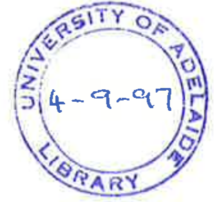
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REGULATION OF MACROPHAGE FUNCTIONS BY POLYUNSATURATED FATTY ACIDS

ZHI HUA HUANG M.B.B.S., M.D.

Thesis submitted for the degree of Doctor of Philosophy

Department of Immunopathology

Women's and Children's Hospital

and

Department of Paediatrics

The University of Adelaide

(Faculty of Medicine)

June 1997

SUMMARY

Recent studies have demonstrated that polyunsaturated fatty acids (PUFAs), such as arachidonic acid (AA, 20:4, *n-6*), eicosapentaenoic acid (EPA, 20:5, *n-3*) and docosahexaenoic acid (DHA, 22:6, *n-3*) can alter major cell responses in lymphocytes and neutrophils. To date, there is very little known on the effects of these fatty acids on macrophage function. Since a major reason for studying the interaction of PUFAs with leukocytes is to better define mechanisms of the inflammatory response and ways of applying PUFAs to treat infectious diseases and autoimmune/allergic diseases, it is important to study the effects of PUFAs on the mononuclear phagocytic system because of its role in the pathogenesis of these diseases.

The thesis focussed on investigations into the effects of PUFA on a key response of the mononuclear phagocyte, namely the respiratory burst involving NADPH oxidase activation. This was determined by the lucigenin-dependent chemiluminescence assay. A major finding was that the PUFAs, AA, EPA and DHA induced little or no production of superoxide in monocytes, macrophages and the monocytic cell line (HL-60) differentiated to macrophages. This is in direct contrast to the well-characterised substantial activation of the NADPH oxidase by these fatty acids in neutrophils. However, such PUFA treated

monocyte/macrophages showed significantly enhanced superoxide production in response to the tripeptide agonist, f-met-leu-phe (fMLP). These synergistic responses were characterized by a more rapid onset of the chemiluminescence response, as an increase in initial peak rate of chemiluminescence and longer duration of the response. It is interesting that, in relation to this synergistic response, the *n-3* PUFAs, EPA and DHA, were as active as AA (20:4, *n-6*) since *n-3* fatty acids have been implicated as anti-inflammatory fatty acids. The role of fatty acid structure in the ability to stimulate the fMLP response was also investigated. The data showed that a free carboxyl group was necessary and that the activity of the fatty acids decreased substantially as the number of double bonds decreased to two or fewer. The addition of a hydroxy- and hydroperoxy-group to the PUFA (products normally formed from the metabolism of PUFA via the lipoxygenase pathway) resulted in total loss of activity.

The mechanisms by which fatty acids induce their effects on mononuclear phagocytes were partially elucidated. The data showed that PUFAs not only enhanced the response of macrophages to a surface receptor acting agonists, fMLP, but also to agonists which act at post receptor levels, such as phorbol myristate acetate (PMA) which directly activates on protein kinase C (PKC) and Ca^{2+} ionophore which acts by increasing intracellular calcium concentration. In macrophages, PUFA stimulated the translocation of the PKC isozymes α, βI, βII and ε to a particulate fraction and the activity of extracellular signal-regulated protein kinase 1&2 (ERK1 and ERK2) of the mitogen-

activated protein kinase (MAP kinase) family. Using inhibitors of PKC and ERK we were able to establish that the priming of macrophages by PUFA occurs via PKC and ERK pathways. By pursuing transfection technology and introducing dominant negative mutants of signalling molecules as well as inhibitors of the upstream regulators of ERKs into macrophages, we were able to establish that ERK activation occurs via PKC, $p21^{ras}$ and raf-1 dependent mechanism.

These findings establish that PUFAs of the *n-6* and *n-3* types, while being poor activators of the NADPH oxidase, prime macrophages to become highly reactive to other agonists/mediators. This was observed as a synergistic superoxide production. The data suggest that this activity is restricted to certain structural elements of the fatty acid molecules. The mechanisms of the biological effects of the PUFAs in terms of intracellular signalling pathway were also partly defined.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis when deposited in the University Library, being available for loan and photocopying.

Z. H. Huang

ACKNOWLEDGMENTS

The study presented in this thesis was carried out at the Department of Immunopathology, Women's and Children's Hospital, Adelaide and the Department of Paediatrics, University of Adelaide, and was supported by grants from the National Health and Medical Research Council of Australia. During the course of this study, the candidate was supported by an Overseas Postgraduate Research Scholarship, a University of Adelaide Scholarship for Postgraduate Research and the Department of Paediatrics Research Scholarship.

I am greatly indebted to my supervisor Professor Antonio Ferrante for his guidance, enthusiasm and encouragement on this project. His constructive suggestions and invaluable comments on the thesis are much appreciated. Many thanks go also to my co-supervisor Professor Don Roberton for his permission to undertake the project in the Departments and his help in procurement of funds for this study. His critical review on the thesis is acknowledged.

I would like to express my sincere gratitude to Dr. Charles Hii for many stimulating, fruitful discussions, his helpful advice on Western blotting, FPLC, ERK assay, PKC assay etc., and also his critical comments on this thesis. It has been a pleasure working with him. Special thanks are due to Professor Andrew Murray for allowing me to carry out some experiments in his lab and his kind supply of anti-ERK and anti-PKC antibodies, to Professor Alf Poulos and Dr. Brenton Robinson for their valuable suggestions on lipids, to Professor Ping Han and Mr. Greg Hodge for their helpful advice on FACS analysis, and to Dr. Lakshmi Kumaratilake for her suggestion on macrophage culture. Contributions made by Professor Alf Polous, Dr. Deborah Rathjen and Mrs. Judy Ferrante to the proof reading of the thesis are gratefully acknowledged.

My humble thanks also go to members of the Department of Immunopathology and Department of Paediatrics. Their technical advice, friendship and help in one way or another are all greatly appreciated. In particular the cell preparation team, without their tireless processing of hundreds of litres of blood during the past 3 years for preparation of monocytes, the outcome of this thesis might have been very different. In addition, I wish to thank Ms. Amanda Watts for her excellent secretarial assistance on typing the manuscripts for the papers and some of the chapters of this thesis with a great patience. Support provided by Dr. David Bates in computing matters is also gratefully appreciated.

Many thanks are extended to all blood donors from the hospital, who made this study possible.

Finally, I would also like to thank my parents and my sister for their belief, encouragement, understanding and support, and to my friends Dr. Stephane Luo, Dr. Miao Yan, Dr. Kang Zhang, Dr. Tao Qian, Miss Anne-Marie Tan and Professor Ian Maddock and Mrs. Diana Maddock for their friendship throughout the good times and bad times.

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ABBREVIATIONS

AA	Arachidonic acid (20:4, *n-6*)
AA-ME	20:4 (*n-6*) methyl ester
CGD	Chronic granulomatous disease
DAG	Diacylglycerol
DG	Diglycerides
DHA	Docosahexaenoic acid (22:6, *n-3*)
DHA-ME	22:6 (*n-3*) methyl ester
DPC	Dipalmitoyl phosphatidylcholine
ECL	Enhanced chemiluminescence
EPA	Eicosapentaenoic acid (20:5, *n-3*)
ERK	Extracellular signal-regulated kinases
FABP	Fatty acid binding protein
FACoA	Fatty acyl coenzymeA
FATP	Fatty acid transporter protein
fMLP	*N*-formyl-Mel-Leu-Phe
FPLC	Fast protein liquid chromatography
GAP	GTPase activating protein

GLA	γ-linolenic acid (18:3, *n-6*)
HBSS	Hank's balanced salt solution
HETE	Hydroxyeicosatetraenoic acids
HPETE	Hydroperoxyeicosatetraenoic acids
IL-1β	Interleukine-1 β
IP_3	Inositol 1,4,5-trisphosphate
LA	Linoleic acid (18:2, *n-6*)
LAL	Limulus Amebocyte Lysate
MAP kinases	Mitogen-activated protein kinases
MEK1	ERK kinase
MEKK	MEK kinase
MKP	MAP kinase phosphatase
MLK	Mixed lineage kinase
MNL	Mononuclear leukocytes
NADPH	Nicotinamide hypoxanthine dinucleotide phosphate
NRPTK	Non-receptor protein tyrosine kinases
NSAIDs	Nonsteroidal anti-inflammatory drugs
ODRS	Oxygen-derived reactive species
PA	Phosphatidic acid
PAK	$p21^{rac}$-activated kinase
PC	Phosphatidylcholine

PE	Phycoerythrin
PI-3 kinase	Phophatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKM	Protein kinase M
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol 12-myristate 13-acetate
PS	Phosphatidylserine
PTK	Protein tyrosine kinases
PUFA	polyunsaturated fatty acid
RACK	Receptors for activated C kinase
ROS	Reactive oxygen species
RPTK	Receptor protein tyrosine kinases
Thr	Threonine
TLC	Thin layer chromatography
TNF	Tumor necrosis factor α
Tyr	Tyrosine
VLDL	Very low density lipoprotein

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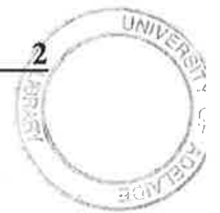
Chapter 1

Introduction



1.1 General introduction

Diets enriched with specific types of polyunsaturated fatty acids (PUFAs) have been of wide-spread interest because of their potential usefulness in treating a range of human diseases and conditions. In particular, it is well appreciated that increasing the ratio of *n-3* over *n-6* PUFAs in membrane phospholipids has some beneficial therapeutic effects (Simopoulos, 1991). Many diseases/conditions in which fatty acid diet manipulations have been used are outlined in Table 1.1. These include those which have an autoimmune and allergic basis. While the mechanisms governing the beneficial effects of certain types of PUFAs in different types of diseases is likely to vary, it is well appreciated that altering the types of PUFAs in diets can modify the immune response. This is thought to be the major mechanism by which PUFAs exert their protective effects in inflammatory and autoimmune disorders.

Some PUFAs, such as arachidonic acid [AA, 20:4, (*n-6)*], form an integral component of the cellular biochemical pathways which govern cell and tissue functions. Cell activation leads to the release of AA from phospholipids and the metabolism of the free fatty acid via the lipoxygenase and cyclooxygenase pathways, in addition to their oxidation via β-, α– and ω-oxidation.

Table 1.1 Outline of diseases/conditions in which PUFA manipulations have been used for treatment

Cardiovascular diseases
Rheumatoid arthritis
Atopic dermatitis
Inflammatory bowel disease
Systemic lupus erythematosus
Asthma
Cystic fibrosis
Infectious diseases
Breast pain and premenstrual syndrome
Transplantation
Allergic condition
Neurological diseases
Psoriasis
Malignant diseases
Diabetes

(Compiled from Sinclair and Gibson, 1992)

Classically, the major interest in AA has been its metabolism via the lipoxygenase and cyclooxygenase pathways which leads to the generation of some highly active eicosanoids with proinflammatory activity. The ability of the lipoxygenase and cyclooxygenase systems to also generate fatty acid metabolites with substantially lower activity (<1000) than the AA-derived eicosanoids has provided the basis for strategies to manipulate the inflammatory reaction. For example, increasing the ratio of *n-3* to *n-6* in membrane phospholipids of leukocytes reduces the production of inflammatory eicosanoids in favour of metabolites with markedly reduced or those which lacked, proinflammatory activity. Thus diets which contain high levels of the *n-3* fatty acids, eicosapentanoic acid [EPA, 20:5, (*n-3*)] and/or docosahexaenoic acid [DHA, 22:6, (*n-3*)] as well as those which contain high levels of their precursors have been used as ways of decreasing inflammatory reactions and relieving the symptoms of these diseases (Simopoulos, 1991).

Clinical studies have shown that *n-3* fatty acids have beneficial effects in autoimmune and inflammatory diseases, such as ulcerative colitis, gingivitis, psoriasis, rheumatoid arthritis, systemic lupus erythematosus and asthma. A high *n-3* fatty acid intake for four months significantly increased the general score and sigmoidoscope score of active ulcerative colitis patients compared with a placebo diet (Greenfield *et al*, 1993; Simopoulos, 1991). The effects were maintained for three months after the fatty acid treatment was discontinued. In a human gingival inflammation model, 28-day treatment with EPA and DHA (1.8g/day) markedly reduced the gingival index in interdental papilla (Campan *et al*,

1996). Dietary supplementation with fish oil fatty acids rich in EPA and DHA in conjunction with conventional treatment (cyclosporin) in psoriasis patients has been shown to improve the skin lesions and decrease the nephrotoxicity of cyclosporin (Simopoulos, 1991). In the treatment of rheumatoid arthritis, the beneficial effects of fish oil are pronounced and reproducible in both animal models and in human trials. It has been shown that feeding mice with the *n-3* PUFAs, EPA and DHA, reduces the incidence and severity of type II collagen-induced experimental arthritis (Leslie *et al*, 1985). Dietary supplementation with *n-3* PUFAs in rheumatoid arthritis patients shows significant relief of joint pain and swelling, duration of morning stiffness and has led to a reduction in requirement for nonsteroidal anti-inflammatory drugs (NSAIDs). Some patients were able to discontinue NSAIDs while receiving *n-3* PUFA treatment (Kremer, 1995; Sperling, 1991). A recent clinical study of γ-linolenic acid dietary manipulation in patients with rheumatoid arthritis also showed evidence of alleviation of disease activity (Zurier *et al*, 1996).

Apart from the antiinflammatory effects of fish oil fatty acids, it also has been demonstrated that *n-3* fatty acids reduce the risk of atherosclerosis, prevent thrombosis and increase the survival rates of patients who had previously experienced myocardial infarction. *n-3* PUFAs inhibit platelet aggregation, and significantly lower VLDL cholesterol and plasma triglyceride levels (provided the content of saturated fatty acids is reduced) in patients with primary hypertriglyceridaemia, and HDL levels are also

increased in type IV hyperlipoproteinaemic patients (Mackness *et al*, 1994; Simopoulos, 1991). Increasing the intake of *n-3* fatty acids as an adjunctive therapy also decreases both systolic and diastolic blood pressure. This effect can be enhanced by limiting sodium intake (Simopoulos, 1991; Howe, 1995; Schmidt and Dyerberg, 1994). Other studies have shown that *n-3* fatty acids (EPA and DHA) have antiarrhythmic effects by preventing ventricular fibrillation which is largely responsible for the motility of acute myocardial infarction. Feeding of rats, dogs, pigs and monkeys with *n-3* fatty acids or administration of fatty acids by intravenous infusion prevented ischaemia-induced ventricular fibrillation. Two clinical studies on subjects who previously had experienced myocardial infarction showed that an oily fish diet or a fat-restricted α-linolenic acid (the precursor of EPA and DHA) rich diet markedly reduced the incidence of sudden cardiovascular deaths (Abeywardena *et al*, 1992; McLennan *et al*, 1985; Kang and Leaf, 1996). A high fish oil diet may also be beneficial for those insulin-dependent diabetics who are at a high risk of suffering from cardiovascular diseases (Simopoulos, 1991).

It has also been reported that an increase in *n-3* fatty acid intake reduces tumor growth in animal models of breast, colon, pancreas and prostate cancer. In contrast, the *n-6* PUFA, AA, promoted tumor development in the same circumstances (Karmali, 1996; Cave, 1996). EPA and DHA have also been shown to prevent cachexia and metastasis of tumors in animal models (Karmali, 1996; Cave, 1996).

Dietary supplementation of *n-3* PUFAs also decreases the symptoms of dysmenorrhoea, a prostaglandin-mediated condition in adolescents. After a two month treatment with fish oil, a significant reduction in the Cox Menstrual Symptom Scale was found compared with a placebo diet. This could be due to alteration of the prostanoid profile by the high *n-3* fatty acid intake (Harel *et al*, 1996; Deutch, 1995).

Essential fatty acids play an important role in brain and retinal development which mainly occurs during the latter half of pregnancy and the postnatal stage. The growth of fetal brain acquires approximately 21g/wk of DHA during the last trimester of pregnancy. Fatty acids are transported from maternal circulation across the placenta and fetal blood-brain barrier into the central nervous system. A deficiency of essential fatty acids during pregnancy leads to a reduced level of DHA in the newborn infants, which is related to a reduction in slow-wave sleep and impaired vision in these infants. Dietary supplementation of *n-3* fatty acids to pregnant women and increasing the amount of DHA in infant formula are beneficial for early neurological development and improve the visual recognition in preterm and term infants (Gibson *et al*, 1996; Connor *et al*, 1996; Uauy *et al*, 1996). It has also been shown that diets rich in *n-3* fatty acids can prevent premature labor and preeclampsia (Olsen *et al*, 1990). Dietary *n-3* fatty acids also reduce the severity and frequency of relapses in patients suffering from multiple sclerosis (Bates, 1990). A significant improvement in schizophrenic symptoms has also been reported (Laugharne *et al*, 1996).

Many recent studies have shown that production of immunological/inflammatory mediators can also be regulated by PUFAs. Diets rich in *n-3* PUFAs significantly reduce the production of the pro-inflammatory cytokines, tumor necrosis factor (TNF), interleukin-1β (IL-1β) and IL-2, as well as the lipid mediator, platelet activating factor (PAF) (Endres *et al*, 1989; Williams *et al*, 1996; Sperling, 1991) (details in 1.5.2).

However, in addition to downregulation of many leukocyte functions, which form the basis of their anti-inflammatory effects, PUFAs of both the *n-6* and *n-3* types stimulate leukocyte function associated with proinflammatory activity and tissue damage. In particular, PUFAs have been shown to stimulate neutrophil adhesion (Bates *et al*, 1993). This is conducive with their ability to activate the intracellular signalling systems of protein kinase C (PKC), phospholipase A_2 (PLA_2), sphingomylinase, extracellular signal-regulated protein kinase (ERK) and the small G protein, rac 2 (Hii *et al*, submitted; Robinson *et al*, 1996 and 1997; Abramson *et al*, 1991).

Many studies to date on the effects of PUFAs on the inflammatory reaction have focussed on neutrophils and lymphocytes. Limited studies have been conducted on the biological effects of PUFAs on macrophages. Macrophages are components of the immune system which may be stimulated specifically by T lymphocytes or nonspecifically by endogenous and exogenous mediators. Their stimulation and/or

activation is essential for immunity against infections on one hand (Locksley and Wilson, 1995) and on the other these changes promote tissue damage in autoimmune diseases and allergic inflammation. Macrophages are known to participate in the formation of atherosclerotic plaque. Consequently, a clear understanding of the effects of *n-6* and *n-3* PUFAs on the key functions of macrophages is likely to give us a better appreciation of the mechanism of regulation, stimulation and activation of macrophages during the inflammatory response and help formulate better diets in nutrition based treatment and management of the type of diseases outlined in Table 1.1.

1.2 Macrophage functions in immunity to infection and in inflammatory diseases

Monocytes/macrophages belong to the mononuclear phagocyte system which consists of bone marrow monoblasts, promonocytes, peripheral monocytes and tissue macrophages. Macrophages originate from bone marrow. Progenitor monoblasts are committed to the monocytic cell lineage to become mature monocytes under the influence of colony stimulating factors and other cytokines. They then extravasate from the blood vessel and enter into tissues as either resident tissue macrophages or immature macrophages. These cells are involved in both the afferent and efferent phases of the immune response (Auger and Ross, 1992).

Macrophages which reside in liver, spleen and lung have been shown to play an important role in clearing microorganisms, such as *Streptococcus pneumoniae*, African *trypanosomes* and intraerythrocytic asexual blood stages of *Plasmodium,* from the blood and tissues (Ferrante, 1976; Allison, 1984; Brown *et al*, 1983). Resting tissue macrophages have probably adapted to deal with small numbers of microorganisms at the initial stage of infection. This is mediated by cross-linking of receptors for complements, mannose and fucose, Fc, and LPS, which is followed by phagocytosis, killing and degradation of phagocytosed organisms via oxygen-dependent and oxygen-independent mechanisms. Products of tissue damage and microbial origin also induce the generation of a variety of inflammatory mediators, such as the cytokines TNF and IL-1, lipid products eg. PAF and LTB_4 and complement products, C5a etc. which attract more macrophages to sites of inflammation and stimulate their microbicidal functions at these sites. This reaction is primarily a non-specific event (Locksley and Wilson, 1995).

Immunity to the extracellular pathogens, such as *Pneumocystis*, *Schistosoma mansoni*, *Trichinella spiralis*, *Candida albicans*, can be better dealt with by the delayed-type hypersensitivity (DTH) response in association with antibody. This involves the release of lymphokines, such as IL-2, IFN-γ and lymphocyte-derived chemotactic factors by activated T cells. The cytokines released by both macrophages and lymphocytes promote the recruitment and stimulation of macrophages, and induction of expression of

class II MHC molecules on these cells which ultimately leads to the activation of macrophages for increased microbicidal activity (Locksley and Wilson, 1995).

At the other end of the spectrum, activated macrophages and sensitized T lymphocytes are essential in the killing of intracellular bacteria and protozoan parasites, such as species of *Mycobacteria, Listeria, Leishmania* and *Toxoplasma*, and killing of tumor cells. This chronic inflammatory reaction is associated with tissue destruction and pathophysiology (Unanue, 1993; Gallin, 1993; Adams and Hamilton, 1988; Allison *et al*, 1978; Locksley and Wilson, 1995).

Cytokines produced by activated T lymphocytes as well as those produced by leukocytes and local tissues form a network of interactions which regulates the activity of macrophages. Among these cytokines, IL-2 and IFN-γ stimulate macrophage functions while IL-4, IL-10 and TGF-β downregulate the same functions (Unanue, 1993). The cytokines also "cross talk" with other types of mediators, such as C5a, LTB_4 and PAF, forming a highly complex network of intercellular signalling molecules. The level of disease activity, microbial killing, wound healing and tissue remodelling seen during inflammation is most likely a reflection of changes in the balance of these intercellular regulatory mediators (Unanue, 1993).

As a result of macrophage migration into inflammatory sites, the cells become actively phagocytic and display increased microbicidal or tissue damaging properties. These changes are manifested by increased expression of Fc and complement receptors on the macrophage surface and increased production of antimicrobial substances. In particular, the cells release substantial amount of oxygen-derived reactive species (ODRS) as a result of the assembly of NADPH oxidase. ODRS are one of the most powerful systems in macrophages for the killing and elimination of pathogens (Kaufmann, 1993).

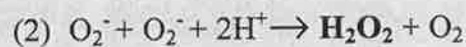
1.2.1 Activation of phagocyte NADPH oxidase and the generation of oxygen- derived reactive species

Phagocytic cells are characterized by the generation of oxygen-derived reactive species (ODRS), such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), singlet oxygen ($^1O_2$) and hypochlorous acid (HOCl) which are released in phagocytic vacuoles and act as antimicrobial substances. This metabolic process is referred to as the respiratory burst which is associated with several fold increases in oxygen consumption by the cells. The superoxide which is generated by the activation of a membrane bound enzyme complex, NADPH oxidase, is the precursor of other reactive oxygen species. Some of these reactions leading to the generation of several ODRS are shown below.

NADPH oxidase

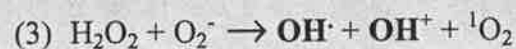
(1) $NADPH + 2O_2 \rightarrow 2O_2^{-} + NADP^{+} + H^{+}$

SOD

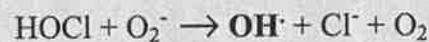


(2) $O_2^{-} + O_2^{-} + 2H^{+} \rightarrow H_2O_2 + O_2$

Fe



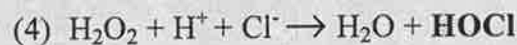
(3) $H_2O_2 + O_2^{-} \rightarrow OH^{\cdot} + OH^{+} + {}^{1}O_2$



$HOCl + O_2^{-} \rightarrow OH^{\cdot} + Cl^{-} + O_2$

$NO + H^{+} + O_2^{-} \rightarrow OH + NO_2$

Myeloperoxidase



(4) $H_2O_2 + H^{+} + Cl^{-} \rightarrow H_2O + HOCl$

The NADPH oxidase consists of (i) the membrane components: cytochrome b_{558} and FAD; (ii) the cytosolic components $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and a small GTP binding protein, rac 2. In quiescent cells, the oxidase components are dormant, dissociated and distributed in the cytosol and membrane. In response to a variety of inflammatory mediators, the cytosolic components become phosphorylated and translocated to the plasmalemma, leading to assembly of the NADPH oxidase complex. This results in the concomitant activation of the NADPH oxidase which then catalyses the transfer of an electron to an oxygen molecule from the donor NADPH molecule (Robinson and Badwey, 1995; Segal, 1996) (Fig 1.1).

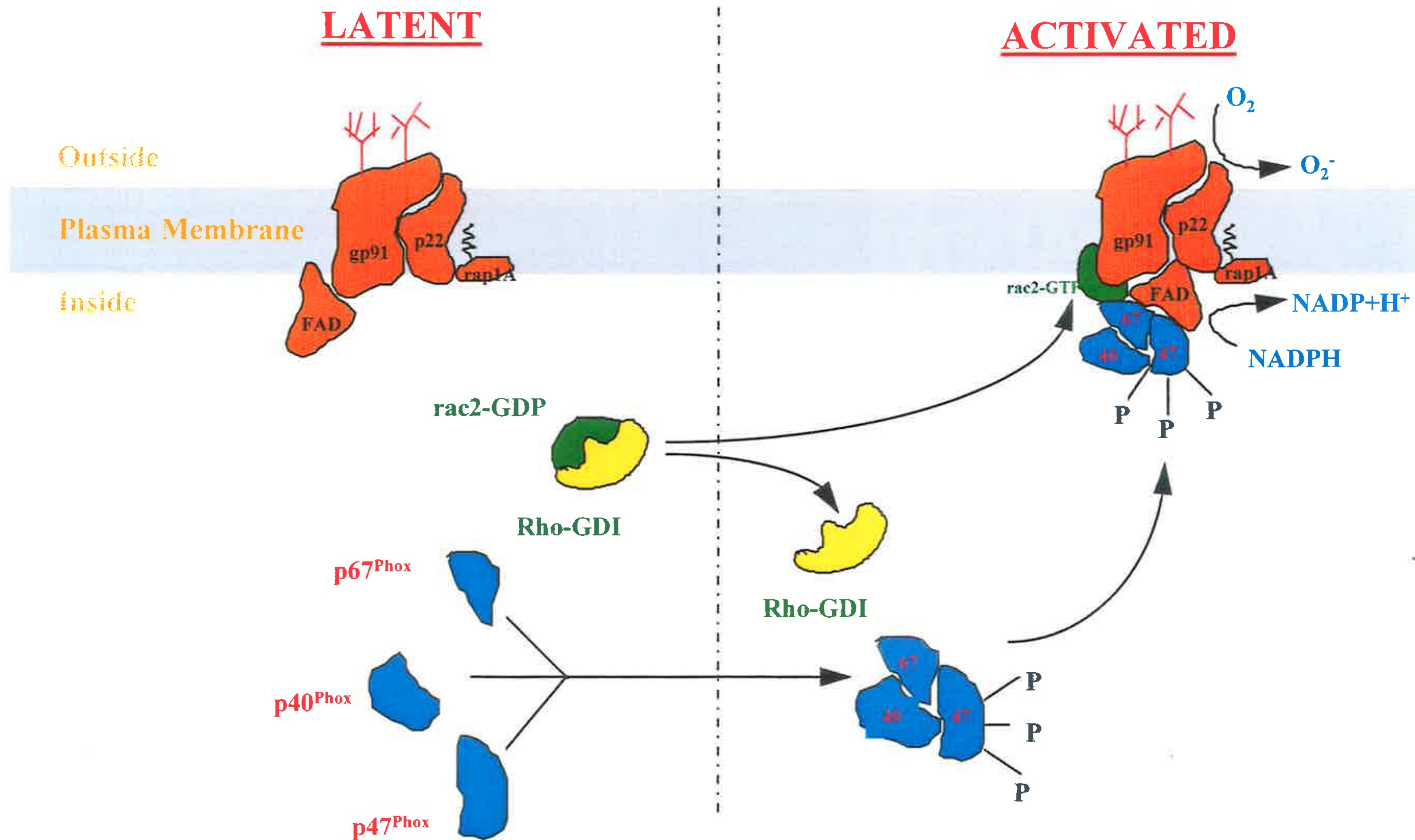


Fig 1.1 Schematic representative of the assembly of the NADPH oxidase. Upon stimulation, the cytosolic components, p47^{phox}, p67 ^{phox} and p40^{phox}, form a 240 kDa complex followed by phosphorylation of p47 ^{phox}. Rac 2 is dissociated from Rho-GDI complex and converted to rac2-GTP to bind to cytochrome b_{558}. This results in activation of the NADPH oxidase and the generation of O_2^- from O_2. (Adapted from Moslen, 1994; De Leo *et al*, 1996).

Patients with chronic granulomatous disease (CGD) have a defective NADPH oxidase in neutrophils and macrophages and lack the ability to produce oxygen radicals. The defect is a result of mutations in NADPH oxidase components, $p47^{phox}$, $p67^{phox}$ and/or cytochrome b_{558}. Neutrophils, macrophages and monocytes from these patients kill phagocytosed *Staphylococcus* and *Aspergillus* species very poorly. Thus the patients usually present with severe and recurrent infections which require aggressive antibiotic treatment (Roos *et al*, 1996).

1.2.1.1 Components of the NADPH oxidase

Cytochrome b_{558} is a heterodimer that is located in the plasmalemma and specific granules of neutrophils. It contains two subunits, 91 kDa $gp91^{phox}$ and 22 kDa $gp22^{phox}$, which are present in a 1:1 ratio and bound to a small amount of FAD (Fig 1.1). Cytochrome b_{558} contains the binding sites for various components during the assembly of the NADPH oxidase complex. Its prosthetic groups are essential for the reduction of oxygen to generate superoxide (Chanock *et al*, 1994).

The $p47^{phox}$ is a cytosolic component of 390 amino acids. It has two SH3 domains which interact with other proteins. Its C-terminal contains many serines and positively charged basic residues which are the target of phosphorylation by PKC and other proline-directed protein kinases (for details see Section 1.3.1 and 1.3.2) (Chanock *et al*, 1994).

The p67^{phox} is another cytosolic component of 526 amino acids. It has two SH3 domains, a highly acidic C terminal and several serine residues flanked by basic groups which serve as phosphorylation sites recognised by the proline-directed protein kinases. This subunit is thought to interact with cytoskeletal elements due to its insolubility in Triton-X-100 (Robinson and Badwey, 1995; Chanock *et al*, 1994).

Rac 2 is a 20 kDa small GTP binding protein identified in human neutrophils and rac 1 is found in guinea pig macrophages. Rac shuttles between an active GTP-bound form and an inactive GDP-bound form. Upon stimulation, rac 2 becomes dissociated from rhoGDI (a GDP dissociation inhibitor) and following GTP loading by an exchange factor, smgGDS, rac participates in the assembly of the NADPH oxidase in the cell membrane (Robinson and Badwey, 1995; Chanock *et al*, 1994).

Rap1A is another small GTP binding protein which copurifies with cytochrome b_{558}. A mutant of rap1A inhibits superoxide production suggesting that rap1A plays a role in the activation of the NADPH oxidase (Chanock *et al*, 1994).

1.2.1.2 Activation of the NADPH oxidase

Activation of the NADPH oxidase occurs following perturbation of phagocyte plasma membranes either specifically or non-specifically. Many surface acting agonists engage a

specific cell surface receptor which is then responsible for initiating a series of intracellular signalling events leading to the activation of the oxidase. Examples of agonists which interact with specific receptors are cytokines (TNF, IFN-γ, IL-1, GM-CSF and IL-8), molecules of microbial origin (fMLP and LPS), complement components (C3bi and C5a) and immunoglobulins (via Fcγ receptor interaction). Some mediators or agents can act less specifically by engaging various cell signalling molecules, eg. diacylglycerol (DAG) activates PKC to cause the assembly of the NADPH oxidase. PMA is a synthetic agent which mimics the action of DAG and has been used as a model agent for activating the oxidase via PKC.

Bacteria and fungi opsonised with either complement or antibody bind to complement and Fc receptors on phagocytes and induce a substantial activation of the NADPH oxidase. Deposition of complement components and antibody on host tissues will lead to a similar binding and activation of phagocyte oxidative respiratory burst. Non-specific induction of the NADPH oxidase activity may occur when phagocyte surfaces contact bacterial or detergent-like molecules.

It is evident that the NADPH oxidase is assembled at a submembranous cytoskeleton site created by cytochrome b_{558}. The two major events in this assembly are (i) phosphorylation of certain components such as $p47^{phox}$ and $p67^{phox}$ and (ii) translocation of all the components to the plasma membrane (Chanock *et al*, 1994).

1.2.1.2.1 Phosphorylation of NADPH oxidase components

In response to stimulation, the cytosolic components of the NADPH oxidase, $p47^{phox}$, $p67^{phox}$ and a not fully characterized component, $p40^{phox}$, form a 240 kDa complex by the interaction of the carboxyl-terminal SH3 domain of $p67^{phox}$ and the carboxyl-terminal proline-rich region of $p47^{phox}$. This is followed by phosphorylation of $p47^{phox}$ on the serine residues. To date, several kinases have been shown to phosphorylate $p47^{phox}$ and activate the NADPH oxidase. These include protein kinase C (PKC), mitogen-activated protein kinases (MAP kinases), protein kinase A (PKA), $p21^{rac}$-activated kinase (PAK) and also a kinase whose activity depended on phophatidylinositol 3-kinase (PI-3 kinase) (El Benna *et al*, 1996a and 1996b; Knaus *et al*, 1995; Didichenko *et al*, 1996). Recent studies show that several purified kinases are able to phosphorylate $p47^{phox}$ on distinct serine residues. MAP kinases (ERK and p38) phosphorylate $p47^{phox}$ on Ser-345 and Ser-348 while PKC phosphorylate Ser-303/304, Ser-315, ser-320, Ser-328, and/or Ser-359/370, but not Ser-345 and Ser 348. PKA is reported to phosphorylate Ser 320 and one or both of the Ser 328 and Ser 359/370 (El Benna *et al*, 1996a). Inhibition of PKC, MAP kinase/ERK or PI-3 kinase activity by their specific inhibitors, GF-109203X, PD 098059 and wortamannin, respectively, attenuates the generation of superoxide (Hii *et al*, submitted; Ahmed *et al*, 1995; Okada *et al*, 1994; Jacobson *et al*, 1995). A calcium-dependent PKC β isozyme has been shown to participate the phosphorylation of this

NADPH component (Majumdar *et al*, 1993). The phosphorylation of $p47^{phox}$ is reversible. The removal of stimulators causes a rapid dephosphorylation of $p47^{phox}$ and dissociation from its membrane binding site (Chanock *et al*, 1994). $p67^{phox}$ undergoes a similar phosphorylation/dephosphorylation event as $p47^{phox}$, but the mechanism is not yet understood.

1.2.1.2.2 Translocation of NADPH oxidase components to the membrane

Activation of the NADPH oxidase requires the assembly of all the components at the plasma membrane. The cytosolic component, $p47^{phox}$, is translocated to the membrane in complex form as well as in free unit form. The translocation of $p67^{phox}$ requires the presence of $p47^{phox}$, whereas $p47^{phox}$ can migrate independently. The association of $p67^{phox}$ with cytochrome b_{558} promotes the electron flow to FAD (Chanock *et al*, 1994).

Rac 2 is first dissociated from its inhibitory complex rhoGDI and converted to an active GTP-bound form, and then redistributed to the membrane to associate with cytochrome b_{558}. The translocation of rac 2 is also dependent on the presence of $p47^{phox}$ but not $p67^{phox}$ (Robinson and Badwey, 1995).

Following the assembly of all the NADPH components at the inner membrane, cytochrome b_{558} undergoes a conformational change that allows electrons to be extracted

from NADPH and transferred via FAD to oxygen to generate superoxide on the opposite side of the membrane (Fig 1.1) (Robinson and Badway, 1995).

1.3 Signalling molecules and activation of the NADPH oxidase

Stimulation of phagocytic cells with microbial products and inflammatory mediators initiates a series of functional responses that serve to eliminate pathogens or cause tissue damage. A number of cellular signalling molecules, including protein kinases, have been found to be crucial for linking events at the cell surface to the microbicidal or inflammatory activities of phagocytic cells. Among these kinases, protein kinase C, mitogen-activated protein kinases, protein tyrosine kinases, PI-3 kinase and calcium/calmodulin dependent kinases have been shown to play a role in triggering the respiratory burst in phagocytic cells.

1.3.1 Protein kinase C (PKC)

PKC is a family of cytoplasmic serine/threonine protein kinases which were first identified in rat brain tissues in 1977 by Nishizuka and colleagues (Inoue *et al,* 1977; Takai *et al*, 1977). It plays a critical role in a wide range of cellular responses, such as muscular contraction, platelet activation, growth factor action, tumor promotion, neurotransmission and hormonal responses (Nishizuka, 1986 and 1995). At present 12

PKC isozymes have been reported in the literature; PKC α, βI, βII, γ, δ, ε, θ, η, μ, ζ, ι and λ. With the exception of PKC β isozyme, the other isozymes are encoded by different genes which are located on different chromosomes. PKC βI and βII isozymes are formed by alternative splicing of the 3' exon of the β gene. According to the structural differences and cofactor requirement, the PKC isozymes are divided into three groups: Group A [conventional PKCs or classical PKCs (cPKCs)] consists of PKC α, βI, βII and γ; Group B [novel PKCs (nPKCs)] consists of PKC δ, ε, θ, η and μ; Group C [atypical PKC (aPKCs)] consists of PKC ζ, ι and λ (Fig 1.2).

1.3.1.1 The structure of PKC

PKC consists of a single polypeptide chain which is divided into two functional domains: the C-terminal catalytic domain (45 kDa) and the N-terminal regulatory domain (20-40 kDa). Each domain is composed of several conserved regions (eg. C1-C5) and variable regions (V1-V5) (Fig 1.2). In cPKCs, the regulatory domain contains C1, C2, V1 and V2 regions, while in nPKCs and aPKCs, the C2 region is absent.

The C1 region contains two cysteine-rich Zn^{2+} fingers. This region is responsible for the binding of Zn^{2+}, phosphatidylserine (PS), diacylglycerol (DAG)/phorbol esters and lipids. nPKCs and aPKCs have only one of the cysteine-rich Zn^{2+} fingers which renders them unresponsiveness to DAG or phorbol ester. The C2 region contains a calcium

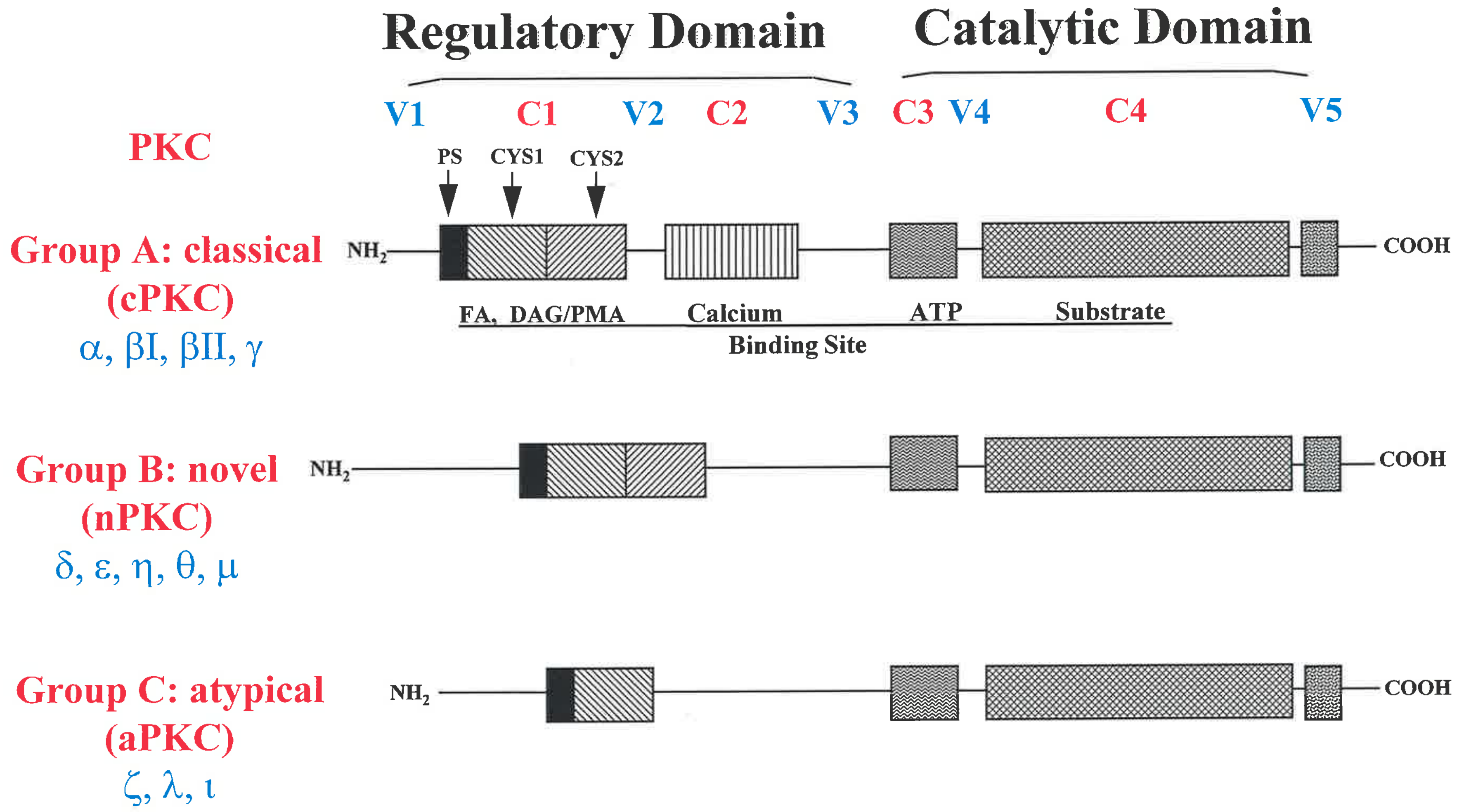


Fig 1.2 Schematic structure of PKC members. PS: pseudosubstrate; CYS: cysteine-rich Zn^{2+} fingers. (Adapted from Mahoney and Huang, 1994)

binding site. Binding of calcium to this site is thought to be responsible for causing the translocation of cPKCs to particulate fractions, thereby facilitating activation of PKC. Novel PKCs have similar structure to classical PKCs but lack the calcium-binding C2 region. However, nPKCs have an extended V1 region and this has been proposed to regulate the translocation of nPKCs to the particulate fractions in a calcium-independent manner.

The regulatory domain also contains a pseudosubstrate motif at the beginning of the C1 region and this motif is present in all PKC isozymes. The pseudosubstrate motif contains residues which are similar to those found in natural substrates but lacks the phosphorylatable serine or threonine residues. Therefore it acts as an autoinhibitory factor by interacting with the substrate binding site in the catalytic domain of PKC to inhibit the basal activity of PKC (Mahoney and Huang, 1994).

The catalytic domain contains sequences for binding ATP (C3) and PKC substrates (C4). The regulatory and catalytic domains are joined by a hinge region, V3, which is the cleavage site for calpain and trypsin. (Mahoney and Huang, 1994; Huang and Huang, 1990; Burns and Bell, 1992).

1.3.1.2 The distribution of PKC

Although PKC is a ubiquitous enzyme, PKC isozymes are distributed in a tissue- and cell type-specific manner (Table 1.2). Beside the distinct tissue distribution, PKC also has specific intracellular localisation. In unstimulated cells, most isozymes are predominantly cytosolic. (Parker, 1994). Upon stimulation, PKC undergoes a selective subcellular redistribution. In phorbol ester-stimulated NIH 3T3 fibroblasts, immuofluorescence studies show that PKC α and ε are located at cell margins, and PKC γ is concentrated at the Golgi apparatus. PKC α also accumulates in the endoplasmic reticulum while PKC βII is associated with actin-rich microfilaments of the cytoskeleton and PKC ε is enriched in nuclear membranes (Parker, 1994; Goodnight *et al*, 1995).

In unstimulated human monocytes, PKC α is found in the cytosolic fraction of cells, however, PKC βI and βII are equally distributed in the cytosolic and the particulate (membrane) fractions of monocytes (Chang and Beezhold, 1993). Very little or no PKC γ, ε and δ are expressed in these cells. In neutrophils, PKC α, βI and βII isozymes have been identified in the cytosolic fraction of quiescent cells (Pontremoli *et al*, 1990; Hii *et al*, submitted). In neutrophils, phorbol esters stimulate the translocation of PKC α and β to the plasma membrane and the nucleus. PKC α is translocated to a particulate fraction in monocytes/macrophages following stimulation with a phorbol ester (Chang and Beezhold, 1993).

Table 1.2 The distribution of PKC isozymes and their activation

	Isozymes	Distribution	Activators
cPKC	α	all cell types	Ca^{2+}, PS, DAG, FFA, LysoPC
	βI	some tissues	as above
	βII	many tissues	as above
	γ	brain only	as above
nPKC	δ	all tissues	PS, DAG
	ε	brain and others	PS, DAG, FFA, PIP_3
	μ	skin, lung and heart	PS, DAG, PIP_3, cholesterol sulfate
	θ	muscle and T cells	?
	η	NRK cells	?
aPKC	ζ	all tissues	PS, FFA, PIP_3, PIP_2
	λ	many tissues	?
	τ	?	?

cPKC: classical PKC group; nPKC: novel PKC group; aPKC: atypical PKC group; FFA: free *cis*-unsaturated fatty acids; DAG: diacylglycerol; PS: phosphatidyl-L-serine; PIP_2: phosphatidylinositol-4,5-bisphosphate: PIP_3: phosphatidylinositol-3,4,5, triphosphate; Lyso PC: lysophosphatidylcholine; NRK cells: normal rat kidney cells. (Adapted from Nishizuka, 1995)

1.3.1.3 Synthesis and degradation of PKC

PKC is first synthesised as an inactive form of 74 kDa. This primary translation product then undergoes transphosphorylation and autophosphorylation by a putative PKC kinase. This yields a mature kinase of 80 kDa. Phosphorylation of PKC increases the sensitivity of PKC for calcium, the binding of phorbol esters to PKC and the rate of phosphorylation of histone. However, PKC at this stage is not active due to the occupancy of the substrate binding site by its pseudosubstrate (Newton, 1995; Azzi *et al*, 1992).

After stimulation, activated PKC becomes susceptible to proteolytic degradation by calpain and trypsin at the V3 hinge region. Following cleavage, the catalytic domain fragment can function as a constitutively active protein kinase M (PKM). In neutrophils, 50% of PKC α and 100% of β can be converted to PKM by calpain digestion (Mahoney and Huang, 1994; Nishizuka, 1995).

Prolonged treatment of cells with phorbol esters causes the degradation and loss of phorbol ester-responsive PKC isozymes by proteolytic degradation (Mahoney and Huang, 1994; Jaken, 1990). This renders the cell unresponsive to agonists which act via

PKC. This phenomenon has been widely exploited to investigate the role of PKC in regulating cellular response.

1.3.1.4 Activation of PKC

PKC is activated by a combination of phospholipids, diacylglycerol/phorbol esters, certain *cis*-unsaturated fatty acids and/or calcium.

1.3.1.4.1 The role of calcium

Receptor-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C (PLC) yields inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Fig 1.3). IP_3 causes the mobilisation of calcium from the intracellular stores. The increase in intracellular calcium concentration promotes the association of classical PKC isozymes with the membrane/particulate fraction where the lipid co-factors, such as DAG and phospholipids, are located to form a pre-activated form of kinase (Burns and Bells, 1992).

1.3.1.4.2 The role of DAG

The generation of DAG by hydrolysis of PIP_2 is transient (seconds). In many instances, this is followed by a sustained DAG generation (minutes to hours). The second phase of

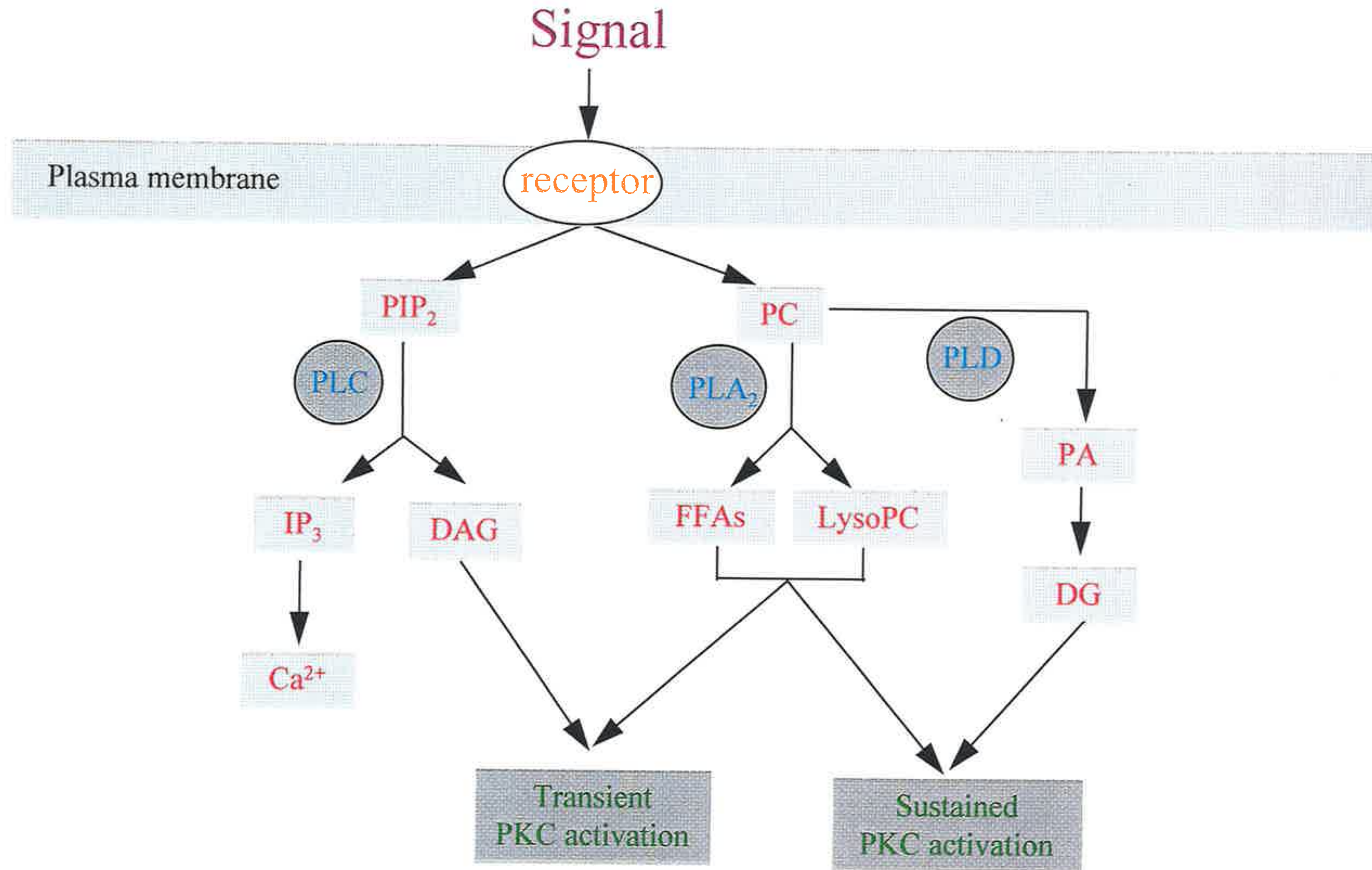


Fig 1.3 Signalling pathway for the activation of PKC. PIP_2: phosphatidylinositol-4,5-bisphosphate; IP_3: phosphatidylinositol-3,4,5,-triphosphate; PC: phosphatidylcholine; PA: phosphotidic acid; PLC: phospholipase C; PLD: phospholipase D; FFA: free fatty acids; PLA_2: phospholipase A_2; LysoPC: lysophosphotidic acid; DG: diglycerides. (Adapted from Nishizuka, 1995)

DAG generation, caused by the breakdown of PC, is likely to be responsible for activation of PKC in cells stimulated with cytokines, phorbol esters or growth factors (Asaoka *et al*, 1992; Nishizuka, 1995) (Fig 1.3). Hydrolysis of PC does not lead to an increase in intracellular calcium and hence, only nPKC, but not, cPKCs are activated (Exton, 1994).

DAG activates cPKC and nPKC but not aPKC isozymes. Activation of nPKC and cPKC by DAG can be mimicked by tumor promoting phorbol esters, eg. phorbol 12-myristate 13-acetate (PMA) (Table 1.3). Both DAG and PMA increase the affinity of PKC for membrane PS and serve as hydrophobic anchors to promote the formation of irreversible membrane-inserted PKC (see below). Phorbol esters compete with DAG in binding to the same interaction site in the C1 region of PKC, but with higher affinity. In addition, unlike DAG which is rapidly metabolized/converted to products, such as phosphatidic acid, phorbol esters are slowly degraded which causes long lasting effects. It is known that phorbol ester is about a thousand times more potent than DAG in the regulation of cell differentiation, proliferation and carcinogenesis. (Rando and Kishi, 1992) (Table 1.3).

Table 1.3 Comparison of activity of DAG and phorbol esters

	DAG	Phorbol ester
Nature	natural lipid	tumor promoter
Turnover	rapid	stable
Binding site in PKC	C1 region	C1 region with higher affinity
	competes with phorbol esters	competes with DAG
Activation of PKC	✓less potent than phorbol esters	✓
Mechanism	↑ affinity of PS to PKC	↑ affinity of PS to PKC
	↑ association of PKC with the membrane	↑ association of PKC with the membrane
	↑ PKC conformational change	↑ PKC conformational change

DAG: diacylglycerol; PS: phosphatidylserine; ↑: increase/induce; ✓: activate.
(Compiled from Burns and Bell, 1992)

1.3.1.4.3 The role of lipids

The activity of PKC is tightly regulated by phospholipids and fatty acids. Among phospholipids, phosphatidyl-L-serine (PS) is the most effective cofactor of PKC (Quest and Bell, 1994). PS is a common factor required for activation of all three groups of PKC isozymes (Table 1.2). Lyso PC, although not effective by itself, is synergistic with DAG to activate PKC (Nishisuka, 1995). PIP_2, a precursor of DAG, activates PKC in the presence of calcium and PS, but to a lower degree than DAG. PIP_2 can also act as a cofactor for activation of PKC and can replace PS in the activation of PKC (Nishizuka, 1995; Mahoney and Huang, 1994).

The role of fatty acids in stimulating the activity of PKC is discussed in section 1.5. Novel PKC ε and η isozymes can be activated by PIP_3, a product of PI-3 kinase and to a lower extent by PIP_2, while the activation of atypical PKC ζ isozyme is dependent on PS, PIP_2, ceramide and PIP_3 as well as *cis*-unsaturated fatty acids (Nishizuka, 1995).

1.3.1.5 Current Model of PKC activation

It is proposed that activation of PKC consists of (i) association of PKC with the membrane; (ii) membrane insertion which leads to a subsequent PKC conformational

change and ultimately, activation of PKC. The model described below is for cPKC (Fig 1.4).

In unstimulated cells, PKC resides in the cytosol. Upon stimulation, the classical PKC isozymes become associated with the membrane via the C2 region. However, PKC is still not active. Following a rise in DAG or the addition of phorbol esters, the affinity of PKC for the membrane is increased remarkably by the insertion of cysteine-rich Zn^{2+} finger in the C1 region of PKC into the bilayer membrane. Then PKC undergoes a conformational change during which the pseudosubstrate of PKC becomes dislodged from its interaction site in the catalytic domain and the substrate binding site becomes available for binding the substrates. This finally leads to a full activation of PKC (Fig 1.4). The novel PKC isozymes, because of the absence of the C2 region, are normally distributed close to the membrane and are associated with the membrane by using the C1 region in a calcium-independent fashion. The activation of atypical PKCs is not fully understood (Burns and Bells, 1992; Newton, 1995).

It has also been suggested that proteins, such as Receptors for Activated C Kinase (RACK), may function as putative receptors for the recruitment of PKC to the membrane. Both 30 kDa and a 33 kDa RACK have been isolated from the detergent-insoluble membrane fractions. The binding of PKC to RACK is saturable and specific, and dependent on the presence of calcium and PS and also can be enhanced by the

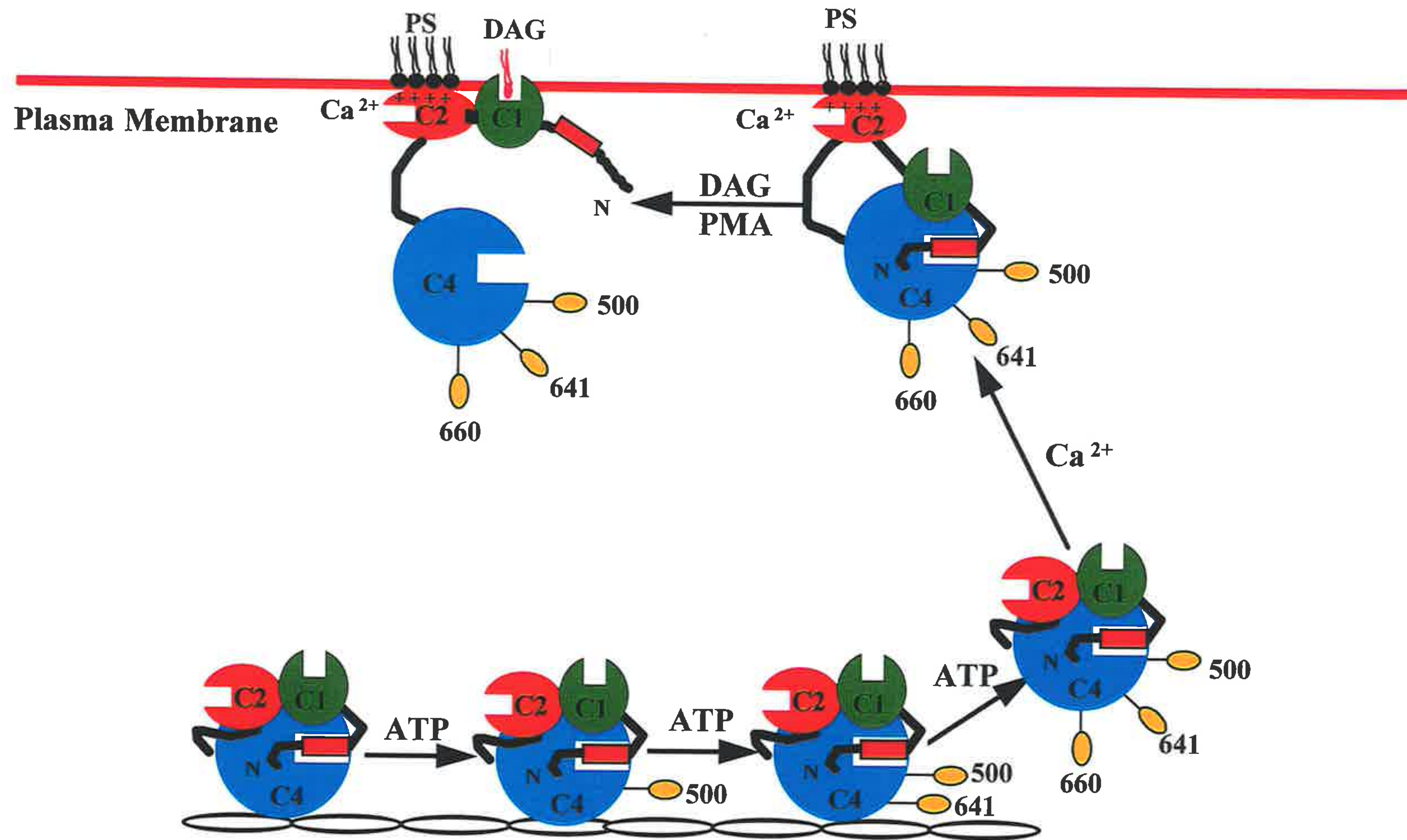


Fig 1.4 Model of activation of calcium-dependent PKC. Newly synthesized PKC is transphosphorylated and autophosphorylated on Thr-500, Thr-641 and Ser-660 residues before being released to the cytosol. Classical PKCs are then translocated to the membrane in the presence of calcium and become associated with the membrane via the calcium binding C2 region. Stimulation by DAG or PMA induces the insertion of PKC to the bilayer membrane via C1 region. This results in a conformational change of PKC and the pseudosubstrate motif is dislodged from its binding site, thereby producing an active molecule of PKC. PS: phosphatidylserine; DAG: diacylglycerol; N:n-terminal. (Adapted form Newton, 1995; Jaken and Kiley, 1994; Hug and Sarre, 1993. It is suggested that membrane association of PKC is via the interaction of C2 region with PS in a calcium-dependent manner).

addition of PMA or DAG (Nishizuka, 1995; Mahoney and Huang, 1994; Mochly-Rosen, 1995; Ron and Mochly-Rosen, 1995).

1.3.2 Mitogen-activated protein kinases (MAP kinases)

Many neutrophil ligands (eg. fMLP, PMA, IL-8 and LTB_4) and monocyte/macrophage ligands (eg. LPS, PMA and IFN-γ) stimulate the activity of the extracellular signal regulated protein kinase (ERK) 1 and 2. These kinases and other closely related kinases are now being referred to as mitogen-activated protein (MAP) kinases. Purified ERKs have been reported to phosphorylate $p47^{phox}$ on Ser-345 and Ser-348 (El Benna *et al*, 1996a). These same sites are also phosphorylated in intact neutrophils which had been stimulated by ligands which stimulate the activity of ERK 1 and ERK 2 (El Benna *et al*, 1994). Hence, ERK may play a role in regulating the production of superoxide.

1.3.2.1 The MAP kinase subfamily

MAP kinases were originally discovered by virtue of their ability to phosphorylate exogenous substrates, such as microtubule-associated protein-2 and myelin basic protein (Ray and Sturgill, 1987; Ahn *et al*, 1990; Hoshi *et al*, 1988; Pelech 1988; Cicirelli *et al*, 1988). To date, seven closely related MAP kinases from mammalian cells have been

identified and sequenced. These are MAPK1/ERK1, MAPK2/ERK2, ERK3, ERK5, ERK6, Jun N-terminal kinases/stress protein kinases (JNK/SAPKs) and p38/HOG1/CSBP/RK. ERK1 and ERK2 were the first two MAP kinase members to be purified and cloned (Boulton *et al*, 1990 and 1991; Gotoh *et al*, 1991). The sequence of ERK1 and ERK2 is 90% identical (Boulton, 1991).

1.3.2.2 Distribution and localization of MAP kinases

Although the kinases were originally thought to be located in the cytoplasm of quiescent cells, recent studies have found that one third of ERKs may be associated with the microtubules in quiescent cells (Morishima-Kawashima and Kosik, 1996; Arendt *et al*, 1995). After stimulation, activated ERK1 and ERK2 become associated with the cytoskeletal elements and the cell membrane, and a proportion of the kinases has been reported to translocate into the nucleus (Cobb *et al*, 1994). ERK3 is widely distributed in nearly all types of tissues and cells, but strongly expressed in heart muscles and brain tissues (Davis, 1995). This kinase is always localised in the nucleus, irrespective of whether the cells are quiescent, exponentially growing or stimulated with growth factors (Cheng *et al*, 1996). ERK5 is abundantly distributed in heart and skeletal muscles. It is found primarily in the cytosol of the cells (Zhou *et al*, 1995). JNK/SAPK and p38/HOG1 are also expressed in nearly all types of tissues and cells. Both cytoplasmic and nuclear localization of JNK and p38 have been reported (Raingeaud *et al*, 1996; Cavigelli *et al*, 1995).

1.3.2.3 Molecular activation and inactivation of ERK

MAP kinases are activated by phosphorylation on tyrosine (Tyr) and threonine (Thr) residues in the TEY motif. Although the tyrosine and threonine residues can be phosphorylated independently of each other, maximal activation of ERK requires the phosphorylation on both residues (Ahn, 1993). Dual phosphorylation of ERK1 and ERK2 is achieved by the action of the dual specificity MAP kinase/ERK kinase (MEK) 1 or MEK 2. Studies with ERK2 reveal that phosphorylation of ERKs by MEK 2 leads to global and local conformational changes in the ERK2 structure (Cobb *et al*, 1994; Cobb and Goldsmith, 1995).

Removal of phosphate from tyrosine and/or threonine residues by phosphatase 2A (specific for serine/threonine), CD45 (specific for tyrosine) or the dual specificity phosphatase, MAP kinase phosphatase (MKP) 1, MKP2 and MKP3 leads to inactivation of ERK (Cobb *et al*, 1994). The dual specificity phosphatases are activated by serum and growth factor treatment and their activation can be blocked by cycloheximide (Cobb *et al*, 1994).

1.3.2.4 Ligands which stimulate the activity of MAP kinases

ERK1 and ERK2 are activated by a variety of stimulators which bind to the G-protein linked seven transmembrane receptors, receptor tyrosine kinases or G_i-linked receptors. These include insulin, epidermal growth factor, platelet derived growth factor, nerve growth factor, phorbol esters, cytokines, T cell antigens, LPS, fMLP, LTB_4, PAF, C5a, nicotine, growth hormone etc. (Guan, 1994). Activation of ERKs not only regulates cell proliferation and differentiation, but may also regulate non-mitogenic responses, for example, the respiratory burst in phagocytic cells (Cobb *et al*, 1994; Chanock *et al*, 1994; El Benna *et al*, 1994, 1996a and 1996b)

1.3.2.4.1 MAP kinase cascades

Currently four MAP kinase cascades have been reported in mammalian cells: (i) ERK (raf-1/MEK/ERK) cascade; (ii) JNK/SAPK cascade; (iii) p38/HOG1 cascade; (iv) ERK5 cascade. These cascades are presented in Fig 1.5. With the exception of ERK5 cascade, the existence of the ERK1 and ERK2, JNK and p38 cascades have been reported in haemopoietic cells (see section 1.4.2.5).

MAP kinase cascades

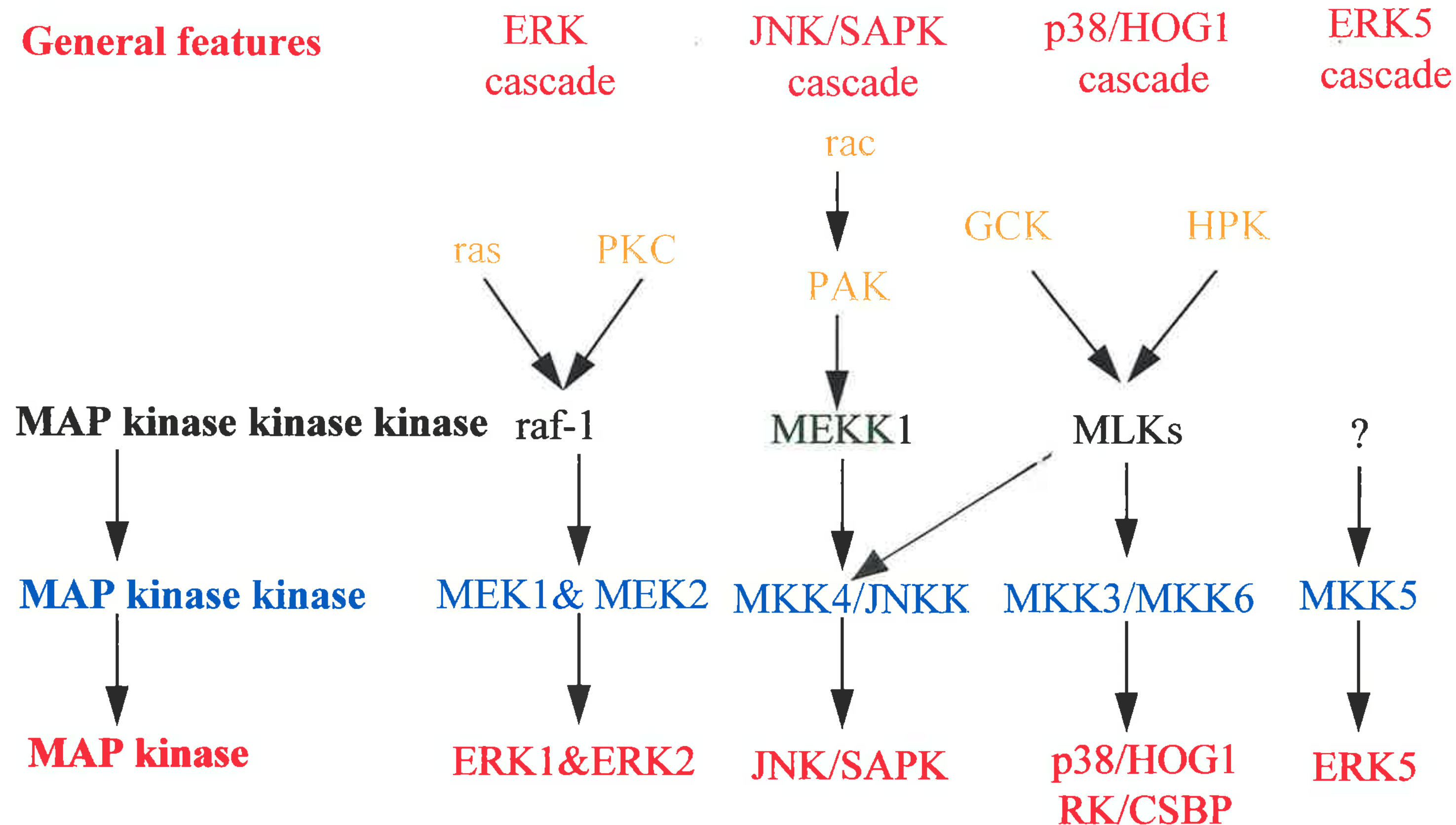


Fig 1.5 MAP kinase cascades in mammalian cells. MAP kinase: mitogen-activated protein kinase; ERK: extracellular signal-regulated protein kinase; MEK: ERK kinase; PAK: $p21^{ras}$-activated protein kinase; MKK: MAP kinase kinase; GCK: germinal center kinase; JNK: jun N-terminal kinase; SAPK: stress-activated protein kinase; JNKK: JNK kinase; RK: reactivating kinase; CSBP: cytokine synthesis anti-inflammatory drug-binding protein; MEKK: MEK kinase; MLK: mixed lineage kinase; HPK1: hematopoietic protein kinase. (Compiled from Cobb and Goldsmith, 1995; Kyriakis and Avruch, 1996; Tibbles *et al*, 1996; Han *et al*, 1996)

1.3.2.4.2 The ERK cascade

The upstream regulators of ERKs in the ERK cascade include MEK 1 and MEK 2, raf-1, the small GTP-binding protein, p21^{ras} and/or PKC.

1.3.2.4.2.1 p21^{ras}

P21^{ras}, a product of a protooncogene, is a 21 kDa small GTP-binding (G) protein which exists in two forms, the active ras-GTP form and an inactive ras-GDP form. In response to stimulation, ras-GDP is converted to ras-GTP by the guanine nucleotide exchange factor, mSOS (mammalian Son of Sevenless), while ras-GTP is then converted to ras-GDP by GTPase activating protein (GAP) and recycled again. GTP loading of p21^{ras} is stimulated by agonists which bind to receptor tyrosine kinases, eg. EGF, or to receptors which stimulate the activity of tyrosine kinases, such as M-CSF, IL-2, IL-3, fMLP and GM-CSF (Maruta and Burgess, 1994; Johnson and Gomez-Cambronero, 1994). The precise mechanism of how p21^{ras} stimulates raf-1 (MEK kinase) is still unclear. However, it has been suggested that p21^{ras} serves as the activating ligand for the oligomeric raf-1 assembly, recruiting c-raf-1 to the membrane and initiating a conformational change that allows raf-1 to interact with a second raf-1 polypeptide (dimerization) maybe via 14-3-3 (Luo *et al*, 1996; Farrar *et al*, 1996). Mutation of p21^{ras} leads to the development of carcinomas, suggesting a critical role of p21^{ras} in the control of cell proliferation and differentiation (Maruta and Burgess, 1994). While p21^{ras} is

widely recognised to be an upstream regulator of the ERK cascade, it has also been reported to stimulate the JNK/SAPK cascade (Kyriakis and Avruch, 1996). The mechanism by which $p21^{ras}$ stimulates the JNK/SAPK cascade is not fully understood. Another small GTP binding protein, rac, has been reported to be upstream of the JNK/SAPK cascade (Kyriakis and Avruch, 1996).

1.3.2.4.2.2 raf-1

The proto-oncogene, raf-1, encodes a serine/threonine kinase which functions as MAP kinase kinase kinase. Three isoforms of raf-1 have been identified, ie. raf-1, A-raf and B-raf which are expressed in a cell-type specific manner and are activated by growth factors and mitogens, such as CSF-1, PDGF, insulin and IL-2 (Dent *et al*, 1992; Kyriakis *et al*, 1992; Howe *et al*, 1992; Ahn, 1993). Raf-1 lies between $p21^{ras}$ and MEK in the ERK cascade. It can also be switched on by PKC, following the ligation of G-protein coupled, seven-transmembrane receptors. Hence, raf-1 acts as a convergent point through which the effects of ligands which bind to receptors with intrinsic tyrosine kinase activity and the seven transmembrane receptors are mediated (Cobb *et al*, 1994; Marquardt *et al*, 1994; Kolch *et al*, 1993; Anderson *et al*, 1991). In addition, activated ERK1 and ERK2 are shown to phosphorylate raf-1 (Lee *et al*, 1991 and 1992; Anderson *et al*, 1991). Activation and phosphorylation of raf-1 are evident during the differentiation of promyelocytic leukemia cells into mature granulocytes (Okuda *et al*, 1994b). Recent studies have suggested that dimerization of raf-1 is important for its function (Farrar *et al*,

1996). MEK kinase (MEKK) 1 and mixed lineage kinase (MLK) 3 act as MAP kinase kinase kinase in the JNK/SAPK and p38/HOG1 cascades, respectively (Tibbles *et al*, 1996; Yan and Templeton, 1994).

1.3.2.4.2.3 MAP kinase kinases (MEK)

The MAP kinase kinases or MEK 1 and MEK 2, located immediately upstream of ERK1 and ERK 2 are dual specificity kinases which phosphorylate ERK1 and ERK 2 on threonine and tyrosine residues in their TEY motif (Guan, 1994). MEKs are phosphorylated by raf-1 on serine residues, for example Ser-218 and Ser-222 for MEK1 (Yan and Templeton, 1994). MEKs can also be phosphorylated by activated ERKs (Guan, 1994). In the JNK/SAPK and p38/HOG1/RK cascades, MKK4 and MKK3/MKK6, respectively, serve as MAP kinase kinase, while in the ERK5 cascade, MKK5 is the MAP kinase kinase. With the possible exception of MKK3 and MKK4, the MAP kinase kinases are cascade-specific (Cobb and Goldsmith, 1995; Stein *et al*, 1996; Zhou *et al*, 1995).

1.3.2.4.2.4 Functional role of ERKs

ERK1 and ERK 2 were originally found to be activated by growth factors and hormones and were hence referred to as mitogen-activated protein kinases. A major role of the ERKs is the regulation of proliferation and differentiation (Cobb *et al*, 1994). Recent

studies have demonstrated that ERK provides a survival signal. Studies from a number of laboratories have shown that the balance between ERK activation, on the one hand, and JNK/p38 activation, on the other hand, plays a pivotal role in determining whether a cell undergoes apoptosis (programmed cell death) or proliferation and differentiation (Dibenedetto and Pittman, 1996).

1.3.2.4.2.5 Substrates of ERKs

Activated ERK1 and ERK2 phosphorylate a number of cytoplasmic substrates, such as 90k S6 kinases ($pp90^{RSK}$), 70K S6 kinases ($pp70^{RSK}$), raf-1, MEK1 and EGF receptor (Guan, 1994). Two cytoskeletal elements, Tau and high molecular weight microtubule associated kinases in yeast, are also the targets of ERK phosphorylation (Robbins *et al*, 1994). In neutrophils, $p47^{phox}$ is a substrate for ERK (El Benna *et al*, 1996a and 1996b). Beside these, the cytosolic phospholipase A_2 (PLA_2) is phosphorylated on Serine-505 by ERK (Lin *et al*, 1993; Abdullah *et al*, 1995). Activated ERKs can also be translocated from the cytoplasm to the nucleus (Fig 1.6). *In vitro* studies have demonstrated that ERKs phosphorylate the transcription factors, c-jun, c-myc, c-fos, c/EBPβ, tal1, Elk-1, NF-IL6 and ATF-2 etc. (Guan, 1994, Matsuda *et al*, 1994) (Fig 1.6). Of these, Elk-1 appears to be the physiological target of ERKs *in vitro* (Cobb *et al*, 1994).

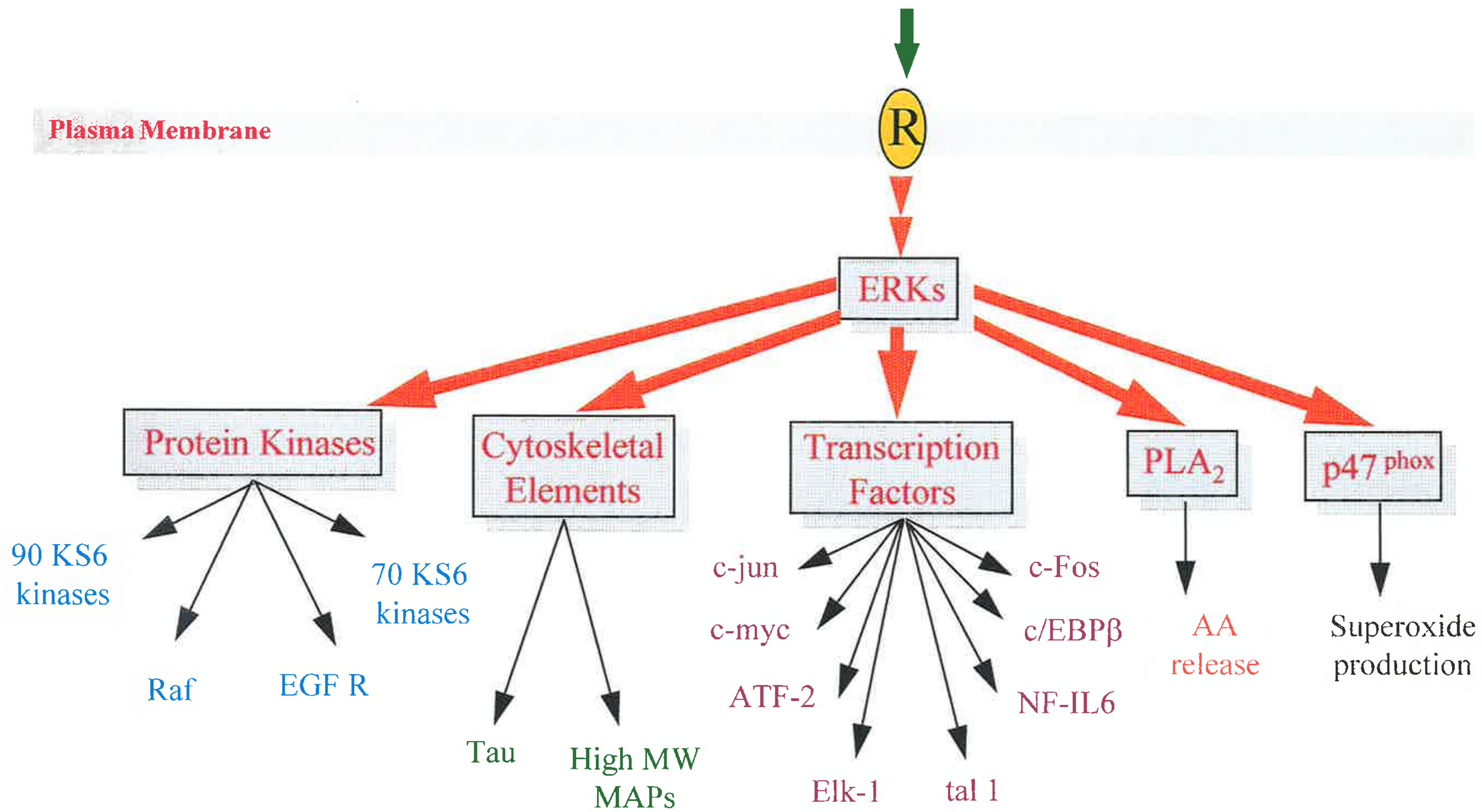


Fig 1.6 ERK1 and ERK2 are pleiotropic regulators of cell functions. ERK: extracellular signal-regulated protein kinase; MAPKAP kinase-2: MAPK-activated protein kinase-2; hsp27: heat shock protein 27; EGF R: epidermal growth factor receptor; AA: arachidonic acid; PLA$_2$: phospholipase A$_2$. (Compiled from Robbins *et al*, 1994)

1.3.2.5 Activation of MAP kinases in leukocytes

Activation of MAP kinases occurs in neutrophils and monocytes/macrophages in response to a wide range of inflammatory mediators and bacterial products such as fMLP and LPS.

In the monocytes, macrophages and monocytic cell lines (THP-1, U937, or RAW 264.7), stimulation with agonists such as LPS, zymosan, PMA, substance P, okadaic acid, A23187, oxidised LDL, TNF, IL-1, IL-6 and IFN-γ as well as the cross-linking of CD120a (TNF receptor p55) result in increased activity of ERKs as observed by an increased ability of cytosolic extracts to phosphorylate the artificial substrate, myelin basic protein. ERK1 and ERK2 have been identified from these cytosolic fractions as two MAP kinase members involved in these cell responses (Ambs *et al*, 1995; Kavelaars *et al*, 1994; Qiu and Leslie, 1994; Winston and Riches, 1995; Deigner and Claus, 1996; Winston *et al*, 1995; Ahlers *et al*, 1994; Liu *et al*, 1994; Belka *et al*, 1995) (Fig 1.7). The activation of two other MAP kinase subfamilies, JNK/SAPK and p38/HOG1, have also been studied in monocytes/macrophages. It has been shown that JNK and p38 kinase can be activated by TNF, IL-1, LPS and stress (Sanghera *et al*, 1996; Lee and Young, 1996). Activation of these two kinases has been proposed to mediate inflammatory responses, stress-related cellular responses and apoptosis (Kyriakis and Avruch, 1996) (Fig 1.7). p38 kinase is an upstream kinase of MAP kinase-activated protein kinase 2 (MAPKAP

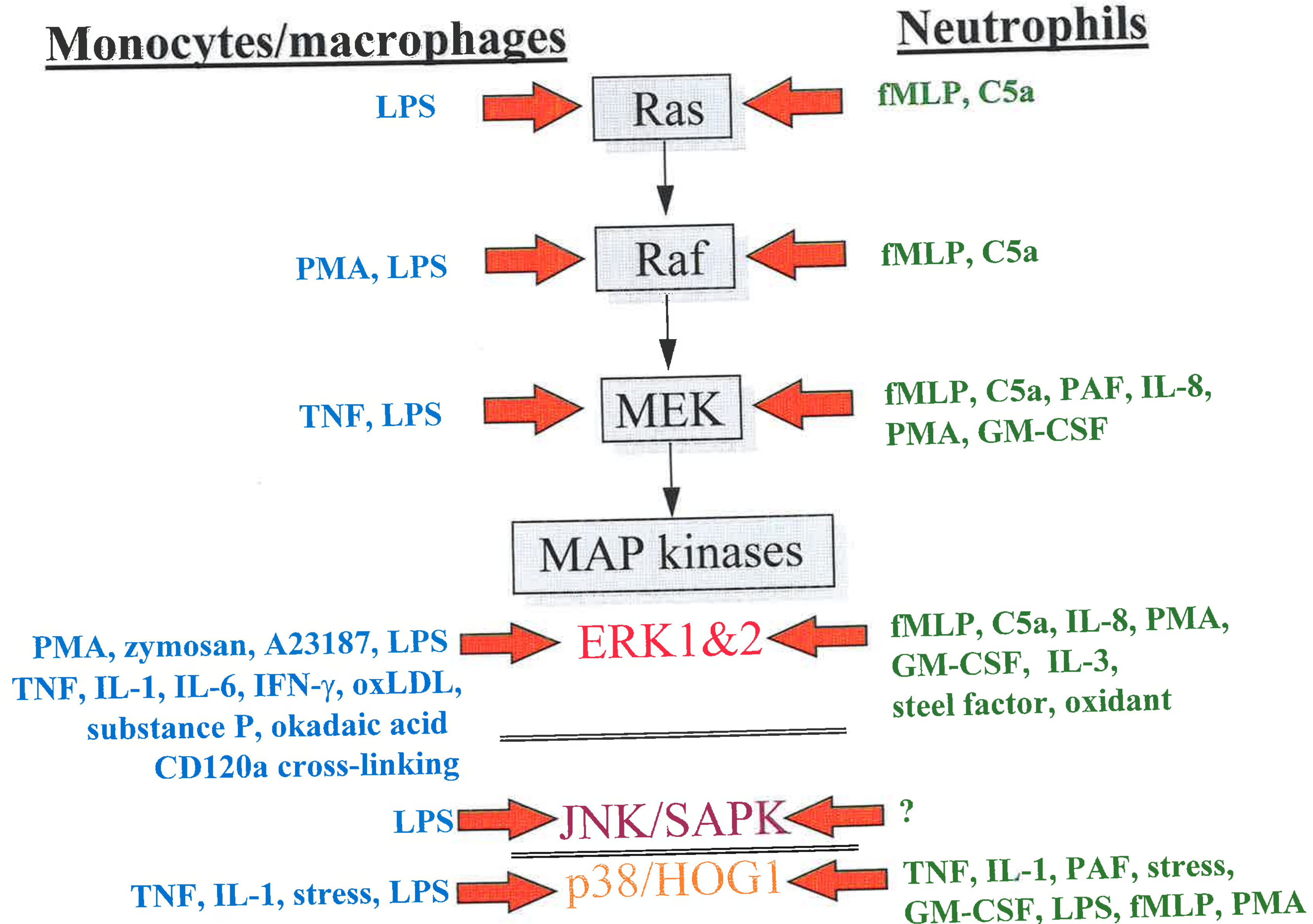


Fig 1.7 Activation of the components of MAP kinase cascade in mononuclear cells and neutrophils. (Compiled from Buhl *et al*, 1995; El Bennna *et al*, 1994; Van-Lint *et al*, 1993; Gomez-Cambronero *et al*, 1993; Fouda *et al*, 1995; Okuda *et al*, 1992; Fialkow *et al*, 1994; Ňahas *et al*, 1996; Ambs *et al*, 1995; Kavelaars *et al*, 1994; Qiu *et al*, 1994; Winston and Riches, 1995; Deigner and Claus, 1996; Winston *et al*, 1995; Liu *et al*, 1994; Belka *et al*, 1995; Sanghera *et al*, 1996; Lee and Young, 1996; Kyriakis and Avruch, 1996; Ahler *et al*, 1994).

kinase 2) which subsequently phosphorylates a small heat shock protein, hsp27 (Ahler *et al*, 1994). The signalling pathway for LPS-stimulated ERK activation in monocytes/macrophages has also been investigated. It has been shown that LPS utilises the pathways, $p21^{ras}$ → raf-1 → MEK → ERK1 and ERK2, to transduce a signal from the cell surface molecule CD14 to induce TNF production (Geppert *et al*, 1994), although other studies have pointed to the p38 cascade being involved in LPS-stimulated TNF production (Lee and Yong, 1996).

In neutrophils, activation of ERK1 and ERK2 occurs in response to fMLP, C5a, IL-8, PAF, PMA and the cytokines, GM-CSF, IL-3 as well as steel factor, oxidants (eg. diamine, H_2O_2 etc) (Buhl *et al*, 1995; El Bennna *et al*, 1994; Fouda *et al*, 1995; Van-Lint *et al*, 1993; Gomez-Cambronero *et al*, 1993; Okuda *et al*, 1992; Fialkow *et al*, 1994) (Fig 1.7). Activation of p38 kinase has also been reported following the treatment of neutrophils with LPS, PAF, TNF, GM-CSF, fMLP, PMA or by environmental stress (Nick *et al*, 1996; Krump *et al*, 1997; Nahas *et al*, 1996). fMLP and PMA have recently been reported to stimulate the activation of MAPKAP kinase 2, the activity of which is dependent on p38 (Zu *et al*, 1996; Krump *et al*, 1997). Stimulation of JNK activity in a number of cell types by ligands, such as TNF, sorbitol and anisomycin has also been demonstrated. Activation of JNK in neutrophils has not been reported. p38 and ERK may play a role in the production of superoxide in neutrophils. Treating neutrophils with fMLP and PMA leads to phosphorylation and translocation of $p47^{phox}$. It has been shown that ERK and p38 phosphorylate $p47^{phox}$ at the same site and at the same rate,

while JNK fails to phosphorylate this NADPH component (El Benna *et al*, 1996b). Inhibiting the activation of ERK kinase, MEK1 with a highly selective inhibitor, PD 098059, or blocking the p38-activated MAPKAP kinase 2 activity with a competitive inhibitory peptide, all prevent the fMLP- or PMA-induced neutrophil superoxide production (Zu *et al*, 1996; Hii *et al*, submitted; Mayer *et al*, 1996). fMLP and PMA are known to stimulate the activity of PKC which can also phosphorylate $p47^{phox}$. However, *in vitro* investigations show that the phosphorylation sites in $p47^{phox}$ by ERK/p38 are distinct from those phosphorylated by PKC. ERK/p38 phosphorylates serine 345 and serine 348 while PKC phosphorylates serine 303, serine 304, serine 320 and serine 348. It indicates that PKC and ERK may act independently to modulate the respiratory burst of neutrophils (Van-Lint *et al*, 1993; El Benna *et al,* 1994, 1996a and 1996b).

1.3.3 Protein tyrosine kinases

Protein tyrosine kinases (PTK) can be divided into two groups: receptor protein tyrosine kinases (RPTK) and non-receptor protein tyrosine kinases (NRPTK). Receptor protein kinases have an extracellular ligand-binding domain, transmembrane sequence and a cytoplasmic kinase domain. NRPTK have diverse structural feature and are located in the cytoplasm, nucleus or associated with the membrane. This includes Src, Syk, Jak, Fps and Abl families. Phosphorylated protein tyrosine kinases are deactivated by phosphotyrosine phosphatases (Hunter *et al*, 1992; Van der Geer, 1994).

Recent studies show that phosphorylation of NRPTK regulates the activity of the NADPH oxidase. In neutrophils, fMLP is known to trigger superoxide production by binding to its G-protein-coupled heptahelical receptors. However, treating the neutrophils with a specific protein tyrosine kinase inhibitor (ST 638, erbstatin or herbimycin A) prevents the fMLP-induced superoxide production while the PMA- and A23187-induced superoxide production are not affected (Berkow *et al*, 1989; Yasui *et al*, 1994; Kusonoki *et al*, 1992). In neutrophils, non-receptor tyrosine kinases, Lyn ($p53/56^{lyn}$), Fgr ($p58^{fgr}$), Fyn ($p59^{fyn}$), Hck ($p59^{hck}$) and Btk ($p77^{btk}$) of the Src family, and Syk ($p72^{syk}$) of the Syk family are found to be phosphorylated/activated by treating the cells with fMLP (Brumell *et al*, 1996). PTK has also been shown to play an important role with other agonists, such as PAF, opsonized zymosan, sodium fluoride, surface bound and soluble aggregated IgG, involved in activation of the NADPH oxidase (Naccache *et al*, 1990; Berkow *et al*, 1989; Kusonoki *et al*, 1992). Another study shows that fMLP-induced translocation of NADPH oxidase component, rac 2, but not $p47^{phox}$ and $p67^{phox}$, can be blocked by PTK inhibitors, herbimycin and genistein in a cell-free system (Dorseuil *et al*, 1995). In addition, inhibition of phosphotyrosine phosphatase activity by vanadate or vanadyl hydroperoxide causes the accumulation of tyrosine phosphorylated proteins and increased activity of the NADPH oxidase (Yamaguchi *et al*, 1995; Bennett *et al*, 1995). In mouse peritoneal macrophages, vanadate increase the extent of tyrosine phosphorylation and potentiates the PMA-induced superoxide production (Goldman and Zor, 1994).

It is also evident that the priming of agonist-induced neutrophil superoxide production by cytokines is mediated by tyrosine phosphorylation. Blocking the activity of PTK by the specific PTK inhibitors (herbimycin and genistein) attenuates the GM-CSF- and TNF-primed superoxide production elicited by fMLP (Utsumi *et al*, 1992; Johnson and Gomez-Cambronero, 1995). In macrophages, LPS-primed, PMA-induced NADPH oxidase activation is also dependent on the activity of protein tyrosine kinases (Jian *et al*, 1995; Mayer and Spitzer, 1994).

1.3.4 Phosphatidylinositol 3-kinase (PI 3-kinase)

PI 3-kinase has recently been reported to play a pivotal role in the regulation of the NADPH oxidase. PI 3-kinase phosphorylates the inositol ring of the inositol phospholipids in the 3 position. PI 3-kinase consists of a 110 kDa catalytic subunit (p110) and a 85 kDa (p85) regulatory subunit. The regulatory subunit contains one SH3, two SH2 and one Rho-GAP (BCR) domains. The kinase is expressed in all tissues. In resting cells, PI 3-kinase is located in the cytoplasm. Upon stimulation, activated receptor tyrosine kinases and receptor-independent tyrosine kinases bind to PI 3-kinase via SH2 and SH3 domains in the regulatory subunit. PI 3-kinase is then translocated to the plasma membrane and phosphorylates its substrates giving rise to phosphatidylinositol-3-phosphate ($PtdIns3P_1$), phosphatidylinositol-3,4-phosphate

[PtdIns(3,4)P_2] and phosphatidylinositol-3,4,5-phosphate [PtdIns(3,4,5)P_3]. These phosphoinositides may have distinct roles as second messengers. For example, PtdIns(3,4,5)P_3 has been shown to activate the PKC ζ isozyme which plays a role in mitogenic signalling.

With the discovery of a potent and selective PI 3-kinase inhibitor, wortmannin, PI 3-kinase has been implicated in diverse cell responses, such as degranulation in platelets, histamine release from the stimulated mast cells/basophils, glucose uptake in insulin-stimulated adipocytes and endotoxin tolerance in macrophages (Nakanishi *et al*, 1995; Bowling *et al*, 1996). Activation of PI 3-kinase also occurs following cross-linking of Fc gamma RI and RII receptors or stimulation with LPS, CSF-1 or GM-CSF in U937 or THP-1 monocytic cells, peripheral monocytes and murine bone marrow-derived macrophages (Ninomiya *et al* 1994; Yusoff *et al*, 1994; Herrera and Reiner, 1996).

Previous studies also suggest that PI 3-kinase regulates the neutrophil responses induced by various inflammatory mediators. Inhibition of neutrophil PI 3-kinase activity by wortmannin has been shown to abolish the fMLP-induced chemiluminescence response, suggesting that PI 3-kinase plays an important role in the neutrophil respiratory burst (Ahmed *et al*, 1995). It is also evident that phosphorylation of the NADPH oxidase component $p47^{phox}$ by fMLP is attributed, in part, to the activation of PI 3-kinase (Didichenko *et al*, 1996). Moreover, the PDGF- and TGFβ-induced, but not the classical

chemoattractant fMLP-induced, neutrophil chemotaxis is dependent on the activity of PI 3-kinase (Thelen *et al*, 1995).

1.3.5 Intracellular calcium

Receptor-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C (PLC) results in formation of inositol 1,4,5-trisphosphate (IP_3) which mobilizes calcium from intracellular stores and DAG, which stimulates the activity of PKC (see section 1.3.1). IP_3-mediated release of calcium leads to a transient elevation in intracellular calcium level. Intracellular calcium concentration can also be increased resulting from calcium influx from the external medium. This is achieved by store depletion-mediated influx and/or through voltage-dependent calcium channels (Clapham, 1995).

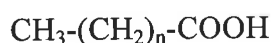
It has been shown that numerous stimulators which activate the NADPH oxidase cause a transient increase of intracellular calcium. Depletion of cellular calcium by calcium chelators and depletion of calcium from intracellular pools lead to inhibition of the response to fMLP and AA (Cross, 1990; Hardy *et al*, 1995). However, it is also evident that a rise of calcium is not an obligatory requirement for the stimulation of superoxide production. For example, PMA stimulates the respiratory burst in calcium-depleted neutrophils. Increasing the intracellular calcium concentration by calcium ionophore,

ionmycin, does not elicit superoxide production. It has been suggested that transient mobilization of calcium alone is not sufficient for triggering the respiratory burst (Cross, 1990). In addition, there is evidence which shows that priming for superoxide production in neutrophils by PAF, fMLP, IL-8 and LTB_4, parallels an increase in intracellular calcium levels, although a rise in calcium is not responsible for the priming effects. Cytokines, such as TNF, IL-1 and GM-CSF prime neutrophils without altering calcium levels. However, optimal priming occurs in buffer containing calcium (Walker and Ward, 1992). Nevertheless, calcium serves as a second messenger in the activation of other cellular responses, eg. secretion. Calcium mobilization is also important for chemotaxis. Activation of classical PKC isozymes also requires the presence of calcium (section 1.4.1) (Cross, 1990).

1.4 Polyunsaturated fatty acids and leukocyte functions

1.4.1 Fatty acid structure and nomenclature

Fatty acids are characterised by an alkyl chain and carboxyl group with the basic formula shown below:



$$CH_3\text{-}(CH_2)_n\text{-}COOH$$

The degree of unsaturation in the molecule is determined by the number of double bonds in the fatty acid backbone. Normally the double bounds are in a *cis* configuration and separated by a methylene group ($-CH_2-$). The positions of the double bounds are numbered from the carboxyl group, with the carboxyl carbon atom as carbon 1. The term, PUFA, refers to fatty acids which have more than one double bound in the carbon chain. According to the number of carbon atoms in the fatty acid backbone, the fatty acids are divided into short chain (<6 carbon atoms), medium chain (6-12 carbon atoms), long chain (14-22 carbon atoms) and very long chain (>22 carbon atoms) fatty acids. The *n-3* PUFAs have their first double bond between the 3rd and 4th carbon atom counting from the ω or methyl end of the chain, while the *n-6* PUFAs have their first double bond between the 6th and 7th carbon atom respectively.

The approved abbreviation of fatty acids involves firstly "the number of carbon atoms" followed by "the number of double bonds", eg. 20:4 refers to a 20 carbon fatty acyl with 4 double bonds. The fatty acids relevant to this thesis are summarised in Table 1.4

1.4.2 Sources of fatty acids and their uptake by tissues

The source of fatty acids in the body can be obtained either through diet or *de novo* synthesis in tissues. Dietary fatty acids can be obtained from animal meats, fish, green

Table 1.4 The structure and nomenclature of fatty acids

Numerical symbol	Structure	Trivial name	Systematic name
14:0	$CH_3-(CH_2)_{12}-COOH$	Myristic Acid	Tetradecanoic acid
16:0	$CH_3-(CH_2)_{14}-COOH$	Palmitic Acid	Hexadecanoic acid
18:2(n-6)	$CH_3-(CH_2)_3-(CH_2-CH{=}CH)_2-(CH_2)_7-COOH$	Linoleic Acid	*cis*-9,12,-octadecadienoic acid
18:3(n-3)	$CH_3-(CH_2-CH{=}CH)_3-(CH_2)_7-COOH$	α-Linolenic Acid	*cis*-9,12,15-octadecatrienoic
20:0	$CH_3-(CH_2)_{18}-COOH$	Arachidic Acid	Eicosanoic acid
20:4(n-6)	$CH_3-(CH_2)_3-(CH_2-CH{=}CH)_4-(CH_2)_3-COOH$	Arachidonic Acid	*cis*-5,8,11,14-eicosatetraenoic acid
15-HETE	$CH_3-(CH_2)_4-CH(OH)-(CH{=}CH)_2-(CH_2-CH{=}CH)_2-(CH_2)_3-COOH$		15-hydroxyeicosatetraenoic acid
15-HPETE	$CH_3-(CH_2)_4-CH(OOH)-(CH{=}CH)_2-(CH_2-CH{=}CH)_2-(CH_2)_3-COOH$		15-hydroperoxyeicosapentaenoic acid
20:4ME	$CH_3-(CH_2)_3-(CH_2-CH{=}CH)_4-(CH_2)_3-COOCH_3$		*cis*-8,11,14-eicosatetraenoic acid methyl ester
20:5(n-3)	$CH_3-(CH_2-CH{=}CH)_5-(CH_2)_3-COOH$		*cis*-5,8,11,14,17-eicosapentaenoic acid
22:6(n-3)	$CH_3-(CH_2-CH{=}CH)_6-(CH_2)_2-COOH$		*cis*-4,7,10,13,16,19-docosahexaenoic acid
22:6ME	$CH_3-(CH_2-CH{=}CH)_6-(CH_2)_2-COOCH_3$		*cis*-4,7,10,13,16,19-docosahexaenoic acid methyl ester

Compiled from "Biochemistry with clinical correlations" by T. M. Devlin. 1993

vegetables, and from oils derived from the above. They mainly occur as triacylglycerols. After absorption by the intestine, the fatty acids are transported to tissues where they may be utilized immediately or stored. Four types of vehicles have been shown to be involved in the transportation of fatty acids: (i) chylomicrons, where dietary triacylglycerols are carried in protein-coated lipid droplets and transported to the whole body from the intestine; (ii) ketone bodies (acetoacetate and β-hydroxybutyrate) and (iii) very low density lipoprotein (VLDL), which are responsible for transporting fatty acids, processed by or synthesised in the liver, to either adipose tissue for storage, or to various tissues to be used for cell structure and metabolism; triacylglycerols in the blood are enzymatically hydrolysed by lipases, such as lipoprotein lipases, on the surface of endothelial cells. The released free fatty acids become bound to serum faty acid binding protein eg. albumin, type IV fatty acid transporter, which carries the released fatty acids in the blood stream to appropriate tissue sites. The free fatty acids in the extracellular fluid continuously exchange with the intracellular fatty acids which are released by the action of phospholipase A_1 and A_2. This process is called intracellular fatty acid turnover (McGarry, 1993) (Fig 1.8).

How fatty acids are taken up by cells remains unclear. It has been proposed that fatty acids firstly become dissociated from albumin and then bind to a fatty acid transporter protein (FAT, 80 kDa) in the plasma membrane or a membrane fatty acid binding protein (FABP, 40 kDa) to cross the membranes. Fatty acids are then transported to various

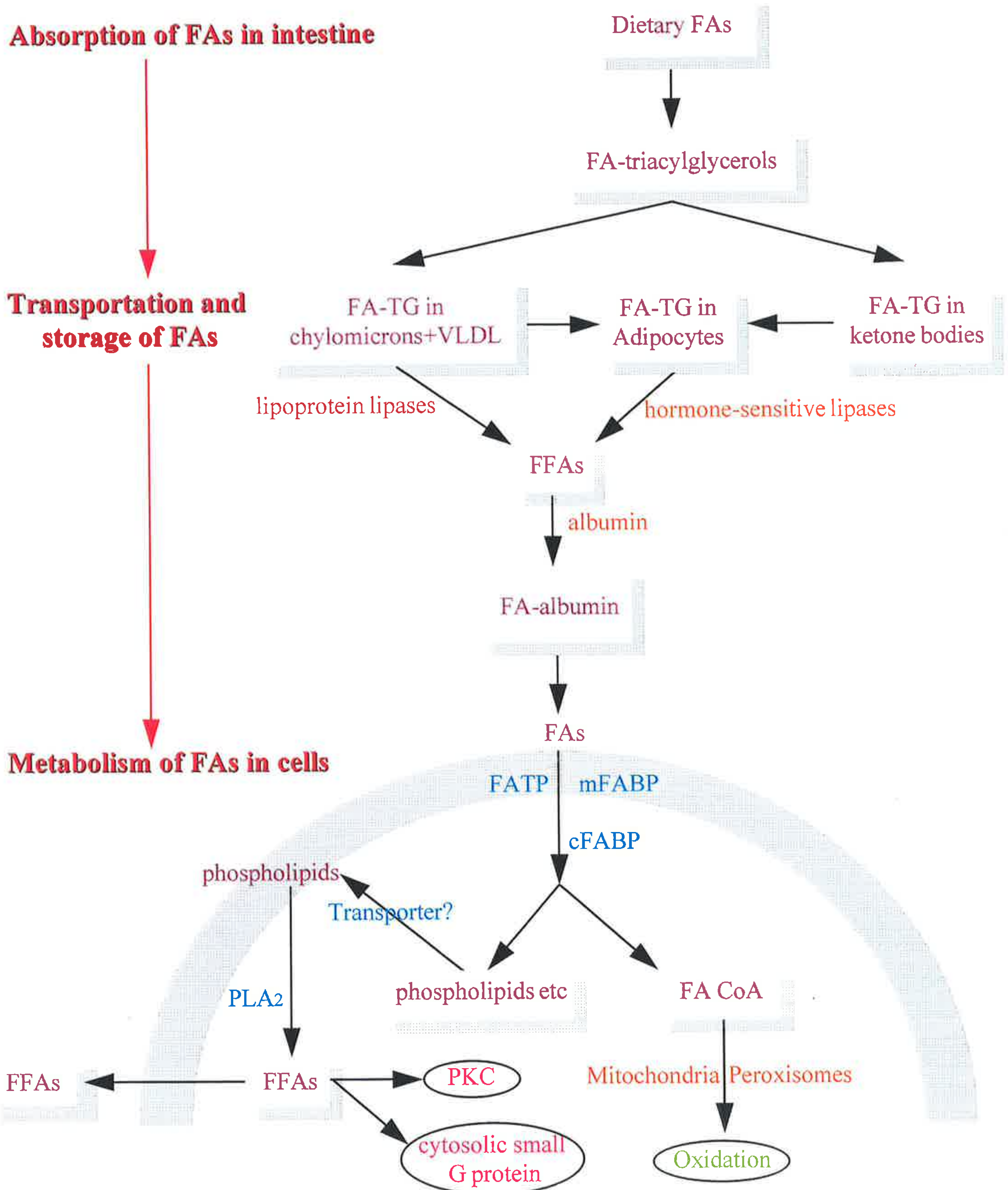


Fig 1.8 Mechanisms of transportation and utilisation of fatty acids in human body. FAs: fatty acids. FFAs: free fatty acids. VLDL: very low density lipoprotein; TG: triacylglycerols; FATP: fatty acid transporting protein. mFABP or cFABP: membrane or cytosolic fatty acid binding protein; FA CoA: fatty acyl coenzyme A. (Compiled from McGarry, 1993)

intracellular sites by the cytosolic fatty acid binding protein (FABP, 14-15 kDa) where they are involved in cellular responses (Fig 1.8) (Spector, 1992; Poirier *et al*, 1996).

Fatty acids taken up by cells are converted to fatty acyl coenzymeA (FACoA) by CoA synthetase. FACoA can be then transported into the inner membrane of mitochondria to undergo β-oxidation. Alternatively, they are incorporated into phospholipids, glycosphingolipids, triglycerides, and cholesteryl esters which are involved in membrane biosynthesis, membrane replacement or energy storage. The exchange of intracellular and extracellular fatty acids is a continuous process essential for normal tissue function. This on-going turnover of cell lipids is regulated by the intracellular and extracellular environments resulting in changing tissue functions through diet manipulation (Fig 1.8) (Spector, 1992).

Stimulation of cells by agonists interacting with specific receptors, such as growth factors, thrombin, bradykinin or f-met-leu-phe (fMLP), leads to activation of phospholipases, eg. PLA_2, and the liberation of fatty acids from the membrane phospholipids into the cytosol and plasma. These free fatty acids (unesterified fatty acids) act as regulators of cellular responses and functions (Fig 1.8).

1.4.3 Fatty acid synthesis and metabolism

Human beings can synthesize most fatty acids except for essential fatty acids. Synthesis of essential fatty acids requires the appropriate precursors supplied in the diet. These are LNA (18:3, *n-3*) and LA (18:2, *n-6*), rich in green leafy vegetables and the seeds of most plants respectively.

From these precursors, the *n-3* and *n-6* fatty acids are synthesized by increasing the carbon chain length (elongation) and the number of double bonds (unsaturation) through the action of desaturases ($\Delta6$, $\Delta5$ and $\Delta4$) and elongases. AA is derived from LA while EPA and DHA are derived from LNA. The *n-3* fatty acids EPA and DHA are abundant in marine oils and fish rich diets are another source of these fatty acids. Grain-fed animals are rich in AA (Fig 1.9) (Simopoulos, 1991).

Unesterified AA (*n-6*) can be metabolized via two major pathways, the lipoxygenase and cyclooxygenase pathways, producing a number of biologically active eicosanoids. The products of AA metabolism via the cyclooxygenase pathway are 2-series prostaglandins and thromboxanes. AA is first converted to PGH_2 which is followed by the formation of PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2 (Fig 1.10). The types of prostaglandins formed in different tissues varies depending on the type of prostaglandin synthases being expressed in tissues. For example PGI_2 is mainly found in the blood, PGE_2 and $PGF_{2\alpha}$ are generated in

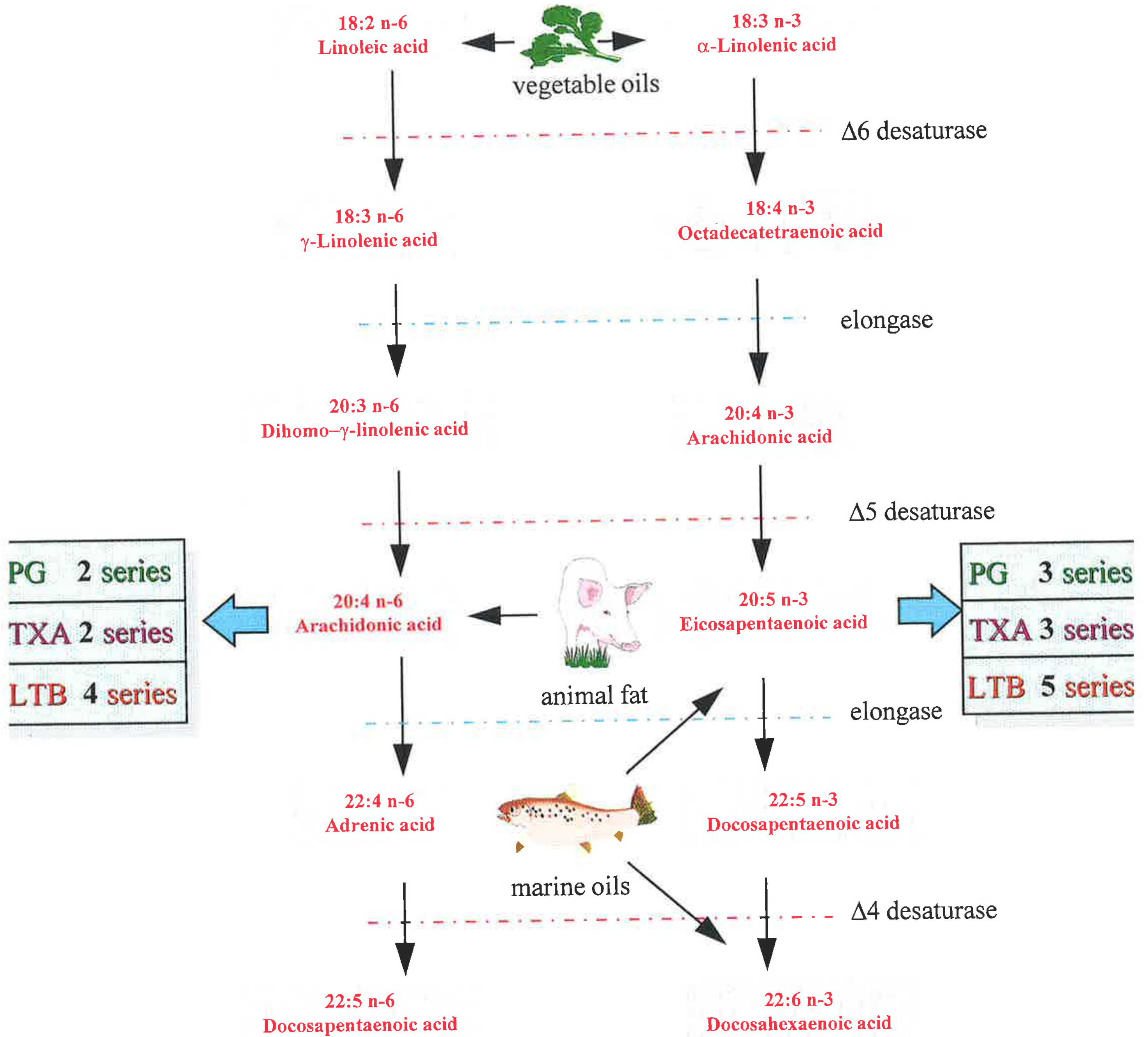


Fig 1.9 Synthesis of *n-3* and *n-6* PUFA and their metabolism. PG: prostaglandin; TXA: thromboxane; LTB: leukotriene. (Adapted from Simopoulos, 1991)

the kidney and spleen, while PGE_2 $PGF_{2\alpha}$ and PGI_2 are synthesised in the heart. The other product of the cyclooxygenase pathway is TXA_2, which is produced from PGH_2 by thromboxane A synthetase. TXA_2 is mainly synthesized in the lung and platelets. The generation of these eicosanoids is believed to play a major role in the inflammatory reaction in rheumatoid arthritis and psoriasis. The 2-series eicosanoids also increase sensitivity to pain, induce fever, platelet aggregation and thrombosis, and act as vasodilators to lower the systemic arterial blood pressure (Simopoulos, 1991).

The metabolism of AA via the lipoxygenase pathway is catalysed by three dioxygenases, 5-, 12- and 15-lipoxygenases which convert AA to either 5-, 12- or 15-monohydroperoxy-eicosatetraenoic acids (HPETES). These HPETEs are the precursors of 5-, 12- or 15-hydroxyeicosatetraenoic acids (HETEs). Leukotrienes are another important group of eicosanoids generated by this pathway. 5-HPETE is converted to an epoxide LTA_4 then to LTB_4 and LTC_4. LTC_4 is then converted to LTD_4 and LTE_4 (Glew, 1992) (Fig 1.10).

Among the metabolites of AA, LTB_4 and 5-HETE stimulate neutrophil chemotaxis, degranulation, respiratory burst, adherence to endothelial cells and the transmigration of neutrophils across vascular barriers (Glew, 1992; Bates, 1995). The products also cause the contraction of smooth muscles in pulmonary airways and the gastrointestinal tract and in this manner promote inflammation and allergic reactions.

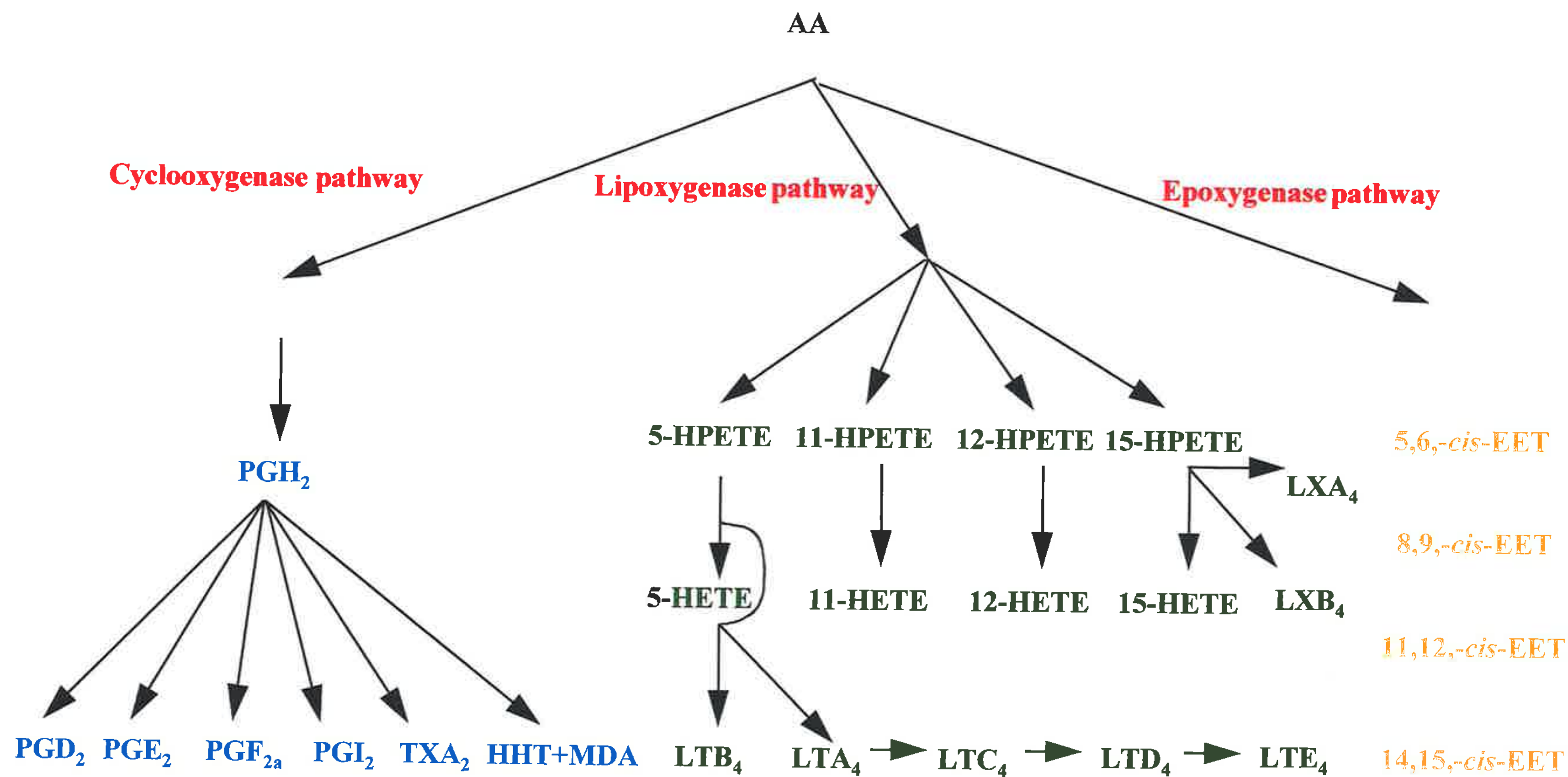


Fig 1.10 Simplified representation of the metabolism of arachidonic acid (AA) via the lipoxygenase and cyclooxygenase pathways. PG: prostaglandin; TXA: thromboxane; LT: leukotriene; HETE: hydroxyeicosatetraenoic acid; HPETE: hydroperoxyeicosatetraenoic acid; EET:epoxyeicosatrienoic acid: MDA: malondialhehyde; HHT: 12-hydroxy 5,8,10-heptadecatrenoic acid; LX: lipoxin. (Adapted from Glew, 1993).

These proinflammatory products of AA metabolism, along with other peptide inflammatory mediators, therefore form a network which modulates cell responses involved in various physiological responses (Lewis and Austen, 1988; Marcus, 1988).

The *n-3* fatty acids, EPA and DHA, can also be metabolised by the lipoxygenase pathway. However, the cyclooxygenase pathway perferentially metabolizes EPA (Fig 1.11). In the presence of *n-3* fatty acids, less metabolites of AA are formed. In contrast to AA, the metabolism of *n-3* fatty acids, such as EPA, by the lipoxygenase and cyclooxygenase pathways give rise to products with different properties from those of AA metabolites. Metabolism of EPA and DHA by the lipoxygenase and/or cyclooxygenase pathways results in the generation of less active metabolites, such as LTB_5, TXB_3, and 5-hydroxyeicosapentaenoic acid (5-HEPE) in the case of EPA and small amount of anti-inflammatory 7-hydroxydocosahexaenoic acid (7-HDHE) in the case of DHA in neutrophils and macrophages (Simopoulos, 1991; Lokesh *et al*, 1988; Ziboh, 1996; Fischer *et al*, 1984). The alteration of metabolic product profiles from proinflammatory to less-inflammatory activity by EPA and DHA are shown to be beneficial in the prevention of disease progress and relieving the symptoms. This switch from the proinflammatory AA-derived metabolites to the less inflammatory DHA or EPA-derived metabolites has been proposed as a mechanism by which *n-3* fatty acids exert their anti-inflammatory actions (Simopoulos, 1991).

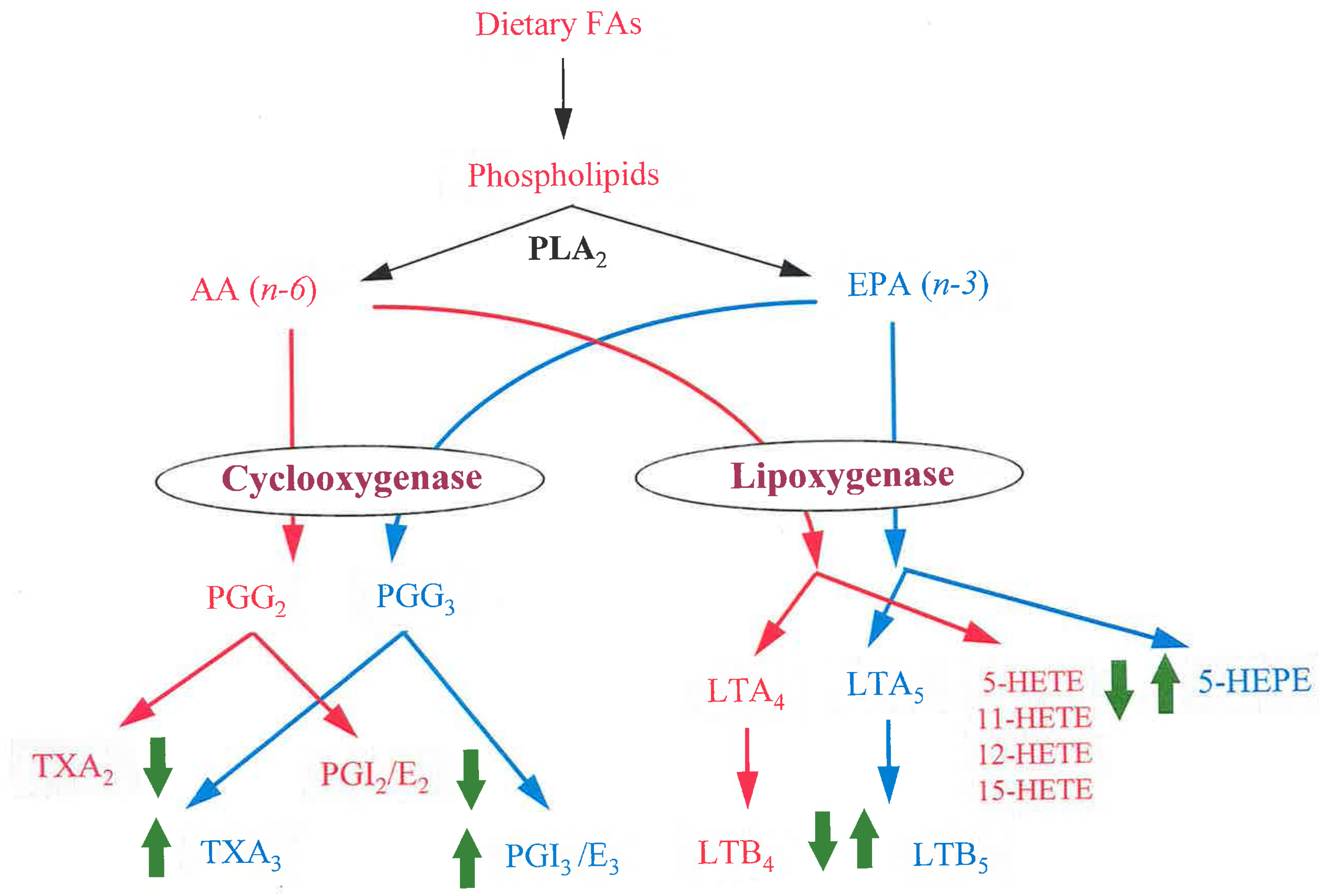


Fig 1.11 Metabolism of *n-3* and *n-6* fatty acids via the lipoxygenase and cyclooxygenase pathways in neutrophils and macrophages. FAs: fatty acids; PLA$_2$: phospholipase A$_2$; LTB: leukotrienes ; TXA: thromboxane: PG: prostaglandin; HETE: hydroxyeicosapentaenoic acid; HEPE: hydroxyeicosatetraenoic acid; green arrows indicate the results of metabolism of *n-3* fatty acids. (Adapted from Simopoulos, 1991)

Dietary supplementation with *n-3* fish oil fatty acid also alter macrophage eicosanoid profile. It has been shown that EPA and/or DHA rich diets reduce the AA metabolites, such as LTB_4, 5-HETE, 11-HETE, 12-HETE, 15-HETE , PGI_2, PGE_2 and TXA_2, and increase the amount of LTB_5, 5-HEPE, PGI_3 6-keto-PGF_{1a} and PGE_3 being produced (Careaga-Houck and Sprecher, 1990; Gadd and Hansbrough, 1990; Watanabe *et al*, 1993; Taylor *et al*, 1987; Lefkowith *et al*, 1990; Kinsella *et al*, 1990; Broughton *et al*, 1991; Lokesh *et al*, 1988; Surette *et al*, 1995; Laegreid *et al*, 1988).

1.4.4 Regulation of cell functions by PUFAs

Stimulation of leukocytes with agonists leads to the activation of phospholipase A_2. This results in the release of AA from membrane phospholipid. While AA is then available to be metabolised via the lipoxygenase and cyclooxygenase pathways to produce a number of biologically active eicosanoids as described above, the fatty acid can also directly stimulate various intracellular molecules.

Cis-unsaturated fatty acids, such as arachidonic acid, oleic acid and linoleic acid directly activate purified total PKC in the absence of calcium and PS. Unsaturated fatty acids, such as AA, DHA, oleic acid, linoleic acid and linolenic acid are active in the stimulation of most of the PKC isozymes except PKC δ and η. Saturated and *trans*-unsaturated

fatty acids are inactive in this action. Unsaturated fatty acids also interact in a synergistic fashion with DAG in causing the activation of PKC α, β, and γ isozymes in the presence of PS and basal level of calcium. The mechanism of PKC activation by fatty acids is not clearly defined. It has been shown that only the free, non-membrane-bound fatty acids are able to activate soluble PKC (Burns and Bell, 1992; Hardy *et al*, 1994b). Recent studies have demonstrated that unsaturated fatty acids can cause the translocation of PKC isozymes in a number of cell types including human neutrophils (Hii *et al*, 1995b; Hii *et al*, submitted).

1.4.4.1 Effects of PUFAs on neutrophils

1.4.4.1.1 PUFA-induced responses in neutrophils

AA has been shown to be an agonist of neutrophils, stimulating a range of functions in this leukocyte. The ability of AA to stimulate the oxygen-dependent respiratory activity has been recognized over a decade (Badwey *et al*, 1984). More recently, other neutrophil functions were shown to be stimulated by AA. Bates *et al* (1995) and Ferrante *et al* (1996) demonstrated that AA induced degranulation of neutrophil specific and azurophilic granules. Other activities of AA included an increase in neutrophil adhesion associated with enhanced expression of CR3 and CR4 but not LFA-1 (Bates *et al*, 1993) and mobilization of calcium (Hardy *et al*, 1995). These altered properties induced by AA

were reflected in an increased neutrophil-mediated damage to endothelial cells and increased killing of parasites (Bates *et al*, 1995; Kumaratilake *et al*, 1997). Ferrante *et al* (1994) demonstrated that AA inhibited neutrophil migration in a chemotactic gradient generated by fMLP.

The *n-3* fatty acids, EPA and DHA, exhibit biological actions which are similar to AA. These include stimulation of superoxide production (Poulos *et al*, 1991; Hardy *et al*, 1991; Hardy *et al*, 1994a), degranulation (Bates *et al*, 1995; Ferrante *et al*, 1996), adhesion and CR3/CR4 expression (Bates et al, 1993), calcium mobilization (Hardy *et al*, 1995) and migration inhibition (Ferrante *et al*, 1994). Neutrophils primed with EPA and DHA caused increased damage to endothelial cells (Bates *et al*, 1995) and showed increased killing of parasites (Kumaratilate *et al*, 1997).

Studies have in fact demonstrated a relationship between the ability of unsaturated fatty acids to stimulate neutrophils and their structure. Fatty acid carbon chain length, degree of saturation and the presence or absence of a free carboxyl group all influence the activity of the fatty acid. PUFAs with more than three double bonds and 20-24 carbon atoms show the greatest neutrophil stimulatory activity (Poulos *et al*, 1991; Hardy *et al*, 1994a and 1995; Bates 1995; Bates *et al*, 1995; Kumaratilake *et al*, 1997). Conversion of the PUFA to the methyl ester form resulted in loss of ability to stimulate neutrophil responses. The addition of a hydroxy or hydroperoxy group to PUFA also alter in loss

of biological activity in the above assays (Hardy *et al*, 1994a; Bates *et al*, 1995; Ferrante *et al*, 1994; Kumaratilake *et al*, 1997).

1.4.4.1.2 Synergism between PUFA with other agonists in neutrophils

Co-treatment of neutrophils with PUFA and another agonist, such as fMLP or PMA, led to a synergistic respiratory burst response suggesting that PUFA may take part in the regulatory network by synergizing with other mediators of inflammation (Poulos *et al*, 1991). This is further supported by the recent finding of Li *et al* (1996) which showed that PUFA and the cytokine TNF were synergistic for the respiratory burst response in neutrophils. Furthermore, pre-exposure of neutrophils to either fMLP or TNF promoted the cells for an increased respiratory burst activity in response to PUFAs (Poulos *et al*, 1991; Li *et al*, 1996). It was also found that pre-exposure of neutrophils to PUFAs primed them for an increased response to a second agonists, such as fMLP (Corey *et al*, 1991; Robinson *et al*, in preparation). Although work from our laboratory on neutrophils has demonstrated that the addition of hydroxy or hydroperoxy group to PUFA resulted in a loss of activity of PUFA, a previous study has shown that 5-HETE is synergistic with suboptimal concentration of PMA in stimulating a neutrophil respiratory burst (Badwey *et al*, 1991).

The ability of PUFA to act synergistically with other agonists was also shown to be highly dependent on fatty acid structure (Poulos *et al*, 1991; Li *et al*, 1996). Methylation of the carboxyl group, introduction of hydroxy or hydroperoxy groups, a decrease in unsaturation and an increase of carbon chain length to more than 22 carbon atoms resulted in a decrease and/or loss of this synergistic activity (Hardy *et al*, 1994a; Li *et al*, 1996).

Recently, 5-oxo-eicosatetraenoic acid (5-oxo-ETE), a metabolic product of AA via the 5-lipoxygenase pathway, was reported to stimulate neutrophil functions (Powell *et al*, 1992; Schwenk *et al*, 1993). 5-oxo-ETE induced calcium mobilization, lysozyme release, chemotaxis, actin polymerization and the generation of IP_3 in neutrophils. However, 5-oxo-ETE was a weak stimulator for neutrophil respiratory burst or priming for fMLP-induced superoxide production (Powell, *et al*, 1993; O'Flaherty *et al*, 1993; Schwenk and Schroder, 1995; Norgauer *et al*, 1996). Pretreating the cells with GM-CSF potentiated the 5-oxo-ETE-induced AA release, superoxide production and degranulation in these cells (O'Flaherty *et al*, 1996; Norgauer *et al*, 1996).

1.4.4.2 Effects of PUFAs on macrophages

Limited research has been conducted on the effects of PUFAs on macrophages. The unsaturated fatty acid, arachidonic, linoleic, linolenic and oleic acids were found to stimulate the NADPH-dependent superoxide production in a cell-free system of peritoneal macrophages derived from guinea pigs (Bromberg and Pick, 1984). Arachidonic

acid was the most active fatty acid (Bromberg and Pick, 1984). The effective concentration was shown to be between 25-125μM (Bromberg and Pick, 1984). In a whole-cell system, the respiratory burst in guinea pig peritoneal macrophages was also elicited by the addition of arachidonic, linoleic, linolenic and oleic acids at concentrations of 25-100μM, in a dose dependent manner (Bromberg and Pick, 1983). Treating the cells with indomethacin, a cyclooxygenase inhibitor, or 15-HETE, a metabolite of AA generated via the lipoxygenase pathway, promoted superoxide generation, suggesting that the effects of fatty acids were mediated by activation of the lipoxygenase pathway (Bromberg and Pick, 1983).

The *ex vivo* studies show that macrophages isolated from mice fed with an *n-3* fatty acid rich diet (menhaden oil) for 8 weeks showed an increased PMA-induced superoxide production (Yaqoob and Calder, 1995).

Studies on macrophage cytokine production in animals and humans which are supplemented with *n-3* fatty acids, EPA and DHA, remain controversial (Endres, 1996). In healthy humans, a diet rich in *n-3* fatty acids resulted in a decrease in the ability of mononuclear cells to produce the cytokines, TNF, IL-1β and IL-6 (Endres *et al*, 1989; Meydani *et al*, 1993). When multiple sclerosis, rheumatoid arthritis and AIDS patients were given dietary supplements of *n-3* fatty acids, it was also found that the production of TNF and IL-1β was suppressed (Endres *et al*, 1989; Watson *et al*, 1993; Gallai *et al*,

1995; Kremer, 1995; Caughey *et al*, 1996; Bell *et al*, 1996; Hellerstein *et al*, 1996). The decrease of TNF and IL-1β production in monocytes was partially regulated by inhibition of TXA_2 due to the increase in the intake of *n-3* fatty acids (Caughey *et al*, 1997). However, the studies with peritoneal macrophages from the *n-3* fatty acid fed rats or mice showed an increase in TNF and IL-1β production and inverse correlation with the presence of prostaglandin E_2 (Hardardottir and Kinsella, 1992; Lokesh *et al*, 1990; Turek *et al*, 1991). The reason for different patterns of cytokine production observed between cells from humans and cells from rodents are currently not known.

1.5 Concluding remarks

There is a considerable interest in the biological properties of PUFAs for several reasons: Firstly, AA, is a major cell product whose concentration increases dramatically during cell activation and inflammation. Secondly, *n-3* PUFAs, such as EPA and DHA, have been considered for use in the suppression of chronic inflammation. However, to date, we still understand very little of the effects of PUFAs on macrophage functions. The objective of the present study is to investigate this interaction in terms of a major macrophage response, the oxidative respiratory burst, and to dissect some of the mechanisms by which these fatty acids bring about their effects on macrophages.

1.6 Aims

(1) To examine the effects of PUFAs on macrophage oxygen radical production.

(2) To examine the fatty acid structure on their biological effects on macrophages

(3) To examine the ability of PUFAs to prime the macrophage respiratory burst in response to other agonists.

(4) To study the mechanisms by which PUFAs induce their effects on macrophages, include the role of protein kinase C and mitogen-activated protein kinases.

Chapter 2

Materials and Methods

2.1 Preparation of fatty acids

2.1.1 *Fatty acids*

The fatty acids used in this study, myristic (14:0), palmitic (16:0), linoleic (18:2, *n-6), γ-* linolenic (18:3, *n-6*), arachidic (20:0), arachidonic (20:4, *n-6*), eicosapentaenoic (20:5, *n-3),* docosahexaenoic (22:6, *n-3)* acid, 20:4 *(n-6)* methyl ester and 22:6 (*n-3*) methyl ester, were purchased from Sigma (Sigma Chemical Company, St Louis, Mo, USA).

15-HPETE [15-(s)-monohydroperoxy 20:4 *(n-6)*] and 15-HETE [15-(s)-monohydroxy 20:4(*n-6*)] were prepared by Dr. B. S. Robinson as described previously (Huang *et al,* 1997). 15-HPETE was obtained by incubating 20:4 (*n-6*) (50mg in 1ml ethanol) with soybean lipoxidase (7 mg, Type 1B, Sigma) in 100ml 0.1M sodium borate, pH 9.0 for 20 min at room temperature (Kuhn *et al,* 1990). The reaction mixture was acidified with 5ml 1M citric acid and fatty acid derivatives were extracted 3 times with 4ml diethylether. The sample was evaporated under nitrogen and taken up in 3ml hexane/diethyl ether (9:1, v/v) before application to a silicic acid column (4g) equilibrated in the same solvent. Nonoxidised 20:4 (*n-6*) was eluted with 120ml of this solvent and 15-HPETE was eluted with 120ml hexane/diethyl ether (1:1, v/v). A portion (5mg) of 15-HPETE was reduced to 15-HETE with 2mg sodium borohydride for 2h at 4'C, and recovered by the addition of 2.5ml water, acidified with formic acid and extracted 3 times with 4ml of diethylether. The purity of 15-HPETE and 15-HETE was always >95% with other isomers making up

the 5%, as determined by thin layer chromatography (TLC) and by gas-liquid chromatography. There was no evidence of 20:4 (*n-6*) in the preparations.

All fatty acids were stored in chloroform or absolute ethanol at -20°C. The purity of fatty acids was routinely checked by silica gel TLC in diethyl ether:hexane:glacial acetic acid (60:40:1 by volume). The plates were visualised with iodine. No oxidative products in commercial fatty acids, 15-HETE or 15-HPETE were found by TLC within the storage period (Fig 2.1).

2.1.2 Preparation of fatty acids

Fatty acids were presented to cells in micelle form. On the day of use, fatty acids (in chloroform) and dipalmitoyl phosphatidylcholine (DPC) (with ratio of fatty acid: DPC=1:4 by weight) were mixed and the solvent was completely evaporated under nitrogen at 30°C in baked glass tubes and sonicated in HBSS for 1min on ice (Ystrom System Ultrasonicator, Westwood, NJ, USA) (power setting, 8, tune setting, 4) giving rise to a clear preparation. Control cells received equivalent micelles of DPC alone. Fatty acids in ethanol were diluted with HBSS to 1-30μM as indicated in the results. The final concentration of ethanol was 0.01-0.1% (v/v). Control cells received an appropriate amount of vehicle (ethanol). The fatty acids were prepared immediately prior to use in the experiments.

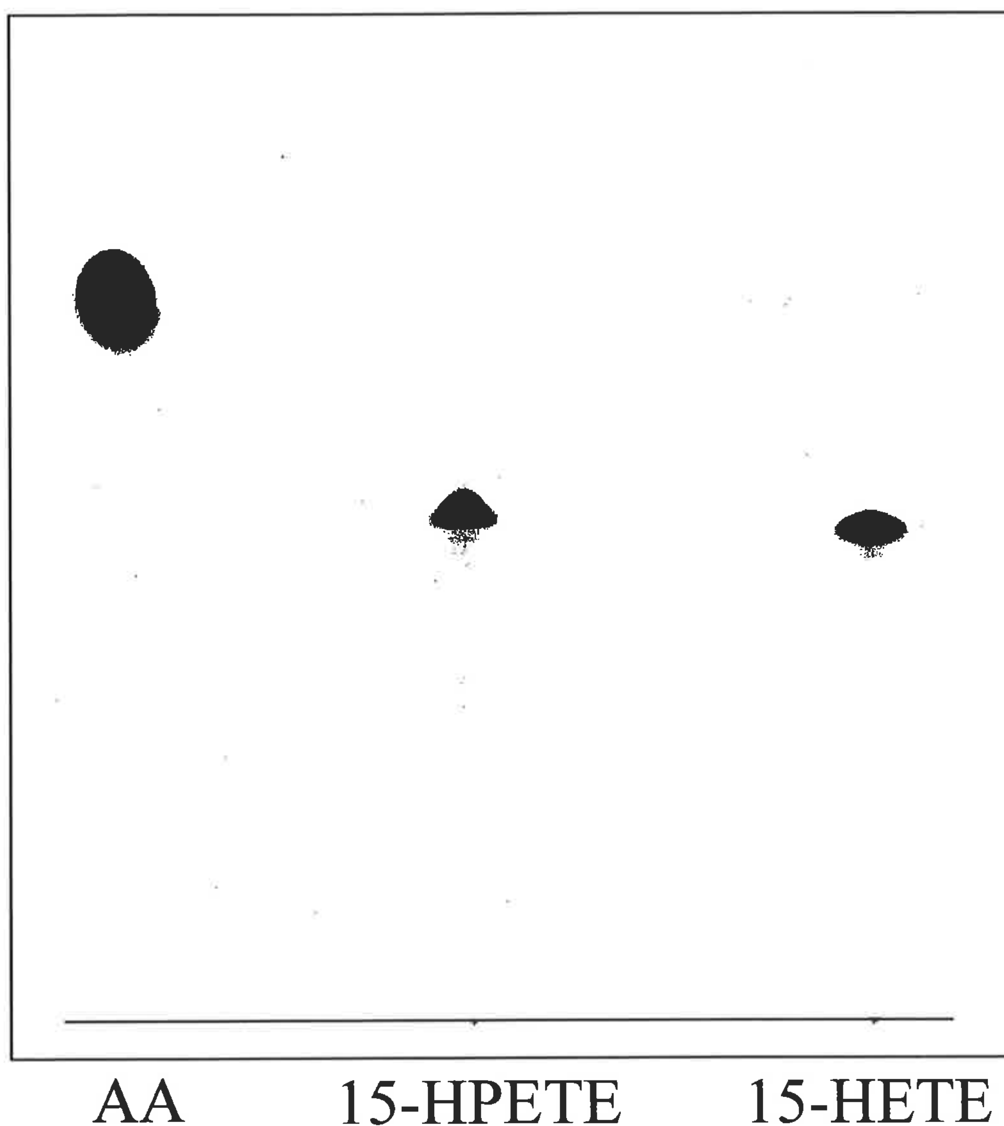


Fig 2.1 A typical TLC result of fatty acid preparations. AA, 15-HETE and 15-HPETE preparations were chromatographed on silica gel thin layer-plate using the solvent system of diethyl ether:hexane:glacial acetic acid (60:40:1, v/v) and then visualised with iodine. No oxidative products in the fatty acid preparations were found to migrate with lower R_f value.

2.2 Cell preparation

2.2.1 Preparation of peripheral blood mononuclear leukocytes and neutrophils

Leukocytes were obtained from heparinized blood (200-240ml) of healthy donors by the rapid single-step method (Ferrante and Thong, 1982). Blood was drawn into tubes containing 25 IU/ml heparin. In each 10ml sterile tube, 6ml of blood was layered onto 4ml of Hypaque-Ficoll medium containing 8% Ficoll 400 (Pharmacia Biotech, Uppsala, Sweden) and brought to a density of 1.114 by adding sodium diatrizoate (Sigma, St Louis, Mo, USA) and Angiografin (Schering, Germany). The tubes were centrifuged at 400g for 30 min at room temperature. After centrifugation the leukocytes become resolved into two distinct bands and red blood cells sedimented to the bottom (Fig 2.2). Mononuclear leukocytes (MNL) were obtained from the top band while neutrophils were collected from the second band, approximately 7mm below the MNL band. Neutrophils were washed twice in M199 (400g for 5min each wash) (Cell Image, Adelaide, Australia). Prior to use, neutrophils were resuspended in HBSS and used in assays within 2h after preparation. The purity of neutrophils by this method was 96-99% and the viability was >99%. MNL were washed with M199 by centrifugation firstly at 400g for 5min, followed by 160g for 5min to remove contaminating erythrocytes and platelets, and then used for purification of monocytes/macrophages.

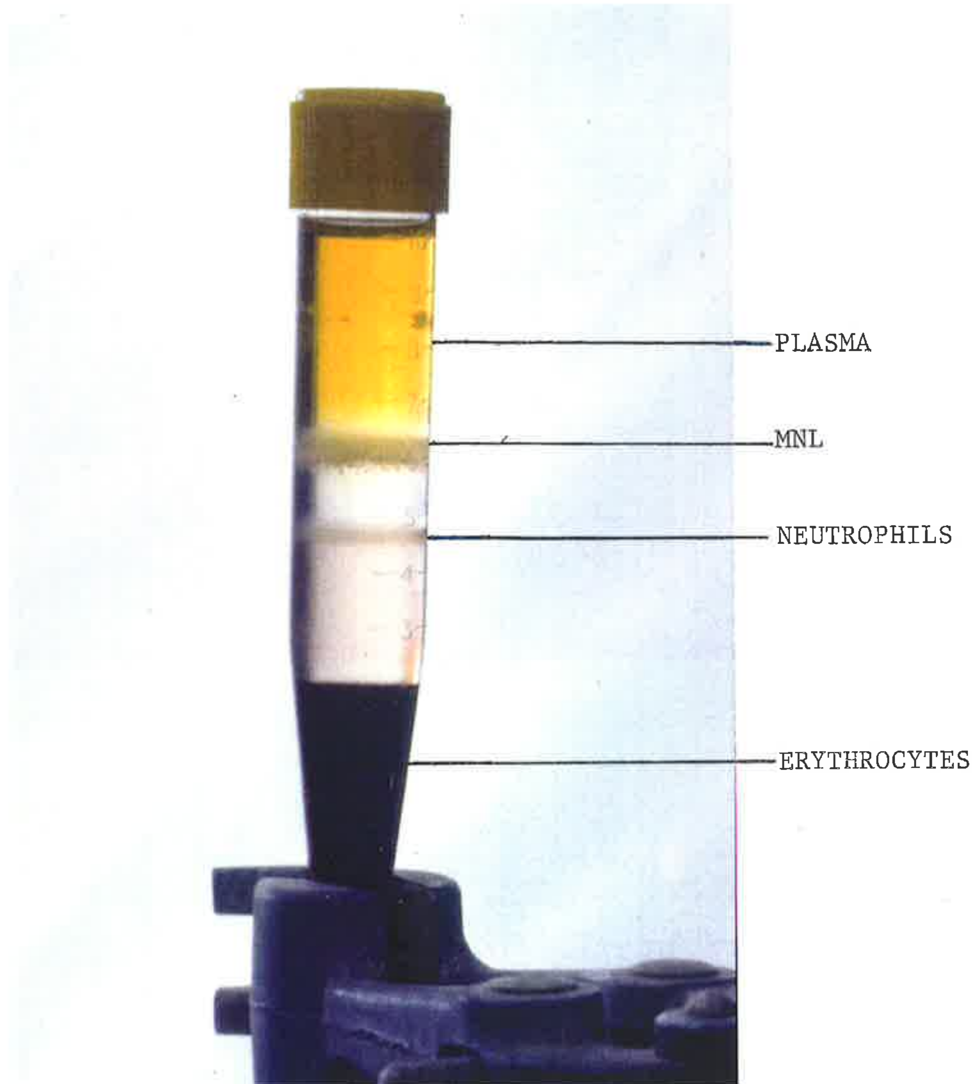


Fig 2.2 Fractionation of leukocytes by the single step method. Neutrophils were collected from the lower band of the leukocyte population while MNL were collected from the upper band.

2.2.2 *Preparation of monocytes and macrophages*

Monocytes or macrophages were prepared from MNL by adherence to cytodex microcarriers as previously described (Kumaratilake and Ferrante, 1978).

2.2.2.1 Preparation of cytodex microcarriers

Collagen-coated cytodex 3 microcarriers (Pharmacia Fine Chemicals, NSW, Australia) were prepared according to the manufacturer's instructions. Briefly, the dry microcarriers were rehydrated in PBS without Ca^{2+} and Mg^{2+} (1g cytodex beads/100ml PBS) for 6h at room temperature. The supernatant was decanted and the microcarriers were washed twice with PBS (Ca^{2+} and Mg^{2+} free). The cytodex beads were then resuspended in the same buffer at 1g cytodex beads/50ml PBS and aliquoted at 10ml per bottle for autoclaving. It has been shown that the autoclaving procedure does not affect the performance of cytodex microcarriers.

2.2.2.2 Purification and preparation of monocytes/macrophages

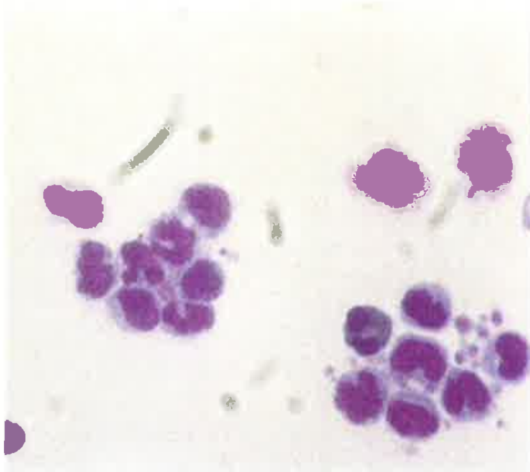
Monocytes were purified by adding the MNL preparation to pre-swollen cytodex microcarriers. Cytodex beads were washed twice with RPMI 1640 and rinsed with warm culture medium containing RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum, 2mM L-glutamine, 50IU/ml penicillin, 50mg/ml streptomycin and 20 mM HEPES. The beads were allowed to settle and the supernatant was discarded. To

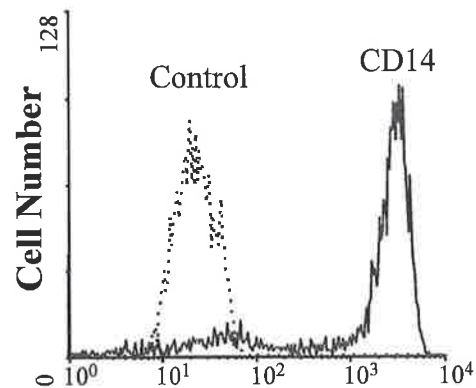
1ml of cytodex beads (packed volume) was added 8ml of culture medium and lml of MNL from 50ml blood. The tubes were incubated at 37°C for 2-4h on a rotating platform. At the end of the incubation, the cytodex microcarriers were allowed to settle at the bottom of the tubes and the supernatant containing non-adherent cells was removed. The beads were thoroughly washed with RPMI 1640. The microcarriers with adhered monocytes were either used for preparing monocytes or transferred to culture flasks for preparation of macrophages. The beads with adhered monocytes were cultured for 5-7 days at 37°C, in a 5% CO_2 humidified atmosphere to differentiate monocytes into macrophages (Kumaratilake and Ferrante, 1978).

Monocytes or macrophages were detached from the microcarriers by vortexing for 1min (Thermolyne mixer, type 37600, Sybron, Laboratory Supply Pty Ltd, NSW, Australia). The released cells were collected immediately after the beads had settled down at the bottom of the tubes and the procedure was repeated 2-3 times. By this method, the yield of monocytes or macrophages was $1.2\text{-}1.5\times10^7$ from 200ml blood. The purity of monocytes or macrophages in the preparations was ≈95% as determined by measuring the CD 14 expression on cell surface using flow cytometry (Fig 2.3). The viability of monocytes or macrophages was always greater than 95% as assayed by the trypan blue exclusion assay. More than 95% of cells in the preparation also stained positive for nonspecific esterase which is strongly expressed in monocytes and macrophages (Fig 2.4).

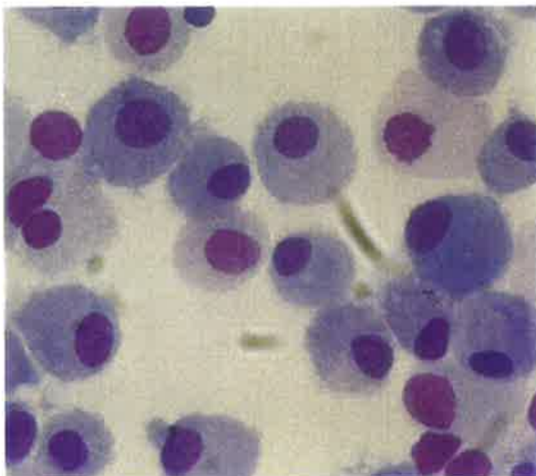
In some experiments, monocytes were purified by adhering MNL to 10cm-diameter Petri dishes (MNL from 40-50ml blood per dish) in culture medium for 2-4h at 37°C in a

A: Monocytes





B Macrophages



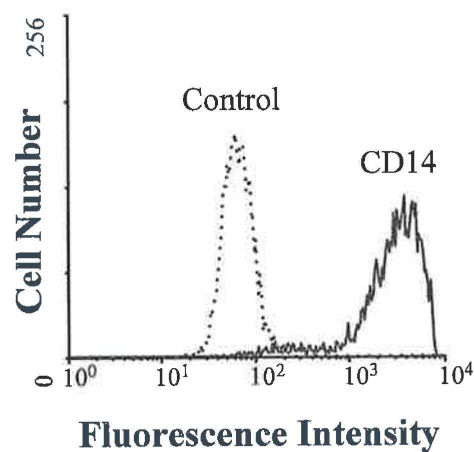
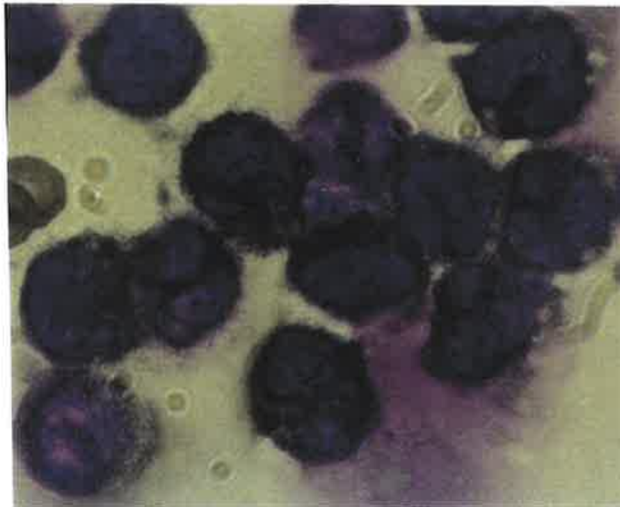


Fig 2.3 Expression of CD14 on monocytes and macrophages. Monocytes (A) and monocyte-differentiated macrophages (B) were cytocentrifuged and slides were stained with May-Grunwald Giemsa. The same preparations were also stained with a PE-labelled CD 14 antibody. Of the cells, 90% were positive for CD 14 staining.

A Monocytes



B Macrophages

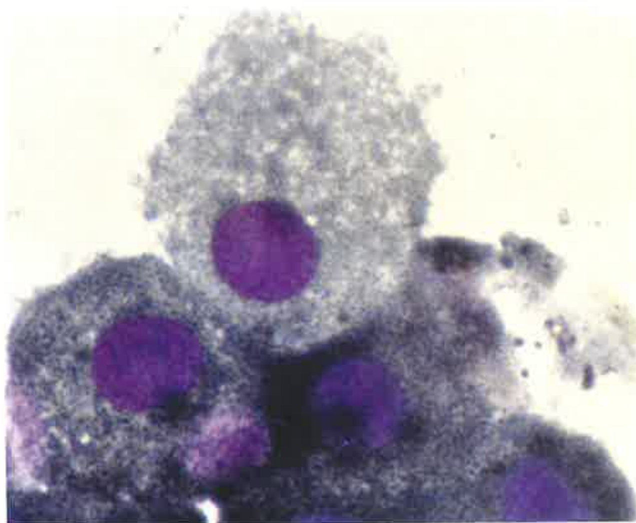


Fig 2.4 Staining for non-specific esterase in monocytes and macrophages. Monocytes (A) or monocyte-differentiated macrophages (B) were stained positive for α-naphthyl acetate esterase (Sigma).

humidified atmosphere of 5% CO_2 : 95% air. Non-adherent cells in the supernatant were removed and the dishes were thoroughly washed three times with warm RPMI 1640. To differentiate monocytes to macrophages, cells in the dishes were cultured in culture medium for 5-7 days. Monocytes or macrophages were released from the surface of the dishes with a rubber policeman. The viability and purity of this preparation were similar to those of cells prepared by the microcarrier adherence method.

2.2.3 Cell lines and their maintenance

2.2.3.1 Culture of cell lines

A human monoblastic leukemia cell line, THP-1, and a human promyelocytic leukemia cell line, HL-60, were obtained from ATCC (Rockville, MD, USA) and maintained at a density of $<1\times10^6$/ml in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum, 2mM L-glutamine, 50IU/ml penicillin, 50mg/ml streptomycin and 20mM HEPES and passaged twice a week (1/10 dilution). WB rat epithelial cells were obtained from Prof. A. W. Murray (Flinders University, Adelaide, Australia:) and were maintained as described previously (Hii *et al,* 1995b). Cells at exponential growth stage with viability of >95% were used for experiments.

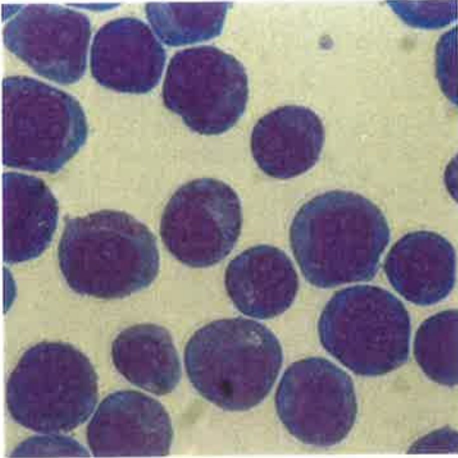
2.2.3.2 Differentiation of HL-60 along the macrophage lineage

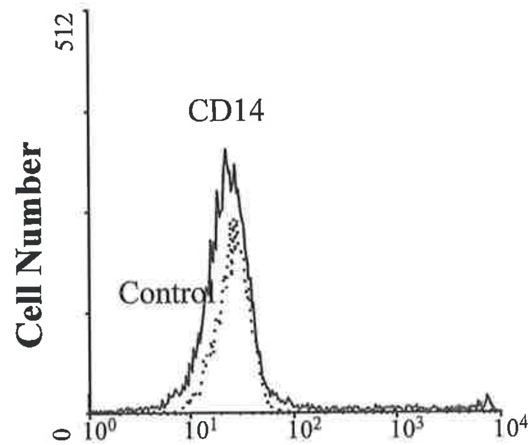
Human promyelocytic leukemia HL-60 cells were differentiated along the macrophage lineage by culturing with 1α,25-dihydroxyvitamin D3 according to the method of Levy *et al* (1990). The cells were treated with 1×10^{-8}M 1α,25-dihydroxyvitamin D3 for 6 days. The medium was replaced on day 3 of culture. Differentiated cells were confirmed by morphological examination, positive fluorescence staining for CD14 expression (Fig 2.5) and positive staining for non-specific esterase enzyme (Fig 2.6). The viability of the differentiated cells was >95% as assayed by trypan blue exclusion. On day 6 of differentiation, cells were harvested, washed and resuspended in HBSS and immediately used for the investigations.

2.3 Chemiluminescence assay

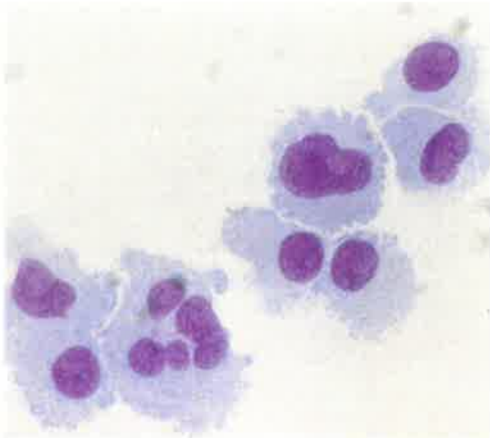
Superoxide production by monocytes, neutrophils or macrophages was measured by a chemiluminescence assay which is based on the reduction of the fluorescent probe, lucigenin, according to the method of Gyllenhammar (1987) and Hardy *et al* (1994a). One million cells in 100μl of HBSS were acclimatised to 37°C and treated with fatty acids, agonists, inhibitors or vehicle in a total volume of 400μl for the time indicated in the Results or Figure legends. Then 500μl of lucigenin (250μg/ml) was added. The test tubes were immediately transferred to chambers in a luminometer (37°C) (Model 1251, Bio-Orbit Oy, Turku, Finland) (Fig 2.7). The light output was recorded in millivolts (mV)

A HL60





B Differentiated HL-60



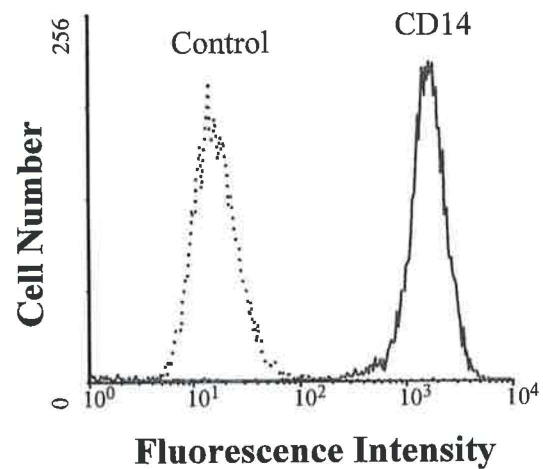
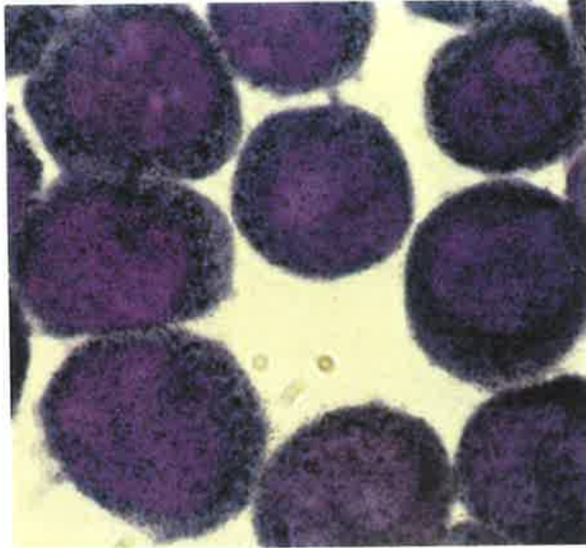


Fig 2.5 Expression of CD14 on HL-60 and 1α,25-dihydroxyvitamin D_3 differentiated HL-60. HL-60 (A) and 1α,25-dihydroxyvitamin D_3 differentiated HL-60 (B) cells were cytocentrifuged and slides were stained with May-Grunwald Giemsa. The same preparations were also stained with a PE-labelled CD 14 antibody. Of the cells, 85% were positive for CD 14 staining.

A HL-60



B Differentiated HL-60

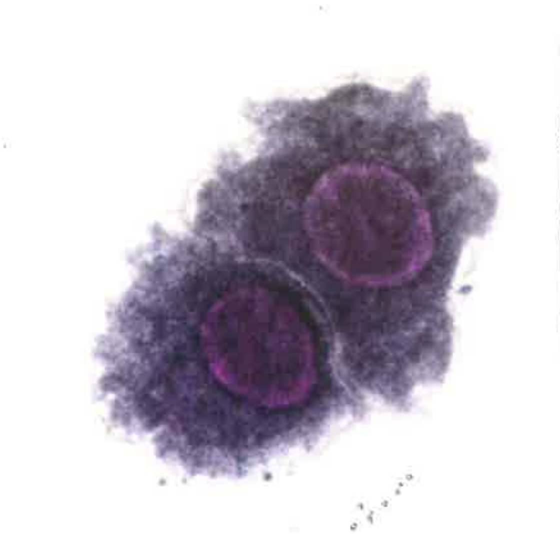


Fig 2.6 Staining for non-specific esterase in HL-60 and 1α,25-dihydroxyvitamin D_3 differentiated HL-60. HL-60 (A) and 1α,25-dihydroxyvitamin D_3 differentiated HL-60 (B) cells were stained positive for α-naphtyl acetate esterase (Sigma).

Fig 2.7 A luminometer (Bio-Orbit, Model 1251, Turku, Finland).

every 10sec by an interface computer and analysed with software Multiuser (Bio-Orbit Oy, Turku, Finland). The results are expressed as initial peak rate (mV) of each activity as well as average accumulated chemiluminescence (mV/sec).

2.4 Translocation of protein kinase C (PKC)

Translocation of PKC from the cytosol to a particulate (membrane) fraction is equated with activation of PKC. The amount of particulate-associated PKC was determined by either assaying for phosphorylation of the PKC substrate, histone type IIIS as previously described (Hardy *et al,* 1994b) or by Western blotting with anti PKC isozyme specific antibodies (Hii *et al,* 1995b).

2.4.1 PKC translocation

Cells in serum free medium were incubated with fatty acids or agonists for the times indicated in Chapter 6. After incubation, cells were washed with cold HBSS and sonicated in 1ml of buffer containing 20mM Tris/HCl pH 7.4, 5mM EGTA, 2mM EDTA, 0.25M sucrose, 10µg/ml leupeptin, 10µg/ml aprotinin, 10mM benzamidine, 10µg/ml pepstatin A, 20mM 2-mercaptoethanol and 10mM phenylmethylsulfonyl fluoride. Following centrifugation at 100,000g for 30min at 4°C, the supernatant was collected as the cytosolic fraction. The pellet was resuspended by sonication in 500µl of sonication buffer which contained 2% Triton X-100. After 30min on ice, the extract was

centrifuged at 100,000g for 30 min at 4°C. This supernatant (particulate fraction) was adsorbed onto 0.5ml DE52 (packed volume) which was pre-equilibrated with 20mM HEPES (pH 7.5) and washed twice with lml cold HEPES buffer. PKC was eluted with 0.12M NaCl. The activity of PKC as a measure of quantity of PKC was determined by assaying for the incorporation of ^{32}Pi into the PKC substrate, histone type IIIS. The reaction mixture (60μl) contained particulate/cytosolic fraction, 20mM HEPES/HCl pH 7.5, 5mM $MgCl_2$, 0.04 mg/ml histone type IIIS, [γ-^{32}P]-ATP [approximately 4.2-8.4 kBq (0.1-0.2 μCi)] and either 0.04mg/ml of L-α-phosphatidyl-L-serine and 5mM $CaCl_2$ or 5mM EGTA. After incubation at 30°C for 20 min, the reaction was terminated by spotting an aliquot of the mixture onto Whatman P81 phosphocellulose paper and this was followed by three washes with cold 75mM orthophosphoric acid (200ml for 5 min per wash). The radioactivity associated with the P81 paper was determined by liquid scintillation spectrophotometry (Beckman LS 3801, Ca, USA). The results were presented as the percentage of increase compared to the control level after subtracting the background level.

2.4.2 Western blotting for PKC isozymes

After extracting PKC with 2% Triton X-100, the supernatant, containing particulate fraction-associated PKC, was collected and mixed with Laemmli buffer (20mM Tris/HCl pH 6.8, 40% sucrose, 6% sodium dodecyl sulphate (SDS) and l0mM 2-mercaptoethanol), boiled for 5 min and stored frozen until use. Twenty μg of denatured protein was loaded onto 10% SDS polyacrylamide gels and the proteins were separated by electrophoresis

using a Bio-Rad mini gel apparatus (200V, 35min), and then eletrophoretically transferred to nitrocellulose (0.2mm thickness, Schleicher and Schuell, Keene, NH, USA) at I00V for 1.5h (Bio-Rad Mini Trans-Blot apparatus). After blocking the nonspecific binding sites on the membrane with a 5% skim milk solution for 1h at 37^{0}C, the membranes were incubated with a rabbit anti PKC isozyme specific antibody for I h at 37°C. The membranes were washed three times in blocking buffer and then incubated with horseradish peroxidase (HRP) conjugated sheep anti rabbit IgG for 1h at 37°C. The immune reactive complex was detected by enhanced chemiluminescence (ECL) according to manufacturer's instructions and quantified by laser densitometry with software Image Quant (Molecular Dynamics, Sunny Vale, CA, USA).

2.5 Assay for ERK activity

The activity of ERKs in the cytosolic fraction (100,000g) was measured by examining the ability of this fraction to phosphorylate an ERK substrate according to the method of Hii *et al* (1995b). Further evidence of ERK activation was also provided by fractionation of enzymes on Mono Q FPLC as well as examining for the presence of phosphorylated ERK isoforms by immune blotting with anti ERK antibodies.

2.5.1 ERK Assay

Three to five million serum starved macrophages or monocytic cells were treated with fatty acids or stimulators at 37°C for the times shown in Chapter 7. After incubation, cells were washed with cold HBSS and sonicated on ice for 1min in 900μl of buffer containing 25mM Tris/HCl pH 7.2, 2mM EGTA, 10μg/ml leupeptin, 10μg/ml aprotinin, 10μg/ml Sigma 104, 1mM sodium orthovanadate, 10μg/ml pepstatin A, 1mM dithiothreitol and 0.2mM phenylmethylsulfonyl fluoride. After centrifugation (100,000g for 20 min at 4°C), ERKs in the supernatant (cytoplasmic fraction) were partially purified by adhesion onto phenyl-Sepharose CL4B (250μl packed volume). The beads were washed twice with 10% ethylene glycol and twice with 35% ethylene glycol (v/v). ERKs were eluted by 60% of ethylene glycol (v/v). Previous studies have demonstrated that ERKs were eluted between 35-60% of ethylene glycol (v/v) (Anderson *et al,* 1990; Hii *et al*, 1995b). The activity of ERKs in the eluate was determined by assaying the incorporation of ^{32}Pi into myelin basic protein in a mixture (50μl) of 25mM Tris/HCl pH 7.2, 50mM β-glycerophosphate. 2mM $MgCl_2$, 1.5mM EGTA, 100μM sodium orthovanadate, 0.33 mg/ml myelin basic protein, [γ-^{32}P]-ATP [approximately 4.2-8.4 kBq (0.1-0.2 μCi)] and cytoplasmic fraction at 30°C for 10min. The reaction was stopped by spotting an aliquot of the mixture onto Whatman P81 paper which was washed three times (5min each) with cold 75mM orthophosphoric acid. The radioactivity associated with the P81 papers was determined by liquid scintillation spectrophotometry. The results are presented as the percentage increase compared to control activity after subtracting the background level.

2.5.2 Western blotting for ERK isoforms

The cytoplasmic fractions were prepared as described in section 2.5.1. Two μg of proteins were then subject to electrophoresis and transferred to nitrocellulose membranes as described in section 2.4.2. After blocking, the membranes were incubated with a rabbit anti ERK polyclonal antibody for 1h at 37°C. The membranes were washed three times in blocking buffer and this was followed by incubation with HRP conjugated sheep anti rabbit IgG for 1h at 37°C. After washing three times, the immune complexes were detected by ECL. These were analysed by laser densitometry.

2.5.3 Fast protein liquid chromatography (FPLC)

Cytosolic extracts from monocytic cells which had been treated with PUFA or vehicle were prepared as for Western blotting (see section 2.5.1). After centrifugation, the extract enriched with ERKs (1ml) was loaded onto 8cm×l.5cm Mono Q anion exchange columns (Pharmacia Biotech Inc., Sweden) pre-equilibrated in a buffer containing 20mM Tris/HCI pH 7.4, 2mM EDTA 5mM EGTA, 1.3mM Benzamidine, 0.5mM PMSF, 2.5mM Dithiothreitol and 0.8mM β-glycerol phosphate. The bound ERKs were eluted (0.5ml/min) with the same buffer using a linear 0-0.5M salt gradient. The fractions (0.5ml) were collected on ice and assayed for ERK activity by using myelin basic protein as substrate according to section 2.5.1.

2.6 Transient transfection of DNA to THP-1 cells

THP-1, a human monocytic cell line, was chosen for transient transfection by using lipofectamine according to manufacturer's instructions (Life Technologies, USA). Five million cells at exponential growth stage were washed and resuspended in 800µl serum-free medium. One µg of dominant negative $p21^{ras}$, raf-1 or control plasmid (pCMV-Green Lantern) was mixed with 5 µl of lipofectamine in 200µl of serum-free medium in 24-well culture plates to allow the formation of DNA-Liposome complexes. After 30min incubation at 37°C, the cell suspension (800µl) was added to the DNA-Liposome mixture (200µl) and incubated for 18h at 37°C 5% CO2. Cells were then washed and fresh medium supplemented with 1% FCS and lµg of the appropriate plasmid was added and the cells were cultured for 48h. ERK activation in these transfected cells which had been stimulated with PUFA was assayed as described in section 2.5.1. The transfection efficiency was examined for the expression of green fluorescent protein under the similar condition.. The results showed that 77% (77.17%±5.18, n=3) of the pCMV-Green Lantern transfected cells expressed green fluorescent protein compared with the control plasmid (pCMV-ERK) transfected cells when analysed by FACS (see Chapter 7).

2.7 Measuring cell surface molecules by flow cytometry

Monocytes (1×10^6) were treated with 30μM of PUFA or vehicle for 30min at 37°C. After incubation, cells were washed and stained with a phycoerythrin (PE)-conjugated anti human CD 11b monoclonal antibody or isotype control antibody (Becton Dickinson, CA, USA) for 30min at 4°C. For CD14 staining, monocytes, macrophages, HL-60 cells or lα,25-dihydroxyvitamin D3 differentiated HL-60 cells were incubated with a PE-conjugated anti human CD14 monoclonal antibody or isotype control antibody (Becton Dickinson, CA, USA) for 30min at 4°C. After incubation, cells were washed twice with Isoton II supplemented with 0.1% BSA and then fixed with 1% paraformaldehyde. Cells were stored cold in the dark prior to FACS analysis. The fluorescence intensity of the samples was measured by flow cytometry (Becton Dickinson, Mountain View, Ca). Ten thousand viable cells were gated for analysis. Data were processed with Lysis II software (Becton Dickinson, CA, USA).

2.8 Materials and reagents

2.8.1 Media, buffers and culture ware

Tissue culture flasks were from Corning (Coring Incorporated, New York, USA). 24- or 96-multiwell plates were from Linbro (Flow Laboratories, McLean Virginia, USA). Petri dishes were from Nunc (Nunc Inc, Naperville IL, USA).

Cell culture media RPMI 1640 and HBSS were prepared by reconstitution of the respective powders (Multicell, Cytosystems, Sydney, Australia) in sterile pyrogen-free water. All batches were quality controlled before experimental use and no endotoxin could be detected by the Limulus Ameobocyte Lysate (LAL) assay.

Monocytes/macrophages and cell lines were maintained in RPMI 1640 containing 10% heat-inactivated foetal calf serum (Multicell, Trace Bioscience Pty Ltd, Australia), 5mM L-glutamine, 50μg/ml streptomycin and 50IU/ml penicillin. All assay buffers and culture media were filtered through 0.2μm filters before use (Sartorius, Gottingen, Germany). The osmolarity and pH of media and buffers were adjusted to the physiological range.

2.8.2 Inhibitors

The PKC inhibitor, GF-109203X, was obtained from Biomol (Biomolecules for research, PA, USA). Other chemicals/inhibitors, leupeptin, aprotinin, Sigma 104, sodium orthovanadate, pepstatin A and phenylmethylsulfonyl fluoride were purchased from Sigma, St Louis, Mo, USA. Dithiothreitol was from Worthington (Worthington Biochemical Incorporation, New Jersey, USA). The MEK1 inhibitor, PD 098059, was obtained from New England Biolabs (Beverley, MA, USA).

2.8.3 Antibodies

Rabbit anti PKC isozyme specific antibodies were obtained from Santa Cruz (Ca, USA). Rabbit anti ERK polyclonal antibodies R1 and R2 were gifts from Dr. S. Pelech, University of British Colombia or were purchased from Upstate Biotechnology Incorporated (Lake Placid, NY, USA). PE-conjugated anti human CD11b and CD14 monoclonal antibodies or control antibody were from Becton Dickinson, CA, USA.

2.8.4 Plasmids

pZIP-ras(I7N)-amp'.kan', pZIP-ras(I5A)-amp'.kan', pCMV-ERK(KR)-amp' and pCGN-raf(1-130)-amp' were the gifts from Dr. C. J. Der, University of North Carolina at Chapel Hill, USA. pCMV-Green Lantern was purchased from GIBCO (Rockville, MD, USA). Lipofectamine for transfection was obtained from Life Technologies (Gaithersburg, MD, USA).

2.8.5 Other reagents

Other reagents, fMLP, TPA, A23187, lucigenin, myelin basic protein, histone type IIIS, 1α,25-dihydroxyvitamin D3 and ethylene glycol, were all purchased from Sigma Chemical Company, St Louis, Mo, USA. L-glutamine, streptomycin and penicillin were obtained from Trace Bioscience Pty Ltd, NSW, Australia. Phenyl-Sepharose CL4B was obtained from Pharmacia (Pharmacia Biotech, Sweden).

DE52 and P81 phosphocellulose papers were obtained from Whatman Ltd, Maidstone, Kent, England. Reinforced nitrocellulose (0.2mm thickness) was from Schleicher and Schuell, Keene, NH, USA. Acrylamide and other reagents for electrophoresis were from Bio-Rad, Ca, USA.

[γ-^{32}P]-ATP (4000 Ci/mmol) was from Bresatec, Adelaide, Australia. Enhanced chemiluminescence (ECL) solution was purchased from DuPont NEN Research Products, Boston, MA, USA.

2.9 Statistics and data analysis

Data are expressed in this thesis as the mean±sem of 3-10 independent experiments. The results were analysed by Student's *t* test (for paired data) using Statwork software (Cricket Software Inc, 1985, Philadephia, USA). $P<0.05$ is regarded as statistically significant; $P<0.01$, as very significant; $P<0.001$, as highly significant. For multiple comparisons, results were processed with analysis of variance (ANOVA) using Systat software (pairedwise comparison, Fisher's least-significance-test, Systat Inc., 1993, IL, USA).

Chapter 3

The Effects of Polyunsaturated Fatty Acids on the Macrophage Respiratory Burst Response Stimulated by a Receptor-Mediated Agonist

3.1 Introduction

A key macrophage response involved in the macrophage-mediated killing of microorganisms is the generation of reactive oxygen species (ROS), such as superoxide, hydroxyl radicals, hydrogen peroxide and hypocholorous acid (Rosen *et al,* 1995). Agonist-induced activation of macrophages and the release of ROS involves the sequential steps of ligand-receptor interaction, leading to the activation of intracellular signalling pathways which are responsible for promoting the assembly of components of the NADPH oxidase.

In both acute and chronic inflammation, a spectrum of lipid and protein mediators are generated which are believed to play a role in orchestrating the development and nature of the inflammatory reaction. Cytokines and other mediators induce activation of phospholipase C, D and A_2 which result in the release of several lipid mediators, such as DAG, IP_3 and lysophosphatidic acid (Nishizuka, 1995; Liscovitch and Cantley, 1994). These lipid molecules act as second messengers and modulate various cell responses (Liscovitch and Cantley, 1994). AA (20:4, *n-6*) which is released by activation of phospholipase A_2 can also be metabolised to eicosanoids such as, LTB_4, PGI_2, TXA_2, which are potent proinflammatory mediators (Simopoulos, 1991; Pruzanski and Vada 1991; Lewis and Austen, 1988; Marcus, 1988; Sumida *et al*, 1993).

While extensive investigations have been conducted on the action of AA on neutrophils, very little work has been done on the effects of this fatty acid on mononuclear

phagocytes. The interaction between AA and mononuclear phagocytes was therefore investigated. We were interested in both the ability of AA to stimulate the NADPH oxidase in macrophages as well as the ability of fatty acids to alter the macrophage response to other agonists.

The *n-3* fatty acids, EPA and DHA *per se* are also biologically active. They are interesting because they give rise to products with less inflammatory activity than those which are generated from the oxidation of AA (Simopoulos, 1991). In some studies, *n-3* fatty acids have been reported to possess immunosuppressive activity by inhibiting the production of macrophage-derived cytokines, such as TNF (Endres *et al,* 1989). Thus, there is a need to compare the biological activities of the *n-3* fatty acids with those of AA on macrophage functions.

3.2 Assaying for the oxygen-dependent respiratory burst: lucigenin dependent chemiluminescence assay

The agonist-induced respiratory burst activity, which is associated with the activation of the NADPH oxidase, was measured as superoxide production. There are two types of assays which are commonly used in the detection of superoxide production by phagocytes: the lucigenin-dependent chemiluminescence assay and the cytochrome C reduction assay. Previous studies have shown that the cytochrome C reduction assay is inappropriate to study fatty acid effects since fatty acids were found to directly bind to

cytochrome C resulting in a significant underestimation of the amount of superoxide which was being produced (Hardy *et al,* 1994a). However, no interference by fatty acid on the lucigenin-detected superoxide production was found (Hardy *et al,* 1994a). Therefore the chemiluminescence assay was chosen to study the respiratory burst of monocytes/macrophages.

3.3 The effect of AA on superoxide production by monocytes

Since AA has been reported to directly stimulate cellular responses, the ability of AA to stimulate the oxygen-dependent respiratory burst in monocytes was examined. Monocytes were prepared by a combination of density centrifugation using hypaque-ficoll to obtain a mononuclear leukocyte fraction and adherence of monocytes to cytodex microcarriers (Chapter 2, Materials and Methods). The cells were resuspended in HBSS (without phenol red). To the luminometer tubes, 100µl of monocytes (1×10^6) and 100µl of AA were added and the volume was made up to 1 ml by addition of lucigenin and HBSS. The concentrations of fatty acid were varied from 1-10µg/ml. In parallel to examining the effects of AA, tubes were set up in which monocytes were stimulated with fMLP (5µM). The reactants were mixed immediately and placed in the prewarmed (37°C) chamber of a luminometor to measure the chemiluminescence emitted. The rate of chemiluminescence detected from the cells over the incubation period was recorded by a computer which interfaced with the luminometer (Chapter 2, Materials and Methods).

The results showed that AA at a concentration of 1-10μg/ml (≈3-30μM) caused a poor and non-significant respiratory burst (chemiluminescence response) (Fig 3.1A). In contrast to monocytes, a substantial activation of the NADPH oxidase was seen in neutrophils which has been exposed to 10μg/ml of AA (Fig 3.1 insert). However, fMLP did cause a significant chemiluminescence response in monocytes (Fig 3.1B).

3.4 The synergistic chemiluminescence responses of monocytes/macrophages to AA and fMLP

3.4.1 Synergistic responses in monocytes

Monocytes were treated with 30μM of AA or vehicle [0.1% of ethanol, (v/v)] in the presence or absence of 5 μM of fMLP [0.02% DMSO, (v/v)] and then examined for the generation of chemiluminescence. These amounts of vehicle or diluent were used in all subsequent experiments. The addition of AA alone to monocytes did not cause a significant chemiluminescence response (Fig 3.2). However, the combined addition of AA and fMLP to the monocytes led to a significant enhancement of the monocyte chemiluminescence response. Thus the initial peak rate of chemiluminescence response in the presence of AA was 4.5 mV (with base level subtracted) and that in response to fMLP was 9.2 mV, giving rise to a sum of 13.7 mV. But cells co-treated with AA and fMLP gave a chemiluminescence response of 22.6 mV.

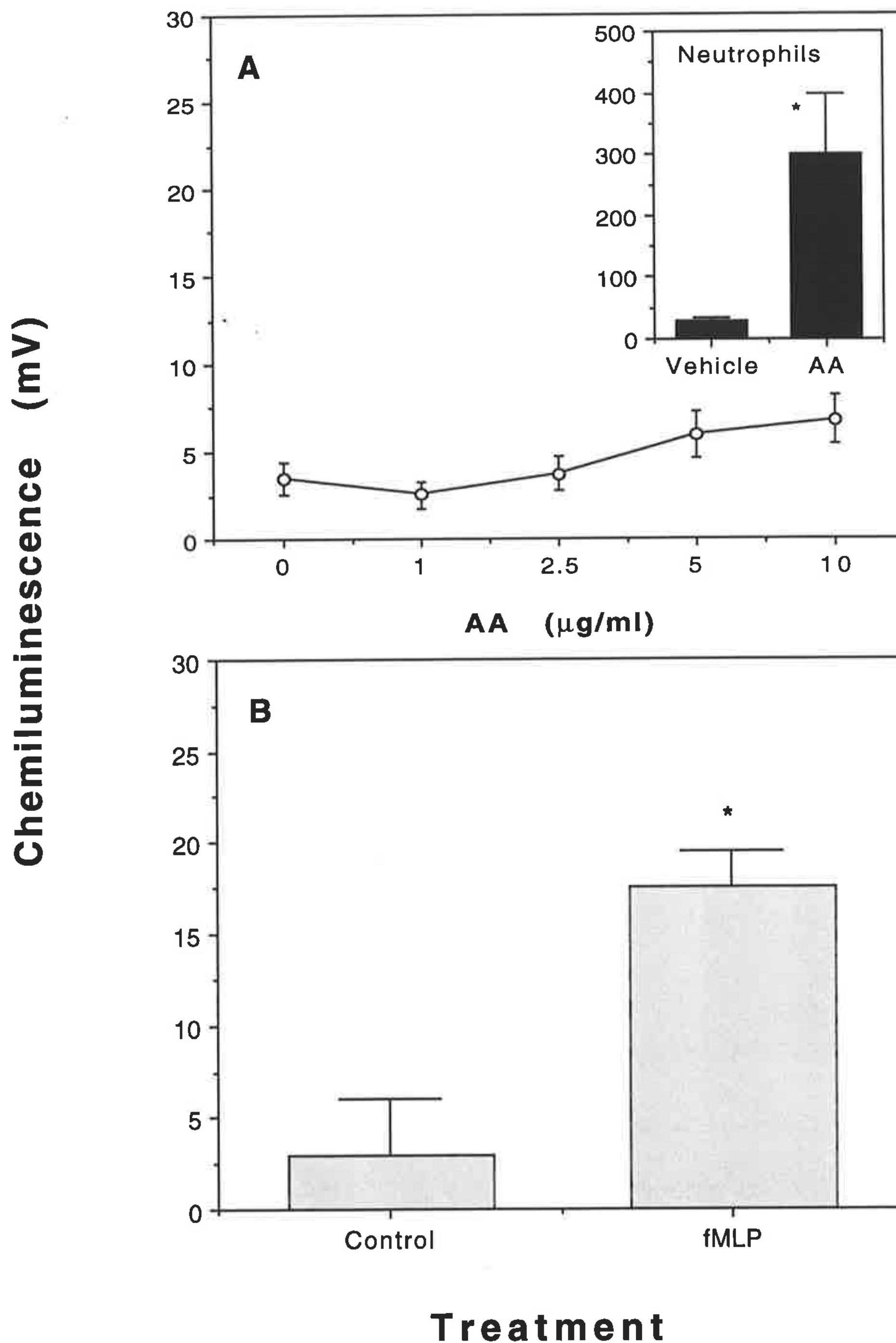


Fig 3.1 The effect of AA on the chemiluminescence response in monocytes. (A) Human monocytes were treated with varying concentrations of AA and the superoxide produced measured by the lucigenin-dependent chemiluminescence. The results are the mean±sem of 4 experiments, each carried out with cells from a different individual. Insert shows a typical chemiluminescence response of neutrophils to 10μg/ml of AA. (B) chemiluminescence response of monocytes in the presence of fMLP (5μM).

The kinetics of superoxide production over the incubation period are shown in Fig 3.2B. It is evident that AA alone caused only a slight increase above basal activity. fMLP caused a significant increase in chemiluminescence response which rapidly (42 sec) reached an initial peak rate of chemiluminescence of 17.35 mV in monocytes (Fig 3.2B). The effect of AA on 'the fMLP response was manifested by an accelerated chemiluminescence response (Fig 3.2B). The other main differences seen were on increased initial peak rate observed in the presence of AA and fMLP (41.19 mV) compared to fMLP alone (17.35 mV) and longer duration of the maximal initial peak response in the presence of AA and fMLP (Fig 3.2B). The synergistic effects seen with the combined treatment of AA and fMLP were also reflected in the total chemiluminescence generated over the entire incubation period (essentially the accumulated chemiluminescence) (Fig 3.2, Table 3.1).

3.4.2 Synergistic response in macrophages

Human macrophages were obtained by differentiation of peripheral blood monocytes on cytodex beads in RPMI 1640 medium containing 10% heat-inactivated FCS at 37°C, in 5% CO_2 : 95% air for 5 days and then used in the following investigations (details are given in Chapter 2).

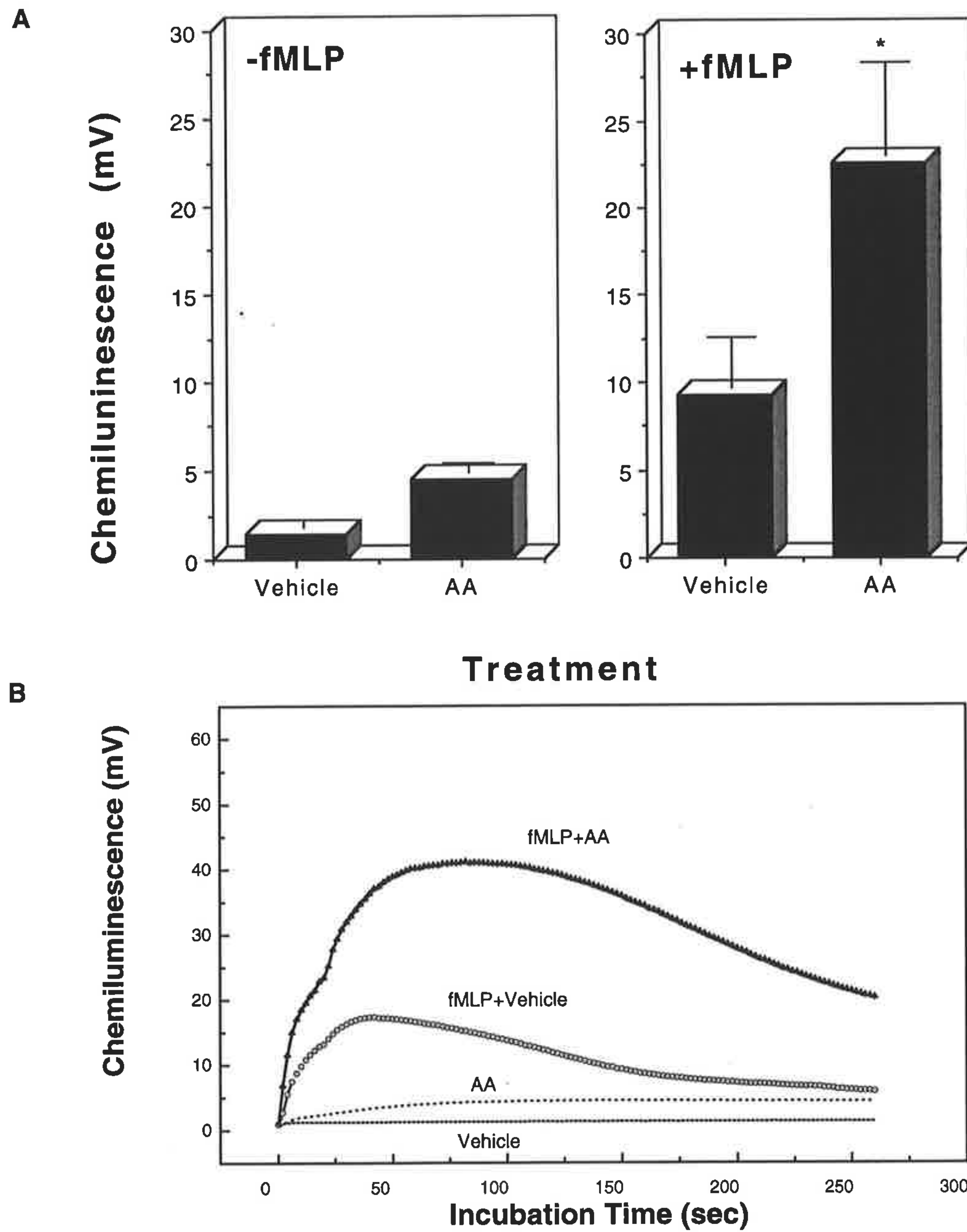


Fig 3.2 The effect of co-addition of AA and fMLP on the monocyte chemiluminescence response. Monocytes were treated with 30μM of AA or the appropriate amount of vehicle in the presence or absence of 5 μM of fMLP. The superoxide produced was measured by the lucigenin-dependent chemiluminescence assay. The results (A) are the mean±sem of 4 experiments, each carried out with cells from a different individual. *: P<0.05 for the difference between the combined treatment with AA and fMLP compared to the sum of the values of AA and fMLP .

Table 3.1 Effects of AA and fMLP on initial peak rate of chemiluminescence and average accumulated chemiluminescence in monocytes

	Initial peak rate (mV)	Average accumulated chemiluminescence (mV/sec)
Control	1.429±0.376	1.348±0.082
AA	4.520±0.472	3.599±0.790
fMLP	9.243±2.904*	6.026±0.873**
fMLP+AA	22.600±5.036#	23.991±4.594##

Monocytes were treated with 30μM AA or vehicle in the presence or absence of 5 μM of fMLP. The superoxide produced from these cells was measured by the lucigenin-dependent chemiluminescence assay. The results are presented as mean±sem of 4 experiments, each conducted with cells from a different individual. *:$P<0.05$, **:$P<0.01$, compared with control; #:$P<0.05$, ##:$P<0.01$, compared with fMLP+vehicle.

Macrophages were treated with either 30μM of AA or vehicle [0.1% ethanol, (v/v)] alone or in combination with 5 μM of fMLP or diluent. As was the case with monocytes, macrophages gave a very poor and non-significant chemiluminescence response to AA (Fig 3.3). Cells co-treated with AA and fMLP showed a synergistic chemiluminescence response (Fig 3.3). Thus while AA caused a chemiluminescence of 4.4 mV (basal activity subtracted) and fMLP 9.3 mV, the co-addition of these two agents produced 46.7 mV of chemiluminescence (Fig 3.3B, Table 3.2).

Examination of the kinetics of chemiluminescence response revealed that while AA caused a small response, fMLP caused a significant increase in superoxide production. But the combination of AA and fMLP caused an accelerated and greater chemiluminescence response compared to the addition of fMLP alone (Fig 3.3B). As with monocytes, in the presence of AA and fMLP the macrophages showed a longer duration of the maximal attainable response (Fig 3.3B). The response as total chemiluminescence production by the macrophages over the entire incubation period supported the observation that AA and fMLP caused synergistic activation of the respiratory burst of macrophages (Fig 3.3, Table 3.2).

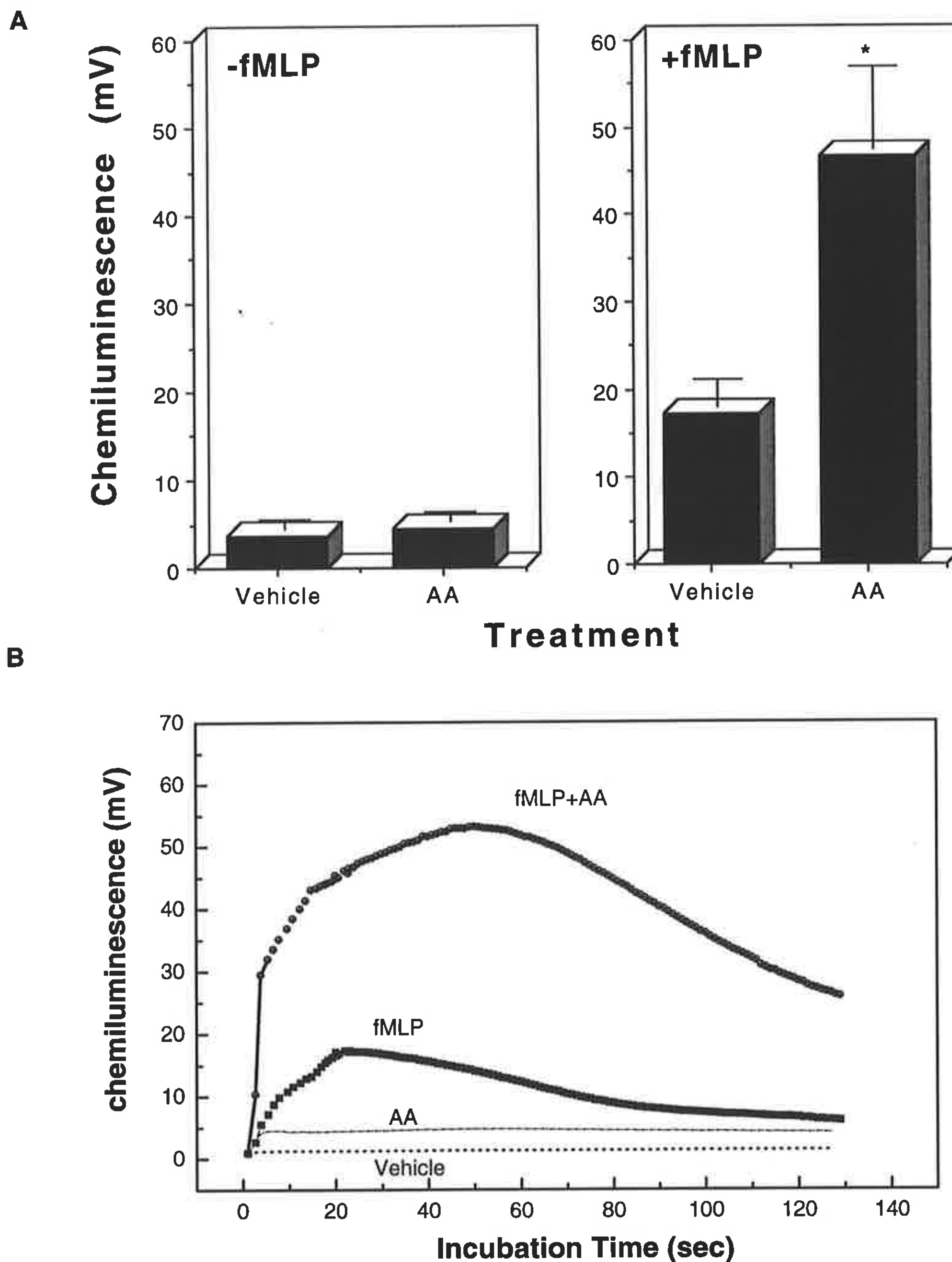


Fig 3.3 The effect of co-addition of AA and fMLP on the macrophage chemiluminescence response. Macrophages were treated with 30μM of AA or an appropriate amount of vehicle in the presence or absence of 5 μM of fMLP. The superoxide produced was measured by the lucigenin-dependent chemiluminescence assay. The results (A) are the mean±sem of 4 experiments, each carried out with cells from a different individual. *: $P<0.05$ for the difference between the combined treatment with AA and fMLP compared to the sum of the values of AA and fMLP alone treatments. (B) shows the kinetics of chemiluminescence production and is a representative experimental run.

Table 3.2 Effects of AA and fMLP on initial peak rate of chemiluminescence and average accumulated chemiluminescence in macrophages

	Initial peak rate (mV)	Average accumulated chemiluminescence (mV/sec)
Control	3.698±0.561	1.928±0.092
AA	4.528±0.988	3.319±0.990
fMLP	17,297±1.200*	9.315±1.210**
fMLP+AA	46.667±9.501#	38.788±5.968##

Macrophages were treated with 30μM AA or vehicle in the presence or absence of 5 μM of fMLP. The superoxide produced from these cells was measured by the lucigenin-dependent chemiluminescence assay. The results are presented as mean±sem of 4 experiments, each conducted with cells from a different individual. *:$P<0.05$, **:$P<0.01$, compared with control; #:$P<0.05$, ##:$P<0.01$, compared with fMLP+vehicle.

3.5 The synergistic chemiluminescence responses to the co-addition of EPA and fMLP in monocytes and macrophages

EPA (20:5, *n-3*), a long chain polyunsaturated fatty acid, is found in abundance in marine oil. When released from membrane phospholipids, EPA like AA, is metabolised via the cyclooxygenase and lipoxygenase pathways (Simopoulos, 1991). However it has been well established that the eicosanoids generated from the metabolism of EPA are several fold less proinflammatory than those generated from AA. In this manner, EPA enrichment of tissues and incorporation into membrane phospholipids have been considered to reduce the generation of proinflammatory mediators and consequently inflammation. While EPA has been shown to be comparable to AA in the stimulation of neutrophil chemiluminescence (Hardy *et al,* 1994a), its effects on monocyte and macrophage function remain to be established.

3.5.1 Synergistic responses in monocytes

Thirty μM of either EPA or vehicle was added to monocytes in the presence or absence of 5 μM fMLP before measuring the monocyte chemiluminescence response. The data presented in Fig 3.4A show that EPA alone was a poor stimulator of superoxide generation in monocytes. However, co-treatment of monocytes with EPA and fMLP induced a substantially greater chemiluminescence response than the sum of the values obtained separately with an individual agonist (Fig 3.4). The initial peak rate of chemiluminescence induced by EPA alone was 5.9 mV (basal value subtracted), and 9.2

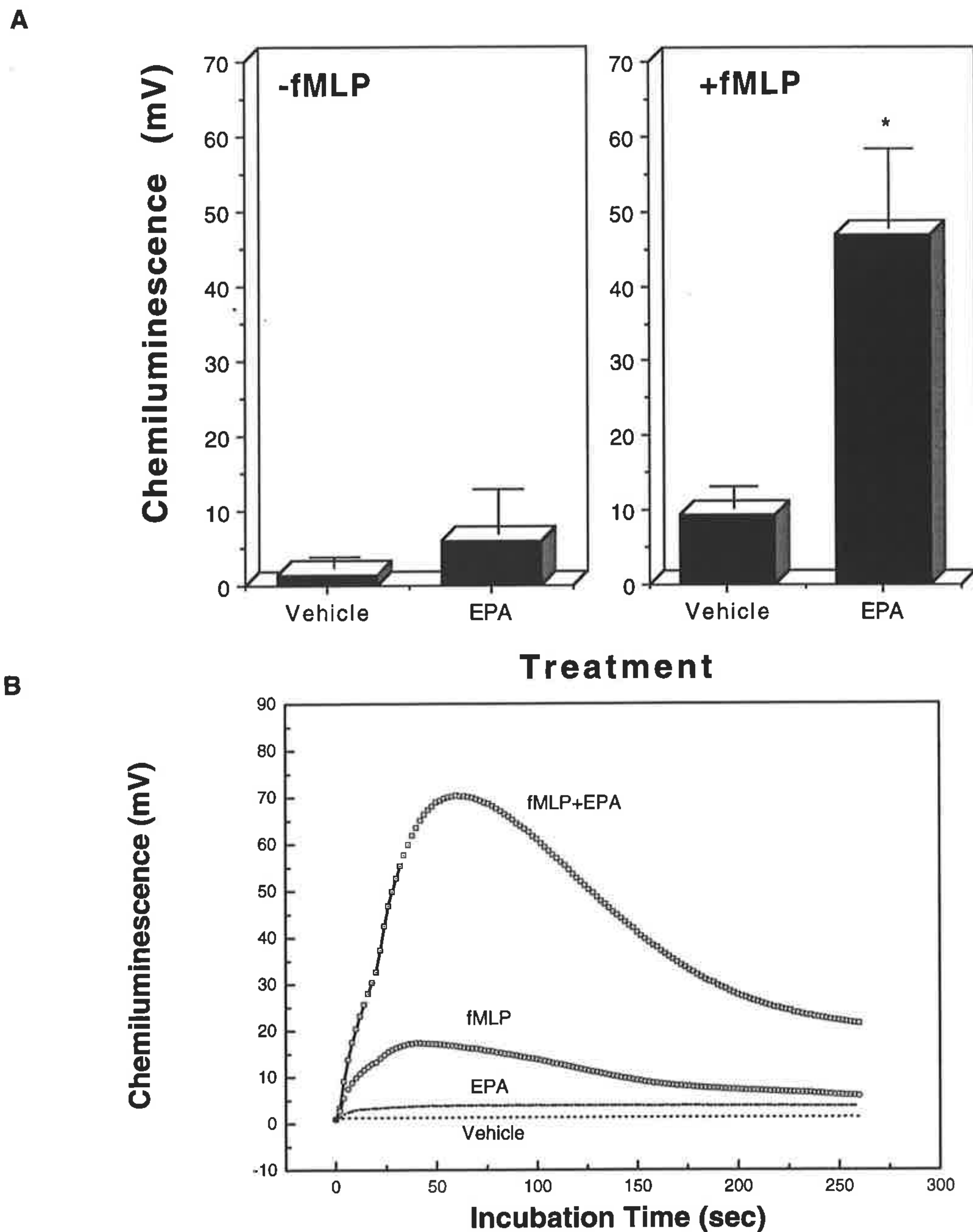


Fig 3.4 The effect of co-addition of EPA and fMLP on the monocyte chemiluminescence response. Monocytes were treated with 30μM of EPA or the an appropriate amount of vehicle in the presence or absence of 5 μM of fMLP. The superoxide produced was measured by the lucigenin-dependent chemiluminescence assay. The results (A) are the mean±sem of 5 experiments, each carried out with cells from a different individual. *: P<0.05 for the difference between the combined treatment with AA and fMLP compared to the sum of the values of AA and fMLP alone treatments. (B) shows the kinetics of chemiluminescence production and is a representative experimental run.

Table 3.3 Effects of EPA and fMLP on initial peak rate of chemiluminescence and average accumulated chemiluminescence in monocytes

	Initial peak rate (mV)	Average accumulated chemiluminescence (mV/sec)
Control	1.429±0.376	1.348±0.082
EPA	5.897±1.098	1.921±0.428
fMLP	9.243±2.904*	6.026±0.873**
fMLP+EPA	46.933±10.35#	21.078±2.016##

Monocytes were treated with 30μM EPA or vehicle in the presence or absence of 5 μM of fMLP. The superoxide produced from these cells was measured by the lucigenin-dependent chemiluminescence assay. The results are presented as mean±sem of 5 experiments, each conducted with cells from a different individual. *:P<0.05, **:P<0.01, compared with control; #:P<0.05, ##:P<0.01, compared with fMLP+vehicle.

mV for fMLP but was 46.9 mV for the co-treatment with EPA and fMLP (Fig 3.4A, Table 3.3)

Examination of the rate of chemiluminescence response over the incubation period showed that the synergistic effect was evident over the duration of this period (Fig 3.4B, Table 3.3). The combination of EPA and fMLP gave rise to an accelerated chemiluminescence response.

3.5.2 Synergistic responses in macrophages

Macrophages were stimulated with either 30μM of EPA or vehicle and 5 μM of fMLP or diluent. As observed in monocytes, EPA caused a poor and insignificant lucigenin-dependent chemiluminescence response in macrophages (Fig 3.5). A substantial response was evident when macrophages were co-treated with EPA and fMLP (Fig 3.5). EPA caused an initial peak rate of chemiluminescence of 6.3 mV, fMLP 9.3 mV, but the co-treatment gave rise to 47.2 mV of chemiluminescence (Fig 3.5A, Table 3.4).

When the rate of chemiluminescence production was plotted against time of incubation, it was evident that the co-treatment with EPA and fMLP led to an accelerated response, and the initial peak rate was maintained for a longer period (Fig 3.5B). The data in Table 3.4 show the accumulated amount of chemiluminescence produced by macrophages over the incubation period. The results are consistent with those of comparisons made between

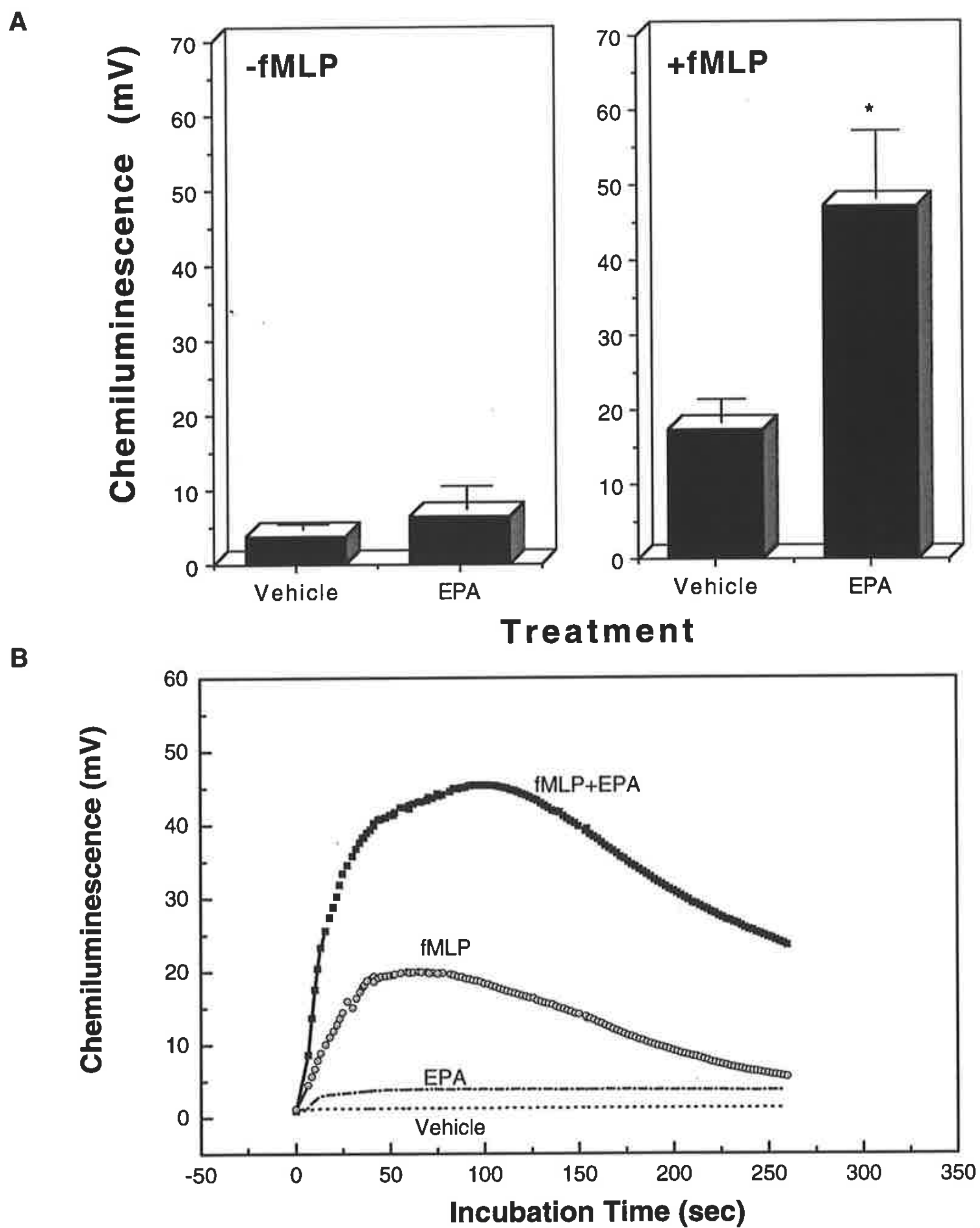


Fig 3.5 The effect of co-addition of EPA and fMLP on the macrophage chemiluminescence response. Macrophages were treated with 30μM of EPA or an appropriate amount of vehicle in the presence or absence of 5 μM of fMLP. The superoxide produced was measured by the lucigenin-dependent chemiluminescence assay. The results (A) are the mean±sem of 5 experiments, each carried out with cells from a different individual. *: $P<0.05$ for the difference between the combined treatment with AA and fMLP compared to the sum of the values of AA and fMLP alone treatments. (B) shows the kinetics of chemiluminescence production and is a representative experimental run.

Table 3.4 Effects of EPA and fMLP on initial peak rate of chemiluminescence and average accumulated chemiluminescence in macrophages

	Initial peak rate (mV)	Average accumulated chemiluminescence (mV/sec)
Control	3.698±0.561	1.928±0.092
EPA	6.347±3.121	1.241±0.828
fMLP	17.297±1.200*	9.315±1.210**
fMLP+EPA	47.200±9.064#	32.473±5.068##

Macrophages were treated with 30μM EPA or vehicle in the presence or absence of 5 μM of fMLP. The superoxide produced from these cells was measured by the lucigenin-dependent chemiluminescence assay. The results are presented as mean±sem of 5 experiments, each conducted with cells from a different individual. *:P<0.05, **:P<0.01, compared with control; #:P<0.05, ##:P<0.01, compared with fMLP+vehicle.

initial peak rates of chemiluminescence, demonstrating that a substantial respiratory burst response could be seen in the presence of EPA and fMLP.

3.6 The synergistic chemiluminescence response in monocytes and macrophages co-stimulated with DHA and fMLP

The above studies were extended to include another *n-3* polyunsaturated fatty acid, DHA (22:6, *n-6*). This fatty acid is also one of the major fish oil fatty acids. Previous reports have shown that DHA is even more effective than EPA in the direct stimulation of neutrophil superoxide production (Hardy *et al,* 1994a).

3.6.1 Synergistic responses in monocytes

Monocytes treated with 30μM of DHA or vehicle showed a poor superoxide response, compared to the chemiluminescence response induced by fMLP. However, co-treatment with DHA and 5 μM of fMLP induced a substantial chemiluminescence in monocytes (Fig 3.6A). Examination of the initial peak rate responses showed that DHA alone induced a chemiluminescence response of 4.8 mV, fMLP 9.2 mV and the co-treatment response was 42.4 mV (Table 3.5).

The kinetics of the chemiluminescence response are presented in Fig 3.6B. It is evident that the co-treatment with DHA and fMLP led to a more rapid response. In addition, the

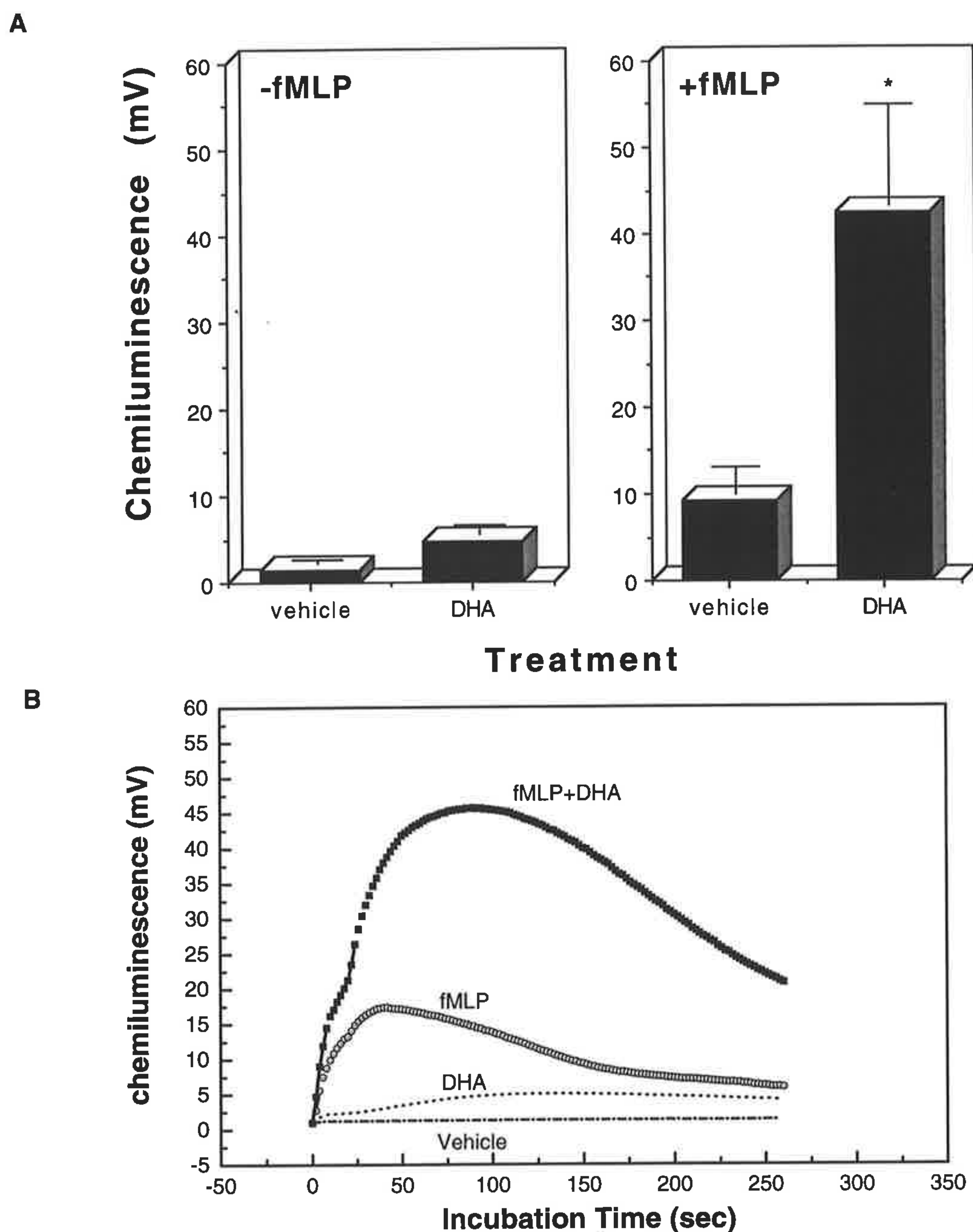


Fig 3.6 The effect of co-addition of DHA and fMLP on the monocyte chemiluminescence response. Monocytes were treated with 30μM of DHA or the an appropriate amount of vehicle in the presence or absence of 5 μM of fMLP. The superoxide produced was measured by the lucigenin-dependent chemiluminescence assay. The results (A) are the mean±sem of 5 experiments, each carried out with cells from a different individual. *: P<0.05 for the difference between the combined treatment with AA and fMLP compared to the sum of the values ofDHAand fMLP alone treatments. (B) shows the kinetics of chemiluminescence production and is a representative experimental run.

Table 3.5 Effects of DHA and fMLP on initial peak rate of chemiluminescence and average accumulated chemiluminescence in monocytes

	Initial peak rate (mV)	Average accumulated chemiluminescence (mV/sec)
Control	1.429±0.376	1.348±0.082
DHA	4.787±0.953	2.252±0.630
fMLP	9.243±2.904*	6.026±0.873**
fMLP+DHA	42.425±11.584#	21.017±1.702##

Monocytes were treated with 30μM DHA or vehicle in the presence or absence of 5 μM of fMLP. The superoxide produced from these cells was measured by the lucigenin-dependent chemiluminescence assay. The results are presented as mean±sem of 5 experiments, each conducted with cells from a different individual. *:P<0.05, **:P<0.01, compared with control; #:P<0.05, ##:P<0.01, compared with fMLP+vehicle.

maximal attainable response persisted for a longer time. The synergistic response induced by DHA and fMLP was evident over the duration of the incubation period (Fig 3.6B). The data in Table 3.5 confirm the augmented responses induced by the co-treatment of monocytes with DHA and fMLP by evaluating and comparing the total chemiluminescence produced by the cells over the incubation period.

3.6.2 Synergistic response in macrophages

The effects of either 30μM of DHA or vehicle in the presence or absence of 5 μM of fMLP or diluent were next examined in macrophages. DHA alone caused a poor lucigenin-dependent chemiluminescence response. Co-treatment of cells with DHA and fMLP caused a substantial chemiluminescence response which was significantly greater than the response elicited by either fMLP or DHA alone (Fig 3.7A). DHA induced 7.2 mV (base level subtracted) of chemiluminescence, fMLP 9.3 mV but the co-treatment of macrophages with DHA and fMLP produced 48.7 mV chemiluminescence response (Fig 3.7A, Table 3.6).

The kinetics of the chemiluminescence response over the incubation time are shown in Fig 3.7B. Co-treatment with DHA and fMLP led to a more rapid response and gave rise to a synergistic chemiluminescence response which lasted over the entire incubation period. It is reflected in the data shown in Table 3.6, representing the total chemiluminescence detected over the incubation period.

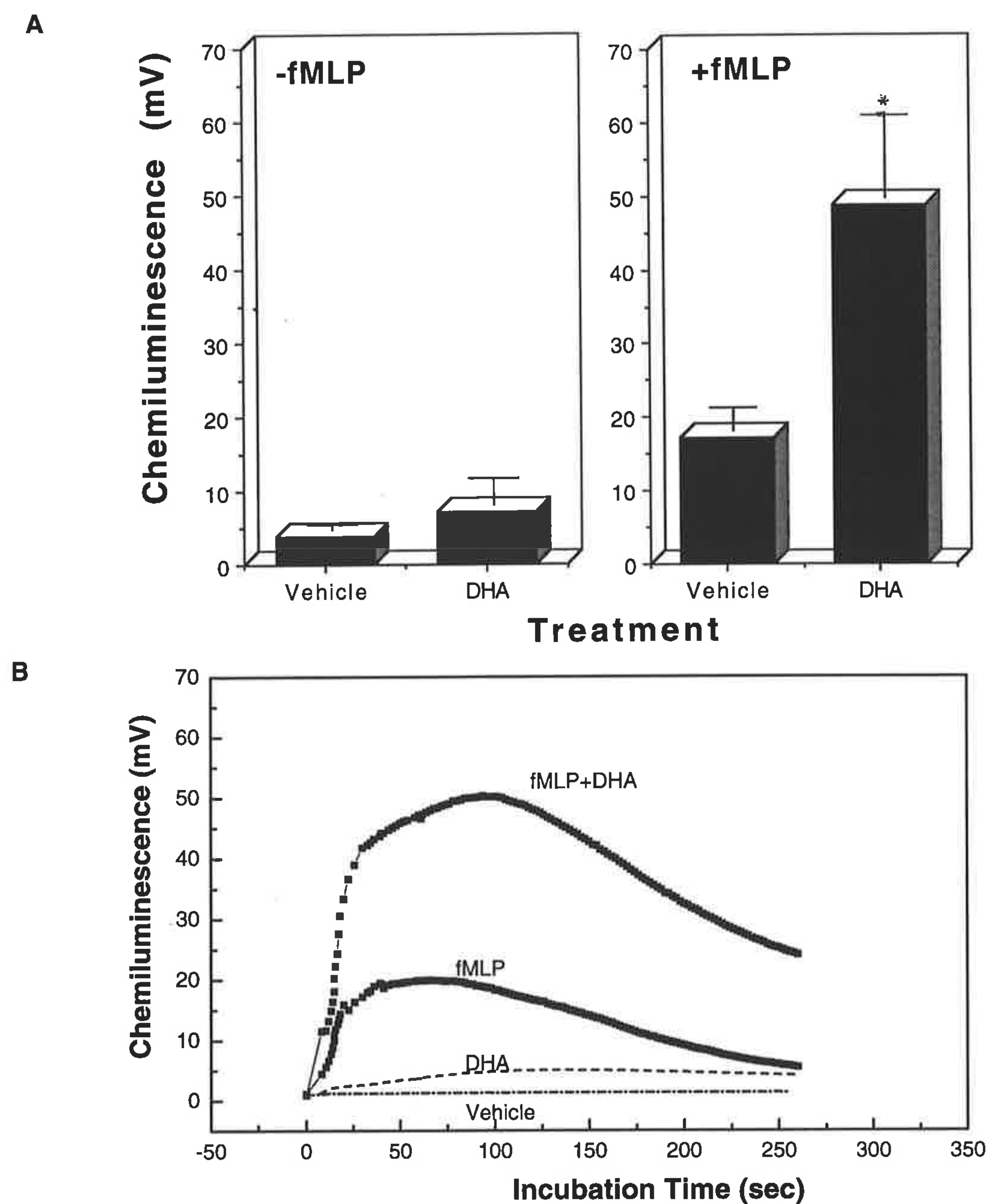


Fig 3.7 The effect of co-addition of DHA and fMLP on the macrophage chemiluminescence response. Macrophages were treated with 30μM of DHA or an appropriate amount of vehicle in the presence or absence of 5μM of fMLP. The superoxide produced was measured by the lucigenin-dependent chemiluminescence assay. The results (A) are the mean±sem of 5 experiments, each carried out with cells from a different individual. *: P<0.05 for the difference between the combined treatment with AA and fMLP compared to the sum of the values of DHA and fMLP alone treatments. (B) shows the kinetics of chemiluminescence production and is a representative experimental run.

Table 3.6 Effects of DHA and fMLP on initial peak rate of chemiluminescence and average accumulated chemiluminescence in macrophages

	Initial peak rate (mV)	Average accumulated chemiluminescence (mV/sec)
Control	3.698±0.561	1.928±0.092
DHA	7.242±3.419	2.982±0.683
fMLP	17.297±1.200*	9.315±1.210**
fMLP+DHA	48.733±11.315#	38.07±6.638##

Macrophages were treated with 30μM DHA or vehicle in the presence or absence of 5 μM of fMLP. The superoxide produced from these cells was measured by the lucigenin-dependent chemiluminescence assay. The results are presented as mean±sem of 5 experiments, each conducted with cells from a different individual. *:$P<0.05$, **:$P<0.01$, compared with control; #:$P<0.05$, ##:$P<0.01$, compared with fMLP+vehicle.

3.7 Priming effects of PUFAs on the agonist-induced respiratory burst in mononuclear phagocytes

The above studies showed that synergistic responses were evident when PUFAs and fMLP were co-added to a monocyte/macrophage culture. It was therefore of interest to determine if pretreatment of mononuclear cells with PUFAs would result in greater or reduced responses, namely to determine whether PUFAs primed monocytes/macrophages for increased responses to agonists such as fMLP.

Monocytes were preincubated with 30µM each of AA, EPA or DHA, or an appropriate amount of vehicle for a period of 0-30 min before being challenged with 5 µM fMLP. The results presented in Fig 3.8A, B and C show that pretreatment of monocytes with AA, EPA and DHA, respectively, potentiated the fMLP-induced superoxide production. The optimal preincubation time which was needed for priming for a maximal chemiluminescence response was 5 min. There was a tendency for a PUFA to reduce the synergistic effects of PUFA and fMLP (at 0 min) as the preincubation time progressed towards 30 min (Fig 3. 8A, B and C). A lower concentration of AA (0.3µM) also primed monocytes for the fMLP-induced chemiluminescence response (data not shown).

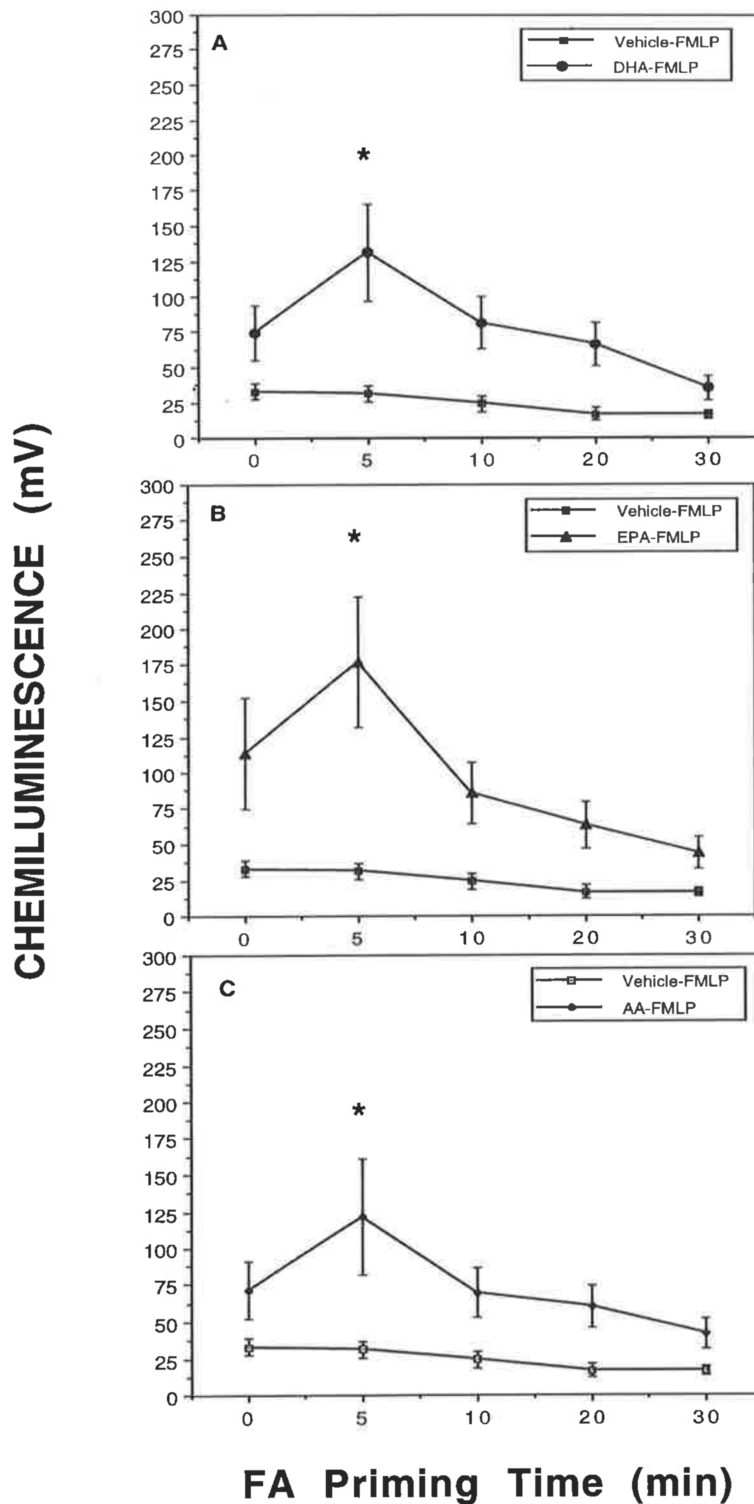


Fig 3.8 The effect of AA, EPA or DHA pretreatment period on the fMLP-induced chemiluminescence response. Monocytes were pretreated with 30µM AA (A), EPA (B) or DHA (C) for the times indicated in the graph and them tested for chemiluminescence response to fMLP (5µM). The results shown are the mean ±sem of 3 experiments. *:P<0.05 when compared with o time.

3.8 The effect of PUFA on the expression of the complement receptor on monocytes

To defend the host against microbial invasion, phagocytic cells are recruited to infectious sites by adhesion and transmigration through the vascular endothelial barrier. This process involves the upregulation of a number of functional receptors, such as the complement receptor CR3, on the surface of phagocytes by inflammatory mediators. CR3 (Mac-1) molecule which contains a αM (CD11b)chain and a β_2 chain (CD18) is the receptor for C3bi, fibrinogen, factor X and ICAM-1. It was of interest to know if the PUFA modulated another membrane-associated activity of monocytes, the CD11b expression.

After incubating monocytes with 30μM of AA for 30 min, the cells were stained with anti CD11b monoclonal antibodies prior to FACS analysis. The histogram shows that AA caused the appearance of a right-shifted peak which was similar to the fMLP-induced expression of CD11b (Fig 3.9). The AA-induced expression of CD11b was statistically significant over the vehicle treatment (Fig 3.9). No alteration was found on the expression of CD11a, CD11c and CD18 by AA in macrophages (data not shown).

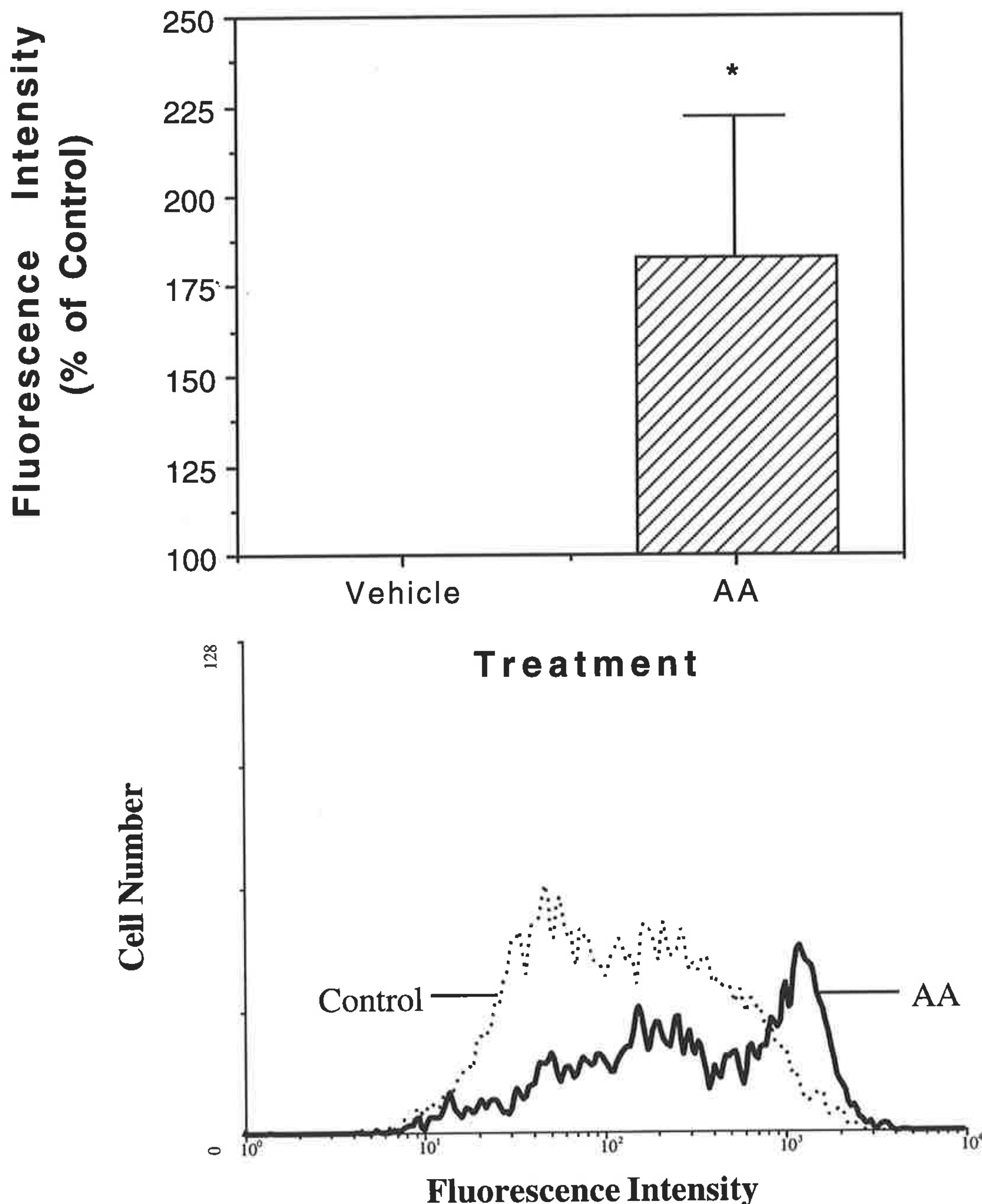


Fig 3.9 The effects of AA on the expression of CD11b on monocytes. Cells were treated with 30μM of AA, 5μM of fMLP or vehicle for 30 min and then stained with phycoerythrin (PE)-conjugated anti CD11b monoclonal antibody for 30 min at 4°C. The bound anti-CD11b antibody was analysed by flow cytometry. The results are presented as mean fluorescence intensity of antibody bound. The histogram in the lower panel is representative of three experimental runs.

3.9 Summary

The studies on the interaction of the *n-6* fatty acid AA or the *n-3* fatty acids, EPA and DHA, with monocytes and macrophages showed that these were poor stimulators of the oxygen-dependent respiratory burst (chemiluminescence). Although the PUFA *per se* had weak effects on the cells, a synergistic response was seen when a PUFA was added together with another agonist, fMLP. The characteristic property of this synergistic response was seen as an increase in the initial peak rate of the chemiluminescence response, an increase in duration of the maximal attainable response and a synergistic effect over the entire incubation period. Studies examining the effect of PUFA pretreatment time on the fMLP-induced monocyte response showed that the chemiluminescence response could be further enhanced if the cells were preincubated with a PUFA for 5 min. However, after this period of preincubation, the effect of the co-treatment with a PUFA and fMLP was reduced. AA also increase the expression of CD11b on monocytes.

Chapter 4

The Effects of Polyunsaturated Fatty Acids on Macrophage Respiratory Burst Stimulated by a Receptor Independent Agonist

4.1 Introduction

The binding of ligands such as fMLP to their G-protein-linked, seven transmembrane type receptors results in the hydrolysis of phosphatidyl inositol 4,5 bisphosphate and the liberation of inositol triphosphate and diacylglycerol (DAG). While the water soluble inositol triphosphate stimulates the release of stored calcium, DAG, together with the released calcium, stimulate the translocation of classical protein kinase C (PKC) isozymes (α, βI, βII and γ) to a particulate fraction where PKC becomes activated. Many ligands, including fMLP, also stimulate the hydrolysis of membrane phosphatidylcholine which liberates diglycerides (DG) and phosphocholine. In the absence of calcium, the liberated DG stimulate the translocation of novel PKC isozymes (δ, ε, θ, η and μ) to a particulate fraction where these isozymes become activated (Asaoka *et al,* 1992).

The actions of DAG/DG are mimicked by the tumor promoting phorbol ester, phorbol 12-myristate 13-acetate (PMA). Hence PMA has been widely used to study the role of PKC in mammalian cells. It is currently believed that PMA acts by directly binding to and stimulating the translocation and activation of PKC. Both PMA and DAG/DG bind to the same site within the C1 region (containing 2 cysteine-rich zinc finger-like motifs) of the classical and novel PKC isozymes (details in Chapter 1). Such binding causes a conformational change in PKC and this exposes the catalytic domain of PKC, thereby, facilitating activation of the enzymes (Azzi *et al,* 1992). Since the atypical PKC

isozymes (ζ, τ and λ) lack one of the cysteine-rich zinc finger-like motifs in the C1 region (Chapter 1), their modes of activation differ from those of the classical or the novel forms.

It is well documented that PMA stimulates the production of superoxide in neutrophils (Myer *et al,* 1985) and causes the phosphorylation of $p47^{phox}$, one of the components of the NADPH oxidase (Chanock *et al,* 1994). It has also been reported that PMA-stimulated superoxide production in neutrophils could be augmented by the co-treatment of cells with EPA, DHA or AA (Poulos *et al,* 1991).

Although AA, EPA or DHA *per se* did not cause a significant chemiluminescence response in monocytes/macrophages (see Chapter 3), it was of interest to investigate if these fatty acids could modulate the PMA-elicited respiratory burst in monocytes or macrophages. This would allow the determination of whether the enhancing effects of PUFA are exerted exclusively on receptor-associated stimulation (Chapter 3) or also on receptor-independent stimulation of monocytes/macrophages.

4.2 The effect of AA and PMA on monocyte/macrophage superoxide production

Cells were incubated with 100nM PMA in the presence or absence of 30μM of AA and superoxide produced was measured by the lucigenin-dependent chemiluminescence assay.

The results show that PMA and AA exerted a synergistic effect on the chemiluminescence responses in monocytes (Fig 4.1A) or macrophages (Fig 4.2A) as has been observed with fMLP and PUFA (Chapter 3).

The rate of chemiluminescence production over the incubation period is presented in Fig 4.1B and Fig 4.2B. While PMA caused a sluggish chemiluminescence response, the presence of AA caused an accelerated response to PMA. In macrophages, a lag period prior to the onset of the response was also observed when the cells were incubated with AA and PMA (Fig 4.2B). The co-treatment of cells with AA and PMA also led to an increase in the overall response. The initial peak rate and the total chemiluminescence produced over the incubation period by the co-treatment were both substantially increased compared to treatment with PMA alone (Table 4.1 and Table 4.2).

The effect of varying concentrations of AA on the response of monocytes to PMA was also investigated. Monocytes were incubated with 100nM of PMA and co-treated with 1-10μg/ml (equivalent to 3-30μM) of AA and the generation of superoxide was measured by the lucigenin-dependent chemiluminescence assay. The data show that AA augmented the PMA-induced monocyte superoxide production in a concentration related manner (Fig 4.3). The synergistic effect of AA on PMA was evident at 1μg/ml and continued to increase up to 10μg/ml.

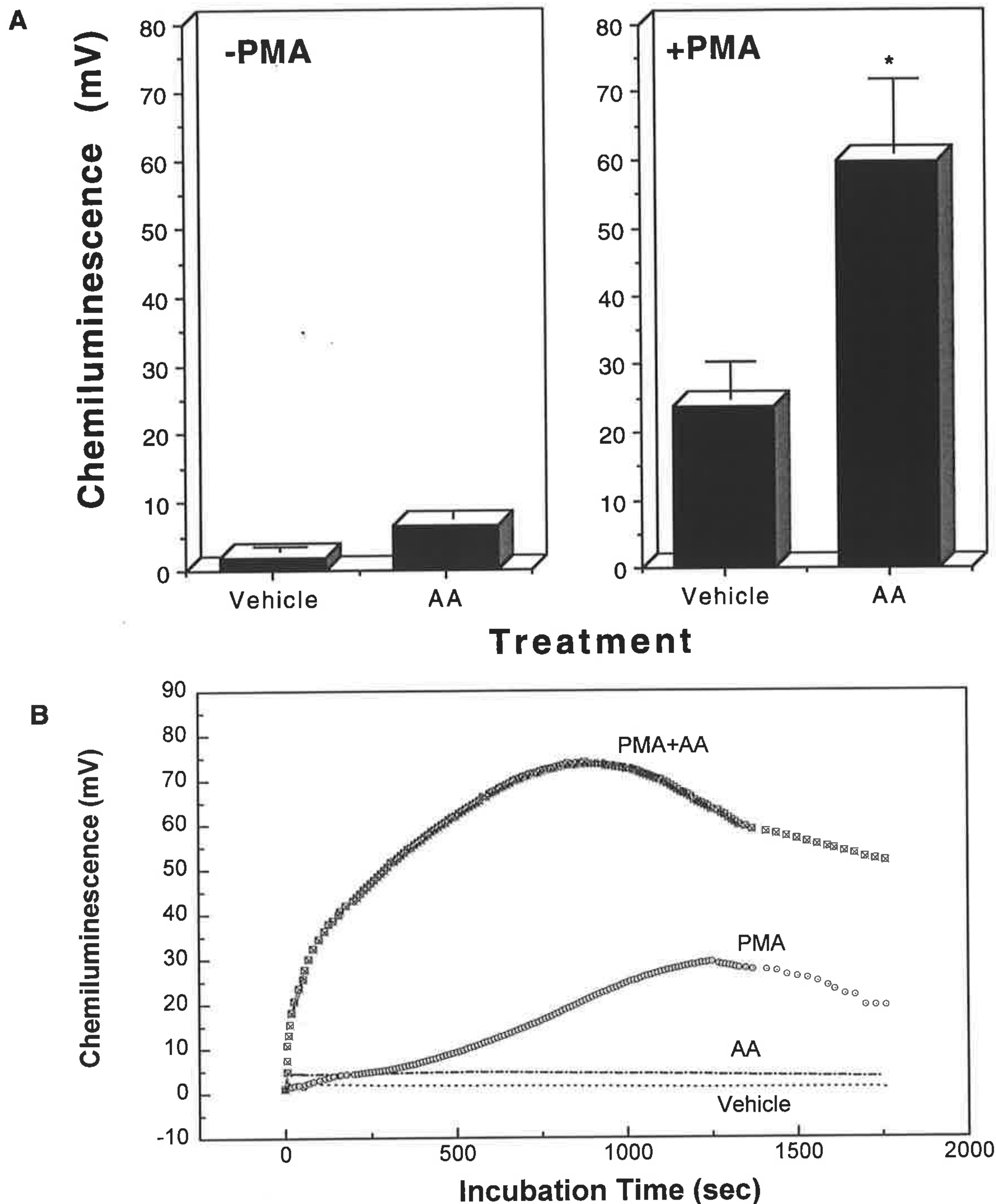


Fig 4.1 The effect of co-addition of AA and PMA on the monocyte chemiluminescence response. The cells were treated with 30μM of AA or an appropriate amount of vehicle in the presence or absence of 100nM of PMA. The superoxide produced was measured by the lucigenin-enhanced chemiluminescence assay. The results (A) are the mean±sem of 4 experiments, each carried out with cells from a different individual. *: $P<0.05$ for the difference between PMA+AA and PMA+vehicle . (B) is a representative experimental run of the rate of chemiluminescence production over the incubation period.

Table 4.1 Effects of AA and PMA on initial peak rate of chemiluminescence and average accumulated chemiluminescence in monocytes

	Initial peak rate (mV)	Average accumulated Chemiluminescence (mV/sec)
Control	1.823±0.664	0.976±0.050
AA	6.56±1.172	3.599±0.790
PMA	23.795±2.904*	21.911±1.486**
PMA+AA	59.801±10.792#	62.041±10.973##

Monocytes were treated with 30μM of AA or an appropriate amount of vehicle in the presence or absence of 100nM of PMA. The superoxide produced from these cells was measured by the lucigenin-dependent chemiluminescence assay. The results are presented as mean±sem of 4 experiments, each conducted with cells from a different individual. *:$P<0.05$, **:$P<0.01$, compared with control; #:$P<0.05$, ##:$P<0.01$, compared with PMA+vehicle.

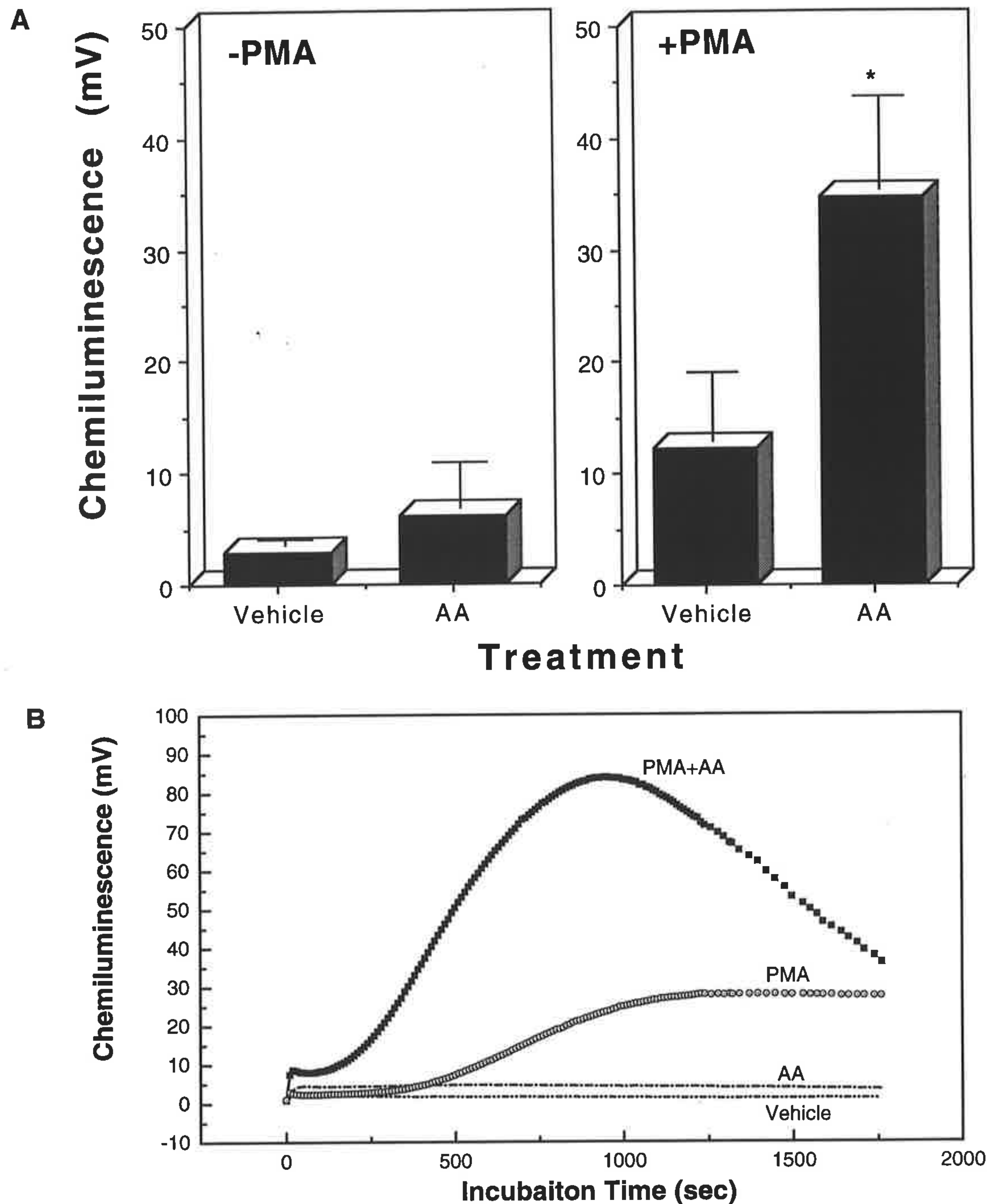


Fig 4.2 The effect of co-addition of AA and PMA on the macrophage chemiluminescence response. The cells were treated with 30μM of AA or an appropriate amount of vehicle in the presence or absence of 100nM of PMA. The superoxide produced was measured by the lucigenin-enhanced chemiluminescence assay. The results (A) are the mean±sem of 4 experiments, each carried out with cells from a different individual. *: $P<0.05$ for the difference between PMA+AA and PMA+vehicle . (B) is a representative experimental run of the rate of chemiluminescence production over the incubation period.

Table 4.2 Effects of AA and PMA on initial peak rate of chemiluminescence and average accumulated chemiluminescence in macrophages

	Initial peak rate (mV)	Average accumulated Chemiluminescence (mV/sec)
Control	2.894±0.473	1.928±0.092
AA	6.102±4.064	3.319±0.990
PMA	12.232±6.188*	11.233±1.495**
PMA+AA	34.640±8.308#	40.767±5.916##

Macrophages were treated with 30μM of AA or an appropriate amount of vehicle in the presence or absence of 100nM of PMA. The superoxide produced from these cells was measured by the lucigenin-dependent chemiluminescence assay. The results are presented as mean±sem of 4 experiments, each conducted with cells from a different individual. *:$P<0.05$, **:$P<0.01$, compared with control; #:$P<0.05$, ##:$P<0.01$, compared with PMA+vehicle.

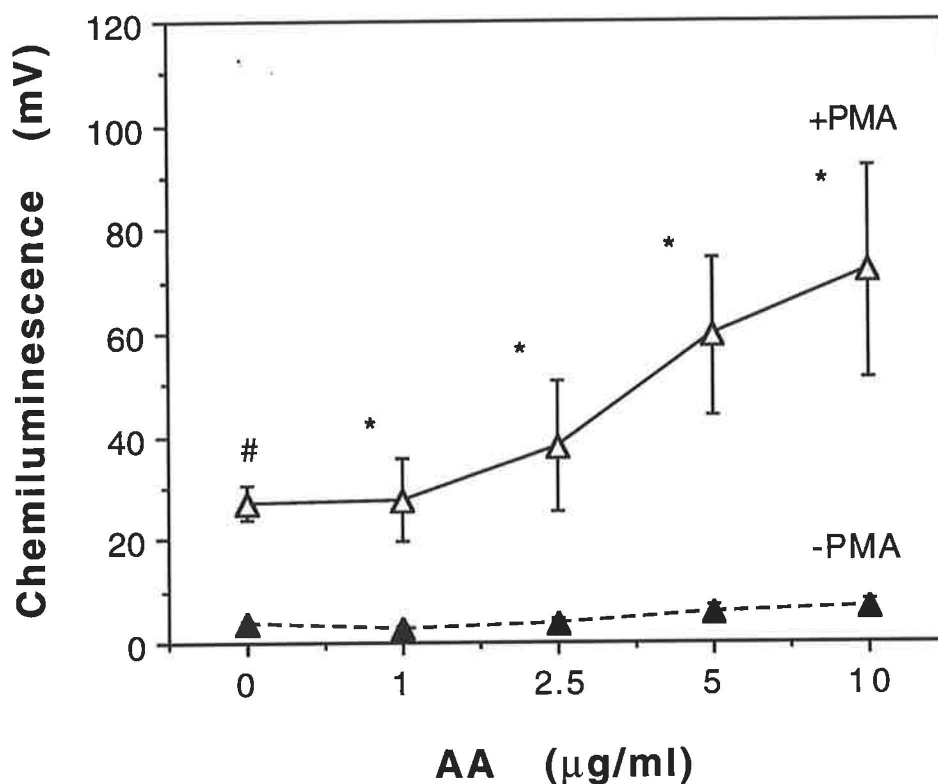


Fig 4.3 The AA concentration related effects on the PMA-induced chemiluminescence response in monocytes. Monocytes were treated with 100nM of PMA and varying concentrations of AA. The superoxide produced was measured by the lucigenin-enhanced chemiluminescence assay. The results are the mean±sem of 5 experiments. #: $P<0.05$ for the difference between PMA and diluent in the absence of AA. *: $P<0.05$ for the difference between PMA and AA co-treatment versus PMA.

4.3 The effect of EPA and PMA on monocyte/macrophage superoxide production

The effect of EPA on the PMA-stimulated respiratory burst was also investigated in monocytes or macrophages. Cells were incubated with 100nM of PMA in the presence of 30μM EPA. As observed with AA, EPA caused a significant enhancement in PMA-stimulated chemiluminescence production in monocytes and macrophages (Fig 4.4A and Fig 4.5A).

An examination of the rate of chemiluminescence production over incubation time in monocytes or macrophages showed that the addition of EPA increased the PMA-induced initial peak rate of chemiluminescence. The enhancing effects of EPA were seen over the entire incubation period (Fig 4.4B and Fig 4.5B). Again, a lag period was observable, either in the presence of EPA and PMA or PMA alone prior to the onset of the chemiluminescence response. This is in contrast to the almost instantaneous response seen in the presence of a PUFA and fMLP (Chapter 3). The data in Table 4.3 and Table 4.4 show the effects of EPA and PMA co-treatment as a function of the total chemiluminescence accumulated over the incubation period and on initial peak rate of chemiluminescence. The presence of EPA greatly enhanced the response to PMA.

The synergistic effect of fatty acids and PMA in the stimulation of the chemiluminescence response in monocytes was examined over 1-10 μg/ml EPA. The

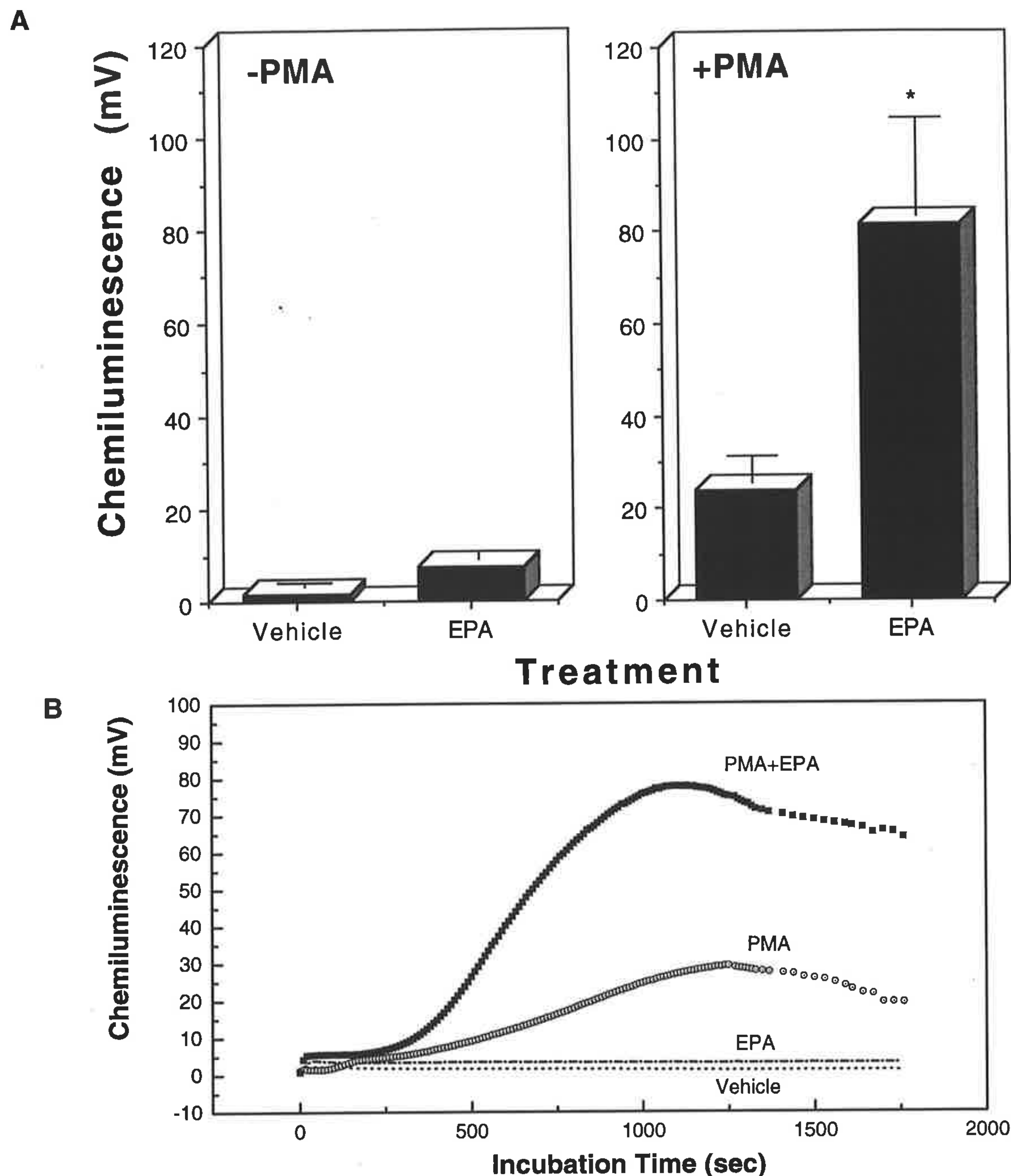


Fig 4.4 The effect of co-addition of EPA and PMA on the monocyte chemiluminescence response. The cells were treated with 30μM of EPA or an appropriate amount of vehicle in the presence or absence of 100nM of PMA. The superoxide produced was measured by the lucigenin-enhanced chemiluminescence assay. The results (A) are the mean±sem of 6 experiments, each carried out with cells from a different individual. *: $P<0.05$ for the difference between PMA+EPA and PMA+vehicle. (B) is a representative experimental run of the rate of chemiluminescence production over the incubation period.

Table 4.3 Effects of EPA and PMA on initial peak rate of chemiluminescence and average accumulated chemiluminescence in monocytes

	Initial peak rate (mV)	Average accumulated Chemiluminescence (mV/sec)
Control	1.823±0.664	0.976±0.050
EPA	7.448±1.389	1.921±0.428
PMA	23.795±2.904*	21.911±1.486**
PMA+EPA	81.267±21.419#	54.127±13.641##

Monocytes were treated with 30μM of EPA or an appropriate amount of vehicle in the presence or absence of 100 nM of PMA. The superoxide produced from these cells was measured by the lucigenin-dependent chemiluminescence assay. The results are presented as mean±sem of 6 experiments, each conducted with cells from a different individual. *:P<0.05, **:P<0.01, compared with control; #:P<0.05, ##:P<0.01, compared with PMA+vehicle.

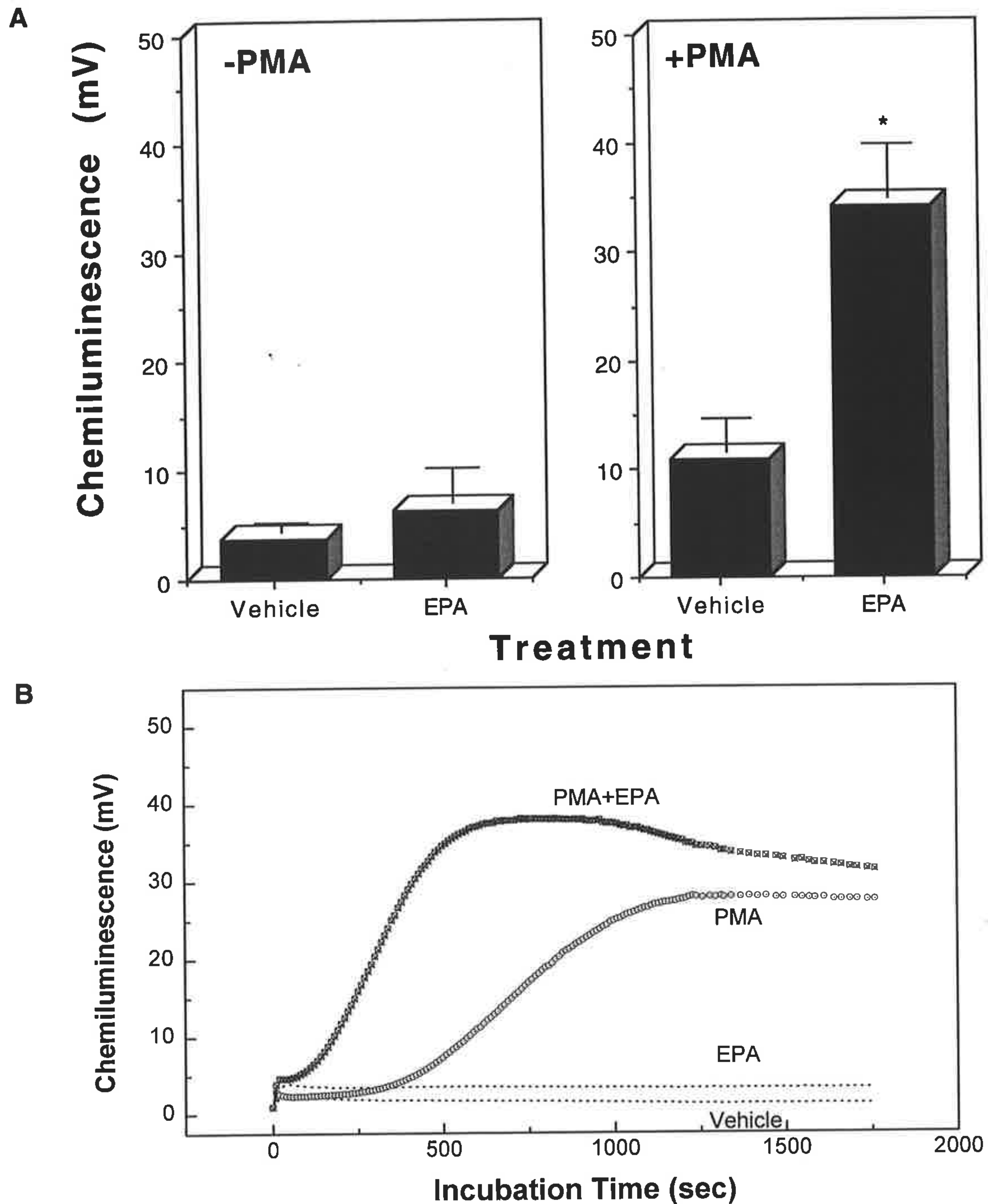


Fig 4.5 The effect of co-addition of EPA and PMA on the macrophage chemiluminescence response. The cells were treated with 30μM of EPA or an appropriate amount of vehicle in the presence or absence of 100nM of PMA. The superoxide produced was measured by the lucigenin-enhanced chemiluminescence assay. The results (A) are the mean±sem of 5 experiments, each carried out with cells from a different individual. *: $P<0.05$ for the difference between PMA+EPA and PMA+vehicle. (B) is a representative experimental run of the rate of chemiluminescence production over the incubation period.

Table 4.4 Effects of EPA and PMA on initial peak rate of chemiluminescence and average accumulated chemiluminescence in macrophages

	Initial peak rate (mV)	Average accumulated Chemiluminescence (mV/sec)
Control	2.894±0.473	1.928±0.092
EPA	6.347±3.121	1.241±0.828
PMA	12.232±6.188*	11.233±1.495**
PMA+EPA	34.125±4.931#	33.291±6.356##

Macrophages were treated with 30μM of EPA or an appropriate amount of vehicle in the presence or absence of 100 nM of PMA. The superoxide produced from these cells was measured by the lucigenin-dependent chemiluminescence assay. The results are presented as mean±sem of 5 experiments, each conducted with cells from a different individual. *:$P<0.05$, **:$P<0.01$, compared with control; #:$P<0.05$, ##:$P<0.01$, compared with PMA+vehicle.

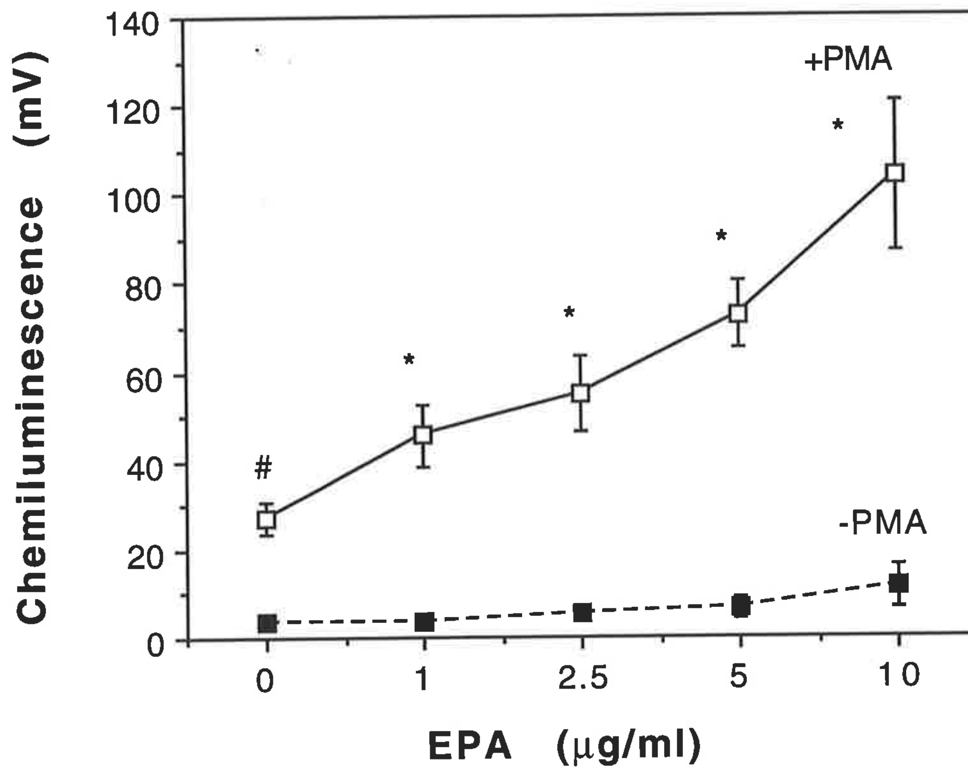


Fig 4.6 EPA concentration related effects on the PMA-induced chemiluminescence response in monocytes. Monocytes were treated with 100nM of PMA and varying concentrations of EPA. The superoxide produced was measured by the lucigenin-enhanced chemiluminescence assay. The results are the mean±sem of 5 experiments. #: $P<0.05$ for the difference between PMA and diluent in the absence of EPA. *: $P<0.05$ for the difference between PMA and EPA co-treatment versus PMA.

results show that EPA over this range of concentrations was not effective in stimulating superoxide production, but it promoted the PMA response. EPA dose-dependently augmented the PMA response, with a 1.6 fold increase at 1μg/ml to >3 fold increase at 10μg/ml (Fig 4.6). The responses of macrophages to EPA over this concentration range were identical to the above data.

4.4 The effect of DHA and PMA on monocyte/macrophage chemiluminescence production

Human monocytes or macrophages were treated with either 10μg/ml of DHA or its vehicle in the presence or absence of 100nM of PMA. The results show that DHA enhanced the PMA-induced chemiluminescence response in monocytes and macrophages (Fig 4.7A and Fig 4.8A).

A plot of the rate of chemiluminescence production over the incubation period showed that DHA increased the initial peak rates (Fig 4.7B and Fig 4.8B) and that the synergistic effect persisted over the incubation period. The presence of DHA also caused an acceleration in the response of PMA. The data in Table 4.5 and Table 4.6 show that the synergistic effect between DHA and PMA was evident on the total chemiluminescence produced and paralleled that on initial peak rates.

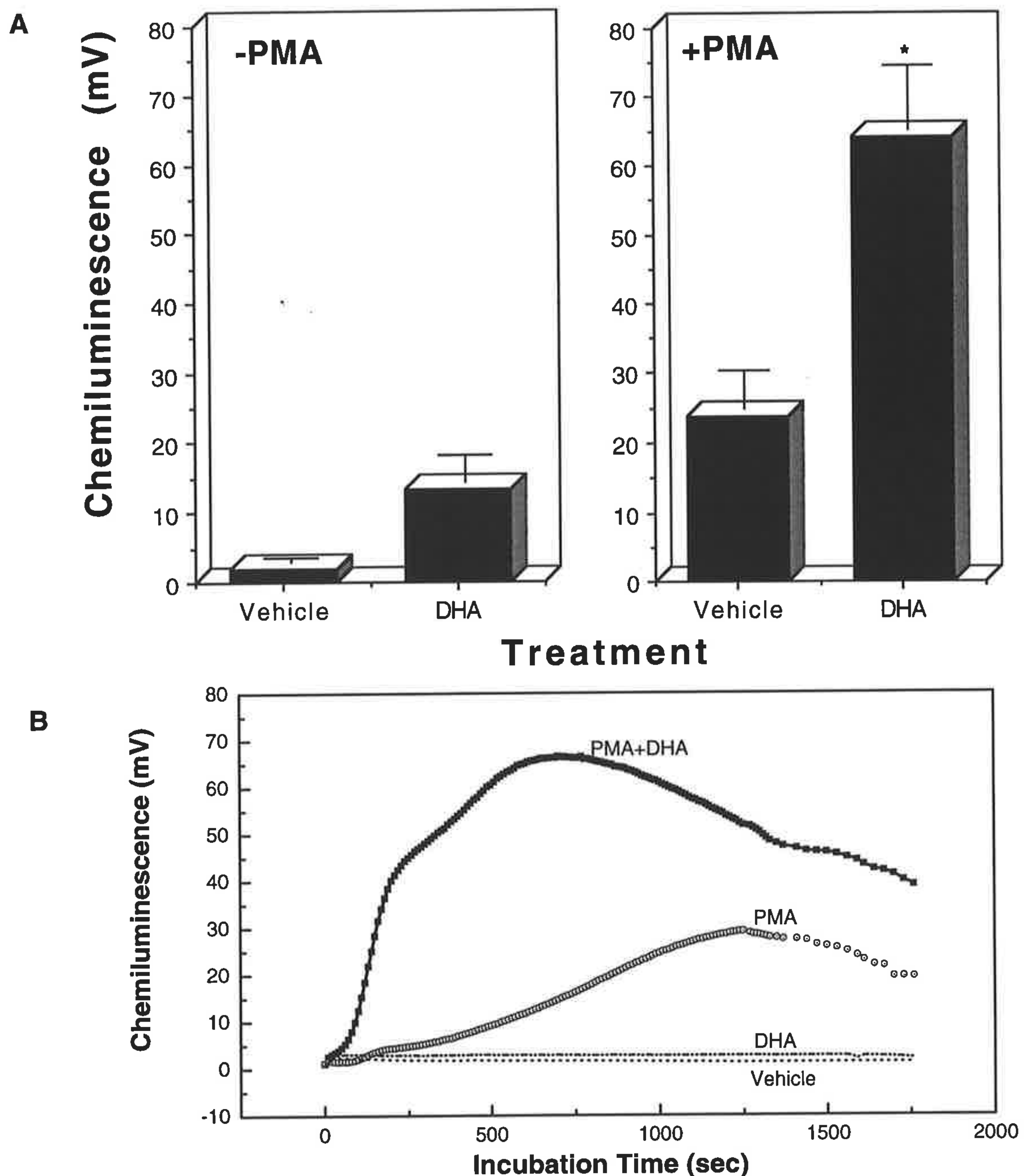


Fig 4.7 The effect of co-addition of DHA and PMA on the monocyte chemiluminescence responses. The cells were treated with 30μM of DHA or an appropriate amount of vehicle in the presence or absence of 100nM of PMA. The superoxide produced was measured by the lucigenin-enhanced chemiluminescence assay. The results (A) are the mean±sem of 9 experiments, each carried out with cells from a different individual. *: $P<0.05$ for the difference between PMA+DHA and PMA+vehicle treatment. (B) shows the rate of chemiluminescence production over the incubation period.

Table 4.5 Effects of DHA and PMA on initial peak rate of chemiluminescence and average accumulated chemiluminescence in monocytes

	Initial peak rate (mV)	Average accumulated Chemiluminescence (mV/sec)
Control	1.823±0.664	0.976±0.050
DHA	13.238±3.821	2.252±0.630
PMA	23.795±2.904*	21.911±1.486**
PMA+DHA	64.021±9.470#	70.164±9.43##

Monocytes were treated with 30μM of DHA or an appropriate amount of vehicle in the presence or absence of 100 nM of PMA. The superoxide production from these cells was measured by the lucigenin-dependent chemiluminescence assay. The results are presented as mean±sem of 9 experiments, each conducted with cells from a different individual. *:P<0.05, **:P<0.01, compared with control; #:P<0.05, ##:P<0.01, compared with PMA+vehicle.

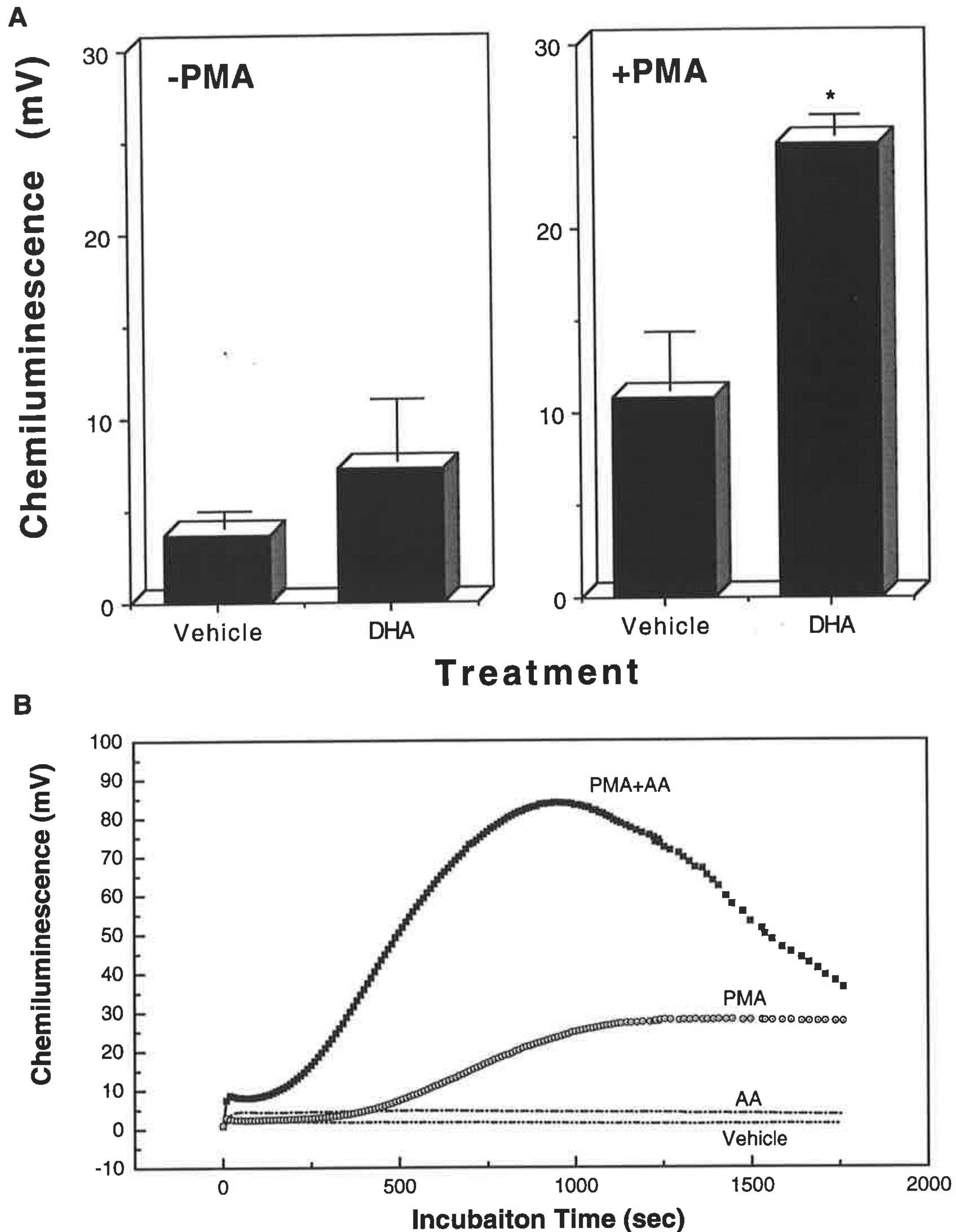


Fig 4.8 The effect of co-addition of DHA and PMA on the macrophage chemiluminescence response. The cells were treated with 30μM of DHA or an appropriate amount of vehicle in the presence or absence of 100nM of PMA. The superoxide produced was measured by the lucigenin-enhanced chemiluminescence assay. The results (A) are the mean±sem of 5 experiments, each carried out with cells from a different individual. *: $P<0.05$ for the difference between PMA+DHA and PMA+vehicle treatment. (B) shows the rate of chemiluminescence production over the incubation period.

Table 4.6 Effects of DHA and PMA on initial peak rate of chemiluminescence and average accumulated chemiluminescence in macrophages

	Initial peak rate (mV)	Average accumulated Chemiluminescence (mV/sec)
Control	2.894±0.473	1.928±0.092
DHA	7.242±3.419	2.982±0.683
PMA	12.232±6.188*	11.233±1.495**
PMA+DHA	24.625±1.089#	30.234±6.323#

Macrophages were treated with 30μM of DHA or an appropriate amount of vehicle in the presence or absence of 100 nM of PMA. The superoxide produced from these cells was measured by the lucigenin-dependent chemiluminescence assay. The results are presented as mean±sem of 5 experiments, each conducted with cells from a different individual. *:P<0.05, **:P<0.01, compared with control; #:P<0.05, compared with PMA+vehicle.

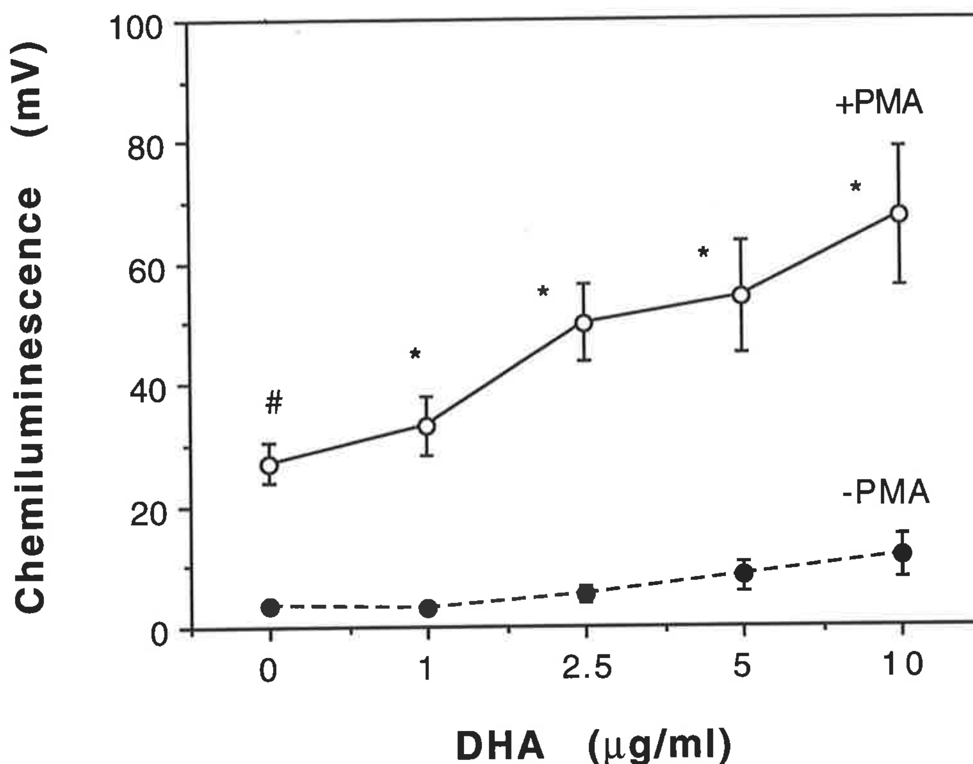


Fig 4.9 The DHA concentration related effects on the PMA-induced chemiluminescence response in monocytes. Monocytes were treated with 100nM of PMA and varying concentrations of DHA. The superoxide produced was measured by the lucigenin-enhanced chemiluminescence assay. The results are the mean±sem of 5 experiments. #: $P<0.05$ for the difference between PMA and diluent in the absence of DHA. *: $P<0.05$ for the difference between PMA and DHA co-treatment versus PMA.

The dose-response relationship of DHA on PMA-induced chemiluminescence was investigated next (Fig 4.9). The results show that the monocyte chemiluminescence response was dose-dependently increased over a DHA concentration range of 1-10 μg/ml in the presence of PMA. (Fig 4.9).

4.5 The synergistic response of 1,α 25-dihydroxyvitamin D3 differentiated macrophages to DHA and PMA

The synergistic effect of PUFA with PMA on the stimulation of a chemiluminescence response was also reproduced in a HL-60-differentiated macrophages. It has been reported that incubation of human promyelocytic leukemia cells, HL-60, with 1,α, 25-dihydroxyvitamin D3 (10^{-8} M) for 5 days promotes the differentiation of HL-60 along the macrophage lineage (Levy *et al,* 1990). By this method, a highly pure population of macrophage-like cells was obtained and these differentiated macrophages produced superoxide in response to 100nM of PMA (Fig 4.10). When these cells were incubated with both PMA and 30μM of DHA, a synergistic chemiluminescence response occurred (Fig 4.10). DHA alone caused a poor chemiluminescence response (Fig 4.10). This is consistent with the result obtained with peripheral blood monocytes/macrophages.

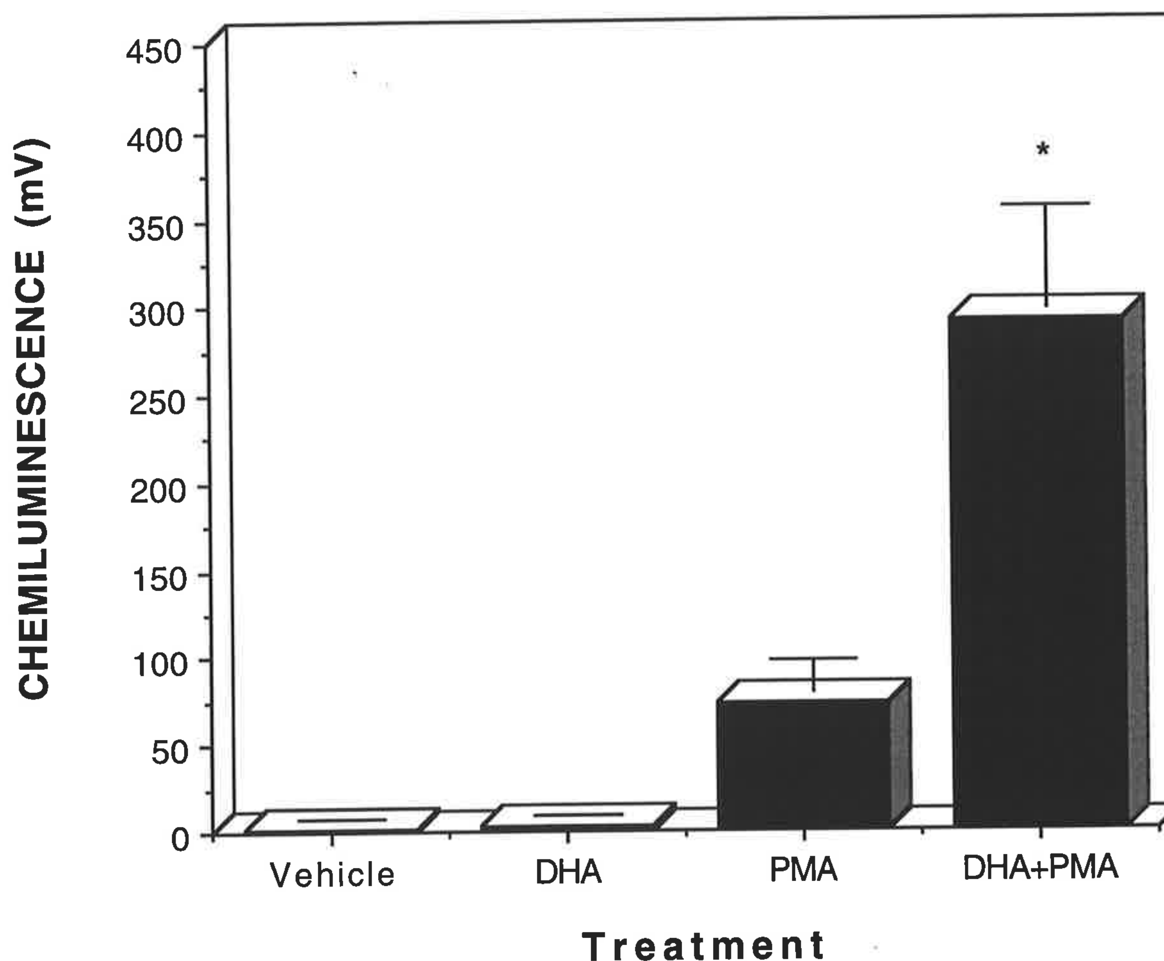


Fig 4.10 Synergistic chemiluminescence response of macrophages derived from HL-60 cells to DHA and PMA. HL-60 were differentiated with 1×10^{-8}M 1-α-25-dihydroxyvitamin D_3 for 5 days to macrophages. The cells were washed and 30μM of DHA or vehicle and 100nM of PMA or diluent were added. The superoxide production was assayed by the lucigenin-enhanced chemiluminescence assay. The results are the mean±sem of 4 experiments. *: $P<0.05$ for the difference between the sum of values for individual treatment of DHA and PMA versus the co-treatment with both agents.

4.6 The effects of PUFAs on the stimulation of superoxide production in human neutrophils

Human neutrophils are another source of reactive oxygen species and they are active in response to various agonists especially during acute inflammatory processes. Neutrophils treated with PUFAs show altered biological functions, such as an increase in integrin expression, adhesion and degranulation as well as the activation of the NADPH oxidase (Bates *et al*, 1993; Bates *et al*, 1995; Hardy *et al*, 1994a; Poulos *et al*, 1991).

Neutrophils which had been incubated with AA, EPA or DHA, showed a strong chemiluminescence response (Hardy *et al,* 1994a). The PUFA-induced superoxide production in neutrophils was much greater than that induced by either PMA or fMLP, and this is in direct contrast to the present results with macrophages, in which the PUFAs were very poor stimulators of the chemiluminescence response (Table 4.7, Chapter 3 and Chapter 4).

Previously it was shown that neutrophils incubated with either 5μM of fMLP or 100nM of PMA in the presence of 30μM of AA, EPA or DHA led to an enhancement in either fMLP- or PMA-induced superoxide production (Table 4.7). This is similar to what was observed in macrophage responses when cells were incubated with a PUFA and fMLP or PMA (Chapter 3 and Chapter 4).

Table 4.7 Modulation of superoxide production by PUFAs in phagocytic cells

Effects	Neutrophils			Macrophages		
	AA	EPA	DHA	AA	EPA	DHA
FA alone	✓	✓	✓	✗	✗	✗
Synergisms						
with fMLP	✓	✓	✓	✓	✓	✓
with PMA	✓	✓	✓	✓	✓	✓
Priming for						
fMLP-induced CL	✓	✓	✓	✓	✓	✓

✓: active; ✗ :inactive/poor response; FA: fatty acids;
CL: chemiluminescence.

Pretreatment of neutrophils with PUFA for 30 min was found to enhance the fMLP-induced chemiluminescence response (Cory *et al*, 1995; Robinson *et al*, in preparation). A similar priming effect but optimal after 5 min preincubation with PUFA was seen (Table 4.7, Chapter 3).

4.7 Summary

The results presented in the previous chapter showed that PUFA acts synergistically with fMLP, an agonist which binds to cell surface receptors. Since a number of compounds can stimulate the NADPH oxidase in intact cells by directly stimulating intracellular signalling pathways, it was of interest to see if PUFAs modified the chemiluminescence response to these compounds which bypass cell surface receptors. In this study PMA, a molecule which acts by directly activating PKC and mimics the actions of intracellularly generated DAG, was used. The data showed that the co-addition of PUFA and PMA to monocytes or macrophages caused a synergistic respiratory burst response. In most cases, in contrast to the responses observed in fMLP-stimulated cells, the chemiluminescence response after the addition of PMA was evident only after a lag period, irrespective of whether a fatty acid was added. This is consistent with the known rapid response induced by fMLP compared to PMA.

The ability of PUFA to enhance the superoxide production has also been demonstrated in neutrophils. As observed in monocytes/macrophages (Chapter 3 and Chapter 4), PUFA synergised with either fMLP or PMA in causing a chemiluminescence response, and primed neutrophils for an enhanced respiratory burst in response to fMLP. Moreover, PUFA directly stimulated the NADPH oxidase in neutrophils, which differed from their effects on monocytes/macrophages.

Chapter 5

The Importance of Fatty Acid Structure

5.1 Introduction

Previous studies have suggested that structural elements of fatty acids are important for their biological activity. For example, the chain length of a fatty acid molecule was shown to determine its ability to activate neutrophils (Hardy *et al*, 1994a). Fatty acids with chain lengths of 20-22 carbon atoms were more highly effective than fatty acids with longer (>22 carbon atom) or shorter chain length (18 carbon atom) in the stimulation of neutrophil superoxide production, adherence to plasma coated surface and lysosomal enzyme release and degranulation (Hardy *et al,* 1994a; Bates *et al,* 1995) as well as in enhancing the neutrophil-mediated killing of the parasite, *Plasmodium falciparum* (Kumaratilake *et al,* 1997). The hydroxyl and hydroperoxy form of PUFA and methylated forms of PUFA lacked the ability to stimulate neutrophils in the above activities. It has also been reported that the saturated fatty acids were inactive in the above effects as well as their ability to mobilise calcium in neutrophils and inhibit migration (Hardy *et al,* 1994a; Bates *et al,* 1995; Ferrante *et al*, 1994; Kumaratilake *et al,* 1997; Hardy, *et al,* 1995).

The work described in the previous chapter (chapter 3) showed that AA (*n-6*), EPA (*n-3*) and DHA (*n-3*) were able to enhance the ability of human monocytes and macrophages to produce superoxide in response to other agonists. In order to gain an understanding of the fatty acid structure-biological activity relationship which gives rise to monocyte/macrophage enhancement in superoxide production, a range of fatty acids with

different structures and chain lengths were examined for this activity. In this study the effects of altering the fatty acid (i) carbon chain length; (ii) number and position of double bonds; (iii) methylation of the carboxyl group; (iv) hydroxylation and hydroperoxylation status of the internal carbons on ability to modulate macrophage function was studied.

5.2 The effects of the 18 carbon chain polyunsaturated fatty acids (18:2, *n-6*; 18:3, *n-6*) on fMLP-induced macrophage superoxide production

The eighteen carbon polyunsaturated fatty acids, linoleic acid (LA, 18:2, *n-6*) and γ-linolenic acid (GLA, γ18:3, *n-6*), were examined and compared to AA, EPA and DHA in their ability to act synergistically with fMLP in the generation of a macrophage respiratory burst. In these experiments, macrophages were treated with 30μM of one of the following fatty acids: LA, GLA, AA, EPA, DHA or vehicle control and 5μM of fMLP. The respiratory burst was measured as superoxide production by the lucigenin-dependent chemiluminescence assay.

LA and GLA were poor stimulators of macrophage superoxide production (data not shown). Co-addition of GLA together with fMLP, as with the results with AA, EPA and DHA, resulted in a synergistic superoxide response (Fig 5.1). However, GLA was less

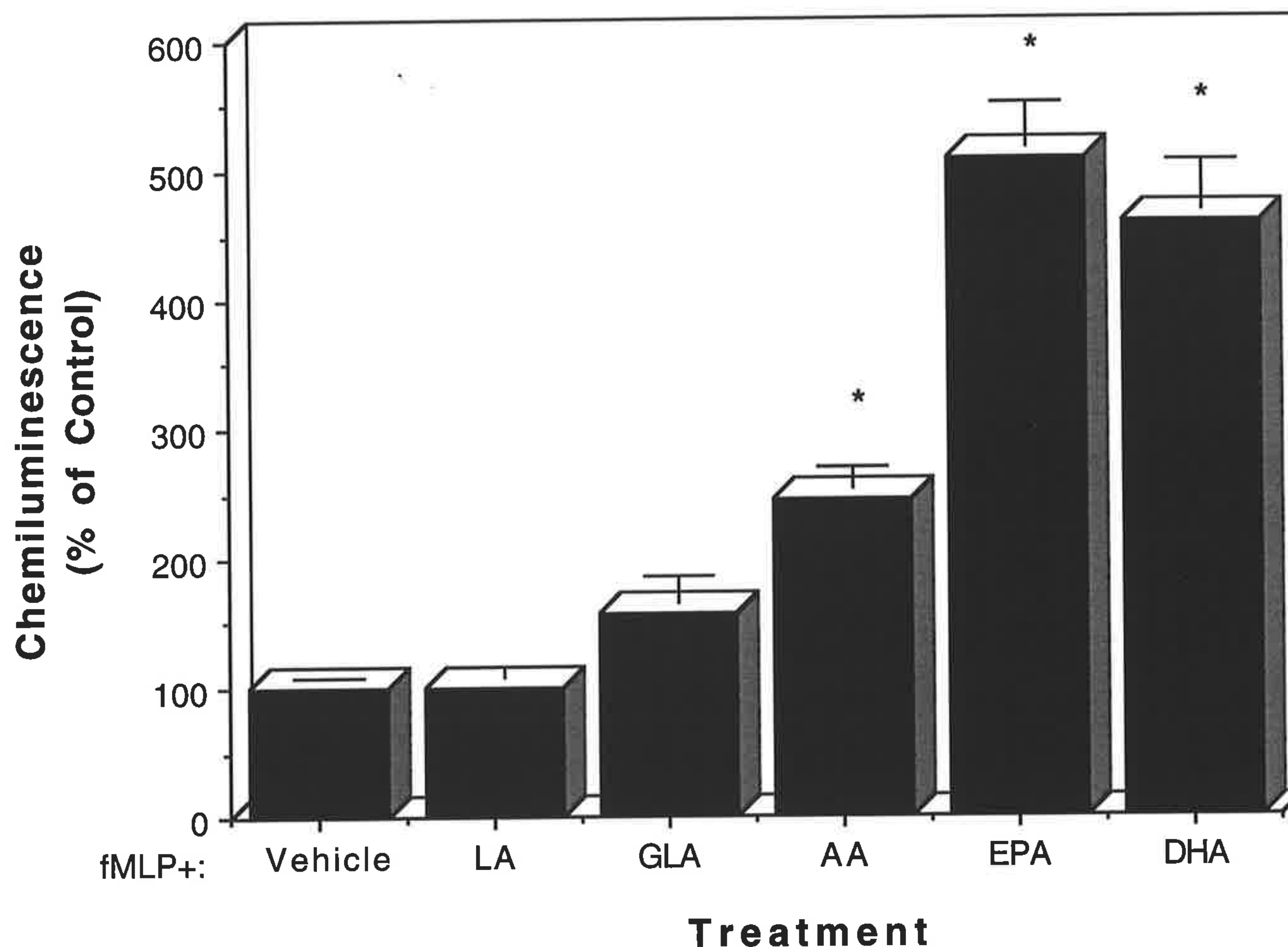


Fig 5.1 The effect of PUFAs on the fMLP-induced superoxide production in human macrophages. Macrophages were treated with 5μM of fMLP and 30μM each of DHA, EPA, AA, linolenic acid (GLA), linoleic acid (LA) or an appropriate amount of vehicle. The superoxide produced from these cells was measured by the lucigenin-dependent chemiluminescence assay. The data are presented as the mean±sem of 3 experiments and as percentage of the fMLP response. *: P<0.05 for the difference between the effect of a fatty acid compared with the vehicle control.

active than those by AA, EPA and DHA. In contrast, the fMLP response was not altered by LA (Fig 5.1).

Examination of the data as total accumulated chemiluminescence over a 60 sec incubation period showed a similar difference between the effects of 18 carbon and the 20-22 carbon fatty acids (Fig 5.2A). The data are displayed as the rate of chemiluminescence production over the time of incubation period in Fig 5.2B. It is evident here that, with the exception of AA, the fatty acids do not appreciably alter the time taken to achieve initial peak rate when added together with fMLP compared to that seen in the presence of fMLP alone. In addition to AA, GLA also caused an increase in the fMLP-induced chemiluminescence produced over the entire incubation period (Fig 5.2B). GLA and AA also accelerated the chemiluminescence response when added together with fMLP compared to the response that was seen with fMLP alone (Fig 5.2B).

5.3 The effects of saturated fatty acids on the macrophage respiratory burst induced by fMLP

The importance of unsaturation on the activity of fatty acids was further studied by examining the effects of the saturated fatty acids, myristic acid (14:0), palmitic acid (16:0) or arachidic acid (20:0) on macrophages. The cells were treated with 30μM of each of above fatty acids in the presence or absence of 5μM fMLP. The results show that

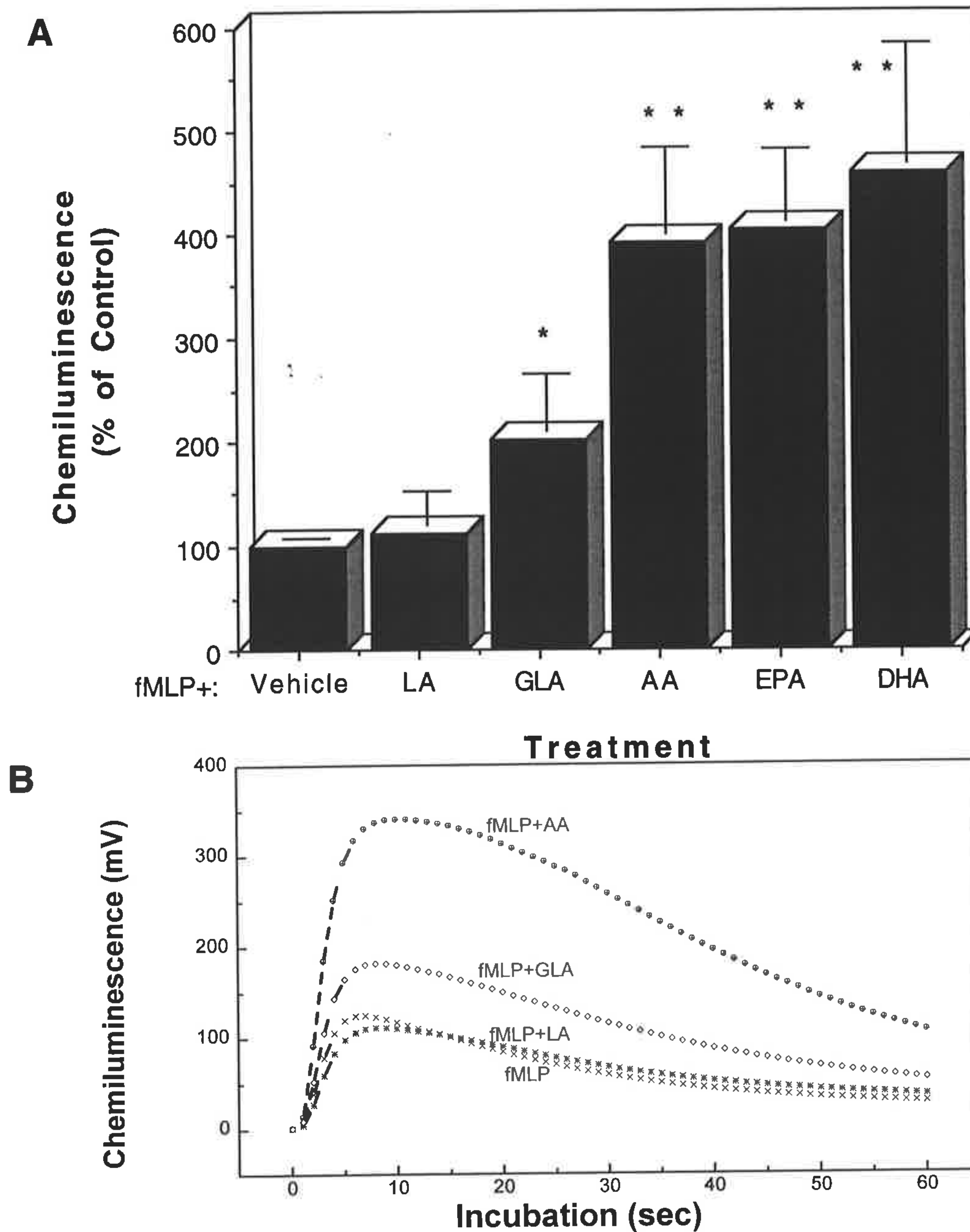


Fig 5.2 The effect of PUFAs on the fMLP-induced superoxide production in human macrophages expressed as total chemiluminescence produced over the incubation period. Macrophages were treated with 5μM of fMLP and 30μM of either DHA, EPA, AA, linolenic acid (LA), linoleic acid (GLA) or vehicle. The total superoxide produced from these cells was measured by the lucigenin-dependent chemiluminescence assay. (A) The data were presented as the mean±sem of 3 experiments and as percentage of the fMLP response. *: $P<0.05$, **: $P<0.01$ are the differences between the effect of a fatty acids compared with the vehicle control. (B) The kinetics of superoxide production from these cells over the incubation period is presented in the lower panel

neither of these fatty acids directly stimulated superoxide production nor did they alter the fMLP-induced chemiluminescence response (Fig 5.3).

5.4 The importance of the carboxyl group on the activity of fatty acids

Previous studies with other cell types have demonstrated that the carboxyl group of the PUFA is critical to many of its activities. These include the production of superoxide by neutrophils, inhibition of neutrophil migration and the inhibition of gap junction communication by PUFA in WB rat epithelial cells (Poulos *et al,* 1991; Ferrante *et al,* 1994; Hii *et al,* 1995a). Here the role of the carboxyl group in the PUFA-induced enhancement of the macrophage respiratory burst to fMLP was examined. This was achieved by converting the PUFAs to their methyl ester derivatives. Both AA and DHA were converted to these forms and then tested for abilities to enhance the chemiluminescence response of macrophages to fMLP. The macrophages were incubated with 30μM each of DHA, DHA methyl ester (DHA-ME), AA, AA methyl ester (AA-ME) or vehicle in the presence or absence of 5μM of fMLP and the resultant chemiluminescence measured. The results show that while both AA and DHA significantly enhanced the fMLP-induced macrophage chemiluminescence production, the methyl ester derivatives were devoid of this activity (Fig 5.4).

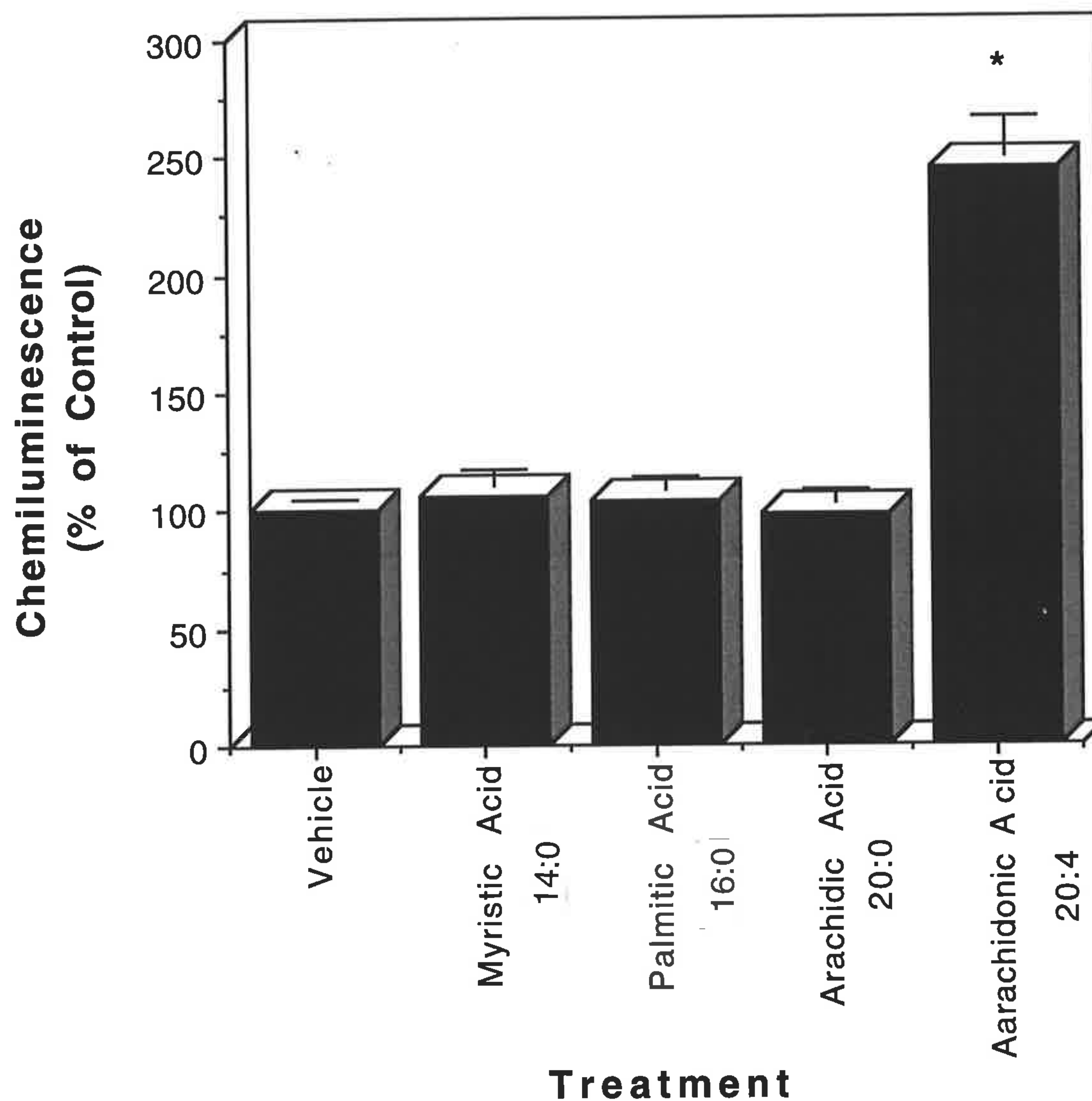


Fig 5.3 Lack of effect of saturated fatty acids on the fMLP-induced superoxide production in human macrophages. Macrophages were treated with 5μM of fMLP and 30μM each of AA, arachidic acid, palmitic acid, myristic acid or an appropriate amount of vehicle. The superoxide produced by these cells was measured by the lucigenin-dependent chemiluminescence assay. The data are presented as the mean±sem of 3 separated experiments and as percentage of the fMLP response. *: $P<0.05$ for the difference between the effect of AA compared with the vehicle control.

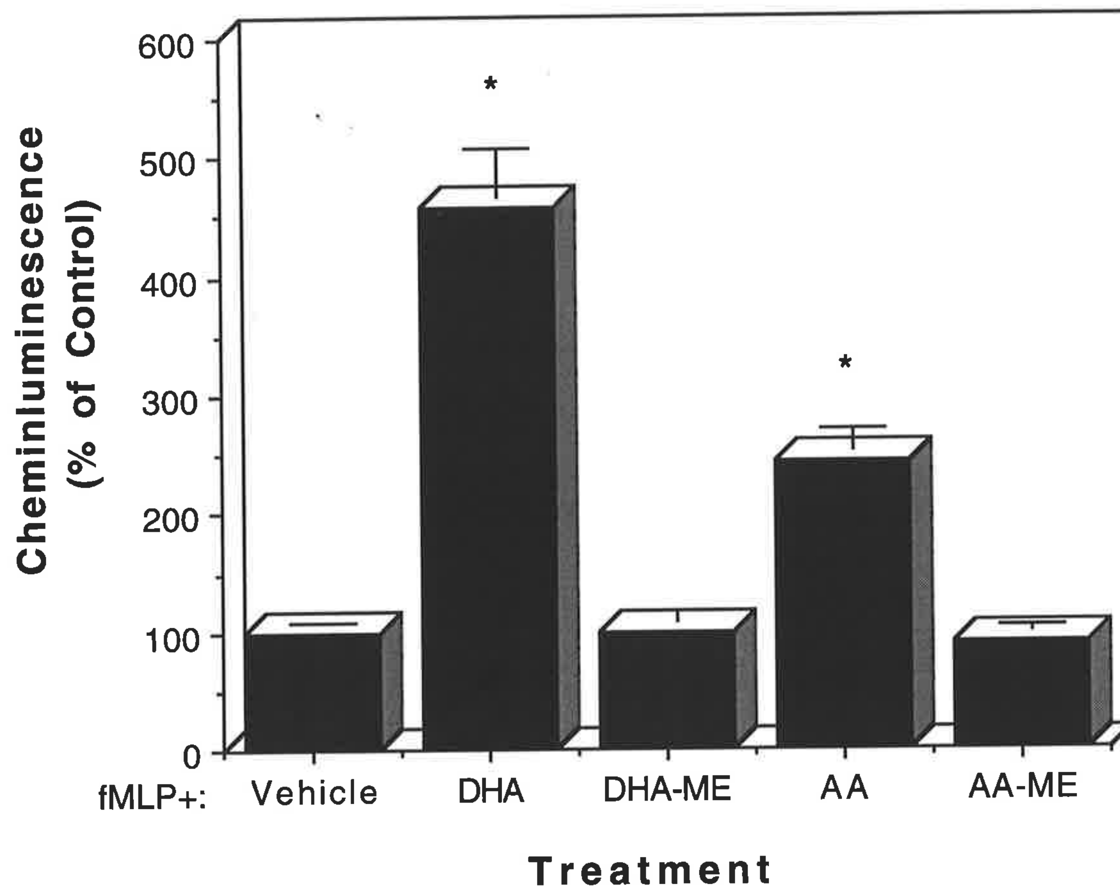


Fig 5.4 The effect of methyl ester derivatives of AA and DHA on the fMLP-induced superoxide production in human macrophages. Macrophages were treated with 5μM of fMLP and 30μM each of DHA, AA, DHA-ME, AA-ME or vehicle. The superoxide produced from these cells was measured by the lucigenin-enhanced chemiluminescence assay. The data are presented as the mean±sem of 3 experiments and as percentage of the fMLP response. *: $P<0.05$ for the difference between the effect of a fatty acid compared with vehicle control.

5.5 The effects of hydroxylation and hydroperoxylation of the PUFAs on macrophage respiratory burst

As previously described, the oxidation of AA via the lipoxygenase pathway leads to the generation of 15-HPETE and 15-HETE which have been shown to modulate some cellular functions. These fatty acids were found to suppress the LPS-induced TNF production from monocytes and the TNF-induced expression of adhesion molecules on endothelial cells (Ferrante *et al*, 1997; Huang *et al*, 1997). However, these fatty acids alone had no effect on altering neutrophil migration and parasite killing by neutrophils (Ferrante *et al*, 1994; Kumaratilake *et al*, 1997). It was therefore of interest to know whether the introduction of an internal hydroxy or hydroperoxy group in AA altered the biological activity. Macrophages were treated with 30µM of AA, 15-HETE or 15-HPETE or vehicle in the presence or absence of 5µM of fMLP. The macrophage chemiluminescence response was measured. The results showed that the ability of AA to enhance the fMLP-induced respiratory burst was totally lost following the conversion of the fatty acid to the hydroxy or hydroperoxy derivatives (Fig 5.5).

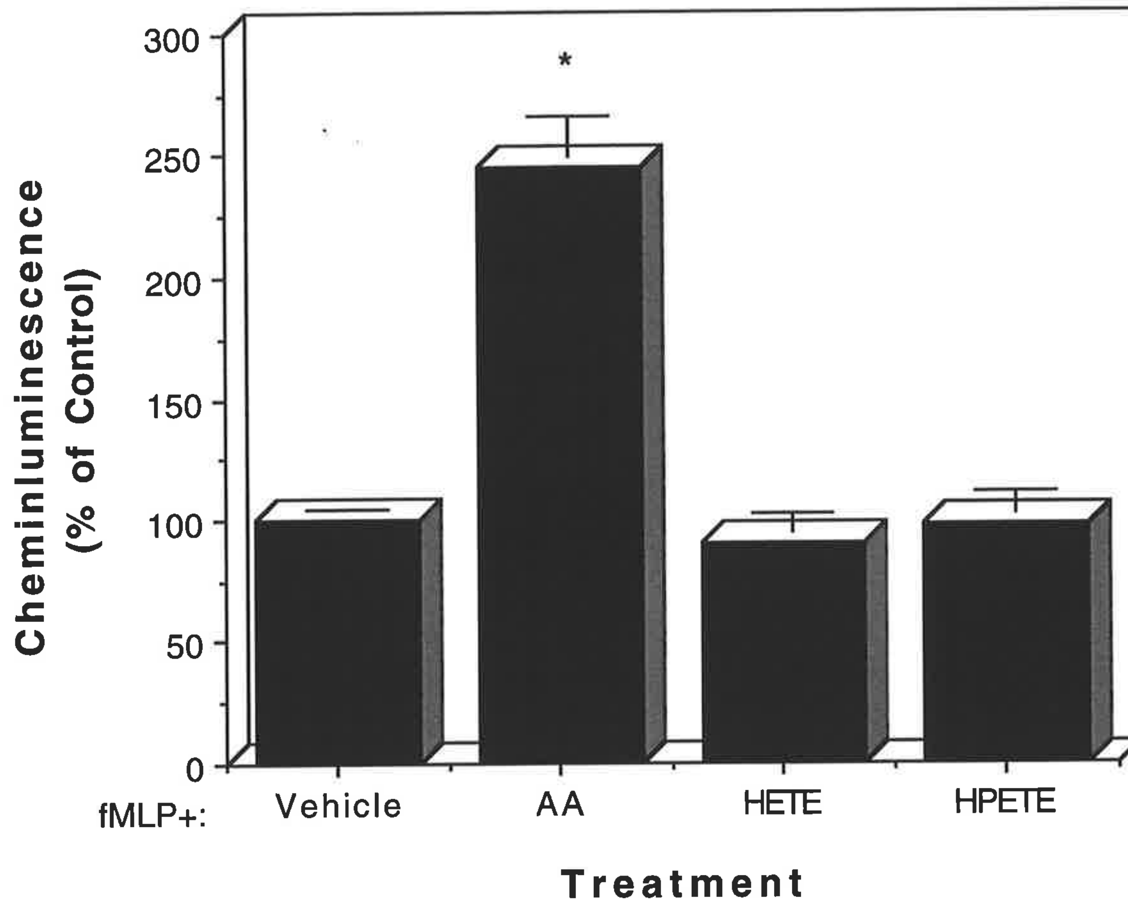


Fig 5.5 The effect of hydroxylated and hydroperoxidated AA on the fMLP-induced superoxide production in human macrophages. Macrophages were treated with 5μM of fMLP and 30μM each of AA, HETE, HPETE or an appropriate amount of vehicle control. The superoxide produced from these cells was measured by the lucigenin-enhanced chemiluminescence assay. The data are presented as the mean±sem of 3 separated experiments and as percentage of the fMLP response. *: $P<0.05$ for the difference between the effect of AA compared with the vehicle control.

5.6. Summary

The data demonstrate that the structure and chain length of the polyunsaturated fatty acids play critical roles in their ability to show synergistic responses with fMLP in the macrophage respiratory response. An eighteen carbon fatty acid was found to be active if it contained more than two double bonds. Thus, while LA (2 double bonds) was ineffective, GLA (3 double bonds) caused a significant increase in the fMLP-induced chemiluminescence response. However AA, EPA and DHA were significantly more active than GLA. These fatty acids all contain more carbon atoms and double bonds than GLA. The importance of unsaturation for the fatty acids to show this activity was also evident from results of studies which examined the activity of the saturated fatty acids, 20:0, 14:0 and 16:0. These saturated fatty acids failed to enhance the fMLP-induced macrophage chemiluminescence response.

The importance of the carboxyl group was evident from studies which examined the effects of the methyl ester forms of DHA and AA on the fMLP-induced response in macrophages. While AA and DHA were synergistic with fMLP on the chemiluminescence response, the methyl ester derivatives showed no activity. Furthermore, maintaining the same number of carbon atoms and degree of unsaturation but introducing an internal hydroxy or hydroperoxy group into the carbon chain of AA resulted in a complete loss of activity of the fatty acid.

Chapter 6

The Activation of PKC by Polyunsaturated Fatty Acids in Macrophages

6.1 Introduction

Protein kinase C (PKC) is a family of serine/threonine protein kinases. The kinases are classified into three groups, (1) classical PKC (α, βI, βII and γ); (2) novel PKC (δ, ε, θ, η and μ) and (3) atypical PKC (ζ, τ and λ). The classical PKC isozymes are activated by the combination of a phospholipid, calcium and diacylglycerol, and the novel PKC isozymes require phospholipid and DAG for activation. The atypical forms require only a phospholipid (reviewed in chapter 1). Activation of PKC is required for a range of macrophage activities, for example activation of the NADPH oxidase which leads to the generation of reactive oxygen radicals (Robinson and Badway, 1994).

Activated PKCs phosphorylate $p47^{Phox}$, a key component of the NADPH oxidase complex, which, in neutrophils and macrophages, has been demonstrated to translocate from the cytosol to the membrane (Thelen *et al,* 1993; Robinson and Badway, 1994). The assembly and activation of the NADPH oxidase complex in the cell membrane elicits the respiratory burst of phagocytic cells (Myers *et al,* 1985). Impairment of PKC integrity or acceleration of PKC degradation caused by the infection of macrophages with Parainfluenza-3 virus, *Legionella pneumophila, Plasmodium falciparum* and *Leishmania donovani* leads to a defect in superoxide production and an inability of these cells to eliminate the pathogen (Dyer *et al,* 1994; Olivier *et al,* 1992; Jacob *et al,* 1994; Schwarzer *et al,* 1993).

Human macrophages express PKC isozymes α (mainly in the cytosol), β (equally distributed in the cytosol and the membrane), little to no ε, δ, γ and ζ, (Chang *et al,* 1993). The phorbol ester, phorbol 12-myristate 13-acetate (PMA) was found to cause PKC α isozyme to translocate from the cytosol to a particulate fraction in macrophages (Chang *et al,* 1993). PMA has been found to mimic the action of diacylglycerol, but activate PKC to a greater degree. Previous studies have shown that PMA binds to PKC C1 region in the regulatory domain, and results in PKC undergoing a conformational change and activation. The identified role of the PKC isozymes in macrophages are summarised in table 6.1.

Fatty acids may induce their effects on macrophages by either directly activating components of the NADPH oxidase or indirectly via activation of PKC. *In vitro* investigations have shown that *cis*-fatty acids, arachidonic, oleic, linoleic, linolenic and docosahexaenoic acids stimulate the activity of PKC α, β, γ, ε and ζ isozymes purified from rat brain in the presence of very low level of calcium and/or PS, whereas saturated and *trans*-fatty acids are without effect (Mcphail *et al,* 1984; Murakami *et al,* 1985&1986; Sekiguchi *et al,* 1987&1988; Nakanishi *et al,* 1993; El Touny *et al*, 1990; Koide *et al*, 1992; Hardy *et al,* 1994b).

Table 6.1 Role of PKC isozymes in monocytic cell functions stimulated by various agents

PKC	Stimulators	Effects	Cell types
cPKC: α	PMA	↑differentiation to macrophages	Mouse myeloid progenitor cells HL-60
	PMA	↑NADPH oxidase activation	MPM
	L. pneumophila	↓ Superoxide production by PMA	Human monocytes
β	LPS	↑Translocation to particulate fraction	TG-elicited MPM
	PMA	↑Superoxide production and AA metabolism	Liver macrophages
	PMA	↑NADPH oxidase activation	MPM
	L. pneumophila	↓ Superoxide production by PMA	Human monocytes
	PMA	↑Differentiation to macrophages	HL-60
γ	PMA	↑NADPH oxidase activation	MPM
nPKC: ε	LPS, IFN-γ	↑ Nitric oxide production	J774
	IL-4	↓ Nitric oxide production	J774
	PMA	↑NADPH oxidase activation	MPM
δ	zymosan	↑PI hydrolysis and PGE_2 formation	Liver macrophages
	PMA	↑Differentiation to macrophages	Mouse myeloid progenitor cells
	PMA	↑NADPH oxidase activation	MPM

cPKC: classical PKC; nPKC: novel PKC: MPM: murine peritoneal macrophages; J774: murine macrophage cell line; TG: thioglycollate; IL-4: interleukine 4; PI: phosphoinositide; ↑: induced; ↓:decreased. (Compiled from: Mischak *et al*, 1993; Duyster *et al*, 1993; Sharma *et al*, 1991; Jacob *et al*, 1994; Fujihara *et al*, 1994; Sands *et al*, 1994; Shinji *et al*, 1994; Tonetti *et al*, 1992 and 1994)

The following investigation addressed the question of whether *cis*-polyunsaturated fatty acids activate PKC in intact human macrophages and its significance to the PUFA-enhanced macrophage superoxide production induced by other agonist.

6.2 The stimulation of total PKC translocation by polyunsaturated fatty acids

Monocytes were isolated and purified from peripheral blood of healthy donors as described in Materials and Methods (Chapter 2). Monocytes were differentiated to macrophages by culturing on cytodex beads for 4-5 days (Kumaratilake and Ferrante, 1988). The cells (5 $\times 10^6$ in 5 ml of serum free medium) were treated with 30μM of either DHA (*n-3*) or AA (*n-6*), 100nM of PMA or vehicle (0.1% ethanol or 0.02% DMSO as appropriate, v/v) for 5 min at 37°C. The cells were sonicated and centrifuged (100,000g at 4°C) as described in Materials and Methods (Chapter 2), and PKC in the particulate fractions was extracted with 2% Triton X-100 (v/v). After adsorption onto DE52 ion exchange beads, PKC was eluted with 120 mM NaCl. The activity, a measure of the amount of PKC in the particulate fraction, was determined by the phosphorylation of histone type IIIS in the presence or absence of calcium and phosphatidyl-l-serine (PS) (see Materials and Methods, Chapter 2). In some experiments, the activity of PKC in the cytosolic fractions was also measured.

The results show that 30μM of DHA (*n-3)* or AA (*n-6*) caused a redistribution of PKC in the cells from the cytosol to a particulate fraction by 5 min incubation (Fig 6.1). Total particulate fraction-associated PKCs were increased by 2 fold over the control value by the fatty acids. The amount of PKC translocated by these fatty acids was less than that achieved by 100nM PMA (Fig 6.1). The increase in particulate fraction-associated PKC was associated with a loss of PKC from the cytosolic fractions (Fig 6.1).

To determine the time related effects of PUFA on PKC translocation, macrophages were treated with 30μM of DHA or vehicle for up to 30 min and PKC translocation was determined as described above. Translocation of PKC to the particulate fraction was observed at 1 min after incubation with DHA and reached a maximum at 5 min (Fig 6.2). The level of particulate fraction-associated PKC rapidly declined after this so that by 10 min, the level of PKC in the particulate fraction had returned to pre-stimulation levels. Although not marked, there was a reduction in the level of PKC in the cytosol at 1 and 5 min after DHA stimulation (Fig 6.2).

6.3 The translocation of individual PKC isozymes by polyunsaturated fatty acids in macrophages

Monocyte-derived macrophages at 3 $\times 10^7$ cells per treatment were incubated with 30μM of DHA or vehicle for 5 min. Particulate fractions from these cells were prepared and PKC

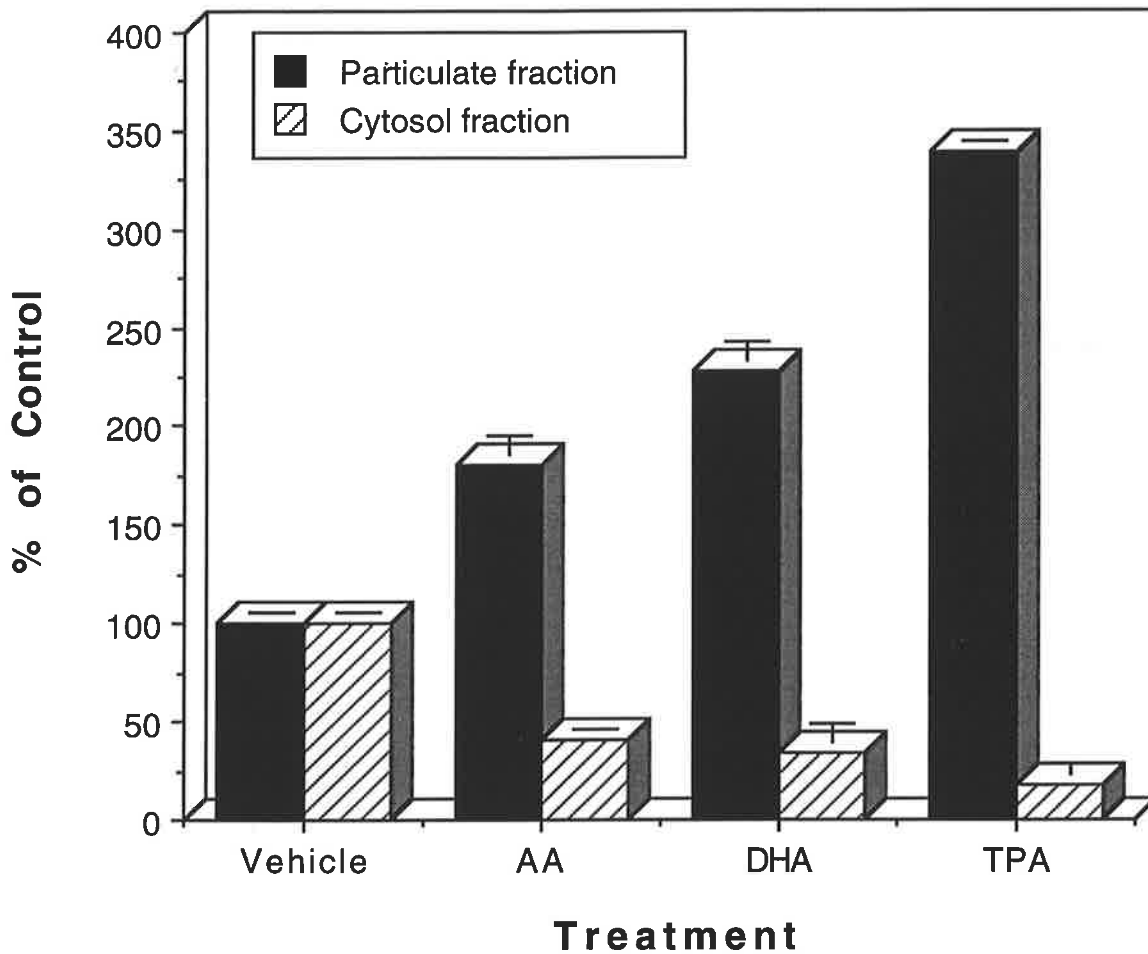


Fig 6.1 The effect of AA (n-6) and DHA (n-3) on the redistribution of PKC in macrophages. The cells were stimulated with 30μM of AA, DHA, 100nM of PMA or vehicle control for 5 min. The amount of PKC in the cytosolic or particulate fractions was measured by phosphorylation of histone type IIIS. The results are presented as range from two experimental runs, each conducted in duplicate.

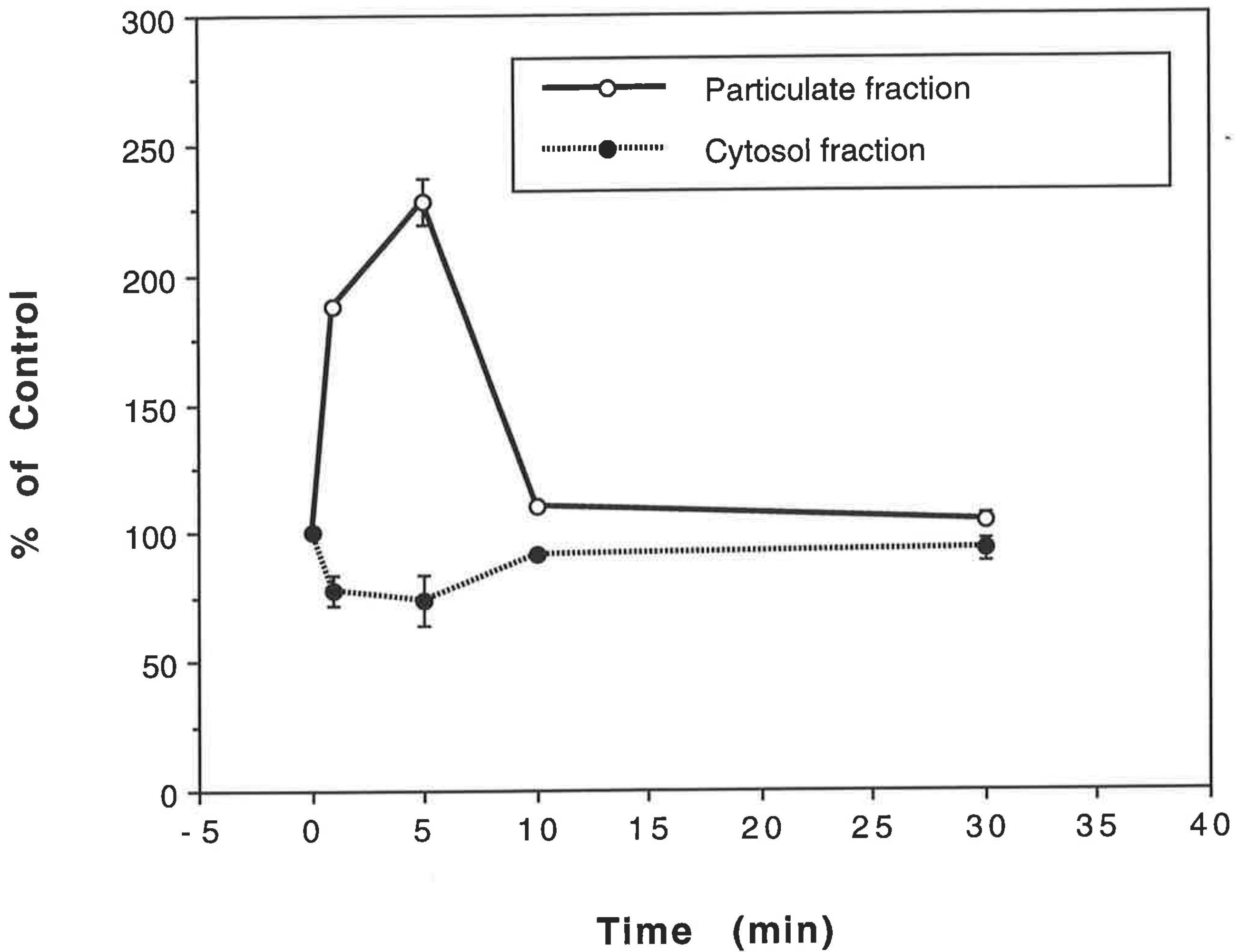


Fig 6.2 Kinetics of PKC translocation in macrophages treated with DHA. The cells were treated with 30μM DHA for the times indicated and PKC translocation determined as describ:d in Chapter 2. The amount of PKC in either cytosolic or particulate fraction was determined by enzyme activity. The results are presented as range of two experimental runs, each conducted in duplicate.

was extracted as described above. After the addition of Laemmli buffer, proteins were separated by SDS polyacrylamide gel electrophoresis, transferred to reinforced nitrocellulose and immunoblotted with polyclonal antibodies to human PKC isozymes. The immunocomplexes were detected by enhanced chemiluminescence (see Chapter 2, Materials and Methods).

The data presented in Fig 6.3 show that in unstimulated macrophages there was little or no PKC α, βI, ε associated with the particulate fraction. However a substantial amount of PKC βII was found to be associated with the particulate fraction in unstimulated macrophages. Stimulation of macrophages with 30μM of DHA for 5 min significantly increased the level of PKC α, βI, βII and ε isozymes in the particulate fraction (Fig 6.3). The PKC immunoreactive bands were scanned and quantified by laser densitometry. The data illustrate that the peak levels of particulate fraction-associated PKC α, βI, βII and ε following stimulation with DHA were 2.4, 2.7, 2.4 and 2.6. folds respectively over the vehicle controls (Table 6.2). PKC ζ was not detected in any of the macrophage fractions (data not shown).

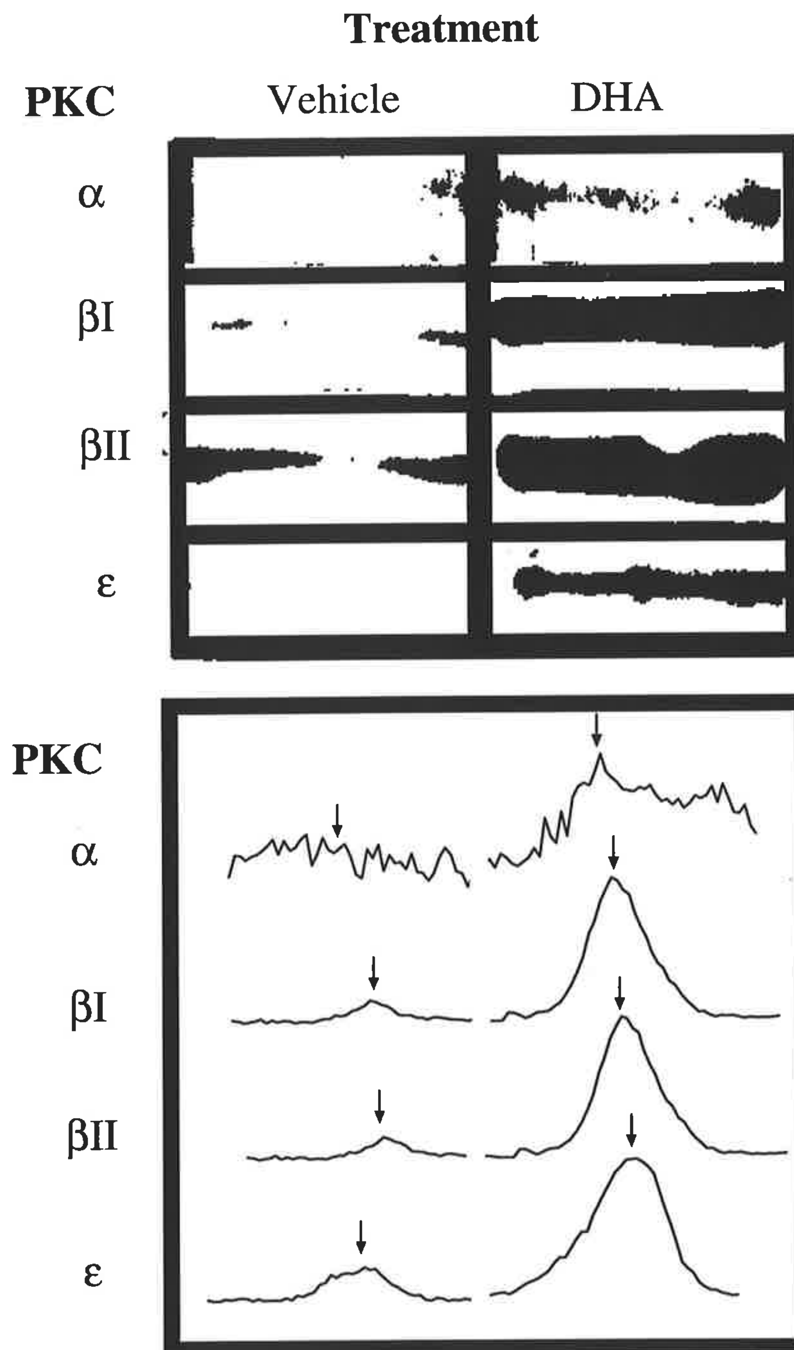


Fig 6.3 Immunoblots of particulate fraction-associated PKC isozymes in extracts from DHA-stimulated macrophages. Macrophages were stimulated with 30μM of DHA for 5 min. The particulate fractions were prepared and PKC was extracted. Proteins were separated by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose. PKC isozymes were then stained with anti PKC α, βI, βII and ε antibodies. The immunocomplexes were detected by enhanced chemiluminescence. The lower panel shows profiles of immunoreactive bands following densitometric analysis of the blots from control cells (arrows on the left) or from DHA-stimulated cells (arrows on the right).

Table 6.2 Translocation of PKC isozymes in macrophages by DHA

	Vehicle	DHA	P value
PKC α	78.8±41.6	190.7±93.8	<0.05
PKC βI	223.4±296.7	594.3±172.1	<0.02
PKC βII	294.8±296.7	697.7±280.5	<0.02
PKC ε	124.8±40.7	318.8±159.5	<0.05

Particulate fractions extracted from the macrophages treated with 30μM of DHA or vehicle for 5 min were blotted with anti PKC isozyme specific antibodies. The immunoblots were quantified by densitometry (Image Quant, Sunny Vale, CA, USA). The results represent arbitrary units and are the mean±sd of 3 experiments. The difference between DHA and vehicle for each isozyme was analysed by the Student's *t* test for paired data.

6.4 The role of PKC in the PUFA-enhanced respiratory burst in macrophages

Although PUFA was shown to stimulate the translocation of PKC to a particulate fraction in monocytes/macrophages, unlike PMA, PUFA *per se* does not appreciably stimulate the respiratory burst (Chapter 4). But nevertheless the activation of PKC by these fatty acids may be essential in the synergistic response with fMLP or PMA.

Examination of the role of PKC in the PUFA-induced priming is made difficult by the fact that the fMLP or PMA response is also dependent on PKC activation, and therefore experiments utilising either PKC inhibitors or PKC depletion can not resolve this question. Thus, calcium ionophore, A23187, a PKC independent agonist (Chakravarthy *et al,* 1995) was chosen for the following experiment.

Monocytes were co-incubated with DHA and A23187. While each of these alone induced a very poor chemiluminescence response, the co-treatment led to a significant synergistic response (Fig 6.4). The sum of chemiluminescence response obtained with DHA and A23187 alone was 3.4 mV compared to that observed during the co-treatment with two agonists which gave a 22.0 mV response (Fig 6.4). Examination of the rate of chemiluminescence production over the incubation period showed that the synergistic effect was seen over the complete incubation period (Fig 6.4B).

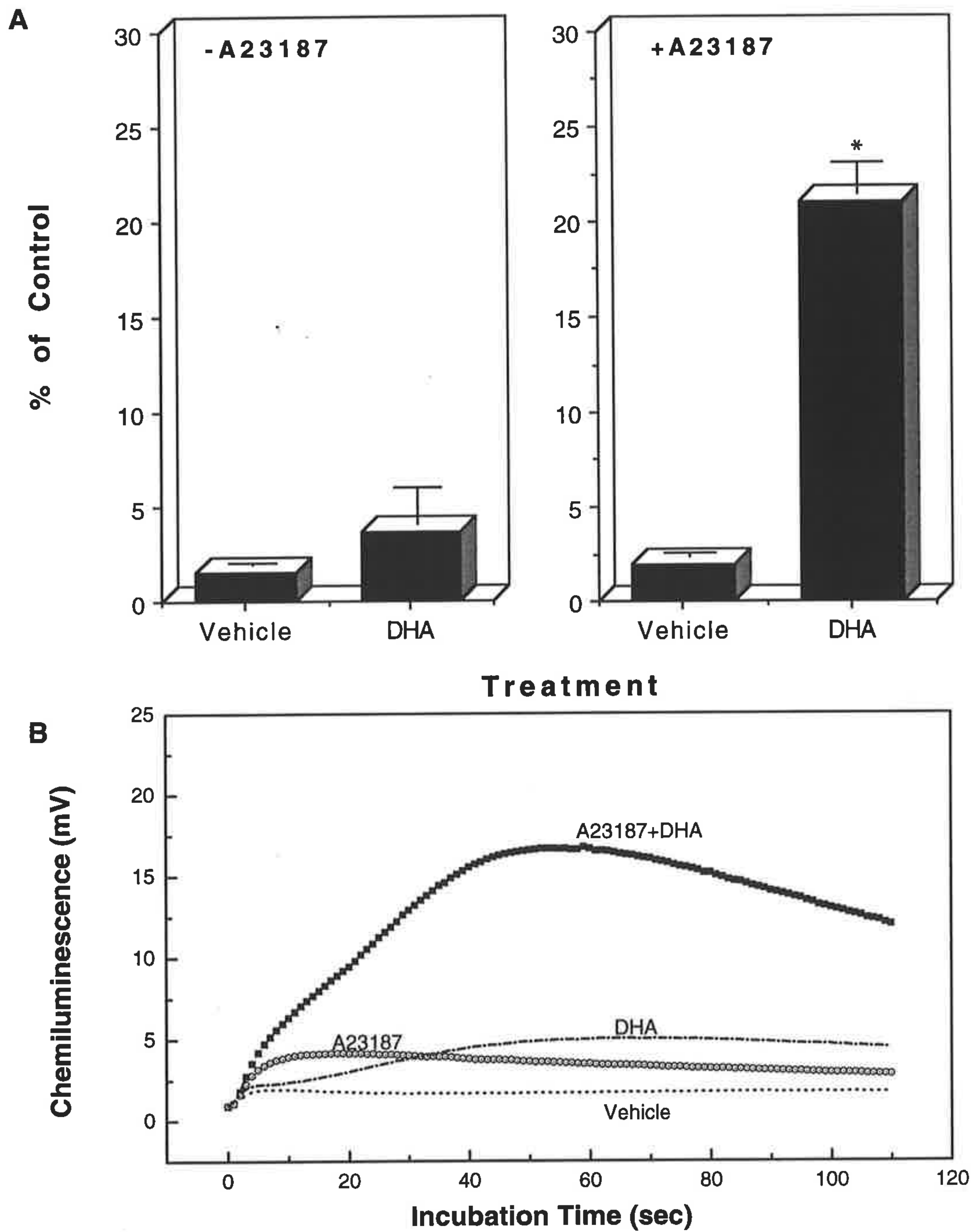


Fig 6.4 The effect of co-addition of DHA and A23187 on the monocyte chemiluminescence response. The cells were treated with 30μM of DHA or vehicle in the presence or absence of A23187. The superoxide produced was measured by the lucigenin-enhanced chemiluminescence assay. The results (A) are the mean±sem of 4 experiments, each carried out with cells from a different individual. *: $P<0.05$ for the difference between A23187+DHA and A23187+vehicle. (B) is a representative experimental run of the rate of chemiluminescence production over the incubation period.

To examine the role of PKC in these experiments, macrophages were pretreated with a 'specific' PKC inhibitor (see chapter 8 discussion), GF-109203X (0.2μM) for 5 min. The ability of these cells to generate superoxide in response to the co-treatment with A23187 and DHA was examined. The results show that the synergistic response to DHA and A23187 was completely abolished by the PKC inhibitor (Fig 6.5). GF 109203X is a structural analogue of staurosporine which binds to the ATP binding site to selectively inhibit the ability of PKC to phosphorylate its substrates. IC_{50} of GF 109203X is 5-70 nM which is 2000 fold less that needed to inhibit PDGF-, EGF- and insulin-induced receptor tyrosine kinases, calmodulin and PKA (Mahoney and Huang, 1994).

Another approach which is widely used to examine for a role of PKC is based on the well-established fact that pretreatment of cells with PMA results in depletion of PKC (Hii, *et al,* 1995b). Macrophages which had been pretreated with PMA (100nM) for 18h contained little or no PKC isozymes as determined in whole cell extracts (data not shown). After this PMA pretreatment, the ability of macrophages to respond to the co-addition of DHA and A23187 was examined. The results show that PMA-pretreated macrophages failed to respond to DHA and A23187 (Fig 6.6).

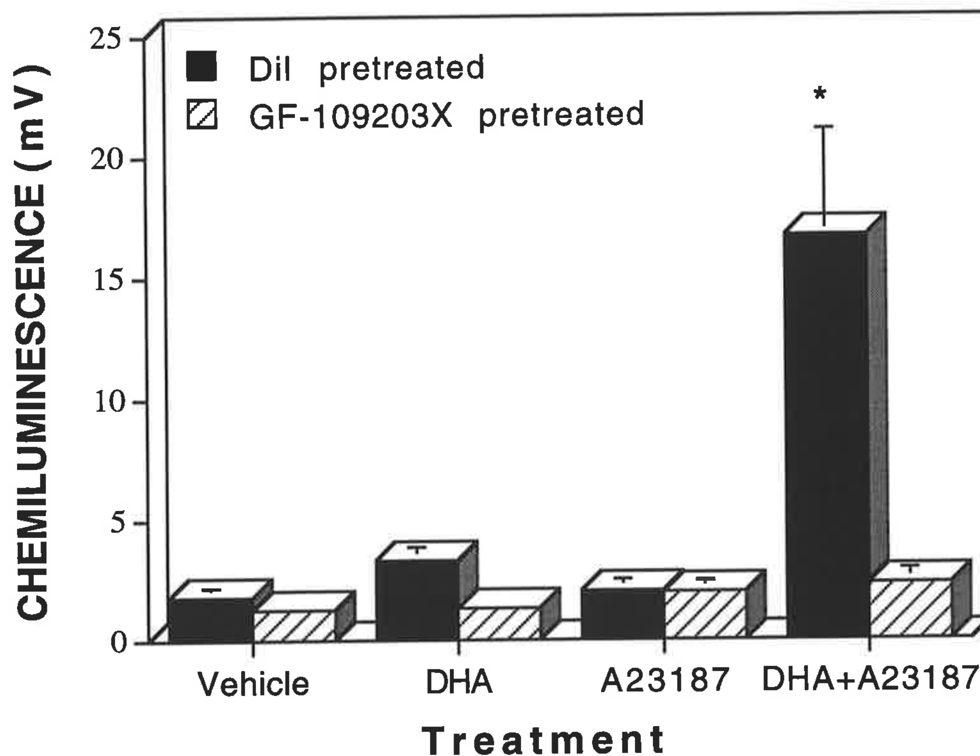


Fig 6.5 The effect of a PKC inhibitor GF-109203X on DHA and A23187-stimulated chemiluminescence response in monocytes. Cells were pretreated with 0.2μM of GF-109203X or diluent (0.02% of DMSO, v/v) for 5 min before the addition of DHA and A23187. The results are the mean±sem of the initial peak rate from four experiments. *: $P<0.05$ for the difference between GF-109203X pretreated and diluent-pretreated monocyte chemiluminescence response by DHA and A23187.

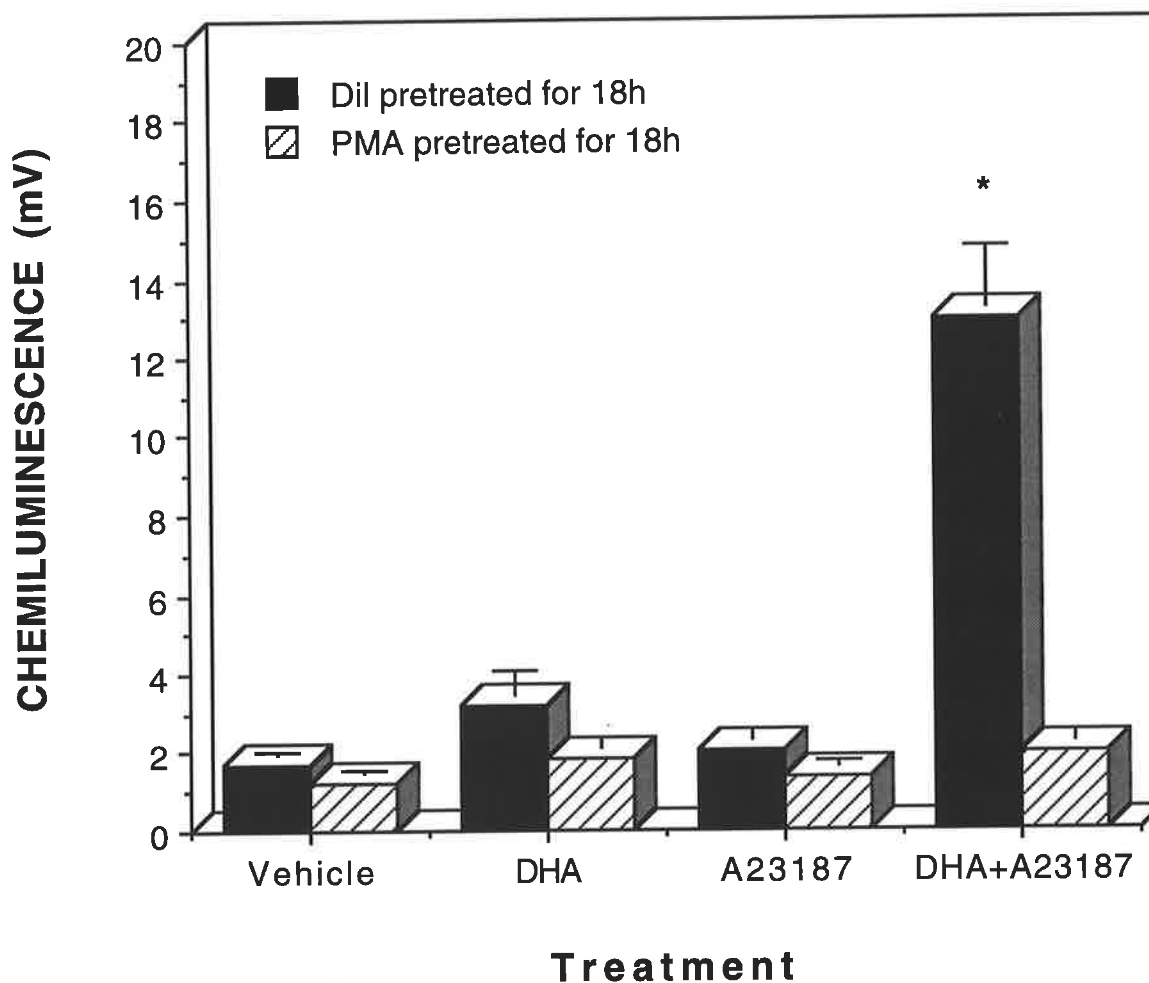


Fig 6.6 The ffect of PMA pretreatment on monocyte chemiluminescence response in the presence of DHA and A23187. The cells were pretreated with 100 nM of PMA or diluent for 18h to deplete the PMA-responsive PKC. Cells were washed and tested for responses to DHA, A23187 or the co-addition of these agents. The results are the mean±sem of the initial peak rates from four experiments. *: $P<0.05$ for the difference between PKC depleted and non-depleted cells when incubated with DHA and A23187.

6.5 The stimulation of PKC translocation by PUFA in neutrophils and WB rat liver epithelial cells

To determine whether the effect of PUFA on PKC translocation is cell-type specific, the ability of PUFA to stimulate PKC translocation was examined in neutrophils and WB rat liver epithelial cells. The cells were incubated with AA for 5 min and the translocation of PKC isozymes to a particulate fraction in each of these cell types was determined by immunoblotting using anti-PKC isozyme specific antibodies. The results show that AA stimulated the translocation of PKC α, βI and βII, and PKC α, ε and δ isozymes to the particulate fractions in neutrophils and WB cells, respectively (Fig 6.7 and Fig 6.8). The redistribution of PKC isozymes in these cells were also quantified by densitometric analysis of the above blots (Fig 6.7 and Fig 6.8). The densitometry profiles indicate increased peak height and/or width due to AA-stimulated translocation of PKC to the particulate fractions in both neutrophils and WB cells (lower panels of Fig 6.7 and Fig 6.8).

6.6 Summary

The PUFAs stimulated the activation and translocation of PKC in macrophages, neutrophils and WB rat liver epithelial cells. In macrophages, PUFA stimulated the

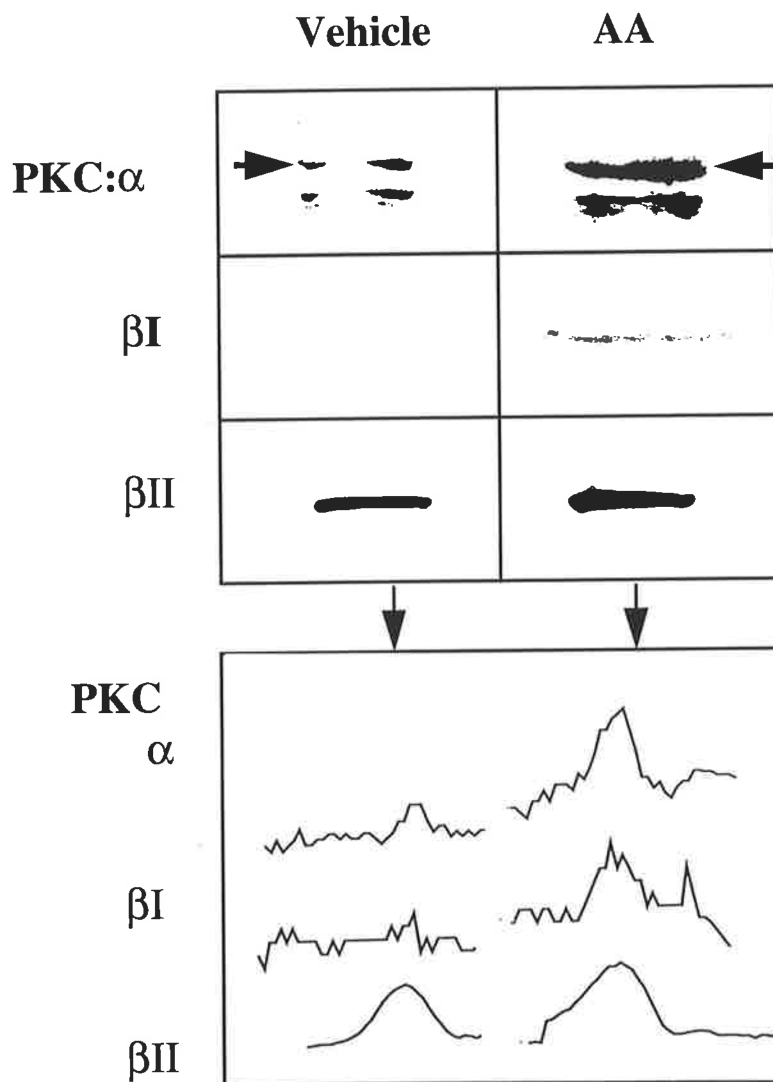


Fig 6.7 Immunoblots of AA induced-translocation of PKC isozymes in neutrophils. Cells were stimulated with 20μM of AA for 5 min. The particulate fractions were prepared and PKC was extracted. Proteins were separated by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose. Proteins were then stained with anti PKC α, βI, and βII antibodies. The immunocomplexes were detected by enhanced chemiluminescence. The lower panel displays the densitometry profiles of the blots. Arrows (left: control; right: AA-treated) indicate the location of immunoreactive PKC bands.

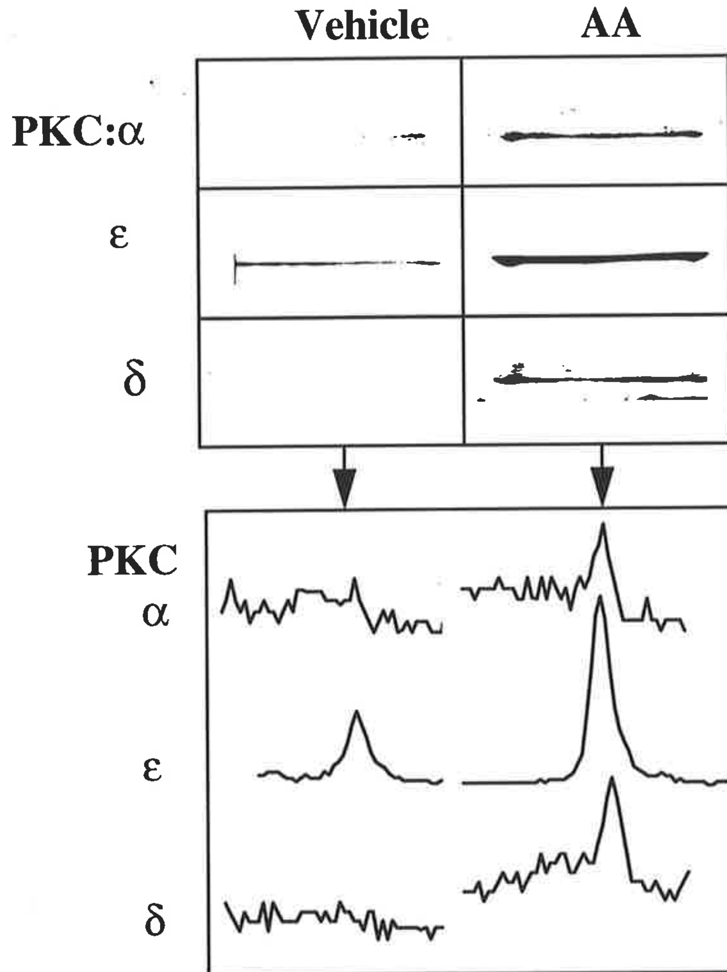


Fig 6.8 Immunoblots of AA induced-translocation of PKC isozymes in WB cells. Cells were stimulated with 20μM of AA for 5 min. The particulate fractions were prepared and PKC was extracted. Proteins were separated by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose. Proteins were then stained with anti PKC α, ε and δ antibodies. The immunocomplexes were detected by enhanced chemiluminescence. The lower panel displays the densitometry profiles of the blots. Arrows (left: control; right: AA-treated) indicate the location of the immunoreactive PKC bands.

translocation of PKC α, βI, βII and ε to a particulate (membrane) fraction. PKC translocation was rapid, peaking at 5 min and returning to pre-stimulation levels by 30 min of incubation with PUFA. A comparison of the ability of fatty acids to stimulate the translocation of various PKC isozymes in macrophages (this study) versus PKC activation in cell free system is summarised in Table 6.3. It shows that the ability of fatty acids to stimulate PKC translocation in an intact cell system is similar to the ability of the lipids to stimulate PKC activity in a cell free system.

PUFA also stimulated the translocation of PKC in human neutrophils and rat liver epithelial WB cells. In neutrophils, PUFA stimulated the translocation of PKC α, βI and βII, and PUFA stimulated the translocation of PKC α, ε and δ in WB cells (Table 6.4).

PUFA and the calcium ionophore, A23187, exerted a synergistic effect on the monocyte/macrophage respiratory burst. On its own, PUFA or A23187 failed to appreciably stimulate the respiratory burst in monocytes/macrophages. Since the effects of A23187 are generally thought to be independent of PKC, this agent, therefore, allows the role of PKC in the action of PUFA to be examined. The results show that treatment of macrophages with a highly selective PKC inhibitor, GF-109203X, completely prevented the synergistic responses. The role of PKC was further demonstrated by the finding that macrophages which had been depleted of PKC by PMA-pretreatment failed to respond in a synergistic manner to the co-addition of PUFA and A23187.

Table 6.3 Activation/translocation of PKC isozymes by unsaturated fatty acids

	Activation in cell free system	Translocation in intact macrophages
cPKC		
α	+	+
β I	+	+
β II	+	+
γ	+	ND
nPKC		
ε	+	+
aPKC		
ζ	+	-

cPKC: classical PKC; nPKC: novel PKC ; aPKC: atypical PKC; +: stimulate/activate; -: not stimulate /not activate; ND: not detected (compiled from the results of this chapter and Quest and Bell, 1994)

Table 6.4 Effects of PUFA on PKC translocation in different cell types

Method of assay	Neutrophils	Macrophages	WB cells
Total PKCs	+	+	ND
Immunoblotting for individual isozymes	α βI βII	α βI βII ε	α ε δ

+: activated, ND: not determined.

Chapter 7

The activation of Mitogen-activated Protein Kinases by Polyunsaturated Fatty Acids in Macrophages

7.1 Introduction

Mitogen-activated protein kinase (MAP kinase) is a family of serine/threonine kinases. Among these kinases, the extracellular signal-regulated kinase (ERK) ERK1, a 44 kDa protein and ERK2, a 42 kDa protein, are two widely studied isoforms. Activated ERKs are located in the cytoplasm in unstimulated cells. Upon stimulation, ERKs are phosphorylated by MAP kinase kinases 1 and 2 on threonine and tyrosine residues in the TEY motif. ERKs also phosphorylate other protein kinases and one of their roles is the transmission of signals into the nucleus. Thus, ERKs are known to translocate into the nucleus in a number of cell types following stimulation (Cobb *et al,* 1994). In the nucleus, ERKs can directly phosphorylate the transcription factor, Elk-1 (Cobb *et al,* 1994). ERK 1 and 2 are, in turn, phosphorylated and activated by raf-1. Raf-1, serving as the MAP kinase kinase kinase, is activated by $p21^{ras}$ or PKC via a mechanism which is still not fully understood (Fig 7.1) (Cobb *et al,* 1994).

It is evident that ERKs are not only involved in mitogen-induced signalling but also serve as a signalling system for mediators of inflammation such as cytokines and lipids (Cobb *et al,* 1994). It has also been suggested that ERKs are likely to participate in the assembly of the NADPH oxidase. Thus, ERKs have been demonstrated to directly phosphorylate $p47^{phox}$ (El Benna *et al,* 1994 and 1996a). Consequently the ability of PUFAs to activate ERKs in macrophages, the role of ERKs in the enhancement of agonist-induced

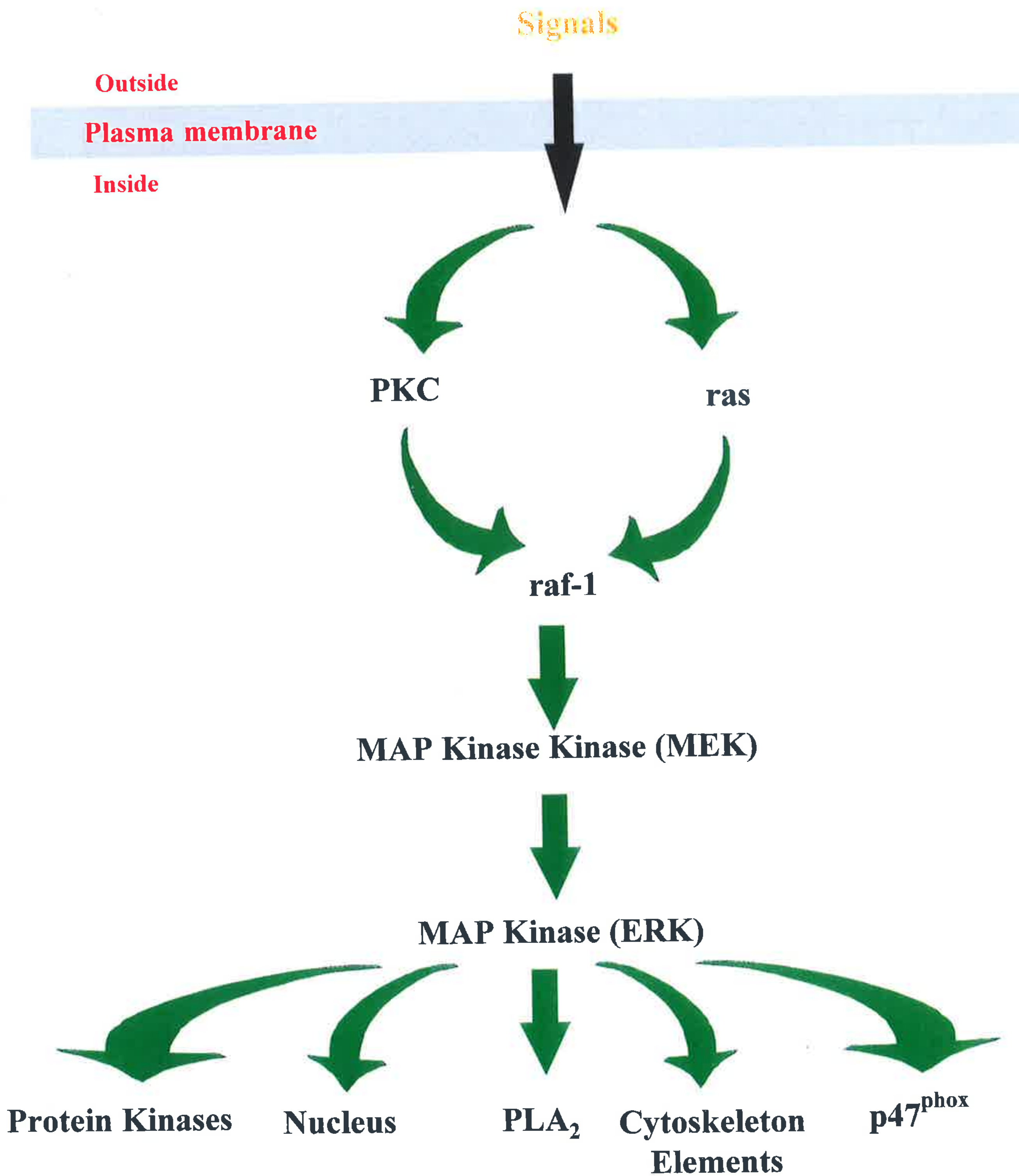


Fig 7.1 The pathway of MAP kinase activation.

respiratory burst and the upstream signalling molecules which are involved in activating ERKs in macrophages were investigated.

7.2 The activation of ERKs by AA in macrophages

Macrophages were serum starved for at least 2h prior to investigation since serum has been reported to stimulate the ERK activity (Gonzalez *et al,* 1993). The cells (5×10^6 in 5 ml) were incubated with 30μM of AA, 100nM of PMA or vehicle for 5 min at 37°C. After sonication, the cytosolic fraction was obtained by ultracentrifugation (100,000g for 30 min) and ERKs were partially purified by adsorption onto phenyl-Sepharose CL4B. After washing the beads twice with 10% ethylene glycol and twice with 35% ethylene glycol, bound material was eluted with 60% ethylene glycol. It has previously been established that ERKs were eluted between 35-60% ethylene glycol (Hii *et al*, 1995b; Anderson *et al*, 1990). The ERK activity in the eluate was assayed by determining the transfer of ^{32}Pi onto myelin basic protein. The results show that AA significantly stimulated the activity of ERKs in macrophages (Fig 7.2). The AA-stimulated ERK activity was approximate 2.8 fold over that observed in control cells and the standard agonist, PMA, induced a 4 fold increase in kinase activity (Fig 7.2).

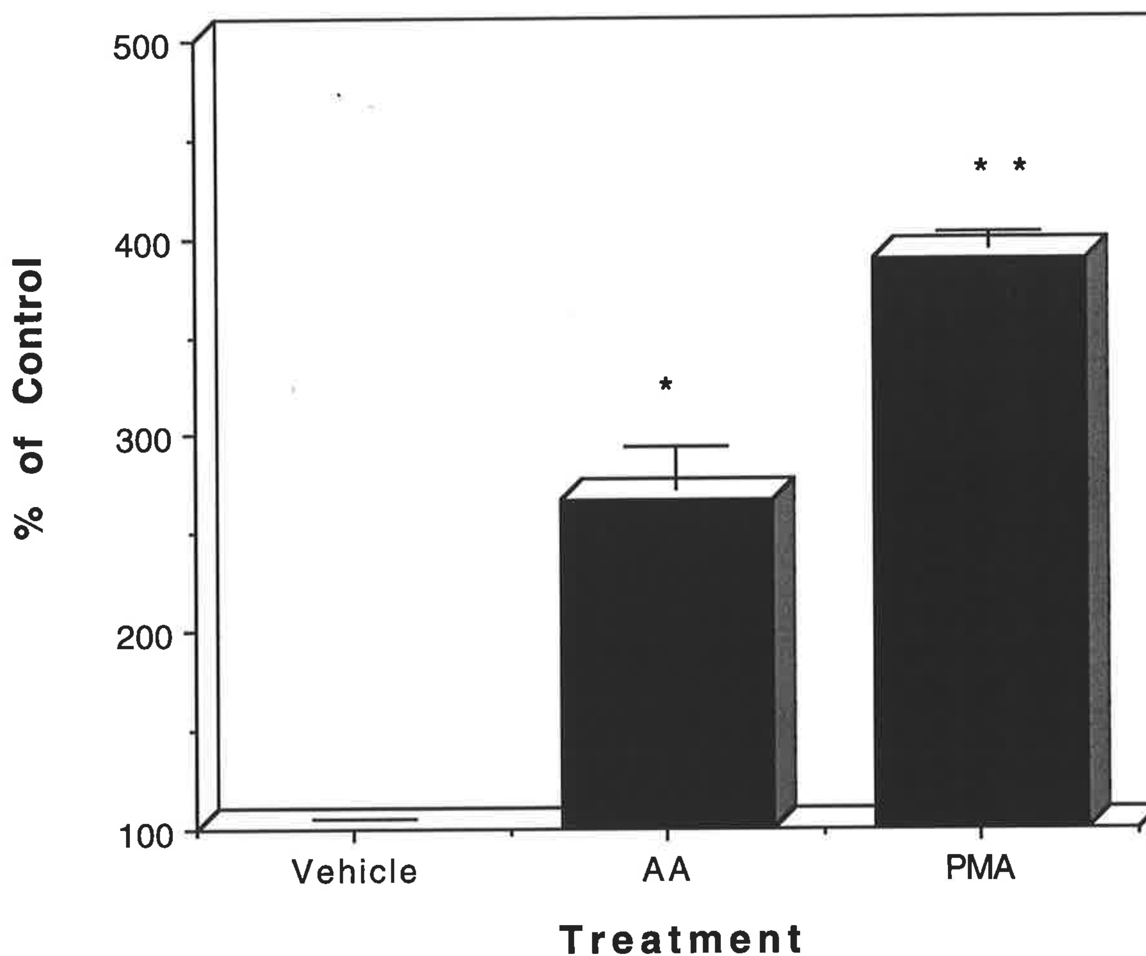


Fig 7.2 The activation of ERKs by AA in macrophages. Macrophages were treated with 30μM of AA, vehicle, or 100nM of PMA for 5 min. The activity of ERKs was assayed by determining the incorporation of ^{32}P into myelin basic protein as described in Materials and Methods. The results, % of vehicle control, are presented as mean±sem of 6 experiments, each conducted in duplicate, each with cells from a different individual. Differences between either AA or PMA and the vehicle treatment: *: P<0.05, **: P<0.01

Activated ERKs display reduced electrophoretic mobility in SDS gels due to phosphorylation. To confirm that AA stimulates the phosphorylation and hence the activity of ERKs, cytosolic fractions from these samples were also subjected to Western blot analysis. Proteins in the fraction (2µg/lane) were separated by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were then incubated with an anti-ERK polyclonal antibody which recognises both isoforms. The immune-complexes were detected by enhanced chemiluminescence. Fig 7.3 shows that there are two isoforms of ERKs in human macrophages with molecular weights of 42 kDa and 43 kDa. Stimulation with PMA resulted in reduced electrophoretic mobility of both bands which migrated with apparent molecular weights of between 42 kDa and 43 kDa and between 43 kDa and 44 kDa. Cells treated with AA also showed increased phosphorylation of the two insoforms of ERKs. This was observed as a loss of the 42 kDa isoform and the appearance of immunoreactive material which migrated with an apparent molecular weight of between 43 kDa and 44 kDa and a minor amount of material with molecular weight of 44 kDa (Fig 7.3). The loss of the 42 kDa isoform is probably due to poor resolution of the phosphorylated bands such that the phosphorylated form of the 42 kDa ERK was not resolved from the phosphorylated 43 kDa ERK which migrated with an apparent molecular weight of between 43 kDa and 44 kDa.

To further confirm that the activity of these two isoforms of ERKs in monocytic cells was stimulated by AA, cytosolic fractions from THP-1 monocytic cells which had been stimulated with 30µM of AA were chromatographed on Mono Q FPLC columns. The

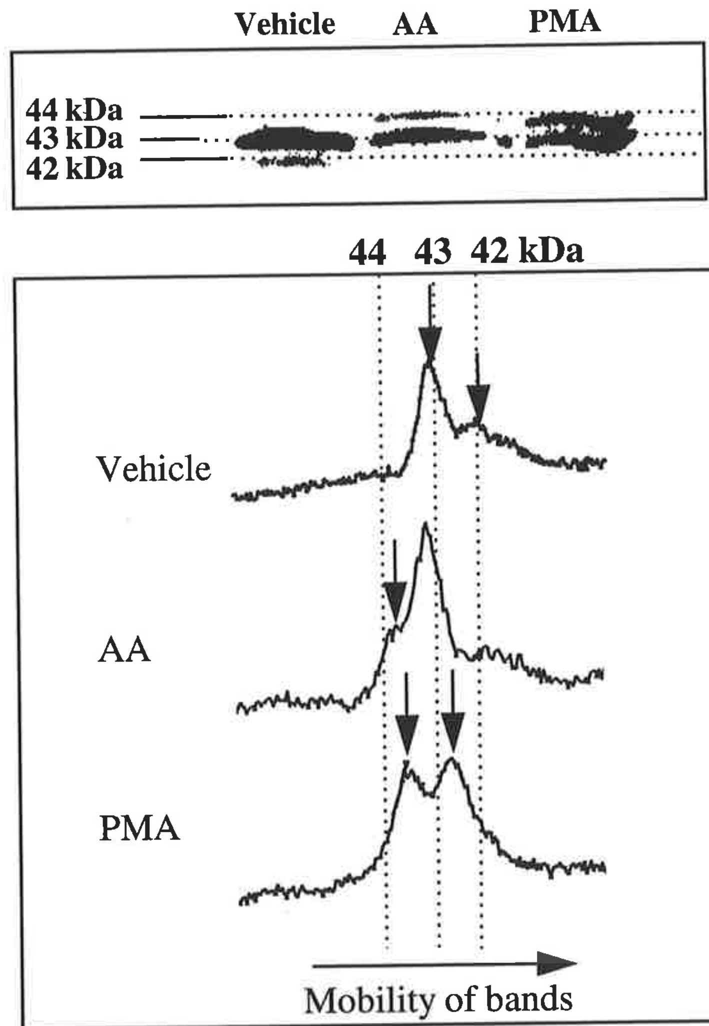


Fig 7.3 The activation of ERK isoforms in human macrophages by AA. Macrophages were treated with 30μM of AA, vehicle or 100nM of PMA for 5 min. Cytosolic fractions were prepared, and ERKs were partially purified and Western blotted. Proteins on nitrocellulose were stained with an anti-ERK antibody and the immune complexes were detected by enhanced chemiluminescence (upper panel). The lower panel displayed the densitometry profile of the immunoreactive bands. Light arrows indicate the location of 42 and 43 kDa bands. Heavy arrows indicate the appearance of slower migrating material.

bound material was eluted with a linear gradient of NaCl (0-500mM). Kinase activity from AA-stimulated cells was resolved into two peaks (fraction 23 and fraction 29), each displaying the ability to phosphorylate myelin basic protein. It has previously been demonstrated that the early peak contains the 42 kDa ERK while the late peak contains the 44 kDa ERK (Hii *et al,* 1995b). The vehicle treated cells showed no activity above basal level (Fig 7.4).

7.3 The kinetics of ERK activation by AA in macrophages

Macrophages were incubated with 30μM of AA for up to 30 min in serum free conditions. At different times, cells were harvested and the ERK activity in the cytosolic fractions was determined as described above. The data show that the stimulation of ERK by AA was transient. Optimal activation of the kinase was seen after 1 min incubation of cells with AA (Fig 7.4). Thereafter, ERK activity steadily declined, reaching basal level by 30 min incubation (Fig 7.5).

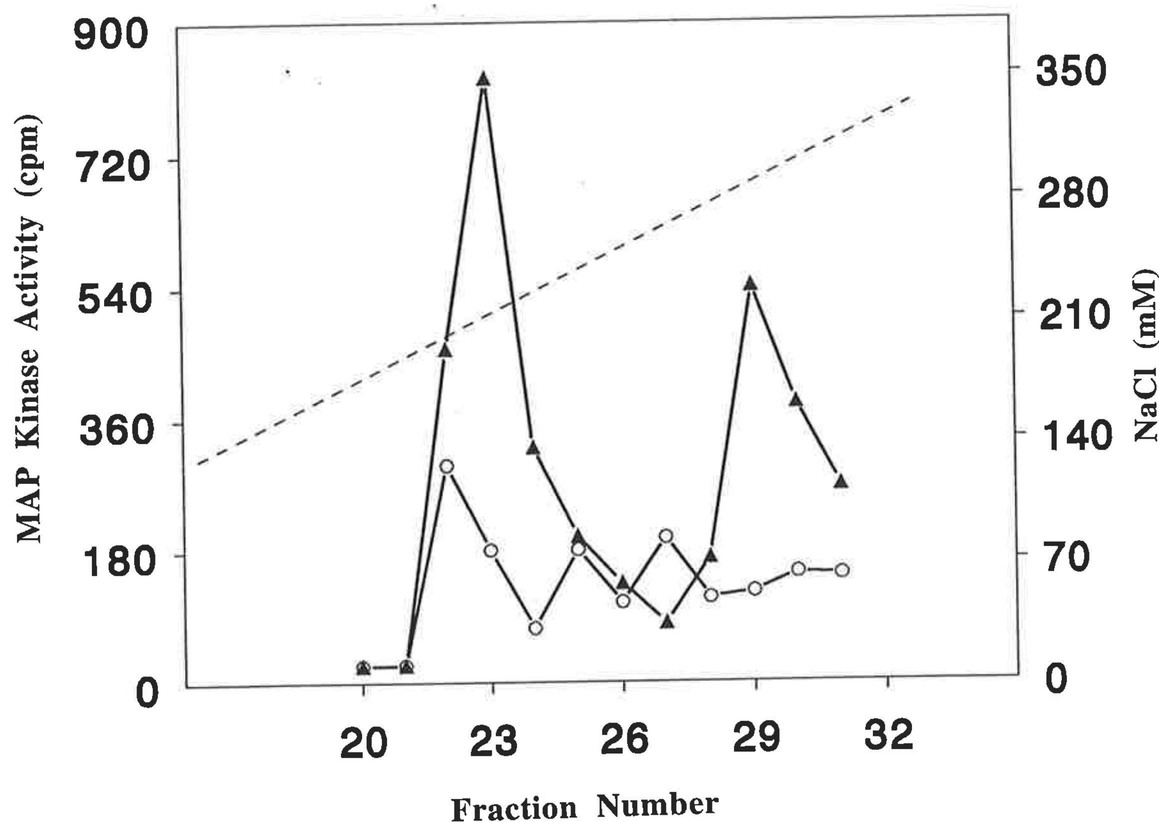


Fig 7.4 Chromatography of ERK isoforms from AA-treated monocytes on Mono Q FPLC. Monocytic THP-1 cells were treated with 30µM of AA (▲) or vehicle control (O) for 5 min. The cytosolic fractions were prepared and chromatographed on Mono Q columns. The columns were washed and the bound protein eluted with a linear NaCl gradient. The fractions were collected and ERK activity determined by measuring the incorporation of ^{32}P into myelin basic protein (detail as described in Materials and Methods).

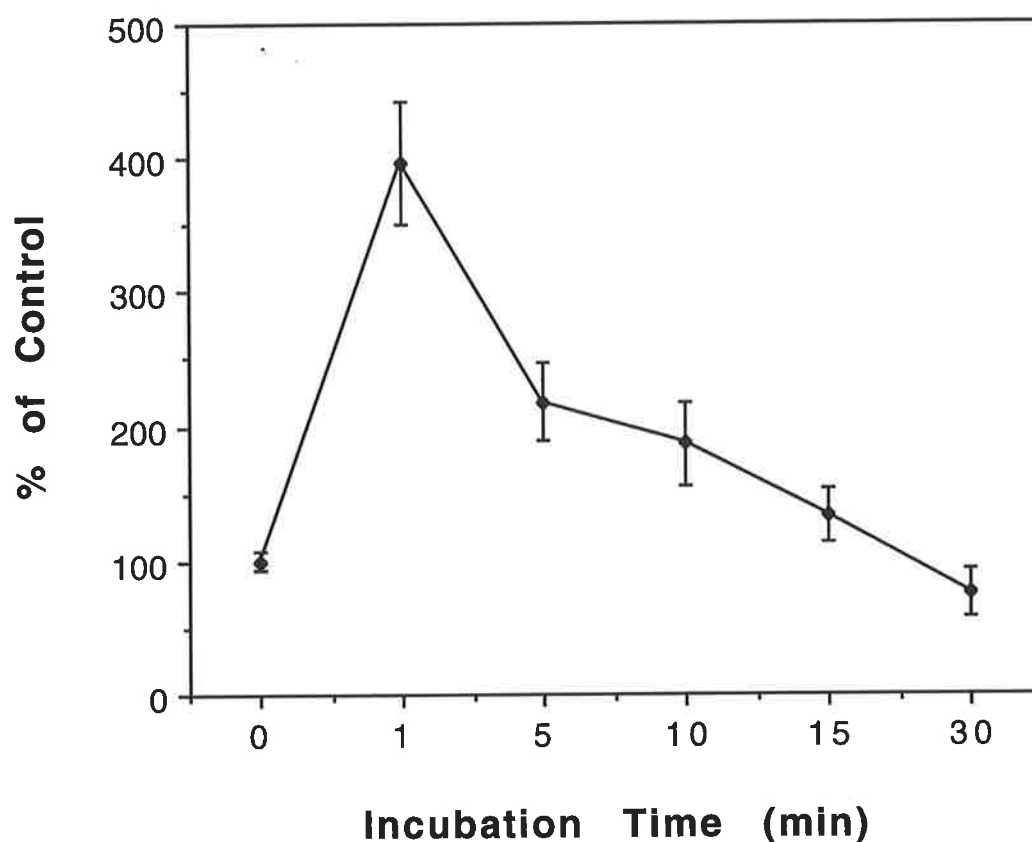


Fig 7.5 Time dependence of ERK activation in macrophages treated with AA. Human macrophages were treated with 30μM of AA or vehicle for up to 30 min. Cells were harvested at the times indicated, and cytosolic fractions prepared and ERKs were partially purified. The ERK activity in these fractions was determined by measuring the incorporation of ^{32}P into myelin basic protein as described in Materials and Methods. The data are representative of 3 experimental runs, each conducted in duplicate.

7.4 The effect of varying the AA concentration on activation of ERKs

A concentration range of 5-30μM of AA was tested for the ability to activate ERK in macrophages. After 5 min treatment of cells with different concentrations of AA, ERK activity in the cytoplasmic fraction was measured by the incorporation of ^{32}Pi into myelin basic protein. The result show that AA dose-dependently stimulated the activity of ERKs (Fig 7.6) and the lowest concentration of AA which stimulated the activity of ERKs in macrophages was 5μM (Fig 7.6).

7.5 The effects of different fatty acid structures on ERK activation

Previous studies, including the above (see Chapter 4) showed that *n-3* PUFA were just as effective as AA in synergizing with fMLP to induce a respiratory burst, but that saturated fatty acids or the methyl ester derivatives of PUFA were not active. In view of this we examined whether differences in fatty acid structures affected the ability of these fatty acids to activate ERKs. Firstly, the *n-3* PUFA, DHA, was compared with AA. Macrophages were treated with 30μM of AA or DHA, or vehicle control for 5 min at 37°C. The activity of ERKs was determined as described above. The results showed that DHA (*n-3*) was as active as AA (*n-6*) in stimulating the activity of macrophage ERKs (Fig 7.7). Similar effects were seen using the human monocytic line, THP-1 cells (Fig 7.7).

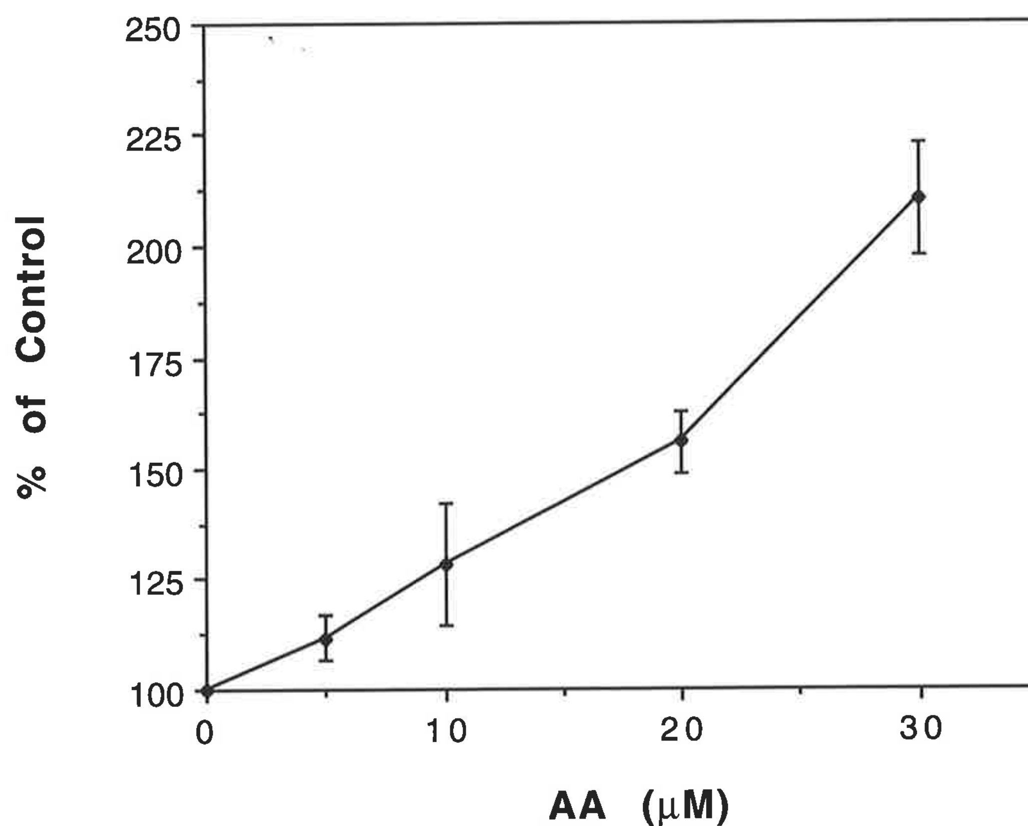


Fig 7.6 The effects of varying the AA concentration on activation of ERKs in macrophages. Macrophages were treated with 5-30μM of AA for 5 min. The cytosolic fractions were prepared and ERKs were partially purified. The ERK activity in these fractions was determined by measuring the incorporation of ^{32}P into myelin basic protein (detail in Materials and Methods). The data are representative of 3 experimental runs, each conducted in duplicate.

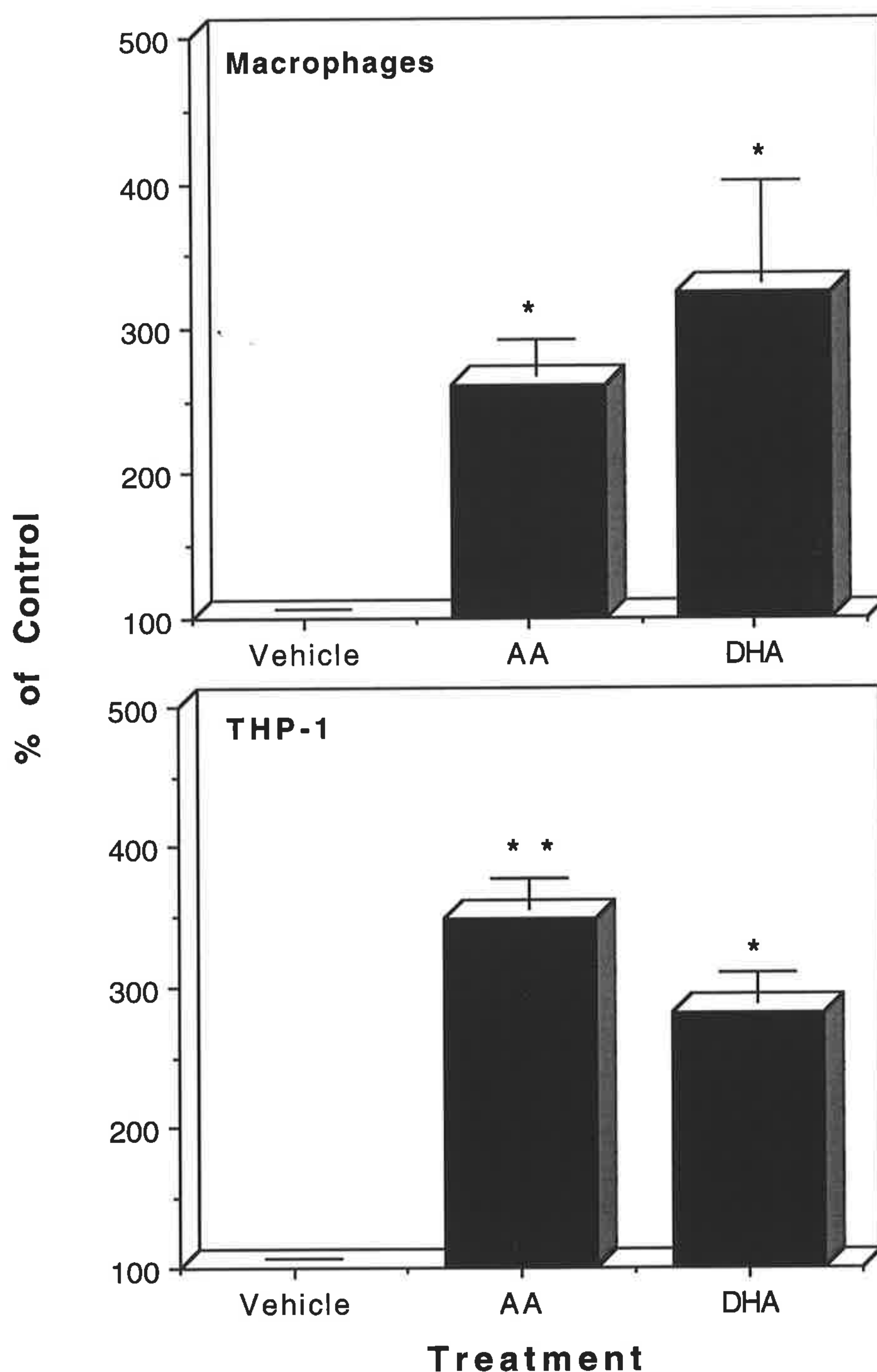


Fig 7.7 The activation of ERKs in macrophages or THP-1 cells by AA and DHA. Macrophages or THP-1 cells were treated with 30μM of AA or DHA, or an appropriate amount of vehicle for 5 min. The cytosolic fractions were prepared and ERKs were partially purified. The ERK activity in the fractions was assayed by the incorporation of ^{32}P into myelin basic protein (details in Materials and Methods). The results are presented as mean±sem of 3-6 experiments, each conducted in duplicate. Differences between either AA or DHA and vehicle control: *: $P<0.05$, **: $P<0.01$.

The ability of the methyl ester forms of DHA or AA, or the saturated fatty acid arachidic acid (20:0) to stimulate the activity of ERKs was also tested in THP-1 cells. Unlike DHA or AA, the methyl ester derivatives of these PUFAs did not significantly stimulate the activity of ERKs in monocytic cells (Fig 7.8). The saturated fatty acid, arachidic acid (20:0), induced a small but non-significant activation of ERK activity (Fig 7.8).

7.6 The role of PKC on the AA-induced activation of ERKs in macrophages

The role of PKC, an upstream regulator of the ERK cascade, in the AA-induced activation of ERKs was investigated. Macrophages were pretreated with a selective PKC inhibitor, GF-109203X (0.2μM) for 5 min, followed by the addition of 30μM of AA and 100nM of PMA or vehicle. The cells were incubated for another 5 min and then the ERK activity in the cytosolic fractions was determined. The data show that GF-109203X totally inhibited the PMA-stimulated ERK activation. The AA-induced ERK activation in macrophages was similarly inhibited by GF-109203X (Fig 7.9).

Further support for a role of PKC was obtained by using macrophages which had been depleted of PKC by pretreatment with PMA. Cells were pretreated overnight with 100nM of PMA and then tested for activation of ERKs by AA. Although the prolonged

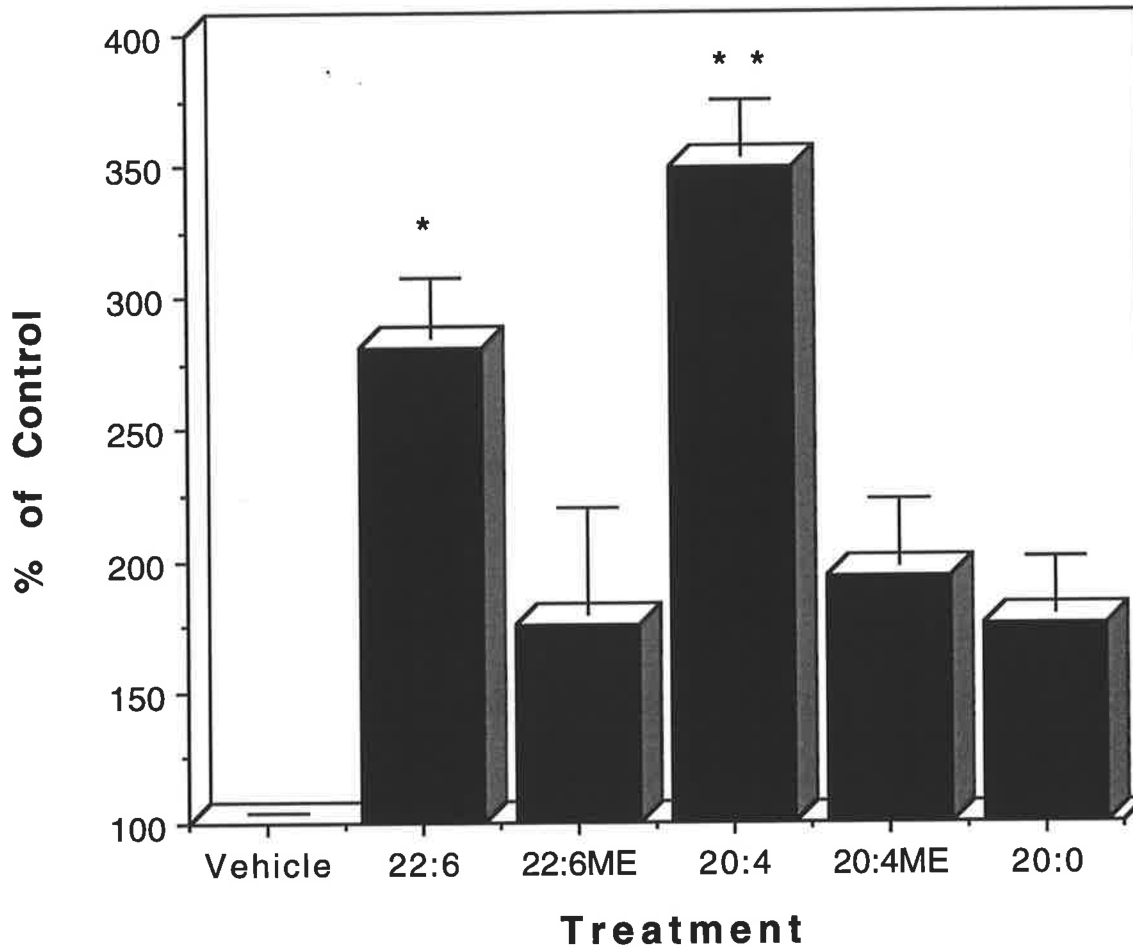


Fig 7.8 The activation of ERKs in THP-1 cells by a saturated fatty acid and the methyl ester of AA (20:4ME) or DHA (22:6ME). Human monocytic THP-1 cells were treated with 30μM of AA (20:4), DHA (22:6) or their methyl esters (ME), arachidic acid (20:0) or vehicle for 5 min. Cytosolic fractions were prepared and ERKs were partially purified. ERK activity was determined by assaying for the incorporation of ^{32}P into myelin basic protein (details in Materials and Methods). The results are presented as the range of four determinations from two experimental runs. Differences between either AA or DHA and the vehicle control: *: $P<0.05$, **: $P<0.01$.

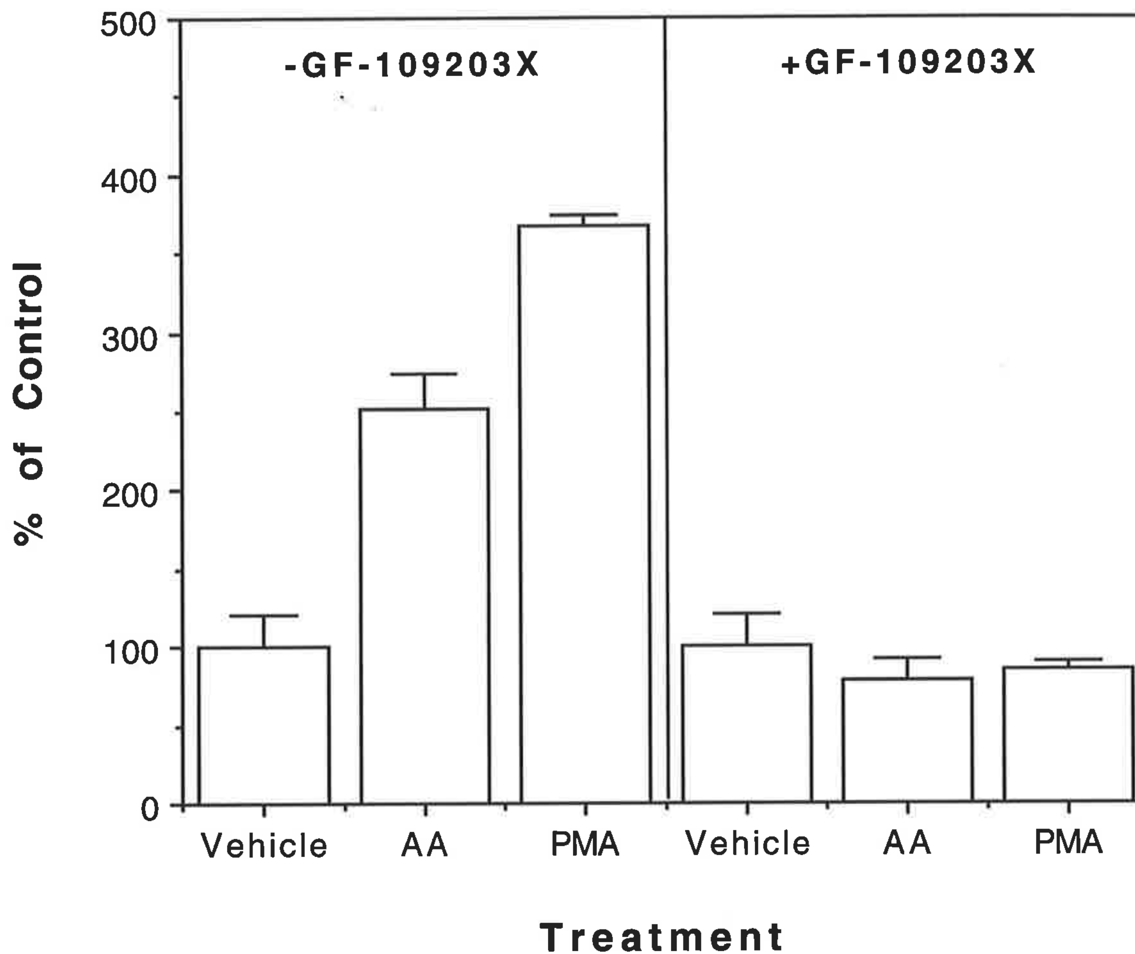


Fig 7.9 The effect of PKC inhibition on the ability of AA to activate ERKs in macrophages. Human macrophages were pretreated with 0.2μM of a specific PKC inhibitor, GF-109203X, for 5 min and then treated with 30μM AA, 100nM PMA or vehicle control. After 5 min of incubation, the cytosolic fractions were prepared and ERKs were partially purified. The ERK activity was determined by assaying for the incorporation of ^{32}P into myelin basic protein (details in Materials and Methods). The results are presented as the range of four determinations from two experimental runs.

treatment with PMA elevated the basal level of ERK activity (from 825±85cpm to 2516±137cpm), no further stimulation of ERK activity by either AA or PMA was detected (Fig 7.10).

7.7 The role of $p21^{ras}$ on the AA- or DHA-induced activation of ERKs in monocytes

Although PKC and the small G protein, $p21^{ras}$, can independently regulate the ERK cascade, studies in Jurkat cells have suggested that stimulation of ERK by PMA, an activator of PKC, is $p21^{ras}$ dependent (Franklin *et al*, 1994; Izquierdo *et al*, 1993). The role of $p21^{ras}$ in PUFA stimulated ERK activity was studied by transiently transfecting monocytic cells, THP-1, with a plasmid carrying dominant negative $p21^{ras}$ ($p21^{ras}$ 17N) under the control of the CMV promoter or a control plasmid (pCMV-Green Lantern) (GIBCO). The dominant negative $p21^{ras}$ construct has been widely used to block signalling via $p21^{ras}$. Cells were transiently transfected using lipofectamine (see Chapter 2 Methods and Materials). The ability of AA to stimulate the activity of ERKs was examined. To determine the efficiency of transfection, cells which had been transfected with pCMV-Green Lantern (GIBCO) or a control plasmid (pCMV-ERK) (a gift from Dr. C.J. Der, North Carolina, USA) were examined for the expression of green fluorescent protein by FACS. It was found that up to 77% (77.17%±5.18, n=3) of the pCMV-Green Lantern-transfected cells expressed green fluorescent protein (Fig 7.11). The fluorescent intensity of the control plasmid-transfected cells was similar to that of

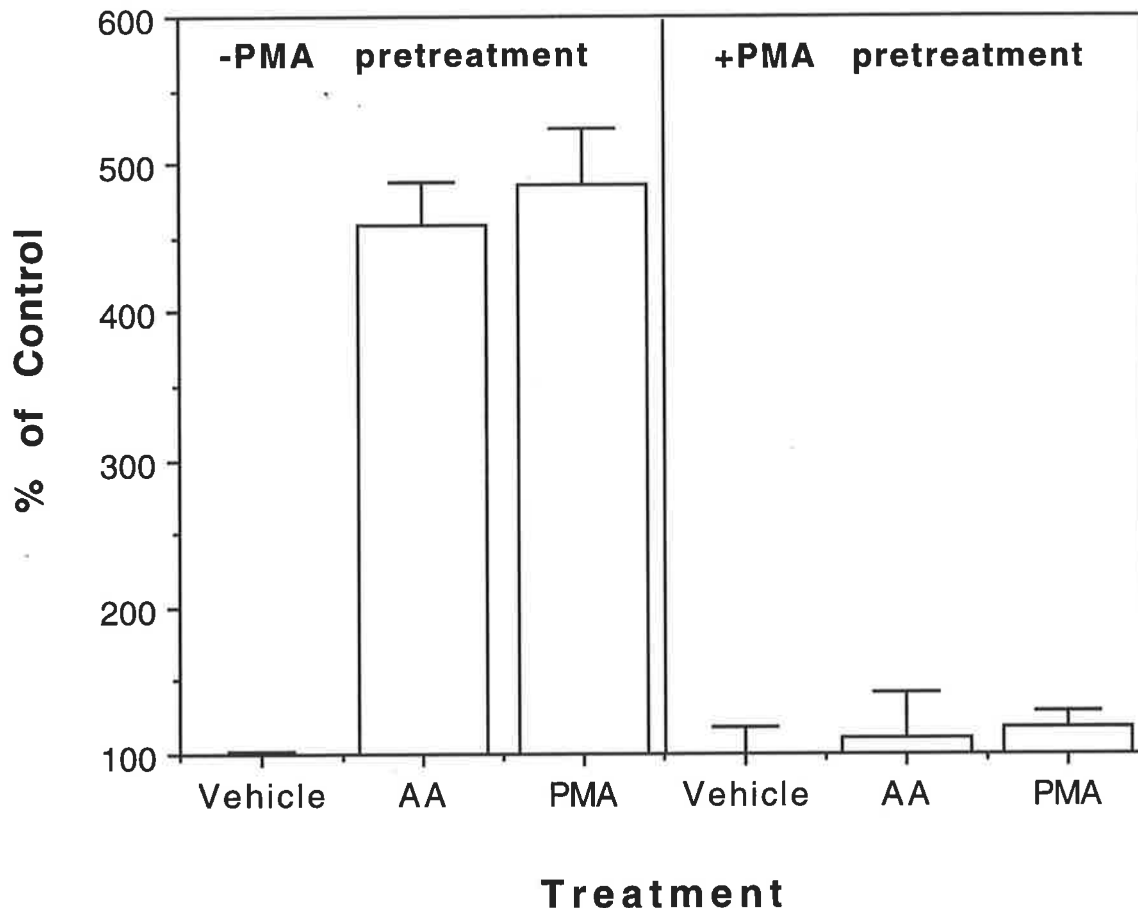


Fig 7.10 The effect of PMA pretreatment (PKC depletion) on the ability of AA to activate ERKs in macrophages. Macrophages were pretreated with 100nM of PMA for 18h and washed before the addition of 30μM of AA, 100nM of PMA or vehicle for 5 min. The cytosolic fractions were prepared and ERKs were partially purified. ERK activity was determined by measuring the incorporation of ^{32}P into myelin basic protein (details in Materials and Methods). The results are presented as the range of four determinations from two experimental runs. Following the 18h pretreatment with PMA the macrophages showed significant loss of total PKC (data not presented).

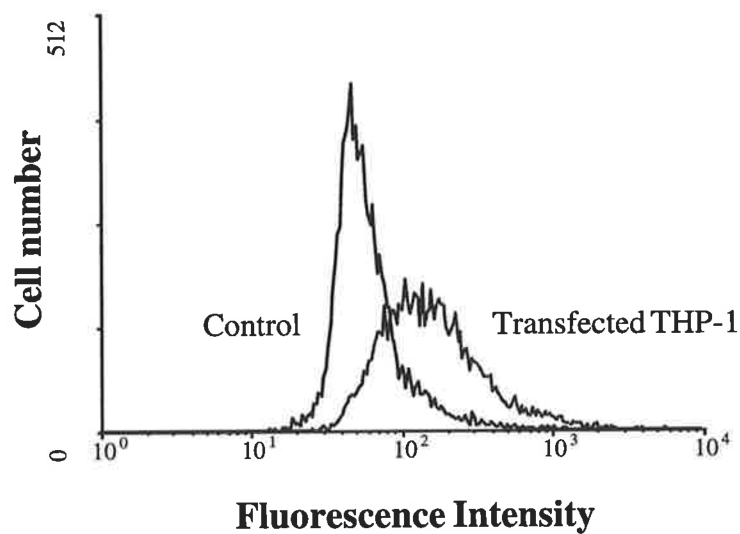


Fig 7.11 The expression of green fluorescent protein in THP-1 cells. Cells were transfected with 1µg of pCMV-Green Lantern or a control plasmid, and then examined for the expression of green fluorescent protein by FACS (details see Chapter 2). The result is the representative of three experimental runs.

nontransfected cells. The results presented in Fig 7.12 show that THP-1 cells transfected with control vector retained the ability to respond to AA in the activation of ERKs. However, cells transfected with the dominant negative p21^{ras} gene showed a poor activation of ERKs by AA (Fig 7.12). The viability of transfected cells was monitored by trypan blue exclusion (which was >90%).

THP-1 cells which were transiently transfected with dominant negative p21^{ras} were also examined in their ability to respond to DHA for activation of ERK. The results show that while DHA caused normal ERK kinase activation in cells which had been transfected with control vector, the response to DHA was significantly reduced in cells which had been transfected with p21^{ras} mutant (Fig 7.13).

7.8 The role of raf-1 on the AA- or DHA-induced activation of ERKs in monocytes

Raf-1 is an upstream kinase in the ERK cascade (Cobb *et al,* 1994). A role of raf-1 in AA-induced activation of ERKs was also investigated. Human monocytic THP-1 cells were transiently transfected with a plasmid carrying either dominant negative raf-1 (raf 1-300) or pCMV-Green Lantern (control). The dominant negative raf-1 was previously shown to block p21^{ras}-dependent activation of ERKs in NIH 3T3 fibroblasts (Cox *et al,* 1994). While transient transfection of cells with control vector did not interfere with

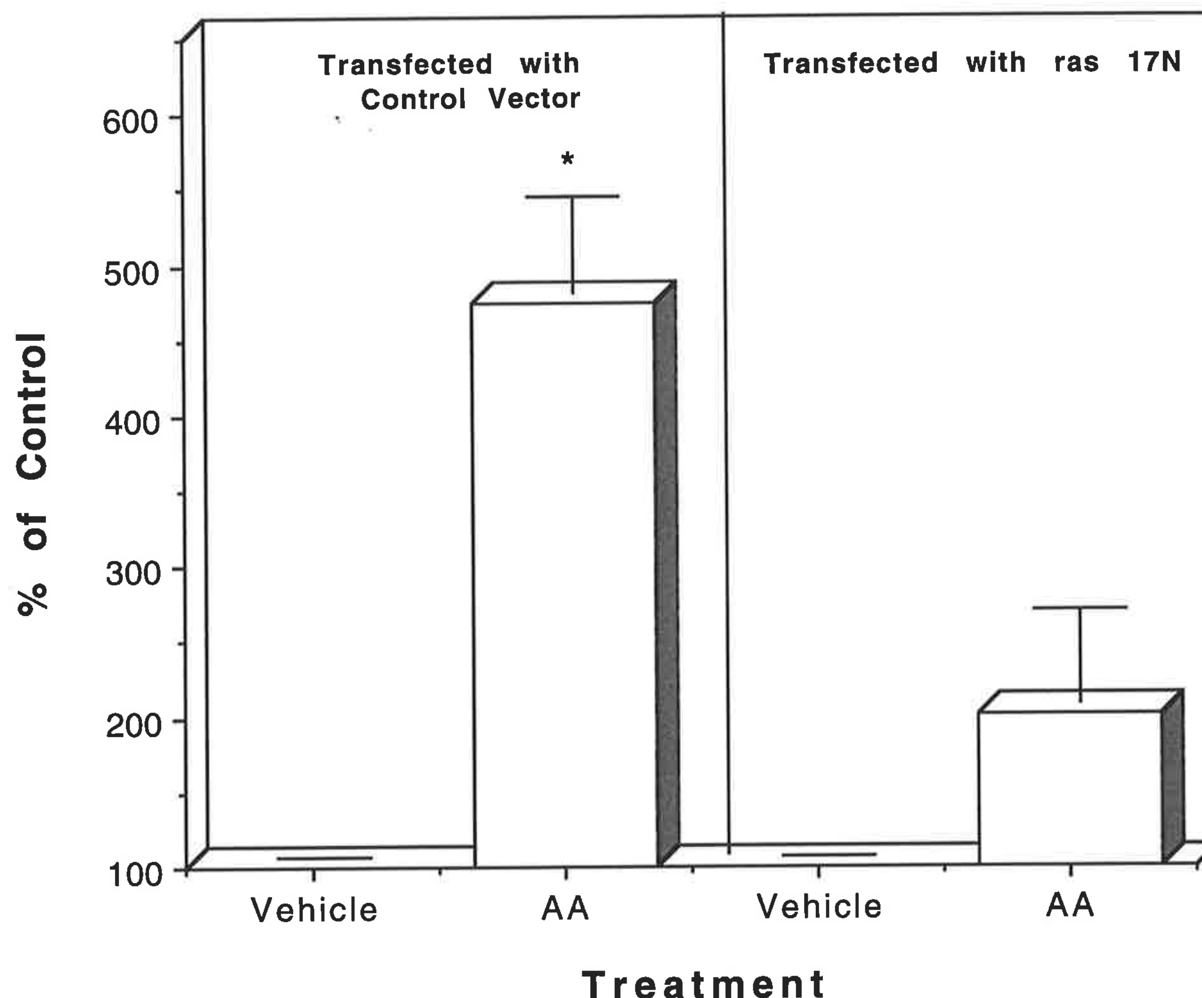


Fig 7.12 The effect of dominant negative p21^{ras} on the ability of AA to activate ERKs in THP-1 cells. The cells were transfected with either dominant negative p21^{ras} (1µg of DNA) or control vector (1µg of DNA) using lipofectamine (see Materials and Methods). The transfected cells were treated with 30µM of AA or vehicle for 5 min and ERK activity was determined by measuring the incorporation of ^{32}P into myelin basic protein (details in Materials and Methods). The results are presented as the mean±sem of 3 experiments, each conducted in duplicate, each with cells from a different individual. *: P<0.05 is the difference between AA and the vehicle treatment.

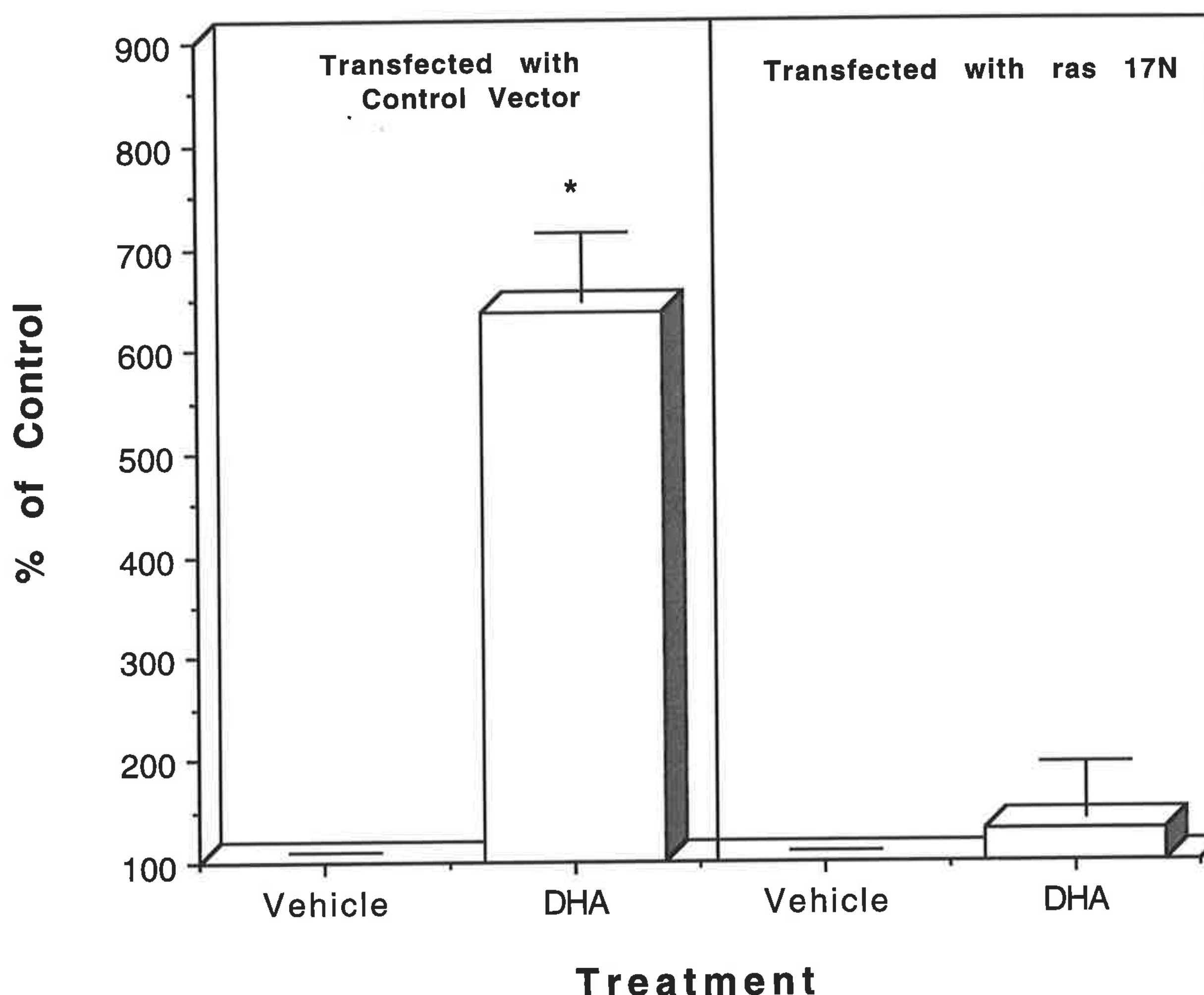


Fig 7.13 The effect of dominant negative p21^{ras} on the ability of DHA to activate ERKs in THP-1 cells. The cells were transfected with dominant negative p21^{ras} (1μg of DNA) or control vector (1μg of DNA) using lipofectamine. The transfected cells were treated with 30μM of DHA or vehicle for 5 min and ERK activity was determined by measuring the incorporation of ^{32}P into myelin basic protein (details in Materials and Methods). The results are presented as the mean±sem of 3 experiments, each conducted in duplicate, each with cells from a different individual. *: P<0.05 is the difference between DHA and the vehicle treatment.

activation of ERKs activity by AA or DHA (Fig 7.14 and Fig 7.15), stimulation of ERKs by AA or DHA was significantly reduced in cells which had been transfected with the dominant negative raf-1 (Fig 7.14 and Fig 7.15).

7.9 The effects of PUFA on activation of ERKs in neutrophils and WB rat liver epithelial cells

Since it has been shown in Chapter 5 that PUFAs stimulate the translocation of PKC in human neutrophils and WB rat liver epithelial cells, the activation of ERKs by PUFAs in these two cell types were also investigated.

The activity of ERKs in both neutrophils and WB cells was found to be stimulated by 20μM of AA (Fig 7.16). The stimulation of ERK activity by AA in WB cells peaked at 5 min of incubation and gradually declined to a basal level after 60 min incubation with AA (Fig 7.17A). However, the effect of AA in neutrophils peaked at 15 min and persisted for at least 30 min of incubation (Fig 7.17B). The PUFA-induced ERK activation was demonstrated to be dependent on the PUFA concentrations (Fig 7.18). The minimal concentration of AA which stimulated the activity of ERKs was 5μM (Fig 7.18).

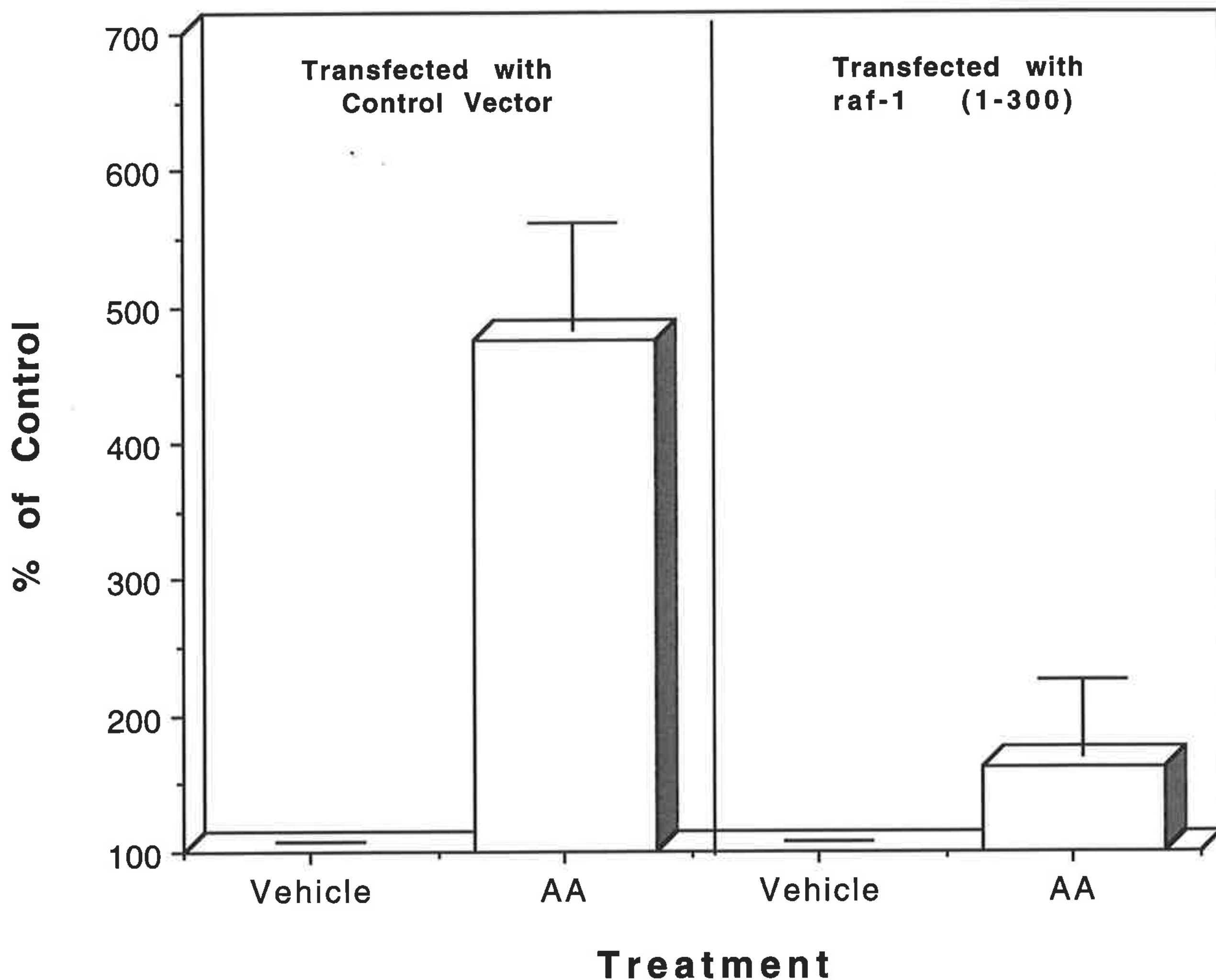


Fig 7.14 The effect of dominant negative raf-1 on the ability of AA to activate ERKs in THP-1 cells. The cells were transfected with dominant negative raf (1μg of DNA) or control vector (1μg of DNA) using lipofectamine. The transfected cells were treated with 30μM of AA or vehicle for 5 min and ERK activity in the fraction was determined by measuring the incorporation of ^{32}P into myelin basic protein (details in Materials and Methods). The results are presented as the mean±sem of 3 experiments, each conducted in duplicate, each with cells from a different individual. *: P<0.05 is the difference between AA and the vehicle treatment.

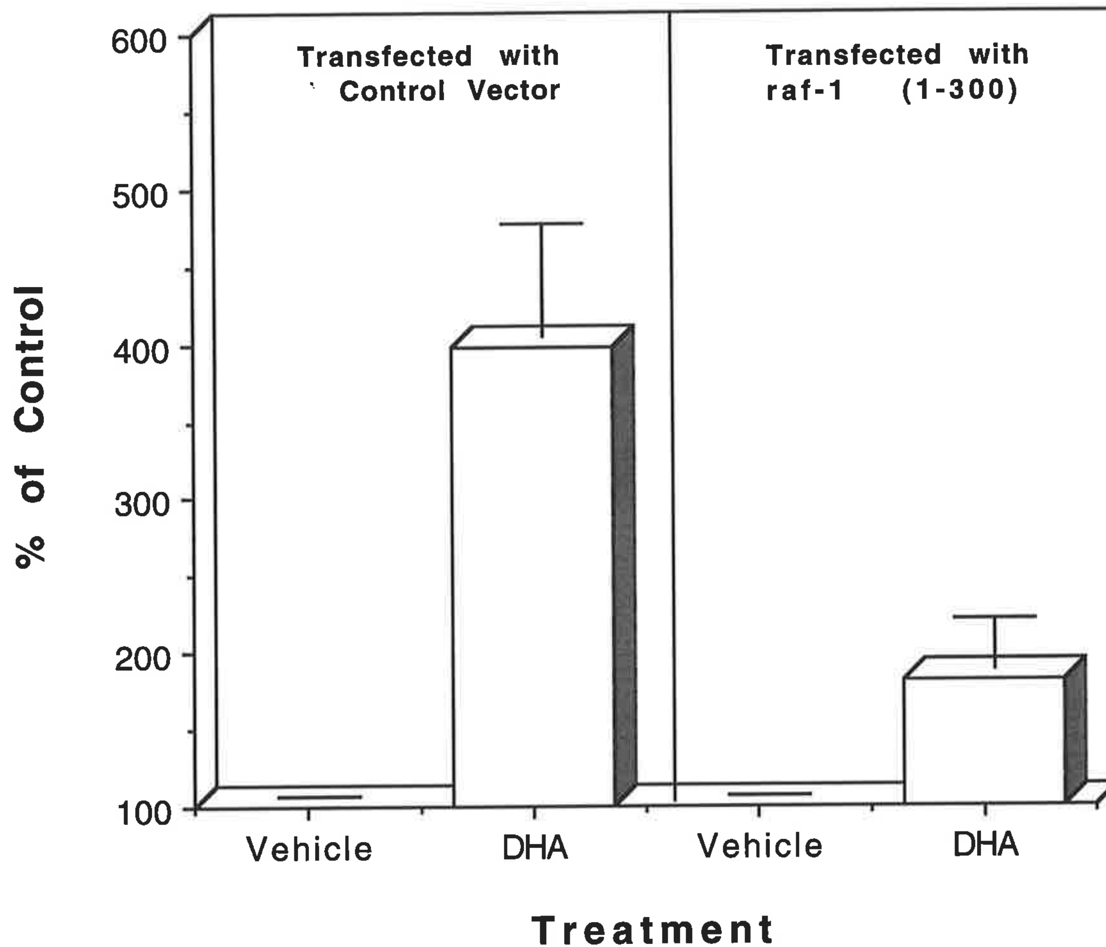


Fig 7.15 The effect of dominant negative raf-1 on the ability of DHA to activate ERKs in THP-1 cells. The cells were transfected with dominant negative raf mutant (1μg of DNA) or control vector (1μg of DNA) using lipofectamine. The transfected cells were treated with 30μM of DHA or vehicle for 5 min and ERK activity was determined by measuring the incorporation of ^{32}P into myelin basic protein (details in Materials and Methods). The results are presented as the mean±sem of 3 experiments, each conducted in duplicate, each with cells from a different individual. *: $P<0.05$ is the difference between DHA and the vehicle treatment.

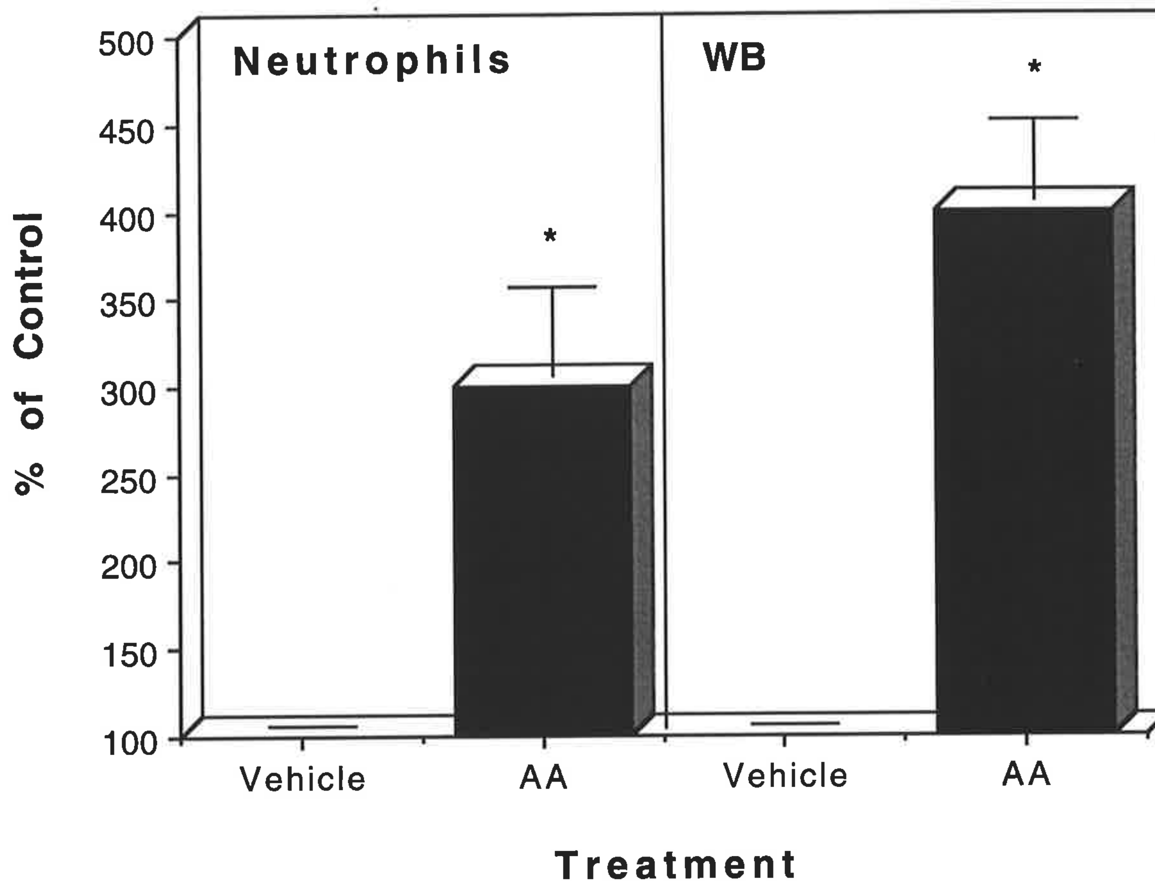


Fig 7.16 The activation of ERKs by AA in neutrophils or WB cells. Cells were treated with either 20μM of AA or vehicle for 5 min and ERK activity was measured by examining the incorporation of ^{32}P into myelin basic protein as described in Materials and Methods. The results are presented as mean±sem of 3-5 experiments. Difference between AA and the vehicle treatment: *: $P<0.05$.

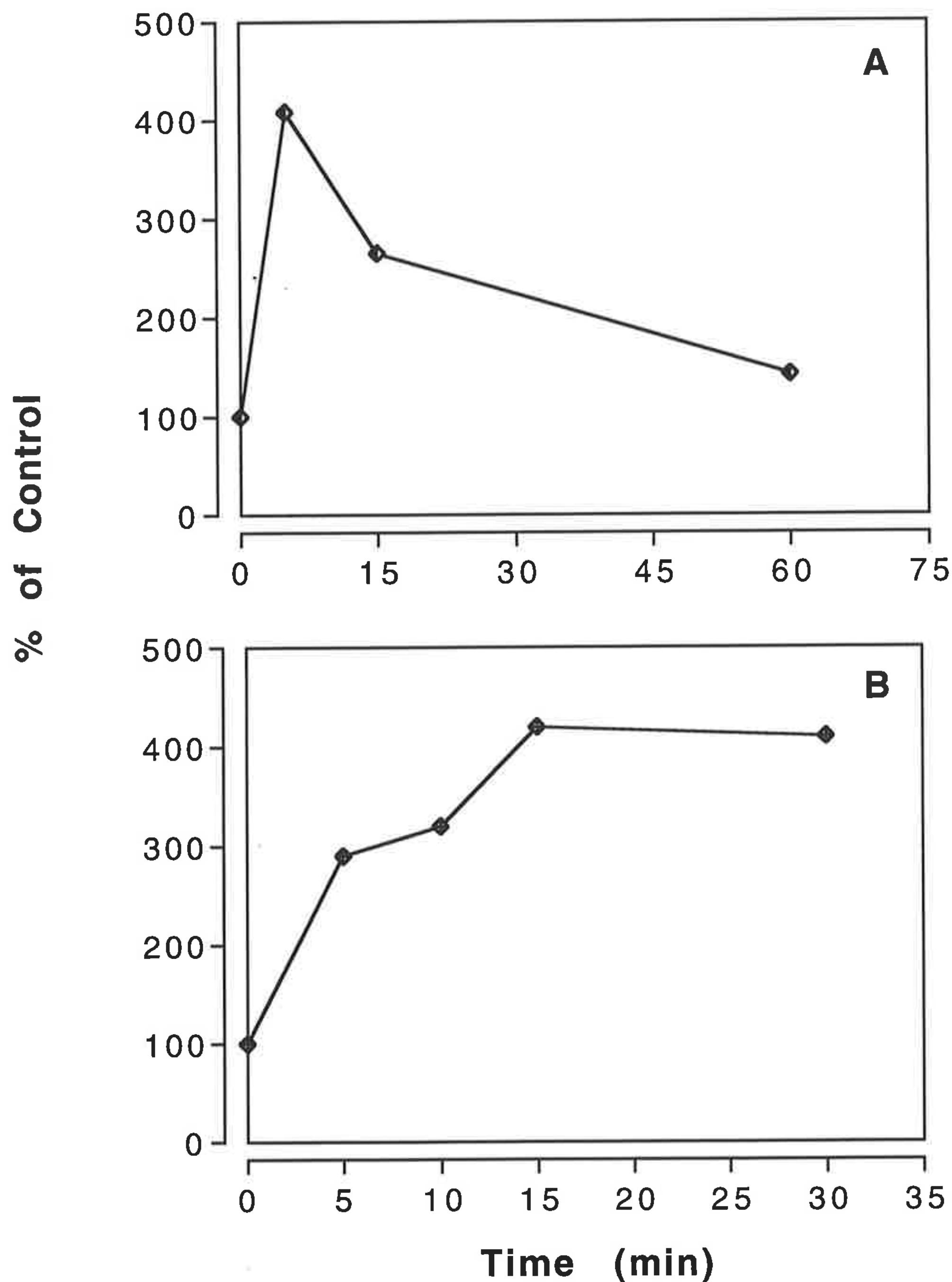


Fig 7.17 The kinetics of AA-stimulated activation of ERKs in WB cells (A) and neutrophils (B). Cells were treated with 20μM of AA or vehicle for 60 or 30min and ERK activity was determined by measuring the incorporation of ^{32}P into myelin basic protein as described in Materials and Methods. The data are representative of three experimental runs, each conducted in duplicate.

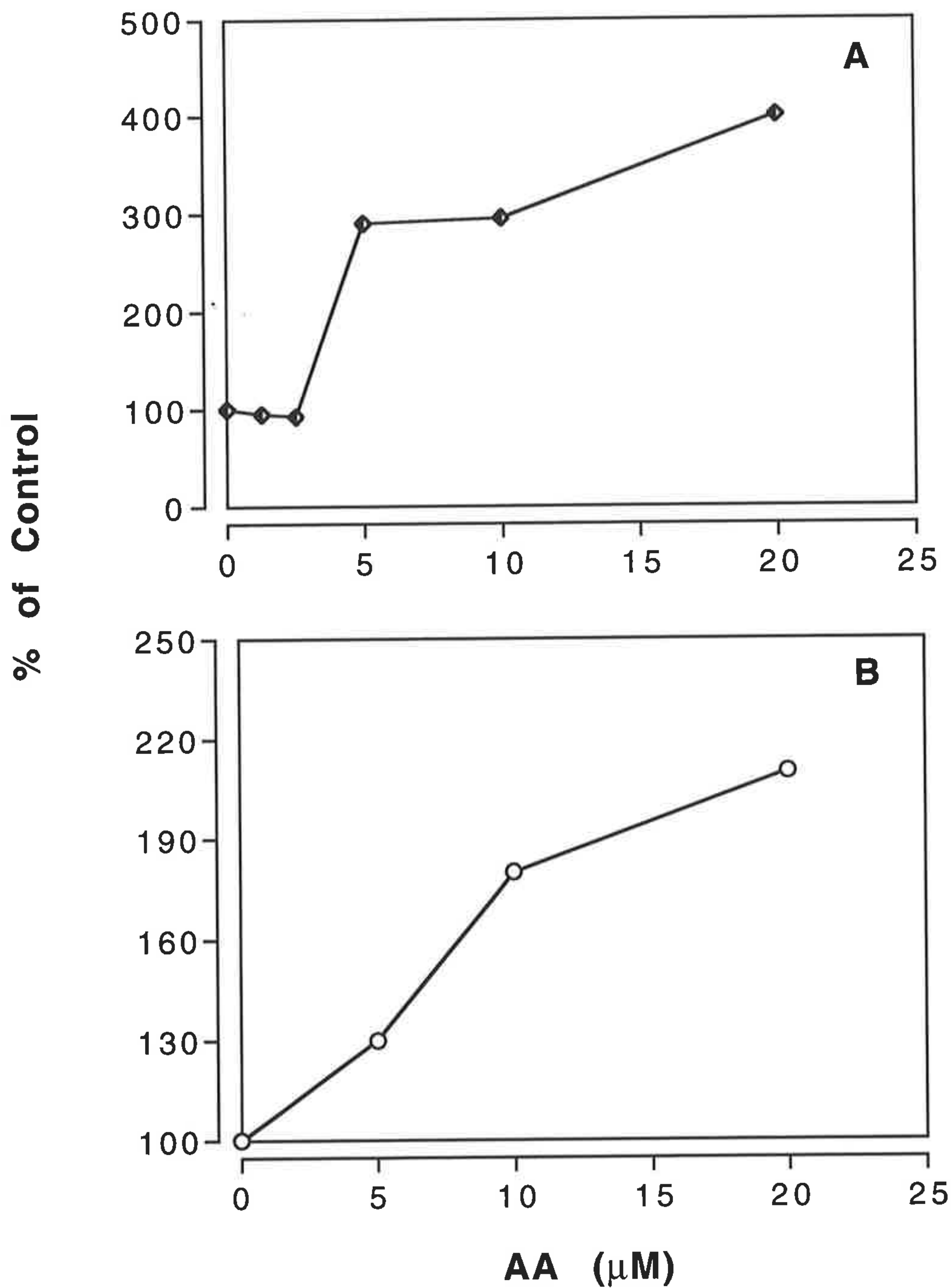


Fig 7.18 The effects of varying the AA concentration on the activation of ERKs in WB Cells (A) or neutrophils (B). Cells were treated with 5-20μM of AA for 5 min and ERK activity was determined by measuring the incorporation of ^{32}P into myelin basic protein (detail in Materials and Methods). The data are representative of three experimental runs, each conducted in duplicate.

Immunoblotting of cytosolic fractions of both neutrophils and WB cells with anti ERK antibodies showed retardation of both isoforms of ERKs: 42 kDa and 44 kDa, in WB cells, and 42 kDa and 43 kDa in neutrophils (Fig 7.19, Fig 7.20). Retardation of the electrophoretic mobility of ERK isoforms in the blots was confirmed by densitometric analysis (the lower panels in Fig 7.19 and Fig 7.20). Fractionation of cytosolic extracts from WB cells on Mono Q FPLC columns resolved the kinase activity in WB cells which had been stimulated with AA into two peaks (Hii *et al,* 1995b). Attempts to chromatograph neutrophil extracts on Mono Q failed, possibly due to high protease activity released from the azurophillic granules during sonication of neutrophils so that active enzymes were degraded during fractionation at room temperature.

7.10 The role of ERKs in the synergistic superoxide production

Recent studies have demonstrated that ERKs directly phosphorylate a key component of the NADPH oxidase, $p47^{phox}$, *in vitro* (Chanock *et al,* 1996). An examination of the sites of phosphorylation revealed that two proline-directed serines, Ser-345 and Ser-348 serve as ERK phosphorylation sites (El Benna *et al,* 1994). These sites are phosphorylated *in vivo* by ERKs in intact cells which had been stimulated with PMA, and *in vitro* by purified ERKs (El Benna *et al,* 1994 and 1996a). Inhibition of ERK activation by a specific ERK kinase inhibitor, PD 098059, blocked the AA-induced respiratory burst in neutrophils (Hii *et al,* submitted). PD 098059 has also been previously demonstrated not

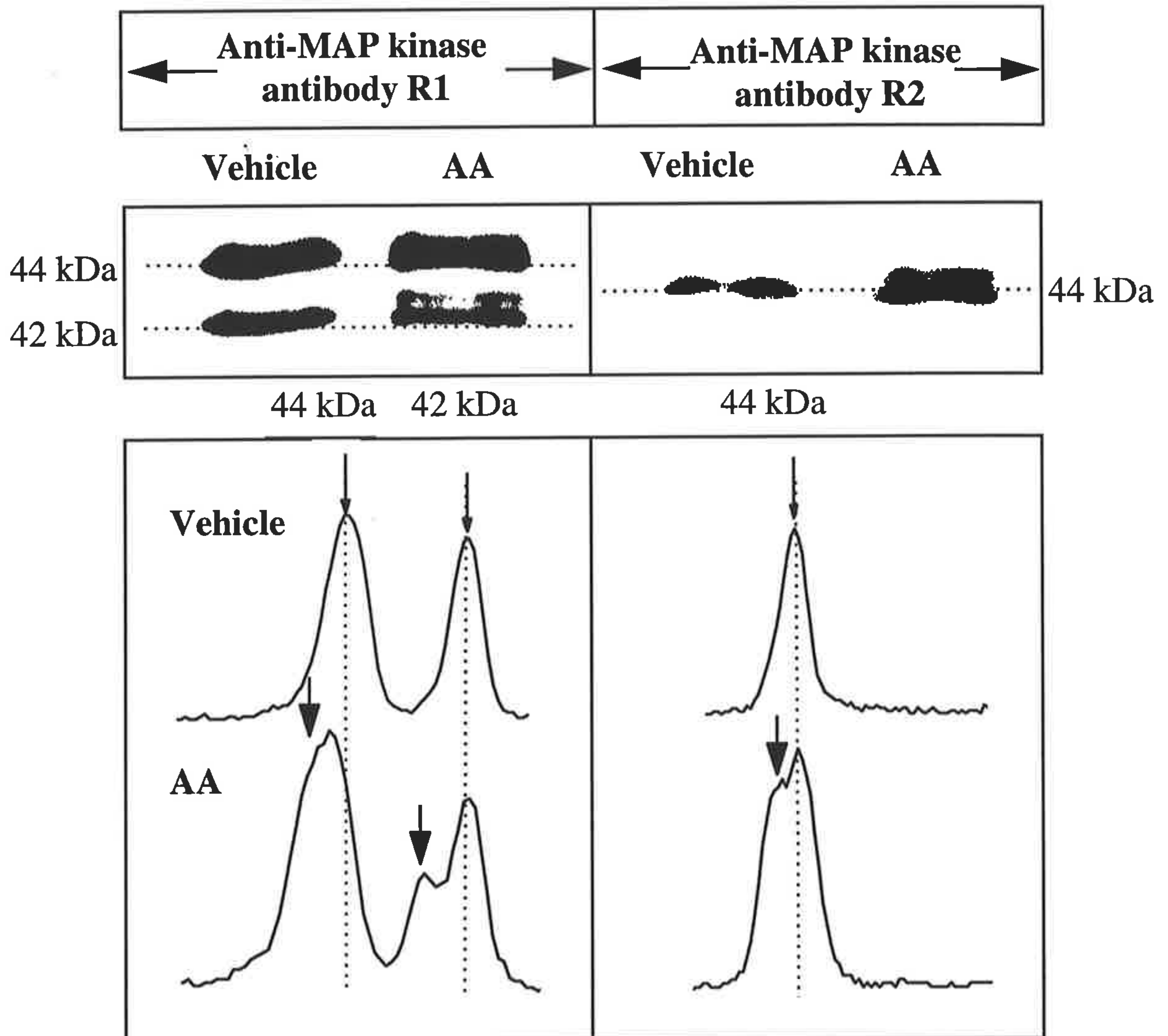


Fig 7.19 The phosphorylation of ERK isoforms in WB cells by AA. Cells were treated with either 20μM of AA or vehicle for 5 min. The cytosolic fractions were prepared and ERKs were Western blotted. Two μg of protein was loaded and separated on a 10% polyacrylamide gel, and then transferred to nitrocellulose membranes. The membranes were stained with anti-ERK antibody R1 (UBI) and R2 and the immune complexes were detected by enhanced chemiluminescence. Light arrows indicate the location of 42 and 44 kDa bands. Heavy arrows indicate the appearance of slower migrating material.

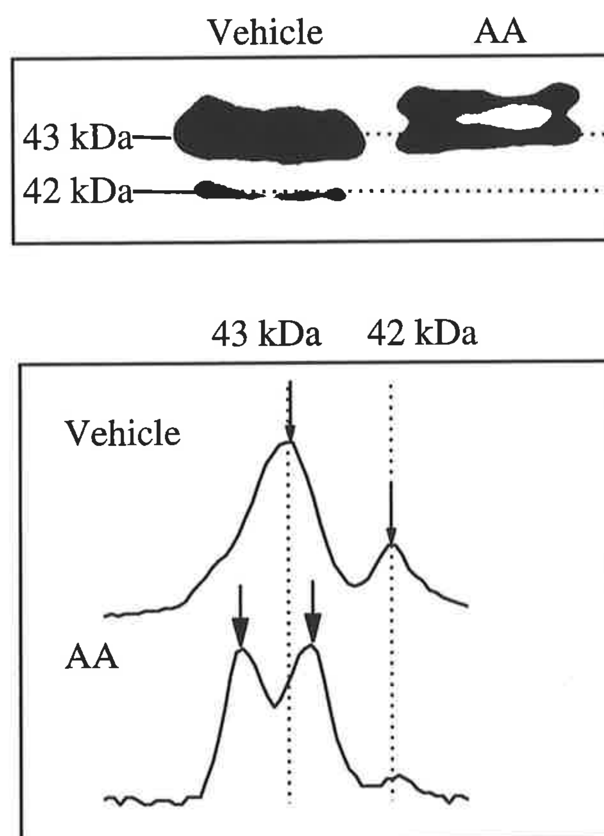


Fig 7.20 The phosphorylation of ERK isoforms in neutrophils by AA. Cells were treated with either 20μM of AA or vehicle for 5 min. The cytosolic fractions were prepared and ERKs were Western blotted. Two μg of protein was loaded and separated on a 10% polyacrylamide gel, and then transferred to nitrocellulose membranes. The membranes were stained with anti-ERK antibody R1 (UBI) and the immune complexes were detected by enhanced chemiluminescence. The lower panel shows the densitometry profile of the immunoreactive bands. Light arrows indicate the location of 42 and 44 kDa bands. Heavy arrows indicate the appearance of slower migrating material.

to affect the activity of 18 kinases tested, including those involved in regulating JNK and p38 cascades, PKC, PKA and calcium/calmodulin dependent kinases (Alessi *et al,* 1995).

In the previous chapter, AA interacted in a synergistic fashion with A23187 to stimulate macrophage chemiluminescence response (Chapter 5). When macrophages were pretreated with 50μM PD 098059 for 45 min, the addition of 30μM of AA and 10μM of A23187 produced a reduced response compared with that observed from cells which had been pretreated with diluent (Fig 7.21).

7.11 Summary

The *n-6* fatty acid, AA, was found to cause significant activation of ERK1 and ERK2 in human macrophages. ERK activation by AA was rapid, peaking within 1 min and decreasing towards basal level by 30 min. Activation of the kinases was evident at 5μM AA concentration.

The *n-3* fatty acid, DHA, activated ERKs to the same extent as AA. However conversion of AA or DHA to their methyl ester derivatives resulted in a loss in the ability of the fatty acids to stimulate kinase activity in macrophages. The saturated fatty acid, 20:0, similarly, did not stimulate the activity of ERKs. These results highlight the

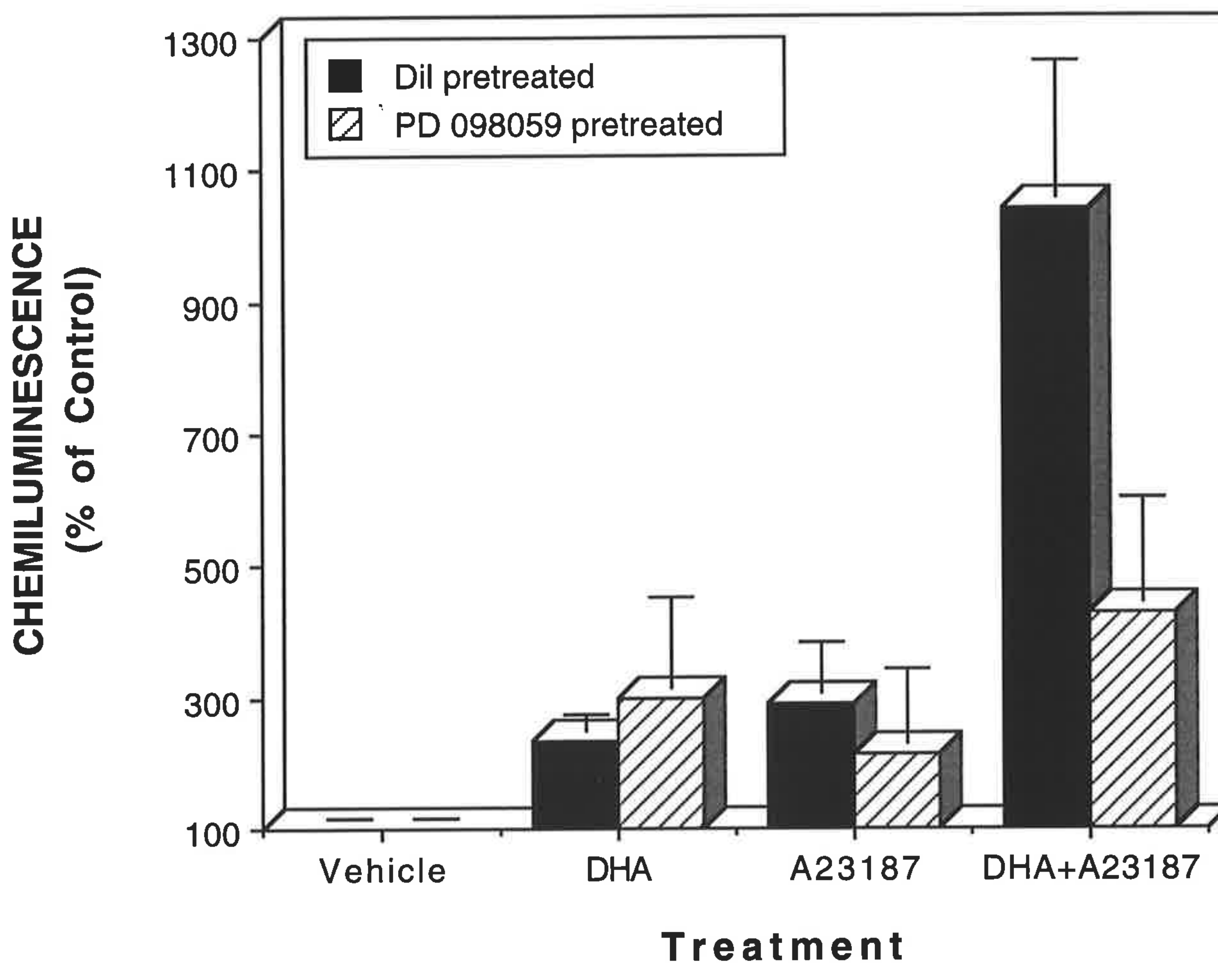


Fig 7.21 The effect of MEK1 inhibitor on the synergistic effect of DHA on the A23187-stimulated macrophage chemiluminescence response. Cells were pretreated with 50μM of MEK1 inhibitor, PD 098059, for 45 min prior to addition of 30μM of DHA and 10μM of A23187. The superoxide produced was measured by the lucigenin-dependent chemiluminescence assay. The result are presented as % of Control from two experimental runs.

importance of the carboxyl group and unsaturation in the ERK stimulating activity of PUFA.

A role for PKC, $p21^{ras}$ and raf-1 in the activation of ERKs in macrophages was demonstrated. Macrophages treated with a PKC inhibitor, GF-109203X, or depleted of PKC by pretreatment with PMA for 18h, failed to show significant activation of ERKs in the presence of AA or DHA. Cells which had been transiently transfected with a dominant negative mutant of $p21^{ras}$ or raf-1 showed a significant reduction in PUFA-induced activation of ERKs.

The stimulation of ERK activation by AA was also observed in both neutrophils and WB rat epithelial cells. The time required to achieve maximum stimulation of ERKs by AA was 5 min for WB cells and 15 min for neutrophils. The AA effect in WB cells rapidly returned to basal level whereas the effect in neutrophils persisted for at least 30 min. Immunoblots showed the phosphorylation of two isoforms of ERKs by AA treatment; 42 and 44 kDa in WB cells and 42 and 43 kDa in macrophages and neutrophils (Table 7.1).

The synergistic effect of PUFA and A23187 on the macrophage respiratory burst was abolished when cells had been preincubated with the specific MAP kinase kinase inhibitor, PD 098059, indicating that the ERK cascade played an essential role in the synergistic interaction between PUFA and A23187.

Table 7.1 ERK activation by PUFA in different cell types

	Neutrophils	Macrophages	WB cells
Activation	+	+	+
Concentration range	5-20μM	5-30μM	5-20μM
Time for maximal peak	15 min	5 min	5 min
Type of response	long-lived	short-lived	short-lived
Isoforms phosphorylated	42 kDa and 43 kDa	42 kDa and 43 kDa	42 kDa and 44 kDa
Resolved by Mono Q FPLC	ND	two peaks	two peaks

+: activated: ND: not detected

Chapter 8

Discussion

Arachidonic acid over a concentration range of 1-10μg/ml (3.3-33μM) induced a very small and in most cases, a non-significant respiratory burst response in human monocytes and macrophages. In contrast, previous studies showed that AA, at the same concentrations, induced a marked respiratory burst response and release of oxygen derived reactive species in neutrophils (Poulos *et al*, 1991; Hardy *et al*, 1994a). The basis for this difference in the effects of the fatty acid between these two cell types is difficult to appreciate. Nevertheless, our studies do illustrate that the fatty acid stimulates the respiratory burst and, to some extent, supports previous results that AA (25-100μM) significantly stimulated the NADPH-dependent superoxide production in a cell free system or in intact guinea pig peritoneal macrophages (Bromberg and Pick, 1983 and 1984; Bromberg *et al*, 1986). The poor production of superoxide by human monocytes/macrophages in the presence of AA was not due to any scavenging or inhibitory effects of the fatty acids on the detection system employed since it has been shown that the chemiluminescence reaction is not affected by this fatty acid (Hardy *et al*, 1994a). It is unlikely that the difference in fatty acid action on neutrophils versus macrophages is due to differences in cyclooxygenase/lipoxygenase products being formed by the two cell types. Previous studies have demonstrated that the ability of fatty acids to stimulate superoxide production in neutrophils was not affected by cyclooxygenase/lipoxygenase inhibitors (Smith *et al*, 1995; Mayer *et al*, 1996; McPhail *et al*, 1996). While it is possible that macrophages may handle fatty acids differently from neutrophils, eg. β-oxidation or ω-oxidation, and that this could be responsible for differences in their action in these cells, the data presented here point to distinct fundamental differences of the regulatory pathways which trigger superoxide production

in the two cell types.

In contrast to this difference in ability to stimulate the NADPH oxidase, the present studies demonstrate that AA stimulates the translocation of PKC in both cell types. Similarly, AA activated ERK1 and ERK2 in both macrophages and neutrophils and was also equally effective at increasing the surface expression of CR3 on macrophages (Chapter 3) and neutrophils (Bates *et al*, 1993). These results suggest that the AA-induced translocation of PKC, ERK activation and increase of the surface expression of CR3 by AA in macrophages is not associated with a corresponding increase in the production of superoxide. Since some superoxide was produced, it is likely that triggering of the NADPH oxidase does occur but either occurs at a level or there is some negative feedback on this response.

Activation of sphingomyelinase and production of ceramide has been shown to act as a downregulator of the agonist-induced respiratory burst in neutrophils (Wong *et al*, 1995). In neutrophils, AA has been shown to stimulate production of ceramide (Robinson *et al*, 1997) and this may be responsible for downregulating the AA-induced superoxide production (Nakamura *et al*, 1994). Imbalances favouring an early increase in ceramide production could very well lead to decreased activation of the NADPH oxidase. However, this is unlikely to be a plausible explanation for the poor superoxide response induced by PUFA in macrophages since CR3 expression was significantly increased, an event also inhibited by ceramide (Hii C.S.T., manuscript in preparation).

Another possible reason for this difference could be related to differences in need to mobilize components of the NADPH oxidase, cytochrome b_{558} and flavoprotein, to the plasma membrane in neutrophils versus macrophages. Stimulation of neutrophils leads to these components being mobilized from the specific granules to the plasma membrane (Calafat *et al*, 1993). This promotes the assembly of the oxidase. However, in macrophages, these components are already primarily expressed in the plasma membrane and there is no requirement for AA to promote their mobilisation (Yamaguchi and Kaneda, 1989).

While the respiratory burst response of monocytes/macrophages to AA was poor, the addition of the fatty acid with another agonist, such as fMLP, resulted in a synergistic respiratory burst activity. This synergistic interaction between the fatty acid and fMLP was characterised by a more rapid response, an increase in the initial peak rate of chemiluminescence and an increase in the total chemiluminescence released over the entire incubation period. These results illustrate that two agents which are poor/moderate inducers of the monocyte/macrophage respiratory burst, when added together to mononuclear phagocytes, induce a substantial respiratory burst response and the release of oxygen radicals. The synergistic effects of AA and receptor-mediated agents/mediators was also evident in neutrophils. Addition of fMLP and AA to neutrophils led to a synergistic respiratory burst response (Poulos *et al*, 1991). Similarly, treatment of neutrophils with AA and TNF resulted in a synergistic response to the two agents (Li *et al*, 1996). This 'cross-talk' between fatty acids and other mediators is an additional consideration in the regulation of leukocyte function in immunity to infection and in pathophysiology. The superoxide generated can be converted to other oxygen-derived

reactive species, such as hydrogen peroxide, hydroxyl radicals and hypochlorous acid. These reactive oxygen intermediates promote oxygen-dependent microbicidal and tumorcidal activities as well as causing tissue damage (Adams and Hamilton, 1992).

When macrophages were pretreated with PUFAs (AA, EPA or DHA), these cells showed increased responses to fMLP in superoxide production. Similar results have been reported for neutrophils, in which PUFA primed these cells for a significant enhancement of fMLP-triggered neutrophil respiratory burst (Corey *et al*, 1995; Robinson *et al*, in preparation). The priming effect for macrophage respiratory burst by PUFA was rapid, with a maximum response seen within a 5 min pretreatment period, while the optimal priming period for neutrophils was 30 min. This is consistent with the activation status of PKC and ERKs by PUFA in these two cell types (Hii *et al*, submitted; Chapter 6 and Chapter 7). PKC and ERKs have been shown to regulate the assembly of the NADPH oxidase in neutrophils by phosphorylation of a key component of the oxidase, $p47^{phox}$, at the different sites (El Benna *et al*, 1994, 1996a and 1996b). In addition, PUFAs have been shown to mobilize intracellular calcium in neutrophils and macrophages which also may, to a certain extent, contribute to their priming effects for the agonist-induced respiratory burst (Hardy *et al*, 1995; unpublished results of Huang Z.H.).

It is of interest that AA (Ferrante *et al*, 1997) and especially DHA (Ferrante *et al*, unpublished results) inhibit LPS-induced TNF production by macrophages. Indeed conversion of a fatty acid to the hydroperoxylated form, such as 15-HPETE, increased the cytokine inhibitory activity of PUFA. Why the PUFAs should inhibit cytokine production, on the one hand, and stimulate the oxidative respiratory burst, on the other, is

difficult to appreciate at present. The inhibitory effects of PUFAs and their derivatives were also evident as a reduction of TNF-induced/increased expression of endothelial cells adhesion molecules under a similar condition (Huang *et al*, 1997).

The findings described above are likely to have important implications in our understanding of the inflammatory reaction, during which inflammatory mediators, including lipids, generated from the surrounding cells, can interact and prime monocytes/macrophages for respiratory burst at suboptimal concentrations of other agonists. Chronic inflammatory diseases are characterised by an accumulation, stimulation or activation of mononuclear phagocytes. Neutrophils may become dominant during acute exacerbation of the disease. Agonist induced activation of leukocytes during inflammation leads to the release of AA (Hoffman *et al*, 1990). The concentrations of fatty acid of 1-10μM which were found to act synergistically with fMLP have been reported as occurring *in vivo* during inflammation and infection. Essien (1993) reported that plasma free AA levels in human malaria patients were >100μM and Yasuda *et al* (1985) found that the free AA levels in brain were 50μM, rising to 500μM under ischaemic conditions. At these fatty acid concentrations, macrophages are likely to be regulated in their responses to other endogenous inflammatory mediators. Concentrations of ≥ 3μM of AA acted synergistically with fMLP to stimulate oxygen radical production in monocytes. As described above, neutrophils also showed a synergistic response to the co-addition of AA and fMLP (Poulos *et al*, 1991). Thus, both neutrophils and macrophages have the potential to be influenced in their behaviour by exposure to AA

and it would be expected that these findings need major consideration in events of acute and chronic inflammation.

AA is released from membrane phospholipids as a consequence of the activation of phospholipase A (PLA_2) in the inflammatory leukocytes and other tissues due to the production of endogenous mediators such as complement products and cytokines or exposure to exogenous antigens (Doerfler *et al*, 1994; Wu *et al*, 1996; Ponzoni 1993; Cybulsky, 1991). Accumulation of AA in inflammatory fluids is thus likely to enhance the responses of neutrophils and macrophages to various mediators. Neutrophils exposed to AA show substantial release of oxygen radicals (Poulos *et al*, 1991; Hardy *et al*, 1994a), and lysosomal enzymes (Bates *et al*, 1995), an increase of surface CR3 expression (Bates *et al*, 1993), increased adherence (Bates *et al*, 1993) and damage to endothelial cells (Bates *et al*, 1995) as well as migration inhibition (Ferrante *et al*, 1994). Thus, fatty acids regulate the basic elements of activation and accumulation of neutrophils at inflammatory foci in the acute inflammatory reaction in association with the regulation of components which promote microbial killing and host tissue damage.

Fixed tissue macrophages, such as Kuppfer cells, splenic macrophages and lung macrophages are given the task of dealing with extracellular bacteria and protozoan parasites particularly when these microbes are present in low numbers. As the number of organisms in tissue increases so does the requirement for blood monocytes to migrate and accumulate at infection foci in tissues. It is likely that AA released as a result of microbial interaction with local tissue and from inflammatory leukocytes plays a role in regulating the activity of mononuclear phagocytic cells as well as neutrophils which constitute the

phagocytes with a role in first line defence. We have already reported that neutrophils primed with AA, EPA and DHA showed an increased killing of asexual intraerythrocytic stages of *Plasmodium falciparum* (Kumaratilake *et al*, 1997) and more recent studies have shown that these fatty acids also increase the neutrophil-mediated killing of *Staphylococcus aureus* (Robinson *et al*, in preparation). In addition, it was found that pretreatment of neutrophils with a combination of PUFA and TNF resulted in a synergistic increase in the killing of *S. aureus* (Robinson *et al*, in preparation). Although microbial killing by macrophages was not investigated in this thesis, the finding that macrophages, like neutrophils, when treated with PUFA showed an increased expression of CR3 and synergized with a second agonist to elicit a respiratory burst suggests that the antimicrobial power of fixed and inflammatory macrophages may be elevated as a result of release of AA as well as other inflammatory mediators.

The effects of AA and other PUFAs were short lived and at this stage it is difficult to relate any of these effects to macrophage activation as this characteristic of the macrophages was not examined. Macrophage activation can be simply measured by examining the ability of the macrophages to kill intracellular bacteria or protozoan parasites. However, it is possible that PUFA may work in synergy with cytokines, such as IFN-γ, to promote macrophage activation and this remains an important question to be addressed in the future.

The ability of AA to stimulate neutrophils in the release of ODRS and lysosomal enzymes (Bates *et al*, 1995) also has implications in the acute inflammatory response and acute exacerbation of chronic inflammatory diseases in which neutrophils accumulate in

substantial numbers. The tissue destructive nature of oxygen radicals and lysosomal enzymes is well appreciated. The finding that macrophages sensitized with AA show significantly increased ODRS release in response to other agonists has important implications in chronic inflammation and tissue damage experienced in chronic inflammatory diseases, such as chronic inflammatory peritoneal diseases, chronic inflammatory diseases of the gastrointestinal tract, rheumatoid arthritis, chronic obstructive pulmonary diseases, hepatitis, Schistosomiasis and Leishmaniasis.

Oxygenation of AA (*n-6*) leads to the generation of HPETE which under the action of an enzyme (or spontaneously) is degraded to HETE via the lipoxygenase pathway. Further products generated include epoxides, LTB_4 and LTC_4, and a recently characterised product, 5-oxo-ETE. Metabolism of AA via the cyclooxygenase pathway leads to the generation of *n-6* prostanoids, such as PGE_2 and TXA_2. Cells may vary in the degree to which AA is metabolised via these two pathways. In addition, the products generated can differ between cell types. Some of these eicosanoids are potent inflammatory mediators which are able to regulate the activity of leukocytes.

Membrane phospholipids are a major source of fatty acids during the activation of cells. The composition of the fatty acid in phospholipids can, under certain circumstances, be changed eg. increased amounts of EPA (*n-3*) and DHA (*n-3*) are observed in membrane phospholipids following diets rich in fish oils (Yaqoob and Calder, 1995). These fatty acids, like AA, are released via PLA_2 during cellular activation (Hoffman *et al*, 1990; Triggiani *et al*, 1990; Shikano *et al*, 1993 and 1994). Since EPA can be metabolised via the lipoxygenase and cyclooxygenase pathways, the metabolism of EPA leads to generation

of LTB_5, which is 25-50 fold less active than LTB_4, and other less inflammatory metabolites, such as PGE_3, TXA_3 and 5-hydroxyeicosapentaenoic acid (5-HEPE) (Taylor *et al*, 1987; Chapkin *et al*, 1988; Kisella *et al*, 1990). PAF, another lipid mediator known to stimulate neutrophil and macrophage functions, is also reduced following an increase in EPA (Lefkowith *et al*, 1990; Sperling *et al*, 1987). Metabolism of DHA, although not clearly defined, also generates inflammatory products with lower activity than AA-derived eicosanoids (Lokesh *et al*, 1988; Ziboh, 1996). The production of these less inflammatory molecules at the expense of the proinflammatory eicosanoids has been proposed to be responsible for the beneficial effect of *n-3* fatty acid rich diets in reducing inflammation in a number of human autoimmune and allergic inflammatory diseases (Simopoulos, 1991). Neutrophils with increased amounts of EPA and DHA in membrane phospholipids also show reduced chemotactic responses (Sperling *et al*, 1993).

More recently it has been appreciated that diets rich in EPA and DHA lead to depressed production of the cytokines, TNF and IL-1β (Endres *et al*, 1989). The results, however, remain to some extent conflicting. Mononuclear cells from either healthy volunteers or patients with multiple sclerosis and rheumatoid arthritis, when given *n-3* fatty acid rich diets, showed decreased production of TNF and IL-1β *in vitro* (Endres *et al*, 1989; Watson *et al*, 1993; Gallai *et al*, 1995; Kremer, 1995; Caughey *et al*, 1996). This is, at least in part, due to the inhibition of TXA_2 by *n-3* PUFAs (Caughey *et al*, 1997). However, peritoneal macrophages from mice fed with *n-3* fatty acids produced increased amounts of TNF and IL-1β which was inversely correlated with a reduction in PGE_2 in

these cells (Hardardottir and Kinsella, 1992; Lokesh *et al*, 1990). At this stage, more studies are required to clarify these conflicting results. .

Previous results with neutrophils and data presented in this thesis show that EPA and DHA are similar to AA in their regulation of the biological properties of neutrophils and macrophages. These include enhancing the respiratory burst response, increasing degranulation, increasing adherence to extracellular matrix and increasing damage to endothelial cells. Thus it would have to be assumed that this property of the *n-3* PUFAs contributes to the unexpectedly modest effects of DHA in suppressing inflammation (Otto *et al*, 1992).

The fatty acids AA (20:4, *n-6)*, EPA (20:5, *n-3*) and DHA (22:6, *n-3*) were very similar in their ability to increase the responses of macrophages to a second agonist, such as fMLP. However, these fatty acids were significantly more active than the 18 carbon PUFAs, LA (18:2, *n-6*) and GLA (18:3, *n-6).* LA in fact did not show synergistic properties with fMLP on the oxidative respiratory burst of macrophages. The saturated fatty acids 20:0, 14:0 and 12:0 also had no activity (Chapter 5). This illustrates the importance of carbon chain length and the degree of unsaturation in the synergistic response of PUFA treated monocytes/macrophages to other agonists.

Previous studies on the direct alteration of neutrophil functions by fatty acids showed a similar structure-function relationship (Poulos *et al*, 1991; Hardy *et al*, 1994a; Bates *et al*, 1995; Ferrante *et al*, 1994). From these investigations, it was evident that 18:1 (*n-9*) had poor activity, but the activity increased with increasing carbon chain length and the

number of double bonds (Poulos *et al*, 1991; Hardy *et al*, 1994a; Bates *et al*, 1995; Ferrante *et al*, 1994). However, once chain length increased to >24 carbon atoms, the biological activity of the fatty acid was dramatically reduced (Hardy *et al*, 1994a). This relationship between fatty acid structure and biological activity was evident with a number of key neutrophil functions: respiratory burst, degranulation, cell migration, microbial and parasite killing, calcium mobilisation and tissue destruction (Poulos *et al*, 1991; Hardy *et al*, 1994a and 1995; Bates *et al*, 1993 and 1995; Ferrante *et al*, 1994; Calder *et al*, 1990; Kumaratilaki *et al*, 1997). A similar effect was seen in relation to the ability of these fatty acids to act in a synergistic manner with other agonists in the stimulation of neutrophil responses. Another study has also shown that PUFAs act synergistically with cytokines, such as TNF, in enhancing the neutrophil respiratory burst and this was also dependent on the number of carbon atoms and degree of unsaturation of fatty acids (Li *et al*, 1996).

Introduction of a hydroxyl group or hydroperoxy group to the PUFA resulted in complete loss of activity on macrophages (Chapter 5). Similar results were also observed with neutrophils (Bates *et al*, 1995; Ferrante *et al*, 1994; Kumaratilaki *et al*, 1997). The importance of the carboxyl group was shown by the finding that conversion of the fatty acid to its methyl ester led to a loss of the PUFA activity on neutrophils (Ferrante *et al*, 1994; Li *et al*, 1996; Kumaratilaki *et al*, 1997) and monocytes/macrophages (Chapter 5). The inability of the methyl ester to stimulate cellular functions suggests that ionic interactions between a fatty acid and the proteins involved in regulating cellular functions is important for biological activity.

The basis of the structural requirement for fatty acid activity remains unknown. It is speculated that different intracellular compartmentation or mobilization of fatty acids and the susceptibility to certain metabolic enzymes might contribute to the differences in the biological activity between fatty acids. In addition, the hydroxylated and hydroperoxylated forms of AA, 15-HETE and 15-HPETE, although lacking the ability to stimulate the neutrophil/macrophage respiratory burst, have been shown to be active in the modulation of other cell responses. For example, 15-HPETE was found to inhibit the TNF production induced by LPS in monocytic cells and suppress TNF-induced/increased expression of endothelial cell adhesion molecules (Ferrante *et al*, 1997; Huang *et al*, 1997). The effects were more potent than that caused by their parental form, AA (Ferrante *et al*, 1997; Huang *et al*, 1997). These AA derivatives were also as active as AA, in their ability to inhibit gap junctional communication in WB cells (Hii *et al*, 1995a). Thus, modification of fatty acid structural elements may alter their biological properties in a function/cell type specific manner. Similar loss in activity upon conversion of a fatty acid to its methyl ester derivative is observed in the inhibition of gap junctional communication and the ability of *n-3* fatty acids to protect against adverse effects of calcium and chemicals on cardiac myocyte contraction (Hii *et al*, 1995a; Xiao *et al*, 1997).

The phenomenon of PUFA augmented agonist-induced NADPH oxidase activation was extended to agonists which act on different intracellular targets. A23187 is a calcium ionophore which elevates the intracellular calcium concentration by facilitating calcium influx. Addition of A23187 to monocytes/macrophages in the presence of PUFA resulted in a synergistic respiratory response (Chapter 6). Similarly, treatment of cells with

PMA, which directly activates PKC, in the presence of PUFA resulted in a synergistic release of superoxide (Chapter 4). These results demonstrate that PUFAs augment responses of agonists which act on quite different targets, in macrophages. In neutrophils, PUFAs also synergise with PMA in the production of superoxide (Poulos *et al*, 1991). During inflammation and cell activation, mediators are released which act via surface receptors, mobilise calcium or activate PKC. These responses may be influenced by PUFAs, such as AA, whereby the fatty acid regulates intercellular and intracellular signalling.

PUFAs activate PKC in cell free systems and in purified PKC preparations *in vitro* (Shimonura *et al*, 1991; Lester *et al*, 1991; Koide *et al*, 1992; Nakanishi *et al*, 1993; Hardy *et al*, 1994a). Our data show that in macrophages, PUFAs induced the translocation of PKCα, βI, βII and ε to a particulate fraction. This is similar to previous findings that AA stimulates the translocation of PKC α, βI and βII in neutrophils and PKC α, δ and ε in rat liver epithelial WB cells (Hii *et al*, 1995b and submitted). Thus, while the PUFAs were capable of activating PKC, an early cell signalling system in various cell types, as in macrophages this was not sufficient in triggering the production of superoxide.

PKC is a multifunctional kinase which plays an important role in regulating many cellular functions. Its role in the assembly and activation of NADPH oxidase is believed to be via phosphorylation and activation of the components of NADPH oxidase, such as $p47^{phox}$ (Chanock *et al*, 1994). However, our results showed that while PUFAs induced the translocation of PKC, poor NADPH oxidase activation occurred in monocytes/macrophages. In neutrophils, the activation of NADPH oxidase was strong

and hence consistent with the PUFA-induced activation of PKC in this cell type. The reason for this difference in the magnitude of the response in the two cell types is unclear. However, differences in the metabolic fates of the PUFA between the two cell types may be one possible explanation. Neutrophils, having poor mitochondrial function, are unlikely to have the same capacity as macrophages to promote β- and ω-oxidation of fatty acids. In this manner, fatty acids may be metabolised via some selective pathways and/or have increased half life in the cytosol, thereby maintaining a greater concentration of PUFAs for reacting with components of the oxidase.

Other possibilities cannot be excluded. It is likely that a number of signalling molecules need to be activated in the assembly of the NADPH complex (see Chapter 1). Although roles for these molecules have been demonstrated in neutrophils, it is currently not known whether similar molecules are involved in regulating the NADPH oxidase in macrophages. The ability of PUFAs to stimulate superoxide production in neutrophils suggests that PUFAs can stimulate the activity of most or all these molecules. It is possible that PUFAs are able to stimulate the activity of only some of these molecules in macrophages. Alternatively, there are fundamental differences in the regulatory pathways which cause the assembly of the NADPH oxidase between the neutrophils and macrophages (as described above).

Examination of the kinetics of activation of PKC by PUFA in macrophages showed a transient response which peaked by 5 min and declined thereafter over a 30 min incubation period. This response correlated well with the ability of the fatty acids to alter the NADPH oxidase activation induced by a second agonist, such as fMLP. Thus

the priming effect of PUFAs on superoxide production was maximal at 5 min of PUFA-pretreatment and diminished with increased pretreatment time. A role for PKC in the PUFA-induced alteration of the macrophage response was deduced from studies in which the PKC activity was inhibited by treatment with a highly selective inhibitor, GF-109203X (Toullec *et al*, 1991). GF-109203X is generally accepted as a highly specific inhibitor of PKC activation in macrophages (Qin and Leslie, 1994). Although a complete concentration curve for this inhibitor was not established in the present study. it has been shown that GF-109203X does not inhibit the activity of PKA, insulin-, EGF- and PDGF-induced receptor tyrosine kinases at the concentrations used in this study (Mahoney and Huang, 1994).

To examine the role of PKC, A23187 was used as the second agonist because it does not require PKC activation to induce a response. GF-109203X treatment of macrophages resulted in a loss of the PUFA-A23187 synergistic respiratory burst. Similarly, macrophages which had been pretreated with PMA for 18h to deplete classical and novel PKC isozymes were unable to respond to PUFA and A23187 to produce a synergistic response (Chapter 6). These results support a major role for PKC in the alteration of the macrophage respiratory burst response by PUFAs. PUFA and derivatives may also target PKC in their influence on other macrophage functions. For example, pretreating monocytic cells with the AA derivative, 15-HPETE, is accompanied by an increase in PKC translocation to a particulate fraction (Ferrante *et al*, 1997). Similarly, the addition of LPS to these monocytic cells caused PKC translocation (Ferrante *et al*, 1997). However, the addition of LPS to 15-HPETE-pretreated cells resulted in a loss of PKC from the particulate fraction when compared to cells exposed to 15-HPETE or LPS alone

(Ferrante *et al*, 1997). This was associated with suppression of LPS-stimulated TNF production by 15-HPETE. The precise mechanism by which PKC was lost in these PUFA-pretreated, LPS-stimulated, cells is unknown.

MAP kinases are a family of serine/threonine kinases which include ERK1, ERK2, ERK3, ERK5, ERK6, Jun N-terminal kinases/stress protein kinases (JNK/SAPKs) and p38/HOG1. Since ERK1 and ERK2 are downstream of PKC, we were interested to know whether PUFA activated ERK1 and ERK2, especially since these have been shown to phosphorylate $p47^{phox}$ (El Benna *et al*, 1996b). AA and DHA both activated ERKs in monocytes/macrophages (Chapter 7). A 42 kDa ERK2 and 43 kDa ERK1 were identified by Western blotting with anti-ERK antibodies. This result was confirmed by using antibodies to the phosphorylated/activated forms of ERK (data not shown). The effect of PUFA on ERK activity with respect to AA and DHA was transient, peaking at 5 min and declining to control level at 30 min. This is consistent with the observation on activation of their upstream kinase, PKC, by PUFA. Examination of the effects of modifying the fatty acid structure on their ability to activate ERKs showed a correlation of structural elements of the fatty acid with their ability to synergise with a second agonist in the stimulation of macrophage superoxide production. Thus, if the PUFAs were saturated, methylated, hydroxylated or hydroperoxylated then they lost the ability to activate ERK as well as the ability to synergise with a second agonist in macrophage superoxide production.

It has been proposed that activation of ERK contributes, at least in part, to the activation of the NADPH oxidase in neutrophils (El Benna *et al*, 1994). This is based on the observation that ERKs phosphorylate p47^{phox}, a key component of the NADPH oxidase, at sites which are different from the sites phosphorylated by PKC (El Benna *et al*, 1994, 1996a and 1996b). Pretreating neutrophils with an inhibitor of MAP kinase/ERK kinase 1 (MEK1), PD 098059, inhibited the PUFA-induced respiratory burst response by approximately 50% (Hii *et al*, submitted). Pretreatment of macrophages with this inhibitor prevented the synergistic activation of the NADPH oxidase by co-treatment with PUFA and A23187 (Chapter 7). This demonstrates that PUFA use the ERK cascade, in part, as the mechanism of the PUFA-induced synergistic response to second agonists in macrophage superoxide production.

Our data also demonstrate that PKC was involved in the activation of ERK1 and ERK2 by PUFA. Pretreatment of cells with a PKC inhibitor, GF 109203X, or depletion of PKC completely abolished the activation of ERKs by PUFA. The role of p21^{ras} and raf-1 in activation of ERKs by PUFAs was studied by using monocytic cells which were transiently transfected with dominant negative mutants of either p21^{ras} (17N) or raf-1 (1-300) DNA. Cells containing these mutant DNAs significantly diminished ERK activation induced by PUFAs, suggesting the involvement of p21^{ras} and raf-1 in the PUFA-stimulated ERK cascade. Pretreating the cells with a MEK1 inhibitor, PD 098059, reduced the PUFA-stimulated activation of ERKs to basal levels which indicated that MEK1 was the immediate kinase for ERK activation in response to PUFA stimulation. These results provide evidence that the activation of ERK1 and ERK2 by PUFA in

macrophages involves the stimulation of upstream regulators/kinases, PKC, $p21^{ras}$, raf-1 and MEK1 (Fig 8.1).

The ability of PUFA to synergise with receptor-dependent and receptor-independent stimuli to initiate a respiratory burst in human monocytes/macrophages has been demonstrated, while PUFA may act via a number of ways to promote this synergism, the use of a calcium ionophore has allowed the unambiguous demonstration that the PKC-$p21^{ras}$-raf-1-MEK1-ERK cascade is involved in mediating the action of PUFA. A similar mechanism may account for the ability of PUFA to synergise with fMLP or PMA. Within the constraints of the data obtained with the calcium ionophore, we propose the following model to explain how PUFA act to amplify the respiratory burst in monocytes/macrophages (Fig 8.1). Although fMLP and PMA, unlike the calcium ionophore, also activate the PKC-ERK cascade, we speculate that the co-presence of PUFA with fMLP or PMA may enhance the flow of signals through this cascade or that this cascade is activated more efficiently by fMLP or PMA in the presence of exogenous PUFA. These exogenous PUFA may also serve to increase the total pool of PUFA in activated cells since fMLP and PMA also stimulate the release of endogenous PUFA. Other mechanisms in addition to the PKC-ERK cascade may also be involved, eg. enhancement of PI3 kinase activity which has been found to be important in mediating the neutrophil respiratory burst.

The thesis has addressed an important issue; the effects of essential and non-essential fatty acids on a major function of the mononuclear phagocytic system. The highlights of the findings were that PUFA primed macrophages for an increased response to a second agonist resulting in a significant increase of superoxide production. Interesting was the observation that both *n-6* and *n-3* PUFAs demonstrated these properties. In addition, a major contribution has been made into the intercellular signalling mechanisms activated and utilised by these PUFAs to prime the mononuclear phagocytic system. Attempts to treat autoimmune and allergic inflammatory diseases with *n-3* PUFAs will need to take into consideration the effects of these fatty acids on the mononuclear phagocytic system.

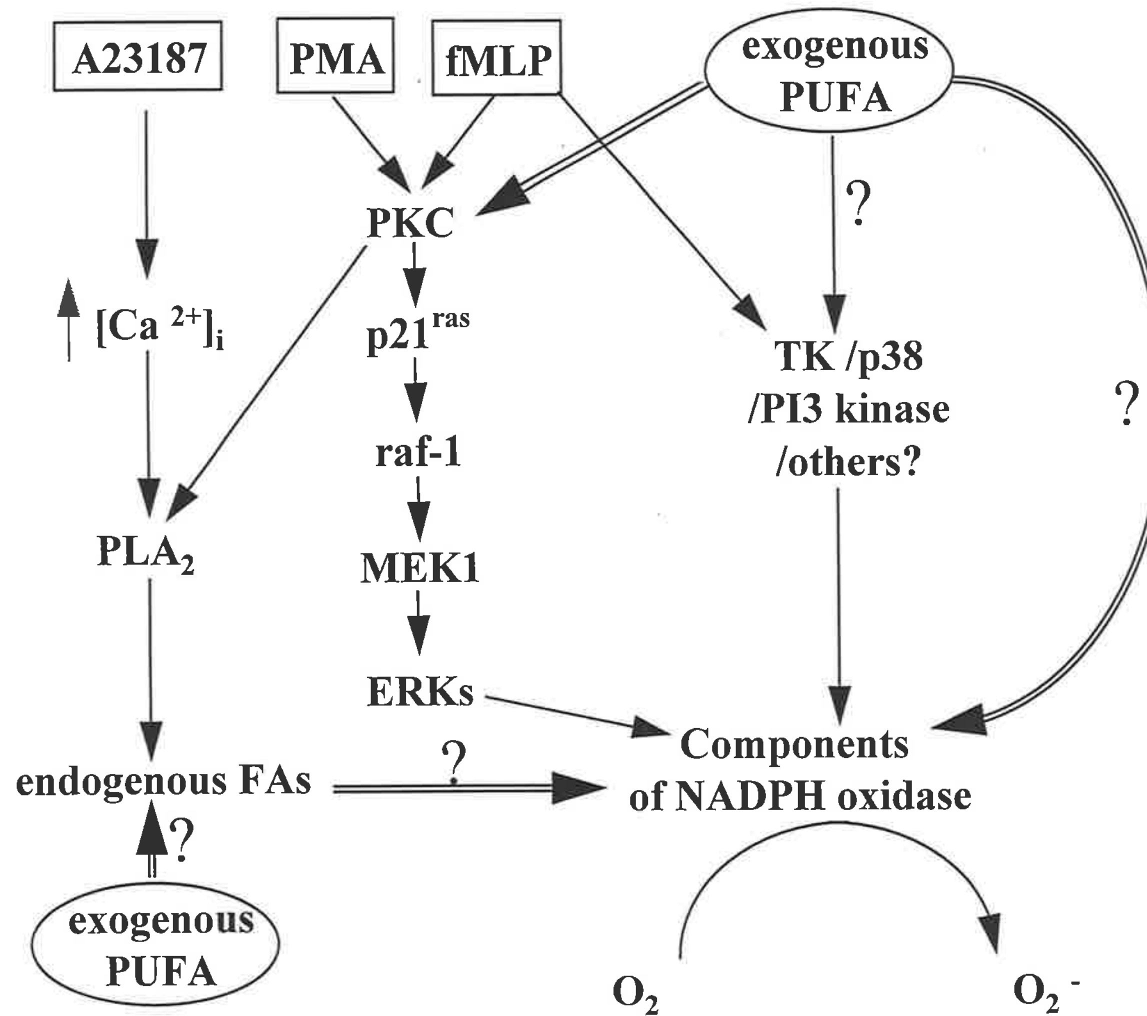


Fig 8.1 Schematic representative of the effects of PUFA on alteration of the agonist-induced superoxide production in macrophages. PUFA were synergistic with second agonists, fMLP, PMA and A23187 to stimulate macrophage superoxide production. The signal pathway of this stimulation is likely to involve phosphorylation /activation p21^{ras}, raf-1, MEK1 and ERKs, and possibly some other kinases such as, p38, PI3 kinase and TK. Exogenous and endogens PUFA may also act on certain components of NADPH oxidase. PUFA: polyunsaturated fatty acid; FA: fatty acid; PLA_2: phospholipase A_2; MEK1: ERK kinase 1; ERKs: extracellular signal-regulated protein kinases; TK: tyrosine kinases.

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