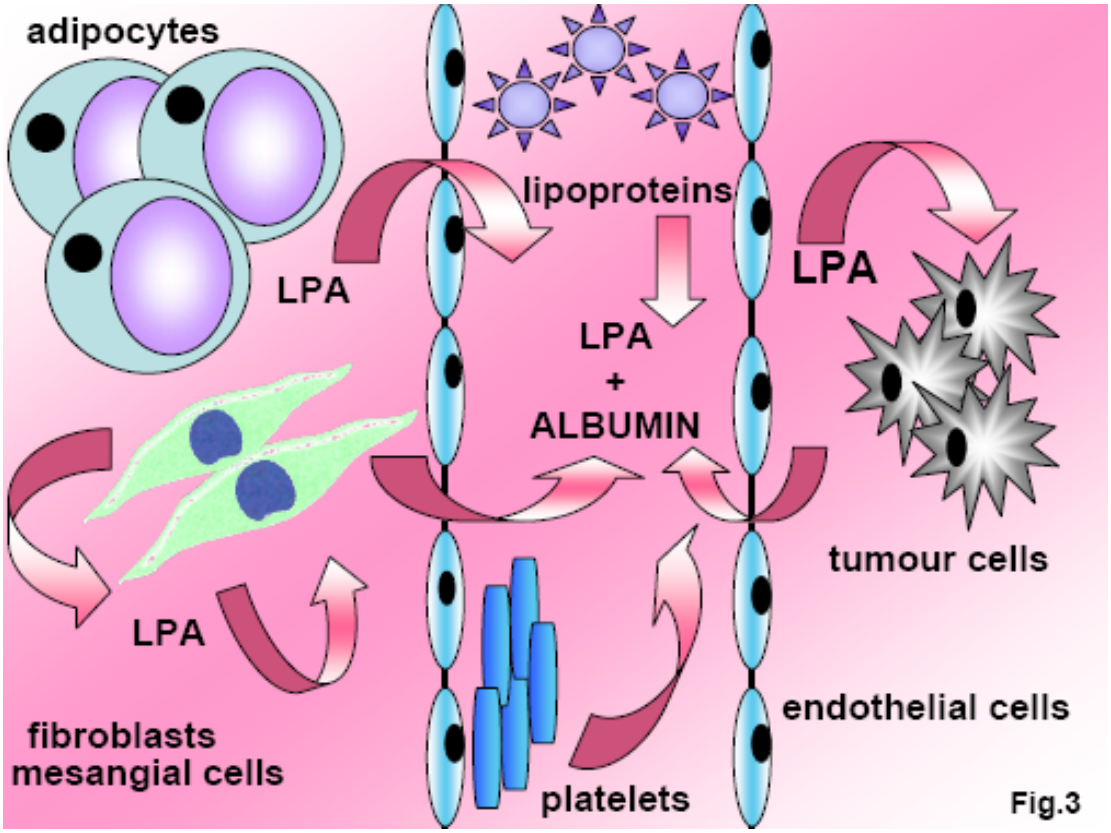
cholesterol acyltransferase (LCAT) and a circulating lysoPLD also known as ***autotaxin***, an esterase cleaving LPC to LPA and choline (Umezu-Goto et al., 2002; Tokumura et al., 2002). LCAT appears to have a critical role in serum LPA production supplying LPC to lysoPLD (Aoki et al., 2002). This pathway can explain part of the platelet-independent serum LPA (Fig. 2).



In follicular fluid, LPA may be produced from LPC by the action of lysoPLD (Tokumura et al., 1999), but the mechanisms of lipid production in other biological fluids such as saliva, seminal fluid and ascites are poorly known.

Since LPA is also found in plasma in several physiopathological conditions as well as in non-plasmatic extracellular fluids, the involvement of cell types other than platelets in its production is possible. Several studies, in fact, have revealed the existence of other cellular and non-cellular origin of LPA

(Fourcade et al., 1995). Particularly, mesangial cells, fibroblasts and adipocytes as well as many cancer cell types can play an important role in LPA generation and release (Pagès et al., 2001). In the blood, activated platelets and, to a smaller degree, red blood cells have been identified as sources of LPA (Eichholtz et al., 1993; Aoki et al., 2002), while in the immune system, activated inflammatory cells such as dendritic cells, mast cells and macrophages are the principal phospholipid sources (Goetzl et al., 2004). Finally, lipoproteins can also be a source of LPA since the lipid has been reported to be produced during mild non-enzymatic oxidation of low density lipoproteins (LDL) (Siess et al., 1999) (Fig. 3).



Due to its hydrophobic character, LPA associates with other lipids and proteins both in biological fluids and within cells (Tigyi and Parrill, 2003). Like

other lipids, intra- and extracellular lipid binding proteins are necessary to avoid LPA toxicity as well as to allow its transport in plasma and from one cellular organelle to another. ***Albumin*** has been identified as a LPA carrier in blood plasma (Dyer et al., 1992; Tigyi and Miledi, 1992): it constitutes the main extracellular LPA binding protein and can bind up to 3 mol LPA/mol of protein with a nanomolar range affinity for the phospholipid (Kinkaid and Wilton, 1993). Albumin serves three functions for LPA. First, it acts as a carrier to solubilize LPA, without affecting its biologic potency (Thumser et al., 1994); second, it is necessary for the biological LPA activity (Tigyi and Miledi, 1992) and, third, it increases LPA physiologic half-life by protecting it from degradation by phospholipases (Jalink et al., 1994).

Intracellularly, LPA binds to ***fatty acid binding proteins*** (***FABPs***). The liver-type FABP (LFABP) has been recognized as an intracellular carrier able to transport mitochondrial LPA to microsomes in order to be acylated to PA (Vancura and Haldar, 1992). LFABP has two LPA binding sites per molecule and it is also known to bind other LPLs with micromolar affinities (Thumser et al., 1994). Interestingly, besides the liver and the intestine, LFABP is expressed in the proximal tubules of the kidney where it could play a role in LPLs reabsorption (Maatman et al., 1991). Moreover, LFABP is hypothesized to be involved in extracting LPA from the membrane as well as in buffering high LPLs concentrations produced by phospholipases in the hepatocytes (Tigyi and Parrill, 2003).

A third protein which could play an important role in LPA binding and transport is ***gelsolin***. It has been discovered in 1979 as an intracellular actin-binding protein involved in the remodelling of cellular actin filaments associated with

cell shape changes and movement (Yin and Stossel, 1979). Intracellularly, this protein to binds and caps actin filaments as wells as LPA with nanomolar affinity (Meerschaert et al., 1998). Gelsolin has been also found in a circulating form. The secretory gelsolin isoform, called plasma gelsolin, circulates in the blood and constitutes a potential circulating carrier for LPA (Kwiatkowski, 1999). A novel hypothesis for the role of plasma gelsolin in inflammatory homeostasis has been proposed. In local injury, activated platelets and leukocytes produce LPA and, at the same time, cell lysis releases actin, which binds in turn to plasma gelsolin. This local gelsolin depletion permits LPA to exert its effects of defence and repair (Lind et al., 1988).

It is obvious that LPA accumulation must be counterbalanced by its inactivation and/or clearance mechanisms. LPA removal from the bloodstream is extremely rapid, thus suggesting that degradation and clearance are very efficient.

LPA degradation includes three major pathways. In the first, exogenous LPA may rapidly be dephosphorylated to yield biologically inactive MAG by the action of membrane-spanning phosphatase enzymes belonging to the phosphatidate phosphatase type 2 (PAP-2 or lipid phosphate phosphohydrolase, LPP) family (Brindley and Waggoner, 1998) as well as by a yet unknown nuclear LPA phosphohydrolase (Baker and Chang, 2000). Although several PAP subtypes have been cloned and characterized in mammals which are all able to catalyze LPA dephosphorylation, the existence of an ecto-LPA phosphatase, the lipid phosphate phosphohydrolase-1 (LPP1), able to efficiently hydrolyse extracellular LPA and to attenuate its biological

activity by reducing receptor activation, must be noticed (Jasinska et al., 1999). The second LPA degradation pathway involves the action of 1-acylglycerol-3-phosphate acyltransferase (AGPAT) enzymes, also called LPA-acyltransferases (LPAATs) (Leung, 2001). In this pathway, LPA is rapidly esterified to PA. This enzyme, found in microsomes and plasma membranes, is crucial for both de novo glycerolipids synthesis and for membrane phospholipids remodelling (Bishop and Bell, 1988). Since LPAAT activity is under the control of cyclic adenosine monophosphate (cAMP)-dependent protein kinase (Schmidt et al., 1999), the existence of a hormonal control of LPA metabolization is possible.

Finally, in the third pathway, LPA may be hydrolysed and converted into G3P by the action of lysophospholipase enzymes. Although several lysophospholipase exist (Wang and Dennis, 1999), an 80 kDa protein with LPA-specific lysophospholipase activity has been purified and characterized from the rat brain (Thompson and Clark, 1994) and could also play an important function controlling LPA availability.

1.2. LPA biological and pathophysiological activities

LPA exhibits a broad spectrum of biological activities in many different cellular lineages and contributes to multiple physiological and pathophysiological processes (Umezu-Goto et al., 2004) (Fig. 4). LPA effects, then, are both pleiotropic and specific, and can be controlled at multiple levels.

The first control level is exerted by several enzymes, whose regulation is complex and tissue/cell type-specific, which regulate LPA metabolism and subsequently its serum levels. Secondly, at the receptor level, LPA functions can be regulated both by temporal and spatial distributions of LPA receptors,

as well as by selective expression of their receptor subtypes (Anliker and Chun, 2004b). Moreover, LPA receptors expression levels are likely to be tightly controlled under developmental and physiological conditions, and an imbalance of these factors may lead to pathological states. Finally, the third level of regulation is at the G proteins level. Different LPA receptors subtypes, in fact, preferentially couple to different types of G proteins, which are further regulated by the cellular context (Contos et al., 2000; Fukushima et al., 2001). LPA behaves both as a blood-born circulating mediator and a locally produced autocrine and/or paracrine factor (Takuwa et al., 2002). Since its initial characterization as a growth factor, the list of cellular responses to LPA has considerably expanded and this lipid has emerged as a critical regulator of several aspects of cell biology.

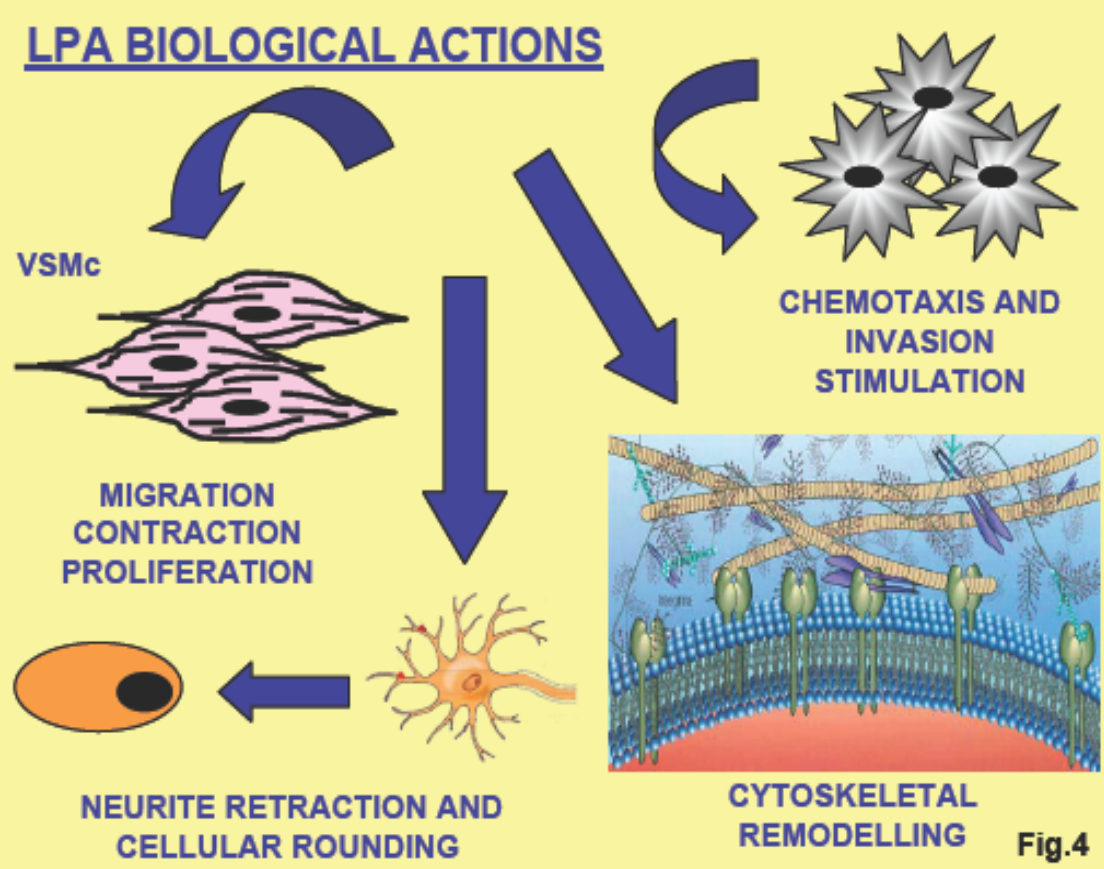


Fig.4

The biological responses to LPA can be roughly subdivided into two major categories: 1) growth-related responses such as cell proliferation stimulation or inhibition, protection from apoptosis, differentiation, and regulation of the growth factors expression and 2) growth-unrelated responses such as aggregation, contraction, cell to cell interaction, rounding, adhesion, chemotaxis, membrane depolarization, and secretion (Luquain et al., 2003). In addition, some LPA actions, such as morphological changes induction, Ca^{2+} mobilization from intracellular stores, cellular motility, chemotaxis and invasion stimulation, gap-junctions closure and tight-junctions opening, are rapid and occurring independently of new protein synthesis (van Corven et al., 1989; Balazs et al., 2001). Other effects, such as the stimulation of cell-cycle progression, incresead cell viability and the production of cytokines including those affecting new vessels formation and maintenance, endothelin and pro-angiogenic factors release and the production or activation of proteases such as urokinase plasminogen activator (uPA) and some metalloproteinases, are instead long-term and secondary to gene transcription (Mills and Moolenaar, 2003; Tigyi and Parrill, 2003). Furthermore, multiple evidences on LPA involvement in complex physiological responses including brain development, immunological competence, wound healing, vascular remodelling, coagulation and blood pressure regulation, have been supplied. On the other hand, LPA appears to contribute to the development and progression of several pathophysiological states such as autoimmunity, immunodeficiency, atherosclerosis and cancer (Sengupta et al., 2004). In this regard, by influencing cellular responses such as proliferation, migration, survival, and cytoskeletal fibers reorganization, LPA actions are concordant with many

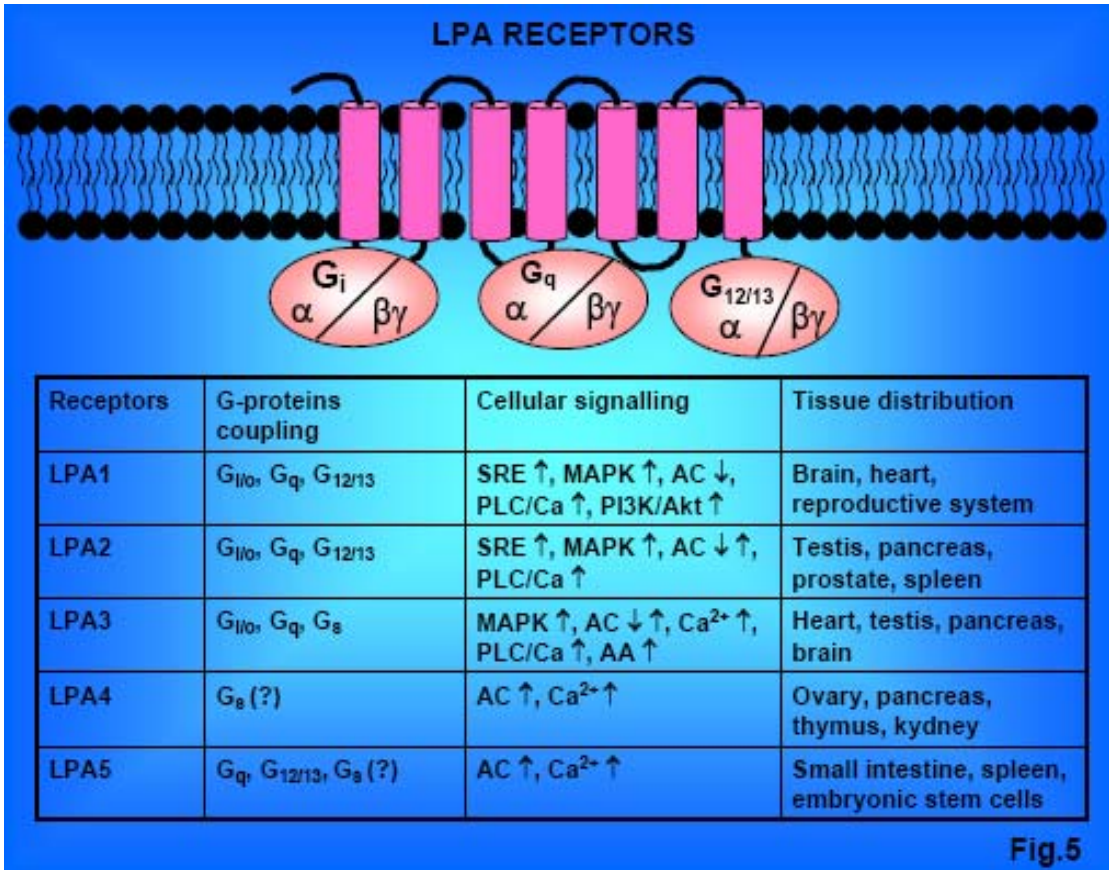
cancer hallmarks (Hanahan and Weinberg, 2000), suggesting a critical role for LPA both as an inducer of tumour initiation and progression and as a potential biomarker of cell aggression. Indeed, LPA levels are significantly increased in malignant effusions and its receptor expression and signalling are aberrant in several human tumours (Xu et al., 1995; Schulte et al., 2001). For this reason, LPA receptors and/or signalling have emerged as promising drug targets for therapeutic and pharmacological intervention, and might result of a fundamental diagnostic value. LPA, then, functions as a double-edge sword and it provides an excellent example of a molecule with dual and opposite effects.

1.3. LPA receptors and signal transduction pathways

The great variety of LPA cellular and biological actions is attributable to the binding to and the activation of specific seven-transmembrane domains G-protein coupled receptors (GPCRs) (Fig. 5). LPA-induced cell responses critically depend on the profile and expression levels of these receptors and the signal transduction pathways being used (Fukushima and Chun, 2001; Takuwa et al., 2002).

To date, five functional cell-surface LPA receptors have been identified and characterized in humans (Ishii et al., 2004). A distinct gene encodes each receptor, and the best known are now called LPA_1, LPA_2 and LPA_3; they were originally termed ***endothelial cell differentiation genes*** (***EDGs***) because the prototypic member of the class was identified as a GPCR that was up-regulated during endothelial cell differentiation. The three LPA receptors belonging to the EDG family are highly homologous in their structure, showing about 50% sequence similarity to one another, with the C-terminal tails being

the most diverging and differing in ligand specificity and signalling pathways (van Leeuwen et al., 2003). The recent identification of a fourth LPA-specific receptor (LPA_4/P2Y9), which is closely related to purinergic P2Y receptors but far distant from the EDG receptors (Noguchi et al., 2003), suggested that LPA receptors have evolved from at least two distinct ancestor genes.



Receptors	G-proteins coupling	Cellular signalling	Tissue distribution
LPA1	$G_{i/o}$, G_q, $G_{12/13}$	SRE ↑, MAPK ↑, AC ↓, PLC/Ca ↑, PI3K/Akt ↑	Brain, heart, reproductive system
LPA2	$G_{i/o}$, G_q, $G_{12/13}$	SRE ↑, MAPK ↑, AC ↓↑, PLC/Ca ↑	Testis, pancreas, prostate, spleen
LPA3	$G_{i/o}$, G_q, G_s	MAPK ↑, AC ↓↑, Ca^{2+} ↑, PLC/Ca ↑, AA ↑	Heart, testis, pancreas, brain
LPA4	G_s (?)	AC ↑, Ca^{2+} ↑	Ovary, pancreas, thymus, kydney
LPA5	G_q, $G_{12/13}$, G_s (?)	AC ↑, Ca^{2+} ↑	Small intestine, spleen, embryonic stem cells

Fig.5

LPA receptors are widely distributed and many cell types express more than one of them. Each receptor isoform displays a unique tissue expression pattern and couples to at least three distinct sets of heterotrimeric G proteins, principally $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$, leading to the activation of a specific panel of multiple intracellular signal transduction pathways (Moolenaar, 1999). Particularly, LPA-induced $G_{i/o}$ stimulation can lead to at least three distinct signalling routes: (i) adenylate cyclase (AC) inhibition with the subsequent fall

in intracellular cAMP levels (van Corven et al., 1989); (ii) mitogenic Ras/mitogen-activated protein kinases (MAPKs) cascade activation leading to cell growth and proliferation stimulation (Fang et al., 2000; Kranenburg and Moolenaar, 2001); (iii) phosphoinositide 3-kinase (PI3K) activation leading to the stimulation of the guanosine diphosphate/guanosine triphosphate (GDP/GTP) exchange factor (GEF) TIAM 1 and of the downstream small G-protein Rac with subsequent cell spreading and movement (van Leeuween et al., 2003). PI3K would also lead to antiapoptotic pathway protein kinase B (PKB)/Akt activation, which promotes cell survival (Kumagai et al., 1993).

LPA can also elicit protein $G_{q/11}$ activation which, in turn, leads to phospholipase C (PLC) β stimulation, with subsequent phosphatidylinositol-bisphosphate (PIP_2) hydrolysis and two major classes of second messengers generation, DAG and inositol-triphosphate (IP_3) leading to protein kinase C (PKC) activation and cytosolic Ca^{2+} concentrations changes, respectively (Exton, 1997). Finally, through one or more specific Rho-GEFs, protein $G_{12/13}$ stimulation induces the small G-protein RhoA activation, which regulates actin stress fibers and focal adhesion formation, and drives cytoskeletal contraction and cell rounding. Thus, the Rho-family GTPases, RhoA and Rac, are activated through two separate G protein pathways, $G_{12/13}$-Rho-GEFs and $G_{i/o}$-PI3K-TIAM1, respectively (van Dijk et al., 1998; Kranenburg et al., 1999). Their multiple downstream effectors affect cell migration and invasion, but also impact on cell-cycle progression (Kumagai et al., 1993; Etienne-Manneville and Hall, 2002). Thus, LPA not only signals through “classic” second messengers such as Ca^{2+}, DAG and cAMP, but it also through Ras, Rac and

Rho-family GTPases, the master switches that, controlling proliferation, migration and morphogenesis, alter cell behaviour and shape.

LPA_1 is the first LPA receptor identified and the most widely expressed (Hecht et al., 1996). In human, LPA_1 mRNA is highly expressed in the brain, followed by the heart and, less abundantly, in the gastrointestinal tract tissues and the reproductive system; lower expression levels in the skeletal muscle and kidneys have been also observed (An et al., 1998).

Human *lpa1* gene, located on chromosome 9, encodes for a 41 kDa protein consisting of 364 amino acids (aa) (Contos and Chun, 1998). LPA_1 receptors couple to three G protein subtypes, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$ and LPA_1-mediated LPA signalling includes serum-response element (SRE) activation, MAPKs and PLC stimulation, AC inhibition, Ca^{2+} mobilization, Akt and Rho activation (Fukushima et al., 2001; Ishii et al., 2004). Through LPA_1 binding, LPA regulates a wide range of cellular responses such as proliferation, migration and cell cycle-dependent morphological changes as well as induces surprising effects on neurogenesis, Schwann cell survival and morphology, and myelination (Weiner and Chun, 1999; Weiner et al., 2001).

LPA_2 encoded by *lpa2* gene, located on chromosome 19, was the second LPA receptor to be identified (Contos and Chun, 2000). It contains, in humans, 351 aa with a predicted molecular mass of ~39 kDa and shows widespread adult tissue expression. LPA_2 transcript distribution is totally differing from LPA_1; indeed, it is readily detectable in testis, pancreas, prostate, spleen, thymus, and peripheral blood leukocytes, but is almost undetectable in brain, heart, and digestive tract tissues (An et al., 1998). As for LPA_1, LPA_2 receptor couples with three G protein types, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$, to mediate many

LPA-induced intracellular signalling pathways. The major LPA_2-mediated signalling routes include: SRE and MAPKs activation, intracellular Ca^{2+} levels increase, PLC stimulation, arachidonic acid (AA) release, and AC inhibition or stimulation that likely depend on cell type (An et al., 1998).

The third specific multifunctional LPA receptor is encoded by *lpa*$_3$ gene located, in humans, on chromosome 1 (Bandoh et al., 1999). **LPA_3** receptor human form consists of 353 aa and the estimated molecular weight is about 40 kDa. Interestingly, it appears that LPA_3 couples with $G_{i/o}$ and $G_{q/11}$ but not $G_{12/13}$ (Ishii et al., 2000) and is much less responsive to LPA species carrying saturated acyl chains (Bandoh et al., 1999). Nonetheless, LPA_3 can mediate several LPA-induced signalling such as PLC activation, cytosolic Ca^{2+} changes (Im et al., 2000), MAPKs activation, AC inhibition or stimulation with respect to analyzed cell lines, and AA release (Ishii et al., 2004). Although is still expressed in many adult tissues, it shows somewhat more restricted expression. Particularly, LPA_3 mRNA, in humans, it is mainly observed in the heart, pancreas, testis, prostate, and brain while moderate expression levels in lung and ovary, have been also found (Bandoh et al, 1999; Im et al., 2000).

LPA_4 was the first LPA receptor which has a divergent sequence compared to other three LPA receptors (Noguchi et al., 2003). It appears to be encoded by a single exon and contains 370 aa with a molecular mass of about 42 kDa. LPA_4 mediates LPA-induced Ca^{2+} mobilization and probably by functional coupling to protein G_s, promotes the AC activation with subsequent cAMP accumulation (Lee et al., 2006). LPA_4 transcripts are expressed at very high levels in the ovary and, to a much lesser extent, in several other tissues such

as pancreas, thymus, kidney and skeletal muscle (Noguchi et al., 2003). Its biological functions are currently unknown.

Very recently, it was demonstrated that the orphan GPR92 receptor is activated by LPA, and it was named **LPA_5** (Lee et al., 2006). These two receptors, LPA_4 and LPA_5, have about 35% amino acid identity with each other and are thus more related to each other than to the LPA-EDG receptors. The LPA_5 receptor mediates stress fibres formation in rat hepatoma cells, neurite retraction through $G_{\alpha 12}$, $G_{\alpha 13}$ and Rho kinase in neuroblastoma cells, and furthermore, via $G_{\alpha q}$, elevates intracellular Ca^{2+} concentration. Although LPA_5 also stimulates increases in cAMP levels, its coupling to G_s proteins remains to be proven. Low levels of LPA_5 transcripts are widely express while LPA_5 high levels are strongly expressed in small intestine, spleen, dorsal root ganglion cells and embryonic stem cells (Lee et al., 2006). It is tempting to speculate that LPA_4 and LPA_5 receptors allow LPA to couple to G_s pathways which are not among the preferred signalling pathways of EDG receptors (Meyer zu Heringdorf and Jakobs, 2006).

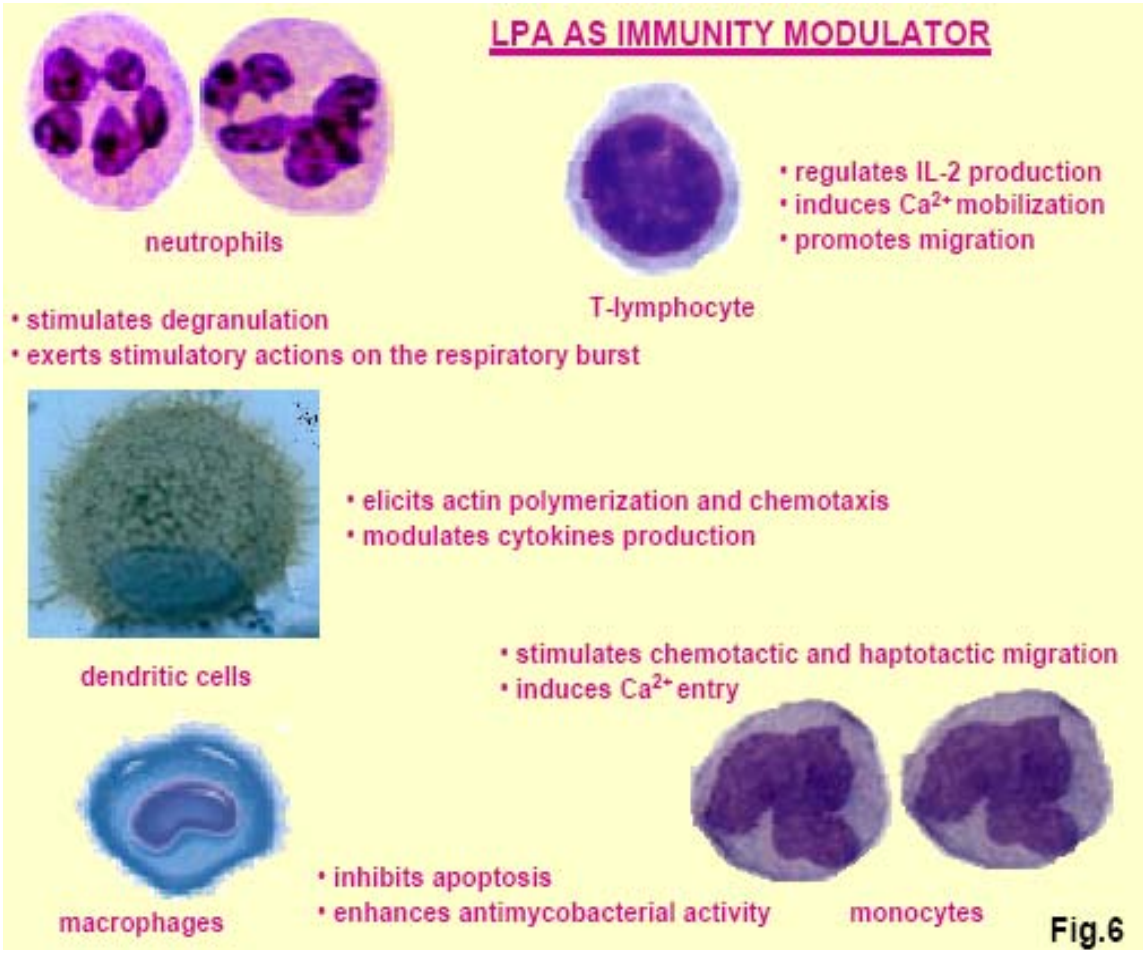
Finally, it has been also recognized LPA ability to serve as a transcellular agonist of the nuclear transcription factor ***peroxisome proliferator-activated receptor-γ*** (***PPAR-γ***), a member of the nuclear hormone receptor superfamily (McIntyre et al., 2003). PPAR-γ controls the transcription of those genes involved in glucose and fatty acid metabolism, adipocyte differentiation, and inflammatory processes in the vasculature (Yki-Jarvinen, 2004). In some cell systems, it has been reported that LPA effects are mediated through PPAR-γ receptors activation. However the question whether under physiological conditions, extracellular LPA migrates to the cytosol in sufficient

concentrations to activate these intracellular receptors or whether intracellularly produced LPA is the only relevant source remains to be determined.

Nearly all mammalian cells, tissues and organs (except the liver) co-express at least two distinct LPA-receptor subtypes of the EDG family, strongly suggesting that LPA receptors signal in a cooperative manner. However, which LPA receptor subtype couples to which G-protein effector system in a given cellular context is still not known. Obviously, receptor-based studies as well as those determining how mutations in *lpa* genes might contribute to human genetic disorders and to other pathological processes, will likely provide a better understanding of functions and roles of this important lipid.

1.4. LPA as immunity mediator

Recently, a new LPA research era began. This has led to the unravelling and biochemical characterization of LPA multiple signalling pathways as well as to the discovery of new biological actions. According with its ubiquitous nature and its role as lipid extracellular mediator able to exert broad and powerful actions, LPA has been recently shown to evoke multiple responses also in immune cells, conferring LPA unsuspected and complex functions in immune and inflammatory responses induction and amplification. Almost all immune system cells express LPA receptors (whose patterns of expression can be altered by cell activation) and their activities and functions can be differentially influenced by the expressed LPA receptor subtypes (Lin and Boyce, 2006) (Fig. 6).



1.4.1. T- and B-lymphocytes

LPA modulates multiple biological functions of both human T- and B-lymphocytes. Particularly, LPA promotes early T cell migration to tissue sites of immune and inflammatory responses and regulates both cell proliferation and several cytokines secretion (Huang et al., 2002). T lymphocytes play a central role in cell-mediate immunity; several different subsets of T cells have been described, each with a distinct function.

T helper (Th) cells are crucial in controlling infections and, once activated, they can be divided into terminally differentiated Th1 or Th2 cells depending on their ability to secrete cytokines (Abbas et al., 1996; Mosmann and Coffman, 1989).

LPA receptors expression in Th cells is highly flexible, in relation to their functional activation state. Unstimulated human Th cells predominantly express LPA_2 receptors and at much lower levels LPA_1, while LPA_3 receptors are not detectable (Goetzl et al., 2000). Prior to mitogenic stimulation, LPA induces Th cell migration by enhancing both chemotactic mobility and matrix metalloproteinases (MMPs) secretion. Moreover, LPA has an inhibitory effect on interleukin-2 (IL-2) generation and secretion. These responses were presumed to be transduced by LPA_2 receptors. Upon mitogens-induced activation, LPA receptors pattern expression rapidly and significantly change leading to LPA_2 down-regulation as well as LPA_1 up-regulation expression (Zheng et al., 2000). In this case, LPA inhibits the migration and enhances both IL-2 production and cell proliferation. Therefore, it is possible that the first LPA effects are to facilitate Th cell movement to the site of an immune response; naïve T cells, then, may initially respond to LPA as a chemoattractant and an inhibitor of cytokine secretion, a process which might be harmful for other cells in the path

of their migration. After their arrival to the antigen presentation site, the transition to effector T cell state may be accompained by a loss of LPA-induced chemotaxis and migrational arrest but, at this stage, LPA may amplify the T cell effector functions such as IL-2 production or cell proliferation probably through LPA_1 receptors (Graler and Goetzel, 2002). Thus, LPA_2 receptors, which are constitutively expressed on naïve Th cells, and LPA_1 receptors, which are only expressed by activated Th cells, transduce opposite LPA effects on some T cells responses.

In contrast, both Th1 and Th2 cells express all LPA receptors (Wang et al., 2004). These cells have also different functions: whereas Th1 cells help in the IgG induction and participate in autoimmune diseases and delayed type hypersensitivity reactions, Th2 cells activate eosinophils and mast cells, and are responsible for allergic diseases (Lukacs, 2000; Lloyd et al., 2000). LPA acts as a powerful chemoattractant but does not induce chemokinesis in both Th1 and Th2 cells (Wang et al., 2004). Interestingly, pertussis toxin (PTx) pre-treatment, which inhibits $G_{i/o}$ protein functions, potentiates the chemotactic response to LPA in Th1 cells while abolishes LPA-induced Th2 cell chemotaxis. These findings suggest that PTx-sensitive G proteins are impeding rather than mediating this response and may represent a promising basis for the therapy of allergy or hypersensitivity.

LPA also induces Ca^{2+} mobilization in T cells; however, while naïve T cells do not flux Ca^{2+} in response to LPA, the lipid induces intracellular Ca^{2+} mobilization in polarized Th cells. Moreover, Ca^{2+} flux response in Th1 cells is PTx-insensitive, whereas it is only slightly sensitive to this pre-treatment in Th2 cells. In both cell types, this response is related to the influx of extracellular Ca^{2+} and

it is opposite to LPA activity in natural killer (NK) cells, where this lipid induces Ca^{2+} release from intracellular sources (Jin et al., 2003).

Finally, LPA proliferative action has not only been demonstrated in T-lymphocytes but also in B-lymphocytes (Rosskopf et al., 1998). In addition, an immortalized human B lymphoblast cell line responded to LPA with transient intracellular Ca^{2+} release, MAPKs activation, enhanced inositol phosphate formation, DNA synthesis, and immunoglobulin formation, probably in a receptor-mediated fashion. However, which LPA receptors are involved in mediating the enhanced immunomodulatory B-lymphoblasts functions remains yet unknown even if it would be of particular interest to investigate whether B-cells are able to switch their responsiveness to LPA by altering their LPA receptors pattern expression during development or upon activation.

1.4.2. Natural Killer (NK) cells

NK cells constitute a special kind of lymphocytes that bridges the adaptive immune system with the innate immunity. They are anti-tumour/anti-microbial effector cells and serve to control viral infections while through the help of the adaptive immune response which generates antigen-specific cytotoxic T cells, can clear the infection (Moretta et al., 2002a; Moretta et al., 2002b).

Either resting or activated human NK cells express all three LPA receptors (Maghazachi, 2003). LPA physiological concentrations induce a strong chemotactic response and the mobilization of intracellular Ca^{2+} in activated NK cells (Jin et al., 2003). LPA-induced chemotaxis is completely inhibited by PTx, whereas Ca^{2+} flux is only partially inhibited, suggesting that PTx-sensitive G proteins-coupled events mediate the chemotactic response whereas PTx-dependent and -independent transduction steps mediate Ca^{2+} mobilization in

NK cells. Moreover, consistently with previous findings in other cell types (An et al., 1998; Bandoh et al., 1999), it seems that LPA_1 receptors are involved in both chemotaxis and Ca^{2+} mobilization, whereas LPA_2 or LPA_3 receptors are involved in Ca^{2+} flux only.

Considering the role of NK cells in controlling viral infections and that LPA is also secreted at infectious and inflamed sites, it can be hypothesized that this lipid may provide an innate stimulus in recruiting NK cells toward these sites. On the other hand, it has been also demonstrated that LPA can enhance interferon-γ (IFN-γ) secretion by activated NK cells (Jin et al., 2003). Since LPA is released in large quantities by several tumour cells (Fang et al., 2002; Xie et al., 2002), it is possible that LPA-mediated IFN-γ enhanced production could contribute to establish a suitable environment for the growth of tumoral cells.

1.4.3. Mast cells (MCs)

Strategically situated in perivascular spaces of mucosal and connective tissues, mast cells (MCs) are specialized at initiating immune responses to allergic, inflammatory, and infectous stimuli, generating and releasing diverse soluble mediators (Austen and Boyce, 2001). Particularly, cytokine and chemokine production by MCs underlies their ability to initiate inflammatory responses related to both host defense and pathological states (Supajatura et al., 2002). Thus, consistently with their roles in host defence and their wide perivascular distribution in all tissues and organs, human MCs express receptors for several soluble signal molecules, including complement fragments and other serum constituents such as LPA (Nilsson et al., 1996). Particularly, in MCs, which express all three LPA receptor-proteins, this lipid powerfully stimulates both their proliferation, providing a synergistic signal with the stromal cell-derived

cytokine stem cell factor (SCF) required for normal MC development, and Ca^{2+} fluxes, through a pathway involving both LPA_2 and LPA_3 receptors (Bagga et al., 2004). LPA is comitogenic with SCF but does not prolong MC survival. Cells pre-treatment with PTx almost completely inhibits LPA-induced proliferation but partially interferes with LPA-stimulated Ca^{2+} fluxes implying the involvement of both G_i-dependent and G_i-independent signalling pathways in this latter response.

Because MCs initiate inflammatory responses from perivascular spaces, where local LPA concentrations are likely to be high, it is possible that LPA could also activate MCs. LPA, in fact, strongly enhances both secretory granules formation and the expression of MC-specific tryptases such as α and β tryptases. In addition, in these cells, LPA exerts direct and indirect effects on differentiation, maturation and development, it modulates their phenotype without providing cytoprotection and it is a powerful agonist inducing their expression and secretion of proinflammatory chemokines such as macrophage inflammatory protein-1 beta (MIP-1β), IL-8 and monocyte chemotactic protein-1 (MCP-1) (Lin and Boyce, 2005). This latter response mainly depends on MCs priming by IL-4, a Th2 pleiotropic cytokine required to switch the mucosal immune responses toward a Th2 response and critical to the induction of type I allergy (O'Byrne, 2006). Particularly, chemokines production in response to LPA requires the IL-4 dependent up-regulation of MAPK kinase-1 (MEK-1) expression by a pathway involving PI3K activation. Moreover, cell pre-treatment with PTx partially abrogates LPA-induced chemokine production, suggesting both G_i family and other G proteins involvement in this response. In contrast with its mitogenic effect, LPA mediates chemokine production independently of LPA_1 and LPA_3

receptors and PPAR-γ but, rather, dependently of LPA_2 receptors. LPA ability to activate MCs through a LPA_2 receptor-dependent pathway further suggests functional distinctions between different LPA receptor family members that are constitutively expressed by cells of a single hemopoietic lineage. In the light of the above reported effects, LPA may both support reactive mastocytosis, a feature observed in several pathological states, and may also serve as an amplifier of mucosal inflammatory responses where MC hyperplasia is mediated by a Th2 cytokine-based mechanism (Lin and Boyce, 2006). Therefore, the apparent convergence of a Th2 cytokine, a resident tissue effector cell, and an endogenous mediator such as LPA may regulate and facilitate trafficking of blood-born leukocytes in the circumstances of mucosal inflammation where LPA is likely to be abundant due to vascular leakage (Lin and Boyce, 2005).

1.4.4. Dendritic Cells (DCs)

Dendritic cells (DCs), the most powerful antigen-presenting cells (APCs), are specialized in naïve T lymphocytes activation and immune responses initiation (Banchereau and Steinman, 1998). DCs originating from hemopoietic stem cells are characterized by the ability to migrate into peripheral tissues, where they are adapted to capture and process antigens thus alerting for danger signals such as microbial products, inflammatory cytokines, and cytoplasmatic molecules released in the extracellular environment as a consequence of cell necrosis (Gallucci and Matzinger, 2001). During antigen processing, DCs undergo maturation, a process involving tightly coordinated changes in cell surface phenotype and several responses to endogenous chemoattractants in order to facilitate their migration to secondary lymphoid organs. Mature DCs expressing high levels of CD83, major histocompatibility complex (MHC) class I

and II and co-stimulatory molecules and producing a broad panel of cytokines such as tumour necrosis factor-α (TNF-α), IL-10, and IL-12, then, migrate to regional lymph nodes to prime T cells to direct immune responses development providing a belated protection against pathogens and tumours (Lanzavecchia and Sallusto, 2001; Sozzani et al., 2000). Although both immature and mature human DCs express all three LPA receptors transcripts, immature DCs responses to LPA are substantially different from mature cells ones (Panther et al., 2002). In immature DCs, LPA elicits PTx-sensitive intracellular Ca^{2+} transient fluxes, actin polymerization, and chemotaxis without affecting the ability to internalize proteins or particles.

Actin reorganization is a prerequisite for leukocytes migration. In this context, LPA causes a rapid polymerization of actin molecules in a dose-dependent manner and chemoattracts immature DCs. The mechanism underlying actin reorganization in response to LPA requires the activation of both G_i proteins and small GTP-binding proteins of the Rho family. These chemotactic responses are, however, lost after cells stimulation with lipopolysaccharide (LPS) to induce their maturation. During the maturation process, in fact, DCs lose their ability to respond to LPA in terms of Ca^{2+} fluxes, actin reorganization, and chemotaxis. However, in maturating DCs, LPA dose-dependently inhibits LPS-mediated IL-12 and TNF-α production, whereas it increases IL-10 release. LPA ability to modulate cytokines production is transduced through PTx-insensitive G proteins such as $G_{q/12/13}$.

As reported above, mRNAs expression for all three LPA receptors has been described in DCs (Panther et al., 2002). However, since mRNA levels do not always correlate with protein amounts or with surface expression, it is also

important to look for protein expression. To this regard, it has been recently shown that both immature and mature DCs only express LPA_2 receptors on cell membrane but not LPA_1 or LPA_3 (Oz-Arslan et al., 2006). In this study, LPA influenced inflammatory cytokines production, such as IL-6 and IL-8, by human DCs in different differentiation stages. Particularly, simultaneous addition of LPA to immature DCs in the presence of LPS enhanced dose- and time-dependent IL-6 and IL-8 secretion in maturing DCs. Conversely, fully matured cells were no longer influenced by LPA addition suggesting that cytokine release modulation by LPA critically depends on cell activation before final maturation. The analysis of signalling mechanisms revealed that both G_i proteins and MAPKs played important roles in mediating LPA-induced release of inflammatory cytokines; however, their involvement seemed to exclusively regard immature or maturating DCs but not fully mature DCs, entertaining the possibility that other signal transduction intermediates may facilitate IL-6 and IL-8 secretion from mature DCs. Interestingly, the insensitivity of mature DCs towards LPA was not caused by the down-regulation or the disappearance of LPA receptors from cell membranes, rather than it was dependent on LPS ability to uncouple LPA receptors from the signal-transducing machinery leading to MAPKs activation during DCs maturation. Moreover, LPS ability to apparently “freeze” LPA receptors on mature DCs surface accounts for the lost of mature DCs ability to respond to LPA with Ca^{2+} fluxes, actin reorganization, and chemotaxis (Panther et al., 2002).

However, LPA did not affect either the endocytic and phagocytic capacities and the surface phenotype of mature DCs. In contrast, LPA enhanced their ability to both present antigens and stimulate naïve T cells proliferation, and inhibited

their capability to induce Th1 differentiation, all of these effects not being observed in immature DCs. Particularly, LPA increased DCs ability to polarize T cells directing the differentiation to a higher percentage of Th2 cells and to a parallel lower percentage of INF-γ single positive cells. The increased T cell stimulatory functions of mature DCs, however, cannot be attributed to a higher expression level of membrane-presenting and co-stimulatory molecules. LPA, in fact, did not induce changes in the expression of CD54, CD80, CD86, CD83, and MHC class I and II molecules in immature or LPS-differentiated DCs. These findings suggest that LPA could favour T cells polarization toward a pro-allergic phenotype during the development of adaptive immunity through DCs mediated effects. Thus, LPA concentrations resembling those present in the inflamed tissues, could exert multiple effects on immature, maturating, and fully mature DCs by modulating their biological functions such as trafficking, cytokine production, and T cell-activating functions.

Recently, LPA has been also demonstrated to influence the immunophenotypic differentiation of human monocytes into DCs (Martino et al., 2006). At the sites of inflammation, chemokines promote the recruitment of DC precursors such as monocytes, which differentiate into DCs or macrophages depending on the environmental factors (Randolph et al., 1998; Martino et al., 2004). In this context, the high LPA content in the inflammatory exudate, could affect DCs differentiation from monocytes by blocking the expression of CD1a molecules on their surface in a dose-dependent manner. Furthermore, in contrast to $CD1a^+$ DCs, this peculiar population has been previously described to be involved in Th2 immunity or to have a suppressive role on immune response (Martino et al., 2004; La Rocca et al., 2004). Thus, LPA may both regulate an

immune program in circulating monocytes which cannot be reverted during DC differentiation and maturation, and modify the immune functions of differentiated DC or their precursors generating a DC population that can, in turn, alter the immune system homeostasis with important consequences in several pathological states (Martino et al., 2006).

1.4.5. Granulocytes

Human granulocytes or polymorphonuclear leukocytes (PMNs) including neutrophils, eosinophils, and basophils, are critical effectors of the innate immune system and provide an essential first line of host defense against invading microrganisms. PMNs are rapidly recruited at infection sites and their ability to effectively remove pathogens is essential for the maintenance of human health (Weber and Dahinden, 1995).

Traditionally associated with parasitic infection or allergic manifestations, eosinophils are thought to be major effector cells in several inflammatory or infectious processes (Desreumaux and Capron, 1996). At inflamed sites, eosinophils accumulation and proinflammatory activity stimulation are probably triggered by several chemotactic agents. Among these, LPA has been shown to induce migration and to stimulate effector functions such as reactive oxygen intermediates (ROI) production and integrins up-regulation, to an extent which was comparable to that obtained by other well-characterized eosinophils activators (Idzko et al., 2004). Human eosinophils express mRNas encoding LPA_1 and LPA_3 receptors but not LPA_2. The experiments performed to prove LPA receptors functional expression revealed that LPA had chemotactic activity towards eosinophils and induced a concentration-dependent CD11b up-regulation, a classical feature of eosinophils migratory response (Tenscher et

al., 1997). Moreover, as it could be expected from a chemotactic agent, LPA was able to influence actin network reorganization causing a rapid and transient actin molecules polymerization. LPA micromolar concentrations also induced a rapid and concentration dependent intracellular Ca^{2+} increase. Since the Ca^{2+} chelator EGTA had no effect on the magnitude or the time course of LPA-induced Ca^{2+} transient changes, it can be suggested that the lipid stimulated Ca^{2+} mobilization from intracellular stores.

During inflammatory reactions, eosinophils are subjected to a process of cellular activation, which leads to degranulation and mediators production and release (Desreumaux and Capron, 1996). Particularly, it is known that eosinophils can mediate their proinflammatory activity in tissues by producing ROI. LPA, at all concentrations tested, evoked a rapid induction of ROI production with a maximum value after 5 minutes.

LPA-elicited eosinophil activation was mediated by specific receptors. In fact, cell pre-treatment with diacylglycerol pyrophosphate (DGPP), a dual-selective antagonist of LPA_1 and LPA_3 receptors, and PTx blockade of $G_{i/o}$ protein-coupled receptors, completely inhibited all LPA-induced cell effects strongly suggesting that, in eosinophils, LPA exerted its effects by specifically activating $G_{i/o}$ proteins-coupled LPA_1 and/or LPA_3 receptors. Moreover, the lack of influence on certain activation markers such as CD44 and CD69 (Triggiani et al., 2003) suggests that LPA induces only a very specific set of cell responses associated with chemotaxis and oxygen radicals production. All of these results point to a novel role for LPA as a powerful eosinophils chemokine as well as a proinflammatory effector functions activator, thus suggesting its involvement in

those diseases characterized by eosinophilic inflammation including bronchial asthma and rhinitis allergica.

Like eosinophils, human peripheral blood neutrophils also respond to LPA (Tou and Gill, 2005). LPA ability to mobilize intracellular Ca^{2+} through specific LPA receptors is widely documented in multiple cell types (Fueller et al., 2003; Idzko et al., 2004). However, although human neutrophils express all four mRNas encoding for LPA receptors, the application of low micromolar concentrations of the lipid triggered a receptor-independent Ca^{2+} influx (Itagaki et al., 2005). In this context, more detailed analysis also revealed that LPA can directly trigger Ca^{2+} entry without depleting Ca^{2+} stores through yet unknown mechanisms.

Since LPA failed to cause neutrophils chemotaxis at all tested concentrations, it is possible that these cells do not express functional LPA receptors even if the lipid exerted stimulatory actions on the respiratory burst in a dose-dependent manner. Moreover, since pharmacological interventions to inhibit G protein-coupled receptors signalling were unsuccessful in abolishing LPA-induced Ca^{2+} influx and oxidative burst, the existence of a potential alternative pathway for neutrophil activation by LPA can be hypothesized. These findings emphasize the possibility that the LPA released during thrombosis or other relevant pathological events could directly activate neutrophil Ca^{2+} entry and inflammatory response without LPA receptors involvement, thus acting as an intracellular second messenger (Itagaki et al., 2005).

Several studies have shown that LPA stimulates PLD activity in a wide variety of both normal and transformed cells (Hess et al., 2000; Xie et al., 2002). PLD plays a central role in regulating neutrophil functions. Particularly, one the functions of PA, the principal lipid product of PLD action, is to enhance

neutrophil degranulation to killing invading bacteria (Kanaho et al., 1991; Brandolini et al., 1996). In this regard, it has been recently documented that high LPA concentrations both time and concentration dependently stimulated PLD activity leading to an increase in intracellular PA levels and the subsequent degranulation of human neutrophils (Tou and Gill, 2005). However, since other pathways may contribute to the LPA-induced enhancement in PA formation, it is possible that also LPA-elicited degranulation process could be at least independent on an increased PLD activity. As it is known, neutrophils can also participate in wound healing process (Werner and Grose, 2003). Therefore, these results suggest that the LPA released from activated platelets during blood clotting may participate in bacterial killing and wound healing process regulation by stimulating neutrophils to release granule enzymes as a defense mechanism against contaminating bacteria. On the other hand, it should be also noticed that an augmented LPA production might be involved in inflammation, causing damage of the host tissues.

To date, nothing is still known concerning LPA biological activities on human basophils.

1.4.6. Monocytes and macrophages

Blood monocytes and mature tissue macrophages are key regulators of host defense against several pathogens and other challenges. However, anomalous changes of their physiological functions can lead to mononuclear phagocytes participation in many pathological conditions such as chronic inflammation and cancer (Sunderkotter et al., 1994). So far, only few studies have been published concerning LPA actions on monocyte-macrophage system, although peripheral blood monocytes and/or tissue macrophages in mice, humans, and rats all

express LPA receptors (Duong et al., 2004; Hornuβ et al., 2001). Human monocytes predominantly express LPA_1 and LPA_2 receptors while a low or absent expression of LPA_3 receptors has been observed (Lee et al., 2002). Previous studies reported that high LPA concentrations could stimulate both a rise in cytosolic Ca^{2+} and the haptotactic migration of human monocytes isolated from peripheral blood (Zhou et al., 1995). However, nanomolar LPA concentrations can also induce Ca^{2+} entry in human monocytic cells (Fueller et al., 2003). Particularly, this study showed that LPA dose-dependently increased cytosolic Ca^{2+} concentration in LPS-activated Mono Mac 6 (MM6) monocytes, a human cell line retaining the phenotypical and functional characteristics of mature blood monocytes (Ziegler-Heitbrock et al., 1988). In MM6 cells, LPA stimulated Ca^{2+} influx predominantly from the extracellular medium, an effect which was dependent on PLC stimulation and mainly mediated by PTx-sensitive G_i proteins activation (Fueller et al., 2003).

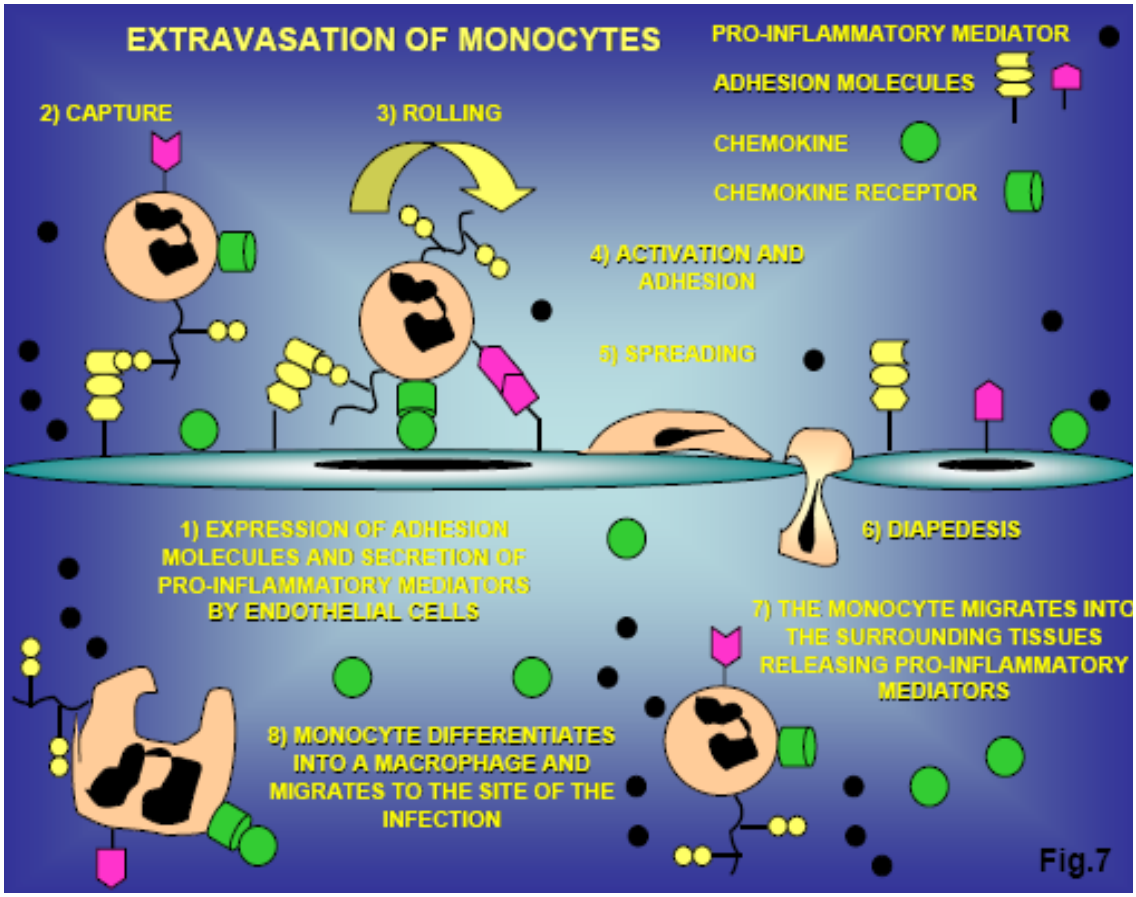
In a further study, nanomolar LPA concentrations have been reported to inhibit murine macrophages apoptosis, identifying LPA as a major non-cytokine serum survival factor (Koh et al., 1998). Results obtained in this report showed, for the first time, an LPA-mediated effect on macrophages, a cell type previously thought not to be responsive to LPA (Jalink et al., 1990, 1994). LPA administration, in fact, protected murine macrophages from serum deprivation-induced apoptosis to an extent similar to other well-known macrophage survival factors such as macrophage colony stimulating factor (MCSF). Furthermore, LPA action as a survival factor was specific: other structurally-related phospholipids, in fact, were all less effective than LPA in promoting macrophages survival. Although murine macrophages only express LPA_1

receptors (Hornuβ et al., 2001), cell pre-treatment with suramin, a broad inhibitor of ligand-receptor interactions, blocked LPA-induced macrophage viability. This result offers a strong support to the hypothesis that LPA ability to inhibit macrophage apoptosis could depend on interaction with a specific receptor (Koh et al., 1998).

Finally, human alveolar macrophages express all three LPA receptors (Hornuβ et al., 2001). To this regard, LPA has been recently shown to enhance the antymicobacterial activity of human macrophages (Garg et al., 2006). Particularly, results demonstrated that LPA reduced intracellular mycobacterial (MTB) growth in monocyte-derived macrophages, in the differentiated THP-1 monocytoid cell line, and in bronchoalveolar lavage (BAL) cells coming from tuberculosis patients. This LPA effect is mediated by host PLD activation which, in turn, promotes intracellular mycobacterial killing by favouring phagosomes acidification, and it is also associated with an enhanced cathepsin-D, an important enzymatic component of lysosome associated antimicrobial machinery, expression and maturation (Richo and Conner, 1994). On these grounds, a possible protective role for LPA and probably for its receptors, can be hypothesized; through the stimulation of the antimycobacterial innate immune response, in fact, the lipid leads to decrease both in vitro and ex vivo Mycobacterium tuberculosis (MTB) intracellular growth, thus evidencing that the understanding of the mechanisms through which LPA promotes an efficient anti-tuberculosis response appears to be fundamental to the development of novel therapeutic approaches against this dramatic disease.

1.5. LPA and inflammation: the role of monocytes

Inflammation is a complex reaction of the body to an injury related to physical or chemical noxious stimuli or to the invasion by different harmful agents which are capable to derange its homeostasis. The discovery of the detailed phases of inflammatory process has revealed a close relationship between inflammation and immune response. The inflammatory process, in fact, consists of specific and non-specific immunological reactions and it is intended to inactivate or destroy invading organisms, remove irritants, and set the stage for tissue repair. The immune system is activated by a complicated sequence of events leading to vasodilatation, fluid and proteins vascular leakage and local infiltration of inflammatory cells (Schmid-Schönbein, 2006) (Fig. 7).



Several evidences suggested a potential function for LPA as a sentinel endogenous mediator for the induction of genes controlling inflammatory and/or immune responses (Cummings et al., 2004). However, although all immune system cells express LPA receptors, the target cells and receptors responsible for LPA putative contribution to inflammation are yet to be defined. Nonetheless, according to its double-edge sword behaviour, recent evidences have shown that LPA can exert dual and opposite roles in inflammatory processes. On one hand, LPA is involved in the development of normal immunological reactions by means of its ability to exert multiple modulating effects on immune responses acting as a powerful immunoregulatory factor (Graler and Goetzl, 2002). In this context, it is well known LPA ability to induce a local or systemic inflammatory response by recruiting and activating macrophages (Balazs et al., 2000) as well as T- and B-lymphocytes (Wang et al., 2004; Rosskopf et al., 1998), thus influencing their interaction with other cell types. On the other hand, it has been also widely documented that LPA has powerful proinflammatory properties and it can initiate and perpetuate several inflammatory-associated pathologies (Sengupta et al., 2004). At inflamed sites, in fact, local vascular leakage and cell activation result in increased LPA tissue concentrations. To this regard, it is known that LPA high concentrations play an important mitogenic role which couples with its stimulatory activity on diverse cell types implicated in the pathogenesis of several human diseases characterized by chronic inflammation, such as bronchial asthma, rhinitis allergica, and sepsis (Siess et al., 1999; Gennero et al., 1999; Panther et al., 2002). Moreover, LPA role in atherosclerotic wounds development is particularly representative, since its pro-atherogenic effect under certain pathological conditions is well known (Siess,

2002; Siess and Tigyi, 2004). In this context, LPA locally released has been widely shown to stimulate both proliferation and proinflammatory activity of several cell types involved in vascular lesions development.

Excessive cell proliferation and activation, in fact, are important processes involved in vascular damage (Andres and Castro, 2003). These processes were initially believed to exclusively occur in smooth muscle cells; at present, however they are known to also influence inflammatory cells including T-lymphocytes and monocytes/macrophages (Ross, 1999).

The monocyte proinflammatory potential can be triggered by various physiological and pathological stimuli, to whom cell respond generating several biologically active molecules, such as arachidonic acid metabolites, ROI, and nitric oxide (NO) (Osterud and Bjorklid, 2003). In particular, ROI produced by NADPH oxidase (NADPHox), an enzyme complex which is primarly responsible for such a production in phagocytic cells (Babior, 1999), have been demonstrated to play an important role in determining oxidative stress at inflammatory sites (Hadjigogos, 2003; Lassegue and Griendling, 2004). Such a phenomenon can be worsened by the release of arachidonic acid metabolites (Lu and Wahl, 2005). To this regard, LPA ability to stimulate arachidonic acid (AA) release is well documented (Inoue et al., 1995; Pebay et al., 1999). AA can be, in turn, directly converted to prostaglandins and leukotrienes, important inflammatory lipid mediators, by cycloxygenases (COX) and 5-lypoxygenase enzymes (5-LOX), respectively (Vila, 2004; Busse, 1998). Particularly, monocytes activation is often associated with a significantly augmented production of leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2), which

accounts alone for approximatively 70% of total prostaglandins released by stimulated monocytes during inflammatory processes (Passwell et al., 1979).

By means of its role as a powerful mediator of inflammatory processes, immune responses, and host defense against infections, LTB_4 promotes events that can likely contribute to the development and progression of several inflammatory-associated pathological states (Crooks and Stockley, 1998; Yokomizo et al., 2001). LTB_4, in fact, is one of the most powerful endogenously-synthesized chemotactic factors for neutrophils, macrophages, and other inflammatory cells, and it also induces the chemokinesis and the adhesion of these cells to the vascular endothelium (De Caterina et al., 1988). Moreover, through its binding to specific receptors on the surface of inflammatory cells, LTB_4 is known to stimulate their complete activation leading to degranulation, lysosomal enzymes release, and ROI production (Busse, 1998). These mechanisms, then, may create a vicious circle in which inflammatory cells, by means of biologically active molecules synthesis and release, cause a local inflammation, perpetuating the recruitment of further inflammatory cells which produce additional proinflammatory mediators.

Another group of arachidonic acid-derived metabolites produced by activated monocytes includes prostaglandins (PGs), particularly PGE_2, which contributes to vasodilation, pain, and fever (Astiz et al., 1996). PGs synthesis is regulated by two main enzyme isoforms, COX-1 and COX-2. COX-1 is constitutively expressed and detectable in most human tissues. In contrast, COX-2, which is normally expressed at low levels, is strongly induced by proinflammatory agents, including cytokines and growth factors. COX-2 induction in cells is responsible for the major increase in PGE_2 production (Dubois et al., 1998).

Depending on the context, then, PGE_2 can exert homeostatic (Sugimoto et al., 2000), inflammatory (Davies et al., 1984), or in some cases anti-inflammatory effects (Takayama et al., 2002). However, PGE_2 has long been considered the principal prostaglandin in acute inflammation and high levels of this powerful mediator are observed in numerous pathological states.

Another major hallmark of proinflammatory monocyte activation is NO and ROI production and release. These mediators constitute the chemical network of inflammatory response and result in most of the clinical and pathological manifestations of inflammation itself (Guzik et al., 2003). They affect virtually every step of inflammatory response development and exert multiple modulatory effects on immune responses. However, the exact effects of NO and ROI on inflammation may be ambiguous, and depend on cellular environment, their concentrations as well as other factors. Particularly, low NO concentrations mostly seem to be cytoprotective, probably due to the characteristic redox properties of such a molecule (Xie and Nathan, 1994), and to inhibit adhesion molecule expression, cytokine and chemokine synthesis and leukocyte adhesion and transmigration. In contrast, large amounts of NO can be toxic and pro-inflammatory. However, it is also important to note that cells producing toxic NO levels, do not suffer from its toxic properties. This response is mainly possible through the induction of specific protection mechanisms (Wink et al., 1996).

In addition to its direct effector functions, NO is also involved in immune regulation through multiple mechanisms including interactions with cell signalling systems. In this context, NO may also lead to the modification of

transcription factors activity and thus modulate the expression of multiple other inflammatory mediators (Langrehr et al., 1993).

NO is involved in innate immunity as a toxic agent towards infectious organisms (Rosa Brunet, 2001), but it can also induce or regulate host immune cells death and functions, thereby regulating specific immunity (Bogdan et al., 2000). Moreover, NO may induce toxic reactions against other host tissues and since it is generated at high levels in certain types of inflammatory diseases, it has been implicated as a pro-inflammatory agent. Equally, it may act as an anti-inflammatory or immunosuppressive agent due its inhibitory or apoptotic effects on cells. Therefore, NO role in immunity and inflammation is still unclear mainly due to the fact that it is produced by several cells participating at all stages of inflammation. However, its actions in immunity regulation are likely to depend on cellular environment and activation state (Coleman, 2001).

Similarly to NO, ROI can also elicit a wide spectrum of cellular responses. Typically, low ROI concentrations are mitogenic and can promote cell proliferation, while mild concentrations result in either temporary or permanent growth arrest, such as replicative senescence. Finally, very severe oxidative stress can induce modifications inhibiting cellular components activity or resulting in a damage ultimately leading to cell death via either apoptotic or necrotic mechanisms (Martindale and Holbrook, 2002).

ROI production also plays an important role in inflammatory reactions modulation. Recent studies, in fact, have shown that ROI generation by monocyte/macrophage system at inflamed sites can affect inflammatory processes (Lassegue and Griendling, 2004; Andreadis et al., 2003). ROI produced by NADPHox all may, in fact, lead to toxic effects but they may also

modulate inflammation in a far more discrete way, when continuously produced at low levels. The physiological ROI production during the respiratory burst, in fact, serves as an important bactericidal mechanism of host defense but an excessive extracellular release can also lead to surrounding tissues damage as well as cell death (Tiidus, 1998). Moreover, the significant amounts of ROI produced by monocytes and other cell types at inflamed sites have often been associated with several diseases initiation or aggravation (Gate et al., 1999).

Finally, a cross talk exists between the cell signalling system and the cellular redox state. In fact, being highly reactive by nature, ROI can directly or indirectly modulate the functions of many enzymes and transcription factors (Martindale and Holbrook, 2002). The observation that ROI can both stimulate multiple signalling pathways and are produced, in a regulated fashion, in response to several stimuli has led to hypothesize possible ROI roles as second messengers in cellular signalling (Kamata and Hirata, 1999). Recently, several evidences have shown that ROI can also act as intracellular signalling molecules, in an autocrine or a paracrine fashion, in inflammatory signal transduction. In this context, it is known that intracellular ROI can modulate the release of other proinflammatory mediators, regulate adhesion molecule expression on endothelium and inflammatory cells (Niu et al., 1994; Fraticelli et al., 1996), thus affecting cell recruitment at inflamed sites and, finally, increase chemokines and cytokines production (Kimura et al., 2003; Brzozowski et al., 2003). At least a part of these effects results from ROI ability to stimulate MAPKs activity which, in turn, leads to several transcription factors activation (Torres and Forman, 2003). MAPKs are essential components of the complex intracellular networks that regulate gene expression and cell function. They are

important serine/threonine signalling kinases that are activated by a phosphorylation resulting in the mediation of cellular responses to extracellular signals by means of gene expression changes in the nucleus. Particularly, the MAP kinase cascade is one of the most ancient and evolutionarily conserved signalling transduction pathway, which is widely involved in controlling a wide array of physiological processes including proliferation, differentiation, stress adaptation, and apoptosis (Schaeffer and Weber, 1999). Basing on structural differences, they are divided into three major subfamilies: 1) the extracellular signal-regulated protein kinases (ERK); 2) the p38 MAPKs, and 3) the c-Jun NH_2-terminal kinases (JNK). The ERK, JNK, and p38 subfamilies have all been shown to be activated in response to oxidant injury and therefore could potentially contribute to influence the cellular responses to oxidative stress (Torres, 2003).

To this regard, LPA-induced ROI generation is known to contribute to MAPKs activation (Schmitz et al., 2002). Recently, using rat aortic smooth muscle cells as a model, we demonstrated LPA ability to promote cell proliferation through signalling transduction pathway involving NADPHox activation and subsequent ROI production followed by p42/44 MAPK stimulation (Baldini et al., 2005). LPA, in fact, has been shown to elicit strong proliferative effects by activating several signalling pathways including the enhancement of phospholipase and PI3K activities and MAPKs stimulation. In this context, although LPA has been found to induce MAPKs activation in several cell types (Seewald et al., 1997; Reiser et al., 1998), their specific roles in cell proliferation are cell types and agonist dependent.

2. AIM AND SCOPE

Excessive proliferation as well as the release of proinflammatory mediators from leukocytes represent common phenomena in several important diseases characterized by chronic inflammation such as atherosclerosis, bronchial asthma, and sepsis. In this context, early monocyte proliferation and activation can contribute to the development and progression of inflammatory processes by means of the synthesis and the release of biologically active molecules such as arachidonic acid metabolites, reactive oxygen intermediates (ROI) and nitric oxide (NO). Several evidences identify lysophosphatidic acid (LPA), a small lipid endowed with pleiotropic activities, as an important modulator of both proliferation and activation of different cell types involved in the development of several inflammatory pathologies. However, its exact role on monocyte proinflammatory activation and regulation is not fully understood yet. Aim of the present study was then to investigate possible LPA effects on human monocytic cell line THP-1 activation, evaluated in terms of proliferation, ROI and NO production, and release of arachidonic acid-derived inflammatory mediators, all of them being considered to be key events in leukocytes inflammatory response. Moreover, in order to shed light on complexes and tangled signalling functions of LPA receptors involved in the several biological activities of this lipid, we tried to clarify the contribution of each receptor isoform in mediating LPA effects on THP-1 cells and, particularly, to elucidate LPA signalling transduction pathways leading to proliferation.

3. MATERIAL AND METHODS

3.1. Cell culture

The human monocytic cell line THP-1, used to avoid inter- and intraspecific variability of peripheral blood mononuclear cells (PBMCs), was obtained from American Type Culture Collection (Manassas, VA), cultured in Falcon flasks with RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS), L-glutamine (2 mM), streptomycin (100 μg/ml), penicillin (100 U/ml), sodium pyruvate (100 μg/ml), and maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cells were serum starved before each experiment to rule out possible interferences with cell growth due to serum components. In all experiments, to determine cell viability, monocyte cultures were stained with Trypan blue at specific timepoints after treatment with different reagents. Stained vs living cells were counted under a microscope using a Neubauer modified chamber.

3.2. DNA synthesis assay

THP-1 cells were cultured in Falcon flasks 75 cm^2 and seeded in 30×15mm dishes with serum-free medium ($1x10^6$ cells/well). Cells were challenged with different concentrations of LPA (0.1-20 μM) for 3, 6 and 9 hours. In a series of experiments, cells were pretreated with Ki16425 (10 μM) for 30 min or with diphenylene iodinium (DPI) (10^{-8} M), PD98059 (50 μM), and SB293580 (10 μM) for 1 h, before LPA addition. [^{3}H]-thymidine incorporation into DNA was used to measure the mitogenic response of THP-1 cells. Cells were pretreated with Ki16425 for 30 min or with DPI (10^{-8} M), PD98059 (50 μM), and SB293580 (10 μM) for 1 h, pulsed with [^{3}H]-thymidine (1 μCi/ml) and then challenged with LPA

for the requested times. Cells were then harvested by centrifugation and treated with 5% trichloroacetic acid (TCA) at 4°C for 15 min. The TCA-insoluble fraction was resuspended in 0.1% SDS in NaOH (200 mM) and samples were counted for radioactivity, after addition of 3.5 ml Optifluor, by a liquid scintillation counter (Tricarb 2180/TR, Packard Instruments, Downers Grave, IL).

3.3. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and LightCycler-PCR analysis

Total RNA isolation and purification were carried out by SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. Briefly, total RNA was extracted from $3x10^6$ THP-1 cells treated or not with LPA (1 μM) for 6 h. Total RNA quantity and quality were assessed by UV absorbance at 260 nm and electrophoresis on agarose/formaldehyde gel, respectively. For RT-PCR analysis, cDNA was retro-transcribed from 2 μg of total mRNA for 1 h at 37°C in an incubation buffer containing deoxynucleotide triphosphate (dNTP, 250 μM each), random primers $p(dN)_5$ (Roche), RNase inhibitor and RNA murine Molooney leukaemia virus reverse transcriptase (200U, Promega). For classic PCR analysis, an aliquot of each RT product (2μl) was amplified in a final volume of 50 μl using PCR buffer containing dNTP (160 μM), primers designed to amplify the mRNA of human LPA receptors (15 pmol each, Invitrogen) and Taq polymerase (1U, Amersham Pharmacia Biotech.). All samples were subjected to the following reaction conditions: 30 cycles at 94°C for 2 min, 94°C for 30 sec, (60°C for 30 sec for LPA_2 and 58°C for 30 sec for LPA_1, LPA_3 and GAPDH), 72°C for 30 sec, followed by a final extension at 72°C for 3 min. Expression levels of receptor mRNAs in THP-1 cells were quantified by normalising their respective mRNAs levels to the housekeeping human GAPDH

mRNA levels. The amplified fragments were then separated by electrophoresis on agarose gel 1-2% in tris-acetate-EDTA (TAE) buffer and visualized by ultraviolet transillumination.

For LightCycler-PCR, an aliquot of each RT reaction product (2μl) was amplified using the LightCycler FastStart DNA master SYBR Green I (Roche Diagnostic) and primers (10 pmol each). The reactions for LPA receptors were undergoing to 95°C for 10 min followed by 45 cycles 94°C for 10 sec, (60°C for 5 sec for LPA_2, 58°C for 10 sec for LPA_3, 60°C for 20 sec for LPA_1), 72°C for 15 sec, and one melting curve analysis from 95°C, 65°C for 15 sec and to 95°C for 10 min, using the LightCycler instrument (Roche). The GAPDH reaction was undergoing to 95°C for 10 min followed by 45 cycles 94°C for 10 sec, 58°C for 20 sec, 72°C for 15 sec, and one melting curve analysis from 95°C, 65°C for 15 sec and to 95°C for 5 min, using the LightCycler instrument (Roche).

The expression levels of receptor mRNAs were quantified by normalizing the mRNA levels to the housekeeping human GAPDH mRNA by an external standard curve (LightCycler operator's Manual). The external standard curve was created with diluitions of genomic DNA, amplified with LPA_3 primers.

Primer	Sequence
LPA_1 sense	5'-CGGCGGGTAGTGGTGGTC-3'
LPA_1 antisense	5'-GTCGCGGTAGGAGTAAATGATG-3'
LPA_2 sense	5'-GTCGAGCCTGCTTGTCTTC-3'
LPA_2 antisense	5'-CCAGGAGCAGTACCACCTG-3'
LPA_3 sense	5'-TCGCGGCAGTGATCAAAAACAGA-3'
LPA_3 antisense	5'-ATGGCCCAGACAAGCAAAATGAGC-3'
GAPDH sense	5'-CATGGGTGTGAACCATGAGAAG-3'
GAPDH antisense	5'-GTGGCTGTTGAAGTCAGAGGAG-3'

3.4. Evaluation of ROI production by 2',7'-dichlorofluorescein diacetate (DCF-DA) fluorescence

The samples ($2x10^6$ cells) were loaded with the fluorescent indicator dichlorofluorescein diacetate (DCF-DA) (10 μM) for 30 min at 37°C in the dark. DCF-DA diffuses through the cell membrane and is hydrolyzed by intracellular esterase to nonfluorescent DCF deacetylated, which is then rapidly oxidized to highly fluorescent DCF in the presence of ROI. The DCF fluorescence intensity is proportional to the amount of intracellular ROI formed (Shen et al., 1996). After the incubation with the fluorescent dye, cells were collected, centrifuged for 5 min at 1,200 rpm at room temperature, resuspended in serum-free medium and challenged with different LPA concentrations (0.1-20 μM) for 3, 6 and 9 h. When requested, THP-1 monocytes were pretreated with DPI (10^{-8} M), a specific inhibitor of NADPH oxidase, and Ki16425 (10 μM), a LPA_1 and LPA_3 receptor antagonist, for 1 h and 30 min, respectively, before LPA addition. In other series of experiments, cells were pretreated with PD98059 (50 μM) and SB293580 (10 μM) for 1 h, before lipid addition. Fluorescence was measured under continuous magnetic stirring and controlled temperature (37°C) in a Perkin-Elmer luminescence spectrometer (Model LS-5) equipped with a chart recorder (Model R 100A), with excitation wavelenght at 485 nm and emission wavelenght at 530 nm using 5 and 10 nm slits respectively, for each light path. Results were expressed as Fluorescence Intensity, reported as Fluorescence Units (F.U.), in respect to cells loaded with only DCF-DA (C).

3.5. ROI generation as assessed by microscopy analysis

ROI generation was carried out on glass chamber slides. $3x10^6$ cells were labelled with the peroxide-sensitive fluorescent dye 2',7'dichlorofluorescein

diacetate (DCF-DA, 10 μM) and incubated for 6 h in the presence or absence of LPA plus or minus DPI (10^{-8} M) or Ki16425 (10 μM), as described above. Intracellular fluorescence was monitored using a fluorescence microscope (Leica DMRB; objective: x 200). Signal-based averaging was used to quantitate the fluorescence signal from five randomly selected fields (Delta System Software).

3.6. [^{3}H]-Arachidonic acid release assays

THP-1 cells were seeded in 30×15mm dishes with serum-free medium ($1x10^6$ cells/well) and labelled for 3 h with 1 μCi [^{3}H]-arachidonic acid (AA) (spec. act. 202,4 Ci/mmol) at 37°C as previously described (Donchenko et al., 1994). To remove non-specific binding of [^{3}H]-AA to cell surface prior to agonist stimulation, culture medium was eliminated, cells resuspended in serum-free medium and challenged with different LPA concentrations (1-20 μM) for 6 h. After treatment, supernatants were collected, centrifuged at 1,200 rpm for 5 min to remove suspended cells and 100 μl aliquots were added to 3 ml Optifluor and analyzed by a liquid scintillator counter. Results were expressed as [^{3}H]-AA cpm/$1x10^6$ cells.

3.7. Measurement of Leukotriene B_4 and Prostaglandin E_2 release

$3x10^6$ cells/well were pretreated or not with Ki16425 (10 μM) for 30 min, challenged or not with LPA (1 μM) for 6 h and, at the end of experimental time, supernatants were collected and Leukotriene B_4 (LTB_4) and Prostaglandin E_2 (PGE_2) levels in the medium were determined by enzyme immunoassay (EIA) (Cayman Chemical), following manufacturer's instructions. Briefly, 50 μl LTB_4 standard or samples were added to the pre-coated mouse monoclonal anti-

rabbit IgG macrotitre plates. Subsequently, 50 μl LTB_4 tracer and 50 μl monoclonal antiserum of LTB_4 were added into each well and the mixture was incubated overnight at 4°C. After incubation, the content in each well was removed and the wells were washed 5 times with phosphate buffer saline (PBS) containing 0.05% Tween-20. An aliquot of 200 μl Ellman's reagent was added into each well, and the mixture was incubated for 2 h at room temperature with occasional shaking. The optical density of the solution was determined by a multi-well spectrophotometer (ELISA reader) at 405 nm. A similar procedure was performed for PGE_2 release determination, using pre-coated goat polyclonal anti-mouse IgG macrotitre plates. LTB_4 and PGE_2 concentration in each sample was calculated as the concentration corresponding to sample's optical density (O.D.) values plotted on respective standard built-in curves. Values were reported as percentile increase in respect to control (C).

3.8. Evaluation of nitrite (NO_2) production by Griess reaction fluorescence

$2x10^6$ cells/well were pretreated or not with Ki16425 (10 μM) for 30 min, challenged or not with LPA (1 μM) for 6 h and, at the end of experimental time, supernatants were collected and nitrite (NO_2) levels in the medium, whose concentration is directly proportional to intracellularly-formed nitric oxide (NO) (Pèrez-Pèrez et al., 1995), were determined by a colorimetric assay. Briefly, each sample was probed in triplicate by adding 100 μl of Griess Reagent (modified) to 100 μl samples' aliquots in a 96-well macrotitre plate. Wells were mixed repeatedly and the reaction was performed at room temperature for 30 min. At the end of this time, the absorbance of each well was read by LETTORE BIOCHIMICA with wavelength set at 550 nm. NO_2 concentration in each sample was calculated as the concentration corresponding to samples' O.D. values

plotted on a standard curve routinely prepared for each experiment using sodium nitrite ($NaNO_2$, 0,43-65 μM) as the reference. Values were then divided by their respective controls and multiplied by 100 to obtain the percentile increase.

3.9. Immunoblots

THP-1 cells were seeded in 6-well plates ($3x10^6$ cell/well), resuspended in serum-free medium and challenged with LPA (1 μM) for 3, 6, and 9 hours. When requested, cells were pretreated with DPI (10^{-8} M) for 1 h before LPA addition. The reactions were stopped, cells were collected, centrifuged at 1,200 rpm for 5 min, washed with PBS, an other time centrifuged and the pellet was lysed with sample buffer containing: 10% sodium dodecyl sulphate (SDS), 10% glycerol, dithiotreitol (100 mM), 5% β-mercaptoetanol, 1% bromophenol blue, EDTA (2 mM), sodium vanadate (1 mM), Tris-HCl (50 mM, pH 6,8), plus a cocktail of protease inhibitors (1 mM phenylmethylsulphonylfluoride, 1 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg/ml aprotenin) in ice for 30 min. The lysate was centrifuged at 13,000 rpm for 5 min; the supernatant was boiled for 5 min and stored at -80°C. The protein content of cell lysates was evaluated by the metod of Lowry. Equal amounts of whole cell lysate (30-50 μg) were subjected to 10% acrylamide SDS-PAGE, transferred into nitrocellulose membranes and probed with primary anti-phospho and total p38 MAP kinase antibodies (both 1:1000) or anti-phospho and total p42/44 MAPKs antibodies overnight at 4°C. A horseradish peroxidase-conjugated secondary antibody (1:2500) was used for 1 h at room temperature. Membranes were then analyzed by ECL assay. Each experiment was repeated at least three times.

3.10. Reagents

RPMI-1640, glutamine, penicillin (100 Ul/ml), streptomycin (100 μg/ml), and sodium pyruvate (100 μg/ml) were from Eurobio Laboratoires. Foetal Bovine Serum (FBS) was from GIBCO (Grand Island NY, USA). LPA (C18:1, 1-oleoyl-sn-glycerol-3-phosphate), Trypan blue, DPI, PD98059, SB203580, SDS, Ditiotreitol, Mercaptoetanol, Glycerol, Sodium Vanadate, Phenylmethylsulfonylfluoride, Pepstatin, Aprotenin, Leupeptin, Tris-HCl, Ki16425, Griess Reagent, and 2',7'-dichlorofluorescein diacetate (DCF-DA) were from Sigma Chemicals. [^{3}H]-thymidine (20 Ci/mmol), [^{3}H]-arachidonic acid (202,4 Ci/mmol) from Amersham Biosciences. Random primers, murine Molooney leukaemia virus reverse transcriptase, LPA receptors and GAPDH primers were purchased from Invitrogen**.** Taq polymerase, horseradish peroxidase-conjugated secondary antibody and chemioluminescence detection of immunoreactive bands ECL reagents were obtained from Amersham Pharmacia Biotech**.** SV Total RNA Isolation System was from Promega. LightCycler FastStart DNA master SYBR Green I kit and all other reagent for LightCycler-PCR were from Roche Diagnostic. Leukotriene B_4 and Prostaglandin E_2 assay kits were purchased from Cayman Chemical. Primary anti-phospho and total p38 MAP kinase antibodies and anti-phospho and total p42/p44 MAPKs antibodies were purchased from Santa Cruz, Biotechnology Inc. (Santa Cruz, CA).

3.11. Statistical analysis

Data distribution was preliminarily verified by the Kolmogorov-Smirnov test. Each experiment set was independently performed and compared with the same control by Student's t-test. Quantitative data were expressed as the mean

± S.D. of at least four replicate determinations, except where otherwise indicated. Differences were regarded as significant when P value was less than 0.05.

4. RESULTS

4.1. LPA effects on THP-1 cell growth

Mononuclear phagocytes, including monocytes and macrophages, can proliferate in situ in response to several soluble mediators (Takahashi et al., 1996). In this context, to evaluate LPA ability to stimulate THP-1 cell growth, cells were challenged with different lipid concentrations (1-5 μM) and assayed for [^{3}H]-thymidine incorporation into the DNA at 3, 6 and 9 h. As shown in Fig. 1, only 1 μM LPA induced an increased DNA synthesis that was statistically significant yet at 3 h and reached a maximum (+50% in respect to untreated cells) at 6 h. Longer experimental times and lower (0.1 and 0.5 μM) or higher (10 and 20 μM) LPA concentrations (data not shown), did not exert any significant effect.

4.2. LPA stimulates ROI production and NADPH oxidase activation

To evaluate LPA ability to stimulate ROI production, a fundamental marker of cell activation (Johann et al., 2006), THP-1 cells were challenged with the lipid (1-5 μM) for different experimental times (3, 6 and 9 h) after labelling with DCF-DA (10 μM). Time-course and dose-response experiments of ROI production are shown in Fig. 2a. Similarly to cell growth, LPA induced a significant increase in ROI production only at 1 μM after 3 h with a maximal effect after 6 h. Longer experimental times and lower (0.1 and 0.5 μM) or higher (10 and 20 μM) LPA concentrations (data not shown), did not exert any significant effect.

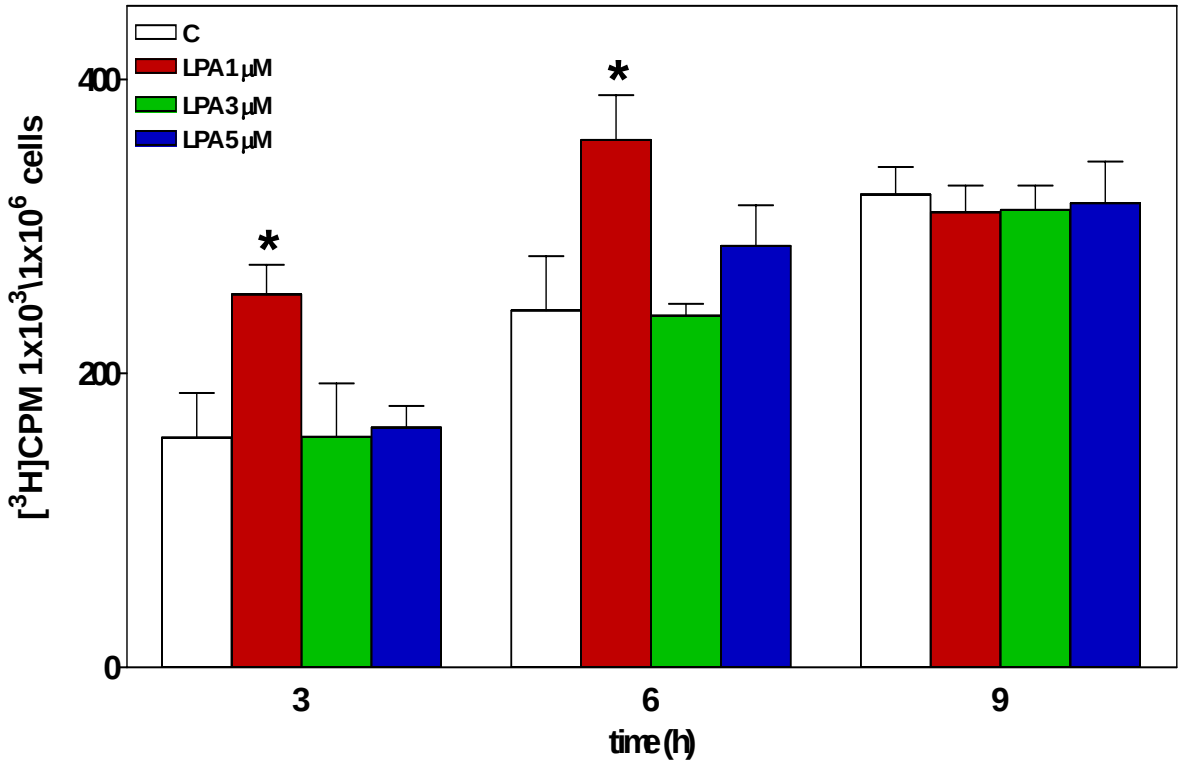


Fig.1. LPA effects on THP-1 cells growth

THP-1 cells were grown in RPMI-1640 plus 10% FBS and serum starved before each experiment to rule out possible interferences with cell growth due serum components. Cells were challenged with different LPA concentrations (1-5 μM) for 3, 6 and 9 h. [^{3}H]-thymidine incorporation into DNA was assessed as reported in the Materials and Methods Section. Data are reported as mean ± S.D. of 4 different experiments. *$P<0.05$, as reported from Student's t test in respect to untreated cells (C).

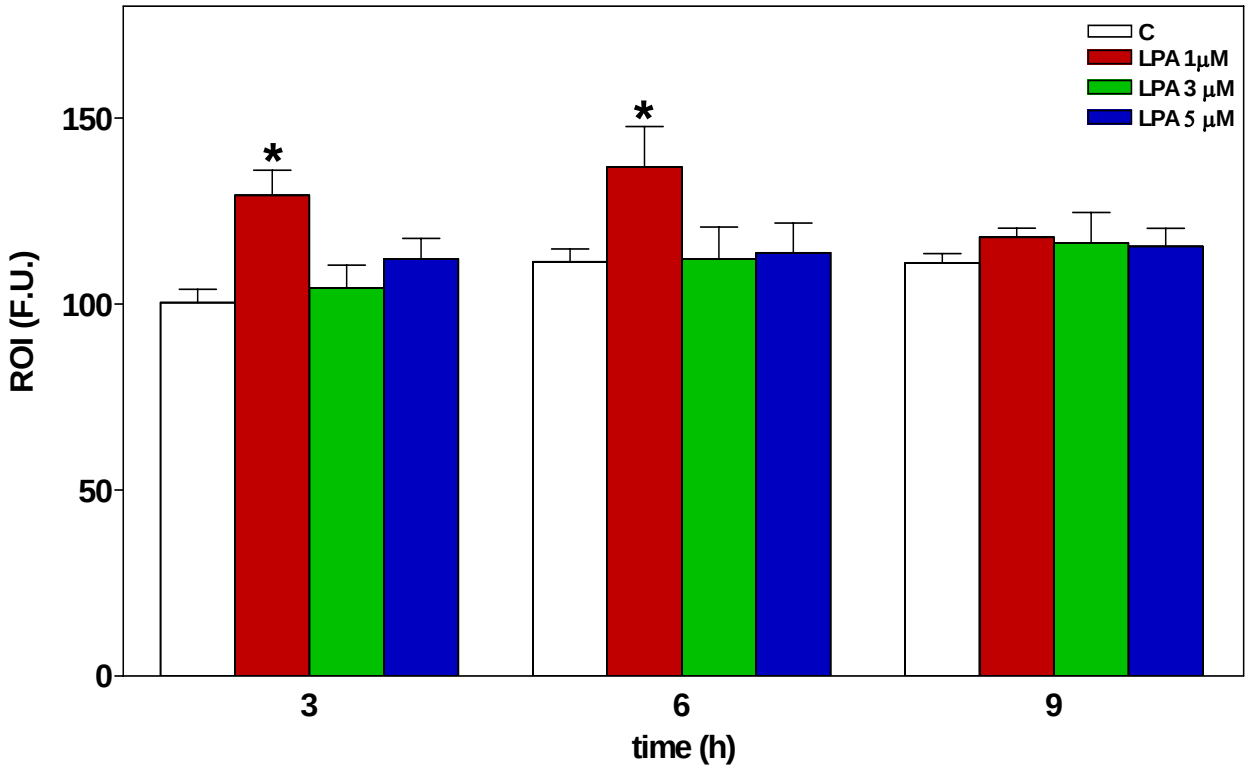


Fig.2. LPA effects on ROI production in THP-1 cells

THP-1 cells were labelled with DCF-DA (10 μM), as reported in the Materials and Methods Section, and ROI production was assessed after cell exposure to different LPA concentrations (1-5 μM) for 3, 6 and 9 h. Results are expressed as Fluorescence Intensity, reported as Fluorescence Units (F.U.), in respect to cells loaded with DCF-DA only (C). Data are reported as mean ± S.D. of 4 different experiments. *$P<0.05$, as reported from Student's t test in respect to untreated cells (C).

Several intracellular sources contribute to ROI generation in phagocytic cells; however, the enzyme complex primarly responsible for this production in monocytes is NADPHox complex (Babior, 1999).

For this reason, the possible involvement of NADPHox in LPA-induced ROI production was investigated. Since the maximal production of ROI occurred after 6 h of stimulation with 1 μM LPA, such a concentration and time were used in the experiments performed in the presence or absence of DPI, a specific inhibitor of NADPHox. As shown in Fig. 3a, cells pre-treatment with 10^{-8} M DPI significantly reduced the production of LPA-induced ROI in respect to untreated cells as further on confirmed by fluorescence microscopy analysis (Fig. 3b).

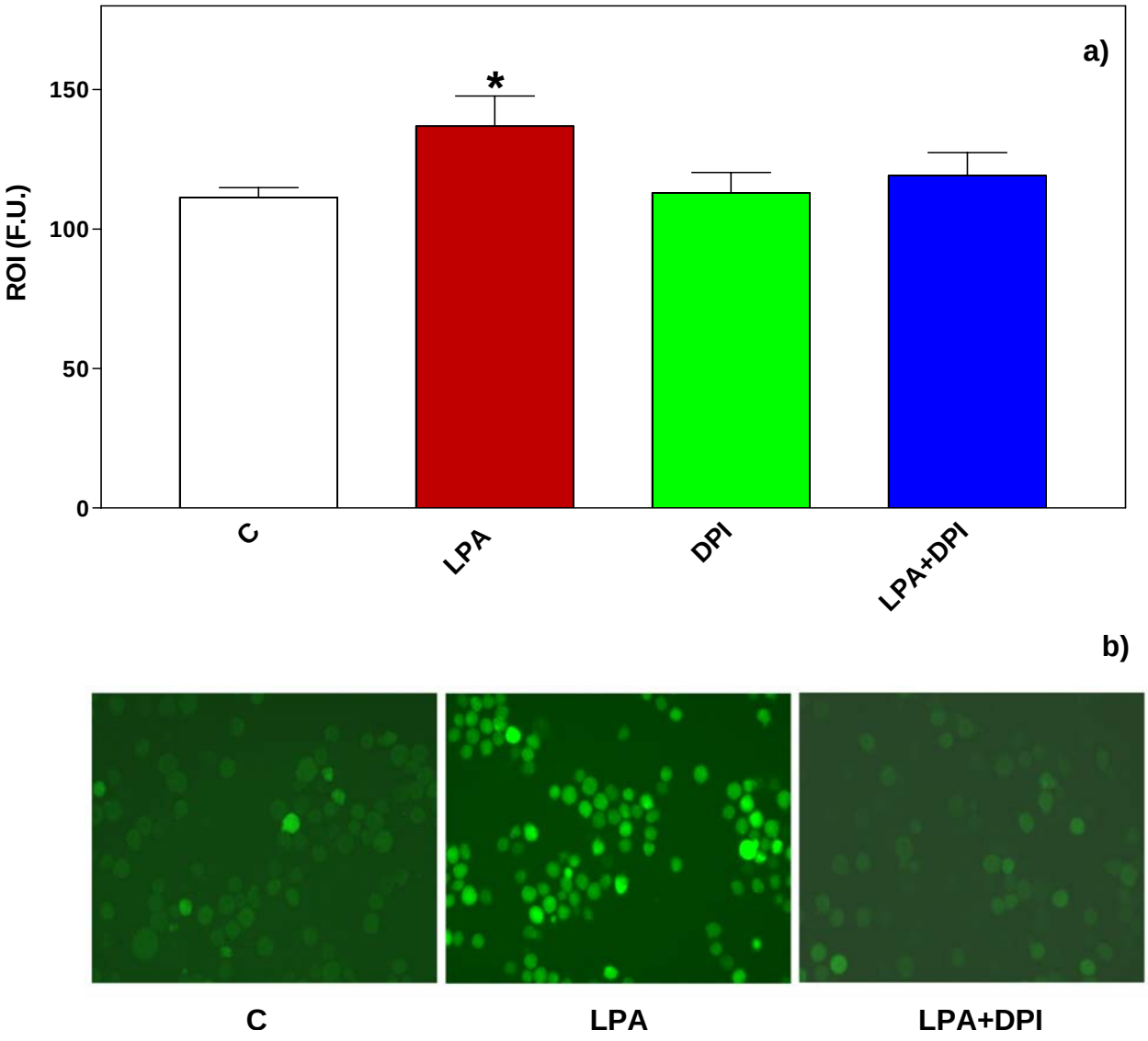


Fig.3. LPA effects on ROI production in THP-1 cells

THP-1 cells were labelled with DCF-DA (10 μM), as reported in the Materials and Methods Section, and ROI production was assessed after cell exposure to LPA (1 μM) for 6 h. In the experiments with DPI (10^{-8} M), cells were pre-treated with the inhibitor for 1 h before LPA addition. Results are expressed as Fluorescence Intensity, reported as Fluorescence Units (F.U.), in respect to cells loaded with DCF-DA only (C). (a) Micrographs were acquired as reported in the Materials and Methods Section. (b) Original magnification: x200. Data are reported as mean ± S.D. of 4 different experiments. *$P<0.05$, as reported from Student's t test in respect to untreated cells (C).

4.3. LPA promotes arachidonic acid, leukotriene B_4 and prostaglandin E_2 release

Monocytes activation also entails the stimulation of different phospholipase enzymes activity, among which are phospholipases A_2, that cleave membrane phospholipids to release arachidonic acid (AA) (Murakami et al., 1997). Since it is known that LPA can promote AA mobilization in certain cell types (Inoue et al., 1995; Pebay et al., 1999), experiments were performed to test the possible LPA ability to stimulate AA release in our cellular model. In the same way of cell growth and ROI production, assays were carried out after cell incubation for 6 h with different LPA concentrations (1-5 μM). Data shown in Fig. 4 demonstrate that all LPA concentrations exerted a significant effect on AA release, the maximal response occurring at 1 μM and progressively decreasing at higher lipid concentrations. These results indicated to assess LTB_4 and PGE_2 release in the presence of 1 μM LPA for 6 h.

Activated monocytes, in fact, play a pivotal role in inflammatory responses also by means of leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2) release, which are considered the principal arachidonic acid-derived lipid regulators (Busse, 1998; Morteau, 2000). As shown in Fig. 5, LPA was able to induce an approximately 15% increase in LTB_4 release (left panel), while PGE_2 production increase was approximately 70% (right panel) suggesting a more efficient activation of the enzymatic prostaglandin synthesizing machinery.

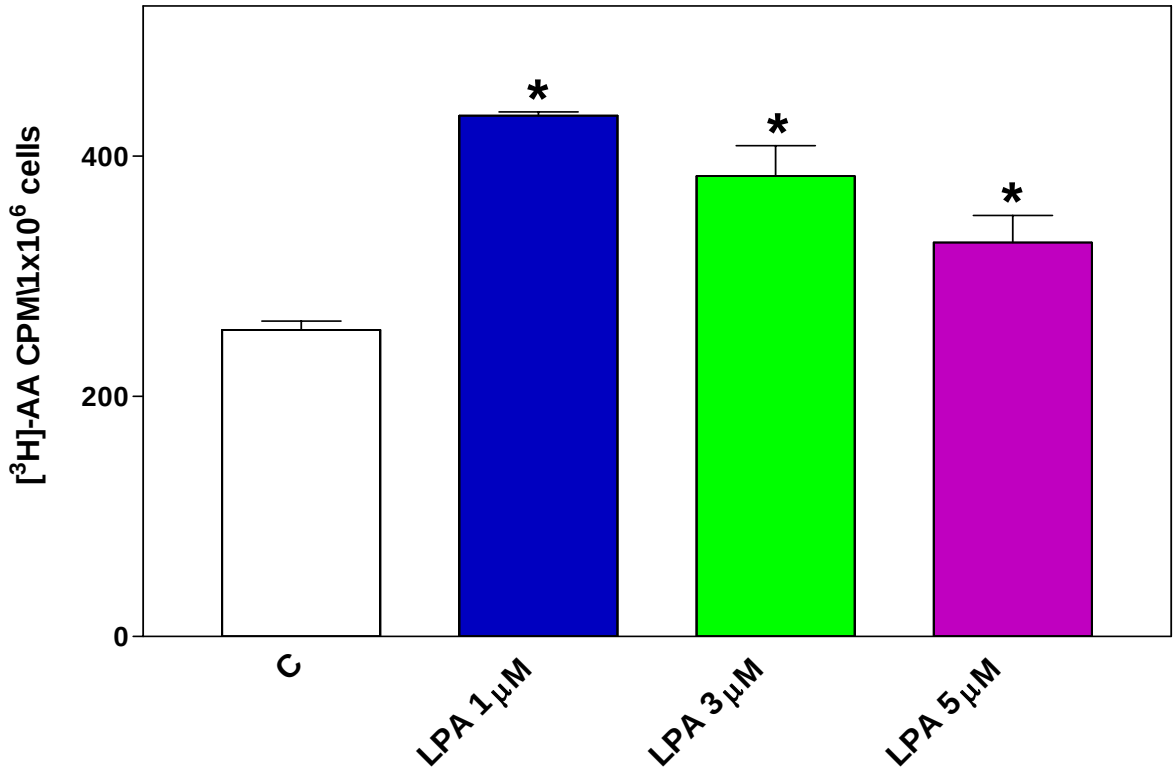


Fig.4. LPA effects on Arachidonic Acid (AA) release in THP-1 cells

THP-1 cells were incubated for 3 h with [^{3}H]-AA (1 μCi/ml), centrifuged, resuspended in serum-free medium and challenged with different LPA concentrations (1-5 μM) for 6 h. The [^{3}H]-AA released was determined as reported in Materials and Methods Section. Data are reported as mean ± S.D. of 4 different experiments. *$P<0.05$, as reported from Student's t test in respect to untreated cells (C).

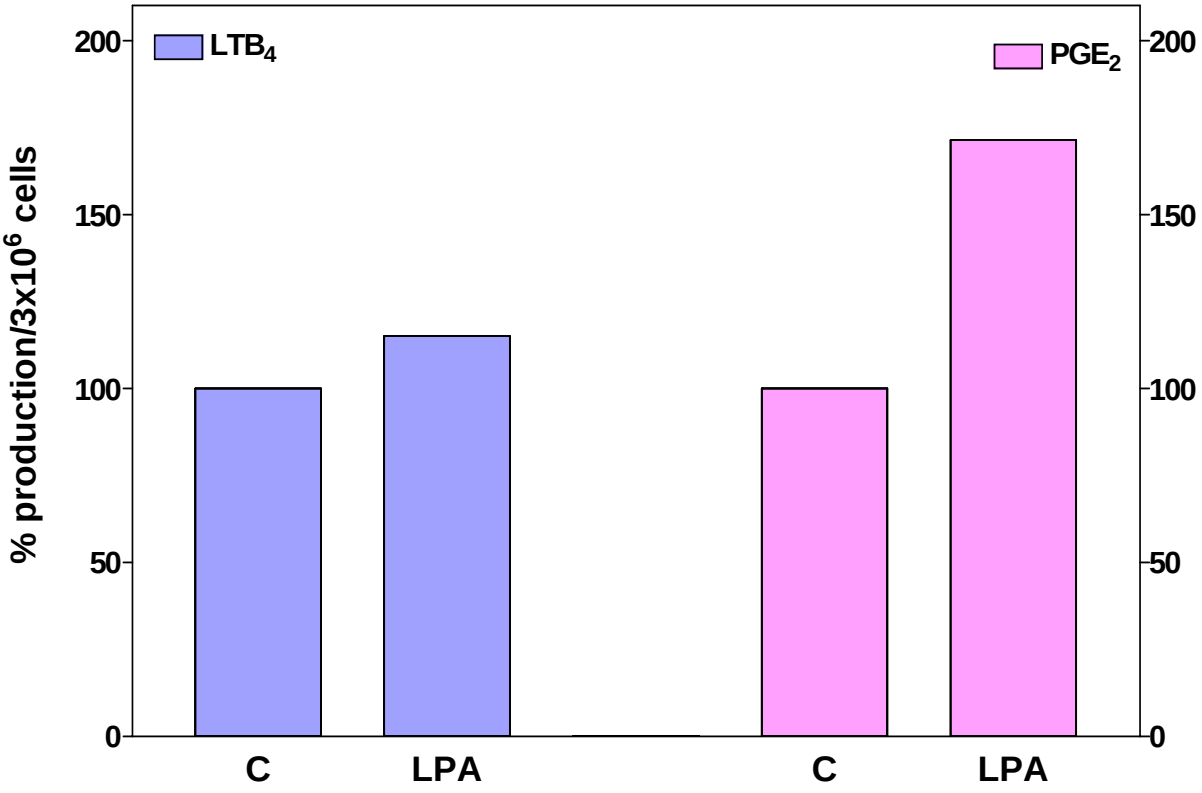


Fig.5. LPA effects on Leukotriene B_4 (LTB_4) and Prostaglandin E_2 (PGE_2) release in THP-1 cells

THP-1 cells were challenged with LPA (1 μM) for 6 h. LTB_4 and PGE_2 released into the medium was measured by EIA assay, as described in the Materials and Methods Section. A representative experiment is shown, which was repeated four additional times with similar results. Values are reported as percentile increase compared to control (C).

4.4. LPA effects on cell growth, ROI production, AA and PGE_2 release in the presence of Ki16425

LPA biological actions are exerted through the binding to and the activation of at least three distinct receptors, LPA_1, LPA_2 and LPA_3, differentially expressed in almost all cell types (Anliker and Chun, 2004b). In this context, we carried out experiments in the presence of Ki16425, a selective LPA_1 and LPA_3 receptor antagonist (Ohta et al., 2003), since these receptors have been currently correlated to cellular activation (Fueller et al., 2003) and malignant tumour proliferation (Nakamoto et al., 2005), respectively. As shown in Fig. 6, the enhanced LPA-induced [^{3}H]-thymidine incorporation was significantly inhibited by Ki16425 (10 μM) pre-treatment. Similarly, Ki16425 cell pre-treatment totally abolished LPA-stimulated ROI production (Fig. 7a), as also confirmed by fluorescence microscopy analysis (Fig. 7b).

In addition, the antagonist pre-treatment totally abrogated LPA effects on AA mobilization (Fig. 8a) and PGE_2 release (Fig. 8b). These results suggested that LPA_1 and LPA_3 receptors play a fundamental role in mediating LPA effects on THP-1 cell growth, ROI production and AA and PGE_2 release.

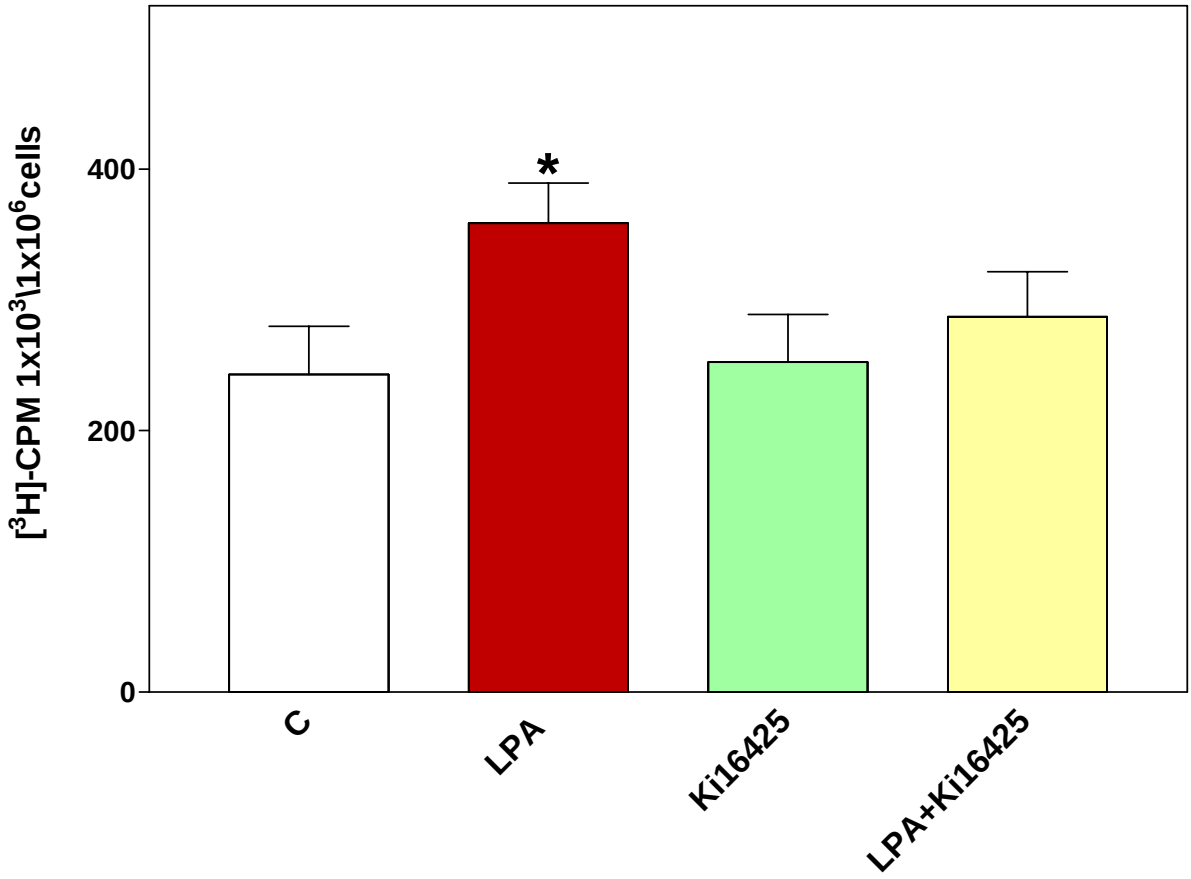


Fig.6. LPA effects on [^{3}H]-thymidine incorporation in the presence of Ki16425 in THP-1 cells

THP-1 cells were pre-treated with Ki16425 (10 μM) for 30 min and then challenged with LPA (1 μM) for 6 h. [^{3}H]-thymidine incorporation into DNA was assessed as reported in the Materials and Methods Section. Data are reported as mean ± S.D. of 4 different experiments. *$P<0.05$, as reported from Student's t test in respect to untreated cells (C).

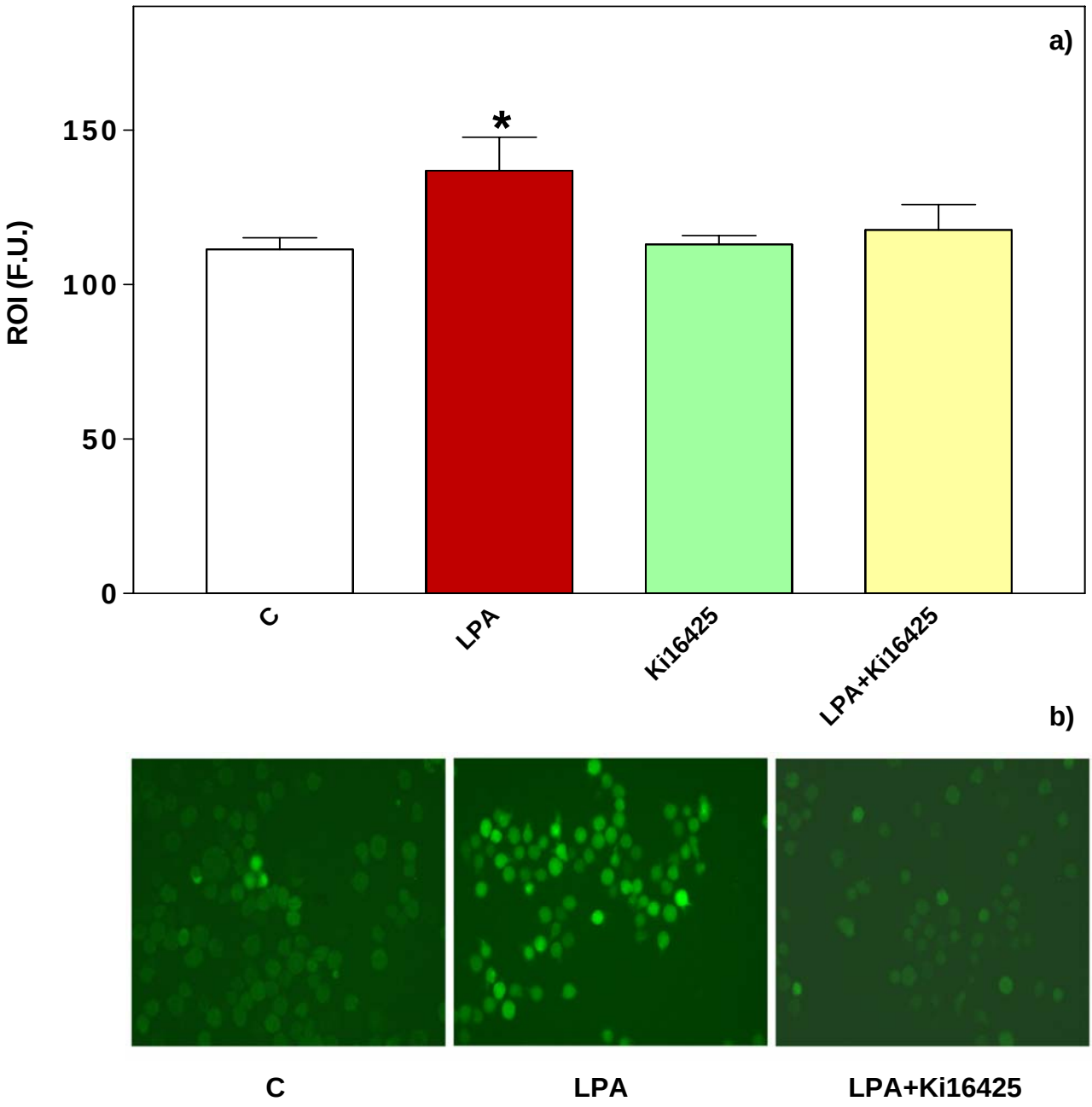


Fig.7. LPA effects on ROI production in the presence of Ki16425 in THP-1 cells

THP-1 cells were labelled with DCF-DA (10 μM), pre-treated with Ki16425 (10 μM) for 30 min and then challenged with LPA (1 μM) for 6 h. ROI production was assessed as reported in the Materials and Methods Section and expressed as Fluorescence Intensity, reported as Fluorescence Units (F.U.), respect to cells loaded with only DCF-DA (C). (a) Micrographs were acquired as reported in the Materials and Methods Section. (b) Original magnification: x200. Data are reported as mean ± S.D. of 4 different experiments. *$P<0.05$, as reported from Student's t test in respect to untreated cells (C).

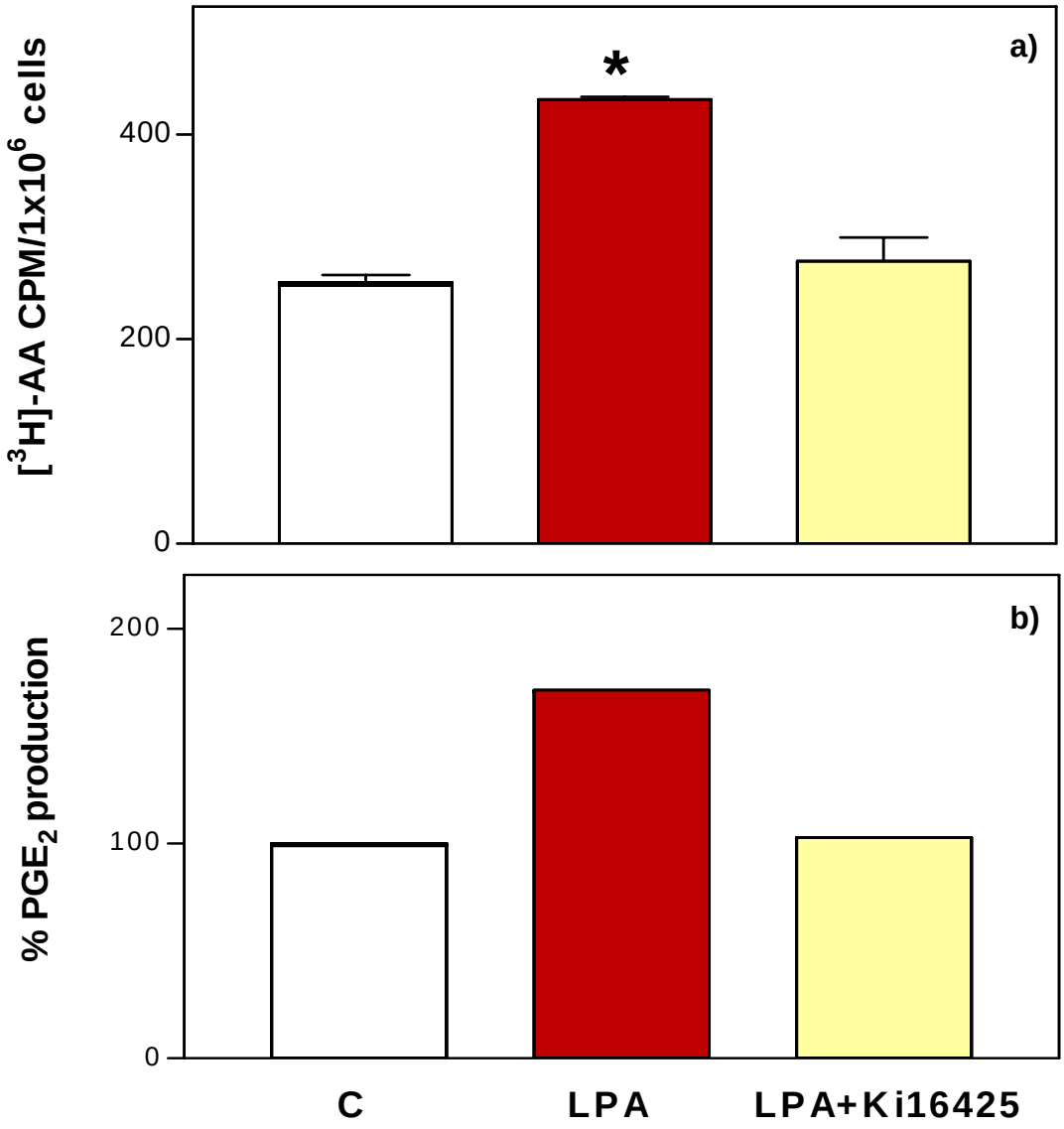


Fig.8. LPA effects on Arachidonic Acid (AA) and Prostaglandin E_2 (PGE_2) release in the presence of Ki16425 in THP-1 cells

a) THP-1 cells were treated as reported in Fig. 3a and challenged with LPA (1 μM) for 6 h after 30 min pre-treatment with Ki16425 (10 μM). The [^{3}H]-AA released was determined as reported in Materials and Methods Section. Data are reported as mean ± S.D. of 4 different experiments. *$P<0.05$, as reported from Student's t test in respect to untreated cells (C).

b) THP-1 cells were treated as reported in Fig. 3b after 30 min pre-treatment with Ki16425 (10 μM). PGE_2 released into the medium was measured by EIA assay, as described in Materials and Methods Section. A representative experiment is shown, which was repeated four additional times with similar results. Values are reported as percentile increase compared to control (C).

4.5. LPA effects on NO release in the presence of Ki16425

Monocytes can mediate their proinflammatory activity in inflamed sites even through NO production, an important mediator generated at high levels during inflammatory reactions (Kharitonov et al., 1994). To test the possibility that, besides ROI, LTB_4 and PGE_2, LPA could also stimulate NO production, THP-1 cells were challenged with 1 μM LPA for 6 h. As shown in Fig. 9, LPA was able to induce a significant increase in NO production (approximately 80%) in respect to untreated cells. Cell pre-treatment with Ki16425 (10 μM) totally abolished LPA-stimulated NO production, suggesting that LPA_1 and LPA_3 receptors might play a fundamental role also in mediating LPA effects on NO release.

4.6. LPA effects on receptor expression profile

Prolonged cell exposure to LPA can alter the receptor expression pattern (Baldini et al., 2005). Moreover, it is well documented that LPA-induced proinflammatory activation of human T-lymphocytes fundamentally depends on the expression profile and relative amount of each LPA receptor (Siess, 2002). Accordingly, PCR analysis was performed in an attempt to further discriminate the relative contribution of each receptor subtype in LPA effects. Our results, obtained by classical RT-PCR analysis (Fig. 10a) showed that all three LPA receptors are expressed in THP-1 cells; however, un-stimulated cells predominantly expressed LPA_1 and LPA_2 mRNAs, while LPA_3 mRNA was poorly expressed. After LPA (1 μM for 6 h) treatment, a significant increase in LPA_3 receptor mRNA levels occurred, while no significant variations in LPA_1 and LPA_2 mRNA levels were detectable.

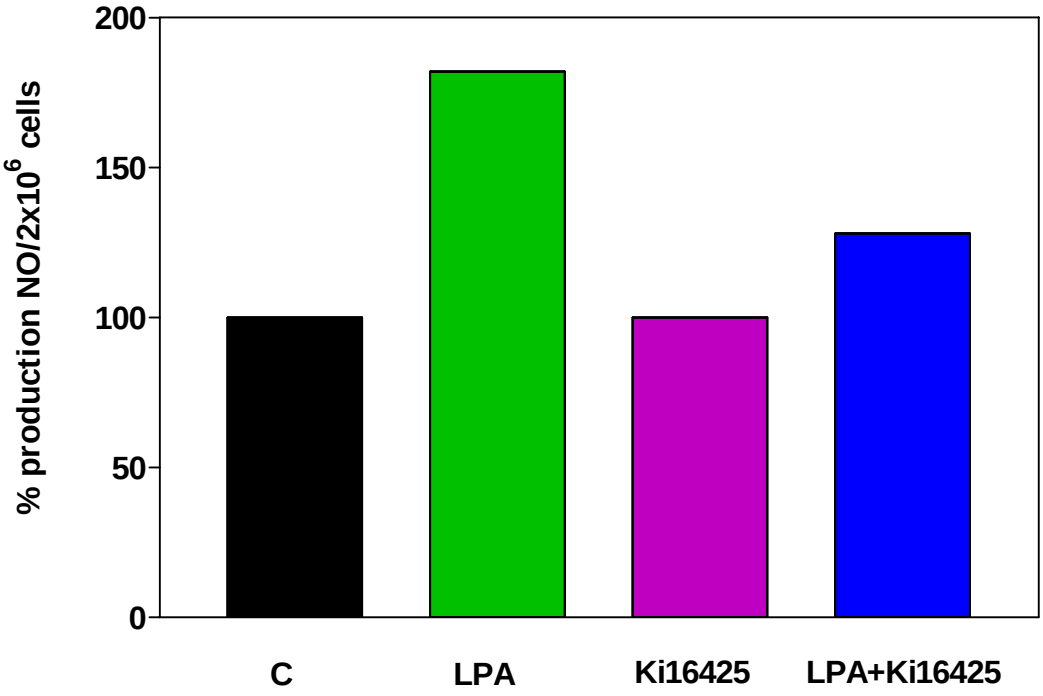
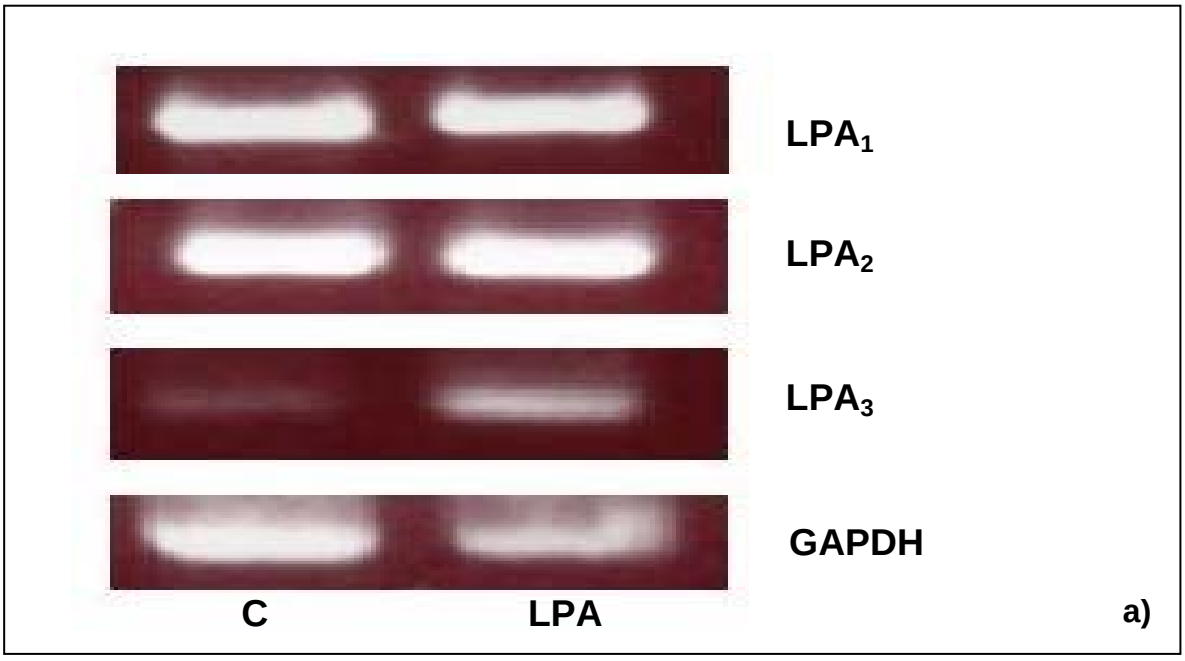


Fig.9. LPA effects on Nitric Oxide (NO) release in THP-1 cells

THP-1 cells were pre-treated with Ki16425 (10 μM) for 30 min and then challenged with LPA (1 μM) for 6 h. NO released into the medium was assessed as reported in the Materials and Methods Section. A representative experiment is shown, which was repeated four additional times with similar results. Values are reported as percentile increase compared to control (C).



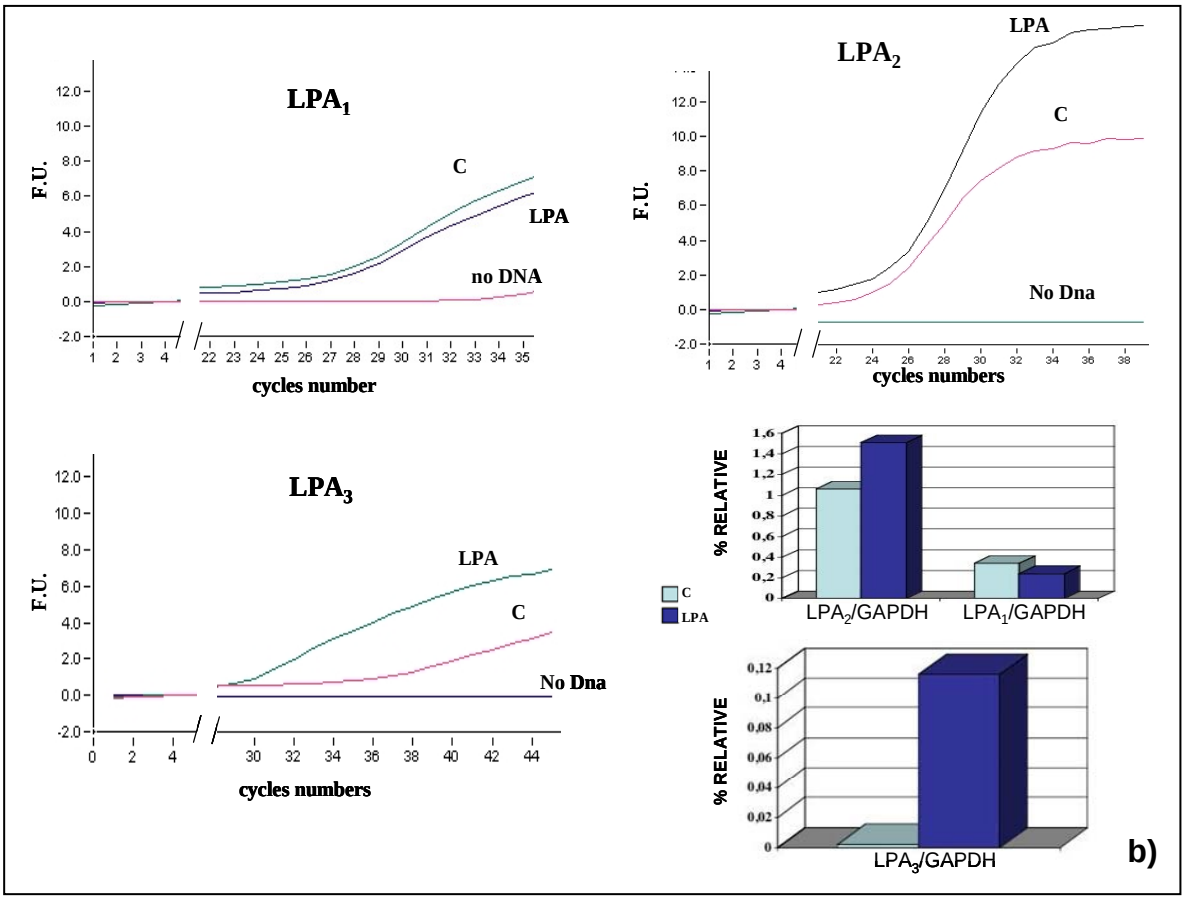


Fig.10. LPA effects on the expression of LPA_1, LPA_2 and LPA_3 receptors in THP-1 cells

Total RNA was extracted from THP-1 cells treated or not with LPA (1 μM) for 6 h. RT-PCR (a) and Real-Time RT-PCR (b) analysis were performed as reported in the Material and Methods Section. The expression levels of LPA receptors in THP-1 cells were quantified by normalising their respective mRNA levels with the housekeeping human GAPDH mRNA levels. A representative experiment is shown, which was repeated three additional times with similar results.

To finely quantify such an increase, Real-Time PCR analysis (Fig. 10b) were performed demonstrating that LPA addition induced a 50-fold up-regulation in the expression of LPA_3 receptor in respect to control, allowing us to hypothesize a pivotal role for this receptor in all the observed effects.

4.7. NADPH oxidase activation is required for LPA-stimulated DNA synthesis

Several evidences have shown that ROI can also act as second messengers modulating the activation of various intracellular signalling molecules and pathways and regulating a wide spectrum of cellular responses such as growth, differentiation, and apoptosis. Moreover, low ROI concentrations are known to be mitogenic and promote cell proliferation (Martindale and Holbrook, 2002). For this reason, we questioned whether NADPHox-derived ROI could be involved in LPA-induced THP-1 cell growth. As shown in Fig. 11, pre-treating cells with DPI (10^{-8} M) significantly abrogated LPA-stimulated [^{3}H]-thymidine incorporation in respect to untreated cells. These results suggest a NADPHox-produced ROI involvement in the mechanism by which LPA induces proliferation in THP-1 cells.

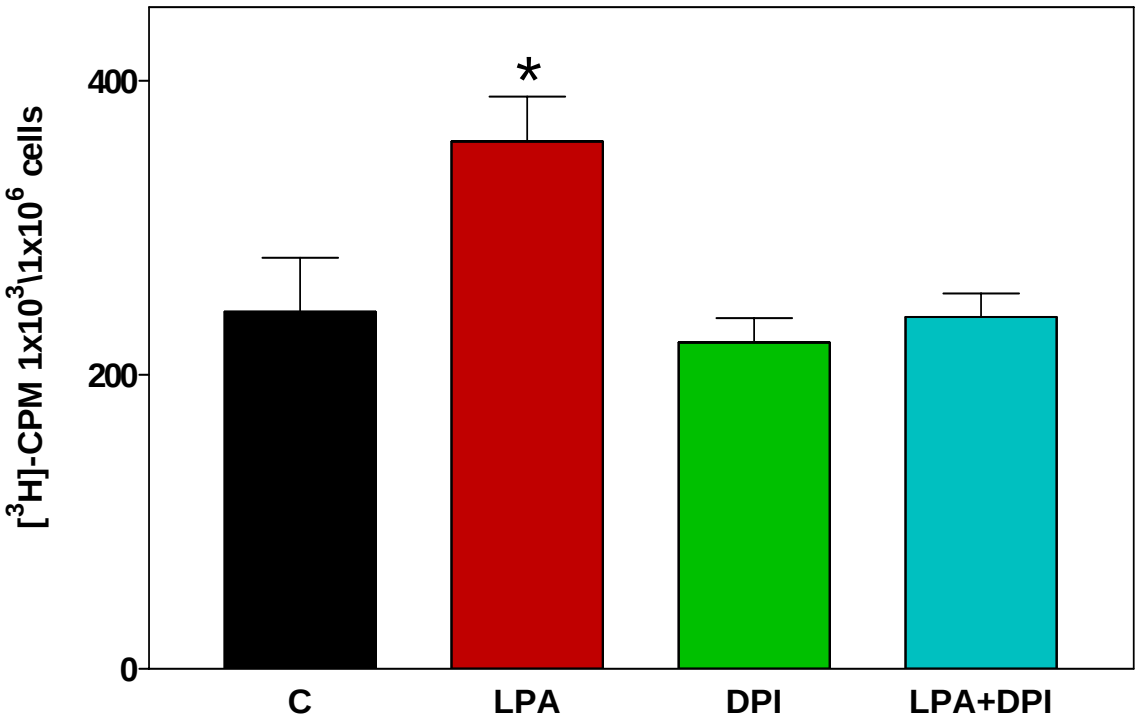


Fig.11. LPA effects on [^{3}H]-thymidine incorporation in the presence of DPI in THP-1 cells

THP-1 cells were pre-treated with DPI (10^{-8} M) for 30 min and then challenged with LPA (1 μM) for 6 h. [^{3}H]-thymidine incorporation into DNA was assessed as reported in the Materials and Methods Section. Data are reported as mean ± S.D. of 4 different experiments. *$P<0.05$, as reported from Student's t test in respect to untreated cells (C).

4.8. LPA effects on MAPKs phosphorylative status

LPA has been found to induce MAP kinases, particularly p38 and p42/44 MAPKs, activation in several cell types (Seewald et al., 1997; Reiser et al., 1998). Thus, we performed experiments to determine whether LPA could also stimulate MAPKs phosphorylation in THP-1 cells. 1 μM LPA effect at 3, 6, and 9 h, on p38 and p42/44 MAPKs activation was assayed by immunoblot using anti-phosphorylated p38 and p42/44 MAPKs antibodies.

Our results show that, at all times tested, LPA induced a detectable activation of p38 MAPK (Fig. 12a) that was statistically significant at 3 h and reached a maximum after 6 h stimulation. Similarly, LPA (1 μM) determined a significant p42/44 MAPKs activation after 3 h stimulation, reaching a maximum after 6 h (Fig. 12b).

4.9. MAPKs activation is required for LPA-induced cell proliferation and ROI production

Although MAPKs activation seems to play an important role in mediating many biological LPA activities linked to proliferation in different cell types (Seewald et al., 1997; Huang et al., 2000), their specific roles in cell proliferation are cell types and agonist dependent. For this reason, having observed LPA ability to trigger MAPKs activation in THP-1 cells, we evaluated their involvement in lipid-induced proliferation and ROI production using SB203580 and PD98059, specific inhibitors of p38 and p42/44 MAPKs, respectively. Fig. 13 shows that SB203580 or PD98059 alone didn't affect DNA synthesis, while pre-treating cells with both inhibitors was able to totally inhibit LPA-induced [^{3}H]-thymidine incorporation.

Parallely, experiments carried out to estimate ROI levels show that SB203580 or PD98059 alone didn’t affect ROI production while only SB203580 was able to totally inhibit ROI release (Fig. 14). Collectively, these results suggest that the activation of both MAPKs represents a critical step in the mechanism through which LPA stimulates the proliferation in THP-1 cells while only p38 MAPK is required for LPA-triggered ROI production.

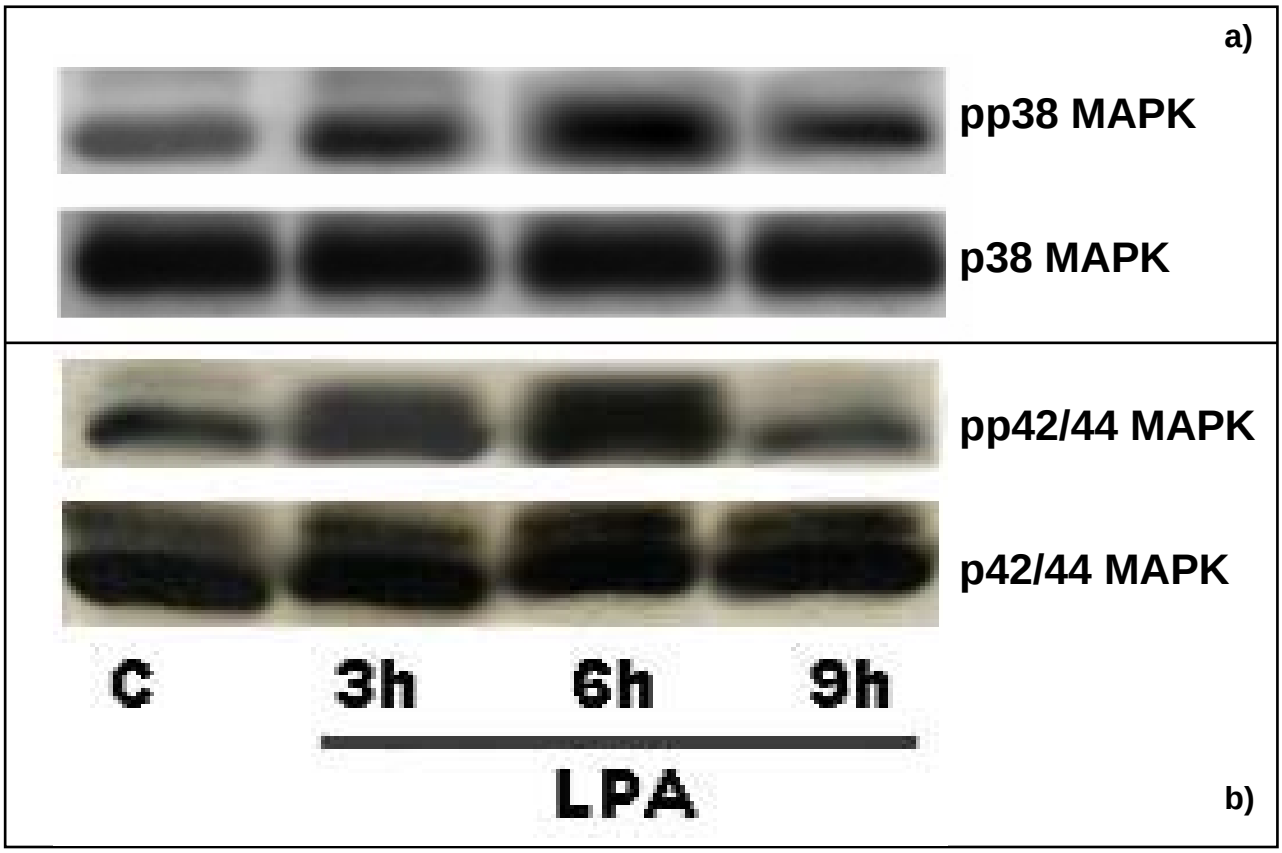


Fig.12. LPA effects on p38 and p42/44 MAP kinases phosphorylation in THP-1 cells

Cell lysates from THP-1 cells were separated by SDS-PAGE. Immunoblotting were performed as described in the Material and Methods Section. Each lane represents lysates from THP-1 cells challengend with LPA (1 μM) for different experimental times (3, 6 and 9 h) or not (C).

a) p38 MAP kinase activation; the figure shows a representative experiment repeated three additional times with similar results. *Upper row:* phosphorilated p38 MAP kinase (pp38 MAPK). *Lower row:* total p38 MAPK (p38 MAPK).

b) p42/44 MAP kinase activation; the figure shows a representative experiment repeated three additional times with similar results. *Upper row:* phosphorilated p42/44 MAP kinase (pp42/44 MAPK). *Lower row:* total p42/44 MAPK (p42/44 MAPK).

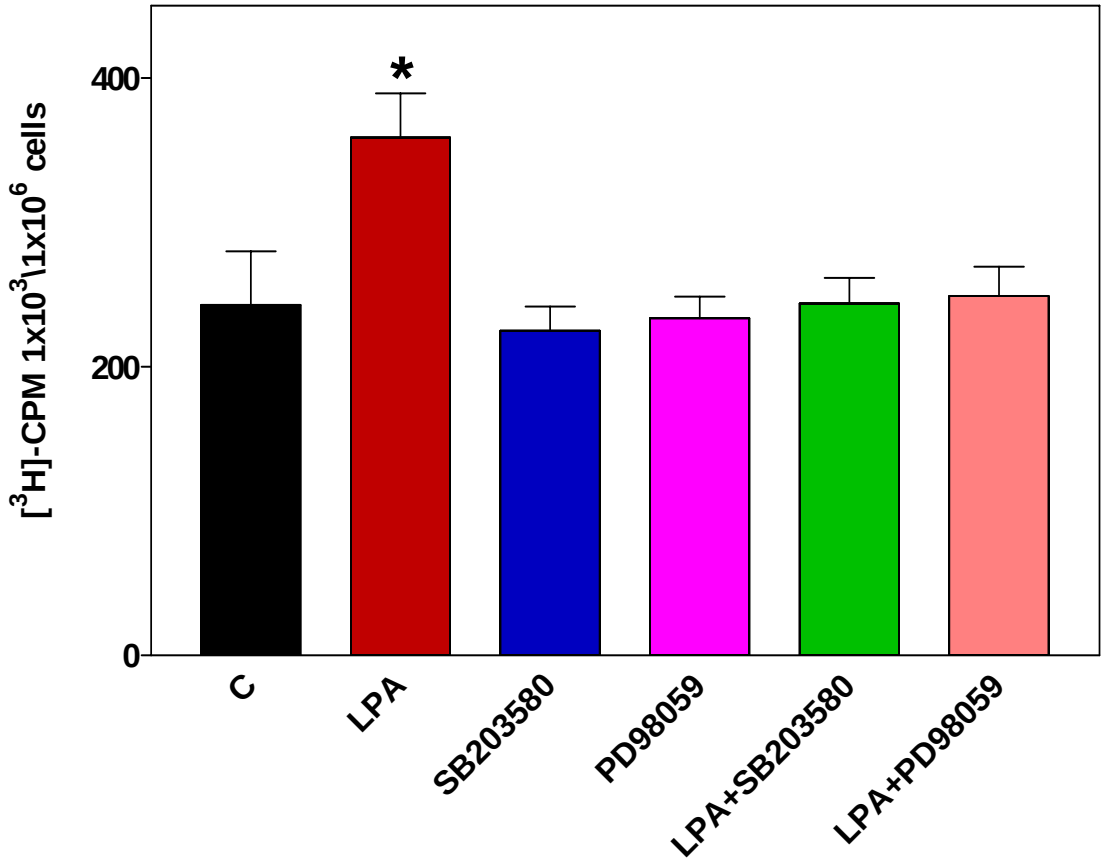


Fig.13. LPA effects on [^{3}H]-thymidine incorporation in the presence of PD98059 and SB203580 in THP-1 cells

THP-1 cells were pre-treated with PD98059 (50 μM) and SB203580 (10 μM) for 1 h and then challenged with LPA (1 μM) for 6 h. [^{3}H]-thymidine incorporation into DNA was assessed as reported in the Materials and Methods Section. Data are reported as mean ± S.D. of 4 different experiments. *$P<0.05$, as reported from Student's t test in respect to untreated cells (C).

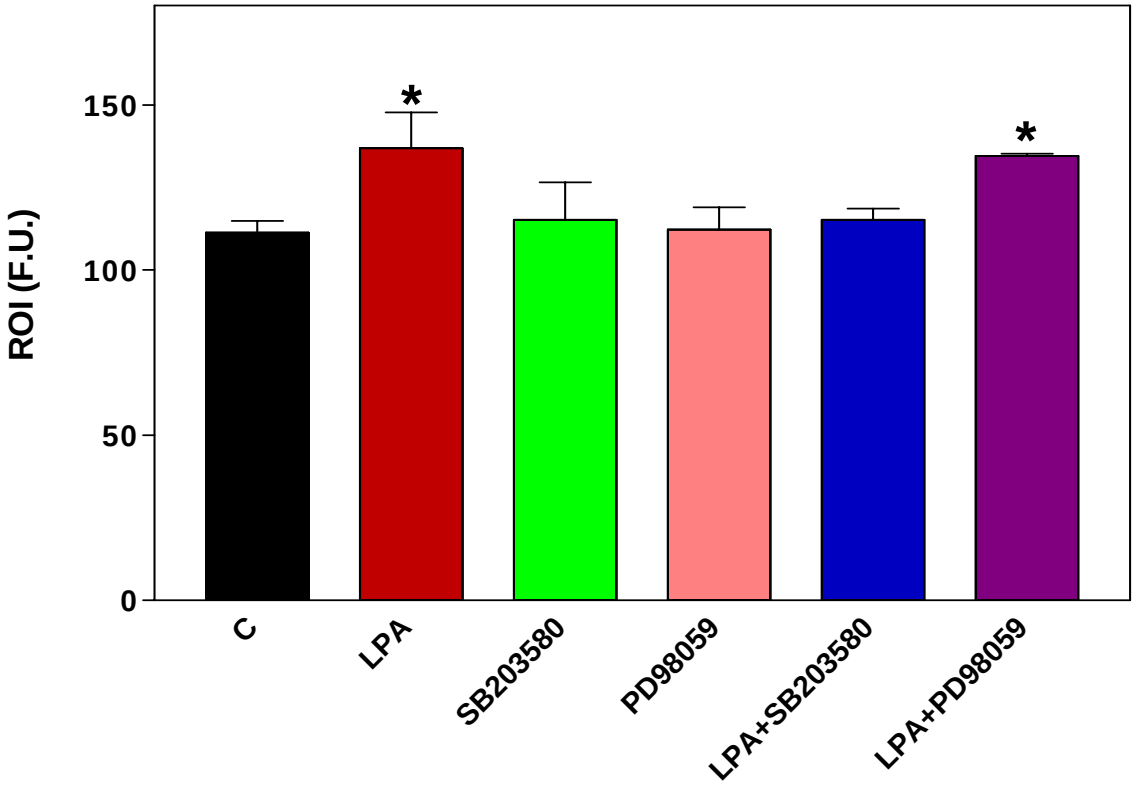


Fig.14. LPA effects on ROI production in the presence of PD98059 and SB203580 in THP-1 cells

THP-1 cells were labelled with DCF-DA (10 μM), pre-treated with PD98059 (50 μM) or SB203580 (10 μM) for 1 h and then challenged with LPA (1 μM) for 6 h. ROI production was assessed as reported in the Materials and Methods Section and expressed as Fluorescence Intensity, reported as Fluorescence Units (F.U.), respect to cells loaded with only DCF-DA (C). Data are reported as mean ± S.D. of 4 different experiments. *$P<0.05$, as reported from Student's t test in respect to untreated cells (C).

4.10. ROI production is requested for LPA-induced p42/44 MAPKs activation

Finally, in order to further evaluate reciprocal relationships between p38 MAPK, ERK and ROI in LPA signal transduction pathway leading to THP-1 cells proliferation, MAPKs phosphorilative status after DPI (10^{-8} M), a specific NADPH oxidase inhibitor, pre-treatment was determined. Fig. 15 evidences that pre-treating cells with DPI completely abolished LPA-induced ERK activation while p38 MAPK was unaffected, thus accounting for the ROI-primed ERK activation as a downstream event in LPA-activated signal transduction pathway. Taken together, these results clearly demonstrate p38 MAPK involvement in LPA-induced monocytes growth stimulation, pointing out its crucial role as an upstream activator of NADPH oxidase. Moreover, they indicate p42/44 MAPKs as the ending point of a signal transduction pathway involving NADPH oxidase activation and subsequently ROI production leading to proliferation of THP-1 monocytic cells in response to LPA.

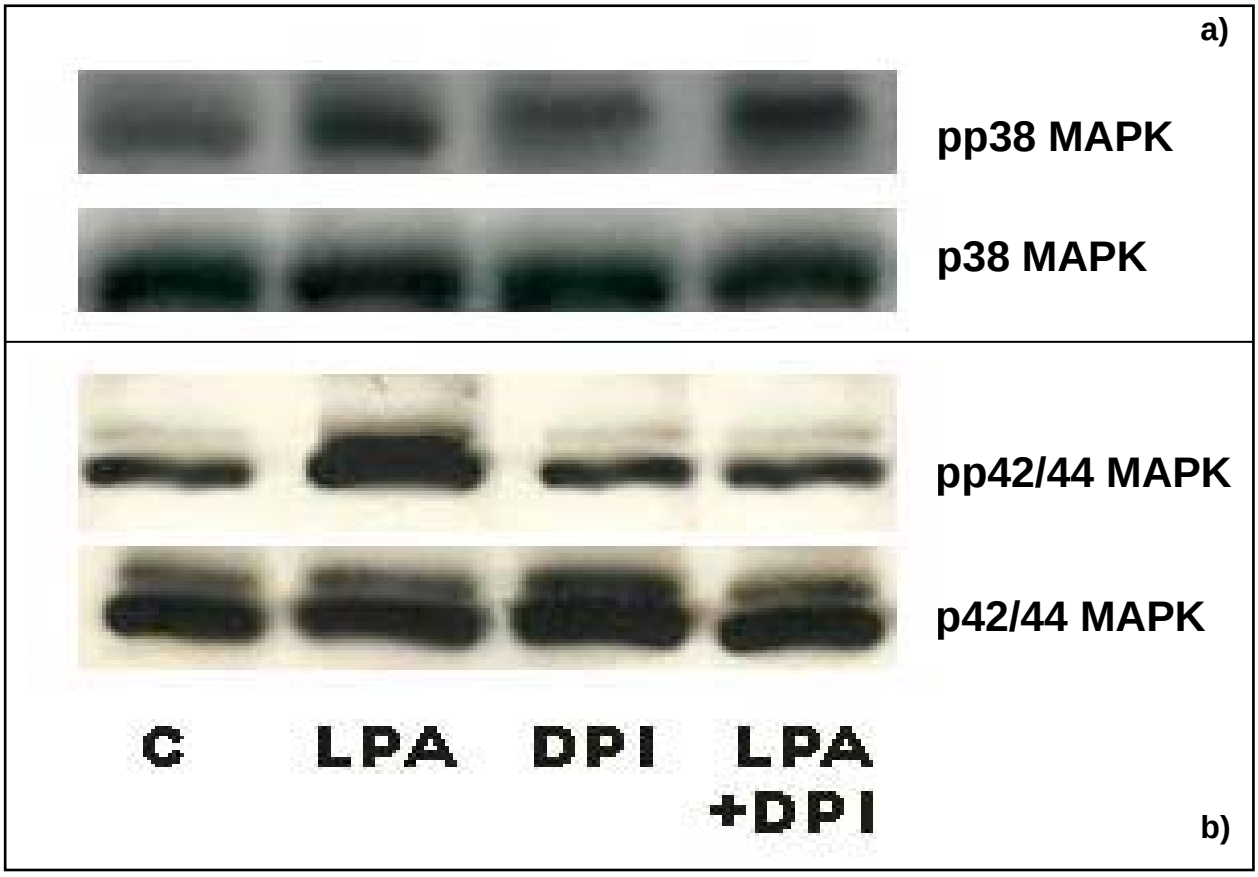


Fig.15. LPA effects on p38 and p42/44 MAP kinases phosphorylation in THP-1 cells in the presence of DPI

Cell lysates from THP-1 cells were separated by SDS-PAGE. Immunoblotting were performed as described in the Material and Methods Section. Each lane represents lysates from THP-1 cells challengend with LPA (1 μM) for 6 h in the presence or absence of DPI (10^{-8} M).

a) p38 MAP kinase activation; the figure shows a representative experiment repeated three additional times with similar results. *Upper row:* phosphorilated p38 MAP kinase (pp38 MAPK). *Lower row:* total p38 MAPK (p38 MAPK).

b) p42/44 MAP kinase activation; the figure shows a representative experiment repeated three additional times with similar results. *Upper row:* phosphorilated p42/44 MAP kinase (pp42/44 MAPK). *Lower row:* total p42/44 MAPK (p42/44 MAPK).

5. DISCUSSION

The present study represents the first characterization of lysophosphatidic acid (LPA) proinflammatory properties in cultured human THP-1 monocytes. Excessive cell proliferation and activation are important processes involved in the inflammatory response to endothelial injuries (Andres and Castro, 2003). Though these processes were initially believed to exclusively occur in smooth muscle cells, it cannot be a priori excluded that monocytes could display a similar aptitude. At present, in fact, they are known to also influence inflammatory cells including T-lymphocytes and monocytes/macrophages (Ross, 1999). Particularly, monocytes are considered major effector cells in several important pathologies characterized by chronic inflammation and their number can be augmented by various activating agents (Pakala and Benedict, 1999). In this context, several substances that are locally released by different activated cell types in the inflamed sites have been demonstrated to affect both cellular proliferation and activation (Rakesh and Agrawal, 2005). Among these, LPA, one of the most attractive phospholipid mediator endowed with pleiotropic activities, might potentially be of great interest resulting to be a powerful mitogenic and activating factor even for immune cells (Panther et al., 2002). Several lines of evidence, in fact, suggest a role for LPA in both proliferation and activation of T-cells (Wang et al., 2004) and B-cells (Rosskopf et al., 1998) and in neutrophils and monocytes recruitment and adhesion to the endothelium (Rizza et al., 1999). However, although LPA is acknowledged to induce both chemotactic and haptotactic migration (Zhou et al., 1995) and to stimulate Ca^{2+} influx (Fueller et al., 2003) in human monocytes, the exact role that the lipid

carries out on monocyte proinflammatory activation and regulation remains to be elucidated.

The present study, for the first time, indicates that micromolar concentrations of LPA are able to stimulate THP-1 monocyte cell growth, evidencing the mitogenic ability of the lipid. Even though monocytes are not accounted for a significant proliferative potential in the absence of sufficient stimuli (Cheung and Hamilton, 1992) or for a high-rate proliferation when seeded at low densities which are considered to be similar to an *in vivo* situation (Antonov et al., 1997), the observed significant increase in proliferation is undoubtedly due to LPA stimulation, although it could be amplified by other collateral factors. The first of these factors is that THP-1 cells are leukaemic monocytes, which would then be more prone to proliferate than peripheral monocytes and possibly more responsive to the lipid than others. The second factor is that THP-1 cells were seeded at high density, suggesting that the presence of autocrine/paracrine factors released from activated cells could empower LPA effects. For this reason, the present should be considered as a preliminary study to be extended to other cell types, such as peripheral monocytes.

One of the most immediate monocyte responses to a variety of activating stimuli is the activation of different phospholipases including phospholipases A_2 (PLA_2) which cleave membrane phospholipids to release arachidonic acid (Murakami et al., 1997). At least two different metabolic pathways proceed from free arachidonic acid: the first pathway is controlled by cycloxygenases (COXs) and generates prostaglandins (PGs), while the latter is controlled by lipoxygenases (LOXs) and produces leukotrienes (LTs) (Funk, 2001). Both pathways exert a key role in inflammatory reactions occurring at inflamed sites, determining the

production of strong local and systemic mediators responsible for leukocyte recruitment and activation as well as tissue response (Vila, 2004; Claria and Romano, 2005). It has been variously reported that a positive factor in PGs and LTs synthesis is represented by an increase in the cytosolic oxidant power (Lu and Wahl, 2005). This is generally due to an enhancement in ROI production, whose primary source differs depending on cell type (Martindale and Holbrook, 2002). In phagocytes, NADPHox enzyme complex represents the principal ROI source (Cathcart, 2004). Upon activation, this enzyme catalyzes hydrogen peroxide (H_2O_2) production, which in turn participates to both inflammation induction and enzyme activity regulation (Nagata, 2005; Allen and Tresini, 2000). Moreover, ROI release at inflamed site causes a broad activation of different cell types, mostly determining the pathological course of reactions in that site (Lassegue and Griendling, 2004; Lu and Wahl, 2005). In this context, the observed LPA ability to increase both PGs and LTs production, particularly exerting a stronger effect on PGE_2 synthesis rather than LTB_4, and to determine NADPHox-dependent ROI production and a remarkable NO release accounts for a possible role of this lipid in the pathogenesis of different diseases characterized by the development of local inflammatory reactions. Moreover, these data are substantiated by the demonstration that these LPA effects occurred at the same concentration and timepoint determining a remarkable increase in cell proliferation.

Besides being normally produced during the phagocyte “respiratory burst” as a defense mechanism against pathogens, ROI are also known to serve as intracellular signalling molecules and to regulate multiple basic cell functions such as growth, differentiation, apoptosis, and proliferation, acting at

transductional and transcriptional level (Martindale and Holbrook, 2002). Recently, using rat aortic smooth muscle cells as a model, we demonstrated LPA ability to promote cell proliferation through NADPHox activation and subsequent ROI production (Baldini et al., 2005). Interestingly, our studies on possible signal transduction mechanisms involved in LPA-induced growth of THP-1 cells demonstrate that, even in such a cell type, the proliferative response is totally dependent on NADPHox activation and ROI production, being absent after DPI, a specific NADPH oxidase inhibitor, pre-treatment.

A mild ROI production is then likely to be considered as a key step in such a signal transduction pathway, due to its ability to induce sulphide potential variations leading to several effectors activation. Among these, MAPKs are known to act as a convergence point for mitogenic signals originating from a variety of extracellular sources (Cobb et al., 1994). Particularly, it is generally thought that both p38 and p42/44 MAPKs mediate the proliferative effects of LPA in several cell types (Seewald et al., 1997; Huang et al., 2000) but direct evidence for their involvement in LPA-induced cell growth in THP-1 monocytes, has still not been reported. To this regard, when LPA-dependent signal transduction system activation was examined using specific MAPKs inhibitors, it clearly resulted that both p38 and p42/44 MAPKs were necessary to induce proliferation. Moreover, sustained phosphorylation is generally required for ERK translocation to the nucleus and subsequent proliferation-related genes transcription (Karin and Hunter, 1995); this could probably determine, in our cellular model, the observed proliferative effect.

Subsequently, the experiments performed in order to further evaluate the reciprocal relationships between p38 MAPK, ERK and ROI indicated that

p42/44 MAPKs are the ending point of a signal transduction pathway involving p38 MAPK and NADPHox activation leading to LPA-induced cell growth in THP-1 monocytic cells. Coherently with literature and our previous works (Baldini et al., 2005), therefore, our findings suggest that ROI production represents a fundamental step in stimulating LPA-dependent p42/44 MAPK activation, as confirmed by the total lack of activation of these kinases when cells were pretreated with DPI. Moreover, several evidences account for p38 MAPK ability to activate NADPHox thus subsequently determining ROI production increase. Our results suggest p38 MAPK involvement in LPA-induced monocytes growth stimulation, pointing out its crucial role as an upstream activator of NADPHox.

Finally, to refine our analysis, we tried to determine which receptor subtype could be mostly involved in mediating LPA effects. LPA, in fact, regulates a broad variety of biological processes by binding to specific receptors, principally LPA_1, LPA_2 and LPA_3, which, in turn, activate pleiotropic signalling pathways (Anliker and Chun, 2004b). To date, however, the relative contributions of these receptors and their differential signalling functions in the diverse biological activities of LPA remain poorly defined.

Since previous studies demonstrated that LPA_1 receptor stimulation was critical for monocyte activation (Fueller et al., 2003) and LPA_3 receptor was responsible for malignant tumours growth and proliferation (Nakamoto et al., 2005), experiments were carried out in the presence of Ki16425, a selective LPA_1 and LPA_3 receptors antagonist (Ohta et al., 2003). Results demonstrated that both receptors were involved in mediating LPA effects, since these were totally abolished by inhibitor addition. Preincubating cells with Ki16425, in fact, a significant inhibition of LPA-induced THP-1 proliferation, ROI and NO

production as well as AA release and PGE_2 production occurred. Since LPA pro-inflammatory action on human T-lymphocytes crucially depends on the individual expression of LPA receptors (Siess, 2002) and a prolonged exposure to mitogens can alter such an expression profile (Baldini et al., 2005), it is possible that, in THP-1 cells, the abundance of specific LPA receptors may significantly differ in resting vs activated cells. To further unravel the relative contribute of each receptor subtype in LPA effects, PCR analysis were performed demonstrating that the lipid caused a 50-fold increase in LPA_3 receptor mRNA expression, while the other receptors did not undergo any significant change. Although further specifically designed investigations will be needed to exhaustively clarify the role of each receptor isoform, these findings suggest a pivotal role for LPA_3 receptor in mediating LPA effects in THP-1 cells even though the relative contribute of LPA_1 receptor cannot be excluded.

Taken together, our results demonstrated that LPA can modulate early THP-1 cells proinflammatory activation. This allows us to hypothesize that locally-released LPA, acting as an autocrine/paracrine factor, could sustain an ongoing complex cascade of inflammatory reactions ultimately leading to a pathological chronic condition.

Moreover, our study demonstrates, for the first time, the existence of a specific LPA-dependent signal transduction pathway involving NADPHox, ROI, and MAPKs cascade activation which seem to be critical for the proliferative response of THP-1 monocytic cells to LPA. These results, therefore, not only identify lysophosphatidic acid as a THP-1 monocyte growth factor, but also furnishe new and further evidences on LPA proinflammatory properties, whose

understanding might help us to develop novel therapeutic strategies to control, treat and possibly prevent monocyte-related human diseases in the future.

6. LIST OF ABBREVIATIONS

AA	Arachidonic acid
AC	Adenylate cyclase
AGPAT	1-acylglycerol-3-phosphate acyltransferase
APCs	Antigen-presenting cells
BAL	Bronchoalveolar lavage
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary desossiribonucleic acid
COXs	Cycloxygenases
COX-1	Cycloxygenase-1
COX-2	Cycloxygenase-2
DAG	Diacylglycerol
DAGK	Diacyglycerol kinase
DCF-DA	Dichlorofluorescein diacetate
DCs	Dendritic cells
DGPP	Diacylglycerol pyrophosphate
DHPA	Dihydroxy acetone phosphate
DNA	Desossiribonucleic acid
dNTP	Deoxynucleotide triphosphate
DPI	Diphenylene iodinium
EDGs	Endothelial cell differentiation genes
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis (2-aminoethylether)-N, N, N', N' - tetraacetic acid
EIA	Enzyme immunoassay

ERK	Extracellular signal- regulated protein kinases
FABPs	Fatty acid binding proteins
FBS	Foetal bovine serum
FU	Fluorescence units
G3P	Glycerol 3-phosphate
G3PAT	Glycerol 3-phosphate acyltransferase
GAPDH	Glyceraldehyde phosphate dehydrogenase
GEF	Guanosine diphosphate/ guanosine triphosphate exchange factor
GPRCs	G-protein coupled receptors
H_2O_2	Hydrogen peroxide
IgG	Immunoglobulin G
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-12	Interleukin-12
INF-γ	Interferon-γ
IP_3	Inositol- trisphosphate
LCAT	Lecithin-cholesterol acyltransferase
JNK	c-Jun NH_2-terminal kinases
LDL	Low density lipoproteins
LFABPs	Liver-type fatty acid binding proteins
LOXs	Lypoxygenases
5-LOX	5-lypoxygenase
LPA	Lysophosphatidic acid

LPAATs	LPA-acyltransferases
LPC	Lysophosphatidylcholine
LPLs	Lysophospholipids
LPP	Lipid phosphate phosphohydrolase
LPS	Lipopolysaccharide
LPS	Lysophosphatidylserine
LTB_4	Leukotriene B_4
LTs	Leukotrienes
LysoPLD	Lysophospholipase D
MAG	Monoacylglycerol
MAGK	Monoacylglycerol kinase
MAPKs	Mitogen-activated protein kinases
MCP-1	Monocyte chemotactic protein-1
MCs	Mast cells
MCSF	Macrophage colony stimulating factor
MEK-1	MAPK kinase-1
MGAT	Monoacylglycerolphosphate acyltransferase
MHC	Major histocompatibility complex
MIP-1β	Macrophage inflammatory protein-1 beta
MMPs	Matrix metalloproteinases
mPA-$PLA_{1\alpha}$	Membrane-associated phosphatidic acid -selective PLA_1 alpha
mPA-$PLA_{1\beta}$	Membrane-associated phosphatidic acid -selective PLA_1 beta
mRNA	Messenger ribonucleic acid
MTB	Mycobacterium tuberculosis
NADPHox	NADPH oxidase

$NaNO_2$	Sodium nitrite
NK	Natural killer cell
NO	Nitric oxide
NO_2	Nitrite
OD	Optical density
PA	Phosphatidic acid
PAP-2	Phosphatidate phosphatase type 2
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PC	Phosphatidylcoline
PE	Phosphatidylethanolamine
PGE_2	Prostaglandin E_2
PGs	Prostaglandins
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol-bisphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PLA	Phospholipase A
PLA_1	Phospholipase A_1
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PLs	Phospholipids
PMNs	Polymorphonuclear leukocytes
PPAR-γ	Peroxisome proliferator-activated receptor-γ

PS	Phosphatidylserine
PS-PLA$_1$	Phosphatidylserine-specific PLA$_1$
PTx	Pertussis toxin
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediates
SCF	Stem cell factor
SDS	Sodium dodecyl sulfate
RT-PCR	Reverse transcriptase- polymerase chain reaction
sPLA$_2$-IIA	Type IIA secretory PLA$_2$
SRE	Serum-response element
TAE	Tris-acetate-EDTA
TCA	Trichloroacetic acid
TG	Triacylglycerol
Th	T helper cells
Th1	T helper 1 cells
Th2	T helper 2 cells
TNF-α	Tumour necrosis factor-α
UPA	Urokinase plasminogen activator

7. REFERENCES

- ABBAS A.K., MURPHY K.M., SHER A. Functional diversity of helper T lymphocytes. Nature. 1996; 383(6603): 787-793.
- ALLEN R.G., TRESINI M. Oxidative stress and gene regulation. Free Radic. Biol. Med. 2000; 28(3):463-499.
- AN S.Z., BLEU T., HALLMARK O.G., GOETZL E.J. Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. J. Biol. Chem. 1998; 273(14):7906-7910.
- AN S.Z., BLEU T., ZHENG Y., GOETZL E.J. Recombinant human G protein-coupled lysophosphatidic acid receptors mediate intracellular calcium mobilization. Mol. Pharmacol. 1998; 54(5):881-888.
- ANDREADIS A.A., HAZEN S.L., COMHAIR S.A., ERZURUM S.C. Oxidative and nitrosative events in asthma. Free Radic. Biol. Med. 2003; 35(3):213-225.
- ANDRES V., CASTRO C. Antiproliferative strategies for the treatment of vascular proliferative disease. Curr. Vasc. Pharmacol. 2003; 1(1):85-98.
- **ANLIKER B., CHUN J. Cell surface in lysophospholipid signalling. Semin. Cell Dev. Biol. 2004a; 15(5):457-465.**
- **ANLIKER B., CHUN J. Lysophospholipid G protein-coupled receptors. J. Biol. Chem. 2004b; 279(20):20555-20558.**
- ANTONOV A.S., MUNN D.H., KOLODGIE F.D., VIRMANI R., GERRITY R. Aortic endothelial cells regulate proliferation of human monocytes in vitro via a mechanism synergistic with macrophage colony-stimulating factor. Convergence at the cyclin E/p27 (Kip1) regulatory checkpoint. J. Clin. Invest. 1997; 99(12):2867-2876.

- **AOKI J. Mechanisms of lysophosphatidic acid production. Semin. Cell Dev. Biol. 2004; 15(5):477-489.**
- AOKI J., TAIRA A., TAKANEZAWA Y., KISHI Y., HAMA K., KISHIMOTO T., MIZUNO K., SAKU K., TAGUCHI R., ARAI H. Serum lysophosphatidic acid is produced through diverse phospholipase pathways. J. Biol. Chem. 2002; 277(50):48737-48744.
- ASTIZ M., SAHA D., LUSTBADER D., LIN R., RACKOW E. Monocyte response to bacterial toxins, expression of cell surface receptors, and release of anti-inflammatory cytokines during sepsis. J. Lab. Clin. Med. 1996; 128(6):594-600.
- AUSTEN K.F., BOYCE J.A. Mast cell lineage development and phenotypic regulation. Leuk. Res. 2001; 25(7):511-518.
- **BABIOR B.M. NADPH oxidase: an update. Blood. 1999; 93:1464-1476.**
- BAGGA S., PRICE K.S., LIN D.A., FRIEND D.S., AUSTEN K.F., BOYCE J.A. Lysophosphatidic acid accelerates the development of human mast cells. Blood. 2004; 104(13):4080-4087.
- BAKER D.L., DESIDERIO D.M., MILLER D.D., TOLLEY B., TIGYI G.J. Direct quantitative analysis of lysophosphatidic acid molecular species by stable isotope dilution electrospray ionization liquid chromatography-mass spectrometry. Anal. Biochem. 2001; 292(2):287-295.
- BAKER D.L., UMSTOT E.S., DESIDERIO D.M., TIGYI G.J. Quantitative analysis of lysophosphatidic acid in human blood fractions. Ann. N. Y. Acad. Sci. 2000; 905:267-269.
- BAKER R.R., CHANG H. A metabolic path for the degradation of lysophosphatidic acid, an inhibitor of lysophosphatidylcholine

lysophospholipase, in neuronal nuclei of cerebral cortex. Biochim. Biophys. Acta. 2000; 1483(1):58-68.

- BALAZS L., OKOLICANY J., FERREBEE M., TIGYI G. Topical application of LPA accelerates wound healing. Ann. N. Y. Acad. Sci. 2000; 905:270-273.
- BALAZS L., OKOLICANY J., FERREBEE M., TOLLEY B., TIGYI G. Topical application of the phospholipid growth factor lysophosphatidic acid promotes wound healing in vivo. Am. J. Physiol. Regul. Integr. Comp. Physiol. 2001; 280(2):R466-R472.
- **BALDINI P.M., DE VITO P., D'AQUILIO F., VISMARA D., ZALFA F., BAGNI C., FIACCAVENTO R., DI NARDO P. Role of atrial natriuretic peptide in the suppression of lysophosphatydic acid-induced rat aortic smooth muscle (RASM) cell growth. Mol. Cell. Biochem. 2005; 272(1-2):19-28.**
- BANCHEREAU J., STEINMAN R.M. Dendritic cells and the control of immunity. Nature. 1998; 392(6673):245-252.
- BANDOH K., AOKI J., HOSONO H., KOBAYASHI S., KOBAYASHI T., MURAKAMI-MUROFUSHI K., TSUJIMOTO M., ARAI H., INOUE K. Molecular cloning and characterization of a novel human G-protein-coupled receptor, EDG7, for lysophosphatidic acid. J. Biol Chem. 1999; 274(39):27776-27785.
- BISHOP W.R., BELL R.M. Assembly of phospholipids into cellular membranes: biosynthesis, transmembrane movement and intracellular translocation. Annual. Rev. Cell Biol. 1988; 4:579-610.
- BOGDAN C., ROLLINGHOFF M., DIEFENBACH A. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. Curr. Opin. Immunol. 2000; 12(1):64-76.

- BRANDOLINI L., BERTINI R., BIZZARRI C., SERGI R., CASELLI G., ZHOU D., LOCATI M., SOZZANI S. IL-1 beta primes IL-8-activated human neutrophils for elastase release, phospholipase D activity, and calcium flux. J. Leukoc. Biol. 1996; 59(3):427-434.
- BRINDLEY D.N., WAGGONER D.W. Mammalian lipid phosphate phosphohydrolases. J. Biol. Chem. 1998; 273(38):24281-24284.
- BRZOZOWSKI T., KONTUREK P.C., KONTUREK S.J. KWIECIEN S., , SLIWOWSKI Z., PAJDO R., DUDA A., PTAK A., HAHN E.G. Implications of reactive oxygen species and cytokines in gastroprotection against stress-induced gastric damage by nitric oxide releasing aspirin. Int. J. Colorectal. Dis. 2003; 18(4):320-329.
- BUSSE W.W. Leukotrienes and inflammation. Am. J. Respir. Crit. Care. Med. 1998; 157(6 Pt 2):S210-S213.
- **CATHCART M.K. Regulation of superoxide anion production by NADPH oxidase in monocytes/macrophages: contributions to atherosclerosis. Arterioscler. Thromb. Vasc. Biol. 2004; 24(1):23-28.**
- CHEUNG D.L., HAMILTON J.A. Regulation of human monocyte DNA synthesis by colony-stimulating factors, cytokines, and cyclic adenosine monophosphate. Blood 1992; 79(8):1972-1981.
- CLARIA J., ROMANO M. Pharmacological intervention of cyclooxygenase-2 and 5-lipoxygenase pathways. Impact on inflammation and cancer. Curr. Pharm. Des. 2005; 11(26):3431-3447.
- COBB M.H., HEPLER J.E., CHENG M., ROBBINS D. The mitogen-activated protein kinases, ERK1 and ERK2. Semin. Cancer Biol. 1994; 5(4):261-268.

- COLEMAN J.W. Nitric oxide in immunity and inflammation. Int. Immunopharmacol. 2001; 1(8):1397-1406.
- CONTOS J.J., CHUN J. Complete cDNA sequence, genomic structure, and chromosomal localization of the LPA receptor gene, lpA1/vzg-1/Gpcr26. Genomics. 1998; 51(3):364-378.
- CONTOS J.J., CHUN J. Genomic characterization of the lysophosphatidic acid receptor gene, lp(A2)/Edg4, and identification of a frameshift mutation in a previously characterized cDNA. Genomics. 2000; 64(2):155-169.
- **CONTOS J.J., ISHII I., CHUN J. Lysophosphatidic acid receptors. Mol. Pharmacol. 2000; 58(6):1188-1196.**
- CROOKS S.W., STOCKLEY R.A. Leukotriene B4. Int. J. Biochem. Cell. Biol. 1998; 30(2):173-178.
- CUMMINGS R., PARINANDI N., WANG L., USATYUK P., NATARAJAN V. Phospholipase D/phosphatidic acid signal transduction: role and physiological significance in lung. Mol. Cell Biochem. 2002; 234-235(1-2):99-109.
- CUMMINGS R., ZHAO Y., JACOBY D., SPANNHAKE E.W., OHBA M., GARCIA J.G., WATKINS T., HE D., SAATIAN B., NATARAJAN V. Protein kinase Cdelta mediates lysophosphatidic acid-induced NF-kappaB activation and interleukin-8 secretion in human bronchial epithelial cells. J. Biol. Chem. 2004; 279(39):41085-41094.
- DAVIES P., BAILEY P.J., GOLDENBERG M.M., FORD-HUTCHINSON A.W. The role of arachidonic acid oxygenation products in pain and inflammation. Annu. Rev. Immunol. 1984; 2:335-357.
- DE CATERINA R., MAZZONE A., GIANNESSI D., SICARI R., PELOSI W., LAZZERINI G., AZZARA' A., FORDER R., CAREY F., CARUSO D.

Leukotriene B4 production in human atherosclerotic plaques. Biomed. Biochim. Acta. 1988; 47(10-11):S182-S185.

- DESREUMAUX P., CAPRON M. Eosinophils in allergic reactions. Curr. Opin. Immunol. 1996; 8(6):790-795.
- DONCHENKO V., ZANNETTI A., BALDINI P.M. Insulin-stimulated hydrolysis of phosphatidylcholine by phospholipase C and phospholipase D in cultured rat hepatocytes. Biochim. Biophys. Acta. 1994; 1222(3):492-500.
- DUBOIS R.N., ABRAMSON S.B., CROFFORD L., GUPTA R.A., SIMON L.S., VAN DE PUTTE L.B., LIPSKY P.E. Cyclooxygenase in biology and disease. FASEB J. 1998; 12(12):1063-1073.
- DUNLOP M.E., LARKINS L.G. Pancreatic islets synthetise phospholipids de novo from glicose via acyl-dihydroxyacetone phosphate. Biochem. Biophys. Res. Commun. 1985; 132(2):467-473.
- DUONG C.Q., BARED S.M., ABU-KHADER A., BUECHLER C., SCHIMTZ A., SCHIMTZ G. Expression of the lysophospholipid receptor family and investigation of lysophospholipid-mediated responses in human macrophages. Biochim. Biophys. Acta. 2004; 1682(1-3):112-119.
- DYER D., TIGYI G., MILEDI R. The effect of active serum albumin on PC12 cells: I. Neurite retraction and activation of the phosphoinositide second messenger system. Brain Res. Mol. Brain Res. 1992; 14(4):293-301.
- EICHHOLTZ T., JALINK K., FAHRENFORT I., MOOLENAAR W.H. The bioactive phospholipid lysophosphatidic acid is released from activated platelets. Biochem. J. 1993; 291(Pt 3):677-680.
- ETIENNE-MANNEVILLE S., HALL A. Rho GTPases in cell biology. Nature. 2002; 420(6916):629-635.

- EXTON J.H. Cell signalling through guanine-nucleotide-binding regulatory proteins (G proteins) and phospholipases. Eur. J. Biochem. 1997; 243(1-2):10-20.
- EXTON J.H. Regulation of phospholipase D. FEBS Lett. 2002; 531(1):58-61.
- FANG X., SCHUMMER M., MAO M., YU S.,TABASSAM F.H., SWABY R., HASEGAWA Y., TANYI J.L., LaPUSHIN R., EDER A., JAFFE R., ERICKSON J., MILLS G.B. Lysophosphatidic acid is a bioactive mediator in ovarian cancer. Biochim. Biophys. Acta. 2002; 1582(1-3):257-264.
- FANG X., YU S., LaPUSHIN R., LU Y., FURUI T., PENN L.Z., STOKOE D., ERICKSON J.R., BAST R.C. Jr, MILLS G.B. Lysophosphatidic acid prevents apoptosis in fibroblasts via G(i)-protein-mediated activation of mitogen-activated protein kinase. Biochem. J. 2000; 352(1):135-143.
- FOURCADE O., SIMON M.F., VIODE C., RUGANI N., LEBALLE F., RAGAB A., FOURNIE B., SARDA L., CHAP H. Secretory phospholipase A_2 generates the novel lipid mediator lysophosphatidic acid in membrane microvesicles shed from activated cells. Cell 1995; 80(6):919-927.
- FRATICELLI A., SERRANO C.V. Jr, BOCHNER B.S., CAPOGROSSI M.C., ZWEIER J.L. Hydrogen peroxide and superoxide modulate leukocyte adhesion molecule expression and leukocyte endothelial adhesion. Biochim. Biophys. Acta. 1996; 1310(3):251-259.
- **FUELLER M., WANG D.A., TIGYI G., SIESS W. Activation of human monocytic cells by lysophosphatidic acid and sphingosine-1-phosphate. Cell. Signal. 2003; 15(4):367-375.**
- **FUKUSHIMA N., CHUN J. The LPA receptors. Prostaglandins. 2001; 64(1-4):21-32.**

- **FUKUSHIMA N., ISHII I., CONTOS J.J., WEINER J.A., CHUN J. Lysophospholipid receptors. Annu. Rev. Pharmacol. Toxicol. 2001; 41:507-534.**
- FUNK C.D. Prostaglandins and leukotrienes: advances in eicosanoid biology. Science. 2001; 294(5548):1871-1875.
- GALLUCCI S., MATZINGER P. Danger signals: SOS to the immune system. Curr. Opin. Immunol. 2001; 13(1):114-119.
- **GARG S.K., VALENTE E., GRECO E., SANTUCCI M.B., DE SPIRITO M., PAPI M., BOCCHINO M., SALTINI C., FRAZIANO M. Lysophosphatidic acid enhances antimycobacterial activity both in vitro and ex vivo. Clin. Immunol. 2006; 121(1):23-28.**
- GATE L., PAUL J., BA G.N., TEW K.D., TAPIERO H. Oxidative stress induced in pathologies: the role of antioxidants. Biomed. Pharmacother. 1999; 53(4):169-180.
- **GENNERO I., XUEREB J.M., SIMON M.F., GIROLAMI J.P., BASCANDS J.L., CHAP H., BONEU B., SIE P. Effects of lysophosphatidic acid on proliferation and cytosolic Ca^{++} of human adult vascular smooth muscle cells in culture. Thromb. Res. 1999; 94(5):317-326.**
- **GOETZL E.J., KONG Y., VOICE J.K. Cutting edge: differential constitutive expression of functional receptors for lysophosphatidic acid by human blood lymphocytes. J. Immunol. 2000; 164(10):4996-4999.**
- **GOETZL E.J., WANG W., McGIFFERT G., HUANG M.C., GRALER M.H. Sphingosine 1-phosphate and its G protein-coupled receptors constitute a multifunctional immunoregulatory system. J. Cell. Biochem. 2004; 92(6):1104-1114.**

- **GRALER M.H., GOETZL E.J. Lysophospholipids and their G protein-coupled receptors in inflammation and immunity. Biochim. Biophys. Acta. 2002; 1582(1-3):168-174.**
- **GUZIK T.J., KORBUT R., ADAMEK-GUZIK T. Nitric oxide and superoxide in inflammation and immune regulation. J. Physiol. Pharmacol. 2003; 54(4):469-487.**
- HADJIGOGOS K. The role of free radicals in the pathogenesis of rheumatoid arthritis. Panminerva Med. 2003; 45(1):7-13.
- HALDAR D., VANCURA A. Glycerophosphate acyltransferase from liver. Methods enzymol. 1992; 209:64-72.
- HAMA K., BANDOH K., KAKEHI Y., AOKI J., ARAI H. Lysophosphatidic acid (LPA) receptors are activated differentially by biological fluids: possible role of LPA-binding proteins in activation of LPA receptors. FEBS Lett. 2002; 523(1-3):187-192.
- HANAHAN D., WEINBERG R.A. The hallmarks of cancer. Cell. 2000; 100(1):57-70.
- HECHT J.H., WEINER J.A., POST S.R., CHUN J. Ventricular zone gene-1 (vzg-1) encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex. J. Cell Biol. 1996; 135(4):1071-1083.
- HESS J.A., BUCHANAN F.G., RYDER S., EXTON J.H. Altered activation of phospholipase D by lysophosphatidic acid and endothelin-1 in mouse embryo fibroblasts lacking phospholipase C-gamma1. Cell. Signal. 2000; 12(1):37-45.
- HIRAMATSU T., SONODA H., TAKANEZAWA Y., MORIKAWA R., ISHIDA M., KASAHARA K., SANAI Y., TAGUCHI R., AOKI J., ARAI H. Biochemical

and molecular characterization of two phosphatidic acid-selective phospholipase A1s, mPA-PLA1 alpha and mPA-PLA1 beta. J. Biol. Chem. 2003; 278(49): 49438-49447.

- HORNUβ C., HAMMERMANN R., FUHRMANN M., JUERGENS U.R., RACKE K. Human and rat alveolar macrophages express multiple EDG receptors. Eur. J. Pharmacol. 2001; 429(1-3):303-308.
- HUANG J.S., CRILLY K.S., KISS Z. Ethanol enhances the stimulatory effects of lysophosphatidic acid on DNA synthesis but not cell proliferation in human and mouse fibroblasts. Arch. Biochem. Biophys. 2000; 381(2):328-324.
- **HUANG M.C., GRAELER M., SHANKAR G., SPENCER J., GOETZL E.J. Lysophospholipid mediators of immunity and neoplasia. Biochim. Biophys. Acta. 2002; 1582(1-3):161-167.**
- **IDZKO M., LAUT M., PANTHER E., SORICHTER S., DÜRK T., FLUHR J.W., HEROUY Y., MOCKENHAUPT M., MYRTEK D., ELSNER P., NORGAUER J. Lysophosphatidic acid induces chemotaxis, CD11b up-regulation, Ca^{2+} mobilization, and actin reorganization in human eosinophils via pertussis toxin-sensitive G proteins. J. Immunol. 2004; 172(7):4480-4485.**
- IM D.S., HEISE C.E., HARDING M.A., GEORGE S.R., O'DOWD B.F., THEODORESCU D., LYNCH K.R. Molecular cloning and characterization of a lysophosphatidic acid receptor, Edg-7, expressed in prostate. Mol. Pharmacol. 2000; 57(4):753-759.
- INOUE C.N., FORSTER H.G., EPSTEIN M. Effects of lysophosphatidic acid, a novel lipid mediator, on cytosolic Ca^{2+} and contractility in cultured rat mesangial cells. Circ. Res. 1995; 77(5):888-896.

- ISHII I., CONTOS J.J., FUKUSHIMA N., CHUN J. Functional comparisons of the lysophosphatidic acid receptors, LP(A1)/VZG-1/EDG-2, LP(A2)/EDG-4, and LP(A3)/EDG-7 in neuronal cell lines using a retrovirus expression system. Mol. Pharmacol. 2000; 58(5):895-902.
- **ISHII I., FUKUSHIMA N., YE X., CHUN J. Lysophospholipid receptors: signaling and biology. Annu. Rev. Biochem. 2004; 73:321-354.**
- ITAGAKI K., KANNAN K.B., HAUSER C.J. Lysophosphatidic acid triggers calcium entry through a non-store-operated pathway in human neutrophils. J. Leukoc. Biol. 2005; 77(2):181-189.
- JALINK K., HORDIJK P.L., MOOLENAAR W.H. Growth factor-like effects of lysophosphatidic acid, a novel lipid mediator. Biochim. Biophys. Acta. 1994; 1198(2-3):185-196.
- JALINK K., VAN CORVEN E.J., MOOLENAAR W.H. Lysophosphatidic acid, but not phosphatidic acid, is a potent Ca^{2+}-mobilizing stimulus for fibroblasts. Evidence for an extracellular site of action. J. Biol. Chem. 1990; 265(21):12232-12239.
- JASINSKA R., ZHANG Q.X., PILQUIL C., SINGH I., XU J., DEWALD J., DILLON D., BERTHIAUME L., CARMAN G., WAGGONER D.W., BRINDLEY D.R. Lipid phosphate phosphohydrolase-1 degrades exogenous glycerolipid and sphingolipid phosphate esters. Biochem. J. 1999; 340(3):677-686.
- **JIN Y., KNUDSEN E., WANG L., MAGHAZACHI A.A. Lysophosphatidic acid induces human natural killer cell chemotaxis and intracellular calcium mobilization. Eur. J. Immunol. 2003; 33(8):2083-2089.**
- JOHANN A.M., VON KNETHEN A., LINDEMANN D., BRÜNE B. Recognition of apoptotic cells by macrophages activates the peroxisome proliferator-

activated receptor-gamma and attenuates the oxidative burst. Cell Death Differ. 2006; 13(9):1533-1540.

- JONAS A. Lecithin cholesterol acyltransferase. Biochim. Biophys. Acta. 2000; 1529(1-3):245-256.
- KAMATA H., HIRATA H. Redox regulation of cellular signalling. Cell. Signal. 1999; 11(1):1-14.
- KANAHO Y., KANAHO K., SAITOH K., NOZAWA Y. Phospholipase D activation by platelet-activating factor, leukotriene B4, and formyl-methionyl-leucyl-phenylalanine in rabbit neutrophils. Phospholipase D activation is involved in enzyme release. J. Immunol. 1991; 146(10):3536-3541.
- KARIN M., HUNTER T. Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. Curr. Biol. 1995; 5(7):747-757.
- KHARITONOV S.A., YATES D., ROBBINS R.A., LOGAN-SINCLAIR R., SHINEBOURNE E.A., BARNES P.J. Increased nitric oxide in exhaled air of asthmatic patients. Lancet. 1994; 343(8890):133-135.
- KIMURA T., IWASE M., KONDO G., WATANABE H., OHASHI M., ITO D., NAGUMO M. Suppressive effect of selective cyclooxygenase-2 inhibitor on cytokine release in human neutrophils. Int. Immunopharmacol. 2003; 3(10-11):1519-1528.
- KINKAID A.R., WILTON D.C. A continuous fluorescence displacement assay for phospholipase A2 using albumin and medium chain phospholipid substrates. Anal. Biochem. 1993; 212(1):65-70.
- KOH J.S., LIEBERTHAL W., HEYDRICK S., LEVINE J.S. Lysophosphatidic acid is a major serum noncytokine survival factor for murine macrophages

which acts via the phosphatidylinositol 3-kinase signaling pathway. J. Clin. Invest. 1998; 102(4):716-727.

- KRANENBURG O., MOOLENAAR W.H. Ras-MAP kinase signaling by lysophosphatidic acid and other G protein-coupled receptor agonists. Oncogene 2001; 20(13):1540-1546.
- KRANENBURG O., POLAND M., VAN HORCK F.P., DRECHSEL D., HALL A., MOOLENAAR W.H. Activation of RhoA by lysophosphatidic acid and Galpha12/13 subunits in neuronal cells: induction of neurite retraction. Mol. Biol. Cell. 1999; 10(6):1851-1857.
- KUMAGAI N., MORII N., FUJISAWA K., NEMOTO Y., NARUMIYA S. ADP-ribosylation of rho p21 inhibits lysophosphatidic acid-induced protein tyrosine phosphorylation and phosphatidylinositol 3-kinase activation in cultured Swiss 3T3 cells. J. Biol. Chem. 1993; 268(33):24535-24538.
- KWIATKOWSKI D.J. Functions of gelsolin: motility, signaling, apoptosis, cancer. Curr. Opin. Cell Biol. 1999; 11(1):103-108.
- LA ROCCA G., ANZALONE R., BUCCHIERI F., FARINA F., CAPPELLO F., ZUMMO G. CD1a and antitumour immune response. Immunol. Lett. 2004; 95(1):1-4.
- LANGREHR J.M., HOFFMAN R.A., LANCASTER J.R. Jr, SIMMONS R.L. Nitric oxide--a new endogenous immunomodulator. Transplantation. 1993; 55(6):1205-1212.
- LANZAVECCHIA A., SALLUSTO F. Regulation of T cell immunity by dendritic cells. Cell. 2001; 106(3):263-266.
- LASSEGUE B., GRIENDLING K.K. Reactive oxygen species in hypertension: an update. Am. J. Hypertens. 2004; 17(9):852-860.

- LEE C.W., RIVERA R., DUBIN A.E., CHUN J. LPA4/GPR23 is an LPA receptor utilizing G_s, G_q/G_i-mediated calcium signaling and $G_{12/13}$-mediated Rho activation. J. Biol. Chem. 2006; [Epub ahead of print].
- LEE C.W., RIVERA R., GARDELL S., DUBIN A.E., CHUN J. GPR92 as a new $G_{12/13}$- and G_q-coupled lysophosphatidic acid receptor that increases cAMP, LPA_5. J. Biol. Chem. 2006; 281(33):23589-23597.
- LEE H., LIAO J.J., GRAELER M., HUANG M.G., GOETZL E.J. Lysophospholipid regulation of mononuclear phagocytes. Biochim. Biophys. Acta. 2002; 1582(1-3):175-177.
- LEUNG D.W. The structure and functions of human lysophosphatidic acid acyltransferases. Front. Biosci. 2001; 6:D944-D953.
- LILIOM K., GUAN Z., TSENG J.L., DESIDERIO D.M., TIGYI G., WATSKY M.A. Growth factor-like phospholipids generated after corneal injury. Am. J. Physiol. 1998; 274(4):C1065-C1074.
- LIN D.A., BOYCE J.A. IL-4 regulates MEK expression required for lysophosphatidic acid-mediated chemokine generation by human mast cells. J. Immunol. 2005; 175(8):5430-5438.
- **LIN D.A., BOYCE J.A. Lysophospholipids as mediators of immunity. Adv. Immunol. 2006; 89:141-167.**
- LIND S.E., SMITH D.B., JANMEY P.A., STOSSEL T.P. Depression of gelsolin levels and detection of gelsolin-actin complexes in plasma of patients with acute lung injury. Am. Rev. Respir. Dis. 1988; 138(2):429-434.
- LLOYD C.M., DELANEY T., NGUYEN T., TIAN J., MARTINEZ-A.C., COYLE A.J., GUTIERREZ-RAMOS J.C. CC chemokine receptor (CCR)3/eotaxin is followed by CCR4/monocyte-derived chemokine in mediating pulmonary T

helper lymphocyte type 2 recruitment after serial antigen challenge in vivo. J. Exp. Med. 2000; 191(2):265-274.

- **LU Y., WAHL L.M. Oxidative stress augments the production of matrix metalloproteinase-1, cyclooxygenase-2, and prostaglandin E2 through enhancement of NF-kappa B activity in lipopolysaccharide-activated human primary monocytes. J. Immunol. 2005; 175(8):5423-5429.**
- LUKACS N.W. Migration of helper T-lymphocyte subsets into inflamed tissues. J. Allergy Clin. Immunol. 2000; 106(5 Suppl):S264-S269.
- LUQUAIN C., SCIORRA V.A., MORRIS A.J. Lysophosphatidic acid signaling: how a small lipid does big things. Trends Biochem. Sci. 2003; 28(7):377-383.
- MAATMAN R.G., VAN KUPPEVELT T.H., VEERKAMPJ.H. Two types of fatty acid-binding protein in human kidney. Isolation, characterization and localization. Biochem. J. 1991; 273(3):759-766.
- MAGHAZACHI A.A. G protein-coupled receptors in natural killer cells. J. Leukoc. Biol. 2003; 74(1):16-24.
- **MARTINDALE J.L., HOLBROOK N.J. Cellular response to oxidative stress: signalling for suicide and survival. J. Cell. Physiol. 2002; 192(1):1-15.**
- MARTINO A., SACCHI A., SANARICO N., SPADARO F., RAMONI C., CIARAMELLA A., PUCILLO L.P., COLIZZI V., VENDETTI S. Dendritic cells derived from BCG-infected precursors induce Th2-like immune response. J. Leukoc. Biol. 2004; 76(4):827-834.
- **MARTINO A., VOLPE E., BALDINI P.M. The influence of lysophosphatidic acid on the immunophenotypic differentiation of human monocytes into dendritic cells. Haemat. 2006; 91(9):1273-1274.**

- MAUCO G., CHAP H., SIMON M.F., DOUSTE-BLAZY L. Phosphatidic and lysophosphatidic acid production in phospholipase C- and thrombin-treated platelets. Possible involvement of a platelet lipase. Biochimie 1978; 60(6-7):653-661.
- McINTYRE T.M., PONTSLER A.V., SILVA A.R., ST HILAIRE A., XU Y., HINSHAW J.C., ZIMMERMANN G.A., HAMA K., AOKI J., ARAI H., PRESTWICH G.D. Identification of an intracellular receptor for lysophosphatidic acid (LPA): LPA is a transcellular PPARgamma agonist. Proc. Natl. Acad. Sci. U.S.A. 2003; 100(1):131-136.
- MEERSCHAERT K., DE CORTE V., DE VILLE Y., VANDEKERCKHOVE J., GETTEMANS J. Gelsolin and functionally similar actin-binding proteins are regulated by lysophosphatidic acid. EMBO J. 1998; 17(20):5923-5932.
- MELO R.C.N., FABRINO D.L., D'ÁVILA H., TEIXEIRA H.C., FERREIRA A.P. Production of hydrogen peroxide by peripheral blood monocytes and specific macrophages during experimental infection with Trypanosoma cruzi in vivo. Cell. Biol. Int. 2003; 27(10):853-861.
- MEYER ZU HERINGDORF D., JAKOBS K.H. Lysophospholipid receptors: Signalling, pharmacology and regulation by lysophospholipid metabolism. Biochim. Biophys. Acta. 2006; [Epub ahead of print].
- **MILLS G.B., MOOLENAAR W.H. The emerging role of lysophosphatidic acid in cancer. Nat. Rev. Cancer. 2003; 3(8):582-591.**
- MOOLENAAR W.H. Bioactive lysophospholipids and their G protein-coupled receptors. Exp. Cell. Res. 1999; 253(1):230-238.
- MOOLENAAR W.H. Development of our current understanding of bioactive lysophospholipids. Ann. N. Y. Acad. Sci. 2000; 905:1-10.

- **MOOLENAAR W.H., KRANENBURG O., POSTMA F.R., ZONDAG G.C.M. Lysophosphatidic acid: G-protein signalling and cellular responses. Curr. Opin. Cell Biol. 1997; 9(2):168-173.**
- MORETTA A., BOTTINO C., MINGARI M.C., BIASSONI R., MORETTA L. What is a natural killer cell? Nat. Immunol. 2002a; 3(1):6-8.
- MORETTA L., BOTTINO C., PENDE D., MINGARI M.C., BIASSONI R., MORETTA A. Human natural killer cells: their origin, receptors and function. Eur. J. Immunol. 2002b; 32(5):1205-1211.
- MORTEAU O. Prostaglandins and inflammation: the cyclooxygenase controversy. Arch. Immunol. Ther. Exp. (Warsz). 2000; 48(6):473-480.
- MOSMANN T.R., COFFMAN R.L. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. 1989; 7:145-173.
- MURAKAMI M., KUDO I. Phospholipase A2. J. Biochem. (Tokyo). 2002; 131(3):285-292.
- MURAKAMI M., NAKATANI Y., ATSUMI G., INOUE K., KUDO I. Regulatory functions of phospholipase A2. Crit. Rev. Immunol. 1997; 17(3-4):225-283.
- NAGATA M. Inflammatory cells and oxygen radicals. Curr. Drug. Targets Inflamm. Allergy. 2005; 4(4):503-504.
- NAKAMOTO T., YASUDA K., YASUHARA M., YOSHIMURA T., KINOSHITA T., NAKAJIMA T., OKADA H., IKUTA A., KANZAKI H. Expression of the endothelial cell differentiation gene 7 (EDG-7), a lysophosphatidic acid receptor, in ovarian tumor. J. Obstet. Gynaecol. Res. 2005; 31(4):344-351.
- NILSSON G., JOHNELL M., HAMMER C.H., TIFFANY H.L., NILSSON K., METCALFE D.D., SIEGBAHN A., MURPHY P.M. C3a and C5a are

chemotaxins for human mast cells and act through distinct receptors via a pertussis toxin-sensitive signal transduction pathway. J. Immunol. 1996; 157(4):1693-1698.

- NIU X.F., SMITH C.W., KUBES P. Intracellular oxidative stress induced by nitric oxide synthesis inhibition increases endothelial cell adhesion to neutrophils. Circ. Res. 1994; 74(6):1133-1140.
- NOGUCHI K., ISHII I., SHIMIZU T. Identification of p2y9/GPR23 as a novel G protein-coupled receptor for lysophosphatidic acid, structurally distant from the Edg family. J. Biol. Chem. 2003; 278(28):25600-25606.
- O'BYRNE P.M. Cytokines or their antagonists for the treatment of asthma. Chest. 2006; 130(1):244-250.
- OHTA H., SATO K., MURATA N., DAMIRIN A., MALCHINKHUU E., KON J., KIMURA T., TOBO M., YAMAZAKI Y., WATANABE T., YAGI M., SATO M., SUZUKI R., MUROOKA H., SAKAI T., NISHITOBA T., IM D.S., NOCHI H., TAMOTO K., TOMURA H., OKAJIMA F. Ki16425, a subtype-selective antagonist for EDG-family lysophosphatidic acid receptors. Mol. Pharmacol. 2003; 64(4):994-1005.
- **OSTERUD B., BJORKLID E. Role of monocytes in atherogenesis. Phys. Rev. 2003; 83(4):1069-1112.**
- OZ-ARSLAN D., RUSCHER W., MYRTEK D., ZIEMER M., JIN Y., DAMAY B.B., SORICHTER S., IDZKO M., NORGAUER Y., MAGHAZACHI A.A. IL-6 and IL-8 release is mediated via multiple signaling pathways after stimulating dendritic cells with lysophospholipids. J. Leukoc. Biol. 2006; 80(2):287-297.

- **PAGÉS C., SIMON H.F., VALET P., SAULNIER-BLANCHE J.S. Lysophosphatidic acid synthesis and release. Prostaglandins Other Lipid Mediat. 2001; 64(1-4):1-10.**
- PAKALA R., BENEDICT C.R. Endothelial cells regulate the proliferation of monocytes in vitro. Atherosclerosis 1999; 147(1):25-32.
- **PANTHER E., IDZKO M., CORINTI S., FERRARI D., HEROUY Y., MOCKENHAUPT M., DICHMANN S., GEBICKE-HAERTERP., DI VIRGILIO F.,GIROLOMONI G., NORGAUER J. The influence of lysophosphatidic acid on the functions of human dendritic cells. J. Immunol. 2002; 169(8):4129-4135.**
- PASSWELL J.H., DAYER J.M., MERLER E. Increased prostaglandin production by human monocytes after membrane receptor activation. J. Immunol. 1979; 123(1):115-120.
- PEBAY A., TORRENS Y., TOUTANT M., CORDIER J., GLOWINSKI J., TENCE M. Pleiotropic effects of lysophosphatidic acid on striatal astrocytes. Glia. 1999; 28(1):25-33.
- PEREZ-PEREZ G.I., SHEPHERD V.L., MORROW J.D., BLASER M.J. Activation of human THP-1 cells and rat bone marrow-derived macrophages by *Helicobacter pylori* lipopolysaccharide. Infect. Immun. 1995; 63(4):1183-1187.
- RAKESH K., AGRAWAL D.K. Cytokines and growth factors involved in apoptosis and proliferation of vascular smooth muscle cells. Int. Immunopharmacol. 2005; 5(10):1487-1506.
- RANDOLPH G.J., BEAULIEU S., LEBECQUE S., STEINMAN R.M., MULLER W.A. Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking. Science 1998; 282(5388):480-483.

- REISER C.O., LANZ T., HOFMANN F., HOFER G., RUPPRECHT H.D., GOPPELT-STRUEBE M. Lysophosphatidic acid-mediated signal-transduction pathways involved in the induction of the early-response genes prostaglandin G/H synthase-2 and Egr-1: a critical role for the mitogen-activated protein kinase p38 and for Rho proteins. Biochem. J. 1998; 330(3):1107-1114.
- RICHO G.R., CONNER G.E. Structural requirements of procathepsin D activation and maturation. J. Biol. Chem. 1994; 269(20):14806-14812.
- RIZZA C., LEITINGER N., YUE J., FISHER D.J., WANG D.A., SHIH P.T., LEE H., TIGYI G., BERLINER J.A. Lysophosphatidic acid as a regulator of endothelial/leukocyte interaction. Lab. Invest. 1999; 79(10):1227-1235.
- ROSA-BRUNET L. Nitric oxide in parasitic infections. Int. Immunopharmacol. 2001; 1:1457-1467.
- **ROSS R. Atherosclerosis-an inflammatory disease. N. Engl. J. Med. 1999; 340(2):115-126.**
- ROSSKOPF D., DAELAMN W., BUSCH S., SCHURKS M., HARTUNG K., KRIBBEN A., MICHEL M.C., SIFFERT W. Growth factor-like action of lysophosphatidic acid on human B lymphoblasts. Am. J. Physiol. 1998; 274(6):C1573-C1582.
- SANO T., BAKER D.L., VIRAG T., WADA A., YATOMI Y., KOBAYASHI T., IGARASHI Y., TIGYI G. Multiple mechanisms linked to platelet activation result in lysophosphatidic acid and sphingosine-1-phosphate generation in blood. J. Biol. Chem. 2002; 277(24):21197-21206.
- SATO T., AOKI J., NAGAI Y, DOHMAE N., TAKIO K., DOI T., ARAI H., INOUE K. Serine phospholipid-specific phospholipase A that is secreted from

activated platelets. A new member of the lipase family. J. Biol. Chem. 1997; 272(4):2192-2198.

- SCHAEFFER H.J., WEBER M.J. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. Mol. Cell. Biol. 1999; 19(4):2435-2444.
- SCHMID-SCHÖNBEIN G.W. Analysis of inflammation. Annu. Rev. Biomed. Eng. 2006; 8:93-131.
- SCHMIDT A., WOLDE M., THIELE C., FEST W., KRATZIN H., PODTELEJNIKOV A.V., WITKE W., HUTTNER W.B., SOLING H.D. Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid. Nature. 1999; 401(6749):133-141.
- **SCHMITZ U., THOMMES K., BEIER I., VETTER H. Lysophosphatidic acid stimulates p21-activated kinase in vascular smooth muscle cells. Biochem. Biophys. Res. Commun. 2002; 291(3):687-691.**
- SCHULTE K.M., BEYER A., KÖHRER K., OBERHÄUSER S., RÖHER H.D. Lysophosphatidic acid, a novel lipid growth factor for human thyroid cells: over-expression of the high-affinity receptor edg4 in differentiated thyroid cancer. Int. J. Cancer. 2001; 92(2):249-256.
- **SEEWALD S., SACHINIDIS A., DUSING R., KO Y., SEUL C., EPPING P., VETTER H. Lysophosphatidic acid and intracellular signalling in vascular smooth muscle cells. Atherosclerosis. 1997; 130(1-2):121-131.**
- **SENGUPTA S., WANG Z., TIPPS R., XU Y. Biology of LPA in health and disease. Semin. Cell. Dev. Biol. 2004; 15(5):503-512.**

- SHEN H.M., SHI C.Y., SHEN Y., ONG C.N. Detection of elevated reactive oxygen species level in cultured rat hepatocytes treated with aflatoxin B1. Free Radic. Biol. Med. 1996; 21(2):139-146.
- SHEN Z., BELINSON J., MORTON R.E., XU Y., XU Y. Phorbol 12-myristate 13-acetate stimulates lysophosphatidic acid secretion from ovarian and cervical cancer cells but not from breast or leukaemia cells. Gynecol Oncol. 1998; 71(3):364-368.
- **SIESS W. Athero- and thrombogenic actions of lysophosphatidic acid and sphingosine-1-phosphate. Biochim. Biophys. Acta. 2002; 1582(1-3):204-215.**
- **SIESS W., TIGYI G. Thrombogenic and atherogenic activities of lysophosphatidic acid. J. Cell. Biochem. 2004; 92(6):1086-1094.**
- SIESS W., ZANGL K.J., ESSLER M., BAUER M., BRANDL R., CORRINTH C., BITTMAN R., TIGYI G., AEPFELBACHER M. Lysophosphatidic acid mediates the rapid activation of platelets and endothelial cells by mildly oxidized low density lipoprotein and accumulates in human atherosclerotic lesions. Proc. Natl. Acad. Sci. U. S. A. 1999; 96(12):6931-6936.
- SIMPSON C.M., ITABE H., REYNOLDS C.N., KING W.C., GLOMSET J.A. Swiss 3T3 cells preferentially incorporate SN-2-arachidonoyl monoacyl glycerol into SN-1 stearoyl-2-arachidonoyl phosphatidyl inositol. J. Biol. Chem. 1991; 266(24):15902-15909.
- SONODA H., AOKI J., HIRAMATSU T., ISHIDA M., BANDOH H., NAGAY Y., TAGUCHI R., INOUE K., ARAI H. A novel phosphatidic acid-selective phospholipase A1 that produces lysophosphatidic acid. J. Biol. Chem. 2002; 277(37):34254-34263.

- SOZZANI S., ALLAVENA P., VECCHI A., MANTOVANI A. Chemokines and dendritic cell traffic. J. Clin. Immunol. 2000; 20(3):151-160.
- SUGIMOTO Y., NARUMIYA S., ICHIKAWA A. Distribution and function of prostanoid receptors: studies from knockout mice. Prog. Lipid Res. 2000; 39(4):289-314.
- SUGIURA T., NAKANE S., KISHIMOTO S., WAKU K., YOSHIOKA Y., TOKUMURA A. Lysophosphatidic acid, a growth factor-like lipid, in the saliva. J. Lipid Res. 2002; 43(12):2049-2055.
- SUNDERKOTTER C., STEINBRINK K., GOEBELER M., BHARDWAJ R., SORG C. Macrophages and angiogenesis. J. Leukoc. Biol. 1994; 55(3):410-422.
- SUPAJATURA V., USHIO H., NAKAO A., AKIRA S., OKUMURA K., RA C., OGAWA H. Differential responses of mast cell Toll-like receptors 2 and 4 in allergy and innate immunity. J. Clin. Invest. 2002; 109(10):1351-1359.
- TAKAHASHI K., NAITO M., TAKEYA M. Development and heterogeneity of macrophages and their related cells through their differentiation pathways. Pathol. Int. 1996; 46(7):473-485.
- TAKAYAMA K., GARCIA-CARDENA G., SUKHOVA G.K., COMANDER J., GIMBRONE M.A. Jr, LIBBY P. Prostaglandin E2 suppresses chemokine production in human macrophages through the EP4 receptor. J. Biol. Chem. 2002; 277(46):44147-44154.
- **TAKUWA Y., TAKUWA N., SUGIMOTO N. The Edg family G protein-coupled receptors for lysophospholipids: their signaling properties and biological activities. J. Biochem (Tokyo). 2002; 131(6):767-771.**

- TENSCHER K., METZNER C. HOFMANN C., SCHÖPF E., NORGAUER J. The monocyte chemotactic protein-4 (MCP-4) induces oxygen radical production, actin reorganization and CD11b up-regulation via a pertussis toxin-sensitive G-protein in human eosinophils. Biochem. Biophys. Res. Commun. 1997; 240(1):32-35.
- THOMPSON F.J., CLARK M.A. Purification of a lysophosphatidic acid-hydrolysing lysophospholipase from rat brain. Biochem. J. 1994; 300(2):457-461.
- THUMSER A.E., VOYSEY J.E., WILTON D.C. The binding of lysophospholipids to rat liver fatty acid-binding protein and albumin. Biochem. J. 1994; 301(3):801-806.
- TIGYI G., MILEDI R. Lysophosphatidates bound to serum albumin activate membrane currents in Xenopus oocytes and neurite retraction in PC12 pheochromocytoma cells. J. Biol. Chem. 1992; 267(30):21360-21367.
- **TIGYI G., PARRILL A.L. Molecular mechanisms of lysophosphatidic acid action. Prog. Lipid Res. 2003; 42(6):498-526.**
- TIIDUS P.M. Radical species in inflammation and overtraining. Can. J. Physiol. Pharmacol. 1998; 76(5):533-538.
- TOKUMURA A., HARADA K., FUKUZAWA K., TSUKATANI H. Involvement of lysophospholipase D in the production of lysophosphatidic acid in rat plasma. Biochim. Biophys. Acta. 1986; 875(1):31-38.
- TOKUMURA A., MAJIMA E., KARIYA Y., TOMINAGA K., KOGURE K., YASUDA K., FUKUZAWA K. Identification of human plasma lysophospholipase D, a lysophosphatidic acid-producing enzyme, as

autotaxin, a multifunctional phosphodiesterase. J. Biol. Chem. 2002; 277(42):39436-39442.

- TOKUMURA A., MIYAKE M., NISHIOKA Y., YAMANO S., AONO T., FUKUZAWA K. Production of lysophosphatidic acids by lysophospholipase D in human follicular fluids of in vitro fertilization patients. Biol. Reprod. 1999; 61(1):195-199.
- TOPHAM M.K., PRESCOTT S.M. Diacylglycerol kinases: regulation and signaling roles. Thromb. Haemost. 2003; 88(6):912-918.
- **TORRES M. Mitogen-activated protein kinase pathways in redox signaling. Front. Biosci. 2003; 8:D369-D391.**
- **TORRES M., FORMAN H.J. Redox signaling and the MAP kinase pathways. Biofactors. 2003; 17(1-4):287-296.**
- TOU J.S., GILL J.S. Lysophosphatidic acid increases phosphatidic acid formation, phospholipase D activity and degranulation by human neutrophils. Cell. Signal. 2005; 17(1):77-82.
- TRIGGIANI M., GRANATA F., BALESTRIERI B., PETRAROLI A., SCALIA G., DEL VECCHIO L., MARONE G. Secretory phospholipases A2 activate selective functions in human eosinophils. J. Immunol. 2003; 170(6):3279-3288.
- UMEZU-GOTO M., KISHI Y., TAIRA A., HAMA K., DOHMAE N., TAKIO K., YAMORI T., MILLS G.B., INOUE K., AOKI J., ARAI H. Autotaxin has lysophospholipase D activity leading to tumor cell growth and motility by lysophosphatidic acid production. J. Cell. Biol. 2002; 158(2):227-233.
- UMEZU-GOTO M., TANYI J., LAHAD J., LIU S., YU S., LAPUSHIN R., HASEGAWA Y., LU Y., TROST R., BEVERS T., JONASCH E., ALDAPE K.,

LIU J., JAMES R.D., FERGUSON C.G., XU Y., PRESTWICH G.D., MILLS G.B. Lysophosphatidic acid production and action: validated targets in cancer? J. Cell. Biochem. 2004; 92(6):1115-1140.

- **VAN CORVEN E.J., GROENINK A., JALINK K., EICHHOLTZ T., MOOLENAAR W.H. Lysophosphatidate-induced cell proliferation: identification and dissection of signaling pathways mediated by G proteins. Cell. 1989; 59(1):45-54.**
- VAN DIJK M.C., POSTMA F., HILKMANN H., JALINK K., VAN BLITTERSWIJK W.J., MOOLENAAR W.H. Exogenous phospholipase D generates lysophosphatidic acid and activates Ras, Rho and Ca^{2+} signaling pathways. Curr. Biol. 1998; 8(7):386-392.
- **VAN LEEUWEN F.N., GIEPMANS B.N., VAN MEETEREN L.A., MOOLENAAR W.H. Lysophosphatidic acid: mitogen and motility factor. Biochem. Soc. Trans. 2003; 31(6):1209-1212.**
- VAN LEEUWEN F.N., OLIVO C., GRIVELL S., GIEPMANS B.N., COLLARD J.G., MOOLENAAR W.H. Rac activation by lysophosphatidic acid LPA1 receptors through the guanine nucleotide exchange factor Tiam1. J. Biol. Chem. 2003; 278(1):400-406.
- VANCURA A., HALDAR D. Regulation of mitochondrial and microsomal phospholipid synthesis by liver fatty acid-binding protein. J. Biol. Chem. 1992; 267(20):14353-14359.
- VILA L. Cyclooxygenase and 5-lipoxygenase pathways in the vessel wall: role in atherosclerosis. Med. Res. Rev. 2004; 24(4):399-424.
- VOGT W. The chemical nature of Darmstoff. J. Physiol. 1957; 137(1):154-167.

- WANG A., DENNIS E.A. Mammalian lysophospholipases. Biochim. Biophys. Acta. 1999; 1439(1):1-16.
- WANG L., KNUDSEN E., JIN Y., GESSANI S., MAGHAZACHI A.A. Lysophospholipids and chemokines activate distinct signal transduction pathways in T helper 1 and T helper 2 cells. Cell. Signal. 2004; 16(9):991-1000.
- WEBER M., DAHINDEN C.A. Basophil and eosinophils activation by CC chemokines. Int. Arch. Allergy Immunol. 1995; 107(1-3):148-150.
- WEINER J.A., CHUN J. Schwann cell survival mediated by the signaling phospholipid lysophosphatidic acid. Proc. Natl. Acad. Sci. U.S.A. 1999; 96(9):5233-5238.
- WEINER J.A., FUKUSHIMA N., CONTOS J.J., SCHERER S.S., CHUN J. Regulation of Schwann cell morphology and adhesion by receptor-mediated lysophosphatidic acid signaling. J. Neurosci. 2001; 21(18):7069-7078.
- WERNER S., GROSE R. Regulation of wound healing by growth factors and cytokines. Physiol. Rev. 2003; 83(3):835-870.
- WINK D.A., HANBAUER I., GRISHAM M.B., LAVAL F., NIMS R.W., LAVAL J., COOK J., PACELLI R., LIEBMANN J., KRISHNA M., FORD P.C., MITCHELL J.B. Chemical biology of nitric oxide: regulation and protective and toxic mechanisms. Curr. Top. Cell. Regul. 1996; 34:159-187.
- XIAO Y., CHEN Y., KENNEDY A.W., BELINSON J., XU Y. Evaluation of plasma lysophospholipids for diagnostic significance using electrospray ionization mass spectrometry (ESI-MS) analyses. Ann. N. Y. Acad. Sci. 2000; 905:242-259.

- XIE Q., NATHAN C. The high-output nitric oxide pathway: role and regulation. J. Leukoc. Biol. 1994; 56(5):576-582.
- XIE Y., GIBBS T.C., MUKHIN Y.V., MEIER K.E. Role for 18:1 lysophosphatidic acid as an autocrine mediator in prostate cancer cells. J. Biol. Chem. 2002; 277(36):32516-32526.
- XU Y., FANG X.J., CASEY G., MILLS G.B. Lysophospholipids activate ovarian and breast cancer cells. Biochem. J. 1995; 309(3):933-940.
- XU Y., GAUDETTE D.C., BOYNTON J.D., FRANKEL A., FANG X.J., SHARMA A., HURTEN J., CASEY G., GOODBODY A., MELLORS A. Characterization of an ovarian cancer of activating factor in ascites from ovarian cancer patients. Clin. Cancer Res. 1995; 1(10):1223-1232.
- YIN H.L., STOSSEL T.P. Control of cytoplasmic actin gel-sol transformation by gelsolin, a calcium-dependent regulatory protein. Nature. 1979; 281(5732):583-586.
- YKI-JARVINEN H. Thiazolidinediones. N. Engl. J. Med. 2004; 351(11):1106-1118.
- YOKOMIZO T., IZUMI T., SHIMIZU T. Leukotriene B4: metabolism and signal transduction. Arch. Biochem. Biophys. 2001; 385(2):231-241.
- YOKOYAMA K., KUDO I., INOUE K. Phospholipid degradation in rat calcium ionophore-activated platelets is catalyzed mainly by two discrete secretory phospholipase As. J. Biochem. (Tokyo). 1995; 117(6):1280-1287.
- YOSHIDA K., NISHIDA W., HAYASHI K., OHKAWA Y., OGAWA A., AOKI J., ARAI H., SOBUE K. Vascular remodelling induced by naturally occurring unsaturated lysophosphatidic acid in vivo. Circulation 2003; 108(14):1746-1752.

- **ZHENG Y., VOICE J.K., KONG Y., GOETZL E.J. Altered expression and functional profile of lysophosphatidic acid receptors in mitogen-activated human blood T lymphocytes. FASEB J. 2000; 14(15): 2387-2389.**
- ZHOU D., LUINI W., BERNASCONI S., DIOMEDE L., SALMONA M., MANTOVANI A., SOZZANI S. Phosphatidic acid and lysophosphatidic acid induce haptotactic migration of human monocytes. J. Biol. Chem. 1995; 270(43):25549-25556.
- ZIEGLER-HEITBROCK H.W., THIEL E., FUTTERER A., HERZOG V., WIRTZ A., RIETHMULLER G. Establishment of a human cell line (Mono Mac 6) with characteristics of mature monocytes. Int. J. Cancer. 1988; 41(3):456-461.

PUBBLICATIONS

1. “Atrial Natriuretic Peptide induces cell death in human hepatoblastoma (HepG2) through the involvement of NADPH oxidase” P.M. Baldini, P. De Vito, D. Antenucci, D. Vismara, **F. D’Aquilio**, P. Luly, F. Zalfa, C. Bagni and P. Di Nardo. **Cell Death and Differentiation (2004) 11: S210-S212;**

2. “Role of Atrial Natriuretic Peptide in the suppression of lysophosphatidic acid-induced rat aortic smooth muscle (RASM) cell growth” P.M. Baldini, P. De Vito, **F. D’Aquilio**, D. Vismara, F. Zalfa, C. Bagni, R. Fiaccavento and P. Di Nardo. **Molecular and Cellular Biochemistry (2005) 272(1-2): 19-28;**

3. “Activatory properties of lysophosphatidic acid on human THP-1 monocytes” **D’Aquilio F**., Procaccini M., Izzi V., Chiurchiù V., Giambra V., Carotenuto F., Di Nardo P. and Baldini P.M. **Inflammation (2006) Dec**; **29(4-6):129-140.**