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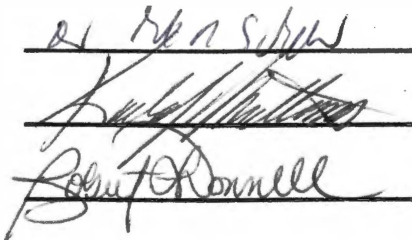


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Vice Provost and
Dean of Graduate Studies

**LYSOPHOSPHATIDIC ACID INDUCTION OF TISSUE FACTOR GENE
EXPRESSION IN VASCULAR SMOOTH MUSCLE CELLS**

A Dissertation
Presented for the
Doctor of Philosophy Degree
The University of Tennessee,
Knoxville

Essam Laag
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DEDICATION

This Dissertation is dedicated to my parents who have given me invaluable educational opportunities and to my wife Ola Mostafa and my children, Mostafa, Abdelrahman and Salma.

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ABSTRACT

LPA is a component of oxidized low density lipoproteins (oxLDL) which has been shown to accumulate in human atherosclerotic plaques. Tissue factor (TF) is the principal initiator of blood coagulation. Tissue factor upregulation in atherosclerotic plaque can lead to undesirable vascular thrombosis. The generation of reactive oxygen species (ROS), which act as signaling molecules in the vascular system, is enhanced in response to injury and has been associated with a procoagulant state and the progression of atherosclerotic disease. Oxidative stress might contribute to the increased expression of proatherosclerotic genes at sites of vascular injury, including TF. Little is known about the regulation of TF by LPA in smooth muscle cells (SMC) which is a major player in the process of atherosclerosis. Data generated by this study demonstrate that LPA markedly induces TF expression in rat aorta smooth muscle cells (RASMCs) and human aorta smooth muscle cells (HASMCs). The signaling pathways involved are multiple. One signaling pathway demonstrates that LPA activates G α i protein which in turn activates PKC and MAPK cascades to induce Egr-1 that is involved in turning on TF gene in the nucleus. The other pathways include LPA-induced release of ROS and calcium, mediated by PLA2 or without PLA2 involvement. Our data suggest that elevated LPA have potential atherogenic and thrombogenic actions by inducing TF expression and ROS release. Understanding the mechanisms involved could provide new insights into atherosclerosis, thrombosis and restenosis after angioplasty.

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LIST OF ABBREVIATIONS

AA: arachidonic acid.

AP-1: activator protein-1.

CRE: cyclic AMP response element.

CREB: cyclic AMP response element binding protein.

EMSA: electrophoretic mobility shift assay.

HASMCs: human aorta smooth muscle cells.

HRP: horseradish peroxidase.

LPA: lysophosphatidic acid.

LDL: low density lipoproteins.

MAPK: mitogen activated protein kinase.

NAC: N-acetyl cysteine.

NF κ B: nuclear factor κ B.

OxLDL: oxidized low density lipoproteins.

PKC: protein kinase C.

PKD: protein kinase D.

PTX: pertussis toxin.

RASMCs: rat aorta smooth muscle cells.

ROS: reactive oxygen species.

SMCs: smooth muscle cells.

SRE: serum response element.

TF: tissue factor.

I. BACKGROUND AND INTRODUCTION

Cardiovascular diseases are the number one cause of death in the United States [Groer, 2001]. Atherosclerosis is a progressive cardiovascular disease characterized by accumulation of lipids and fibrous elements in the arteries. Atherosclerosis primarily affects elastic arteries (e.g., aorta) and large and medium-sized muscular arteries (e.g., coronary arteries). This disease is the primary cause of heart disease and stroke. In Westernized societies, it is the underlying cause of about 50% of all deaths [Lusis, 2000].

THE NORMAL ARTERY:

The arterial wall normally consists of three well-defined concentric layers: the innermost layer is called the intima, the middle layer is called the media, and the outermost layer is known as the adventitia. These three layers are demarcated by concentric layers of elastin known as the internal elastic lamina (which separates the intima from the media), and the external elastic lamina (which separates the media from the adventitia).

The luminal surface of arteries is lined by a single contiguous layer of endothelial cells that sits upon a basement membrane of extracellular matrix, and is bordered by the internal elastic lamina. The amount of extracellular matrix and elastin in the internal elastic lamina is most prominent in medium and large sized arteries.

The media consists of a single cell type, smooth muscle cells. There can be one or many layers of smooth muscle cells depending on the size of the artery [Keaney Jr, 2000]. The vascular smooth muscle has been shown to be a pleiotropic cell capable of phenotypic changes associated with the synthesis of many biologically active molecules

that mediate cell growth, death, and migration, as well as matrix modulation and inflammation. These activities of vascular smooth muscle play important roles in physiological vascular functions, such as vascular remodeling, and in pathological disorders, such as atherosclerosis, restenosis, transplant vasculopathy, and other vascular diseases [Alexander and Dzau, 2000]. The media can vary in size considerably based on the function of a given artery. For example, in very small arteries the media may only be one cell layer thick, or not present at all, whereas in large arteries like the aorta the media may be many cell layers thick and consist of considerable elastin as part of the extracellular matrix.

Beyond the external elastic lamina, the adventitia is the outermost layer of the artery. The adventitia typically consists of a loose matrix of elastin, smooth muscle cells, fibroblasts, and collagen. This layer contains small vessels that gives rise to vasa vasorum. Most of the neural input into blood vessels also traverse the adventitia [Keaney Jr, 2000; Avrum et al, 1999]

ATHEROSCLEROSIS:

The early lesions of atherosclerosis (fatty streaks) consist of subendothelial accumulations of cholesterol-engorged macrophage, called foam cells. These lesions are not clinically significant, but they are the precursors of more advanced lesions (atheromatous plaques). An atheromatous plaque consists of a raised focal plaque within the intima, having a necrotic core of lipid and a covering fibrous cap. The superficial fibrous cap is composed of smooth muscle cells with a few leukocytes and relatively dense connective tissue. The lipid necrotic core lies deeper to the fibrous cap and it

consists of a disorganized mass of lipid material, cholesterol clefts, cellular debris, lipid-laden foam cells, fibrin, thrombus in various stages of organization, and other plasma proteins. The lipids in the core are primarily cholesterol and cholesterol esters. Foam cells predominantly derive from lipid-laden macrophages, but smooth muscle cells can also take up lipids to become foam cells. A primary initiating event in atherosclerosis is the accumulation of LDL in the subendothelial matrix. Accumulation is greater when levels of circulating LDL are raised [Schoen and Cotran, 1999]. Native LDL is not taken up by macrophages rapidly enough to generate foam cells, and it has been shown that trapped LDL does undergo modification, including oxidation, lipolysis, proteolysis and aggregation. These modifications contribute to inflammation and foam cell formation [Boren et al, 1998]. If the hypercholesterolemia persists, smooth muscle cell proliferation and extracellular matrix deposition in the intima continue and are the major processes that convert a fatty streak into a mature fibrofatty atheroma (plaque), accounting for the progressive growth of atherosclerotic lesions [Schoen and Cotran, 1999]. Plaques can become increasingly complex, with calcification, ulceration at the luminal surface, and hemorrhage from small vessels that grow into the lesion from the media of the blood vessel wall. The most important clinical complication is acute occlusion due to superimposed thrombosis which usually occurs on disrupted lesions (those with rupture, ulceration, erosion, or hemorrhage) [Lusis, 2000; Schoen and Cotran 1999]. Acute thrombosis can lead to arterial occlusion and consequently provoke myocardial infarction, unstable angina, stroke and sudden death [Tremoli et al, 1999].

TISSUE FACTOR:

Pathological studies have identified tissue factor (TF), present in cells and in the extra cellular debris within the atherosclerotic plaque, as a candidate molecule responsible for the thrombogenicity associated with the plaque rupture [Fuster et al, 1997]. TF, a small (47kd) transmembrane glycoprotein, is the principal initiator of the clotting cascade and is considered to be a major regulator of hemostasis and thrombosis [Marmur et al, 1994; Edgington et al, 1991]. TF initiates the extrinsic pathway of blood coagulation [Schonbeck et al, 2000]. The ultimate events of this cascade are thrombin generation and thrombin-catalyzed events which lead to thrombus formation and stabilization [Carson and Brozna, 1993]. TF might also affect intimal area by increasing matrix accumulation and stimulating smooth muscle cell (SMC) migration and proliferation contributing to athermatous plaque development [Hasenstab et al, 2000]. The data so far available from samples retrieved by coronary atherectomy suggests that TF plays a critical rule in determining atherosclerotic plaque thrombogenicity. Thrombus evident on histology has been found only in TF-positive plaques [Marmur et al, 1994] and TF antigen and activity were higher in the plaques with complex morphology or coronary thrombosis at angiography [Ardissino et al, 1997]. TF concentration and activity were also higher in the plaques taken from patients with unstable angina or myocardial infarction, and in patients with de novo angina as compared to stable patients [Ardissino et al, 1997; Annex et al, 1995].

In normal arteries, little or no TF is found in the intima or media, whereas TF is abundant in the adventitia [Merlini et al, 1999]. TF is not constitutively expressed by the normal artery endothelium or by cellular components of blood. In healthy tissue, this distribution allows the endothelium to provide an anticoagulant surface lining the vessel lumen, and the adventitia a procoagulant surface around the vessel, ensuring prompt initiation of coagulation in the event of vascular rupture [Mc Vey, 1999]. Vascular smooth muscle cells (SMCs) is a major source of arterial TF [Speidel et al, 1995]. In animal models of arterial injury, TF is rapidly induced in the SMCs of the media and accumulates in the SMCs of the developing neointima [Merlini et al, 1999; Gertz et al, 1998; Annex et al, 1995].

TF is also abundant in macrophages, and SMCs of atherosclerotic plaques [Hatakeyama et al, 1997; Wilcox et al, 1989]. It has also been detected in the extracellular matrix and in the acellular lipid rich core in atherosclerotic plaques [Moreno et al, 1996; Wilcox et al, 1989]. Induction of TF in human endothelial cells and monocyte/macrophages has been extensively studied [Siess et al, 1999; Thiruvikraman et al, 1996]. In contrast, little is known about the regulation of its expression in human arterial SMC and in the cell and lipid-rich core of the atheromatous plaque.

LYSOPHOSPHATIDIC ACID (LPA):

Oxidized LDL is a key factor in the pathogenesis of atherosclerosis. Lysophosphatidic acid is formed during mild oxidation of LDL and is the active compound in mildly oxidized LDL and minimally modified LDL. It has been found that LPA also accumulates in the lipid-rich core of atherosclerotic plaques [Fernandez-Ortiz et al, 1994].

Experimental evidence indicates that the lipid-rich atheromatous core and not the exposed collagen is thrombogenic initiator in ruptured plaques [Goetzl et al, 1998]. Our group has recently reported that LPA dramatically induces TF expression in RASMCs (TF mRNA was induced eight folds and TF protein and TF total activity were also increased). These results indicate that LPA might be a thrombogenic risk factor that plays an important role in pathogenesis of atherosclerosis. SMC is known to be one of the important players in atherosclerotic lesion development. However, little is known about the regulatory mechanisms governing the induction of TF expression by LPA in RASMCs.

ROLE OF REACTIVE OXYGEN SPECIES IN THE ARTERIAL WALL:

Reactive oxygen species (ROS) are a family of molecules including molecular oxygen and its derivatives produced in all aerobic cells. Many ROS possess unpaired electrons and thus are free radicals. These include molecules such as superoxide anion (O_2^-), hydroxyl radical ($HO^\cdot$), nitric oxide ($NO^\cdot$), and lipid radicals. Other reactive oxygen species, such as hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and hypochlorous acid ($HOCl$), are not free radicals per se but have oxidizing effects that contribute to oxidant stress [Cai and Harrison, 2000].

Reactive oxygen species (ROS) play an important role in many cardiovascular pathologies involving inflammatory processes, including atherosclerosis. Several pathogenic effects of increased ROS production have been identified. These include the oxidation of core lipids of lipoproteins and cell membranes which then modify apolipoproteins and other proteins, leading to their recognition by scavenger receptors. Similar modifications also influence DNA binding of regulatory proteins in the nucleus.

Exposure of cells to ROS induces a local inflammatory response and the release of cytokines and growth factors, such as tumor necrosis factor- α , interleukin1 β , and interferon γ . These factors may then provide a positive feedback mechanism, for example by stimulating ROS-producing enzymes. Although cells are normally well protected from ROS by antioxidant defenses, including oxygen-radical scavenger enzymes, the rate of ROS formation can exceed the local antioxidant defense capacity and thus induce oxidant stress in arterial cells [Napoli et al, 2001].

Vascular smooth muscle cell (SMC) hyperplasia and hypertrophy are characteristic of atherosclerotic, restenotic, and hypertensive vascular diseases [Ross, 1993]. The net balance between proliferation and apoptosis determines the extent of SMC growth. ROS can mediate phenotypes in vascular endothelial and smooth muscle cells that may be considered both physiological and pathophysiological. Among these are growth, apoptosis, and survival. A full understanding of how ROS regulate mitogenesis and apoptosis in vascular smooth muscle and endothelial cells will permit the development of novel strategies to modify or prevent vascular diseases in which these phenotypes predominate [Irani, 2000].

II. OBJECTIVES AND HYPOTHESIS

Tissue factor is the primary initiator of clotting, both normal and pathologic, in a large number of clinical settings and animal models [Osterud, 1997]. Tissue factor upregulation in atherosclerotic plaque can lead to undesirable vascular thrombosis [Vallet, 2001]. Balloon angioplasty of arteries causes endothelial denudation and, as a consequence of the exposure of the endothelium to the blood, provokes an instantaneous activation of platelets and the coagulation cascade [Gasperetti, 1993 and Hamon, 1995]. Tissue factor is rapidly induced in the arterial smooth muscle injured by this procedure [Marmur, 1993]. An increase in the generation of reactive oxygen species (ROS) by the cells of the vascular wall has been observed at sites of balloon injury [Nunes, 1997]. Oxidative stress might contribute to the increased expression of proatherosclerotic genes at sites of vascular injury, including TF [Taubman, 1997]. Our group has recently reported that LPA (a component of oxLDL present within the intima of atherosclerotic lesions) dramatically induces TF expression in RASMCs and it has been reported that it can also induce ROS release in RASMCs [Schmitz, 2002]. Little is known about the regulation of TF by LPA in SMC which is a major player in the process of atherosclerosis. The objectives of this study are to elucidate the signal transduction pathways involved in tissue factor induction by LPA in SMC. Understanding the mechanism of LPA induction of TF in SMC could contribute to proper understanding of atherosclerosis, thrombosis and restenosis after vascular procedures.

III. MATERIAL AND METHODS

MATERIALS:

LPA used in this study was (18:1) oleoyl LPA from Avanti Polar Lipids, Inc. pertussis toxin, RO-31, GO-6979, U73122, U73343, PD-98059, UO-126, Chelerythrin chloride, GF, Resveratrol, SB, Sp, BPB (4-bromophenol bromide), Aristolochic acid, BAPTA-AM, and MAFP were from Biomol Research Laboratories Inc. Arachidonic Acid, N-acetyl cysteine, sodium formate, desferoxamine, DPI, BEL (bromophenol lactone), thapsigargin, Luminol, and HRP (horse reddish peroxidase) were from Sigma Inc. MJ-33 was from Calbiochem Inc. ³H-arachidonic acid was purchased from American Radiolabeled Chemical Inc. (ARC).

TISSUE CULTURE:

Rat Aorta smooth muscle cells (RASMCs) were prepared from explants of excised aortas of rats as described previously [Brock et al., 1985]. RASMCs between passages 7 and 17 were used in these studies. Cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were made quiescent by incubation in serum-free Dulbecco's modified Eagle's medium for 48 h prior to the addition of LPA or other agonists or drugs as described previously [Schechter et al., 1997 and Penn et al., 1999]. Human smooth muscle cells (HASMCs) were purchased from BioWhittaker, A Camprex Company. HASMCs between passages 4 and 7 were used.

NORTHERN BLOT ANALYSIS FOR DETECTION OF RASMCS TF MRNA:

Total cellular RNA was isolated by using TRIzol reagent (Gibco BRL.) according to the manufacturer's instructions. Total RNA (5-6 µg) was subjected to denaturing electrophoresis in formaldehyde/agarose gels. RNA was blotted onto HybondTM-N; nylon (Amersham) membranes and hybridized with radiolabeled cDNA probes [Virca et al., 1990]. A 685-bp *Eco*RI fragment of rat TF cDNA (GenBankTM accession number U07619) was a gift from Dr. Mark B. Taubman (Mount Sinai School of Medicine, New York) and was used to detect TF mRNA, whereas glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as an internal control.

NORTHERN BLOT ANALYSIS FOR DETECTION OF RASMCS EGR-1 AND HASMCS TF MRNA:

Total cellular RNA was isolated by using TRIzol reagent (Gibco BRL.) according to the manufacturer's instructions. RNA samples (8 µg per lane) were separated by electrophoresis through 1.2% agarose gels after denaturation of the RNA with glyoxal and dimethylsulfoxide [Morawietz et al., 1999]. RNA was transferred with 20x SSC buffer to Hybond-N nylon membranes (Amersham Life Science Inc). RNA was then hybridized with radiolabeled cDNA probes (641 bp *Eco*RI fragment of human TF cDNA or 1.5 kb of mouse Egr-1) in: 1 mol/L NaCl, 1% SDS, 10% dextran sulfate, and 100 µg/mL denatured salmon sperm DNA at 65°C for 16 hours. The membranes were washed twice for 15 minutes with 2xSSC, 0.1% SDS at 60°C and once for 15 minutes with 0.2xSSC, 0.1% SDS at 55°C.

ADENOVIRUS CONSTRUCTS AND ADENOVIRAL INFECTION OF RASMCs:

Adenoviruses encoding mouse PKC isotypes (δ , ϵ , or ζ) were constructed as previously described [Ohba et al., 1998 and Matsumoto et al., 2001]. The adenoviral vectors were amplified in 293 cells to obtain a high titre of the virus before infecting RASMCs. The cells were infected by vectors expressing the following proteins : Lac-Z (control), wild type PKC ϵ (PKC ϵ -wt), kinase deficient PKC ϵ (PKC ϵ -kd), wild type PKC δ (PKC δ -wt), dominant negative PKC δ (PKC δ -dn), PKC ζ (PKC ζ -wt), and dominant negative PKC ζ (PKC ζ -dn) for 24 h. the cells were then starved for 48 hours and the stimulated by LPA for 1.5 h or unstimulated.

WESTERN BLOT ANALYSIS:

Total cellular proteins were obtained from RASMCs grown in 100-mm dishes. At the end of each incubation, cell lysates were washed twice with PBS. Combined floating and attached cells were lysed by sonication for 20 s on ice in Western blot lysis buffer (50 mM Tris-HCl, pH 6.8, 8 M urea, 5% β -mercaptoethanol, 2% SDS, and protease inhibitors). After addition of 4 $\times$ SDS sample buffer and incubation at 65 °C for 15 min, samples were subjected to SDS-polyacrylamide gel 10% electrophoresis and transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore). The membranes were then probed with antibodies against Egr-1 (C19), CREB, P-CREB, MEK, and P-MEK (Santa Cruz) followed by a 1:3000 dilution of a peroxidase-labeled secondary antibody. The signal was visualized by ECL-Plus (Amersham Biosciences) as described previously [Xu et al., 1999].

ELECTROPHORETIC MOBILITY SHIFT ASSAYS:

Cell lysates were prepared from RASMCs grown in 100-mm dishes. At the end of each incubation, cell lysates were washed twice with PBS. Combined floating and attached cells were lysed by sonication for 20 s on ice in luciferase assay lysis buffer (Promega). Oligonucleotides containing a prototypic Sp1 site, an AP-1 site, and NFκB site were obtained from Promega Corp. An oligonucleotide containing a prototypic EGR-1 site was obtained from Operon Technologies (Alameda, CA). Oligonucleotides were radiolabeled using [³²P]dCTP (Amersham Corp.) as described [Oeth et al., 1994]. Gel shift assays were performed as follows: cell lysates (4-5 μg) were incubated with radiolabeled DNA probes (about 10ng; 1 ×10⁶ cpm) for 20 min at room temperature in a 20-μl binding reaction containing 20 mM Hepes, pH 7.9, 50 mM KCl, 0.5 mM EDTA, 5% glycerol, 1 mM dithiothreitol, 1 mg/ml bovine serum albumin, 0.1% Nonidet P-40, and 12.5 μg/ml poly (dI-dC). Protein-DNA complexes were separated from free DNA probe by electrophoresis through 6% nondenaturing acrylamide gels (Novex, San Diego, CA) in 0.5 ×Tris borate-EDTA. For competition and antibody supershift experiments, binding reactions were incubated with unlabeled double-stranded oligonucleotides (50 molar excess) or monospecific antibodies for 20 min before the addition of the radiolabeled oligonucleotide. Anti-EGR-1 (C19), anti-SRF, and anti CREB antibodies were obtained from Santa Cruz Biotechnology, (Santa Cruz, CA).

TRANSIENT TRANSFECTION ASSAY:

RASMCs were cultured in 60-mm dish 24 h prior to transfections. SuperFect reagent from Qiagen was used in transfecting the cells in accordance with the

manufacturer's recommendations. One set of RASMCs was transfected with a plasmid containing the wild type TF promoter cloned upstream of the luciferase reporter gene (5µg) [pTF (-258)]. Another set of cells was cotransfected with the same plasmid [pTF (-258)] and another plasmid expressing LacZ (pcDNA3.1/LacZ) (5µg). A third set of cells was cotransfected with pTF (-258) and another plasmid expressing Egr1 (pSVKrox-24) (5 µg) which was kindly provided by P. Charnay [LeMaire et al., 1990]. Cells were starved for 48 hours before assaying luciferase activity. Luciferase activity was determined using the Luciferase Assay System (Promega) and ML2250 luminometer (Dynatech).

DELETION ANALYSIS AND FUNCTIONAL ANALYSIS OF THE EGR-1 PROMOTER:

A series of transfection studies have been conducted to determine the LPA primary response regions in the Egr1 promoter. According to the published rat Egr1 promoter sequence, a set of oligonucleotides have been synthesized as primers to clone a series of Egr1 promoter regions with selected deletions by PCR techniques using the rat genomic DNA. These derivatives were cloned upstream of the luciferase reporter gene in the plasmid pGL2. LPA responsiveness of the promoter region of the Egr1 gene was evaluated by transiently transfecting RASMCs with the deleted Egr1 promoter constructs (hooked to a luciferase reporter gene) and subsequently treating these cells with LPA (25µM) for 3 hours after starving it for 48 hours. The construct pGL2/Egr1 (-140/+21) contains a luciferase reporter gene driven by the rat Egr1 promoter fragment -140/+21 which have 2 SRE and 2 CRE areas. To perform Functional analysis of the Egr-1 promoter, We constructed a plasmid having the 2 SRE areas mutated [pGL2/Egr1 (-

140/+21)dmSRE] and another plasmid having the 2 CRE areas mutated [pGL2/Egr1(-140/+21)dmCRE]. RASMCs were transiently transfected by those constructs, starved for 48h and then either untreated or stimulated with LPA (25 μ M) for 3 hours.

ROS RELEASE DETECTION:

A variety of oxygen radicals, including superoxide anion and the hydroxyl radical, as well as hydrogen peroxide are potential mediators of luminal-dependent chemiluminescence. Luminol (amino-2,3-dihydro-1,4-phthalazinedione) undergoes a dioxygenation reaction in the presence of reactive oxygen species to yield a cyclic peroxide that disintegrates to yield the electronically excited aminophthalate anion HRP (horseradish peroxidase) is used to enhance the the chemiluminescence signal [Allen, 1982]. Rat Aorta smooth muscle cells (RASMC) were grown in Dulbecco's minimal essential medium (DMEM) containing 10% fetal bovine serum. Cells were serum starved for 24 hours in serum-free DMEM prior to use for experiments. ROS were detected by the luminal and HRP-dependent chemiluminescence assay [Goldman et al, 1998]. Serum-starved cells were rinsed with PBS, trypsinized, suspended in Hanks balanced salt solution (HBSS), and were washed twice with HBSS. Cells (1×10^6) were incubated in a test tube (75 $\times$ 12 mm, Sarstedt no. 55.476) in 0.5 ml HBSS containing 0.2% fatty acid-free BSA, 100 μ M luminol, and 0.5 unit of HRP at 37 $^{\circ}$ C for 10 min. Cells were then placed in the luminometer and LPA (25 μ M) was added at time 0. Chemiluminescence was recorded immediately at 1 minute intervals.

DETERMINATION OF ^3H -ARACHIDONIC ACID (^3H -AA) RELEASED FROM PRELABELED RASMC EXPOSED TO LYSOPHOSPHATIDIC ACID (LPA):

To determine the effect of LPA on AA release in RASMC, cells were seeded into six well culture plates at a density of 3×10^5 cells/well and grew them to 75% confluence. Release of ^3H -AA into the medium was determined as described previously [Tithof et al, 1998]. Briefly, the cells were labeled with ^3H -AA for 24 h. Release of AA was determined by incubating cells with compounds in Hank's Balanced Salt Solution with 0.1 % albumin to inhibit reacylation and metabolism of released AA. Therefore, the data reflect cumulative deacylation from membrane phospholipids.

When the cells were 90 % confluent, release of ^3H -AA was determined after exposure to LPA (25 μM) for 1 h. At the end of the incubation period, the medium was collected into scintillation vials containing: xylene, 2,5-diphenyloxazole, polyoxyethylene, octyl phenol, Bis-MSB, and oleic acid. The radioactivity was determined by scintillation counting. Radioactivity in the cellular fraction was also determined, and ^3H -AA release was expressed as a percentage of total cellular radioactivity.

IV. Results

INDUCTION OF TF MRNA BY LPA:

LPA significantly increased TF mRNA in quiescent RASMCs in a concentration dependent manner with maximal accumulation at 25 to 50 μ M LPA [Figure 1 (figures: 1-45 are located in the appendix)]. Maximal levels of TF mRNA were observed at 1.5-2h of LPA stimulation. The increased accumulation of TF mRNA induced by LPA was transient and declined significantly between 4 and 8 hours (figure 2).

IDENTIFICATION OF THE TRANSCRIPTION FACTORS THAT BIND TO THE RAT TF PROMOTER UPON INDUCTION BY LPA:

Our group's preliminary transfection data suggest that the region from -258 to +106 of the Rat TF promoter is important for the TF gene induction by oxLDL. This area contains several transcription factor binding sites (two AP-1 sites, one NF κ B site, four Sp1 sites and one Egr-1 site). Electrophoretic mobility shift assays (EMSA) were used to examine whether binding activities of the above mentioned transcription factors were induced by LPA. Radiolabeled oligonucleotides containing the above mentioned sites in the rat TF promoter were incubated with cell lysates from quiescent untreated cells or cells treated with LPA for the indicated times. As shown in Fig.3 (C), at the Egr-1 site, in addition to the slower migrating protein-DNA complex (complex I) which remains unchanged during treatment, an increase in the signal intensity of another faster migrating protein-DNA complex (complex II) was induced by LPA. In contrast, no detectable changes were observed in Sp1, NF κ B, or Ap1 binding activities (figure 3 A, B, D). The identity of this complex (complex II) was confirmed by using an antibody that

specifically recognizes the transcription factor Egr1. The Egr1 complex (complexII) observed in LPA-stimulated cells [figure 4 (lane 2)] was abolished by pre-incubation of the RASM cell lysate with the Egr1 specific antibody, indicating that the induced band is Egr-1 complex.

EGR1 MRNA IS INDUCED BY LPA:

LPA (25 μ M) significantly increased Egr1 mRNA in quiescent RASMCs. Maximal levels of Egr1 mRNA were observed at about 35-50 minutes of LPA stimulation. The increased accumulation of TF mRNA induced by LPA was transient and decreased significantly after about 3.5 hours (figure 5).

EGR1 TRANSACTIVATES THE TF PROMOTER ACTIVITY:

RASMCs were trasfected with a plasmid containing the wild type TF promoter cloned upstream of the luciferase reporter gene [pTF (-258)]. Another set of cells was cotransfected with the same plasmid and another plasmid expressing LacZ (pcDNA3.1/LacZ). A third set of cells was cotransfected with both pTF (-258) and another plasmid expressing Egr1 (pSVKrox-24). The results show increased fold of luciferase activity in the cells cotransfected with both pTF/-258 and pSVKrox-24 compared to the other two sets indicating that Egr-1 transactivates the TF promoter, suggesting that Egr1 is a transcriptional activator of the TF gene in RASMCs (figure 6).

INCREASED EGR1 TRANSCRIPTIONAL BINDING ACTIVITY IS TRANSIENT AND IS DUE TO DE NOVO PROTEIN SYNTHESIS:

To investigate if increased Egr-1 binding activity induced by LPA is due to de novo protein synthesis or activation of pre-existing Egr-1 protein by post –translational modification, levels of Egr1 protein in cell lysates from quiescent and LPA-stimulated RASMCs were assessed by western blotting using an Egr1-specific antibody. As shown in figure 7, Egr1 protein was not detectable in quiescent cells. However, Egr1 protein expression was transiently induced following stimulation with LPA and expression level increased after about 35-50 minutes and then declined.

LOCALIZATION OF THE LPA-RESPONSIVE REGION IN THE EGR1 PROMOTER:

A series of transfection studies was conducted to determine the LPA primary response regions in the Egr1 promoter. Using the published mouse Egr1 promoter sequence, a set of oligonucleotides were synthesized as primers to clone a series of rat Egr1 promoter regions with selected deletions by PCR techniques using the rat genomic DNA. LPA responsiveness of the promoter region of the Egr1 gene was evaluated by transiently transfecting RASMCs with the deleted Egr1 promoter constructs which were inserted upstream of the luciferase gene. The cells were subsequently treated with LPA (25 μ M) for 3 hours after starvation for 48 hours. Four-fold induction by LPA was observed until deletions reached -140 bp. Further deletion resulted in a significant loss of activity (figure 8, 9). Sequence analysis revealed that this area of the Egr1 promoter contains two SRE (serum response elements) and two CRE (cyclic AMP response elements) sites.

FURTHER CHARACTERIZATION OF THE REGULATORY ELEMENTS IN THE EGR1 PROMOTER (SRE AND CRE ARE INVOLVED IN MEDIATING LPA INDUCTION OF THE EGR-1 PROMOTER ACTIVATION:

Transcriptional regulation of gene expression is mediated through DNA binding proteins, termed transcription factors. Response elements are the recognition sites of certain transcription factors. Most of them are located within 1 kb from the transcriptional start site. The Serum Response Element (SRE) present in the promoter region of several genes, including Egr-1, requires the binding of SRF (serum response factor) to activate expression of the gene downstream. [Lamb et al., 1996]. Another response element in the Egr-1 promoter is the cyclic AMP response element (CRE) which binds the transcription factor cyclic AMP response element binding protein (CREB) [Silva et al, 1998].

EMSA (electrophoretic mobility shift assays) were performed by incubating ³²P-labeled oligonucleotides containing SRE and CRE sites with cell lysates from untreated and LPA-stimulated RASMCs for various times. SRE binding activity was transiently induced, peaked between 7 and 15 minutes and decreased to background level at about 30 minutes (figure 10). Similarly, CRE binding activity was induced at about 10 minutes (figure 11). To determine whether the binding of the cell lysates to these regions of the Egr1 promoter was specific, competition studies using 50-fold molar excess of unlabeled SRE and CRE oligonucleotides were performed. Both SRF and CREB binding activities were completely blocked (no induced complex was formed), indicating the specificity of these bindings [Figure 12, 13 (lanes 5, 6)]. When radiolabeled oligonucleotides

containing mutated SRE and mutated CRE were also incubated with cell lysates from untreated and LPA-stimulated RASMCs for 15 and 10 minutes respectively. As shown in Figure 12 and 13, Both mutated SRE and CRE sites failed to bind to corresponding transcriptional factor confirming again the specificities of these binding activities observed with wild type SRE and CRE. Further confirmation was achieved by using antibodies that specifically recognize SRF and CREB. The protein-DNA complexes observed using LPA-stimulated cells were supershifted by pre-incubation of the RASMC lysates with these specific antibodies (fig12, 13).

FUNCTIONAL ANALYSIS OF THE EGR1 PROMOTER CONFIRMED THE INVOLVEMENT OF SRE AND CRE IN TURNING ON THE EGR1 PROMOTER BY LPA:

The construct pGL2/Egr1 (-140/+21) contains a luciferase reporter gene driven by the fully active rat Egr1 promoter fragment -140/+21 which contains two SRE and two CRE sites. We also constructed a plasmid having the two SRE sites mutated [pGL2/Egr1 (-140/+21) dmSRE] and another plasmid having the two CRE sites mutated [pGL2/Egr1(-140/+21) dmCRE]. RASMCs were transiently transfected with those constructs, starved for 48h and then either untreated or stimulated with LPA (25 μ M) for 3 hours. The results revealed that the double SRE and double CRE mutations reduced the LPA induction of the Egr1 promoter from 2.71-fold to 1.17-fold and 1.11-fold respectively (figure 14). These data together with the EMSA results indicates that both SRE and CRE sites are required for mediating LPA-induction of Egr-1 which ultimately regulate TF expression.

PERTUSSIS TOXIN SENSITIVE G-PROTEIN, PKC, PKD, AND MAPK ARE INVOLVED IN LPA-INDUCED TF AND EGR1 SIGNALING PATHWAYS IN RASMCS:

Several LPA receptors have been cloned and all of them have been identified as G protein coupled receptors [Goetzl and An, 1998]. To define the intracellular signaling pathways involved in LPA-induced TF gene expression, RASMCs were preincubated with the following drugs: pertussis toxin (G α i inhibitor), UO-126, PD98059 (MAPK inhibitors), SB-203580 (p38 inhibitor), GF-109, RO-31, chelerethrine chloride (pan-PKC inhibitors), GO-6976 (α , β PKC inhibitor), Sp-600125 (JNK inhibitor), U-73122 (PLC inhibitor), U73343 (negative control for U73122) and Resveratrol (PKD inhibitor). Northern blot analyses revealed that LPA-induction of TF and Egr1 mRNA accumulation were blocked by PTX (G α i inhibitor), indicating the involvement of a PTX-sensitive G-protein (G α i) in the signaling pathway mediating LPA induction of TF and Egr1 gene expression. It was also found that LPA-induced TF and Egr1 mRNA accumulation were blocked in the cells pretreated with UO-126, PD98059 (MAPK inhibitors), GF-109, RO-31, chelerethrine chloride (PKC inhibitors) and Resveratrol (PKD inhibitor), whereas, they were not blocked by GO6976, SB-203580 (p38 inhibitor), Sp-600125 (JNK inhibitor) and U73343, (PLC inhibitor). These results suggest that MAPK, PKC and PKD are involved in the signaling pathway of LPA-induced TF and Egr1 gene expression whereas PKC α , β , p38, JNK and PLC are not involved (figures 15-18).

PKC ϵ IS INVOLVED IN LPA-INDUCTION OF TF MRNA EXPRESSION:

The above mentioned data suggest that some PKC isoforms (except for α , β) are involved in the signaling pathway of LPA-induced TF and Egr1 mRNA expression. PKC exists as a family composed of at least 10 isoforms. They are classified into three major groups [conventional PKC isoforms (α , β 1, β 2, and γ), novel PKC isoforms (δ , ϵ , η , and θ) and atypical PKC isoforms (ζ and λ) (Chiu and Rozengurt, 2001). The possible roles of δ , ϵ , and ζ isoforms on LPA induction of TF mRNA expression in RASMS have been examined. An adenovirus vector system which allows high expression of the genes encoding for these proteins in almost all the cells infected for at least 24 hours (kindly provided by T. Kuroki) was used. RASMCs were infected by vectors expressing the following proteins : Lac-Z (control), wild type PKC ϵ (PKC ϵ -wt), kinase deficient PKC ϵ (PKC ϵ -kd), wild type PKC δ (PKC δ -wt), dominant negative PKC δ (PKC δ -dn), PKC ζ (PKC ζ -wt), and dominant negative PKC ζ (PKC ζ -dn) for 24 h. the cells were starved for 48 hours and then stimulated by LPA for 1.5 h or unstimulated. Total RNA was harvested and Northern blot analyses were performed. TF mRNA was only blocked in cells infected with the adenovirus expressing kinase deficient PKC ϵ suggesting that PKC ϵ is involved in LPA-induced TF mRNA expression, whereas, PKC δ and PKC ζ are not involved in this signaling pathway (figures 19, 20).

LPA STIMULATES ROS RELEASE IN RASMCS:

Reactive oxygen species (ROS) in specified amounts play an important role in normal metabolic and signalling processes. However, excess ROS can cause severe cardiovascular damage. ROS cause various types of damage to ion channels and pumps

and this damage is associated with vascular diseases such as atherosclerosis and hypertension (Walia et al, 2003). To determine whether LPA increases ROS release in RASMCs, serum-starved RASMCs were incubated with luminal (100 μ M) and HRP (see material and methods for details). Low chemiluminescence was detected in the absence of LPA. Addition of LPA (25 μ M) caused a rapid and marked increase in the chemiluminescence signal within a couple of minutes to return to the basal level after about 5 to 6 hours (figure 21). To determine if the chemiluminescence signal was generated from ROS, cells were pre-treated with N-acetyl cysteine (a general antioxidant) in a concentration of 10 mM for 30 minutes and then treated with LPA (25 μ M). Figure 21 shows that NAC diminishes the LPA-stimulated chemiluminescence signal, indicating that the chemiluminescence signal is a valid measure for ROS release. These results indicate that LPA stimulates ROS release in RASMCs.

ANTIOXIDANTS BLOCK LPA INDUCTION OF TF MRNA:

The above mentioned data indicates that LPA induces TF mRNA expression and at the same time stimulates ROS release. To determine whether LPA-induced ROS release is also involved in TF mRNA induction, quiescent RASMCs were stimulated with LPA (25 μ M) for 1.5 h alone or after preincubation with the following antioxidants: sodium formate [Na F. (specific blocker for free hydroxyl radicals release)] in a concentration of 100 mM for 24 h, N-acetyl cysteine [NAC (general antioxidants)] in a concentration of 10 mM for 30 min., desferoxamine mesylate (hydrogen peroxide release blocker) in a concentration of 50 mM for 30 min., and DPI [diphenyleneiodonium chloride (specific blocker for free oxygen radical release)] in a concentration of 25 μ M

for 30 min. Northern blot analysis revealed that LPA-induction of TF mRNA was blocked in the cells pre-treated with sodium formate, NAC and DPI suggesting that ROS is involved in LPA induction of TF mRNA (figure 22).

MAPK IS INVOLVED, BUT, PTX AND PKC ARE NOT INVOLVED IN LPA-INDUCTION OF ROS RELEASE IN RASMCS:

In the above mentioned data, PTX, PKC, and MAPK were shown to be involved in LPA-induction of TF mRNA. ROS release was measured in quiescent RASMCs after stimulation with LPA (25 μ M) alone or after pretreatment with PTX (120ng/ml) for 16 h, GF (10 μ M), or UO126 (10 μ M) (for 30 minutes each) to determine if the same G proteins-coupled receptors signaling pathway (involved in LPA-induced TF mRNA) is also involved in LPA-stimulated ROS release. As shown in figures 23, 24, and 25, PTX (G α i inhibitor) and GF (pan-PKC inhibitor) failed to block this induction. Only UO126 (MAPK inhibitor) was effective. These data suggests that LPA-induction of ROS release may involve an alternative signaling pathway which does not involve PTX-sensitive G-protein.

LPA STIMULATES AA RELEASE IN RASMCS:

Arachidonic acid (AA) is a polyunsaturated fatty acid present in cell membranes that modulates diverse physiologic and pathologic responses. These effects are primarily mediated by its metabolic products. Their effects, which depend on the nature of compounds, principally concern inflammation and thromboregulation (Marcus, 1994). It is well known that several transduction pathways are activated by LPA including arachidonic acid release (Moolenaar, 1995). To determine whether LPA increases AA

release in RASMCs, the cells were labeled with ^3H -AA for 24 h. Release of AA was determined by incubating cells with LPA (25 μM) in Hank's Balanced Salt Solution with 0.1 % albumin to inhibit reacylation and metabolism of released AA. When the cells were 90 % confluent, release of ^3H -AA was determined after exposure to LPA (25 μM) for 1 h. At the end of the incubation period, the medium was collected into scintillation vials and the radioactivity was determined by scintillation counting. Radioactivity in the cellular fraction was also determined, and ^3H -AA release was expressed as a percentage of total cellular radioactivity. Figure 26 shows a time course for the release of AA in RASMCs upon stimulation by LPA.

AA INDUCES TF MRNA:

AA is known to enhance the TF expression in mononuclear cells (Cadroy et al, 1998). To determine whether AA induces TF mRNA in RASMCs, quiescent RASMCs were stimulated with AA (10 μM) for different periods of time. Northern blot analysis was performed. Maximal levels of TF mRNA were observed after about 1.5 h of LPA stimulation. The increased accumulation of TF mRNA induced by LPA was transient and decreased to basal level in 8 hours (figure 27).

AA STIMULATES ROS RELEASE IN RASMCS:

It was reported that AA may play an essential part in activation of the enzyme NADPH oxidase, which is responsible for the generation of superoxide anion by neutrophils (Dana et al, 1994; Henderson et al, 1993; and Badwey et al, 1981). To determine whether AA increases ROS release in RASMCs, serum-starved RASMCs were incubated with luminol and HRP. Low chemiluminescence was detected in the absence of

AA. Addition of AA (10 μ M) caused a rapid increase in the chemiluminescence signal within a couple of minutes. (figure 28). To determine if the chemiluminescence signal was generated from ROS, cells were pre-treated with N-acetyl cysteine (a general antioxidant) and then treated with AA (10 μ M). Figure 28 shows that NAC diminished the AA-stimulated chemiluminescence signal. Together, these results indicate that AA can stimulate ROS release in RASMCs.

ANTIOXIDANTS BLOCK AA INDUCTION OF TF MRNA IN RASMCs:

The data presented above indicates that AA induces TF mRNA and at the same time stimulates ROS release in RASMCs. To determine whether AA-induced ROS release is also involved in TF mRNA induction, quiescent RASMCs were stimulated with AA (50 μ M) for 1.5 h alone or after pre-treatment with the following antioxidants: sodium formate [Na F. (specific blocker for free hydroxyl radicals release)] in a concentration of 100mM for 24h, N-acetyl cysteine [NAC (general antioxidants)] in a concentration of 10 mM for 30 min., desferoxamine mesylate (hydrogen peroxide release blocker) in a concentration of 50 mM for 30 min., and DPI [diphenyleneiodonium chloride (specific blocker for free oxygen radical release)] in a concentration of 25 μ M for 30 min. Northern blot analysis revealed that AA-induction of TF mRNA was blocked in the cells pre-treated with sodium formate, and DPI, suggesting that ROS is involved in AA induction of TF mRNA (figure 29).

PHOSPHOLIPASE A₂ (PLA₂) IS INVOLVED IN RELEASING AA FROM MEMBRANE PHOSPHOLIPIDS:

PLA₂ cleaves phospholipids at the sn-2 position to produce AA (Van Corven et al, 1989). Several different isoforms of PLA₂ have been described [Murakami and Kudo, 2002]. To investigate whether PLA₂ is involved in releasing AA from membrane phospholipids in RASMCs upon stimulation by LPA, quiescent RASMCs were stimulated with LPA (25 µM) for 1.5 h alone or after pretreatment with the following PLA₂ inhibitors: MAFP (10 µM), ARA (aristolochic acid, 10 µM), BPB (10 µM), Bel (10 µM), and MJ33 (10 µM), each for 30 minutes. It was found that LPA-induction of TF mRNA was blocked in the cells pre-treated with ARA, Bel, and MJ 33, suggesting that at least some of PLA₂ isoforms are involved (figure 30). The same PLA₂ inhibitors also blocked ROS release in RASMCs (figure 31, 32, 33) suggesting that LPA-induction of ROS release in RASMCs involves PLA₂-mediated AA release.

ANTIOXIDANTS DO NOT BLOCK LPA-INDUCTION OF MAPK SUGGESTING THAT LPA-INDUCED ROS RELEASE IS DOWNSTREAM OF MAPK INDUCTION IN RASMCS:

The above mentioned data (figure 25) suggests that MAPK is involved in LPA-induction of ROS release in RASMCS. To further confirm that MAPK acts upstream of ROS, quiescent RASMCs were stimulated with LPA (25µM) for 3 minutes alone or after preincubation with the following antioxidants: sodium formate [Na F. (specific blocker for free hydroxyl radicals release)] in a concentration of 100mM for 24h, N-acetyl cysteine [NAC (general antioxidants)] in a concentration of 10 mM for 30 min.,

desferoxamine mesylate (hydrogen peroxide release blocker) in a concentration of 50 mM for 30 min., and DPI [diphenyleneiodonium chloride (specific blocker for free oxygen radical release)] in a concentration of 25 μ M for 30 min. Cell lysates were collected and Western blot analysis was performed. Figure 34 shows that the anti-oxidants, which were shown to block ROS production, do not block LPA-induction of MAPK suggesting that LPA-induced ROS acts downstream of LPA-induced MAPK activation or independent of MAPK activation.

UO126 BLOCKS AA-INDUCTION OF ROS RELEASE CONFIRMING THAT ROS ACTS DOWNSTREAM OF MAPK:

Serum-starved RASMCs were incubated with luminol and HRP. They were then stimulated with AA (10 μ M) alone or after pre-incubation with UO126 (10 μ M) for 30 minutes. Addition of AA (10 μ M) caused a rapid increase in the chemiluminescence signal within a couple of minutes. UO126 pretreatment diminished AA-stimulated chemiluminescence signal (figure 35) confirming that MAPK mediates AA-induction of ROS release.

CALCIUM MOBILIZATION IS INVOLVED IN LPA-INDUCTION OF TF EXPRESSION:

It is well known that LPA induces calcium mobilization (Jalink et al., 1995 and AN et al., 1998). A previous study also indicated that calcium is required for ROS release in keratinocytes (Goldman et al., 1998). To determine whether calcium mobilization is involved in the LPA-induced TF mRNA pathway, quiescent RASMCs were stimulated

with Thapsigargin alone [endoplasmic reticulum Ca ATPase inhibitor (10 μ M for 1.5 h)], with LPA alone (25 μ M for 1.5 h) or after preincubation with BAPTA-AM [intracellular calcium chelator (40 μ M for 30 minutes)]. Northern blot analysis was performed. The results show that thapsigargin alone was able to induce TF mRNA. At the same time, BAPTA-AM was able to block LPA-induced TF mRNA suggesting that calcium mobilization is involved in induction of TF mRNA by LPA (figure 36).

CALCIUM MOBILIZATION IS INVOLVED IN LPA-INDUCTION OF ROS RELEASE:

To determine whether calcium mobilization is also involved in LPA-induced ROS release, serum-starved RASMCs were incubated with luminol and HRP. They were then stimulated with LPA (25 μ M) alone or after pre-incubation with BAPTA-AM (40 μ M) for 30 minutes. Addition of LPA (25 μ M) caused a rapid increase in the chemiluminescence signal within a couple of minutes. BAPTA-AM pretreatment diminished the LPA-stimulated chemiluminescence signal (figure 37), suggesting a role for calcium mobilization in LPA-induction of ROS release.

CALCIUM MOBILIZATION IS ALSO INVOLVED IN BOTH AA-INDUCED TF MRNA AND AA-INDUCED ROS RELEASE:

We also determined whether calcium mobilization is involved in AA-induction of TF mRNA and ROS release. The same previous 2 experiments (determining the relation between calcium mobilization and both TF mRNA and ROS release induction by LPA) were repeated exchanging AA (50 μ M) for LPA (25 μ M). Similar results were observed,

suggesting that calcium mobilization is also involved in AA-induction of TF mRNA and ROS release in RASMCs (figure 38, 39).

USE OF HUMAN AORTA SMOOTH MUSCLE CELLS (HASMCs):

Northern blot analyses were performed using Quiescent HASMCs to see whether the results obtained by using RASMCs can be reproduced in human cells. LPA significantly increased TF mRNA in quiescent HASMCs in a concentration dependent manner with maximal accumulation at 5 μ M LPA (compared to 25-50 μ M in RASMCs), indicating that HASMCs are more sensitive to LPA (compare figure 1 and figure 39). The results also indicate that responses to pertussis toxin and kinases inhibitors were similar, whereas the human cells were responding mainly to antioxidants blocking the release of free oxygen radicals (figures 40-45) (RASMCs responded also to antioxidants that block the release of free hydroxyl radicals).

V. DISCUSSION

Tissue Factor is the principal initiator of blood coagulation [Morrissey, 1997]. Upregulation of intravascular TF occurs in many models of inflammation and stress, and its upregulation is important in pathologic thrombotic events [Kibbe et al., 2003]. LPA is a component of oxLDL which has been shown to accumulate in human atherosclerotic plaques [Seiss et al., 1999]. Our group has recently reported that LPA markedly induces TF mRNA, TF protein, and TF surface activity in RASMCs [Cui et al., 2003]. It has also been reported that LPA induces ROS release in RASMCs [Schmitz, 2002]. The generation of ROS, which act as a signaling molecule in the vascular system, is enhanced in response to injury and has been associated with a procoagulant state and the progression of atherosclerotic disease [Berk, 1999; Kunsch and Medford, 1999; and Ruef et al., 1999]. The results of our study provide a link between these observations and contribute to dissecting the relevant signaling pathways involved.

The importance of oxidized LDL (OxLDL) in atherogenesis is currently well appreciated [Kita et al., 2001]. LPA is formed during mild oxidation of LDL and it was reported to be a major component of both the lipid core and the intima of atherosclerotic lesions [Seiss et al., 1999]. LPA may play a role in the early phase of atherosclerosis by inducing monocyte adhesion to the vascular endothelium, as well as in the late phase by triggering platelet activation and intra-arterial thrombus formation upon rupture of the atherosclerotic plaque. Moreover, LPA, by stimulating the proliferation of fibroblasts and by enhancing the survival of inflammatory cells, is likely to play a central role in the

excessive fibro-proliferative and inflammatory response to vascular injury that characterizes the progression of atherosclerosis. Furthermore, LPA can cause the phenotypic de-differentiation of medial smooth muscle cells that leads to the formation of the neointima [reviewed in Seiss, 2002]. Our study demonstrates that LPA significantly increases TF mRNA in both RASMCs and HASMCs. These data indicate another important aspect of the athero- and thrombogenic actions of lysophosphatidic acid.

A recent study of our group indicated that LPA-induced increase in TF mRNA is controlled at the transcriptional level [Cui et al., 2003]. The transcriptional regulation of TF expression appears to be both cell type-specific and stimulus-specific [reviewed in Bavendiek et al., 2002 and Mackmam, 1997]. Functional studies of the human TF promoter in various cell types identified two promoter regions implicated in TF gene expression. The first region, termed the LPS-response element (LRE), contains two AP-1 (activator protein-1) and one NF- κ B (nuclear factor κ B)-like binding sites. The LRE mediates inducibility of the TF promoter in response to pro-inflammatory stimuli (e.g. IL-1 β and TNF α), LPS, and PMA. The second region, the serum response region (SRR), contains three Egr-1 binding sites. This region mediates the induction of TF gene expression via Egr-1 in response to serum, PMA, shear stress, LDL, hypoxia, vascular endothelial growth factor, and LPS. A distal enhancer (-227 to -172) containing two AP-1 sites and an NF- κ B site mediates induction of the human TF promoter in monocytic cells and endothelial cells. Similarly, two AP-1 sites (-220 to -200) regulate induction of the murine TF promoter in fibroblast-like cells. Proximal enhancers in the human (-109 to -59) and rat (-103 to -80) TF promoters containing Egr-1 and Sp1 sites mediate induction

in epithelial-like cells and vascular smooth muscle cells, respectively. Our results indicate that LPA significantly induces TF and Egr-1 mRNA in RASMCs. Our western blot analysis and gel-shift results indicate that LPA induces immediate and de novo Egr-1 mRNA and protein synthesis. Importantly, our data demonstrate that LPA induces the binding activity of Egr-1 to the TF promoter. These results suggest that LPA-induction of TF gene expression in RASMCs is mediated by the transcription factor Egr-1.

Egr-1 is a ubiquitous transcription factor that participates in a wide range of physiological and pathophysiological processes [Gashler and Sukhatme, 1995]. The Egr-1 promoter contains many regulatory elements, including two Sp1 sites, five serum response elements (SREs) with adjacent Ets-like motifs, two cAMP response elements (CREs). The SREs in the Egr-1 promoter have been extensively studied. The distal SREs are critical for induction of Egr-1 by GH (growth hormone), PDGF (platelet-derived growth factor), GM-CSF (granulocyte macrophage colony-stimulating factor), urea, and stress. The proximal CRE is required for stimulation by GM-CSF and the distal CRE is required for stimulation by SAPKs (p38/stress-activated protein kinases) [reviewed in Duan et al., 2002]. To localize the LPA-responsive region in the Egr-1 promoter, a deletion analysis was performed. Our results indicated that the fold induction by LPA was relatively constant until deletions reached -140 bp. Further deletion resulted in a significant loss of activity, suggesting that the LPA response region is located within -140bp. This area contains two SREs and two CREs. EMSA and super shift experiments indicated that specific SRF and CREB binding activities to the SRE and CRE of Egr-1 promoter were induced by LPA. Functional analysis of the Egr-1 promoter confirmed the

EMSA results and indicated that both SREs and CREs are responsible for LPA induction of the Egr-1 promoter activation in RASMCs.

In vascular tissues, LPA binds to members of a family of G protein-coupled receptors, EDG (endothelial differentiation genes). Eight distinct subtypes of EDG receptors have been identified in mammalian cells. EDG-2 receptors are activated by LPA and the responses mediated by this subtype are blocked by pertussis toxin [Kou et al., 2002]. LPA was shown to sequentially activate PKC and MAPK in mesangial cells [Coy et al., 2002]. LPA was also reported to stimulate protein kinase C (PKC) in a pertussis toxin sensitive pathway in vascular smooth muscle cells [Seewald et al., 1999]. PKC- ζ was shown to mediate angiotensin II-induced and platelet derived growth factor (PDGF)-induced MAP kinase activation in vascular smooth muscle cells and Rat fibroblasts respectively [Liao et al., 1997 and van Dijk et al., 1997]. PKC- ϵ was also reported to activate MAPK in cat oesophagus smooth muscle cells upon induction by C2-ceramide [Shin et al., 2002]. Xuereb et al., 2000 reported that they were able to inhibit LPS-induced tissue factor synthesis in human vascular smooth muscle cells by disruption of ERK kinases and PKC signaling pathways. A group of kinases with PKC-like functional structures has been described. This kinase family consists of PKC μ , its mouse homologue termed PKD [Johannes et al., 1994 and Valverde et al., 1994]. These kinases share structural homology to PKCs with respect to the catalytic domain and to the presence of amino-terminal cysteine fingers, defining the structural basis for lipid binding. PKC μ /PKD differs from PKC isozymes by an acidic domain, a PH domain within the regulatory region and the lack of a typical pseudo-substrate site [Gschwendt et al., 1997 and Gibson et al., 1994]. PKC μ /PKD activation occurs through several

mediators via a PKC-dependent pathway [Zugaza et al., 1996]. PKC-epsilon was reported to colocalize with, phosphorylates, and activates PKC-μ in HEK293 cells [Brandlin et al., 2002]. In another study [Hausser et al., 2001], PKC-μ was shown to selectively activate the MAPK pathway. A previous study indicates that LPA induces expression of the Egr-1 transcription factor in mesangial cells. This induction was pertussis toxin sensitive and was blocked by a MAPK inhibitor. Oxidized phospholipids were shown to stimulate TF expression in human endothelial cells via activation of PKC/ERK/Egr-1 and calcium [Bochkov et al., 2002]. Our results also show that Pertussis toxin sensitive G-protein (Goi), PKC-ε, PKD, and MAPK are involved in LPA-induction of TF and Egr1 mRNA. Taken together, our results (together with the published data) suggest a signaling pathway in RASMCs starting by LPA activating Goi protein which in turn activate PKC- epsilon. PKC-epsilon then activates PKC-μ / PKD which phosphorylates MAPK. Activated MAPK induces Egr-1 which is involved in turning on TF gene in the nucleus (figure 46). Many cells which comprise the vasculature, generate reactive oxygen species (ROS) [Irani, 2000]. Oxidative stress is a term used to describe an imbalance between the production and destruction of reactive oxygen species (ROS), thereby leading to cellular and tissue injury [Rice-Evans and Burdon, 1994]. The common risk factors for atherosclerosis increase production of reactive oxygen species (ROS) by endothelial, vascular smooth muscle, and adventitial cells [Harrison et al., 2003]. The damage inflicted by ROS on cellular and extra-cellular targets such as membrane lipids, proteins, and DNA clearly contribute to tissue and organ dysfunction in many pathological states.

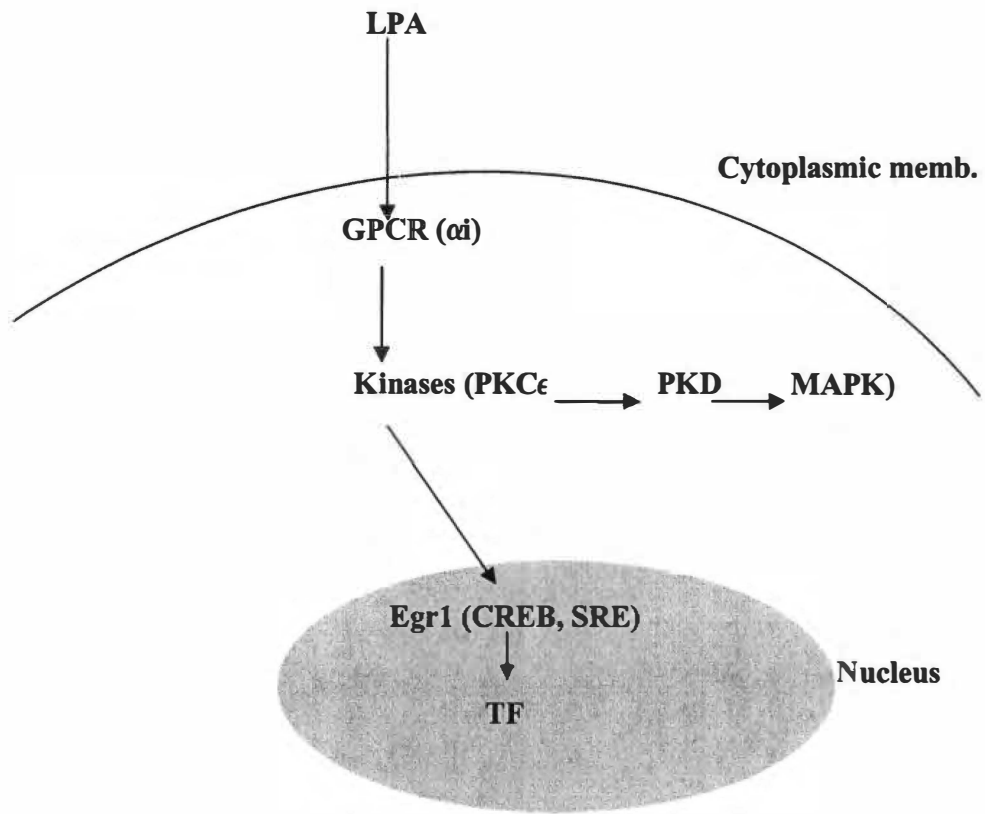


Fig.46. A diagram showing one of the signaling pathways for LPA induction of TF mRNA.

ROS has been identified as signaling molecules in a variety of cell types, including smooth muscle cells and other vascular cells. In particular, the oxidation of low-density lipoproteins (LDL) in the vascular wall is widely accepted to play a fundamental role in the pathogenesis of atherosclerosis [Shihabi et al., 2002]. Lysophosphatidic acid (LPA) is formed during mild oxidation of LDL and is the active compound in mildly oxidized LDL and minimally modified LDL [Siess et al., 1999]. Because of the importance of both LPA and ROS in vascular disease, we were interested in studying their interactions and relations at the molecular level. LPA was reported to stimulate ROS production in Hela cells [Chen et al., 1995]. Our results indicate that LPA induces ROS release in RASMCs, suggesting an important role for ROS in the athero- and thrombogenic actions of lysophosphatidic acid.

Increased ROS production is observed at sites of vascular injury and has been related to the development of restenosis/atherosclerosis [Berk, 1999]. Moreover, the enhanced generation of ROS promotes a procoagulant state [Ruef, 1999]. A role of ROS in the control of coagulation has been suggested, and the key activator of the extrinsic coagulation cascade, tissue factor (TF), has been shown to be modulated by ROS in several cell types, including vascular cells [Herkert and Gorlach, 2002]. Our results indicate that LPA-induction of TF mRNA was blocked by antioxidants suggesting a role for ROS in the control of LPA-induction of TF expression in RASMCs. To further elucidate the signaling pathways involved in ROS-mediated LPA-induction of TF gene expression in RASMCs, ROS release was measured in quiescent RASMCs after stimulation with LPA (25 μ M) alone or after pretreatment with PTX (G α i inhibitor), GF

(PKC inhibitor), and UO126 (MAPK inhibitor) to determine if the same G proteins-coupled receptors signaling pathway (involved in LPA-induced TF mRNA) is also involved in LPA-stimulated ROS release. Our results indicate that PTX (G α i inhibitor) and GF (pan-PKC inhibitor) failed to block this induction. In contrast, the MAPK inhibitor, UO-126, blocked LPA-induction of ROS production. These data suggest that LPA-induction of ROS release may involve an alternative signaling pathway which is different from the G α i protein-coupled receptor pathway.

It is well known that several signaling pathways are activated by LPA including calcium mobilization and AA release. It has been reported that LPA stimulates ROS release in human keratinocytes by a pathway involving intracellular calcium release, phospholipase A2 (PLA2) stimulation, and arachidonic acid (AA) release [reviewed in Sekharam et al., 2000]. Arachidonic acid serves as precursor for the generation of bioactive lipid mediators known as eicosanoids that include prostaglandins, thromboxanes, and leukotrienes [Tithof et al., 1998]. Arachidonic acid metabolites are important regulators of inflammation, blood coagulation, and thrombosis [Marcus, 1994; Petrukhina and makarov, 1997]. Eicosanoids modulate physiologic processes in the vessel wall contributing to atherosclerosis and thrombosis, including platelet aggregation, control of vascular tone, and the local inflammatory response [Linton and Fazio, 2002]. Proliferation of vascular smooth muscle cells (VSMCs) is implicated in the pathogenesis of primary atherosclerosis, and restenosis following interventional revascularization procedures such as balloon angioplasty, arterial stenting, and by-pass surgery [Anderson et al., 1997]. AA has been reported to induce smooth muscle cell proliferation and

migration by releasing reactive oxygen species in rabbit cultured aortic smooth muscle cells [Nishio and Watanabe, 1997]. AA has also been reported to induce apoptosis in rat glioma cells and human promyelocytic leukemia cells (HL-60) by accumulating reactive oxygen species [Higuchi and Yoshimoto, 2002; Arita et al., 2001]. LTB₄ [leukotriene B₄ (an arachidonic acid metabolite)] induces ROS generation in Rat-2 fibroblasts, neutrophils, eosinophils, and other fibroblasts [reviewed in Woo et al., 2002]. AA was shown to enhance tissue factor expression in mononuclear cells by the cyclo-oxygenase pathway [Cardoy et al., 1998]. Eicosanoids (AA metabolites) were also shown to enhance LPS and TNF- α –evoked expression of monocyte TF [Eilertsen et al., 2003]. Our results indicate that LPA stimulates rapid release of AA in RASMCs. These results also indicate that AA stimulates ROS release and induces TF mRNA, suggesting its involvement in ROS-induction of TF expression in RASMCs upon stimulation by LPA.

Phospholipase A₂ (PLA₂) catalyzes the hydrolysis of the sn-2 position of membrane glycerophospholipids to liberate arachidonic acid (AA). The same reaction also produces lysophospholipids, which represent another class of lipid mediators. So far, at least 19 enzymes that possess PLA₂ activity have been identified and cloned in mammals [Kudo and Murakami, 2001]. The secretory PLA₂ (sPLA₂) family, in which 10 isozymes have been identified, consists of low-molecular weight, Ca²⁺-requiring secretory enzymes that have been implicated in a number of biological processes, such as modification of eicosanoid generation, inflammation, and host defense. The cytosolic PLA₂ (cPLA₂) family consists of three enzymes, among which cPLA₂ α has been paid much attention by researchers as an essential component of the initiation of AA

metabolism. The activation of cPLA2 α is tightly regulated by Ca²⁺ and phosphorylation. The Ca²⁺-independent PLA2 (iPLA2) family contains two enzymes and may play a major role in phospholipid remodeling. The platelet-activating factor (PAF) acetylhydrolase (PAF-AH) family contains four enzymes that exhibit unique substrate specificity toward PAF and/or oxidized phospholipids. Degradation of these bioactive phospholipids by PAF-AHs may lead to the termination of inflammatory reaction and atherosclerosis [Kudo and Murakami, 2001].

Atherosclerosis can be regarded as a localized inflammation of the artery wall, and plaque progression is substantially driven by inflammatory mediators. Lipoprotein-associated phospholipase A₂ (Lp-PLA₂) is a calcium-independent serine lipase that, in plasma, is mostly associated with low density lipoprotein (LDL) particles. Its substrates include platelet-aggregating factor (PAF), and the enzyme is widely referred to in the literature as PAF acetylhydrolase. Oxidised LDL (formed under conditions of oxidative stress, such as exist in the artery wall) contains a variety of lipid peroxidation products, which are efficiently hydrolysed at the SN2 position by Lp-PLA₂. The products are oxidised fatty acids plus lyso-phosphatidylcholine (PC); both have a variety of proinflammatory effects in many cell types, including monocyte. A proposed proatherogenic mechanism for this enzyme is that associated Lp-PLA₂, becomes oxidised within the intima, providing a substrate for the enzyme. The resulting hydrolysis products promote the chronic inflammation that is associated with macrophage accumulation. Macrophages also produce and secrete further Lp-PLA₂, representing one of the positive feedback mechanisms driving disease progression [Leach et al., 2001].

LPA may be synthesized by cells either *de novo* from glucose through pathways of lipid metabolism in the endoplasmic reticulum or through liberation of precursor phospholipids and subsequent enzymatic conversions in membrane microvesicles [Goetzl, 1998; Jalink, 1994; and Gaits, 1997]. PLD first converts phosphatidylcholine to PA and PLA₂ then hydrolyzes the *sn*-2 (*sn*-1) ester bond of PA to generate LPA. Exogenous LPA can also induce PLA₂ to generate LPA (positive feed back mechanism) [Eder et al., 2000]. Our results demonstrate that LPA-induced TF mRNA was blocked in the cells pre-treated with ARA, Bel, and MJ 33 (PLA₂ inhibitors), suggesting that at least some of PLA₂ isoforms are involved in LPA-induced AA release. The same PLA₂ inhibitors also blocked ROS release in RASMCs suggesting that LPA-induced ROS release in RASMCs involves AA release by PLA₂.

LPA is known to activate arachidonic acid release, MAPK activation and calcium mobilization [Sekharam et al., 2000]. AA and its metabolites are implicated in the control of calcium homeostasis, including modulation of calcium release and calcium entry via voltage-gated and nonvoltage-gated pathways by AA itself or by metabolites synthesized via cyclooxygenase (COX), lipoxygenase (LOX), or mono-oxygenase (cytochrome P450, MOX) pathways. In general, AA and its metabolites stimulate calcium mobilization [Liu and Rittenhouse, 2000]. AA was shown to activate MAPK in vascular smooth muscle cells [Kalyankrishna and Malik, 2003]. MAPK was also reported to induce ROS generation in human lung endothelial cells and other cells [Parinandi et al., 2002 and Kroj et al., 2003]. Increased intracellular calcium concentration was shown to induce TF expression in vascular endothelial cells [Taubman et al., 1993 and Wakita et al., 1994].

Our results show that thapsigargin (intracellular calcium mobilization inducer) alone was able to induce TF mRNA. At the same time, BAPTA-AM (intracellular calcium chelator) was able to block both LPA-induced TF mRNA and LPA-induced ROS release in RASMCs. Our western blot results also show that antioxidants do not block LPA-induction of MAPK suggesting that LPA-induction of ROS release acts downstream of MAPK activation in RASMCs. Taken together, these data show the diverse ways and signaling mechanisms by which LPA can induce TF in RASMCs (figure 47). In one pathway, LPA activates PLA2 which releases AA from the membrane phospholipids. AA can then activate MAPK, ROS release, and calcium (all of them can induce TF). In another pathway, LPA activates MAPK which induces ROS release inducing TF. LPA can also induce ROS release to induce TF.

We used HASMCs to reproduce some of the main experiments performed initially in RASMCs. HASMCs are more expensive and more difficult to grow compared to RASMCs. We were able to reproduce most of the important data we had in RASMCs, including the induction of both TF mRNA and ROS release by LPA and AA. These results indicate that RASMCs still represent an authentic model for studying vascular smooth muscle cell biology.

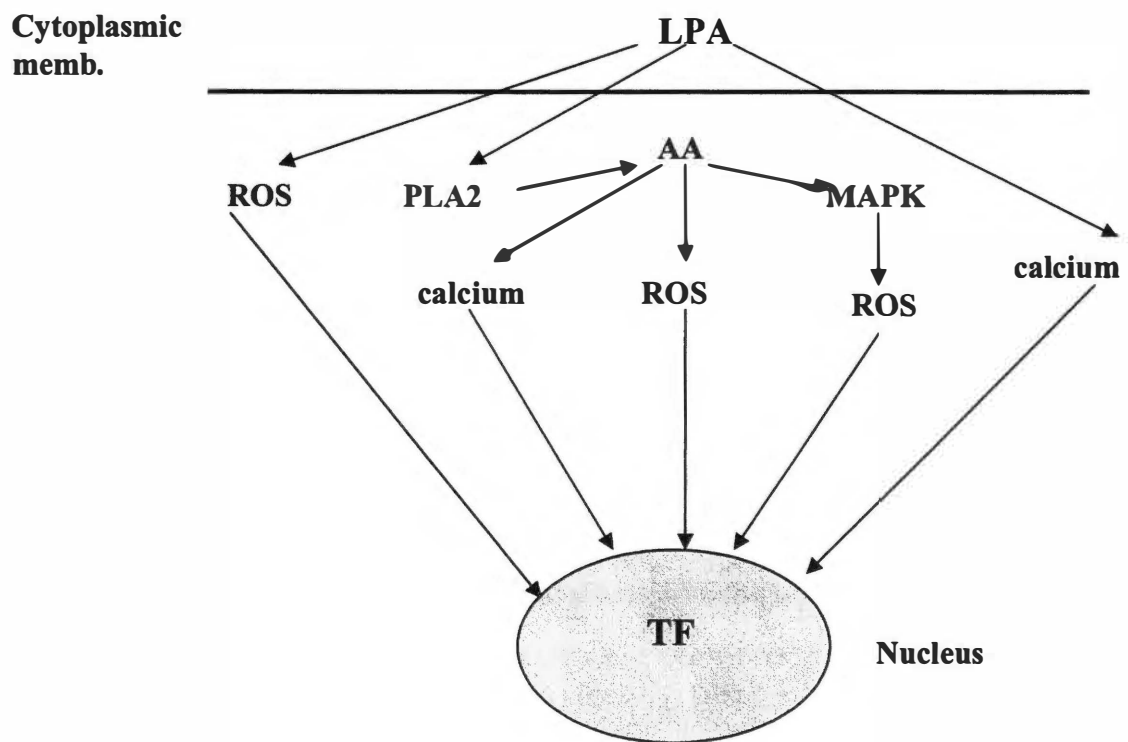


Fig. 47. A diagram showing another set of signaling pathways by which LPA induces TF.

VI. SUMMARY AND CONCLUSION

Lysophosphatidic acid (LPA) is a potent bioactive phospholipids with specific and multiple effects on blood cells and cells of the vessel wall. LPA is a component of oxidized low density lipoproteins (oxLDL) which has been shown to accumulate in human atherosclerotic plaques. Tissue factor is the principal initiator of blood coagulation. The active form of TF has been shown to be present in specimens of human coronary artery in association both with acellular lipid areas and with macrophages and smooth muscle cells, which suggests that TF plays a major role in determining plaque thrombogenicity. The generation of ROS, which act as a signaling molecule in the vascular system, is enhanced in response to injury and has been associated with a procoagulant state and the progression of atherosclerotic disease.

Our group has recently reported that LPA markedly induces TF mRNA, TF protein, and TF surface activity in RASMCs. It has also been reported that LPA induces ROS release in human keratinocytes. Little is known about the regulation of TF by LPA in SMC which is a major player in the process of atherosclerosis. The present study investigated the regulation of TF expression by LPA in RASMCs. Data generated by this study demonstrate that LPA markedly induces TF expression in RASMCs and HASMCs. The signaling pathways involved are multiple. One signaling pathway demonstrates that LPA activates G α i protein which in turn activate PKC- epsilon that activates PKC- μ / PKD which phosphorylates MAPK to induce Egr-1 that is involved in turning on TF gene in the nucleus. Another pathway indicates that LPA activates PLA2 which releases AA from the membrane phospholipids. AA can then activate MAPK, ROS release, and

calcium (all of them can induce TF). In another pathway, LPA activates MAPK which induces ROS release inducing TF. LPA can also induce ROS release to induce TF. Our data suggest that elevated LPA have potential atherogenic and thrombogenic actions by inducing TF expression and ROS release. Understanding the mechanisms involved could provide new insights into atherosclerosis, thrombosis and restenosis after angioplasty. It could also help in designing therapeutic strategies for these disease conditions.

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APPENDIX

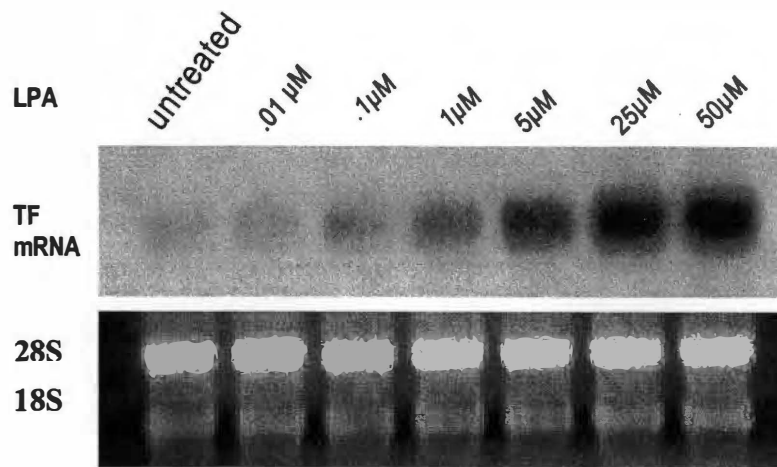


Fig.1. Induction of TF mRNA in response to LPA in RASMC. LPA was added to quiescent RASMC at the concentrations indicated above each lane. Total RNA (6 μ g per lane) was isolated from cells incubated with LPA for 1.5 h, and TF mRNA levels were determined by northern blotting. Rat TF cDNA fragment (685 bp) was used as a probe. 28S and 18S rRNA were visualized by ethidium bromide staining.

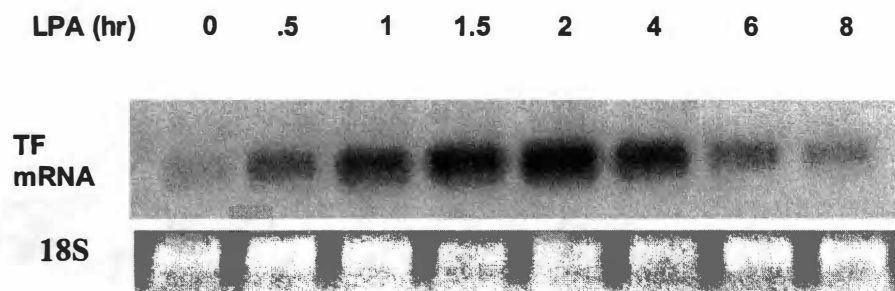


Fig. 2. Time course of LPA induction of TF mRNA. LPA (25 μ M) was added to quiescent RASMC at the times indicated. Total mRNA was isolated, and TF mRNA levels were determined by Northern blot analysis. A rat TF cDNA fragment (685bp) was used as a probe. 18S rRNA was visualized by ethidium bromide staining.

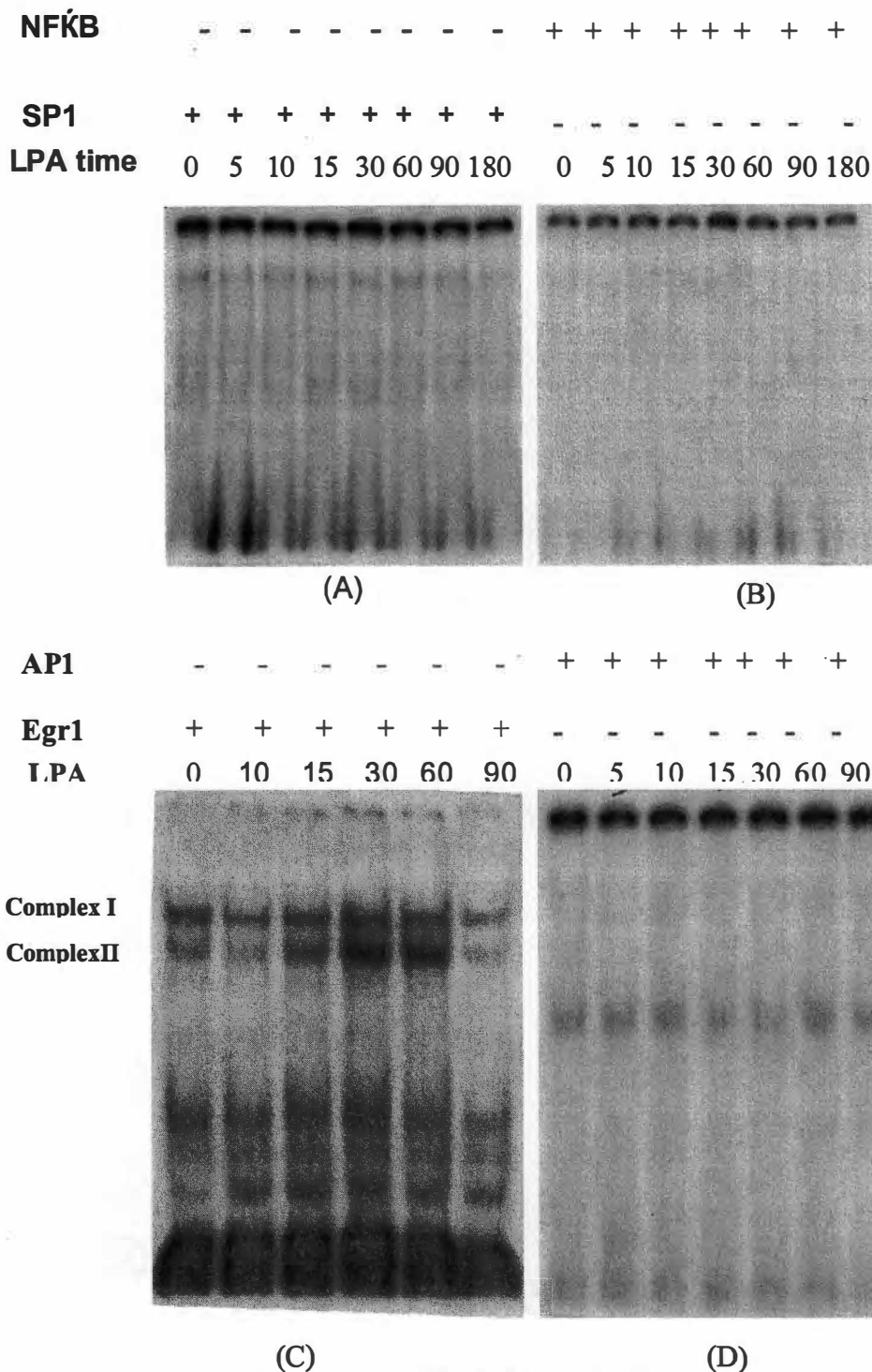


Fig.3. Nuclear protein binding activity is induced by LPA at the Egr1 site (not at SP1, NFκB, AP1). Four oligonucleotides (SP1, NFκB, AP1, and Egr1) were individually incubated with cell lysates from unstimulated RASMCs, or cells stimulated with LPA for the times indicated. Protein-DNA complexes were analyzed by EMSA using a 6% polyacrylamide gel.

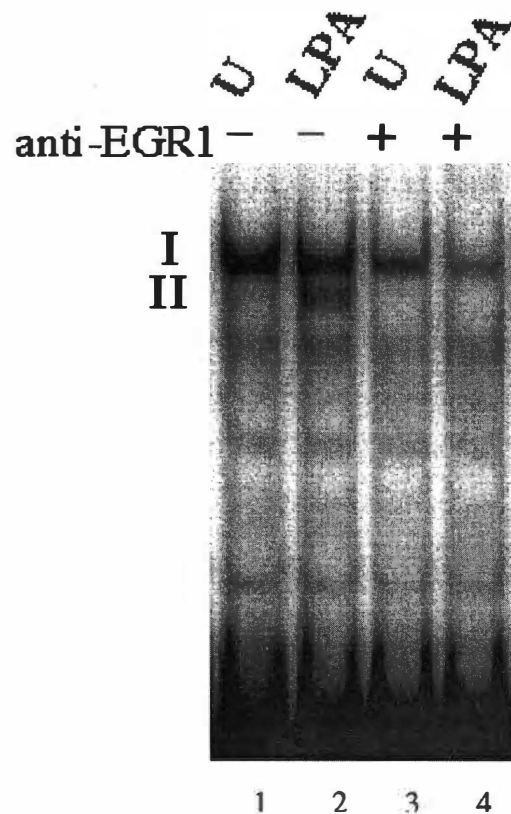


Fig.4. Identification of the protein present in Egr1 protein_DNA complex II using Egr1 specific antibody. Egr1 oligonucleotides were incubated with nuclear extracts from LPA-stimulated (1h) RASMCs in the absence or presence of 2 μ l of Egr1 antibody. The Egr1 complexes are separated using 6 % nondenaturing acrylamide gel.

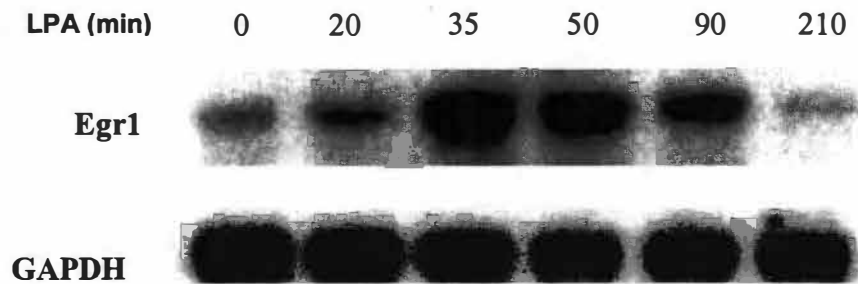


Fig. 5. Time course of LPA induction of Egr1 mRNA. LPA (25 μ M) was added to quiescent RASMC at the times indicated. Total mRNA was isolated, and Egr1 mRNA levels were determined by Northern blot analysis. A rat Egr1 cDNA fragment (1.5kb.) was used as a probe.

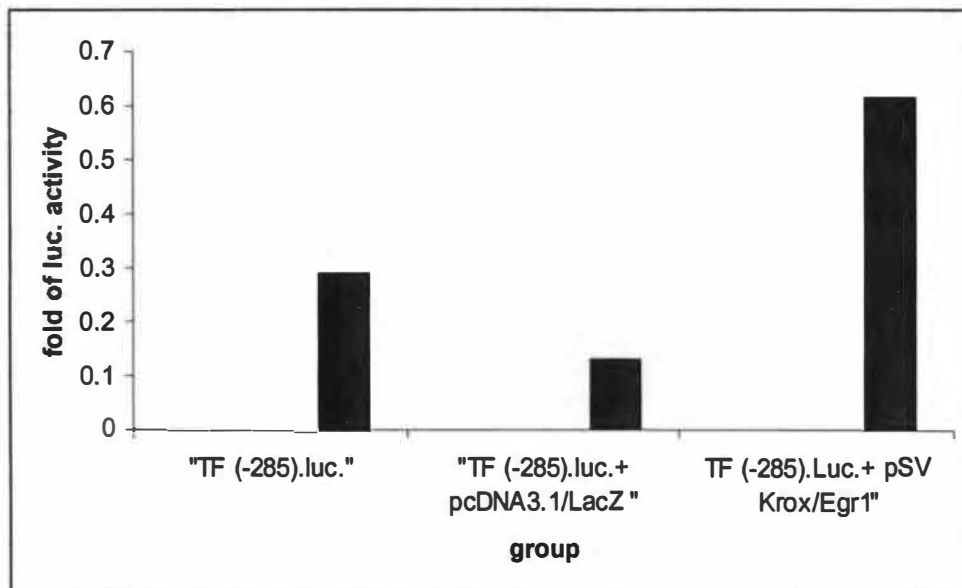


Fig.6. Egr1 can transactivate the TF promoter. RASMCs were transfected with a plasmid containing the wild type TF promoter cloned upstream of the luciferase reporter gene [pTF (-258)]. Another set of cells was cotransfected with the same plasmid and another plasmid expressing Lac Z (pcDNA3.1/LacZ). A third set of cells was cotransfected with both pTF (-258) and another plasmid expressing Egr1 (pSVKrox-24). Cells were starved for 48 hours before assaying luciferase activity.

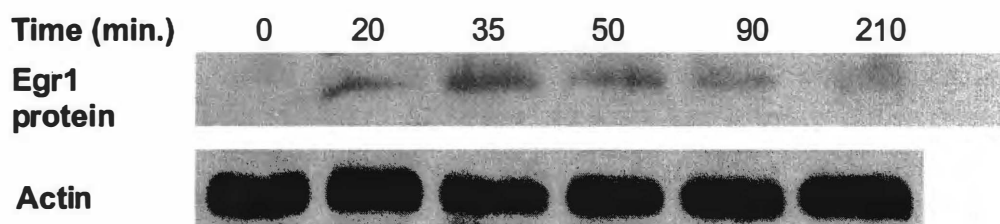


Fig.7. Time course of LPA induction of Egr1 protein in RASMCs. Quiescent RASMCs were stimulated with 25 μ M of LPA, and at the times indicated, cell lysates were analysed by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Egr1 protein was detected with antibody against aegr1(C-19). Actin protein was used to confirm equal protein loading.

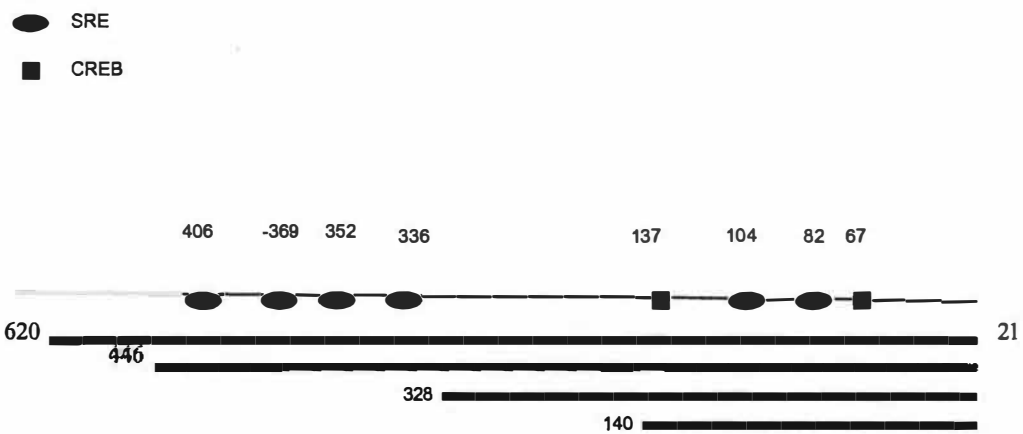


Fig.8. A diagram representing mouse *Egr1* promoter fragment (-620/+21).

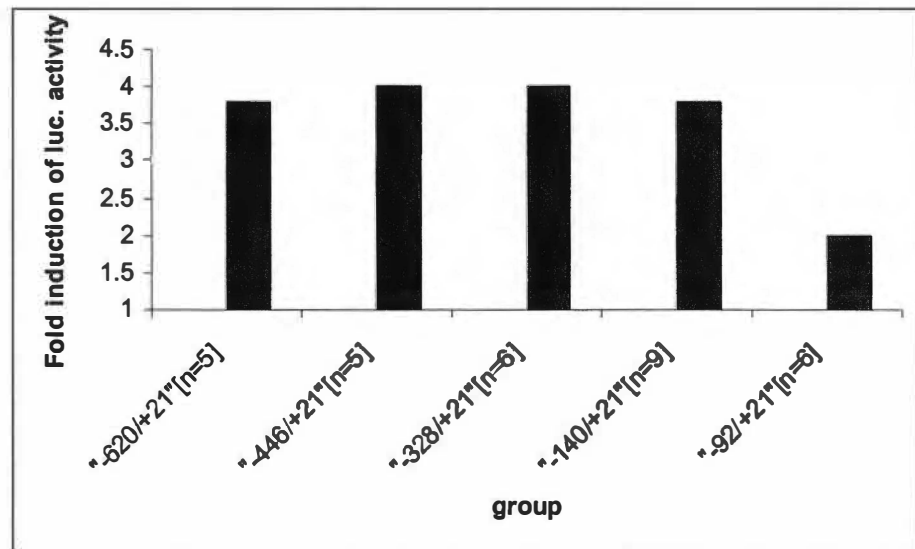


Fig.9. Localization of the LPA-responsive region in the Egr1 promoter. A series of deleted Egr1 promoter constructs was made (see “experimental procedures”). Transfected RASMCs were made quiescent for 48 h before the addition of 25 μ M LPA for 3.5 h. Fold induction is the luciferase (luc.) of transfected cells stimulated with LPA compared with unstimulated contrls.

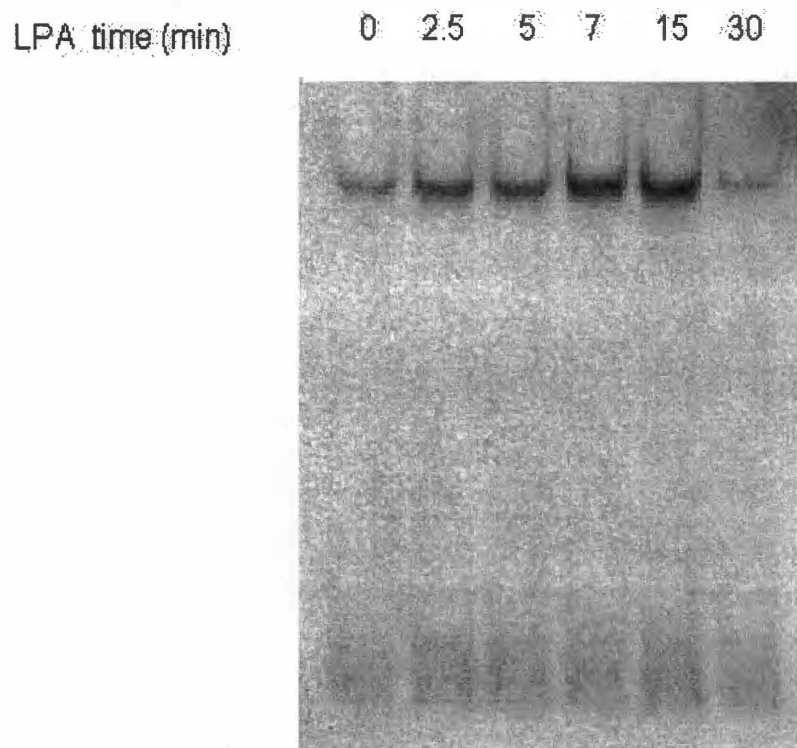


Fig.10. Nuclear protein binding activity is induced by LPA at the SRE site. Radiolabelled SRE oligonucleotides were incubated with cell lysates from unstimulated RASMCs, or cells stimulated with LPA for the times indicated. Protein-DNA complexes were analyzed by EMSA using a 6% polyacrylamide gel.

LPA time (min)

0 5 7 10

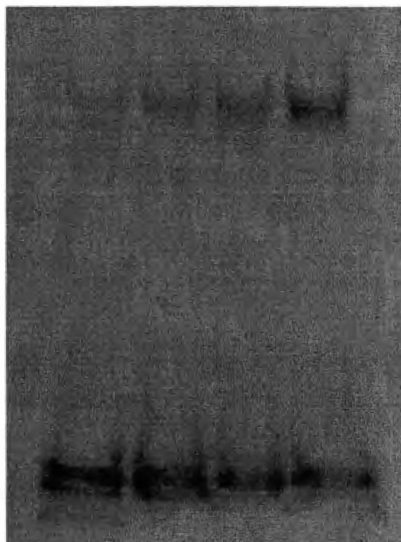


Fig.11. Nuclear protein binding activity is induced by LPA at the CRE site. Radiolabelled CRE oligonucleotides were incubated with cell lysates from unstimulated RASMCs, or cells stimulated with LPA for the times indicated. Protein-DNA complexes were analyzed by EMSA using a 6% polyacrylamide gel.

P-SRE Ab.	-	-	-	-	-	-	-	-	+	+
SRE Ab.	-	-	-	-	+	+	-	-	-	-
Mut. SRE	-	-	-	-	+	+	-	-	-	-
Cold SRE	-	-	+	+	-	-	-	-	-	-
SRE	+	+	+	+	-	-	+	+	+	+
LPA	0	10	0	10	0	10	0	10	0	10

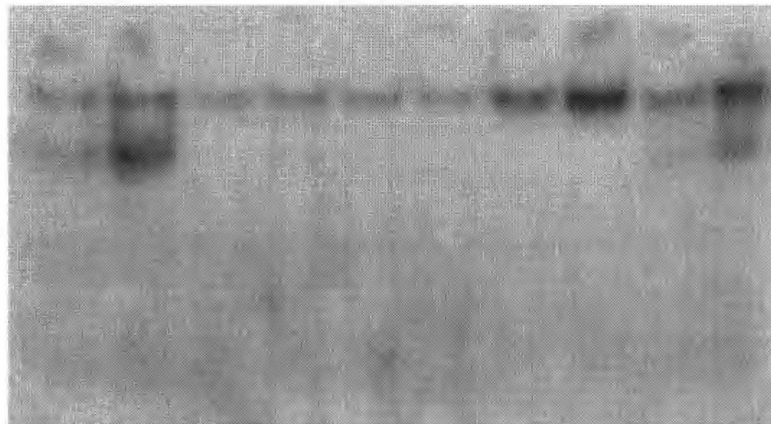


Fig.12. Specific nuclear protein binding activity is induced by LPA at the SRE site. Cell lysates from quiescent RASMCs or RASMCs stimulated with LPA for 10 minutes were pre- incubated for 20 minutes with: 50 molar excess of unlabelled SRE oligonucleotides (3, 4), radiolabelled mutated SRE oligonucleotides (5, 6), 2 μ l of SRF antibody (6, 7), 2 μ l of phosphorylated SRF antibody (9, 10). They were then incubated with radiolabelled SRE oligonucleotides for another 20 minutes (1, 2, 3, 4, 7, 8, 9, 10). Protein DNA complexes were then analyzed by EMSA using a 6% polyacrylamide gel.

Anti-P-CREB	-	-	-	-	-	-	-	-	+	+
Anti-CREB	-	-	-	-	-	-	+	+	-	-
m-CREB	-	-	-	-	+	+	-	-	-	-
Cold CREB	+	+	-	-	-	-	-	-	-	-
CREB	-	-	+	+	-	-	-	-	-	-
LPA Time (min)	0	10	0	10	0	10	0	10	0	10

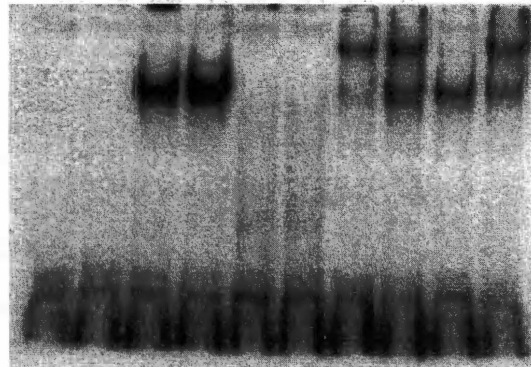


Fig.13. Specific nuclear protein binding activity is induced by LPA at the CRE site. Cell lysates from quiescent RASMCs or RASMCs stimulated with LPA for 10 minutes were pre- incubated for 20 minutes with: 50 molar excess of unlabelled CRE oligonucleotides (1, 2), radiolabelled mutated SRE oligonucleotides (5, 6), 1 μ l of CRE antibody (6, 7), 1 μ l of phosphorylated CRE antibody (9, 10). They were then incubated with radiolabelled CRE oligonucleotides for another 20 minutes (1, 2, 3, 4, 7, 8, 9, 10). Protein DNA complexes were then analyzed by EMSA using a 6% polyacrylamide gel.

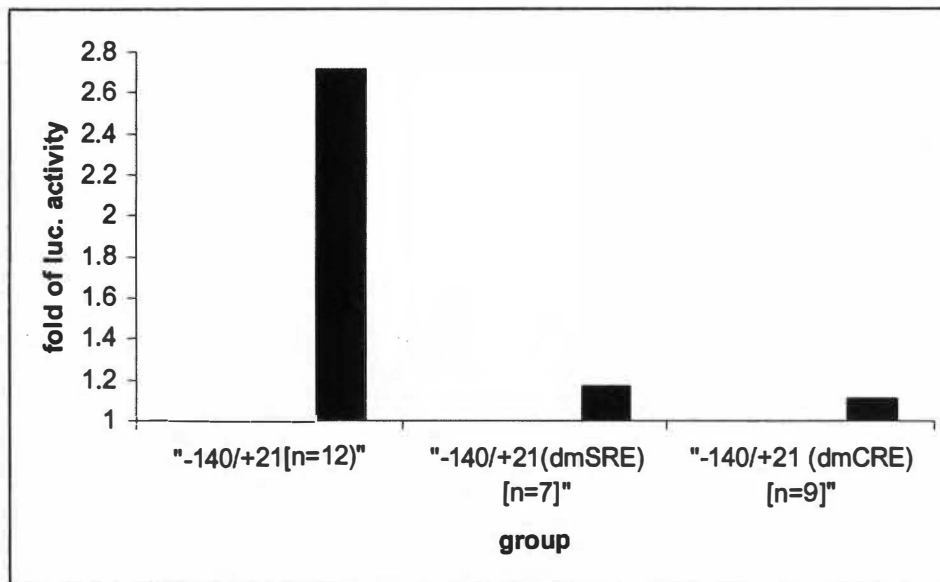


Fig.14.Functional analysis of the Egr1 promoter. RASMCs were transfected with th native Egr1 promoter (-140/+21) or mutated Egr1 promoter constructs [(-140/+21)dmSRE and (-140/+21)dmCRE)]. Transfected cells were starved for 48h before the addition of LPA (25 μ M) for 3h. Fold induction is the luciferase activity of transfected cells stimulated with LPA compared with unstimulated controls (n = number of experiments; in each, transfections were performed in duplicates).

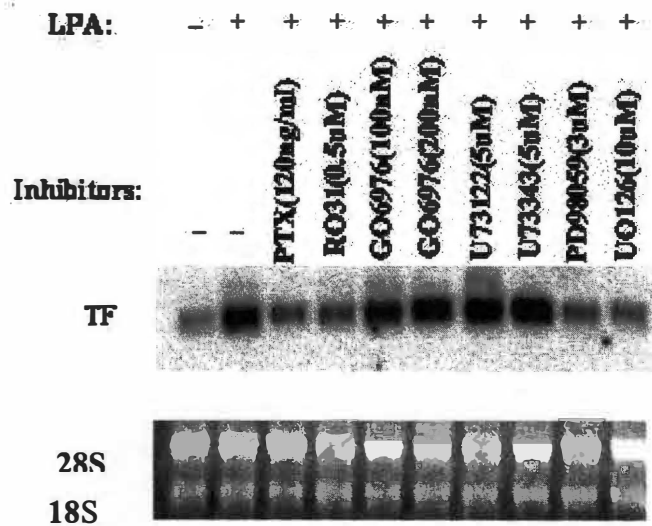


Fig.15. Northern blot analysis shows that pretreatment of quiescent RASMCs with PTX, RO31, PD98059, UO126 block LPA-induced TF mRNA, whereas, GO73122, U73122, and U73343 were not effective.

Quiescent RASMCs were stimulated with LPA (25μM) alone for 1.5 hor after pretreatment with PTX (16h), RO31, PD98059, UO126, GO6976, U73122, and U73343 (each for 30 min.) in the concentrations indicated above. A rat TF cDNA fragment (685bp) was used as a probe. 28S and 18S rRNA were visualized by ethidium bromide staining.

Chelerythrin chloride	-	-	+	-
(3 μ M)				
GF (10 μ M)	-	-	-	+
LPA (25 μ M)	-	+	+	+

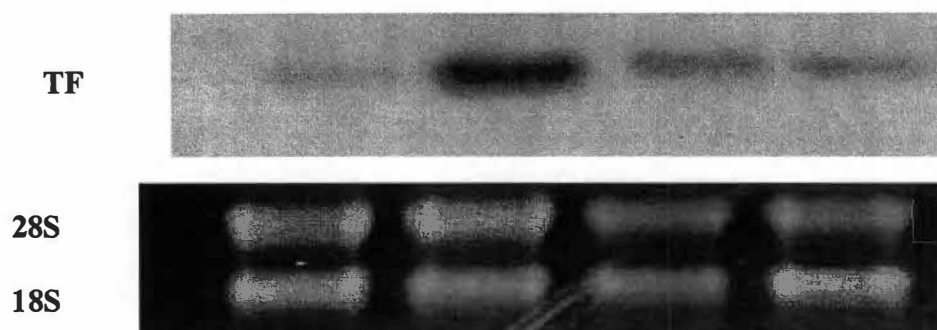


Fig.16. Northern blot analysis shows that pretreatment of quiescent RASMCs with chelerythrin chloride and GF block LPA-induced TF mRNA. Quiescent RASMCs were stimulated with LPA (25 μ M) alone for 1.5 h or after pretreatment with chelerythrin chloride and GF (each for 30 min.) in the concentrations indicated above. A rat TF cDNA fragment (685bp) was used as a probe. 28S and 18S rRNA were visualized by ethidium bromide staining.

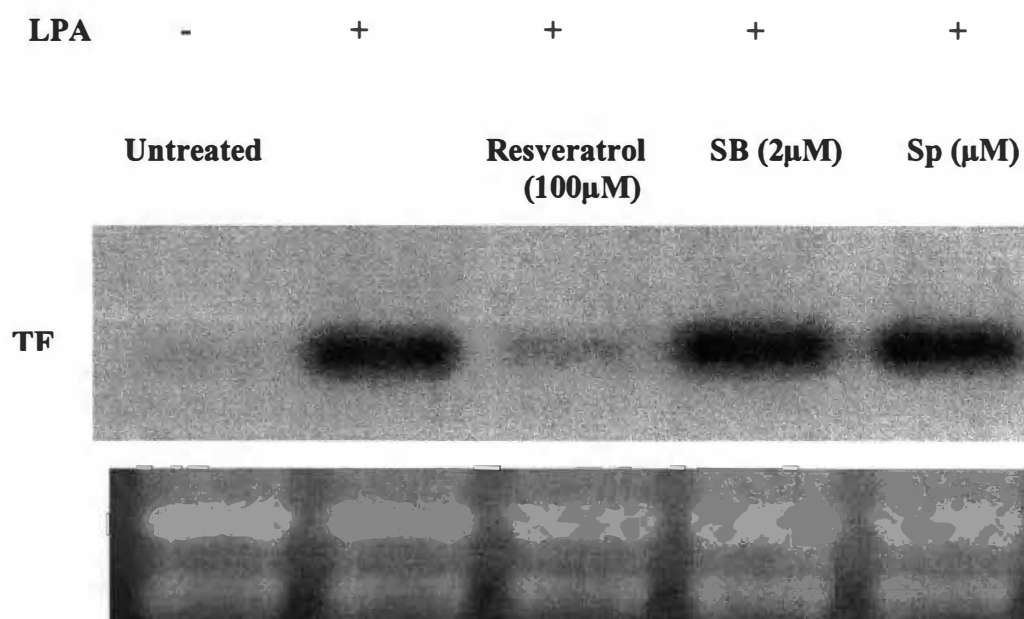


Fig.17. Northern blot analysis shows that pretreatment of quiescent RASMCs with UO-126, SB, and Sp block LPA-induced TF mRNA. Quiescent RASMCs were stimulated with LPA (25 μ M) alone for 1.5 h or after pretreatment with Resveratrol, SB, Sp (each for 30 min.) in the concentrations indicated above. A rat TF cDNA fragment (685bp) was used as a probe. 28S and 18S rRNA were visualized by ethidium bromide staining.

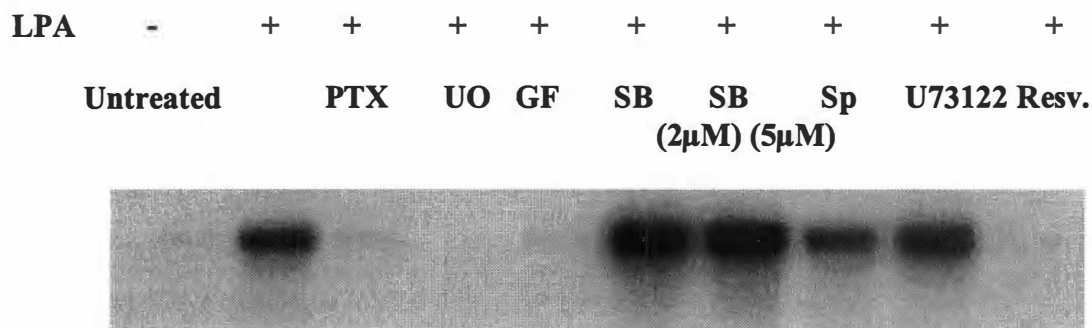


Fig.18. Northern blot analysis shows that pretreatment of quiescent RASMCs with PTX (120ng/ml), UO-126 (10 μ M), GF (μ M), and Resveratrol (100 μ M) block LPA-induced Egr-1 mRNA, whereas, SB (2,5 μ M), Sp (2 μ M), U73122 (5 μ M) was not effective. Quiescent RASMCs were stimulated with LPA (25 μ M) alone for 1.5 h or after pretreatment with PTX (120ng/ml) for 16h, UO-126 (10 μ M), GF (μ M), and Resveratrol (100 μ M), SB (2,5 μ M), Sp (2 μ M), U73122 (5 μ M) (each for 30 min.). Total mRNA was isolated, and Egr1 mRNA levels were determined by northern blot analysis. A rat Egr1 cDNA fragment (1.5kb.) was used as a probe.

LacZ	-	+	-	-	-	-	-	-	-	-
PKCδ-wt	-	-	+	+	-	-	-	-	-	-
PKCδ-dn	-	-	-	-	+	+	-	-	-	-
PKCϵ-dn	-	-	-	-	-	-	+	+	-	-
PKCϵ-wt	-	-	-	-	-	-	-	-	+	+
LPA	-	+	-	+	-	+	-	+	-	+



Fig.19. Northern blot analysis shows that LPA-induced TF mRNA was only blocked in quiescent RASMCs infected by PKC ϵ -dn. Total mRNA was isolated, and TF mRNA levels were determined by Northern blot analysis. A rat TF cDNA fragment (685bp) was used as a probe.

LacZ	+	+	-	-	-	-
PKC ζ -wt	-	-	+	+	-	-
PKC ζ -DN	-	-	-	-	+	+
LPA	-	+	-	+	-	+

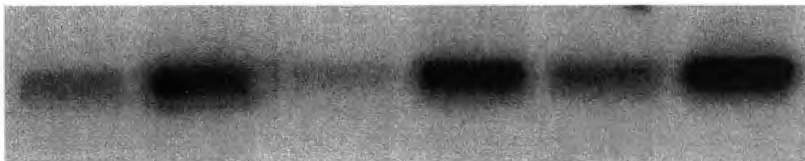


Fig.20. Northern blot analysis shows that LPA-induced TF mRNA was not blocked in quiescent RASMCs infected by PKC ζ . Total mRNA was isolated, and TF mRNA levels were determined by Northern blot analysis. A rat TF cDNA fragment (685bp) was used as a probe.

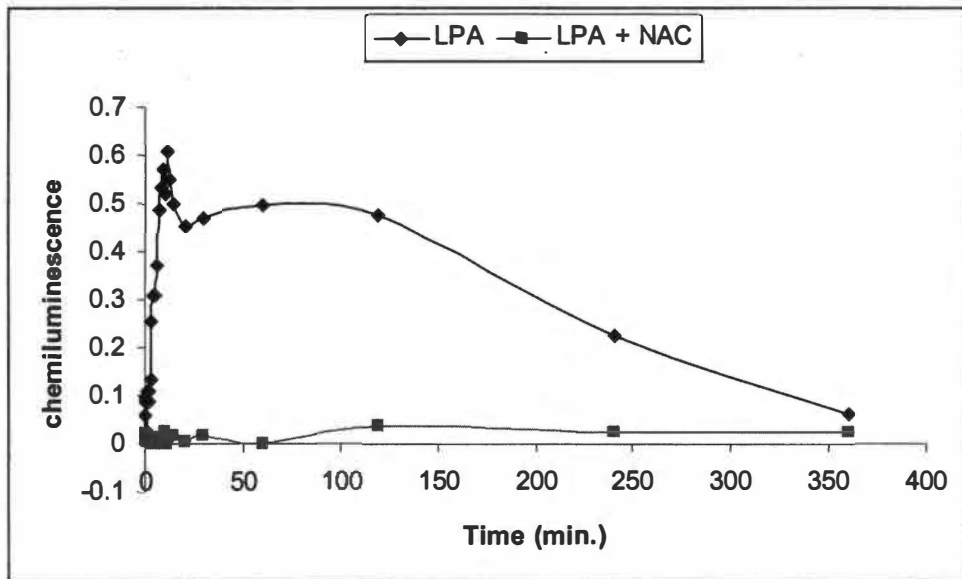


Fig.21. LPA stimulates ROS release in RASMCS. Serum starved cells were incubated with luminol and HRP and treated with either LPA (25 μ M) alone or pre-treated with NAC for 30 min and then treated with LPA (25 μ M). chemiluminescence was recorded immediately at 10 seconds intervals and then every 5 minutes after 30 minutes. For clarity, data from some time points are not shown in the graph.

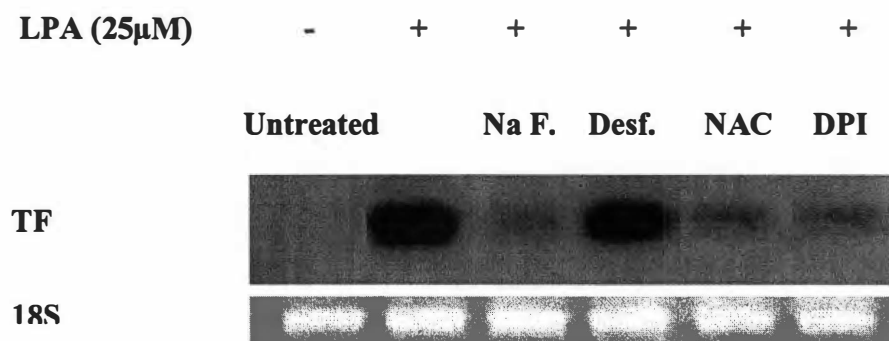


Fig.22. Northern blot analysis shows that LPA induction of TF mRNA is blocked by antioxidants. Quiescent RASMCs were stimulated with LPA (25 μ M) for 1.5 h alone or after pretreatment with Na F. [sodium formate (100mM)] for 24h, Desf. [Desferoxamine (50mM)], NAC [N-Acetyl Cysteine (10mM)], DPI [diphenyleneiodonium chloride (25 μ M)] for 30 minutes each. A rat TF cDNA fragment (685bp) was used as a probe. 28S and 18S rRNA were visualized by ethidium bromide staining.

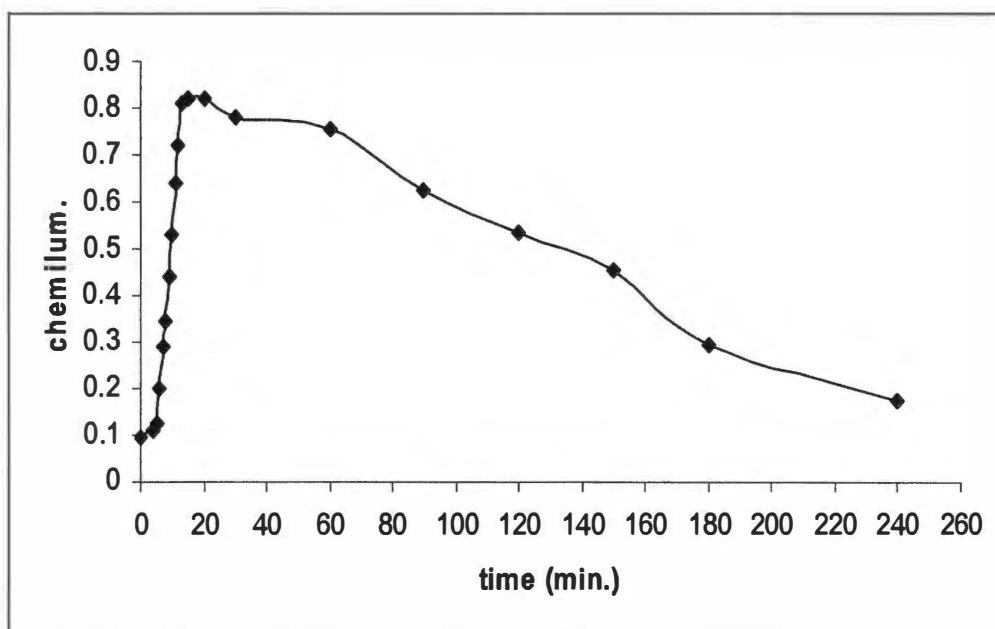


Fig.23. PTX (Gai inhibitor) does not block LPA-induced ROS release in RASMCS. Quiescent RASMCs were incubated with luminol and HRP, pre-treated with PTX for 16 h and then treated with LPA (25 μ M). Chemiluminescence was recorded immediately at 10 seconds intervals and then every 5 minutes after 30 minutes. For clarity, data from some time points are not shown in the graph.

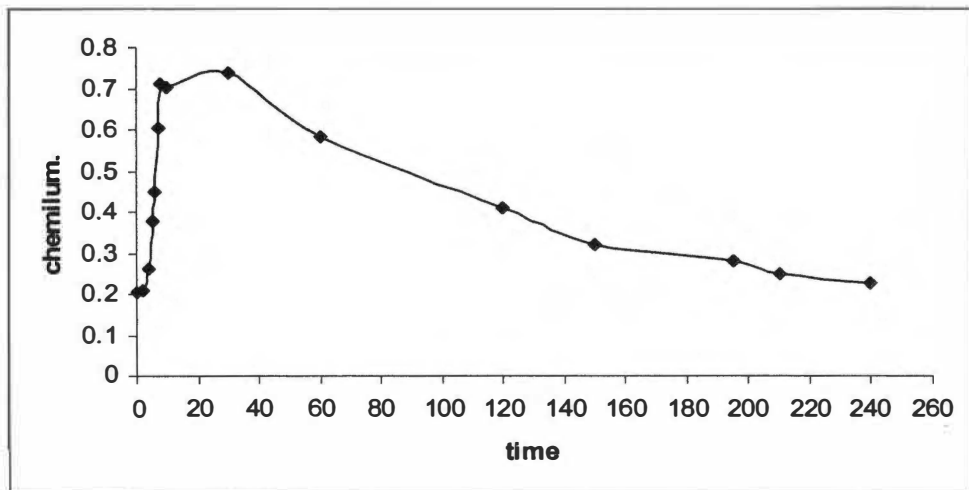


Fig.24. GF (PKC inhibitor) does not block LPA-induced ROS release in RASMCs. Quiescent RASMCs were incubated with luminol and HRP, stimulated with LPA (25 μ M) alone or after pre-treatment with GF for 30 minutes. Chemiluminescence was recorded immediately at 60 seconds intervals and then every 5 minutes after 30 minutes. For clarity, data from some time points are not shown in the graph.

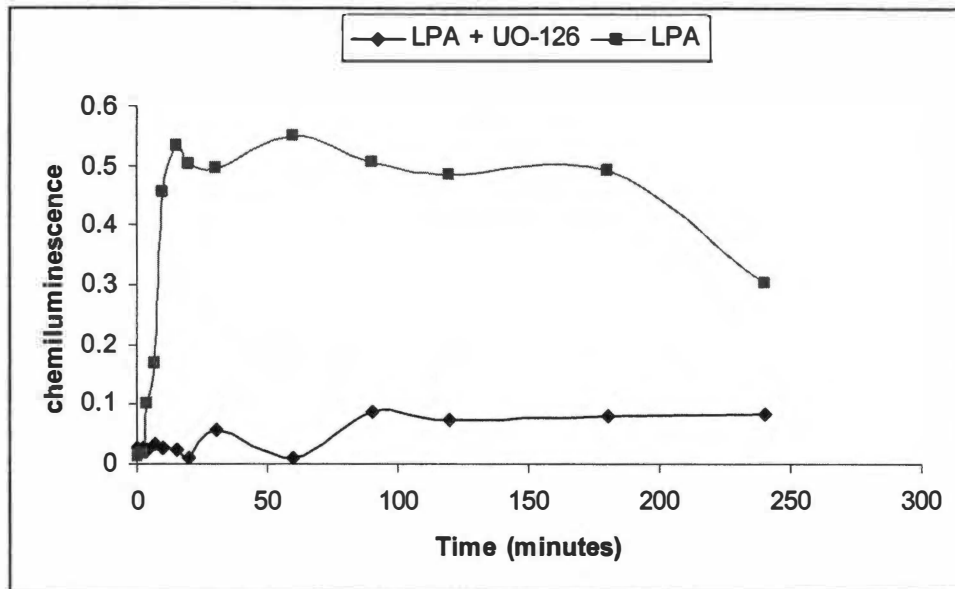


Fig.25. UO126 (MAPK inhibitor) blocks LPA-induced ROS release in RASMCS. Quiescent RASMCs were incubated with luminol and HRP, stimulated with LPA (25 μ M) alone or after pre-treatment with UO126 (10 μ M) for 30 minutes. Chemiluminescence was recorded immediately at 60 seconds intervals and then every 5 minutes after 30 minutes. For clarity, data from some time points are not shown in the graph.

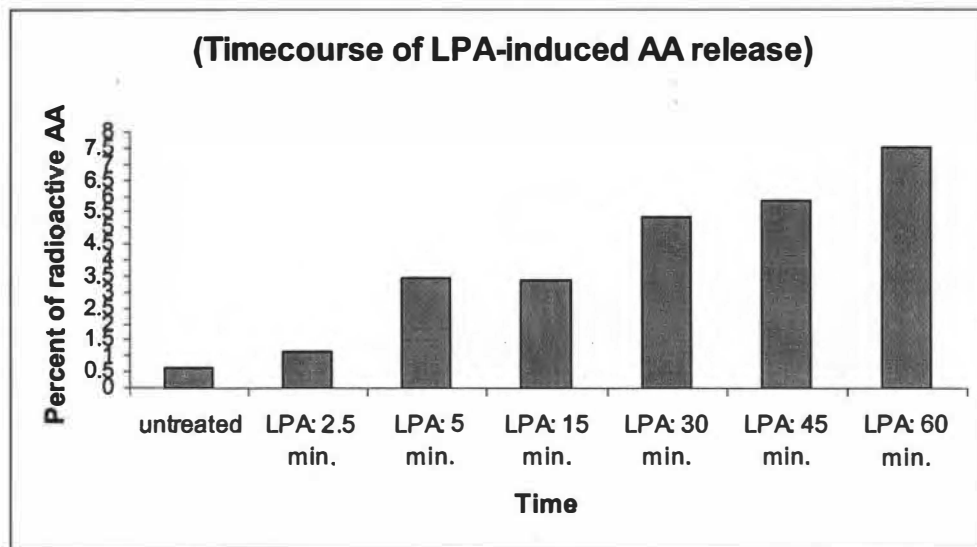


Fig.26. LPA stimulates AA release in RASMCS. Release of [^3H]AA in the extracellular medium was measured in the presence of 0.1% albumin. Results are expressed as the percent of total incorporated cellular radioactivity.

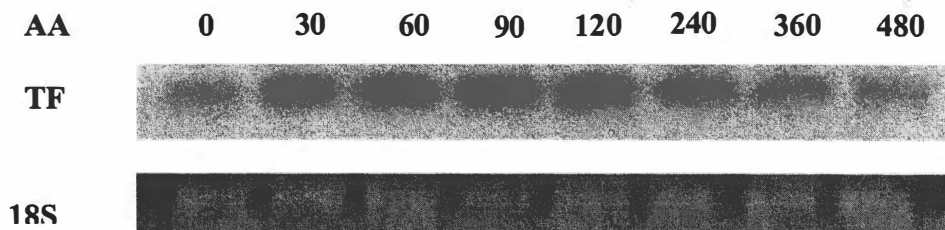


Fig.27. Time course of AA induction of TF mRNA. AA ($10\mu\text{M}$) was added to quiescent RASMC at the times indicated. Total mRNA was isolated, and TF mRNA levels were determined by Northern blot analysis. A rat TF cDNA fragment (685bp) was used as a probe. 18S rRNA was visualized by ethidium bromide staining.

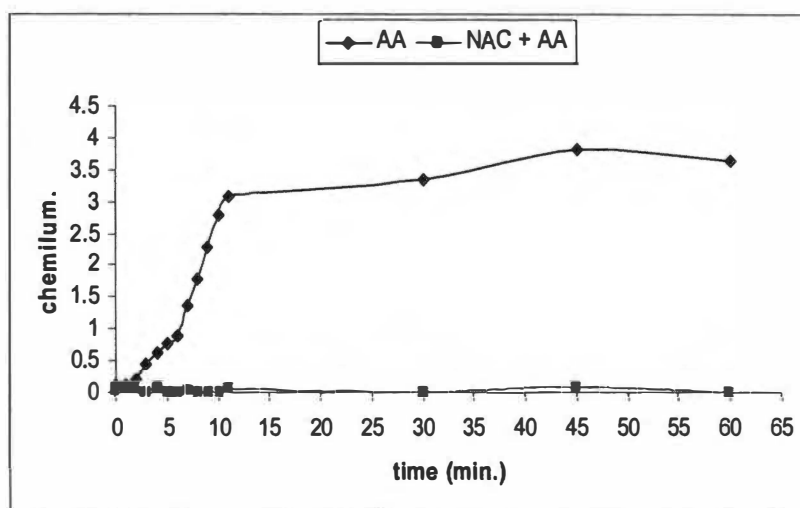


Fig.28. AA stimulates ROS release in RASMCS. Serum starved cells were incubated with luminol and HRP and treated with either AA ($10\mu\text{M}$) alone or pre-treated with NAC ($10\mu\text{M}$) for 30 min and then treated with AA ($10\mu\text{M}$). chemiluminescence was recorded immediately at 10 seconds intervals and then every 5 minutes after 30 minutes. For clarity, data from some time points are not shown in the graph.

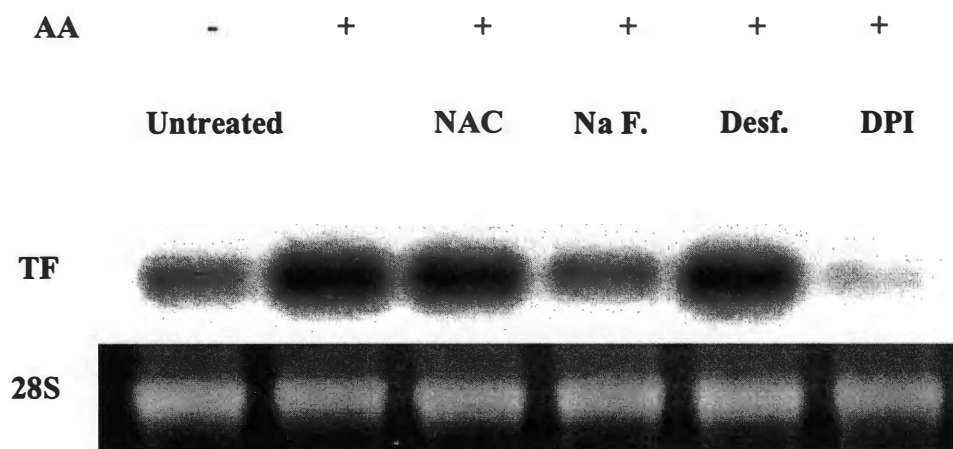


Fig.29. Northern blot analysis shows that AA induction of TF mRNA is blocked by antioxidants. Quiescent RASMCs were stimulated with AA (50 μ M) for 1.5 h alone or after pretreatment with Na F. [sodium formate (100mM)] for 24h, Desf. [Desferoxamine (50mM)], NAC [N-Acetyl Cysteine (10mM)], DPI [diphenyleneiodonium chloride (25 μ M)] for 30 minutes each. A rat TF cDNA fragment (685bp) was used as a probe. 28S rRNA was visualized by ethidium bromide staining.

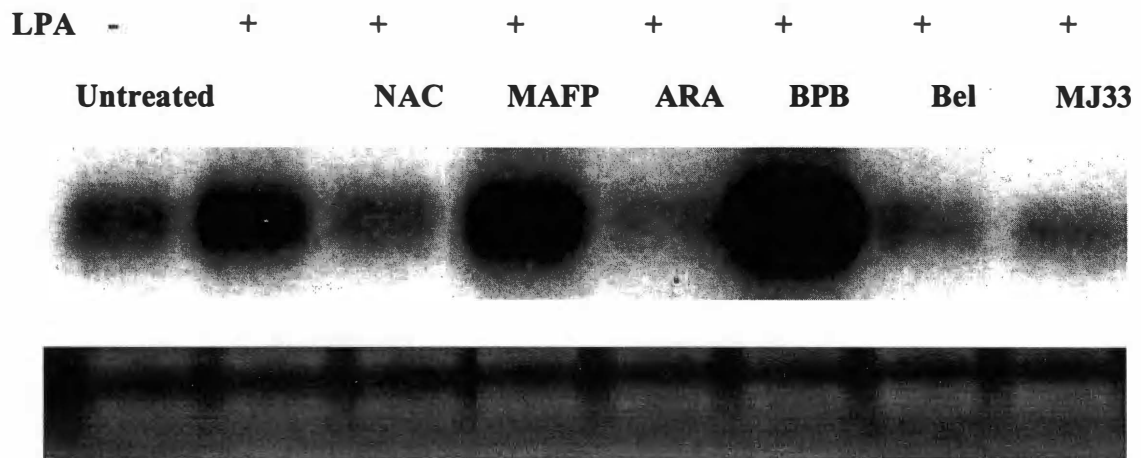


Fig.30. Northern blot analysis shows that LPA induction of TF mRNA is blocked by PLA₂ inhibitors. Quiescent RASMCs were stimulated with LPA (25μM) for 1.5 h alone or after pretreatment with the following PLA₂ inhibitors: MAFP (10μM), ARA (aristolochic acid, 10μM), BPB (10μM), Bel (10μM), and MJ33 (10μM), each for 30 minutes. A rat TF cDNA fragment (685bp) was used as a probe. 28S and 18S rRNA were visualized by ethidium bromide staining.

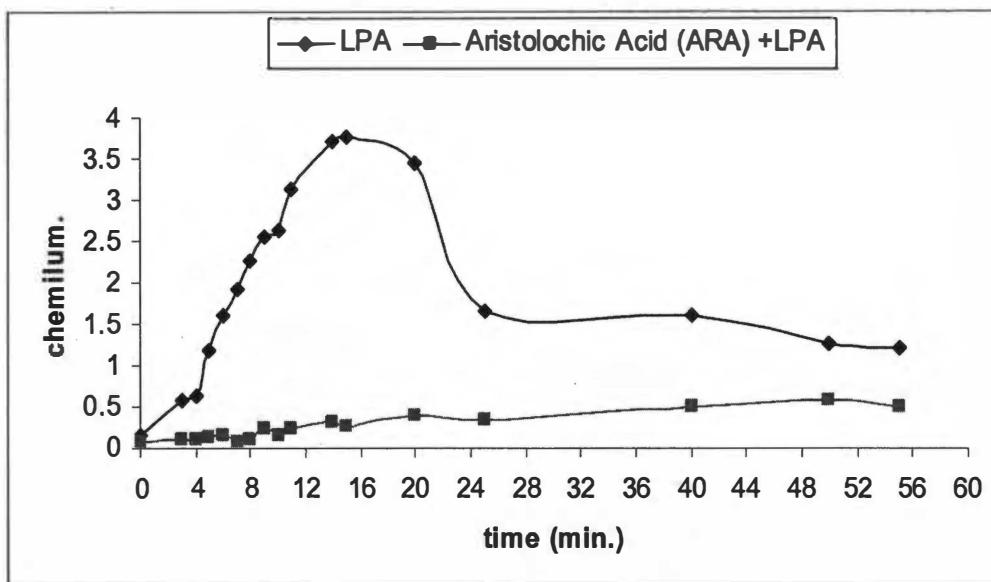


Fig.31. ARA (PLA₂ inhibitor) blocks LPA-induced ROS release in RASMCs. Quiescent RASMCs were incubated with luminol and HRP and treated with either LPA (25 μ M) alone or pre-treated with ARA (10 μ M) for 30 min and then treated with LPA (25 μ M). Chemiluminescence was recorded immediately at 60 seconds intervals and then every 5 minutes after 30 minutes. For clarity, data from some time points are not shown in the graph.

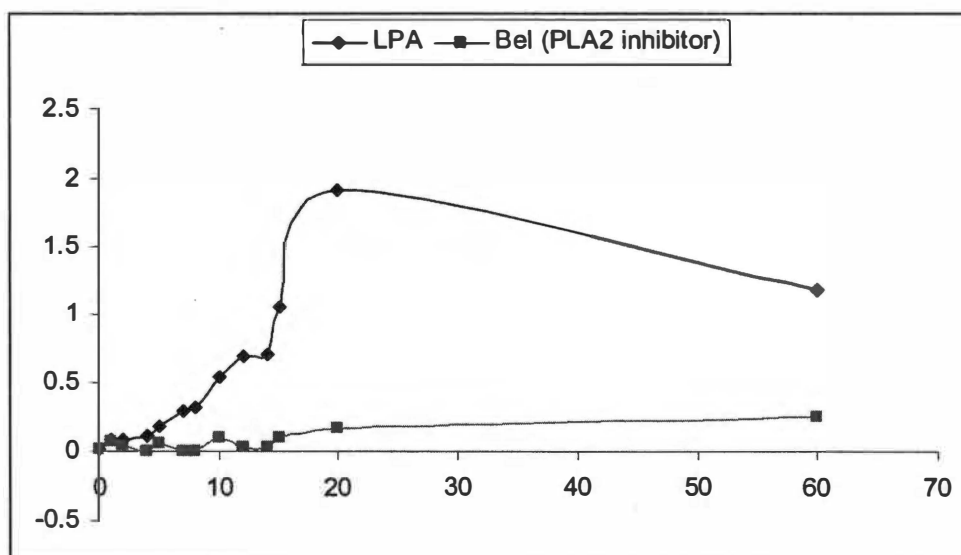


Fig.32. Bel (PLA₂ inhibitor) blocks LPA-induced ROS release in RASMCs. Quiescent RASMCs were incubated with luminol and HRP and treated with either LPA (25 μ M) alone or pre-treated with Bel (5 μ M) for 30 min and then treated with LPA (25 μ M). Chemiluminescence was recorded immediately at 60 seconds intervals and then every 5 minutes after 30 minutes.

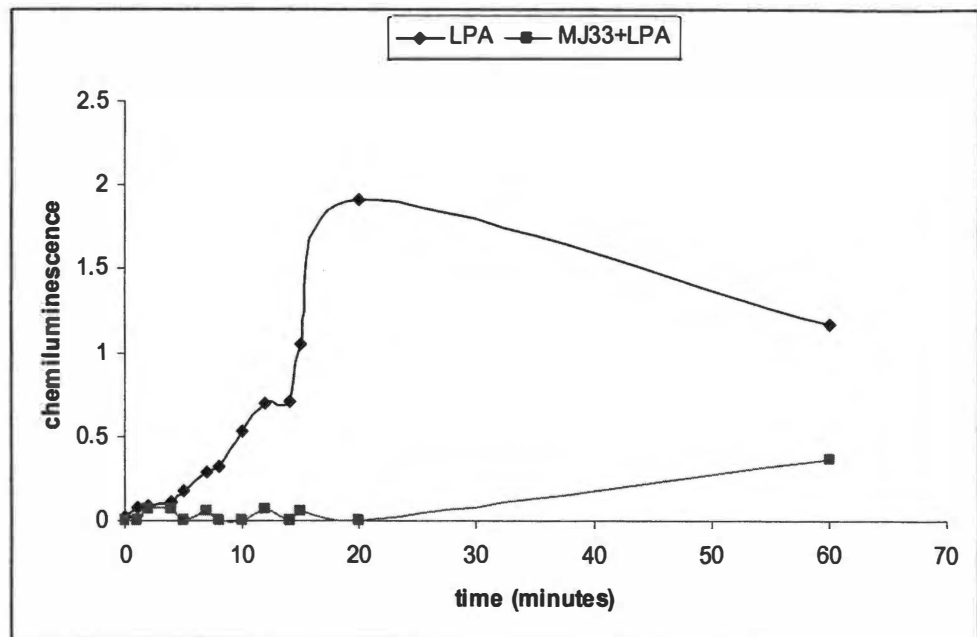


Fig.33. MJ33 (PLA₂ inhibitor) blocks LPA-induced ROS release in RASMCs. Quiescent RASMCs were incubated with luminol and HRP and treated with either LPA (25 μ M) alone or pre-treated with MJ33 (10 μ M) for 30 min and then treated with LPA (25 μ M). Chemiluminescence was recorded immediately at 60 seconds intervals and then every 5 minutes after 30 minutes. For clarity, data from some time points are not shown in the graph.

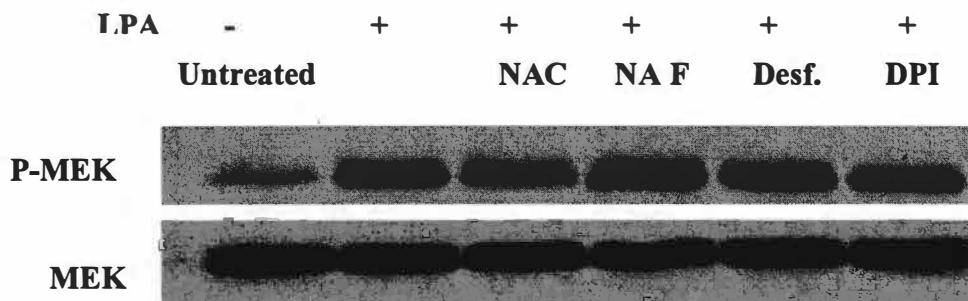


Fig.34. Western bolt analysis shows that Anti-oxidants do not block LPA-induced MAPK. Quiescent RASMCs were stimulated with LPA (25 μ M) alone for 3 minutes or after pretreatment with Na F. [sodium formate (100mM)] for 24h, Desf. [Desferoxamine (50mM)], NAC [N-Acetyl Cysteine (10mM)], DPI [diphenyleneiodonium chloride (25 μ M)] for 30 minutes each. Cell lysates were analysed by 8% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Phosphorylated MEK protein was detected by its specific antibody.

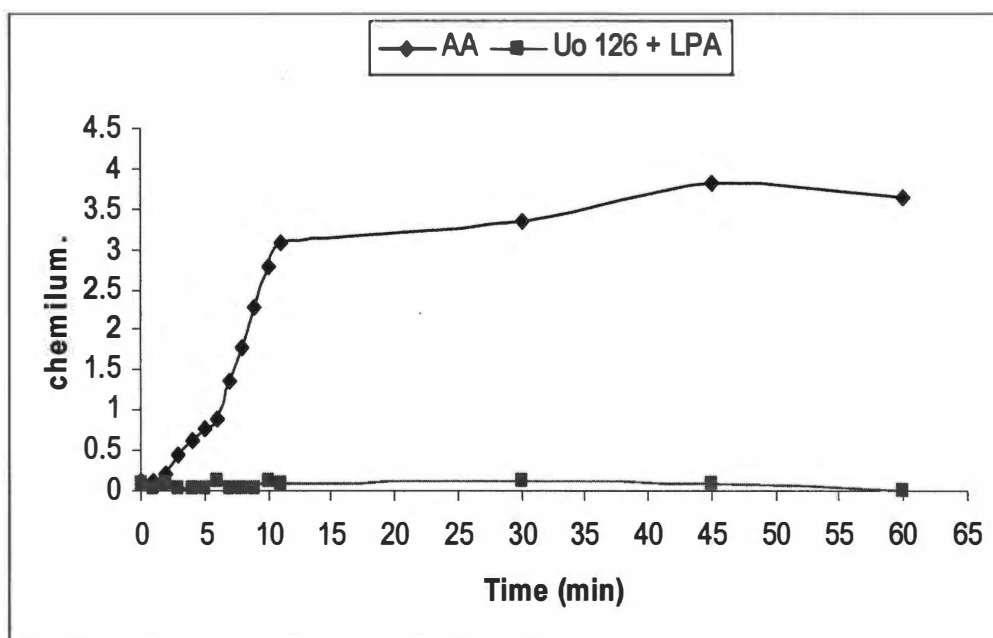


Fig.35. UO-126 (MAPK inhibitor) blocks AA-induced ROS release in RASMCs. Quiescent RASMCs were incubated with luminol and HRP and treated with either AA (10 μ M) alone or after pre-treatment with UO-126 (10 μ M) for 30 minutes. Chemiluminescence was recorded immediately at 60 seconds intervals and then every 5 minutes after 30 minutes. For clarity, data from some time points are not shown in the graph.

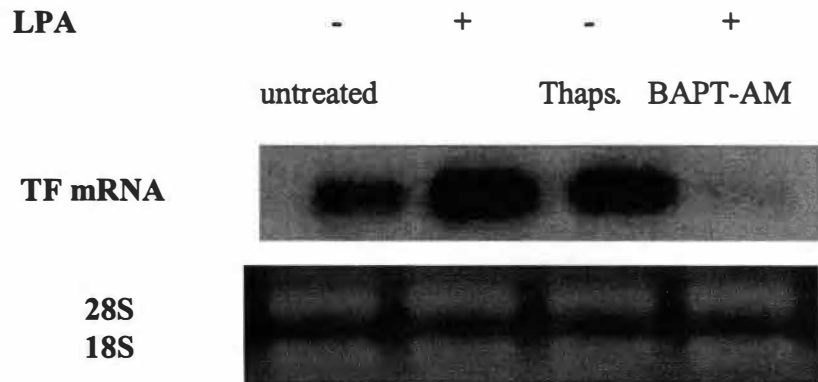


Fig.36. Northern blot shows that calcium is involved in LPA-induced TF mRNA. Quiescent RASMCs were stimulated with thapsigargin alone (10 μ M) for 1.5 h, LPA alone (25 μ M) for 1.5 h or after pretreatment with BAPTA-AM (40 μ M) for 30 minutes. A rat TF cDNA fragment (685bp) was used as a probe. 28S and 18S rRNA were visualized by ethidium bromide staining.

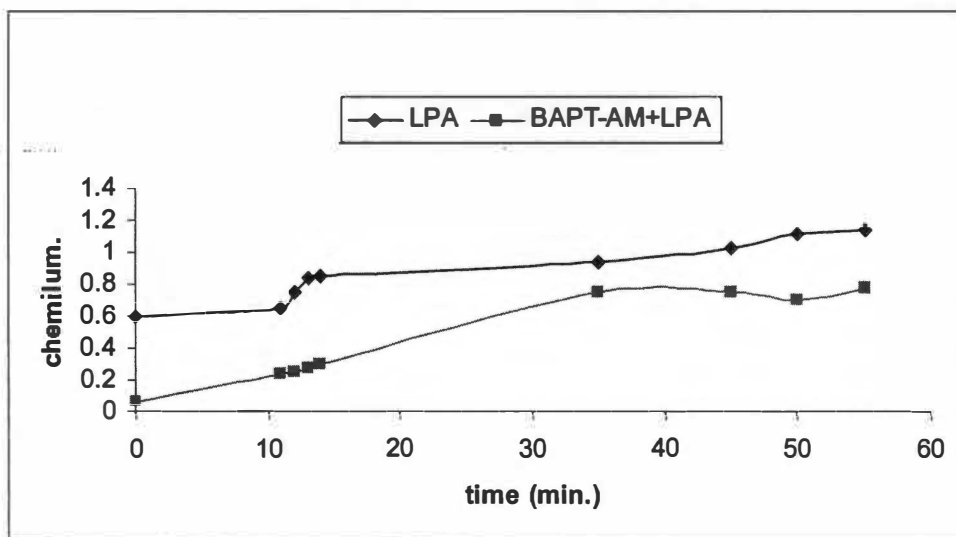


Fig.37. BAPT-AM (intracellular calcium chelator) blocks LPA-induced ROS release in RASMCs. Quiescent RASMCs were incubated with luminol and HRP and treated with either LPA (25 μ M) alone or pre-treated with BAPT-AM (40 μ M) for 30 min and then treated with LPA (25 μ M). Chemiluminescence was recorded immediately at 60 seconds intervals and then every 5 minutes after 30 minutes. For clarity, data from some time points are not shown in the graph.

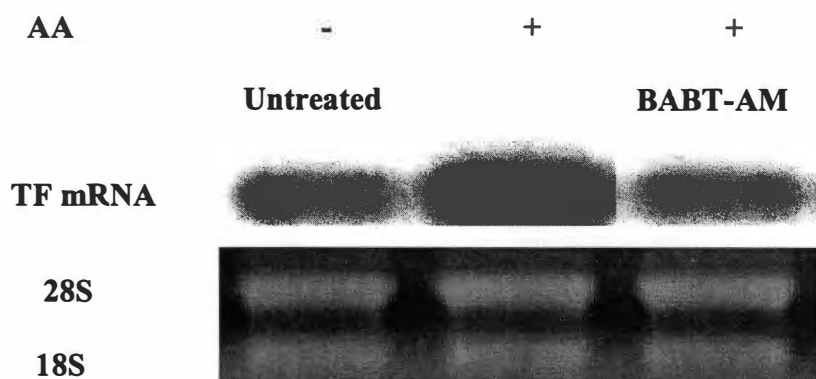


Fig.38. Northern blot shows that calcium is involved in AA-induced TF mRNA. Quiescent RASMCs were stimulated with AA alone (50 μ M) for 1.5 h or after pretreatment with BAPTA-AM (40 μ M) for 30 minutes. A rat TF cDNA fragment (685bp) was used as a probe. 28S and 18S rRNA were visualized by ethidium bromide staining.

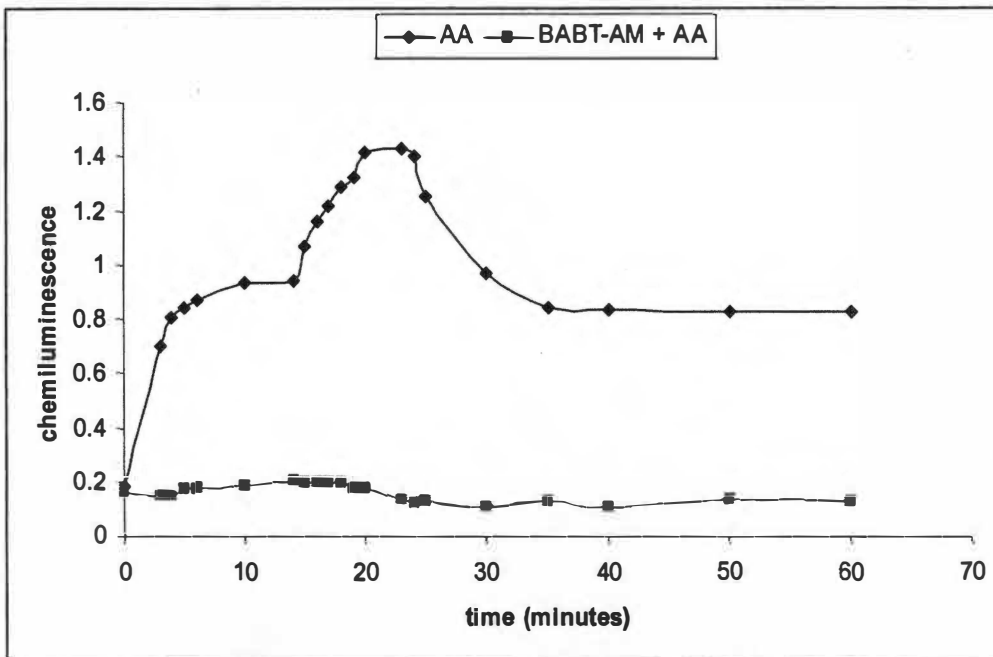


Fig.39. BAPT-AM (intracellular calcium chelator) blocks AA-induced ROS release in RASMCs. Quiescent RASMCs were incubated with luminol and HRP and treated with either AA (50 μ M) alone or pre-treated with BAPT-AM (40 μ M) for 30 min and then treated with AA (50 μ M). Chemiluminescence was recorded immediately at 60 seconds intervals and then every 5 minutes after 30 minutes. For clarity, data from some time

LPA 0 1 5 25 50



Fig.40. Induction of TF mRNA in response to LPA in HASMC. LPA was added to quiescent HASMC at the concentrations indicated above each lane. Total RNA (6 μ g per lane) was isolated from cells incubated with LPA for 1.5 h, and TF mRNA levels were determined by northern blotting. Human TF cDNA fragment (641 bp) was used as a probe.

LPA	-	+	+	+	+	+	+
	untreated	PTX	GF(5μM)	GF(10μM)	Resv.	GO	U73122

TF

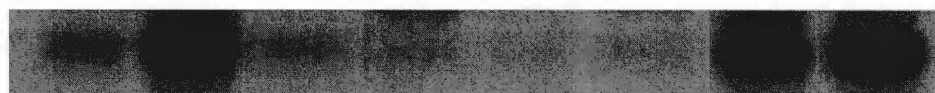


Fig.41. Northern blot analysis shows that pretreatment of quiescent HASMCs with PTX, GF, Resveratrol block LPA-induced TF mRNA, whereas, GO73122, and U73122 were not effective. Quiescent HASMCs were stimulated with LPA (25μM) alone for 1.5 h or after pretreatment with PTX (120ng/ml for 16h), GO6976 (100μM), Resveratrol (100μM), and U73122 (5μM) (each for 30 min.) A human TF cDNA fragment (641bp) was used as a probe.

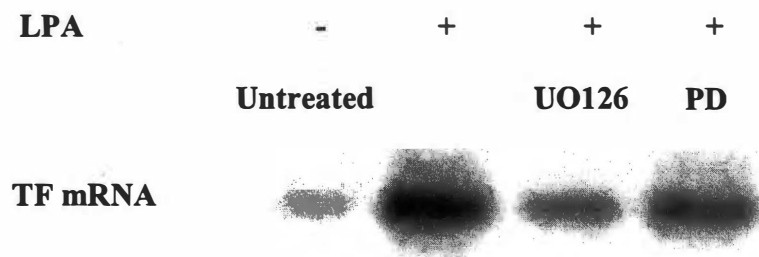


Fig.42. Northern blot analysis shows that pretreatment of quiescent HASMCs with UO126 and PD block LPA-induced TF mRNA. Quiescent HASMCs were stimulated with LPA (25 μ M) alone for 1.5 h or after pretreatment with UO126 (10 μ M) and PD (3 μ M). A human TF cDNA fragment (641bp) was used as a probe.

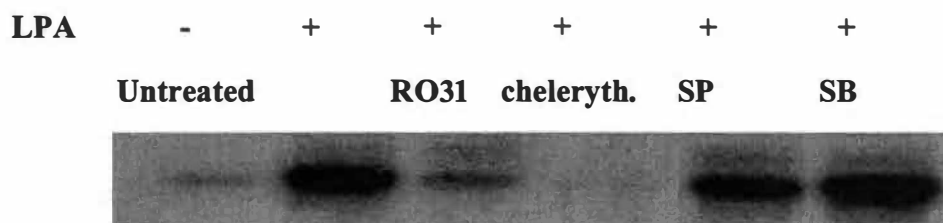


Fig.43. Northern blot analysis shows that pretreatment of quiescent HASMCs with RO31 and chelerythrin chloride block LPA-induced TF mRNA, whereas SP and SB were not effective. Quiescent HASMCs were stimulated with LPA (25 μ M) alone for 1.5 h or after pretreatment with RO31 (.5 μ M), chelerythrin chloride (3 μ M), SP (5 μ M), and SB (2 μ M). A human TF cDNA fragment (641bp) was used as a probe.

LPA (25μM)	-	+	+	+	+	+
	Untreated		NAC	Na form.	Desf.	DPI



Fig.44. Northern blot analysis shows that LPA induction of TF mRNA is blocked by DPI. Quiescent RASMCs were stimulated with LPA (25μM) for 1.5 h alone or after pretreatment with Na F. [sodium formate (100mM)] for 24h, Desf. [Desferoxamine (50mM)], NAC [N-Acetyl Cysteine (10mM)], DPI [diphenyleneiodonium chloride (25μM)] for 30 minutes each. A human TF cDNA fragment (641bp) was used as a probe.

AA (50μM)	-	+	+	+	+	+
	Untreated		NAC	Na form.	Desf.	DPI



Fig.45. Northern blot analysis shows that AA induction of TF mRNA is blocked by DPI in HASMCs. Quiescent HASMCs were stimulated with AA (50μM) for 1.5 h alone or after pretreatment with Na F. [sodium formate (100mM)] for 24h, Desf. [Desferoxamine (50mM)], NAC [N-Acetyl Cysteine (10mM)], DPI [diphenyleneiodonium chloride (25μM)] for 30 minutes each. A rat TF cDNA fragment (685bp) was used as a probe.

Vita

Essam Laag was born in Tanta-Egypt in 1962. He graduated from Tanta University Faculty of Medicine, Egypt in December, 1986. He then practiced medicine for 10 years as a pediatrician. He got his Masters in Pediatrics in 1996. In 1990, he was appointed as a teaching assistant in the department of Histology at Tanta University Faculty of Medicine, Egypt where he received his Masters in Histology in 1994 and was appointed as an assistant lecturer. He won the national exam done by the Egyptian ministry of higher education to pursue graduate education in the US in 1995. He came to the US in 1996 and got his Masters in Human Genetics from the department of Human Genetics at the Medical College of Virginia-Virginia Commonwealth University, Richmond, Virginia. He then came to the University of Tennessee in 2000 to get his Ph. D in Comparative and Experimental Medicine. He is presently a graduate research assistant in the department of Pathobiology at the Vet. School, University of Tennessee, Knoxville.