# CELLULAR SIGNALLING AND REGULATION OF NITRIC OXIDE SYNTHESIS

A Thesis submitted to the University of Glasgow

for the degree of Doctor of Philosophy

by

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January 1996

## SUMMARY

Murine macrophages synthesise nitric oxide (NO) from L-arginine, and the reaction is catalysed by NO synthases (NOS). Macrophages stimulated by interferon-γ (IFN-γ) and low dose of lipopolysaccharide (LPS) or a high concentration of LPS alone can produce large amounts of NO. Using a murine macrophage cell line, J774, we have investigated the signalling mechanisms for NO production.

Transient increases in cyclic adenosine monophosphate (cAMP) did not affect the production of NO. However, prolonged elevation of intracellular cAMP levels, using the prostaglandin $E_2$ and phosphodiesterase inhibitors isobutylmethylxanthine or rolipram, was found to inhibit NO production. This inhibition did not occur at the level of gene transcription, but at the post transcriptional level.

Interferon-γ and LPS treatment resulted in protein kinase C (PKC) activation and translocation into the plasma membrane, which correlated with NO production and NOS activity. The activation and the translocation of PKC was inhibited by IL-4, a physiological inhibitor of NO synthesis. IL-4 and the PKC inhibitor, Ro31-8220, inhibited the transcription of inducible NOS (iNOS) gene. IL-4 did not induce an elevation of cAMP levels in macrophages. These data suggest that IL-4 acts by inhibiting the activation of PKC, which is essential in the induction of iNOS gene transcription, but not via the induction of cAMP generation.

We also showed that pertussis toxin (Ptx) sensitive-G-proteins are not involved in the induction of iNOS expression in macrophages, and modification of G-proteins by Ptx and cholera toxin (Ctx) did not alter NO production in J774 cells. NO

production, stimulated by IFN-γ and LPS, was not inhibited by Ctx, even although Ctx induced significant levels of intracellular cAMP.

Furthermore, since phospholipase $A_2$ ($PLA_2$) inhibitors, such as 4-4-octadecyl-4-oxobenzene butenoic acid, 4-bromophenacyl bromide and dexamethasone, have no effect on NO production by J774 cells, it is suggested that arachidonic acid metabolites and $PLA_2$ are not involved in NO production in macrophages.

## ACKNOWLEDGEMENTS

I would like to thank my supervisors, Professor F.Y. Liew, Dr. A. Severn and Dr. William A. Sands for advice, help and encouragement throughout these studies.

I am grateful to the department of Immunology, Glasgow University for allowing me to carry out this work and Firat University for financial support.

I also like to thank my colleagues, Dr. Kate O'Donnell, Dr. Lorna Proudfoot, Dr. Damo Xu, Mrs. Jackie Thomson, Dr. Guijie Feng and Mr. William Reece for their support and friendship.

Finally, I thank my parents and my wife for their support and encouragement.

## ABBREVIATIONS

| | |
|---|---|
| ADP | Adenosine diphosphate |
| ATP | Adenosine triphosphate |
| BSA | Bovine Serum Albumin |
| cAMP | Cyclic adenosine monophosphate |
| cGMP | Cyclic guanosine monophosphate |
| CPM | Counts per minute |
| CRE | cAMP-regulated Enhancer |
| CREB | cAMP Response Element-Binding Protein |
| CRP | cAMP receptor protein |
| Ctx | Cholera toxin |
| DAG | Diacylglycerol |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic acid |
| DMEM | Dulbecco's modified Eagles Medium |
| EDTA | Ethylene diamine tetra acetic acid |
| EGTA | Ethylene glycol tetra acetic acid |
| Epo | Erythropoietin |
| FAD | Flavin Adenine Dinucleotide |
| FCS | Foetal calf serum |
| FIM | Factor Increasing Monocytopoiesis |
| FMN | Flavin Mononucleotide |
| g | Gravity |
| GAF | Interferon-γ Activated Transcription Factor |
| GAS | Interferon-γ Activated Site |

| | |
|---|---|
| GDP | Guanosine diphoshate |
| GM-CSF | Granulocyte Monocyte Colony-Stimulating Factor |
| GTP | Guanosine triphosphate |
| hrs | Hours |
| I | Inhibitor |
| IBMX | 3-isobutyl methyl xanthine |
| ICER | Inducible cAMP Early Repressor |
| IFN- | Interferon |
| Ig | Immunoglobulin |
| IL- | Interleukin |
| IP | Inositolphosphate |
| i.p. | intraperitoneal |
| IRF | Interferon Response Factor |
| IRS | Insulin Receptor Substrate |
| ISGF- | Interferon-Stimulated Gene Factor |
| ISRE | Interferon-Stimulated Response Element |
| Jak | Janus kinase (Just Another Kinase) |
| L-arg | L-arginine |
| L-cit | L-citrulline |
| LPS | Lipopolysaccharide |
| mAb | Monoclonal antibody |
| 2-ME | 2-mercaptoethanol |
| M-CSF | Macrophage-Colony Stimulating Factor |
| NADPH | Nicotinamide adenine dinucleotide phosphate reduced form |
| NaF | Sodium fluoride |
| NF | Nuclear Factor |
| NK | Natural Killer |

| | |
|---|---|
| NO | Nitric oxide |
| $NO_2^-$ | Nitrite |
| $NO_3^-$ | Nitrate |
| NOS | Nitric oxide synthase |
| O.D. | Optical density |
| PA | Phosphatidic Acid |
| PAF | Platelet Activating Factor |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate-buffered saline |
| PDBu | Phorbol dibutyrate |
| PGE | Prostaglandin E |
| PI | Phosphatidyl Inositol |
| PKA | Protein kinase A (cAMP-dependent protein kinase) |
| PKC | Protein kinase C |
| PLA | Phospholipase A |
| PLB | Phospholipase B |
| PLC | Phospholipase C |
| PLD | Phospholipase D |
| PMA | Phorbol myristate acetate |
| PPD | Purified Protein Derivatives |
| PTK | Protein-Tyrosine kinases |
| Ptx | Pertussis toxin |
| RNA | Ribonucleic Acid |
| RNI | Reactive Nitrogen Intermediates |
| ROI | Reactive Oxygen Intermediates |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| SEM | Standard error of the means |

| | |
|---|---|
| STAT | Signal transducers and activators of transcription |
| STF | Signal Transducing Factor |
| TGF | Transforming Growth Factor |
| Th | T helper |
| TNF | Tumour necrosis factor |
| TK | Tyrosine Kinases |

## PUBLICATIONS

1) **V. Bulut**, A. Severn and F.Y. Liew, (1993) Nitric Oxide Production by Macrophages is Inhibited by Prolonged Elevation of cyclic AMP. *Biochemical and Biophysical Research Communications* **195**, 1134-1138.

2) W.A. Sands, **V. Bulut**, A. Severn, D. Xu and F.Y. Liew, (1994) Inhibition of Nitric Oxide Synthesis by Interleukin-4 may Involve Inhibiting the Activation of Protein kinase C epsilon. *Eur. J. Immunol.* **24**, 2345-2350.

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# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1. THE MACROPHAGE

### 1.1.1. Macrophage biology:

The term "macrophage" was first used more than 100 years ago by Elie Metchnikoff in Messina, to describe the large mononuclear phagocytic cells he observed in tissues [Karnovsky, 1981]. Ontogenetically, macrophages originate in the yolk sac [Moore and Metcalf, 1970], but in adult man arise from the bone marrow [Furth, 1989]. They are found in lymphoid organs, the liver, lungs, gastrointestinal tract, central nervous system, serous cavities, bone, synovium and skin. Macrophages are the major differentiated cells of the mononuclear phagocyte system originating from monoblasts in the bone marrow. Having arrived at their target organs, monocytes differentiate into macrophages [Furth and Cohn, 1968]. In the absence of a local inflammation, the migration of monocytes from blood into different tissues appears to be a random process [Terr, 1994]. Differentiation of promonocytes to monocytes is induced by retinoic acids, hydroxyvitamin $D_3$, TGF-β and IFNs as physiological agents and phorbol esters experimentally [Reviewed by Eilers et al., 1995]. Many cytokines play a role in this differentiation process. These include macrophage colony-stimulating factor (M-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), IL-3 and factor increasing monocytopoiesis (FIM) [Waarde et al., 1977; Shum and Galsworthy, 1982] [Reviewed by Auger and Ross, 1992].

Under light microscopy, macrophages are generally large, irregularly shaped cells measuring 25-50 μm in diameter, with cytoplasm containing fine granules and multiple large, azurophylic granules. Cytoplasmic vacuoles are often seen near the cell periphery, indicating active pinocytosis. The nucleus of a macrophage is round or kidney-shape, and possess one or two nucleoli and finely dispersed nuclear chromatin [Reviewed by Auger and Ross, 1992].

Electron microscopy shows an eccentric nucleus of variable shape, with chromatin disposed in fine clumps. Cytoplasm contains scattered strands of rough endoplasmic reticulum, and a well-developed Golgi complex in a juxtanuclear position. It has also large mitochondria, variable numbers of vesicles, vacuoles and pinocytic vesicles, and electron-dense, membrane bound lysosomes [Reviewed by Auger and Ross, 1992].

### 1.1.2. Plasma membrane structure and receptors of the macrophage:

The macrophage surface is covered in ruffles and microvilli, and small surface blebs [Auger and Rose, 1992]. Mononuclear phagocytes bear at least 50 distinct types of surface receptors that are coupled to various second messengers. These receptors determine and control activities, such as growth, differentiation, activation, recognition of foreign antigens and cytokines, endocytosis, migration, and secretion. These receptors can be classified into several groups, such as Fc and complement receptors, cytokine receptors, lipoprotein receptors, advanced glycosylation end-products receptors, Lectin-like receptors, and adhesion receptors. There are at least 4 distinct Fc receptors, including a high affinity receptor for immunoglobulins of the $IgG_{2a}$ isotype ($Fc_{\gamma}2aR$) and a distinct receptor for immunoglobulins of the $IgG_1$ and $IgG_{2b}$ isotypes ($Fc_{\gamma}1R/Fc_{\gamma}2bR$) [Springer and Unkless, 1984].

In general, cytokine receptors can be classified into several distinct families, according to similarities of the extracellular amino acid sequences and intracellular signalling pathways [Miyajima et al., 1992]:

1) Receptors without tyrosine kinase activity:

1a) Type 1 cytokine receptors: (e.g. Haematopoietic growth factor receptors):
These include IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, GM-CSF, G-CSF, and Epo (Erythropoietin). These receptors have a conserved motif containing cysteine and tryptophan residues in their N terminal portion. A typical sequence of Trp-Ser-X-Trp-Ser (W-S-X-W-S) is present in the C terminus.

The IL-2R possess three polypeptide chains, designated α, β, and γ, and it is known that a heterodimer of β and γ chains is responsible for IL-2 signal transduction [Takeshita et al., 1992; Taniguchi and Minami, 1993]. It was reported that the cytosolic domain of the IL-2Rβ chain cytosolic domain has a binding site for the Src family nonreceptor tyrosine kinases [Minami et al., 1993]. The IL-2Rγ chain is shown to interact with IL-4 and IL-7, and probably with IL-13 [reviewed by Kishimoto, 1994].

The IL-4 receptor is typically present at low levels on the cell surface (a few hundred to a few thousand molecules per cell) [Mosley et al., 1989]. The IL-4 receptor is a high affinity receptor, and is expressed on a variety of cells including nonhaematopoietic cells such as fibroblasts, neuroblasts, keratinocytes, hepatocytes and stromal cells [Lowenthal et al., 1988a]. The murine and human IL-4Rs are about 50% identical at the amino acid levels and contain the common motif of cytokine receptors [Idzerda et al., 1990; Galizzi et al., 1990]. There are three kinds of IL-4 receptor reported, so far. The molecular mass of the membrane bound form of IL-4Rα chain is 140 kDa. It has been shown the γ subunit of the IL-2 receptor interacts with IL-4R, and forms part of the membrane bound IL-4 receptor complex [reviewed by Kishimoto, 1994].

The second type of IL-4 receptor lacks the cytoplasmic region, enabling it to internalise ligand, but not to induce downstream signalling in the cell. A mouse cDNA encoding a soluble extracellular domain of the IL-4R has also been isolated, and this soluble IL-4R is capable of binding IL-4 with high affinity, and is expressed in several cell lines and tissues, nevertheless, the physiological importance is not known [Mosley et al., 1989], although it might be a carrier protein for IL-4 in the serum [Fernandez-Botran and Vitetta, 1991]. Some of the soluble receptors, e.g., soluble IL-4R and TNFR, serve as inhibitors for their respective ligands, at least in vitro [Engelmann et al,. 1989; Mosley et al., 1989].

The IL-4 receptor on resting B cells is upregulated by LPS or IL-4 itself, and the IL-4R on resting T cells is increased by stimulation with ConA or IL-4 [Lowenthal et al., 1988b; Ohara and Paul, 1988].

1b) Type 2 receptors: (e.g. IFN-α/β and IFN-γ receptors):

IFN-α and IFN-β are produced by virus infected cells, whereas, IFN-γ is produced by activated T cells and natural killer (NK) cells [Weismann and Weber, 1986]. IFN-α and IFN-β share a common receptor, but the IFN-γ receptor is distinct [Aguet et al., 1984; Merlin et al., 1985]. IFN-γ receptors are expressed only in limited numbers (200-10,000/cell), like other cytokine receptors, but they bind ligand with high affinity ($K_a$=2x$10^9$-2x$10^{10}$ M) [Gray et al., 1989]. The IFN-γ receptor consists of two chains, α and β (accessory factor -1, AP-1) [Soh et al., 1994]. IFN-γ receptor α chain has the ability to bind to IFN-γ with high affinity. Nevertheless, The presence of IFN-γRβ chain is required for down-stream signalling, such as the activation of signal transducing factor-IFN-γ (STF-IFN-γ) and the induction of interferon response factor-1 (IRF-1) [Cook et al., 1994; Hemmi et al., 1992]. The murine IFN-γ receptor α chain has serine rich polypeptides of 471 amino acids, with a predicted molecular mass of 52.5 kDa, and

is synthesised with an additional 17 amino acid signal peptide. The extracellular domain has 228 aa. and the intracellular domain has 200 aa. that are separated by a single 23 or 21 aa. transmembrane domain. The intracellular domains of IFN-γ receptors contain an unusually high content (20-25%) of serine and threonine residues [Gray et al., 1989].

1c) Type 3 receptors: (e.g. TNF receptors, Fas, CD40, NGF binding protein):

Since TNF-α and TNF-β are structurally and functionally homologous proteins [Goedell et al., 1986], two distinct receptors of TNF of 55 and 75 kDa bind TNF-α and TNF-β equally well [Schall et al., 1990]. Although the relationship between TNFR and Fas is not yet clear, it is known that an anti-Fas antibody can mimic TNF function and induce apoptosis [Yonehara et al., 1989].

1d) Type 4 receptors: (e.g. Immunoglobulin like receptors, IL-1R):

IL-1R has higher affinity for IL-1β than for IL-1α in T cells. However, it shows higher affinity for IL-1α than for IL-1β in B cells. IL-1R has an Ig-like motif in the extracellular domain. [Reviewed by Miyajima et al., 1992]. The cytoplasmic domain of the human 80 kDa IL-1R contains a sequence Lys-Lys-Ser-Arg-Arg (429-433 aa.) resembling a PKC phosphorylation site [Sims et al., 1988].

2) Receptors with tyrosine kinase activity:

2a) Receptors of the tyrosine kinase family (e.g. M-CSF and stem cell factor, SCF):

These receptors are also called proto-oncogenes, because they relay mutative signals. The M-CSF receptor (M-CSFR) extracellular domain contains five Ig-like

repeats, and the large cytoplasmic domain has a tyrosine kinase, having a highly homologous aa. sequence with platelet derived growth factor receptor (PDGFR).

2b) Receptors with nonreceptor tyrosine kinase activity: Although IL-2 is a member of type 1 receptors, Lck, which is a tyrosine kinase, is associated with IL-2Rβc (common) chain in the absence of IL-2 stimulation, and activated rapidly upon ligand binding to IL-2R [Hatakeyama et al., 1991; Horak et al., 1991]. There is clear evidence that type I and type II IFN systems interact with Jak1 and Tyk2, and Jak1 and Jak2, respectively [Reviewed by Darnell et al., 1994; Taniguchi, 1995].

LPS receptors; the best known LPS receptor is CD14, which is a 55 kDa phosphatidylinositol (PI)-linked protein, and is present on monocytes and macrophages [reviewed by Lynn and Golenbock, 1992]. A soluble form of the protein also exists and can be found in human serum [Bazil et al., 1989]. There is a 50 kDa protein secreted by hepatocytes, namely, lipopolysaccharide binding protein (LBP). It binds to LPS and increases the endotoxin effect and mediates cellular responses even at subthreshhold LPS concentrations via binding to CD14 and increasing the binding affinity of LPS to CD14 [Schumann, 1992]. An LPS resistant mutant (called LR-9) of the J774.1 cell line was isolated, recently, which has no CD14 expression on the cell surface, but possess normal CD14 mRNA levels. In these cells, low concentrations of LPS (1-10 ng/ml) induced TNF-α production and arachidonate metabolite release in normal J774.1 cells, but failed to induce TNF-α production and arachidonate metabolite release in the LR-9 mutant cells. Interestingly, high concentrations of LPS (100-1000 ng/ml) could successfully induce TNF-α production and arachidonate metabolite release in both cell lines, indicating other LPS induction pathways rather than the crucial CD14-mediated stimulation [Nishijima et al., 1994]. It has been suggested that LPS can be taken up by nonspecific pinocytosis by macrophages, and soluble LPS receptors in the cytosol or microtubules, such as β-tubulin and microtubule associated protein-2

in the cytoskeleton [reviewed by Ding and Nathan, 1992] may be responsible for the response to high concentrations of LPS in the LR-9 mutant cell line.

The information about the cytosolic domains and their functions in intracellular signalling pathways of cytokines, especially IFNs and IL-4, and LPS receptors will be given below under the section "Signalling pathways in macrophage activation". Some of the macrophage receptors are shown in table 1.1.

**Table 1.1: Surface receptors of monocytes and macrophages:**

| |
|---|
| Fc receptors |
| $IgG_1$, $IgG_2$, $IgG_3$, $IgG_4$, IgA, IgE |
| Complement receptors |
| C3b, C3bi, C5a, C1q |
| Cytokine receptors |
| MIF, LIF, CF, MFF, IL-1, IL-2, IL-3, IL-4, IL-10, IL-13, TGF-β, IFN-α, IFN-β, IFN-γ, Colony-stimulating factors (GM-CSF, M-CSF/CSF1) |
| Receptors for peptides and small molecules |
| $H_1$, $H_2$, 5HT, 1,2,5-dihydroxy vitamin D3, N-formulated peptides, Enkephalins/endorphins, substance P, Arg-vasopressin |
| Hormone receptors |
| Insulin, Glucocorticosteroids, Angiotensin |
| Transferrin and lactoferrin receptors |
| Lipoprotein lipid receptors |
| Anionic low density lipoproteins, $PGE_2$, LTB4, $LTC_4$, $LTD_4$, Apolipoproteins B and E |
| Receptors for coagulants and anticoagulants |
| Fibrinogen/fibrin, Coagulation factor VII, α1-Antithrombin, Heparin |
| Fibronectin receptors |
| Laminin receptors |
| Mannosyl, fucosyl, galactosyl residue, AGE receptors |
| α2-Macroglobulin-proteinase complex receptors |
| Others |
| Cholinergic agonists, α1-Adrenergic agonists, β2-Adrenergic agonists |

AGE, advanced glycosylation end-products; GM, granulocyte macrophage; CSF, colony stimulating factor; VLDL, very low density lipoprotein; PG, prostaglandin; LT, leukotriene; Ig, immunoglobulin; C, complement; MIF, macrophage inhibitory factor; MAF, macrophage activating factor; LIF, leukocyte migration inhibition factor; MFF, macrophage fusion factor; IL, interleukin; H, Histamine; 5HT, 5-hydroxytryptamine [Reviewed by Auger and Rose, 1992].

### 1.1.3. Macrophage functions:

Functional, morphological, and phenotypic heterogeneity of macrophages may reflect the local environment of the macrophages and involvement in various physiological or pathological processes. Macrophage nomenclature depends on their tissue distribution, like Kupffer cells in the liver, alveolar histiocytes in the lung or giant cells in the central nervous system. Macrophages in the immune system carry out many functions, such as phagocytosis and destruction of micro-organisms, chemotaxis, through pseudopodia movements in response to chemical substances, antigen processing and presentation, secretion of substances including some cytokines and tumour cell control. Some important secretory and release products of macrophages are IFNα, IFNβ, M-CSF, GM-CSF, G-CSF, TNFα, IL-1, IL-6, IL-8, IL-12, TGFβ, reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI), PAF and cyclo-oxygenase products, such as $PGE_2$, prostacyclin and thromboxane [reviewed by Nathan, 1987; Auger and Ross, 1992].

Macrophages play important roles in health and disease. These roles can be classified into at least 9 areas: The acute phase protein response, haematopoiesis, haemostasis, the destruction of micro-organisms, the disposal of damaged or senescent cells, wound healing, tissue repair and remodelling, atherogenesis, they also play an important role in autoimmunity and central nervous system diseases [Auger and Ross, 1992].

### 1.1.4. Macrophage activation:

The activation of macrophages is a very important step in cellular immunity, since their secretory products can drive the responses of T cells and natural killer (NK) cells, and the phagocytosis and destruction of foreign particules, like micro-organisms and parasites [reviewed by Auger and Ross, 1992]. Macrophage

activation involves the enhancement and suppression of various genes encoding for proteins essential for biological functions. This process can be regulated by many cytokines some of which are listed in table 1.2.

IFN-γ acts on macrophages by using high affinity surface receptors [Arai et al., 1990]. Treatment of macrophages with IFN-γ induces a state which was termed "primed". Primed macrophages become cytolytic when pulsed with low levels of LPS (ng/ml). These cytolytic macrophages are termed "fully activated" [Adams and Hamilton, 1992]. The role of signalling mechanisms will be explored in section 1.3.

Bacterial endotoxin is generally accepted as a prototypic macrophage activator. LPS, complexed to the 60 kDa serum glycoprotein lipopolysaccharide binding protein (LBP), can bind to the 55 kDa membrane glycoprotein CD14 and directly to the CD11/CD18 complex, which are expressed by monocytes and macrophages [Wright et al., 1990; Lynn and Golenbock, 1992].

In fully activated macrophages, cytotoxic effects are mainly dependent on two kinds of secretory products of activated macrophages; reactive oxygen intermediates (ROI), such as superoxide [Dradth and Karnovsky, 1975], hydrogen peroxide [Nathan and Root, 1977], hydroxyl radical [Weiss et al. 1977], and reactive nitrogen intermediates (RNI), such as NO, nitrite, and peroxynitrite [Stuehr and Marletta, 1985; reviewed by Beckman and Tsai, 1994].

**Table 1.2: Selected extracellular ligands with activating or suppressive effects on macrophages:**

| Ligand | Macrophage activation | Macrophage suppression |
|---|---|---|
| Cytokines | | |
| IFNα | + | + |
| IFNβ | + | |
| IFNγ | + | |
| TNFα | + | + |
| IL-1 | + | |
| IL-2 | + | |
| IL-4 | + | + |
| IL-6 | + | |
| IL-13 | | + |
| Colony-stimulating factors | | |
| CSF-1 | | - |
| GM-CSF | + | |
| Growth factors | | |
| TGFβ | - | + |
| MCP-1 | + | |
| Other molecules | | |
| LPS | + | + |
| mal-BSA | + | + |
| $PGE_2$ | | + |
| Dexamethasone | | + |
| Serotonin | | + |

adapted from Adams and Hamilton, 1992;

+; positive effect, -; negative effect.

Activated macrophages encountering micro-organisms may affect them in at least three ways. Firstly, the intracellular environment can be changed to one which is unsuitable for reproduction of micro-organisms. For instance, the intracellular pH may be decreased or nutrition factors may be denied to micro-organisms in the phagolysosome. Secondly, a killing reaction may be activated against dividing organisms. Thirdly, microbiostatic or microbicidal effector molecules like NO may be produced by activated macrophages. Therefore, some intracellular organisms are maintained in a non-replicating state.

## 1.2. NITRIC OXIDE

### 1.2.1. Historical review and the role of NO in organisms and the immune system :

NO has the lowest molecular weight of any known bioactive mammalian secretory product. Therefore, its diffusion in tissues is very fast, and interactions in signalling pathways in and between cells are possible. The first report came from Mitchell and colleagues in 1916 suggesting that mammals produce oxides of nitrogen [Mitchell et al., 1916]. Nevertheless, it was ignored until the 1980s. However, the existence of endogenous nitrite/nitrate synthesis in mammals was observed by some authors in the 1980s [Green, et al., 1981; Stuehr and Marletta, 1985]. Before 1987, NO had been known mainly as an atmospheric pollutant. Since then, NO synthases have been found in a very diverse range of other species from slime moulds through snails, horseshoe crabs, starfish, insects, chickens, mice, cows and humans [reviewed by Knowles, 1994]. Hibbs and colleagues reported the connection between L-arginine, L-citrulline and nitrite/nitrate release by activated macrophages [Hibbs et al., 1987a,b]. It was declared that NO is endothelial derived relaxing factor (EDRF) [Furchgott, 1988; Ignarro et al., 1987]. Moncada and co-workers demonstrated the widespread vasoregulatory effects of

NO [Palmer et al., 1987, reviewed by Moncada et al., 1991]. Since then, progress in the biology of NO and signalling mechanisms has been increasingly fast. NO is a mediator for neurotransmission [Bredt and Snyder, 1992], penile erection [Rajfer et al., 1992; Burnett et al., 1992] and host defence mechanisms. In 1992, Billiar and colleagues showed that NO produced by activated macrophages may regulate the immune response [Billiar et al., 1992; Mills, 1991]. Until 1987, it was believed that activated macrophage cytotoxicity was due to the synthesis of reactive oxygen intermediates (ROI) by NADPH oxidase. Drapier and co-workers reported in 1991 that using electron paramagnetic resonance spectroscopy, nitrosyl-iron complexes are present in tumour cells contiguous to NO-generating macrophages.

It is possible that NO is a paracrine effector molecule. A combination of NO with the superoxide anion in an acidic environment (e.g. in a phagosome), could yield toxic hydroxyl radicals [Mauel et al., 1992]. On the other hand, it was suggested that by reacting with the superoxide anion, NO may have a protective role in tissues, during inflammation [Billiar et al., 1992]. In addition, many authors suggested that NO production has potent cytotoxic effects for the facultative intracellular fungal pathogen *Cryptococcus neoformans* [Granger et al., 1988], schistosomula of *Schistosoma mansoni* [James and Glaven, 1989], intracellular amastigotes of *Leishmania major* [Green et al., 1990a,b; Liew et al., 1990a,b; Mauel et al., 1991], bacteria *Francisella tularensis* [Fortier et al., 1992] and mycobacteria [Adams et al. 1991] as well as intracellular trophozoites of *Toxoplasma gondii* [Adams et al., 1990]. Moreover, it was demonstrated by Liew and colleagues in 1990 that *Leishmania major* was killed directly by exposure to NO. Furthermore, it is important to consider other NO effects at the molecular level. Hibbs and co-workers showed in 1987 that the oxidation of L-arginine into nitrogen oxides is an effector mechanism causing target cell cytostasis as well as the reversible inhibition of the Krebs cycle enzyme aconitase by removal of its labile iron atom [Beinert and Kennedy, 1989] from the (4Fe-4S) cluster [Drapier and

Hibbs 1986]. Nitric oxide also leads to the loss of activity of complexes I and II of the electron transport chain in both macrophages and co-cultured tumour target cells [Drapier and Hibbs, 1988; Hibbs et al. 1988; Stuehr and Nathan, 1989]. It was reported that NO produced by peritoneal macrophages in response to IFN-γ did not have killing activity on *Brucellae abortus*, since it does not possess iron-sulphur-containing enzymes that are targets for NO [Jiang et al., 1993].

Finally, NO can regulate the following processes in organisms: transcription factor activation; translation of mRNAs controlling Fe metabolism; mutagenesis; apoptosis; glycolysis and mitochondrial electron transport; protein acylation; deoxynucleotide synthesis; fusion of myoblasts; adhesion of platelets and neutrophils; proliferation of myeloid progenitors; T cells, keratinocytes, and tumour cells; release of pituitary hormones; the tone of bronchi and sphincters in the gastrointestinal system; the contractions of stomach, intestines, uterus, and heart; erection of the penis; opioid dependence; tolerance, and toxicity; memory; sleep; sunburn; and the pressure of the blood [Reviewed by Nathan and Xie, 1994].

### 1.2.2. NO synthases isoenzymes and NO synthesis:

It has been shown that L-arginine is a precursor for nitrate biosynthesis in humans [Leaf et al., 1989]. Experiments with $^{15}N$-labelled arginine showed that both nitrite and nitrate were derived from the terminal guanidino nitrogen atom of this amino acid [Iyengar et al., 1987]. The oxidation products of L-arginine in murine macrophages have been characterised to be L-citrulline and NO, which is rapidly converted into nitrite and nitrate in the presence of molecular oxygen [Marletta et al., 1988; Stuehr and Marletta, 1987]. It should be added that the half life of NO in oxygenated media in vitro ranges from 5 to 50 seconds. Its half life in tissues is probably much shorter [Kelm and Schrader, 1990]. The enzymes, which catalyse the production of NO from L-arginine, are known as NO synthases (EC

1.14.13.39). In spite of the fact that the term NO is used for simplicity, the cells containing NOS possess a mixture of NO, $NO_2$, $N_2O_3$, $NO_2^-$, $NO_3^-$, nitrosamines, nonprotein nitrosothiols, and S-nitrosylated proteins [reviewed by Nathan, 1992].

Some analogues of L-arginine were reported as inhibitors of NO production in macrophages activated by IFN-γ and LPS. These include $N^{\omega}$-methyl-L-arginine (L-NMMA) [Iyengar et al., 1987], N-iminoethyl-L-ornithine (L-NIO) [McCall et al., 1988], $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME) and $N^{\omega}$-nitro-L-arginine (L-NNA). L-NAME and L-NNA effects were fully reversible [Gross et al., 1990; McCall et al., 1991]. In addition, L-canavanine was shown to be an inhibitory effector molecule of the NO producing pathway [Iyengar et al., 1987].

Studies using $^{18}O_2$ and mass spectrometry have shown that the inducible NOS (iNOS) incorporates molecular oxygen into both NO and citrulline in activated J774 cells [Leone et al., 1991]. It is clear that the inducible NOS, like the constitutive enzyme, can be classified as a dioxygenase.

Three distinct isoforms of NOS enzymes (more formally L-arginine, NADPH: oxidoreductase, NO-forming, EC 1.14.13.39) are accepted to be responsible for NO production from L-arginine and molecular oxygen. All of them have a consensus sequence for the binding of reduced nicotinamide adenine nucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and calmodulin and protein kinase A phosphorylation. The endothelial NOS (*e* NOS) also has an N-terminal consensus sequence for myristoylation [reviewed by Knowles, 1994]. Two of them, *e* NOS and neuronal NOS (*n* NOS), are constituvely expressed and calcium-dependent and generate low levels of NO, which are typically found in endothelial cells and neurones, respectively. The other is inducible by cytokines and/or microbial products like LPS [reviewed by

Moncada et al., 1991] and generates high levels of NO. This isoform (iNOS) is not dependent on calcium concentrations in the physiological range, probably because calmodulin is tightly bound. The prototype of iNOS is found in macrophages that can be induced by cytokine treatment to 20 times its basal level. This induction takes several hours until maximal activity is reached, and cells produce nitrogen oxides over a period of several days [Stuehr and Marletta, 1987: Marletta et al, 1988]. To date, NOS's have been purified from rat, pig, cow and man, rat, mouse and bovine cells [Reviewed by Nathan, 1992]. Some cell types may have both pathways [Kilbourn and Belloni, 1990], such as macrophages [Reviewed by Nathan and Xie, 1994]. The isoform purified and cloned from endothelial cells is also expressed in neurones [Dinerman et al, 1994]. The iNOS purified and cloned from macrophages is also expressed in neurones, endothelial cells, vascular smooth muscle cells and others [Oswald et al, 1994].

In general, the homology between equivalent isoforms from different species averages 90 ± 6 %, while within species, the homology between isoforms averages 53 ± 2 % [Nathan and Xie, 1994].

The control mechanisms of iNOS synthesis may be at different levels of downstream signalling before the nucleus, at the transcriptional or post transcriptional levels. Ferric ions in the J774 macrophages inhibit iNOS transcription [Weiss et al., 1994]. Examples of post transcriptional control include TGF-β destabilization of iNOS mRNA in mouse macrophages. Cycloheximide stabilises iNOS mRNA in the vascular smooth muscle cells, while it prevents iNOS mRNA expression in mouse macrophages [Nathan and Xie, 1994]. The function of both types of NOS enzyme can be influenced by phosphorylation of their specific sequences for protein kinases, such as PKC, PKA and calcium-calmodulin-dependent protein kinases. It was shown that NOS purified from rat brain, which is $Ca^{2+}$ and calmodulin-dependent, was phosphorylated by calcium calmodulin-

dependent protein kinase II and PKC [Nakane et al., 1991]. iNOS displays a consensus site for phosphorylation by PKA located at a site different for the comparable sequence in cNOS. In both cNOS and iNOS this is a weak phosphorylation sequence, Lys-Arg-X-X-Ser, for PKA [Lowenstein et al., 1992]. Brune and Lapetina (1991) showed that PKA selectively and stoichiometrically phosphorlates NOS isolated from rat and porcine cerebellum. Table 1.3. shows the similarities and differences between the two isozymes of NOS.

### 1.2.3. Induction of NO production in macrophages:

The first observation about nitrate production came from rats treated with bacterial LPS; a nine-fold increase in nitrate excretion in their urine was observed [Wagner et al., 1983]. In vitro studies have also shown that murine macrophages treated with LPS produced nitrite and nitrate [Stuehr and Marletta, 1985]. IFN-$\gamma$ alone or in combination with TNF-$\alpha$, TNF-$\beta$ or LPS leads to the formation of nitrite and nitrate in murine macrophage cultures and in a number of murine macrophage cell lines [Stuehr and Marletta, 1987]. Once iNOS is induced, NO may be released into tissues for days.

The mechanism which terminates high-output NO release has not been elucidated. However, there is evidence showing that NO itself can cause feedback inhibition [Assreuy et al., 1993]. The mechanism of this feedback inhibition is unknown at present .

It was reported that calcium ionophore A23187 could mimic the effects of IFN-$\gamma$ as a priming stimulus when applied together with LPS [Buchmuller-Rouyiller and Mauel, 1991]. Endogenously formed TNF-$\alpha$ and $PGE_2$ are coinducers in rat Kupffer cells [Gaillard et al., 1991]. On the other hand, There are some cytokines,

**Table 1.3: Similarities and differences between the two NO synthases:**

| Constitutive | Inducible |
|---|---|
| Cytosolic* | Cytosolic |
| NADPH dependent | NADPH dependent |
| Dioxygenase | Dioxygenase |
| Inhibited by L-arginine analogues | Inhibited by L-arginine analogues |
| $Ca^{2+}$/calmodulin dependent | $Ca^{2+}$/calmodulin independent |
| Picomoles NO released | Nanomoles NO released |
| Short-lasting release | Long-lasting release |
| Unaffected by glucocorticoids | Induction inhibited by glucocorticoids |

(*: The *e* NOS enzyme has an N-terminal consensus sequence for myristoylation, and can anchor the plasma membrane)

which were reported capable of blocking both NO synthesis and macrophage antimicrobial action. TGF-β, macrophage deactivating factor [Nelson et al., 1991; Nathan, 1992] and IL-4 [Liew et al., 1991] have inhibitory effects on NO production in macrophages stimulated by IFN-γ and LPS. In addition, it was shown that glucocorticoids prevent induction of NOS in smooth muscle cells and macrophages [Rees et al., 1990; Knowles et al., 1990]. On the other hand, forskolin, an activator of adenylate cyclase, or dibutrylyl adenosine 3', 5'-cyclic monophosphate induces iNOS only modestly in vascular smooth muscle cells but synergistically induces iNOS when applied together with IFN-γ, IL-1, or TNF in the same cells [Koide et al., 1993]. iNOS expression can also be inhibited by endogenous hormones such as corticosteroids, cortisol and corticosterone.

Exogenous application of the potent corticosteroid, dexamethasone, can almost completely inhibit iNOS expression in a number of cell types and reduces iNOS expression in hepatocytes [Geller et al., 1993]. The inhibitors of NO synthases are shown in table 1.4.

## 1.3. SIGNALLING PATHWAYS IN MACROPHAGE ACTIVATION:

The binding of IFN-γ to its receptor has been shown to produce at least one rapidly acting second messenger [Prpic et al., 1989]. Specifically, addition of IFN-γ to macrophages causes a marked rise in intracellular pH accompanied by a concomitant influx of $Na^+$ ions within seconds. This effect is inhibited by amiloride, suggesting that the exchange was mediated via the $Na^+/H^+$ exchanger or antiport [Smith et al., 1988].

In mutant cells lacking Jak1 and Jak2 (non-receptor tyrosine kinases), the cells failed to respond to IFN-γ. IFN-γ treatment of cells resulted in the activation of p91

**Table 1.4: Inhibitors of NOS's:**

| Class | cNOS | iNOS |
|---|---|---|
| Inhibitors of action | | |
| Substrate analogues | $N^{\omega}$-nitro-L-arginine<br>N-iminoethyl-L-ornithine<br>$N^{\omega}$-methyl-L-arginine<br>$N^{\omega}$-amino-L-arginine<br>$N^{\omega}$-nitro-L-arginine methyl ester | $N^{\omega}$-nitro-L-arginine<br>$N^{\omega}$-methyl-L-arginine<br>$N^{\omega}$-amino-L-arginine |
| Flavoprotein binders | Diphenylene iodonium | Diphenylene iodonium<br>Di-2-thienyl iodonium |
| Calmodulin binders | Calcineurin<br>Trifluoroperazine<br>N-(4-aminobutyl)-5-chloro-2-naphthalene-sulphonamide | None described |
| Heme binder | Carbon monoxide | Carbon monoxide |
| Depleter of tetrahydrobiopterin | 2,4-Diamino-6-hydroxy-pyrimidine | |
| Inhibitors of induction | None described | Corticosteroids<br>TGF-$\beta_{1, 2, 3}$<br>IL-4<br>IL-10<br>IL-13<br>Macrophage deactivation factor |

protein, named cytokine activated transcription factor-1, signal transducers and activators of transcription (Stat1), which is a component of IFN-stimulated gene factor-3 (ISGF-3) induced by IFN-α [Darnell et al., 1994]. The requirement of accessory factor (AF-1) for the induction of Stat1 activation has also been reported [Cook et al., 1994]. It was suggested that Jak1 and Jak2 are associated with the IFN-γR, and Jak1 and Tyk2 are associated with the IFN-αR. After the activation of these kinases Stat1 and Stat2 can be phosphorylated [Darnell et al., 1994]. The IFN-γR might be phosphorylated on tyrosine residues in response to IFN-γ binding to its receptor, and Stat1 may bind to the phosphorylated IFN-γR [Darnell et al., 1994]. On the other hand, the interferon-stimulated gene factor-3 (ISGF-3) formation, consists of Stat1 and Stat2, has been reported in response to IFN-α treatment of cells, leads to nuclear transcription activation, binding to the interferon-stimulated response element (ISRE) [reviewed by Darnel et al., 1994]. Other protein complexes induced by IFN-α treatment are ISGF-1 and ISGF-2 [David and Larner, 1992]. In monocytes (U937), instead of the tyrosine phosphorylation of Stat1 carried out by Jak kinases, serine phosphorylation has been found to be important for the elevated DNA-binding and transcriptional potential of IFN-γ-activated transcription factor (GAF). In GAF activation, the Jak-dependent tyrosine phosphorylation and dimerization of Stat1 result in the potential binding of GAF to the IFN-γ activation site (GAS) on the gene promoter [Eilers et al., 1995].

A variety of signals, including the lipopolysaccharide component of various bacterial cell walls, high concentrations of crude lymphokines, supernatants of tumour cells, heat-killed gram positive micro-organisms such as *Listeria monocytogenes*, and liposome-encapsulated muramyl dipeptide, drive primed macrophages to the fully activated stage [Adams and Hamilton, 1984; Fidler, 1986]. Macrophages are a potent source of arachidonic acid metabolites. When macrophages interact with an appropriate stimulus, such as bacterial LPS, immune complexes, PMA, the $Ca^{2+}$ ionophore A23187 and zymosan particles [reviewed

by Aderem and Cohn, 1986], phospholipases are activated, resulting in the liberation of arachidonate from membrane phospholipids and its quantitative oxygenation via either the cyclooxygenase or lipoxygenase pathways. In macrophages, one of the major cyclooxygenase products synthesised is $PGE_2$ [Bonney et al. 1978, Scott et al. 1980].

One of the signalling pathway initiated by LPS in macrophages is the breakdown of polyphosphoinositides. Stimulation of macrophages with small concentrations of LPS (in the range of ng/ml) can result in the hydrolysis of phosphatidylinositol-4,5-bisphosphate ($PIP_2$), the formation of inositol-1,4,5,-tris-phosphate ($IP_3$), and tetra-*kis*-1,3,4,5,-inositol phosphate ($IP_4$) as well as the formation of 1,2-diacylglycerol (DAG) [Prpic et al., 1987]. LPS has been shown to produce a rapid and sustained elevation in $Ca^{2+}$, dependent on an extracellular supply of calcium [Prpic et al., 1987]. In Raw 264.7 cells (murine macrophage cell line), LPS-induced NOS activity is dependent upon PKC, tyrosine kinase (TK) activities and endogenous poly-ADP ribosylation factors [Paul et al., 1993]. It also appears that the TK inhibitor, genistein inhibits iNOS activity and iNOS mRNA expression in J774 cells induced by IFN-γ and LPS [A. Severn, personal communication]. Recently, it has been shown that LPS can provoke cellular responses by mimicking the action of ceramide [Wright and Kolesnick, 1995], which is a sphingolipid-derived intracellular messenger. However, LPS could not be substituted by ceramide in J774 cells [W.A. Sands, personal communication]. In myeloid cells, LPS, which has structural similarity to ceramide, has been shown to activate ceramide-activated protein kinase [Joseph et al., 1994]. It is suggested that TNF and IL-1 may mediate immune responses through ceramide generation [Kolesnick and Golde, 1994]. Treatment of cells with LPS results in nuclear factor κB activation (NF-κB), as a result of phosphorylation of the inhibitor κB (IκB) [Muroi and Suzuki, 1993]. NF-κB exists in the cytoplasm associated with IκB,

and the phosphorylation of NF-κB/IκB complex leads to release of the active NF-κB molecule [Henkel et al., 1993].

The cytoplasmic domain of the IL-4R contains 553 residues and is separated from the extracellular domain (137 residue) with the 24 residue transmembrane domain. The threonine at position 298 is potentially a protein kinase C phosphorylation site, which could play a role in receptor regulation and signal transduction [Edelman et al., 1987]. However, using a mutant hIL-4 receptor, it has been shown that amino acids in the cytoplasmic domain between positions 96 and 138 are important for signal transduction for the cell growth effect of IL-4 [Reviewed by Izuhara et al., 1994b].

It has been shown by some authors that tyrosine kinases may interact with IL-4R, which could participate in the downstream signalling pathway in cells [Reviewed by Miyajima et al, 1992]. There is clear evidence that IL-4 treatment of HT2 cells results in protein tyrosine phosphorylation of 140, 110, 100 and 92 kDa proteins. The 92 kDa protein was recognised by anti-c-fes antibody, suggesting that c-fes proto-oncogene might be involved in the IL-4 signal transduction pathway [Izuhara et al., 1994a]. Moreover, the IL-4R cytosolic domain can be phosphorylated upon IL-4 stimulation [Izuhara et al., 1994b]. Results of studies by Izuhara and colleagues indicated that PI3 kinase is associated with the IL-4R, after IL-4 binds to its receptor. Involvement of PKC, cAMP elevation and $Ca^{2+}$ influx in IL-4-mediated signalling in B cells have also been suggested [Finney et al., 1990]. It has been shown that PKCζ isoform can be activated by phosphatidyl inositol kinase-3 (PI-3 kinase) products, such as phosphatidylinositol 3,4,5-tris-phosphate ($PIP_3$), and it can participate in the signal transduction of IL-4 [Nakanishi et al., 1993].

At the nuclear level, the effect of IL-4R activation results in the activation of a 100 kDa cytosolic protein, called interleukin-4-induced transcription factor (IL-4 Stat or

Stat-6), since the sequence of amino acids is similar to the Stat family [Wakao et al., 1994]. It was reported that the binding of IL-4 to IL-4R causes IL-4 Stat dimerization by the involvement of tyrosine phosphorylation at the C-termini [Hou et al., 1994], allowing them to leave the receptor and migrate to the nucleus [Hill and Treisman, 1995].

### 1.3.1. G-proteins and signal transduction:

G-protein-linked receptors are the largest family of cell-surface receptors. GTP is required for the activation of adenylate cyclase by β–adrenergic agonists. Other known functions regulated by G-proteins are the inhibition or activation of adenylyl cyclase, the stimulation of retinal cGMP phosphodiesterases, the stimulation of phosphoinositide hydrolysis and the regulation of ion channels [reviewed by Hepler and Gilman, 1992; Houslay and Milligan, 1990]. G-proteins can be subclassified into three main classes, as trimeric GTP-binding proteins, G-proteins which direct ribosomal protein synthesis, and monomeric GTP-binding proteins. Table 1.5. shows the classification and functions of trimeric G-proteins.

The binding of GTP causes activation of the G-protein and the hydrolysis of GTP initiates deactivation. It is clear that the α subunit of the trimeric G-protein is not the only subunit active in cellular signalling, but the β/γ subunits also play a role in some cells, such as cardiac atrial muscle ($G_S$) and bovine rod outer segment cells (TD1) by activating phospholipase $A_2$ [Kim et al, 1989; Jelsema and Axelrod, 1987]. Meanwhile, in HL-60 cells, phospholipase C-β activity is stimulated by the G-protein β/γ subunits [Camps et al, 1992]. Moreover, G-protein α subunits, which are hydrophilic, can not be anchored to the phospholipid rich membrane without β/γ subunits [Sternweis, 1986, 1993]. The β subunit alone is involved in coupling specific receptors to calcium channels [Kleus et al., 1992]. β/γ subunits of $G_i$ protein inhibit adenylyl cyclase activity by scavenging α subunits from the

**Table 1.5: The structure and function of trimeric G-proteins:**

| G-protein | Sensitivity to ADP-ribosylation by bacterial toxins | Function | Distribution |
|---|---|---|---|
| $G_s$ | Yes, Cholera toxin | Stimulation of adenylate cyclase, activation of dihydropyridine-sensitive $Ca^{2+}$ channels | Universal |
| $G_{olf}$ | Yes, Cholera toxin | Stimulation of adenylate cyclase | Olfactory sensory neurones |
| $G_i1$ | Yes, Pertussis toxin | Undefined | Limited; high levels in brain |
| $G_i2$ | Yes, Pertussis toxin | Inhibition of adenylate cyclase, stimulation of PLC | Universal |
| $G_i3$ | Yes, Pertussis toxin | Regulation of $K^+$ channels | Undefined; Universal |
| $G_o$ | Yes, Pertussis toxin | Regulation of $Ca^{2+}$ channels | Limited |
| $G_z$ | No | Undefined, stimulation of PLC | Undefined, but restricted |
| TD1 | Yes, Cholera and and pertussis toxin | Activation of cGMP phosphodiesterase | Rod outer segments |
| TD2 | Yes, Cholera and pertussis toxin | Activation of cGMP phosphodiesterase | Cone outer segments |

Adapted from Milligan and Wakelam, 1989.

cytosol and breaking the cycle of GTP-mediated activation. All trimeric G-proteins share other unique or unusual characteristics. For example, they are activated by $AlF_4$, because GDP-$AlF_4$ mimics the effect of GTP [reviewed by Gilman, 1987]. In 1993, it has been reported that NO signalling in human peripheral blood mononuclear cells (PBMC) can regulate G-protein activity [Lander et al., 1993].

### 1.3.2. Cyclic nucleotides as second messengers:

Cyclic nucleotides are involved in the regulation of several macrophage functions, including phagocytosis, motility, responses to lymphokines, and DNA synthesis. Bromberg and Pick (1980) reported that macrophages treated with NO-generating agents showed an increase in cyclic GMP levels. Cyclic GMP is generated from GTP in response to the activity of guanylyl cyclase. NO generated in the macrophage cytosol stimulates soluble guanylate cyclase and results in cGMP elevation in several cells, such as vascular endothelial and smooth muscle cells present in the vascular wall [Mulsch et al., 1990].

The activation of adenylyl cyclase results in the formation of cAMP from ATP. In general, the $\alpha$ subunit of $G_s$ activates adenylyl cyclase. However, type II and IV adenylyl cyclases have been shown to be stimulated by G-protein $\beta/\gamma$ subunits in the presence of $\alpha_s$ subunit, and type III adenylyl cyclase has very little sensitivity to $\beta/\gamma$ subunits [Tang and Gilman, 1992].

Gene regulatory effects of cAMP are well established in eukaryotes [Roesler et al., 1988; Karin, 1989]. The PKA enzyme can activate the cAMP response element-binding protein (CREB) [reviewed by Hill and Treisman, 1995] by phosphorylating it at Ser-133 amino acid point, then leads to the association of CREB with other cofactors, like cAMP receptor protein (CRP), for transcriptional activation. The CREB can be activated by calmodulin kinase and neurone growth

factor, independently. The attenuation of the CREB effect can be caused by the inducible cAMP early repressor (ICER), which competes with CREBs to bind the cAMP-regulated enhancer (CRE) target sites on the gene promoter [Molina et al., 1993].

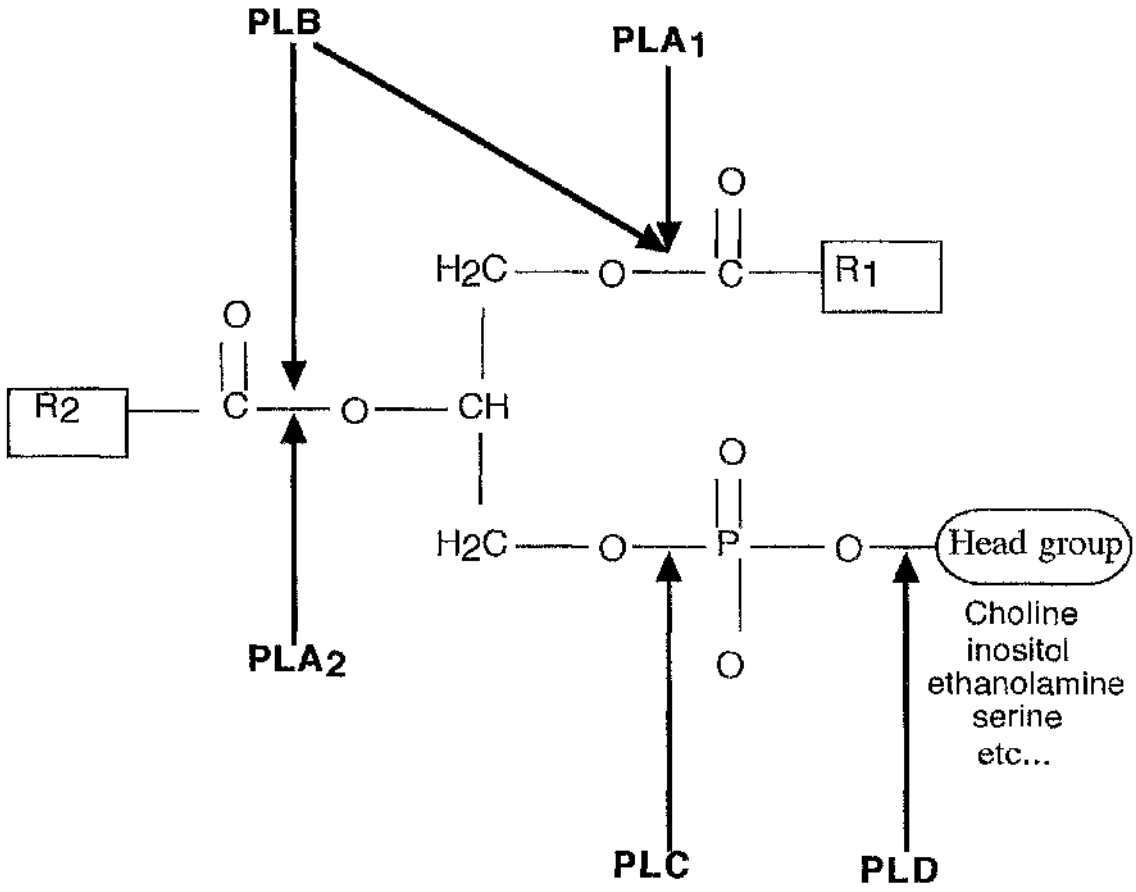
### 1.3.3. Phospholipases as generators of second messengers:

The phospholipids, which are substrates for phospholipases, can be divided into two main groups: the glycerophospholipids and the sphingophospholipids. Glycerophospholipids have either acyl (ester linkage) or alkyl (ether linkage) groups at the C(1) hydroxyl of the glycerol backbone and predominantly acyl groups at the C(2) hydroxyl. A hydrophilic phosphate at the C(3) position gives phospholipids the amphipathic character that is important for the formation of lipid bilayers and also provides a site for phosphodiester linkage to a variety of head groups, including choline, serine, ethanolamine and inositol. Phospholipids can be hydrolysed to generate a variety of potential second messengers by phospholipases that are classified on the basis of where the phospholipid is hydrolysed. Phospholipases A (PLA) hydrolyse ester linkages between the acyl group and the glycerol backbone at C(1) for $PLA_1$ or C(2) for $PLA_2$. The products of PLAs are a fatty acid and a lyso-phosphatidyl derivative. Phospholipases C (PLC) hydrolyse the phosphodiester linkage between the glycerol backbone and the phosphate to generate a diacylglycerol (DAG) and a phosphorylated head group. Phospholipases D (PLD) hydrolyse the phosphodiester linkage between the phosphate and the head group to generate phosphatidic acid (PA) and the head group. [Reviewed by Foster, 1993]. Sites of hydrolysis of phospholipids by various phospholipases are shown in figure 1.1.

### 1.3.4. Sphingolipids as second messengers:

Sphingolipids play roles in many biological processes, such as cell-cell interaction, proliferation, differentiation, oncogenic transformation, immune recognition and responsiveness, and serve as receptors for microbial membrane lectins and viral, bacterial toxins [reviewed by Ballou, 1992]. So far, over 300 types of sphingolipids have been reported. Sphingolipids are derived from ceramide. Sphingomyelin has a phosphocholine head group attached to ceramide via a phosphodiester bond [van Holde, 1992]. The PKC inhibition has been reported in response to sphingosine, but not to ceramide [Hannun et al., 1986].

The enzyme, sphingomyelinase, hydrolyses sphingomyelin to produce ceramide and phosphorylcholine [Merril and Jones, 1990]. Ceramide and sphingosine induce $PGE_2$ synthesis, adenylate cyclase activation and cAMP elevation in macrophages [reviewed by Ballou, 1992].




Adapted from Bonventre, 1992.

**Figure 1.1: Site of hydrolysis of phospholipids by various phospholipases:**

$PLA_1$ hydrolyses the 1-acyl ester; $PLA_2$, the 2-acyl ester. Phospholipase B hydrolyses at the both 1- and 2- acyl ester bonds. Phospholipase C cleaves the glycerol-phosphate bond, whereas the base is removed by phospholipase D. $R_1$ and $R_2$ represent fatty acids.

### 1.3.5. Protein-serine/threonine kinases and intracellular signalling:

Phosphorylation of proteins at serine and threonine residues is one of the most frequent forms of post-translational modification leading to changes in biological function.

Protein-serine/threonine kinases can be classified into four main groups:

1) cyclic nucleotide-dependent protein kinases, i.e. cAMP-dependent and cGMP-dependent protein kinases (respectively PKA and PKG).

2) $Ca^{2+}$-dependent protein kinases, such as phosphorylase kinase C, myosin light chain kinase and other calmodulin-dependent protein kinases, and the conventional protein kinase C family (cPKC)

3) messenger-independent protein kinases; many enzymes are in this group, including casein kinases I and II, glycogen synthase kinase-3, ribosomal protein S6 kinases, and β-adrenergic receptor kinases.

4) Others; mitogen activated protein kinases (MAPK), the novel PKC (nPKC) and the atypical PKC families.

All these protein kinases can use ATP as the phosphoryl donor. A few of them can also utilise GTP, for example casein kinase II and glycogen synthase kinase-3 [Reviewed by Wang and Roach, 1993].

#### 1.3.5.1. Cyclic AMP-dependent protein kinases (PKA):

The PKA enzyme is composed of two regulatory (R) and two catalytic (C) subunits that together constitute an inactive holoenzyme of form $R_2C_2$. Binding of cAMP to the regulatory subunit dimer results in release and concomitant activation of the catalytic subunits. The hypothesis has been advanced that eukaryotic R subunits after binding of cAMP might directly function as transcriptional regulators [Nagamine and Reich, 1985; Constantinou et al., 1985]. On the other hand, it has

been speculated that transcriptional regulation of eukaryotic cAMP-responsive genes may depend on the phosphorylation of trans-acting gene regulatory proteins by the C subunit of the kinase [Jungmann and Kranias, 1976; Johnson, 1982]. Moreover, previous studies have shown that increased intracellular cAMP levels can block receptor-induced PKC activation [Takai et al., 1982a; Nishizuka, 1984a,b; Coggeshal and Cambier, 1984; Patel et al., 1987].

### 1.3.5.2. Protein Kinase C:

Protein kinase Cs (PKCs) are phospholipid-dependent serine/threonine kinases. They can be divided into three main groups: the $Ca^{2+}$- and phospholipid-dependent or conventional PKCs (cPKCs), α, βI, βII and γ isoforms, $Ca^{2+}$-independent or novel PKCs (nPKC), δ, ε, η and θ isoforms, and $Ca^{2+}$- and DAG-independent atypical PKCs (aPKCs), ζ, and ι (also called λ in the mouse) [reviewed by Hug and Sarre, 1993; Stabel and Parker, 1991; Limatola et al., 1994]. The former two classes of protein kinase C are activated by DAG. Phorbol esters dramatically increase the affinity of cPKCs for $Ca^{2+}$, resulting in its full activation at physiological $Ca^{2+}$ concentrations [Castagna et al., 1982; Fujiki et al., 1984]. cPKCs are "Biochemically"/calcium-dependent, but "physiologically"/ calcium-independent [Nishizuka, 1986; Kikkawa et al., 1983]. Atypical PKCs are resistant to even phorbol ester treatment [Limatola et al., 1994; Ways et al., 1992]. PKC-ζ can be activated by phosphatidylinositol 3,4,5-tris-phosphate ($PIP_3$) [Nakanishi et al., 1993]. Phosphatidic acid (PA), bisPA and various other acidic lipids, such as phosphatidylserine (PS), lysoPA, PI, phosphatidylglycerol (PG) and cardiolipin (CL), have been reported as physiological activators of PKC-ζ in the absence of $Ca^{2+}$ [Limatola et al., 1994]. Interestingly, it was reported that milimolar concentrations of $Ca^{2+}$ inhibit PKC-ε and ζ [Schaap and Parker, 1990; Nakanishi and Exton, 1992; Limatola et al., 1994]. It was suggested that PKC-ζ is not translocated to the membrane and is not affected by phorbol esters [Schaap and

Parker, 1990]. The PKC-ι isoform has 72 % homology with PKC-ζ. Like PKC-ζ, it has no $Ca^{2+}$ binding domain (C2), and contains only one zinc-finger [Selbie et al., 1993]. The PKC-μ isoform has 44-68 % aa. homology with other PKC isoforms, and contains two zinc-fingers (C1) [Johannes et al, 1994].

Of the various phospholipids tested, phosphatidylserine appears to be indispensable for the activation of PKCs, whereas other phospholipids may show either positive or negative cooperativity [Kaibuchi, et al., 1981]. It is possible that PKC might be activated in the absence of inositol phospholipid turnover. Some hormones and growth factor can stimulate phosphatidylcholine hydrolysis through the activation of PLC or PLD. DAG can be generated either directly by PLC or indirectly by PLD to yield phosphatidic acid, which is then hydrolysed to DAG [Reviewed by Pfeffer and Tan, 1991]. For instance, IFN-γ rapidly activates PC turnover, which results in DAG production in HeLa cells [Pfeffer et al., 1990]. Regulation of phosphorylation of 68 kDa protein by PKC in response to LPS in peritoneal macrophages has been observed [Rosen et al., 1989]. There are many controversial findings about PKC isoforms and their activation which require to be clarified.

The structures of PKCs may explain their different biochemical properties and functions. Figure 1.2. shows the regulatory and catalytic domains and molecular masses of 9 isoforms of PKC. PKC isozymes, excluding ζ and ι isoforms, contain a cysteine rich C1 domain (DAG/phorbol ester binding site), which has two zinc motifs each with 6 cysteine residues. Nevertheless, the ζ and ι isoforms contain only one zinc finger, which is probably the reason for the constitutive protein kinase activity exhibited by this isoform. PKC α, βI/βII, and γ isozymes contain the $Ca^{2+}$ binding site (C2). The V3 variable chain (hinge region) separates the catalytic and the regulatory domains, and is sensitive to proteolytic cleavage by trypsin or the $Ca^{2+}$-dependent neutral proteases, calpain I and II. The C3 region (ATP-binding site) is found in all isoforms, and is necessary for the

**Figure 1.2: Domain structure of PKC isoenzymes:**

(Adapted from Hug and Sarre, 1993; Nishizuka, 1992)

**V**: Variable region, **C**: Conserved domain.

C1: Cystein-rich zinc fingers (DAG/Phorbol ester binding site)

The ζ isoform contains only one zinc finger

C2: $Ca^{2+}$ binding site

V3: Hinge region separates the catalytic and the regulatory domains

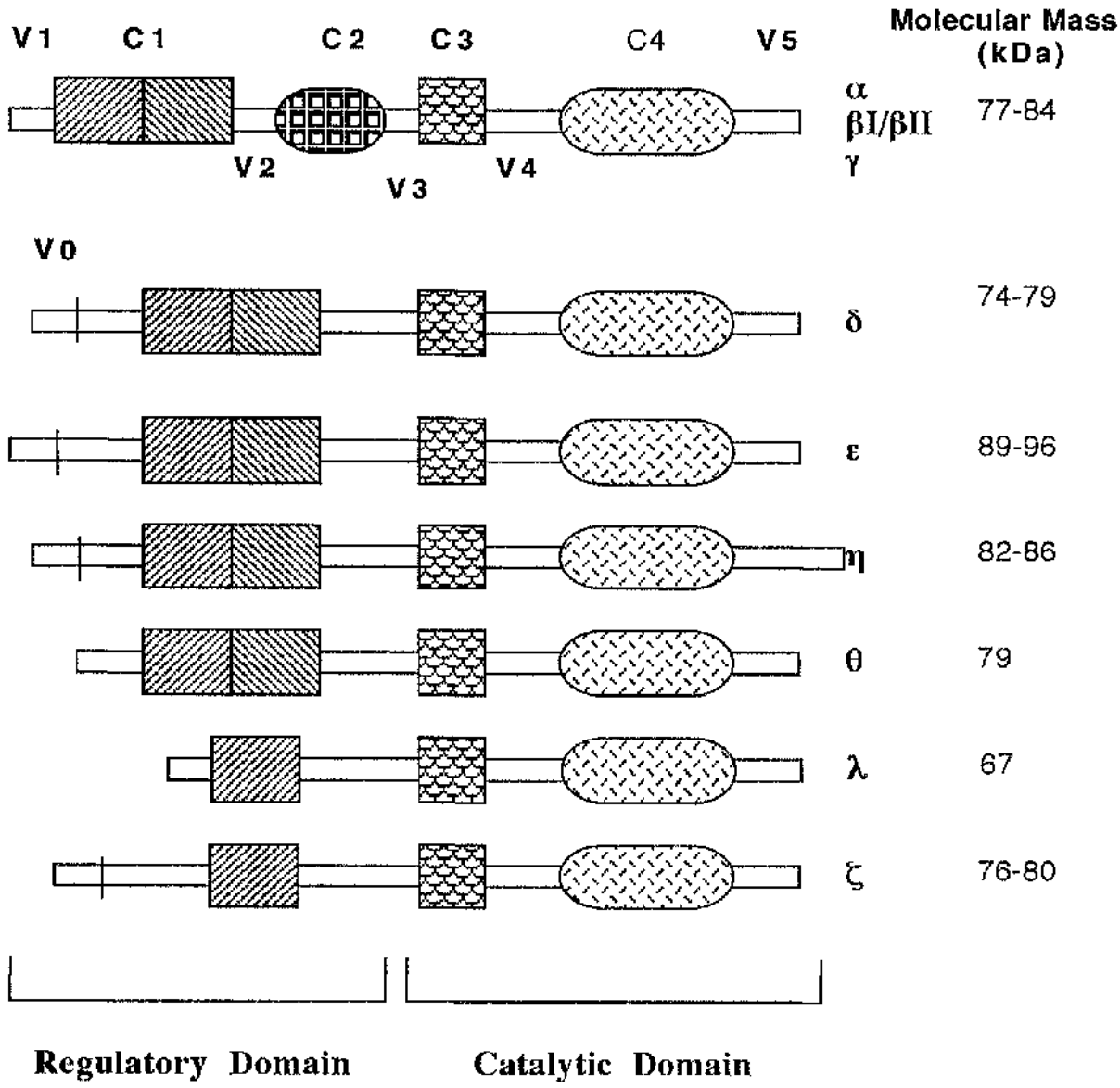
C3: ATP-binding site

V4: Region separates C3 and C4 domains

C4: Substrate binding site/phosphate transfer region)

V0: PKC δ, ε, ζ and η isoforms isozymes contain, but not PKC α, βI/βII, and γ isozymes.

V5: poorly conserved


V1
C1
C2
C3
C4
V5
V2
V3
V4
Molecular Mass (kDa)
α
βI/βII
γ
77-84
V0
δ
74-79
ε
89-96
η
82-86
θ
79
λ
67
ζ
76-80
Regulatory Domain
Catalytic Domain

phosphorylation of substrates. The V4 region separates C3 and C4 domains, and is present in all isoforms. The C4 region (substrate binding site/phosphate transfer region) is also present in all isoforms and is highly conserved. Additionally, PKC δ, ε, ζ and η isoforms contain a V0, domain which is absent in α, βI/βII, and γ isozymes. The V5 extension is poorly conserved.

PKC has a broad substrate specificity, phosphorylating seryl and threonyl residues, but not tyrosyl residues, of many endogenous proteins in most tissues in vitro [Nishizuka, 1980; Takai et al., 1982b; Nishizuka et al., 1984b]. The consensus sequence for the phosphorylation site for PKC is xRxxS/TxRx found in the substrates [Rewieved by Hug and Sarre, 1993].

It was reported that inhibition of PKC activity, or down-regulation of PKC by prolonged incubation of cells with phorbol esters, reduces NO production in response to IFN-γ and LPS [Severn et al., 1992b]. Furthermore, stimulation of macrophages with these agents results in translocation of PKC to the cell membrane [Severn et al., 1992b]. It was shown that J774 cells constitutively express PKC βII, ε, and ζ specific mRNAs and their translational products, but PKCα, γ, and δ specific mRNAs as well as their translation products were not detectable, and down-regulation of PKC βII, ε, and ζ by using PMA pre-treatment resulted in reduced NO production and iNOS gene transcription in response to LPS in J774 cells [Fujihara et al., 1994]. These observations suggest that PKC activation is essential in the induction of NO production by IFN-γ and LPS.

### 1.3.6. Protein-tyrosine kinases:

Stimulation of cells by IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, GM-CSF, and Epo has been shown to activate intracellular tyrosine kinases and induce the tyrosine-specific phosphorylation of cellular proteins [Taga et al., 1992; Ip et al., 1992; Otani et al., 1992; Kanakura et al., 1990; Nakajima and Wall, 1991; Linnekin et al., 1992]. PTKs transduce intracellular signals that lead to cell division. However, PTK

activity does not always produce cell division signals [Reviewed by Foster, 1993]. It was reported by Foster and co-workers that PTKs (v-Src) may activate three distinct intracellular signalling mechanisms, namely, serine/threonine-protein kinases, Raf-1 and PKC, by using gene expression as an output signal for the PTK activity [Foster, 1993]. PTKs can be divided into two main groups, namely receptor tyrosine kinases and non-receptor tyrosine kinases. The binding of ligand to the extracellular domain activates the tyrosine kinase in the cytoplasmic domain, which leads to downstream activation of a number of common signalling molecules. Some intracellular proteins, such as PLC-γ, phosphatidylinositol 3-kinase (PI3-kinase), GTPase-activating protein, pp60c-src, p21Ras, Raf-1 kinase, ERK1 and ERK2 (referred as MAP kinases) kinases, and S6 ribosomal kinases, are activated by the cytoplasmic domain of receptor PTKs [Fantl et al., 1993]. Since PTKs are not the subject of this project, the only nomenclature of PTKs is shown in table 1.6.

## 1.4. INTERACTIONS BETWEEN SECOND MESSENGER SYSTEMS:

Several of the second messenger systems have been shown to interact with one another. It is known that IFN-γ and low concentration of LPS can synergise their many effects on macrophages [Green et al., 1990b]. First, IFN-γ has been shown to cause PKC activation. When macrophages were stimulated by LPS or PAF or PMA after pre-treatment with IFN-γ, phosphorylation of some target proteins was potentiated [Hamilton et al., 1985; Becton et al., 1985]. Secondly, IFN-γ potentiates DAG generation [Sebaldt et al., 1990]. As a result of these changes, pre-treatment of macrophages with IFN-γ potentiates the activation of $Na^+/H^+$ exchange induced by either LPS or PAF by making the shift of the intracellular pH into the alkaline more rapid and by raising the degree of alkalinization [Prpic et al., 1989].

**Table 1.6: Nomenclature of protein tyrosine kinases:**

1) Receptor tyrosine kinases

i) The epidermal growth factor receptor family:

-Endothelia growth factor receptor (EGFR)

-p185neu (erbB-2 or Her2) -erbB-3

ii) The insulin receptor family:

-insulin-like growth factor receptor -insulin-related receptor

iii) The PDGF/MCSF-1/Steel receptor family:

-platelet-derived growth factor (PDGF) α–and β receptor

-the macrophage colony stimulating factor-1 (MCSF-1) receptor

-c-kit protein

iv) The vascular Endothelial Cell Growth Factor Receptor Family

v) The Hepatocyte Growth Factor Receptor:

vi) The neurotrophin receptor family:

-Nerve growth factor (NGF)

-Brain-derived neurotrophic factor (BDNF)

-neurotrophin-3 (NT-3) -neurotrophin-4 (NT-4)

-neurotrophin-5 (NT-5)

vii) The fibroblast growth factor receptor family:

-acidic and basic FGF -the product of the int-2 oncogene

-the product of the hst oncogene -FGF5 -FGF6

-Keratinocyte growth factor (KGF)

viii) The Eph-Like Receptor Tyrosine Kinases:

-Eph -Eck -Elk -Eek -Erk -Cek4 -Cek5

ix) The Axl Receptor Tyrosine Kinase

to be continued

2) Non-receptor tyrosine kinases:

-Abl, Arg -Janus family, Jak1, Jak2, Jak3 -Tyk2

-Fak -Fes/Fps, Fer -Spleen tyrosine kinase (Syk) and Zap

-Interleukin-2 inducible kinase (Itk/Tsk)

-B cell progenitor kinase (Bpk/Atk) -Tec kinase

-Src gene family, Src, Yes, Fyn, Lyn, Lck, Blk, Hck, Fgr and Yrk tyrosine

-Csk kinase

$PGE_2$, is a potent suppressor agent of macrophage function, and can lead to elevated levels of cAMP. It has been found that at least one effect of cAMP is to suppress the activation of $Na^+/H^+$ exchange in macrophages [Cowlen et al., 1990].

The signal transduction mechanisms initiated by $Fc\gamma 2aR$ and $Fc\gamma 1R/Fc\gamma 2bR$ remain to be fully clarified. Activation of $Fc\gamma 2aR$ has been reported to raise intracellular levels of cAMP approximately 5-fold within 30 s in the P388D1 macrophage cell line, while activation of the $Fc\gamma 1R/Fc\gamma 2bR$ on this cell line leads to enhanced release of arachidonic acid. Suzuki and colleagues (1982) have isolated from the same cell line phosphatidylcholine (PC)-binding proteins, which also bind immunoglobulins of the $IgG_{2b}$ isotype and which have phospholipase $A_2$ activity [reviewed by Nitta et al, 1984]. Additionally, the activation of Fc receptors can stimulate protein kinase C [Johnston, 1984].

As described above, $PGE_2$, acting via elevations in AMP can dampen responses to both IFN-γ and to LPS by suppressing activation of $Na^+/H^+$ exchange [Figueiredo et al., 1990]. It has been found that LPS may block the $PGE_2$-mediated enhancement of cAMP activities possibly by activating PDEs that accelerate degradation of cAMP [Okonogi et al., 1991]. In many cells, the SH2-containing proteins, PI3 kinase and PLC-γ interact with TK, and are activated [Reviewed by Foster, 1993].

## 1.5. CYTOKINES, NITRIC OXIDE AND IMMUNE REGULATION:

Th1-like cells produce lymphotoxin (LT), IL-2 and IFN-γ, mediate cell-mediated immune responses important for the response to various infectious diseases, whereas Th2-like cells secrete IL-4, IL-5, IL-10 and IL-13, which are the predominant helpers of B cell responses, and are clearly implicated in allergic

responses [Mosmann and Coffman, 1989]. IFN-γ and LT activate macrophages and increases expression of Fc receptors for IgG2a antibodies [Warren and Vogel, 1985]. These receptors can then bind the increased IgG2a levels produced in response to IFN-γ [Snapper and Paul, 1987], leading to increased antibody-dependent macrophage cytotoxicity. Purified protein derivatives (PPD), prepared from tubercle bacillus, in the presence of IL-4 have been shown to stimulate Th2 development from peripheral blood human T cells [reviewed by O'Garra and Murphy, 1995]. IFN-γ has been shown to inhibit Th2 clones' proliferative response to either IL-2 or IL-4 [Fernandez-Botran et al., 1988; Gajewski and Fitch, 1988]. IL-1, TGF-β and TNF-α are other cofactors for the production of Th1 cells [Hsieh et al., 1993].

IL-2 induces macrophages to produce TGF-β, and then TGF-β can inhibit T cell proliferation, NO and $PGE_2$ production, and microbicidal activity of macrophages. This inhibitory effect of TGF-β can be reversed by IFN-γ and TNF-α [Reviewed by Doherty, 1995]. IL-10 inhibits a number of different macrophage activities, including cytokine production, such as IL-1, IL-6 and TNF-α [Bogdan et al., 1991; Fiorentino et al., 1991], respiratory burst [Bogdan et al., 1991], Ia expression in monocytes [Malefyt et al., 1991], and NO production by macrophages induced by IFN-γ and TNF-α [Oswald et al., 1992].

IL-12 was clearly identified as an inducer of the differentiation of Th1-like cells producing IFN-γ [reviewed by Wolf et al., 1994]. IL-12 is a 75 kDa heterodimer, composed of 40 kDa and 35 kDa glycoproteins, secreted by phagocytic cells [D'Andrea et al., 1992; reviewed by Reiner and Seder, 1995], having multiple activities on natural killer (NK) cells and T cells [reviewed by Wolf et al., 1994]. In vivo Th1 differentiation has been shown to be enhanced by IL-12. However, IL-12 needs to work with IFN-γ in Th1 development, as a co-stimulator.

IL-13 affects macrophages but not T cells, then it can be accepted as macrophage-specific regulator without altering T cell functions [reviewed by Doherty, 1995]. Recently, it has been shown that IFN-γ induces IL-15, a novel cytokine which has similar properties to IL-2, produced by macrophages [Reviewed by Doherty, 1995]. The functions of IL-15 are yet to be investigated.

There are several diseases related to disturbances in Th1/Th2 ratios. For instance, in a helminthic disease, *Nippostrongylus brasiliensis*, high IgE levels, eosinophilia, high IL-4, IL-5 levels and low IL-2, and IFN-γ levels indicate a Th2 dominant response. Interestingly, *Leishmania* infection shows different patterns dependent on the mice strains. BALB/c mice suffer from fatal, progressive disease and can not develop a Th1-dominant response. In contrast, C57BL/6 mice produce a very effective Th1-mediated response, with low levels of IL-4 and IgE and high IFN-γ, and develop only a limited disease which heals spontaneously [reviewed by Mosmann and Coffman, 1989].

Recently, the regulation of T cell functions by NO was reviewed by Liew [1995]. NO might inhibit the production of IFN-γ by Th1 cells [Taylor-Robinson et al., 1994]. It is clear that in vivo and in vitro murine models showed the inhibitory effect of NO on the T cell proliferation. L-NMMA, a competitive inhibitor of NOS activity, reversed or elevated many T cell functions, such as cytokine production in experimental models of malaria [Taylor-Robinson et al., 1994]. Although there are some controversial reports using different experimental models and NO donors, it is likely NO has a dual effect on T cell proliferation. Thus, physiological concentrations of NO produced by constitutive NOS enzymes might result in T cell proliferation, but high levels of NO in response to cytokine stimulations in the immune system might inhibit T cell proliferation. The inhibition of Ia antigen expressions on macrophages, as antigen-presenting cells (APCs), in response to NO has been reported. [Sicher et al., 1994].

A proliferative effect of NO on B cells has not been observed yet. Nevertheless, the antiproliferative effects of hydrogen peroxide and $PGE_2$ have been observed [Albina et al., 1991]. On the other hand, a constitutive low level of NO production is present in the Epstein-Barr virus transformed human B cells and Burkitt's lymphoma cell lines [Mannick et al., 1994]. Figure 1.3. shows the interplay among the immune system cells and cytokines.

## 1.6. AIM OF PROJECT:

The aim of this project is to investigate the signalling pathways in macrophages, leading to nitric oxide production, with special interest in the role of serine/threonine kinases, such as cAMP-dependent protein kinase, and protein kinase C. We will concentrate on how IL-4, a physiological agent, regulates NO synthesis in the macrophage-like cell line, J774.

Specifically, we will investigate the following points to explain the NO producing pathways in response to IFN-γ and LPS in murine macrophages;

1) The effect of IL-4 on PKC activity and NO synthesis induced by IFN-γ and LPS in macrophages.

2) The effect of intracellular cAMP elevation, transiently or sustained, on NO production and PKC activity in response to IFN-γ and LPS.

3) The exploration to see whether or not $PLA_2$ activation and arachidonic acid metabolites are involved in the NO-producing pathway in response to IFN-γ and LPS in macrophages.

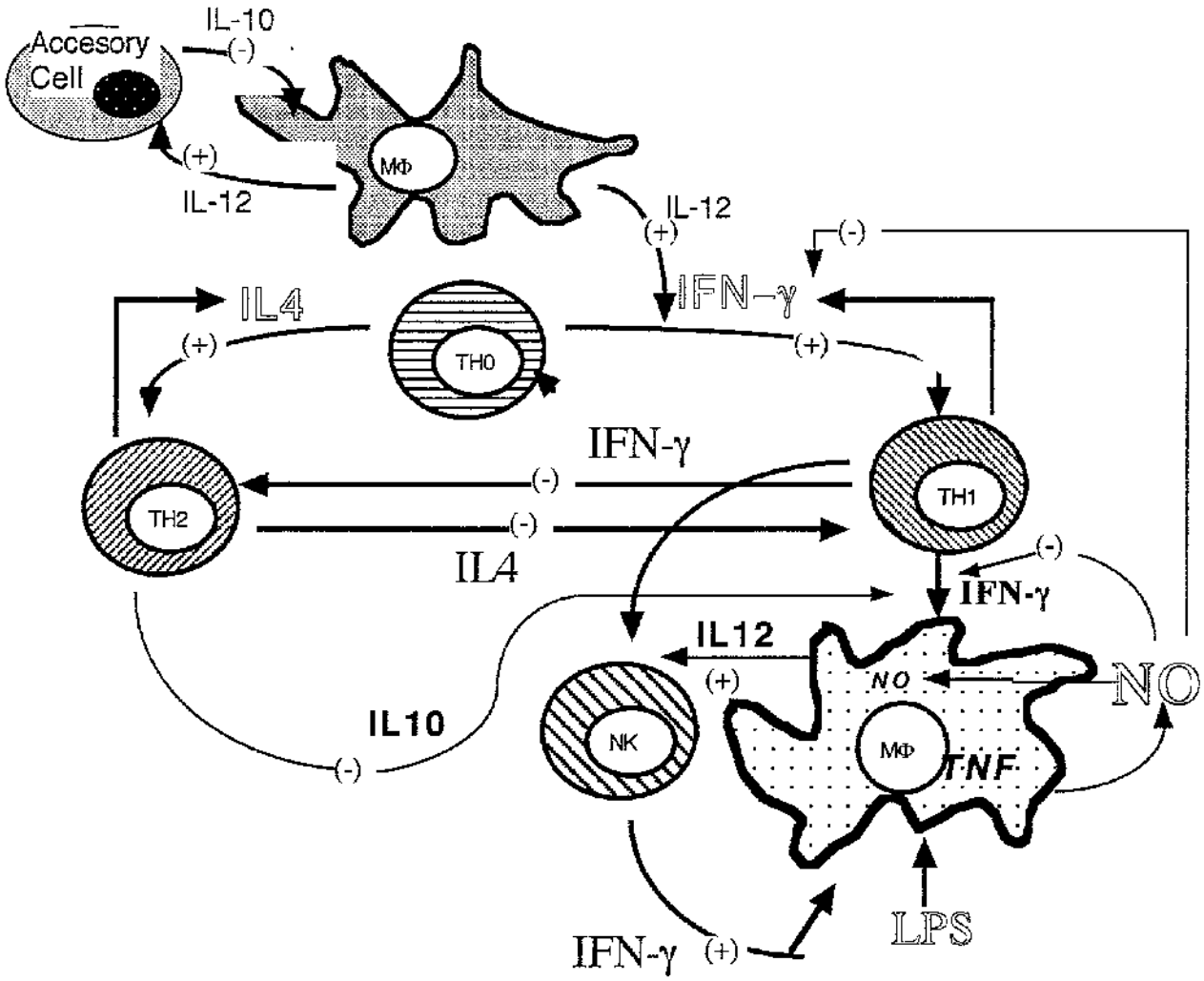



**Figure 1.3: The interplay among Th1, Th2, NK cells, macrophages and cytokines.**

# CHAPTER 2

# MATERIALS AND METHODS

## 2. MATERIALS AND METHODS:

### 2.1. Materials:

J774 and RAW 264 cells were obtained from the American Type Culture Collection (ATCC). Murine recombinant IFN-γ (activity $10^7$ unit/ml) was kindly provided by Dr. G. Adolf, Ernst-Boehringer Institut fur Arzneimittel-Forschung, Vienna, Austria. Murine recombinant IL-4 (activity $10^7$ unit/mg; 100μg/ml=$10^6$ unit/ml) and monoclonal antibodies for IL-4 receptor (mAb IL-4r) were obtained from Genzyme (Cambridge, MA.). The PKC inhibitor, Ro31-8220 [Davis et al., 1989], was kindly provided by Dr. G. Lawton, Roche Products Limited, Hertfordshire, UK. Rolipram, a specific cAMP phosphodiesterase inhibitor, was obtained from Schering Ahtiengesellschaft (Germany). LPS from *Salmonella enteritidis*, prepared by phenol extraction, was obtained from Sigma. It was dissolved at 1 mg/ml in RPMI 1640 medium as a stock solution and stored at -20°C. Prostaglandin $E_2$ ($PGE_2$) was obtained from Sigma and stored at 5 mg/ml in ethanol at -20°C. Adenosine-3' : 5'- cyclic phosphorotiolate-Rp (Rp-cAMP), a PKA inhibitor, was purchased from Calbiochem-Novabiochem (UK) Ltd. [$^3$H]- phorbol dibutyrate ([$^3$H]-PDBu), radio-labelled cAMP-binding assay kits for the measurement of cAMP, and PKC assay kits were obtained from Amersham International plc. (Amersham, England). Primers for the polymerase chain reaction (PCR) were kindly provided by Wellcome Research Laboratories (Beckenham, Kent, England). RNAzol B was obtained from Biogenesis (Bournemouth, England). Moloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT) and reverse transcriptase buffer were obtained from Gibco BRL (Paisley, Scotland). Other reagents used in reverse transcription and PCR were from Promega (Madison, WI, USA). All other reagents were obtained from Sigma (Poole, Dorset, England).

## 2.2. Methods:

### 2.2.1. Cell storage and culture:

Cell lines, J774.1 or RAW 264.7 macrophages, were stored in RPMI 1640 containing 10% dimethylsulphoxide (DMSO) in freeze vials in a liquid nitrogen tank at approximately -168$^{O}$ C. When needed, cells were obtained from this store; they were thawed suddenly under cold tap water and centrifuged at 1200 RPM (approximately 250 g) for 5 minutes and placed in RPMI 1640 medium (Flow Laboratories, Irvine, Scotland), without DMSO, supplemented with 10% heat inactivated-foetal calf serum (HI-FCS) (Gibco, Paisley, Scotland), 2mM L-glutamine (Flow Laboratories), 100 U/ml penicillin and 100 µg/ml streptomycin (Flow Laboratories). For routine culture, cells were grown in 25 or 75 ml flasks (Costar) and incubated at 37$^{O}$C in 5% $CO_2$. Every 3-4 day, cells were subcultured by scraping and placing into new flasks and diluted 5-10 times to supply nutrition and space enough for growth.

### 2.2.2. Obtaining fresh peritoneal macrophages from CBA mice:

Four days after intraperitoneal injection with 4% Brewer's thioglycollate broth, peritoneal cells were harvested with RPMI 1640 medium from female CBA mice (Harlan Olac, Bicester, Oxon, UK.), 8-12 week old. The cells were cultured with RPMI 1640 medium containing 2mM L-glutamine, 50U/ml penicillin/100 µg/ml streptomycin and 10% heat inactivated FCS in either flasks or plates at 37$^{O}$C in an atmosphere containing 5% $CO_2$. The cells were allowed to adhere for 2 hours. After 2 hours, nonadherent cells were removed by washing the plates and replacing medium.

### 2.2.3. Induction and measurement of NO synthesis:

J774 cells were cultured at $5x10^5$/ ml or $1x10^6$/ ml in 96 or 24-well flat-bottomed plates (Costar), respectively, with various reagents as detailed in the Result chapters and incubated at $37^oC$ in 5% $CO_2$. Supernatants were collected after 24 or 48 hr incubation, and $NO_2^-$ concentrations determined by chemiluminescence as described previously [Palmer et al., 1987] and summarised below or by the Griess Method. The oxidation products of L-arginine in murine macrophages have been characterised to be L-citrulline and NO, which is rapidly converted into nitrite and nitrate in the presence of molecular oxygen [Marletta et al., 1988; Stuehr and Marletta, 1987]. Nitrite and nitrate levels detectable in supernatants of activated murine macrophages thus reflect the formation of NO. Therefore, these concentrations are indicative of the amount of NO produced by the cells [Marletta et al., 1988].

#### 2.2.3.1. Chemiluminescence:

Luminescence is the emission of light or radiant energy when an electron returns from an excited or higher energy level to a lower energy level. There are several types of luminescence phenomena including fluorescence, phosphorescence and chemiluminescence [Tietz, 1986]. In chemiluminescence, the excitation event is caused by a chemical reaction and not by photoillumination. The physical event of the light emission in chemiluminescence is similar to fluorescence in that it occurs from an excited singlet state and the light is emitted when the electron returns to the ground state. Chemiluminescence involves the oxidation of an organic compound, such as nitric oxide, by an oxidant. In this event, the oxidising agent is oxygen itself with the emission of light occurring from the excited product formed in the chemical reaction [Tiffany, 1986]. Supernatants from samples and standard solutions, obtained by diluting $10^{-2}$ M stock $NaNO_2^-$ solution, were injected by

Hamilton syringes (100 µl) into a boiling flask at approximately 95°C, containing %6 NaI (sodium iodide) and glacial acetic acid at the ratio of one in three (v/v), respectively. Nitric oxide (NO) in samples or standards were released in this boiling media, then NO and evaporated glacial acetic acid passed through tubes in a dry ice box, where the acid was trapped and NO alone passed through the plastic tubes. Gas, containing NO to be measured, comes into the machine's reaction chamber, which is connected to the ozone generator, and the NO rapidly reacts with ozone to generate a chemiluminescence product. Then, the chemiluminescence product is propelled with high speed into the detection chamber. An optical filter is inserted between the reaction chamber and the photomultiplier tube to provide a gas seal and yet allow the photons to pass from the reaction chamber to the photomultiplier tube. The photomultiplier tube is an integral part of the detector cell. The function is to convert to the low-level light (photons) produced by the gas phase chemiluminescent reaction between ozone and the NO in the reaction chamber into electrical energy and to amplify this electrical energy, and this is detected by the chemiluminescence device as parts per billion (ppb). which is shown on a digital displayer. Standard curves were obtained by using 2, 5, 10, 20, 50, and 100 µM concentrations of $NaNO_2^-$, then samples were compared with the curve values to obtain their values as molar concentrations.

**2.2.3.2. Griess reaction:**

In this technique, the nitrite production by macrophages is determined colorimetrically. Supernatants, obtained as detailed above, were mixed with equal volumes of the Griess reagent (one volume of 0.1% (w/v) naphthylenediamine dihydrochloride and one volume 1% (w/v) sulphanilamide in 5% (v/v) phosphoric acid) in 96-well flat-bottomed plates. The mixture was incubated at room temperature for 10-30 min and the absorbances at 490 or 570 nm of each well were measured with a plate reader (Dynatech MR5000), and evaluated by Mikrotek 5.0

Laborsysteme GMBH, version 4.0 software, using a 3865 XP/16 IBM compatible Toshiba Laptop computer. Printouts were taken from (Star LC-20, Dot Matrix printer). Standard curves were obtained by using $NaNO_2^-$ solution at 6.25, 12.5, 25, 50 and 100 μM.

### 2.2.4. Determination of NO synthase activity:

J774 cells were incubated at 1 x $10^6$ cells/ml in 24-well plates for 12 hours with various reagents as detailed in Results. NOS activity in cell extracts was determined by a modification of a previously described method, based on the conversion of L-[U$^{14}$C] arginine to L-[U$^{14}$C] citrulline and NO [Severn et al., 1993; Salter et al., 1991]. Medium was then removed, and extraction buffer (0.1 M Hepes, pH:7.4 with 1 mM DTT and protease inhibitors; pepstatin 1 μM, amastatin 5 μM, Soya bean trypsin inhibitor 10 μg/ml, leupeptin 5 μM and aprotinin 2 μg/ml) added at 100 μl per $10^6$ cells. The cells were subjected to three freeze-thaw cycles at -20$^o$C and 37$^o$C, and NO synthase activity was measured in the cell extracts. Assay buffer (100 μl), consisting of 50 mM potassium phosphate, pH 7.2, 50 mM L-valine, 100 μM NADPH, 1 mM L-citrulline, 20 μM L-arginine and L-[U$^{14}$C] arginine (132 μl/10 ml), 1 mM $MgCl_2$, 0.2 mM $CaCl_2$ and 5 μM tetrahydrobiopterin ($H_4B$) was dispensed into Eppendorf tubes. Cell extract (20 μl) was added to each tube, with and without 12 μl of L-NMMA solution (0.01 M in distilled water) and the tubes were incubated at room temperature for 1 hr. The reaction was terminated by the addition of 1.5 ml water/Dowex-50W ($H^+$ cation exchanger) (1:1). The Dowex was allowed to settle for 10 min, centrifuged at 14 000x g at room temperature for 3 minutes, then 0.5 ml of supernatant was removed from each tube, and L-[U$^{14}$C] citrulline levels determined by liquid scintillation counting (Wallac Oy). ΔCPM values were obtained by subtracting the value of tube with L-NMMA from the value of tube without L-NMMA. Specific activity was calculated by using total count tubes (100 μl of the assay buffer including L-[U$^{14}$C] arginine, 2nmol). Results

from triplicate cultures were expressed as pmole citrulline formed per mg protein per minute.

$$\frac{[\text{Tube without L-NMMA - Tube with L-NMMA}] \times [\text{Amount of L-Arginine added}]}{\text{Total counts} \times \text{Time} \times [\text{Amount of protein added}]}$$

Where: Total counts=The total numbers of counts in 100 µl of the assay buffer.

Time= Incubation time (60 minutes)

L-[U$^{14}$C] arginine was obtained from Amersham Int. and $H_4B$ from Dr. B. Schircks, Jona, Switzerland. All other reagents used in this assay were from Sigma. Protein concentrations were measured by a Coomassie blue dye binding method using a protein assay kit from Pierce (Rockford, IL), as recommended by the manufacturer.

### 2.2.5. Measurements of protein concentrations in cell extracts:

#### 2.2.5.1. Coomassie dye binding:

A Coomassie based protein assay was used that incorporates a modification of Bradford's technique [1976], based on the shift in absorbance maximum of Coomassie brilliant blue G, from 465 to 595 nm, when it binds to protein. The reagent was supplied by Pierce and Warringer. Protein standards were diluted from 2 mg/ml bovine serum albumin (BSA) stock solution in sterile distilled water, including an equal volume of sample buffer to; 200, 100, 50, 25 and 12,5 µg/ml. Samples to be measured were diluted in distilled water as 1 in 10. Fifty µl of standards and extracts of cells, were put in wells of 96-well plates. Similar volumes of Coomassie solution were added wells. The plate was incubated at room temperature for 30 minutes and the absorbance at 570 nm of each well was

measured with a plate reader (Dynatech MR5000), and evaluated by software Mikrotek 5.0 (Laborsysteme GMBH, version 4.0), using a 3865 XP/16 IBM compatible Toshiba laptop computer.

#### 2.2.5.2. Micro BCA (Bicinchoninic acid) assay:

Reagents were prepared fresh before use as follows:

Reagent A: 4% bicinchoninic acid (BCA)

Reagent B: 8% $Na_2CO_3$, 2% NaOH, 1.0 % Sodium tartarate and sufficient $NaHCO_3$, to adjust pH to 11.25

Reagent C: 4% $CuSO_4$. $5H_2O$

Reagent D: 100 volumes Reagent A+ 100 volumes Reagent B + 4 volumes Reagent C.

Standard solution: Standard solutions were prepared from 2 g/ml stock solution of bovine serum albumin (BSA), as follows: 500, 250, 125, 62.5 and 31.25 µg/ml in lysis buffer.

Fifty µl of samples were mixed with an equal volume of reagent D and heated at $60^{O}$C for 1 hr. Samples were brought to room temperature and put into 96 well-plates as 100 µl volumes. Absorbances were read at 570 nm against a reagent blank (lysis buffer) in a plate reader (Dynatech MR5000), and evaluated by software Mikrotek 5.0 (Laborsysteme GMBH, version 4.0), using a 3865 XP/16 IBM compatible Toshiba laptop computer.

### 2.2.6. Measurement of intracellular cAMP concentrations:

cAMP was extracted from J774 cells by a modification of a previously described method [Renz et al., 1988]. Cells were cultured at 5 x $10^5$ or $10^6$ / well in 1 ml culture medium in 24-well tissue culture plates (Costar, Cambridge, MA.), then the medium was removed and the cells loosened by scraping with a disposable cell

scraper. 200-250 µl boiling assay buffer (0.05 M Tris and 4 mM EDTA, pH:7.5) was added to each well. The cells were transferred to eppendorf tubes (Scotlab, Coatbridge, Scotland) and boiled for 10 minutes, to coagulate proteins. The cells were then sonicated, the tubes centrifuged at 12000 x g for 10-20 minutes at $4^{o}C$, and cAMP measured in the supernatants, using a radio labelled binding assay kit (Amersham International plc, Amersham, UK), as recommended by the manufacturer. Results were expressed as pmol/$10^{6}$ cells.

### 2.2.7. Determination of [$^{3}$H]-PDBu binding:

Specific binding of $^{3}$H-phorbol dibutyrate ([$^{3}$H]-PDBu) to J774 cells was determined by a modification of a previously described method [Severn et al., 1992b; Schutze et al., 1990]. Binding of [$^{3}$H]-PDBu to whole cells predominantly reflects binding to the cell membrane [Schutze et al., 1990]. Scatchard analysis indicates that roughly one molecule of PDBu binds to one molecule of PKC in the presence of a physiological concentration of $Ca^{2+}$ and an apparent excess of phospholipid [Kikkawa et al., 1983]. Cells (2-3 x $10^{5}$/well) were incubated at $37^{o}C$ in 96-well flat-bottomed plates, in the presence and absence of IL-4 (100 U/ml) for 18 hrs, as detailed in results. Then cells were stimulated with IFN-γ (40U/ml) and LPS (10 ng/ml) for 3 minutes. The medium was removed and the cells were washed three times with cold PBS. Cells were then incubated for 10 minutes at $37^{o}C$ with [$^{3}$H]-PDBu (0.6 µCi/well), with or without 3nmol/well unlabelled 4α-phorbol-12,13-didecanoate (PDD) (Sigma), which does not stimulate PKC activation [Castagna et al., 1982]. Cells were harvested using an automatic harvester (Wallac Oy, Finland) and bound radioactivity counted in a betaplate counter (Wallac Oy). Specific binding of [$^{3}$H]-PDBu was determined by subtracting binding in the presence of excess PDD (80 µg/ml) from total binding.

### 2.2.8. Determination of PKC Activity:

Low-passage J774 cells were incubated for 48-72 hrs in 25 ml flasks in RPMI medium supplemented, as previously described. Medium was renewed daily. Cells in control flasks were counted and approximately 2 x $10^7$ cells per flask was accepted as a sufficient amount of cells for each experiment. Then, cells were washed 3 times with cold PBS and scraped with disposable cell scrapers into eppendorf tubes and centrifuged for 5 minutes at 10,000 x g. Supernatant was removed and 1 ml of extraction buffer (50 mM Tris/HCl, pH: 7.5 with 1 μM pepstatin A, 5 μM amastatin, 10 μg/ml soya bean trypsin inhibitor, 5 μM leupeptin, 2 μg/ml aprotinin, 10 mM benzamidine, 50 μl/ml phenylmethylsulphonylfluoride, 5 mM EDTA, 10 mM EGTA, 0.3% w/v β-mercaptoethanol and 50 μg/ml digitonin) added. The cell suspensions were then sonicated 5 times for 10 sec. each with an ultrasonic generator (Branson Sonifier, model 250) with a tip diameter of 3 mm, output adjusted to 20 and tuning 1, and cells were kept on ice between sonication periods. Eppendorf tubes were centrifuged for 30 min at 10,000 x g. The supernatants were used for PKC determination using an assay kit (Amersham Int. Plc), as recommended by the manufacturers.

### 2.2.9. RNA extraction and reverse transcription:

$3x10^6$ J774 cells were incubated at $37^oC$ for 3-5 hours, then total RNA was extracted from J774 cells using RNAzol B (Biogenesis, Bournemouth, UK.), as recommended by the manufacturer. The RNA isolation was carried out by a single-step procedure, as described by the inventors [Chomczynski and Sacchi, 1987]. The RNAzol B method is completed within 1.5-2 hrs. Firstly, cells were homogenised by using RNAzol B solution (400 μl), then 80 μl chloroform was added to extract RNA, and tubes were shaken for 15 seconds. After 15-20 minutes on ice, the tubes were centrifuged at 13000xg in a microfuge for 15 minutes.

Supernatants were removed, and an equal volume of isopropanol was added to precipitate RNA. After a 20 minute incubation on ice or in a cold room (+4$^{O}$C), tubes were centrifuged at 13000xg for 20 minutes, then pellets often visible were obtained, forming a white-yellow pellet at the bottom of the tube. Pellets were washed by using ice-cold ethanol (800 µl for each tube). Finally, the tubes were centrifuged for 10 minutes, then pellets were dried by using a vacuum drier. Pellets were dissolved in sterile distilled water and absorbances were measured. Samples, having a 260/280 ratio between 1.6 and 2.1, were used for reverse transcription. Two µg in a total volume of 20 µl was reversed transcribed, using Moloney leukaemia virus reverse transcriptase and random primers (Promega, Madison, WI, USA). The reaction mixture, including 0.5 µl RNAsin (40 U/ml), 4 µl 5xRT buffer, 2 µl dithiothreitol (DTT) (100 mM), 2 µl dextro-nucleoside triphosphates (dNTPs) (10 mM), 1 µl random primer, 1 µl reverse transcriptase and sample RNA, was left at room temperature for 10 minutes to allow annealing of the primer to its target sequence, then incubated at 37$^{O}$C for 60 minutes.

### 2.2.10. Polymerase chain reaction:

Four microliters of reverse transcription mixtures from each tube were used for PCR, as cDNAs. Polymerase chain reaction (PCR) amplification of cDNA was then carried out, using primers for murine iNOS (3'-TTCCGAAGTTTCTGGCAGCA; 5'-ATAGGAAAAGACTGCACCGAAGAT) and β-actin (3'-CTCTTTGATGTCACGCACGATTTC; 5'-GTGGGCCGCT CTAGGCACCAA). Primers for iNOS were synthesised at the University of Glasgow by Damo Xu, and primers for β-actin were obtained from British Biotechnology (Oxon, UK.). Two microliter of the reverse transcription reaction was made up to 100 µl with 1 x Taq buffer containing 0.5 µg of the 5' primer, 0.5 µg 3' primer and 2.5 U Taq polymerase (Promega, Madison, WI, USA). β–actin 3' and 5' primers were used as a control. 2.5 mM dNTPs were used for each tube.

Thirty-five amplification cycles were carried out, each cycle consisting of 1.5 minutes at 94$^{O}$C, 2 minutes at 55$^{O}$C and 3 minutes at 72$^{O}$C. PCR products were separated by electrophoresis through 1% agarose containing ethidium bromide, and viewed by UV illumination.

### 2.2.11. Detection and analysis of proteins: SDS Page and Western Blotting:

J774.1 cells were set up in 25 ml culture flasks and adjusted to 1 x $10^7$ cells/group. RPMI 1640 medium supplemented by some factors as described before was renewed before treatments as detailed in related experiments. Medium was removed and then cells were washed by ice cold PBS twice. Cells were scraped into 5-10 ml sterile PBS and centrifuged at 250 x g for 3-5 minutes, and proteins were dissolved in extraction buffer (400 µl), containing 150 mM NaCl, 1 % NP-40 (Nonidet-P-40), 1 mM EDTA, 2 mM EGTA, 1 mM DTT, 50 µg/ml leupeptin, 50 µg/ml aprotinin and 50 µg/ml PMSF in 25 mM Tris-HCl buffer (pH:7.5). Cells in extraction buffer were homogenised and kept on ice for 20-30 minutes, then centrifuged at 13 000xg for 10 minutes. Supernatants were used immediately for protein measurements and running SDS-polyacrylamide gel. Protein measurements were carried out as described below in this chapter.

Gel tanks were washed and cleaned by using ethanol before use. 30% acrylamide mix, 10% SDS, 1.5 M Tris-HCl (pH:8.8), 10% ammonium persulphate and tetramethylethylenediamine (TEMED) in distilled water were solutions to prepare the lower gel (resolving gel), which was prepared by mixing these solution components in different portions to obtain 7.5% of SDS-polyacrylamide gel for samples as detailed in related experiments. The mixture was poured into the gap between the glass plates, leaving sufficient space for the stacking gel (Upper gel) immediately after mixing it, because polymerisation will begin as soon as the

TEMED has been added. 1-2 ml distilled water was added onto the gel to prevent oxygen diffusion into the gel. The gel was placed in a vertical position at room temperature. After gel polymerisation is completed (approximately 30 minutes), the overlay was poured off and the top of the gel was washed several times with deionized water to remove any unpolymerized acrylamide. The stacking gel, including appropriate volumes of 30% acrylamide, 1.0M Tris-HCl (pH:6.8), 10 % SDS, 10% ammonium persulphate and TEMED in distilled water, was prepared, as detailed in related experiments. The stacking gel solution was poured directly onto the surface of the polymerised resolving gel. A clean Teflon comb was inserted into the stacking gel solution, being careful to avoid trapping air bubbles. The gel was then placed in a vertical position at room temperature. After polymerisation is completed, approximately in 30 minutes, the Teflon comb was removed carefully. The wells were immediately washed with deionized water, using a squirt bottle, to remove any unpolymerized acrylamide.

The gel tank was mounted in the electrophoresis apparatus. Tris-glycine buffer (pH: 8.3) was prepared, using 25 mM Tris, 250 mM glycine and 0.1 % SDS, to add to the top and the bottom reservoirs after samples were loaded. Samples were loaded 10-20 μg protein/well after denaturation by boiling for 5 minutes in SDS gel-loading buffer, containing bromophenol blue as a tracking dye to follow the process. Rainbow markers were used to determine the weights of the protein bands investigated. The positive electrode was connected to the bottom and the negative one to the top reservoir, and the current of 20 mA was used to separate the proteins. After the dye front has reached the bottom of the resolving gel, the power supply was turned off.

For western blotting, the gel was transferred into transfer buffer immediately. Transfer buffer contains 39 mM glycine, 48 mM Tris base and 20 % methanol. Proteins were transferred onto a nitro-cellulose membrane (BioRad, Hempstead,

USA), applying 300 mA current for 1-2 hours. Nonspecific binding sites were blocked with 2% BSA in wash buffer 1, containing 0.01 M Tris HCl (pH:7.5), 0.1 M NaCl and 0.1% Tween-20, at 4°C overnight. Next day, the primary antibody, mouse ab α iNOS (1/200), was added in 1% BSA, then the membrane was left shaking for 1 hour. The membrane was washed with wash buffer 1 twice for 10 minutes, and wash buffer 2, including 0.01 M Tris HCl (pH:7.5), 0.5 M NaCl and 0.1% Tween-20, twice for 10 minutes each, finally, wash buffer 1 again. The secondary antibody, mouse ab α-HRP (1/5000), was added onto the membrane, and incubated for 1 hour. A further cycle of washing was carried out (WB1x2, WB2x2, WB1x2) for 10 minutes each. Finally, enhanced chemiluminescence (ECL) solutions were used to visualise proteins by luminescence and exposing it onto a Kodak film in a dark room. ECL solution A and B (1:1, v/v) were added into a tank, containing membrane, and shook for 1 minute, then used to expose the film.

### 2.2.12. Cell viability tests:

All reagents to modify cell functions were assayed for any possible cytotoxic effects by several techniques; Trypan blue exclusion test and glucose uptake assays.

#### 2.2.12.1. Trypan Blue Exclusion Test:

96-well plates were used for this test. Wells including cells treated by IBMX or other appropriate agents for 24 hours or controls were washed by warm PBS twice after removing RPMI 1640 medium supplemented as indicated above. Fifty µl trypan blue dye (1 % in saline, w/v) was added into wells. Then, the plate was incubated for 3-5 minutes in room temperature. The trypan blue solution was removed and cells were observed with a Zeiss microscope. Dead and surviving cells in at least nine randomly selected areas were counted. Results have been shown as the ratio of [total cells] - [stained cells] x 100/ [total cells]. Percentages

for cell viability expressed with SD values were indicated in the related experiments.

### 2.2.12.2. Glucose uptake:

Cells were adjusted to a density of $1x10^6$ cells/well in 1 ml RPMI 1640 medium to check, if cells were damaged by various substances. Two μCi/ml [$^3$H]-glucose was used to determine this effect. Cells were treated with 2 μCi/ml [$^3$H]-glucose in RPMI 1640 medium. Then test medium (1 ml) containing appropriate substances was added. Cells were treated with agents, such as cholera and pertussis toxins, IBMX, and untreated cells were used as controls, and the incubation was continued at 37$^{o}$C for 24 hrs. After, 24 hours, this medium was removed, the wells were washed four times with sterile warm PBS. This was sufficient to eliminate unincorporated radioactive [$^3$H]-glucose from wells, counting β-radiation each time. The cells remaining in each well were treated with 0.1 % Triton X-100 in PBS for 5 min to lyse the cells. One ml of lysate was also added to scintillation vials and counted for [$^3$H] on a scintillation counter. Sodium azide (0.01 M) treated wells were used as positive controls. The percentage uptake of [$^3$H]-glucose was presented as CPM values for each group [Andreoli et al., 1985].

## 2.3. Statistical Analysis:

All experiments were carried out at least two or three times. Statistical significance (**: $p<0.01$, *: $p<0.05$, †: $p<0.1$, $n=3$) was analysed by student's paired *t* -test. Standard error of means (SEM) or standard deviation (SD) bars of sample measurements have been shown where required.

# CHAPTER 3

# THE EFFECT OF PROLONGED ELEVATION OF cAMP LEVELS ON NITRIC OXIDE PRODUCTION BY J774 MACROPHAGES

## 3.1. INTRODUCTION

Nitric oxide plays an important role in the tumoricidal and anti-microbial activities of macrophages [Stuehr and Marletta, 1985]. However, as its cytotoxic effects are non-specific, it is important that its production is regulated. NO production is inhibited by cytokines such as IL-4 [Liew et al., 1991] and TGF-β [Ding et al., 1990]. The production of TNFα and IL-1 by macrophages is inhibited by agents which increase intracellular cAMP concentrations, including adenylyl cyclase activators, such as $PGE_2$ [Kunkel et al., 1986; Renz et al 1988; Hart et al., 1989a] and β–adrenergic agonists [Severn et al., 1992a], and nonspecific cAMP phosphodiesterase inhibitors [Strieter et al., 1988]. It is important to indicate that $PGE_2$ is the major cyclooxygenase product synthesised in macrophages activated by bacterial LPS [reviewed by Bonney and Humes, 1984]. It has been reported that increased intracellular cAMP levels direct a negative signal for several immune responses [Smith et al., 1971; Bourne et al., 1974; Kammer et al., 1989; Chouaib et al., 1987], such as cytotoxicity and conjugate formation by mouse CTL [Gray et al, 1988] and the TcR-induced increase of adhesion [Dustin and Springer, 1989].

Cyclic AMP activates PKA, which phosphorylates many proteins, some of which function in the nucleus to alter gene transcription, such as CREB protein. It has been reported that cAMP-elevating agents alter transcriptional induction of iNOS mRNA in rat glomerular mesangial cells [Kunz et al., 1994]. The PKA enzyme is composed of two regulatory (R) and two catalytic (C) subunits that together constitute an inactive holoenzyme of an $R_2C_2$ structure. Binding of cAMP to the regulatory subunit dimer results in release and concomitant activation of the catalytic subunits. The catalytic subunit requires that substrates have basic amino acids that precede the site of phosphotransfer with the consensus sequence for recognition; being Arg-Arg-**X**-Ser [reviewed by Bramson et al., 1984]. iNOS displays a consensus site for phosphorylation by PKA located at a site different for the

comparable sequence in cNOS. In both cNOS and iNOS this is a weak phosphorylation sequence, Lys-Arg-**X**-**X**-Ser [Lowenstein et al., 1992].

We have therefore investigated the effect of increased cAMP levels on NO production by using a murine macrophage cell line, J774. Prostaglandin $E_2$ alone induced a brief increase in intracellular cAMP concentrations and did not affect NO production, but $PGE_2$ and a nonspecific phosphodiesterase inhibitor IBMX, in combination induced a prolonged elevation of cAMP levels and inhibited NO production. It should be added that the phosphodiesterases consist of a family of more than 30 members, all of which may be inhibited by IBMX [reviewed by Beavo et al., 1994]. Rp-cAMP, a nonhydrolysable inhibitor of cAMP-dependent kinase (PKA) [Botelho et al., 1988] prevented the inhibition of NO production by $PGE_2$ and IBMX, confirming that the effect was dependent on cAMP itself. In addition, by using rolipram, which is a cAMP-specific PDE inhibitor [Reviewed by Beavo and Reifsnyder in 1990], we demonstrated that there is no other effect of IBMX involved in this inhibition.

## 3.2. RESULTS

### 3.2.1. cAMP-elevating agents inhibit NO production:

In macrophages, it is known that cAMP generation induced by $PGE_2$, cholera toxin and IBMX, or cAMP analogues added into medium, inhibits macrophage motility, phagocytosis and cytokine secretions. We tested the effect of cAMP elevation, induced by $PGE_2$ or 8-bromo-cAMP alone, and in combination with IBMX, on NO produced in response to high concentrations of LPS or IFN-γ and low dose LPS. J774 cells, 5 x $10^5$ /ml in RPMI 1640 medium, were cultured in 96-well plates. Thirty minutes after adding cAMP-elevating agents, cells were stimulated by 1 μg/ml LPS or IFN-γ (40 U/ml) and low dose LPS (10 ng/ml), then incubated for 24 hrs. Supernatants were used for measurement of $NO_2^-$ by using chemiluminescence.

Stimulation of J774 cells with 1 μg/ml LPS induced the production of NO (Figure 3.1.a). Production of NO was not affected by $PGE_2$ ($10^{-6}$ M) or 8-Br-cAMP ($10^{-5}$ M) alone, but was significantly inhibited by the combination of $PGE_2$ or 8-Br-cAMP with IBMX ($10^{-4}$ M) ($p<0.01$). The PKA inhibitor, Rp-cAMP (4 x $10^{-4}$ M), used to evaluate if this effect is due to cAMP itself, completely prevented inhibition.

NO production induced by IFN-γ and low dose LPS was also inhibited by $PGE_2$ or 8-Br-cAMP, in combination with IBMX, and the inhibitory effect of cAMP-elevating agents was prevented by Rp-cAMP ($p<0.01$) (Figure 3.1.b).

In the absence of agents which increase cAMP, Rp-cAMP had no effect on NO production. IBMX alone, in cells stimulated by IFN-γ and low dose LPS or high dose LPS alone did not alter NO production. Since IBMX (100 mM) was dissolved

in DMSO, controls carried out showed that the results were not affected by DMSO (maximum concentration 0.1 % v/v).

Cell viability was examined by trypan blue exclusion in cells treated with IBMX, and it has not been found that IBMX ($10^{-4}$ M) has a toxic effect on cells (CV>96%). Moreover, cells treated with IBMX were able to adhere and spread in a similar fashion to untreated cells, and the general morphology appeared normal.

### 3.2.2. $PGE_2$ and IBMX pretreatment of J774 cells is required to inhibit NO production in response to IFN-γ and low doses of LPS:

It is required to observe the best time point for usage of cAMP-elevating agents to inhibit NO production in response to IFN-γ and LPS in macrophages. Then, it might be possible to predict where and how this effect alters NO production.

The response of J774 cells to produce NO, following stimulation by IFN-γ (40 U/ml) and LPS (10 ng/ml), was assessed by using $PGE_2$ ($10^{-6}$ M) and IBMX ($10^{-4}$ M) at different time points. Cells were treated with $PGE_2$ and IBMX at different time points (-18, -4, -1/2, 0, +4 hours) and stimulated by IFN-γ and LPS, and then cultured for 24 hours. Supernatants were used for measurement of $NO_2^-$ by using chemiluminescence.

The best inhibition, approximately 35 %, was observed, when cells were treated with $PGE_2$ and IBMX 30 minutes before stimulation ($p<0.01$) (Figure 3.2). This evidence shows that the inhibitory effect of cAMP elevation on NO producing macrophages requires pretreatment of J774 cells with cAMP-elevating agents. However, the treatment of cells with $PGE_2$ and IBMX after 4 hrs stimulation inhibited NO production in response to IFN-γ and LPS. This inhibition was less than the inhibition caused by pretreatment of cells, but was statistically significant ($p<0.05$).

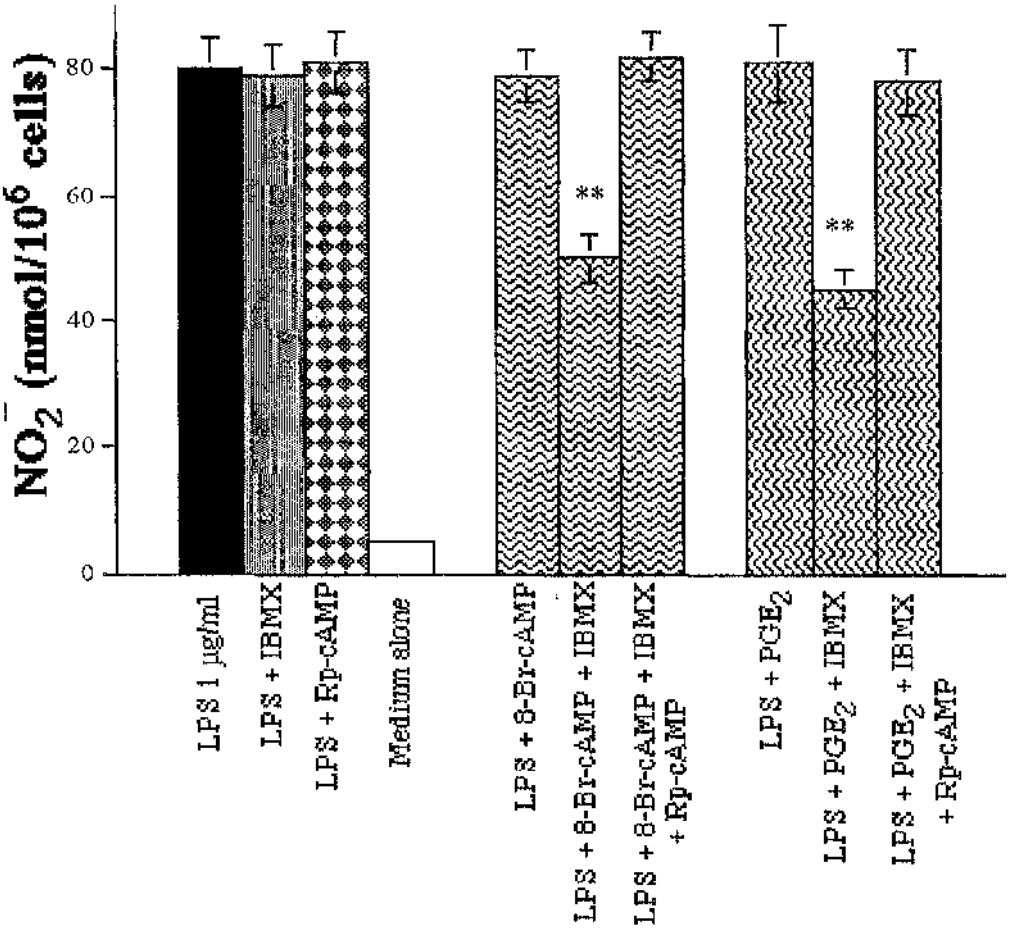



**Figure 3.1.a:** The effect of $PGE_2$ ($10^{-6}$ M) or 8-Br-cAMP ($10^{-5}$ M) with IBMX ($10^{-4}$ M) on NO production in response to LPS (1 μg/ml), and the effect of Rp-cAMP ($4 \times 10^{-4}$ M) in J774 cells. Cyclic-AMP elevating agents were added 30 min after adding Rp-cAMP and 30 min before stimulation with LPS (1 μg/ml). The means ± SEMs of triplicate samples are shown. Results are representative of three separate experiments.

(**: $p<0.01$, n=3)

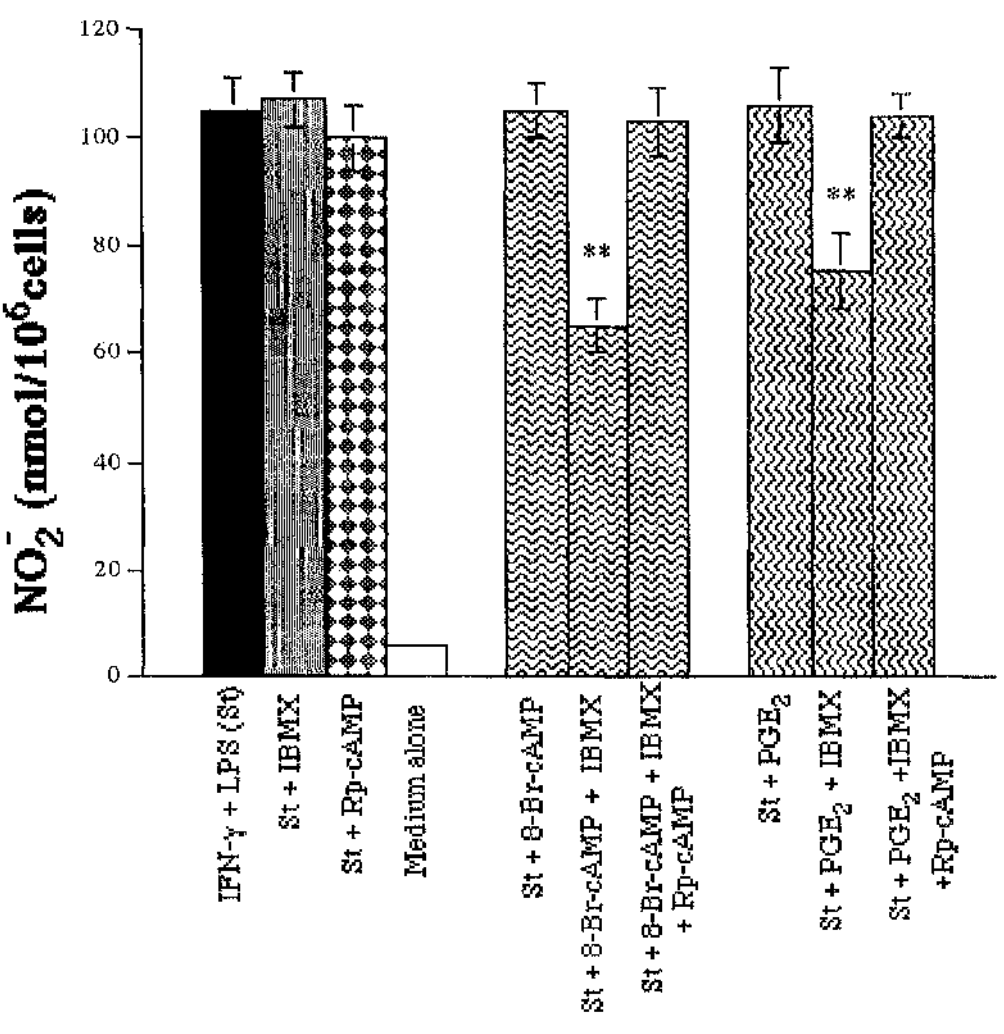



**Figure 3.1.b:** The effect of $PGE_2$ ($10^{-6}$ M) or 8-Br-cAMP ($10^{-5}$ M) with IBMX ($10^{-4}$ M) on NO production in response to IFN-γ (40 U/ml) and LPS (10 ng/ml), and the prevention by Rp-cAMP of this inhibition in J774 cells. Cyclic AMP-elevating agents were added 30 min after adding Rp-cAMP ($4 \times 10^{-4}$ M) and 30 min before stimulation with IFN-γ (40 U/ml) and LPS (10 ng/ml). The means ± SEMs of triplicate samples are shown. Results are representative of three separate experiments.

(**: $p<0.01$, n=3)

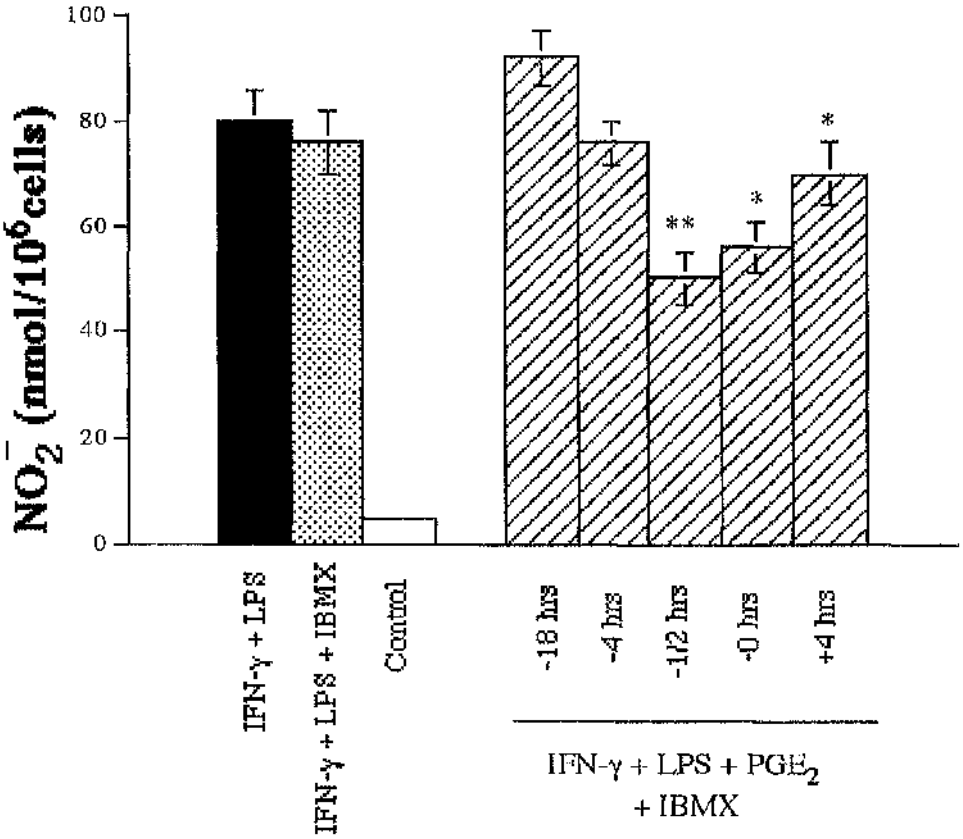



**Figure 3.2:** The effect of $PGE_2$ ($10^{-6}$ M) and IBMX ($10^{-4}$ M) used at different time points on stimulation with IFN-γ (40 U/ml) and LPS (10 ng/ml) in J774 cells (5 x $10^5$ cells/ml). Control shows nitrite measurements by chemiluminescence in samples obtained from resting J774 cells. Since IBMX (100 mM) is dissolved in DMSO, controls carried out showed that the results were not affected by DMSO (maximum concentration 0.1 % v/v). The means ± SEMs of triplicate samples are shown. Results are representative of three separate experiments.

(**: $p<0.01$, *: $p<0.05$, n=3)

### 3.2.3. The effect of $PGE_2$ and IBMX on intracellular cAMP levels:

Cells were incubated with $10^{-6}$ M $PGE_2$, alone or with $10^{-4}$ M IBMX, and cAMP was extracted and measured, as described in Chapter 2. $PGE_2$ alone resulted in an increased cAMP level, detectable at 15 minutes. However, $PGE_2$ alone causes transient cAMP elevation in cells, which disappears 1 hour after treatment. The combination of $PGE_2$ and IBMX induced a greater rise in cAMP, and the level was still elevated after 8 hours. This result shows the difference between use of $PGE_2$ alone and together with IBMX. It is clear that the inhibitory effect of cAMP requires elevated and sustained levels of cAMP. Results are expressed in pmol/$10^6$ cells, and means and standard deviations of triplicate samples are shown (Table 3.1).

### 3.2.4. Short-term cAMP-elevating agents do not have an effect on NO production:

Cells were adjusted to 5 x $10^5$ cells in each well in RPMI 1640 medium supplemented with 10 % FCS, L-glutamine and antibiotics. 8-Br-cAMP at graded concentrations, was added into the medium 30 min before stimulation. Then, cells were stimulated with high concentrations of LPS (1 μg/ml) or IFN-γ (40 U/ml) and low dose LPS (10 ng/ml), and incubated for 24 hrs. Supernatants were used for $NO_2^-$ measurement by chemiluminescence, as detailed in Chapter 2.

8-Br-cAMP did not affect NO production by J774 cells in response to high concentrations of LPS (1 μg/ml) or IFN-γ (40 U/ml) and low dose LPS (10 ng/ml) (Figure 3.3). This result demonstrate 8-Br cAMP, which is a membrane analogue of cAMP, does not affect J774 cells at any concentration.

**Table 3.1: Intracellular cAMP levels in response to cAMP elevating agents:**

cAMP (pmol/ $10^6$ cells)

| Time | $PGE_2$ | $PGE_2$ + IBMX | IBMX alone | Controls |
|---|---|---|---|---|
| 0 | < 1 | < 1 | < 1 | < 1 |
| 15 mins | 32.5 ± 2.01 | 65.87 ± 6.00 | < 1 | < 1 |
| 1 hr | < 1 | 19.60 ± 3.96 | < 1 | < 1 |
| 2 hrs | < 1 | 13.07 ± 1.22 | < 1 | < 1 |
| 4 hrs | < 1 | 11.47 ± 0.92 | < 1 | < 1 |
| 8 hrs | < 1 | 9.87 ± 1.85 | < 1 | < 1 |
| 12 hrs | < 1 | < 1 | < 1 | < 1 |
| 24 hrs | < 1 | < 1 | < 1 | < 1 |

The comparison of cAMP levels elevated by $PGE_2$ ($10^{-6}$ M) and IBMX ($10^{-4}$ M) or $PGE_2$ ($10^{-6}$ M) alone at different time points. J774 cells were cultured at $10^6$ cells in 1 ml medium, and then cAMP was extracted and measured as described. Results are expressed in pmol/$10^6$ cells. Results are representative of three separate experiments. The means and standard deviations of triplicate cultures are shown. (n=3)

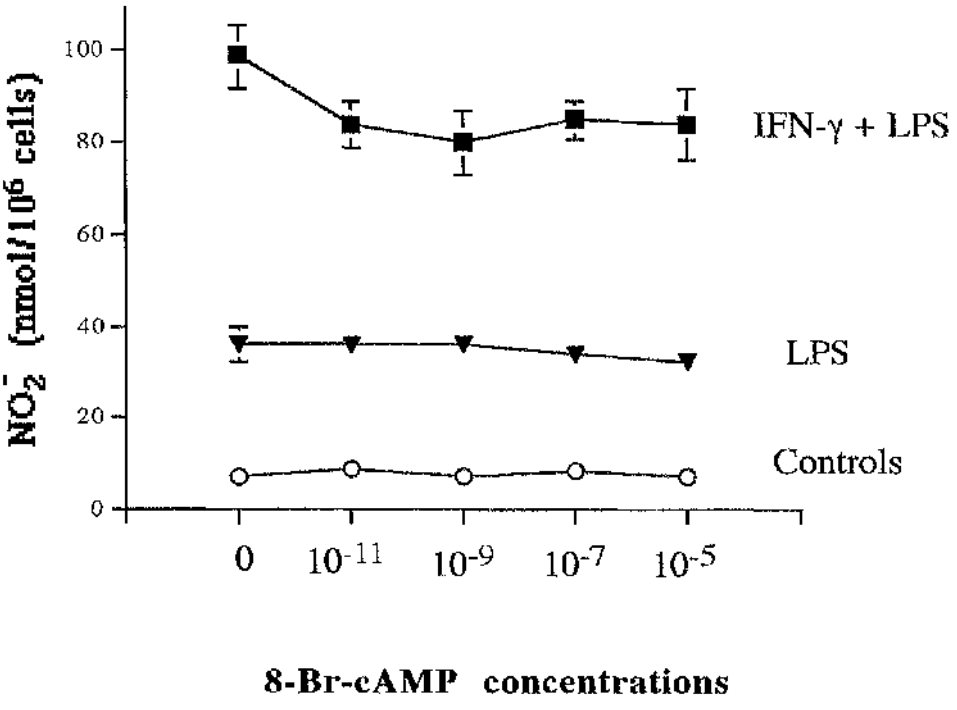



**Figure 3.3:** Short-term cAMP-elevating agents do not affect NO production in J774 cells. 8-Br-cAMP, which is a membrane analog of cAMP, is used, and there is no inhibitory effect of the agent at a variety of concentrations on NO production by J774 cells in response to high concentrations of LPS (1 μg/ml) alone or IFN-γ (40 U/ml) and low dose LPS (10 ng/ml). Controls show nitrite measurements by chemiluminescence in supernatants, which have been obtained from resting J774 cells. Results are representative of three separate experiments. The means ± SEMs of triplicate samples are shown. (n=3)

### 3.2.5. Prolonged elevation of cAMP levels in J774 cells inhibits iNOS activity in response to IFN-γ and LPS:

It is required to investigate the effect of cAMP elevation on iNOS activity. The effect of cAMP-elevating agents on iNOS activity produced by J774 cells in response to IFN-$_\gamma$ (40 U/ml) and LPS (10 ng/ml) was assessed, as detailed in Chapter 2. Cells were adjusted to 1 x $10^6$ cells in 1 ml medium. cAMP-elevating agents were added 30 min before stimulation and 30 min after adding Rp-cAMP (4 x $10^{-4}$ M), then cells were stimulated by IFN-$_\gamma$ (40 U/ml) and LPS (10 ng/ml) for 12 hours. Cytosols were extracted, as detailed in Chapter 2, and iNOS activity was measured by the citrulline assay.

The effect of $PGE_2$ ($10^{-6}$ M), in combination with IBMX ($10^{-4}$ M), resulted in a significant decrease in iNOS activity ($p<0.05$). The combination of 8-Br-cAMP ($10^{-5}$ M) and IBMX ($10^{-4}$ M) also inhibited iNOS activity ($p<0.05$). The PKA inhibitor, Rp-cAMP (4 x $10^{-4}$ M), completely prevented inhibition in both cases (Figure 3.4). These results indicate clearly that sustained elevated intracellular cAMP inhibits NO production by inhibiting iNOS activity.

### 3.2.6. There is no difference in the level of inhibition between usage of IBMX, a nonspecific PDEs inhibitor, and Rolipram, a cAMP-specific-PDE inhibitor:

Since IBMX is a nonspecific PDEs inhibitor, and may additionally inhibit some PLC isoforms, it is necessary to clarify that the inhibition of NO production by J774 cells is due to cAMP elevation itself, but not other interferences in signalling pathway. The effects of $PGE_2$ or 8-Br-cAMP alone, and in combination with IBMX or rolipram, on NO production were assessed. J774 cells, 5 x $10^5$ /ml in RPMI 1640 medium, were cultured in 96-well plates, then cells were treated with

IBMX ($10^{-4}$ M) or rolipram ($10^{-6}$ M), and $PGE_2$ ($10^{-6}$ M) or 8-Br-cAMP ($10^{-5}$ M) for 30 minutes. Cells were stimulated by IFN-$\gamma$ (40 U/ml) and LPS (10 ng/ml) or LPS (1 μg/ml) alone. After 24 hours incubation, $NO_2^-$ was determined, using chemiluminescence, in supernatants.

Both IBMX and rolipram, together with $PGE_2$ or 8-Br-cAMP, caused the inhibition of NO production in macrophages stimulated by IFN-$\gamma$ and low dose LPS or high dose LPS alone ($p<0.01$-0.1) (Figure 3.5). In the absence of agents which increase cAMP, IBMX or rolipram had no effect on NO production. Because IBMX (100 mM) and rolipram (10 mM) were dissolved in DMSO, controls were carried out, and the results were not affected by DMSO (maximum concentration 0.1 % v/v).

Cell viability was examined by trypan blue exclusion in cells treated with IBMX or rolipram and it was found to be >95%. Moreover, cells treated with IBMX and rolipram were able to adhere and spread similarly to untreated cells, and the general morphology appeared normal.

### 3.2.7. $PGE_2$ and IBMX do not have an effect on iNOS mRNA transcription in J774 cells stimulated by IFN-γ and LPS:

The inhibitory effect of cAMP elevation might be transcriptional or posttranscriptional. Transcriptional effects involve either downstream signalling induced by IFN-$\gamma$ and LPS or a direct nuclear effect of PKA activation, whereas posttrancriptional effects involve iNOS phosphorylation and alterations of iNOS mRNA stability. Cells were adjusted to $5 \times 10^6$ cells/ml and cultured in 25 ml flasks. Cells were treated with $PGE_2$ ($10^{-6}$ M) and IBMX ($10^{-4}$ M) with or without Rp-cAMP ($4 \times 10^{-5}$ M), a PKA inhibitor, for 30 min before stimulation with IFN-$\gamma$ (40 U/ml) and LPS (10 ng/ml). Rp-cAMP was added 30

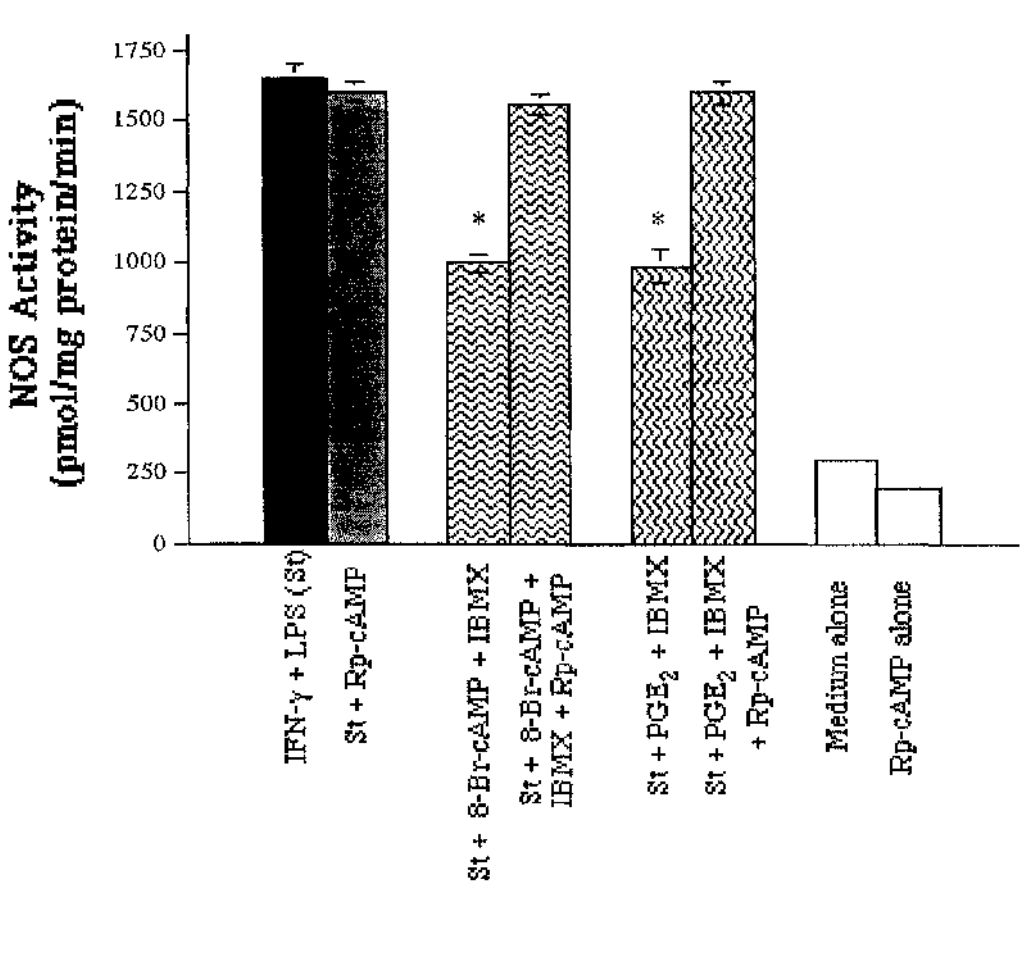
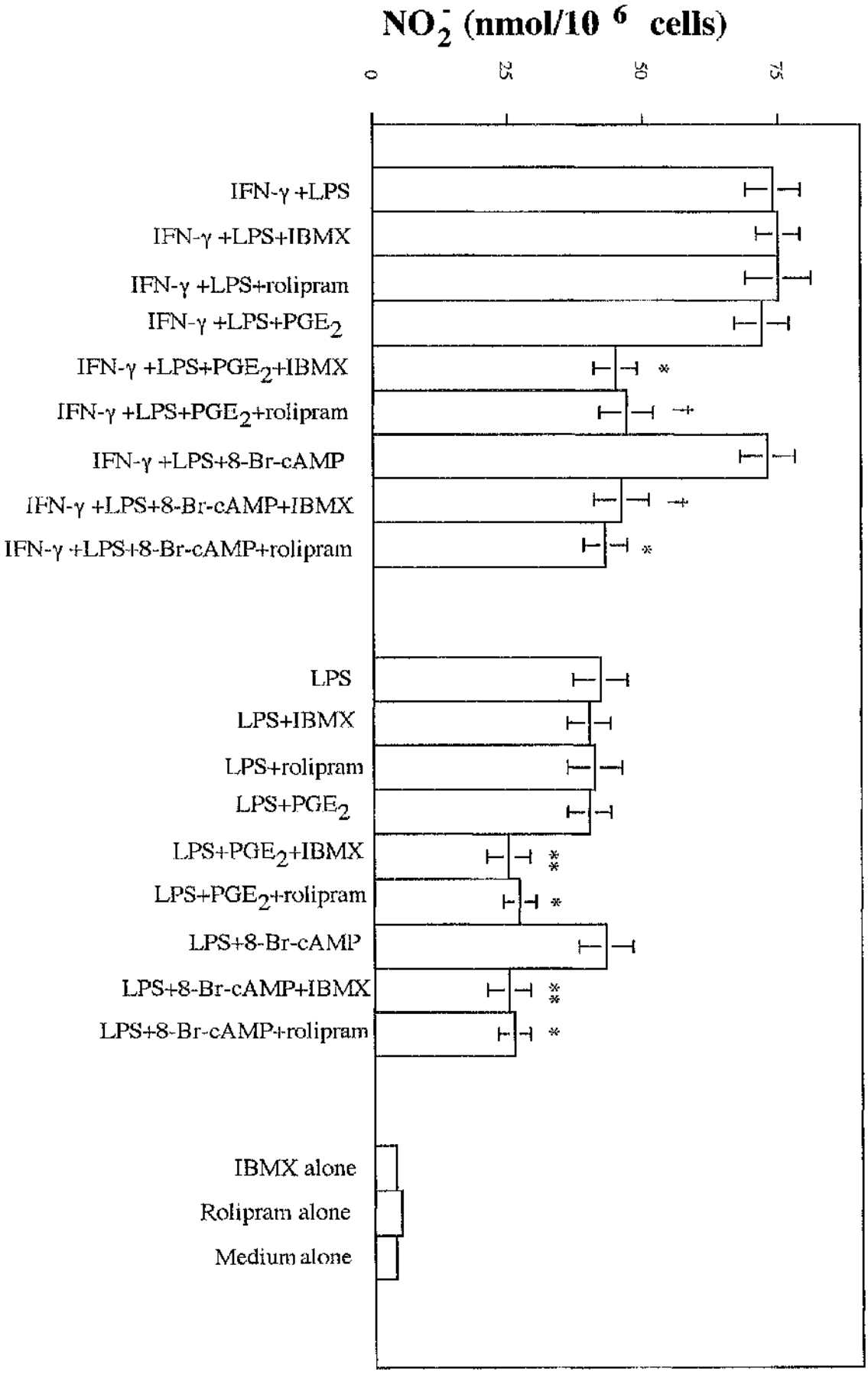



**Figure 3.4:** The sustained elevation of cAMP levels inhibits iNOS activity in response to IFN-γ and LPS. The effect of cAMP-elevating agents on iNOS activity produced by J774 cells in response to IFN-γ (40 U/ml) and LPS (10 ng/ml) was assessed. Cyclic AMP-elevating agents were added 30 min after adding Rp-cAMP ($4 \times 10^{-4}$ M) and 30 min before stimulation, then cells were stimulated by IFN-γ (40 U/ml) and LPS (10 ng/ml) for 12 hours. Controls show NOS activity measurements by citrulline assay in resting cells and Rp-cAMP treated cells. The means ± SEMs of triplicate samples are shown. Results are representative of three separate experiments.

(*: $p<0.05$, n=3)

**Figure 3.5:** The comparison of IBMX with a cAMP-specific PDE inhibitor, rolipram. Cells were treated with IBMX ($10^{-4}$ M) or rolipram ($10^{-6}$ M), and $PGE_2$ ($10^{-6}$ M) or 8-Br-cAMP ($10^{-5}$ M) for 30 minutes. Then, cells were stimulated by IFN-$\gamma$ (40 U/ml) and LPS (10 ng/ml) or LPS (1 μg/ml) alone. After 24 hours incubation, $NO_2^-$ was determined, using chemiluminescence, in supernatants. Controls were obtained from wells untreated, treated with rolipram and IBMX alone. Results are representative of three separate experiments. The means ± SEMs of triplicate samples are shown.

(**: $p<0.01$, *: $p<0.05$, †: $p<0.1$, n=3)


$NO_2^-$ (nmol/10 $^6$ cells)
0
25
50
75
IFN-γ +LPS
IFN-γ +LPS+IBMX
IFN-γ +LPS+rolipram
IFN-γ +LPS+$PGE_2$
IFN-γ +LPS+$PGE_2$+IBMX
IFN-γ +LPS+$PGE_2$+rolipram
IFN-γ +LPS+8-Br-cAMP
IFN-γ +LPS+8-Br-cAMP+IBMX
IFN-γ +LPS+8-Br-cAMP+rolipram
LPS
LPS+IBMX
LPS+rolipram
LPS+$PGE_2$
LPS+$PGE_2$+IBMX
LPS+$PGE_2$+rolipram
LPS+8-Br-cAMP
LPS+8-Br-cAMP+IBMX
LPS+8-Br-cAMP+rolipram
IBMX alone
Rolipram alone
Medium alone

min before adding the cAMP-elevating agents. Cells were incubated for 5 hrs and medium removed, then cells were washed three times with sterile warm PBS buffer. Cells were gently scraped and total RNA was extracted for reverse transcription and PCR, as described in Chapter 2. Reverse transcription and PCR have shown that cAMP-elevating agents have no effect on iNOS gene transcription. (Figure 3.6).

### 3.2.8. $PGE_2$ and IBMX reduce iNOS protein synthesis in J774 cells stimulated by IFN-γ and LPS:

Having shown that there was no the effect of cAMP-elevating agents on iNOS message, protein extraction, SDS-PAGE separation and western blotting were carried out to explore the post transcriptional alterations in NO production. Cells were adjusted to $5 \times 10^6$ cells/group and cultured in 25 ml flasks. Cells were treated with $PGE_2$ ($10^{-6}$ M) and IBMX ($10^{-4}$ M) together or IBMX alone, then incubated for 10-12 hrs and medium removed. Cells were washed three times with sterile warm PBS buffer, and scraped into the extraction buffer, then cytosolic proteins were extracted for SDS-Page, as described in Chapter 2. Having run SDS-Page gel, proteins were transferred to solid phase, nitro-cellulose membranes as described in Chapter 2. The next day, the primary antibody, mouse anti-iNOS (1/200), was added in 1% BSA. Then the membrane was washed, as detailed in chapter 2. Secondary antibody, mouse ab α-HRP (1/5000), was added onto the membrane, and incubated for 1 hour. The membrane was washed once again. Finally, film was developed by ECL detection method.

The treatment of J774 cells with $PGE_2$ and IBMX, when applied together, results in iNOS synthesis inhibition in response to IFN-γ and LPS. $PGE_2$ ($10^{-6}$ M) and IBMX ($10^{-4}$ M) were used 30 minutes before stimulation with IFN-γ (40 U/ml) and LPS (10 ng/ml). In order to determine inhibition level of iNOS protein

synthesis by $PGE_2$ and IBMX, the extract of cells stimulated by IFN-$\gamma$ and LPS were diluted at different ratios (1/2 and 1/4). Densitometer results have shown approximately 65 % inhibition of iNOS protein synthesis, when the cells were treated with $PGE_2$ and IBMX. Moreover, IBMX alone did not affect iNOS synthesis in stimulated cells. (Figure 3.7).


**Lane 1:** IFN-$\gamma$ + LPS

**Lane 2:** Resting cells (Controls)

**Lane 3:** IFN-$\gamma$ and LPS + $PGE_2$ + IBMX

**Lane 4:** IFN-$\gamma$ and LPS + $PGE_2$ + IBMX + Rp-cAMP

**Figure 3.6:** The effect of $PGE_2$ and IBMX on iNOS gene transcription in J774 cells stimulated by IFN-$\gamma$ and LPS. Cells were treated with $PGE_2$ ($10^{-6}$ M) and IBMX ($10^{-4}$ M) with or without Rp-cAMP ($4 \times 10^{-5}$ M), which is a PKA inhibitor for 30 min before stimulation with IFN-$\gamma$ (40 U/ml) and LPS (10 ng/ml). Rp-cAMP was used 30 min before adding cAMP-elevating agents. Reverse transcription and PCR have shown that cAMP-elevating agents have no effect on iNOS gene transcription. Controls show β-actin bands in J774 cells. Experiments have been done three times.

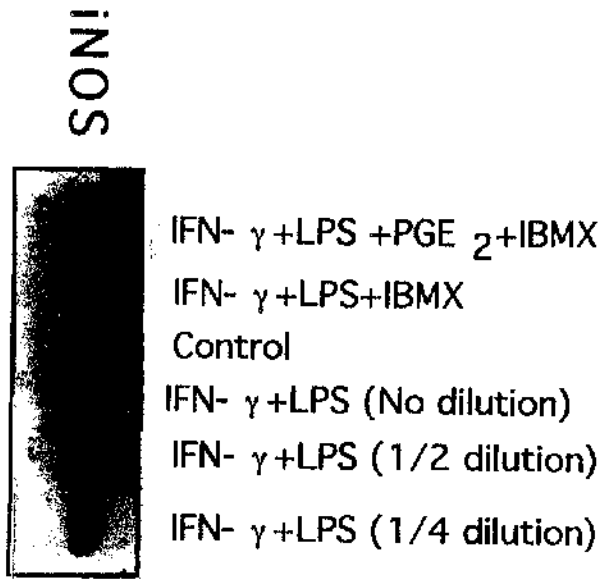



**Figure 3.7:** Western blot analysis as the effect of $PGE_2$ and IBMX on iNOS synthesis in response to IFN-$\gamma$ and LPS. $PGE_2$ ($10^{-6}$ M) and IBMX ($10^{-4}$ M) were used 30 minutes before stimulation with IFN-$\gamma$ (40 U/ml) and LPS (10 ng/ml). To determine inhibition level of iNOS protein synthesis by $PGE_2$ and IBMX, the extract of cells stimulated by IFN-$\gamma$ and LPS were diluted at different ratios (1/2 and 1/4) to measure protein levels by densitometer. Experiments have been repeated twice.

## 3.3. DISCUSSION:

Data presented here demonstrate that NO production by murine macrophages is inhibited by a prolonged increase in intracellular cAMP concentrations. A non-specific, toxic effect of $PGE_2$ and IBMX was excluded by the use of a PKA inhibitor, which completely prevented the inhibition. Bromberg and Pick [1980] reported that macrophages treated with NO-generating agents showed an increase in cyclic GMP levels. Furthermore, NO generated by macrophage cytosol stimulates soluble guanylate cyclase [Mulsch et al., 1990]. These data suggest that macrophage cytotoxicity may also be related to the formation of cGMP. Since IBMX is a nonspecific PDE inhibitor, it might affect intracellular cGMP generation. IBMX has also an antagonist effect for adenosine receptors [Beavo and Reifsnyder, 1990]. Therefore, it was required to be confirmed by using Rp-cAMP that the inhibitory effect of cAMP-elevating agents was directly due to cAMP elevation. Meanwhile, $PGE_2$ was also inhibitory for the NO production, the inhibition observed in the presence of 8-Br-cAMP is more likely be due to its action as a cAMP analogue and not to nonspecific effects of its breakdown products. Additionally, rolipram, which is a cAMP-specific PDE inhibitor, was used with cAMP-elevating agents $PGE_2$ or 8-Br-cAMP, and no difference was found between the usage of IBMX and rolipram (also called ZK62711) [Reviewed by Beavo and Reifsnyder in 1990]. One high affinity cAMP phosphodiesterase can be activated by cAMP-dependent protein kinase (PKA) [Anderson et al., 1989; Gettys et al., 1987; Kilgour et al., 1989; Macphee et al., 1988]. Therefore, IBMX use in experiments seems to be necessary to inhibit PDEs nonspecifically.

What could be the role of the cAMP pathway in the inhibition of NO production in macrophages? According to PCR, SDS-Page and western blotting results, it is clear that the inhibitory effect of cAMP elevation on NO production occurs at the post transcriptional or translational levels of iNOS gene and iNOS protein synthesis

respectively, but not at the level of transcription. In fact, the mechanism by which cAMP inhibits NO production is still unclear, but the requirement for a prolonged increase in cAMP suggests that it acts at a translational or post-translational level. The macrophage iNOS has a consensus sequence for PKA phosphorylation [Lowenstein et al., 1992], and it may be that phosphorylation by this enzyme reduces the activity of NOS. Activated macrophages produce large quantities of $PGE_2$ [Nathan, 1987], and this may contribute to the regulation of NO production, via the elevation of cAMP. On the other hand, PKA activity can cause phosphorylation of many other kinases, such as Raf kinases and other proteins which are involved in NO producing process in macrophages. It is clear that cAMP-dependent protein kinases (PKA) regulate the expression of numerous eukaryotic genes by agents such as epidermal growth factor and mobilised calcium [Sheng et al., 1988].

$PGE_2$ may have a very important role in immune regulation. For instance, $PGE_2$ inhibits IL-2 and IFN-γ production from Th1 clones, but not IL-4 and IL-5 production from Th2 clones [Betz and Fox, 1991]. Addition of IFN-γ to macrophages results in a marked rise in intracellular pH within a few seconds, which depends on an influx of $Na^+$ ions. To date, the enhanced exchange of $Na^+/H^+$ induced by IFN-γ has been most closely linked to the functional effects of IFN-γ [Prpic et al., 1989]. Some evidence indicates that cAMP levels elevated by $PGE_2$ suppress $Na^+/H^+$ exchange [Uhing et al., 1992].

LPS treatment of macrophages results in the activation of various genes, including the genes for TNF-α, IL-1, IFN-β, NOS as well as proto-oncogenes like c-fos, c-jun, c-fms and c-myc. The activation of these genes is at the transcriptional level, and controlled by some DNA-binding transcriptional factors such as NF-κB or AP-1 [Lenardo and Baltimore, 1989]. It has been showed that LPS treatment of J774 cells leads to the activation of the fast-moving (called B1) and slow-moving (called

B2) NF-κB, and this activation is inhibited by H89, which is a PKA inhibitor, but not by H7, which is a PKC inhibitor [Muroi and Suzuki, 1993]. Furthermore, the treatment of J774 cells with dibutyryl cAMP, forskolin, cholera toxin and IBMX induced the activation of the both slow-moving and fast-moving NF-κB proteins. However, there is a difference between the NF-κB activation time points induced by cAMP-elevating agents and LPS. Cyclic AMP-elevating agents induce early activation of both NF-κB proteins, synchronously, although LPS induce them at different time points. The correlation between NF-κB activation and NO production in macrophages has not been shown clearly yet. Further studies are required to elucidate the mechanism of the effect of the NF-κB activation on NO production.

# CHAPTER 4

# THE EFFECTOR MECHANISM OF IL-4 ON NITRIC OXIDE SYNTHESIS

## 4.1. INTRODUCTION

Several cytokines, including IL-4 [Liew et al., 1991], IL-10 [Cunha et al., 1992; Gazzinelli et al., 1992] and transforming growth factor-β [Ding et al., 1990], have been shown to inhibit NO production. However, the pathways involved in the regulation of NO synthesis have not yet been fully elucidated.

IL-4 is a 20 kDa polypeptide also known as B-cell stimulatory factor-1 (BSF-1) or B cell growth factor (BCGF). IL-4 is produced by activated Th2-like cells [Mosmann et al., 1986; Swain et al., 1988], and has many actions on a variety of cell types. For example, the proliferation of B cells, the stimulation of the growth of T cells, mast cells, and macrophage differentiation and activation [Reviewed by Farrar et al., 1990]. On the other hand, there are reports suggesting that IL-4 induces expression of class II major histocompatibility complex molecules on resting B cells [Roehm et al, 1984] and that IL-4 induces IgE responses and maintains them [Finkelman et al, 1988].

The mechanism of signal transduction by IFN-γ is not yet known. However, it was found that IFN-γ stimulation involved PKC activity in peritoneal macrophages [Hamilton et al., 1985]. It has been reported that inhibition of PKC activity, or down-regulation of PKC by prolonged incubation of J774 cells with phorbol esters, reduces NO production in response to IFN-γ and LPS. Furthermore, stimulation of macrophages with these agents results in translocation of PKC to the cell membrane [Severn et al., 1992b]. These observations suggest that PKC activation is essential in the induction of NO production by IFN-γ and LPS.

IL-4 induces a transient activation of PLC, followed by a delayed sustained cAMP elevation, in human B lymphocytes [Finney et al., 1990]. Agents which increase intracellular cAMP levels inhibit cytokine production by macrophages. In our early

studies, it was shown that prolonged elevation of cAMP levels induced by $PGE_2$ and IBMX (a phosphodiesterase inhibitor) reduces NO production and iNOS activity and expression, but does not inhibit the expression of iNOS mRNA. Therefore, increased intracellular cAMP levels in response to IL-4 might effect NO production.

Using a murine macrophage cell line, J774, we have investigated the mechanism of inhibition of NO production by IL-4. Translocation of PKC is inhibited by IL-4, as is PKC activity in cell extracts. Both IL-4 and the PKC inhibitor, Ro31-8220, inhibited iNOS gene transcription. The $IC_{50}$ of this compound against PKC isolated from rat brain is 20 times and 200 times lower than those against isolated protein kinase A (from bovine heart) and $Ca^{2+}$/calmodulin-dependent protein kinase (from rat brain), respectively [Davis et al., 1989].

On the other hand IL-4 did not induce changes in cAMP levels in macrophages. These data suggest that IL-4 may act by inhibiting the activation of PKC, which is essential in the induction of iNOS gene transcription, and not by inducing cAMP generation.

## 4.2. RESULTS:

### 4.2.1. Pretreatment of cells with IL-4 is necessary to inhibit NO production in response to IFN-γ and LPS:

In our experimental model, it is necessary to know by which mechanism IL-4 inhibits NO production in macrophages. In order to do that, it is necessary to investigate the best effective time point for IL-4 treatment in J774 cells. Then, we may be able to predict if the effect of IL-4 requires early or late phase events compared to the relay of signalling by stimulating agents, like IFN-γ and LPS.

The effect of IL-4 (100 U/ml) was investigated in a time response study in J774 cells. J774 cells were adjusted to 5 x $10^5$ cells per ml RPMI 1640 medium supplemented, as detailed in Chapter 2. IL-4 was used at 18 hrs and 4 hrs before stimulation with IFN-γ (40 U/ml) and LPS (10 ng/ml), as well as at the same time as IFN-γ and LPS and 4 hrs after stimulation. Then, cells were incubated at $37^{o}$C, in a $CO_2$ incubator (5 % $CO_2$) for 24 hrs. $NO_2^-$ was measured in supernatants after 24 hrs incubation by using chemiluminescence, as previously described.

It was observed that the best time point to treat the cells with IL-4 is 18 hrs before stimulation by IFN-γ and LPS, indicating that the inhibitory effect of IL-4 on NO production by J774 cells in response to IFN-γ and LPS requires early phase events, such as activation of constitutively expressed proteins, new protein synthesis or consumption of substances, which are required for IFN-γ and LPS-induced downstream signalling, or that IL-4 requires time to work ($p<0.05$). Results are shown in figure 4.1.

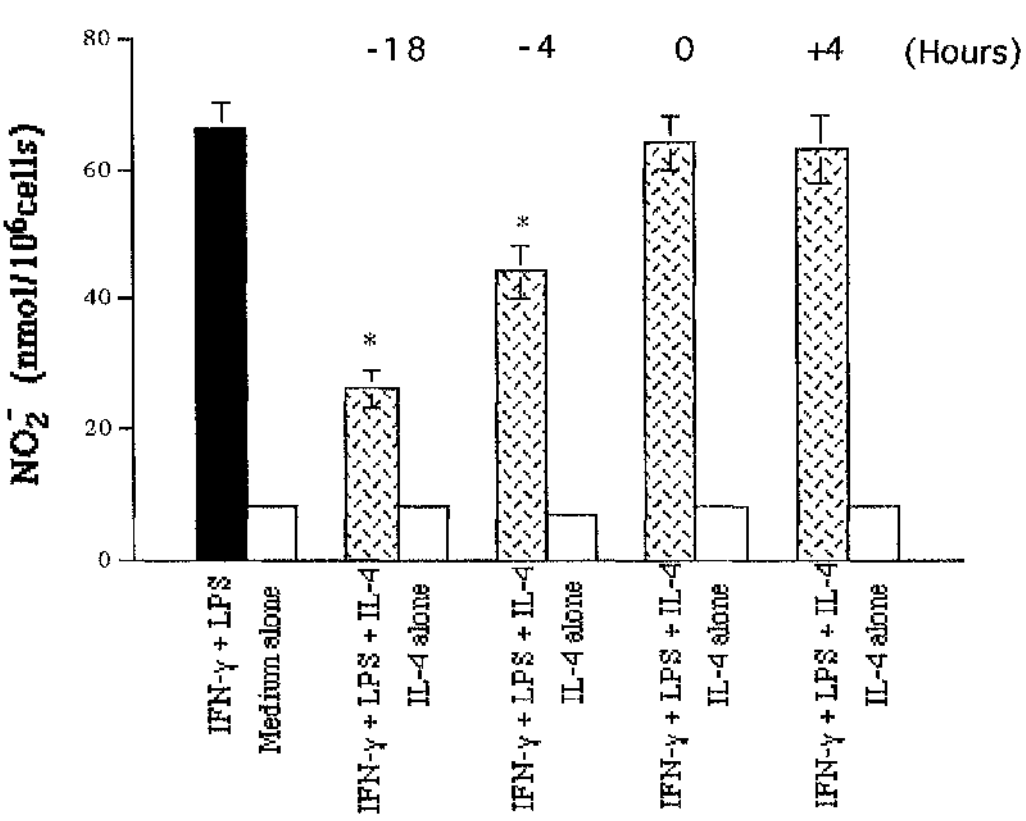



**Figure 4.1:** The effect of IL-4 (100 U/ml) at different time points on NO production in response to IFN-γ (40 U/ml) and LPS (10 ng/ml) in J774 cells. IL-4 was used at 18 hrs and 4 hrs before stimulation with IFN-γ (40 U/ml) and LPS (10 ng/ml), as well as at the same time as the stimulatory agents and 4 hrs after the stimulatory agents. Cells treated with IL-4 and stimulated by IFN-γ (40 U/ml) and LPS (10 ng/ml) at the same time, were incubated for 24 hrs. $NO_2^-$ was measured in supernatants after 24 hrs incubation of cells by using chemiluminescence, as previously described. The means ± SEMs are shown in triplicate samples. Results are representative of three separate experiments.

(*: $p<0.05$, $n=3$)

### 4.2.2. The best inhibitory concentration of IL-4 is 100 U/ml:

In the experimental model used, the finding of the best IL-4 concentration for sufficient inhibition of NO production in response to IFN-γ and LPS would be helpful for future work. The inhibitory effect of IL-4 on NO production, at a variety of concentrations, was investigated in J774 cells. Cells were adjusted to 5 x $10^5$ cells in 1 ml RPMI 1640 medium supplemented, as described in Chapter 2. IL-4 was given to the cells 18 hrs before stimulation. Cells were given IL-4 at a variety of concentrations, and were incubated at 37°C, in a $CO_2$ incubator (5 % $CO_2$) for 18 hrs and then stimulated by IFN-γ (40 U/ml) and LPS (10 ng/ml). Supernatants were measured, after a further 24 hrs incubation of the cells, by chemiluminescence, as previously described. As shown in figure 4.2., the most efficient concentration of IL-4 that inhibits NO release by macrophages is 100 U/ml ($p$ value<0.01). Therefore, in our following experiments, IL-4 will be used at 100 units per ml.

### 4.2.3. A monoclonal antibody against the IL-4 receptor neutralises the inhibitory effect of IL-4 on NO synthesis by J774 cells:

It was necessary to evaluate whether the inhibitory effect of IL-4 is specific for IL-4 itself or is due to nonspecific interactions, such as changes in membrane fluidity. The specificity of the inhibitory effect on NO production caused by IL-4, was tested by using the monoclonal receptor blocking antibody Ab-IL-4R at a range of concentrations (0-20 μg/ml). Anti-IL-4R monoclonal antibody, which is an $IgG2_{a\kappa}$ class antibody, was supplied by Genzyme. It was reported that J774 cells have 355 ± 120 IL-4 receptors per cell on their surface [Beckmann et al., 1990], and the association and dissociation $t_{1/2}$ are 2 min and 4 hrs, respectively [Lowenthal et al,. 1988a].

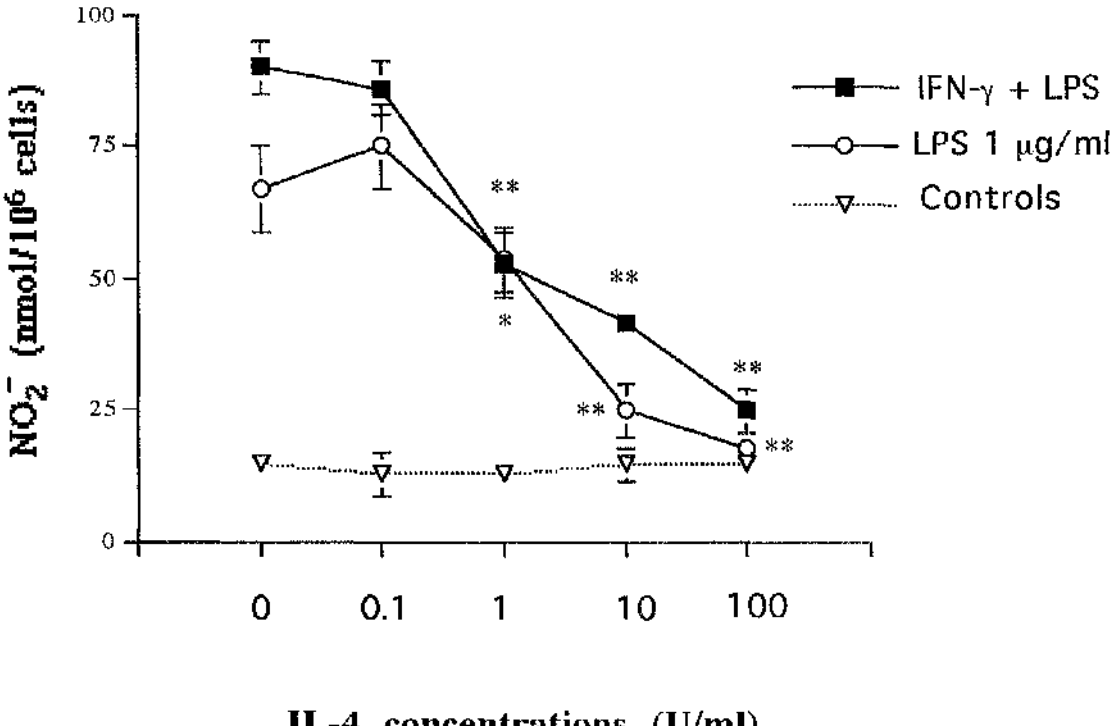


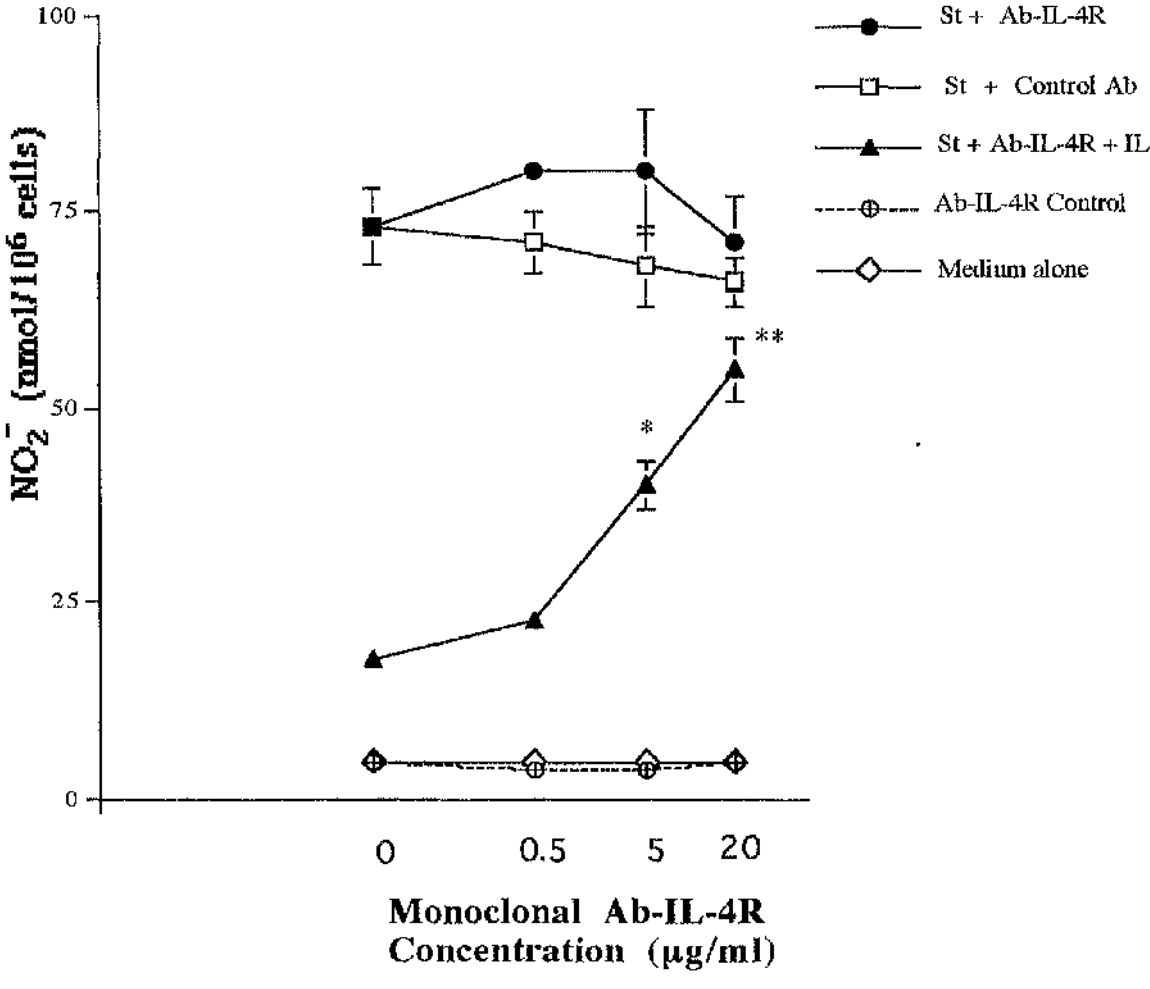

**Figure 4.2:** The inhibitory effect of IL-4 on NO production, at a variety of concentrations, was investigated in J774 cells. IL-4 was used 18 hrs before stimulation. Cells were given IL-4 at variety of concentrations and incubated for 18 hrs before stimulation with IFN-γ (40 U/ml) and LPS (10 ng/ml) or LPS (1 μg/ml) alone. Supernatants were measured after a further 24 hrs incubation of the cells by chemiluminescence. Controls were obtained from the supernatants of unstimulated cells. Results are representative of three separate experiments. The means ± SEMs are shown in triplicate samples.

(**: $p < 0.01$, *: $p < 0.05$, n=3)

The mAb-IL-4R was used at a range of 0-20 μg/ml. Cells were treated with IL-4 1 hr after adding mAb-IL-4r and 18 hrs before stimulation with IFN-γ and LPS. Cells stimulated by IFN-γ (40 U/ml) and LPS (10 ng/ml) were incubated for a further 24 hrs in incubation conditions, as previously described. As a control, an irrelevant monoclonal antibody to CD8 (YTS 169 IgG) was used at a concentration between 0-20 μg/ml (produced by C. O'Donnell of Western Infirmary, Immunology department, Glasgow, UK). Supernatants were used for $NO_2^-$ measurement by using chemiluminescence. As shown in figure 4.3., the binding of monoclonal Ab-IL-4R to IL-4 receptors on the cell surface prevents the inhibition of NO production by IL-4 in a dose-dependent manner ($p<0.01$-$0.05$). These data indicate that the inhibition of NO production in response to the combination of IFN-γ and LPS or high concentration of LPS alone is due to specific IL-4-mediated signalling in J774 cells. The next step concentrates on the mechanisms employed by IL-4 through the IL-4R.

### 4.2.4. IL-4 inhibits iNOS activity in cells stimulated by IFN-γ and LPS:

The effect of IL-4 on the iNOS enzyme activity was determined. J774 cells were adjusted to 1 x $10^6$ cells/ml in RPMI medium in 24-well plates (Costar), then treated with or without IL-4 (100 U/ml) for 18 hrs before stimulation with IFN-γ (40 U/ml) and LPS (10 ng/ml). Cells were incubated, as described previously, for a further 12 hrs. Medium was removed and the cells were washed three times with warm PBS, and the cytosol was extracted, as described in Chapter 2. The cytosol was used for the measurement of citrulline produced from L-arginine in 1 hr by using the technique detailed in Chapter 2. As shown in Figure 4.4., pretreatment of J774 cells with IL-4 results in the reduction of iNOS activity induced by IFN-γ and LPS ($p<0.05$). IL-4 alone does not have any effect in resting cells.




St: Stimulation (IFN-γ +LPS)

**Figure 4.3:** The effect of the IL-4R blocking monoclonal Ab-IL-4R at a range of concentrations (0-20 µg/ml) was assessed for its ability to block IL-4-mediated inhibition of NO production in J774 cells. Cells were treated with IL-4 1 hr after adding mAb-IL-4R and 18 hrs before stimulation. Cells stimulated by IFN-γ (40 U/ml) and LPS (10 ng/ml) were incubated for a further 24 hrs. As a control, an irrelevant monoclonal antibody to CD8 (YTS 169 IgG) was used at a variety of concentrations (0-20 µg/ml). Supernatants were used for $NO_2^-$ measurement by chemiluminescence. Results are representative of three separate experiments. The means ± SEMs of triplicate samples have been shown.

(*: $p<0.05$, **: $p<0.01$, n=3)

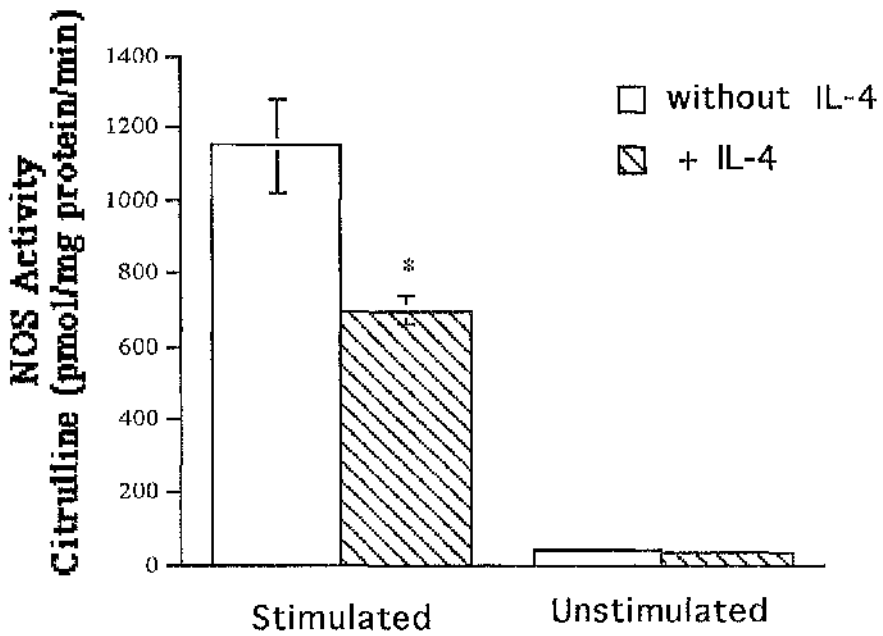



**Figure 4.4:** The effect of IL-4 on iNOS activity in resting and stimulated cells by IFN-γ and LPS. J774 cells were adjusted to 1 x $10^6$ cells in ml RPMI medium in 24-well plates (Costar), then treated with or without IL-4 (100 U/ml) for 18 hrs before stimulation with IFN-γ (40 U/ml) and LPS (10 ng/ml). Cells were incubated, for a further 12 hrs. The cytosol was used for measurement of citrulline produced from L-arginine in 1 hr by using the technique detailed in Chapter 2. Results are representative of three separate experiments, and the means ± SEMs of triplicate samples are shown.

(*: $p<0.05$, n=3)

### 4.2.5. IFN-γ and LPS induce PKC translocation to the membrane at two different time points:

The activation of PKC in response to IFN-γ and LPS in J774 cells has been reported [Severn et al., 1992b]. The translocation of some PKC isoforms induced by IFN-γ and LPS can be easily explored by using phorbol ester binding assays. In this study, phorbol dibutyrate (PDBu) was used. Therefore, cells were adjusted to $2 \times 10^5$ /ml in medium and incubated in 96-well plates, as described in Chapter 2. Then, cells were stimulated by IFN-γ (40 U/ml) and LPS (10 ng/ml) for different times. Medium was removed and the cells were washed three times with ice cold PBS. Cells were treated with [$^3$H]-PDBu (0.6 μCi/ml) for 10 minutes at 37$^{o}$C. [$^3$H]-PDBu binding was measured, as detailed in Chapter 2. As shown in Figure 4.5., the stimulation of J774 cells with IFN-γ and LPS results in the translocation of PKC into membrane at two different time points, 3 minutes and 30 minutes after stimulation ($p<0.01$). Now, we can measure the effect of IL-4 on PKC activation, and the translocation induced by IFN-γ and LPS can be investigated.

### 4.2.6. IL-4 inhibits PKC translocation to the cell membrane:

The exploration of the effect of IL-4 on PKC translocation is a vital step in this project to explain the effector mechanisms mediated by IL-4. Therefore, we examined the effect of IL-4 on PKC translocation in macrophages. J774 cells were incubated in medium at 37$^{o}$C, as described in Chapter 2. for 18 hours with or without IL-4 (100 U/ml), then IFN-γ (40 U/ml) and LPS (10 ng/ml) added for a further 3 minutes. Then, medium was removed and cells were washed 3 times with ice cold PBS buffer (pH:7.4), and the binding of [$^3$H]-PDBu to the cells determined, as described in Chapter 2. As expected, stimulation of the cells by IFN-γ and LPS has markedly increased [$^3$H]-PDBu binding compared to unstimulated cells.

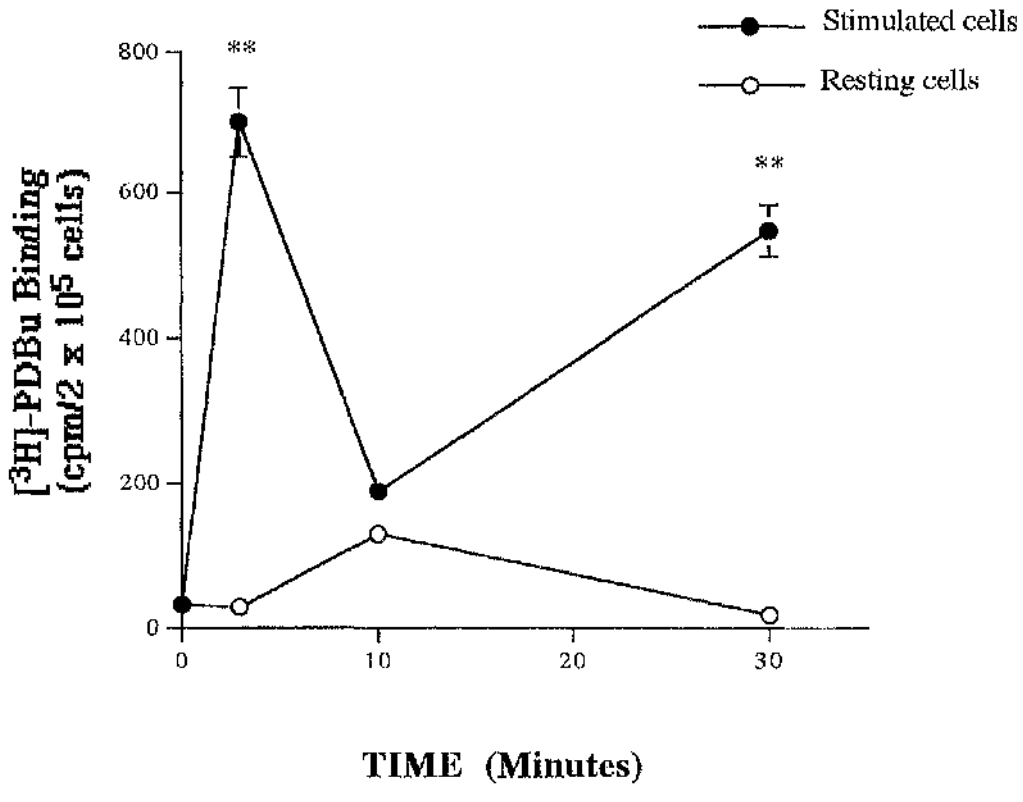



**Figure 4.5:** The effect of IFN-γ (40 U/ml) and LPS (10 ng/ml) on PKC translocation into the plasma membrane in J774 cells. The cells ($2x10^5$ cells/well) were stimulated by IFN-γ (40 U/ml) and LPS (10 ng/ml), and resting cells were used as controls. Cells were stimulated by IFN-γ (40 U/ml) and LPS (10 ng/ml) at different time points (0, 3, 10 and 30 minutes). Medium was removed and cells were washed three times with ice cold PBS. Cells were treated with [$^3$H]-PDBu (0.6 μCi/ml) for 10 minutes at $37^0$C. [$^3$H]-PDBu binding was measured, as detailed in Chapter 2. The means ± SEMs of triplicate cultures are shown. Results are representative of three separate experiments.

(**: $p<0.01$, n=3)

IL-4 reduced binding to both resting and stimulated cells ($p<0.01$). The binding of [$^3$H]-PDBu to the cell surface may be due to constitutively membrane bound PKC, as J774 cells can proliferate and PKC is known to be involved in this process.

### 4.2.7. IL-4 inhibits total PKC activity:

The determination of [$^3$H]-PDBu binding to the translocated PKC enzymes acting as a receptor on the cell surface may not be sufficient to explain the mechanisms of IL-4 action. Because, some isoforms of PKC, like the PKC-ζ isoform, may be activated, but not translocated in J774 cells, and PKC-ζ does not bind PDBu. Therefore, a broad specificity PKC assay system was used.

J774 cells were incubated in the presence or absence of IL-4 (100 U/ml) for 18 hrs, then stimulated with IFN-γ (40 U/ml) and LPS (10 ng/ml) as in the previous experiments. Cytosol was extracted, as detailed in Chapter 2. Then, results were obtained by using Amersham PKC assay kit system. Cells activated with IFN-γ and LPS showed a marked rise in PKC activity which was significantly inhibited by the pretreatment of cells with IL-4 ($p<0.01$). The fact that, IL-4 did not inhibit PKC activity in resting cells, may be due to the basal activity of PKC, which is resistant to the IL-4 treatment. (Figure 4.7).

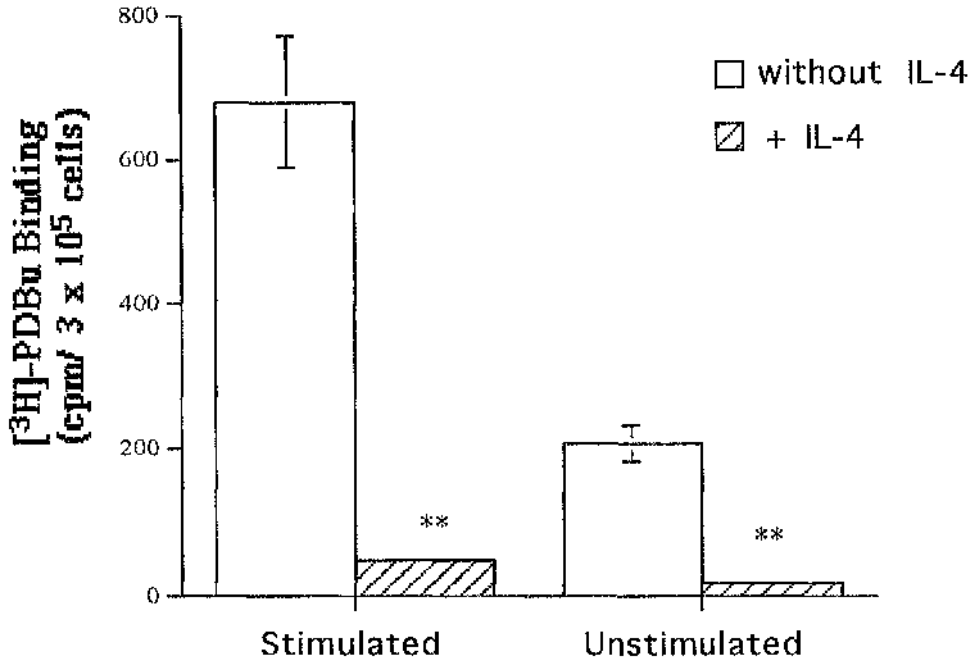



**Figure 4.6:** The effect of IL-4 on PKC translocation to the cell membrane. J774 cells ($3x10^5$ cells/well) were incubated in medium for 18 hours with or without IL-4 (100 U/ml), then IFN-γ (40 U/ml) and LPS (10 ng/ml) added for a further 3 minutes. Then, medium was removed and cells were washed 3 times with ice cold PBS buffer (pH:7.4), and the binding of [$^3$H]-PDBu to the cells was determined. Results are representative of three separate experiments, and the means ± SEMs of triplicate samples are shown.

(**: $p<0.01$, $n=3$)

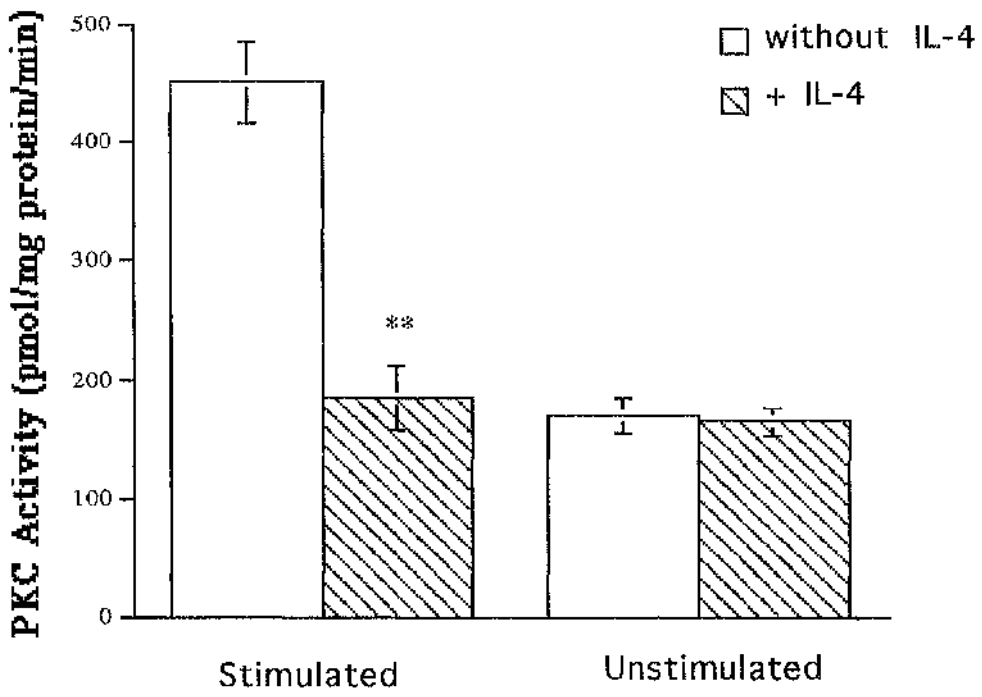


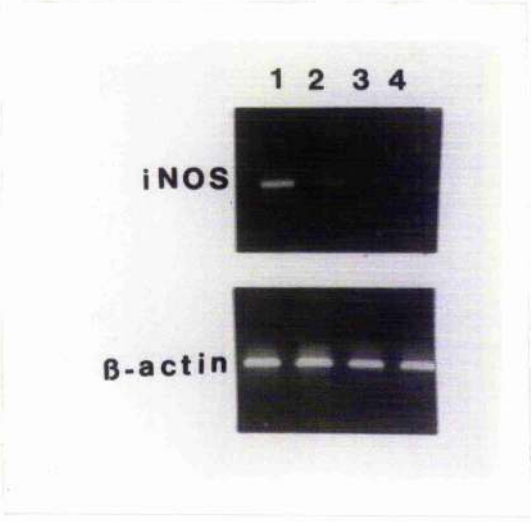

**Figure 4.7:** The effect of IL-4 on total PKC activity. J774 cells were incubated in the presence or absence of IL-4 (100 U/ml) for 18 hrs, then stimulated with IFN-γ (40 U/ml) and LPS (10 ng/ml). Total protein kinase activity was measured in detergent extracts as described in Chapter 2. Results are representative of three separate experiments, and the means ± SDs of duplicated samples are shown.
(**: $p<0.01$, $n=2$)

### 4.2.8. iNOS gene transcription is inhibited by IL-4:

In order to determine the level at which IL-4 inhibits NO production, we investigated the effect of IL-4 on iNOS gene transcription. Cells were incubated for 18 hours, with or without IL-4, then incubated for 6 hours with IFN-γ and LPS, or without stimuli. Total RNA was then extracted, and reverse transcription and PCR were performed. Preliminary experiments had established that the peak iNOS mRNA expression is between 3-6 hrs after stimulation in the present system (G.J. Feng, personal communication). Transcription of the iNOS gene was induced by IFN-γ and LPS, and this was inhibited by pre-incubation with IL-4. iNOS mRNA was not detected in cells not exposed to IFN-γ and LPS, regardless of whether IL-4 was present (Figure 4.8). These data indicate that IL-4 regulates NO production by altering iNOS mRNA expression in response to IFN-γ and LPS by J774 cells.

### 4.2.9. iNOS gene transcription is reduced by a PKC inhibitor:

The involvement of PKC activation in NO production is still to be determined, in order to do this, the selective PKC inhibitor, Ro31-8220 was used. If the inhibition of NO production by IL-4 is via the inhibition of PKC translocation and activation, it would be predicted that inhibition of PKC activity by other means would also inhibit the iNOS gene transcription. The effect of Ro31-8220 on iNOS mRNA levels was therefore investigated. Results shown in Figure 4.9. demonstrate that the transcription of iNOS by J774 cells activated with IFN-γ and LPS was inhibited by the presence of Ro31-8220 at 50 μM, a concentration not cytotoxic to the J774 cells [Severn et al., 1992b; Davis et al., 1989].



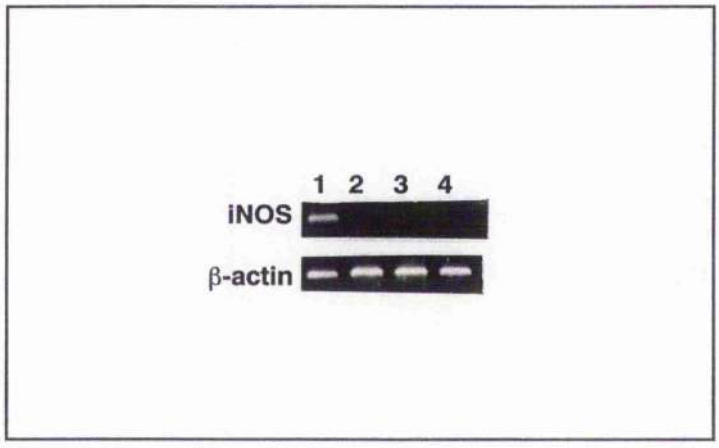

**Lane 1:** IFN-γ (40 U/ml) and LPS (10 ng/ml)

**Lane 2:** IFN-γ (40 U/ml) and LPS (10 ng/ml) + IL-4 (100 U/ml)

**Lane 3:** IL-4 (100 U/ml) alone

**Lane 4:** Resting Cells (Controls)

**Figure 4.8:** The effect of IL-4 on iNOS gene transcription. Cells were incubated for 18 hours, with or without IL-4, then incubated for 6 hours with IFN-γ and LPS, or without stimuli. iNOS gene transcription was induced by IFN-γ (40 U/ml) and LPS (10 ng/ml), and this was inhibited by pre-incubation with IL-4 (100 U/ml). Controls show β-actin bands in J774 cells. Results are representative of three separate experiments.




**Lane 1:** IFN-γ (40 U/ml) + LPS (10 ng/ml)

**Lane 2:** IFN-γ (40 U/ml) + LPS (10 ng/ml) + Ro-31-8220 (50 μM)

**Lane 3:** Ro-31-8220 (50 μM) alone

**Lane 4:** Resting cells (Control)

**Figure 4.9:** The effect of the selective PKC inhibitor, Ro31-8220, on iNOS gene transcription. Results shown in Figure 4.9. demonstrate that the transcription of iNOS by J774 cells activated with IFN-γ (40 U/ml) and LPS (10 ng/ml) was inhibited by the presence of Ro31-8220 (50 μM) added half an hour before stimulation with IFN-γ and LPS. Results are representative of three separate experiments.

### 4.2.10. IL-4 does not increase cAMP levels in J774 cells:

In B cells, IL-4 treatment causes an intracellular elevation of cAMP. The investigation of intracellular cAMP responses to IL-4 treatment is necessary to clarify the regulatory function of IL-4 in J774 cells. The effect of IL-4 (100 U/ml) on cAMP levels in J774 cells was therefore investigated.

Cells were adjusted to $5 \times 10^5$ cells in 24-well plates and treated with reagents for different time points. $PGE_2$ ($10^{-6}$ M) was used to stimulate cAMP production in J774 cells for 15 min as a positive control. After removing the medium, cells were washed three times with ice cold PBS. Cytosol was extracted for cAMP measurement by using an Amersham radio-labelled binding kit system, as detailed in Chapter 2.

No increase in cAMP concentrations was detected in cells incubated with IL-4 (100 U/ml) (Table 4.1). In control cultures, $PGE_2$ induced a large increase in cAMP levels. These results presented here demonstrate that IL-4 does not regulate NO production by inducing intracellular cAMP levels in J774 cells.

**Table 4.1:** IL-4 (100 U/ml) does not cause cAMP elevation in J774 cells. Cells were adjusted to 5 x $10^5$/ml in 24-well plates and treated with reagents for different time points. $PGE_2$ ($10^{-6}$ M) was used to activate J774 cells for 15 min as a control. IL-4 (100 U/ml) was used at different time points. No increase in cAMP concentrations was detected in cells incubated with IL-4 (100 U/ml). In positive control cultures, $PGE_2$ induced a large increase in cAMP levels. Basal cAMP concentrations were obtained from resting cells. Results are representative of three separate experiments. The means and standard deviations of triplicate cultures are shown (n=3)

**cAMP (pmol/$10^6$ cells)**

| Substance | 15 min | 2 hrs | 6 hrs | 24 hrs | 48 hrs |
|---|---|---|---|---|---|
| IL-4 | <1 | <1 | <1 | <1 | <1 |
| $PGE_2$ | 80.00 ± 5.00 | --- | --- | --- | -- |
| Controls | <1 | <1 | <1 | <1 | <1 |

## 4.3. DISCUSSION:

It is generally recognised that the balance between Th1-like and Th2-like cells determines the outcome of a number of infectious and autoimmune diseases [Liew, 1989; Sher and Coffman, 1992; Romagnani, 1991; Mason and Fowell, 1992; Bloom et al., 1992]. They do so via their characteristic cytokines. One mechanism is as follows: Th1 cells produce IFN-γ which, together with other molecules such as LPS and TNFα, activates macrophages to produce NO which mediates cytopathic and microbicidal effects [Nathan and Hibbs, 1991; Hibbs et al., 1990; Liew and Cox, 1991; Green et al., 1990]. Th2-like cells, on the other hand, produce IL-4 and IL-10 which inhibit the synthesis of NO by macrophages [Liew et al., 1991; Cunha et al., 1992; Gazzinelli et al., 1992]. The mechanism of interaction of IFN-γ and IL-4 in the regulation of NO synthesis is unknown. This is clearly an important area, as an understanding of this mechanism may lead to effective control of this important biological mediator and therapy for several important diseases. Data presented here contribute to the understanding of this mechanism.

It has been previously demonstrated that the activation of PKC is required for the induction of macrophage iNOS gene transcription [Severn et al., 1992b]. In this study, IL-4 inhibits the synthesis of NO via the blocking of the PKC activation pathway. This is supported by our finding that transcription of the iNOS gene was inhibited by IL-4 as well as the PKC inhibitor, Ro31-8220. It has been proposed that PKC-epsilon (PKC-ε) is the predominant isoform in the macrophages activated with IFN-γ and LPS [Sands et al., 1994a]. It should be noted that IL-4 inhibits NO synthesis only when macrophages are pretreated with this cytokine before activation with IFN-γ and LPS [Liew et al., 1991]. Inhibition of PKC activation also required incubation with IL-4 for several hours before stimulation. IL-4 did not effect an immediate reduction in [$^3$H]-PDBu binding to whole cells, and addition of IL-4 to

the cells at the same time as or after IFN-γ and LPS did not prevent an increase in PDBu binding [Sands et al., 1994a]. In human monocytes, IL-4 induces translocation of PKC to the nucleus, resulting in reduced activity in the cytoplasm [Arruda and Ho, 1992]. Thus the mechanism of the interaction of IL-4 and IFN-γ in the control of NO synthesis is complex and factors other than PKC are probably involved. However, this change occurs within minutes, and it is unlikely that the same process is occurring in J774 cells. Another recent report has described inhibition of PKC by IL-4 in lymphocytes [Hart et al., 1989b]. It is not yet clear how IL-4 inhibits PKC activation. It may inhibit generation of DAG, which is the physiological activators of PKC [Nishizuka, 1992], by acting on a phospholipase.

IL-4 has other inhibitory effects on macrophages, reducing the production of TNF-α, IL-1 and prostaglandin $E_2$ [Politis and Vogel, 1990]. Several macrophage activities have been shown to be dependent on PKC activation [Kawanishi, 1993]. The blocking of the PKC activation pathway by IL-4 may, therefore, explain its general inhibitory effects on macrophages. A report has also described inhibition of PKC by IL-4 in B lymphocytes [Harnett et al., 1991]. Additionally, In B cells, IL-4-mediated regulation of gene expression is controlled by the Janus family of kinases Jak1 and Jak3, which mediate tyrosine phosphorylation of signal-transducers and activators of transcription (STATs), such as the IL-4 nuclear activated factor (IL-4 NAF) and the signal-transducing factor of IL-4 (STF-IL-4) [Keegan et al., 1994; Johnston et al., 1994; Witthuhn et al., 1994].

An increase in intracellular cAMP concentration as a mechanism of inhibition of NO production by IL-4 has been excluded. In human B cells, IL-4 does induce a delayed rise in cAMP [Finney et al., 1990]. This was not detected in J774 cells. However, differences between the signalling pathways of human and murine IL-4 have been reported [McGarvie and Cushley, 1989]. Additionally, some other differences in iNOS regulation can be observed between different types of

macrophage. For instance, IL-4 was reported to strongly reduce iNOS mRNA at 24-72 hrs post stimulation, but not 4-6 hrs after exposure to IFN-γ and LPS in primary peritoneal mouse macrophages [Bogdan et al., 1994].

In this study, Data suggest that IL-4 reduces NO production and iNOS mRNA synthesis by inhibiting PKC, but not inducing intracellular cAMP levels. The monoclonal Ab-IL-4R was used to block the effect of IL-4 on macrophages.

In conclusion, we have further defined the role of PKC in the induction of NO production by murine macrophages, showing its activation to be essential for iNOS gene transcription. We have also partly elucidated the mechanism by which IL-4 inhibits NO production. The inhibition of PKC activation by IL-4 may explain its other effects on macrophages.

# CHAPTER 5

# THE EFFECT OF G-PROTEIN MODIFIERS, PLATELET ACTIVATING FACTOR AND PHOSHOLIPASE $A_2$ ACTIVITY ON NO SYNTHESIS

## 5.1. INTRODUCTION:

It has been reported that cAMP-elevating agents, such as $PGE_2$ and 8-Br-cAMP, when applied together with the nonspecific PDE inhibitor isobutyl-methylxanthine (IBMX), causes a sustained elevation of intracellular cAMP and inhibits NO production in macrophages stimulated by IFN-γ and LPS [Bulut et al, 1993]. Platelet activating factor, which is a potent ether linked phosphocholine (1-alkyl-2-acetylglycerophosphocholine) [Prpic et al., 1988], is a secretory product of activated macrophages, like Prostaglandin $E_2$ ($PGE_2$) [Adams and Hamilton, 1992]. A high affinity receptor on macrophages for PAF has been described, and binding of PAF to macrophages results in the rapid breakdown of polyphosphoinositides, resulting in diacylglycerol production and activation of protein kinase C [Rosen et al, 1989]. Moreover, like $PGE_2$, PAF induces an elevation of intracellular cAMP levels. Therefore, it might inhibit NO production via the elevation of cAMP in cells.

As indicated in Chapter 4, IL-4 reduces NO and iNOS message synthesis by inhibiting PKC activity [Sands et al, 1994a]. It was observed that phosphatidylcholine-specific PLC (PC-PLC), which causes DAG release via hydrolysis of membrane bound phosphatidylcholine, is involved in PKC activation in macrophages, and IL-4 acts by inhibiting PC-PLC [Sands et al., 1994b]. On the other hand, phospholipase $A_2$ ($PLA_2$) comprises a family of enzymes that hydrolyse the acyl bond at the sn-2 position of phospholipids to generate free fatty acid and lysophospholipids. In other word, $PLA_2$ activity selectively cleaves arachidonyl phosphatidylcholine to initiate eicosanoid and PAF production in macrophages [Suga et al., 1990]. The free arachidonic acid generated by $PLA_2$ activity is converted to a large number of products by cyclooxygenases, lipoxygenases and monooxygenases [reviewed by Bonventre, 1992], and have both intercellular and intracellular messenger effects. $PLA_2$ has been identified in the cytosol of U937 cells [Clark et al, 1991], RAW 264.7 cells [Channon and

Leslie, 1990], the P388D$_1$ macrophage-like cell line [Lister et al., 1989], rat brain [Yoshihara and Watanebe, 1990], kidney [Gronich et al, 1990], and platelets [Takayama et al., 1991]. The presence of the enzyme in U937 cells, brain, kidney, platelets, RAW 264.7 and P388D$_1$ macrophage cell lines, would strongly suggest that this enzyme is ubiquitous in cells of the reticulo-endothelial system and is also likely to be present in the J774 macrophage cell-line. Early studies had shown that $Ca^{2+}$/ diacylglycerol may be the main regulators of PLA$_2$ [Billah and Siegel, 1987, and Volpi et al., 1985], both products of the phospholipase C pathway. Furthermore, the PLA$_2$ activity may also be altered by the following events: intracellular pH change, protein kinase activation, heterotrimeric guanine nucleotide-binding regulatory proteins (G-proteins) activation, inhibitory proteins of PLA$_2$, such as lipocortins, and PLA$_2$ activating proteins (PLAP) induced by TNF and leukotriene D$_4$ (LTD4), metabolic product inhibition, and transcriptional control by forskolin, which is an adenylyl cyclase activator, glucocorticoids and TNF [reviewed by Bonventre, 1992; Cockroft, 1992]. For instance, IL-1, TNF-$\alpha$, forskolin and LPS enhance the secretion of PLA$_2$ in rat mesangial cells [Schalkwijk et al., 1991], and it was also reported that glucocorticoids inhibit the increase of PLA$_2$ mRNA expression induced by forskolin in cultured smooth muscle cells [Nakano et al., 1990]. A synthetic glucocorticoid, dexamethasone, which inhibits PLA$_2$ activity, leads to reduced amounts of NO production in vascular endothelial cells and hepatocytes [Radomski et al., 1990, Geller et al., 1993].

Therefore, an investigation of the effects of PAF and PLA$_2$ inhibitors on NO production in macrophages would be useful for understanding the mechanism, which is involved in NO production.

Meanwhile, Studies in neutrophils have shown that the formyl peptide, formylmethionylleucylphenylalanine (fMetLeuPhe)-mediated release of arachidonic acid is sensitive to inhibition by pertussis toxin treatment [Okajima and Ui, 1984].

Moreover, it has been demonstrated that $PLA_2$ activity can be induced by $\beta/\gamma$ subunits of TD1, which is a G-protein, in bovine rod outer segments [Jelsema and Axelrod, 1987]. This evidence shows that $PLA_2$ activation can be in a G-protein-dependent manner. Therefore, it is necessary to investigate the relationship of G-proteins to LPS and cytokine receptors, such as IFN-γ and IL-4.

Since well-characterised covalent modifications of G-proteins are the ADP-ribosylation reactions carried out by toxins produced by *V. cholerae* and *B. pertussis* [Gill and Meren, 1978; Cassel and Pfeuffer, 1978; Moss and Vaughan, 1977; Katada and Ui, 1982]. In this study we have examined the effects of the G-protein modifiers, Pertussis toxin (Ptx) and Cholera toxin (Ctx), $PLA_2$ activity and its product PAF on NO production.

Pertussis toxin (Ptx), produced by *Bordetella pertussis*, is composed of two subunits. The B oligomer is responsible for the binding of the toxin to the cell membrane, whereas the A oligomer catalyses ADP-ribosylation and inactivation of GTP binding proteins coupling membrane receptors to either adenylate cyclase, phospholipase-C, phospholipase $A_2$, $Ca^{2+}$ or $K^+$ channels [reviewed by Stryer and Bourne, 1986; Gilman, 1987; Spiegel, 1988; Dunlap et al, 1987; Exton, 1988]. In contrast, early studies have shown that Ptx induces an early increase in intracellular $[Ca^{2+}]$ resulting from an influx across the plasma membrane and release of $Ca^{2+}$ from intracellular stores in Jurkat T-cells, and activates a phospholipase C-dependent pathway [Modesto et al., 1991; Gray et al., 1989]. It was reported that Ptx enhances LPS-induced TNF-α production while at the same time inhibits LPS-dependent NO production, and these effects are correlated with Ptx-mediated ADP-ribosylation of a 41-kDa protein [Zhang and Morrison, 1993].

Cholera toxin (Ctx) is composed of an A subunit and multiple B subunits. The A subunit induces ADP ribosylation, with consequent activation, of the α subunit of

the stimulatory G-protein, $G_s\alpha$. This can result in the activation of adenylyl cyclase in most cells [Mathews and von Holde, 1992; Cassel and Pfeuffer, 1978; Milligan, 1989], and Ctx also causes activation of dihydropyridine-sensitive $Ca^{2+}$ channels [Milligan, 1989]. As detailed in Chapter 1, Ctx, can activate $G_{olf}$, which is found in olfactory sensory neurones, as well as, TD1 and TD2, which are distributed in Rod outer and inner segments, respectively [Reviewed by Milligan and Wakelam, 1989]. The B subunits have high affinity for the ganglioside $GM_1$, which is thought to be the membrane receptor for Ctx [Sommermeyer et al., 1989].

In this chapter, the effects of cholera and pertussis toxins have been investigated to show if G-protein activation regulates the activity of downstream effector proteins involved in NO production. Pertussis toxin does not alter NO production in response to IFN-γ and LPS or LPS alone. However, cholera toxin inhibits NO produced in response to high doses of LPS alone or IFN-γ and a low dose of LPS together in J774 cells in a dose-dependent manner. Unfortunately, this effect was found to be nonspecific for cholera toxin, because the toxin solution contains sodium azide at a concentration of 0.15 mM. Data presented here suggests that the downstream signals from the ligand-receptor complex to the nucleus to produce NO does not involve G-protein-mediated signalling mechanisms in J774 cells.

Meanwhile, PAF does not affect NO production by the J774 macrophage-like cell line. Having found PAF does not affect NO production, some $PLA_2$ inhibitors, such as 4-bromophenacyl bromide (4-BPB), 4-4-octadecyl-4-oxobenzene butenoic acid (OBAA) [Madi et al., 1991] and dexamethasone were used to see, if $PLA_2$ activity is involved in NO production in J774 cells. In this chapter, data suggest that $PLA_2$ activity is not involved in the NO production in response to IFN-γ and low doses of LPS in J774 cells.

## 5.2. RESULTS:

### 5.2.1. PAF does not alter NO production in response to IFN-γ and LPS in J774 cells:

PAF is a useful molecule to investigate NO production, because of its LPS mimicking effects, like the induction of cAMP generation and polyphosphoinositides hydrolysis, DAG generation and PKC activation in macrophages [Nibbering et al., 1992; Adams and Hamilton, 1992]. In addition, PAF may induce arachidonic acid hydrolysis products. Because of this, we used PAF to start the exploration of $PLA_2$-mediated signalling events.

J774 cells, 5 x $10^5$ /ml in RPMI 1640 medium, were cultured in 96-well plates. Cells were pretreated with a variety of concentrations of PAF in the range of $10^{-6}$-$10^{-10}$ M. 30 min after adding PAF, cells were stimulated by IFN-γ (40 U/ml) and a low concentration of LPS (10 ng/ml) or with LPS alone (1 μg/ml), then incubated at 37°C for 24 hrs. $NO_2^-$ levels were measured by chemiluminescence in supernatants. Stimulation of J774 cells with IFN-γ (40 U/ml) and a low concentration of LPS (10 ng/ml) or with LPS alone (1 μg/ml) induced the production of NO. Nitric oxide production was not affected by PAF alone. In cells treated with PAF and stimulated with IFN-γ and LPS, there was no significant inhibition of NO production (Figure 5.1). Because PAF (100 mM) was dissolved in chloroform, controls were carried out, and the results were not affected by chloroform (maximum concentration 0.1 % v/v).

Cell viability was examined by trypan blue exclusion and evaluated as normal in cells treated with PAF (>96%). Moreover, cells treated with PAF were able to adhere and spread in a similar manner to untreated cells, and the general morphology also appeared normal.

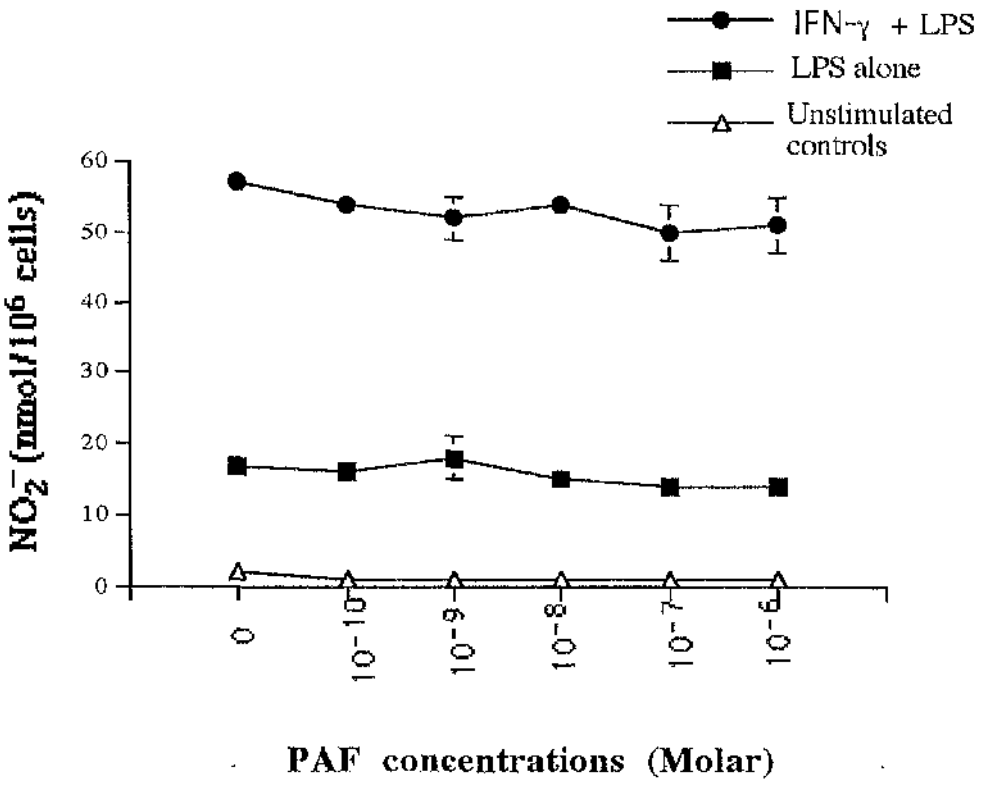



**Figure 5.1:** The effect of PAF at a variety of concentrations on NO production in response to IFN-γ and LPS in J774 cells. The effect of PAF at a variety of concentrations ($10^{-6}$-$10^{-10}$ M) on NO production in resting and stimulated cells was investigated. J774 cells, 5 x $10^5$, were cultured in 96-well plates. Cells were pretreated with a variety of concentrations of PAF. 30 min after adding PAF, cells were stimulated by IFN-γ (40 U/ml) and a low concentration of LPS (10 ng/ml) or with LPS alone (1 μg/ml), then incubated for 24 hrs. $NO_2^-$ levels were measured by chemiluminescence in the supernatants. Since PAF was dissolved in chloroform (100 mM), controls carried out showed that the results were not affected by chloroform (max. 0.1 % v/v). Control unstimulated cells did not produce NO in response to PAF. Results are representative of three separate experiments. The means ± SEMs of triplicate cultures are shown. (n=3)

### 5.2.2. PAF does not alter NO production in response to various concentrations of LPS in J774 cells:

In the previous experiment, PAF was used with a high concentration of LPS alone (1 μg/ml). This concentration of LPS alone is sufficient to induce NO production. If there is a common pathway used by both PAF and LPS, PAF may cause delayed events, consequently, some required factors may have been consumed by LPS-mediated signalling. For instance, LPS induces a twofold to threefold rise in the intracellular cAMP levels in the presence of IBMX in resident macrophages, as PAF elevates the intracellular cAMP concentrations in macrophages [Nibbering et al., 1992; Adams and Hamilton, 1992]. Meanwhile, The effects of LPS on polyphosphoinositide hydrolysis can be mimicked by PAF in peritoneal macrophages [Prpic et al., 1988]. Therefore, the effect of PAF on NO production induced by a variety of concentrations of LPS alone was assessed. J774 cells, 5 x $10^5$ /ml in RPMI 1640 medium, were cultured in 96-well plates. Thirty minutes after adding PAF (1 μM), cells were stimulated with a variety of concentrations of LPS, then incubated for 24 hrs at 37°C. $NO_2^-$ levels were measured by chemiluminescence in supernatants. Stimulation of J774 cells with LPS, at the range of 10 ng-1 μg/ml, induced the production of NO. Production of NO was not affected by PAF (μM), and PAF alone and medium alone controls did not produce any NO in the supernatants. No effect of the chloroform carrier was observed (Figure 5.2).

Cell viability was examined by trypan blue exclusion in cells treated with PAF (>97%). Moreover, cells treated with PAF were able to adhere and spread in a similar manner to untreated cells, and the general morphology appeared also normal.

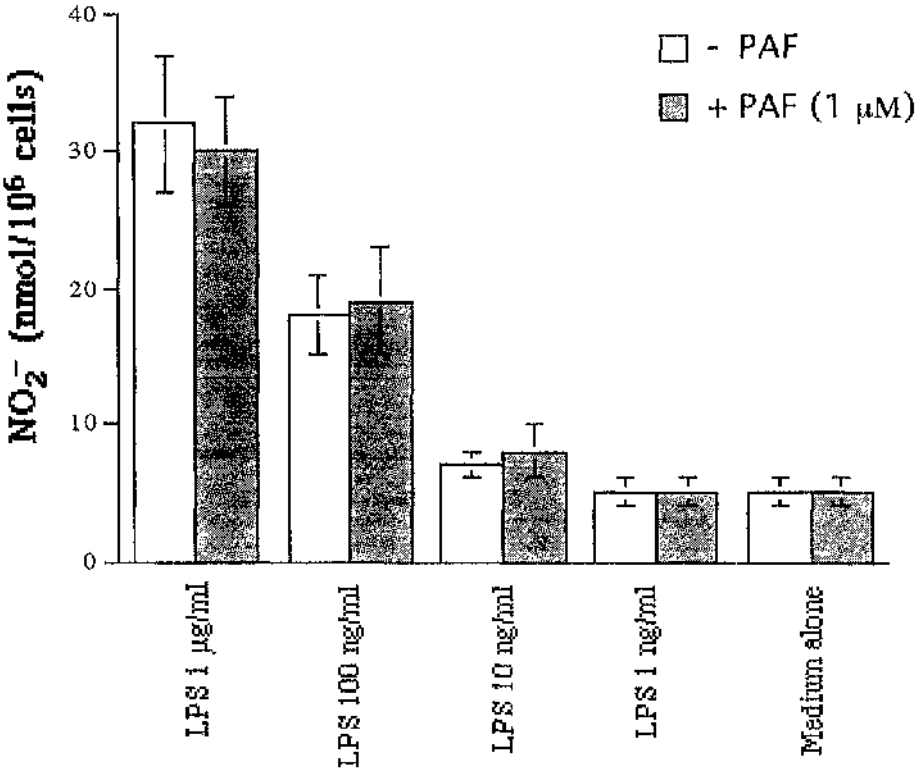



**Figure 5.2:** The effect of PAF on NO production in response to LPS at a variety of concentrations in the J774 macrophage-like cell line. J774 cells were cultured in 96-well plates. 30 min after adding PAF (1 μM), cells were stimulated by variety concentrations of LPS (10 ng-1 μg), then incubated for 24 hrs. $NO_2^-$ levels in the supernatants were measured by chemiluminescence. Controls were obtained from unstimulated resting cells (medium alone). Results are representative of three separate experiments. The means ± SEMs of triplicate cultures are shown. (n=3)

### 5.2.3. NO production was not influenced by PAF pretreatment at different time points in J774 cells:

There is a possibility that J774 cells might require pretreatment with PAF to influence NO production. In order to investigate whether PAF requires time to alter NO production, the following experiment was performed. J774 cells, 5 x $10^5$ /ml in RPMI 1640 medium, were cultured in 96-well plates. Having added PAF ($10^{-6}$ M) to the wells at different time points (-18 hrs, -4 hrs, -1 hr and 0), cells were stimulated by IFN-γ (40 U/ml) and 10 ng/ml LPS , then incubated for a further 24 hrs at 37° C. $NO_2^-$ levels were measured by chemiluminescence in supernatants. Because PAF was dissolved in chloroform (100 mM), required controls were carried out, and results were not affected by chloroform (max. 0.1 % v/v).

As shown in Figure 5.3., stimulation of J774 cells with IFN-γ and a low concentration of LPS (10 ng/ml) induced the production of NO. However, in stimulated and PAF treated cells, there was no significant influence on NO production at any time point.

These data suggest that PAF-mediated signalling is not involved NO production in J774 cells.

### 5.2.4. The $PLA_2$ inhibitor, 4-4-octadecyl-4-oxo-benzene butenoic acid, inhibits NO production in response to IFN-γ and low concentration LPS by a cytotoxic mechanism on J774 cells:

Having found that PAF does not affect NO production, some $PLA_2$ inhibitors, such as 4-bromophenacyl bromide (4-BPB), 4-4-octadecyl-4-oxobenzene butenoic acid (OBAA) and dexamethasone were used to investigate whether $PLA_2$ activity is

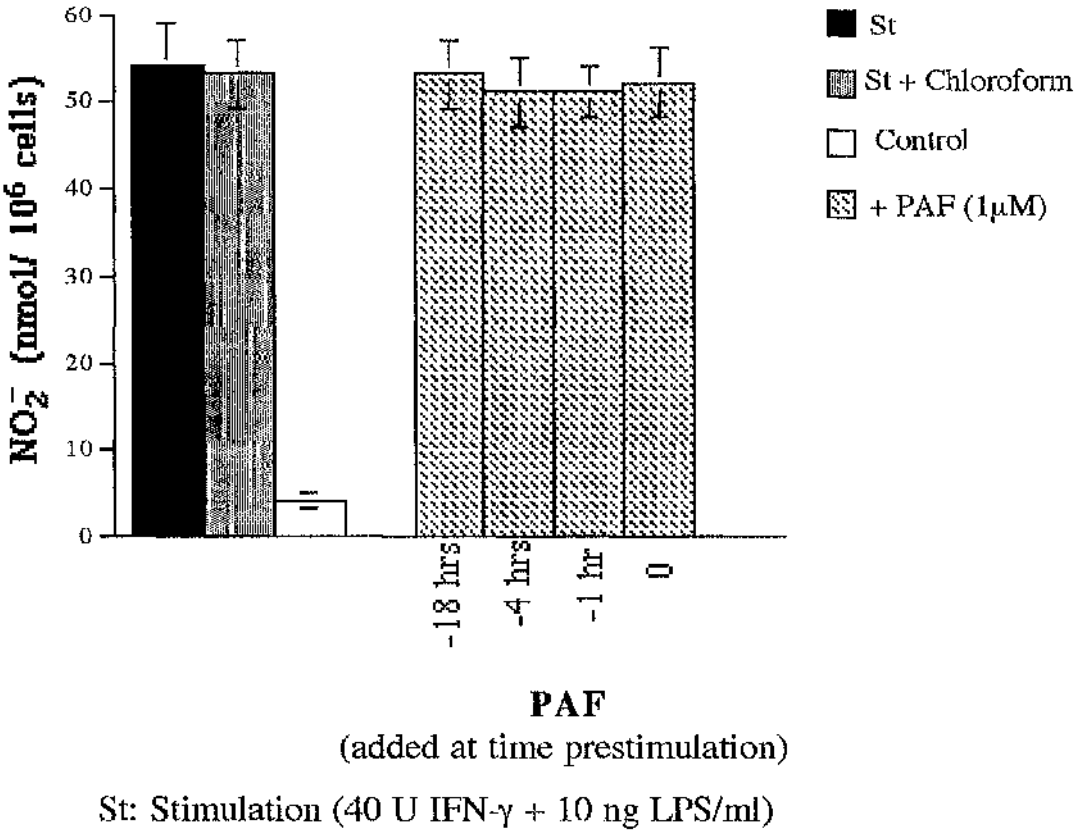



**Figure 5.3:** The effect of PAF added to cells at different time points prestimulation with IFN-γ and LPS on NO production in J774 cells. J774 cells were cultured in 96-well plates. PAF ($10^{-6}$ M) was added to the wells at different time points (-18, -4, -1 and 0 hrs) prestimulation, and cells were stimulated by IFN-γ (40 U/ml) and 10 ng/ml LPS, then incubated for a further 24 hrs. Controls show $NO_2^-$ levels in untreated well supernatants. $NO_2^-$ levels in the supernatants were measured by chemiluminescence. Results are representative of three separate experiments. The means ± SEMs of triplicate cultures are shown. (n=3)

involved in NO production in J774 cells. So that, the function of $PLA_2$-mediated signalling may be eliminated. Again, J774 cells, 5 x $10^5$ /ml in RPMI 1640 medium, were cultured in 96-well plates. 30 min after adding OOBA at a variety of concentrations, cells were stimulated by IFN-γ (20 U/ml) and 10 ng/ml LPS, for 24 hrs at 37°C. $NO_2^-$ levels in the supernatants were measured by the Griess reaction, as detailed in Chapter 2. The $PLA_2$ inhibitor OOBA was dissolved in DMSO (10 mM). Controls, using DMSO (0.1 % v/v). were carried out, and the results were not affected.

Stimulation of J774 cells with IFN-γ (20 U/ml) and 10 ng/ml LPS induced the production of NO. Production of NO was inhibited by OOBA in a dose-dependent manner. However, there was a correlation between its toxic effect and NO inhibition (Figure 5.4.a).

Cell viability was examined by trypan blue exclusion in cells treated with OOBA. The correlation between NO production and cell viability in cells treated with OOBA and stimulated by IFN-γ (20 U/ml) and 10 ng/ml LPS is presented in figure 5.4.b.

### 5.2.5. The $PLA_2$ inhibitor, 4-bromophenacyl bromide (4-BPB), does not affect NO production in response to IFN-γ and LPS at noncytotoxic concentrations in J774 cells:

Since OOBA is a toxic substance for cells, another structurally unrelated $PLA_2$ inhibitor 4-bromophenacyl bromide (4-BPB) was used to see if $PLA_2$ activity is involved in NO production in J774 cells. J774 cells, 5 x $10^5$ /ml in RPMI 1640 medium, were cultured in 96-well plates. 30 min after adding 4-BPB at a variety of concentrations, cells were stimulated by IFN-γ (20 U/ml) and a low concentration of LPS (10 ng/ml), then incubated at 37°C for 24 hrs. $NO_2^-$ levels were measured

**Figure 5.4.a:** The effect of the $PLA_2$ inhibitor, 4-4-octadecyl-4-oxo-benzene butenoic acid, on NO production in response to IFN-γ and LPS in J774 cell line. J774 cells were cultured in 96-well plates. 30 min after adding OOBA at a variety of concentrations ($10^{-8}$-$10^{-5}$ M), cells were stimulated by IFN-γ (20 U/ml) and 10 ng/ml LPS, then incubated for 24 hrs. $NO_2^-$ levels in the supernatants were measured by the Griess reaction. OOBA was dissolved in DMSO (10 mM), required controls were carried out, and results were not affected by DMSO (max. 0.1 % v/v). Results are representative of three separate experiments carried out. The means ± SEMs of triplicate cultures are shown. (n=3)

**Figure 5.4.b:** The correlation between cell toxicity and NO production in cells treated with OOBA. Cell viability was examined by trypan blue exclusion in cells treated with OOBA. The correlation between NO production and cell viability in cells treated with OOBA and stimulated by IFN-γ (20 U/ml) and a low concentration of LPS (10 ng/ml) is shown. The data obtained in figure 5.4.b. are used here to display the fact that OOBA inhibits NO production by simply killing cells (r value, the correlation coefficiency=0.931). Results are representative of three separate experiments. (n=3)

**Figure 5.4.a.**

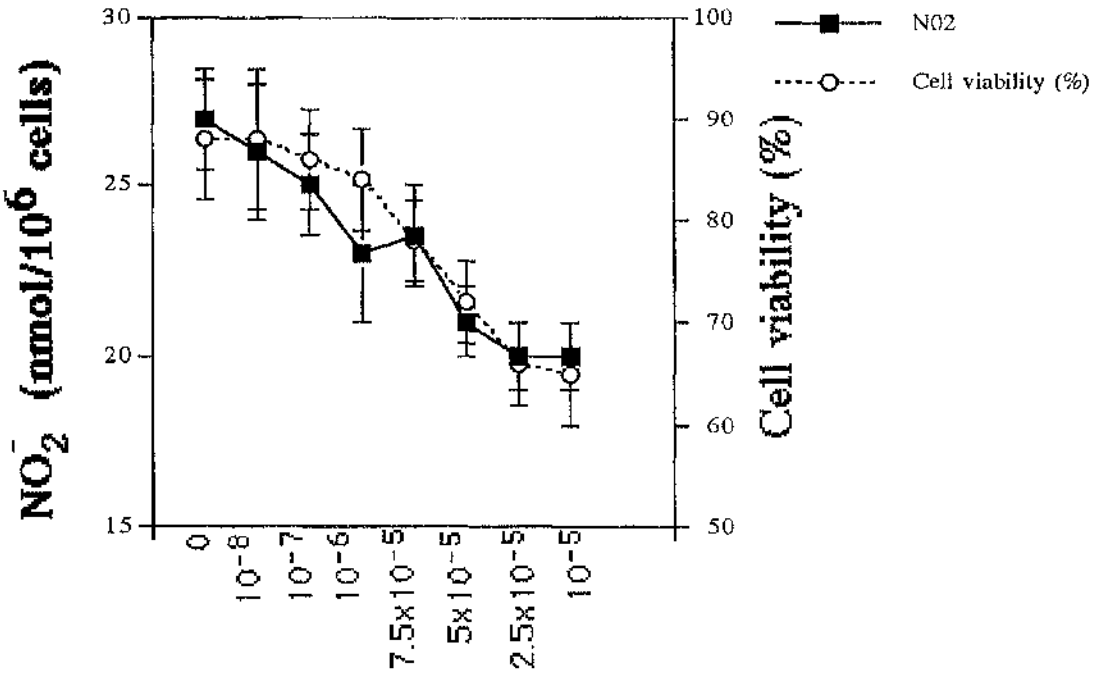
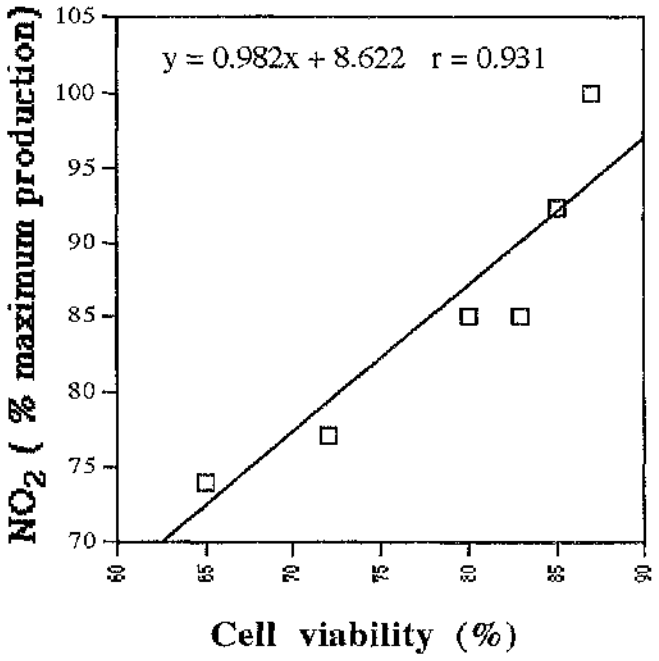



**Figure 5.4.b.**



by the Griess reaction in supernatants, as detailed in Chapter 2. Because 4-BPB was dissolved in DMSO, controls were carried out, using DMSO (0.1 % v/v), showing that DMSO does not affect the cells. Stimulation of J774 cells with IFN-γ (20 U/ml) and 10 ng/ml LPS induced the production of NO. Production of NO was not inhibited by 4-BPB at noncytotoxic concentrations (Figure 5.5). Nevertheless, When 4-BPB was used at concentrations in excess of 1 μM, the cell viability was diminished.

Cell viability was examined by trypan blue exclusion in cells treated with 4-BPB and the values were indicated where required in Figure 5.5.

Data presented here demonstrate that $PLA_2$ may not be involved the signalling that leads to NO production in J774 cells.

### 5.2.6. Dexamethasone does not affect NO production in response to IFN-γ and LPS:

Dexamethasone is a very well known $PLA_2$ inhibitor, used as a treatment in many aspects of medicine. In order to firm up the evidence that $PLA_2$ is not involved in NO production, an inhibitor irreleated to OOBA, dexamethasone was used.

Dexamethasone has been reported to inhibit NO production in cells expressing iNOS. J774 cells, 5 x $10^5$ /ml in RPMI 1640 medium, were cultured in 96-well plates, and 30 min after adding dexamethasone at a variety of concentrations, cells were stimulated by IFN-γ (20 U/ml) and 10 ng/ml LPS, then incubated at $37^{O}$C for 24 hrs. $NO_2^-$ levels were measured in the supernatants by the Griess reaction, as detailed in Chapter 2. Because dexamethasone is dissolved in chloroform (10 mM), controls were carried out, and the results were not affected by chloroform (max. 1 % v/v).

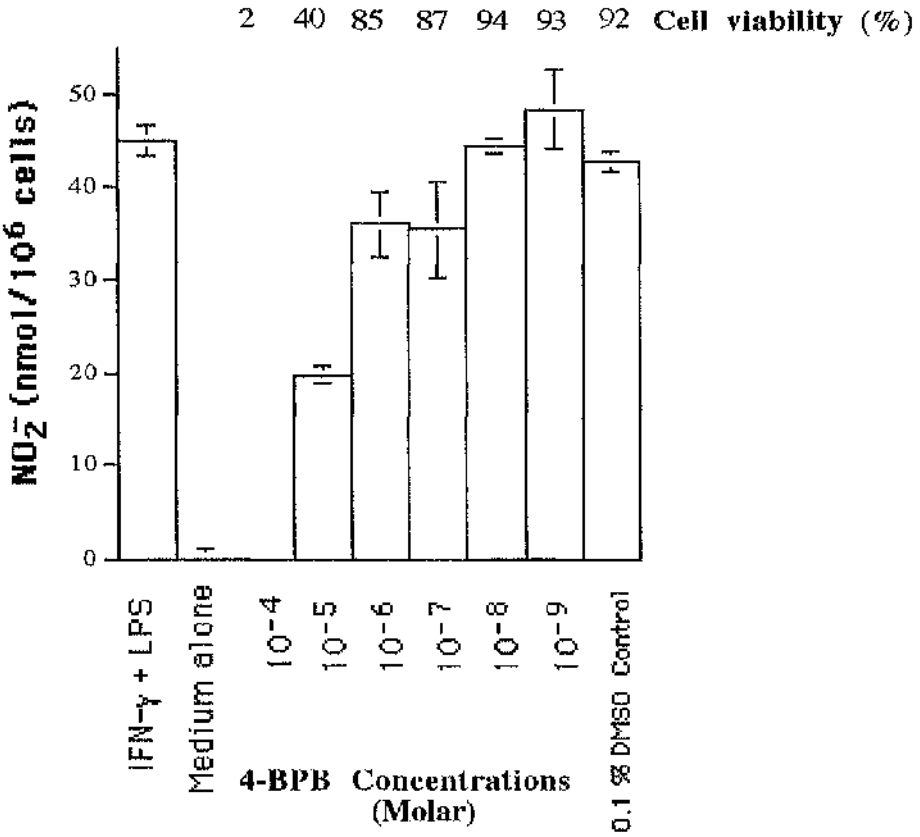



**Figure 5.5:** The effect of the $PLA_2$ inhibitor, 4-bromophenacyl bromide (4-BPB), at non-cytotoxic concentrations, on NO production in response to IFN-γ and LPS in the J774 cell line. J774 cells were cultured in 96-well plates. 30 min after adding 4-BPB at a variety of concentrations, cells were stimulated by IFN-γ (20 U/ml) and LPS (10 ng/ml), then incubated for 24 hrs. $NO_2^-$ levels in the supernatants were measured by the Griess reaction. Because 4-BPB was dissolved in DMSO (100 mM), required controls were carried out. When 4-BPB was used at concentrations in excess of 1 μM, the cell viability was significantly diminished. Cell viability was examined by trypan blue exclusion in cells treated with 4-BPB. Results are representative of three separate experiments. The means ± SEMs of triplicate cultures are shown. (n=3)

Stimulation of J774 cells with IFN-γ (20 U/ml) and 10 ng/ml LPS induced the production of NO. Production of NO was inhibited by dexamethasone at 100 μM. (Figure 5.6). However, it was not found consistent and reproducible.

Cell viability was examined by trypan blue exclusion and evaluated as normal in cells treated with dexamethasone. Moreover, cells treated with dexamethasone were able to adhere and spread in a similar manner to untreated cells, and the general morphology appeared also normal.

### 5.2.7. Cholera toxin does not affect NO production in response to IFN-γ and LPS in the J774 cell-line:

In order to investigate whether there is a role of G-proteins in NO production, cholera toxin, which activates $G_S$, was used as a G-protein modifier. Therefore, J774 cells, 5 x $10^5$ /ml in RPMI 1640 medium, were cultured in 96-well plates. Thirty minutes after adding cholera toxin (Ctx) at a variety of concentrations (1-100 μg/ml), dissolved in the same medium, cells were stimulated by IFN-γ (40 U/ml) and LPS (10 ng/ml), then incubated at 37°C for 24 hrs. $NO_2^-$ levels were measured by the Griess reaction in supernatants, as detailed in Chapter 2. Stimulation of J774 cells with IFN-γ (20 U/ml) and 10 ng/ml of LPS induced the production of NO. Production of NO was inhibited by Ctx in a dose-dependent manner. Unfortunately, this affect was found to be nonspecific for cholera toxin, because the toxin solution contains 0.15 mM sodium azide. Sodium azide controls gave the same level of inhibition when applied alone in stimulated cells. On the other hand, the acceptable concentrations of cholera toxin (500 ng-1 μg/ml) according to the literature, using the cell systems did not effect NO production. (Figure 5.7.a). Cell viability was checked out by using trypan blue dye exclusion and determined as normal (>95%). In addition, glucose uptake was used to evaluate cytotoxic effect of Ctx and sodium Azide.

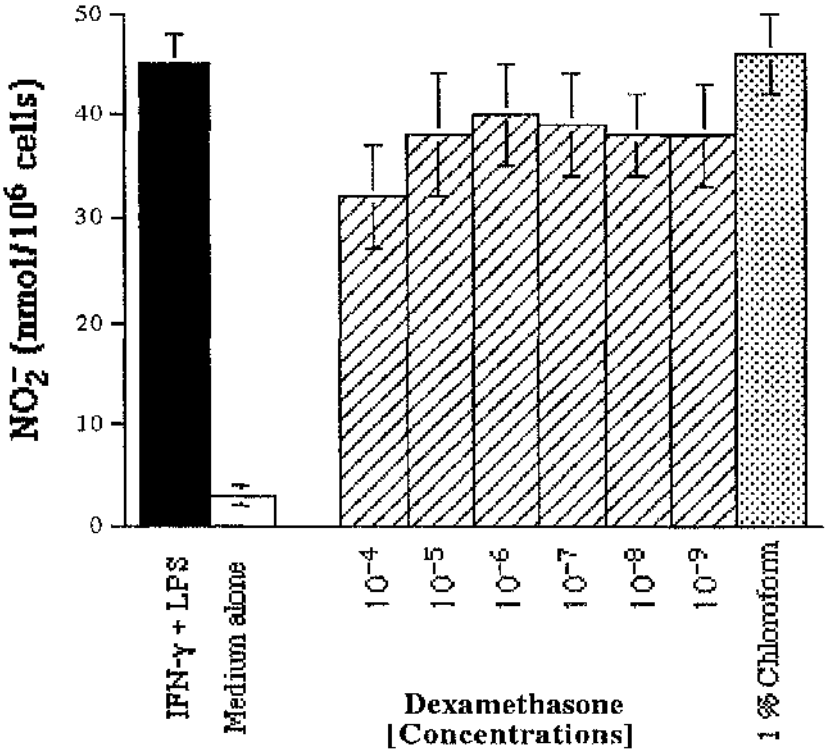



**Figure 5.6:** The effect of dexamethasone on NO production in response to IFN-γ and LPS. J774 cells were cultured in 96-well plates. 30 min after adding dexamethasone at a variety of concentrations, cells were stimulated by IFN-γ (20 U/ml) and LPS (10 ng/ml), then incubated for 24 hrs. $NO_2^-$ levels in the supernatants were measured by the Griess reaction.

Because dexamethasone is dissolved in chloroform (10 mM), controls were carried out, and data obtained showed no effect of chloroform (maximum concentration, 1 % v/v) on NO production. The negative controls were obtained from resting cell supernatants. Results are representative of three separate experiments. The means ± SEMs of triplicate cultures are shown. (n=3)

Cells were adjusted to a density of $1x10^6$ cells/well in 1 ml RPMI 1640 medium. Cells were treated with 2 μCi/ml [$^3$H]-glucose and in RPMI 1640 medium. Then test medium (1 ml) containing substances, IFN-γ, LPS, Ctx and sodium azide, was added. Cells were treated with IFN-γ and LPS, Ctx with and without stimulation. Untreated cells were used as negative controls, and sodium azide treated wells were used as positive controls. The uptake of [$^3$H]-glucose was presented as CPM values for each group, as detailed in Chapter 2. (Figure 5.7.b).

Cells treated with sodium azide and Ctx showed a low level of glucose uptake compared to resting cells. However, the same level of inhibition in glucose uptake has been observed in cells stimulated with IFN-γ and LPS. This may be due to the toxic effect of NO produced in response to IFN-γ and LPS by macrophages.

### 5.2.8. Cholera toxin treatment of J774 cells results in sustained intracellular cAMP elevation:

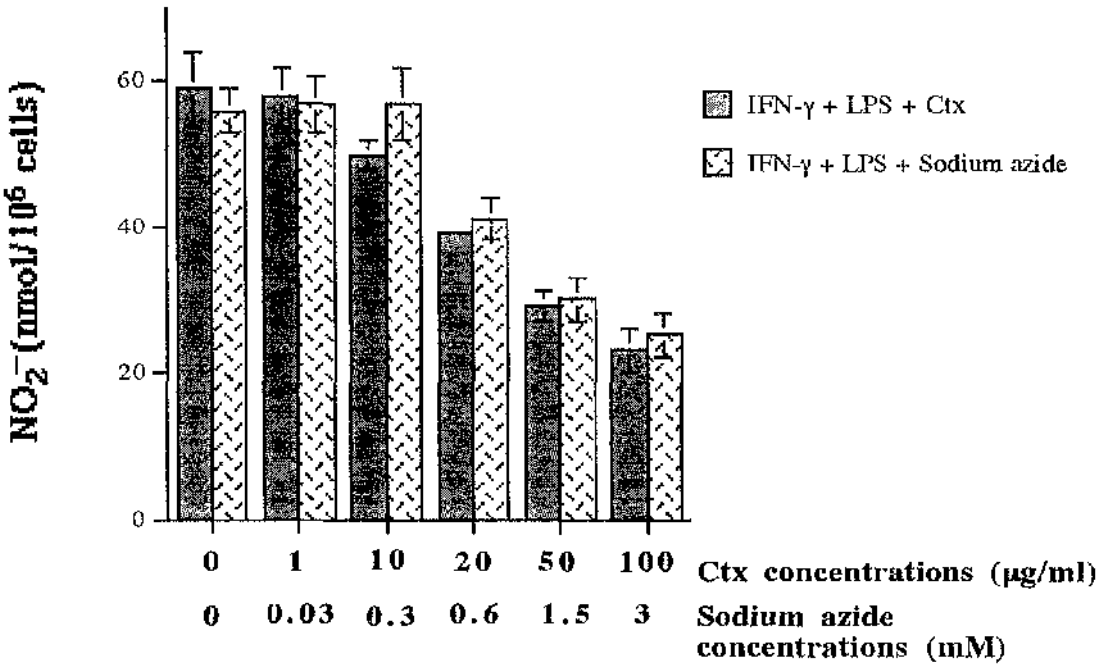
Cholera toxin is an enzyme that catalyses the transfer of ADP ribose from intracellular $NAD^+$ to the α subunit of the $G_s$-protein. This causes intracellular cAMP generation induced by adenylyl cyclase [Lai, 1980]. Therefore, the effect of Ctx on intracellular cAMP levels was assessed, using 50 μg/ml Ctx. Cells were treated with Ctx at 37$^o$C for 15 minutes, 8 and 24 hrs. Cytoplasm was extracted, as detailed in Chapter 2, and intracellular cAMP levels were determined by using Amersham kit system.

Results are shown as pmol/$10^6$ cells in table 5.1. Ctx caused sustained intracellular cAMP elevation, and untreated control cells did not produce cAMP.

**Figure 5.7.a:** The effect of cholera toxin on NO production in response to IFN-γ and LPS on the J774 cell-line. J774 cells were cultured in 96-well plates. 30 min after adding cholera toxin (Ctx) at a variety of concentrations (1-100 μg/ml), cells were stimulated by IFN-γ (40 U/ml) and LPS (10 ng/ml), then incubated for 24 hrs. $NO_2^-$ levels were measured by the Griess reaction in supernatants. Because the toxin solution contains 0.15 mM sodium azide controls were carried out, using sodium azide. Results are representative of three separate experiments. The means ± SEMs of triplicate cultures are shown. (n=3)

**Figure 5.7.b:** The effect of cholera toxin on glucose uptake of J774 cells were evaluated. Cells were adjusted to $1x10^6$ cells/well. Cells were treated with 2 μCi/ml [$^3$H]-glucose and in RPMI 1640 medium. Cells were treated with IFN-γ (40 U/ml) and LPS (10 ng/ml) with and without Ctx (50 μg/ml) and untreated cells were used as controls, then the incubation was continued at 37°C for 24 hrs. After 24 hours, cells were counted for [$^3$H] on a scintillation counter. Sodium azide treated wells were used as positive controls. The uptake of [$^3$H]-glucose was presented as CPM values for each group. Results are representative of three separate experiments. The means ± SDs are shown.

(*: $p< 0.05$, †: $p< 0.1$, n=3)


$NO_2^-$ (nmol/$10^6$ cells)
60
40
20
0
IFN-γ + LPS + Ctx
IFN-γ + LPS + Sodium azide
0 1 10 20 50 100
Ctx concentrations (μg/ml)
0 0.03 0.3 0.6 1.5 3
Sodium azide concentrations (mM)

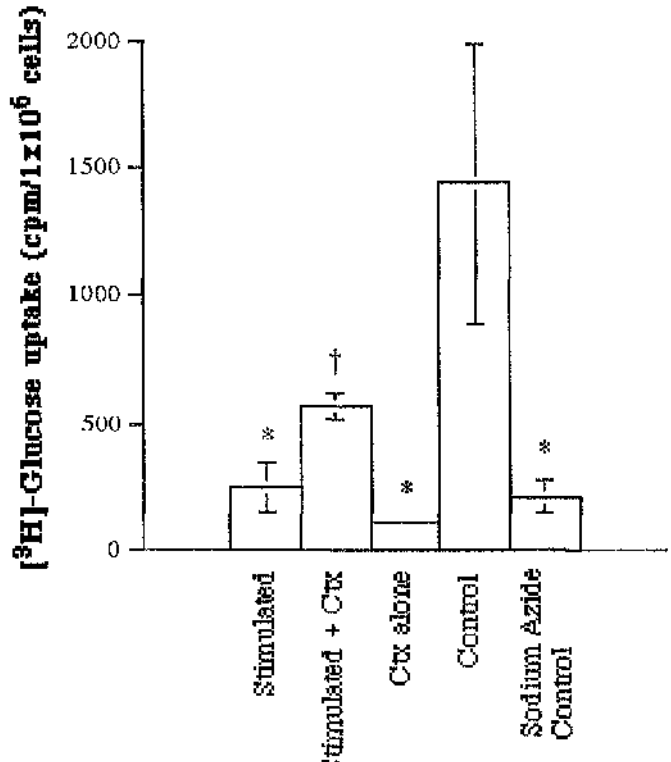

[$^3$H]-Glucose uptake (cpm/1x$10^6$ cells)
2000
1500
1000
500
0
*
†
*
*
Stimulated
Stimulated + Ctx
Ctx alone
Control
Sodium Azide Control

**Table 5.1: Cholera toxin induces a sustained intracellular elevation of cAMP in J774 cells:**

J774 cells were adjusted to 1x $10^6$ cells/ml in RPMI 1640 medium and incubated in 24-well plates. Ctx was used at the concentration of 50 µg/ml as final concentration. Cells were incubated at 37$^{O}$C for different time points (15 mins, 8 and 24 hrs). Untreated cells were used as controls. Results are representative of three separate experiments. The means ± SDs are shown in triplicate samples. (n=3)

cAMP pmol/$10^6$ cells

| Time | Ctx treated cells | Control cells |
|---|---|---|
| 15 mins | 44.33±1.15 | 0.167±0.275 |
| 8 hrs | 20.70±2.30 | 4.330±1.53 |
| 24 hrs | 6.00 ± 0.57 | 5.83 ± 1.01 |

**5.2.9.a. Pertussis toxin does not alter NO production in J774 cells and CBA mouse peritoneal macrophages:**

The role of pertussis toxin (Ptx) sensitive G-proteins was investigated with respect to NO production. J774 cells or peritoneal macrophages obtained from CBA mice treated with thioglycollate (ip) for 3-4 days, as detailed in Chapter 2, were used. CBA peritoneal lavage in RPMI medium was centrifuged at 1000 RPM for 5 minutes, and the pellet was washed three-times with sterile warm PBS. Cells were resuspended in RPMI 1640 medium by using sterile plastic Pasteur pipettes. Cells were adjusted to 5 x $10^5$ /ml in RPMI 1640 medium, were cultured in 96-well plates, then incubated at 37°C for 2 hours. Cell adhesion was examined by using a Zeiss microscope. Medium was changed to remove non-adherent and dead cells. Ptx, dissolved in RPMI 1640 medium, was added one and half hours before stimulation with IFN-γ and LPS.

$NO_2^-$ levels were measured by the Griess reaction in supernatants, as detailed in Chapter 2. Ptx, at a variety of concentrations (10-500 ng), did not affect NO production induced by IFN-γ (40 U/ml) and LPS (10 ng/ml) or LPS alone (1 μg/ml) in CBA mice peritoneal macrophages and J774 cells. In control wells, Ptx in the absence of other stimuli did not induce NO production. (Figure 5.8.a). Cell viability was examined by trypan blue exclusion and evaluated as normal in cells treated with Ptx.

**5.2.9.b: Pertussis toxin does not alter NO production in response to LPS:**

The effect of Ptx on NO production in response to LPS was studied to observe whether LPS at excessive concentrations might result in ineffective Ptx-mediated signalling, or whether there is no involvement of Ptx-sensitive proteins in signal

transduction mechanisms leading to NO production in response to LPS. J774 cells or peritoneal macrophages obtained from CBA mice were used. Cells were adjusted to 5 x $10^5$ /ml in RPMI 1640 medium, were cultured in 96-well plates, then the effect of Ptx on NO production in response to LPS at a variety of concentrations by J774 cells were investigated. Ptx (100 ng/ml), dissolved in RPMI 1640 medium, was added one and half hours before stimulation with LPS. $NO_2^-$ levels were measured by chemiluminescence in supernatants. Ptx did not affect NO production induced by LPS in J774 cells. In control wells, Ptx in the absence of LPS did not induce NO production (Figure 5.8.b). Cell viability was examined by trypan blue exclusion and evaluated as normal in cells treated with Ptx. Moreover, cells treated with Ptx were able to adhere and spread in a similar manner to untreated cells, and the general morphology appeared also normal.

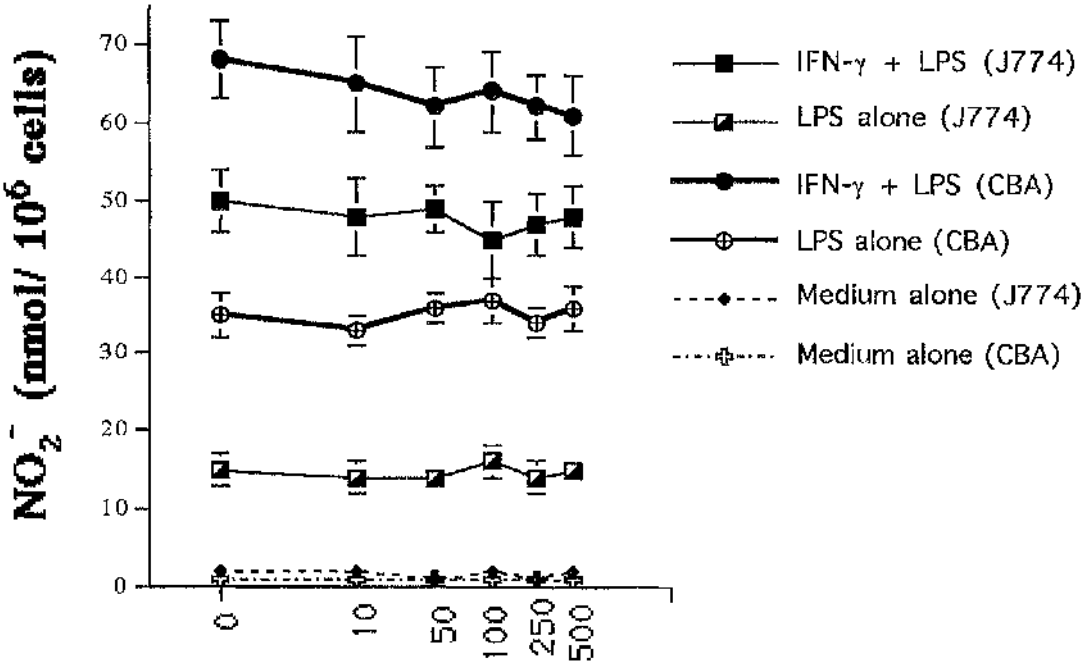
### 5.2.10. Pertussis toxin does not alter NO production in response to IFN-γ at a variety of concentrations and to a low concentration of LPS:

Finally, it is required to observe the effect of Ptx on NO produced by cells in response to IFN-γ and LPS, using a wide range of concentration of IFN-γ. Therefore, J774 cells were adjusted to 5 x $10^5$ /ml in RPMI 1640 medium, and cultured in 96-well plates, then the effect of Ptx on NO production in response to different doses of IFN-γ at different concentrations, from 0.4 to 400 units, and to LPS (10 ng/ml) by J774 cells were investigated. Pertussis toxin (100 ng/ml), dissolved in RPMI 1640 medium, was added one and half hours before stimulation with LPS. $NO_2^-$ levels were measured by chemiluminescence in supernatants. Ptx did not affect NO production induced by IFN-γ at variety activity units and LPS in J774 cells. In control wells, Ptx in the absence of IFN-γ and LPS did not induce NO production (Figure 5.9). Cell viability was examined by trypan blue exclusion and evaluated as normal in cells treated with Ptx (>95%).

**Figure 5.8.a:** The effect of pertussis toxin on NO production in J774 cell lines and CBA murine peritoneal macrophages. J774 cells or peritoneal macrophages were cultured in 96-well plates. Pertussis toxin, over a wide range of concentrations (10-500 ng), was added one and half hours before stimulation with IFN-γ (40 U/ml) and LPS (10 ng/ml) or LPS alone (1 μg/ml). $NO_2^-$ levels were measured by the Griess reaction in supernatants. Results are representative of three separate experiments. The means ± SEMs of triplicate cultures are shown. (n=3)

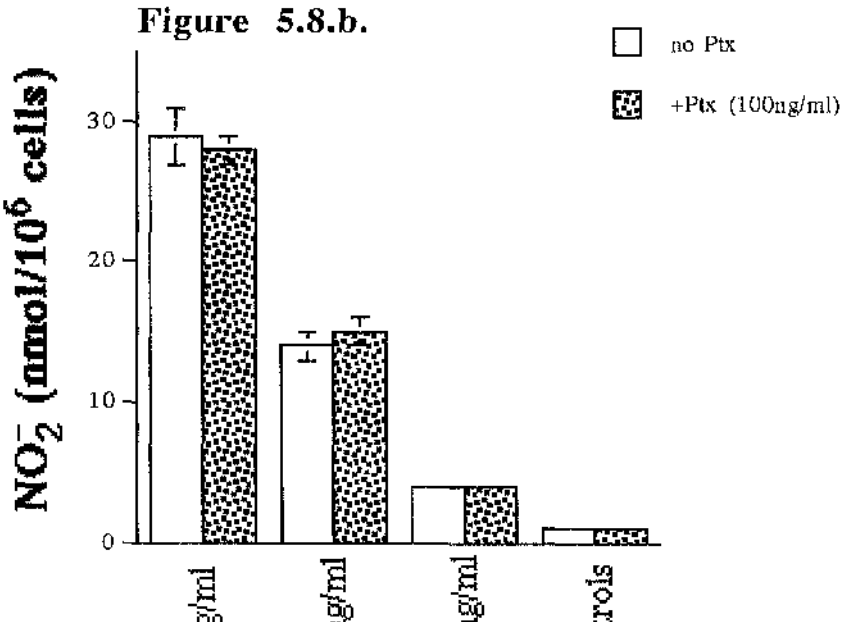
**Figure 5.8.b:** The effect of pertussis toxin on NO production in response to LPS at variety concentrations. J774 cells were cultured in 96-well plates. Pertussis toxin (100 ng/ml), was added one and half hours before stimulation with LPS at a variety of concentrations (10 ng-1 μg/ml). $NO_2^-$ levels were measured by chemiluminescence in supernatants. Results are representative of three separate experiments. The means ± SEMs of triplicate cultures are shown. (n=3)

**Figure 5.8.a.**




**Ptx Concentrations (ng/ml)**

**Figure 5.8.b.**




**LPS Concentrations**

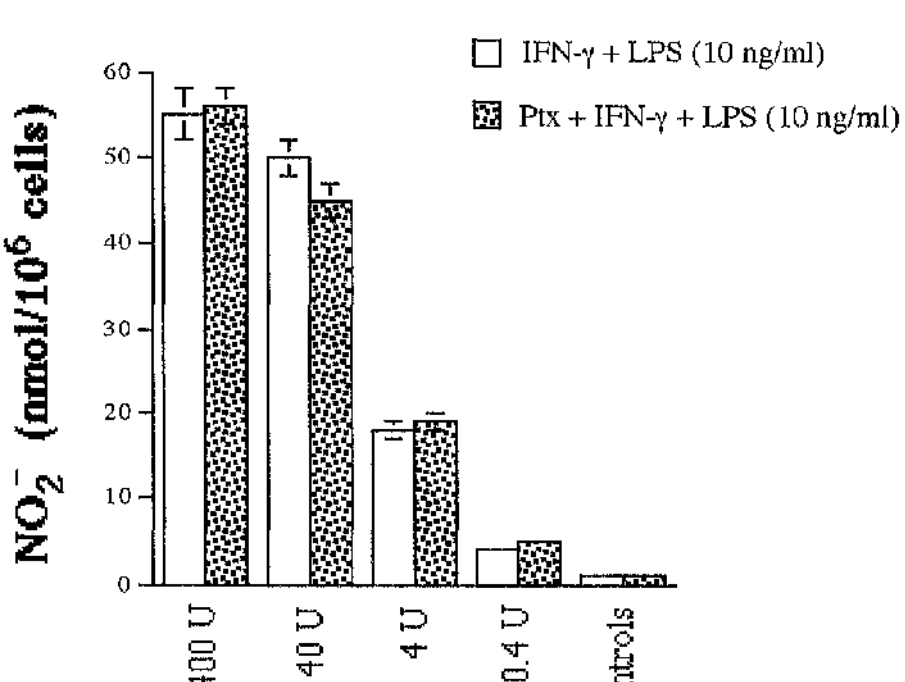



**Figure 5.9:** The effect of pertussis toxin on NO production in response to IFN-γ at a variety of concentrations and LPS (10 ng/ml). J774 cells were cultured in 96-well plates, then the effect of Ptx (100 ng/ml), added one and half hours before stimulation with LPS, on NO production in response to IFN-γ at a variety of concentrations (0.4-400 U/ml), and 10 ng/ml LPS by J774 cells were investigated. $NO_2^-$ levels were measured by chemiluminescence in supernatants. Cell viability was examined by trypan blue exclusion and evaluated as normal in cells treated with Ptx. The means ± SEMs of triplicate cultures are shown. Results are representative of three separate experiments. (n=3)

**DISCUSSION:**

It has previously been shown that the induction of NO production by IFN-γ and LPS is dependent on PKC activation [Severn et al, 1992b]. It may have been possible that Ptx activates G-protein-associated phospholipase C (PLC), producing DAG which then activates PKC. Data presented in this chapter suggest that the Ptx sensitive-G-proteins, like $G_{plc}$, are not involved in the induction of NO in macrophages, and modification of G-proteins does not alter NO production in the J774 macrophage-like cell line. Therefore, it is clear that the signalling events leading to NO production are independent on G-protein-mediated effectors.

In Pertussis disease, there is a necrosis of the basal and midzonal portions of the bronchial epithelium and a marked inflammatory infiltrate which consists mainly of PNL [Ritchie, 1990]. This lesion may be caused by increased NO production which is produced by cells in the necrotic area, since the role of NO in epithelial autotoxicity in pertussis disease has also been reported [Heiss et al., 1994]. In the P388D1 macrophage like cell line, it has been reported that LPS induces G-protein activity, which can be blocked by Ptx [Jakway and De Franco, 1986].

PAF induces an elevation of intracellular cAMP levels, like $PGE_2$ does. There is evidence showing that NO plays a protective role in the acute gastrointestinal damage induced by PAF [Boughton-Smith et al, 1992; MacKendrick et al, 1993]. However, data presented here also suggest that PAF does not alter NO production in J774 cells.

NO production, stimulated by IFN-γ and LPS, is not inhibited by Ctx, even although Ctx induces intracellular cAMP levels. A sustained increase in intracellular cAMP levels was induced by Ctx in J774 cells, at concentrations which inhibited NO production. However, it was not significant because of sodium azide found in

the Ctx solution. We have shown that other agents which increase cAMP levels inhibit NO production (Bulut et al. 1993).

Glucocorticoids, dexamethasone, hydrocortisone and cortisol, inhibit the induction of iNOS, but not its activity, in response to LPS and IFN-γ, in vitro in vascular endothelial cells [Radomski et al., 1990] and the J774 macrophage cell line [Di Rosa et al., 1990]. However, in this study, it was not found to be a robust finding, using a variety of concentrations of dexamethasone in J774 cells.

The role of $PLA_2$ in activation of PKC has been reported, so that, Cis unsaturated fatty acids, including oleic, linoleic, linolenic, arachidonic and docosahexaenoic acids may be hydrolysed by $PLA_2$, and the products can enhance PKC activity. In OK-432 (picibanil) treated peritoneal exudate macrophages, NO production was inhibited by the $PLA_2$ inhibitors, dexamethasone and 4-BPB, the lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA). Nevertheless, caffeic acid and esculetin, inhibitors of 5- and 12-lipooxygenase respectively, were not inhibitory [Ryoyama et al., 1993]. Data demonstrated here suggest that $PLA_2$ inhibitors are ineffective on NO production in response to IFN-γ and LPS in the J774 cell-line.

# CHAPTER 6

# GENERAL DISCUSSION

## 6. DISCUSSION:

Until the 1990s, it was accepted that the classical criteria for macrophage activation are Ia antigen (Immune response: IR; MHC class II) expression, the induction of reactive oxygen intermediate production and the ability to inhibit the proliferation of intracellular protozoa. Besides that the secretion of reactive nitrogen intermediates, such as NO, $NO_2^-$, $ONOO^-$, is now considered as an additional criterion in macrophage activation [Nibbering et al., 1992].

Nitric oxide produced by macrophages at high concentrations plays an important role in the regulation of T lymphocytes, although there is no clear evidence for B lymphocyte regulation. Recently, it was suggested that NO produced at physiological concentrations by constitutive NOS activity is required for proliferation of T cells. However, high concentrations of NO inhibit the proliferation of T cells and IFN-γ secretion by Th1-like cells [reviewed by Liew, 1995]. The balance between Th1-like and Th2-like cells determines the outcome of a number of infectious and autoimmune diseases [Liew, 1989; Sher and Coffman, 1992; Romagnani, 1991; Mason and Fowell, 1992; Bloom et al., 1992]. Their characteristic cytokines play important roles for these effects. For instance, Th1-like cells produce IFN-γ which, together with other molecules such as LPS and TNFα, activates macrophages to produce NO which mediates cytopathic and microbicidal effects [Nathan and Hibbs, 1991; Hibbs et al., 1990; Liew and Cox, 1991; Green et al., 1990]. On the other hand, Th2-like cells produce IL-4 and IL-10 which inhibit the synthesis of NO by macrophages [Liew et al., 1991; Cunha et al., 1992; Gazzinelli et al., 1992].

This is clearly an important area, as an understanding of this mechanism may lead to effective control of this important biological mediator, NO, and therapy for

several important diseases, such as parasitic diseases, rheumatoid arthritis, and tumours.

Data presented in this thesis contribute to the understanding of NO production and regulation mechanisms. These data suggest that cytokine signalling pathways inside macrophages are non-linear and form a network with cross-talk occurring among different signalling pathways. Therefore a ligand binding unit of the receptor may not be the sole determinant of the cellular responses. Accordingly, a single receptor may couple to multiple signal transduction systems, for example, IL-4 treatment of human B cells results in the hydrolysis of $PIP_2$, elevation of DAG and $IP_3$, as well as delayed cAMP production [Finney et al., 1990]. Likewise, multiple receptors may couple to the same signal transduction pathway via common transducers, for instance, both β-adrenergic receptors and $PGE_2$ induces cAMP generation by the activation of adenylyl cyclases.

It has been previously demonstrated that the activation of PKC is required for the induction of the macrophage iNOS gene transcription [Severn et al., 1992b]. In this study, IL-4 inhibits the synthesis of NO via the blocking of PKC activation. This is supported by our finding that transcription of the iNOS gene was inhibited by IL-4 as well as the PKC inhibitor, Ro31-8220. It was demonstrated that PKC-epsilon (PKC-ε) is the predominant isoform in the macrophage that translocates to the membrane when the cells are activated with IFN-γ and LPS [Sands et al., 1994a]. It should be noted that IL-4 inhibits NO synthesis only when macrophages are pretreated with this cytokine before activation with IFN-γ and LPS [Liew et al., 1991]. Inhibition of PKC activation also required incubation with IL-4 for several hours before stimulation. IL-4 did not effect an immediate reduction in [$^3$H]-PDBu binding to whole cells, and addition of IL-4 to the cells at the same time as or after IFN-γ and LPS did not prevent the rise in [$^3$H]-PDBu binding [Sands et al., 1994a]. Thus, the mechanism of the interaction of IL-4 and IFN-γ in the control of

NO synthesis is complex and factors other than PKC are probably involved. An earlier report has described inhibition of PKC by IL-4 in lymphocytes [Hart et al., 1989b], but it is not yet clear how IL-4 inhibits PKC activation. It may inhibit generation of DAG, the physiological activators of PKC, [Nishizuka, 1992], by acting on a phospholipase. Meanwhile, There was no observed $Ca^{2+}$ elevation and $IP_3$ generation in macrophages induced by IFN-γ and LPS [Sands et al., 1994b], and it was reported that phosphatidylcholine-specific PLC (PC-PLC) is involved in DAG generation and PKC activation induced by IFN-γ and LPS in macrophages [Sands et al., 1994b]. The blocking of the PKC activation pathway by IL-4 may, therefore, explain its general inhibitory effects on macrophages. IL-4 induces a novel signal transduction cascade involving an immediate and transient elevation of inositol 1,4,5-triphosphate and $Ca^{2+}$ levels, followed by a sustained rise in cellular cyclic AMP levels [Hart et al, 1992]. In human B cells, IL-4 does induce a delayed rise in cAMP [Finney et al., 1990]. Therefore, we have excluded an increase in intracellular cAMP concentration as a mechanism of inhibition of NO production by IL-4. This was not detected in J774 cells. However, differences between the signalling pathways of human and murine IL-4 have been reported [McGarvie and Cushley, 1989]. Additionally, other differences in iNOS regulation can be observed between different types of macrophage. For instance, IL-4 was reported to strongly reduce iNOS mRNA at late times post stimulation (24-72 hrs), but not at early times post stimulation (4-6 hrs after exposure to IFN-γ and LPS) in primary peritoneal mouse macrophages [Bogdan et al., 1994]. Figure 6.1 shows a proposed pathway involved in the inhibition of NO production by IL-4 and cAMP elevation.

The roles of other kinases such as myosin light chain kinase or calmodulin-dependent kinase in tumoricidal activation appeared to be limited, since the inhibitors of these enzymes, ML-7 or W-7, failed to block LPS triggered macrophage activation [Novotney et al., 1991].

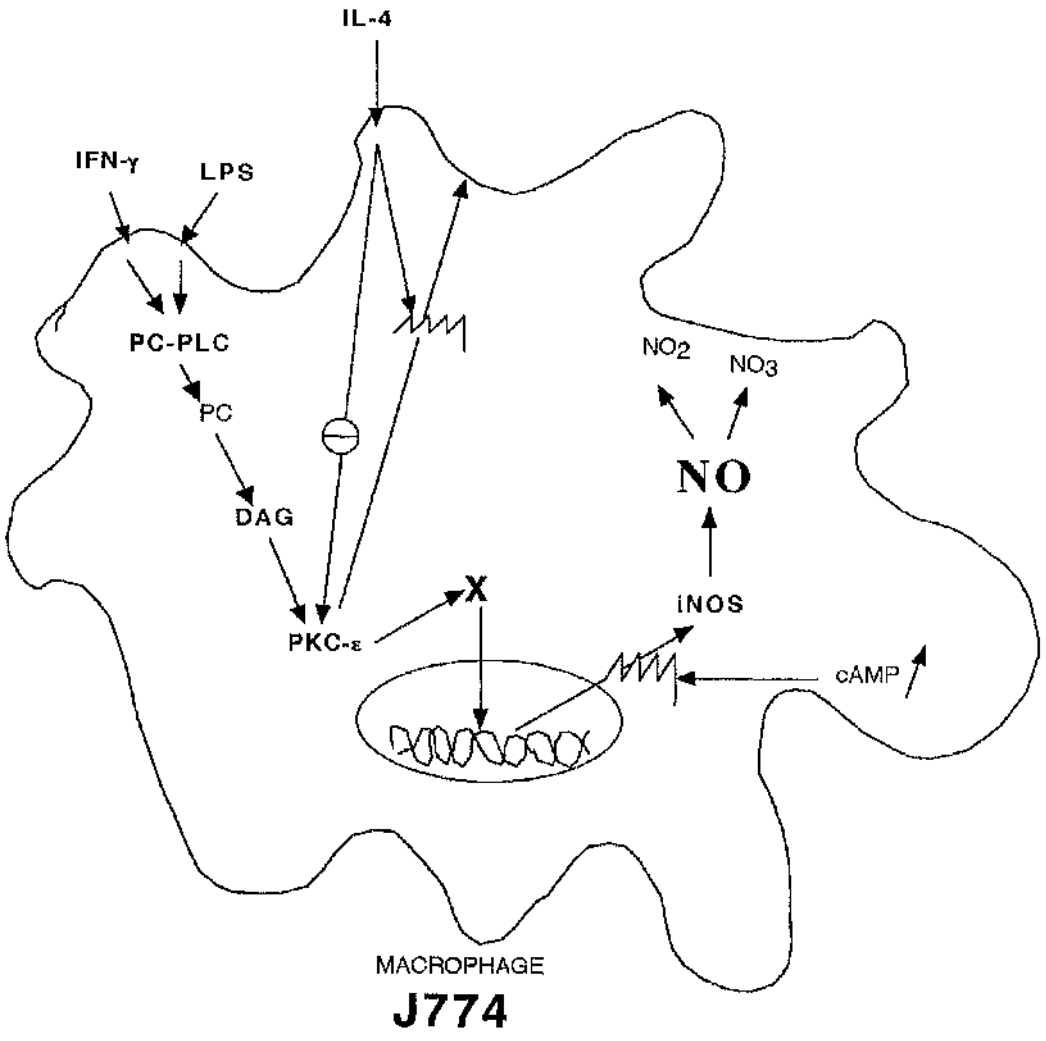



**Figure 6.1: Regulatory mechanisms of NO production by IL-4 and cAMP elevation**

IFN-γ is the most potent known lymphokine for activating mononuclear phagocytes [Nathan et al., 1984]. IFN-γ results in eicosanoid production, such as $PGE_2$, in macrophages [Uhing et al., 1990]. The receptor of murine IFN-γ has been cloned and the specific phosphorylation site for PKA, Arg-Arg-Ser-Phe-Ser, between 351-357 amino acids has been identified [Gray et al., 1989]. Therefore, the effect of the intracellular cAMP elevation is required to explore NO production regulating mechanisms.

Data presented in this thesis demonstrate that NO production by murine macrophages is inhibited by a prolonged increase in intracellular cAMP concentrations. It was reported that cyclic nucleotides are involved in the regulation of several macrophage functions, including phagocytosis, locomotion, responses to lymphokines, and DNA synthesis [Reviewed by Moncada et al., 1991]. The role of cAMP elevation and PKA activation is to produce or direct negative signals in the cells, whereas PKC directs positive signals [Reviewed by Nishizuka, 1986].

What could be the role of the cAMP pathway in the inhibition of NO production in macrophages? According to our PCR, SDS-Page and western blotting data, it is clear that the inhibitory effect of cAMP elevation on NO production is involved in post transcriptional or translational levels of the iNOS gene and iNOS protein synthesis, and not at the level of transcription.

Meanwhile, the use of IBMX, a nonspecific PDE inhibitor, which might inhibit the PLC enzyme as well, is questionable. However, the specific inhibitor of cAMP, Rp-cAMP, reversed the inhibitory effect of cAMP elevation on NO production. Since the cells can exhibit an IBMX-resistant cAMP phosphodiesterase activity, we also used rolipram, which is a specific cAMP-specific PDE inhibitor, to evaluate if there is a difference between the effects of IBMX and rolipram. There was no difference found between the use of IBMX and rolipram, suggesting that the use of

IBMX is sufficient to observe the effect of cAMP-elevating agents on NO production in macrophages.

J774 macrophage NOS has a consensus sequence for PKA [Lowenstein et al., 1992], and it may be that phosphorylation by this enzyme reduces the activity of NOS. Activated macrophages produce large quantities of $PGE_2$ [Nathan, 1987], and this may contribute to the regulation of NO production, via the elevation of cAMP. On the other hand, PKA activity can cause phosphorylation of many other kinases, such as Raf kinases, and other proteins, involved in NO producing process in macrophages. It is clear that cAMP-dependent protein kinases (PKA) regulate the expression of numerous eukaryotic genes by agents such as epidermal growth factor and mobilised calcium [Sheng et al., 1988]. Intracellular cAMP elevation can block receptor-induced PKC activation [Takai, et al. 1982a]. $PGE_2$ may have a very important role in immune regulation. For instance, $PGE_2$ inhibits IL-2 and IFN-γ production from Th1 clones, but not IL-4 and IL-5 production from Th2 clones [Betz and Fox, 1991]. It has been reported that human alveolar macrophages suppress the proliferation of peripheral blood lymphocytes via their secretory product $PGE_2$ [McCombs et al., 1982]. Meanwhile, Kawabe and co-workers [1992] reported that NO secreted by activated alveolar macrophages was a major suppressive agent in this system.

Finally, LPS treatment of macrophages results in the activation of various genes, including the genes for TNF-α, IL-1, IFN-β, NOS as well as the proto-oncogenes c-fos, c-jun, c-fms and c-myc. The activation of these genes is regulated at the transcriptional level by some DNA-binding transcriptional factors such as NF-κB or AP-1 [Lenardo and Baltimore, 1989]. It has been shown that LPS-treatment of J774 cells leads to the activation of the fast-moving (called B1) and slow-moving(called B2) NF-κB, and this activation is inhibited by H89, which is a PKA inhibitor, but not by a PKC inhibitor, H7 [Muroi and Suzuki, 1993].

Furthermore, the pretreatment of J774 cells with dibutyryl cAMP, forskolin, cholera toxin and IBMX induced NF-κB activation, and LPS treatment results in type 1 PKA activation in macrophages. The correlation between NF-κB activation and NO production in macrophages has not been clearly shown.

Data presented here also suggest that the Ptx sensitive-G-proteins are not involved in the induction of NO in macrophages, and modification of G-proteins does not alter NO production in J774 cells. It might be possible Ptx activates G-protein-associated phospholipase C (PLC), producing DAG which then activates PKC. The role of NO in epithelial autotoxicity in pertussis disease has also been reported [Heiss et al., 1994]. In the P388D$_1$ macrophage like cell line, it has been reported that LPS induces G-protein activity, which can be blocked by Ptx [Jakway and De Franco, 1986].

NO production, stimulated by IFN-γ and LPS, is not inhibited by Ctx, even although Ctx induces elevated levels of intracellular cAMP. A sustained increase in intracellular cAMP levels was induced by Ctx in J774 cells, at concentrations which inhibited NO production. This difference might be dependent on multipotent effect of Ctx or cAMP elevation may be transient in response to Ctx, since PDEs are activated in response to high level cAMP levels and PKA activation [reviewed by Houslay, 1990].

Glucocorticoids, dexamethasone, hydrocortisone and cortisol, inhibit the induction of iNOS, but not its activity, in response to LPS and IFN-γ, in vitro in vascular endothelial cells [Radomski et al., 1990] and the J774 macrophage cell line [Di Rosa et al., 1990]. However, in our study, it was not found to be a robust finding, using a range of concentrations of dexamethasone at different time points in J774 cells. The inhibition of NO synthesis by glucocorticoids explains many therapeutic actions of these agents in antierythema, anticdema, endotoxin shock, asthma and

rheumatoid arthritis, where NO may be responsible for pathological vasodilatation and tissue damage. Moreover, NO synthesis inhibition may help to explain why glucocorticoids cause the spread of infections [Schaffner and Schaffner, 1987] and malignancy [Schultz et al., 1985; Hogan and Vogel, 1988].

Platelet activating factor (PAF), which is a secretory product of activated macrophages, has a high affinity receptor on the surface of macrophages, and the binding of PAF to the receptor results in the rapid breakdown of polyphosphoinositides, diacylglycerol production, and phosphorylation via protein kinase C [Rosen et al., 1989]. Moreover, PAF induces elevated levels of intracellular cAMP, like $PGE_2$ does [Nibbering et al., 1992; Adams and Hamilton, 1992]. There is evidence showing that NO plays a protective role in the acute gastrointestinal damage induced by PAF [Boughton-Smith et al., 1992; MacKendrick et al., 1993]. Meanwhile, NO is involved in the process of neutrophil and platelet activation in the rabbit [May et al., 1991]. Therefore, an investigation of the effects of PAF on NO production in macrophages would be useful for understanding the mechanism, which is involved in the NO producing pathway. However, PAF was not found to affect NO production.

The role of $PLA_2$ in activation of PKC has been reported, so that, Cis unsaturated fatty acids, including oleic, linoleic, linolenic, arachidonic and docosahexaenoic acids may be hydrolysed by $PLA_2$, and the products can enhance PKC activity. In OK-432 (picibanil) treated peritoneal exudate macrophages, NO production was inhibited by the $PLA_2$ inhibitors, dexamethasone and 4-BPB, lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA). Nevertheless, caffeic acid and esculetin, inhibitors of 5- and 12-lipooxygenase respectively, were not inhibitory. The cyclooxygenase inhibitors, such as indomethacin and ibuprofen, have not been found to be inhibitors for NO production [Ryoyama et al., 1993]. Conversely, Data

demonstrated here suggest that $PLA_2$ inhibitors are ineffective on NO production in response to IFN-γ and LPS in J774 cell line.

## 6.2. CONCLUSIONS:

In conclusion, we have further defined the role of PKC in the induction of NO production by murine macrophages, showing its activation to be essential for iNOS gene transcription. We have also partly elucidated the mechanism by which IL-4 inhibits NO production. The inhibition of PKC activation by IL-4 may explain its other effects on macrophages.

The inhibitory effect of cAMP-elevating agents on NO production in macrophages stimulated by IFN-γ and LPS can add some useful information to explain how cAMP-elevating agents direct the negative signalling in macrophages.

In our study, it has also been shown that toxin sensitive-G-proteins and $PLA_2$ activation are not involved in NO production in J774 cells.

## 6.3. FUTURE WORK:

Understanding of macrophages signal transduction is still far from complete. Future work will undoubtedly uncover more receptor kinases and phosphatases that are involved in macrophage activation.

Further applications can be the investigation of the role of PKC and PKA phosphorylation products in iNOS synthesis and NO production by macrophages treated with IFN-γ and LPS. Meanwhile, the injection of ras protein or G-protein $\alpha_S$ subunit into permeabilized macrophages may be useful to study G-protein functions in downstream signalling in the NO synthesis pathway.

The role of IL-4 inhibiting the downstream signalling in IFN-γ and LPS-mediated NO production in macrophages needs further studies. This includes exploring the role of tyrosine kinases and gene transcription regulators, such as signal-transducers and activators of transcription (STATs), NF-κB and CREB proteins. In B cells, IL-4-mediated regulation of gene expression is related to Janus family kinases Jak1 and Jak3, which mediate tyrosine phosphorylation of STATs, such as IL-4 nuclear activated factor (IL-4 NAF) and signal-transducing factor of IL-4 (STF-IL-4) [Keegan et al., 1994; Johnston et al., 1994; Witthuhn et al., 1994].

The investigation of PKC regulated signalling mechanisms related to NO production will also be useful for understanding the activation and suppression of macrophages. Autophosphorylation of PKC and decreased $Ca^{2+}$ affinity of PKC through the autophosphorylation have been reported [Huang et al., 1986]. In intracellular downstream signalling, traffic can be directed by PKC phosphorylated substrates, such as myristoylated alanine rich C kinase substrate (MARCKS) proteins and NF-κB. MARCKS is an acidic protein, 87 kDa, synthesised an myristoylated in response to LPS induction [Aderem and Allen, 1992].

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